J. exp. Biol. **106**, 91–117 (1983)
Printed in Great Britain © *The Company of Biologists Limited 1983*

POTASSIUM ION TRANSPORT ATPase IN INSECT EPITHELIA

BY WILLIAM R. HARVEY, MOIRA CIOFFI, JULIAN A. T. DOW AND MICHAEL G. WOLFERSBERGER

Department of Biology, Temple University, Philadelphia, PA 19122, U.S.A.

SUMMARY

K^+ transport by the epithelia of midgut, salivary glands, Malpighian tubules, sensory sensilla, possibly rectum, and other organs of certain insects appears to use a unique K^+ ATPase. Ouabain inhibition of transport-related events has not been demonstrated in these epithelia. The K^+ pump is unlike the Na^+, K^+ pump but resembles the H^+ pump of phosphorylating membranes in its transport orientation, efficient thermodynamics, speculated two K^+ per one $MgATP^{2-}$ stoichiometry, electrogenicity, and structure. Older electrochemical, tracer flux, and conductance evidence suggested that the K^+ pump was on the apical plasma membrane of transporting cells in these epithelia. New X-ray microanalytical studies (XMA), reveal that the K^+ concentration in all cells is more than 100 mM. Together with new microelectrode data these XMA results confirm the apical K^+ pump location, resolve the K^+ transport route, and suggest that the goblet cell cavity facilitates the generation of a large apical PD which may be used in nutrient absorption and pH regulation. K^+ portasomes, which resemble F_1-F_o ATPase particles, stud these K^+ transporting apical membranes and are thought to be the unit of active K^+ transport. We have suggested a K^+ transport mechanism in which two cations ($2\,K^+$) are abandoned in an isolated domain of the portasomes during ATP^{2-} hydrolysis and are repelled to the opposite membrane side *via* a K^+ channel. Small peptides hydrolysed from the δ-endotoxin of *Bacillus thuringiensis* inhibit the K^+ transport and may be useful as K^+ pump inhibitors, apical membrane probes and insecticides. Goblet cell apical membrane fragments (GCAM) as well as fragments from columnar cell apical membrane (CCAM), lateral membrane (LM) and basal membranes (BM) were isolated as clean fractions using ultrasound, aspiration, and both differential and density gradient centrifugation; purification was monitored by electron microscopy. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) reveals that GCAM, CCAM, LM and BM have very different protein compositions. Preliminary enzymology is consistent with the K^+ ATPase being on the apical plasma membrane of the goblet cells of midgut and enveloping cells of sensilla.

INTRODUCTION

Definition of ion transport ATPase

An ion transport ATPase is an enzyme responsible for the primary coupling of ATP hydrolysis to ion movement across a biomembrane. Ion movement is vectorial;

Key words: X-ray microanalysis, transport mechanism, membrane isolation.

likewise ATP hydrolysis is vectorial at the molecular level; but in the absence of membrane-transport ATPase assembly the vectoriality is lost and the hydrolysis is scalar in bulk solution (Mitchell, 1979). The role of a transport ATPase, then, is not only to catalyse ATP hydrolysis but to translate the ion movement inherent in the vectorial hydrolysis reaction into a net flux of an ionic species across a membrane. The definition of primary coupling between ATP hydrolysis and ion movement is made explicit by the cross coefficient, R_{kr}, in equation (1) a general flux equation from non-equilibrium thermodynamics (Kedem & Katchalsky, 1961).

$$J_k = \frac{\Delta\bar{\mu}_k}{R_{kk}} - \Sigma\frac{R_{kj}}{R_{kk}}J_j - \frac{R_{kw}}{R_{kk}}J_w - \frac{R_{kr}}{R_{kk}}J_r. \qquad (1)$$

The term $\Delta\bar{\mu}_k/R_{kk}$ asserts that the flux of an ionic species, J_k, is driven by and is proportional to, a difference in the electrochemical potential of that species across a membrane, $\Delta\bar{\mu}_k$, it being understood that this difference has two components, a chemical concentration difference given by $RT\Delta \ln c_k$ and an electrical potential difference, given by $zF\Delta\Psi$. The straight coefficient, R_{kk}, is a resistance term i.e. a coefficient which converts the proportionality to an equality. A flux coupled solely to $\Delta\bar{\mu}_k$ is universally accepted as being a 'passive' flux. We will consider a flux coupled to the flow of any other solute, J_j, ($R_{kj} \neq 0$) or a flux coupled to the flow of water, J_w, ($R_{kw} \neq 0$) to be secondary to the flow of the other solute or of water. By primary active ion transport we mean the coupling of an ion movement directly to the flow of a chemical reaction, J_r; for such a flux the cross coefficient in the last term, $R_{kr} \neq 0$. Such a primary active ion flux is coupled to a reaction flux, in this case ATP hydrolysis, by mechanisms whose structural orientation within a biomembrane is crucial.

This definition of primary ion transport is illustrated by the sketch, Fig. 1. The relationship between the variables, numbered 1, 2 and 3 in the sketch and defined in its legend, is given by Ussing's (1949) and Linderholm's (1952) well known flux ratio equation from diffusion theory. Thus if there is no coupling of the ion flux to the metabolism or to other solute or solvent flow (R_{kj}, R_{kw} and R_{kr} all = zero) then $J_kR_{kk} = \Delta\bar{\mu}_k$ and:

$$\ln\frac{\Phi_{12}}{\Phi_{21}} = \ln\frac{c_1}{c_2} + \frac{zF}{RT}(\Psi_1 - \Psi_2). \qquad (2)$$

However, if there is coupling between flux and metabolism then we can collect all of the terms of equation (2) and set them equal to the free energy change for ATP hydrolysis multiplied by a coefficient R'_{kr} to express the efficiency of the coupling. The resulting equation (3) shows explicitly the relationship between unidirectional fluxes (Φ_{12}, Φ_{21}), concentrations (c_1, c_2) and electrical potentials (Ψ_1, Ψ_2) on two sides of a biomembrane and the ATP hydrolysis which is responsible for the differences (Ussing, in Zerahn, 1956; see Harvey, 1982; K_e and Γ are the equilibrium constant and products/reactants ratio respectively for ATP hydrolysis).

$$\ln\frac{\Phi_{12}}{\Phi_{21}} - \ln\frac{c_1}{c_2} - \frac{zF}{RT}(\Psi_1 - \Psi_2) = -R'_{kr}\ln\frac{K_e}{\Gamma} \qquad (3)$$

In summary, the cross coefficient, R_{kr}, can be viewed as a formal expression of an ion transport ATPase.

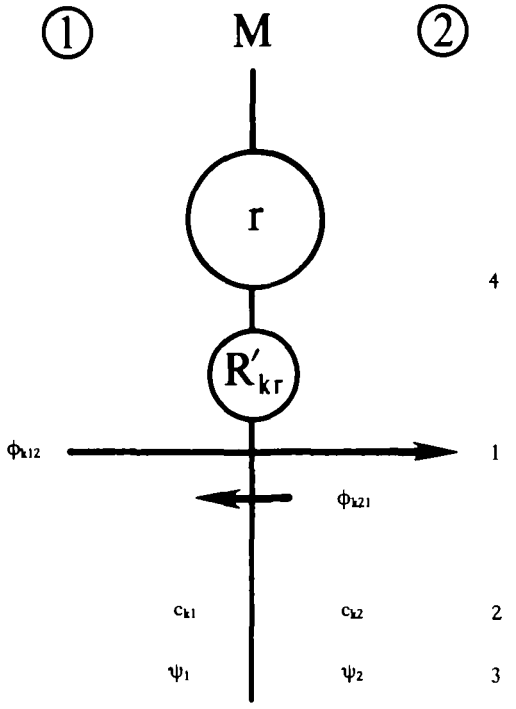


Fig. 1. Diagram showing the relationship between the three terms of equation (2). Reaction r is coupled by coefficient R'_{kr} to the transport of electrolyte k across membrane M. In the example shown, the coupling is such that the unidirectional flux Φ_{k12} is larger than the unidirectional flux Φ_{k21}, with the result that concentration c_{k2} becomes greater than concentration c_{k1}, leading to potential Ψ_2 developing with respect to potential Ψ_1.

