A P-glycoprotein-like mechanism in the nicotine-resistant insect, *Manduca sexta*.

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To Mom and Dad, Jim and Wendy, Frank and Olivia

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PREFACE

The work presented in this thesis was carried out in the Department of Biology at the University of Ottawa under the supervision of Dr. C.E. Morris and the co-supervision of Dr. J.T. Arnason. The work described is my own except for the *Rhodnius* Malpighian tubule studies described in Chapter 4, which were performed in collaboration with Dr. S. Maddrell at the Department of Zoology at Cambridge University. Reference is made in the text to autoradiographical studies of insect tissue (Murray et al., 1992) that were performed by Michelle Quaglia, a graduate student in the laboratory of Dr. Morris.

ABSTRACT

The phenomenon of multi-drug resistance in tumor cells is mediated by the overexpression of a membrane protein called P-glycoprotein. Since its initial discovery in tumor cells, P-glycoprotein-like molecules have been found to transport a wide range of substrates in organisms from microorganisms to humans. To test the hypothesis that a P-glycoprotein-like mechanism is operating in insects, three insect species were used: *Manduca sexta*, *Periplaneta americana* and *Rhodnius prolixus*, the nicotine-resistant *M. sexta* was the model toxin-resistant insect. Immunostaining with a monoclonal antibody, C219, which recognizes a highly conserved epitope in all P-glycoprotein molecules, demonstrated positive immunolabelling of the Malpighian tubules of *M. sexta* and *R. prolixus* and at the blood-brain barrier of the central nervous systems of *M. sexta* and *P. americana*.

To assess the extent of P-glycoprotein involvement at the blood-brain barrier of *M. sexta*, I took advantage of the three different stages of the insect's life cycle, the actively feeding larval stage and the non-feeding pupal and adult stages. Using an extracellular recording technique to monitor levels of neural activity in isolated abdominal nerve cords, I demonstrated that, in contrast with larval *M. sexta*, the CNS of both the pupal and adult stages are both highly nicotine-sensitive. Immunostaining for P-glycoprotein in the metamorphosing CNS illustrated that the distribution of P-glycoprotein in the barrier region changes dramatically when the need for a barrier to dietary neurotoxins is lost.

M. sexta Malpighian tubules provided an ideal *in vitro* assay system to directly test our hypothesis that a P-glycoprotein-like molecule is involved in nicotine transport. Using an isolated Malpighian tubule preparation, the tubules were assayed for their ability, first, to concentrate nicotine in the tubule lumen (confirming the presence of an active transporter in the tubules) and, second, to determine if various drugs interfere with nicotine transport. Tubules bathed in 0.5 mM nicotine were found to concentrate nicotine in the secreted fluid at least 9-fold. Nicotine transport was inhibited with verapamil, a known inhibitor of P-glycoprotein. In addition, nicotine transport was competitively inhibited by another alkaloid, atropine, suggesting that the *M. sexta* pump may be a non-selective alkaloid pump. To further confirm that the insect pump is a P-glycoprotein-like multi-drug pump, the tubules were tested for their ability to transport a known P-glycoprotein substrate, vinblastine. The tubules were not only able to transport radiolabelled vinblastine, but this transport was also inhibited by verapamil.

P-glycoprotein is localized to the digestive tracts of mammals and of invertebrates, where it is thought to act as a defensive mechanism to protect organisms from dietary toxins. Immunohistochemical staining for P-glycoprotein demonstrated that it is also expressed in the *M. sexta* gut. In a parallel study, a monoclonal antibody against a P450 from a DDT-resistant housefly (Waters et al., 1989) was used to localize a cytochrome P450 monooxygenase enzyme (P450 or PSMO) to the *M. sexta* midgut. The co-localization of P450 and P-glycoprotein to the midgut suggests that larval *M. sexta* use a combination of P450-mediated metabolism coupled with transport to process dietary toxins.

ABRÉGÉ

Le phénomène de resistance à plusieurs drogues dans les cellules cancereuse est causé par une surexpression d'une membrane protéine appellée P-glycoprotein. Depuis sa découverte initiale dans les cellules cancereuses, des molécules semblables au P-glycoprotein ont été trouvées dans les organismes de microorganismes aux humains. Afin d'évaluer l'hypothèse qu'un mécanisme semblable au P-glycoprotein opère chez les insectes, j'ai utilisé les trois insectes suivantes: *Manduca sexta*, *Periplaneta americana* et *Rhodnius prolixus*. *M. sexta* fut utilisé comme insecte modèle, puisqu'il est résistant à la nicotine. Immunochemie avec une anticorp contre le P-glycoprotein, C219, demontre une réaction positive dans les tubes Malpighian est le système nerveux central.

Afin d'évaluer à quel point le P-glycoprotein est engagé au niveau de la system nerveux du *M. sexta*, j'ai pris avantage des trois étapes du cycle de vie de l'insecte, l'étape de la larve (qui mange beaucoup tobacco), l'étape pupal (qui ne mange pas) et l'étape adulte (qui ne mange pas). En utilisant une technique électrophysiologique afin de contrôler les niveaux d'activité dans les cordes nerveuses isolées de l'abdomen, j'ai demontré que contraiviement à l'étape de larve le système nerveux central des stages pupal et d'adulte ont une haute sensitivité à la nicotine. L'immunochemie pour P-glycoprotein a montré que la distribution du P-glycoprotein change dramatiquement lorsque le besoin d'une barrière à la diète neurotoxine est perdu.

Les tubes de Malpighian du *M. sexta* on fourni l'idéal système pour évaluer

directement notre hypothèse qu'une molécule semblable au P-glycoprotein est impliquée dans le transport de la nicotine. En utilisant une preparation isolée, les tubes furent testés pour leur habileté, premièrement, à concentrer la nicotine dans l'intérieur des tubes et deuxièment de déterminer si diverses sortes de drogues interfèrent dans le transport de la nicotine. Les tubes baignés dans 0.5 mM nicotine ont eu une concentration de nicotine dans les liquides sécrétés à moin 9-fois. Le transport de la nicotine fut interdit avec verapamil, un inhibiteur connu de P-glycoprotein. De plus, le transport de la nicotine fut interdit par un autre alcaloïde, atropine, suggèrant que la pompe *M. sexta* est une pompe alcaloïde non-selective. Afin de confirmer d'avantage que la pompe chez les insectes semblable au P-glycoprotein, est une pompe multi-drogue, les tubes furent testés pour leur habité à transporter vinblastine, un substrat du P-glycoprotein. Les tubes Malpighian pouvaient transporter le vinblastine et le transport fut supprimé par le verapamil.

P-glycoprotein est localisé dans les voies digestives des mammifères et des sous-organismes, où on croit que P-glycoprotein va agir comme mécanisme de défense afin de protéger l'organisme d'un régime toxique. L'immunochemie pour P-glycoprotein a démontré qu'elle est aussi évidente dans le ventre du *M. sexta*. Dans une étude parallèle, un anticorps contre cytochrome P450 (P450 ou PSMO) d'une mouche commune (*Musca domestica*; Waters et al., 1989) fut utilisé pour localisé P450 dans le ventre du *M. sexta*. La localisation du P450 et P-glycoprotein dans le ventre suggère que la larve du *M. sexta* utilise une combination de P450 couplé avec le transport pour tolérer effectivement le régime toxique.

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ABBREVIATIONS

ABC:	avidin-binding complex
BSA:	bovine serum albumin
CNS:	central nervous system
DAB:	diaminobenzadine
DIG:	digoxigenin
GABA:	gamma aminobutyric acid
hr:	hour
5-HT:	5-hydroxytryptamine
min:	minute
mw:	molecular weight
P450:	cytochrome P450-dependent monooxygenase
PB:	phosphate buffer
PBS:	phosphate buffered saline
PSMO:	polysubstrate monooxygenase
RT:	room temperature
SDS:	sodium dodecyl sulphate
s.e.:	standard error
sec:	second
SSC:	sodium citrate
V:	voltage

CHAPTER 1

GENERAL INTRODUCTION

Overview

The current thesis is based on the hypothesis that a P-glycoprotein-like mechanism is operating in insects, protecting them from host plant toxins. P-glycoprotein, an integral membrane protein, confers multi-drug resistance in mammalian tumor cells and also appears to protect tissues from endogenous or exogenous toxins. P-glycoprotein homologs have been identified in numerous organisms, both invertebrate and vertebrate, where they have a variety of transport functions including that of a xenobiotic transporter. To test the hypothesis that a P-glycoprotein-like transporter is operating insects, three insect species were used: *Manduca sexta*, *Periplaneta americana* and *Rhodnius prolixus*, with *M. sexta* being the model toxin-resistant insect. More specifically the objectives were:

1. To test the hypothesis that an alkaloid pump is an insect homolog of P-glycoprotein (Chapter 2).
2. To establish the relative sensitivities of isolated larval, pupal and adult abdominal nerve cords to nicotine and to examine how the expression of P-glycoprotein changes in the metamorphosing central nervous system (Chapter 3).
3. To assess the potential role of P-glycoprotein on nicotine transport by insect

Malpighian tubules (Chapter 4).

4. To establish whether a P-glycoprotein-like molecule is present in the gut of *M. sexta* (Chapter 5).

The following introduction reviews the behavioural, physiological, biochemical and genetic mechanisms that underly insect resistance to either host plant toxins or synthetic insects and is followed by a literature review of P-glycoprotein in mammals and in lower organsims.

1.1 Insecticide resistance: A challenge to pest management and basic research

Insect pests continue to cause severe crop damage and serve as major vectors for disease despite an estimated 4.3 billion dollars spent annually worldwide on insecticide production and application programs (Georghiou, 1990). The major obstacle to dependable control of insects is their ability to develop resistance to conventional insecticides at phenomenal rates. Resistance is defined as "the development of an ability in a strain of an organism to tolerate doses of a toxicant which would prove lethal to the majority of individuals in a normal (susceptible) population of that species" (WHO, 1976). In 1980, 260 species of agricultural pests had insecticide-resistant strains (Georghiou and Mellon, 1983). According to recently published figures, at least 504 species of insects and mites are resistant to one or more insecticides, with the majority of these species displaying multi-insecticide resistance (Georghiou, 1990). Multi-insecticide resistance is a particularly pernicious

phenomenon, in that once an insect develops resistance to one class of insecticide, it will simultaneously demonstrate resistance to other, often structurally unrelated, insecticides. In order to preserve the efficacy of current and future insecticides, it is essential that we more clearly understand the mechanisms by which insects acquire resistance so that we can design strategies to delay its onset.

The development of insecticide resistance is not surprising if viewed from an ecological and evolutionary standpoint. Herbivorous insects have coexisted for millions of years with higher plants. Plants, in response, have developed a chemical defence strategy based on the so-called allelochemicals (e.g. alkaloids, terpenes and phenols) to deter foraging (Harborne, 1990). These chemicals are often appreciably toxic and have favoured the evolution of defensive strategies in insects that allow them to overcome the chemical defenses of their host plants (Ehrlich and Raven, 1964). It has been suggested that insects which have become tolerant to toxins in their host plants may be preadapted to deal with applied synthetic insecticides since they already posses the molecular machinery to develop resistance (Brattsten et al., 1986; Brattsten, 1988).

An understanding of how insects tolerate host plant toxins may provide insight into how insects deal with synthetic insecticides. Herbivorous insects have evolved a multitude of defensive strategies to overcome the chemical defenses of their host plants. These adaptations can be classified as either behavioural, biochemical and/or physiological.

i) Behavioural resistance mechanisms

An insect's primary defensive strategy against plant toxins is simply avoidance of the toxin. Although this aspect of plant-insect interactions has received relatively little attention, there is growing evidence that phytophagous insects exhibit an array of avoidance behaviours which are derived from slight extensions of various foraging and feeding behaviours already in place (Tallamy, 1986).

For example, since many plant species produce toxins only in response to tissue damage by insects or pathogens, some insects have developed strategies to prevent the subsequent translocation of these defensive chemicals to feeding sites. Carroll and Hoffman (1980) have shown that certain beetles in the genus *Epilachna* chew a circular trench which isolates the leaf area on which they are feeding. This behaviour is thought to prevent the rapid translocation of a bitter-tasting allelochemical, thereby preserving the palatability and quality of the leaf. Another strategy used by some species of Lepidoptera and Coleoptera is to cut the leaf or petiole vein to prevent the flow of toxins into the leaf on which they are feeding (Compton, 1987; Edwards and Wanjura, 1989).

Some plants also possess compounds whose toxicity is greatly enhanced in the presence of UV light (Downum, 1986). Some insects successfully exploit these so-called phototoxic plants by avoiding photoactivation of the toxins by sunlight. Probably the best documented example of light avoidance behaviour is that displayed by leaf rollers or leaf miners (Berenbaum, 1978). These insects conceal themselves from the sunlight by feeding between two leaves or rolling the leaf over top of them

while they are feeding. Fields et al. (1990) have shown that larvae of two coleopteran insects that feed on the phototoxic plant *Hypericum perforatum* (St. John's wort) avoid potential photoactivation by feeding early or late in the day, when light intensity is lowest. Guillet et al. (1994) have demonstrated that, in the presence of light, larvae (*Sparganothis reticulatana*) that feed on the common daisy build an opaque shelter by bending and tying the ray florets over the receptacle so they can safely feed on the phototoxic tissue.

ii) Biochemical defense mechanisms

Once a toxic compound is ingested, insects have evolved an arsenal of detoxification enzymes that metabolize the toxin before it is able to reach its target site. The most important enzyme detoxification systems known in insects are the cytochrome P450-dependent polysubstrate monooxygenases (PSMOs; reviewed in Hodgson, 1985; Ahmad et al., 1986). Other enzymes important in detoxification include the glutathione transferases, carboxylesterases and epoxide hydrolases (Brattsen et al., 1979; Hodgson, 1985; Ahmad et al., 1986). The role of PSMO's in the metabolism of host plant toxins in insects is well established (Brattsen et al., 1977; Brattsten, 1979). Their importance in feeding behaviour was first recognized by Kreiger et al., (1971); they observed, in polyphagous lepidopterans which encounter a broad spectrum of plant toxins, dramatically higher levels of PSMO activity than in monophagous or oligophagous species.

The majority of xenobiotics that enter the body, whether they are toxins from

host plants or synthetic insecticides, are lipophilic and thus able to penetrate lipid membranes and accumulate in tissues where they interfere with fundamental biochemical processes. Since insects, like most other organisms, have a water-based excretory system, they have difficulties eliminating these non-polar compounds from their tissues. Thus, insects have evolved an arsenal of enzymes that convert lipophilic compounds into hydrophilic metabolites that can be more easily excreted. The overall metabolic process is divided into two phases (Ahmad et al., 1986). The phase I reactions are oxidation reductions catalyzed predominantly by PSMOs that increase hydrophilicity by insertion of one oxygen atom into the substrate. The monooxygenases are ideally suited for their role as primary metabolizing enzymes since they are relatively nonspecific in accepting a large variety of compounds as substrates (Brattsten et al., 1977). The resulting primary product is more water- soluble than the parent compound. These products of primary metabolism are either directly excreted or they may undergo further metabolism by phase II reactions catalyzed by glutathione transferases, epoxide hydrolases or carboxylesterases. Phase II reactions result in the formation of water-soluble products through conjugation of primary products with endogenous substrates such as glucose, sulphate, phosphate or amino acids and, with few exceptions, lead to harmless metabolites that are easily excreted at this point by transport mechanisms such as anion or cation transport mechanisms (Pratt, 1990).

Several molecules have been shown to inhibit the PSMOs in insects (Hodgson, 1985; Ahmad et al., 1986). The inhibitory effect of these molecules has received considerable attention because of the potential practical importance in pest control.

Generally, these inhibitors function by acting as electron sinks, diverting electrons away from the central heme protein, cytochrome P-450, or they can form irreversible complexes directly with the cytochrome (Hodgson, 1985). These compounds act as synergists since they are not toxic by themselves, but enhance the toxicity of a simultaneously applied plant toxin or insecticide (Metcalf, 1967). The most potent natural synergists are those containing the 1,3-benzodiole-methylenedioxyphenyl group (Ahmad et al., 1986). For example, sesame oil, which has long been known to improve the insecticidal action of pyrethrum (Brattsten, 1979), contains two compounds, sesamin and sesamolin, both of which contain a methylenedioxyphenyl group. The most successful commercially used synergist, piperonyl butoxide, also contains a methylenedioxyphenol group, and has been shown to be a potent inhibitor of the PSMOs in insects; however, its use as a large scale insecticide synergist is unlikely since it also inhibits the PSMO system of other organisms, including mammals (Guengerich, 1987).

iii) Physiological resistance mechanisms

Physiological resistance mechanisms to host plant toxins or insecticides include the removal of the toxin from sensitive sites by increased excretion, sequestration, and/or altered sensitivity of target sites.

Previous studies have demonstrated that, in many resistant insects, a large percentage of the ingested compound is rapidly excreted, suggesting that the toxin is cleared from the body before it has a chance to interfere with target sites. In a study

comparing the toxicity of the phototoxic allelochemical, α-T, in three lepidopteran insects, Iyengar et al. (1987) concluded that toxicity was inversely related to the rate of elimination of the ingested α-T. In a similar study, Hasspieler et al. (1988) found that variations in the susceptibility amongst mosquito strains to α-T were due to differences in their ability to excrete the compound. For example, the low sensitivity to α-T of the larval mosquito *Culex tarsalis* is attributed to its ability to eliminate twice the α-[^{3}H]T in the first hour after exposure, compared to the more sensitive mosquito *Aedes aegypti.* Rapid elimination appears to be an important defense against other toxins since larvae of the black swallowtail, which are specialized to feed on toxic furanocoumarin-containing plants, excrete the compounds and their metabolites 50 times faster than by non-specialized insects such as the fall armyworm, (Ivie et al., 1983; Bull et al., 1984). This metabolism eliminates the compounds from black swallowtail tissue before a toxic dose can accumulate, whereas non-specialized insects suffer poisoning.

If the toxin is not excreted, the insect can remove it from sensitive sites and actively or passively transport it to less sensitive sites (Duffy, 1980). This sequestration of toxins can both provide the insect protection from the toxic effects of the chemical, and serve as a source of chemical defense against its predators. For example, female Baltimore checkerspot butterflies lay their eggs on the leaves of iridoid glycoside-containing plants. These toxins act as feeding cues for the larvae upon emergence. Once ingested, the larvae sequester the toxins and retain them during metamorphosis, at which point they are redistributed to the wings and other tissue and

act as a defense against bird predators (Bowers, 1980, 1981).

Many lepidopterous larvae have large proteins in their hemolymph, called lipophorins and arylphorins, that bind hydrophobic toxins (Shapiro et al., 1988). These protein-toxin complexes can act as temporary forms of storage, reducing free (unbound) toxin concentrations to non-lethal doses. If a toxin is released slowly from the protein, detoxifying enzymes have an improved opportunity to metabolize it, since there will not be enough toxin at any one time to saturate the enzymes, thereby keeping doses non-lethal (Brattsten, 1991).

Many examples of target site insensitivity exist. Organophosphorous insecticides, for example, bind to acetylcholinesterase (AChE), the enzyme responsible for breaking down the neurotransmitter acetylcholine (ACh) at synapses, by competitively inhibiting ACh (Berenbaum, 1986). While the ACh/AChE complex breaks down in a few milliseconds to free the enzyme, the organophosphorous-enzyme complex breaks down very slowly, in minutes to hours. Since the enzyme is "pre-occupied", ACh remains available in the synaptic space and so binds to the receptor, continuing to depolarize the post-synaptic cell and thus producing tremors, convulsions and other physiological disturbances. Bonning (1990) reported seven mosquito species that have AChEs that are insensitive to these insecticides. Extensive overuse of these insecticides is thought to have resulted in the selection of an AChE with an altered catalytic subunit. This altered AChE has a decreased activity and provides an increased opportunity for detoxification enzymes to operate on the insecticides. Another example of target site insensitivity is seen in the milkweed bug, *Oncopeltus fasciatus*, which

feed on ouabain-containing plants. Ouabain, a cardiac glycoside, inhibits the Na^+/K^+ ATPase which is essential for maintaining the correct distribution of Na^+ and K^+ across neuronal membranes. The milkweed bug posseses a Na^+/K^+ ATPase that is resistant to ouabain; consequently, milkweed bugs display 200-fold less sensitivity to inhibition by ouabain than other species not specialized to feed on ouabain-containing plants (Moore and Scudder, 1986).

iv) Genetics of resistance

Resistance mechanisms, whether behavioural, biochemical or physiological, all ultimately have a molecular basis. With advances in the field of molecular biology, much of the current focus is being directed towards understanding the molecular basis of resistance. Resistant populations arise through heritable changes originating in the genomes of individual insects. Usually, a resistant allele is derived from the wild-type susceptible gene by mutation, resulting in an altered gene product that confers some degree of resistance to its carrier (Price, 1991). Mutations may include substitutions in DNA base pairs, chromosomal breakage events such as inversions or deletions, or amplifications of preexisting genes that confer defense mechanisms (Wilson, 1993). The mutational events that result in resistant alleles in an insect population have been poorly understood.

Recently, however, ffrench-Constant et al. (1993) demonstrated that in *Drosophila* a single point mutation in a GABA channel confers cyclodiene resistance. Cyclodiene insecticides exert their toxic effects by binding to GABA receptors in

insects (Matsumura et al., 1987). Sequencing and functional expression of a GABA channel cloned from a cyclodiene-resistant strain of *Drosophila* (ffrench-Constant et al., 1991), demonstrated that cyclodiene resistance arises from a single point mutation (alanine to serine) within the second membrane-spanning domain (ffrench-Constant et al., 1993). This single amino acid substitution is thought to confer enough steric or electrostatic hindrance to prevent the insecticide from reaching its binding site (ffrench-Constant et al., 1993).

Similarly, Knipple et al. (1994) demonstrated that houseflies resistant to DDT and pyrethroids possess a mutation in a voltage-sensitive sodium channel gene. Both of these insecticides act by targeting the voltage-sensitive sodium channel, altering the normal conduction of nerve action potentials, ultimately leading to paralysis (Sattelle and Yamamoto, 1988).

Gene amplification has also been identified as a molecular mechanism for generating insecticide resistance (Devonshire and Field, 1991). The amplification process results in an increase in the number of gene copies that code for a particular enzyme or enzyme system in a cell (Terriere, 1983). Gene amplification is clearly an important mechanism that increases levels of the resistance-conferring detoxification enzymes in aphids. In the green peach aphid *Myzus persicae*, resistance is positively correlated with increased carboxylesterase activity (Needham and Sawicki, 1971; Devonshire and Moores, 1992); amplification of the E4 and FE4 esterase genes results in an increased expression of esterase protein (Field et al., 1988).

1.2 P-glycoprotein: The multi-drug resistance transporter

Insects face continual exposure to both natural and synthetic insecticides in the environments in which they live and reproduce. Insect species that demonstrate resistance to unrelated insecticides with different modes of action are said to be multi-insecticide resistant. Insects continue to develop resistance to conventional insecticides. For example, some populations of the diamondback moth, *Plutella xylostella*, and the Colorado potato beetle, *Leptinotarsa decemlineata*, demonstrate resistance to virtually all of the available insecticides (Georghiou and Mellon, 1983; Georghiou, 1990). Recently, resistance to abamectin, a member of the newest class of pesticides to be introduced (Clark et al., 1994), has been reported in a field population of the diamondback moth and in two strains of Colorado potato beetle. Mechanisms by which insects can tolerate such a wide array of structurally unrelated insecticides remains poorly understood.

Multi-insecticide resistance is paralleled by a similar phenomenon, multi-drug resistance, in mammalian tumor cells. Cell lines displaying the multi-drug resistance phenotype are selected for resistance against a single drug, yet they simultaneously display unpredictable cross-resistance to a wide variety of unrelated cytotoxic drugs. The drugs most often involved in multi-drug resistance are alkaloids or antibiotics of plant or fungal origin or semi-synthetic analogs of these compounds (reviewed by Endicott and Ling, 1989; Gottesman and Pastan, 1993). Since many of these drugs are used extensively in chemotherapy, multi-drug resistance has had tremendous implications on the success of cancer chemotherapy.

Initial pharmacological and biochemical studies demonstrated that drug-resistant cells differ from drug-sensitive cells in the ability of resistant cells to maintain a lowered intracellular drug concentration. Further studies demonstrated that resistant cells overexpressed (30-fold increase compared with controls) a 140-170 kD integral membrane protein, subsequently called P-glycoprotein or the multi-drug resistance transporter, which was proposed to actively pump drugs out of the cell (Juliano and Ling, 1976). Proof of its role in drug resistance came from transfection experiments in which cDNA sequences encoding either the human or mouse P-glycoprotein was enough to confer a complete multi-drug resistance phenotype in otherwise drug sensitive cells (Gros et al., 1986b; Ueda et al., 1987).

Clinically, the discovery of P-glycoprotein has provided some insight into the failure of chemotherapy in cancer patients. Cancerous tumors derived from kidney, colon, adrenal gland, liver and pancreas often show intrinsically high levels of P-glycoprotein expression (Gottesman et al., 1991). Tumors that show initial sensitivity to chemotherapy usually have undetectable levels of P-glycoprotein expression and include tumors of the breast, ovaries, head and esophagus, tissues which do not normally express P-glycoprotein. However, increased expression of P-glycoprotein is commonly seen in these tumors following treatment with chemotherapy and, in the event of relapse, these cancers often become clinically resistant to chemotherapy (Gottesman et al., 1991). Because of the clinical importance of multi-drug resistance in the success of cancer chemotherapy, it is not surprising that there has been a surge of information published on P-glycoprotein in recent years.

i) Cloning and sequence analysis of P-glycoprotein genes

P-glycoprotein is encoded by a small family of homologous genes in mammals, referred to as the *mdr* gene family (Endicott and Ling, 1989). Members include two genes in humans, *MDR1* and *MDR2* (also called *MDR3*; Chen et al., 1986; Roninson, et al., 1986; van der Bliek et al., 1988), three members in mice, *mdr1* (or *mdr1b*), *mdr2*, and *mdr3* (or *mdr1a*) (Gros et al., 1986a; Gros et al., 1988; Devault and Gros, 1990; Hsu et al., 1990), and two members in hamster, *pgp1* and *pgp2* (Endicott et al., 1991), for which full-length cDNA clones have been obtained.

Sequence comparisons demonstrate considerable sequence identity among *mdr* family members. The human *MDR1* and *MDR2* genes share 76% identity (Gottesman and Pastan, 1993) and the mouse *mdr1* and *mdr2* sequences are 71% identical (Gros et al., 1988), yet transfection experiments have demonstrated that these genes are functionally different, since only mouse *mdr1* and *mdr3* and human *MDR1* can confer multi-drug resistance while their *mdr2* and *MDR2* counterparts cannot. Comparisons between the mouse *mdr3* and human *MDR1* sequences, both genes that encode proteins with similar function, share 88% identical sequences (Gottesman and Pastan, 1993).

Sequence analysis of the mouse *mdr3* and human *MDR1* full-length cDNAs led to the current structural model of P-glycoprotein (Gottesman and Pastan, 1988; see Fig. 1.1). This model proposes that P-glycoprotein is composed of 1280 amino acids, has 12 transmembrane domains in two homologous halves each containing six transmembrane regions and a large intracytoplasmic loop encoding an ATP binding site

(Gros et al., 1986a). Among the *mdr* genes that function as multi-drug transporters, the regions with greatest homology are the ATP-binding regions, and the first and second intracytoplasmic loops in each half of the molecule. The least conserved regions are the first extracytoplasmic loop, where even glycosylation sites are not preserved, the intracellular linker region connecting both halves of the molecule, and the amino and carboxy termini.

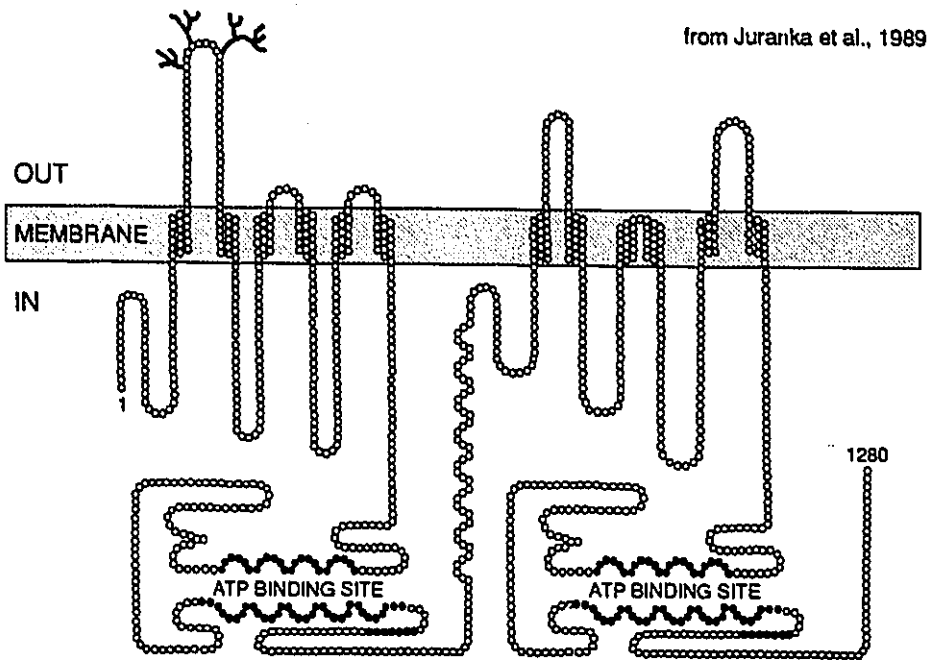


Figure 1.1. Proposed topology of the multi-drug resistance transporter, P-glycoprotein. The model is based on the human *MDR1* sequence, although all P-glycoprotein molecules share a similar structure. The protein is composed of 1280 amino acids (amino acids represented by circles), divided in two halves. Each half consists of six membrane-spanning regions, each with an ATP-binding region, represented by filled circles. The two halves are highly homologous (43% sequence identity; Gottesman and Pastan, 1993), although the first half has a putative glycosylation site in the first transmembrane domain (represented by black-branched structure). The epitope for the monoclonal antibody, C219, has been mapped (Georges et al., 1990) and is delineated by grey circles.

ii) P-glycoprotein is an ATP-dependent transporter

Studies with photoactivatable analogues of ATP (eg. azidopine) have demonstrated that P-glycoprotein binds ATP (Cornwell et al., 1987a; Schurr et al., 1989). Chimeric molecules constructed by exchanging homologous domains between *mdr* genes has shown that either ATP-binding domain can be exchanged without loss of function; for example, the ATP-binding folds of the non-drug resistant isoform, *mdr2*, can substitute for those of *mdr1*, without any loss of multi-drug resistance function in the latter (Buschman and Gros, 1991). However, Hamada and Tsuruo (1988b) have demonstrated that mutations introduced into either of the ATP-binding domains abrogated the ability of P-glycoprotein to transport drugs. The mutant P-glycoproteins were still, nonetheless, able to bind ATP analogues, indicating that a step subsequent to ATP binding, perhaps ATP hydrolysis, was impaired in these mutants.

Since resistant cells have lower intracellular drug concentration as compared to sensitive cells, it is thought that P-glycoproteins actively efflux drugs out of the cells. It has been proposed that drugs bind directly to P-glycoprotein and then are actively effluxed from the cell using energy derived from ATP hydrolysis (Endicott and Ling, 1989; Gottesman and Pastan, 1993). Indeed, P-glycoprotein binds photoactivatable drug analogs (Schurr et al., 1988) and purified P-glycoprotein preparations have ATPase activity (Hamada and Tsuruo, 1988a; Doige et al., 1992). Sharom et al. (1993) have partially purified P-glycoprotein from the membranes of a multi-drug resistant Chinese hamster ovary cell line and reconstituted the protein into phospholipid bilayers. The resulting proteoliposomes displayed an ATP-dependent uptake of colchicine, a

known P-glycoprotein substrate. Colchicine uptake into the proteoliposomes occurred against a 5.6-fold concentration gradient, a characteristic further indicative of an ATP-dependent transporter.

iii) Proposed mechanisms of action for P-glycoprotein

The most puzzling and controversial aspect of P-glycoprotein-mediated multi-drug resistance is the mechanism by which a single transporter can discriminate among and subsequently transport such a wide variety of drugs without transporting normal cellular constituents. Most current models for a wide variety of active transport mechanisms assume that domains of the protein form a hydrophilic pore-like structure where the substrate interacts with the protein from the aqueous phase, gaining access to the other side of the membrane by passing through the pore. For P-glycoprotein, however, an alternative hypothesis has recently been proposed (Higgins and Gottesman, 1992; Higgins, 1994). The key tenet of this model is that, rather than interacting with the transporter directly from the aqueous phase, the substrate (drug) initially interacts with the lipid bilayer, gaining access to the core of the membrane-associated transporter directly from the lipid phase. It is then proposed that the drug interacts with drug-binding sites within the pore; using the energy of ATP hydrolysis, the drug is then expelled out of the cell. How drug expulsion occurs remains highly speculative, although a current theory suggests that drug is flipped from the inner leaflet to the outer leaflet of the lipid bilayer (the flippase model; Higgins and Gottesman, 1992; Higgins, 1994).

Although it has been generally accepted that P-glycoprotein functions as a drug-efflux pump, alternative mechanisms have been proposed that suggest that P-glycoprotein does not function as a drug transporter *per se*, but functions to alter normal pH and/or electrochemical gradients across cellular membranes that in turn will modify the distribution of drugs across the membrane. These models are based on the observation that multi-drug resistant cells often have an elevated intracellular pH (Keizer and Joenje, 1989) and a lowered electrical membrane potential (Roepe, 1992; Roepe et al., 1993). One model suggests that the charged hydrophobic drugs, a characteristic of drugs involved in multi-drug resistance, may be differentially retained in these cells as described by the Nernst and/or Henderson-Hasselbach equilibrium.

Alternative and/or additional biological functions have also been attributed to P-glycoprotein. Valverde et al. (1992) suggest that P-glycoprotein functions not only as a drug transporter but is also a volume-sensitive Cl^- channel. Cells (NIH3T3 fibroblasts) transfected with a human P-glycoprotein gene possess a volume-activated, ATP-dependent, Cl^- selective channel that is not expressed in non-transfected cells. Cl^- channel activity was abolished in the presence of drugs that are substrates for P-glycoprotein (Gill et al., 1992). The possibility remains that Cl^- channel and multi-drug resistance activity are properties of the same protein, although it seems more likely that P-glycoprotein modulates an endogenous Cl^- channel (Higgins, 1995).

Similarly, Abraham et al. (1993) provide evidence that P-glycoprotein functions as an outwardly directed ATP-conducting channel. Indeed, multi-drug resistance cells have an increased rate of ATP release into the medium. Efflux of anionic ATP would

generate an electrochemical gradient providing the driving force for the movement of drugs out of the cells (Abraham et al., 1993).

Recently, Stein et al. (1994; see also Zeuthen and Stein, 1994 and Gottesman and Pastan, 1993), have proposed a model that encompasses facets of the various epiphenomena described in multi-drug resistant cells, as discussed above. They suggest that ATP hydrolysis is linked to the transport of protons into an internal space within the P-glycoprotein molecule, with anions following passively. Once within the transporter, ions will draw water in by osmosis, with amphipathic drugs (which have diffused from the membrane to the same internal space) being washed out of the cell with the water. Alternatively, if it where Cl^- being transported as a result of ATP hydrolysis, a proton, or other cation (including cationic drugs) following passively.

The extent to which P-glycoprotein, operating in any of these postulated modes, contributes to the observed P-glycoprotein-mediated reduction of cellular drug accumulation remains unclear. Further insight should come from the development of suitable reconstitution systems and expression systems.

iv) Expression and proposed functions of P-glycoproteins in normal tissues

Although P-glycoprotein gained notoriety for its role in multi-drug resistance in cancerous tumour cells, it has since been determined that P-glycoprotein is also expressed in normal tissues, where it is thought to have a protective role. Expression of the *mdr* genes in various normal tissues has been determined by detection of *mdr* mRNA using Northern blot analysis, and by immunocytochemical localization of P-

glycoprotein (e.g., Fojo et al., 1987; Thiebaut et al., 1987). The human *MDR1* P-glycoprotein is prominently expressed in the apical surface of the small and large intestines, at the biliary surface of hepatocytes, at the brush border of kidney proximal tubules (Thiebaut et al., 1987), and at the apical surface of capillary endothelial cells of the brain and testes (Cordon-Cardo et al., 1989; Thiebaut et al., 1989). In mice, both *mdr1* and *mdr3* RNA is found in the kidney, although *mdr3* RNA is predominant in the intestine, liver, brain and testes. Based on this distribution, it has been suggested that human *MDR1* and mouse *mdr1* and *mdr3* P-glycoproteins function to protect tissues either from toxins ingested in the diet (e.g. plant or microbial toxins) or produced by the body (e.g. by-products of normal metabolism) by actively extruding these compounds into the blood, bile, urine or intestinal lumen, preventing their accumulation in critical organs (Ames et al., 1990).

The proposal that P-glycoprotein functions to protect organisms from toxins has, until recently, remained merely speculative. Transgenic mice are now providing a model system for studying the role of the *mdr* genes (Smit et al., 1993; Schinkel et al., 1994). Schinkel et al., 1994, have successfully generated mice with a disruption in the *mdr3* gene. Both *mdr3* RNA and *mdr3* protein were absent in the intestinal tissue of these mice, a tissue that normally expresses abundant levels of *mdr3*-encoded P-glycoprotein. These mice were viable, fertile and phenotypically normal compared to control mice. However, when the transgenic mice were exposed to either the pesticide ivermectin or to the anti-cancer drug vinblastine, they were found to accumulate these toxins in many tissues, especially the brain. In fact, mice exposed to ivermectin,

suffered from severe neurotoxicity and death at a concentration routinely used to safely control mite problems in rats (Schinkel et al., 1994). This study is interesting on two counts: first, it demonstrates that a pesticide is a substrate for P-glycoprotein and, second, it is the first study to directly support the hypothesis that P-glycoprotein plays a critical role in protecting tissues from toxins.

Human *MDR1* and mouse *mdr3* are also highly expressed in the adrenal gland (Thiebaut et al., 1987; Croop et al., 1989) and in the endometrium of the gravid uterus (Arceci et al., 1988). This distribution has led to the speculation that P-glycoprotein may have a role in steroid secretion (Bradley et al., 1990). Several steroid hormones, notably progesterone, have indeed been shown to compete with other substrates for binding to mouse *mdr1* P-glycoprotein (Yang et al., 1989). Efflux studies suggesting that cortisol is actively transported by P-glycoprotein (van Kalken et al., 1993) lend further support to this speculation.

Until recently, the role of the human *MDR2* and its mouse homolog, *mdr2*, has remained largely a mystery, since, despite close homology to their drug-resistance producing counterparts (*MDR1* and *mdr1*, *mdr3* respectively), *MDR2* and *mdr2* failed to confer drug resistance when transfected into drug-sensitive cells (Gros et al., 1988; Devault and Gros, 1990). Human *MDR2* and mouse *mdr2* are expressed in liver, heart, skeletal muscle and spleen, both in humans (van der Bliek et al., 1987; Chin et al., 1989) and in mice (Croop et al., 1989). Immunohistochemical studies with antibodies specific for mouse *mdr2* protein (Cordon-Cardo et al., 1990; Buschman et al., 1992) and human *MDR2* protein (Smit et al., 1994) demonstrate that P-glycoprotein

expression is localized to the liver, specifically to the apical surface of the epithelial cells lining the lumen of bile ducts and caniculi, suggesting that this isoform functions in biliary excretion (Smit et al., 1993, 1994). To test this hypothesis, Smit et al., (1993) produced transgenic mice with disruptions in the *mdr2* gene. Histological and biochemical examinations of these mice demonstrated severe liver pathology and a complete absence of phosphatidylcholine in the bile. Phosphatidylcholine is the major phospholipid of the bile and is normally produced by epithelial cells of the liver canaliculi to emulsify bile acids secreted by hepatocytes. These findings indicate that *mdr2* encodes a protein essential for phospholipid transport into the bile.

