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**Interactions of Malpighian tubules of the tobacco hornworm
(*Manduca sexta*) with P-glycoprotein substrates**

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Thesis submitted to the
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Title: Interactions of Malpighian tubules of the tobacco hornworm (*Manduca sexta*) with P-glycoprotein substrates

Author: Lorin S. Gaertner

Abstract

I examined transport characteristics of isolated Malpighian (excretory) tubules from the tobacco hornworm (*Manduca sexta*), a Lepidopteran which tolerates high levels of dietary nicotine, to test the hypothesis that alkaloid (*e.g.* nicotine) transport in the tubules is the consequence of a multidrug transport system similar to that mediated by P-glycoprotein in multidrug resistant cancer cells. P-glycoprotein substrates were applied to the basal (blood) side of the tubule and their subsequent distribution monitored. First, using cannulated tubules in a modification of the Ramsay assay, [³H]-vinblastine levels were measured in samples of bathing and luminal solutions. After 1 h, the tubules had concentrated vinblastine in the lumen 3-fold (from 1 μ M). This accumulation was independent of a transepithelial potential, and was inhibited by verapamil, a P-glycoprotein inhibitor, and by nicotine. Second, fluorescent drugs, including daunomycin, rhodamine 123 and acridine orange, were applied to living tubules in a well of saline on a coverslip and examined by confocal microscopy. All of these drugs stained the cells of the tubule within 1 min, but, contrary to expectation, none of the drugs appeared in the lumen even after 1–2 h of incubation. Neither verapamil nor nicotine altered the pattern of daunomycin staining. The fast and intense Malpighian tubule staining was in sharp contrast to that of other tissues, which (for most drugs) stained lightly and only after prolonged exposures. Fluid-phase markers appeared to penetrate the tissue, but more slowly, and remained largely extracellular. The results suggest that the Malpighian tubule clears hemolymph of xenobiotics by passive filtration, active transepithelial transport involving a P-glycoprotein-like pump which handles alkaloids, and a scavenger-like absorption of compounds.

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Preface

The work presented in this thesis was carried out at the Loeb Research Institute at the Ottawa Hospital under the supervision of Dr. C.E. Morris. All the work presented in this thesis is my own.

Originally, I wrote (with Dr. Morris' advice and assistance) Chapter 2 as a manuscript which described work performed by myself and by C.L. Murray. It was published in the *Journal of Experimental Biology* (Gaertner *et al.* 1998) which grants permission for authors to use published material in theses. Much of the work presented in Chapter 3 was recently (Aug 18, 1998) submitted to *Tissue & Cell* as "Accumulation of daunomycin and fluorescent dyes by drug-transporting Malpighian tubule cells of the tobacco hornworm, *Manduca sexta*" by L.S. Gaertner and C.E. Morris.

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Abstract

P-glycoprotein (P-gp) has been proposed to be a multidrug pump capable of transporting a tremendous variety of drugs, typically lipophilic and weakly basic, out of the cell across the plasma membrane. In addition to its much-studied role in the multidrug resistance of cancer cells, there is evidence suggesting that this protein is deployed at excretory and blood-tissue barrier sites in many taxa, where it provides a defence against harmful xenobiotics. I examined transport characteristics of isolated Malpighian (excretory) tubules from the tobacco hornworm (*Manduca sexta*), a Lepidopteran which tolerates high levels of dietary nicotine, in order to test the hypothesis that alkaloid (*e.g.* nicotine) transport in the tubules is the consequence of a multidrug transport system similar to that mediated by P-gp in multidrug resistant cancer cells. In two different assays, various P-gp substrates were applied to the basal (blood) side of the tubule and their subsequent distribution monitored. First, using cannulated tubules in a modification of the Ramsay assay, [^3H]-vinblastine levels were measured in samples of bathing and luminal solutions. After 1 h, the tubules had concentrated vinblastine in the lumen 3-fold (from 1 μM). This accumulation was independent of a transepithelial potential, and was inhibited by verapamil, a P-gp inhibitor, and by nicotine. Second, fluorescent drugs, including daunomycin, rhodamine 123 and acridine orange, were applied to living tubules in a well of saline on a coverslip and examined by confocal microscopy. All of these drugs stained the cells of the tubule within 1 min, but, contrary to expectation, none of the drugs appeared in the lumen even after 1–2 h of incubation. Neither verapamil nor nicotine altered the pattern of daunomycin staining. For some of the drugs the fast and intense Malpighian tubule staining was in sharp contrast to that of other tissues, which stained lightly and only after prolonged exposures. Fluid-phase markers appeared to penetrate the tissue, but more slowly, and remained largely extracellular. The results *in toto* suggest that clearing the hemolymph (blood) of xenobiotics is a role the Malpighian tubule meets through a combination of mechanisms including passive

filtration, active transepithelial transport involving (but not exclusively) a P-gp-like pump which handles alkaloids and a 'scavenger-like' absorption of many other compounds.

Résumé

On a proposé que la P-glycoprotéine (P-gp) soit une pompe moléculaire qui transporte une énorme diversité de composés chimiques, typiquement amphiphiles et cationiques, hors des cellules, à travers la membrane cellulaire. En plus de son rôle dans la résistance pléiotropique (MDR) des cancers, les indications suggèrent que cette protéine, située aux sites d'excrétion et aux barrières hémato-tissulaires, confère à beaucoup d'organismes une défense contre les xénobiotiques nocifs. J'ai examiné *in vitro* des caractéristiques de transport des tubules de Malpighi (rénal) chez la larve de *Manduca sexta*, un Lépidoptère qui supporte des niveaux élevés de nicotine dans son régime alimentaire, afin de mettre à l'épreuve l'hypothèse que le transport des alcaloïdes (*par exemple*, la nicotine) est le résultat d'un système semblable à celui de la P-gp responsable du phénotype MDR. Deux analyses furent utilisées, dans lesquelles des substrats de la P-gp furent ajoutés au côté basal (sanguin) du tubule et leurs répartitions subséquentes furent observées. En premier lieu, d'après la méthode de Ramsay, une canule à été mise en place, et les niveaux de [³H]-vinblastine mesurés dans des échantillons du bain et de la lumière du tubule. Après 1 h, les tubules avaient accumulé (d'une concentration initiale de 1 µM) la vinblastine dans la lumière à un niveau 3-fois celle du bain. Cette accumulation était indépendante du potentiel électrique transépithélial, et était sensible au vérapamil, un inhibiteur de la P-gp, et, à la nicotine. En deuxième lieu, des agents fluorescents substrats de la P-gp, comprenant la daunomycine, la rhodamine 123 et l'acridine orange, furent appliqués à des tubules vivants. Ceux-ci étaient maintenus dans une solution physiologique salée sur une lamelle, puis visualisés en microscopie de fluorescence confocale. Tous ces agents ont marqué

les cellules du tubule dans moins d'une minute, mais, contrairement à ce qu'on attendait, ne sont point apparus dans la lumière, même après 1–2 h d'incubation. Ni le vérapamil, ni la nicotine ont changé cette évolution caractéristique. Pour certains des agents, la coloration rapide et vive des tubules de Malpighi a grandement contrasté la coloration presque imperceptible des autres tissus. Des sondes extracellulaires (*par exemple*, dextrane à poids moléculaire ~3000) ont aussi pénétré les tubules, mais d'une manière plus lente, et sont demeurés largement dans les compartiments extracellulaires. Les résultats, *in toto*, suggèrent que les tubules de Malpighi accomplissent leur tâche d'enlever les agents étrangers de plusieurs façons, comprenant une filtration passive, le transport actif transmembranaire des alcaloïdes, dans lequel une molécule semblable à la P-gp est impliquée, et ont une fonction d'épuration pour plusieurs autres composés.

Abbreviations

AO, acridine orange

ATP, adenosine triphosphate

CNS, central nervous system

HPLC, high performance liquid chromatography

LY, Lucifer yellow

MDR, multidrug resistance or multidrug resistant

NA, numerical aperture

NMN, N¹-methylnicotinamide

NMR, nuclear magnetic resonance

PCR, polymerase chain reaction

P-gp, P-glycoprotein

R123, rhodamine 123

RT-PCR, reverse-transcriptase polymerase chain reaction

SDS-PAGE, sodium dodecyl sulphate- polyacrylamide gel electrophoresis

TEA, tetraethyl ammonium

TR-dextran, Texas-redTM-conjugated dextran (MW ~3000)

1. Introduction

Overview

What do cancer cells that develop resistance to chemotherapy and insects that feed on toxic plants have in common? The answer may be an enigmatic protein, called P-glycoprotein and proposed to be a “multidrug pump”: a molecular entity apparently capable of protecting sensitive cells, tissues and consequently organisms, from toxic chemicals. A goal of this laboratory has been to explore the possible connection between P-glycoprotein and the ability of insects to feed on toxin-laden plants, and, potentially, to become resistant to insecticides. Recent studies have examined the blood-brain barrier of the tobacco hornworm (*Manduca sexta* L.) for involvement of P-glycoprotein in the protection of the nervous system from nicotine. The goal of the present study is to investigate the potential role of P-glycoprotein in the excretory system of the same insect. The Malpighian (excretory) tubules of a number of insects including the tobacco hornworm have previously been shown to actively transport nicotine and other alkaloids. The hypothesis of this work is that alkaloid (particularly nicotine) transport in the Malpighian tubule of *Manduca sexta* is the consequence of a multidrug transport system similar to that mediated by P-glycoprotein in multidrug resistant cancer cells.

As an introduction to the experiments presented in **Chapters 2 and 3**, this chapter examines first the tissue under study, the Malpighian tubule. Its structure and function, as well as the physiology of transport processes of the tubule are described. The focus then turns to P-glycoprotein about which much is known and yet much is still a mystery. In order to understand the potential role of P-glycoprotein in the insect Malpighian tubule, background information summarizing the current understanding of this protein, in the field of multidrug resistance and in more “natural” systems, is presented. Next, some recent research supporting a link between the insect excretion and P-glycoprotein, and thus the stated hypothesis, is presented. Finally, the

experimental approach and specific, testable hypotheses that are addressed in the subsequent chapters are summarized.

The Malpighian tubule

THE INSECT EXCRETORY SYSTEM

Role

The primary goal of an excretory system is to maintain suitable blood (hemolymph) composition in the face of environmental changes and normal activities. This involves the “elimination or segregation of unwanted substances present in the blood, and the retention or reabsorption of constituents needful to the organism” (Wigglesworth 1972). In this way the insect is no different from humans, so it is not surprising that there are a number of similarities between insect and vertebrate excretory systems. Both ‘filter’¹ the body fluid and subsequently pass the filtrate along a tube where useful substances are reabsorbed and where excess or harmful substances may be actively transported into it. The transport mechanisms involved are also suggested to be quite similar between the two groups (*e.g.* Bresler *et al.* 1990). On the other hand, there are some important differences. In most vertebrates, urine formation occurs by hydrostatic filtration of the blood in the glomerulus followed by modification of the filtrate in various segments of the renal tubule. With their open circulatory system, insects rely on a secretion/reabsorption system whereby the Malpighian tubules *secrete* a fluid (the primary urine) which is passed to the hindgut and rectum where selective reabsorption occurs (Maddrell & Gardiner 1974). Consistent with their diverse lifestyles and habitats, insects exhibit considerable variability in structure and function in their excretory systems, so that caution is warranted when

¹ insect physiologists describe the primary urine as a ‘filtrate’ of the hemolymph, since cells and large molecules are held back (Maddrell 1981, Bradley 1985); however there is no classic, pressure-driven filtration, as shall be explained

applying results from one species to another. However, where no information exists for caterpillars, results from other animal groups will be presented.

Anatomy

Malpighian tubules² of insects consist of a long tubular epithelium, closed at one end, and emptying into the alimentary tract (between the midgut and the hindgut) at the other, the so-called proximal end (Bradley 1985). Larvae of *Manduca sexta* are typical of Lepidoptera in having six Malpighian tubules which lie free in the hemocoel throughout most of the body but whose blind (*i.e.* distal) ends are closely associated with the rectum (Nijhout 1975). This association of tubules and rectum, found only in Coleoptera, larval Lepidoptera, and a Dipteran, is termed the cryptonephridium or rectal complex (Ramsay 1976, Bradley 1985). As the names suggest, the exact structure and function of this arrangement has been, and indeed still is, somewhat unclear. It is thought to act as a bidirectional filter that extracts and recycles water and salts from, and excretes excess body salt into, the fecal fluids (Dr. S. Caveney, *pers. com.*). There is a net flow of tubule fluid out of the rectal complex towards the proximal portions.

The disposition of the Malpighian tubules after exiting the rectal complex, which is not shown, is given in **Figure 1.1**. First there is a short rectal lead, followed by the iliac plexus, a series of serpentine coils lying on the ileum. The tubule then proceeds anteriorly along the midgut in what is known as the ascending straight segment, medial tubule or white region (for *Calpodes*) according to Moffett (1994), Nijhout (1975) or Ryerse (1979) respectively. At the first or second abdominal segment the Malpighian tubule doubles back along the midgut. This non-fluid-secreting portion known as the descending straight segment, proximal tubule or yellow region (again referring to its colour in *Calpodes*), is the principle subject of the investigations presented in **Chapters 2 and 3**. The three tubules on each side of the body then join at a

²named for Marcello Malpighi who, in 1668, first described them in the silkworm (Wigglesworth 1972)

common reservoir, or ampulla, which empties into the gut at the ventrolateral edge of the pylorus (Eaton 1988).

The Malpighian tubule is a “simple epithelium, in the histological sense, *i.e.* only one cell lies between the fluid on the basal surface (hemolymph or ‘blood’ side) and that on the apical side (urine)” (Bradley 1985). The lumen is usually ringed by two or three epithelial cells. These form bulbs or knob-like diverticula (**Figure 1.1**) giving the tubules a very distinctive appearance, and presumably increasing the surface area exposed to the hemolymph compared to a smooth tubule. These diverticula are more knob-like at the distal end of the tubule, while the proximal end, where the tubule enters the gut, is much smoother. It should also be noted that in large fifth-instar larvae, the tubules can have diameters on the order of 500 μm or more, and the cells are correspondingly large. At this stage the lumen is replete with uric acid, which appears as a white precipitate and spills out if the tubule is damaged (Nijhout 1975). At the hemolymph side the epithelium is covered by a basal lamella containing elastic fibres for tubule distension (Ryerse 1979). The finely branched trachea which supply oxygen to the tubule along its length always lie outside this layer (Bradley 1985).

Ultrastructurally, the cells of the Malpighian tubule possess many features frequently associated with water and solute transport (Smith 1968). Notably, the cells are highly polarized, with an apical surface that is produced into a series of microvilli, facing into the lumen. The basal cell surface is also “more or less extensively infolded” (Smith 1968). Mitochondria are abundant, often associated with the basal and apical folds, and even tightly packed into the microvilli. Intercellular spaces are fairly widely distributed, and cells are most commonly joined by septate junctions (Bradley 1985). Once presumed to be transepithelial barriers, these junctions encircle the apical two thirds of the intercellular cleft, and appear to slow diffusional movement via a paracellular pathway, but not to stop it (Skaer *et al.* 1987).

Morphological and functional specialization of different regions of the tubule is quite common, for example, division of fluid-secreting and non-fluid-secreting portions. There may

also be morphologically distinct cell types within a given region. For example, the Lepidopteran *Calpodex* has, in its yellow segment, so-called stellate cells distributed among the more typical principal cells (Bradley 1985). In flies, different cell types appear to be specialized for secretion or reabsorption (Meulemans & De Loof 1992, Sözen *et al.* 1997). Klein *et al.* (1991) state that there is only one cell type in the tubules of *Manduca* -- my observations, described in **Chapter 3**, support this statement, at least for the proximal portion of the tubule. Finally, in Malpighian tubules of some insects (including Lepidoptera), nuclei may be highly branched (Wigglesworth 1972) and polytene (Bradley 1985). The larval cells persist during Lepidopteran metamorphosis (Wigglesworth 1972), presumably obviating the need to separate such irregular nuclei. Micrographs and further discussion of Malpighian tubule microstructure are found in **Chapter 3**.

To reemphasize a point made above, the insect excretory system consists of two parts. The first is the Malpighian tubule whose major physiological function is the production of a hemolymph 'filtrate' or "primary urine", which serves as the fluid vehicle for carrying excretory wastes, toxic compounds and excess ions down the tubule (Bradley 1985). The second is the hindgut/rectum where this fluid is processed, largely by reabsorption, and where ionic homeostasis is ultimately achieved (Phillips *et al.* 1988). The next section examines how the Malpighian tubule achieves its objective.

PHYSIOLOGY OF THE MALPIGHIAN TUBULE

A model of hemolymph 'filtration'

As was suggested above, the generation of the primary urine in insects does not occur by a hydrostatic pressure filter, but rather by an active secretion process. One would therefore not expect to find substances in the urine unless they are specifically excreted (Ramsay 1958), which raises the question of how such a mechanism can allow the insect to achieve its goal of removing any and all unwanted substances while retaining those it requires. Using radiolabelled compounds, several investigators have noted that Malpighian tubules have an appreciable, but

low, permeability to a large number of organic compounds including amino acids, sugars, and even molecules as large as inulin (Ramsay 1958, Maddrell & Gardiner 1974). Because the urine to hemolymph ratio is inversely proportional to molecular weight and is less than one, it is assumed that these compounds enter the lumen passively (Bradley 1985). Maddrell (1981) proposes that this slow filtration occurs via paracellular routes and saves insects energy in producing the primary urine and in reabsorbing useful substances from it. At the same time, all unwanted products are *eventually* cleared from the body passively. Because clearance occurs slowly, the insect must be relatively tolerant of toxins in its hemolymph. Maddrell (1981) suggests that insects can tolerate changes in their internal milieu, at least for short periods of time, because they lack blood-borne respiratory pigments (with the exception of aquatic larvae of certain Chironomidae, whose haemoglobin releases oxygen under conditions of extreme oxygen paucity) and have special protective epithelia covering the most sensitive tissues. To supplement passive movement, there are a number of active transport systems for eliminating many organic and inorganic compounds, presumably those which are most problematic for the insect, and for reabsorbing sugars. Another feature of this model is that by increasing the rate of fluid secretion in the Malpighian tubule and of reabsorption in the hindgut, the insect can facilitate the passive entry of compounds into the tubule. Indeed, caterpillars maintain a high rate of water recycling from the rectum (Reynolds & Bellward 1989) that presumably aids in flushing toxins obtained in the diet from the body.

Ion transport linked to fluid secretion

The generation of fluid flow in the Malpighian tubule can be impressive; secretion rates for water and ions in the tubules of the blood-sucking bug *Rhodnius prolixus* are estimated to exceed those of any other known tissues (Maddrell & O'Donnell 1992). The process of fluid secretion is directly linked to the active transport of ions (Na^+ or K^+ or both) and Cl^- movement, from the hemolymph to the lumen (Pannabecker 1995). There is now excellent evidence that active cation transport is the result of a H^+ V-ATPase in the apical plasma membrane which

generates a proton motive force utilized by a cation/H⁺ antiporter (Maddrell & O'Donnell 1992, Harvey & Wieczorek 1997). This V-ATPase is a multi-subunit protein of at least nine polypeptides, and has been immunolocalized to the apical membrane of the Malpighian tubule in *M. sexta* (Klein *et al.* 1991, Pietrantonio & Gill 1995). It is still a matter of debate whether the apparent preference for Na⁺ or K⁺ transport (which depends on the species and the physiological conditions) is due to the selectivity of a single cation cotransporter, to separate Na⁺/H⁺ and K⁺/H⁺ cotransporters, or to basal channels (Nicolson 1993).

A number of other membrane transfer mechanisms are thought to be involved in ion transport, and so presumably with fluid secretion. An obvious necessity is for a conductance mechanism for the major counter ion, Cl⁻. Proposed transepithelial routes for Cl⁻ include primary and secondary active transport at the basolateral membrane, apical Cl⁻ channels, and a paracellular ("shunt") pathway (Nicolson 1993, Pannabecker 1995).

A basal Na⁺-K⁺ ATPase and a Na⁺-K⁺-2Cl⁻ cotransporter also participate in the movement of ions across the epithelium. The presence of a classical Na⁺-K⁺ ATPase in insect tissues has been controversial due to the apparent insensitivity, in some species, of fluid secretion to ouabain. In fact, in the milkweed bug *Oncopeltus fasciatus*, ouabain is passively secreted then reabsorbed by a downstream segment of the Malpighian tubules (Nicolson 1993). On the other hand, modest effects of ouabain on membrane potentials and fluid secretion have been observed in some species, including *Drosophila* from which a cDNA for the Na⁺-K⁺ ATPase α -subunit has been cloned (Nicolson 1993, Pannabecker 1995). Most likely, it is present in most insects but does not play a major role in fluid secretion; Bresler *et al.* (1990) suggest that it may be important in the secretion of organic acids. A Na⁺-K⁺-2Cl⁻ cotransporter was recently cloned from the Malpighian tubules of the tobacco hornworm and Northern blot analysis suggested it may be an isoform specific to this tissue (Reagan 1995).

It is widely believed that the movement of water from hemolymph to lumen follows ion transport by a form of local osmosis (*e.g.* Bradley 1985, Maddrell & O'Donnell 1992,

Pannabecker 1995); however the precise mechanism of coupling of ion and water movements in the Malpighian tubule remains controversial (Nicolson 1993). Bradley (1985) summarizes the evidence that urine formation in insects is driven by osmotic forces thus: the fluid produced is always isosmotic or slightly hyperosmotic to the bathing medium, varying individual ion secretory rates does not change this osmotic relationship, and tubules will secrete faster in dilute than in concentrated solutions. An interesting alternative to the conventional view that water movement is driven solely by differences in its chemical potential is proposed by Zeuthen & Stein (1994). Their idea, which has experimental support (Reuss 1996, Zeuthen *et al.* 1996), is that ions and water may be cotransported, such that water transport would occur by a secondary active process. While they do not discuss insect tissues specifically, this model should definitely be considered in light of the apparently uphill movement of water in the insect rectum, where the fecal matter is simultaneously depleted of ions and made hyperosmotic (Bradley 1985).

Other ion transport processes

In addition to the much-studied movement of Na^+ , K^+ and Cl^- into the lumen, reabsorption of these ions may occur (*e.g.* Haley & O'Donnell 1997) and a number of other inorganic ions are actively transported into or out of the Malpighian tubule of some insects. These include calcium, magnesium, phosphate and sulphate ions (Bradley 1985, Pannabecker 1995). Such transport is in keeping with the maintenance of ionic homeostasis but presumably is a minor contributor to fluid secretion.

Transport of organics -- acidic/anionic compounds

Although many organic compounds accumulate passively in the lumen (as discussed under 'a model of hemolymph filtration' above), another large group is actively transported. Indeed, clearance of acidic and basic dyes by Malpighian tubules of *Periplaneta* and *Forficula* was noted by L. Lison in the 1930s (Bradley 1985). Maddrell *et al.* (1974) studied the excretion of the dyes indigo carmine and amaranth as well as radiolabelled *p*-aminohippuric acid (PAH) in a number of species, including *M. sexta*, although they do not specify if the adult or larva was

used. Amaranth and indigo carmine are both rapidly secreted and PAH achieves a secreted-to-bath ratio of 2.5 in this species. Based on more extensive experiments in *Rhodnius* and *Calliphora* they conclude that insects possess two separate mechanisms for the excretion of organic anions, one for acylamines such as PAH and one for sulphonates such as indigo carmine (Maddrell *et al.* 1974). This is disputed by Bresler *et al.* (1990), who studied fluorescein transport in four species including the larva of the moth *Galleria mellonella*. They showed that active transport of this dye in all species examined can be inhibited by carboxylic acids and by a sulphonate. They also observed that fluorescein transport is sodium dependent and postulated that sodium-coupled cotransport across the basolateral membrane may be involved (Bresler *et al.* 1990). Before leaving the subject of acidic (anionic) dye transport, the observations of Nijhout (1975) should be noted. He injected water soluble dyes (20 mM, 100 to 150 μ l) into *M. sexta* larvae, and visually examined their progress of excretion by sacrificing animals up to 6 hours after injection. Interestingly, acidic dyes (including amaranth, indigo sulphonate and fluorescein) were found to enter not only the medial section of the Malpighian tubules, but also the midgut. This apparent ability of the midgut and Malpighian tubules to excrete dyes gradually decreases as the larva stop feeding and prepare to enter the pupal stage (Nijhout 1975).

Transport of organics -- basic/cationic compounds and alkaloids³

The movement of basic dyes, cationic compounds, and alkaloids is of special interest to this thesis. Using the approach described in the preceding subsection, Nijhout (1975) showed that many basic dyes, particularly methyl green and methylene blue, rapidly enter the proximal, and to a lesser extent the medial, Malpighian tubules but not the midgut of *Manduca* larvae. He also found that a number of basic dyes are not visibly excreted over a 6 h period. A more recent study examined the pathway of another dye, rhodamine 123, after injection into *Sarcophaga* flies

³An alkaloid is “any of numerous colourless, complex and bitter organic bases containing nitrogen and usually oxygen, that occur especially in seed plants” (Merriam-Webster dictionary), or, as defined by Roberts & Wink (1998), “a cyclic compound containing nitrogen in a negative oxidation state which is of limited distribution in living organisms”.

and after *in vitro* labelling of the Malpighian tubules (Meulemans & De Loof 1992). Uptake *into the cells* from either the hemolymph or the lumen, depending on the cell type, was observed -- results of particular interest for comparison to those described in **Chapter 3**.