The relationship between the irreversible thermodynamics treatment (equation 1) and the diffusion theory treatment (equations 2 and 3) is shown by rewriting equation (3) with all terms expressed as potential differences and below it writing equation (1) with all terms expressed as potential differences in the case when R_{kj} and $R_{kw} = 0$.

$$\frac{RT}{zF}\ln\frac{\Phi_{12}}{\Phi_{21}} - \frac{RT}{zF}\ln\frac{c_1}{c_2} - (\Psi_1 - \Psi_2) = -R'_{kr}\frac{RT}{zF}\ln\frac{K_e}{\Gamma} \qquad (3')$$

$$J_k\frac{R_{kk}}{zF} - \frac{RT}{zF}\ln\frac{c_1}{c_2} - (\Psi_1 - \Psi_2) = -Jr\frac{R_{kr}}{zF} \qquad (1')$$

It is clear that the voltage developed by an ATPase pump is given by $J_r R_{kr}/zF$ and that it depends not only upon the rate of ATP hydrolysis, J_r, but also upon the coupling achieved by the membrane orientated transport ATPase, expressed in the term R_{kr}, i.e. upon the internal resistance of the pump. The pump e.m.f. depends upon the free energy of ATP hydrolysis i.e. upon (RT/zF) $(\ln K_e/\Gamma)$. The equations are general; we have applied them to an ATP driven pump because we are discussing transport ATPases.

Criteria for demonstrating primary ion transport and ion transport ATPases

Recognizing the importance of membrane structure in orientating transport ATPases and from equations (1) and (3) we can formulate four classes of criteria for demonstrating primary active ion transport and transport ATPases: (1) *Indirect evidence* such as the presence of F_1-F_o particles, net flux against an electrochemical gradient, or an oxygen or ATP requirement for transport. (2) A *net flux*, J_k, *under short-circuit conditions* (from equation 2 when $c_1 = c_2$ and $\Psi_1 - \Psi_2 = 0$ then $\ln \Phi_{12}/\Phi_{21} = 0$; the flux ratio should equal one and the net flux should equal zero if the transport is passive). However, short-circuit conditions do not eliminate the possibility that the flux, J_k, is coupled to the flux of the other solutes, J_j, or of water, J_w, and so direct coupling of J_k to a reaction flux, J_r, such as ATP hydrolysis, cannot be deduced directly from the analysis of fluxes under short-circuit conditions. (3) *Specific inhibitors* such as ouabain for the Na^+,K^+ ATPase or oligomycin and dicyclohexylcarbodiimide (DCCD) for the H^+ ATPase. (4) *Isolation and reconstitution* – a definitive demonstration of a transport ATPase requires that the enzyme be identified, isolated and characterized. An important step is to show that isolated membrane vesicles transport ions when provided with ATP. The final proof requires that the transport system be reconstituted in defined lipid bilayer membranes such as liposomes or planar black lipid membranes.

Cation transport ATPases which meet all of these criteria are (for references see Schuurmans Stekhoven & Bonting, 1981; Maloney, 1982; Sachs *et al.* 1976; Rabon *et al.* 1983):

Na^+,K^+ ATPase	e.g. from plasma membranes,
H^+ATPase (F_1-F_o)	e.g. from phosphorylating membranes,
Ca^{2+} ATPase	e.g. from sarcoplasmic reticulum,
H^+,K^+ ATPase	e.g. from parietal cells of gastric mucosa.

Aptness of chemiosmotic model for K^+ transport

The epithelia of certain insects contain an active K^+ transport system which seems to have more in common with the H^+ ATPase (F_1-F_o) of phosphorylating membranes than with the Na^+,K^+ ATPase which is the basis for alkali metal ion transport in most vertebrate epithelia. The Na^+,K^+ ATPase normally is concentrated on the basolateral membrane in epithelia and is inhibited from the K^+ input side by ouabain. Na^+ is always pumped out of cells and K^+ into cells; Na^+ is required but all other alkali metal ions including Na^+ can replace K^+; typically three Na^+ are pumped out for every two K^+ moving in so the pump is electrogenic for 1/3 of the Na^+ ions pumped. A phosphorylated intermediate couples ATP hydrolysis to Na^+ and K^+ movements (review by Post, 1979; Schuurmans Stekhoven & Bonting, 1981).

The H^+ ATPase (F_1-F_o) is located principally in phosphorylating membranes such as the mitochondrial inner membrane, thylakoid membranes and bacterial plasma membranes. It normally operates as an ATP synthetase, using a proton electrochemical gradient to make ATP. However, when the thermodynamic gradient is reversed these normally phosphorylating systems operate as proton pumps. H^+ is always pumped away from the site of ATP binding; there is no phosphoryla

ermediate; ATP hydrolysis is coupled directly to the formation of a proton gradient; the H^+ pump is therefore said to be chemiosmotic (Mitchell, 1961). There is no counterion requirement; the pump is fully electrogenic, the input side being negative; the pump e.m.f. is utilized to make both an electrical potential difference (PD) and a proton concentration difference; the latter therefore substracts from the expression of electrogenicity. The ATPase activity is located in 9 nm spheres, the so-called F_1 particles, which are anchored to the membrane by F_o units (Kagawa, Racker & Hauser, 1966). The H^+ transport in mitochondrial inner membrane is inhibited by oligomycin and in bacterial plasma membranes by DCCD (reviews by Penefsky, 1979; Fillingame, 1980; Maloney, 1982).

K^+ transport in certain insect epithelia appears to use a unique K^+ ATPase. In these epithelia it has not been possible to demonstrate ouabain inhibition of K^+ transport-related events. The K^+ pump is quite unlike the Na^+,K^+ pump and appears to be like the H^+ pump of phosphorylating membranes, as judged from similar orientation, thermodynamics, speculated stoichiometry and structure (Harvey, Cioffi & Wolfersberger, 1981, 1983). We will review the characteristics of this K^+ pump in midgut, salivary glands, Malpighian tubules and sensory sensilla, review speculation that it is located in K^+ portasomes which resemble F_1-F_o ATPase particles, and present a speculative model for its operation. We will explain why ouabain is not a useful inhibitor for this K^+ pump and examine evidence that small peptides from the insecticidal δ-endotoxin of *Bacillus thuringiensis* may be useful as K^+ pump inhibitors or at least as apical membrane probes. We will describe new evidence from X-ray microanalysis that the K^+ pump is located on the apical plasma membrane of midgut goblet cells. We will close with a description of our successful efforts to isolate clean membrane fragments from four different regions of the plasma membrane of midgut epithelial cells.

K^+ PUMP OF CERTAIN INSECT EPITHELIA

Active K^+ transport was first deduced from ion distributions against electrochemical gradients in the Malpighian tubules of several insects by Ramsay (1953) and confirmed by measurements of net ^{42}K fluxes in short-circuited lepidopteran midgut by Harvey & Nedergaard (1964). Subsequently electrical measurements (Prince & Berridge, 1972) and more recently X-ray microanalysis (XMA) coupled with K^+ selective electrode measurements demonstrated that the K^+ pump is located on the apical plasma membrane in isolated dipteran salivary glands (Gupta, Berridge, Hall & Moreton, 1978). The K^+ pump is also involved in generating receptor currents in fly sensilla (see Thurm & Küppers, 1980). The early work was reviewed by Keynes (1969) who classified the insect K^+ transport system as the Type V pump distinct from the Na^+,K^+ system which he classified as the Type I pump. The properties of this insect K^+ pump have been reviewed repeatedly (Harvey & Zerahn, 1972; Maddrell, 1971, 1977*a,b*, 1978; Zerahn, 1977, 1978; Harvey, 1980, 1982; Harvey *et al.* 1981, 1983; Wolfersberger, Harvey & Cioffi, 1982). Its role in transepithelial active K^+ transport is summarized in Fig. 2, which is a diagram of the posterior midgut of *Manduca sexta*; the K^+ transport is similar in most essentials to that in pteran salivary glands, various Malpighian tubules, and dipteran sensilla. The