Ruetz and Gros (1994b) have directly tested the hypothesis that mouse *mdr2* protein is a phosphatidylcholine transporter. They were able to successfully express mouse *mdr2* into the membranes of secretory vesicles isolated from yeast, producing an elegant system for testing its capacity to function as a drug or lipid transporter (Ruetz and Gros, 1994a). An assay involving a fluorescent phosphatidylcholine analog was then used to quantitate asymmetric lipid distribution in the outer and inner leaflets of the lipid bilayer of these vesicles. Expression of *mdr2* resulted in an ATP-dependent enhancement of phosphatidylcholine translocation to the inner leaflet of the membrane.

1.3 P-glycoprotein: member of a superfamily of membrane transporters

P-glycoprotein shares extensive sequence homology with numerous other energy-dependent transport proteins in bacteria and invertebrates (Juranka et al., 1989).

As a group, these proteins have now been classified as a superfamily of membrane transporters, referred to as the ABC (ATP binding cassette) superfamily (for review see Higgins, 1992). Members are generally identified by a highly homologous sequence (varying between 30 to 50% sequence identity depending on the transporters being compared) of approximately 200 amino acids that extends over the entire ATP-binding domain of members and includes a short motif associated with many nucleotide binding proteins (the Walker motif; Walker et al., 1982).

Gerlach et al. (1986) were the first to report a striking homology between mammalian P-glycoprotein and the HlyB protein, a 66 kD *Escherichia coli* membrane protein required for the transport of hemolysin. HlyB has six potential transmembrane domains and shows extensive sequence homology with the nucleotide binding domains of P-glycoprotein, leading some to propose that P-glycoproteins originated from duplication of the bacterial membrane transporter gene (Blight and Holland, 1990; Hyde et al., 1990). There are currently more than 40 members of this ABC superfamily in bacteria, including nutrient, peptide, polysaccharide and toxin transporters (Ames, 1986; Higgins, 1992). Eukaryotic members include a protein in *Drosophila* (Dreesen et al., 1988) which transports pigment precursors into pigment-producing cells in the eye, a transport protein appearing to mediate chloroquine resistance in *Plasmodium falciparum* (Foote et al., 1990), and a transporter, STE6 in the yeast, *Saccharomyces cerevisiae*, responsible for pumping a pheromonal mating factor (the **a** peptide; McGrath and Varshavsky, 1989). The yeast STE6 transporter shares significant homology with the mammalian P-glycoproteins, including 57%

sequence homology, conservative amino acid substitutions and a similar predicted structure and membrane topology (McGrath and Varshavsky, 1989; Kuchler et al., 1989), suggesting they may transport similar substrates. To test this possibility, Raymond et al., (1992) mutated the STE6 gene in yeast so that the pump was no longer able to transport the **a** peptide mating pheromone and the yeasts were thereby rendered defective in mating. The expression of mouse *mdr3* cDNA in the mutated yeast restored the ability of the yeast to export the **a**-mating factor and to subsequently mate.

ABC members of clinical significance include the cystic fibrosis transmembrane conductance regulator (CFTR), a chloride channel whose altered form results in cystic fibrosis (Riordan et al., 1989; Bear et al., 1992), and a peroxisomal membrane pump (Kamijo et al., 1990), which when in mutant form results in a fatal cerebro-hepato-renal dysfunction (Gartner et al., 1992).

1.4 P-glycoprotein in xenobiotic resistance

Many organisms successfully live and reproduce in highly polluted environments. Multixenobiotic transport activity may be one of several defense mechanisms that organisms use to help them resist natural or synthetic toxins in their environment. The following section reviews several invertebrate and parasitic systems in which a P-glycoprotein-like mechanism has been implicated in xenobiotic resistance.

i) Expression of P-glycoprotein in marine organisms

Many marine invertebrates successfully live and reproduce in water polluted with both natural and man-made xenobiotics. For example, marine sponges which are sediment dwellers and filter feeders, are indiscriminately exposed to a wide array of cytotoxic hydrophobic compounds that settle in marine bottom water. It is thus essential that sponges be equipped with a well-functioning defense system(s) against environmental chemicals. Because concentrations of pollutants are lower in tissues and body fluids than in surrounding sediments it has been proposed that the resistance of marine invertebrates to xenobiotics can be explained by the reduced accumulation of pollutants in the body (Graham-Bryce, 1987). 'Multi-xenobiotic resistance' and lack of accumulation are recognized as characteristics of P-glycoprotein mediated multi-drug resistance in tumor cells. Applying immunochemical and molecular biological techniques, Kurelec et al. (1992) tested the hypothesis that a multi-drug resistance like mechanism may be operating in two marine sponges, *Geodia cydonium* and *Verongia aerophoba*. Western blot studies revealed that polyclonal antibodies raised against hamster P-glycoprotein cross-reacted with a sponge polypeptide of molecular weight 125 kD (P125). Immunofluorescence staining with a polyclonal antibody and with the monoclonal antibody C219, which recognizes a highly conserved epitope found in all P-glycoprotein molecules (Endicott and Ling, 1989; Georges et al., 1990), demonstrated that P125 is a membrane bound protein. In addition, Northern blot analysis with a cDNA probe to human P-glycoprotein hybridized with a 4.2 kb transcript that compares appropriately to the 4.5 kb human transcript.

To test whether this protein has a similar function to P-glycoprotein, cubes of sponge tissue were incubated with the pollutant 2-acetylaminofluorene (AAF) and with or without verapamil, a ligand that binds to P-glycoprotein, inhibiting drug efflux and thus restoring the cell's sensitivity to drugs (Yusa and Tsuro, 1989). Verapamil (10 μg/ml) inhibited the bioaccumulation of AAF, corroborating the hypothesis that P125 functions in resistance to aquatic pollutants.

Similar results were obtained in the marine mussel *Mytilus edulis* and in the oyster *Crassostrea gigas* (Minier et al., 1993), both sessile bottom feeders that thrive in heavily polluted waters. DNA-DNA hybridization experiments demonstrated that mussels and oysters possess a highly conserved region constituting one or more genes of the ABC family. Western blot analysis with the monoclonal antibody, C219, revealed two proteins (220 and 240 kDa) which varied quantitatively with the level of organic pollution present at the sites where the mussels and oysters were collected.

In the marine worm, *Urechis caupo*, embryos survive and develop normally in highly polluted sediments of marine waters. *Urechis* embryos were shown by Western blot analysis to express a P-glycoprotein homologue (140-145 kDa) and to transport rhodamine (Holland-Toomey and Epel, 1993), a known substrate of mammalian P-glycoproteins (Neyfakh, 1988). In addition, transport was abolished in the presence of the P-glycoprotein inhibitor, verapamil.

ii) A P-glycoprotein homologue is involved in chloroquine resistance in Plasmodium falciparum, and in drug resistance in Leishmania

The lethal form of human malaria caused by *Plasmodium falciparum* is virtually uncontrollable in many areas of the world due to the development of drug, and in particular chloroquine, resistance (WHO, 1989). Chloroquine has previously been the most widely used and effective antimalarial drug. It acts against *Plasmodium* by accumulating in acidic vesicles in the digestive food vacuole where it is thought to interfere with digestive proteases (Cowman, 1991). Parasites resistant to chloroquine expel the drug rapidly in an unaltered form, thereby reducing levels of accumulation in the vesicles (Krogstad et al., 1987). In subsequent experiments, both verapamil (Martin et al., 1987) and another inhibitor of mammalian P-glycoprotein, desipramine (Bitonti et al., 1988), completely reversed resistance in chloroquine-resistant *Plasmodium* strains with no effect on chloroquine-sensitive parasites. This finding has led to the proposal that chloroquine efflux may involve a P-glycoprotein pump similar to that in mammalian multi-drug resistant tumor cell lines.

A probe to the highly conserved hydrophilic regions of human *mdr1* has identified two *mdr* genes in *P. falciparum* (*pfmdr1* and *pfmdr2*; Foote et al., 1989; Wilson et al., 1989). Comparison of *pfmdr1* with the human *mdr1* gene sequence shows that *pfmdr1* is 54% homologous. The predicted polypeptide is a 160 kDa protein and, as with mammalian P-glycoproteins, consists of 12 transmembrane domains with 2 ATP binding sites. Sequence analysis of *pfmdr2* suggests only 10

transmembrane domains with a single ATP-binding site encoding a protein of approximately 110 kDa (Zalis et al., 1993). Comparisons between chloroquine-resistant and sensitive isolates of *Plasmodium* showed *pfmdr1* is expressed at a higher level and is present in higher copy number in the resistant isolates (Wilson et al., 1989). There is on the other hand no evidence for a change in *pfmdr2* expression or gene copy number in resistant versus sensitive parasites (Zalis et al., 1993), and the role of *pfmdr2* has yet to be determined.

The *pfmdr1* gene product has been termed the P-glycoprotein homologue 1 (Pgh1) and has been localized in the parasite using affinity-purified antibodies made to a peptide encoded by a portion of the *pfmdr1* gene (Cowman et al., 1991). Western blot analysis demonstrated that Pgh1 is expressed throughout the asexual erythrocytic life cycle of the parasite, with the highest expression observed in the mature stages, the period (Cowman et al., 1991) when chloroquine exerts its antimalarial effect (Ward, 1988).

Immunofluorescence and immunoelectron microscopy have demonstrated that in mature parasites, Pgh1 is localized to the membrane of the digestive vacuole. This subcellular localization supports the contention that Pgp1 confers resistance by active efflux of chloroquine from the digestive vacuole (Cowman, 1991). Recently, *pfmdr1* has been implicated in mediating resistance to a second antimalarial drug, mefloquine, since overexpression of *pfmdr1* mRNA correlates with mefloquine resistance (Volkman et al., 1993).

P-glycoprotein gene homologs have also been isolated in drug-resistant parasites of the genus *Leishmania*. Infection with *Leishmania* results in leishmaniasis, a condition that results in necrosis of the skin and, in extreme cases, the visceral organs. Drug-resistance in *Leishmania* is frequently associated with amplification of a 68kb extrachromosomal circular DNA, called the H circle, which encodes P-glycoprotein-like genes (Samuelson et al., 1990; Ouellette et al., 1990; Callahan and Beverly, 1991). The role of these genes in drug resistance has recently been verified by Papadopoulou et al. (1994), who demonstrated that transfection of the H circle into drug-sensitive *Leishmania* confers resistance.

iii) Isolation of Drosophila mdr gene homologs

Five *mdr* gene homologs have been isolated to date from *Drosophila melanogaster*. The first to be identified were the white and brown genes whose protein products appear to transport pigment precursors into pigment-producing cells in the eye (Dreesen et al., 1988). Structurally, the white and brown protein are similar to the bacterial hemolysin transporter, HlyB, each consisting of a nucleotide-binding domain, which shares sequence similarity with the corresponding portion of P-glycoprotein, and a hydrophobic region with six putative transmembrane segments. However, unlike HlyB, the nucleotide binding domain in *Drosophila* white and brown genes is located at the N-terminus and the hydrophobic domain at the C-terminus, opposite to the order of domains in both HlyB and P-glycoprotein. This observation has led some to speculate that these opposite orientations in protein structure are related to the opposite

directions of transport mediated by these proteins (i.e. efflux versus influx; Juranka et al., 1989).

Three *Drosophila mdr* gene homologs have been identified and are referred to as *mdr49*, *mdr65* (Wu et al., 1991) and *mdr50* (Gerrard et al., 1993), designated according to their chromosomal location. The predicted amino acid sequence of the *Drosophila mdr49* and *mdr65* homologues is strikingly similar to mammalian P-glycoproteins. Both *Drosophila mdr49* and *mdr65* homologues are identical to the human and murine polypeptides at 42 to 45% of the amino acid positions along the entire length of the polypeptide.

Although the function of the *Drosophila mdr* homologs is not certain, disruption of the *mdr49* gene conferred colchicine sensitivity in a strain of *Drosophila* selected for resistance to colchicine, suggesting *mdr49* may be involved in drug resistance (Wu et al., 1991).

1.5 The tobacco hornworm as a model for the study of insecticide resistance

Larvae of the tobacco hornworm (*Manduca sexta*) successfully feed on nicotine-rich tobacco leaves, but can also subsist on other alkaloid-containing plants including atropine-laden belladona (deadly nightshade; Maddrell and Gardiner, 1976). *M. sexta* larvae display characteristics of the multi-drug resistance phenotype since both alkaloids, which are highly neurotoxic to most insects, show no apparent toxicity in *M.*

sexta, although they act on different CNS target sites: nicotine is an agonist at nicotinic acetylcholine channels and atropine an antagonist at muscarinic acetylcholine receptors (Satelle, 1978).

Nicotine, a powerful neurotoxic alkaloid present in the leaves of tobacco plants, is toxic to most insects but not to *M. sexta* larvae. The sensitivity of nicotine in other insect species stems from its molecular mimicry of the neurotransmitter, acetylcholine (ACh). Nicotine, binding to ACh receptors in the CNS, evokes a massive discharge of neural activity leading, eventually, to the blockage of synaptic transmission (Flattum and Sternburg, 1970). How does the *M. sexta* larva protect sensitive cholinergic synapses? First, excretion (Maddrell and Gardiner, 1976) and detoxification (Snyder et al., 1994) of nicotine by non-neural *M. sexta* tissues play a critical role in reducing blood (hemolymph) levels of nicotine, and second, the CNS has local mechanisms to protect itself. The most straightforward demonstration of this last point is that the isolated nerve cord from larval *M. sexta* is 100-fold less sensitive to nicotine than nerve cords isolated from the cockroach, *Periplaneta americana* (Morris, 1984).

Maddrell and Gardiner (1976) demonstrated that high levels of nicotine in the hemolymph are reduced by rapid excretion of unmetabolized nicotine by a nicotine transporter present in the Malpighian tubules. This transport system is saturable, concentrating nicotine in the secreted fluid to more than four times its concentration in the surrounding hemolymph (Maddrell and Gardiner, 1976). A similar nicotine transport system was also described in the Malpighian tubules of other insects, including *Rhodnius prolixus*, *Calliphora erythrocephala*, *Musca domestica* and *Pieris*

brassicae (Maddrell and Gardiner, 1976). In addition to nicotine, Maddrell and Gardiner (1976) found that *Rhodnius prolixus* tubules also transport atropine and morphine. In these studies, atropine competitively inhibited transport of both nicotine and morphine, indicating that these alkaloids are transported by the same system.

In addition to the rapid excretion of unmetabolized nicotine, Synder et al. (1994) have recently demonstrated that *M. sexta* larvae are able to metabolize nicotine and that this metabolism is reversibly induced by dietary nicotine. Insects are known to possess detoxifying enzymes, most notably the PSMO enzyme system, and their role in metabolism of host plant toxins and synthetic insecticides is clearly established (see Hodgson, 1985 for review). In *M. sexta* larvae, some metabolism of nicotine by the CNS and by tracheae has been reported (Morris, 1983a), although additional metabolism of nicotine by other tissues has, until the recent study by Synder et al. (1994), not been detected (Self et al., 1964b). In an earlier whole animal study, Self et al. (1964a) demonstrated a rapid excretion (< 5 min) of nicotine by *M. sexta* larvae but found no evidence for metabolism (Self et al., 1964b). Discrepancies between the current and previous studies could be explained by several factors. First, it is possible that Snyder's group may have inadvertently selected for a population of *M. sexta* in which nicotine-inducible PSMO is very pronounced. Second, Snyder et al. (1994) used high-performance liquid chromatography to identify cotinine-N-oxide (a PSMO metabolite) as the major nicotine metabolite produced by the larvae and they argue that the paper chromatography technique used by Self et al. (1964b) may have failed to resolve this metabolite from parent nicotine. Morris (1983a), however, demonstrated

that cotinine-N-oxide can be separated from nicotine, both in polar and apolar thin layer chromatographic systems and cotinine-N-oxide was not one of the CNS metabolites.

Whatever the basis for the discrepancy, it seems unlikely that *M. sexta* relies on gut or hemolymph detoxification of nicotine to protect its CNS. Snyder et al., (1994) show that the ability of non-neural *M. sexta* tissue to metabolize nicotine is not constitutive, but rather is induced by dietary nicotine. Thus, after 6 hours of feeding on a radiolabelled nicotine diet, approximately 60% of the label recovered from the feces was unmetabolized nicotine, indicating that a mechanism for excreting parent nicotine is needed. Maddrell and Gardiner (1976) demonstrated that the Malpighian tubule transport of nicotine by *M. sexta* larvae is not dependent on the presence of nicotine in the diet. Hence, at the larval Malpighian tubules at least, the ability to transport nicotine is not an inducible mechanism. By contrast, tubules from adult *M. sexta*, a non-feeding stage, presumably have no need to excrete nicotine and were found to be unable to transport nicotine (Maddrell and Gardiner, 1976). As with the larval Malpighian tubules, the ability of the larval CNS to metabolize nicotine does not require induction (Morris, 1983a; Snyder et al., 1993); despite the absence of nicotine in the diet, *M. sexta* nerve cords (and tracheae) metabolized nicotine. Therefore, it appears that the ability to metabolize nicotine is a constitutive property of the larval CNS, just as nicotine pumping is a constitutive property of the Malpighian tubules. For the newly hatched tobacco-feeding larvae, a protective mechanism that is "up-and-running" immediately without any latency is critical since the larvae commence feeding

immediately.

In spite of the rapid clearance of nicotine from the hemolymph, nicotine levels in tobacco-feeding larvae still remain high enough (ie. ~2.3 X 10^{-5} M) to adversely effect the CNS if it were not further protected (Self et al., 1964a).

Nicotine is an amphipathic molecule, producing in solution a pH-dependent equilibrium mixture of water-soluble (protonated) and lipid-soluble (unprotonated) forms. The hemolymph of *M. sexta* has a pH of 6.6 and a pKa of 8, and consequently over 98 percent of the nicotine is present in the water-soluble form (Self et al., 1964a). Structural analysis of the CNS of *M. sexta* shows that tight junctions between perineurial cells that surround the central neuropile of the CNS form a peripheral diffusion barrier to water-soluble molecules (Pichon et al., 1972; Lane and Swales, 1979). Developmental analysis of this blood-brain barrier in larval ganglia demonstrated that tight junctions between perineurial cells first form not in the larva, as is the case in other insects, but in the embryo of *M. sexta* (Lane and Swales, 1979). This timing is probably instrumental in allowing the larvae to commence feeding on tobacco immediately upon hatching, since a "loose" blood-brain barrier would be difficult to defend.

The small percentage of nicotine that exists in the lipid-soluble form in *M. sexta* hemolymph (2% at pH 6.6) can circumvent barriers to paracellular diffusion, crossing transcellularly the perineurial region of the CNS. This penetrating nicotine will bring brain nicotine into Henderson-Hasselbach equilibrium with blood nicotine. Thus, even though it is a small percentage, the lipid-soluble species is tremendously important and

must be dealt with metabolically, that is, it must be detoxified, excreted or both, before reaching nicotine-sensitive ACh receptors in the neuropile.

Metabolism of nicotine in the *M. sexta* CNS, presumably at the blood-brain interface, has been demonstrated (Morris, 1983a; Snyder et al., 1993; Murray et al., 1994). Metabolism alone cannot, however, account for the observed insensitivity of the *M. sexta* CNS. Morris (1983a) demonstrated that incubation of isolated nerve cords in 1 mM nicotine (a concentration that failed to alter the level of endogenous neural activity; Morris, 1984) resulted in greater than half of the penetrating nicotine remaining unmetabolized, suggesting that, at this high concentration, nicotine saturates metabolizing enzymes. Residual unmetabolized nicotine should penetrate beyond the perineurial cells and, by simple diffusion, gain access to sensitive ACh receptors in the neuropile. But evidently it does not.

This argues that additional mechanisms must be operating to prevent nicotine from equilibrating in the extracellular space, since this compartment is contiguous with the sensitive neuropile. Morris (1983a,b,c) postulated that a transporter, similar to the one expressed at the insect's Malpighian tubules, could function to remove nicotine from the extracellular space, perhaps transferring it to cellular compartments where it can be held until it is metabolized.

What evidence do we have for the existence of a nicotine transporter in the *M. sexta* CNS? In the CNS, nicotine uptake appears to be saturable, and efflux rates respond to ambient nicotine levels and to temperature in a manner that is not consistent with free diffusion of nicotine (Morris, 1983a,b). Atropine, which strongly inhibits

nicotine transport in *Rhodnius prolixus* Malpighian tubules (Maddrell and Gardiner, 1976), significantly decreased the extent and rate of CNS uptake of nicotine, and increased the efflux rate of radiolabelled nicotine (and its metabolites) from the CNS (Morris, 1983a,b). N'-methylnicotinamide (NMN), a tertiary amine with a structure similar to nicotine, also increased the rate of nicotine metabolite efflux from the *M. sexta* CNS (Morris, 1983c). A conclusion that could be drawn from these studies is that the CNS has a slowly exchanging compartment into which it transports any nicotine that crosses the blood-brain barrier interface. Movement into this compartment (which could, for example, be a perineurial endomembrane compartment) has the effect of preventing nicotine from reaching the extracellular space. The flux studies lend support to the notion that a transport system, similar to that in the Malpighian tubules, is operating in the *M. sexta* CNS.

Electrophysiological experiments have demonstrated that co-application of nicotine and NMN increased the sensitivity of the *M. sexta* CNS to nicotine (Morris, 1984; Trimmer and Weeks, 1989) even though NMN does not affect CNS metabolism of nicotine (Morris, 1983a). These results, taken with NMN's effect on efflux, can therefore be interpreted as NMN competitively inhibiting the sequestration of nicotine in a compartment that is discontinuous from the nicotine-sensitive regions in the neuropile.

Some cases of apparent nicotine sequestration could be explained by factors which either increase the intracellular pH or lower the extracellular pH. Under conditions of decreased extracellular pH more of the unprotonated/membrane-soluble

nicotine leaving a cell will assume the ionized (lipophilic) form and therefore be unavailable for diffusion back into the cell. The rate at which radiolabelled nicotine is able to reach the periphery of the cell, where sampling occurs, is therefore increased. NMN is however a neutral amide and therefore unlikely to alter efflux kinetics through pH effects. Atropine and nicotine are weak bases, not neutral compounds; Morris (1983b) points out that if atropine and nicotine were exerting their effects simply by altering intracellular pH, then the effect should be additive. However, nicotine-plus-atropine produced no effect beyond that of atropine alone (Morris, 1983b).

Most recently, M. Quaglia, a student in Dr. Morris' lab used frozen-section autoradiography to provide visual evidence first, for the existence of a blood-brain barrier to nicotine, and second, for the metabolic nature of this barrier. This technique involved exposure of the freshly isolated *M. sexta* CNS to radiolabelled nicotine to assess the distribution of nicotine in the CNS. Radiolabelled nicotine was detected throughout the cortical regions but was absent from the neuropile, providing unequivocal evidence for the existence of a barrier to nicotine (see Murray et al., 1994). Co-incubation of nerve cords with radionicotine and piperonyl butoxide, a potent inhibitor of the PSMO detoxifying enzymes, resulted in the uniform distribution of label across the ganglion, suggesting that PSMOs constitute a saturable component of the metabolic barrier. If the PSMOs are working in concert with a nicotine pump, as predicted by Morris (1983a,b,c), then inhibition of the PSMOs should shift the metabolic burden on the nicotine pumps, at some point overwhelming their capacity to protect the neuropile. Unmetabolized intracellular nicotine would saturate the

pumps, and nicotine would continue to diffuse towards the neuropile.

Hence, the evidence provided to date demonstrates that the larval *M. sexta* CNS possesses a highly effective barrier to nicotine, consisting of: (1) a physical barrier depending on tight junctions between perineurial cells that surround the sensitive neuropilar region (Lane and Swales, 1979; Morris and Harrison, 1984) and 2) a metabolic barrier that consists of detoxifying enzymes and may also include nicotine pumps (Morris, 1983a,b,c; Morris, 1984).

1.6 Research objectives

The expression of proteins homologous to the mammalian multi-drug resistance transporter in drug-resistant parasites and in marine organisms living in polluted environments suggests that active transport of xenobiotics may be a common defense mechanism against toxic compounds. Herbivorous insects face continual exposure to a wide range of xenobiotics in their environments, including plant toxins and synthetic insecticides. The current thesis is based on the following hypothesis: a P-glycoprotein-like mechanism is operating in insects, protecting them from host plant toxins. To test this hypothesis larval *M. sexta* was used as a model xenobiotic-resistant insect. *M. sexta* larvae provide an ideal model system to address this question, since they tolerate chronically high levels of the potentially neurotoxic alkaloid, nicotine. Nicotine resistance in *M. sexta* has been extensively studied, and resistance appears to require the contribution of an as-yet unidentified nicotine transporter. From hereafter *M. sexta*,

will be referred to as *Manduca*.

An immunohistochemical study, using antibodies directed against highly conserved regions of all P-glycoprotein molecules, was performed to determine if a P-glycoprotein-like molecule is expressed in *Manduca*. The *Manduca* CNS and Malpighian tubules were immunopositive for P-glycoprotein. Immunostaining in the CNS co-localized with a nicotine barrier region previously described by M. Quaqlia (see Murray et al., 1994).

To assess the extent of P-glycoprotein involvement at the blood-brain barrier I took advantage of the different developmental stages of the *Manduca* lifecycle. Unlike the larval stage, the pupal and adult stages are both non-feeding stages. An electrophysiological study was undertaken to establish the relative sensitivities of the CNS of these stages to nicotine. A parallel immunohistochemical study was performed to determine if and how P-glycoprotein expression changes in the metamorphosing CNS.

To directly test our hypothesis that a P-glycoprotein-like mechanism is involved in nicotine transport, an isolated malpighian tubule preparation was used. Drugs known to interfere with P-glycoprotein function in other preparations were tested for their ability to interfere with nicotine transport.

Finally, immunohistochemistry was used to further assess the extent of P-glycoprotein expression in *Manduca* tissue, and an *in situ* hybridization study was performed to assess the expression and distribution of the PSMO detoxifying enzymes.

CHAPTER 2

A PUTATIVE NICOTINE PUMP AT THE METABOLIC BLOOD-BRAIN BARRIER OF THE TOBACCO HORNWORM

2.1 Introduction

The immunohistochemical detection of P-glycoprotein in capillaries at the human (Cordon-Cardo et al., 1989; Thiebaut et al., 1989) and rat (Cordon-Cardo et al., 1989) blood-brain barrier led to the suggestion that this pump restricts entry of circulating drugs and other xenobiotics into the mammalian central nervous system (CNS; Cordon-Cardo et al., 1989; Thiebaut et al., 1989). Transgenic mice with a knockout of the gene, *mdr3*, which encodes P-glycoprotein, were incapable of excluding the cytotoxic alkaloid, vincristine, and the insecticidal compound, ivermectin (both substrates for P-glycoprotein) from the brain when these xenobiotic substances were present in the blood (Schinkel et al., 1994). This study is the most direct piece of evidence to date that P-glycoprotein prevents the accumulation of toxins in the brain, and hence, is the best evidence implicating P-glycoprotein as a functional component of the mammalian blood-brain barrier.

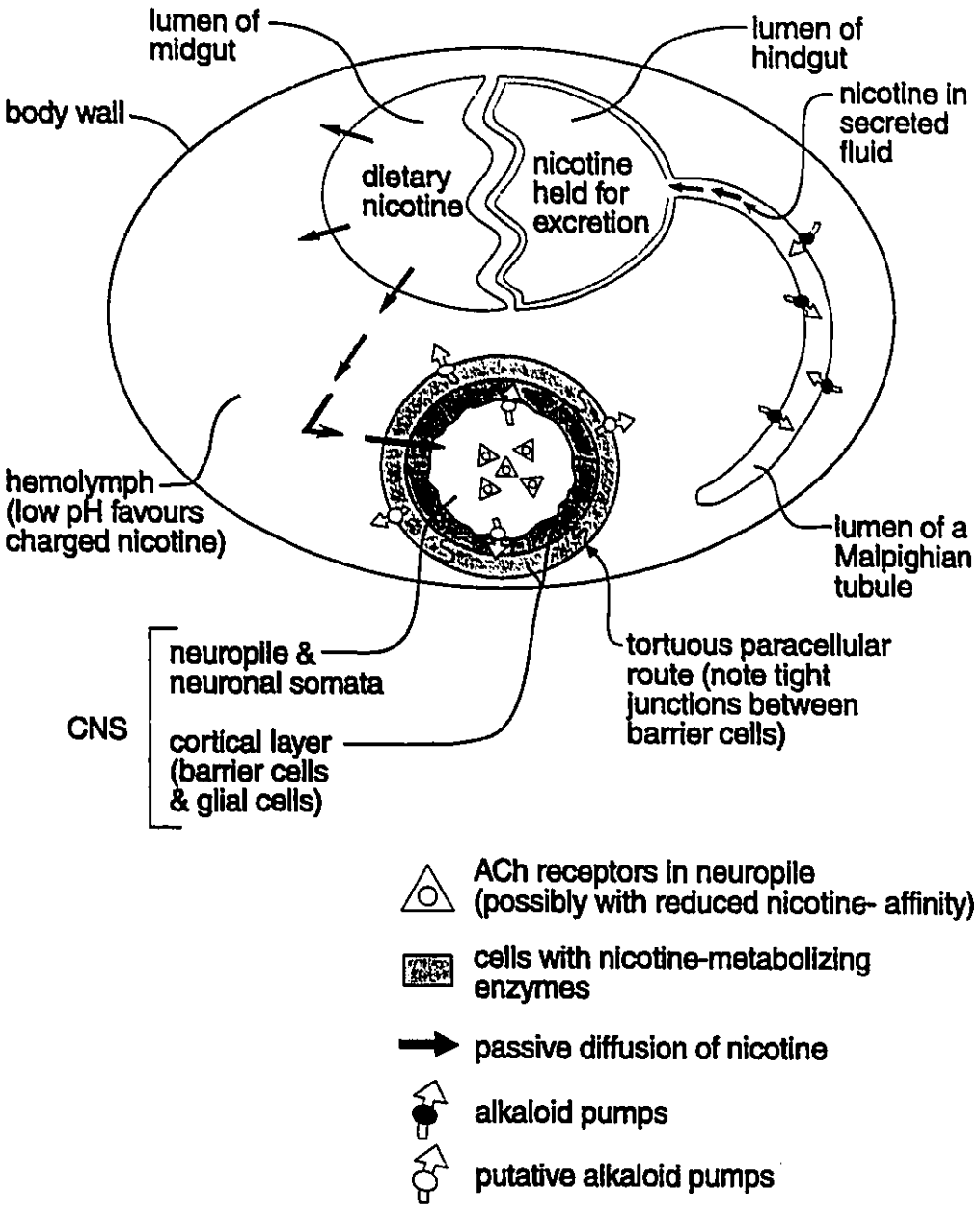
Anatomically, the mammalian blood-brain barrier depends on tight junctions

between the endothelial cells of the blood capillaries of the brain, and consequently is a structure as diffuse as the CNS capillaries themselves. Insects also have a highly developed blood-brain barrier but its topology is simpler; instead of ramifying through the CNS as in mammals, the barrier is an epithelium-like structure surrounding a solid avascular CNS (Fig. 2.1). Herbivorous insects require a sophisticated blood-brain barrier since their plant diets often produce blood (hemolymph) inimical to neural function, high in K^+ and Mg^{2+} and laden with neurotoxic allelochemicals. A prime example is the tobacco hornworm (*Manduca sexta*), which tolerates high levels of blood nicotine. The intact CNS of this caterpillar tolerates 1 mM nicotine (Morris, 1984), whereas, for most insects, 10 μM nicotine produces massive neuroactivation (Sattelle, 1978; Morris, 1984). A barrier associated with the cortex of the *Manduca* CNS appears to be critical in restricting nicotine, since 50 μM nicotine becomes toxic following disruption of the cortical layer surrounding the nicotine-sensitive neuropile (Morris, 1984; Morris and Harrison, 1984). The electrophysiological evidence (Morris, 1984) for a blood-brain barrier to nicotine has been confirmed by autoradiographic evidence that nicotine is excluded from the neuropile of *Manduca* but not from that of a nicotine-susceptible insect, the cockroach *Periplaneta americana* (see Murray et al., 1994). Exposure of the CNS to 100 μM radiolabelled-nicotine resulted in heavy labelling throughout the *Periplaneta* CNS, whereas in *Manduca* label was confined to the cortical regions and was absent from the neuropile.

Ultrastructural analysis of the *Manduca* CNS has revealed that tight junctions between cortical perineurial cells form a peripheral diffusion barrier to hydrophilic

compounds such as the cationic form of nicotine (Lane and Swales, 1979; Morris and Harrison, 1984). Blood (hemolymph) nicotine is, however, a mixture of cationic and neutral lipophilic forms. To reach the neuropile, cationic nicotine must circumvent tight junctional barriers either directly or through deprotonation of its tertiary nitrogen. The deprotonated molecule is lipophilic and so can penetrate transcellularly. To protect the neuropile, penetrating nicotine must be detoxified, extruded or both. Previously, Morris (1983a-c) demonstrated in *Manduca* CNS that penetrating nicotine is not fully detoxified and hence argued that a nicotine pump is required as part of the blood-brain barrier machinery (see Fig. 2.1; Morris 1983a-c; Morris, 1984; Morris and Harrison, 1984). The excretory Malpighian tubules of *Manduca* have been shown to possess an alkaloid pump for which nicotine is a known substrate (Maddrell and Gardiner, 1976). To test the hypothesis that this alkaloid pump is an insect homolog of the P-glycoprotein multi-drug pump, and that it is present at the blood-brain barrier of the insect CNS, an immunohistochemical study was performed using the C219 antibody which recognizes a highly conserved epitope present in all P-glycoprotein molecules discovered to date.

Figure 2.1 Schematic cross-section of the tobacco hornworm, depicting known and postulated means of protecting the cholinergic CNS from nicotine. Following ingestion of tobacco, blood (hemolymph) nicotine levels are reduced by a powerful transport system, pumping nicotine into the lumen of the Malpighian tubules where it is excreted in its unmetabolized form (Maddrell and Gardiner, 1976). Nicotine levels nonetheless remain high enough to have adverse effects on unprotected nicotine-sensitive CNS synapses [Morris, 1984; some cholinergic synapses may be non-nicotinic (Trimmer and Weeks, 1989)]. Physical barriers (the barrier or perineurial cells) and detoxifying metabolism of nicotine provide the next lines of defense for the neuropile (Morris, 1983a; Morris, 1984; Morris and Harrison, 1984). Some nicotine evades detoxification (Morris, 1983a-c); it should permeate to the neuropile by simple diffusion, but does not (see Murray et al., 1994). This suggests that a pump similar to that in the Malpighian tubules works in parallel, transporting nicotine away from the neuropile. Putative pumps could, as depicted, function either by moving nicotine from extracellular CNS space to cortical cell cytoplasm or by moving it from perineurial cytoplasm into the blood space and/or (not shown) into perineurial cell vacuoles. If the nicotine pumps function like known multi-drug pumps, they would act in the latter way, i.e. out of the cytoplasm (Higgins, 1992).


lumen of midgut
lumen of hindgut
body wall
nicotine in secreted fluid
dietary nicotine
nicotine held for excretion
hemolymph (low pH favours charged nicotine)
lumen of a Malpighian tubule
neuropile & neuronal somata
tortuous paracellular route (note tight junctions between barrier cells)
CNS
cortical layer (barrier cells & glial cells)
ACh receptors in neuropile (possibly with reduced nicotine- affinity)
cells with nicotine-metabolizing enzymes
passive diffusion of nicotine
alkaloid pumps
putative alkaloid pumps

2.2 Materials and methods

i) Insects

Manduca sexta were obtained from colonies reared at the University of Ottawa and the Loeb Research Institute. Larvae were reared on a meridic diet (Bell and Joachim, 1976) and kept in a 25 ± 1°C humidified chamber with a 16:8 light:dark photoperiod.

ii) Light microscopy

Abdominal nerve cords from 5th instar larvae were dissected under saline (150 mM NaCl, 3 mM KCl, 5 mM $CaCl_2$ and 2 mM sodium phosphate buffer, pH 6.6), fixed for 1 hr at RT in Lana's fixative (15% v/v picric acid, 4% paraformaldehyde in 0.5 M sodium phosphate buffer), and rinsed in phosphate buffered saline (PBS; pH 7.4). For Giemsa staining, tissue was cryoprotected in 5, 10 and 15% sucrose in PBS for 1 hr each, embedded in TissueTek OCT medium (Miles, Elkhart, IN) and then quick-frozen in liquid isopentane at -40°C to -60°C. Frozen cryostat sections (12 μm) were cut, collected on gelatin-coated slides and kept at -20°C. Just prior to staining, sections were brought to RT, rinsed in 0.1 M phosphate buffer (pH 7.4; PB), and then incubated in Giemsa solution at 60°C for 2-3 min. Giemsa (BDH, Toronto, Canada) was diluted 1:10 from the bottle in PB. After incubation in Giemsa, sections were thoroughly rinsed in PB and mounted in Aquamount (BDH).