Of particular relevance to this thesis are the experiments of Maddrell and Gardiner (1976) which examine the excretion of nicotine and other alkaloids in isolated Malpighian tubules. They found that nicotine is actively transported in the Malpighian tubules of the tobacco-feeding larva of *M. sexta*, as might be expected (see also 'nicotine and the tobacco hornworm', below). Malpighian tubules of larvae of the lepidopteran *Pieris brassicae*, which does not feed on nicotine-containing plants, were also found to be capable of nicotine transport. That *Pieris* is nonetheless intolerant of dietary nicotine suggests that active excretion of nicotine alone is not sufficient to explain the adaptation of the tobacco hornworm (Snyder *et al.* 1994) as discussed below. Even more surprisingly, the blood-sucking bug *Rhodnius prolixus* is also capable of transporting nicotine in its Malpighian tubules at a high rate, despite the lack of any manifest need for such an ability. Further investigation showed that *Rhodnius* Malpighian tubules are also capable of transporting atropine and morphine; adult flies *Calliphora* and *Musca* also transport both nicotine (or its metabolite) and atropine. A common mechanism for the transport of all three of these alkaloids (nicotine, atropine and morphine) was suggested based on mutual competition of transport in *Rhodnius*: atropine suppresses nicotine and morphine transport while nicotine interferes with morphine transport. It is important to stress that while the above results are often taken to suggest that a common alkaloid transporter is found in many insects, direct evidence for this has been lacking. A recent addition is the observation that nicotine transport in *Manduca* Malpighian tubules is sensitive to atropine (Murray 1996, Gaertner *et al.* 1998).

NICOTINE AND THE TOBACCO HORNWORM

The ability of certain insects to feed on plants containing toxic allelochemicals that deter most herbivores has attracted a great deal of interest over the years. This is true for the tobacco

hornworm, which is remarkable in being able to live entirely on tobacco (*Nicotiana tabacum*), a plant which may contain up to 8% nicotine (dry weight), a toxin to insects and humans alike. A number of factors, including excretion, contribute to this resistance.

Excretion

Excretion was early identified as a key factor in the adaptation of tobacco hornworms, when Self *et al.* (1964a) showed that nicotine, which enters the hemolymph from the diet, rapidly (90% within 4 h) leaves the body via the feces.

Using 3 cm lengths of the proximal Malpighian tubules from *Manduca sexta* fifth-instar larvae, Maddrell & Gardiner (1976) found that ¹⁴C-labelled nicotine accumulates in the secreted fluid (what little that is produced after 90 min undisturbed) to a concentration 14 times the initial bath concentration of 4 µM. Using perfused proximal and medial tubules from fourth-instar larvae they showed that such nicotine transport does not depend on the presence of nicotine in the larval diet; however the Malpighian tubules of adults, which feed on nectar, do not appear to actively transport nicotine (Maddrell & Gardiner 1976). The ability of isolated Malpighian tubules to actively transport nicotine has recently been confirmed in our laboratory (Murray 1996, Gaertner *et al.* 1998).

Metabolism

Although Self *et al.* (1964b) did not detect any breakdown of nicotine in *Manduca*, more recent reports suggest that *Manduca* does indeed break down nicotine, and this may be a significant part of its adaptation to the drug. Morris (1983a), for example, found that a component of the central nervous system (CNS) constitutively metabolises the bulk of the nicotine that is taken up by the tissue. Snyder *et al.* (1993) confirmed this activity, and also found significant midgut microsomal metabolism that was inducible by nicotine at typical dietary levels. This correlated with the induction of 9 of 12 midgut cytochrome P-450 activities tested. By reverse-phase high-performance liquid chromatography (RP-HPLC) they identified the main metabolites as cotinine-*N*-oxide and, tentatively, nicotine-1-*N*-oxide. Similar RP-

HPLC profiles were also found when Malpighian tubules were incubated for 1 h *in vitro* with nicotine, although the amount of nicotine metabolism was “very low” (Snyder *et al.* 1993). It should be noted that increased cytochrome P-450 activity is a very significant cause of insecticide resistance (Hodgson *et al.* 1993).

The significance of an inducible mechanism, presumably metabolism, is that nicotine-fed fifth-instar larvae show increased tolerance to subsequent nicotine injection when compared to larvae fed a control diet (Snyder *et al.* 1993). Furthermore, the larvae appear to limit their consumption of nicotine-containing diet until P450 activities have increased (Glendinning 1996, Snyder & Glendinning 1996). Another aspect of the induction of nicotine metabolism is that it may be reversed within 24 h of returning to a nicotine-free diet (Snyder *et al.* 1994).

Blood-brain barrier

Since nicotine exerts its toxic effects in the nervous system at cholinergic receptors in synaptic junctions (Morris 1984), and since excretion and metabolism only reduce hemolymph levels of nicotine slowly, it is logical to suspect that the CNS of *Manduca* is somehow less susceptible to nicotine. Although some cholinergic synapses may be nonnicotinic, “the isolated CNS of *Manduca* is competent to respond to a variety of cholinergic agents, including nicotine” (Morris 1984). However, this is not a liability as a sophisticated blood-brain barrier has evolved. Components of this barrier include a physical barrier (the perineurium, with ultrastructural features that resist intercellular movement of (charged) nicotine; Morris & Harrison 1984), detoxifying enzymes (Morris 1984, Snyder *et al.* 1993) and a putative nicotine pump (Morris 1983a-c, Murray *et al.* 1994). The latter has been proposed to be similar to that present in the Malpighian tubules, and more specifically to be a P-glycoprotein homologue. This leads us to a discussion of this protein and the evidence that it is present in insects.

P-glycoprotein

A MEMBRANE PROTEIN IMPLICATED IN MULTIDRUG RESISTANCE

Discovery of P-glycoprotein as a player in multidrug resistance

The term P-glycoprotein (hereafter abbreviated P-gp) describes a cell-surface glycosylated protein of approximately 170 kDa whose appearance correlates with pleiotropic drug resistance and reduced penetration of drugs in many cell lines (Juliano & Ling 1976). Because of the problem of drug resistance in clinical cancer treatment, P-gp and P-gp-mediated multidrug resistance have received a great deal of attention, with thousands of papers on the subject; reviews include Endicott & Ling 1989, Gottesman & Pastan 1993, Bosch & Croop 1996 and Gottesman *et al.* 1996. As shall be seen, despite over 20 years of extensive research on this one molecule, many fundamental questions remain unanswered.

The term multidrug resistance (MDR) describes cross-resistance to a number of cytotoxic agents following exposure to one such agent. The drugs include a wide variety of compounds, many of which are important anti-cancer agents, with little to unite them either structurally or functionally (see 'drug profile' below). The generation of multidrug resistant cell lines, typically by continuous culture in the presence of increasing concentrations of a selecting drug, is generally accompanied by overexpression of P-gp, also called the MDR protein. Studies in which cells are transfected with the cDNA for P-gp (*e.g.* Gros *et al.* 1986) and biochemical studies with the purified protein clearly indicate that P-gp alone *can* mediate the MDR phenotype, although other cellular changes are frequently involved (Roepe *et al.* 1996).

Molecular biology of P-gp

In the mid 1980s, cDNAs encoding P-gp were isolated from rodent and human cell lines. Based on sequence similarities, particularly in two regions that correspond to ATP-binding sites, the P-glycoprotein genes are placed in the ATP-binding cassette (ABC) superfamily, a large and

diverse group of proteins which includes transport proteins from both prokaryotes and eukaryotes (Higgins 1992).

P-gp is itself a small, highly conserved multigene family, with 2 or more members found in most organisms examined to date (see 'distribution of P-gp among other taxa', pg 20). For mammals, the different family members can be divided into 3 classes based upon functional and sequence similarity. It should be noted that not all P-gps convey multidrug resistance: class III P-gp encodes a phosphatidylcholine translocase (or "flippase") important to the secretion of bile in the liver (Ruetz & Gros 1994, van Helvoort *et al.* 1996). Large divergences in sequence prevent prediction of the biological significance of the various P-gp genes in non-mammals.

The protein encoded by the P-gp gene is predicted to be an integral membrane protein of ~1280 amino acids, with two homologous halves (Juranka *et al.* 1989). Each half contains 6 membrane spanning α -helices, and a cytoplasmic domain containing a highly conserved ATP-binding site. Glycosylation occurs on the first extracellular loop (Zhang 1996) and may (Kramer *et al.* 1995) or may not (Gottesman & Pastan 1993, Senior *et al.* 1995) be important in the protein's activity. The significance of phosphorylation of P-gp at predicted sites is also debated (Gottesman *et al.* 1996). The predicted topology has been supported by antibody binding studies and site-directed mutagenesis (*e.g.* Kartner *et al.* 1985, Loo & Clarke 1995). Although dimers of P-glycoprotein have been observed, there is also evidence to suggest that the protein can function as a monomer (Loo & Clarke 1996).

ACTION OF P-GP IN MDR

Drug profile

As stated, there is little doubt that expression of P-glycoprotein can impart to otherwise sensitive cells a multidrug resistance phenotype. The fundamental characteristic of this phenotype, at least from the point of view of the cell, is decreased sensitivity to normally cytotoxic drugs. While there are many ways for a cell to adapt to a given toxic insult, the peculiar feature of multidrug-resistant cells is that they become immune to a very wide variety of

chemicals. There is no common structure, nor is there a common intracellular target; the list of agents to which the MDR cells are resistant “includes dozens, and perhaps hundreds or more, of hydrophobic natural products (i.e. derived from plants or microorganisms), semi-synthetic analogs of such products, and synthetic organic compounds” (Gottesman & Pastan 1993; **Table 1.1**). Most commonly the compounds are relatively small, lipophilic and cationic at physiological pH (Bosch & Croop 1996); however there are exceptions even to this vague rule.

Another striking characteristic of the MDR phenotype is that sensitivity can be restored in the presence of less cytotoxic drugs, often at low concentrations. These “chemosensitizers” or “resistance reversal agents” also compose an extraordinarily broad group of chemicals, as shown in **Table 1.1**. They include substrate analogs, calcium channel blockers, calmodulin inhibitors, steroids and detergents. Typical effective concentrations are in the range of 2–20 μM ; the degree of reversal is almost complete in some cases, but only partial in others. Generally, “their previously identified activities do not appear to play a role in the mechanism [by] which they reverse multidrug resistance” (Bosch & Croop 1996). Suggestions for what this mechanism may be include competition for substrate binding sites, direct non-competitive inhibition, alteration of membrane properties, or indirect changes via intracellular pH alterations. Clearly, the mechanism ascribed to P-glycoprotein action (see ‘proposed mechanisms for P-gp’, below) will have an important bearing on that ascribed to its inhibition.

Cellular aspects

A universally recognized feature of MDR cells is that they have reduced accumulation and/or a redistribution of the drugs from sensitive intracellular sites. Much more controversial is whether this results from increased efflux, reduced influx, altered partitioning or some combination of these mechanisms. There are, in fact, many studies supporting each of these conjectures (Simon & Schindler 1994), and this is part of the problem in attempting to pin down a mechanism for how P-gp works. As Roepe (1995) notes, “use of the term ‘increased efflux’ is pervasive... but does not necessarily denote increased rate of outward-direct transport”.

The vast majority of P-gp is located at the cell (plasma) membrane, consistent with its proposed role as a transporter (Willingham *et al.* 1987). P-gp has also been immunolocalized to the luminal side of Golgi stack membranes (Willingham *et al.* 1987, Molinari *et al.* 1994), to the nucleus (Baldini *et al.* 1995) and to the cytoplasm (Broxterman *et al.* 1989, Baldini *et al.* 1995, Abbaszadegan *et al.* 1996). In polarized (secretory) epithelia, P-gp is consistently found on the apical surface (*e.g.* Thiebaut *et al.* 1987); this polarity of expression is retained in transfected epithelial cell cultures (Pastan *et al.* 1988).

Biochemical aspects

Because of the inherent complexity of using whole cells, studies of P-gp in enriched fractions, semi-purified preparations, or reconstituted membrane systems have a certain attraction. A number of these studies have demonstrated that a drug-stimulatable vanadate-sensitive ATPase activity with a large constitutive component is associated with P-gp (*e.g.* Doige *et al.* 1992, Dong *et al.* 1996). Partially purified P-gp has been reconstituted in proteoliposomes which display ATP-dependent transport of [³H]-colchicine (Sharom *et al.* 1993). The contribution of this and numerous other biochemical studies to our understanding of the thermodynamics and kinetics of P-gp is considered at some length by Roepe (1995).

PROPOSED MECHANISMS FOR P-GP

The current controversy over what model, if any, best describes P-gp's action is hardly surprising given the broad substrate "specificity" evident by P-gp, many apparently contradictory results, and the difficulty of separating effects irrefutably due to P-gp from those due to selection in cytotoxic drugs or to measurement artefacts. There are two main streams of thought: the first, that P-gp acts as a pump, recognizing substrates directly and removing them from the cell membrane or the cytoplasm; the second, that P-gp overexpression leads to an alteration of the cell's electrical or pH balance which then alters the partitioning and intracellular levels of drugs.

Pump model

In the pump model the hydrolysis of ATP is proposed to be directly coupled to the translocation of substrates in a manner analogous to classical ion transporters (Gottesman *et al.* 1996). This model has for support the sequence homology to known transporters, such as the yeast *STE6* and bacterial *hlyB* gene which are responsible for the secretion of an oligopeptide mating factor and hemolysin, respectively (Juranka *et al.* 1989, Dean & Allikmets 1995).

The main evidence for a direct interaction between P-gp and various drugs comes from photolabelling studies. Plasma membranes vesicles or cell suspensions are incubated with radiolabelled (and photoactivatable) azido analogues of vinblastine, ATP, verapamil, *etc.* and irradiated. Compounds to which the photoactivatable compounds have bound are then identified by immunoprecipitation, SDS-PAGE and/or western blotting, and show direct interactions between P-glycoprotein and many of these compounds (*e.g.* Safa 1988). Photolabelling studies are, however, criticized for employing membrane preparations so highly enriched in P-gp that collision between the hydrophobic probe and “a particularly plentiful and large target in the membrane” is statistically probable even in the absence of any biologically relevant interaction (Roepe 1995). Additional support for a direct interaction comes from point mutations in P-gp that affect drug binding and alter drug resistance profiles (Bosch & Croop 1996).

In view of the observation that drugs are “detected and ejected before they reach the cytoplasm” (Gottesman *et al.* 1996), a variation on the pump model has been proposed whereby P-gp removes substrates directly from the lipid bilayer, thus acting as a “hydrophobic vacuum cleaner” or “flippase” (Higgins & Gottesman 1992, Gottesman & Pastan 1993). As a flippase, P-gp would energize the movement of lipid-soluble drugs from the inner leaflet of the lipid bilayer to the outer or to the external medium (Higgins & Gottesman 1992). The non-MDR mediating P-gp of mice and humans has been convincingly demonstrated to translocate phospholipids in this way (Ruetz & Gros 1994, van Helvoort *et al.* 1996), and recently human

MDR-mediating P-gp has been shown to be a lipid translocase of broad specificity (van Helvoort *et al.* 1996).

The principle thorn-in-the-side of the pump model is that the concept of substrate specificity, a “fundamental tenet of active transporters” (Roepe *et al.* 1996), has virtually no meaning for P-gp. Proponents suggest only that “a complex substrate recognition region or regions” use “uncharacterized molecular cues” to recognize substrates (Gottesman *et al.* 1996). The ability of the pump model to account for the coupling of ATP hydrolysis to drug movement, and calculated drug turnover rates to explain decreased accumulation is also hotly disputed (Roepe *et al.* 1996).

Altered partitioning model

An alternative view is embodied in the altered partitioning model, which envisions that P-gp overexpression leads to altered electrical membrane potential ($\Delta\Psi$) and/or elevated intracellular pH (Roepe *et al.* 1996). This no longer violates the concept of enzyme specificity since any hydrophobic charged or weakly basic drug, which includes most drugs to which MDR cells are resistant, will be affected. For the neutral drug colchicine, it is pointed out that binding of intracellular target is pH- and possibly even $\Delta\Psi$ -dependent (Roepe *et al.* 1996). Consistent with this alternative model are many documented pH_i and $\Delta\Psi$ alterations in MDR cells (*e.g.* Keizer & Joenje 1989, Simon *et al.* 1994, and reviewed by Roepe 1995). Furthermore, the MDR-reversing agent verapamil reverses the pH alteration in a lung tumour cell line (Keizer & Joenje 1989). An interesting perspective is that by becoming more alkaline, MDR tumour cells revert to a more “normal” state *i.e.* they have reduced hypersensitivity rather than increased resistance (Simon & Schindler 1994). The pH gradient between the cytoplasm and intracellular compartments which allows cytotoxic drugs to be protonated, sequestered and secreted is also disrupted in tumour cells, but restored in drug-resistant derivatives, again supporting the altered partitioning model (Schindler *et al.* 1996).

How P-gp might affect pH or electrical gradients is an open question. Supporters of the altered partitioning model point to a number of roles attributed to P-gp, including that of Cl⁻ transporter (channel/exchanger/co-transporter or pump), ion transporter regulator, or ATP transporter (Roepe *et al.* 1996). Several of these theories have been largely discredited; the remainder are highly controversial (Wine & Luckie 1996, Gottesman *et al.* 1996, Roepe *et al.* 1996). Opponents of the altered partitioning model point out that these changes are not found in all P-gp expressing cells, and question if the magnitude of the changes are sufficient (Gottesman *et al.* 1996). Proposals that P-gp may be a channel regulator, a rather vague concept which could describe any influence, or that it can act as both a pump and a channel, have arisen in an attempt to reconcile contradictory results, especially those of electrophysiology studies.

In summary, it appears that the complexity of the interactions between P-glycoprotein, cytotoxic drugs, and drug-resistant cells will continue to defy simple models for some time.

A NATURAL ROLE FOR P-GP

Although most of the attention on P-gp has been for its role in multidrug resistance of cancer cells, its “raison d’être” in the natural world is clearly not to thwart chemotherapy efforts. Early speculation based largely on the distribution of the protein in normal mammalian tissues was that P-gp functions as “a broad-specificity transport system involved in clearing the body of exogenous and endogenous molecules” (Juranka *et al.* 1989). Recent research with transgenic animals and with P-gp substrates and inhibitors continues to point towards a role for this protein in excluding exogenous molecules (xenobiotics) from sensitive organs and excreting them from the body.

Distribution of P-gp in mammals

Using a wide variety of techniques to detect mRNA and/or protein, P-gp has been localized to distinct regions, usually at secretory surfaces, of the biliary canaliculi, the proximal tubules of the kidney, intestinal and colonic epithelium, pancreatic ducts, and pregnant uterine epithelium (Thiebaut *et al.* 1987). It is also found in the cortical regions of the adrenal gland,

which, along with the observation that P-gp is capable of transporting cortisol and aldosterone *in vitro*, suggests that hormones may be endogenous substrates for P-gp (Ueda *et al.* 1992).

P-gp has also been found strongly expressed in endothelial cells of capillary blood vessels of the brain and other blood-tissue barriers (Cordon-Cardo *et al.* 1989, Thiebaut *et al.* 1989, Stewart *et al.* 1996). P-gp is also expressed in cultured brain capillary endothelial cells, where it mediates efflux of various drugs (reviewed in Tsuji & Tamai 1997). However, a recent report shows that P-gp co-localizes with an astrocyte “marker” protein, glial fibrillary acidic protein, but not with an endothelial marker protein, glucose transporter GLUT1 (Pardridge *et al.* 1997). This contradicts the assertion that P-gp plays a functional role at the luminal membrane of the brain capillary endothelium *in vivo*, but is not incongruent with a role for this protein in the overall regulation of brain drug uptake, via expression at the astrocyte foot processes (Pardridge *et al.* 1997).

In the kidney, P-gp is found at the apical surface of epithelial cells of the proximal tubules (Thiebaut *et al.* 1987). As for the blood-brain barrier, a number of cell lines derived from kidney have provided a more tractable model for studying the transport activity of P-gp, as discussed under ‘renal cell lines’, pg 23. The original immunocytochemistry has been confirmed by standard techniques to isolate the brush-border membrane, while RT-PCR has suggested that P-gp expression may not be restricted to the proximal tubule (reviewed by Simmons *et al.* 1997).

Distribution of P-gp among other taxa

As mentioned, P-gp is an ancient molecule which appears to be conserved among all organisms. Homologues of mammalian P-gp are found throughout most phyla; one or more genes have been cloned from vertebrates (humans, rodents, fish), insects (*Drosophila*), nematodes (*Caenorhabditis*), protozoans (*e.g.* *Entamoeba histolytica*), and even plants (*e.g.* *Arabidopsis thaliana*) (Childs & Ling 1996). A number of aquatic invertebrates, including freshwater and marine mussels and marine sponges, also show evidence of P-gp presence. The

evidence for its presence in insects, and specifically in *M. sexta*, is further described in the section, 'the insect/P-glycoprotein connection', pg 26.

The tissue-level distribution has been studied for only some of these organisms. Expression in teleost fish is similar to that in mammals, being present in bile canaliculi, renal tubules, intestinal lumen *etc.*, but also in gill transverse septa (Hemmer *et al.* 1995). In *Caenorhabditis*, P-gp is expressed in both the apical membrane of the excretory cell and in the apical membrane of intestinal cells (Broeks *et al.* 1995). It is found in the gills of mussels (Kurelec 1995) and in the intestine of frog (Castillo *et al.* 1995). In *Arabidopsis* it is present in all plant parts examined, but is particularly abundant in the peduncles (Dudler & Hertig 1992).

In summary, the widespread distribution of this protein strongly suggests that it has been an important part of the survival of life on earth. The variability of its use and tissue-specific expression suggests that it can be put to flexible use to serve the specific needs of the individual organism. At the same time, P-gp seems to be consistently expressed at excretory and blood-tissue barrier sites, which supports the idea that it has evolved for the transport of xenobiotics.

Role for P-gp in protection from xenobiotics

By far the clearest evidence that P-gp can protect organisms from xenobiotics comes from mice homozygous for a disruption of one or two of their P-gp genes. These mice develop and breed normally, with no obvious physiological, anatomical or histological abnormalities. However, after being sprayed with a dilute solution of ivermectin in a routine treatment for a mite infestation, the knockout mice, but not the wild type, died with paralytic symptoms (Schinkel *et al.* 1994). Follow-up showed that their increased sensitivity to ivermectin is correlated with increased drug levels in many tissues, especially in brain, and with decreased drug elimination. Altered pharmacokinetics and increased brain penetration in these mice have since been shown for a number of unrelated drugs including opiates, vinblastine and digoxin (Schinkel *et al.* 1996, Schinkel *et al.* 1997).

Knock-out studies have been performed with two other metazoan species, and these too suggest that P-gp may confer increased fitness through protection from xenobiotics. Deletion of one of the four P-gp genes in *Caenorhabditis* increased the sensitivity of the nematode to colchicine and chloroquine; wild-type resistance was largely restored by reintroducing the wild-type P-gp by microinjection (Broeks *et al.* 1995). In *Drosophila*, fly strains bearing disruptions in a P-gp gene show increased sensitivity to colchicine; however somewhat mysteriously, more complete loss of the gene does not cause increased sensitivity (Wu *et al.* 1991).

In aquatic organisms, some of which survive and develop normally in highly polluted water, a number of studies have built a convincing case for protection from xenobiotics through expression of P-gp. For example, two species of marine sponge express P-gp at cell membranes and accumulate increased levels of drugs, including vincristine and daunomycin, in the presence of verapamil (Kurelec *et al.* 1992). Very similar results are found for both freshwater and marine mussels (Kurelec 1992) and embryos of a marine echiuran worm (Toomey & Epel 1993). The amount of P-gp immunoreactive protein in mussels and oysters varies quantitatively with the level of organic pollution at the sampling sites (Minier *et al.* 1993), and vincristine accumulation in the gills of mussels from polluted sites is much reduced compared to those from unpolluted sites (Kurelec 1995). Finally, common pollutants have been shown to increase drug (vincristine or rhodamine B) accumulation in mussels, which suggests that they may be competing for a common transporter (Cornwall *et al.* 1995, Kurelec 1995). The ability of P-gp to mediate drug excretion in the kidneys of teleost fish is further considered below.

Discussion of the evidence suggesting P-gp protects insects from xenobiotics is deferred to the section on 'the Insect/P-gp connection', pg 26.

TRANSEPITHELIAL TRANSPORT: P-GP IN (VERTEBRATE) EXCRETORY SYSTEMS

Since P-gp acts as a transporter in tumour cell lines, protects whole organisms from xenobiotics, and is substantially expressed in the kidney, it is quite plausible that P-gp should have a renal function as a transporter of xenobiotics. Since this thesis examines the proposal that

P-gp functions as a transepithelial transporter in the insect Malpighian tubule, it is worthwhile examining the precedent for transepithelial transport in vertebrate excretory systems.

Renal cell lines

Cell lines such as the Madin-Darby canine kidney (MDCK), LLC-PK₁ (from pig) and opossum kidney (OK) exhibit substantial differentiation *in vitro*, including apical microvilli, tight junctions and basolateral infoldings. When grown on permeant matrices (filters) they provide convenient models for measurement of vectorial transport (Ardailou *et al.* 1996, Simmons *et al.* 1997). Native (*i.e.* untransfected) MDCK cells have small but detectable amounts of P-gp and exhibit net basal to apical transepithelial flux of the typical P-gp substrates vinblastine, daunomycin, vincristine and actinomycin D (Horio *et al.* 1989). Vinblastine transport can be inhibited by verapamil (Horio *et al.* 1989, Hunter *et al.* 1991a, 1993) as well as by other P-gp substrates (Horio *et al.* 1989, 1990). When cell lines are transfected with P-gp cDNA, basal-to-apical flux of drugs (vinblastine, daunomycin *etc.*) increases dramatically while apical-to-basal flux remains low (Horio *et al.* 1989, Tanigawara *et al.* 1992, Pan *et al.* 1994).

It should be noted that kidney cell lines are not unique in their ability to exhibit apparently P-gp-mediated transepithelial transport, just as the kidney is not the only site of P-gp expression. The intestinal cell lines HCT-8, T84 and Caco-2 express P-gp and are capable of polarized vinblastine transport which is sensitive to verapamil (Hunter *et al.* 1991b, Wils *et al.* 1994).