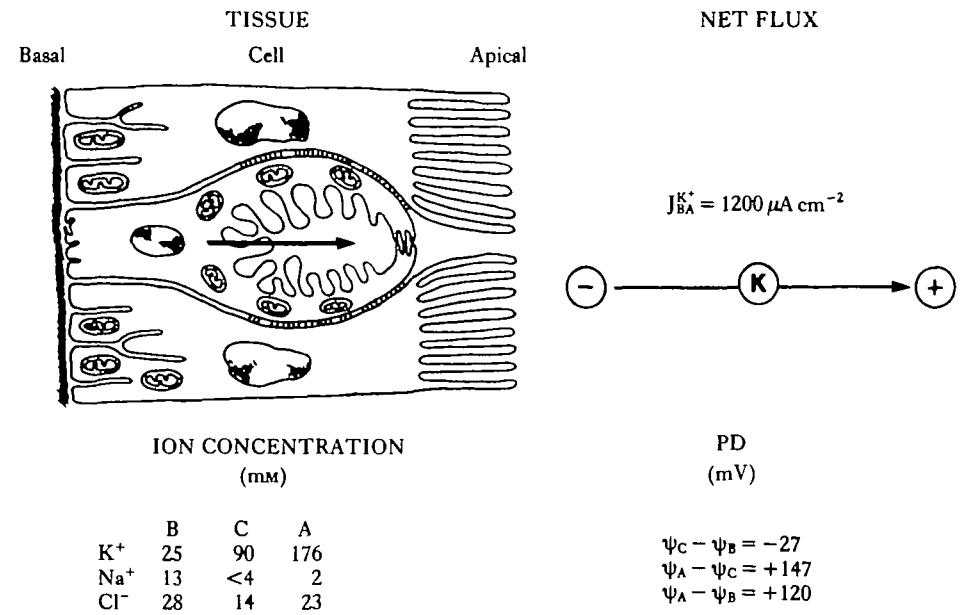


Fig. 2. Diagram of posterior midgut epithelium of *Manduca sexta*, with an arrow showing the suspected location of the potassium ion pump in the invaginated apical plasma membrane of goblet cells. Diagram also includes typical values for the net flux, direction of transport, electrical polarity and ionic concentrations. B, basal; C, cell; A, apical.

isolated lepidopteran midgut consists of a one cell thick epithelium composed almost entirely of columnar cells and goblet cells, separated from the blood side solution by a basement membrane and discontinuous muscle layers and tracheae. On the luminal side a peritrophic membrane is present *in vivo* but it is removed during isolation; the apical plasma membrane of the midgut epithelial cells is thus in direct contact with the luminal bathing solution. The structure of the other insect epithelia is described below. In each of these epithelia the K^+ pump is thought to be located in a particle-studded region of the apical plasma membrane. The particles are thought to be the site of the ATPase as discussed below. In all of these insect epithelia K^+ is actively transported from basal to apical side against a PD in excess of 60 mV and against a large K^+ concentration difference. The K^+ pump is electrogenic in that a K^+-stimulated current is observed under short-circuit conditions and a K^+-stimulated PD is observed under open-circuit conditions; there is no counter ion requirement. Indeed when the K^+ and Ca^{2+} concentration is low any one of the alkali metal ions is transported. However, there is no synergistic interaction between alkali ions and all alkali ions are transported in the same direction – from cytoplasm across the apical membrane to the lumen. Properly then the mechanism is an alkali metal ion pump but it is referred to simply as a K^+ pump.

K^+ pump in lepidopteran midgut

In a recent study of *M. sexta* posterior midgut (Cioffi & Harvey, 1981) the short-circuit current averaged 1159 $\mu A\,cm^{-2}$ at 60 min after isolation and was accounted

[redacted]hin experimental error by a K^+ basal to apical flux of 43·1 and back flux of 1·19 μequiv $cm^{-2} h^{-1}$ (1156 and 32 $\mu A\, cm^{-2}$ respectively). The K^+ transport and transepithelial PD are reversibly inhibited by oxygen lack and irreversibly inhibited by 10^{-10} M δ-endotoxin from *Bacillus thuringiensis* (Bt) (Harvey & Wolfersberger, 1979). Inhibition of the short-circuit current by low molecular weight Bt peptides is described below. An X-ray microanalytical study (reviewed below) reveals that the goblet cavity K^+ concentration decreases in anoxia confirming earlier electrical and tracer deductions that the K^+ pump is on the apical plasma membrane. Unexpectedly a large K^+ concentration step from basal medium to cytoplasm was measured; this step suggests that the entry of K^+ into the cells might be active not passive as heretofore thought. An ATPase whose K_m for ATP is decreased threefold by K^+ has been identified in plasma membrane fractions of the midgut. Goblet cell apical membrane, columnar cell apical membrane, lateral membrane and basal membrane fragments have been isolated in pure form (reviewed below). A separate analysis of each of these fractions should provide a definitive answer to the location of the K^+ ATPase.

K^+ pump in dipteran salivary gland

The K^+ pump in dipteran salivary glands is also thought to be located on the apical plasma membrane on the basis of conductance measurements (Prince & Berridge,

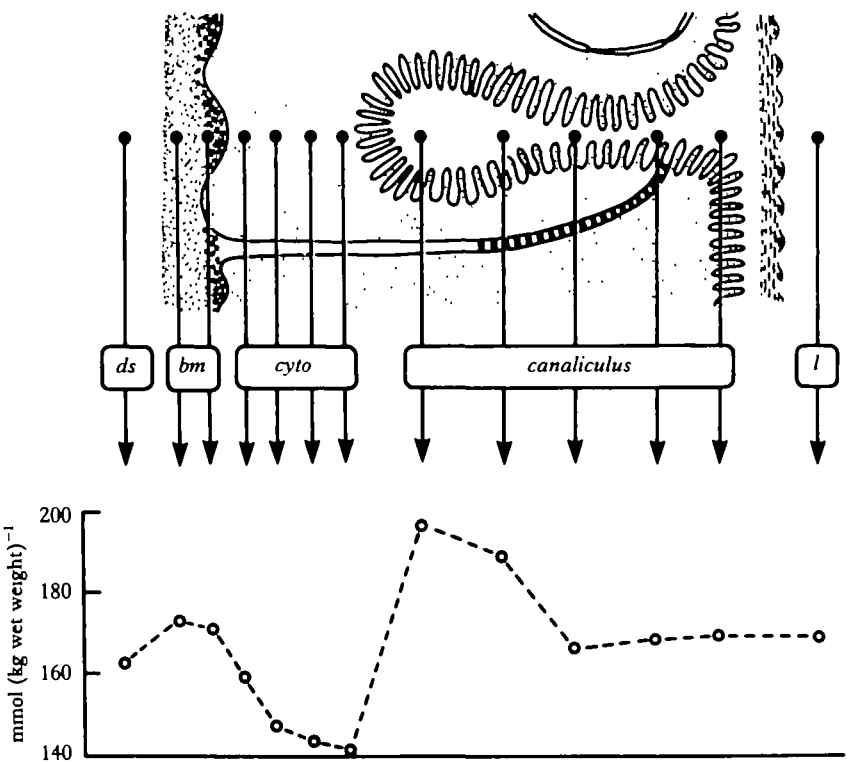


Fig. 3. A profile of sodium concentrations, measured with the microprobe, across the epithelial wall of salivary gland stimulated with 5-hydroxytryptamine in a potassium-free medium: *bm*, basement membrane; *ds*, dextran saline; *cyto*, cytoplasm; *l*, lumen. (From Gupta, Berridge, Hall & Moreton, 1978.)

1972; Berridge, Lindley & Prince, 1975) and the presence of K^+ portasomes (Oschman & Berridge, 1970). However, the most convincing demonstration of the apical location of any insect K^+ pump is from the combined microelectrode and X-ray microanalysis of salivary gland by Gupta *et al.* (1978). A key experiment, in which all of the cellular K was replaced by Na and then a large Na concentration step across the apical membrane was demonstrated by X-ray microanalysis, is shown as Fig. 3. The salivary gland has been useful in analysis of the role of Ca^{2+} and cyclic AMP in controlling fluid secretion coupled to the K^+ pump (Berridge, 1980).