For haemolysin and eosin staining, preparations were fixed in 4% paraformaldehyde

in PBS for 2 hrs at RT. Following fixation, samples were incubated in 70% ethanol overnight, and then dehydrated in a graded ethanol series (1 hr in 70%, 95% and 100% ethanol). Samples were incubated in xylene (twice for 1 hr each), placed in a solution of 50% xylene and 50% paraffin (Paraplast Plus, Monofect Scientific, St. Louis, MO) for 3 hrs and then placed into 100% paraffin overnight in a 60°C oven. The following day, samples were embedded in fresh paraffin. Paraffin sections (12 μm) were cut on a microtome and collected on gelatin coated slides. To improve adherence of the sections to the slides, slides were incubated in a 60°C oven overnight and stored at RT until use. Just prior to staining, sections were deparaffinized in xylene (twice for 5 min each) and then rehydrated in a graded ethanol series (briefly in 100% ethanol, 2 min each in 100%, 95% and 70% ethanol, and then in distilled water for 2 min). Sections were incubated in Mayer's haematoxylin (0.1% of haematoxylin in 0.2 M aluminium potassium sulphate, 1 mM sodium iodate, 5 mM citric acid and 0.15 M chloral hydrate) for 15-20 min, and washed in running tap water for 20 min. After rinsing, sections were stained in 1% aqueous eosin for 3 min and rinsed in tap water for 1 min. Sections were then incubated twice in 95% ethanol and twice in 100% ethanol (2 min each), cleared in two changes of xylene and mounted in Permount.

iii) Immunohistochemistry

The distribution of P-glycoprotein was assessed immunohistochemically using both the avidin-biotin peroxidase method (ABC method) and the immunogold labelling technique. For the ABC method, nerve cords and Malpighian tubules from 5th instar

Manduca larvae and fixed for 1 hr in Lana's fixative, rinsed in PBS, cryoprotected and quick-frozen in embedding medium. Cryostat sections (16 μm) were cut and placed on gelatin-coated slides and incubated for 20 min in 0.3% H_2O_2/methanol to quench endogenous peroxidase. Tissue sections were thoroughly rinsed in PBS + 0.3% Triton X-100 (rinsing buffer) and then incubated in 10% normal goat serum in 1% BSA/PBS + 0.3% Triton X-100 (incubation buffer) for 1 hr to block non-specific binding. Serum was removed by carefully blotting with a piece of filter paper and then sections were incubated with the primary antibody C219 (Centocor, Malvern, PA) at 10 μg/ml in incubation buffer overnight at 4°C. Sections were rinsed three times (10 min each) in rinsing buffer and incubated with biotinylated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, Pennsylvania) at 1:20 for 1 hr at RT. Following secondary antibody incubation, sections were incubated with avidin-biotin peroxidase complex (Vector Laboratories, Berlingame, CA), according to manufacturer's instructions. The peroxidase complex was visualized by a 5 min incubation with 3,3'-diaminobenzidine tetrahydrochloride (DAB; 1 mg/ml; Sigma) with 0.003% H_2O_2. Controls included (i) omission of the primary antibody solution, (ii) substitution of the primary antibody with non-immune mouse serum at 10 μg/ml and (iii) incubation of the primary antibody solution with a 100-fold excess of an oligopeptide containing the C219-epitope (Georges et al., 1990; gift from V. Ling, Toronto) 60 min prior to tissue incubation. At least 3 replicates of each experiment were done. Sections in Fig. 2.2 were typical of staining patterns for the given experimental condition and were specifically chosen on the basis of the following criteria: good tissue preservation, an orientation that adequately demonstrated the differential staining at the

barrier region and, coupled with these, low background "noise". Tissue sections were viewed by bright field microscopy on a Zeiss Axiophot.

iv) Confocal microscopy

For immunogold immunohistochemistry, *Manduca* nerve cords were fixed in 0.25% gluteraldehyde, 4% paraformaldehyde, 0.2% picric acid and 6% sucrose in 0.1 M sodium phosphate buffer, pH 7.4. Cryostat sections were made as above. Sections were rinsed in the 0.5 M PBS and incubated in goat serum and primary antibody solution as above followed by incubation in 5 nm gold-conjugated secondary antibody (Sigma; diluted 1:50 in incubation buffer) overnight at 4°C. Sections were rinsed and incubated in *IntenSE* Silver Enhancement Kit (Amersham) according to the manufacturers' instructions to enhance gold particles for viewing. Sections were analyzed by a Leica Confocal microscope and imaging system. We took advantage of the high reflectivity of gold particles relative to biological material and adjusted the optical path through the confocal microscope to use reflected rather than transmitted light.

2.3 Results

i) General morphology

The morphological features of the *Manduca* CNS were characterized using histochemical stains (Fig. 2.2). Giemsa stains Nissl substance in neuronal cell bodies purple, haemolysin and eosin stain the nucleus and cytoplasm blue and pink, respectively.

These cellular stains revealed that, in the *Manduca* nerve cord, neurons are localized to the peripheral regions of the ganglion. The core of the ganglion was devoid of cell bodies and consisted of fibrous processes constituting the neuropile.

ii) Immunostaining for P-glycoprotein co-localizes with the nicotine barrier

Immunostaining of *Manduca* CNS for the multi-drug pump, P-glycoprotein, using C219, a monoclonal antibody directed against a highly-conserved cytoplasmic epitope, yielded intense reactivity in the cortical cells of the CNS, and none in the neuropile or in the axon-bearing centre of the connectives (Fig. 2.3A). Controls (Fig. 2.3D-F), including one in which antigenic tissue sites compete with a soluble form of the epitope (Georges et al., 1990), indicate that the immunostaining is specific for P-glycoprotein.

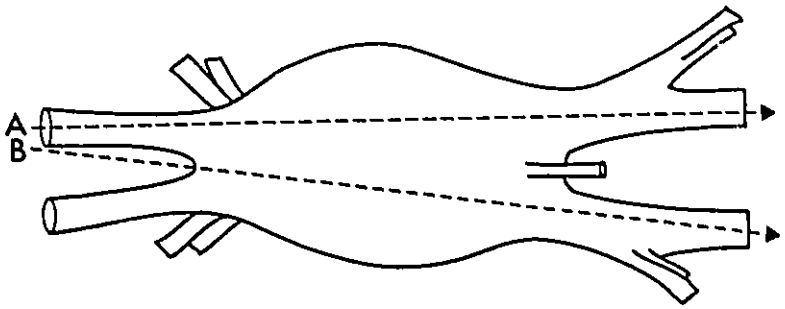
While physiological evidence for a nicotine pump at the CNS is indirect (Morris 1983a-c, 1984), that for the proximal segment of Malpighian tubules is direct (Maddrell and Gardiner, 1976). Immunostaining of the proximal Malpighian tubules for P-glycoprotein with C219 was positive (Fig. 2.3B). As in the CNS, the Malpighian tubule staining was C219-specific since it was abolished by preincubating the immune serum with C219 oligopeptide (Fig. 2.3C). Controls in which the primary antibody solution was replaced with non-immune mouse serum (Fig. 2.3D) or was omitted entirely (not shown) were not immunopositive.

The annular staining pattern for P-glycoprotein at the blood-brain interface is not dependent on the particular monoclonal antibody used nor is it dependent on the ABC staining method. Immunohistochemical staining with a polyclonal antiserum, *mdr*(Ab-

1; Oncogene Science, Uniondale, NY) resulted in an identical staining pattern (see Chapter 3). In addition, staining with the monoclonal antibody, MRK16 (gift of T. Tsuruo), which recognizes an external epitope specific to the human P-glycoprotein (Hamada and Tsuruo, 1986) resulted in no staining (not shown).

A drawback of the ABC technique for insect tissue is the high level of background staining associated with the activity of endogenous peroxidases. The immunogold labelling technique is a more direct method than ABC in that it omits a tertiary amplification step. Immunogold staining of *Manduca* CNS with C219 yielded images of P-glycoprotein in the barrier region that, because of the virtual absence of background, were even more striking than those with ABC (Fig. 2.4).

Figure 2.2 Morphological features of the *Manduca* CNS. Bright field micrographs of longitudinal sections through *Manduca* ganglia stained with the cellular stains haemolysin and eosin (**A**), and the neuronal specific stain, Geimsa (**B**). The dashed lines in the schematic diagram indicate the plane in which the sections, illustrated in panels A and B, were taken. Note that in **A**, the blue and pink colours characteristic of the haemolysin and eosin stain appear purply-brown and mauve respectively, since they were photographed under a green filter. The insect CNS consists of a chain of ganglia connected by the interganglionic connectives (c). The fibrous central region of the ganglion, the neuropile (np), is flanked by cell bodies (asterisks in **A**, arrows in **B**). The entire nervous system is surrounded by an acellular neural lamella (nl) and an inner perineurial layer (p). Outside the nervous system proper, tracheae (t) of various sizes are scattered throughout the neural lamella.


A
B

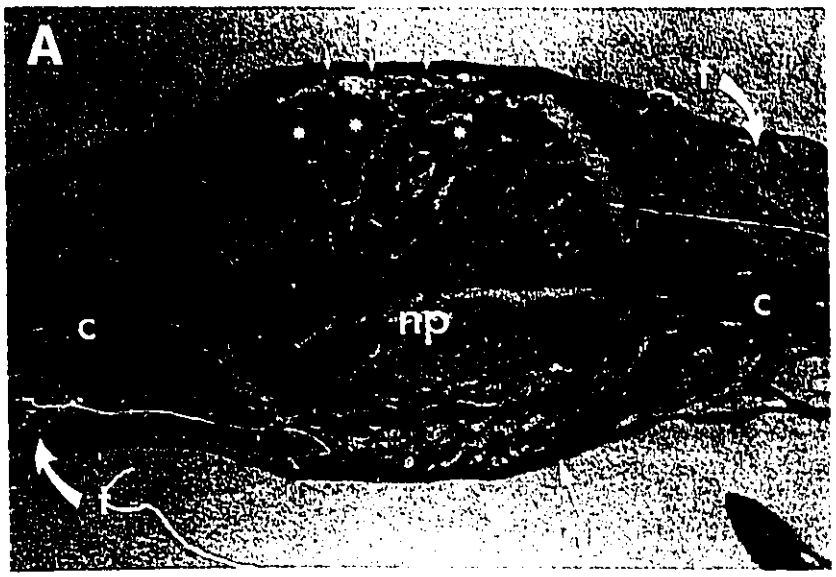

A
c
np
c

Figure 2.3 Bright field micrographs of *Manduca* ganglia and Malpighian tubule sections stained for P-glycoprotein with antibody C219. A longitudinal section through the terminal fused ganglia of *Manduca* illustrates that C219 staining was positive in the cortical layer but not in the neuropile (**A**). A control experiment showed that specific staining seen in the cortical layer (**E**) was abolished when the primary antibody solution was preincubated for 60 min with a 100-fold excess of C219 epitope-containing oligopeptide (**F**) or when normal mouse serum was used in place of the primary antibody (**G**). A cross section through a Malpighian tubule stained with C219 (**B**); **C**, a serial section pretreated with C219-oligopeptide and (**D**) mouse serum control. Bar = 100 μm for **A**, **E-G** and 15 μm for **B-D**.

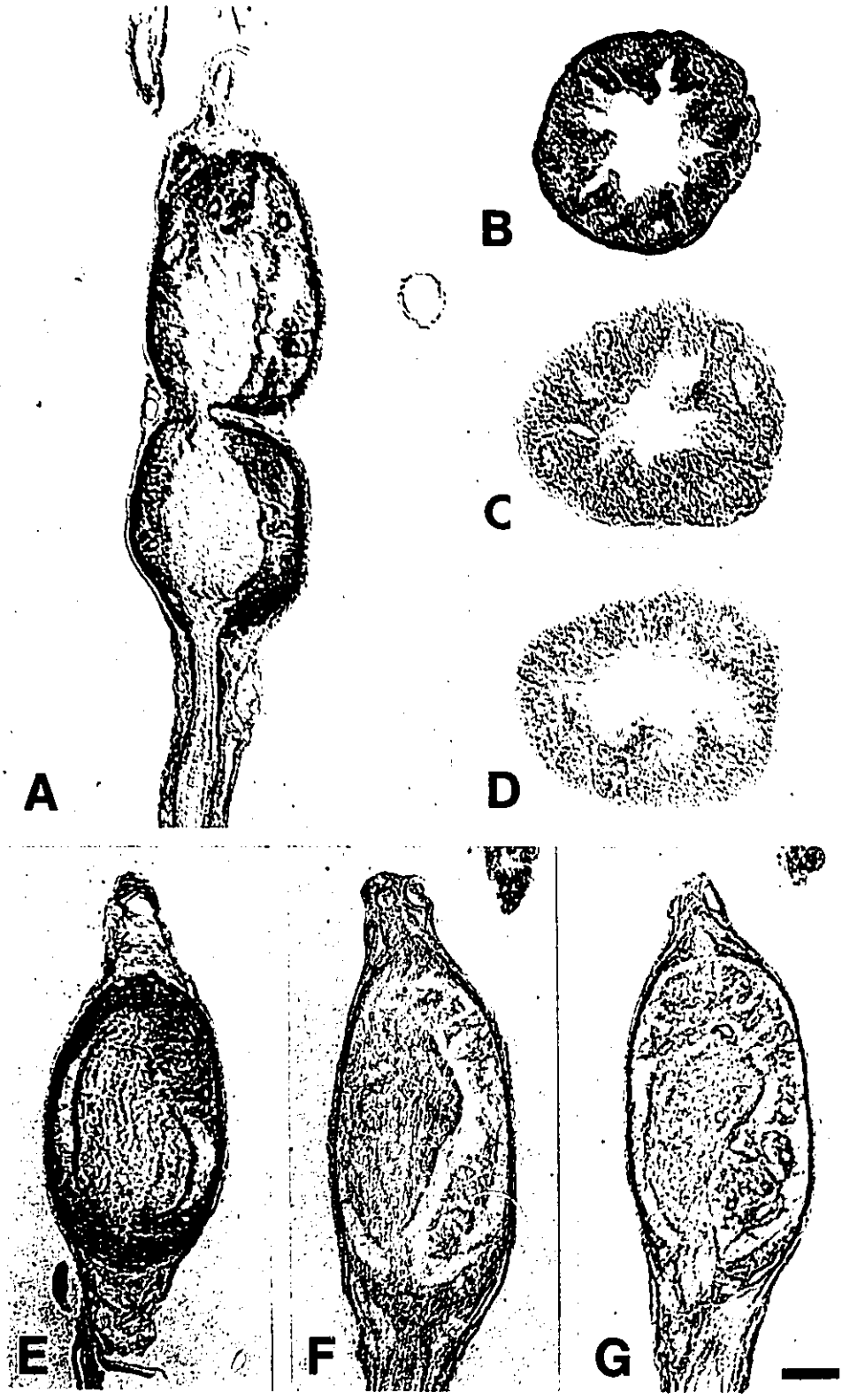
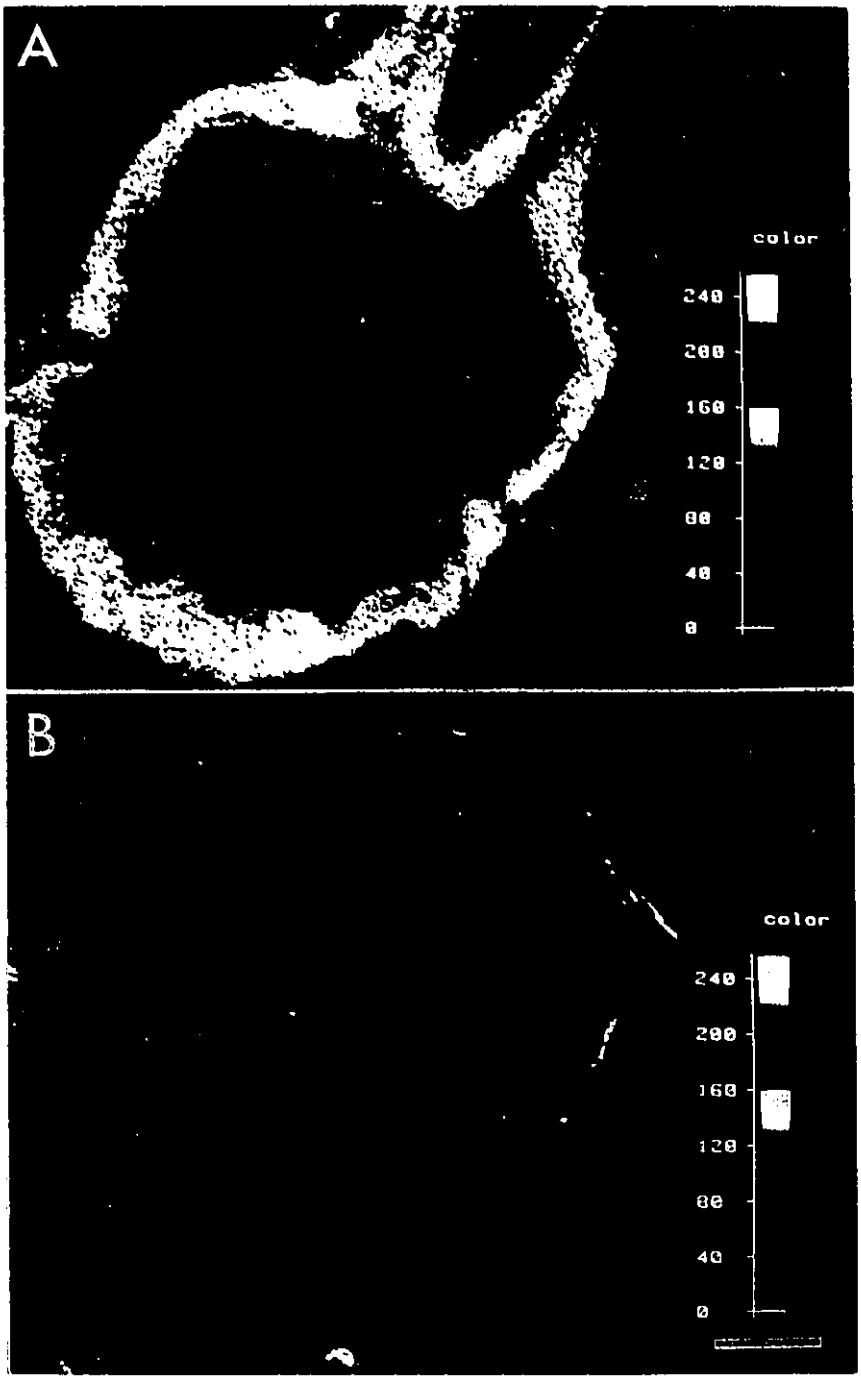

A
B
C
D
E
F
G

Figure 2.4 Pseudocolor confocal images of an immunogold labelled *Manduca* abdominal ganglion stained for P-glycoprotein with monoclonal antibody C219. **A**, longitudinal section through an abdominal ganglion was C219-positive in the cortical layer but not in the central neuropile region. **B**, control, showing that staining of the cortical layer was abolished when the primary antibody solution (but not the gold conjugate) was omitted. The color bar to the right relates the pixel brightness (and hence, amount of reflectance) of the digitized image to a colour scale. Bar = 100 μm.


A
color
240
200
160
120
80
40
0
B
color
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200
160
120
80
40
0

2.4 Discussion

Previous work has established that the *Manduca* barrier to nicotine depends both on physical and biochemical mechanisms and that together these function to restrict nicotine entry into the neuropile, the site of nicotine-sensitive acetylcholine receptors (Morris, 1983a-c; Morris and Harrison, 1984). Why does nicotine fail to reach the neuropile? Ultrastructural analysis of the *Manduca* CNS has revealed that tight junctions between perineurial cells form a peripheral diffusion barrier to hydrophilic compounds such as inorganic or organic cations, which would include the cationic form of nicotine (Pichon et al., 1972; Lane and Swales, 1979; Morris and Harrison, 1984). Nicotine has a cationic form but also a neutral form; it is an amphipathic molecule, producing in the hemolymph a mixture of cationic hydrophilic and neutral lipophilic forms. Because the latter form easily penetrates membranes, it can gain access to the neuropile transcellularly. Consequently, distinctly different barrier mechanisms are required for the two forms of nicotine. The barrier components that can counteract transcellular entry of nicotine are metabolic in nature, depending on at least two classes of enzymes. The first are the detoxifying enzymes, the PSMOs, responsible for metabolizing nicotine to polar compounds that can be more readily excreted, possibly, in some cases, as conjugates (Morris, 1983a; Murray et al., 1994). The second, we propose is a nicotine pump, which our evidence suggests is a P-glycoprotein homolog.

Immunohistochemical staining of the *Manduca* CNS with the C219 antibody to P-glycoprotein revealed positive staining which was localized to the blood-brain barrier

region. This region consists of a layer of modified glial cells called perineurial cells, overlying a layer of neuronal cell bodies and accompanying glia (as illustrated in Fig. 2.5). In the *Manduca* CNS, the perineurium lies beneath the leaky acellular neural lamella. Incubation of the isolated larval nerve cord in the exogenous tracer, lanthanum (Lane and Swales, 1979; Morris and Harrison, 1984), demonstrates that cations can penetrate the ganglion only as far as the base of the intercellular perineurial clefts. Lanthanum is excluded from the underlying extracellular spaces of the CNS by the presence of tight junctions between the innermost perineurial cell borders. In the fifth instar larva the perineurium is composed of two cell types, the type I and II perineurial cells (McLaughlin, 1974a,b). The type I perineurial cells immediately underlie the neural lamella, forming a continuous, interdigitating layer of cells, joined together by occasional gap and tight junctions (McLaughlin, 1974a,b). The type I layer is attached by desmosomes to the thin underlying type II cell layer, the cells of which are joined at their base by tight junctions. The type II cells project thin cytoplasmic processes extending between and around the underlying glial cells, forming desmosomal attachments and gap junctions with the glial membranes (McLaughlin, 1974a,b; see Fig. 2.5). Although both type I and II perineurial cells constitute the anatomical portion of the blood-brain barrier, it is impossible, without immunoelectron microscopy, to specify whether the annular pattern of P-glycoprotein immunostaining is associated with either one or both cell types. In addition to the annular ring of immunostaining, label was also observed in the cell body-rich cortex beneath the perineurial region. This region consists of neuronal and glial cell bodies and accompanying glial wrappings (McLaughlin, 1974a,b; Lane and Swales, 1979;

Morris and Harrison, 1984; see Fig. 2.4), pervasive glial cell extensions and, occasionally, glial cell processes deeply indenting a neuronal cell body, forming a trophospongial configuration (Morris and Harrison, 1984). Thus, P-glycoprotein immunolabelling in this cortical region of the ganglia could be associated with glial cell or neuronal cell membranes, or both. It is clear, however, that neither glial or neuronal processes in the neuropile are immunopositive, thus providing convincing evidence that the regional localization of label is specific. The inability to localize C219 staining to the cellular and subcellular level is a limitation of the light microscopical technique used. Ultrastructural localization of P-glycoprotein staining using immunoelectron microscopy would be required to precisely localize C219 immunostaining in the ganglia.

At the mammalian blood-brain barrier, immunostaining with C219 indicates that P-glycoprotein is localized to capillary endothelial cells (Cordon-Cardo et al., 1989; Thiebaut et al., 1989), specifically to the luminal (i.e. blood-facing) side of the cells (Tsuji et al., 1992; Tatsuta et al., 1992). The mammalian blood-brain barrier, like the insect barrier, has an anatomical barrier characterized by the presence of tight junctions between the endothelial cells, which function to limit the passage of polar compounds into the brain. Some nonpolar (lipophilic) compounds which enter the circulation, such as ethanol, caffeine and nicotine, readily cross the mammalian blood-brain barrier by simple diffusion. Other lipophilic compounds (e.g. many anticancer drugs), however, have unexpectedly low permeation across brain capillary endothelial cells and it is unclear how the brain is able to protect itself from the inward passage of these compounds (Levin, 1980; Cefalu and Pardridge, 1985). Since many of these lipophilic drugs are also substrates for P-glycoprotein

(e.g. vincristine, vinblastine, actinomycin D), it has been postulated that P-glycoprotein expression at brain capillary endothelial cells functions to protect the brain by transporting drugs and other potentially harmful xenobiotics out of the endothelial cells, back into the blood stream (Cordon-Cardo et al., 1989; Thiebaut et al., 1989; Tsuji et al., 1992).

To study the physiological function of P-glycoprotein expressed in brain capillary endothelium, Tatsuta et al. (1992) used isolated mouse brain capillary endothelial cells to examine the cellular uptake and efflux of the antitumor agent, vincristine. Capillary endothelial cells were incubated for 2 hours in radiolabelled vincristine (45 nM), a known P-glycoprotein substrate, in the presence or absence of the P-glycoprotein inhibitor, verapamil (10 nM). Accumlation of vincristine increased 6.5-fold in the presence of verapamil. Further examination of the efflux of vincristine from the cells indicated that after 90 min (following initial incubation in radiolabelled vincristine), more than 90% of the vincristine was lost from the cells in the presence of verapamil, compared to a 50% loss in the absence of verapamil. These results are consistent with P-glycoprotein pumping vincristine out of the endothelial cells. Now, direct evidence that P-glycoprotein plays a role in protecting the brain from potentially harmful xenobiotics *in vivo* (Schinkel et al., 1994) has been provided. Schinkel et al. (1994), using transgenic mice which failed to express the *mdr1* form of P-glycoprotein, demonstrated that this strain of mutant mice displayed an increased sensitivity to the neurotoxic pesticide, ivermectin, compared to control rats. Ivermectin, a pesticide that acts on GABA channels in the CNS, is routinely used in the control of mites in laboratory mice. However, in the strain of mutant mice, application of ivermectin resulted in severe tremors, paralysis and then death, symptoms

consistent with ivermectin binding to GABA receptors in the brain. Analysis of the tissue distribution of ^{3}H-ivermectin and ^{3}H-vinblastine (a P-glycoprotein substrate; Beck,1987), indicated that after 24 h, the levels of these compounds in the brain of mutant mice were 90-fold and 12-fold higher, respectively, compared to control rats.

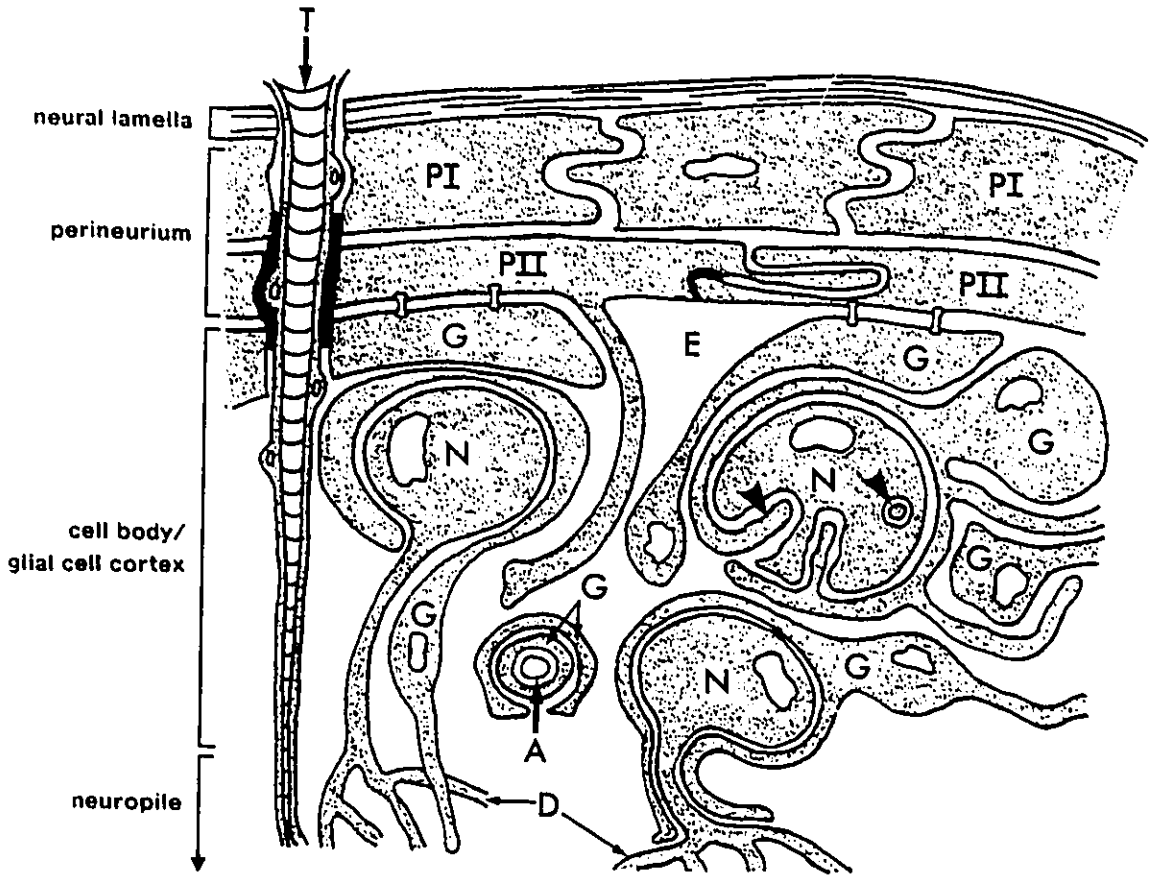
What indicator have we that C219 immunostaining at the insect blood-brain barrier represents a pump capable of transporting nicotine? Maddrell & Gardiner (1976) demonstrated that *Manduca* Malpighian tubules have a nicotine pump which transports nicotine against a large concentration gradient. A related pump in *Rhodnius* Malpighian tubules is capable of transporting the alkaloids atropine and morphine as well as nicotine (Maddrell and Gardiner, 1976). The *Rhodnius* transporter can be called a multi-drug pump since atropine inhibits the transport of morphine and nicotine, indicating that all three alkaloids are transported by a common mechanism. Inhibition of transport by structurally diverse alkaloids is often observed in studies of the multi-drug transporter in other systems (Gottesman and Pastan, 1993). If the Malpighian tubule pump is a P-glycoprotein-like multi-drug pump, I would expect immunostaining with C219 as shown in Fig. 2.3 B, C and D. In addition, preliminary Western blot analysis of both *Manduca* Malpighian tubule and CNS tissue indicates that C219 reacts with a protein band of approximately 140 kD (data not shown), a molecular weight consistent with that of P-glycoprotein molecules in other organisms (Holland-Toomey and Epel, 1993; Greenberger et al., 1988). Data presented in Chapter 4, and obtained subsequent to the immunostaining, also directly support the hypothesis that the Malpighian tubule alkaloid pump is a P-glycoprotein.

Thus, my results are consistent with the idea that the CNS and Malpighian tubule alkaloid pump is a P-glycoprotein-like molecule. The C219 antibody recognizes a highly conserved epitope located near the ATP-binding domains in all P-glycoprotein molecules. C219 immunoreactivity has been observed in proteins from other invertebrate organisms that display resistance to environmental xenobiotics. Cells prepared from tissue from marine sponges living in polluted seawater were C219 immunopositive at their plasma membranes (and perhaps endomembranes) and the cells displayed a verapamil-sensitive ability to bind the P-glycoprotein substrates, vincristine and daunomycin (Kurelec et al., 1992). In the sediment-dwelling marine worm *Urechis caupo*, Western blot analysis indicated that a C219-immunoreactive protein of approximately 140 kD molecular weight was found in *Urechis* eggs (Holland-Toomey and Epel, 1993). In addition, the uptake of the P-glycoprotein substrate, rhodamine (5 μM) by *Urechis* eggs was enhanced at least 2-fold in the presence of verapamil (22 μM), providing physiological evidence that a P-glycoprotein homolog may be functioning in the egg.

Immunoreactivity with C219 antibody suggests that the structure of P-glycoprotein is taxonomically highly conserved, but sequence comparisons with other P-glycoprotein-like molecules must await cloning of the *Manduca* gene(s). Another insect, *Drosophila melanogaster*, has two P-glycoprotein gene homologs with high sequence identity to mammalian (human and mouse) P-glycoprotein (Wu et al., 1991). Southern blot analysis of *Manduca* genomic DNA using probes from highly conserved regions of *Drosophila* and Chinese hamster *mdr* genes (Wu et al., 1991; Riordan et al., 1985), and subsequent

analysis of PCR-amplified fragments indicate that *Manduca* has at least one homolog of the multi-drug transporter gene (G. Drouin, M. Ell, personal communication).

Figure 2.5 Schematic representation illustrating the general organization of a typical larval *Manduca* ganglion from the blood-brain interface to the edge of the neuropile. The ganglion is ensheathed in the acellular neural lamella which overlies the perineurial layer. Tracheae (T) penetrate the neural lamella and branch extensively throughout the ganglion. The perineurium is composed of two cell types, the type I and II perineurial cells. The type I (PI) cells form a thick layer of highly interdigitating cells that lie immediately under the neural lamella and overlie the type II perineurial cell (PII) layer. The type II cells form a thin cellular layer composed of only a few cells that form a continuous 'bracelet' around the ganglion. Type II cells are joined laterally, near the basal surface, by tight junctions (indicated in black) forming the anatomical portion of the blood-brain barrier (Lane and Swales, 1979; Morris and Harrison, 1984). The type II cells form gap junctions with the underlying glial cells and often send finger-like processes deep into the underlying cell body and glial cell layer. Neuronal cell bodies (N) are ensheathed with glial cell (G) processes that occasionally penetrate the neuronal cell body cytoplasm to form trophospongial configurations (arrow heads). Axons (A), with accompanying glial cell investments, extend from the neuronal cell bodies into the synaptic region of the ganglion, the neuropile. Glial associations are absent where neuronal processes branch into pre- and postsynaptic extensions (D) in the neuropile. Note that tracheae penetrate deep into the neuropile, forming a continuous pathway for the diffusion of substrates from the external environment to the neuropile; the blackened area around tracheolar cell in the perineurium represents the requirement for a blood-tracheal barrier to impede the movement of potentially toxic substances into the CNS via paracellular routes adjacent to the tracheal epithelium. The unstipled areas represent extracellular space (E).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CHAPTER 3

CHANGES IN NICOTINE SUSCEPTIBILITY DURING THE LIFE CYCLE OF *MANDUCA SEXTA* CORRELATES WITH REDISTRIBUTION OF P-GLYCOPROTEIN AT THE BLOOD-BRAIN BARRIER

3.1 Introduction

By virtue of being a holometabolous insect, *Manduca* assumes three distinct forms during its life cycle: the actively feeding larval instars and the non-feeding pupal and adult stages. Larval *Manduca* feed on nicotine-laden tobacco plants and possess a blood-brain barrier to protect their CNS from this very permeant neurotoxic alkaloid. The larval CNS barrier to nicotine depends both on physical mechanisms (i.e. tight junctions between perineurial cells; McLaughlin, 1974a,b; Lane and Swales, 1979; Morris and Harrison, 1984), and on biochemical mechanisms that together function to restrict nicotine entry into the neuropile, the site of nicotine-sensitive acetylcholine receptors. We have previously demonstrated that the biochemical mechanisms depend on at least two classes of enzymes, first, the PSMO detoxifying enzymes (Morris, 1983a; Murray et al., 1994) and, second, a nicotine pump, which we propose to be a homolog of the mammalian multi-drug resistance transporter P-glycoprotein (see

Chapter 2; Murray et al., 1994).

Using an extracellular recording technique, Morris (1984) demonstrated that the isolated nerve cord from larval *Manduca* is highly insensitive to nicotine, tolerating up to 100 μM nicotine with no adverse effects. A preliminary electrophysiological study (Morris, 1977) suggested that, unlike the nicotine-insensitive larval CNS (Morris, 1984), both the pupal and adult nervous systems are nicotine-sensitive.

Using a similar global recording technique previously used by Morris (1977, 1984), I undertook an electrophysiological study to establish the relative sensitivities of isolated abdominal nerve cords of the three developmental stages to nicotine. Although the effects of nicotine on the larval nerve cord have been extensively studied by Morris (1984), larval nerve cords were included in the present study for comparative purposes. As reported in this chapter, my results confirm that the pupal and adult nerve cords are sensitive to nicotine. Finally, to determine if P-glycoprotein expression at the barrier decreases along with the increasing sensitivity to nicotine, a parallel immunohistochemical study was performed.

3.2 Materials and methods

i) Insects

Manduca sexta were obtained from a culture reared at the University of Ottawa and Loeb Research Institute. Larvae were fed an artificial diet and housed in a 25°C humidified incubator with 16:8 light dark cycle. Late fifth-instar larvae which were

entering the wandering stage (marked by the appearance of the dorsal vessel) were transferred into individual clear plastic vials and closely observed for cuticular changes marking the onset of pupal ecdysis, which constitutes the start of pupation (designated as Day 0 for pupation). From the wandering larval stage and throughout pupation until just prior to eclosion (approximately 18 days after onset of pupation), the insects were kept in the dark in vermiculite, at which time they were transferred to a chamber for eclosion. For both electrophysiology and immunohistochemistry, the abdominal nerve cords were dissected from fifth instar larvae, Day 12 pupae and from 24 h post-emergence adults.

Periplaneta americana were reared at the Loeb Research Institute. *Periplaneta* were housed in aquariums supplied with layers of corrugated cardboard to provide cover for the insects, kept on a natural light/dark cycle at RT and fed a diet of laboratory chow and water *ad libitum.*

ii) Electrophysiology

Action potentials were monitored using an extracellular recording technique described previously (Morris, 1984; see Figure 3.1). Abdominal nerve cords from all three developmental stages of *Manduca* were dissected under saline (150 mM NaCl, 3 mM KCl, 5 mM $CaCl_2$, 2 mM $MgCl_2$ and 5 mM HEPES, pH 6.6). Nicotine was added to solutions in the form of nicotine bitartrate (Sigma, St Louis, MO) and the pH was adjusted, where necessary, with NaOH. Any connective tissue and fat body surrounding the nerve cords was carefully removed with fine forceps to ensure good

contact between the electrodes and nerve cords. Nerve cords were carefully placed in the recording chamber so that a chain of the distal ganglia floated just below the surface of the saline in compartment B and a pair of connectives passed from compartment B to A via a silicone grease seal in the wall gap. This pair of connectives (between the 5th and 6th abdominal ganglia for larvae and between the 5th and the fused 6th, 7th and 8th abdominal ganglia for pupae and adults) was placed over platinum wire recording electrodes (see Figure 3.1). These differential recording electrodes were connected via a X100 Grass AC preamplifier, and a Tektronix oscilloscope to a Rackal FM tape recorder. The ganglia anterior to the connectives in the recording chamber (A) were thoroughly crushed, ensuring that all activity recorded from the connectives was evoked from ganglia in compartment B. The crushed ganglia served as a wick drawing saline from the bottom of the damp chamber A, preventing drying of the connectives. The nerve cord in chamber B was perfused with saline at a rate of 3 ml/min using a Harvard Apparatus perfusion pump with a 50 cc syringe; the inlet to chamber B was at the bottom (see Fig. 3.1). Nicotine solutions were injected into chamber B using a syringe attached to a two-way non-return valve which was also connected to the Harvard apparatus. To reduce the dead space when switching solutions, this valve was positioned close (~3 cm) to chamber B. Perfused solutions were aspirated from the distal end of chamber B via a piece of filter paper place on a small shelf adjacent to chamber B. This arrangement greatly reduced electrical noise produced when perfused solutions were aspirated directly from chamber B. For each experiment the level of neural activity was monitored while nerve cords

were perfused first with normal saline to establish a baseline, then with 5 min pulses of varying concentrations of nicotine. For nicotine pulses, perfusion was stopped and the normal saline was aspirated from chamber B and quickly replaced with nicotine solution. Perfusion with normal saline was then resumed. The pulse duration was determined to be 5 min from examining the rate of solution exchange with Fast-green dye. The chamber was cleared of dye in approximately 5 min after filling chamber B with fast-green dye and perfusing with dye-free saline. The approximate concentration of nicotine solution during this 5 min pulse was determined by measuring the absorbance of the dye solution at various time points during the pulse (carried out by Stephane Carpentier, an honours student in the lab). The dye concentration (sampled from mid-chamber) was found to decay exponentially, decreasing to one-third the original concentration in approximately 30 sec. Hence, an added drug would achieve approximately 2/3 its final concentration in approximately 30 sec. Each nicotine pulse was followed by at least a 5 min wash period. Continuous recordings of neural activity were made on the FM tape recorder, along with voice notes.

iii) Analysis

Recorded currents were digitized by replaying the tape through a TL-1 interface (Axon Instruments, Claremont, CA) to a PC computer. PClamp v6 (Axon Instruments) was used to analyze the digitized currents - the analog signal was digitized with Fetchex, the digitized record idealized using Fetchan, and pStat was used to construct a continuous plot of the relative level of neural activity as described below. For each

experiment, the number of action potentials whose amplitude was greater than, or equal to, a preset threshold was determined in Fetchex. Variability in the background level of spontaneous neural activity made it necessary to vary the threshold setting for each preparation. Once a threshold was established it was held constant throughout. The digitized records from pClamp were exported into Sigmaplot 4.1 (Jandel Scientific, Carte Madera, CA), where they were plotted.