Other kidney preparations

Intact renal proximal tubules from teleost fish have proven to be a useful preparation for studying secretory transport mechanisms, since they are easily isolated and, when isolated, “broken ends rapidly reseal to form a closed, fluid-filled luminal compartment that only communicates with the medium through the tubular epithelium” (Pritchard & Miller 1991). Miller and colleagues have shown that the inherently fluorescent P-gp substrate daunomycin, as well as fluorescent analogues of cyclosporin and rapamycin, all accumulate in the cells and

lumen of proximal tubules (Miller 1995, Schramm *et al.* 1995, Miller *et al.* 1997). At equilibrium, fluorescence levels in the lumen are two to four times that of cellular fluorescence. Verapamil or cyclosporin A inhibit secretion into the lumen but cellular fluorescence intensity is not affected by any of the treatments used (Miller 1995, Schramm *et al.* 1995, Miller *et al.* 1997). These results are consistent with drugs crossing the basolateral membrane by simple diffusion and subsequently being secreted into the tubular lumen by P-gp. This is the model of secretion that is also supported by the work with renal cell lines (Simmons *et al.* 1997). Essentially, by expressing P-gp in a polarized fashion, the mechanism which simply extrudes drugs from the cytoplasm in typical cancerous MDR is adapted by these epithelia to mediate vectorial transport. It should be noted that at a lower buffer pH the transport of daunomycin appears to be more typically organic cation transporter-mediated than P-gp-mediated (Miller 1995; see also 'related transporters in the kidney', below).

An *in vivo* study of rat renal function provides further evidence for P-gp operating at the whole animal level. By measuring urine flow, glomerular filtration rate, and colchicine clearance, Speeg *et al.* (1992) showed net secretion of colchicine into the urine. Cyclosporin A, which reverses MDR *in vitro*, inhibits renal colchicine secretion without affecting glomerular filtration or net secretion of the organic cation ranitidine (Speeg *et al.* 1992). On the other hand, urinary excretion of digoxin is not impaired in mice in which both MDR-mediating P-gp genes are disrupted (Schinkel *et al.* 1997). This leads us to the question of whether other transporters may overlap, in terms of substrate specificities, with P-gp.

Related transporters in the kidney

Two remarkably effective secretory transport systems have long been recognized in the kidney: one for organic anions and another for organic cations (Pritchard & Miller 1996). Since P-gp is a pleiotropic efflux pump localized to the secretory epithelium of the kidney, it was natural to question if P-gp and the 'organic cation transporter' might be the same entity. A

variety of evidence proves that there are *at least* two separate proteins, one of which is P-gp, but it is not always possible to separate which is acting on a given substrate.

The “classical” organic cation transport facility is characterized by potential-driven facilitated diffusion at the basolateral membrane, luminal organic cation/proton exchange, and, probably, intracellular sequestration (Pritchard & Miller 1996). Typical substrates are quaternary amines such as tetraethyl ammonium (TEA) and N¹-methylnicotinamide (NMN), although positively charged primary, secondary or tertiary amines are also reported to be substrates (Sica & Schoolwerth 1996). Cimetidine and quinine are considered relatively specific inhibitors (Dutt *et al.* 1992).

The most blatant proof that P-gp is not the classical organic cation transporter is the recent cloning of a number of organic cation transporters from kidney, unrelated to P-gp, such as OCT1 (Gründemann *et al.* 1994) and OCT2 (Gründemann *et al.* 1997). Dutt *et al.* (1992) demonstrated that neonate development of cimetidine-sensitive uptake of NMN and TEA did not correlate with the timing of expression of P-gp. The pharmacological observations indicate different but overlapping substrate specificities. For example, vinblastine (yet not actinomycin D or colchicine) reduces TEA uptake in proximal tubular brush border membrane vesicles (McKinney & Hosford 1993), and TEA secretion in flounder renal proximal tubule primary monolayer cultures is inhibited by vinblastine (Sussman-Turner & Renfro 1995). At the same time, TEA and tetramethyl ammonium have no effect on apical to basal transport of [³H]-vinblastine in MDCK cells (Horio *et al.* 1989) and TEA and NMN have no effect on *in vivo* colchicine clearance in rats (Speeg *et al.* 1992). Cimetidine, an inhibitor of organic cation transport, acts like a P-gp substrate in that P-gp enriched vesicles display enhanced cimetidine (but not TEA) transport (Dutt *et al.* 1994), P-gp transfected MDCK cells have increased capacity to transport cimetidine from basal to apical medium (Pan *et al.* 1994), and its secretion by LLC-PK1 cells is reduced by verapamil and nifedipine (Dudley & Brown 1996).

The Insect/P-glycoprotein connection

The hypothesis that alkaloid transport in the tobacco hornworm's Malpighian tubule is mediated by a P-glycoprotein-like mechanism has as part of its foundation the assumption that P-glycoprotein is present in this animal. Its conservation throughout the biological world argues strongly for its presence in *Manduca sexta*, but there is much more than this to suggest a P-glycoprotein/insect connection.

DROSOPHILA

As already mentioned, P-glycoprotein has been cloned from the fruit fly *Drosophila melanogaster* (Wu *et al.* 1991, Gerrard *et al.* 1993). In fact, three homologues have been identified: *Mdr49*, *Mdr65* and *Mdr50*, named for their chromosomal positions (unlike the mammalian P-gps, they are found on two separate chromosomes).

Aside from the evidence implicating the *Mdr49* gene in colchicine resistance (Wu *et al.* 1991, see 'Role for P-gp in protection from xenobiotics', pg 21), it is not known what function the *Drosophila* gene products may have. Although there is approximately 40% amino acid identity between *Drosophila* and human P-gps, this is too divergent to postulate a function based on sequence. When *Mdr65* or *Mdr49* was functionally expressed in Sf9 cells (an insect cell line) ATP channel activity was detected using patch-clamp techniques (Bosch *et al.* 1996). This activity was also reported by the same laboratory for murine P-gp and for the cystic fibrosis transmembrane conductance regulator (CFTR, another ABC protein), but has also been disputed (Gottesman *et al.* 1996, Reddy *et al.* 1996).

TOBACCO BUDWORM (*HELIOTHIS*)

Another tobacco-feeding Lepidopteran, *Heliothis virescens* or the tobacco budworm, has developed resistance to nearly every class of pesticides. Since the phenomenon of 'cross-resistance' in this species is conceptually similar to multidrug resistance, Lanning *et al.*

(1996a,b) examined pesticide resistant and sensitive strains of the tobacco budworm for MDR-like features.

They found that treating resistant larvae with the P-gp inhibitor quinidine increased the sensitivity of the larvae to topically applied thiodicarb; the LD₅₀ was reduced by a factor of 12.5. The increase in mortality corresponded to increased thiodicarb accumulation in the larvae when treated with quinidine. Resistant larvae also tolerate a 4-fold greater concentration of the P-gp substrate vinblastine in their diet and this resistance is reduced by treatment with quinine or quinidine.

Antibodies to P-gp were also used to correlate resistance with the presence of P-gp immunoreactivity. Immunoblots with 3 different antibodies showed that the total level of P-gp was at least 2–6 times higher in resistant as compared to susceptible larvae (Lanning *et al.* 1996a,b). Immunoblots using the C494 antibody demonstrated that, when larvae were dissected into three major parts, P-gp was most abundant (per total protein) in cuticle, then in fat body, with relatively little in the midgut. The amount of P-gp expression was found to increase with age of resistant larvae; sensitive larvae had minimal expression at all stages.

MANDUCA

Immunostaining

Using various antibodies raised against mammalian P-gps, the Malpighian tubules, central nervous system and midgut of *Manduca* larvae have all been shown to possess P-gp like proteins.

The Malpighian tubules are immunopositive to the C219 antibody, a monoclonal directed against a conserved cytoplasmic epitope, although the exact location of staining is not clear (Murray *et al.* 1994, Murray 1996). Preincubation of the primary serum with the C219 epitope abolishes the specific staining. In the tubules of *Rhodnius prolixus*, C219 immunostaining is more strongly associated with the apical face of the tissue (Murray 1996).

At the larval blood-brain barrier, P-gp immunostaining, using both C219 and a polyclonal antibody, mdr1(Ab-1), dramatically colocalizes with a barrier to nicotine revealed by autoradiography (Murray *et al.* 1994). Intense P-gp immunoreactivity is seen in the cortical cells, but none in the neuropile or in the axon-bearing centre of the connectives. Similar staining is seen in pupal and adult CNS, although there seems to be some disassembly of the barrier region in the pupa, and a much thinner layer of staining in the adult. The cockroach, *Periplaneta americana*, ganglion also shows a thin layer of P-gp immunoreactivity (Murray 1996). Since the CNS of pupal and adult *Manduca*, and adult *Periplaneta*, are more susceptible to applied nicotine than larval *Manduca*, there appears to be good correspondence between the functional barrier and the distribution of P-glycoprotein (Murray 1996).

Preliminary Western blot analysis of both Malpighian tubules and CNS from *Manduca* indicate that C219 reacts with a protein band of approximately 140 kDa, consistent with the size of P-gp in other organisms (Murray 1996).

Genetics

Attempts to clone a P-gp homolog from a *Manduca sexta* Malpighian tubule cDNA library in our laboratory have so far been unfruitful (McIntosh 1997). Several probes derived from hamster, *Drosophila* and other species, as well as expression screening with the C219 antibody (T. Ginsberg, *pers. com.*), were tried. Some preliminary work on the P-glycoprotein gene by M. Ell and G. Drouin (University of Ottawa) is described by McIntosh (1997). A high stringency Southern blot of *Manduca* genomic DNA with a hamster and *Drosophila* probes revealed one and several bands respectively. Polymerase chain reaction (PCR) experiments with degenerate oligonucleotide primers and genomic *Manduca* DNA yielded a 431-bp fragment with high sequence similarity to a region in the nucleotide binding domain of other P-glycoprotein genes, and also contains a 66 bp intron. However, use of this fragment as a probe for the tubule cDNA library was also unsuccessful in finding a longer P-gp cDNA (McIntosh 1997).

P-GP AND ALKALOID TRANSPORT

The insect alkaloid transporter revisited

As previously mentioned, the Malpighian tubules of *M. sexta* are capable of transporting nicotine at a high rate (Maddrell & Gardiner 1976, Murray 1996) and this transport is inhibitable by excess atropine (Gaertner *et al.* 1998). The alkaloids nicotine, atropine and morphine are *all* transported in a mutually inhibitable fashion in some insects, particularly *Rhodnius* (Maddrell & Gardiner 1976). Thus it has been postulated that a multi-alkaloid pump is a feature common to many insect Malpighian tubules.

There is also some evidence for a multi-alkaloid pump in the blood-brain barrier of the tobacco hornworm. It has been argued that, since the neuropile is protected from nicotine and detoxification does not account for all of this action, a nicotine pump is required as part of the blood-brain barrier machinery (Murray *et al.* 1994). In light of the interactions between atropine and nicotine in Malpighian tubules, it is interesting to note that both atropine and nicotine seem to affect the same mechanism of nicotine (and/or nicotine metabolite) efflux in *Manduca*'s ventral nerve cord (Morris 1983b), although the efflux of atropine itself follows a different pattern from that of nicotine/metabolite (Morris 1983c). The putative nicotine pump at the blood-brain barrier appears to trap nicotine (and its metabolites) in the periphery, thus sequestering it away from the sensitive inner regions. This is corroborated by the observation that DNP, a metabolic inhibitor, and NMN, a tertiary amine like nicotine, both increase nicotine efflux from the tissue (Morris 1983b).

Alkaloids as P-gp substrates

Although none are "classical" P-gp substrates there are a number of independent lines of evidence to suggest that the alkaloids nicotine, atropine and morphine interact with, and may be substrates of, P-gp.

The structural characteristics of P-gp substrates, such as they exist, are all met; namely, these compounds are heterocyclic and relatively hydrophobic, and possess a tertiary nitrogen that

can become cationic at low or moderate pH. Nicotine, being a relatively small molecule (MW 162), is somewhat anomalous as a P-gp substrate, although the narcotic meperidine (MW 247), also has only a single aromatic ring and basic nitrogen atom. Meperidine inhibits extrusion of vinblastine in drug-resistant cells, and displaces specific binding of vinblastine and the photoaffinity labelling of P-gp, which are all characteristics of P-gp substrates, as described below (Callaghan & Riordan 1993).

One of the simplest and most commonly applied criteria for identifying P-gp substrates, namely that P-gp expressing cells be less sensitive to their toxic effects, can only be marginally met by alkaloids that act on plasma membrane receptors (such as those under consideration) due to their low toxicity in typically-used cell lines. However, atropine does enhance by more than 5-fold the cytotoxicity of vinca alkaloids in a multidrug-resistant human leukemic cell line (Zamora *et al.* 1988).

Another method of identifying substrates is to measure the binding of drugs to plasma membranes and their penetration into the cell, since drug exclusion from the cell is a hallmark of MDR. Morphine was studied this way and was found to bind specifically and saturably to CHO-B30 plasma membranes in a verapamil- and vinblastine-sensitive fashion, although it does not displace the binding of vinblastine to B30 membranes (Callaghan & Riordan 1993). Morphine accumulates 3-fold less in multidrug resistant CHO-B30 cells than in controls and such accumulation increases in the presence of $\geq 15 \mu\text{M}$ verapamil (Callaghan & Riordan 1993).

Both atropine and nicotine were assayed for their effect on the ATPase activity and [^3H]-colchicine uptake of plasma membrane vesicles from MDR cells (H. McDiarmid & F.J. Sharom, unpublished results). P-glycoprotein ATPase was unaffected by atropine but stimulated by millimolar levels of nicotine. Colchicine transport was effectively blocked by high concentrations (10 mM) of atropine, but nicotine stimulates colchicine uptake at this concentration. Unfortunately, such seemingly contradictory results are not uncommon with

these methods; for example, the same drug may stimulate or inhibit ATPase activity in the hands of different experimenters or at different concentrations (Doige *et al.* 1992, Sharom *et al.* 1993).

Substrates, or at least compounds that interact with P-gp, can also be identified by their ability to interfere with the photolabelling of P-gp (Akiyama *et al.* 1988). Whilst atropine does not inhibit photolabelling by a vinblastine analog (Akiyama *et al.* 1988), morphine weakly inhibits photolabelling of P-gp with iodomyacin (Callaghan & Riordan 1993).

Finally, morphine is listed as a P-gp substrate (albeit a poor one compared to daunomycin or vinblastine for example) by Schinkel *et al.* (1996), based on its transepithelial transport in P-gp-transfected LLC-PK₁ cells.

From such varied results, the strongest conclusion to be made is that alkaloids such as nicotine, atropine and morphine *could* be substrates for *mammalian* P-gp. Even less is known about the substrate profiles of non-mammalian P-glycoproteins, although the results that do exist (*e.g.* accumulation of drugs in aquatic organisms as described on pg 22) suggest that they are similar. The experiments described below and in the next chapters were designed principally to gather further information on P-glycoprotein's possible role in alkaloid transport in the tobacco hornworm.

Experimental approach

As stated in the overview to this chapter, the hypothesis of this work is that alkaloid (particularly nicotine) transport in the Malpighian tubule of *Manduca sexta* is the consequence of a multidrug transport system similar to that mediated by P-glycoprotein in multidrug resistant cancer cells. To test this hypothesis, two complementary approaches were taken, both of which employed archetypal substrates and inhibitors of P-gp.

If previously reported P-gp immunoreactivity represents a molecule which is functioning as P-gp does in other systems, the Malpighian tubule should be capable of transporting known

P-gp substrates. Therefore, experiments were performed to study the movement of [^3H]-vinblastine in an *in vitro* assay which enabled near-simultaneous sampling of bath and luminal concentrations (**Chapter 2**). As vinblastine was found to actively accumulate in the lumen of the tubule, the cellular and tissue-level distribution of daunomycin, rhodamine 123 and other fluorescent P-gp substrates was then examined by confocal microscopy (**Chapter 3**). Unexpectedly, the Malpighian tubule tissue was found to rapidly accumulate dyes in a manner unlike other insect tissues, but no movement into the lumen was evident.

If P-gp is the same entity as the nicotine (alkaloid) pump, one would expect that P-gp substrate transport could be inhibited by both nicotine and by a typical P-gp inhibitor. Therefore the effect of verapamil and nicotine was examined for both vinblastine transport -- where these compounds had a significant effect, when applied from the basal side (**Chapter 2**) and for daunomycin partitioning (**Chapter 3**) -- where they had no observable effect.

As described in **Chapter 4** (Discussion), there is more than one way to interpret the results, which are clearly equivocal with respect to our hypothesis. Ongoing research by others continues to add complexity to our understanding of how P-gp works and the interactions of P-gp "substrates" with other transport systems. It is therefore proposed that new approaches to examining the role of P-glycoprotein in insect xenobiotic resistance will be most worthwhile.

Table 1.1

Compounds identified as substrates or inhibitors of P-glycoprotein, adapted from Bosch & Croop. 1996. Compounds used in this thesis are indicated in **boldface**. *This list is not comprehensive.*

P-glycoprotein “substrates” included in the multidrug resistance phenotype		Compounds which inhibit P-glycoprotein mediated multidrug resistance	
Doxorubicin	Actinomycin D	Verapamil	Cyclosporin A
Daunorubicin (=daunomycin)	Puromycin	Nifedipine/AHC-52	RU-486
	Valinomycin	Diltiazem	SDZ PSC 833
Mitoxantrone	Mithramycin	Niguldipine	FK506
Idarubicin	Gramacidin D	Dihydropyridine analogs	Rapamycin
Vinblastine	Emetine	Thioridazine	Chloroquine
Vincristine	Rhodamine 123	Trifluoperazine	Quinidine
Colchicine	Ethidium bromide	Chlorpromazine	Quinine
Taxol	DiOC2	Benzquinamide	Dipyridamole/BIBW 22
Etoposide	Hoechst 33342	Progesterone	Quinoline/MS-209
Tenoposide	Lipophilic peptides	Tamoxifen	Reserpine/Yohimbine
Cortisol	Hydrophobic acetoxy-	Megestrol acetate	Amiodarone
[^{99m} Tc]Sestamibi	methyl esters	Fluorocyclopropyl-	Acridonecarboxamides
Ether lipids		dibenzosuberane	Valinomycin
		Triazinoaminopiperidine	Tween 80

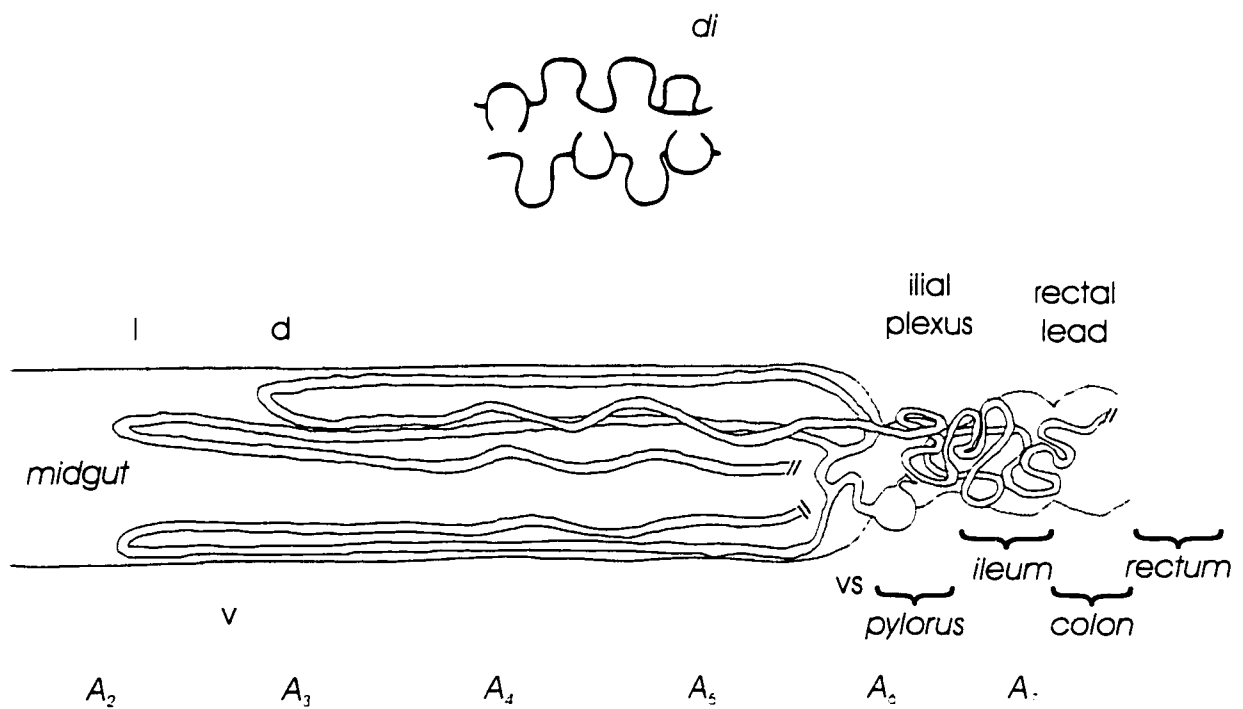


Figure 1.1 Lateral view of excretory system of *Manduca sexta* larva anterior to rectal complex. The distal segment is shown for the dorsal tubule only. (Adapted from Nijhout 1975; see also Eaton 1988). A₂-A₇ positions of external abdominal segments; d, dorsal tubule; di, diverticulum; l, lateral tubule; v, ventral tubule; vs, vesicle.

2. Transepithelial transport of [³H]-vinblastine in isolated Malpighian tubules of the tobacco hornworm (*Manduca sexta*) suggests a P-glycoprotein-like mechanism

Summary

Accumulative transport properties of the Malpighian (excretory) tubules of the tobacco hornworm were examined to test the hypothesis that a P-glycoprotein-like multidrug transporter is active and responsible for excretion of dietary nicotine in this tissue. Isolated tubules were cannulated and exposed to the radiolabelled P-glycoprotein substrate vinblastine in the bathing (basal surface) fluid for 60 min. The luminal (apical) contents were flushed at this time, and lumen-to-bath ratios were measured. These ratios provide conservative estimates of the physiological ability of Malpighian tubules to move compounds from blood to lumen: nevertheless, tubules concentrated vinblastine 3-fold (from 1 μ M). Vectorial transport of vinblastine was eliminated by 25 μ M verapamil (a P-glycoprotein inhibitor), and was not dependent on a transepithelial electrical potential. Nicotine, which is also transported by *Manduca* Malpighian tubules in a verapamil-sensitive manner (Gaertner *et al.* 1998), significantly reduced vinblastine transport at concentrations ≥ 50 μ M. Verapamil was effective at reducing vinblastine transport when applied to the basal side alone, but not when applied to the apical side alone. Together these results are consistent with the idea that the tobacco hornworm's active excretion of nicotine and other alkaloids is mediated by a P-glycoprotein-like mechanism.

Introduction

The insect Malpighian tubule is often compared to the vertebrate kidney, since both perform the role of eliminating wastes from the body fluid. In both systems, non-specific filtration is supplemented by active transport mechanisms. The transporters responsible appear to be few in number but broad in specificity. For insects, one has been characterized as a multi-alkaloid transporter, based on mutually competitive transport of nicotine, atropine and morphine in Malpighian tubules of *Rhodnius prolixus* (Maddrell & Gardiner 1976), a blood-sucking bug (Hemiptera) unlikely to encounter any of these particular compounds in nature. The tobacco hornworm, *Manduca sexta* (Lepidoptera: Sphingidae), which as a caterpillar can live entirely on tobacco, also has the facility to transport nicotine across its Malpighian tubules (Maddrell & Gardiner 1976) and, significantly, to prevent influx of nicotine across its blood-brain barrier (Murray *et al.* 1994). Since both of these alkaloid-handling tissues are also immunopositive for P-glycoprotein (Murray *et al.* 1994), often called the multidrug pump, a P-glycoprotein homologue is a plausible candidate for the insect multi-alkaloid pump.

P-glycoprotein (P-gp) is a membrane protein of the ATP-binding cassette (ABC) transporter family conserved among taxa as diverse as protozoa, plants, insects and mammals (Higgins 1992, Childs & Ling 1996). Despite extensive study since its discovery as a mediator of multidrug resistance in tumour cells, its mode of action remains controversial (Roepe *et al.* 1996). What is clear is that, by direct or indirect means, P-gp expression can protect cells from a variety of unrelated toxic compounds by lowering intracellular concentrations of these drugs. A role in protecting organisms from xenobiotics is supported by a number of different studies. For example, “knock-out” mice genetically deficient in P-gp are phenotypically normal except that they show increased tissue (particularly brain) penetration and decreased elimination of various drugs, leaving them vulnerable compared to their wild-type counterparts. Such drugs include vinblastine and the centrally neurotoxic pesticide ivermectin (Schinkel *et al.* 1994, 1997, van

Asperen *et al.* 1996). Likewise, when a P-gp gene is deleted in *Caenorhabditis elegans*, the nematode becomes sensitive to colchicine and chloroquine (Broeks *et al.* 1995), and *Mdr49*-deleted *Drosophila* show increased sensitivity to colchicine (Wu *et al.* 1991). Other lines of evidence implicating P-gp in protection from environmental toxins include P-gp-inhibitor-sensitive exclusion of multiple P-gp substrates, *e.g.* from gills of mussels (Cornwall *et al.* 1995, Kurelec 1995) and from larvae of a marine worm (Toomey & Epel 1993). In the tobacco budworm, *Heliothis virescens* (Lepidoptera: Noctuidae), western analysis shows increased P-gp expression in cuticle and fat body correlated with increased pesticide resistance (Lanning *et al.* 1996b). In the same insect, quinidine, a P-gp inhibitor, increases cuticular sensitivity to thiodicarb (Lanning *et al.* 1996a).

Evidence for P-gp functioning as part of the excretory system comes from a number of angles. In vertebrates, P-gp is highly expressed at secretory surfaces such as the bile canalicular face of hepatocytes, the intestinal surface epithelium, and the apical surface of kidney proximal tubules (Thiebaut *et al.* 1987). Both intact renal proximal tubules (*e.g.* from killifish; Miller 1995) and renal cell lines (*e.g.* MDCK; Hunter *et al.* 1991b) are capable of active outward transport of P-gp substrates such as daunomycin and vinblastine. A similar role for P-gp in whole-body elimination of xenobiotics in insects is suggested by the finding that Malpighian tubules are P-gp immunopositive (Murray *et al.* 1994).

The Malpighian tubule is amenable to analysis of transepithelial transport since it is relatively easily isolated from the insect and can survive *in vitro* without elaborate oxygenation or temperature regulation (Maddrell & Overton 1990). Furthermore, the two fluid compartments, the bath and the lumen, are well defined and relatively accessible; through stimulation of physiological fluid secretion (in some species) or artificial perfusion (as is necessary for *Manduca*) the luminal contents are readily collected. The basics of the assay, in which an isolated tubule is kept in a droplet of physiological saline under mineral oil, were first described by Ramsay (1958).