K^+ pump in Malpighian tubules

Evidence that the K^+ pump is located on the apical plasma membrane of the epithelial cells in distal Malpighian tubules of *Calliphora, Carausius,* and *Rhodnius* includes demonstration of fluxes against electrochemical gradients (Maddrell, 1977*a,b*), close association of mitochondria with apical plasma membrane and portasomes on that membrane. When *Rhodnius* Malpighian tubules are stimulated by diuretic hormone their fluid secretion rate increases 1000-fold and approaches $5\,\mu l\,cm^{-2}\,min^{-1}$ (Maddrell, 1969). The secretion rate *in vivo* is equal to the total cellular volume every 16 s (Maddrell, 1972).

K^+ pump in dipteran sensilla

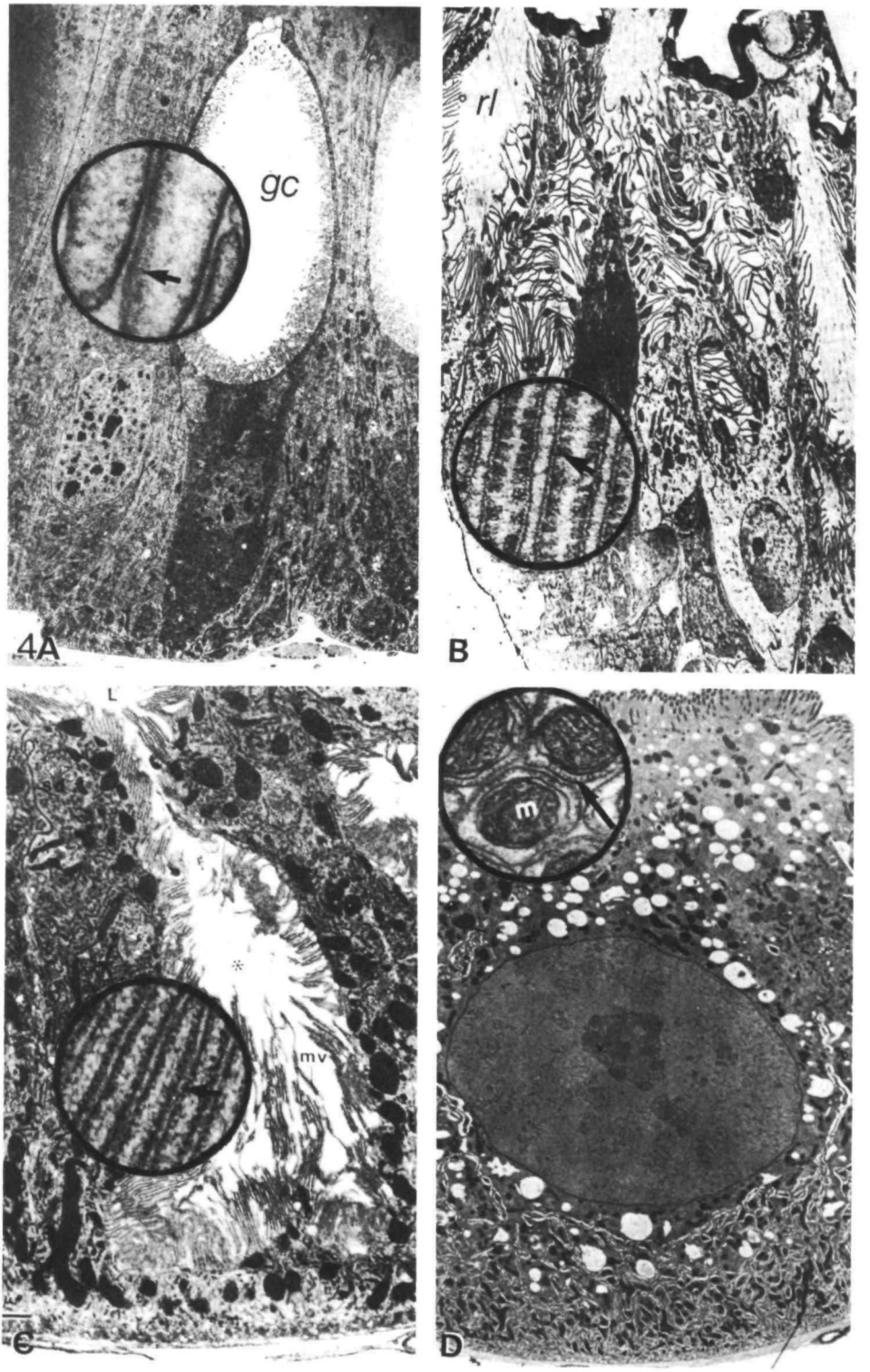
Receptor currents in the sensory sensilla of the labellum of flies are thought to depend on an electrogenic K^+ pump located in the apical membrane of the trichogen and tormogen cells, the so-called enveloping cells. The transepithelial PD is approximately 100 mV, outside positive, and is generated by a K^+ transport from cytoplasm into receptor lymph cavity. The enveloping cells are studded on their cytoplasmic surface by 10 nm particles. Both transepithelial PD and K^+ pump are rapidly inhibited by oxygen lack but are not inhibited by ouabain (Thurm & Küppers, 1980). Wieczorek (1982) has identified a K^+-stimulated ATPase in sensilla-rich labella and shown by density gradient centrifugation that the enzyme is present in plasma membrane enriched fractions and is not of mitochondrial origin.

K^+ pump in other insect epithelia

K^+ transport has been studied intensively in insect rectal tissues but remains controversial; contrast Küppers & Thurm (1980, 1982) and Hanrahan & Phillips (1983). Analysis of electrochemical gradients and the presence of particles on their cytoplasmic surface suggests that in labial glands too the K^+ pump is on the apical membrane (Kafatos, 1968; Hakim & Kafatos, 1974). K^+ is pumped from the basal to the apical side of the isolated integument of *M. sexta* under short-circuit conditions (Jungreis & Harvey, 1975).

K^+ portasomes as transport particles

Electron micrographs of dipteran salivary gland, lepidopteran midgut, orthopteran Malpighian tubules and dipteran sensillum are shown in Fig. 4. Visible on the cytoplasmic surface of the apical plasma membrane (inset) in each case are the 10 nm particles which we have called portasomes. In the midgut (Fig. 4A) the goblet cell


gc
4A
rl
B
L
mv
C
m
D

apical plasma membrane is invaginated, forming the goblet cavity, and additionally folded outwardly, forming apical projections into the cavity. The portasomes in the midgut are restricted to this goblet cell apical plasma membrane (Fig. 4A, inset). In fly taste hairs the trichogen and tormogen cell apical membranes together form a lymph cavity (Fig. 4B). The 10 nm portasomes are restricted to the cytoplasmic surfaces bordering the cavity (Fig. 4B inset). In the secretory region of the dipteran salivary gland there are specialized cells (Fig. 4C) in which the apical plasma membrane is infolded, forming canaliculi, and additionally folded outwardly, forming small leaflets which cover the apical surface of the cell and line the canaliculi. Again portasomes are restricted to the apical surface of the cell including the lining of the canaliculi (Fig. 4C inset). In distal Malpighian tubules the apical plasma membrane of every cell forms a brush border of microvilli (Fig. 4D); again the cytoplasmic surface of this apical plasma membrane is studded with portasomes (Fig. 4D, inset). There are larger (15 nm) particles on the cytoplasmic surface of the apical plasma membrane of many rectal epithelia, see Noirot & Noirot-Timothée, 1971. In *Schistocerca* rectum this membrane is thought to transport Cl^- rather than K^+ (Hanrahan & Phillips, 1983). However, Küppers & Thurm (1980, 1982) argue that an electrogenic K^+ pump is located on the apical membrane of the rectum in many insects. In midgut, salivary gland, Malpighian tubules and sensilla, and possibly in rectum as well, the apical, portasome-studded membrane is thought to be the K^+ transporting membrane. Therefore, it is reasonable to postulate that the portasomes are the unit of active K^+ transport by insect epithelia (Harvey, 1980; Harvey *et al.* 1981, 1983; Küppers & Thurm, 1982). In Fig. 5 the orientation of the K^+ portasomes with respect to K^+ transport in insect membranes is compared to the orientation of F_1-F_o particles with respect to H^+ transport in phosphorylating membranes. In each case the transport is away from the ATP binding side, the electronegative side, and the side with low transported cation concentration. In lepidopteran midgut there appear to be two K^+ transported per ATP hydrolysed (Harvey *et al.* 1981; see Wieczorek, 1982). This similarity in what we had previously thought to be dissimilar systems i.e. K^+-transporting and H^+-transporting membranes, together with the well known ionic character of ATP, suggested a mechanism for insect K^+ transport and for oxidative phosphorylation.