Dose-response curves were made for larval, pupal and adult nerve cords. Sigma plot was used to obtain a best fit of a 4-parameter logistic equation to the activity versus concentration data. The equation of the line is $y=(a-d)/[1+(x/c)^b]+d$, from Sigmaplot, where a is asymptotic max, b is slope parameter, c is inflexion point, d is asymptotic minimum, x is the drug concentration and y is the response as a % of maximum.

iv) Immunohistochemistry

The distribution of P-glycoprotein was assessed immunohistochemically using the monoclonal antibody, C219 (Signet Laboratories Inc., Dedham, MA), or the polyclonal antibody mdr(Ab-1) (Oncogene Science, Uniondale, NY), both of which recognize epitopes in P-glycoprotein's C-terminal cytoplasmic domain. Abdominal nerve cords from larval, pupal and adult *Manduca* and from adult *Periplaneta* were dissected under saline (*Periplaneta* saline: 210 mM NaCl, 3 mM KCL, 5 mM $CaCl_2$ and 2 mM sodium phosphate buffer, pH 7.2) and fixed for 1 h in Lana's fixative [15% v/v picric acid, 4% paraformaldehyde in 0.5 M PB] at RT. Nerve cords

immunolabelled with the C219 antibody were processed as described in Chapter 2. For the mdr(Ab-1) antibody, fixed nerve cords were dehydrated through a graded ethanol series and embedded in Paraplast Plus (Monofect Scientific, St. Louis, MO). Paraffin sections (10 μm) were cut with a rotary microtome and placed on coated glass slides (Fisher). Prior to immunolabelling, sections were deparaffinized, rehydrated and then incubated in 0.3% H_2O_2 in methanol for 20 min at RT to quench endogenous peroxidase. Tissue sections were thoroughly rinsed in PBS + 0.3% Triton X-100 (rinsing buffer) and then incubated in 10% normal goat serum in 1% bovine serum albumin (BSA) in PBS + 0.3% Triton X-100 (incubation buffer) for 1 h at RT to block nonspecific binding. Serum was removed by carefully blotting with filter paper and sections were incubated with the primary antibody at 10 μg/ml in incubation buffer overnight at 4°C. Sections were rinsed three times (10 min each) in rinsing buffer and incubated with biotinylated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, Pennsylvania) at 1:20 for 1 h at RT. Following secondary antibody incubation, sections were incubated with avidin-biotin peroxidase complex (Vector Laboratories, Berlingame, CA), according to the manufacturer's instructions. The peroxidase complex was visualized using a modification of the conventional DAB intensification method (see Murray et al., 1994). Tissue sections are incubated in a solution of 0.1M acetate buffer 3,3'-diaminobenzidine tetrahydrochloride (1 mg/ml; Sigma) with 0.003% H_2O_2. The addition of glucose oxidase instead of hydrogen peroxide to the final incubation solution generates small amounts of hydrogen peroxide at a constant rate, which increases the sensitivity of immunostaining and appears to suppress background

staining (Sakanaka et al., 1987). Tissue sections were mounted in Aquamount (BDH, Poole England). Sections were not dehydrated and cleared since this procedure resulted in the loss of cellular detail which was important in assessing the distribution of label. Sections were viewed by a Zeiss Axiophot microscope. Controls included pre-incubation of the primary antibody with a 100-fold excess of the antigen (Oncogene), or substitution of the primary antibody with normal rabbit serum (Jackson) at 10 µg/ml.

v) Western blotting

Tissue preparation

Immunohistochemical results with the monoclonal and polyclonal antibodies revealed slightly different, but potentially significant, staining patterns. To examine the specificity of the two antibodies, Western blotting was performed on a tissue known to express P-glycoprotein. Rat liver is a good source of P-glycoprotein (Thiebault et al., 1987; 1989) and the liver from one rat provided sufficient material for at least two experiments. The livers from adult male rats were dissected, quick- frozen in liquid N_2 and stored at -70°C. Frozen tissue was minced with a clean razor blade and then homogenized with a glass homogenizer. Cellular material was pelleted by low speed centrifugation and washed with PBS. Six volumes of an extraction buffer containing 10 mM Tris-HCL, pH 8.6, 1.5 mM $MgCl_2$, 0.14 M NaCl, 1% (w/v) NP-40 nonionic detergent, and the protease inhibitors, 2 mM PMSF (freshly diluted from a 200 mM stock in ethanol), 10 µg/ml leupeptin and 200 U/ml aprotinin, were added to the

washed pellet. Following vigorous mixing, the samples were centrifuged at 31 000 x g for 20 min at 4°C to remove intact nuclei and large cellular debris. The supernatant containing membrane and cytoplasmic fragments, as well as solubilized components, was retained, quick frozen and stored at -70°C. The protein concentrations of the samples were determined using the Bicinchoninic Acid Protein Assay Kit (Sigma).

Electrophoresis and Immunoblotting

Samples were solubilized in 0.0625 M Tris HCl (pH 6.8), 2% (w/v) SDS, 10% glycerol, 5% 2-ß-mercaptoethanol and 0.001% (w/v) bromophenol blue (final concentrations; Laemmli, 1970). For each sample, 60 to 100 µg of protein was loaded per well. Proteins were separated on a 7.5% polyacrylamide as described by Laemmli (1970). Gels typically were run at 100 V (constant voltage) for 1.5 hrs using the Bio-Rad Mini-gel system. Proteins were electrophoretically transferred onto nitrocellulose membrane using the Bio-Rad Mini-blot system run at 100 V for approximately 2 h. Gels were then stained with Coomassie blue to ensure that all the proteins had transferred.

Following protein transfer, nitrocellulose membranes were incubated in a blocking solution of 3% BSA in PBS (pH 7.4) overnight at 4°C to block free protein binding sites. Without rinsing, membranes were incubated with either C219 or mdr(Ab-1) primary antibody solutions diluted 1:1000 in 1% BSA/PBS for 2 h at RT. Membranes were rinsed three times (5 min each) in PBS + 0.1% Triton X-100 followed by incubation in biotinylated goat anti-mouse (for C219) or goat anti-rabbit

[for mdr(Ab-1)] both diluted at 1:500 in 1% BSA/PBS for 1 h at RT. Following rinsing, membranes were incubated in an avidin-biotin complex diluted one part avidin to one part biotin in 1% BSA/PBS for 30 min, and the immune complex visualized using DAB.

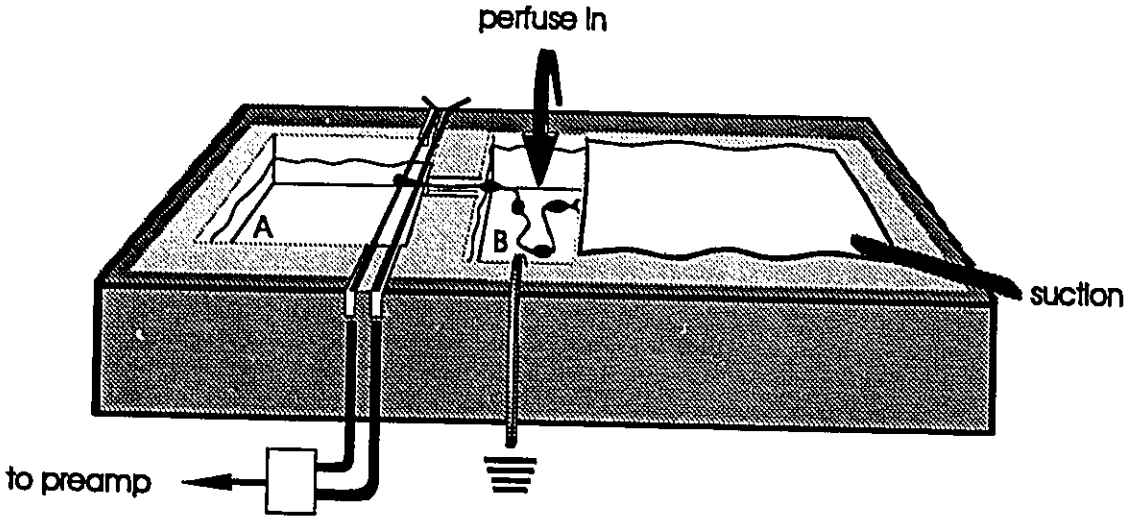


Figure 3.1 Schematic drawing of the recording chamber used for extracellular recording of action potentials in isolated abdominal nerve cords of *Manduca*. A two-compartment recording chamber was used. Compartment A is a damp chamber, containing a pair of platinum recording electrodes slotted through narrow, shallow grooves in the side walls. Compartment B is separated from A via a wall containing a small groove which allows the connectives to pass from A to B. Compartment A is humidified by filling the chamber with saline to below the level of the recording electrodes. The compartment is sealed by covering with a glass coverslip, affixed with a layer of silicone grease (not illustrated). Compartment B is perfused continuously with normal saline or with drug (nicotine) solution.

3.3 Results

i) Sensitivity of the metamorphosing CNS to nicotine

To gauge the relative sensitivities of larval, pupal and adult *Manduca* nervous tissue to nicotine, a global extracellular recording technique was used to monitor the effect of applied nicotine on the level of neural activity from isolated intact abdominal nerve cords. This recording technique monitored changes in the frequency of action potentials, such that a response to nicotine was identified as a change from the background level of spontaneous neural activity. For each developmental stage four nerve cords were tested, dose-response curves constructed, and the EC_{50}'s calculated. A continuous record depicting a typical example of the relative neural activity in response to 0 μM, 10 μM, 100 μM, 1 mM and 10 mM nicotine is illustrated for each developmental stage.

Isolated intact abdominal nerve cords from larval *Manduca* (Fig. 3.2) were unresponsive to 10 μM and 100 μM in all nerve cords tested (ie. 4 of 4). Two of the four nerve cords tested were also unresponsive to 1 mM nicotine (Fig. 3.2A; actual raw data traces in B). In the remaining two nerve cords, application of 1 mM produced a slight increase in activity (eg. Figure 3.2). In all cases, however, application of 10 mM nicotine consistently produced a substantial increase in neural activity. The EC_{50}, as calculated from the dose response curve, was 1.9 ± 0.08 mM nicotine.

For the pupal nerve cord, exposure to 10 μM nicotine resulted in a slight increase in neural activity, and increasing responses were observed with increasing

concentrations of nicotine (Fig. 3.3A). The effect of exposure to increasing concentrations of nicotine are also apparent in the original extracellular recording traces (Fig. 3.3B). During the wash period following both the 100 μM and 1 mM nicotine pulse, the neural activity initially returned to baseline levels but then increased to a higher level before subsiding again (eg. Fig. 3.3A). This pattern was typical in the pupal nerve cord and has also been described in nerve cords from the nicotine-sensitive cockroach, *Periplaneta americana*, following exposure to higher concentrations of nicotine (Morris, 1984). For the *Manduca* pupal nerve cord, the EC_{50} was found to be 0.28 ± 0.2 mM nicotine.

Nerve cords isolated from adult *Manduca* showed a similar response pattern to applied nicotine as that observed in the pupal nerve cords (eg. Fig. 3.4A and B). It should be noted, however, that in the example given for the adult nerve cord the baseline activity is higher than in the example given for the pupal nerve cord (Fig. 3.3A and B). This gives the false impression that the adult response is much greater than the pupal response. In fact, the EC_{50} of the adult nerve cord was 0.27 ± 0.1 mM nicotine, a value which is indistinguishable to the EC_{50} of the pupal nerve cord. More important than the dose response, however, is threshold; since no larva responded to 100 μM nicotine and all adults and pupae responsed to 10 μM nicotine, the difference in the threshold is greater than 10-fold.

ii) Distribution of P-glycoprotein in the metamorphosing CNS

The distribution of P-glycoprotein was assessed immunohistochemically, initially

by frozen section immunohistochemistry with the monoclonal antibody, C219. Positive immunolabelling was observed in ganglia from all three developmental stages (Fig. 3.5), and the distribution of label varied between stages. In the larval ganglion, label was seen in the perineurial layer and in the cell body/glial cell cortex, with no label associated with the neuropile (Fig.3.5 A and B). Staining in the pupal ganglion was associated with the neural lamella and continued to be present in the perineurial layer; label was no longer observed in the cell body/glial cell cortex (Fig.3.5 D and E). In the adult ganglion, only a thin layer of staining associated with the perineurium was observed (Fig.3.5 G and H). Staining was virtually absent in control sections from all three developmental stages (Fig.3.5 C,F and I).

To examine the pattern of P-glycoprotein expression in a nicotine-sensitive insect, immunolabelling of the abdominal nerve cord from the cockroach, *Periplaneta*, was performed. In the cockroach ganglion a thin layer of staining was associated with the perineurium (Fig. 3.6), a pattern similar to that observed in the adult *Manduca* ganglion.

Results with the C219 antibody in *Manduca* indicated that changes in P-glycoprotein expression are occurring during development. However, since the thickness of the tissue often varies between frozen sections, it was not possible to precisely compare the immunolabelling between the three stages. Hence, a parallel study was performed using paraffin sections, which have the advantage compared to frozen sections of more uniform thickness. Additionally, paraffin-embedded tissue exhibits superior morphological preservation to frozen tissue. This feature is critical

when attempting to localize label to specific regions in the metamorphosing CNS.

Immunolabelling of paraffin sections with C219 antibody resulted in no staining (not shown), indicating that the epitope had been altered during tissue processing. This failure of C219 to label paraffin sections has been previously reported (Toth et al., 1994). At this point in the study, the polyclonal antibody mdr1(Ab-1), which recognizes conserved epitopes on the cytoplasmic side of the protein (Chen et al., 1986; Gros et al., 1986), became available. Immunostaining of *Manduca* ganglia from the three developmental stages with the polyclonal antibody resulted in similar staining patterns to those observed with the monoclonal antibody (compare Fig. 3.5 to Fig. 3.7). However, tissue labelled with the former resulted in less background staining, with a higher signal to background ratio (see also Western blot results below). The polyclonal antibody was therefore considered to be superior to that of the monoclonal antibody for this assay.

In the larval *Manduca* ganglion (Fig. 3.7A) immunostained with the mdr(Ab-1) antibody, label was observed in the perineurium, in glial cells surrounding neuronal cell bodies and in the glial layer immediately adjacent to the neuropile. No staining was observed in the neural lamella or in the neuropile. In a control experiment, no staining was observed when the primary antibody was incubated with an excess of the peptide encoding the epitope (Fig. 3.7B).

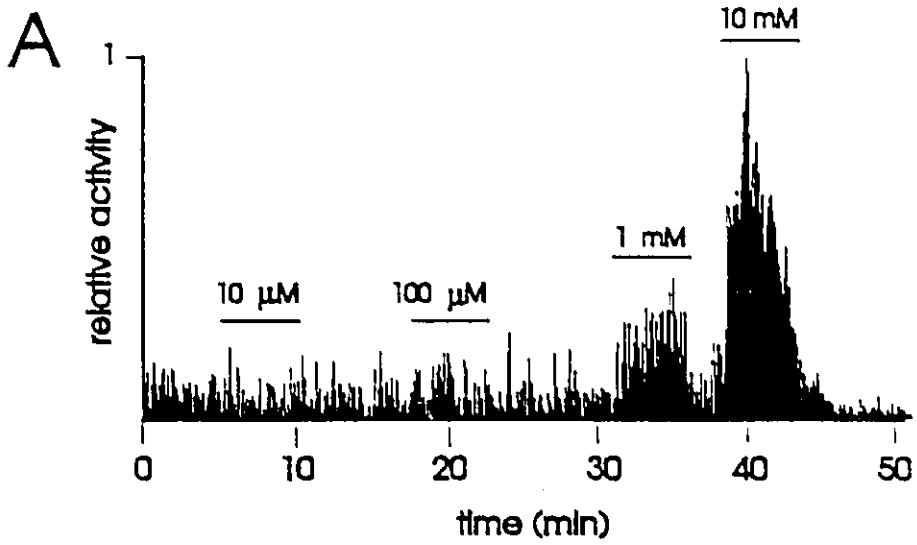
In the pupal ganglion (Fig. 3.7C), the barrier region appears to have disassembled, consistent with the profound rearrangment of the CNS (McLaughlin, 1974a; Lane and Swales, 1979). Label remained, however, associated with the

perineurial layer. Elsewhere in the ganglion, staining was not localized to a specific cellular layer, but appeared speckly and randomly distributed. A control experiment in which the primary antibody was omitted and replaced with normal rabbit serum resulted in no staining (Fig. 3.7D).

In the adult ganglion (Fig. 3.7E), the integrity of the outermost barrier region appears to have re-established itself. Immunolabelling for P-glycoprotein was reduced, with only a faint layer of label associated with the perineurium. This staining was abolished in an adjacent section which was incubated with normal rabbit serum in place of the primary antibody (Fig. 3.7F).

The immunohistochemical results presented in Figures 3.5 and 3.7 are representative of the typical staining patterns that were observed in all preparations examined (n = at least 7 for each developmental stage). Small differences observed in the intensity and distribution of P-glycoprotein immunolabelling between that obtained with the monoclonal antibody, C219, and that obtained with the polyclonal antibody, mdr(Ab-1), could be attributed to the two antibodies having different specificities. Western blot studies of rat liver, a tissue known to express high levels of P-glycoprotein (Thiebault et al., 1987; 1989), with the C219 and mdr(Ab-1) antibodies demonstrated that the mdr(Ab-1) antibody had a higher signal-to-noise ratio ratio and was more specific than the C219 antibody (Fig. 3.8).

Figure 3.2 The effect of nicotine on isolated nerve cords from fifth instar larvae. **A**, a continuous plot of relative neural activity in response to nicotine. Vertical lines represent the relative activity for 1 s periods. Preparations were washed with normal saline for at least 5 min between each nicotine application (horizontal bars). **B**, extracellular voltage records illustrating the effect of 10 μM, 100 μM, 1 mM and 10 mM nicotine on neural activity. **C**, normalized dose response curve where each point is the mean of data obtained from 4 experiments. The curve was fit such that 100 represents the maximum response observed with nicotine application and 0 represents the baseline level of neural activity.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1
relative activity
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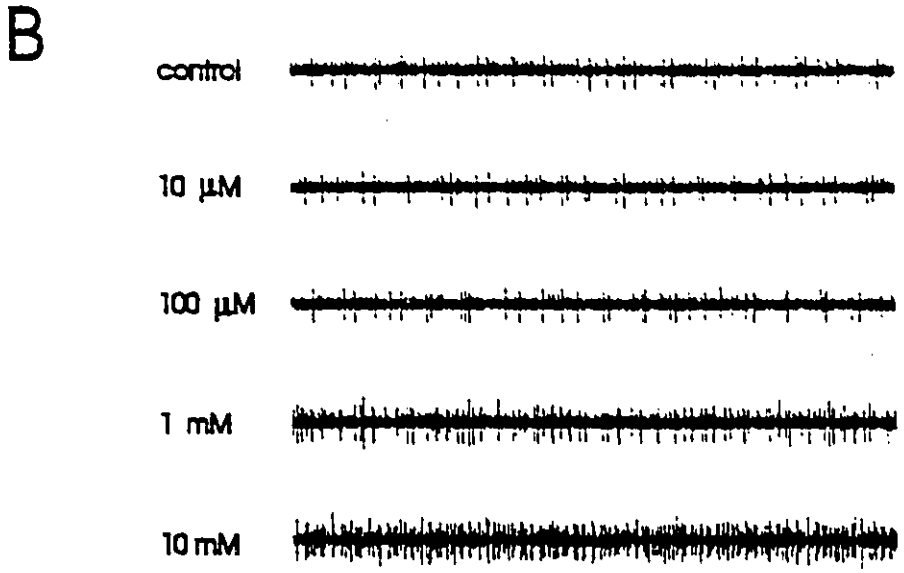
1 mM
10 µM
100 µM
0
10
20
30
40
50
time (min)


B
control
10 µM
100 µM
1 mM
10 mM

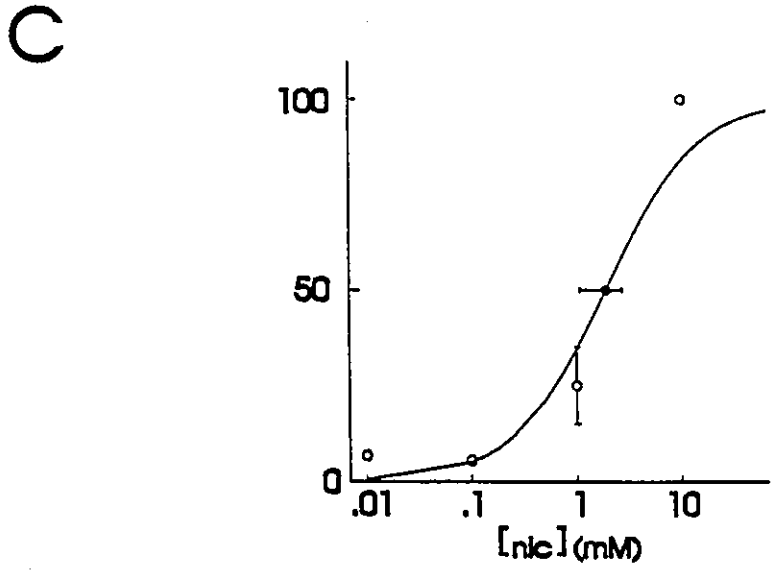

C
100
50
0
.01
.1
1
10
[nic] (mM)

Figure 3.3 The effect of various concentrations of nicotine on the abdominal nerve cord isolated from Day 12 pupa. **A**, a continuous record depicting the effect of 10 μM, 100 μM, 1 mM and 10 mM nicotine on the relative level of neural activity. Note that following application of 100 μM and 1 mM nicotine, activity initially returns to baseline but suddenly increases (arrows) before subsiding again. **B**, actual data traces illustrating the effect of nicotine application on neural activity. **C**, dose response curve constructed from the mean of 4 pupal nerve cords.

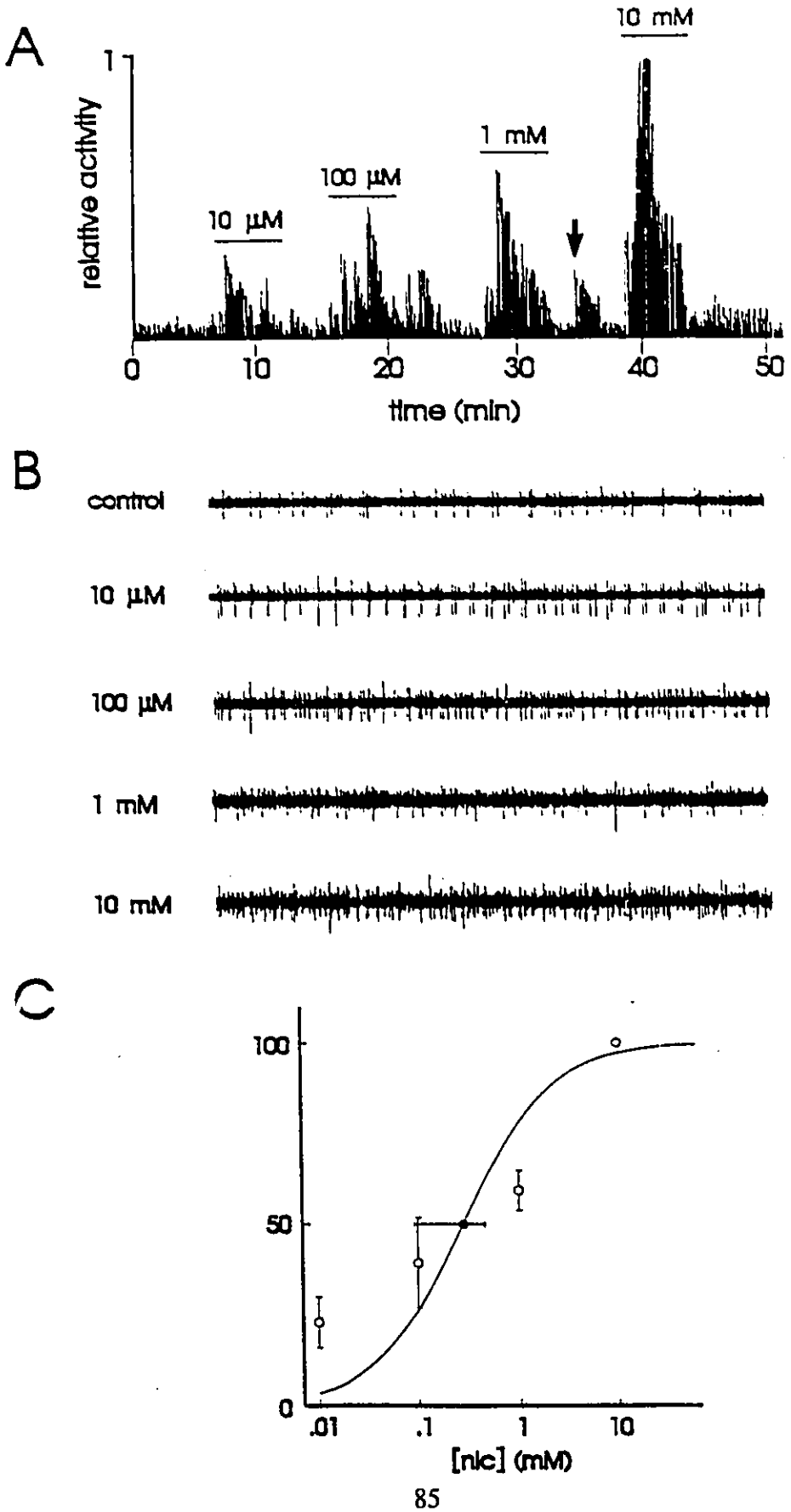
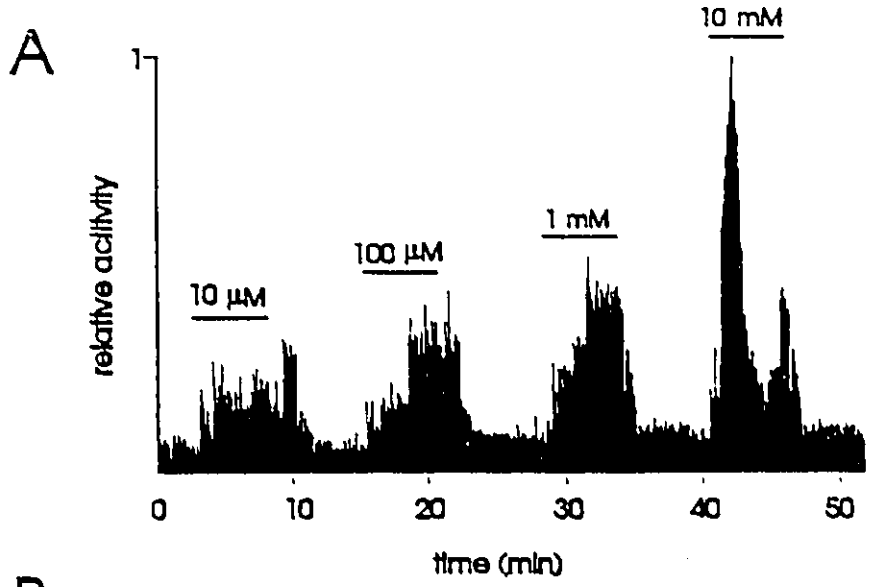
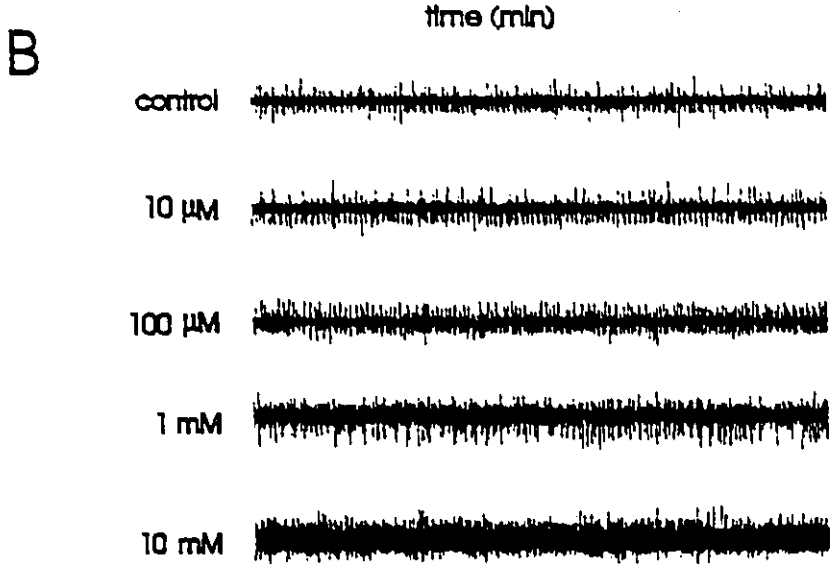

A
10 mM
1
relative activity
1 mM
100 µM
10 µM
0
10
20
30
40
50
time (min)
B
control
10 µM
100 µM
1 mM
10 mM
C
100
50
0
.01
.1
1
10
[nic] (mM)

Figure 3.4 The effect of nicotine on isolated abdominal nerve cords from adult *Manduca*. **A**, continuous record monitoring level of neural activity in response to increasing concentrations of nicotine. **B**, actual data traces to demonstrate how increasing nicotine concentrations alter the frequency of action potentials. **C**, a normalized dose response curve from the mean of 4 adult nerve cords.


A
10 mM
1
1 mM
100 µM
10 µM
relative activity
0
10
20
30
40
50
time (min)


B
control
10 µM
100 µM
1 mM
10 mM

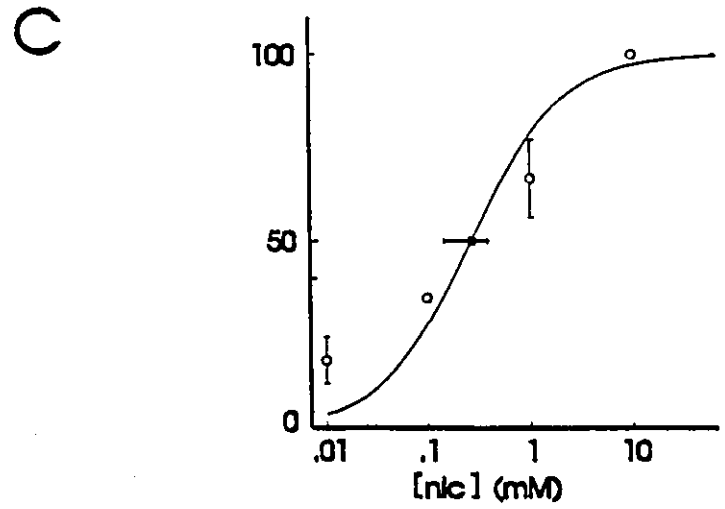
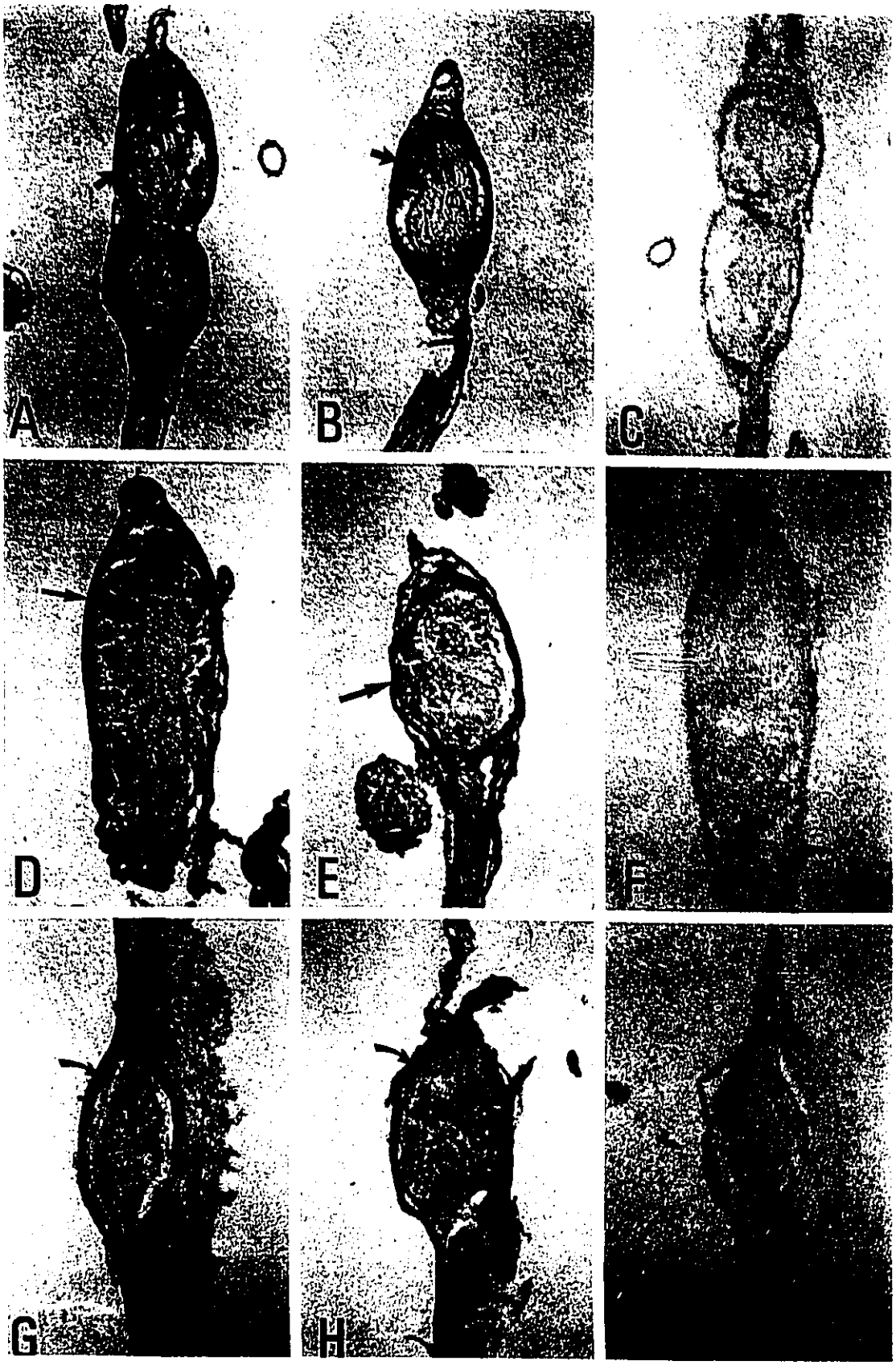

C
100
50
0
.01
.1
1
10
[nic] (mM)

Figure 3.5 Bright-field micrographs of frozen sections of abdominal ganglia from larval, pupal and adult *Manduca* immunolabelled with the monoclonal antibody, C219. In the larval ganglion (**A** and **B**), immunolabel is associated with the perineurial layer and with the underlying neuronal cell body/glial cell region. Staining in the pupal ganglia (**D** and **E**) continues to be localized to the perineurium but appears also to be associated with the neural lamella. Label is no longer associated with either cell bodies or glial processes as observed in the larval ganglia. In the adult (**G** and **H**), staining appears to be restricted to the perineurial cell layer. In all cases, staining is excluded from the neuropile. Controls (**C**, **F** and **I**) in which the primary antibody has been omitted. Bar=150 μm.


A
B
C
D
E
F
G
H

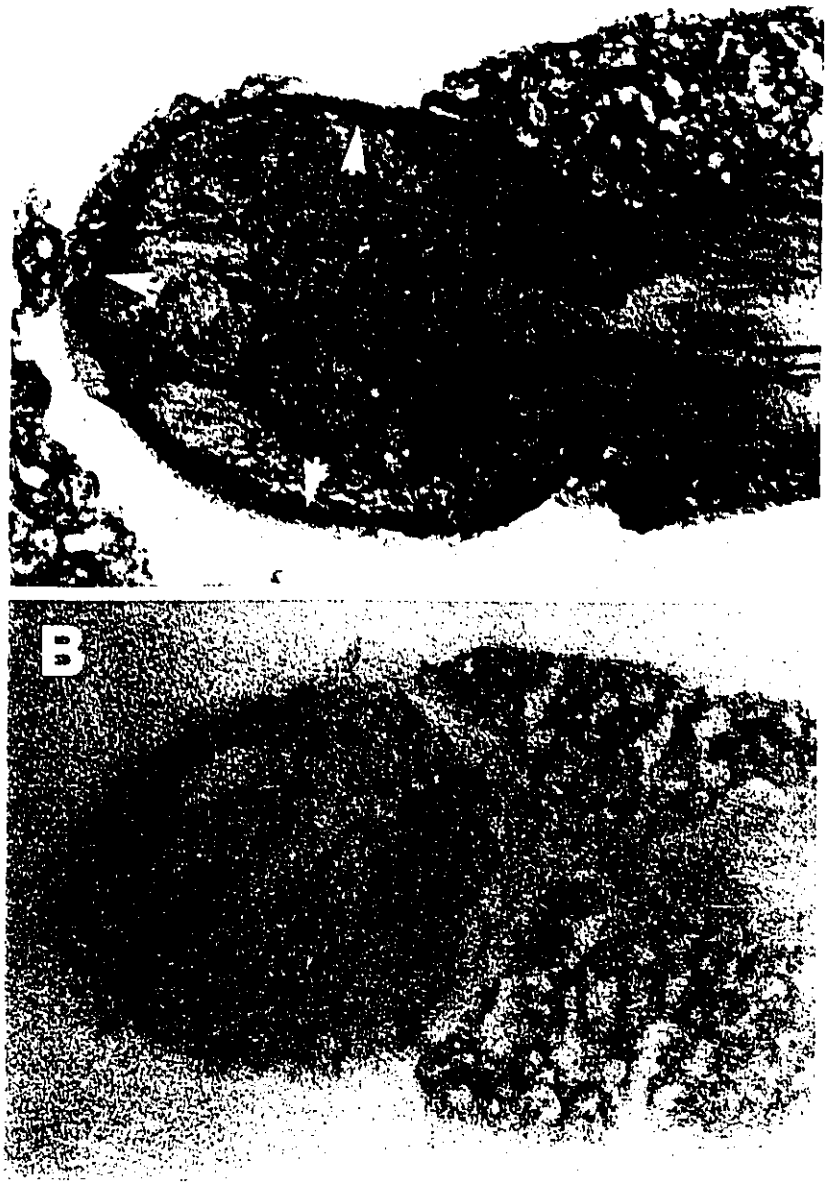
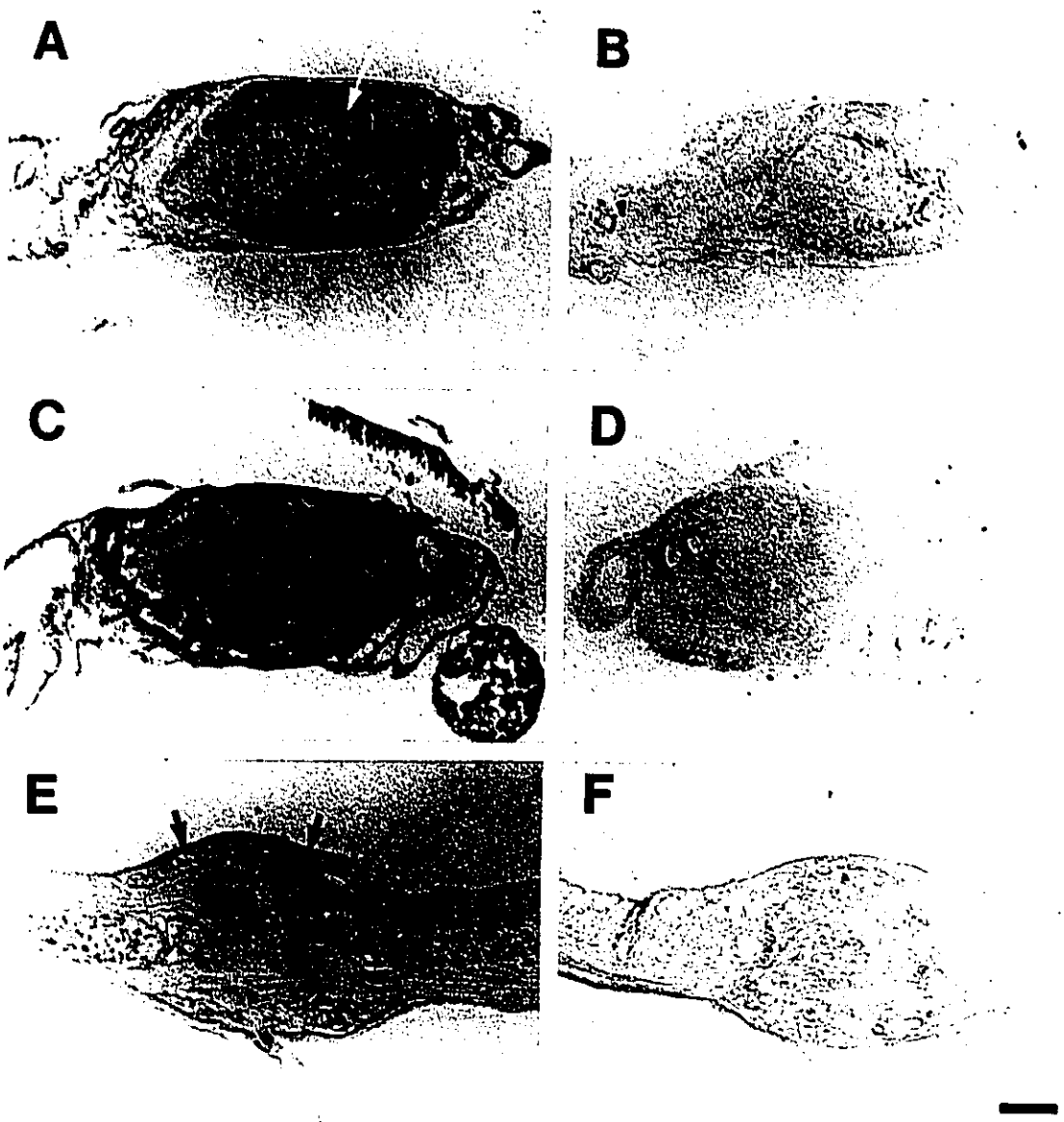


Figure 3.6 P-glycoprotein immunostaining in the CNS of the cockroach *Periplaneta americana*. **A**, a longitudinal section through the abdominal ganglion stained with the monoclonal antibody C219. A thin layer of labelling is associated with the perineurium (arrows). **B**, a serial section demonstrating that staining was abolished when the primary antibody was replaced with normal mouse serum. Bar=250 μm.