Using this isolated tubule assay, nicotine transport was studied by Murray (1996; Gaertner *et al.* 1998). Within 5 min, Malpighian tubules concentrated nicotine 10-fold, from an initial bath concentration of 0.5 mM, confirming the results of Maddrell & Gardiner (1976). Other concentrations (0.05 mM and 5 mM) of nicotine also resulted in lumen-to-bath ratios greater than 1. The accumulation of nicotine in the lumen was significantly inhibited by both verapamil (25 μ M) and atropine (3 mM). These results support the idea of a common 'alkaloid' transporter in *Manduca*, as in *Rhodnius* (Maddrell & Gardiner 1976), and suggest that P-gp may be involved.

However, if active transport of xenobiotics, such as nicotine, depends on P-gp, then the Malpighian tubules should also transport vinblastine, an archetypical P-gp substrate (and alkaloid), and this transport should be sensitive to the standard P-gp inhibitor, verapamil. If nicotine and vinblastine are transported by a common mechanism, excess nicotine should inhibit vinblastine transport. The experiments described below tested these ideas. The results suggest that a P-gp-like mechanism is indeed active in the insect Malpighian tubule, and raise the intriguing possibility that nicotine is one of its substrates. If this is correct, it may have practical implications for such wide-ranging issues as multi-insecticide resistance (Denholm & Rowland 1992), nicotine interference with drug clearance in clinical practice, and nicotine tolerance among tobacco users.

Materials and Methods

ANIMALS

Tobacco hornworm (*Manduca sexta*) eggs were obtained from Carolina Biological Supply Company (Burlington, NC), and raised at room temperature (22–23°C) with photoperiod 16L:8D and ambient humidity. Larvae were raised individually on a nicotine-free artificial diet, based on either wheat germ, particularly for preliminary experiments (modified from Bell &

Joachim 1976), or cornmeal (Carolina Biological Supply). Fifth-instar larvae were used before they entered the wandering stage (determined by appearance of the pulsating dorsal vessel).

SALINE AND CHEMICALS

“*Manduca* saline”, was designed to mimic physiological (high-K) hemolymph and is hereafter referred to as “saline”. It was modified from that of Maddrell and Gardiner (1976) and was used for dissecting, bathing and perfusing the Malpighian tubule tissue, and for final dilution of all applied drugs. Its composition, in mM, was: NaCl (15), KCl (30), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (2), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (30), HEPES (N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid], $\text{C}_8\text{H}_{18}\text{N}_2\text{O}_4\text{S}$) (5), D-glucose (10), maltose (10), sodium citrate (5) and glycine (10). It was adjusted to pH 7.2 with 5 M KOH, filter-sterilized and stored at 4°C.

Vinblastine, as [G- ^3H]-vinblastine sulphate (Amersham; 11.4 Ci/mmol in methanol) was mixed with unlabelled vinblastine sulphate (Sigma-Aldrich, Oakville, ON; made as 17 mM stock in (2:1) ethanol:saline) to make a 2X working solution of vinblastine (2 μM , 0.75 Ci/mmol). The final concentration applied to the tubules was thus 1 μM vinblastine with 0.3% methanol and 0.004% ethanol.

Stock solutions of ($\pm$)verapamil hydrochloride (Sigma) were made in water and diluted 400-fold to their final concentration in saline. Stock solutions (5 or 11 mM) of nicotine hydrogen tartrate (Sigma) were made in saline not more than 5 days prior to use, adjusted to pH 7.2 with 5 M KOH and stored at 4°C. Further dilutions, when necessary, were also made in saline.

Dillapiol, 1-allyl-2,3-dimethoxyl-4,5-(methylenedioxy)-benzene, was purified ($\geq 99\%$ as determined by NMR spectra) as an oil from natural sources by T. Durst (University of Ottawa), and a 100 mM stock was made in ethanol:water (5:4). Even after vortexing, tiny oil droplets were visible, so the stock solution and dilutions in saline were sonicated (Bransonic 12, Branson Cleaning Equipment Co., Shelton, CT) for 1 min immediately prior to use. The final concentration of ethanol was less than 0.06%.

TRANSPORT ASSAY

Larvae were sedated by refrigerating at 4°C, decapitated with scissors, then pinned, dorsal side up, to a dissection tray and covered with ice-cold saline. The dorsal surface was cut lengthwise, exposing four of the six Malpighian tubules. A 3 to 4 cm section of a single Malpighian tubule was carefully dissected free of tracheal connections and removed. The regions of tubule defined as proximal (nearest the entry point to the gut) and medial (Nijhout 1975) or as descending and ascending straight segments respectively (Moffett 1994) were used. Maddrell and Gardiner (1976) showed that either the proximal alone or proximal plus medial regions exhibited nicotine transport. Except for preliminary experiments, where proximal and medial regions may have been used, only the proximal region was used.

In a modification of the method described by Maddrell and Overton (1990), the isolated Malpighian tubule was immersed in a 100 µl bath droplet in a wax-lined, mineral oil-filled petri dish, and cannulated to allow artificial perfusion of the tubule, as shown in **Figure 2.1**.

Cannulae were made by pulling glass, non-heparinized microhematocrit capillary tubes (Fisherbrand, internal diameter 1.1–1.2 mm) with a pipette puller (List-Medical model L/M-3P-A) to a fine tip and then breaking the tip to 30–60 µm outer diameter, and removing jagged edges by fire-polishing close to a red-hot wire. The cannula was bent 20–50° to the horizontal so that it would enter the bath droplet at a convenient angle when mounted on a micromanipulator. The latter held the cannula in a pipette holder, and flexible tubing connected the cannula to a 1 ml syringe driven by a perfusion pump (Harvard Apparatus, South Natick, MA, Pump 22).

One end of the Malpighian tubule segment was pulled onto the cannula with two pairs of fine forceps, and secured with a short piece of waxed dental floss (3–5 individual strands). The other end was pulled out of the bath droplet and pierced with a fine pin to allow fluid to escape. To hold the tissue in place, a fine pin was pushed through the end, into the wax. Because fluid secretion is naturally slow in this segment, the orientation of the tubule was not considered

critical. However, in an effort to improve reproducibility after the preliminary experiments, only the more distal end was cannulated and fluid was collected from the proximal end, mimicking the natural movement of wastes towards the hindgut (Nijhout 1975).

After cannulation, saline (or saline plus drugs) was perfused for several minutes at 2.5 $\mu\text{l}/\text{min}$ through the tubule, refilling it and flushing out the contents. This step was necessary because the proximal tubules became deflated during dissection and natural fluid secretion is very slow (Maddrell & Gardiner 1976). A control sample, for background radioactivity, was taken from the luminal contents at this time. Once it was established that there were no leaks in the tissue, based on throughput at the expected rate of 2.5 $\mu\text{l}/\text{min}$, artificial perfusion was halted, and the radiolabelled drug ($\pm$ inhibitors) was added to the basal (bath) side of the tissue. This was done by replacing half of the bath with drugs at two times the desired final concentration.

In studies of vinblastine transport inhibition, verapamil (25 μM) and dillapiol (50 and 100 μM) were included both in the bath droplet, exposing the basal surface of the tubule cells, and in the perfusate, exposing the apical surface, and thus pre-exposing both surfaces for ~ 20 min. When nicotine (5 to 500 μM) was tested as a potential inhibitor of vinblastine transport, it was added simultaneously with the vinblastine at the basal side only. To further characterize verapamil action on vinblastine transport, experiments were performed in which verapamil was applied to only one surface of the Malpighian tubule. To expose the apical surface, the tubule was cannulated and pre-perfused for 10 min with 25 μM verapamil, flushing through at least 15 μl . During this time 50 μl (50%) of the bath was replaced 3 times with fresh saline, in case some verapamil had entered the bath droplet from the cannula prior to its insertion into the lumen. For basal application, the tubule was also pre-incubated for 10–12 min.

For experiments to determine if a transepithelial electrical potential might be driving the movement of (charged) drugs, a 0.25 mm chlorided silver wire was passed from inside the cannula to the bath (see **Figure 2.1**) in order to short-circuit any electrical potential between the bath and the lumen.

SAMPLING

Once the apical and basal solutions were established as above, the fluids were left undisturbed for the duration of the experiment, then the lumen was flushed and samples collected after an appropriate length of time, which was determined in preliminary experiments to be 60 minutes. Typically, 2.0 μ l samples of the bath were taken 1, 15, 30 and 45 min after addition of the labelled drug. At 60 min, a 5.0 μ l sample of the bath was taken and the perfusion pump restarted (at 2.5 μ l/min) to flush the contents of the lumen. The first of three 5.0 μ l samples collected sequentially from the cut end of the Malpighian tubule was compared to the bath sample to determine the lumen-to-bath ratio.

Samples were dispensed into 3 mL of scintillation cocktail (EcoLite (+)TM, ICN, Costa Mesa, CA) and counted in a liquid scintillation counter (Beckman LS1701). Background counts were negligible compared to all samples and were not subtracted.

It was found that a considerable amount (average ~15% of total activity) of radiolabelled vinblastine adhered to the plastic pipette tips, and was thus not normally counted. However, there was no difference in the lumen-to-bath ratios obtained when this was taken into account.

STATISTICAL TREATMENT

Results are presented as the mean $\pm$ standard error (number of samples). Two-tailed Student's *t* tests at the 5% level of significance were performed to test for differences in means.

Results

PRELIMINARY EXPERIMENTS

Experiments to test whether vinblastine might be transported through the Malpighian tubule epithelium, as nicotine is, were first attempted by Murray (1996) using 100 μ M bath vinblastine. This concentration was thus chosen for my preliminary experiments. Lumen-to-bath ratios for these experiments were variable, but generally less than one. During the course of these

experiments, various experimental details were changed and the protocol described in materials and methods (for example sampling the lumen after 60 min and cannulating the proximal end consistently) was the final result. Suspecting that 100 μM vinblastine may be somewhat toxic to the tissue, the amount of unlabelled vinblastine was reduced such that 1 μM was applied to the tissue. With this change, the *lowest* lumen-to-bath ratio observed was 2.0; prior to that (with 100 μM) the *highest* ratio attained was 1.7 (results not shown). This suggests that vinblastine at higher concentrations does indeed interfere with transport processes, a theory understandable in light of the drug's ability to disrupt microtubules (Dhamodharan *et al.* 1995).

VINBLASTINE ACCUMULATES IN THE LUMEN OF MALPIGHIAN TUBULES

While the accumulation of nicotine in the lumen of Malpighian tubules was readily evident after only five min, preliminary trials showed that vinblastine (MW = 811), accumulated much more slowly than nicotine (MW = 162). This necessitated a change in the experimental protocol: instead of perfusing and collecting the perfusate 5 min after adding the radiolabelled alkaloid, collection was done at 60 min. For vinblastine, therefore, our principle measure of uptake into the lumen was the ratio of lumen radiolabel in the first 5 μl sample collected at 60 min to a 5 μl sample of the bath taken at the same time. The mean ratio (lumen-to-bath) was 3.0 ± 0.2 (n=24) (**Figure 2.2**). The mean concentration of vinblastine in the first 5 μl collected from the lumen was $1.3 \pm 0.1 \mu\text{M}$ (**Figure 2.3**). **Figure 2.3** also illustrates that the first 5 μl sample collected most, but not all, of the luminal contents, since the next two 5 μl samples pushed through each contained some activity. The fraction of the total luminal activity collected in the first 5 μl sample would have depended on the size and, presumably, on the morphology of the tubule.

The bath vinblastine levels, monitored by sampling 2 μl of the bathing droplet at 15 min intervals during the experiment, were variable for any given preparation, but from the whole set of tubules (n=24) it became evident that, over the course of the 60 min, the bath droplet concentration fell significantly ($32 \pm 2 \%$) (**Figure 2.3**).

VERAPAMIL INHIBITS VINBLASTINE ACCUMULATION WHEN APPLIED TO THE BASAL SURFACE

When 25 μ M verapamil was applied to both surfaces of the Malpighian tubule, the accumulative transport of vinblastine was effectively inhibited (**Figure 2.2**). The mean lumen-to-bath ratio of [3 H]-vinblastine under these conditions (a pre-incubation period of $\sim$ 20 min for both apical and basal surfaces) was 1.0 ± 0.1 ($n=10$). When verapamil was applied only to the basal (bath) side of the tubule (with a pre-incubation period of $\sim$ 10 min), a similar result (0.9 ± 0.1 , $n=5$) was obtained. In contrast, when verapamil was applied to the apical (lumen) side only, by perfusing it into the lumen (with pre-exposure of $\sim$ 10 min) the resulting vinblastine transport (lumen-to-bath ratio 2.5 ± 0.3 , $n=9$) was not significantly different from that without any verapamil (**Figure 2.2**).

Thus verapamil inhibited accumulative vinblastine transport 3-fold when applied from the hemolymph side, but was ineffective when applied at the same concentration from the luminal side.

NICOTINE INHIBITS THE ACCUMULATION OF VINBLASTINE

At concentrations greater than or equal to 50 μ M, nicotine (applied basally as described in Methods) significantly reduced the lumen-to-bath ratio of [3 H]-vinblastine (**Figure 2.2**). Nicotine at 5 μ M, however, had no effect on [3 H]-vinblastine accumulation. Evidently the effect of nicotine was already saturated at 50 μ M, since there was no significant difference in the inhibition caused by 50, 160 or 500 μ M nicotine.

Verapamil was a more effective inhibitor of vinblastine transport than was nicotine; the level of inhibition achieved by 25 μ M verapamil exceeded that achieved by nicotine at any concentration.

DILLAPIOL HAS NO EFFECT ON THE ACCUMULATION OF VINBLASTINE

Dillapiol is a natural product of Indian dill (*Anethum sowa*) and other plants, which has been shown to have synergistic action for pyrethrum and carbamate pesticides (Tomar *et al.* 1979), not unlike piperonyl butoxide. Since a possible mode of synergism could be interference with

excretion of these xenobiotics we postulated that dillapiol might interfere with vinblastine transport. When the [^3H]-vinblastine assay was performed in the presence of 50 or 100 μM dillapiol (on both sides of the tissue), the mean lumen-to-bath ratios were 2.1 ± 0.05 ($n=3$) and 2.1 ± 0.3 ($n=3$), respectively, not significantly different from the control (**Figure 2.2**).

TRANSPORT OF VINBLASTINE IS NOT THE RESULT OF A TRANSEPITHELIAL POTENTIAL

Because vinblastine in aqueous solution is predominantly cationic at the pH used in this study, it is possible that the observed accumulation was due to an electrical potential (negative inside) set up across the epithelium. To address this issue, experiments were performed in which a chlorided silver wire was passed from inside the cannula to the bath (see **Figure 2.1**) in order to short-circuit any electrical potential between the bath and the lumen. Accumulative vinblastine transport was observed with a shorting wire in place (**Figure 2.2**); the mean lumen-to-bath ratio was 2.2 ± 0.1 ($n=3$), not significantly different from experiments without the short-circuit. From this small sample, one can not claim that there is *no* effect of transepithelial potential, only that accumulative uptake persists in the presence of a short circuit.

Discussion

Recent results from our laboratory using isolated and cannulated Malpighian tubules from the tobacco hornworm confirmed and expanded Maddrell and Gardiner's (1976) finding that "blood" nicotine rapidly accumulates in the Malpighian tubule lumen (Gaertner *et al.* 1998). The assay was simplified from Maddrell and Gardiner's (1976) in that the tissue was not continuously perfused but flushed out only once, after an appropriate incubation period. Unlike nicotine, which is rapidly excreted, vinblastine required more time, approximately 60 minutes. Differences in lipid solubility, in absolute substrate concentration and in molecular weight may all be factors, although this is a matter of conjecture. It is also possible that the medial-plus-proximal region used for nicotine experiments was more effective than the proximal-only region

used for vinblastine experiments, though there is nothing to suggest this from Maddrell and Gardiner's (1976) earlier work.

The demonstration that nicotine transport can be inhibited by atropine in *Manduca* (Gaertner *et al.* 1998) extends the generality of the observation previously seen only in *Rhodnius* (Maddrell & Gardiner 1976). The fact that vinblastine transport was inhibited by nicotine suggests that these two drugs may also be transported by a common mechanism. The verapamil sensitivity of both nicotine (Gaertner *et al.* 1998) and vinblastine (present results) accumulation into the lumen also suggests that they may have a common transporter, with P-glycoprotein being a likely candidate.

Both nicotine and vinblastine, with pK_a 's of 7.9 and 7.4 respectively, would be predominantly (>60%) cationic in the pH 7.2 solutions used in this study. Although Malpighian tubules usually maintain the lumen positive with respect to the basal side (O'Donnell *et al.* 1996), it is conceivable that an unusual transepithelial potential (negative inside) non-selectively drove the alkaloid accumulation we observed. This conjecture can be ruled out, however, since placement of a wire connecting bath and lumen, to dissipate any transepithelial potential (**Figure 2.1**), did not prevent concentrative uptake into the lumen (**Figure 2.2, short-out**). Non-selective accumulation could also occur if drugs passively penetrated the epithelium in their lipophilic neutral forms but were protonated and hence trapped once in the lumen. While this could be a factor *in vivo* for the proximal region where luminal pH is evidently <6.8 in *Manduca*, it would not apply for the medial region where pH is reported to be >8.4 (Moffett 1994). Moreover, in our setup, both bath and lumen fluids were replaced with HEPES-buffered saline of pH 7.2; the solutions used by Maddrell and Gardiner (1976) were also well-buffered. Finally, there is no ready explanation for how 'pH-trapping', as described above, would be abolished by verapamil, atropine and nicotine, drugs shown here and by Maddrell and Gardiner (1976) to inhibit vinblastine and/or nicotine transport.

Our aim was to determine whether uphill transport of xenobiotic substrates was occurring. To meet this aim we looked for accumulation against a gradient, rather than measuring the rate of appearance of substrate in the lumen (estimated by continuously sampling tubule perfusate, *e.g.* Maddrell & Gardiner 1976). We chose not to measure the rate of transport for two reasons. First, unidirectional transport rates alone do not establish whether transport is energetically uphill, and indeed, by maintaining a favourable concentration gradient can emphasize the downhill components of epithelial transport (an unknown fraction of which may be via transporters). Second, while instantaneous rates are useful for within-tubule comparisons of pharmacological agents, they are most meaningful when transport is from an infinite pool. In our setup, the substrate pool (bath droplet) became appreciably depleted of substrate during the course of the experiment. While a single bath aliquot allowed us to compensate for this depletion in ratio experiments, multiple aliquots would be needed for rate experiments, further exacerbating the depletion.

Though accumulative transport was unequivocally established for vinblastine (as for nicotine, Gaertner *et al.* 1998), the lumen-to-bath ratio of 3.0 probably underemphasizes the physiological ability of tubules to move these xenobiotics from blood to lumen. An obvious factor leading to underestimates of accumulation is that part of the tubule was in oil and so was not exposed to tracer (see **Figure 2.1**), yet the entire luminal contents were sampled when the lumen was flushed out. A second factor is that unstirred layers at both apical and basal surfaces (O'Donnell *et al.* 1982) create unfavourable local concentration gradients against which the transport system must work. These effects are presumably peculiar to the *in vitro* model since *in vivo*, continual body movements should keep unstirred layers to a minimum. Illustrating this point directly is the observation that simply by a gentle agitation of the tissue, nicotine uptake ratios could be increased from 4 to 12, a 3-fold improvement (Gaertner *et al.* 1998). Additionally, with vinblastine, I found that a substantial fraction of lumen radioactivity was only flushed out in second and third sequential samples (**Figure 2.3**). The difficult-to-remove tracer

may in part represent tracer “trapped” between the microvilli and among the folds and pockets of the diverticula. If counts from second and third lumen samples are added to the first, the ratio at 60 minutes becomes 5.5 instead of 3.0.

The verapamil-sensitive accumulative transport of vinblastine shown here is currently the best evidence for the operation of a P-gp-like xenobiotic efflux mechanism in the tobacco hornworm Malpighian tubule. Vinblastine, an alkaloid from *Catharanthus roseus*, is important in cancer chemotherapy (Rowinsky & Donehower 1991) and a well-characterised P-gp substrate. It is vectorially transported across P-gp-expressing epithelia, including those of renal cells (Horio *et al.* 1990), it stimulates the ATPase activity of recombinant P-gp (*e.g.* Dong *et al.* 1996) and it interacts directly with P-gp in photoaffinity labelling studies (Akiyama *et al.* 1988, Bruggemann *et al.* 1992). Verapamil, a widely studied inhibitor of P-gp activity, interferes with drug transport independent of its action on calcium channels (Huet & Robert 1988, Tsuruo 1991, Bosch & Croop 1996). Photoaffinity labelling suggests that, like vinblastine, verapamil interacts directly with P-gp, perhaps competing for binding sites (Safa 1988). The relevance of any of these interactions is, however, disputed, due largely to disagreements on how P-gp actually operates (see **Chapter 1** for details).

Our discovery that verapamil’s effect was from the basal side was somewhat unexpected, because in polarized epithelia, such as the mammalian kidney proximal tubule, P-gp is predominantly located on the apical surface (Thiebaut *et al.* 1987). In studies of transepithelial vinblastine transport, verapamil has typically been applied to both sides simultaneously (*e.g.* Horio *et al.* 1990), but Hunter *et al.* (1991b), using intestinal adenocarcinoma cell layers, noted that at high concentrations (200 μ M), verapamil inhibition of vectorial transport of vinblastine was similar with verapamil on either side. The same authors subsequently reported, however, that verapamil’s effectiveness as a chemosensitizer (inhibitor) fell as access to the basolateral surface decreased (Hunter *et al.* 1993). Consistent with our finding, this suggests that the site of verapamil inhibition is more accessible from the basal side. P-gp immunostaining of Malpighian

tubules of *Manduca* (Murray *et al.* 1994) and *Rhodnius* (Murray 1996) was not limited to apical regions. In some human tumour cells P-gp immunolocalizes to intracellular sites (Broxterman *et al.* 1989, Molinari *et al.* 1994, Baldini *et al.* 1995), a point of particular interest, since alkaloid efflux studies at the insect blood-brain interface suggested temporary sequestration at intracellular sites (Morris 1983b,c). A remote possibility for the complete lack of effect of apically applied verapamil is that the preincubation period is critical; application of verapamil through the cannula may not be as thorough as replacement of bath medium on the basal side.

In insects, P-gp might be responsible for the transport of alkaloids present in the diet. P-gp occurs in all major taxa (Childs & Ling 1996) and appears to protect many organisms from xenobiotics (*e.g.* Kurelec 1992, Broeks *et al.* 1995). Plants and phytophagous insects have an evolutionary history of “chemical warfare” and any “multidrug pump” should be welcome in the insect arsenal. In *Rhodnius* Malpighian tubules, the alkaloids nicotine, atropine and morphine seem to share a common transport mechanism (Maddrell & Gardiner 1976). By virtue of their chemical structure, all three are candidate substrates for P-gp, *i.e.* they are heterocyclic and relatively hydrophobic, and possess a tertiary nitrogen cationic group. Atropine enhances the cytotoxicity of vinca alkaloids in a multidrug-resistant human leukemic cell line (Zamora *et al.* 1988), though it does not inhibit photolabelling of P-gp by a vinblastine analog (Akiyama *et al.* 1988). Morphine accumulates 3-fold less in multidrug resistant CHO-B30 cells than in controls, and binds specifically and saturably to CHO-B30 plasma membranes in a verapamil- and vinblastine-sensitive fashion (Callaghan & Riordan 1993). In *Manduca*, the blood-brain barrier to nicotine co-localizes with P-gp immunostaining (Murray *et al.* 1994). At a biochemical level, nicotine/P-gp interaction is suggested by the observation that nicotine stimulated the ATPase activity of plasma membrane vesicles from the multidrug resistant Chinese hamster ovary cell line CH^RB30 (H. McDiarmid & F.J. Sharom, *pers. com.*).

We now add to the weight of these suggestions that P-gp is the insect alkaloid transporter the pharmacological findings that nicotine transport is inhibited by verapamil and atropine (Gaertner

et al. 1998) and that vinblastine transport is inhibited by verapamil and nicotine. This is consistent with P-gp activity being directly or indirectly responsible for the luminal accumulation of nicotine and atropine. At this point, however, we can not entirely rule out several alternatives. In vertebrate kidney, xenobiotics are also excreted by an organic cation transport system whose model substrates are quaternary ammonium compounds (Pritchard & Miller 1996). While the “classic” cation and P-gp transport pathways involve distinct proteins (Dutt *et al.* 1992, Gründemann *et al.* 1994), their substrates are chemically similar and some appear to interact with both; for example, vinblastine reduces tetraethylammonium uptake into renal brush border membrane vesicles (McKinney & Hosford 1993, Dutt *et al.* 1994). Uncertainties about mechanisms of drug efflux in whole tissue are likely to persist until there is more agreement about mechanisms in cellular models. Although P-gp is described as “the multidrug transporter” (Gottesman & Pastan 1988), there is no consensus on how it acts. Moreover, for charged and pH-sensitive drugs there is evidence that the multidrug-resistance phenotype can be brought about, with or without P-gp, by altered electrical or pH gradients across plasma and intracellular membranes (Simon & Schindler 1994, Roepe *et al.* 1996).

If nicotine is indeed transported by P-gp in the insect Malpighian tubule, as the present and recent results suggest, it raises the intriguing possibility that P-gp could also affect the distribution of nicotine in humans, via its activity both at the blood-brain barrier (Schinkel *et al.* 1994) and in the kidney (Pritchard & Miller 1996). This could have implications for nicotine addiction since delivery of the drug to the central nervous system and its rate of elimination from the body are important factors in determining self-administration, tolerance and physical dependence (Busto *et al.* 1989).