Chemiosmotic model for insect K^+ transport: abandoned cation hypothesis

At the Mg^{2+} concentration and pH thought to exist in cell cytoplasm it is likely that ATP is present as $MgATP^{2-}$, that ADP is present as $MgADP^-$, and that phosphate (Pi) is present as $H_2PO_4^-$. When $MgATP^{2-}$ is hydrolysed to $MgADP^-$ and $H_2PO_4^-$ the nucleotide becomes less negative because an electron moves from it to phosphate.

Fig. 4. Electron micrographs showing four different insect epithelia in which K^+ portasomes are present. Tissues shown are: (A) posterior midgut of *Manduca sexta*, modified from Cioffi (1979), ×1500, inset ×80 000; (B) sensory sensillum of *Musca domestica*, modified from Thurm (1974), ×2750, inset from *Calliphora erythrocephala* ×150 000, courtesy of U. Thurm; (C) salivary gland of *C. erythrocephala*, modified from Berridge & Oschman (1972), ×8000, inset ×70 000. (D) Malpighian tubule from *C. erythrocephala*, from Berridge & Oschman (1969), ×8000, inset ×84 000. Insets show that in all four tissues portasomes (arrows) are present on the cytoplasmic side of the apical plasma membrane. *gc*, goblet cell cavity; *rl*, receptor lymph cavity; *l*, lumen of gland; •, canalicular cavity; mv, microvilli; m, mitochondrion.

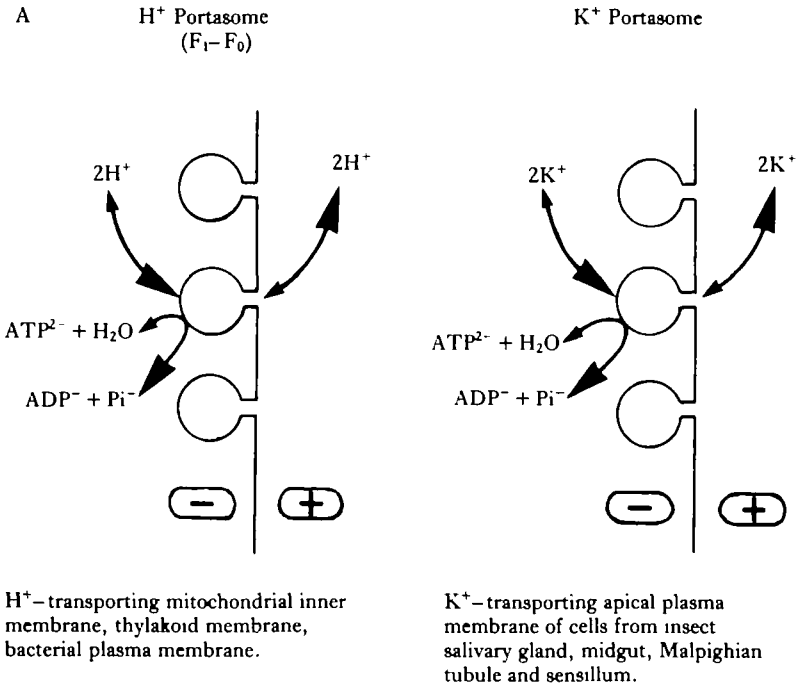


Fig. 5. Summary of location of portasomes with respect to electrical and chemical gradients in phosphorylating membranes and potassium-transporting membranes. The portasomes are located on the low cation concentration, electronegative side in H^+-transporting mitochondrial, thylakoid and bacterial plasma membranes and in K^+-transporting insect plasma membranes where they 'push' the cations across the membrane. (From Harvey, Cioffi & Wolfersberger, 1981.)

Thus during ATP hydrolysis a divalent anion becomes a monovalent anion. Even if we have deduced the cellular charged forms of nucleotide incorrectly the principle holds true – ADP is less negatively charged than ATP. This fact must be dealt with in any serious mechanism for the coupling of ATP hydrolysis to ion transport. If ATP is held by the ATPase in a particular orientation within an isolated membrane domain during hydrolysis then the energy change during the charge separation as ADP^- is repelled from Pi^- can be coupled *directly* to a compensating cation movement; this deduction follows from the inherent vectoriality of ATP hydrolysis mentioned at the outset (Mitchell, 1979). Since during ion transport, ATP hydrolysis always occurs on the particle-studded side of membranes and since electroneutrality must always be preserved (e.g. by transport cations) this observation that nucleotide negativity is decreased on the input side of transporting membranes during ATP hydrolysis led us to postulate two transport rules (Harvey *et al.* 1981) and we now add a third rule:

Rule 1: Cations will always be pushed away from the side of ATP hydrolysis.

Rule 2: Anions will always be pulled toward the side of ATP hydrolysis.

Rule 3: Neutral molecule movement cannot be coupled directly to ATP hydrolysis.

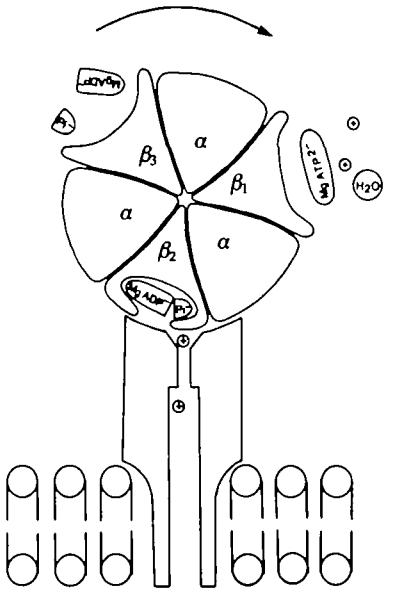
These rules were postulated for chemiosmotically coupled ion transport but they probably apply to all electrogenic ion transport. Let us illustrate them with respe