Figure 3.7 Bright-field micrographs of paraffin sections of abdominal ganglia from larval, pupal and adult *Manduca* labelled for P-glycoprotein with the polyclonal antibody, mdr(Ab-1). Larval ganglion (**A**) shows most intense staining at the perineurial layer, with some staining also occuring in the neuronal somata/glial cell region immediately adjacent to the neuropile. In a ganglion from a 12-day pupa (**C**) the barrier region appears to have disassembled, consistent with the profound rearrangment of the CNS. Label continues to be associated with the perineurial layer but is absent from the neuronal cell body/glial cell region adjacent to the neuropile. In the adult (**E**), the integrity of the outermost barrier region appears to have re-established. A thin layer of staining is present at the perineurial region, but at a much diminished level. Controls represent serial sections in which the primary antibody solution was pre-incubated with peptide encoding the mdr1(Ab-1) epitope (**B**), or in which the primary antibody was replaced with normal rabbit serum (**D** and **F**). Bar=150 μm.


A
B
C
D
E
F

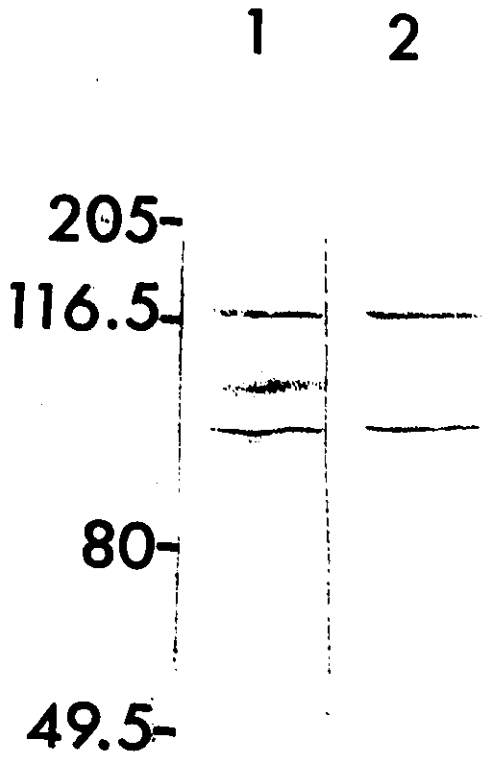


Figure 3.8 Western blots of rat liver extracts labelled using the monoclonal antibody, C219 (lane 1) and the polyclonal antibody, mdr(Ab-1) (lane 2). Both antibodies label two prominent bands, one at ~120 kD and another at ~100 kD. Note that more background labelling, and a lower signal-to-noise ratio is obtained with the C219 antibody compared to that obtained with mdr(Ab-1). Western blot analysis of mammalian multi-drug resistant cell lines indicate that P-glycoprotein can have a molecular weight of 120 to 170 kD. Differences in the molecular weights between preparations can be attributed to the existence of two P-glycoprotein precursors, one of which undergoes differential N-linked glycosylation (Greenberger et al., 1987). In addition, P-glycoprotein has been shown to migrate differently in different gel systems (Greenberger et al., 1988). Discrepancies in the molecular weight of P-glycoprotein are compounded in tissue extracts, where proteases, released when the tissue is being processed, may cause some denaturation.

3.4 Discussion

It was the focus of the work presented in this chapter to gain insight into the functional role of a P-glycoprotein homolog at the *Manduca* blood-brain barrier. To test the hypothesis that P-glycoprotein functions to transport nicotine at the *Manduca* blood-brain barrier, one approach would be to use inhibitors of P-glycoprotein function; if P-glycoprotein is functioning to pump nicotine at the barrier, inhibition of the pump should increase the sensitivity of the CNS to nicotine. However, any effect produced by the inhibitory compounds would be difficult to interpret since the majority of these compounds produce secondary effects that could impact on neural activity. In fact, many would be neurotoxic themselves. I therefore took another, albeit more indirect approach, in which the metamorphosing *Manduca* CNS was monitored, first, for relative nicotine sensitivity and, second, for expression of P-glycoprotein. Using an electrophysiological recording technique to monitor the level of neural activity in isolated abdominal nerve cords from *Manduca*, I have demonstrated that, in comparison to the nicotine-insensitive larval *Manduca* CNS, the pupal and adult CNS are highly sensitive to nicotine. Immunostaining for P-glycoprotein in the metamorphosing *Manduca* CNS indicates that the distribution of P-glycoprotein in the ganglion changes dramatically when the need for a barrier to nicotine is lost. Hence, P-glycoprotein distribution at the blood-brain barrier participates in metamorphosis in a manner consistent with the hypothesis that P-glycoprotein plays a role in protecting nicotine-sensitive acetylcholine receptors in the neuropile.

The electrophysiological results presented here on the CNS of larval *Manduca* are generally consistent with the previous findings of Morris (1984). In both studies, the application of 100 μM nicotine to the intact isolated larval abdominal nerve cord in all cases tested was totally without effect, that is, failed to cause any change in the spontaneous level of neural activity. By increasing the nicotine concentration ten-fold to 1 mM nicotine, Morris (1984) reported that in most cases there was no effect, although occasionally a slight increase in the level of neural activity was observed. In the current study, 1 mM caused a slight increase in activity in two of the four nerve cords tested. The level which would activate, at least weakly, all isolated larval CNS is evidently between 1 and 2 mM nicotine, since Morris (1984) found that doubling the concentration to 2 mM nicotine evoked in all cases strong responses within the 5- min exposure period.

In a preliminary study by Morris (1977), isolated abdominal nerve cords from pupal and adult *Manduca* were both more responsive to application of 1 mM nicotine than the larval tissue. In the present study, pupal and adult nerve cord responsiveness was also demonstrated at a nicotine concentration 100-fold less, 10 μM nicotine, a concentration that was totally without effect in the larval CNS. Since all pupal and adult cords tested responded unequivocally to 10 μM nicotine, the response threshold is about 2 orders of magnitude lower than for larvae.

Another comparison of sensitivity is the midpoint of the dose-response curve. It is a less sensitive indicator of the biologically relevant sensitivity (i.e. the concentration that would begin to produce neural effects). Nevertheless, the EC_{50}

values also indicate reduced nicotine sensitivity in the larvae. The shortcoming of EC_{50} values is that the data were obtained from nerve cords sequentially incubated with increasing concentrations of nicotine, and hence fails to account for desensitization of the nerve cords. Thus, a more accurate indication of the sensitivities is the threshold for response to nicotine. The threshold level at which the larval, pupal and adult cords can tolerate nicotine, was not however determined with greater precision because the "true" thresholds would only be relevant if obtained *in vivo*. It can be said from the "near-threshold" results presented here that the pupal and adult cords were at least 100-fold more sensitive to nicotine than the larval cords.

The observed increase in the sensitivity to nicotine in the pupal and adult CNS suggests that the barrier mechanisms present in the larval nervous system are either disrupted or non-functional in the pupal and adult CNS. The pupal and adult stages of the *Manduca* life-cycle are non-feeding stages, and hence are not exposed to nicotine. It is therefore not surprising that they do not expend the energy necessary to maintain a barrier to nicotine. Recall that the larval CNS barrier to nicotine is multi-faceted, depending both on physical and biochemical mechanisms that prevent hemolymph nicotine from reaching the neuropile. The physical barrier to nicotine is due to tight junctions between perineurial cells; it prevents the intercellular diffusion of nicotine in its water-soluble form. The biochemical barrier depends on metabolism of nicotine (Morris, 1983a) and perhaps on nicotine pumps (Morris, 1983a-c; Murray et al., 1994) that prevent the transcellular penetration into the neuropile of the lipid-soluble form of nicotine. Hence, both the pupal and adult CNS blood-brain barriers

could be deficient in one or all of the barrier components.

It appears, however, that the increase in sensitivity to nicotine is not due significantly to a breakdown of the physical barrier, since ultrastructural analysis of the pupal (McLaughlin 1974a,b; Lane and Swales, 1979) and adult (Pichon et al., 1972; Lane, 1972; Lane et al., 1977) CNS indicate that the perineurium remains intact throughout metamorphosis. An ultrastructural examination of the interganglionic connectives of the abdominal nerve cord of pupal *Manduca* demonstrates that, although the type I perineurial cells become hypertrophied and vacuolated in the early days following larval-pupal ecdysis (i.e. 3-7 days post-ecdysis), the type II cell layer remains intact, with appropriate tight junctions (McLaughlin, 1974a). By 8 to 15 days following ecdysis, the type II perineurial cells decrease in size, regaining their flattened, interdigitating appearance and cellular junctions associated with both type I and II cells are as described in the larval ganglion. By observing the movements of the extracellular tracers, horseradish peroxidase and lanthanum, McLaughlin (1974b) was also able to demonstrate that neither of the tracers penetrated beyond the perineurial type II cell tight junctions. The studies by McLaughlin (1974a,b) were performed on the interganglionic connectives rather than the ganglion proper and it could be argued that the ultrastructural changes occurring in the connectives do not necessarily reflect what is occurring in the ganglion. Lane and Swales (1979) report, however, that, although the pupal perineurial blood-brain barrier breaks down in the early pupal stages, when the 2nd and 3rd thoracic ganglia fuse with the 1st and 2nd abdominal ganglia (i.e. approximately 8 days following ecdysis; Ames and Mesce, 1993), both the

perineurium and its junctions and the permeability barrier are reformed in later pupal life (i.e. > than 8 days post-ecdysis). Since my results were obtained from pupae that were 12 days post-ecdysis, I was assured that the perineurial blood-brain barrier was intact and functional.

In the adult *Manduca* ganglion, ultrastructural analysis demonstrates the presence of tight junctions at the base of the perineurium (Pichon et al., 1972; Lane et al., 1977) and the extracellular tracer, peroxidase, does not penetrate beyond the perineurium. Tight junctions at the base of the perineurium are also found in the adult cockroach, *Periplaneta* (Maddrell and Treherne, 1967). Here too the tight junctions proved to be the sites of restriction to entry of the extracellular tracer peroxidase (Lane and Treherne, 1970). Electrophysiological results demonstrated that the CNS of *Periplaneta* is also sensitive to nicotine at 10 μM (Morris, 1984). Hence, in spite of having an intact blood-brain barrier, the pupal and adult *Manduca* CNS as well as the CNS of *Periplaneta* are nicotine-sensitive.

The larval *Manduca* CNS is able to metabolize nicotine (Morris, 1984a), but it has not been determined if pupal and adult nerve cords are also able to do so. It remains possible, therefore, that the observed increase in nicotine sensitivity in the pupal and adult CNS is due to a decrease in the metabolizing ability of the tissue. Indeed, Morris (1984a) demonstrated that, although the nicotine-sensitive *Periplaneta* CNS is able to metabolize nicotine, it does so to a much lesser extent than *Manduca*. The ability of the larval *Manduca* CNS to metabolize nicotine does not depend on

nicotine being present in the diet (Morris, 1984; Snyder et al., 1993; Murray et al., 1994). This is in contrast to other *Manduca* tissue, notably the midgut, where the PSMO metabolizing enzymes that can handle nicotine require induction by dietary nicotine (Snyder et al., 1993).

The co-localization of P-glycoprotein immunostaining with the *Manduca* blood-brain barrier (Chapter 2; Murray et al., 1994) suggests that a transport pump is also a component of *Manduca*'s metabolic barrier. Experiments done subsequent to the work presented in this chapter (see Chapter 4) demonstrate that the Malpighian tubules, which were also P-glycoprotein immunopositive (Murray et al., 1994), possess a verapamil-sensitive ability to transport nicotine. Hence, it is highly plausible that P-glycoprotein immunostaining at the insect CNS represents a pump capable of transporting nicotine. The results presented here demonstrate that immunostaining for P-glycoprotein in the metamorphosing *Manduca* CNS changes throughout metamorphosis, decreasing when the need for a barrier is lost. It could be reasoned that if, as I have hypothesized, nicotine pumps at the CNS are functioning to transport nicotine or its metabolites away from the neuropile (perhaps into sequestering sites), then the observed increase in nicotine sensitivity is due either to decreased expression of P-glycoprotein (that is, overwhelming the pumps so that 'free' nicotine continues to diffuse into the neuropile), or that, although expressed, the pumps are non-functional.

The CNS of all three *Manduca* developmental stages as well as the CNS of *Periplaneta* show some degree of P-glycoprotein immunolabelling in a thin layer of cells associated with the perineurium. Nicotine in the extracellular space could be

transported into the perineurial cells to be metabolized or stored. Considering, however, that the total area of the perineurial cells is small in comparison to the rest of the ganglion, it is conceivable that in the pupal and adult CNS of *Manduca* and the CNS of adult *Periplaneta*, nicotine simply overloads the cells, with excess nicotine available to continue diffusing towards the neuropile. In the larval *Manduca* CNS, P-glycoprotein immunostaining is also associated extensively with the glial cell and cell body cortex that underlies the perineurial layer. For the larva facing chronic exposure to nicotine, a fail-safe system to deal with nicotine that gets by the perineurium would be to have nicotine carriers on glial cells that could move the alkaloid from the extracellular space into the cells en route to the neuropile.

CHAPTER 4

DIRECT EVIDENCE FOR THE INVOLVEMENT OF A P-GLYCOPROTEIN-LIKE MECHANISM IN NICOTINE EXCRETION BY THE MALPIGHIAN TUBULES OF *MANDUCA SEXTA*

4.1 Introduction

i) Insect Malpighian tubules: structure and function

The Malpighian tubules of insects form an integral component of the insects excretory system. The tubules consist of a single layer of epithelial cells that form long slender tubules with an open-end that empties into the alimentary tract at the junction between the mid- and hind-gut, and a closed-end that lies free in the hemocoel. The primary physiological function of the tubules is the formation of an isosmotic filtrate of the hemolymph, the so-called primary urine. As the primary urine flows through the tubule to the hindgut and rectum, useful molecules such as sugars and amino acids are selectively reabsorbed into the hemolymph, whereas excretory wastes, toxic compounds and excess ions are voided. This non-selective committal of useful substances to the excretory flow and their later reabsorption seems, at first glance, to be metabolically inefficient. For the insect, however, it is a particularly clever arrangement since any previously unencountered toxic molecules acquired from

the environment will be automatically excreted insofar as there is no mechanism for their reabsorption.

The primary urine is formed by a combination of active and passive processes. The active transport of K^+, or occasionally Na^+ (in bloodsucking insects where blood is rich in NaCl; see Maddrell, 1971), from the hemolymph into the tubule lumen establishes an electropotential gradient favouring the passive movement of anions into the lumen. The net result of ion movement into the lumen is the formation of an osmotic gradient which draws water through the epithelium. Organic molecules of low molecular weight including urea, amino acids and sugars follow passively, diffusing down their concentration gradients into the tubule lumen; this rate of diffusion appears to be inversely related to molecular weight (Ramsey, 1958; Maddrell and Gardiner, 1974). The exact route for the passage of these molecules is not clear, but Maddrell and Gardiner (1974) suggested that molecules move into the lumen via a leaky intercellular route, perhaps through septate junctions. Studies on the Malpighian tubules of *Rhodnius prolixus* indicate that the septate junctions are permeable to a wide variety of substances; particularly revealing is that lanthanum ions can penetrate these junctions (Skaer et al., 1987).

ii) Evidence for active transport of toxic alkaloids by insect Malpighian tubules

In addition to passive clearance processes, insect Malpighian tubules possess active transport mechanisms for the rapid clearance of toxins and/or metabolic waste products. For example, the Malpighian tubules from the blood-sucking insect *Rhodnius*

prolixus actively transport nicotine, atropine and morphine, all highly toxic alkaloids, in the excretory fluid. In addition, atropine competitively inhibits nicotine and morphine transport, suggesting that these alkaloids are being transported by a common mechanism (Maddrell and Gardiner, 1976).

To establish that a substrate is being actively transported across a permeation barrier, it must be demonstrated that the net movement of the substrate is occurring against an electrochemical gradient. For example, for a substrate such as nicotine, which can exist in a positively charged, ionized form, two forces act on it to establish its distribution across a permeation barrier: first, the chemical gradient arising from differences in the concentration of the substrate on the two sides of the barrier and, second, the electrical gradient, that is, the difference in potential across the barrier, experienced by the charged form of the substrate as it traverses the barrier. Hence, to establish that the movement of a substrate across a permeation barrier is due to an active process, it must be demonstrated that the net movement of the substrate is occurring against an electrochemical gradient.

Using an isolated Malpighian tubule preparation, Maddrell and Gardiner (1976) established that nicotine is indeed actively transported into the lumen of *Rhodnius* Malpighian tubules. *Rhodnius* tubules bathed in a nicotine solution produced secreted fluid with nicotine concentrations at least 5 times higher than that in the bathing medium. Under these conditions, transepithelial recordings showed that the lumen was approximately 30-40 mV negative to the bathing solution. Clearly, nicotine is being transported against a concentration gradient but the possibility remains that, since the

potential of the tubule lumen is negative to the bathing solution, some concentration of nicotine occurs passively. However, in an experiment where nicotine in the secreted fluid was found to be concentrated 40-fold, the tubule lumen was only 15 mV negative to the bathing solution. Such a small transepithelial potential could not possibly support such a large concentration gradient; according to the Nernst equilibrium (e.g. Hille, 1992), a 40-fold gradient of a monovalent ion needs to be counter balanced by 93 mV potential difference. It was concluded that the movement of nicotine into the lumen of *Rhodnius* tubules is thermodynamically very much uphill and must be occurring by active transport.

The ability of insect Malpighian tubules to transport alkaloids is not restricted to *Rhodnius*. Tubules from larval *Manduca sexta* and *Pieris brassicae*, the large white, are also able to transport nicotine (Maddrell and Gardiner, 1976). Likewise, tubules from adult *Calliphora erythrocephala* and *Musca domestica*, two dipteran species, transport nicotine and atropine (Maddrell and Gardiner, 1976). In this case, however, the secreted compound is not parent nicotine, as is the case for *Manduca*, *Rhodnius* and *Pieris*, but a nicotine metabolite. For the herbivorous *Manduca* and *Pieris*, such a pump likely evolved as a counter-measure to alkaloids they encounter on the plants on which they feed. These transport mechanisms are constitutive rather than induced in these insects, since the ability of the tubules to transport nicotine was not dependent on the presence of nicotine in the diet.

For *Rhodnius*, *Musca* and *Calliphora*, all of which do not have an alkaloid-rich diet, it is not clear why they would require the ability to transport alkaloids. It may

be that such a transporter acts to eliminate toxic organic bases encountered in their environment. For example, in the blood-sucking *Rhodnius*, it is conceivable that some metabolic by-products of digestion are excreted by this transport system.

iii) Is the alkaloid transporter of the Malpighian tubules a P-glycoprotein-like protein?

My previous results demonstrated that the barrier region of the *Manduca* CNS is immunopositive for P-glycoprotein, consistent with the hypothesis that a pump-like mechanism in the CNS is involved in nicotine resistance (Chapter 2; Murray et al., 1994). Lending further support to this hypothesis is the finding that the larval insect's Malpighian tubules, which have been shown to possess a pump for which nicotine is a substrate (Maddrell and Gardiner, 1976), are also immunopositive for P-glycoprotein. Functionally, the Malpighian tubule pump displays hallmarks of a multi-drug transporter such as P-glycoprotein since it is able not only to transport nicotine, but also morphine (a known substrate of mammalian P-glycoprotein; Callaghan and Riordan, 1993) and atropine (Maddrell and Gardiner, 1976). Owing to their simple geometry (i.e. experimentally accessible bath and lumen compartments separated by an epithelial barrier layer), the isolated Malpighian tubules provide an ideal *in vitro* assay system to directly test our hypothesis that a P-glycoprotein-like molecule is involved in nicotine transport. Directly testing the hypothesis in the CNS is not feasible since, unlike the Malpighian tubules, the CNS has a complicated geometry with no bulk fluid compartment equivalent to the lumen.

To assess the potential role of P-glycoprotein on nicotine transport by insect Malpighian tubules, an initial study was performed in the laboratory of Dr. Simon Maddrell (Department of Zoology, Cambridge University) to assess the effect of P-glycoprotein inhibiting drugs on nicotine transport by *Rhodnius* Malpighian tubules. This study was followed by a study of *Manduca* tubules which required the development of a fast and easy technique to monitor concentrative uptake of alkaloid and, subsequently, to assess the effect of P-glycoprotein inhibiting drugs on alkaloid transport.

4.2 Materials and methods

i) Insects

Fourth instar *Rhodnius prolixus* were obtained from laboratory stocks reared in the Department of Zoology, University of Cambridge, Cambridge, England. *Rhodnius* were unfed at the time of experiments but had been fed a blood-meal at least 6 months prior. *Manduca sexta* larvae were obtained from cultures reared either at the University of Ottawa or at the Loeb Research Institute as described in Chapter 2.

ii) Salines and Chemicals

The physiological salines used had the following compositions: for *Rhodnius*, NaCl (129 mM), KCl (8.6 mM), $MgCl_2$ (8.5 mM), $CaCl_2$ (2 mM), $NaHCO_3$ (10.2 mM),

NaH_2PO_4 (4.3 mM), HEPES (8.6 mM), glucose (20 mM) adjusted to pH 7 with NaOH; for *Manduca*, NaCl (15 mM), KCl (30 mM), $CaCl_2$ (2 mM), $MgCl_2$ (30 mM), HEPES (5 mM), glucose (10 mM), maltose (10 mM), sodium citrate (5 mM), glycine (10 mM) adjusted to pH 7.2 with KOH, with an osmolarity of 200 mOsm.

For experiments on *Rhodnius*, [N-methyl-^{3}H]-nicotine (specific activity: 80 Curies/mmol) was supplied by NEN Research Products. Unlabelled nicotine hydrogen tartrate (Sigma) was added to yield a stock solution of 50 mM nicotine. Nicotine stock solution was diluted 1:100 with saline to give a working solution of 0.5 mM nicotine. The following P-glycoprotein inhibiting compounds were used (at final concentration): verapamil (20 μM), vincristine (100 μM), quinidine (10 μM), nifedipine (50 μM), daunomycin (20 μM), actinomycin D (100 μM). Drug concentrations were chosen based on those previously demonstrated to reverse multi-drug resistance in a variety of drug-resistant tumor cell lines. Atropine (3 mM) was used in a competition study, and 5-hydroxytryptamine (5-HT; 0.5 μM) was used to stimulate fluid secretion in *Rhodnius* tubules. All drugs were purchased from Sigma. Drug stock solutions were prepared in saline or water, if water-soluble. For those drugs not soluble in water, stock solutions were prepared in ethanol, with an ethanol concentration below 0.1%, a concentration without effect on fluid secretion (Dr. S. Maddrell, personal communication).

For studies on *Manduca* Malpighian tubules [N-methyl-^{3}H]-nicotine (84 Curies/mmol) was purchased from Amersham (Oakville, ON). A 5 mM stock solution of unlabelled nicotine hydrogen tartrate (Sigma; pH 7, adjusted with KOH) was used

to make solutions of 0.05, 0.5 and 5 mM nicotine and ^{3}H-nicotine was added (final concentration of 70 nM) to the unlabelled nicotine solutions. For vinblastine experiments, vinblastine sulphate salt (Sigma) was added to ^{3}H-vinblastine (Amersham; 11.2 Curies/mmol) to give a final concentration of 100 μM vinblastine. For inhibition studies, stock solutions of verapamil and atropine were made in water, and diluted in *Manduca* saline to yield final concentrations of 3 mM and 25 μM, respectively.

iii) Rhodnius Malpighian tubule preparation

The Malpighian tubules from fourth instar *Rhodnius* were dissected using the technique described by Maddrell (1980). Briefly, the insect's head was crushed with a pair of forceps and the insect was pinned dorsal side up in a dissecting dish with saline. The dorsal portion of the abdominal wall was peeled back and the crop and midgut removed to reveal the coiled mass of Malpighian tubules. A pair of fine glass needles were used to pull away adherent tracheae and unravel the tubules. The freed tubules (two on each side in *Rhodnius*) measured approximately 50-100 μm in diameter and 30 mm in length, with one end of the tubule attached to the junction of the hindgut and rectum and the other distal, closed-ended portion, floating freely in the insect's body cavity. The portion of the tubule proximal to the rectum is termed the lower tubule and the distal end termed the upper tubule. The junction between the upper and lower tubule is easily distinguishable: the upper tubule is more opaque, appearing grey-white in colour, whereas the lower tubule is transparent, with white uric acid crystals clearly visible in the lumen.

Two methods were used to monitor nicotine secretion by *Rhodnius* Malpighian tubules:

Single-droplet method

For this technique, illustrated in Figure 4.1A, only the upper part of the Malpighian tubule was used. Each dissected tubule was transferred (by wrapping it around the glass needle) to a 100 µl drop of saline with 5-HT (0.5 µM; to stimulate secretion) and 0.5 mM ^{3}H-nicotine, under liquid paraffin (4-5 mm) in a 10 cm wax-lined petri dish. To prevent the saline droplet from rolling, it was positioned in a circular well in the wax. The cut end of the tubule was pulled from the droplet, out into the paraffin, and wrapped once or twice around a glass pin (~0.2 mm in diameter) pushed into the wax (see Figure 4.1A). In each petri dish, four such preparations could be arranged so that four tubules could be tested per experiment. Tubules generally began to secrete in less than 5 min.

At timed intervals, the secreted droplet diameter (d) was measured by pulling the droplet away from the glass pin and using a calibrated ocular micrometer and, assuming the droplet to be spherical, the volume calculated ($V=\pi d^3/6$). The fluid secretion rate (nl/min) was then calculated. The droplet was then placed in 3 ml of scintillant (EcoScint, ICN Pharmaceuticals, Montreal, P.Q.) and the nicotine concentration determined by measuring on a Packard scintillation counter. The secretion rates of nicotine transport (pmol/min) were calculated by multiplying the nicotine concentration by the fluid secretion rate. Unfortunately, the addition of any of the P-glycoprotein inhibiting drugs to the bath, at concentrations previously

demonstrated to inhibit P-glycoprotein activity in other systems, resulted in the inability of the tubules to continue to secreted fluid. Consequently, a modified technique involving two saline droplets was adopted.

Two-droplet method

For this technique, illustrated in Figure 5.1B, the entire length of tubule was used. The upper (fluid secreting) part of the tubule was placed in one droplet (droplet A) and the lower (non-fluid secreting) portion of the tubule was placed in a second droplet (droplet B). Droplet B was placed just to the right of droplet A in the petri dish and the cut end of the tubule pulled out into the paraffin and secured with a glass pin. Droplet A contained normal saline and 5-HT (0.5 μM) to stimulate secretion. Droplet B contained normal saline, ^{3}H-nicotine (0.5 mM) and, in some cases drug. This method proved successful in overcoming the problem encountered during trials with the single-drop method. In the two-droplet method, fluid secreted by the upper part of the tubule flushes the lumen of the lower part. The lower tubule, like the upper tubule, has previously been shown to pump nicotine (Maddrell and Gardiner, 1976). Nicotine transported into the lumen is swept out and appears in the secreted droplet formed at the cut end of the tubule. Drugs were added to droplet B and the ability of the tubule to pump nicotine was assayed by collecting secreted droplets at timed intervals and measuring the amount of ^{3}H-nicotine on a Packard scintillation counter.

iv) Manduca Malpighian tubule preparation

The Malpighian tubules of larval *Manduca* produce fluid at very low rates, yielding only a negligible volume of secreted fluid after 90 min (Maddrell and Gardiner, 1976). It was therefore necessary to cannulate and artificially perfuse the tubules with saline. The technique used is illustrated in Figures 4.2 and 4.3.

Fifth instar larval *Manduca* were pinned through the head and tail, the dorsal surface cut lengthwise and pinned open. For these experiments the tubules that lie on the midgut, the proximal and medial tubules, were used. An approximately 3 cm length of the tubule was dissected using a blunt-ended glass rod to manipulate the tubule while fine tip iris scissors were used to cut tracheal connections. The dissected tubule was then transferred to a drop of 100 µl saline immersed in 4-5 mm of paraffin oil in a wax-lined 10 cm petri dish. A glass cannula held by a microelectrode holder mounted on a micromanipulator was positioned close to the droplet. Glass cannulae (~4 cm in length) were made from glass capillary tubes (Fisher Scientific; internal diameter 1.1-1.2 mm and 0.2 mm ± 0.02 mm wall thickness) using a pipette puller adjusted to produce a cannula tip of 30-40 µm in diameter. Using a low flame, the cannula was bent approximately 2.5 cm from the tip to a 50° angle. The cannula was positioned in the micromanipulator such that the straight part (distal to the tip) approached the bath drop at a 45° angle and the tip entered the droplet almost horizontal to the dish. Using two pairs of fine forceps, one end of the tubule was carefully pulled onto the cannula, taking care not to puncture the wall of the tubule. A 1 cm length of waxed dental floss (Johnson & Johnson, 3 to 5 fine strands), that had

been loosely looped around the cannula prior to the cannulating step, was gently slipped over the tubule and using a pair of fine forceps pulled tight enough to firmly secure the tubule onto the cannula (see Fig. 4.3).

Fluid was perfused through the lumen of the tubule by a 1 cc syringe mounted on a Harvard perfusion pump (see Fig. 4.2). The cannula was attached to the syringe by an 8 cm length of polyvinyl tubing (P.E.#50), ensuring there were no air bubbles. Cannulated tubules were perfused with either normal saline or normal saline plus drugs at a rate of 2.5 µl/min, until the distended tubules filled with fluid. A leak could easily be detected by an increase in the volume of the bath droplet. Once it was ensured that there was no leak in the system, perfusion was stopped and the bath droplet was removed, replaced with radiolabelled solution and allowed to sit for 5 min. Perfusion was then restarted to flush the contents of the tubule lumen (~3 min), and the secreted droplet collected (5 µl) and dispensed into 3 ml of scintillation fluid (Ecoscint).

Preliminary experiments indicated that, when tubules were bathed in high specific activity radionicotine at nanomolar concentrations, the tubules rapidly cleared the bath of all radionicotine. Ideally, the nicotine concentration in the bath should remain invariant over the course of an experiment and transport should be unidirectional. To achieve this, the bath would need to be a well-stirred large volume, containing low concentrations of nicotine at extremely high specific activity. Practically, however, the radiotracer is too expensive to use in large volumes. Hence, to ensure that the nicotine concentration in the bath remained close to invariant over the course of an experiment, unlabelled nicotine was added to the labelled solution,

increasing the nicotine concentration to either 0.05, 0.5 or 5 mM nicotine. The high specific activity of the radiotracer was therefore sacrificed in order to maintain an almost-fixed nicotine concentration in the bath.

A preliminary indication that nicotine is actively transported by the tubules is that the ratio of the concentration of nicotine in the secreted droplet to that in the bath medium exceeds unity. However, to ensure drug transport was not a result of an electrochemical potential gradient, a shorting circuit was provided in some experiments to eliminate any potential differences between the lumen of the tubule and the bath. One end of a chloridized silver wire (0.005") was inserted into the glass cannula (threading it via the pipette holder's port) to just before the cannula tip, and the other end of the wire was placed securely in the bath droplet. Although the silver wire was not actually inserted all the way into the lumen, fluid perfused over the wire was continuous with that in the lumen. The tubule was then monitored as usual for its ability to accumulate ^{3}H-nicotine under these conditions.

To determine if either atropine or verapamil interfered with nicotine transport by the tubules, drugs were included both in the bathing droplet, exposing the basal surface of the tubule cells, and in the perfusate, exposing the apical (lumenal) surface of the tubule cells (resulting in pretreatment of the apical surface of approximately 2 min).

v) Immunohistochemistry

Rhodnius Malpighian tubules were dissected as described above and fixed in

Lana's fixative for 1 h at RT. Tubules were processed and immunolabelled with the anti-P-glycoprotein antibody, C219, as described for the *Manduca* Malpighian tubules in Chapter 2.

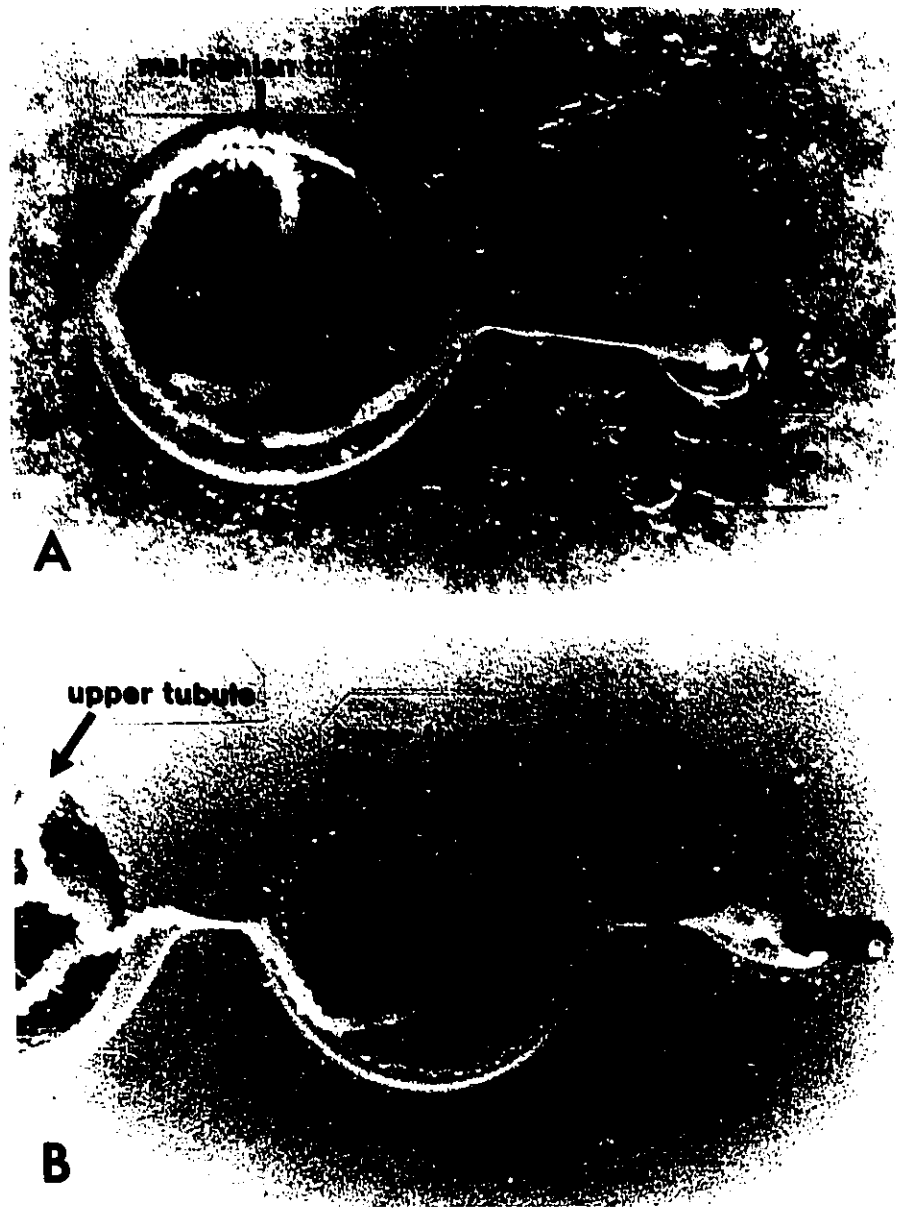
vi) Immunoelectron Microscopy

The Malpighian tubules from fifth instar *Manduca* were removed under saline and fixed in 0.25% glutcraldchyde, 4% paraformaldehyde, 0.2% picric acid and 6% sucrose in 0.1 M PB, pH 7.4 for 1 h at RT followed by a thorough rinse (3 X 5 min each) in PB with 6% sucrose. Following fixation, tissue samples were dehydrated by incubating for 50 min each in 30, 50, 70 and 90% ethanol at 4°C. Tissue was then incubated in equal parts LR White resin (LR Resin Co., London) to 90% ethanol for 45 min at -20°C and then transferred into 100% LR White for 12 h at -20°C. Tissue was transferred into gelatin capsules (on ice) and filled with fresh 100% LR White. The capsules were then transferred into a UV chamber at 4°C and allowed to polymerize overnight (approximately 12 h). Once polymerized, tissue blocks were trimmed and ultrathin sections (gold to silver interference colour) were cut with an ultramicrotome and placed on non-coated nickel grids.

For immunolabelling, grids were incubated in solutions by placing the grid, tissue side down onto a droplet of solution. To block non-specific binding sites, grids were incubated in 1% normal goat serum (Jackson Immunochemicals) in 1% BSA/PBS + 0.05% Tween 20 (incubation buffer) for 1 h at RT. Blocking solution was gently blotted off grids with filter paper and grids were incubated in primary antibody, *mdr1*

(Oncogene) at 30 μg/ml in incubation buffer, for 2 h at RT. Tissue was rinsed by first dipping grids into a beaker of filtered PBS and then with a gentle stream of filtered PBS from a clean squirt bottle. Grids were then incubated in 5 nm gold-conjugated goat anti-rabbit secondary antibody diluted 1:50 in incubation buffer and allowed to incubate for 1 h at RT. Sections were then rinsed (as described above) in PBS followed by two rinses in filtered distilled water (dH_2O). Sections were stained first with Reynold's lead citrate for 1 min, rinsed in dH_20, and then incubated in 1% uranyl acetate in 50% methanol for 5 min. Sections were then rinsed with filtered 50% methanol solution. Tissue sections were viewed on a Phillips 300 electron microscope.