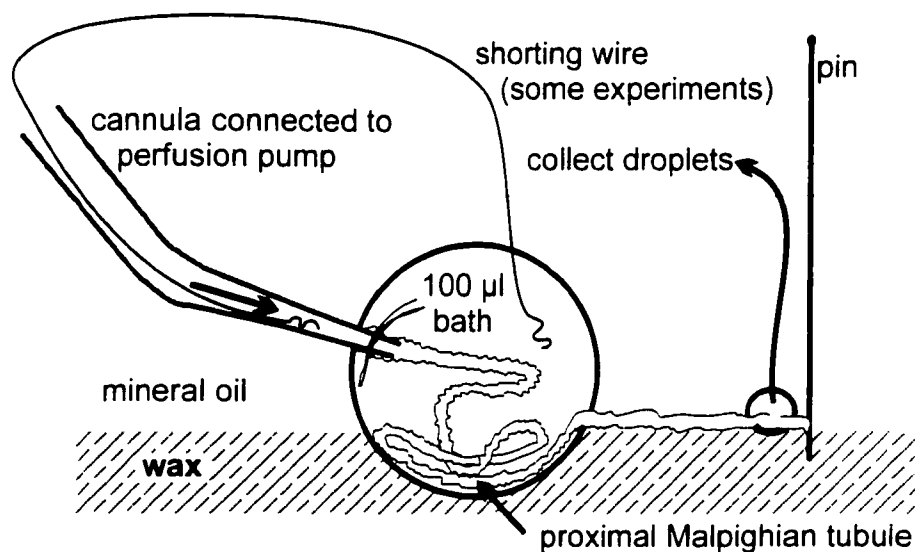


Figure 2.1 Schematic illustrating the procedure for measuring the transport of vinblastine or nicotine across the Malpighian tubule epithelium. A 3–4 cm piece of the proximal Malpighian tubule was cannulated and incubated in a 100 µl bath droplet containing radiolabelled drug. Cannulation enabled the rapid collection of luminal contents from a cut in the tubule and also allowed for drugs to be selectively applied to one or both of the apical (luminal) or basal (bath) side of the tissue. For some experiments a chlorided silver shorting wire was inserted, as shown, to dissipate any transepithelial electrical potential.

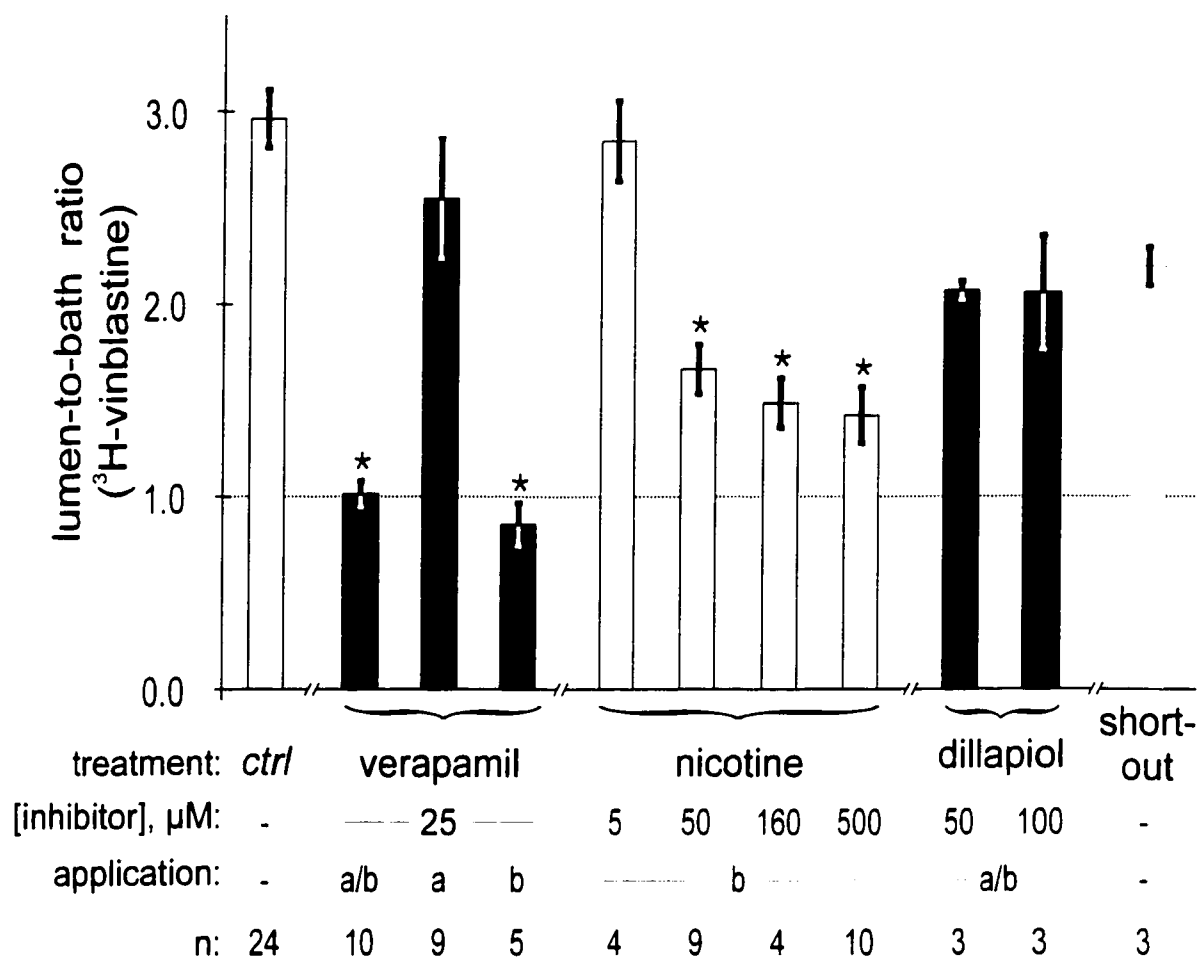


Figure 2.2 Vinblastine is actively transported across the Malpighian tubule. This transport is sensitive to verapamil, and to nicotine at concentrations $\geq 50 \mu\text{M}$ but is not significantly affected by dillapiol at 50 or 100 μM , nor is it the result of a transepithelial potential (short-out tubules not significantly different from control). The initial concentration of vinblastine in the bath was 1 μM . The dotted line indicates a lumen-to-bath ratio of 1, which would be expected for equilibration of the two compartments. *, significantly different from control (*ctrl*); **a**, apical application of inhibitor; **b**, basal application of inhibitor; **a/b**, inhibitor added to both sides of tissue, as described in Methods

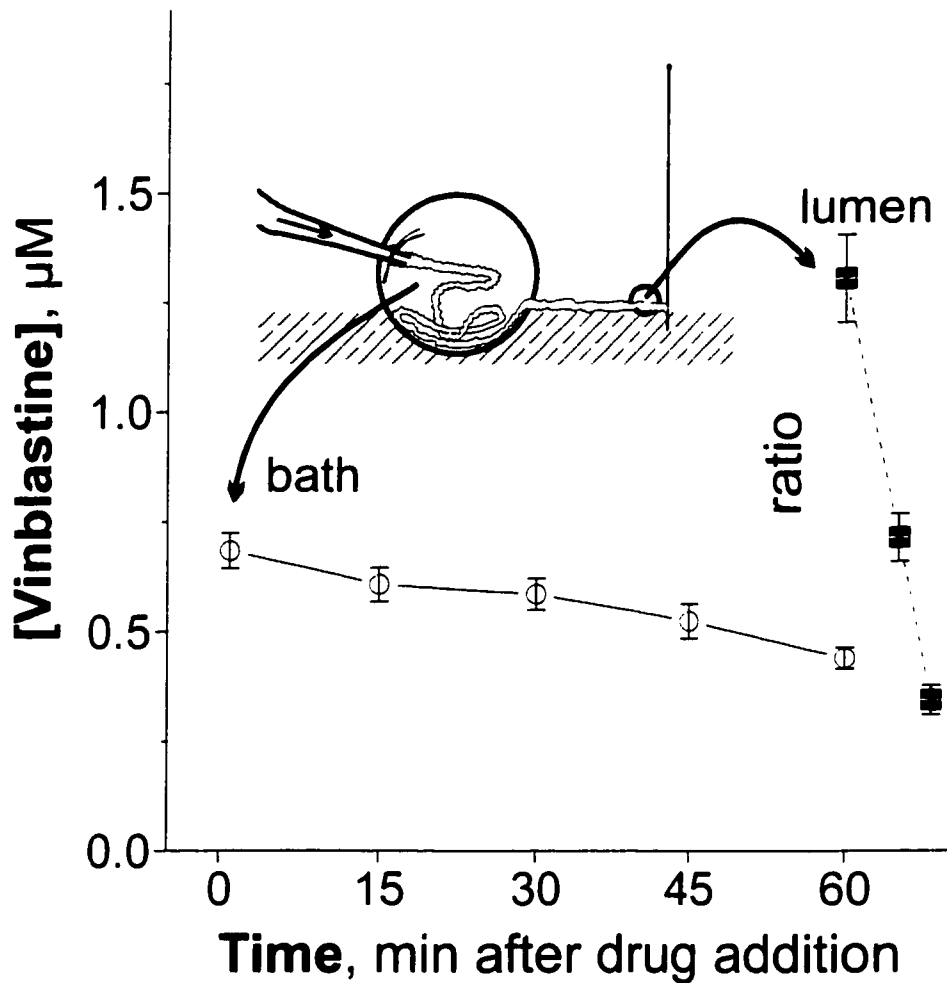


Figure 2.3 Vinblastine concentrations in bath (open circles) and lumen (black squares) samples, showing that vinblastine moves from the bath into the lumen over the course of an hour (n=24). As shown (**ratio**) the first 5.0 μ l sample flushed from the lumen at 60 min was compared with the 5.0 μ l bath sample at 60 min to give the values shown in **Figure 2.2**. This illustrates that the first 5.0 μ l collects appreciably less than the total luminal radioactivity.

3. Accumulation of daunomycin and fluorescent dyes by drug-transporting Malpighian tubule cells of the tobacco hornworm, *Manduca sexta*

Summary

Insect Malpighian tubules actively excrete a variety of xenobiotics and it has been proposed that P-glycoprotein, or the multidrug transporter, may be involved. In order to characterize transport properties of the Malpighian tubule, and to test the hypothesis that P-glycoprotein is involved, the interaction of known P-glycoprotein substrates with Malpighian tubules from tobacco hornworm (*Manduca sexta*) larvae was examined. The fluorescent compounds daunomycin, rhodamine 123, acridine orange and others were applied to the basal side of live tubules (proximal portion) mounted on coverslips and their subsequent distribution was monitored using laser scanning confocal microscopy. Contrary to expectation, none of the compounds appeared in the lumen even after 1–2 hours of incubation, although the cells of the tubule were intensely stained by the fluorescent compounds within 1 minute. Daunomycin is a P-glycoprotein substrate but neither verapamil, a P-glycoprotein inhibitor, nor nicotine, known to be transported by these tubules, altered the pattern of daunomycin staining. In sharp contrast to the fast and intense Malpighian tubule staining, muscle, nervous and other tissues showed only light staining with daunomycin, and only after prolonged periods. These results suggest that the Malpighian tubule acts as a xenobiotic scavenger but do not point to involvement of P-glycoprotein in this process.

Introduction

The insect Malpighian tubule is analogous to the vertebrate kidney in that its function is to remove unwanted chemicals, both endogenous and exogenous, from the body fluid. In the Malpighian tubule, filtration of the body fluid (hemolymph) and production of the primary urine occurs by a secretory process driven by the active transport of ions into the tubule lumen (Pannabecker 1995). Transport of some compounds occurs passively (Maddrell & Gardiner 1974), but others, including organic acids (Maddrell *et al.* 1974, Bresler *et al.* 1990) and alkaloids (Maddrell & Gardiner 1976) are transported actively. In both the kidney and the Malpighian tubule, the myriad of potential substrates appear to be excreted with the help of a relatively small number of active transporters with broad substrate specificity (Bradley 1985, Ullrich 1994). We have proposed that P-glycoprotein (P-gp) is one such transporter in insects as in vertebrates, and have chosen the tobacco hornworm, *Manduca sexta*, as a model species in which to investigate this possibility (Murray *et al.* 1994, Gaertner *et al.* 1998).

P-gp, originally studied for its role in conferring multi-drug resistance (MDR) on cancer cells (Gottesman & Pastan 1993), appears to have an important role in protecting organisms, from bacteria to mammals, from xenobiotics. Its expression in renal tissue has been taken as evidence for its normal role in whole-body elimination of xenobiotics (Simmons *et al.* 1997). It has been implicated in colchicine resistance in *Drosophila melanogaster* (Wu *et al.* 1991), and with pesticide (thiodicarb) resistance in the tobacco budworm, *Heliothis virescens* (Lanning *et al.* 1996b), where resistance was correlated with decreased cuticular penetration rather than excretion (Lanning *et al.* 1996a). In *Manduca*, P-gp-like immunoreactivity has been localized to the blood-brain barrier and the Malpighian tubules (Murray *et al.* 1994). That P-gp may play a role in insect excretion is further suggested by the finding that vinblastine, an archetypical P-gp substrate, is actively transported by isolated larval *Manduca* Malpighian tubules (**Chapter 2**).

Furthermore, this active transport is blocked by basal application of verapamil, a P-gp inhibitor and by nicotine (**Chapter 2**), a substrate of the alkaloid transporter (Maddrell & Gardiner 1976).

In order to further explore the potential role of P-gp in Malpighian tubule physiology, the interaction of this tissue with fluorescent P-gp substrates was examined by laser scanning confocal microscopy. By this means, subcellular distribution could be followed in real time in living, isolated proximal tubule. Daunomycin was the principal substrate examined, as it is an anti-cancer drug whose P-gp mediated transport (Sardini *et al.* 1994) and altered cellular distribution (Weaver *et al.* 1991) in cell cultures is frequently studied by conventional or confocal fluorescence microscopy. Also, in intact killifish renal proximal tubules, daunomycin accumulates in the cells and the tubular lumen, with steady state luminal fluorescence two to three times greater than cellular fluorescence; movement into the lumen, but not the cell, is inhibited by verapamil (Miller 1995). To ascertain if the Malpighian tubule behaves similarly, daunomycin was added to the basal (blood) side of isolated tubules from *Manduca* larvae. The effect of verapamil and, because of its relevance to this species, of nicotine, was tested. In typical MDR studies comparisons are often made between paired resistant and sensitive (parental) cell lines; in this study comparisons were made with tissues presumably not specialized for excretory transport, derived from the same insect.

The fluorescent dyes rhodamine 123, acridine orange and Hoechst 33342, all of which stain MDR cells poorly in comparison to sensitive parental cells, and thus behave like P-gp substrates (Neyfakh 1988, Canitrot *et al.* 1996), were also applied to Malpighian tubules. We hypothesized that these dyes and daunomycin would be transported through the epithelium and accumulate in the lumen of the tubule, as found with nicotine and vinblastine in this tissue and daunomycin in the killifish. Contrary to expectation, however, while all of the drugs accumulated rapidly in Malpighian tubule cells, none were observed to accumulate in the lumen. For daunomycin this was in sharp contrast to the lack of penetration in other cells. The fluorescent fluid-phase markers Lucifer yellow CH and Texas-redTM-conjugated dextran (MW ~3000) confirmed the

previously described low but appreciable passive permeability of Malpighian tubules to large molecules (Ramsay 1958, Maddrell & Gardiner 1974) and highlighted the difference between this slow, presumably paracellular movement and the rapid accumulation of the other drugs. The present results also add to our knowledge of Malpighian tubule microstructure in the living state.

Materials and Methods

ANIMALS

Tobacco hornworm (*Manduca sexta*) eggs were obtained from Carolina Biological Supply Company (Burlington, NC), and raised at room temperature (22–23 °C) with photoperiod 16L:8D and ambient humidity. Larvae were raised individually on a cornmeal-based, nicotine-free artificial diet (Carolina Biological Supply). Under these conditions, larvae reach fifth instar approximately 15 days after hatching and enter the wandering (prepupal) stage after another 6 days. Only fifth-instar larvae were used, before they entered the wandering stage (determined by appearance of the pulsating dorsal vessel).

SALINE AND CHEMICALS

“*Manduca* saline”, as described in **Chapter 2**, was again used for dissecting and bathing the Malpighian tubule tissue, and for final dilution of all applied drugs.

Stock solutions of drugs were made in H₂O, except where noted, and diluted to the final concentration in *Manduca* saline. Unless otherwise specified, daunomycin (Calbiochem, La Jolla, CA) and Hoechst 33342 (Sigma-Aldrich, Oakville, ON) were applied at 10 µM; rhodamine 123 (R123; Sigma) and acridine orange (AO; Molecular Probes, Eugene, OR) at 5 µM; verapamil (as (±)verapamil hydrochloride; Sigma) at 25 µM. Some preliminary observations of tissue morphology were made with Nile red (Sigma), which was made as a 2 mM stock in DMSO and used at 4 µM final concentration. Texas-Red™-conjugated dextran

of MW ~3000 (TR-dextran; Molecular Probes) and Lucifer yellow (LY, Molecular Probes) were used at 0.08 mg/ml and 100 μ M final concentration, respectively. Ethidium homodimer (Molecular Probes) was obtained as a 2 mM solution in DMSO:H₂O (1:4) and used at a final concentration of 4 μ M. Nicotine (as nicotine hydrogen tartrate; Sigma) stock solutions were made in saline and brought to pH 7.2 with 5 M KOH.

SPECIMEN PREPARATION

Malpighian tubules were dissected from larvae as previously described (**Chapter 2**). A 2–4 cm portion of the proximal section (as defined by Nijhout 1975) of a single Malpighian tubule was carefully dissected free of tracheal connections and removed. Other tissues used for comparison purposes were longitudinal muscle (pieces $\leq$ 1 cm), ventral nerve cord (~2 cm section including several abdominal ganglia and interconnectives), salivary gland (~3 cm section), and fat body (~ 1 cm²). Samples were placed directly in saline contained within a silicone grease (Dow Corning high vacuum grease) well (about 20 x 20 mm) on a 24 x 60 mm glass coverslip. For most experiments, the cut ends of the Malpighian tubule were either ligated with a small piece of human hair or draped over the edges of the well such that there was no direct connection between the bath and the lumen of the tubule.

MICROSCOPY

Confocal microscopy was performed on a BIO-RAD MRC1024 confocal system attached to an Olympus IX-70 inverted microscope with Olympus LCPlanFl 20X (NA=0.4) and PlanApo 60X (NA=1.4 oil) objectives. Dyes were excited with the 488 nm (568 nm for TR-dextran) line of a 15 mW krypton/argon laser attenuated with neutral density filters blocking 0 to 99% of the light (**Table 3.1**). Confocal images, generally 512 x 512 pixels, were collected with LaserSharp 2.3 Acquisition software. Pictures were usually taken from an average of 3 or more scans to reduce noise. Brightfield images were collected simultaneously using a transmitted light detector. Images were subsequently processed using Confocal Assistant 4.02 (freeware written by T.C. Brelje) and Corel Draw 7.

Because of the low-wavelength of Hoechst 33342 excitation (**Table 3.1**), a mercury arc lamp as excitation source and conventional epi-fluorescence was used. The microscope was an upright Zeiss Axiophot with Zeiss Plan-Neofluor 10X (NA 0.3), 40X (NA 0.75) and 100X (NA 1.3 oil) objectives. Photographs were taken on Kodak TMAX400 professional black & white print film and subsequently digitized.

Drug application

Once the unstained tissue was observed for autofluorescence, the saline was replaced with saline plus dye at its final concentration, and a second (22 x 22 mm) coverslip was placed over the well to prevent evaporation of the bath and drifting of the tissue. Insect tissue is remarkably tolerant of low-oxygen conditions and Malpighian tubules function in unstirred saline at rates similar to *in vivo* rates (Maddrell & Overton 1990); our previous work indicates the cells are metabolically active (capable of active transport) for at least one hour in less than 100 μ l of saline without special oxygenation (**Chapter 2**). Verapamil (25 μ M) and nicotine (1 mM) were applied to the dissected tissue both before and concomitantly with daunomycin application. Note that except for some R123 imaging, the bath dye was not washed away.

In order to apply LY or TR-dextran to the luminal side of the tissue, the proximal Malpighian tubule was cannulated as previously described (**Chapter 2**, methods), except that the 0.1–0.2 ml drop of saline was not covered by mineral oil. At least 100 μ l of dye solution was pushed through the tubule in an attempt to flush out the contents.

Results

MORPHOLOGY AND MICROSTRUCTURE OF MALPIGHIAN TUBULE CELLS

Although it qualifies as a simple tubular epithelium with only one cell layer between the fluid on the basal and apical sides (Bradley 1985), the morphology of the Malpighian tubule and its cells is relatively complex. In *Manduca*, the proximal Malpighian tubules are composed of a

single morphological cell type, in contrast to those from Diptera (*e.g.* Meulemans & De Loof 1992). These cells are very large, often 300 μm or more in width, such that only 2 or 3 would be seen in a cross section of the tubule (**Fig 3.1a**). Generally, each “bulb” or “diverticulum” (Nijhout 1975) of the tubule is composed of a single cell (**Fig 3.1a,d**). Despite their large size, they are only 3 to 25 μm thick, and their surface area to volume ratio is further increased by extensive infoldings (**Figs 3.1a-c,f**) of the membrane. On the apical (luminal) surface, these took the form of large microvilli-lined folds or “macrovilli”, which appeared as fuzzy islands in confocal sections (**Fig 3.1c**). In electron micrographs, the basal surface is also seen to be highly convoluted (Klein *et al.* 1991), but this was not obvious in the images obtained in this study. The lumen of Malpighian tubules from a healthy fifth-instar larva is normally replete with uric acid, and while some spilled out during dissection, these 1.5–2.5 μm diameter particles were often seen in confocal or conventional brightfield imaging (**Fig 3.1f**).

HOECHST 33342 STAINING OF NUCLEI

Although Hoechst 33342 has been used to detect P-gp mediated MDR in cell lines (Neyfakh 1988, Lautier *et al.* 1993), we used it primarily to confirm the location of nuclei in the tubules, as it is a bright, specific stain for nucleic acids in living cells.

The nuclei, as demarcated by Hoechst 33342 staining, were convoluted and branching within the constraints of the cell (**Fig 3.2a**). This unusual nuclear morphology may result from polyteny (Bradley 1985) and has been reported previously for Lepidoptera (McMahon 1971, Wigglesworth 1972). The larval Malpighian tubule cells persist during Lepidopteran metamorphosis (Wigglesworth 1972), thus obviating the need for a difficult nuclear division. At higher resolution, the staining was revealed to be non-homogeneous; it was composed of many small (<1 μm diameter) regions of intense fluorescence (**Fig 3.2b**). More typical round nuclei were found in muscle tissue from the same insect, stained in the same way, and tiny dots were again seen at high power (**Fig 3.2c**).

DAUNOMYCIN RAPIDLY ENTERS MALPIGHIAN TUBULE CELLS

Daunomycin staining of the cells was consistently seen at the soonest time possible after 10 μ M drug addition. Times earlier than approximately 1 min were missed because of time required to locate an appropriate field of view and adjust focus. The intensity of cellular fluorescence greatly exceeded bath fluorescence even after this short period. **Fig 3.4** (top left image), taken after two minutes, is representative of the earliest staining. Although the cellular environment can change a dye's fluorescence properties (see discussion) the intensity of cellular staining suggests that the tissue accumulated dye to high levels very rapidly.

In sharp contrast to the staining of Malpighian tubules, other tissues, including longitudinal muscle, nervous tissue (abdominal ganglia and interconnectives), and fat body (a metabolically active organ), when incubated in 10 μ M daunomycin, did not take up the drug even after an hour or more. **Fig 3.3** shows staining at ~10 min and after ~1 hour. Daunomycin fluorescence was often seen associated with tracheal coils (*e.g.* **Fig 3.3**, ganglion, *arrow*) presumably due to adsorption rather than absorption or any active process. Faint bath fluorescence can also be seen in the contrast enhanced images of **Fig 3.3** (*asterisks*), demonstrating that the lack of staining in other tissues is not due to the Malpighian tubule absorbing all of the dye, although this may be a contributing factor.

While the low magnification images of Malpighian tubules suggest that daunomycin flooded the entire cell (**Figs 3.3,3.4**), at higher resolution certain regions were seen to stain more brightly than others. In particular, the nucleus was usually demarcated as a region of brighter fluorescence (**Fig 3.5**). As with Hoechst 33342 staining (**Fig 3.2**), the nucleus did not appear homogeneous, although with daunomycin the small points were ones of *less* intense fluorescence (**Fig 3.5**). This may relate to the observation of Simon *et al.* (1994) that "binding to tightly packed DNA in chromatin results in quenching of the daunomycin fluorescence, whereas binding to nucleoli yields fluorescent structures". The cytoplasmic fluorescence was likewise uneven, often taking on a granular appearance, and sometimes showing individual regions of

lower intensity. Daunomycin appeared to penetrate as far as the microvilli, but beyond that -- in the lumen -- no fluorescence was seen (**Fig 3.5**). This was true even when very high (100–400 μ M) concentrations of daunomycin were used (*e.g.* **Fig 3.6**).

An advantage of digital imaging is the potential for quantification of the images. The intensity of fluorescence at each location in the image is stored as a number ranging from 0 to 255. To get a numerical profile of the fluorescence in the bath, the cell and the lumen, a region of interest was selected and the intensity plotted as a function of x-distance. In general, this approach confirms the observation clearly seen by eye, namely that the dye is accumulating in the cell, but not the lumen (**Fig 3.6b**). Some fluorescence in the lumen can be attributed to out-of-focus light from underlying layers (**Fig 3.6a**). Quantitative comparisons between samples would have been much more difficult to perform because factors such as distance from the coverslip, variation in tissue, and the stability of the laser output were hard to control. Since the nature of our observations were largely qualitative anyway, quantitative analysis of our results was not pursued.

NEITHER VERAPAMIL NOR NICOTINE AFFECT DAUNOMYCIN STAINING

To test if P-gp might be involved in daunomycin accumulation, the P-gp inhibitor verapamil was pre- and co-applied to the Malpighian tubule at 25 μ M. This concentration is known to inhibit the active transport of both nicotine and vinblastine in this tissue (**Chapter 2**). No evident change was seen in the speed or intensity of staining (**Fig 3.4**), or in subcellular distribution (not shown). Likewise, the pre-incubation and co-addition of 1 mM nicotine with daunomycin visibly affected neither speed nor location of staining (**Fig 3.4**).