K^+ transport; two K^+ will neutralize $MgATP^{2-}$ allowing it to bind to the electronegative side of a membrane. But what happens when $MgATP^{2-}$ becomes $MgADP^-$ and Pi^-. These newly formed monovalent anions will repel each other and they will move apart. If they are properly orientated by a K^+ ATPase (portasome) in such a way that they can no longer neutralize the two K^+, then the two K^+ become *abandoned cations* within an isolated domain. If a K^+-selective channel is attached to the K^+ ATPase then the two abandoned K^+ will exit to the opposite side of the membrane through the channel, rendering the output side positive to the input side. This is the energy-requiring, electrogenic step. If neutralizing anions (A^-) follow the K^+ through the membrane by a separate route then the result is a net K^+,A^- flux... i.e. active K^+ transport. On the other hand if a Cl^- channel is attached to the ATPase then two Cl^- ions will be pulled in from the opposite side by the abandoned cations (C^+). This ion movement would constitute an active C^+,Cl^- transport inward and the ATPase would be called a Cl^- ATPase. However, uncharged molecules cannot neutralize the abandoned cations and therefore cannot be coupled directly to ATP hydrolysis by this mechanism. Finally, if the channel is an H^+ channel (F_o), if the ATPase is an H^+ ATPase (F_1), and if the high proton concentration is on the side away from F_1, then unneutralized protons will attract ADP^- and Pi^- and we have a mechanism for ATP synthesis during oxidative phosphorylation. This 'abandoned cation' model accommodates the concept of affinity differences since the charge across the membrane will make the apparent affinity for ions quite different on the two sides; it can explain observed conformation changes (e.g. Chang & Penefsky, 1974) since the provisions for moving the $MgADP^-$ and Pi^- away from the membrane channel will involve changes in the shape of the ATPase molecule. Examples of cation movements away from the side of ATP hydrolysis are H^+ transport in phosphorylating membranes, K^+ transport by certain insect membranes and Ca^{2+} transport by sarcoplasmic reticulum. Even Na^+ movement by the Na^+,K^+ ATPase follows these rules because it is the Na^+ which is actively transported away from the side of ATP hydrolysis, whereas K^+ movement is secondary and can be viewed as a neutralizing ion moving inwardly through a pathway which happens to be incorporated into the ATPase molecule. Possible examples of anion movements toward the side of ATP hydrolysis are Cl^- transport in rectal epithelia of insects (Hanrahan & Phillips, 1983) and I^- movement into thyroid cells (Wolff, 1964). The iodide movement is thought to be coupled to Na^+,K^+ transport because it is ouabain sensitive but direct coupling of iodide transport to ATP hydrolysis has not been ruled out. The coupling of sugars and other neutral molecules, not directly to ATP hydrolysis, but secondarily to Na^+ movement, is well known e.g. in mammalian small intestine. Like all hypotheses this one is not meant to be immortal but to provoke attempts to uncover exceptions to the three rules.

A mechanism for chemiosmotic K^+ transport and for $MgATP^{2-}$ synthesis which illustrates these rules was proposed by Harvey *et al.* (1981, 1983). A speculative sketch of the mechanism is shown in Fig. 6 and is described in the legend.

CATION-MODULATED ATPases OF INSECTS

If the insect K^+ pump uses a potent K^+ ATPase then K^+ modulation of ATPase



$$MgATP^{2-} + H_2O + 2K^+_{in} \rightarrow MgADP^- + Pi^- + 2K^+_{out}$$

Fig. 6. Proposed mechanism by which portasomes couple ATP hydrolysis to cation translocation. Head of portasome is composed of three identical regulatory subunits (α) and three identical catalytic subunits (β). Stalk on which head portion rotates constitutes a gated channel, a portion of which extends through the lipid bilayer of the membrane. $MgATP^{2-}$ hydrolysis occurs only at the catalytic site of the β-subunit aligned over the gate. However, hydrolysis at the catalytic site positioned over the gate does not occur readily until product ($MgADP^- + Pi^-$) has been released from the catalytic site of the subunit in which hydrolysis most recently occurred and substrate ($MgATP^{2-}$) has been bound at the catalytic site of the subunit in which hydrolysis will next occur. Two monovalent cations accompany $MgATP^{2-}$ as it binds to the catalytic site thereby reducing the energy required for binding a negatively-charged nucleotide ion at the electronegative side of the membrane. The two cations remain held to the bound nucleotide by electrostatic attraction. When $MgATP^{2-}$ is hydrolysed electrostatic repulsion between negatively-charged phosphates is conserved in a conformational change that indexes the active site of the subunit to which $MgATP^{2-}$ was most recently bound over the stalk of the portasome, bringing two new electrostatically-bound cations into the domain of the gate and into electrostatic repulsion between the cations abandoned in the gate during the conformational change. The abandoned cations leave the gate through the transmembrane channel. (Modified from Harvey, Cioffi & Wolfersberger, 1983.)

activity should be detectable in homogenates of transporting epithelia. The problem is to distinguish it from Na^+,K^+ ATPase activity and from mitochondrial K^+ ATPase activity.

Na^+,K^+ ATPase; ouabain sensitivity and insensitivity

A ouabain-inhibited Na^+,K^+ ATPase occurs so widely in vertebrate tissue that it is considered ubiquitous (Type I pump; Keynes, 1969). If an experimental parameter is inhibited by ouabain then that parameter is acknowledged to be directly or indirectly dependent on a Type I pump and Na^+,K^+ ATPase. However, if ouabain has no effect then a number of explanations are possible. (1) Ouabain may be binding to and inhibiting a Type I pump, but the parameter under study may not be directly dependent on Type I pump activity; an example is impulse transmission in squid axons. In locust caecum ouabain inhibits active Na^+ absorption against an electrochemic

gradient but inhibition of coupled K^+ secretion is masked by a large passive K^+ movement from the K^+-rich gut lumen into the blood (Dow, 1981); the net K^+ flux is therefore not dependent on Type I pump activity in the caecal tissue. (2) Ouabain may be binding to the Type I pump but failing to inhibit it. (3) Ouabain may be reaching the Type I pump but is not binding to it because the assay conditions are inappropriate. Thus ouabain binding is temperature sensitive, both in vertebrate (Ahmed & Judah, 1965) and insect (Peacock, Bowler & Anstee, 1976) preparations, presumably because K^+ affinity to pump increases at low temperatures; ouabain binding might thus be masked below 30 °C. Similarly, the $Na^+:K^+$ ratio of the incubation medium and in cellular pools is critical because high K^+ levels reduce the effectiveness of ouabain inhibition (Skou, 1965). (4) Ouabain may be reaching a Type I pump which lacks the ouabain-binding site. The affinity of the binding site varies widely between ATPases from different species (Anstee & Bowler, 1979) and even between those isolated from different tissues of the same animal (Keynes, 1969). ATPases have been isolated from mutants which, although stimulated by $Na^+ + K^+ + Mg^{2+}$, are almost completely insensitive to ouabain at concentrations below 10^{-4} M (Chan & Little, 1978). Because binding and inhibition eventually occur at very high concentrations the ouabain affinity of the binding site seems to have been modified while the transport process has not (Robbins & Baker, 1977). It is possible that similar mutations may have been selected for in organisms which normally feed on diets containing cardiac glycosides. Possible examples include the milkweed bug (*Oncopeltus fasciatus*) and the monarch butterfly (*Danaus plexipus*) both of which feed on the cardenolide-rich milkweed (*Asclepias curassavica*) (Brower & Glazier, 1975; Vaughan, 1979). (5) The Type I pump, although present, may not be accessible to ouabain, unless a chaotropic agent such as NaI is employed to produce a random orientation of the transport protein (Anstee & Bowler, 1979). This problem would be especially serious in the insect epithelia which pump K^+ out of cells because the ouabain binding site might then be intracellular. Finally (6) the Type I pump may be absent from the cells.

Ouabain sensitivity of several parameters in insect tissues is clearly established (review by Anstee & Bowler, 1979). Recent examples include Na^+ and K^+ net fluxes and water movement in *Schistocerca* midgut caecum (Dow, 1981), fluid absorption by *Rhodnius* midgut (Farmer, Maddrell & Spring, 1981), and ATPase activity in homogenates of several gut regions of *Glossina* and *Sarcophaga* (Peacock, 1981, 1982) and in homogenates of Malpighian tubules and hindgut of *Homorocoryphus* (Peacock *et al.* 1976).