Figure 4.1 Experimental set-up used for monitoring nicotine transport by isolated Malpighian tubules of *Rhodnius prolixus*. (**A**) Single-droplet method. The upper Malpighian tubule is bathed in a droplet of saline containing 5-HT (to stimulate fluid secretion) and ^{3}H-nicotine, under liquid paraffin. The cut-end of the tubule is pulled out of the droplet and secured with a glass pin pushed into the wax lining the bottom of the petrie dish. Fluid secreted by the tubule emerges from the cut-end (see secreted droplet). (**B**) The two-droplet method. The upper part of the tubule is bathed in a droplet of saline containing 5-HT (droplet A) and the lower portion of the tubule is bathed in a second droplet (droplet B) containing ^{3}H-nicotine. Fluid produced in the upper tubule flushes the lumen of the lower tubule. The open-end of the tubule is pulled out of droplet B and secured with a glass pin.


A
upper tubule
B

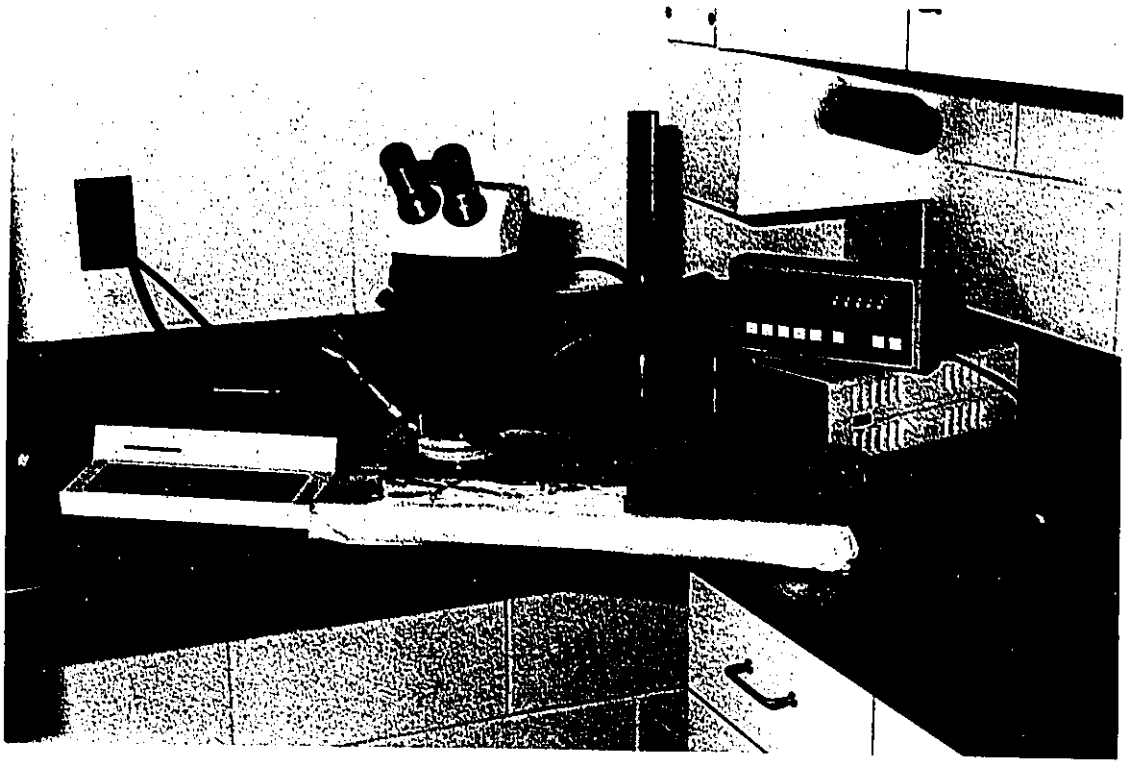
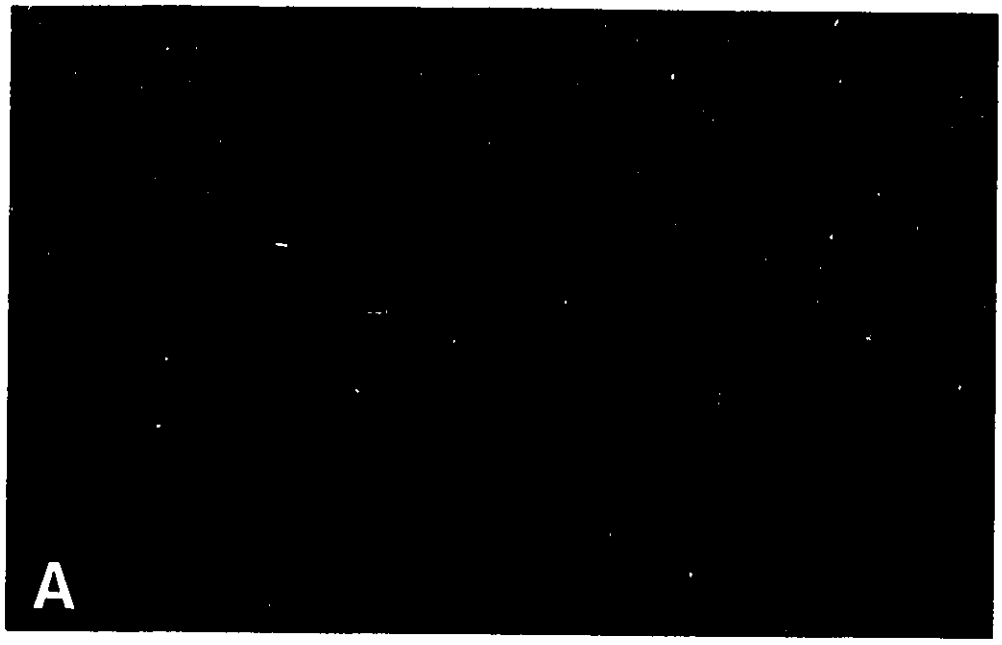
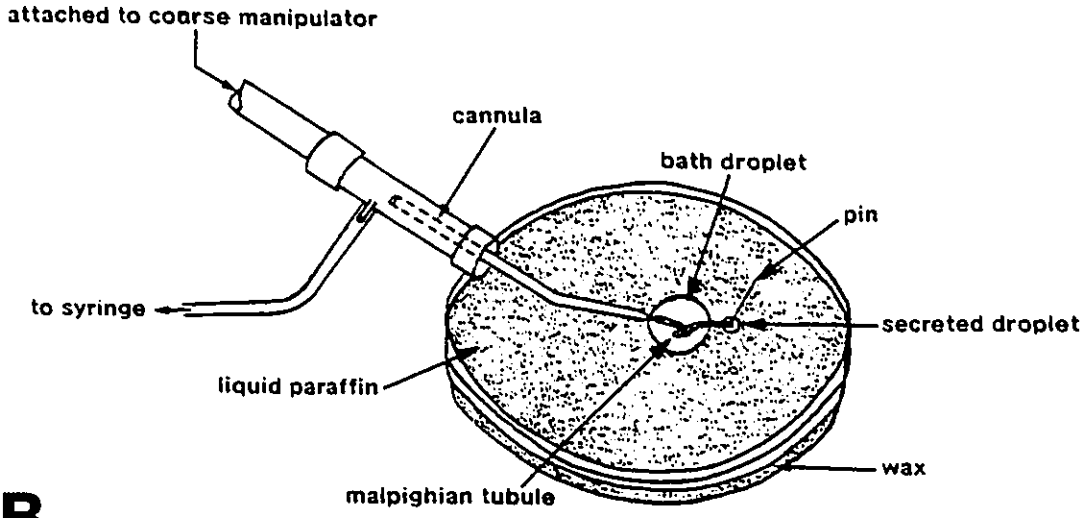


Figure 4.2 Photograph of the set-up used to monitor nicotine transport by *Manduca* Malpighian tubules. The syringe-mounted perfusion pump (left side of photo) is used to perfuse fluid through a cannulated tubule situated in the petri dish. All manipulations are performed under a dissecting microscope illuminated with fiber optics. A large digital timer (right side of photo) is used to accurately monitor the time.

Figure 4.3 Close-up of the experimental set-up used to monitor nicotine transport by the Malpighian tubules of *Manduca sexta*. (A) Photograph of a cannulated Malpighian tubule preparation. The isolated tubule sits in a droplet of saline immersed in paraffin oil (arrow). One end of the tubule is pulled onto a glass cannula while the other end is pulled out of the saline droplet and secured with a pin pushed into the bottom of the wax-lined petri dish. Fluid is perfused through the tubule by a syringe-mounted perfusion pump which is attached to the glass cannula by polyvinyl tubing. The cannula is held by a coarse manipulator, which is used to position the cannula close to the tubule. (B) Schematic representation of A. (C) A close-up view illustrating how the tapered end of the cannula is inserted into the lumen of the tubule and secured with a piece of dental floss.


A


attached to coarse manipulator
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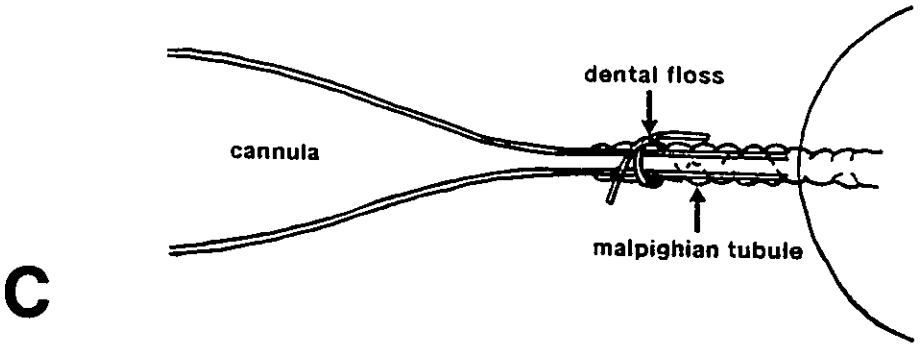
B


dental floss
cannula
malpighian tubule
C

4.3 Results

i) Effect of P-glycoprotein inhibitors on fluid secretion by the Malpighian tubules of Rhodnius

My initial studies were performed on isolated Malpighian tubules of the blood-sucking insect, *Rhodnius prolixus* in the laboratory of Dr. S. Maddrell (Department of Zoology, University of Cambridge). At this stage of the project it was not clear, either from our immunohistochemical results or from Dr. Maddrell's earlier physiological studies (Maddrell and Gardiner, 1976), whether the nicotine pump was located on the basal (hemolymph side) surface and/or the apical (lumenal) surface of the tubule. Although Maddrell and Gardiner (1976) did show that basally applied atropine inhibited nicotine transport, this result alone is not sufficient evidence for a basally situated pump since the high permeability of atropine would not prevent it from reaching the apical surface. We therefore approached these experiments assuming the technically simplest scenario, namely, that the transporter can be accessed by inhibitors supplied from the basal surface, allowing the tubules to be simply bathed in drug solutions rather than perfused (see Fig.4.1). Perfusion directly exposes the apical surface to drug solutions, but owing to the small size of the tubules (50-100 μM outer diameter), perfusing tubules is not an insignificant task.

Rhodnius tubules, unlike those from *Manduca*, secrete fluid at tremendously high rates when stimulated with the diuretic hormone, 5-hydroxytryptamine (5-HT; eg. 50 nl/min; Maddrell, 1991). Since *Rhodnius* tubules possess an alkaloid pump for

which nicotine, atropine and morphine are known substrates (Maddrell and Gardiner, 1976) they gave promise of being an ideal preparation to study the effect of P-glycoprotein inhibitors on nicotine transport. Initial experiments were performed to assess the effect of P-glycoprotein inhibitors on fluid transport by the tubules. For these experiments, the tubules were bathed in a single droplet of radionicotine, 5-HT and drug (as illustrated in Fig. 4.1A). Results demonstrated that P-glycoprotein-inhibiting drugs at concentrations routinely used to inhibit P-glycoprotein activity in other preparations, inhibited fluid secretion. Specifically, verapamil (20 μM; Fig. 4.4A), vincristine (100 μm), quinidine (10 μm), daunomycin (20 μm) (data not shown), and actinomycin D (100 μM), when added to the bath solution all produced an immediate (< 1 min) decrease in the rate of fluid secretion. These results suggest that the drugs were either directly (i.e. interfering with 5-HT induced signalling) or indirectly (e.g. causing cellular damage) interfering with the cell's ability to secrete fluid.

To overcome this problem, the two-droplet method was adopted (see Fig. 4.1B). The lower tubule, like the upper tubule, is able to transport nicotine (Maddrell and Gardiner, 1976). Hence, in this technique, the lower tubule was bathed in a solution of radiolabelled nicotine plus P-glycoprotein inhibiting drugs, relying on fluid secreted by the upper tubule to flush the lumen of the lower tubule. Transported nicotine is flushed out and appears in the fluid emerging from the cut end of the tubule. As illustrated in Figure 4.4B, using this method, the addition of drug, in this case verapamil, had a minimal effect on the rate of fluid secretion (similar results were

obtained with other inhibitors, data not shown).

ii) Failure of P-glycoprotein inhibitors to alter nicotine transport by Rhodnius tubules

For these experiments, the two-droplet method was used. In control experiments in which the tubules were bathed in ^{3}H-nicotine alone (i.e. without P-glycoprotein inhibitors), *Rhodnius* tubules were found to concentrate nicotine in the secreted droplet at least 3-fold (average of 11 tubules chosen haphazardly). Exposure of the basal surface to P-glycoprotein-inhibiting drugs, however, failed to significantly alter the ability of the tubules to transport nicotine. The results of four of the drugs (actinomycin D, daunomycin, vincristine, verapamil) tested are illustrated in Figure 4.5.

iii) Inhibition of nicotine transport in Rhodnius tubules by atropine

In light of the negative results obtained with the P-glycoprotein inhibitors on *Rhodnius* tubules, an experiment was performed to repeat the earlier finding that atropine inhibits nicotine transport in *Rhodnius* tubules (Maddrell and Gardiner,1976). Results from this experiment were similar to those obtained by Maddrell and Gardiner (1976). Addition of 3 mM atropine to the fluid bathing the lower tubules reduced nicotine transport by approximately 82% of control within 20 min (or a 4-fold decrease in the rate of nicotine transport by the Malpighian tubules; Fig. 4.6). This result confirmed that the negative results obtained with the P-glycoprotein inhibitors were not due to problems in the experimental system.

iv) Immunostaining for P-glycoprotein in Rhodnius and Manduca Malpighian tubules

Since the previous experiments of Maddrell and Gardiner (1976) provided physiological evidence for the presence of an alkaloid pump in *Rhodnius* Malpighian tubules, an immunohistochemical study was performed to determine if the tubules are P-glycoprotein immunopositive. Immunolabelling of cryostat sections of *Rhodnius* Malpighian tubule with the C219 antibody demonstrated that the tubules were indeed immunopositive for P-glycoprotein. Staining was not however homogeneous throughout the epithelium, but was associated with the apical (lumenal) rather than basal face of the tissue (Figure 4.7). Specifically, the epithelial layer is approximately 80 μM across and staining was localized to the region approximately 40 μM from the basal surface to the tubule lumen. The P-glycoprotein-positive region of the tubule is an area covered with microvilli, nearly all of which have been found to contain mitochondria (Skaer et al., 1987; Maddrell, 1991). The localization of P-glycoprotein to the apical face of the tubule may explain why the previous transport experiments (described above), where drugs were applied to the basal surface, failed to reveal any effect on nicotine transport. Presumably, atropine is more permeant than the other drugs, and therefore able to achieve inhibitory concentrations at the apical surface, even when applied basally.

For *Manduca* tubules, the previous immunohistochemical study with conventional light microscopy failed to identify the precise subcellular localization of P-glycoprotein (see Chapter 2; Murray et al., 1994), an important piece of information

to have prior to the design of transport experiments. Hence, an immunoelectron microscopy study was performed at this time. For these experiments, the polyclonal antibody, mdr1, was used (Figures 4.8 to 4.10) since the tissue-processing technique for electron microscopy abolished the C219 epitope (results not shown). Immunogold electron microscopy of Malpighian tubules labelled with mdr1 revealed gold particles to be distributed throughout the cell; gold particles were associated with the cytoplasm, with microvillar membranes at the apical cell surface, with mitochondria located within the microvilli and mitochondria located beneath the apical cell surface. The localization of P-glycoprotein to microvillar membranes of tubule epithelium concurs with the distribution of P-glycoprotein in mammalian epithelia. However, since the density of gold particles appeared to be equal throughout the tubule cell, we were unable to make any firm conclusions about the specificity of staining. It should be noted that recent findings demonstrate that mammalian P-glycoproteins, in addition to being located at the cell surface, can also be found on membranes of Golgi (Molinari et al., 1994), suggesting that P-glycoprotein immunoreactivity in the tubule cell cytoplasm may represent transport of the protein out to the membrane. However, at this time, immunogold experiments were deemed to be inconclusive and for this reason, drugs used for transport experiments were applied from both the apical and basal sides of *Manduca* tubules.

v) Transport of nicotine by Manduca Malpighian tubules

Before testing the hypothesis that the *Manduca* Malpighian tubule nicotine

transporter is a P-glycoprotein molecule, we had to demonstrate, first, that our experimental system allowed us to measure concentrative uptake of nicotine, and second, that the *Manduca* transporter is an active transporter. For these experiments we used a modification of the perfusion technique described previously by Maddrell and Gardiner (1976). In their experiments, Maddrell and Gardiner (1976) demonstrated that isolated *Manduca* Malpighian tubules produce insufficient fluid even after 8 hr for any to emerge from the cut ends. Difficulties collecting secreted fluid from *Manduca* tubules arises from their distensible nature and from the fact that after dissection the tubules are deflated; fluid secretion, which is slow in any case, has first to refill the tubules before fluid emerges from the cut ends. Consequently, Maddrell and Gardiner (1976) devised a perfusion system where isolated tubules are cannulated and perfused with solution, so that the lumenal contents can be flushed out and collected for analysis. Using this technique they demonstrated that *Manduca* tubules bathed in 5 mM nicotine while continuously perfusing the lumen (at a rate of 150 nl/min) resulted in a lumenal nicotine concentration of 21.5 mM, four times that of the bath. For our purposes, however, we chose not to continuously perfuse the tubule, since continuous flushing of the lumen with nicotine-free solution would increase the bath to lumen nicotine gradient, promoting the passive component of transport. Hence, for our experiments, the tubules sat undisturbed (i.e. still-pool) for the duration of the experiment so that we could be confident that active accumulation, and not passive efflux, was being observed. Tubules were then rapidly perfused with normal saline to flush out the lumenal contents.

Results of experiments using the 'still-pool/flush' technique are summarized in Tables 4.1 to 4.3. Larval *Manduca* tubules were bathed in either 0.05, 0.5 or 5 mM nicotine, left undisturbed for 5 min, and then the lumenal contents collected by perfusing with normal saline. The flushed perfusate contained nicotine at concentrations of 0.12 ± 0.02 mM, for tubules bathed in 0.05 mM nicotine (Table 4.1), 2 ± 0.2 mM, for tubules bathed in 0.5 mM nicotine (Table 4.2) and 11 ± 0.8 mM, for tubules bathed in 5 mM nicotine (Table 4.3) mM, resulting in lumenal to bath nicotine concentration ratios of approximately, 4, 9, and 2, respectively. Considering that these values were obtained from tubules starting not from a 1:1 gradient but from 0 mM nicotine in the lumen, these values are impressive.

The present results and those of Maddrell and Gardiner (1976) demonstrate that nicotine transport into the lumen occurs against a concentration gradient, i.e. the ratio of the concentration of nicotine in the lumen to that in the bath ratio was > 1, providing tentative evidence that nicotine is actively transported. To confirm that nicotine is actively transported into the tubule lumen it must be demonstrated that the increased concentration in the lumen is not caused by nicotine moving down an electrical gradient. That is, it is possibile that the concentration gradient of nicotine across the tubule epithelium sets up a transepithelial potential which is large enough to drive nicotine into the lumen at a rate comparable to that which would be observed with active transport. This was not, however, the case since when any possible electropotential differences between the bath and the lumen were abolished with a short-circuit, tubules were still able to concentrate nicotine to the same degree as

tubules not subjected to a short-circuit (see Table 4.2). Thus, the net accumulation of nicotine by *Manduca* Malpighian tubules occurs by an active process.

A comparison of results from tubules bathed in 5 mM nicotine using the 'still-pool/flush' technique, with that of the continuous perfusion technique of Maddrell and Gardiner (1976), shows that the concentration of nicotine in the secreted droplet is approximately ten times greater from tubules that have been continuously perfused. Since nicotine is a relatively small molecule (molecular weight of 162.2), and at physiological pH easily permeates tubule cells (Maddrell and Gardiner, 1974), once transported into the lumen it will tend to diffuse back into the cells, the more so the greater the luminal concentration. With the still-pool/flush technique, nicotine transported into the lumen will accumulate in a stagnant pool adjacent to the cells, promoting back diffusion into the cells. The net effect of more back diffusion will be a lower lumenal nicotine concentration in tubules that are flushed, than had the lumen been continuously stirred. Our aim, however, was not to establish the physiological magnitude of pumping, but to establish a reliable way to observe concentrative uptake of nicotine mediated by active transport.

Several factors suggest that in our *in vitro* preparation, the concentration of nicotine in the flushed perfusate is lower than what would be found in the *in vivo* secreted fluid. While a tubule is flushed to collect lumenal contents, laminar flow of fluid through the tubule would display a classical phenomenon referred to as the 'unstirred layer effect', in which fluid flowing adjacent to the center of flow is relatively stagnant compared to that flowing in the center. In the Malpighian tubule,

nicotine is pumped across the epithelium into the outer edge of the lumen, where fluid flow is most stagnant. Hence, the concentration of nicotine in the flushed perfusate will be lower than that of the fluid immediately adjacent to the apical surface and there provides an underestimate of the concentration against which an active transport mechanism must work to sustain the lumenal accumulation.

Manduca Malpighian tubules possess prominent outpocketings of epithelial membrane, referred to as diverticuli (Nijhout, 1975), along the length of the tubule. Nicotine transported into the lumenal region of a diverticulum, away from the center of flow, will act as a reservoir for transported nicotine. This diversion will have two effects. First, flushing of the tubule will fail to collect the nicotine trapped in the diverticuli. Second, thermodynamically, nicotine accumulating in the stagnant layer will counteract net active transport of nicotine. The net result will be less nicotine in the secreted droplet. In the living system with constant flow and with convection from body/gut movements, this would be less of a problem.

We tested this notion by gently agitating the *Manduca* tubules bathed in 0.05 mM nicotine to stir the lumenal contents. Stirring had a major effect, increasing the concentrating value from 4-fold for undisturbed tubules to 12-fold (Table 4.1). However, tubules were left undisturbed for the remaining experiments, since agitating the tubules in a consistent manner for all experiments was not feasible.

Comparisons of the nicotine concentration in the bath droplet at the start with that at the end of the experiments (i.e. after 5 min.), revealed that significant bath depletion occurred during the course of the experiment. Owing to the finite size of the

bath droplet used in this preparation, bath depletion was inevitable. As explained below, discrepancies in the concentration of nicotine in the flushed perfusate of tubules exposed to different concentrations of nicotine in the bath were noted. They can be attributed, at least in part, to bath depletion. When the starting bath concentration was 0.5 mM nicotine, a 52% depletion in the nicotine concentration in the bath was observed after 5 min (Table 4.2). When the initial bath concentration was 5 mM nicotine, the bath had depleted by 30% after 5 min (Table 4.3). This is not unexpected; on thermodynamic grounds, bath depletion should be less for the higher concentration. The higher the bath concentration, the greater the nicotine concentration in the lumen and the greater back diffusion of nicotine into the cells. Increasing the concentration of nicotine in the cell will reduce the bath to cell nicotine gradient, resulting in less diffusion of nicotine from the bath into the cells. Conversely, at lower bath concentrations, depletion should be greater. However, when the bath concentration was 0.05 mM nicotine, after 5 min the bath depleted by only 40%. If the unstirred layer effect is important in determining the net nicotine transport into the lumen, this discrepancy is not unexpected. At low bath nicotine there should be less back diffusion, and nicotine accumulating in the stagnant layer should reduce net nicotine transport into the lumen. In fact, when tubules bathed in 0.05 mM nicotine were jiggled to stir the lumenal contents, bath depletion increased to 60% after 5 min.

Hence, the lumen/bath ratios obtained in these experiments underestimate that ability of tubules *in vivo* to concentrate nicotine. Our aim, however, was not to establish the physiological magnitude of pumping, but to establish a reliable way to test

for inhibition of alkaloid or nicotine transport.

vii) Inhibition of nicotine transport in Manduca tubules

For these experiments, tubules were bathed in 0.5 mM nicotine since it was at this concentration that the technique was best suited to detecting concentrative uptake. To ascertain if nicotine transport could be inhibited, drug solutions were applied both to the bath and perfused through the tubule, exposing both the apical and basal surfaces of the epithelia, ensuring complete exposure of the tubules to drugs. If drugs interfere or compete with the nicotine transporter, there should be a decrease in the concentration of nicotine in the flushed perfusate and hence a lower lumen/bath ratio.

In *Rhodnius* tubules, Maddrell and Gardiner (1976) demonstrated that the addition of 3 mM atropine to tubules bathed in 0.5 mM nicotine resulted in a greater than 85% inhibition of nicotine transport. Likewise, if nicotine and atropine are transported by a common mechanism in *Manduca* Malpighian tubules it should be possible to demonstrate competition between them. Indeed, when 0.3 mM atropine was included, along with 0.5 mM nicotine, in the medium bathing the tubules (Table 4.4), the result was a lumen/bath ratio of 0.93 ± 0.1, a greater than 90% inhibition of nicotine transport compared to nicotine alone.

If the nicotine transporter is a P-glycoprotein-like molecule, then it should be possible to demonstrate inhibition of nicotine transport with a P-glycoprotein inhibitor. The addition of 25 μM, of the P-glycoprotein inhibitor, verapamil (Table 4.5), decreased the lumen/bath ratio to 1.2 ± 0.1, a greater than 87% decrease compared to

control. Inhibition by atropine and verapamil are summarized in Table 4.6.

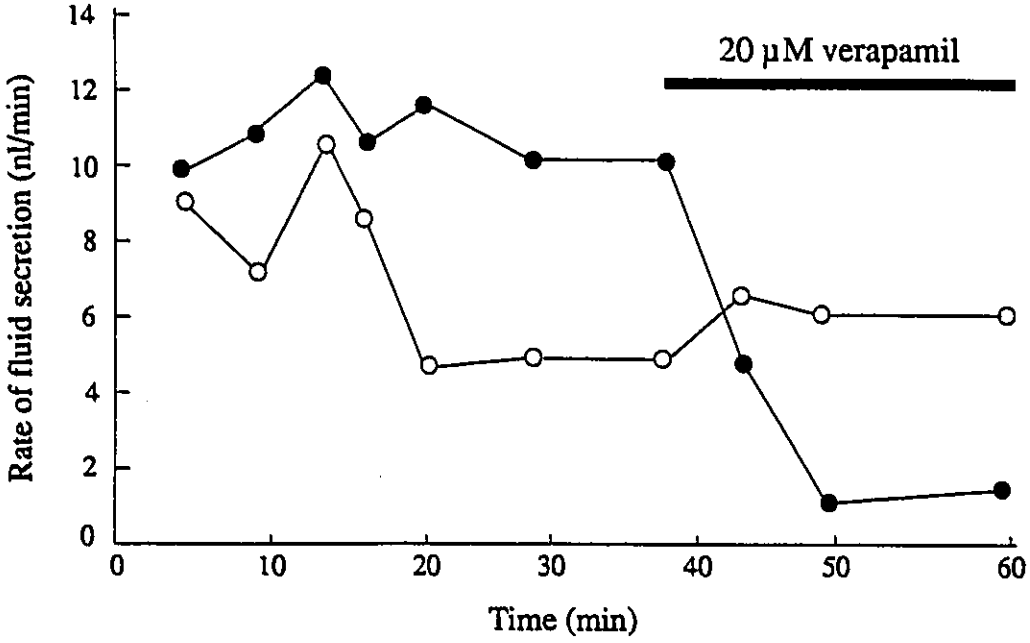
viii) Transport of the P-glycoprotein substrate, vinblastine, by an insect Malpighian tubule

To further establish whether the insect pump is a P-glycoprotein-like multi-drug pump, the tubules were tested for their ability to transport a known P-glycoprotein substrate, vinblastine. Vinblastine (mw 811), like nicotine, is an alkaloid, but is considerably less permeant than nicotine. Hence, for these experiments tubules were bathed in vinblastine at an initial concentration of 100 μM and incubated for 75 min. Results presented in Table 4.7, demonstrated that after 75 min, the concentration of vinblastine in the flushed perfusate was 237 ± 66 mM, approximately 8-fold more concentrated than the bath vinblastine at 75 min. Since our criterion for active transport is concentrative uptake, it appears that *Manduca* tubules are able to actively transport vinblastine.

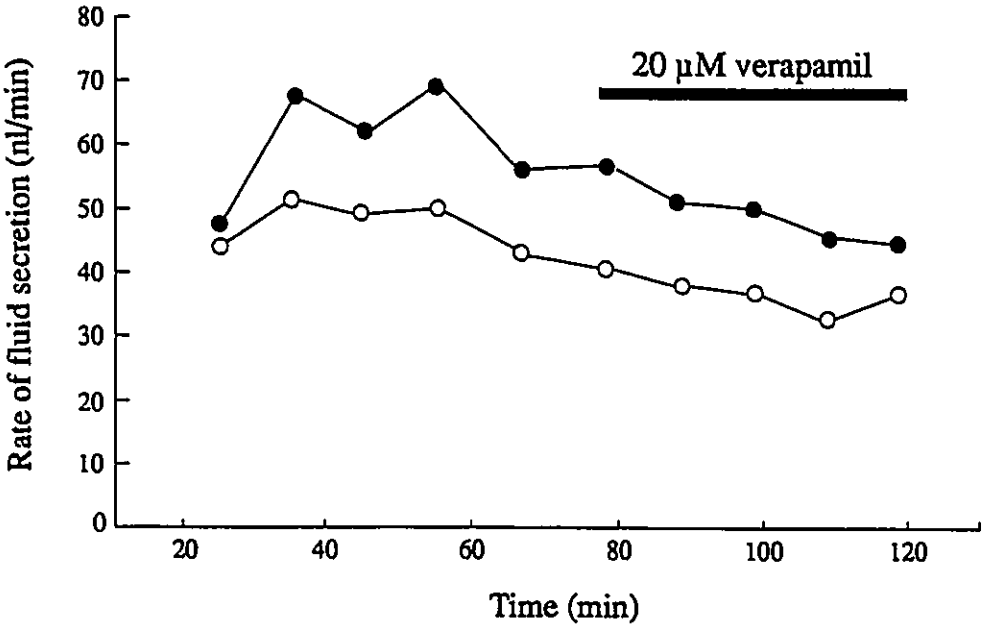
When verapamil (25 μM) was added to the perfusate, the concentration of vinblastine in the secreted fluid after 75 min, was 51 ± 1 mM, resulting in a lumen/bath ratio at 75 min of 0.69 ± 0.0055 mM, a greater than 90% inhibition of vinblastine transport compared to vinblastine alone (Table 4.8). These results provide the most convincing evidence to date, that the insect pump is a P-glycoprotein homolog.

Figure 4.4 The effect of verapamil on the rate of fluid secretion by *Rhodnius* Malpighian tubules. (A) With the single-drop method, the addition of 20 μm verapamil to the bath droplet (bar indicates period when verapamil was present) resulted in a marked decrease in the rate of fluid secretion (closed circles). Whereas, in a control tubule the rate of fluid secretion remained relatively constant over the same time period (open circles). (B) With the two-droplet method, the addition of 20 μM verapamil to droplet B (see Fig. 4.2 B) has no effect on fluid secretion (closed circles). Open circles depict a control tubule. Results are representative of 4 experiments.

A


20 μM verapamil
Rate of fluid secretion (nl/min)
Time (min)
0
2
4
6
8
10
12
14
0
10
20
30
40
50
60

B


20 μM verapamil
Rate of fluid secretion (nl/min)
Time (min)
0
10
20
30
40
50
60
70
80
20
40
60
80
100
120

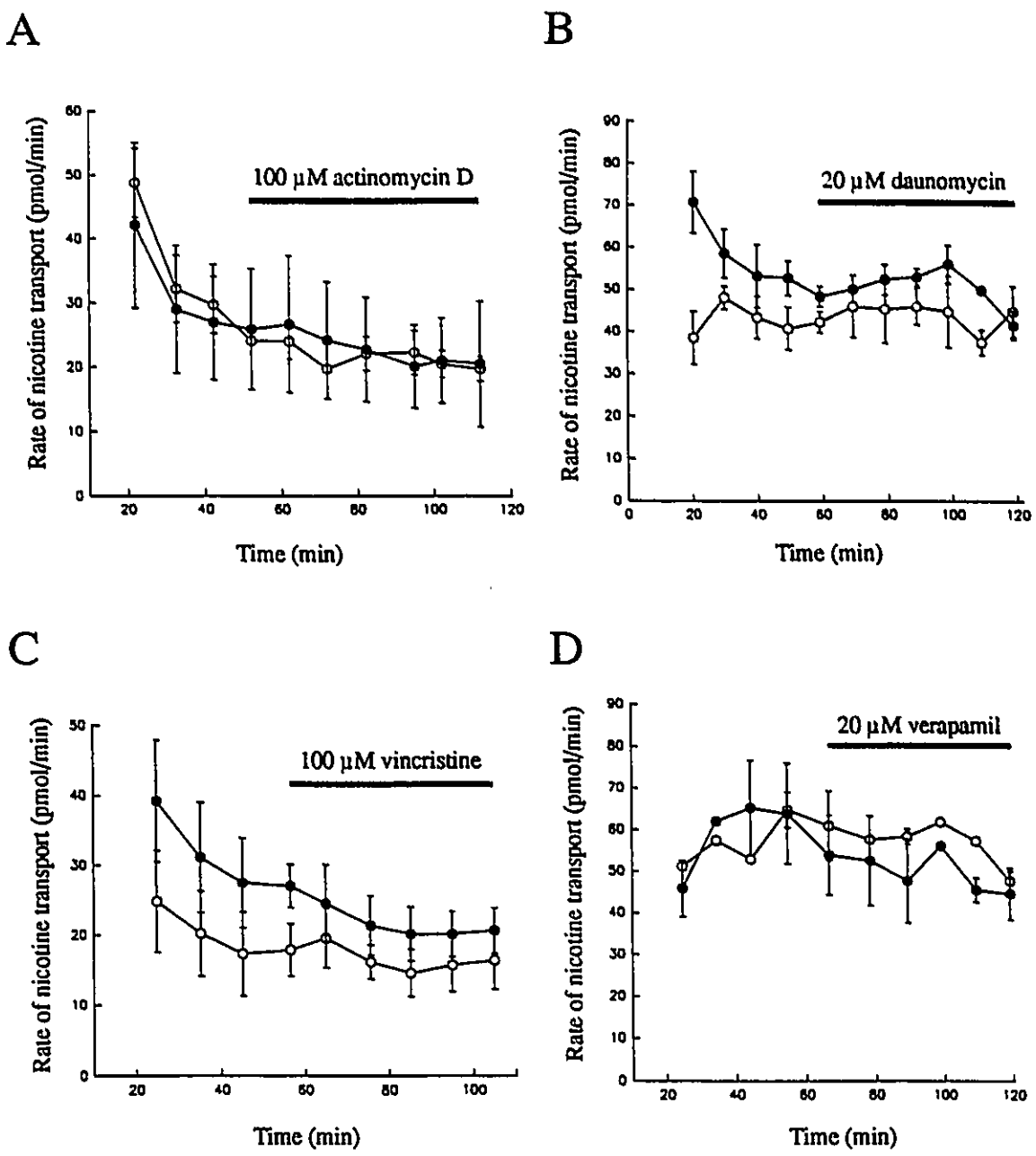


Figure 4.5 Failure of P-glycoprotein inhibitors to interfere with nicotine transport by *Rhodnius* Malpighian tubules. All experiments were performed using the two-drop method. Closed circles depict tubules in which drug was added at times indicated by the solid bar. No change in the rate-of fluid secretion was observed following the addition of 100 µM actinomycin (**A**), 20 µM daunomycin (**B**), 100 µM vincristine (**C**) or 20 µM verapamil (**D**). Open circles depict control tubules.

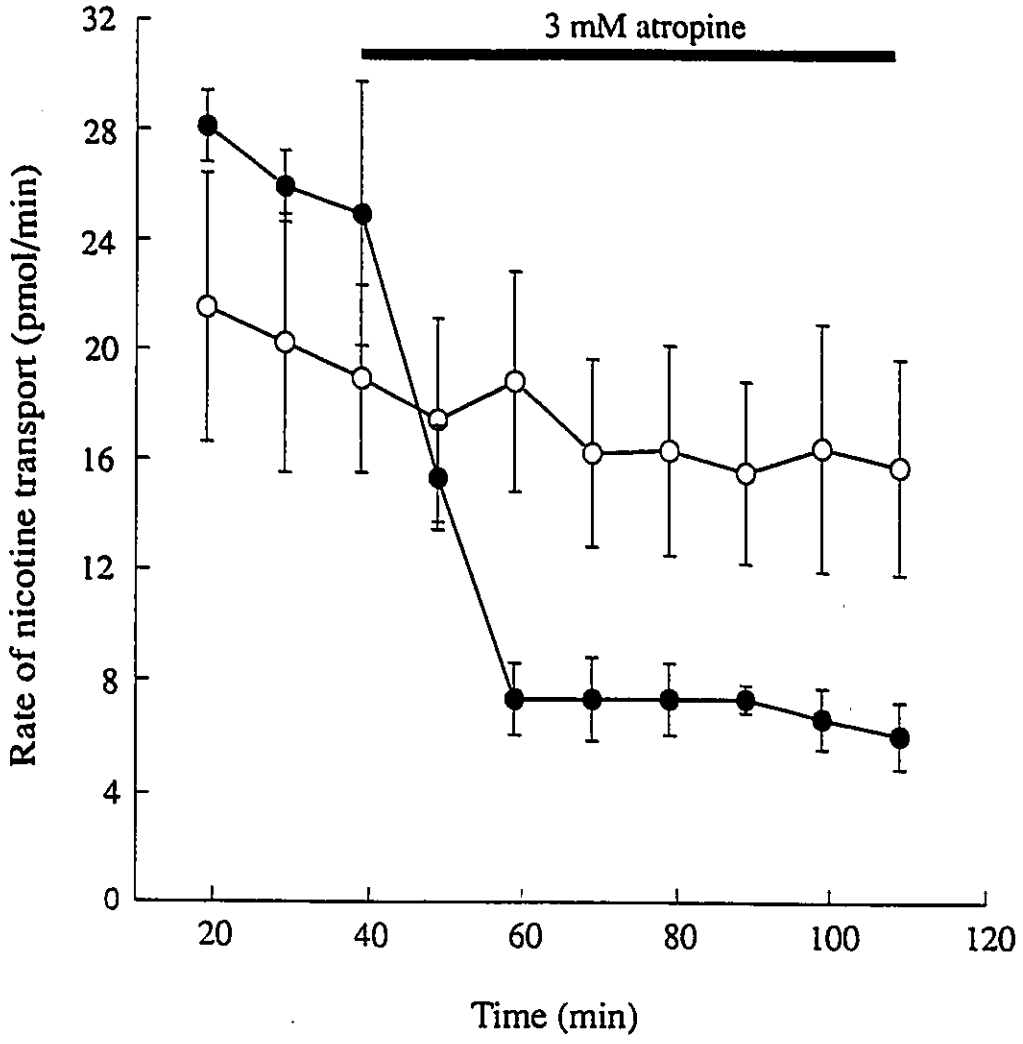


Figure 4.6 The effect of atropine on nicotine transport by the lower Malpighian tubules of *Rhodnius*. All tubules (n=6) were bathed in a solution containing 0.3 mM ^{3}H-nicotine. Three of the tubules were treated with 3 mM atropine (closed circles); bar indicates the time during which atropine was present in the bath. Three control tubules were left untreated (open circles). Each point represents the mean value + s.e.