RHODAMINE 123 STAINING

Like daunomycin, rhodamine 123 (R123) rapidly entered the cell such that within 2 min the tissue was much brighter than the bath (**Fig 3.7a**). Cellular staining was sometimes so bright that subcellular structures could not be distinguished. When finer detail was seen, typical mitochondria were evident in the cytoplasm. Clearer pictures of R123 distribution were

obtained when the tissue was rinsed in saline after a 5 min incubation (**Figs 3.7b,c**). The microvilli, and a layer immediately below them, were much brighter than the cytoplasm (**Fig 3.7b**). As R123 is selective for mitochondria (Johnson *et al.* 1980, Canitrot & Lautier 1995), these images from living tissue are consistent with electron micrographs (*e.g.* Klein *et al.* 1991) which show mitochondria extending into the microvilli and densely packed at their base. As with daunomycin, no fluorescence was seen in the lumen, even after an hour or more in the dye.

As mitochondria can be fairly mobile in some cells and undergo Brownian movement in metabolically compromised cells, images were collected to characterize any motions of these organelles. Images collected for 2–3 min at 30 s intervals from the same location showed virtually no movement between frames. This is not due to lack of sensitivity since, by replaying frames as a movie and as shown in **Fig 3.8**, we were able to detect a single particle of < 200 nm diameter moving approximately 300 nm over the course of 90 s, one of only two movements in a field of view of 60 x 60 μm (3600 μm^2). To see if vinblastine, a microtubule inhibitor, might disrupt this apparent stability, a Malpighian tubule was stained for 5 min in R123 and then placed in 170 μM vinblastine. Again, time series collected 10, 15 or 30 s apart for several minutes showed no appreciable movement, as in the no-vinblastine control (results not shown).

As with daunomycin, staining of Malpighian tubules occurred much more rapidly than staining of other tissues (results not shown). Interestingly, while the salivary gland and muscle did eventually become stained when left in the dye (~1 hour), only the periphery of the nervous tissue (the site of the blood-brain barrier) was ever seen to take up R123.

ACRIDINE ORANGE

Acridine orange (AO), too, stained Malpighian tubules rapidly (**Fig 3.9a,b**); and in this case, other tissues including salivary gland (**Fig 3.9c**), nervous tissue (**Fig 3.9d**), and muscle (**Fig 3.9e**) were also stained in the same time (later, more intensely fluorescent images are shown in **Fig 3.9c-e** because they better illustrate the more dimly stained features). In the Malpighian tubules, the cytoplasm was brightly stained with brighter points which seemed to be located in the

nucleus. There were also occasional, larger vesicles (7–9 μm) (**Fig 3.9b**). The microvilli were also visible. In the other tissues (**Figs 3.9c-e**), the cytoplasm was diffusely stained but there were also many bright vesicles which were not seen so frequently in the Malpighian tubule, other than in the nuclear region. Although in general the images collected in green and red filters showed the same features, with the green being brighter, the brighter vesicles were especially evident in the longer wavelengths ($>585\text{ nm}$), consistent with the propensity for AO to accumulate and fluoresce red in acidic vesicles such as lysosomes (Schindler *et al.* 1996).

ETHIDIUM HOMODIMER

Ethidium homodimer dye, while purportedly cell impermeant (Haugland 1996), stained a Malpighian tubule within one minute (**Fig 3.10a**). This was in contrast to the lack of staining seen in muscle, nervous tissue, and salivary gland, except where the latter was cut. The entire cytoplasm was faintly stained, but the nucleus was especially bright, while microvilli were only very dimly stained (**Fig 3.10b**). Once again the nuclear staining was not homogeneous, but consisted of small brighter regions and some larger (1–1.5 μm diameter) regions of even greater intensity. This observation is presented in support of our contention that the Malpighian tubule acts as a drug “scavenger”, absorbing xenobiotics from the hemolymph (see Discussion, p. 65), however a more thorough examination would be required to be certain that ethidium homodimer uptake is a consistent feature of living Malpighian tubule cells.

FLUID-PHASE MARKERS

To evaluate the potential role of bulk endocytosis or of non-specific permeability to large molecules in the accumulation or transport of xenobiotics, experiments were performed with the fluid-phase markers Lucifer yellow (LY) and Texas-Red™-conjugated-dextran (TR-dextran, MW ~3000). When added to the basal surface of the tissue (*i.e.* in the bath as for other dyes), these dyes initially remained in the extracellular space (**Fig 3.11a**). However, after several minutes, TR-dextran slowly entered the tissue, where it was found primarily at the luminal surface (**Fig 3.11b**). Bright staining of fibres, perhaps fine tracheoles, was seen on the basal

surface (**Fig 3.11b**). Fluorescence is also seen to a lesser extent in the cytoplasm; this may represent endocytosis of the dextran, or could arise from unconjugated TR dye.

When either TR-dextran or LY was injected into the lumen, via the cannula, the convoluted pattern of the luminal space could be made out (**Fig 3.11c**). Interestingly, the large bulk space of the lumen remained dark compared with the lumen near the tissue border (**Fig 3.11d**). With LY-injected tubules, bright vesicles occasionally seen in the tissue (**Fig 3.11d, arrows**) were probably autofluorescent since under the same excitation and collection conditions (which includes 100% laser power), similar structures were sometimes seen with no added dye.

Discussion

The ability to monitor the distribution of fluorescent dyes or drugs in tissues by rejecting out-of-focus fluorescence is one of the chief advantages of confocal microscopy (White *et al.* 1987). In this study, confocal sectioning allowed the cellular and subcellular distribution of the fluorescent drugs daunomycin, rhodamine 123 (R123), acridine orange and others to be determined in a living tissue, the Malpighian tubule, which is not only relatively thick but also of complex geometry. These drugs are extensively used in the study of P-glycoprotein (P-gp)-mediated transport in other systems and so it was felt they could be informative with respect to our hypothesis that P-gp mediates transport of xenobiotics in the Malpighian tubule. In particular, daunomycin is a chemotherapeutic drug which accumulates in nuclei of drug-sensitive cells (Seidel *et al.* 1995) and to which resistance is frequently cited as part of the MDR phenotype (*e.g.* Gottesman & Pastan 1993). It is vectorially transported by P-gp in a variety of cell lines, including kidney-derived MDCK cells (Horio *et al.* 1989), and in isolated proximal tubules from killifish kidney (Miller 1995). R123, in addition to being an excellent vital stain for mitochondria (Johnson *et al.* 1980), is also recognized as a P-gp substrate and dramatically decreased accumulation in cells has been used as a marker for the presence of P-gp (Altenberg *et*

al. 1994, Canitrot & Lautier 1995, Bosch & Croop 1996). Reduced staining intensity in MDR cells in comparison to their sensitive parentals is also seen with both acridine orange and Hoechst 33342 (Neyfakh 1988, Kessel *et al.* 1991, Canitrot *et al.* 1996), but these dyes are more often used to stain specific structures. Acridine orange accumulates in acidic vesicles, where at high concentrations it shows a red fluorescence, and it intercalates with DNA and RNA where its fluorescence is typically green (Schindler *et al.* 1996). Hoechst 33342 is a vital stain of nuclei but unlike acridine orange fluoresces only upon DNA binding and does not intercalate with nucleic acids (Haugland 1996).

All dyes tested rapidly and intensely stained the Malpighian tubule, often visibly depleting the immediately adjacent bath of dye. However, none of the dyes appeared to enter the lumen, contrary to expectations. If the dyes were transported, as they are in other systems (as cited above) and as nicotine and vinblastine are in this tissue (Murray *et al.* 1994, Gaertner *et al.* 1998, **Chapter 2**), we would expect to see accumulation in the lumen. If they were not handled by the tissue at all, one might expect a gradual equilibration between the bath and the lumen. What we saw instead was that the tissue handled these drugs by accumulating them in the cell.

Before concluding that the tubule is incapable of transepithelial transport of any of the drugs tested, several alternative explanations of the results might be envisioned. One possibility is that fluorescence in the lumen was eclipsed by the intense cellular fluorescence. Because the intensity of bath fluorescence was also very low, even a small amount of luminal fluorescence could indicate a dye lumen-to-bath ratio greater than unity. A quantitative look at some of the images, using the assumption that intensity is proportional to concentration and that the confocal 'section' truly rejects all out of focus light, nonetheless confirmed what visual inspection suggested. A second possibility is that the environment of the lumen changed the fluorescence properties of the dyes. This was almost certainly the case for Hoechst 33342, which is only appreciably fluorescent when bound to DNA. Luminal pH would have an effect on acridine orange fluorescence (Palmgren 1991), but it would still have been detectable in the lumen, while

daunomycin's spectrum is unaffected by pH (Simon *et al.* 1994). The peak of the R123 fluorescence spectrum is reported to shift by 7–15 nm depending on its environment (Kessel *et al.* 1991, Canitrot & Lautier 1995), but again our filter band widths would still have allowed its detection. R123 is apparently not broken down in tumour cells (Canitrot & Lautier 1995) but extracellular metabolism of the drugs could still be a factor. While little or no nicotine metabolism is seen in Malpighian tubules (Maddrell & Gardiner 1976, Snyder *et al.* 1993), Li *et al.* (1997) report that the luminal fluid from *Manduca* Malpighian tubules (but not hemolymph) contains all the factors needed to cleave and oxidize a native peptide hormone, Mas-DH. A final scenario that can be entertained is that dyes entered the lumen but could not be seen because the exciting and/or emitted light was quenched, reflected or absorbed by the opaque uric acid crystals that are present in the lumen, even after flushing dye-laden saline into and through the tubule. This would not block visualization of the cells close to the objective, and could explain why the cell fluorescence on the far side of the lumen was not observed. The observation that lumen-injected dye was not detectable in the deeper regions of the lumen (**Fig 3.11d**) might also support this theory while at the same time showing that dye washed directly into the luminal region close to the tissue can be visualized.

Despite these potential objections it seems clear that there is no movement of dyes on the scale of that seen for daunomycin in killifish renal proximal tubules by Miller (1995), who reported a *lumen-to-cell* fluorescence ratio of 2.3.

The use of dyes to examine Malpighian tubule function has a long history, with L. Lison noting clearance of acidic dyes by Malpighian tubules of *Periplaneta orientalis* and *Forficula auricularia* over 60 years ago (Bradley 1985). Nijhout (1975) injected 20 mM solutions of 24 water-soluble dyes into *Manduca sexta* larvae and visually observed the progress of excretion by sacrificing animals at intervals from 5 min to 6 h after injection. While all acidic dyes tested were found in the lumen of the midgut and medial Malpighian tubules, basic dyes, into which category the dyes of the present study fall, were largely excreted in the proximal and medial

tubules, if at all. Just as we found, “basic dyes caused an often intense staining of the cytoplasm of the Malpighian tubules”; but after puncturing or ripping open the tubule, Nijhout (1975) concluded that dye was also present in the lumen. Although Bresler *et al.* (1990) report that in larvae of the Lepidopteran *Galleria mellonella*, fluorescein, an acidic dye, first appears in the cytoplasm of the Malpighian tubule cells and then penetrates into the tubule lumen, they present only fluorimetric determinations of *tissue-to-bath* ratios which do not separate the contributions of tissue and luminal fluorescence. As part of an elegant genetic analysis defining different domains of *Drosophila* tubules, Sözen *et al.* (1997) reported R123 transport into the lumen of the ‘main segment’ of tubules, but like previous authors, did not rule out the possibility that the fluorescent signal came primarily from the cells surrounding the lumen. Meulemans and De Loof (1992) reported interactions of R123 with Malpighian tubules in the flies *Sarcophaga*, *Calliphora* and *Drosophila* which are more consistent with our observations. These authors found that, initially, secondary cells were unstained while primary cells were intensely stained. After a period of 2 hours, this pattern was reversed. They further showed that the secondary cells had accumulated the R123 *from the lumen*, while primary cells only absorbed dye from the basal side. The cells of *Manduca* proximal tubules, which are all of one type (Klein *et al.* 1991), thus resemble the primary cells of flies in that they rapidly absorb R123 from the basal side. *In vivo* injection of R123 in flies allowed long term distribution to be followed, and deposition onto granules in lumen was seen only in anterior Malpighian tubules after 30 hours (Meulemans and De Loof 1992). This demonstrates that initial cellular accumulation is not incompatible with eventual excretion.

While in typical MDR cells P-gp generally ‘excludes or extrudes’ cytotoxic drugs from the entire cell (Gottesman *et al.* 1996), in polarized mammalian epithelia P-gp is expressed only at the apical surface where it is proposed to actively transport out of the cell drugs that enter through passive or facilitated entry at the basal surface (Simmons *et al.* 1997). If this is occurring in the Malpighian tubule, what we have observed is just the first step of this procedure,

i.e. the facilitated entry of drugs into the cell, which presumably does not involve P-gp. In this scenario it would not be surprising that verapamil, a P-gp inhibitor (Bosch & Croop 1996), had no effect on the *accumulation* of daunomycin. Since P-gp expression is also seen at intracellular sites and may produce a subcellular redistribution of drugs (*e.g.* Baldini *et al.* 1995), this could also occur in the Malpighian tubule. However, this did not appear to be a factor since verapamil did not alter the intracellular distribution of daunomycin.

In the nicotine transport assay, Murray (1996; Gaertner *et al.* 1998), like Maddrell and Gardiner (1976), used elevated levels (up to 5 mM) of nicotine to measure accumulation in the lumen because at low micromolar levels nicotine evidently accumulated in the cells and failed to move into the lumen in appreciable quantities. However, even application of 0.1 to 0.4 mM daunomycin did not result in any detectable fluorescence in the lumen, so if there is a threshold level below which transepithelial daunomycin transport is not observed, it must be >0.4 mM. Furthermore, the addition of 1 mM nicotine had no effect on daunomycin accumulation or subcellular distribution which suggests that the mechanism(s) responsible for the rapid trans-epithelial accumulation of nicotine (Maddrell & Gardiner 1976, Gaertner *et al.* 1998) differs from that responsible for the rapid cellular accumulation of daunomycin.

While all the drugs tested rapidly entered Malpighian tubule cells, other tissues from the same insect did not absorb drugs so rapidly -- in the case of daunomycin, accumulation in tissues other than the Malpighian tubule was barely detectable even after 1 hour. If the distribution of this cytotoxic drug is typical of other xenobiotics, this suggests that the Malpighian tubule may help to protect sensitive tissues by rapidly absorbing drugs from the hemolymph. This would be an example of “storage excretion” which occurs in lepidopterous adults that store nitrogenous compounds in their body cuticle and wing scales (Cochran 1985). Since the drugs used are positively charged at physiological pH the different tissue distributions might be partly explained by appropriate electrical potential gradients and facilitated transport at the basal membrane of the tubule. Another determinant may be the presence of protective covering epithelia, such as

the specialized blood-brain barrier which excludes nicotine from the sensitive neuropile (Morris 1984, Murray *et al.* 1994). It is interesting to note that the blood-brain barrier appears to immobilize nicotine and its metabolites by temporary cellular sequestration in the barrier cells (Morris 1983b).

In view of the low but significant permeability of tubules to compounds as large as inulin (MW 5000) (Maddrell & Gardiner 1974), Maddrell (1981) suggests that slow passive removal of substances is the principle of excretion in insects. Our results with the fluid phase markers Lucifer yellow and TR-dextran (MW ~3000) support the idea that large molecules are capable of entering the lumen of the tubule, probably via a paracellular route. At the same time, the accumulation of dyes within the cells makes it clear that transcellular transport may be important for smaller compounds but leaves unanswered the question of why they do not also take a paracellular route. It may be that the cells absorb smaller dyes equally well from the lumen as from the bath, although this was not tested. While we do not know if these drugs eventually reach the lumen (it may be sufficient for the insect to sequester xenobiotics in the tissue indefinitely), if they do, the large concentration gradient from the tubule to urine and the extensive apical surface produced through “macrovilli” and microvilli would promote movement across the apical membrane. As Maddrell (1981) emphasizes, insect tissues tolerate, or are protected from, many potentially deleterious aspects of their chemical environment: the present results suggest that scavenger-like accumulation of xenobiotics by the Malpighian tubule may be part of this broad adaptation.

Table 3.1 Collection conditions and characteristics of dyes used in this study

dye	excitation wavelength (nm)	typical laser power (%)	emission filter wavelengths (nm)	excitation / emission maxima (nm)	reference
acridine orange	488	3	506–538, >585	487 / 520 or 650*	Haugland 1996
daunomycin	488	30	>560	488 / 595†	Simon <i>et al.</i> 1994
ethidium homodimer	568	≥ 10	>585	528 / 617	Haugland 1996
Hoechst 33342	~365	n/a	>420	350 / 461	Haugland 1996
Lucifer yellow	488	30–100	506–538, >515	430 / 540	Swanson 1989
rhodamine 123	488	1–3	506–538	500 / 525	Canitrot & Lautier 1995
Texas-red™-dextran	568	30–100	589–621	595 / 615	Haugland 1996

n/a = not applicable

* red emission spectrum when aggregated, for example in acidic vesicles or at high concentrations with RNA, and green when in monomeric state, for example when bound to double-stranded nucleic acids (Delic *et al.* 1991, Schindler *et al.* 1996)

† 595 nm is the emission maximum when excited at 488 nm, however 488 nm may not be the excitation maximum

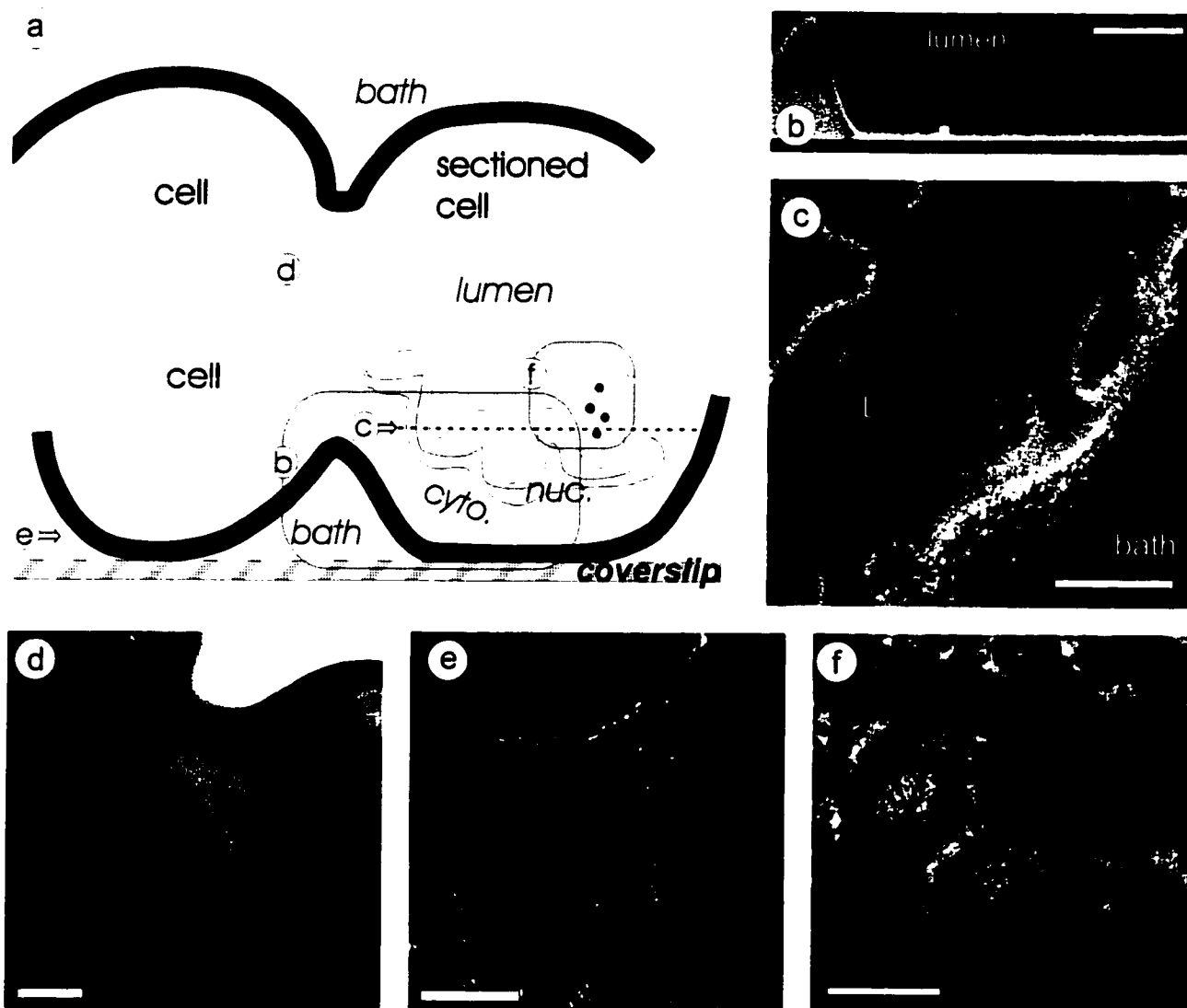


Figure 3.1 Internal and external morphology of the Malpighian tubule. (a) Schematic (not to scale) showing disposition of cells, macrovilli and location of features represented in parts b-f. Typical tubules from fifth-instar *Manduca* were 200–500 μm in diameter, but could be even larger. (b) Vertical section through cell which has been partly flattened against a coverslip. Fluorescence arises from Texas-red™-conjugated dextran (MW ~3000) added to the bath 40 min prior to this image (see also Fig 3.11). While the dextran remains largely outside the cell, it also appears to be bound to the basal lamella and to the apical face of the tissue, and perhaps also to be found within the cell. bar = 40 μm (c) Microvilli-lined macrovilli, stained here with Nile Red and rhodamine 123 resemble “fuzzy islands” in cross section. bar = 20 μm (d) Brightfield image showing region where several cells meet. bar = 100 μm (e) Trachea running along the basal surface of cell supply oxygen to the tissue (stained with Nile red and rhodamine 123). bar = 20 μm (f) Brightfield image showing constantly moving 1.5–2.5 μm spherules of uric acid (arrows) among the macrovilli in the lumen. bar = 20 μm

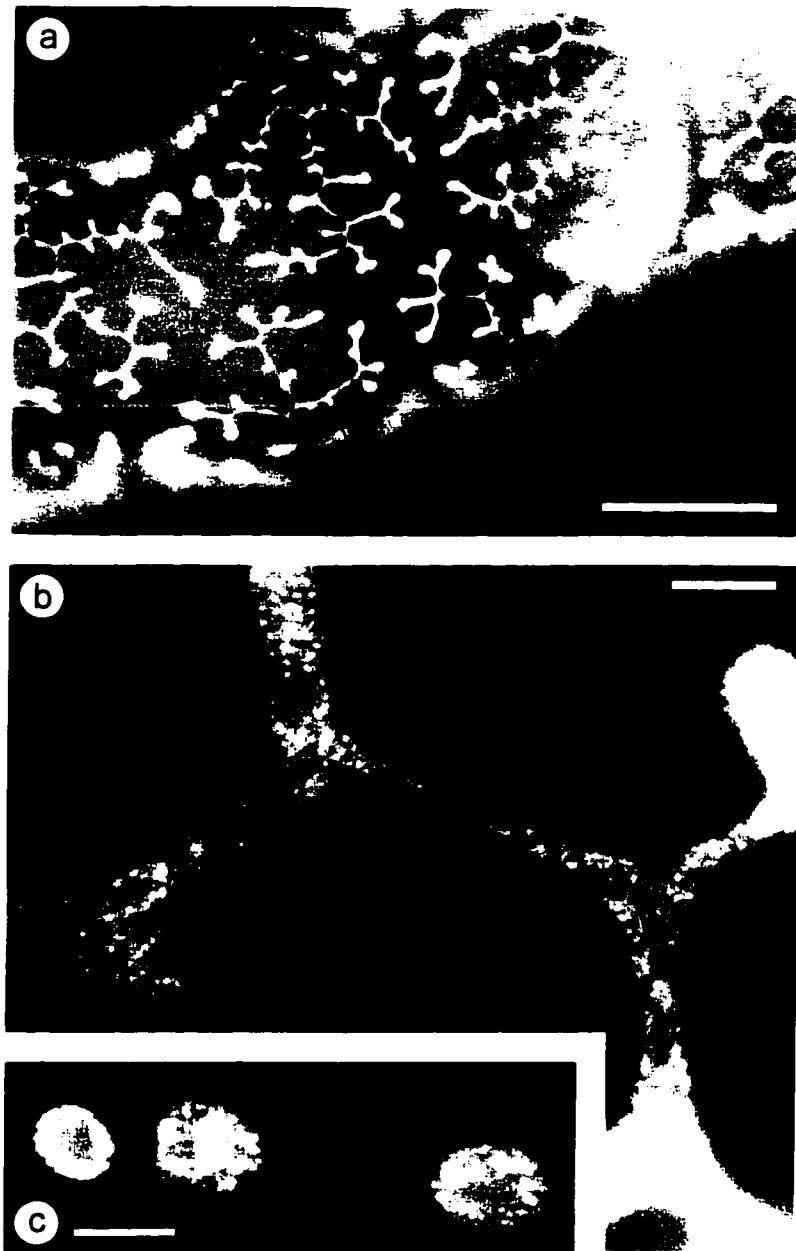


Figure 3.2 Hoechst 33342 staining of nuclei in living tissues of *Manduca sexta* larvae. (a) Malpighian tubule. Each lobe of the tubule is formed by a cell containing a highly branched nucleus. (Arrows point to 2 separate nuclei.) Typical tubules from fifth- instar *Manduca* were 200 to 500 μm in diameter, but could be even larger. bar = 300 μm (b) Detail of Malpighian tubule nucleus showing staining composed of many discrete, highly fluorescent regions of < 1 μm diameter (arrow). bar = 10 μm . (c) More typical round nuclei from muscle tissue also have a non-homogeneous staining pattern when seen at high magnification. bar = 10 μm .