On the other hand failure of ouabain to inhibit the short-circuit current in lepidopteran midgut and of ouabain binding in midgut but not brain homogenates (Jungreis, 1977; Jungreis & Vaughan, 1977) has not been directly explained. Similarly Peacock (1981) did not explain the ouabain-insensitivity of ATPase in homogenates of dipteran ileum and rectum. Under conditions specifically chosen to demonstrate ouabain sensitivity, isolated *M. sexta* midgut bathed in a solution containing 80 mM-Na^+ and 8 mM-K^+ at 30 °C had a large short-circuit current which was not affected by 10^{-3} M ouabain even after 2 h, but was completely and reversibly inhibited by O_2 lack in a few minutes (Dow, cited in Harvey *et al.* 1983). Moreover, Wolfersberger found neither synergistic $K^+ + Na^+$ stimulation nor ouabain inhibition of ATPase activity in midgut

Table 1. *Na^+- and K^+-stimulated ATPase activity in insect tissues*

Species	Tissue	Activity				
		Complete	$-Na^+$	$-K^+$	$-Na^+$ $-K^+$	+ Ouabain
Jamaicana flava	Hindgut	33·9	15·5	13·3	15·1	15·2
Schistocerca gregaria	Hindgut	71·5	8·3	6·2	8·3	7·6
Locusta migratoria	Malpighian tubules	229·8	77·9	28·8	29·3	24·5
Manduca sexta	Posterior midgut	30·0	36·7	30·0	33·3	31·7

Complete assay mixtures all contained 100–120 mM NaCl, 10–20 mM KCl, 3–5 mM $MgCl_2$, 3–5 mM ATP, 30–50 mM buffer (pH 7·3–7·5), and tissue extract. Specific activities are all expressed in units of nmol min^{-1} mg $protein^{-1}$. Data for *J. flava* and *S. gregaria* from Peacock, Bowler & Anstee, (1972), data for *L. migratoria* from Anstee & Bell (1975) and data for *M. sexta* from Harvey, Cioffi & Wolfersberger (1983).

extracts prepared and assayed according to procedures reported to maximize expression of Na^+,K^+ ATPase activity in other insect tissues (Harvey *et al.* 1983). Although they do not constitute positive proof these results strongly suggest that midgut contains little or no Na^+,K^+ ATPase.

K^+-modulated ATPases of insects

Several studies on cation modulation of ATPase activity in homogenates of insect epithelial tissue have been reported. Most of these studies are difficult to interpret because the effects of Na^+ alone, K^+ alone, and Na^+ and K^+ together were not studied. However, such data are available for homogenates of hindgut from *Jamaicana* and *Schistocerca* (Peacock *et al.* 1972), for Malpighian tubules from *Locusta* (Anstee & Bell, 1975), and for posterior midgut from *Manduca* (Harvey *et al.* 1983); they are summarized in Table 1. In hindgut the ATPase appears to be a Na^+,K^+ MgATPase; it is stimulated when both Na^+ and K^+ are present but not by either one alone; the Na^+,K^+-stimulated MgATPase activity is completely inhibited by ouabain. The Malpighian tubules of *Locusta* appear to have both a Na^+,K^+-stimulated, ouabain-inhibited ATPase and in addition a K^+ ATPase because the ATPase is stimulated more than 2·5-fold by K^+ alone. However, in *Manduca* midgut extracts, even under conditions optimal for detecting Na^+,K^+ ATPase in other tissues, there is no decrease in activity when both Na^+ and K^+ are omitted or when ouabain is present whereas the activity is modestly stimulated by K^+ alone (Wolfersberger, cited in Harvey *et al.* 1983). The affinity of the midgut enzyme for ATP is increased three-fold by K^+ (Wolfersberger, *et al.* 1982).

These studies are limited to ATPase assays of tissue extracts or homogenates. To demonstrate a membrane transport ATPase enzyme measurements on cleanly isolated, K^+ pump-containing, plasma membranes are required (see Towle, 1983). A specific inhibitor of the K^+ ATPase, analogous to ouabain for the Na^+,K^+ ATPase, would facilitate such studies. It is possible that *Bacillus thuringiensis* may provide such an inhibitor.

IS *BACILLUS THURINGIENSIS* δ-ENDOTOXIN THE 'OUABAIN OF K^+ ATPase'?

Action of Bt on apical membrane

Indirect evidence that the δ-endotoxin from *Bacillus thuringiensis*, Bt, acts on the midgut cells of susceptible insects (Fast, 1981) includes reports of metabolic disturbances (Fast & Donaghue, 1971) and of swelling of the apical plasma membrane of midgut epithelial cells followed by cell lysis (Heimpel & Angus, 1959) and lysis of tissue culture cells (Murphy, Sohi & Fast, 1976). The earliest sign of Bt action is increased glucose uptake by midgut epithelial cells within 1 min after oral administration of toxin to *Bombyx mori* larvae (Fast & Donaghue, 1971). This result implies that the toxin acts on the apical cell surface, since cell lysis and the appearance of non-toxic dipeptide fragments in blood is observed much later (Fast, 1981). Direct evidence that Bt acts on the surface of tissue culture cells was provided by Fast, Murphy & Sohi (1978). They bound labelled toxin to Sephadex beads and demonstrated that the label remained on the bead surface. Then they incubated the toxin-bead preparation with tissue culture cells and found that the cellular ATP level dropped as much as 50 %, whereas a much smaller reduction occurred in control experiments in which the endotoxin was inactivated by specific antibodies or heat. Because the beads are much larger than the cells, Fast concluded that the bead-bound toxin must be acting on the cell surface. More recently Percy & Fast (1983) have published electron micrographs showing that microfilaments in the microvilli of columnar cells in *Bombyx mori* larval midgut disappear within 1 min after exposure to toxin.

Bt prepared by alkaline hydrolysis irreversibly inhibits the short-circuit current (SCC) of the isolated midgut of *M. sexta* by 70 % at 10^{-10} M (Harvey & Wolfersberger, 1979); Bt prepared by exposure to gut juice inhibits completely (M. G. Wolfersberger, unpublished results). The toxin acts 100 times better when applied to the apical rather than basal bathing solution. In recent X-ray microanalysis studies (B. L. Gupta, J. A. T. Dow, T. A. Hall & W. R. Harvey) the K^+ concentration after Bt is added drops from 129 mM to 37 mM in goblet cell cavity under conditions in which columnar and goblet cytoplasm K^+ concentrations are only slightly reduced. The goblet cavity also swells and becomes less electron dense. Again Bt acts on the apical side of the gut and in this case on the goblet cell apical membrane. In summary, there is general agreement among Bt workers that the endotoxin acts on the apical plasma membrane of midgut cells; the evidence from *B. mori* favours an early action on columnar cell apical membrane, whereas that from *M. sexta* is compatible with an early action on goblet cells. Whether the Bt action is directly on the K^+ pump or on some other site specific to the apical membranes is the object of intensive research.

Bioactivity of small Bt peptides

The native δ-endotoxin from *Bacillus thuringiensis* var. *kurstaki* is a 230 000 Da crystalline protein which is non toxic to insects. It becomes toxic when it is degraded to smaller polypeptides but the nature of the toxic peptides is controversial. The concensus of researchers currently working with Bt can be summarized as follows (Huber & Lüthy, 1982). The 230 000 Da native protein is a dimer held together by

disulphide bonds; it is broken down by dithiothreitol into two, 115 000 Da monomers. These monomers can be digested by trypsin or by short exposure to gut enzymes, releasing 70 000–80 000 Da polypeptides, and by longer exposure to gut enzymes releasing 30 000–60 000 Da polypeptides. The 30 000 Da peptides are widely thought to represent the smallest Bt units which retain toxicity to insects. Fast & Martin (1980) have a different view; they treated the 230 000 Da endotoxin with high concentrations (2–4 M) of potassium thiocyanate (KSCN) in N-morpholinopropane buffer containing dithiothreitol and found peptides ranging from 3000 to 1000 Da which retain toxicity to silkworm larvae. The molecular weights of the peptides were determined by equilibrium centrifugation. Fast & Martin (1980) argue that the entire crystal is composed of short peptides held together by disulphide bonds and non-covalent interactions and that the small peptides are not products of peptide chain hydrolysis. The toxicity to insects of the small peptides ($LD_{50} = 0{\cdot}025\ \mu g$/mg larva) was the same as that of the crystalline protein from which they came ($LD_{50} = 0{\cdot}026\ \mu g$/mg larva).

Working in collaboration with Dr John R. Williams, Mr Charles Lin, Dr Frank N. Chang and Mr Erich Mackow, Wolfersberger & Harvey now have evidence that Bt peptides no larger than 3500 Da are effective in inhibiting the short-circuit current in isolated *M. sexta* midgut. Because of the controversial nature of Fast's results we have avoided KSCN and have degraded the endotoxin with an immobilized *M. sexta* gut enzyme (see Murphy *et al.* 1976). We separated the peptides by high performance liquid chromatography and assayed the peptides for inhibition of SCC in isolated *M. sexta* midgut. We have confirmed the molecular weights of the gut inhibitory peptides by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) using silver staining to visualize the peptides and using insulin and somatostatin as molecular weight standards. Whether the small peptides act directly on the K^+ ATPase or on some other specific site on the midgut cell apical plasma membrane remains to be determined.