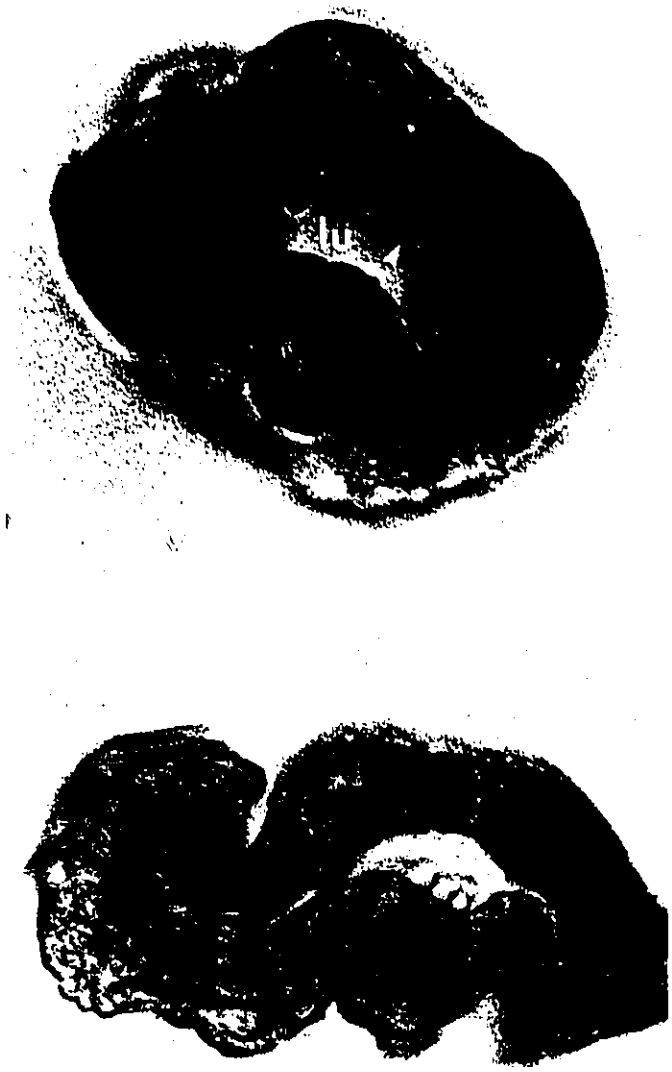


Figure 4.7 P-glycoprotein immunostaining of *Rhodnius* Malpighian tubules. **(A)** frozen cryostat section (16 μm) of a cross-section through the upper Malpighian tubule immunolabelled with the monoclonal antibody C219, showing label associated with the apical (lumenal) side of the tubule. **(B)** A control serial section in which the primary antibody was substituted with mouse serum shows no immunolabelling. Bar=10 μm.

Figure 4.8 Electron micrograph of a cross section of a *Manduca* Malpighian tubule. Characteristic of epithelial tissue, the Malpighian tubule has a microvillied luminal (lu) surface and an acellular basement membrane (bm). Note that because of the mild processing technique utilized to maintain antigenicity (e.g. absence of osmium tetroxide in the staining procedure), membranes are not easily visible and individual cells cannot be resolved. The cytoplasm, particularly at the apical side, has an abundance of mitochondria which often extend into the microvilli. Bar=0.2 μm.

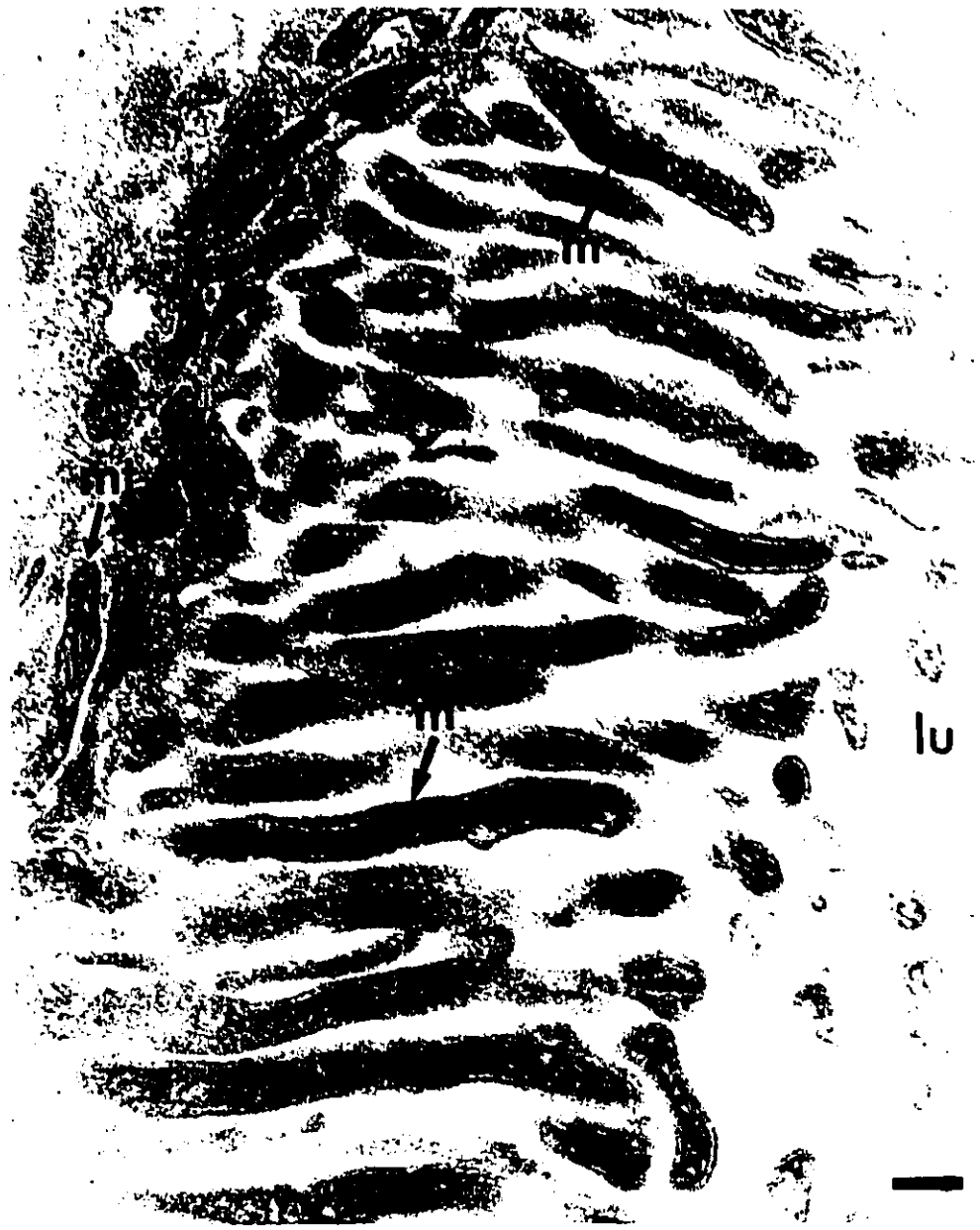


Figure 4.9 Immunogold labelling of a *Manduca* Malpighian tubule with the polyclonal antibody, *mdr1*. Gold particles are associated with the microvillar surface and to mitochondria, both within the microvilli (m) and in the cytoplasm (mt). Gold particles can also be observed in the cytoplasm. The presence of gold particles in the cytoplasm may represent non-specific binding, or immunoreactivity with protein molecules being transported to target membrane. Bar=0.5 μm.

Figure 4.10 High magnification of the luminal surface. (A) A longitudinal section through the tubule illustrates gold particles associated with mitochondrion containing microvilli (arrows). (B) Cross-section of a tubule showing absence of gold labelling in a control experiment in which the primary antibody was omitted. Bar=0.5 μm.

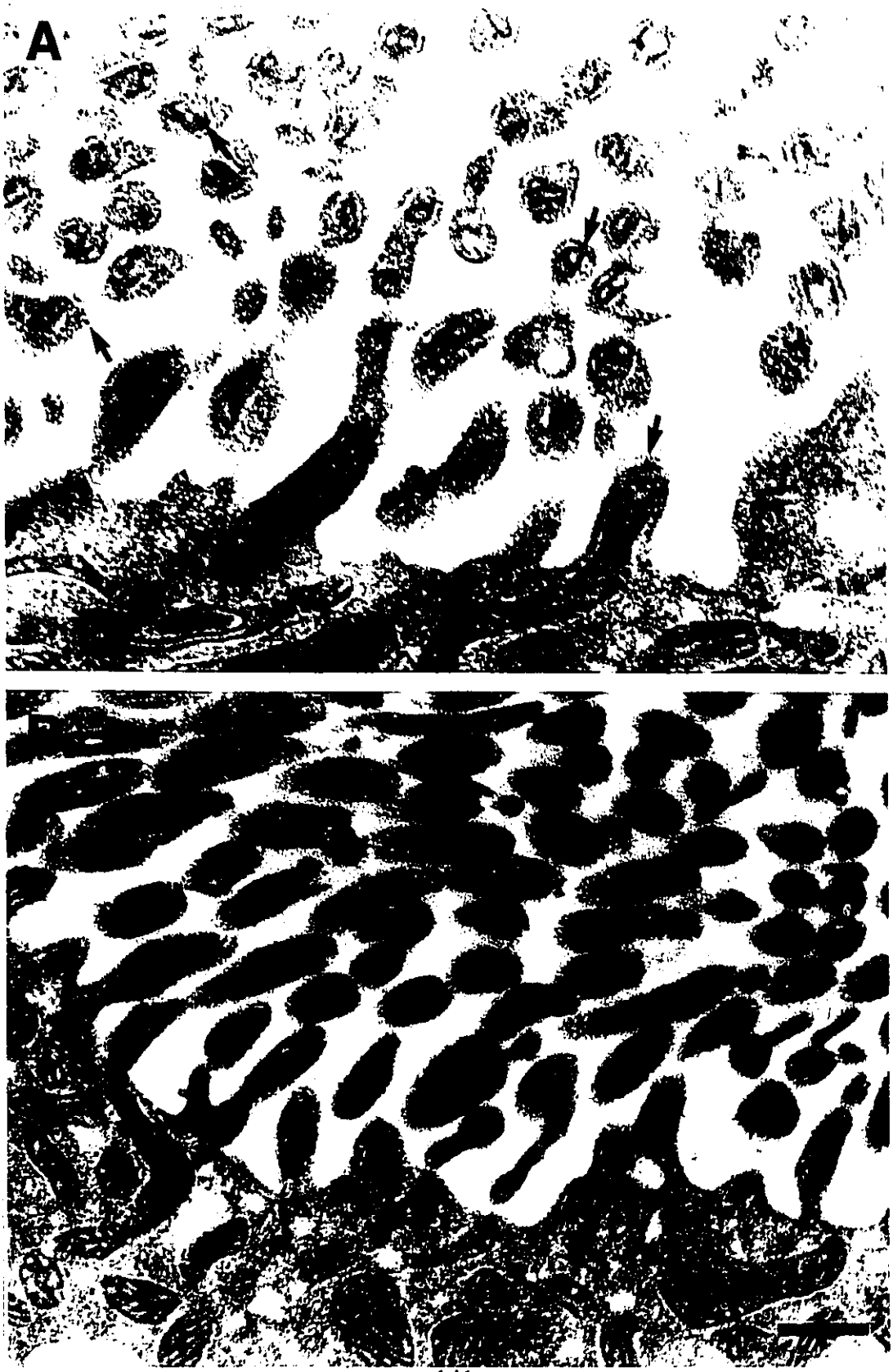

A

Table 4.1. Nicotine transport by *Manduca sexta* malpighian tubules bathed in 0.05 mM nicotine

	[Nicotine] in bath (mM) at t = 5 min	[Nicotine] in flushed perfusate (mM) at t = 5 min	X Concentrated	% bath depletion
Tubule #				
1	0.04	0.08	2.0	30
2	0.03	0.16	5.3	44
3	0.03	0.09	3.3	42
4	0.03	0.13	4.3	40
5	0.03	0.15	5.0	42
*6	0.02	0.30	15	64
*7	0.03	0.33	11	50
*8	0.02	0.34	11	66
Mean±SE	**0.03 ± 0.002**	**0.12 ± 0.02**	**4.0 ± 0.60**	**40 ± 2.5**
	***0.02 ± 0.003**	***0.32 ± 0.01**	***12 ± 1.3**	***60 ± 5.0**

*** tubules jiggled while perfusing**

Table 4.2. Nicotine transport by *Manduca* malpighian tubules bathed in 0.5 mM nicotine

	[Nicotine] in bath (mM) at t = 5 min	[Nicotine] in flushed perfusate (mM) at t = 5 min	X Concentrated	% bath depletion
Tubule #				
1	0.18	1.6	8.9	64
2	0.17	2.8	17	66
3	0.21	1.5	7.1	58
4	0.22	1.7	7.7	56
5	0.18	2.1	12	64
6	0.30	2.4	8.0	40
*7	0.33	1.9	5.8	33
*8	0.32	2.2	6.9	39
Mean±SE	**0.24 ± 0.02**	**2.0 ± 0.2**	**9.1 ± 1.2**	**52 ± 5**

*** tubules from shorting-out experiments**

Table 4.3. Nicotine transport by the malpighian tubules of *Manduca sexta* bathed in 5 mM nicotine

	[Nicotine] in bath (mM) at t – 5 min	[Nicotine] in flushed perfusate (mM) after 5 min	X Concentrated	% bath depletion
Tubule #				
1	3.7	10.8	2.0	26
2	3.1	13.4	2.7	38
3	3.6	9.6	2.0	28
4	3.7	10.7	2.0	26
Mean ± SE	**3.5 ± 0.1**	**11.1±0.8**	**2.2±0.2**	**30 ± 2.9**

Table 4.4. Effect of atropine (3mM) on nicotine secretion by the malpighian tubules of *Manduca sexta*

Tubule #	[Nic] in bath (mM) at t = 5 min	[Nic] in flushed perfusate at t = 5 min	X Concentrated
1	0.41	0.51	1.0
2	0.35	0.30	0.6
3	0.38	0.28	0.6
Mean ± SE	**0.38 ± 0.02**	**0.34 ± 0.07**	**0.73 ± 0.13**

Table 4.5. Effect of verapamil (25 μM) on nicotine secretion by the malpighian tubules of *Manduca sexta*

Tubule #	[Nic] in bath (mM) at t = 5 min	[Nic] in flushed perfusate at t = 5 min	X Concentrated
1	0.38	0.42	1.1
2	0.36	0.36	1.0
3	0.32	0.33	1.0
4	0.41	0.62	1.5
5	0.38	0.49	1.3
Mean ± SE	**0.37 ± 0.02**	**0.44 ± 0.05**	**1.2 ± 0.097**

Table 4.6. Inhibition of nicotine transport in the malpighian tubules of *Manduca sexta*

Nicotine concentration in bath	Drug and concentration used	Effect	Number of tubules tested
0.5 mM	3 mM atropine	>90% inhibition of nicotine transport	3
0.5 mM	25 μM verapamil	>87% inhibition of nicotine transport	5

Table 4.7. Transport of vinblastine (100 μM) by *Manduca* malpighian tubules

Tubule #	[Vinblastine] in bath (μM) after 75 min	[Vinblastine] in flushed perfusate (μM) after 75 min	X Concentrated
1	24	322	13.4
2	49	107	2.2
3	37	281	8.0
Mean ± SE	37 ± 7	237 ± 66	8.0 ± 3

Table 4.8. Inhibition of vinblastine transport by verapamil in *Manduca* malpighian tubules

Tubule #	[Vinblastine] in bath (μM) after 75 min	[Vinblastine] in flushed perfusate (μM) after 75 min	X Concentrated
1	80	50	0.63
2	70	52	0.74
Mean ± SE	75 ± 5	51 ± 1	*0.69 ± 0.055

* results in > 76% inhibition of vinblastine transport

4.4 Discussion

Results from experiments on larval *Manduca* Malpighian tubules demonstrate that the 'still-pool/flush' technique provides a reliable and rapid assay to monitor concentrative uptake of substrate into the tubule lumen. Using this technique we were able to demonstrate that (1) *Manduca* Malpighian tubules actively transport nicotine into the tubule lumen, (2) nicotine transport is inhibited by atropine, (3) *Manduca* tubules concentrate vinblastine in the tubule lumen, and (4) both nicotine and vinblastine transport are inhibited with the P-glycoprotein-inhibitor, verapamil. Physiologically, the *Manduca* Malpighian tubule alkaloid transporter displays the hallmarks of a P-glycoprotein transporter.

Two lines of evidence demonstrate that larval *Manduca* Malpighian tubules actively transport nicotine into the tubule lumen. First, movement of nicotine across the tubules occurred against a concentration gradient, that is, the ratio of the concentration of nicotine in the secreted fluid to that in the bath medium exceeded 1, and second, this ability of the tubules to concentrate nicotine was not altered when any electrical potential difference across the tubule wall was short-circuited. Likewise, transport of another alkaloid, vinblastine, into the tubule lumen occured against a concentration gradient. Although these finding do not establish, unequivocally, that vinblastine is actively transported, the finding that verapamil inhibited nicotine and vinblastine transport implies that both alkaloids are transported by a common mechanism. Likewise, the finding that atropine inhibits nicotine transport is consistent

with the idea that both are transported by a common mechanism. The *Manduca* Malpighian transporter appears then to be a multi-alkaloid transporter.

Like *Manduca* Malpighian tubules, *Rhodnius* tubules possess a multi-alkaloid pump (Maddrell and Gardiner, 1976) and are immunopositive for P-glycoprotein (Fig. 4.7). To directly establish, however, that the insect transporter is indeed a P-glycoprotein-like molecule it must be demonstrated that inhibition of P-glycoprotein is associated with an inhibition in alkaloid transport. Currently, there are a variety of drugs used to reverse P-glycoprotein-mediated multi-drug resistance in tumor cells. These drugs can be grouped into two catagories, P-glycoprotein substrates and chemosensitizers. The mechanisms by which these agents reverse multi-drug resistance are not fully understood but many appear to function as competitive inhibitors of drug binding and/or transport by P-glycoprotein (Cornwell et al., 1987b; Akiyama et al., 1988).

P-glycoprotein substrates are compounds to which multi-drug resistant cells are resistant, i.e. those substrates that are transported out of the cell by P-glycoprotein. These include a diverse group of compounds that are structurally and functionally unrelated but are generally all hydrophobic, tertiary nitrogen compounds. Examples include many natural anti-cancer drugs such as the alkaloids vinblastine and vincristine, the anthracyclines adriamycin and daunomycin, as well as a variety of other agents such as colchicine and taxol.

Chemosensitizers include calcium channel blockers such as verapamil and nifidepine, non-ionic detergents, cyclosporins, and steroid hormones. These drugs are

not generally substrates for P-glycoprotein, and little is known about their mechanisms for reversing multi-drug resistance. Verapamil, however, which is used extensively in the clinic and laboratory to reverse P-glycoprotein mediated multi-drug resistance, has been shown to bind to P-glycoprotein (Cornwell et al., 1987b; Safa, 1988; Yusa and Tsuruo, 1989). It appears that verapamil competively inhibits binding of substrates to P-glycoprotein, leading to the inhibition of drug transport.

The inhibition of nicotine and vinblastine transport by verapamil provides the most convincing evidence to date that the *Manduca* Malpighian tubule transporter is a P-glycoprotein molecule. Verapamil, however, is also known to block L-type calcium channels (Fleckenstein, 1985) and it could be argued that a mechanism other than P-glycoprotein might be involved in inhibition of nicotine or vinblastine transport. This, however, is not likely the case since recent studies have demonstrated that the application of verapamil (20 μM, Venant et al., 1994; 50 μM, Matsunaga et al., 1994) has no effect on calcium entry in epithelial cells. Additionally, it has been demonstrated in a mammalian tumor cell line that the ability of verapamil to reverse multi-drug resistance is not mediated by blocking calcium channels since nifedipine, a more potent blocker of L-type calcium channels, was found to be a poor reversing agent when compared to verapamil (Cornwell et al., 1987b).

In *Rhodnius* Malpighian tubules, verapamil added to the bath had no effect on transport of nicotine. Further experiments on *Rhodnius* tubules demonstrated that bath application of another calcium channel blocker, nifedipine, and the P-glycoprotein substrates vincristine, actinomycin D, daunorubicin, failed to alter the ability of the

tubules to transport nicotine. The immunohistochemical study, performed subsequent to the transport studies revealed P-glycoprotein immunoreactivity at the apical (lumenal) surface of the tubule. The P-glycoprotein reversing agents used in this study were of widely varying pKa's and lipid solubilities, with molecular weights ranging from 346.3 (for nifedipine) to 824.9 (for vincristine). These factors would invariably affect the permeability of the reversing agents across the tubule epithelium. Since drugs were applied only to the basal surface of *Rhodnius* tubules, it is conceivable that the drugs could be more effective if applied apically. Cannulation of *Rhodnius* tubules would be required to ensure drugs come into direct contact with the apical surface. Since *Manduca* tubules are larger than, and hence easier to cannulate than *Rhodnius* tubules, they are more amenable preparation for further studies.

The vast majority of the drugs that act as P-glycoprotein substrates (and those most often involved in cancer chemotherapy) are derived from plants. The huge diversity of compounds found in plants has been hypothesized to be a result of millions of years of co-evolution with herbivorous insects (Erlich and Raven, 1964). Plants have evolved chemical defences and insects, in turn, have evolved very presise mechanisms to enable them to tolerate potentially toxic compounds in the plants they consume. One such mechanism appears to be a P-glycoprotein-like molecule.

Our findings have contributed to an understanding of the natural functions of P-glycoproteins. It has long been postulated that a primary function of P-glycoproteins is the transport of exogenous toxins (i.e. ingested toxins from foods) away from sensitive sites in the body. In fact, a recent study demonstrated that transgenic mice

with a knockout of the gene, *mdr3*, which encodes P-glycoprotein, were unable to exclude the neurotoxic alkaloid, vincristine, from the brain (Schinkel et al., 1994), providing the most direct evidence to date that P-glycoprotein protects the brain from exogenous toxins.

Although functionally the insect transporter and mammalian P-glycoprotein appear similar, morphine and nicotine appear to be only low-affinity substrates, while atropine does not appear to be a substrate for purified reconstituted mammalian P-glycoprotein (Frances Sharom, personal communication). Therefore, based on drug interactions, there may be interesting differences between the insect transporter and mammalian P-glycoprotein. Because each protein has evolved separately, it is possible that each is designed to handle different substrates depending on the needs of the organism. To determine the degree of homology between the mammalian P-glycoprotein and the insect transporter, we must await the cloning of the gene(s) encoding the insect transporter. It is also possible that special physical attributes of the insect tissues (e.g. intracellular compartments, intracellular pH, membrane lipids) facilitate the transport of small highly permeant alkaloids like nicotine.

CHAPTER 5

CO-LOCALIZATION OF P-GLYCOPROTEIN AND CYTOCHROME P450 IN THE MIDGUT OF *MANDUCA SEXTA*

5.1 Introduction

i) P-glycoprotein in epithelia

In mammals, P-glycoprotein is prominently expressed on the apical surfaces of epithelial cells of the digestive tract (Thiebaut et al., 1987; Trezise et al., 1992) and excretory system (i.e. kidney; Thiebaut et al., 1987; Georges et al., 1990). Most recently, P-glycoprotein molecules have been identified in the digestive tracts of the soil-dwelling nematode, *Caenorhabditis elegans* (Lincke et al., 1993; Broeks et al., 1995) and the aquatic frog, *Xenopus laevis* (Castillo et al., 1995), both organisms whose natural habitats are rich in xenobiotics. The expression of P-glycoprotein in the digestive tracts of mammals and in lower organisms suggests that P-glycoprotein operates in these tissues as a defensive mechanism to protect organisms from dietary toxins.

In *C. elegans*, four P-glycoprotein gene homologs have been identified (*pgp-1* to *-4*; Lincke et al., 1992). Analysis of the predicted protein structure of *pgp-1* and *pgp-3* indicate that they share extensive structural homology with their mammalian

counterparts which are composed of two homologous halves, each encoding six transmembrane domains and an ATP-binding site (Lincke et al., 1992). To study the expression of the pgp-1 and pgp-3 genes in *C. elegans*, Lincke et al. (1993) generated transgenic worms carrying the *pgp-1* and *pgp-3* sequences fused to a bacterial *lacZ* reporter gene; by staining for ß-galactosidase activity they were able to study the tissue-specific expression of these genes. Results demonstrated that *pgp-1* and *pgp-3* are both expressed exclusively by the intestinal cells. To investigate the tissue distribution at the protein level, Broeks et al. (1995) performed an immunohistochemical study using the monoclonal antibody, C219, which recognizes a highly conserved epitope in all P-glycoprotein isoforms (Georges et al., 1990). *C. elegans* are multi-cellular organisms and as a result the total amount of protein in wild-type nematodes is very low. To overcome this, Broeks et al. (1995) generated two transgenic strains, one which over-expressed the *pgp-1* protein product (PGP-1) and the other, the *pgp-3* protein product (PGP-3). Immunohistochemical staining demonstrated that PGP-1 was localized to the apical membrane of the intestinal cells, whereas, PGP-3 was localized to the apical membrane of both the intestinal and excretory cells. To determine the function of PGP-3, *C. elegans* strains in which the *pgp-3* gene had been deleted were produced. Exposure of the knock-out strains to colchicine, a known P-glycoprotein substrate, results in an inability of the organisms to develop normally. Broeks et al. (1995) concluded that the colchicine susceptibility was caused by deletion of *pgp-3* and not by a linked mutation, since introduction of the wild-type *pgp-3* gene

into the mutant strain by microinjection restored resistance to colchicine.

In *Xenopus*, Castillo et al. (1995) have identified a P-glycoprotein homolog (*Xe-mdr*) encoding a protein that is 66% identical to the mouse *mdr1b* and 68% identical to the human *mdr1*, both of which are expressed in the intestine. An *in situ hybridization* and immunohistochemical study demonstrated that, in *Xenopus*, *Xe-mdr* mRNA and its protein product are localized exclusively to the apical membrane of the intestine. To examine the function of *Xe-mdr*, membrane vesicles were prepared from the small intestine. Vesicles were shown to transport vinblastine, a known P-glycoprotein substrate, and this transport was decreased in the presence of the P-glycoprotein inhibitor, verapamil (Castillo et al., 1995).

Previously, we have demonstrated that the excretory Malpighian tubules of the insect, *Manduca sexta* possess a P-glycoprotein-like molecule. Labelling with the antibody, C219, demonstrated that the Malpighian tubules were immunopositive for P-glycoprotein (Murray et al., 1994; Chapter 2) and experiments using an isolated tubule preparation demonstrated that the transport of the plant alkaloids, nicotine and vincristine, was inhibited with the P-glycoprotein reversing agent, verapamil (Chapter 4). For a toxin to come into contact with the Malpighian tubule transporter it must be present in the hemolymph, having either penetrated the cuticle or diffused from the gut contents. However, for insects whose diet consists of plant material laden with potentially toxic compounds it would seem logical that the first level of protection to take place would be in the gut, since it is the first tissue to come into contact with the toxin once it has been ingested. *Manduca* larvae subsist on a diet of tobacco and as

a result consume vast quantities of the highly toxic alkaloid, nicotine. In the current study, an immunohistochemical study was performed to determine if a P-glycoprotein-like mechanism is present in the *Manduca* gut. Results demonstrated P-glycoprotein immunolabelling at the apical surface of the *Manduca* midgut, suggesting that, as in other organisms, a P-glycoprotein-like mechanism is operating in the insect digestive system.

ii) Complementary role for cytochrome P450-dependent monooxygenases and P-glycoprotein in resistance?

Although in recent years the term drug resistance has become synonymous with P-glycoprotein, in reality, the process is the result of a wide range of protective mechanisms. One such mechanism is the detoxification of drugs by a specialized group of metabolic enzymes, the most important of which are the cytochrome P450-dependent monooxygenases (P450s), otherwise known as the polysubstrate monooxygenases (PSMOs). Like the P-glycoproteins, the PSMOs are a ubiquitous enzymes widely distributed in species from bacteria to mammals and higher plants (Guengerich, 1993). The PSMO enzymes catalyze the oxidation of exogenous or endogenous toxins, producing metabolites that are more polar than the parent compounds, at which point they can either be excreted or serve as substrates for further metabolism (Guengerich, 1993).

Surprisingly, the importance of a co-interaction between PSMOs and P-glycoproteins has not been well studied, despite a number of striking similarities

between PSMOs and P-glycoproteins. In mammals, PSMOs and P-glycoproteins are often simultaneously expressed in the same tissues and an examination of the compounds that act as either substrates and/or inhibitors of mammalian PSMOs and P-glycoproteins reveal a striking overlap (Wacher et al., 1995), suggesting that these enzymes have complementary roles. For example, in the case of digitoxin, a drug commonly used to treat heart problems, the parent compound is a PSMO substrate (Eberhart et al., 1991), whereas a metabolite, digoxin, has been shown to be transported by P-glycoprotein (deLannoy and Silverman, 1992; Tanigawara et al., 1992).

In insects, the role of the PSMOs in the detoxification of xenobiotics including plant toxins and synthetic insecticides has been well documented (see Oppenoorth, 1985 and Ahmad et al., 1986 for reviews). PSMOs are generally found in the midgut and fat body and occasionally in the Malpighian tubules. In *Manduca*, PSMO-mediated metabolism of nicotine appears to be an integral component of the insect's ability to consume tobacco. Tate et al. (1982) demonstrated, using spectral and enzymatic assays, PSMO activity in the *Manduca* midgut and fat body. PSMO activity was highest in the actively feeding fifth instar and decreased as the insect approached the non-feeding pupal stage, implying that PSMO-based metabolism plays an important role in the insect's ability to tolerate dietary nicotine. In addition to the midgut and fat body, the *Manduca* CNS, the site of nicotine-sensitive ACh-receptors, is also able to metabolise nicotine, and by the nature of the metabolites, this metabolism appears to be mediated by PSMOs (Morris, 1984). More recently, it has been demonstrated (see Murray et al., 1994), that co-incubation of freshly isolated *Manduca* CNS in a

solution of radiolabelled nicotine and piperonyl butoxide, a potent inhibitor of PSMO detoxifying enzymes (Wilkinson et al., 1984), resulted in the uniform distribution of radionicotine across the CNS. This finding suggests that PSMO-based metabolism of nicotine is a critical constituent of the insect's blood-brain barrier, a barrier that restricts nicotine from reaching sensitive neuronal receptors.

Until recently, research on insect PSMOs relied exclusively on enzymatic assays to access PSMO activity in tissues. However, recent advances in purification techniques (e.g. Wheelock and Scott, 1989) have facilitated the development of antibodies and molecular probes to cytochrome P450, cytochrome b_5 and cytochrome P450 reductase, the individual components of the PSMO system. In the current study, an oligonucleotide probe and a monoclonal antibody to cytochrome P450 mRNA and protein, respectively, were used to assess the distribution of PSMO in *Manduca* midgut. The midgut was chosen over other tissues since it is exposed to high concentrations of dietary nicotine and previous research has demonstrated that, along with the fat body, midguts have the highest PSMO activity (Tate et al., 1982).

5.2 Materials and methods

i) Insects

Manduca were reared as described previously (see Chapter 2). For some experiments, larvae were fed directly on the leaves of tobacco plants for 24 hrs prior

to dissection. To assess the cross-species sensitivity of the oligonucleotide probe, another insect, the fall armyworm (*Spodoptera frugiperda*) was also examined for cytochrome P450 expression. *Spodoptera* larvae were reared on a soybean-based diet and maintained in the same environmentally controlled chamber as *Manduca*.

ii) Immunohistochemistry

Paraffin sections were used in this study owing to the superior morphological preservation over frozen sections. The midguts and fat body tissue from fifth instar *Manduca* larvae were dissected under saline and fixed in freshly prepared 4% paraformaldehyde for 2 hrs at RT. Tissue was dehydrated in ethanol and xylene prior to embedding in paraffin. Paraffin sections (12 μM) of the insect tissue were cut and collected on gelatin coated glass slides.

The distribution of P-glycoprotein and cytochrome P450 was assessed immunohistochemically using the ABC method described in Chapter 2. The monoclonal antibody P450B1 (gift from Dr. L.T. Waters, Oak Ridge National Laboratory, Tennessee) was used to detect cytochrome P450 (1:100 dilution). To detect P-glycoprotein, the polyclonal antibody mdr1(Ab-1) was used since the embedding technique destroys the C219 epitope. Like C219, the mdr1(Ab-1) epitope also recognizes a highly conserved epitope. Control experiments included omitting the primary antibody or replacing the primary antibody with non-immune mouse serum or with an unrelated antibody. In this case we used a monoclonal antibody against vasopressin, a neurosecretory hormone found in the mammalian CNS (gift of Dr. L.P.

Renaud, Neurosciences, Loeb Research Institute).

iii) In situ hybridization

Probe selection and synthesis

A synthetic 21-mer oligonucleotide probe, complementary to the coding sequence 5' AACTGCCTAGGTATGCGGTTT 3' was synthesized. The probe sequence was based on a highly homologous region from the 5' region of the cytochrome P450 cDNA of three pesticide-resistant insects: (1) a diazinon-resistant housefly (*Musca domestica*; Feyereisen et al., 1989), (2) a DDT-resistant fruitfly (*Drosophila melanogaster*; Waters et al., 1992), and (3) furanocoumarin-resistant larvae of the black swallowtail butterfly (*Papilio polyxenes*; Cohen et al., 1992). GENBANK was used to compare the full-length sequences of the three insects and determine the region of highest homology. The probe shared 100% sequence identity with larvae of the lepidopteran black swallowtail and 87% homology with both housefly and fruitfly cytochrome P450 cDNA sequences. To ensure that the probe sequence was uniquely complementary to the target mRNA, GENBANK was also used to search the databank for any incidentally homologous sequences. This included a comparison to a cytochrome P450 cDNA from a cockroach that encodes a form of cytochrome P450 that is putatively involved in hormone synthesis (Bradfield et al., 1991). The cockroach cytochrome P450 was found to be 24% identical to the black swallowtail sequence. An unrelated probe to a peptide from the marine mollusc *Aplysia californica*, sensorin-A (Brunet et al., 1991), was used as a control (from Dr. I.

Steffensen, Loeb Research Institute).

Probe Labelling

Both sense and antisense probes were labelled using the Genius Non-radioactive Labelling Kit (Boehringer Mannheim). Briefly, a terminal transferase catalyzes the addition of nucleotide analog, digoxigenin (DIG)-11-dUTP to the 3'-hydroxyl terminus of the DNA probe. The labelled probe was kept at -20°C until use.

Paraffin section in situ hybridization

Tissue from fifth instar larvae of *Manduca* and *Spodoptera* were fixed in freshly prepared 4% paraformaldehyde for 2 hrs at RT. In experiments using *Spodoptera* larvae, the insects were first cooled in a refrigerator, then cut into pieces using a razor blade. Tissue was fixed overnight in 4% paraformaldehyde, and subsequently processed for paraffin embedding. Paraffin sections (12 μM) were cut on a microtome. Tissue sections were incubated at 37°C in 5 μg/ml proteinase K for 15 min, to ensure accessibility of the tissue. Sections were subsequently rinsed in sterile water and allowed to air-dry thoroughly prior to incubation in DIG-labelled cytochrome P450 oligonucleotide probe (500 pg/μl) diluted in hybridization buffer [50% formamide, 6X sodium citrate (SSC), 50 mM Tris-HCl, 2X Denhardt's solution, 0.2% sodium dodecyl sulphate (SDS)]. Tissue was incubated in probe overnight in a humidified box with 50% formaldehyde. The following morning, slides were rinsed in 2 changes of 2X sodium citrate (SSC; 10 min each) followed by 2 X 10 min washes in 0.5 X SSC.

SSC solution was replaced with buffer #1 (1M Tris-HCl diluted in 1.5M NaCl, pH 7.5) and sections were incubated in 10% normal sheep serum in buffer #2 (0.3% Triton X-100 in buffer #1) for 30 minutes at RT. Blocking solution was carefully removed and sections were incubated in alkaline phosphatase DIG antibody (1:500) in buffer #2 for 2 hrs at RT. Slides were rinsed 2 X 10 min in buffer #1 then briefly in buffer #3 (1M Tris-HCl in 1M NaCl, 1M $MgCl_2$, pH 9.5). The hybridization signal was developed by incubation in a solution of NBT (4-nitro blue tetrazolium chloride), BCIP (5-bromo-4-chloro-3-indolyl-phosphate-4-toluisin salt) and 1 mM levamisole (to quench endogenous peroxidase) in buffer #3. The colour reaction was allowed to proceed overnight (in the dark at RT); the reaction was stopped by rinsing 2 X 10 min with buffer #4 (1M Tris-HCl, 100 mM EDTA, pH 8.0), followed by water rinse. The tissue was dehydrated, cleared and mounted with Permount and viewed by bright field microscopy.

5.3 Results

i) Immunostaining for P-glycoprotein and Cytochrome P450 in Manduca

Immunostaining for P-glycoprotein with the polyclonal antibody, mdr1(Ab-1), demonstrated that the *Manduca* midgut was immunopositive for P-glycoprotein. Label was associated with the lumenal surface of the midgut, specifically with the apical membrane of the epithelial cells (Fig. 5.1 A-B). A control experiment in which the primary antibody was omitted (Fig.5.1 C) resulted in an absence of staining.

The *Manduca* midgut is composed of two cell types, the goblet cells and the columnar cells (Cioffi, 1979); a diagrammatic representation of a goblet cell flanked by two columnar cells is provided in Figure 5.2. Without immunoelectron microscopy, it is not clear whether P-glycoprotein staining is localized to one or both of these cell types.

In addition to the midgut, another *Manduca* tissue, the fat body, was assessed for P-glycoprotein immunoreactivity. The insect fat body is organized into thin lobes of highly tracheated tissue suspended in the hemolymph, typically close to the gut or adjacent to the integument (Haunerland and Shirk, 1995). As such, the fat body is continually exposed to any toxins that have penetrated the integument or diffused from the gut and collected in the hemolymph. The insect fat body is not only a storage tissue but is also the site of considerable metabolic activity, including PSMO-based metabolism (Keeley, 1985). Immunohistochemical staining with mdr(Ab-1) demonstrated that *Manduca* fat body is immunopositive for P-glycoprotein, with label predominantly localized to the plasma membrane of the fat body cells (Fig. 5.1D-E).

An antibody directed against the cytochrome P450 of a DDT-resistant *Drosophila* (Waters et al., 1989) was used to label the *Manduca* midgut for PSMOs. This antibody has been successfully used to detect cytochrome P450 in Western blots of tissue from other Lepidoptera, specifically, *Spodoptera frugiperda* (Waters et al., 1989) and the tobacco budworm, *Heliothis virescens* (Dr. L. Waters, personal communication). Labelling of the *Manduca* midgut with the *Drosophila* cytochrome

P450 antibody demonstrated that the midgut is indeed immunopositive (Fig. 5.3A). Staining appeared to be associated predominantly with the goblet cells of the midgut. It would be interesting to see if in these cells label is associated with the smooth endoplasmic reticulum, the subcellular site of the PSMOs (see Hodgson, 1985). Control experiments in which the primary antibody was omitted (Fig. 5.3B) resulted in an absence of label.