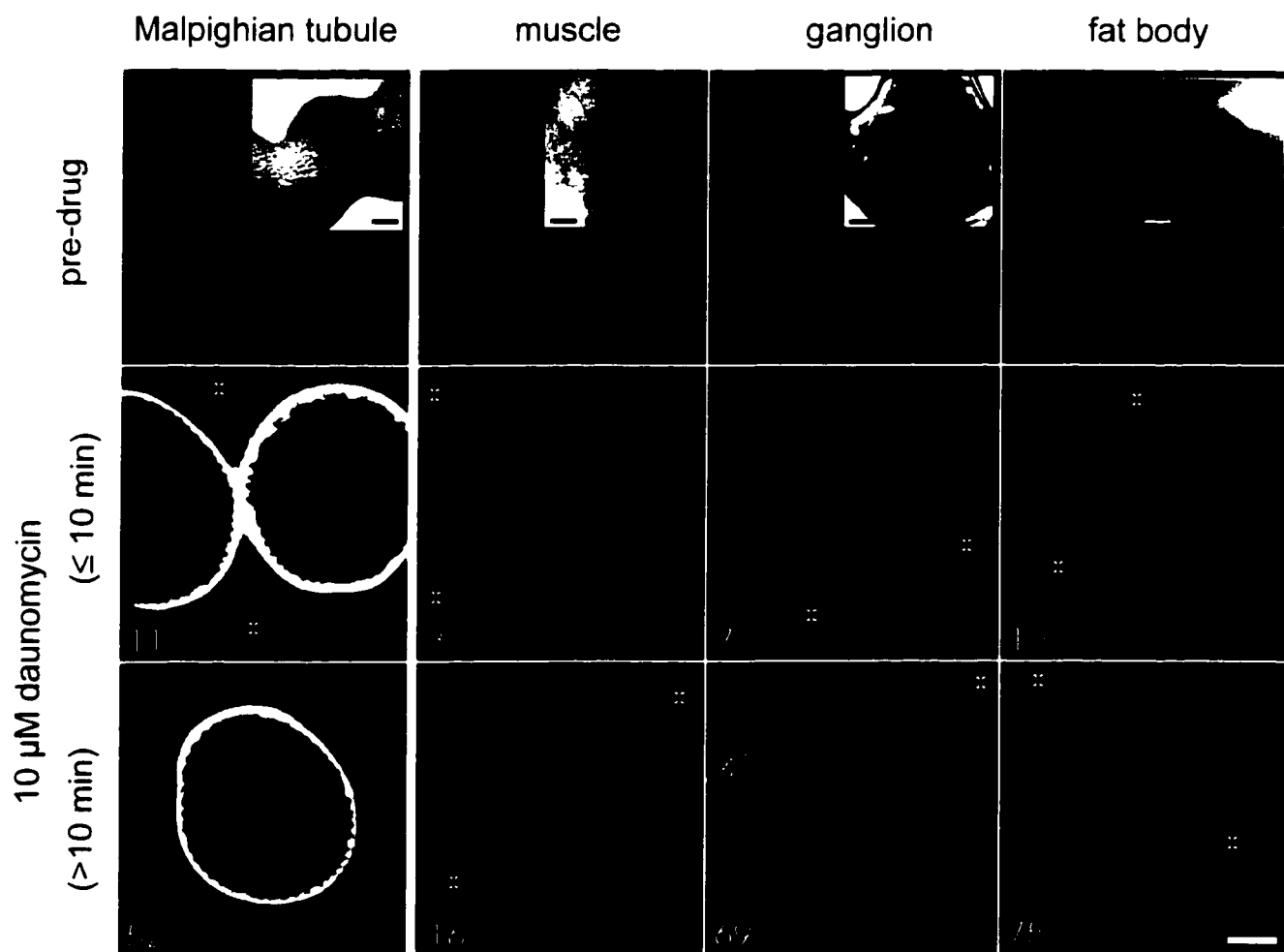


Figure 3.3 Malpighian tubules rapidly and extensively took up daunomycin, while other tissues did not. Sections of Malpighian tubule, longitudinal muscle, abdominal nerve cord (ganglion shown here) and fat body from the same insect were all placed in a well on the same coverslip and imaged identically both before and after replacing saline with 10 μ M daunomycin. The fluorescence of daunomycin in the bath is almost imperceptible in comparison to the tubule fluorescence, but is faintly seen surrounding the other tissues (*asterisks* show position of bath). bar = 100 μ m. **Insets**, transmitted light (brightfield) images of the control for autofluorescence (prior to drug application). bar = 100 μ m.

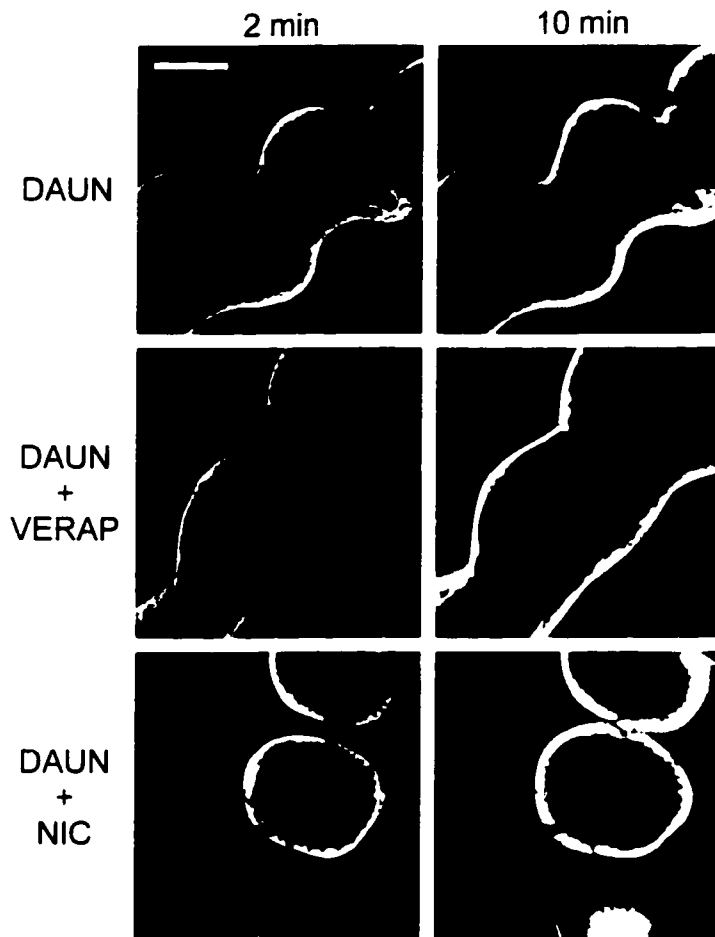


Figure 3.4 Neither verapamil (*verap*) nor nicotine (*nic*) affected the intensity or speed of daunomycin (*daun*) staining of Malpighian tubules. Daunomycin (10 μ M) was applied to live tissues as described in Methods. Brightness and contrast were not adjusted for these images. The images for daunomycin + nicotine are taken from a z-position where the lobes (diverticula) of the tubule are seen separately; the dark lines result from out-of-focus tracheoles. bar = 150 μ m.

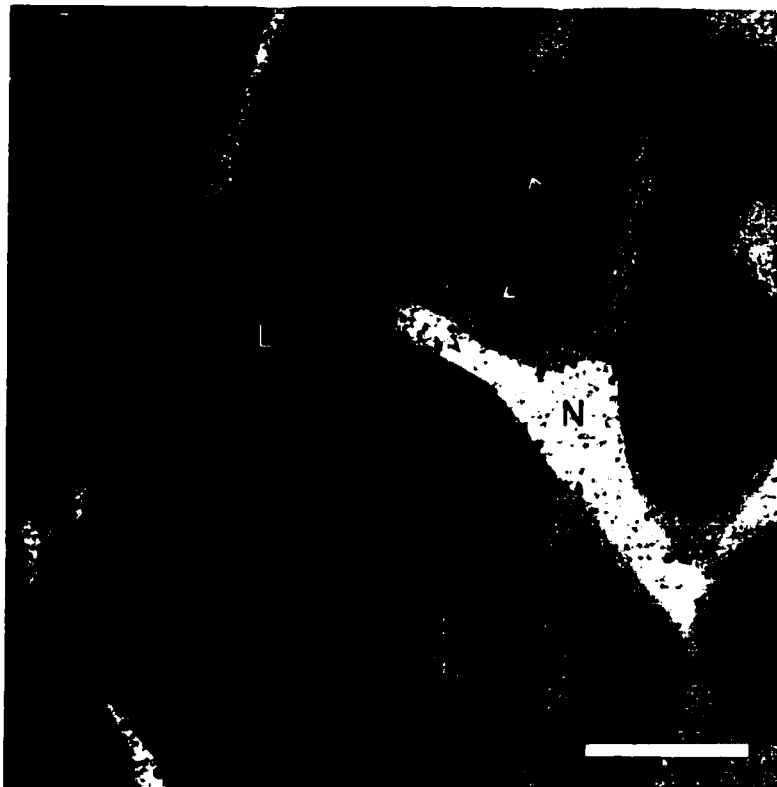


Figure 3.5 Subcellular location of daunomycin fluorescence in Malpighian tubules. Tissue stained in 10 μ M daunomycin for 2 hours. *N*, nucleus; *L*, lumen; *arrows*, microvillar border. bar=20 μ m.

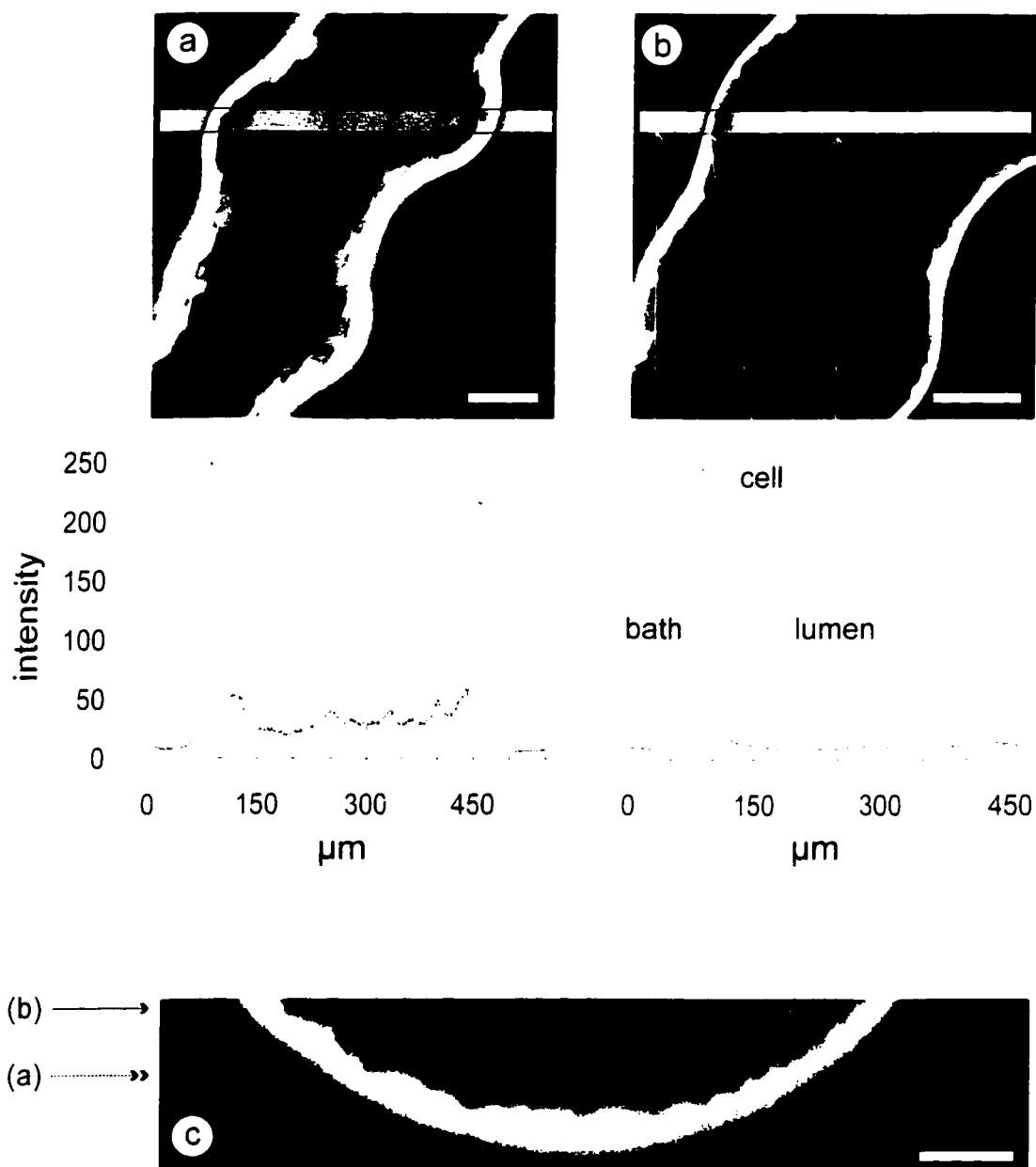


Figure 3.6 Quantitative processing of digital images confirmed what visual inspection suggested: there is little fluorescence in the bulk of the lumen. The average intensity of the highlighted regions in (a) and (b) is plotted directly below the corresponding region of the image, giving a profile of the fluorescence in the bath, the cell and the lumen. In (a), it appears that the lumen contains more dye than the bath, however this is probably due to out-of-focus cellular fluorescence. This suggestion is borne out in (b), which is scanned at a deeper level in the tissue, with a smaller iris diameter. A vertical (z-) scan (c) shows the approximate locations of the x-y scans shown in (a) and (b). Tissue was stained in 100 μM daunomycin for 10 (a) to 20 (b) minutes. bar = 100 μm (a,b); 50 μm (c)

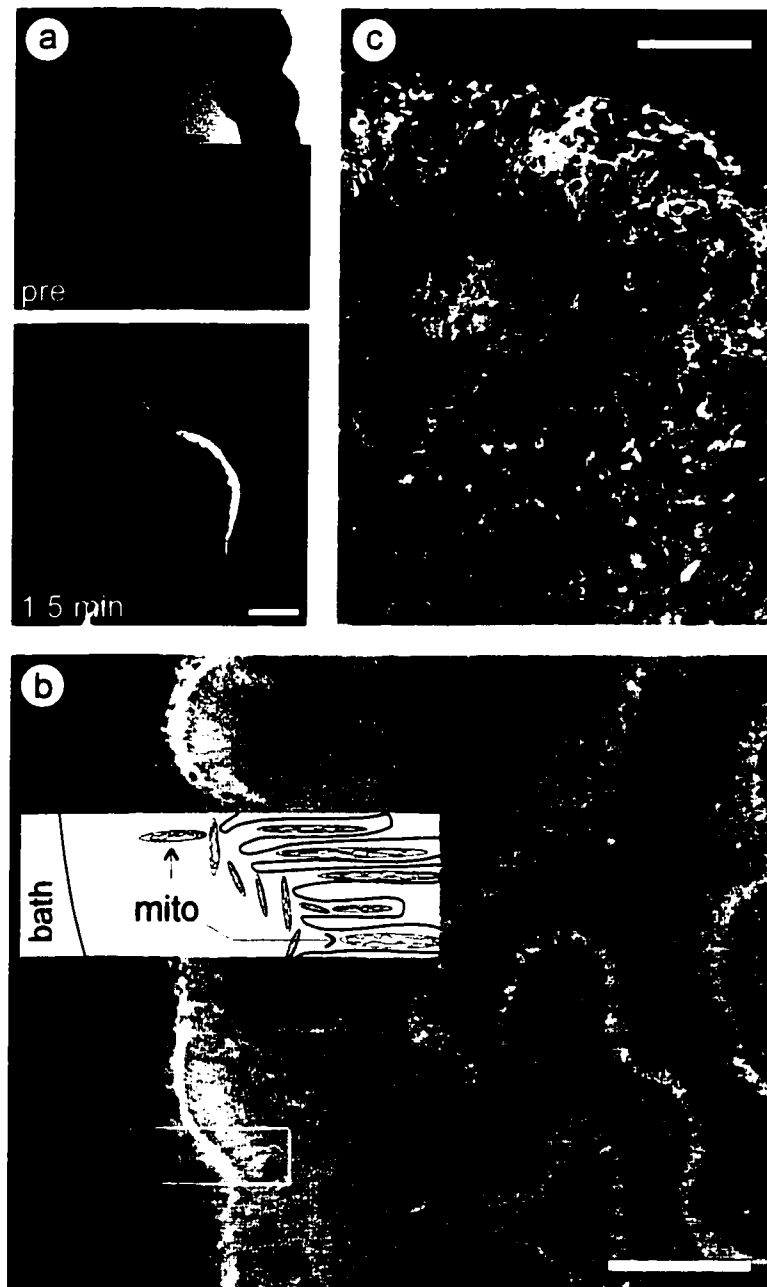


Figure 3.7 Rhodamine 123 (R123) staining of Malpighian tubules. (a) Appearance of tissue at low magnification before (*pre*) and 1.5 minutes after addition of 5 μ M R123. Note that the dye-laden bath has not been replaced but appears black in contrast to the much brighter tissue. bar = 100 μ m. **Inset**, brightfield image of control tissue. bar = 100 μ m. (b) High resolution confocal image showing R123 staining in cytoplasm (at left), in microvilli and adjacent to the microvilli (*arrow*) and absence of drug in lumen proper (*L*). bar = 10 μ m. **Inset**, interpretation supported by electron micrographs (Klein *et al.* 1991), not to scale. *mito*, mitochondria. (c) Typical mitochondrial staining in the bulk cytoplasm. bar = 10 μ m.

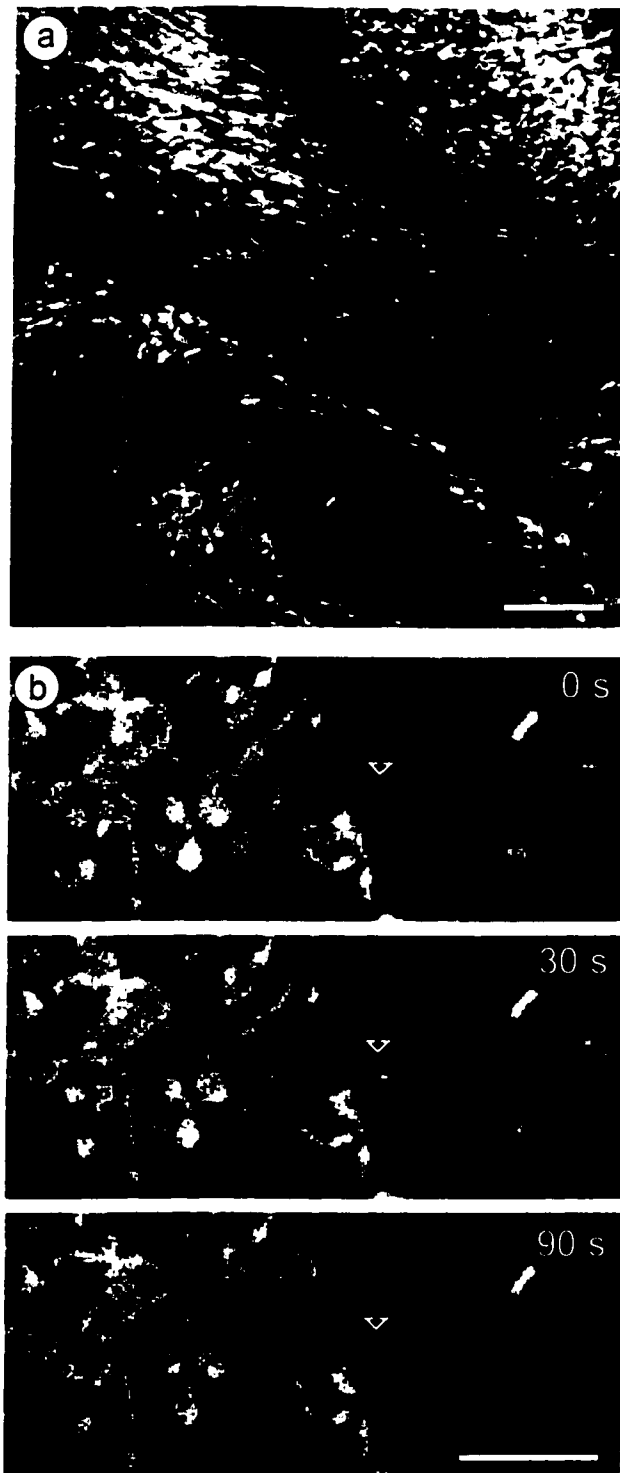


Figure 3.8 Lack of movement of mitochondria in Malpighian tubules. (a) Example of field of view examined for movement. (b) Enlargement of rectangle shown in (a) illustrating sensitivity of method to detect the small motion of particle (mitochondrion?) moving ~ 300 nm with respect to a fiducial mark. Bars = $10\ \mu\text{m}$ (a); $5\ \mu\text{m}$ (b)

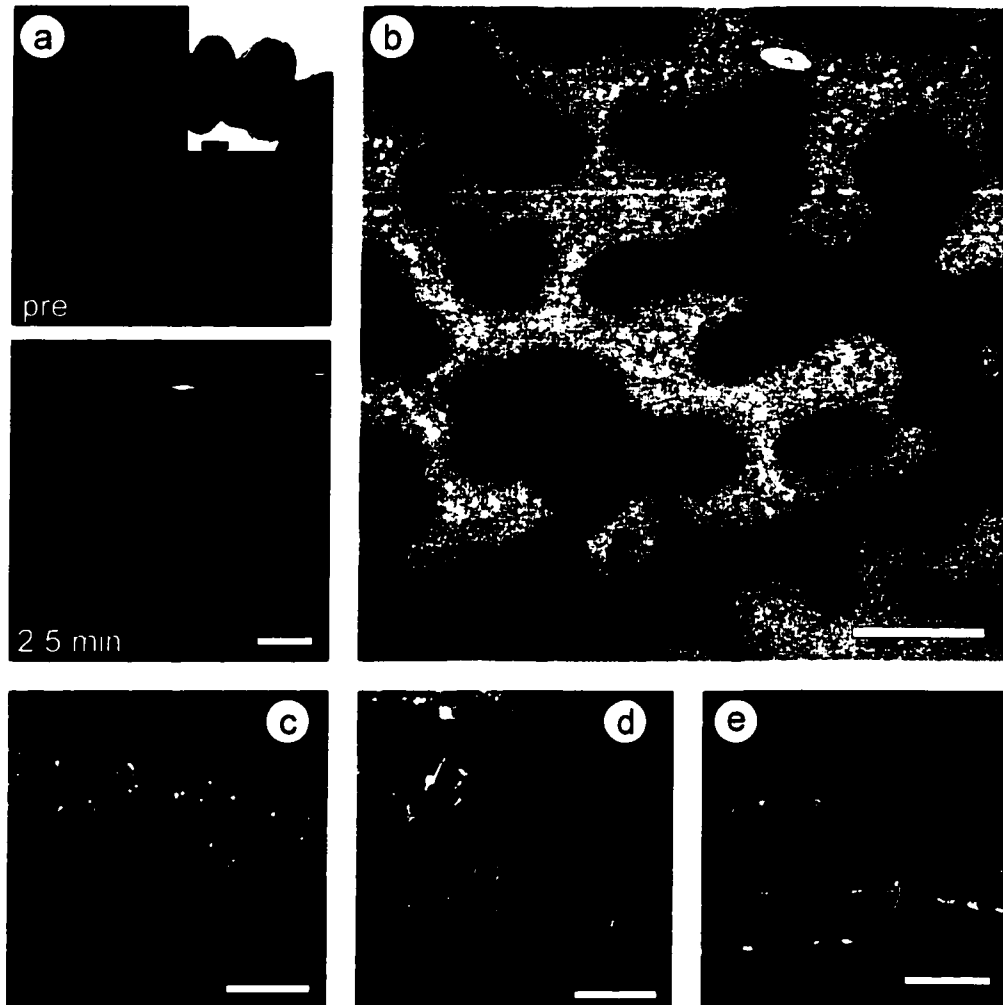


Figure 3.9 Acridine orange (AO) staining of Malpighian tubules (*a-b*) and other tissues (*c-e*). (**a**) Appearance of tissue at low magnification before (*pre*) and 2.5 min after addition of 5 μ M AO. Both pictures have been contrast enhanced to the same extent. bar = 100 μ m. **Inset**, brightfield image of control. bar = 100 μ m. (**b**) Detail of staining after 52 min in AO. The brighter staining vesicles tended to be found where we expected the nucleus. bar = 25 μ m. (**c**) salivary gland after 40 min in AO. bar = 25 μ m. (**d**) nervous tissue (ganglionic interconnectives) after 25 min in AO. bar = 50 μ m. (**e**) muscle after 13 min in AO. bar = 75 μ m. Pictures *c* to *e* are merged images from red and green filters.