To ensure that the antibody was not simply adhering non-specifically to mucous present in the goblet cells, an additional control experiment was performed using a monoclonal antibody against vasopressin, a neurosecretory hormone found within the mammalian CNS, in place of the cytochrome P450 antibody. Results from this experiment demonstrated a virtual absence of staining in the midgut, providing strong evidence that the *Drosophila* cytochrome P450 antibody recognizes a specific target in *Manduca* tissue (Fig. 5.3C).

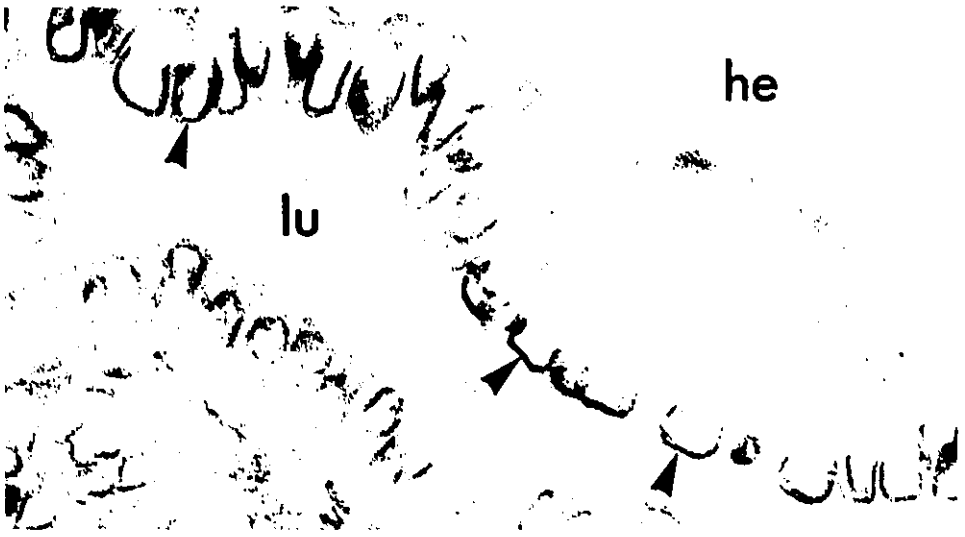
ii) In situ hybridization for cytochrome P450 in insect midgut

An *in situ* hybridization study was performed to determine if an oligonucleotide probe against a conserved region of the cytochrome P450s from three insecticide-resistant insect strains could be used to assess the expression of PSMOs in *Manduca.* Unfortunately, the antisense probe failed to detect cytochrome P450 mRNA in tissue sections of *Manduca* midgut (Fig. 5.4A). In light of a recent study that demonstrated that the presence of nicotine in the diet significantly increases PSMO activity in the *Manduca* midgut (Snyder et al., 1993), a subsequent experiment was performed using

the midgut from *Manduca* that had been fed tobacco leaves for 24 hrs prior to dissection. However, even under these conditions no signal was detectable in midgut sections from nicotine-fed *Manduca* (not shown).

In insects, as in other organisms, there exists a number of different cytochrome P450 isoforms that vary not only between insect strains but often within the same tissue (Hodgson, 1985). It is therefore conceivable that the cytochrome P450 isoform present in *Manduca* midgut does not contain the sequence encoded by our cytochrome P450 oligonucleotide probe. To determine if our probe could detect cytochrome P450 mRNA in another lepidopteran species, *in situ* hybridization was performed on the midgut of the fall armyworm, *Spodoptera frugiperda*. Results demonstrated that cytochrome P450 mRNA appears to present in the midgut of *Spodoptera* (Fig. 5.4B). In a control experiment, hybridization of midgut tissue with an unrelated probe (Fig. 5.4C) resulted in less intense staining compared to midgut sections probed with the antisense strand.

Figure 5.1 P-glycoprotein immunoreactivity in the midgut (**A-C**) and fat body (**D-E**) of larval *Manduca*. **A**, transverse section through the *Manduca* midgut illustrating P-glycoprotein immunoreactivity associated with membrane of the midgut epithelial cells facing the lumen (lu) of the gut (i.e. the apical surface, arrows). No label was associated with the membrane facing the hemolymph (h). **B**, cross section of *Manduca* midgut illustrating apical P-glycoprotein staining. **C**, controls in which the primary antibody was omitted resulted in an absence of staining. **D**, fat body tissue immunolabelled for P-glycoprotein shows label localized to the plasma membranes of the fat body cells (arrows), while staining was absent in control sections (**E**) in which the primary antibody solution was omitted. Bar=50 μm for **A**, 100 μm for **B-C** and 125 μm for **D-E**.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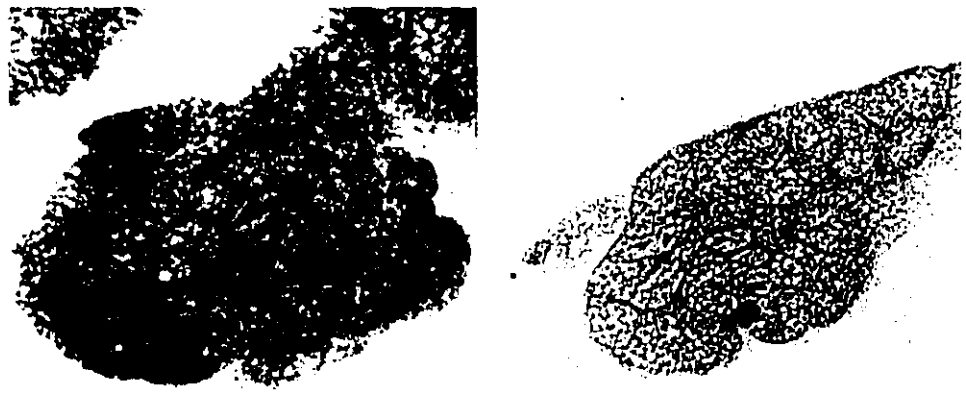


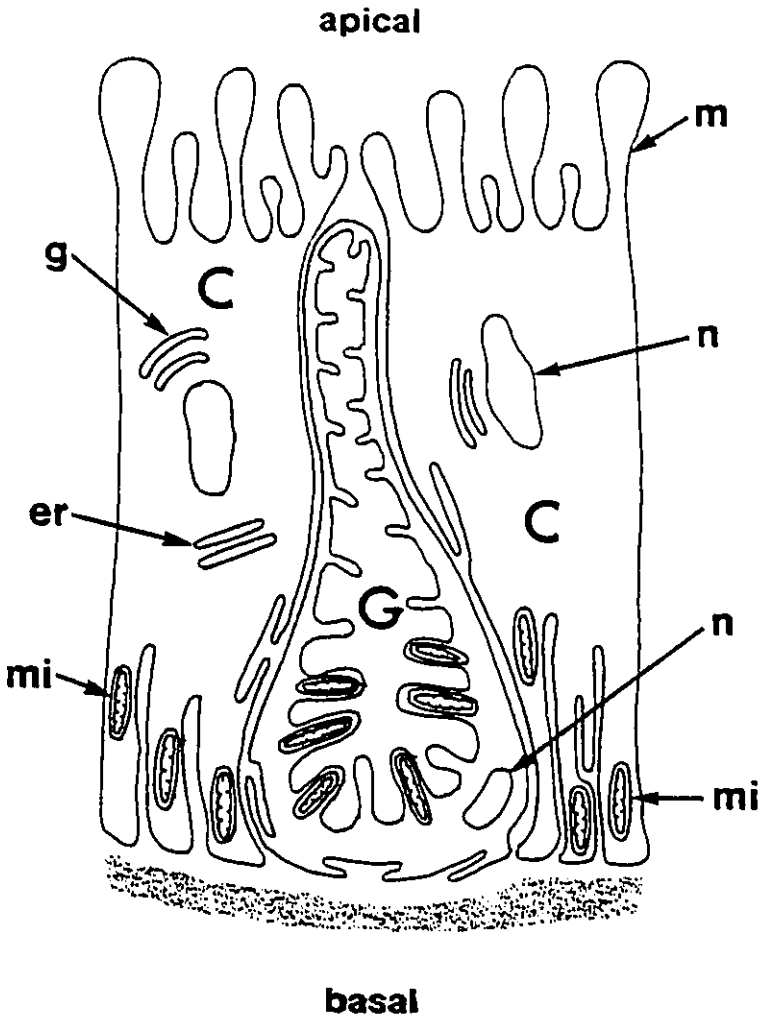


Figure 5.2 Diagrammatic representation illustrating a goblet cell flanked by two columnar cells from the anterior region of the midgut (adapted from Cioffi, 1979). The columnar cells (C) are characterized by a large centrally located nucleus (n) and an apical brush border of microvilli (m). The basal membrane is infolded to form narrow channels that penetrate the cell and are closely associated with mitochondria (mi). The goblet cells (G) are characterized by a large cavity formed by an invagination of the apical membrane. This membrane is thrown into projections which extend into the goblet cavity, and elongated mitochondria are present in the projections. In both cell types the cytoplasm contains golgi (g) and endoplasmic reticulum (er).

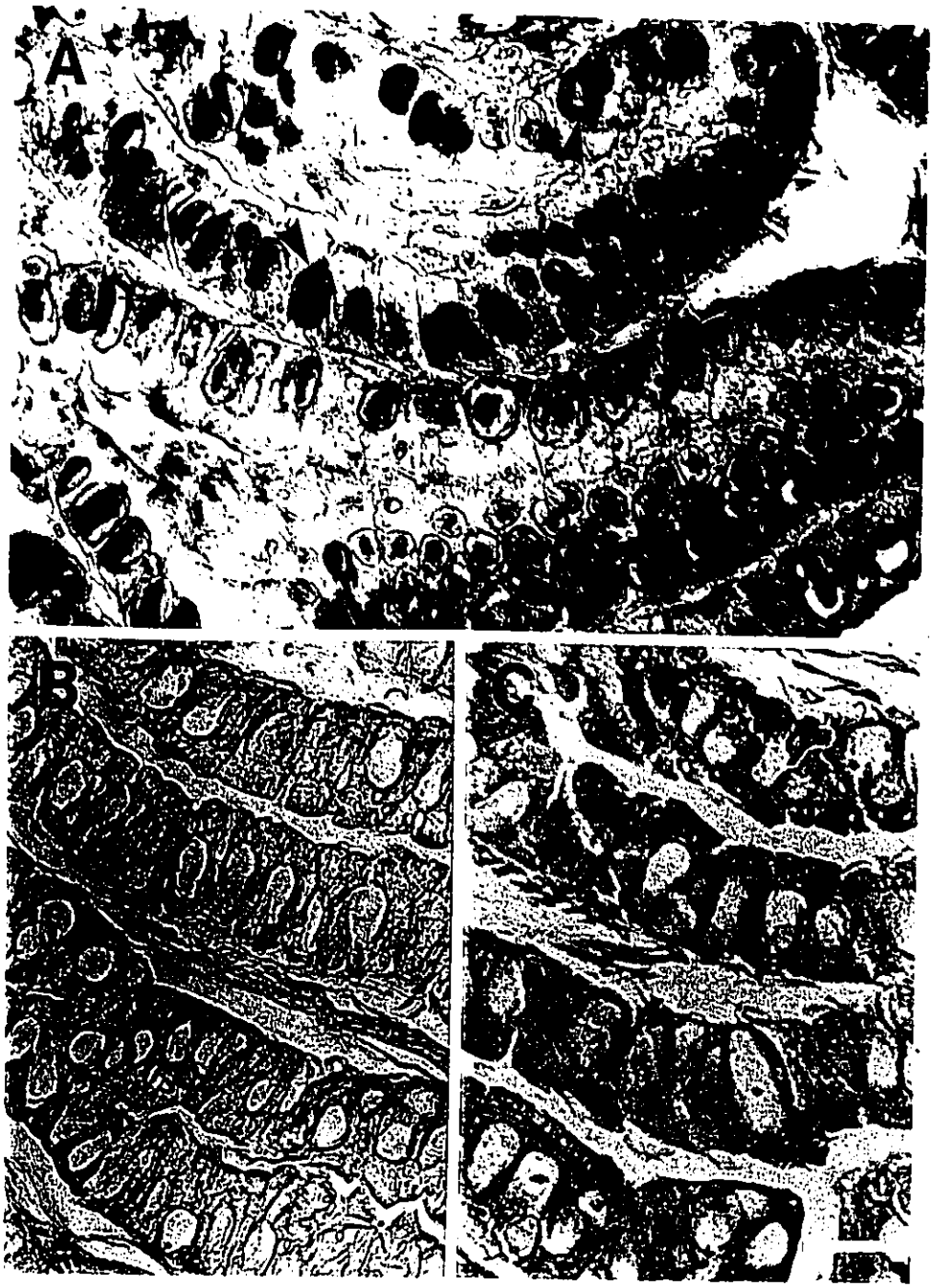
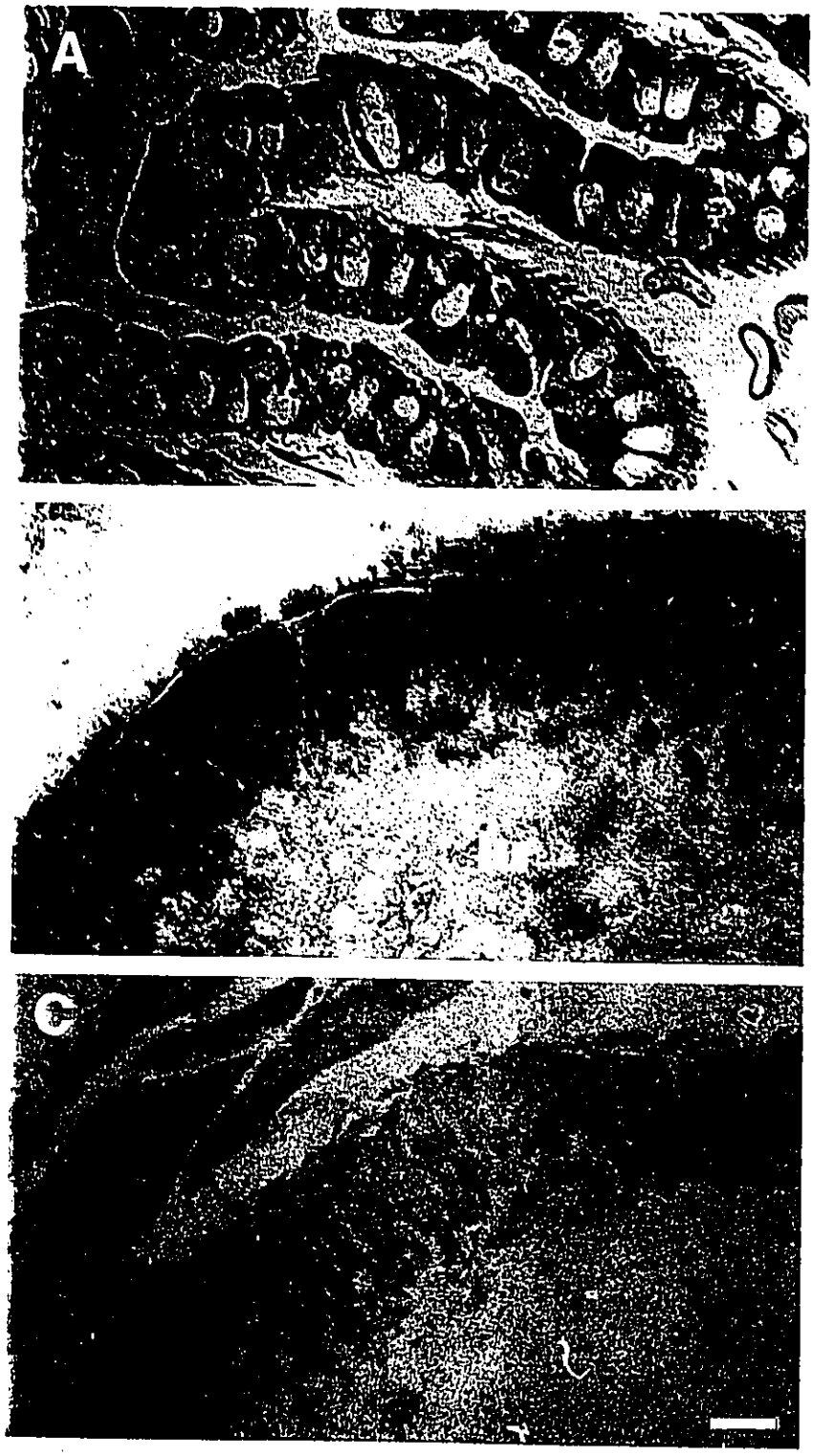


Figure 5.3 Localization of cytochrome P450 immunoreactivity in the *Manduca* midgut. **A**, section of *Manduca* midgut illustrating cytochrome P450 labelling to be associated predominantly with goblet cells (arrows). Controls in which the primary antibody was omitted (**B**) or in which the cytochrome P450 antibody solution was replaced with an anti-vasopressin antibody (**C**), resulted in an absence of labelling (**B**) or low-level labelling (**C**). Bar=40 μm.

Figure 5.4 *In situ* hybridization analysis of *Manduca sexta* (**A**) and *Spodoptera frugiperda* (**B-C**) midgut using a DIG-labelled antisense oligonucleotide probe to insect cytochrome P450. Hybridized probe was visualized using an alkaline phosphatase antibody against DIG. **A**, no alkaline phosphatase activity was observed in sections of *Manduca* midgut indicating a failure of the cytochrome P450 probe to hybridize to cytochrome P450 mRNA. **B**, a strong hybridization signal was observed in midgut sections from *Spodoptera* incubated with the cytochrome P450 antisense probe when compared to control sections (**C**) in which tissue sections were hybridized with an unrelated probe. The lumen (lu) and the hemolymph (h) sides of the gut are labelled in (**B**) for orientation. Bar=30 μm for **A** and 10 μm for **B-C**.


A
C

5.4 Discussion

Larval *Manduca* feed almost continuously in the weeks prior to pupation, so that the insect gut, the first tissue to come into contact with ingested tobacco, should provide some level of protection against nicotine. Shah and Guthrie (1970) demonstrated that nicotine is readily absorbed through the midgut, indicating that the epithelial cells of the midgut are exposed to high levels of nicotine in tobacco-feeding larvae.

The *Manduca* midgut appears to participate in the handling of chronic exposure to nicotine in two ways. First, PSMO enzyme activity has been observed in the *Manduca* midgut (Tate et al., 1982; Snyder et al., 1993) and in the current study we demonstrate immunohistochemically that cytochrome P450 is expressed in the midgut. In addition, examination of the tissue distribution of PSMO-mediated nicotine metabolites in *Manduca* larvae has demonstrated that the highest levels of metabolites are found in the gut contents and feces (Snyder et al., 1994). Thus, through PSMO-mediated metabolism, the mid-gut is able to produce metabolites which are more polar than parent nicotine (Snyder et al., 1994), and hence more easily excreted.

Second, our finding that the *Manduca* midgut is immunopositive for P-glycoprotein implies that nicotine transport is also a protective mechanism available to the midgut. Our previous findings demonstrated that *Manduca* Malpighian tubules are immunopositive for P-glycoprotein and physiologically, nicotine transport by the tubules displays hallmarks of being via a P-glycoprotein transporter. Hence, P-

glycoprotein immunoreactivity in other *Manduca* tissues, including the midgut implies that these tissues are capable of transporting nicotine. The localization of P-glycoprotein primarily to the apical membrane of the midgut suggests that absorbed nicotine and/or its metabolites are transported across the apical membrane into the lumen where it is excreted in the feces. The energy required for active transport across the midgut membranes could be provided by the abundant mitochondria present on the apical surface of *Manduca* midgut cells (Cioffi, 1972).

The expression of P-glycoprotein at the digestive tract appears to be an evolutionarily conserved mechanism to deal with ingested toxins. P-glycoproteins have been localized to the intestinal tracts of humans (Thiebaut et al., 1987), and mice (Georges et al., 1990), but also in the intestines of the lower organisms, *Xenopus* (Castillo et al., 1995) and *C. elegans* (Lincke et al., 1992; 1993; Brocks et al., 1995).

The co-expression of PSMO enzymes and P-glycoprotein in the midgut suggests that decreases in bioavailability resulting from PSMO-mediated metabolism are complemented by active extrusion of absorbed nicotine. The importance of metabolism coupled with pumping has been demonstrated in the *Manduca* CNS. Incubating isolated *Manduca* nerve cords in 1 mM nicotine results in greater than half of the penetrating nicotine remaining unmetabolized, suggesting that, at this concentration, nicotine saturates metabolizing enzymes (Morris, 1983a). Since nerve cords with intact blood-brain barriers are insensitive to 1 mM nicotine (i.e. 1 mM nicotine does not alter the level of endogenous neural activity in isolated nerve cords; Morris, 1984), another mechanism must be operating to prevent unmetabolized nicotine from reaching

sensitive ACh receptors. Several lines of evidence indicate that a nicotine transporter is functioning in conjunction with metabolizing enzymes at the *Manduca* CNS. First, in electrophysiological experiments, Morris (1984) demonstrated that co-application of nicotine and N-methylnicotinamide (NMN), a tertiary amine with a structure similar to nicotine, increases the sensitivity of the *Manduca* CNS to nicotine even though NMN does not affect CNS metabolism of nicotine (Morris, 1983a). An examination of uptake and efflux of radiolabelled nicotine into the CNS demonstrated that NMN increased the rate of nicotine efflux from the *Manduca* CNS (Morris, 1983c). Together these results suggest that NMN competitively inhibits the sequestration of nicotine in compartments within the CNS. Second, atropine, which strongly inhibits nicotine transport in *Rhodnius* (Maddrell and Gardiner, 1976) and *Manduca* (see Chapter 4) Malpighian tubules, significantly decreased the extent and rate of CNS uptake of nicotine, and increase the efflux rate of nicotine (and metabolites) from the CNS (Morris, 1983a,b). Atropine, like NMN, has no effect on *Manduca* CNS metabolism of nicotine (Morris, 1983a). Hence, these results coupled with our current findings suggest that larval *Manduca* use a combination of nicotine metabolism coupled with transport to effectively tolerate dietary nicotine.

CHAPTER 6

GENERAL DISCUSSION

Since its discovery in mammalian tumor cells by Ling and colleagues (Juliano and Ling, 1976), P-glycoprotein has emerged as a ubiquitous transport protein with homologs found in numerous organisms, both eukaryotic and prokaryotic (reviewed in Juranka et al., 1990; Higgins, 1992). The apparently long evolutionary history and conserved structure of P-glycoprotein transporters raises two possibilities regarding its original function: one is that P-glycoprotein evolved specifically as a xenobiotic transporter and second is that P-glycoprotein functioned in some transport process critical to the physiology or development of the organism. A putative normal function of "xenobiotic" P-glycoprotein remains to be elucidated whereas it is highly plausible that defense against dietary and environmental toxins provided a significant selective force early in the evolution of P-glycoproteins. Plants and microbes produce potentially toxic compounds to defend themselves against predators. The multidrug pumps appear to provide a protective mechanism to eliminate xenobiotics, including toxins present in the environment, in an organisms diet (Ames et al., 1990), and perhaps toxic metabolites, supplementing other mechanisms, such as the well-characterized cytochrome P450-catalyzed oxidative detoxification pathways (Wacher et al., 1995).

For insects, especially plant-eating insects, plant toxins are the major xenobiotics

that need to be dealt with; consequently, there has been a strong co-evolutionary interaction between toxin-producing plants and plant-eating insects (Spencer, 1988). Insects demonstrate resistance to plant toxins that is often accompanied by resistance to synthetic insecticides, most of which, like the pyrethroids, are potent neurotoxins. A pressing aspect of the resistance problem is that "resistance" often means unexplained cross-resistance to a variety of structurally unrelated insecticides (Georghiou, 1990). The parallel between multi-insecticide resistance and the phenomenon of multidrug resistance in mammalian tumor cells led us to hypothesize that a P-glycoprotein-like mechanism is operating in insects, protecting them from host plant toxins and synthetic insecticides.

To test our hypothesis, larvae of the nicotine-resistant insect, *Manduca sexta* was used as a model toxin-resistant insect. *Manduca* proved to be an ideal model system since nicotine resistance in *Manduca* has been extensively studied, and resistance requires the contribution of a nicotine transporter. Using immunohistochemical and physiological techniques we provide evidence that the *Manduca* nicotine transporter is P-glycoprotein-like molecule. I demonstrate: (1) that *Manduca* is immunopositive for P-glycoprotein (2) that the sensitivity of the *Manduca* CNS to nicotine increases as expression of P-glycoprotein at the blood-brain barrier changes and (3) that the *Manduca* excretory Malpighian tubules are able to transport nicotine and the P-glycoprotein substrate vinblastine, and that transport of both of these compounds is inhibited by the P-glycoprotein inhibitor verapamil.

Although the physiological adaptations of *Manduca* cannot form the basis of a

generalization applicable to all insects, they can, however, offer insight into how other insects tolerate dietary toxins as well as synthetic insecticides. The possibility that P-glycoprotein is operating in other insect species needs to be further explored. The finding that, in addition to *Manduca* (Order Lepidoptera), the cockroach (*Periplaneta americana*; Order Othoptera) and the blood-sucking *Rhodnius* (Order Hemiptera), are immunopositive for P-glycoprotein suggests that P-glycoprotein may be a widespread transport mechanism in insects. The current results may have potential implications not only in the understanding of how insects tolerate plant toxins but also perhaps into the continuing problem of multi-insecticide resistance.

6.1 Evidence for a P-glycoprotein-like molecule in *Manduca*

i) Immunohistochemical Evidence

Using the mammalian P-glycoprotein antibodies, monoclonal antibody C219 and/or polyclonal mdr(Ab-1), P-glycoprotein was localized to the *Manduca* blood-brain barrier, Malpighian tubules, midgut and fatbody. Since the monoclonal antibody C219 recognizes a highly conserved internal epitope (VVQEALD) present in the C-terminal domains of all P-glycoprotein isoforms, it is recognized as an effective tool for studying the tissue-specific distribution of P-glycoprotein molecules in a wide range of organisms. The C219 antibody has been used to localize P-glycoprotein in human (Cordon-Cardo et al., 1989; Thiebaut et al., 1989); van der Valk et al., 1990), rat (Cordon-Cardo et al., 1989 (Theibault et al., 1987) and hamster (Georges et al., 1990)

tissue, as well as to tissues in lower organisms including the soil-dwelling nematode, *C. elegans* (Brocks et al., 1995), numerous aquatic organisms (Holland-Toomey and Epel, 1993; Minier et al., 1993) and most recently, the teleost fish, *Poecilia reticulata* (Hemmer et al., 1995).

Immunoreactivity with the C219 antibody is, however, lost when tissue has been gluteraldehyde fixed or paraffin-embedded (Toth et al., 1994). Hence, the C219 antibody was useful only for studies using frozen tissue sections but not for either immunoelectron microscopy studies or for studies requiring paraffin-embedded tissue. For the development study, for example, paraffin sections were used rather than frozen sections since paraffin sections are of uniform thickness and have superior morphological preservation over frozen sections, both critical points when attempting to do a comparative study. Fortunately, at the time the developmental study was initiated, the polyclonal antibody mdr(Ab-1), which maintains immunoreactivity in gluteraldehyde-fixed and paraffin-embedded tissue, became available. The mdr(Ab-1) antibody was generated using a 21-aa peptide from the C-terminal domain of mammalian P-glycoprotein. This peptide also contains the highly conserved epitope sequence recognized by the C219 antibody and hence mdr(Ab-1) is also a useful antibody for detecting P-glycoprotein in non-mammalian organisms (Holland-Toomey and Epel, 1993; Hemmer et al., 1995).

In addition to being an effective antibody for studying the developmental expression and ultrastuctural localization of P-glycoprotein, use of mdr(Ab-1) served the purpose of acting as an internal control through comparison with C219 while also

permitting evaluation of the staining efficacy of the more cost-effective polyclonal antibody. In parallel experiments, the staining pattern observed using C219 on frozen tissue sections of *Manduca* CNS correlated with the staining pattern observed using mdr(Ab-1) on paraffin-embedded CNS.

ii) Physiological and pharmacological evidence

Previous studies have established that *Manduca's* resistance to nicotine relies, at least partly, on a nicotine pump at the excretory Malpighian tubules and at the insect's blood-brain barrier, two sites which we have demonstrated to be immunopositive for P-glycoprotein.

Immunostaining for P-glycoprotein in the metamorphosing CNS illustrated that the distribution of P-glycoprotein in the barrier region changes dramatically when the need for a barrier to dietary nicotine is lost. A parallel electrophysiological study demonstrated that unlike the tobacco feeding larval *Manduca*, the CNS of the non-feeding pupal and adult stages are highly nicotine-sensitive. Thus, changes in the distribution of P-glycoprotein at the blood-brain barrier during metamorphosis are consistent with the hypothesis that P-glycoprotein plays a role in protecting the neuropile.

It is difficult to test directly the hypothesis that P-glycoprotein plays a functional role at the *Manduca* blood-brain barrier. One approach could have been to use P-glycoprotein inhibitors and observe any effects on the sensitivity of larval *Manduca* to nicotine; however, any effect produced by P-glycoprotein reversing agents would be

difficult to interpret since the majority of these compounds would produce secondary effects that could affect neural activity.

The *Manduca* Malpighian tubule proved to be an ideal preparation to directly test if the P-glycoprotein immunreactivity in *Manduca* is associated with a functional P-glycoprotein. *Manduca* Malpighian tubules are easily dissected and once isolated will continue to carry to secrete fluid when bathed in saline. Cannulating one end of the tubule served two purposes: first, it provided a rapid way to flush and collect lumenal contents to monitor alkaloid secretion and second, it provided a delivery route for inhibitory compounds. Using this technique I demonstrated that the *Manduca* transporter displays hallmarks of being a P-glycoprotein transporter. First, the *Manduca* transporter is a multi-drug pump, actively transporting nicotine, atropine and vinblastine. Second, transport of nicotine and vinblastine is inhibited by the P-glycoprotein inhibitor, verapamil, providing the most direct evidence that the *Manduca* transporter is a P-glycoprotein-like transporter.

Definitive proof that *Manduca* larvae possess nicotine-transporting P-glycoprotein homolog(s) must await the successful isolation and cloning of P-glycoprotein gene(s) and subsequent functional reconstitution.

6.2 A working hypothesis for the role of a P-glycoprotein-like mechanism in *Manduca*

i) Blood-brain barrier

The mammalian blood-brain barrier, like the insect blood-brain barrier, is both

structural and metabolic in nature. Structurally, the mammalian barrier depends on tight junctions between endothelial cells of capillaries that penetrate the brain that acts as a continuous physical barrier, blocking the passive passage of hydrophilic solutes in either direction. Metabolically, immunohistochemical and functional evidence indicate that P-glycoprotein is an integral component of the ability of the mammalian barrier to restrict entry of certain lipid-soluble compounds, which should theoretically simple diffuse from the capillary lumen to the brain interstium. P-glycoprotein has been detected in brain capillary endothelial cells in mouse (Hegmann et al., 1992; Tatsuta et al., 1992), rat (Thiebault et al., 1989), hamster (Bradley et al., 1990), pig (Hegmann et al., 1992), cattle (Tsuji et al., 1992) and humans (Cordon-Cardo et al., 1989; van der Valk et al., 1990). Tatsuta et al. (1992) demonstrated that in mouse brain endothelial cells P-glycoprotein is localized to the apical surface and transport of the P-glycoprotein substrate, vincristine, is from the basal (brain) side to the apical (blood) side. Similar reports have been reported in cow (Hegmann et al., 1992) and pig (Tatsuta et al., 1992) endothelial cells. Schinkel et al. (1994) have provided the most direct evidence to date for P-glycoprotein involvement at the blood-brain barrier using transgenic mice deficient for the gene encoding P-glycoprotein. Following injection with vinblastine, transgenic mice displayed signs of neurotoxicity and had in fact accumulated 22.4-fold higher vinblastine levels in the brain compared to wild-type mice.

How would P-glycoprotein operate in the endothelial cells of the mammalian blood brain barrier? The blood-brain barrier represents a modification from other

epithelial type cell layers where "apical" is the blood-facing surface. Based on *in vitro* and *in vivo* functional studies, it appears that P-glycoprotein regulates the ability of certain hydrophobic agents to reach the brain, presumably by actively transporting compounds from the cytoplasm of the endothelial cell back into the blood. For this to be occurring, P-glycoprotein would be required to pump at a rate which would maintain cell drug concentrations low enough to prevent the diffusion of drug into the brain. The presence of metabolizing enzymes, including the P450 enzyme system, in cerebral capillaries (Britto and Wheland, 1992; Ghersi-Egea et al., 1994) could mean that a portion of the penetrating drug is metabolized, thereby relieving P-glycoprotein of some of the metabolic burden. It is the most likely scenario that P-glycoprotein and detoxifying enzymes act in concert to maintain the metabolic blood-brain barrier.

A similar scenario likely exists in *Manduca*, where the insect's blood-brain barrier to nicotine appears to rely on the combined actions of detoxifying enzymes and P-glycoprotein-mediated transport. Any interpretation of how a P-glycoprotein transporter could be functioning in the *Manduca* blood-brain barrier needs to take into account the topology of the insect's blood-brain barrier. Unlike in the mammalian blood-brain barrier where a toxin must permeate a single layer of endothelial cells to reach the brain interstium, in *Manduca*, nicotine may traverse several layers of glial cells to reach the neuropile. Intepretation of how P-glycoprotein is contributing to the *Manduca* blood-brain barrier to nicotine is further complicated by the amphipathic nature of nicotine. The *Manduca* hemolymph has a pH 6.6 and, consequently, the bulk of the nicotine will exist as the water-soluble form while a small, but significant

amount will exist as the lipid-soluble form. For water-soluble nicotine diffusing along the tortuous extracellular route, two things must occur in order to maintain sub-toxic concentrations of nicotine in the neuropile. First, the tight junctions between perineurial cells must provide a physical diffusion barrier to prevent nicotine from reaching the underlying extracellular space, where it can easily gain access to the neuropile. It is unlikely, however, that in the *in vivo* situation, where the *Manduca* CNS faces chronic exposure to nicotine, the tight junctions will provide a barrier of sufficiently high resistance to prevent some leakage of nicotine into the extracellular space especially since carbachol, a polar molecule with a similar molecular weight to nicotine, can get past the tight junctions and reach the neuropile (Murray et al., 1994). Nicotine that leaks past the tight junctions must be dealt with, that is, it must be transported out of the extracellular space into cells perhaps where it can be metabolized. Second, the lipophilic form of nicotine penetrating adjacent cytoplasm must require the combined actions of metabolism and transport from the perineurium to the hemolymph. If P-glycoprotein is involved in nicotine transport in the *Manduca* CNS, as we suggest, it means that P-glycoprotein may function in one or both of two ways: it may transport water-soluble nicotine from the extracellular space in the neuropile into glial cells or, more plausibly, transport lipophilic nicotine out of glial cell cytoplasm into vacuoles or into the blood.

ii) Epithelia of the gut and Malpighian tubules

Consistent with findings in mammalian epithelia (Cordon-Cardon et al., 1989;

Thiebaut et al., 1989) as well as in epithelia of lower organisms (Brocks et al., 1995; Hemmer et al., 1995; Castillo et al., 1995), P-glycoprotein was localized predominately to the apical surface of epithelium of the midgut and Malpighian tubules in *Manduca*. P-glycoprotein in epithelia can function to transport compounds in one of two directions: across the apical membrane into the lumen or from the lumen back into blood. In *Manduca* Malpighian tubules, P-glycoprotein appears to reduce blood nicotine concentrations by actively transporting nicotine across the apical membrane into the lumen to be excreted. To ensure the continual influx of nicotine at the basal side of the tubule cell P-glycoprotein must be pumping at a rate to maintain the diffusion gradient across the cell. To account for the *in vivo* clearance rates of nicotine by *Manduca* tubules (Self et al., 1964a; Snyder et al., 1994) P-glycoprotein would surely be under an enormous metabolic load. One possible scenario is that detoxifying enzymes present in both Malpighian tubule and midgut epithelial cells (Tate et al., 1982; Snyder et al., 1993) metabolize some of the penetrating nicotine, relieving P-glycoprotein of some of the metabolic burden. The possibility also exists that the low-level P-glycoprotein immunostaining observed at the basal (blood) surfaces of both Malpighian tubule and midgut tissue represents pumps that could actively transport nicotine from the hemolymph into the epithelial cell. This would, however, represent a departure from the general understanding about how P-glycoprotein functions i.e. as a cellular efflux pump.

6.3 P-glycoprotein: Potential insight into insecticide resistance

Insects have developed resistance to all the major classes of insecticides. From an ecological and evolutionary point of view the ability of insects to develop resistance is not surprising. Insects and plants have co-existed for millions of years, and as a result insects have evolved a multitude of adaptations allowing them to exploit plants as a food source. Resistance to host plant toxins could have predisposed insects to resist insecticides. Much of the current focus in the field of insecticide research has focused on the development of new insecticides with novel modes of action. One such example has been the development of biopesticides; however, resistance has already been reported to two of the most widely used biopesticides, toxin-proteins produced by the bacteria *Bacillus thuringiensis* (Tabashnik et al., 1990; Ferre et al., 1991), and the macrocyclic lactones (avermectins; Clark et al., 1994) produced by *Streptomyces avermetilis*. It appears that the emphasis should not be on the development of new insecticides but rather on fundamental research into the nature of insect resistance mechanisms and on programs aimed at the management of insecticides to prevent the development of resistance to existing or new insecticides.

How far can we exploit our knowledge of resistance mechanisms to combat the resistance problem in the field? Resistance to xenobiotics is multifactorial, relying on several mechanisms including behavioural avoidance, reduced cuticular penetration, increased detoxification, reduced affinity of the target site and/or sequestration, or excretion. Targeting one mechanism may sufficient to neutralize the resistance.

Resistance to pyrethroids, for example, appears to involve at least three mechanisms (Gunning et al., 1991): target-site insensitivity, delayed cuticular penetration, and detoxification. In *Heliocoverpa armigera*, targeting the detoxication mechanism with piperonyl butoxide, an inhibitor of P450-mediated oxidative metabolism, neutralizes resistance to pyrethroids, enabling the continued use of this insecticide (Denholm and Rowland, 1992).

Resistance of *Manduca* to nicotine is dependent on metabolism (Morris, 1983a; Snyder et al., 1993), excretion (Self et al., 1964a; Maddrell and Gardiner, 1976) and perhaps reduced target site sensitivity (Trimmer and Weeks, 1989). We demonstrate that the excretion of nicotine by the Malpighian tubules is mediated by a P-glycoprotein transporter. If P-glycoprotein is deployed in economically and ecologically important pest insects, it may be possible to neutralize resistance by targeting this transporter.

6.4 Insight into the addiction syndrome

The range of alkaloids currently known to be transported by the insect alkaloid pump is also of special interest for the mammalian CNS. Nicotine and morphine, which readily cross the mammalian blood-brain barrier, are both neurotoxic and are both addictive. P-glycoprotein is deployed at the mammalian blood-brain barrier (Cordon-Cardo et al., 1989; van der Valk et al., 1990), and there is evidence that it has a transporter function there (Schinkel, 1994). Morphine as well as nicotine appear to

be substrates for the mammalian P-glycoprotein (Callaghan and Riordan, 1993; Dr. F. Sharom, personal communication), indicating that multidrug pump activity at the blood-brain barrier and at the kidney could have important implications for the phenomenon of drug tolerance, which is part of the addiction syndrome.

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