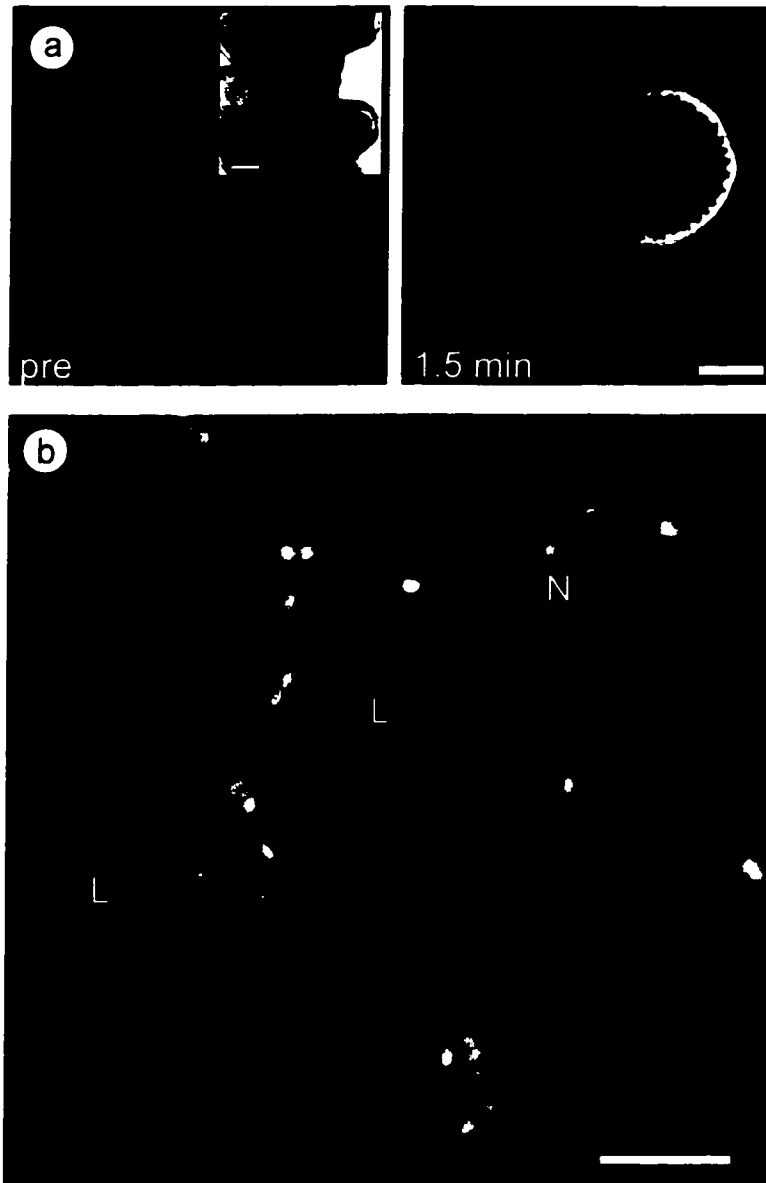


Figure 3.10 While cell impermeant in most systems, ethidium homodimer-1 was rapidly absorbed by the Malpighian tubule. (a) Appearance of tubule before (*pre*) and 1.5 min after addition of 4 μ M ethidium homodimer. (b) Detail of tubule staining -- the nucleus (N) was stained most strongly, but the cytoplasm was also faintly labelled. L= lumen. bar = 100 μ m (a); 10 μ m (b)

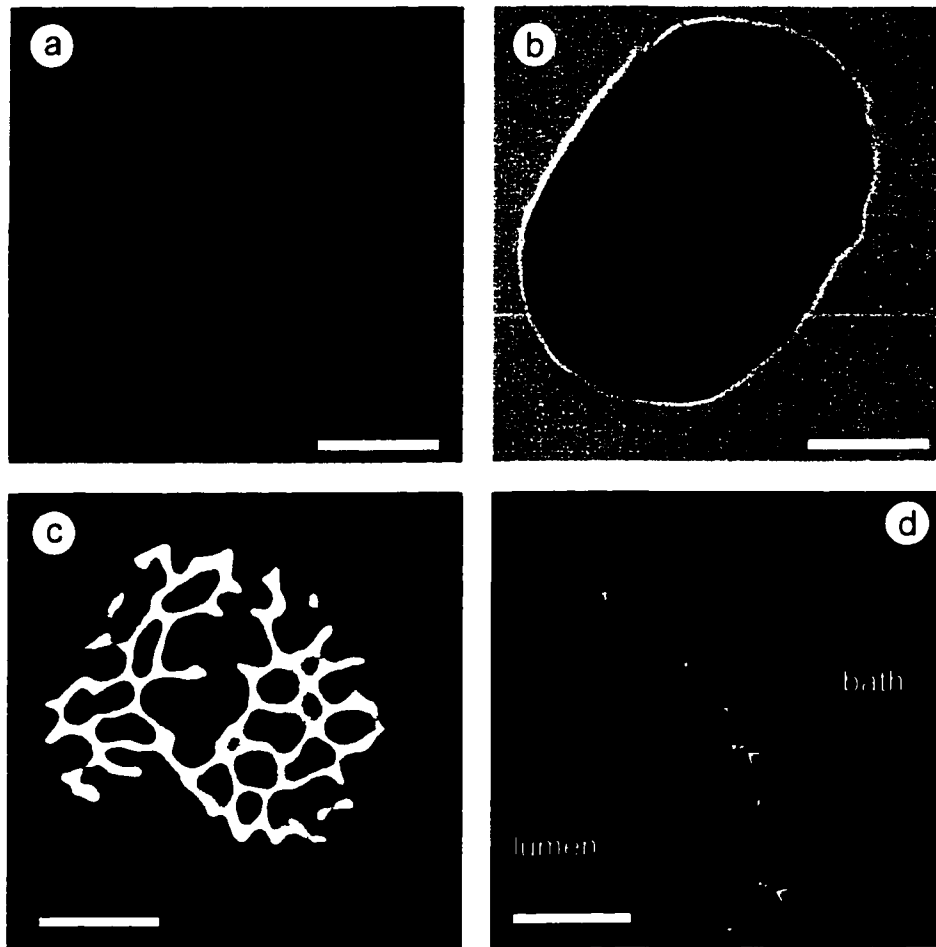


Figure 3.11 Fluid-phase markers added to the basal (*a, b*) or lumenal (*c, d*) side of the Malpighian tubule. (*a*) In this image Texas-red conjugated dextran (MW ~3000) added to the basal side is found largely outside the tubule (after 12 minutes). bar = 75 μm . (*b*) At higher resolution and after longer time periods (this image after 18 minutes in TR-dextran) the dextran was still mostly extracellular but appeared to be bound to the basal lamella, and also to the apical face of the tissue. It was also seen to a lesser extent in the cytoplasm: it is possible that this represented unconjugated Texas red dye. bar = 50 μm (Note **Fig. 3.1b** shows the same features in a vertical section.) (*c*) TR-dextran injected into the lumen outlines the convolutions of the macrovilli. bar = 50 μm (*d*). Lucifer yellow applied via a cannula to the lumen of the tubule. Arrows indicate vesicles presumed (from unstained controls) to be autofluorescence. Note that while dye was added to the centre of the lumen, it was only visible in the region immediately adjacent to the tissue. bar = 50 μm .

4. General Discussion

Motivation

The idea of a multi-drug pump, a molecular entity which, when expressed at the plasma membrane, could exclude or expel from the cell a tremendous variety of potentially toxic agents, seems at first rather fantastic. However, that is exactly what P-glycoprotein (P-gp) has been proposed to be (Gottesman & Pastan 1988). Although most studied in the context of multidrug resistance of cancer cells, the protein is ubiquitous and may have evolved as a means of protecting organisms from xenobiotics through strategic expression at secretory or blood-tissue barrier sites.

The need for protection from xenobiotics is clearly evinced by herbivorous insects which are involved in so-called “chemical warfare” with their food plants (Ehrlich and Raven 1964). The tobacco hornworm, *Manduca sexta*, is an interesting example of such an insect since the larva is capable of feeding exclusively on leaves of tobacco, *Nicotiana tabacum*, a plant poisonous to most animals, including humans, due to high levels of nicotine, a neurotoxic alkaloid. For this reason, and because of practical considerations (it is easily raised, grows to a large size and is widely used as a laboratory insect), our laboratory has been examining the possibility that P-gp plays a role in the tobacco hornworm’s resistance to nicotine (*e.g.* Murray 1996, McIntosh 1997).

Prior to the commencement of this thesis, the evidence supporting the hypothesis that P-gp is present in *Manduca* and functions as a multi-alkaloid pump was highly suggestive but limited and indirect. For example, P-gp immunoreactivity in *Manduca* had been seen at the blood-brain barrier, Malpighian tubules and midgut (Murray *et al.* 1994, Murray 1996). The first two tissues are sites of proposed (blood-brain barrier; Morris 1983a-c, Murray *et al.* 1994) or demonstrated (Malpighian tubule; Maddrell & Gardiner 1976, Murray 1996) nicotine transport. Increased

sensitivity to nicotine of the CNS of *Periplaneta* and of pupal and adult *Manduca* was correlated with decreased levels of P-gp immunolabelling (Murray 1996). In the Malpighian tubule, nicotine transport was found to be inhibited by verapamil, a P-glycoprotein inhibitor (Murray 1996). A fragment of a putative *Manduca* P-gp homologue was amplified from *Manduca* genomic DNA by PCR; hybridization of hamster and *Drosophila* P-gp probes suggested that there may be two or more homologues (M. Ell unpublished results reported by McIntosh 1997). Evidence from other species strongly suggested that P-gp is capable of mediating transepithelial alkaloid transport and of protecting organisms from xenobiotics, but was scarcely informative about its role in *Manduca*. For example, Schinkel *et al.* (1994) found that “knock-out” mice deficient in P-gp were abnormally sensitive to ivermectin and vinblastine, and that this correlated with increased levels of these drugs in the brain. In P-gp-transfected, and to a lesser extent untransfected, kidney cell lines, vectorial transport of P-gp “substrates” such as vinblastine and daunomycin had been clearly shown (Horio *et al.* 1989, Tanigawara *et al.* 1992, Pan *et al.* 1994). As discussed in **Chapter 1**, there was also evidence that the alkaloids nicotine, atropine and morphine, which are known to be transported by *Rhodnius* Malpighian tubules, could be P-gp substrates, although they were not (and are not) commonly considered as such.

A number of important questions were thus unanswered or required further examination when the experiments described in this thesis were contemplated. The first was, is there P-gp-like activity in the Malpighian tubule? This is different from the question of whether P-gp is present in the tubule. The latter had been addressed by immunohistochemistry and molecular methods, although the results still leave room for doubt. In particular, our laboratory’s effort to clone a P-gp homologue from a *Manduca* cDNA library proved unsuccessful (McIntosh 1997). Also, while the immunohistochemistry was performed with two antibodies, the monoclonal C219 and the polyclonal mdr(Ab-1), numerous reports have shown cross-reactivity between antibodies supposedly specific for P-gp (including C219) and other proteins (Thiebaut *et al.* 1989, Rao *et al.* 1994, 1995, Beaulieu *et al.* 1995). To find out if there is P-gp-like activity in

the Malpighian tubule, isolated tubules were tested for their ability to accomplish vectorial movement of typical P-gp substrates. Using radiolabelled vinblastine, a quantitative estimate of bath to lumen movement could be determined, while confocal microscopy gave the potential for examining transepithelial movement of daunomycin and other fluorescent P-gp substrates. Movement of these drugs against a concentration gradient, and inhibition of this movement by the P-gp inhibitor verapamil, would strengthen the evidence that P-gp is present in the Malpighian tubule and also provide evidence that it was functionally active, as it is in vertebrate epithelia.

Prior to these experiments, it was also unclear if the “multi-alkaloid pump” described in *Rhodnius* (Maddrell & Gardiner 1976) was also present in *Manduca*, in other words if the known nicotine-transporting ability of *Manduca* Malpighian tubules was the result of a pump of broader specificity. If the insect nicotine/alkaloid transporter is a P-gp homologue, a hypothesis of this thesis, one would expect nicotine transport to be inhibited by a P-gp inhibitor; one would also expect that nicotine, by competing for the same molecular pump, could inhibit the transport of a P-gp substrate. Murray (1996) began examining these questions, and showed that nicotine transport in *Manduca* could be suppressed by atropine, as in *Rhodnius*. She also presented preliminary data indicating that vinblastine might also accumulate in the lumen of *Manduca* tubules. My experiments sought to validate those data and test if vinblastine transport could be inhibited by nicotine.

Another issue that was unresolved was the cellular/subcellular location of P-gp expression in the Malpighian tubule, as well as the much more difficult matter of how it accomplishes nicotine transport, if it does. The prevailing understanding of P-gp-mediated *transepithelial* transport in mammalian systems is that drugs cross the basolateral membrane of polarized epithelia by (facilitated) diffusion, often assisted by a favourable electrical gradient. The distribution of P-gp is limited to the apical membrane, where it purportedly couples ATP hydrolysis to uphill movement of drug from the cell to the exterior. (As was discussed in **Chapter 1**, the concept of

P-gp as a classical transporter/pump is hotly disputed, since these normally have a narrow substrate specificity, quite unlike P-gp.) The C219 immunoreactivity of *Rhodnius* and *Manduca* Malpighian tubules, detected with the avidin-biotin peroxidase method, did not indicate strong polarization of expression: in *Manduca*, the entire cross-section was stained, with a darker layer at the basal lamella, while in *Rhodnius* staining was slightly stronger in the tissue closer to the lumen (Murray *et al.* 1994, Murray 1996). Immunoelectron microscopy of *Manduca* tubule cross-sections performed with gold-conjugated *mdr1* antibodies was also “inconclusive” with respect to subcellular distribution of the epitope (Murray 1996). While it was not a primary goal of my experiments, clues as to the location and mechanism of transport were sought in two ways. First, cannulation of the Malpighian tubule allowed drugs to be applied to either the basal or apical side selectively, which could conceivably shed light on the location of transport. Second, by confocal microscopy, the path that (fluorescent) P-gp substrates take through an epithelium might be revealed.

Findings

VINBLASTINE CROSSES THE MALPIGHIAN TUBULE EPITHELIUM (CHAPTER 2)

When 1 μ M vinblastine was applied to the basal surface of 3 to 4 cm lengths of Malpighian tubule *in vitro*, the radiolabel accumulated in the lumen of the tubule such that the mean lumen-to-bath ratio after 1 h was 3.0.

A natural interpretation of the results is that Malpighian tubule cells actively transport vinblastine into the lumen, but this is not proven. First of all, since we detected only radiolabel, we have not proved that the substance that accumulates in the lumen is unchanged vinblastine. Some attempt was made to find a thin-layer chromatography system that would effectively distinguish vinblastine from metabolites (not shown); a preferred method for analysis would be HPLC with fluorescence detection, which has been used to establish the pharmacokinetic profile

of *Vinca* alkaloids in animal studies (van Tellingen *et al.* 1993). Second, we do not at this point know what energizes the movement we observed. It is apparently not a transepithelial electrical potential since accumulation was not significantly reduced by placement of a shorting wire between the bath and the lumen. (However the small sample size does not allow us to eliminate the possibility that electrical potential *contributes* to the movement). Another possibility is that “proton-trapping” is responsible for accumulation. Moriyama (1996) describes this as a new type of active transport, which is energy-dependent but transporter-independent; amphiphilic compounds (acridine orange and rhodamine 123 were chosen as examples) permeate through the membrane in a neutral form and are accumulated as a protonated form inside acidic compartments. As previously discussed (**Chapter 2** discussion) this would be possible if the lumen was acidic relative to the bath and tissue, but we do not believe this to be the case. Some sort of active transport thus seems likely, but this still leaves many questions: is transport powered by ATP directly, or indirectly through co-transport? is there a pump of broad specificity or does vinblastine mimic some compound for which *Manduca* has evolved a specific transport mechanism? does the uphill component of transport occur at the basal or apical surface? what happens at the other surface? what happens inside the cell?

The question of the intracellular fate of vinblastine is of particular interest due to this drug’s cytotoxicity. A potent inhibitor of cell proliferation that acts by disrupting spindle microtubule function (Jordan *et al.* 1991), vinblastine also suppresses dynamics of individual microtubules in living interphase cells (Dhamodharan *et al.* 1995). These effects can be seen in mammalian cells at low nanomolar concentrations, implying that the 1 μ M levels applied to the Malpighian tubule, to say nothing of 100 μ M initially attempted, provide a serious challenge to the cell. Whether this is met by sequestering the drug away from microtubules (*e.g.*, in vesicles), resistance (*e.g.*, via altered tubulin), or if the effects the cell suffers in this short (1 hour) exposure time go undetected, is unknown. The same questions apply equally to the trans-

epithelial transport of vinblastine that has previously been observed in mammalian cell lines (Horio *et al.* 1989,1990, Hunter *et al.* 1991a,b,1993).

INHIBITION OF VINBLASTINE ACCUMULATION (CHAPTER 2)

When 25 μ M verapamil was added to both sides of the Malpighian tubule tissue, the vinblastine lumen-to-bath ratio was reduced to 1.0, a level which would be expected from simple equilibration of the two compartments. This effect was also seen if verapamil was applied only to the basal side of the tissue, but when applied to the apical side alone verapamil had no significant effect on vinblastine accumulation. Nicotine, when applied to the basal side of the tissue at 50 μ M or more, also significantly reduced vinblastine accumulation in the lumen.

These results (along with the observation that nicotine transport is verapamil-sensitive, Gaertner *et al.* 1998) are the most supportive to date of the hypothesis that the tobacco hornworm has a P-glycoprotein-like transporter and that nicotine is one of its substrates. At the same time, alternate explanations are acknowledged (see **Chapter 2** discussion). While verapamil is extensively used as a P-gp inhibitor, it is also recognized as an L-type Ca^{2+} channel blocker (Matsunaga *et al.* 1994), although there are no reports of such channels in epithelia. Verapamil is also reported to indirectly affect intracellular pH, perhaps through activation of a normally quiescent Na^+/H^+ exchanger (Keizer & Joenje 1989).

As recent studies with mammalian renal systems show, the presence of multiple transport systems with overlapping specificity is a real possibility, and would complicate the presentation of simple models (Simmons *et al.* 1997; see also **Chapter 1**). There is evidence that both vinblastine (McKinney & Hosford 1993, Sussman-Turner & Renfro 1995) and verapamil (Ott & Giacomini 1993) inhibit renal transport of TEA, a typical organic cation transporter substrate.

Thus, while the evidence does not allow us to be categorical about nicotine as a P-gp substrate, the possibility should be acknowledged, and the implications considered. One of the first questions that has yet to be answered is whether the *Manduca* version of P-gp has been modified in order to transport nicotine more effectively. Single amino acid substitutions have

been shown to change the substrate specificity of mammalian P-gps (Gros *et al.* 1991), so it would not be surprising to find that mammalian and insect P-gps have different specificities. In fact, considering the millions of years of divergence, the remarkable similarity in P-gp drug profiles from such diverse organisms as sponges, fish and humans (see **Chapter 1**) and now apparently insects, is much more surprising. The molecular techniques of cell-free or heterologous expression, production of chimeras, *etc.* could provide some insight into the relationship between DNA sequence and substrate profile, but in the case of *Manduca*, must await the cloning of a full-length coding sequence.

If nicotine is a substrate for human P-glycoprotein, there would be implications on several fronts. Since P-gp is thought to be involved in drug clearance, the possibility of mutual interference between nicotine and therapeutic drugs (such as vinblastine, colchicine and many other P-gp substrates) should be considered for smokers undergoing chemotherapy. In terms of renal clearance, this may not be a large concern since in humans nicotine appears to be transported by the organic cation transporter (Bendayan *et al.* 1990, Wong *et al.* 1992), but the effects of competition in hepatic clearance or at blood-tissue barriers could be more pronounced. While the “recent advances in studies on the blood-brain barrier transport of xenobiotics... have led to a change in the classical concept that the BBB is highly impermeable for hydrophilic compounds, but permeable for lipophilic ones” (Tsuji & Tamai 1997), the concept of a P-gp-mediated blood-brain barrier to nicotine would require a further adjustment. Although nicotine does clearly penetrate into the brain, it is possible that it does so only after overwhelming those barriers in place to keep it out. Given that nicotine drives use of tobacco, estimated to be the single largest cause of premature death in developed countries (cited by Pidoplichko *et al.* 1997), and since delivery of the drug to the central nervous system and its rate of elimination from the body are important factors in determining self-administration, tolerance and physical dependence (Busto *et al.* 1989), the possibility warrants investigation.

DAUNOMYCIN AND OTHER DRUGS ACCUMULATE IN THE CELLS BUT NOT THE LUMEN OF THE MALPIGHIAN TUBULE (CHAPTER 3)

Fluorescent drugs, including daunomycin, rhodamine 123 and acridine orange, were applied to living tubules in a well of saline on a coverslip, and examined by confocal microscopy. All of these drugs stained the cells of the tubule within 1 min, but none of the drugs appeared in the lumen even after 1-2 h of incubation. Neither verapamil nor nicotine altered the pattern of daunomycin staining. For some of the drugs the fast and intense Malpighian tubule staining was in sharp contrast to that of other tissues which stained lightly, and only after prolonged exposures. Fluid-phase markers appeared to penetrate the tissue, but more slowly, and remained largely extracellular.

In accord with my results with vinblastine and a number of studies with vertebrate renal epithelia, we expected that daunomycin in particular, and the other fluorescent dyes which are also considered P-gp substrates but for which transepithelial transport has not been described, would cross the Malpighian tubule tissue and accumulate in the lumen. As discussed in **Chapter 3**, although the possibility exists that some transport into the lumen occurred but was not visible, it is nonetheless apparent that the principle property of the Malpighian tubule with respect to handling daunomycin, rhodamine 123, acridine orange, Hoechst 33342 and ethidium homodimer-1, is rapid *cellular* accumulation. This is all the more interesting for the fact that other tissues from the same insect did not possess this property (for most of the drugs tested).

Although other investigators have noted intense staining of Malpighian tubule cells by many dyes (*e.g.* Nijhout 1975, Bresler *et al.* 1990, Meulemans & De Loof 1992, Sözen *et al.* 1997) the emphasis in the literature is generally on the expected, but not always convincingly demonstrated, movement of these dyes into the lumen. While there is no doubt that such transport exists for certain compounds, I propose that cellular accumulation without (immediate) movement into the lumen may be a normal and sensible approach taken by the Malpighian tubule to deal with many compounds. This proposed scavenging/sequestering function could be

an example of “storage excretion” which is already known in Lepidopterous adults that store nitrogenous compounds in their body cuticle and wing scales (Cochran 1985). It is also possible that the compounds slowly enter the urine by diffusion. This would be promoted by the large concentration gradient that is achieved and by the normal flow of fluid through the tubule, which is absent *in vitro*.

Global Evaluation and Perspectives

As described in the first section of this discussion, several unanswered questions concerning the relationship between P-glycoprotein and insect Malpighian tubule xenobiotic excretion were of interest to us and motivated the experiments described in this thesis. Let us now examine to what extent these questions have been answered, and what new approaches might lead to a greater understanding of the issues of interest.

The first question was, given the P-gp immunoreactivity and preliminary indications that P-gp is present in *Manduca sexta*, do the Malpighian tubules possess P-gp-like transport activity? The answer is undoubtedly yes: transport of a standard P-gp substrate (vinblastine), and its inhibition by a standard P-gp inhibitor (verapamil) at a standard concentration (25 μ M) qualifies the tubule as having P-gp-like activity.

The more pointed question is whether P-gp *is responsible* for the observed transport of vinblastine and nicotine in *Manduca*, and of alkaloids in insect Malpighian tubules in general. Although my results (and those of Murray 1996) are consistent with that proposition, to make such a claim at this point would be overextending the evidence. The use of further MDR substrates and inhibitors, of which there are many, both fluorescently and radioactively labelled, could strengthen the case. On the other hand, due to the current state of controversy regarding what P-gp actually does and the aforementioned interactions of P-gp substrates and inhibitors

with other molecular transport mechanisms, this substrate/inhibitor approach would still not settle the matter.

Since we have not “proven” that P-gp is a transporter in the Malpighian tubule, the question of where and how P-gp works in this system also remains wide open. The finding that verapamil’s effect was from the basal side (**Chapter 2**) indicates that *the site of inhibition* is more accessible from the basal side. However, it does not tell us where P-gp is located; at most it calls into doubt an apical localization (typical of most epithelia), for P-gp in the Malpighian tubule. Our confocal microscopy was intended to shed some light on the transepithelial route taken by P-gp substrates, but the fluorescent drugs used did not move through the tissue. Three possible conclusions arise: (1) P-gp is not present, and the results with vinblastine/verapamil must be explained in another way (2) P-gp is present, but the *Manduca* homologue does not have the capacity for daunomycin transport that vertebrate P-gps do, or (3) the methods used were inadequate to detect P-gp-mediated transport. “Cross-over” experiments, for example using radiolabelled daunomycin in the cannulated tubule assay, or fluorescently labelled vinblastine (which is not commercially available) under the confocal microscope, might help eliminate options (2) or (3). However, for reasons already alluded to, a more convincing demonstration of P-gp’s involvement in alkaloid transport will require a different approach.

Probably the most promising avenue to explore would be to use the genetic techniques which are well developed in *Drosophila*. A full coding sequence for a *Manduca* P-gp homologue would also be an asset to science, but attempts to attain this have so far been frustrating (McIntosh 1997). In *Drosophila melanogaster*, at least three P-gp gene homologues (*Mdr49*, *Mdr65* and *Mdr50*) have already been cloned and sequenced; however their functional properties have not yet been identified (Bosch *et al.* 1996). To the best of my knowledge, the only published work on these genes has been an appraisal of the sensitivity to colchicine of flies bearing mutations in *Mdr49* (Wu *et al.* 1991) and an assessment of the role of *Mdr65* and *Mdr49* in the electrodiffusional movement of ATP in Sf9 cells (Bosch *et al.* 1996). It would be of great

interest to know if other *Drosophila* P-gp mutants show sensitivity to other compounds, in particular to pesticides such as thiodicarb, to which resistance has been correlated with increased P-gp expression in the tobacco budworm (Lanning *et al.* 1996a,b). Other questions which could be addressed through molecular techniques such as PCR, *in situ* hybridization and deletion lines include: what is the distribution of P-gp in the fly? which isoform(s) is (are) expressed in the Malpighian tubules, if any? can P-gp expression be induced by exposure to drugs? what transport properties do the insect P-gps have when transfected in mammalian epithelial cell lines? These types of experiments seem much more likely to produce interesting answers to questions of broad interest than further work with supposed P-gp substrates and inhibitors in any system.

The hypothesis of this thesis was that alkaloid (particularly nicotine) transport in the Malpighian tubule of *Manduca sexta* is the consequence of a multidrug transport system similar to that mediated by P-glycoprotein in multidrug resistant cancer cells. As just discussed, my results taken together are equivocal with respect to P-gp's role in the Malpighian tubule. Research into the role of P-gp in multidrug resistance of cancer cells has also produced a large number of conflicting results and the inevitable conclusion is that the biological systems under study are more complicated than had been presumed (or hoped). With this in mind, I propose that my results *in toto* illustrate that clearing the hemolymph (blood) of xenobiotics is a role the Malpighian tubule meets through a combination of mechanisms. These include passive filtration, active transepithelial transport involving (but not exclusively) a P-glycoprotein-like pump which handles alkaloids, and a 'scavenger-like' absorption of many other compounds.

~ the end ~

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