IDENTIFICATION OF K^+ PUMP-CONTAINING MEMBRANE

Clearly the location of the K^+ pump on the apical plasma membrane of the midgut goblet cells is basic to the interpretation of K^+ transport using the midgut as model. We will discuss older evidence and report on a new electron probe X-ray microanalytical (XMA) study which confirms this location.

Microelectrode studies

Early microelectrode studies (Wood, Farrand & Harvey, 1969) reported a single potential profile, with a basal step of −27 mV and an apical step of +125 mV as a microelectrode was advanced from blood side reference solution to the lumen in *Hyalophora cecropia*. Under anoxia only the apical step decreased, implying that the K^+ pump is apically located. However, PD measurements by themselves are open to criticism. Using improved electrodes Blankemeyer & Harvey (1977, 1978) resolved a low PD profile (LPD) with a basal step of only −9 mV in *M. sexta* and a more frequent, high PD (HPD) profile which resembled the earlier profile reported by Wood *et al.* (1969). Importantly, the resistance ratio between the microelectrode and

basal: apical solution was found to be 1:20 and was insensitive to oxygen lack for HPD impalements but was 1:5 for LPD impalements in oxygen and rose to 1:20 in nitrogen. Blankemeyer & Harvey argued from a frequency histogram of the results that LPD impalements were intracellular recordings from goblet cells and that the goblet cell apical plasma membrane was thus the site of K^+ transport; the HPD impalements were interpreted as intracellular recordings from columnar cells. Recently Blankemeyer (1981) supported the latter conclusion by dye recovery in columnar cells following six HPD impalements, but was unable to recover dye after LPD impalements. Recent studies (Moffett, Hudson, Moffett & Ridgway, 1982) suggest that only HPD impalements represent intracellular recordings from intact cells, and that goblet and columnar cells can both display HPD profiles. This may explain problems in dye recovery from LPD sites. Estimates of transport pool size by tracer kinetic methods (Harvey & Zerahn, 1969) likewise suggest that the K^+ pump is on the apical membrane of the goblet cells (see Blankemeyer & Harvey 1977, 1978; Cioffi & Harvey, 1981) but this conclusion is also controversial (see Discussion in Harvey, 1982 or Wolfersberger *et al.* 1982 compared to Zerahn, 1977, 1978). In view of its importance a convincing demonstration that the goblet cell apical membrane is the K^+ transport site was needed to verify or discredit this indirect evidence.

X-ray microanalysis of midgut

If the K^+ pump is on the apical plasma membrane of the goblet cell and is pumping K^+ from cytoplasm to goblet cavity then the K^+ concentration in the goblet cavity should fall when the transport is stopped by anoxia. Moreover, the K^+ concentration profile across columnar and goblet cells during normal transport and after inhibition by anoxia might give information regarding the K^+ transport route. We have seen the value of electron probe X-ray microanalysis (XMA) in demonstrating that the K^+ pump is on the apical plasma membrane in dipteran salivary gland (Fig. 3; from Gupta *et al.* 1978). A recent XMA study of the midgut (J. A. T. Dow, B. L. Gupta, T. A. Hall, W. R. Harvey, unpublished observations) is summarized here.

The posterior midguts from feeding larvae of *Manduca sexta* were short-circuited in a special chamber, which allowed the gut to be removed and frozen in supercooled liquid freon to −185 °C in less than 1 s. Tissues were then stored under liquid nitrogen until required for microanalysis. Frozen-hydrated sections were cut with steel knives in a cryomicrotome at −60 to −80 °C, at a thickness of 1 μm, and examined in a JEOL JXA-50A microprobe analyser. Data acquisition was performed with an energy-dispersive spectrometer and a Link Systems multichannel analyser, connected to a Link Systems computer.

Values obtained for K are expressed as transcellular concentration profiles in Fig. 7. It can be seen that, while anoxia affects the potassium concentration in the cytoplasm of goblet and columnar cells only slightly, goblet cavity and apical projection [K] is substantially depressed. During the 10 min for which the experimental group was deprived of oxygen, the short-circuit current (which is an exact measure of the net K^+ transport) fell by 90% compared with controls. Because the goblet cavities and their apical projections were the only regions whose K distribution was seriously affected by pump inhibition, we can conclude that there is a K^+ pump in the

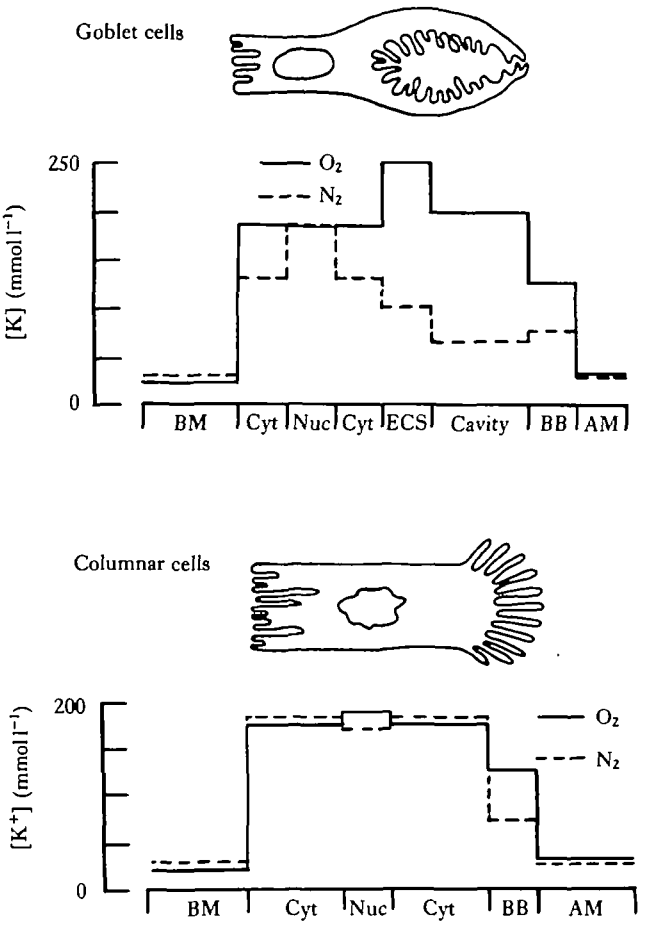


Fig. 7. Distribution of potassium, in mmol (litre local water)$^{-1}$ in the goblet and columnar cells of *Manduca* midgut, after 45 min short-circuiting; bubbled with oxygen throughout the experiment (O_2), or with oxygen for the first 35 min, and then with nitrogen until sampling (N_2). Tissues were frozen in liquid freon at −189 °C, sectioned at 1 μm, and analysed in frozen hydrated and dried states by XMA. (Data from J. A. T. Dow, B. L. Gupta, T. A. Hall & W. R. Harvey, unpublished observations.) Abbreviations: BM, basal medium; Cyt, cytoplasm; Nuc, nucleus; ECS, extracellular fraction of goblet microvillar field (calculated value); BB, extracellular fraction of columnar microvillar field (calculated value); AM, apical medium.

goblet cell apical membrane. This finding confirms the conclusions of earlier indirect studies discussed above. The hypothesis that the goblet cavity is electrically isolated, as is the lumph cavity of sensilla (Thurm & Küppers, 1980), and that the resulting PD in excess of 180 mV across the GCAM may be used for amino acid uptake (Giordano, Sacchi & Hanozet, 1982) and pH regulation will be discussed in a future publication.

Unexpectedly the goblet cell K concentration (approximately 110 mM) was much higher than that which had been measured previously (approximately 20 mM; Moffett, 1979); in fact the potassium level in goblet cytoplasm was nearly the same as that in columnar cytoplasm. We estimate that the Nernst equilibrium potential for K^+ across the basal membrane of both cell types is approximately −27 mV, identical

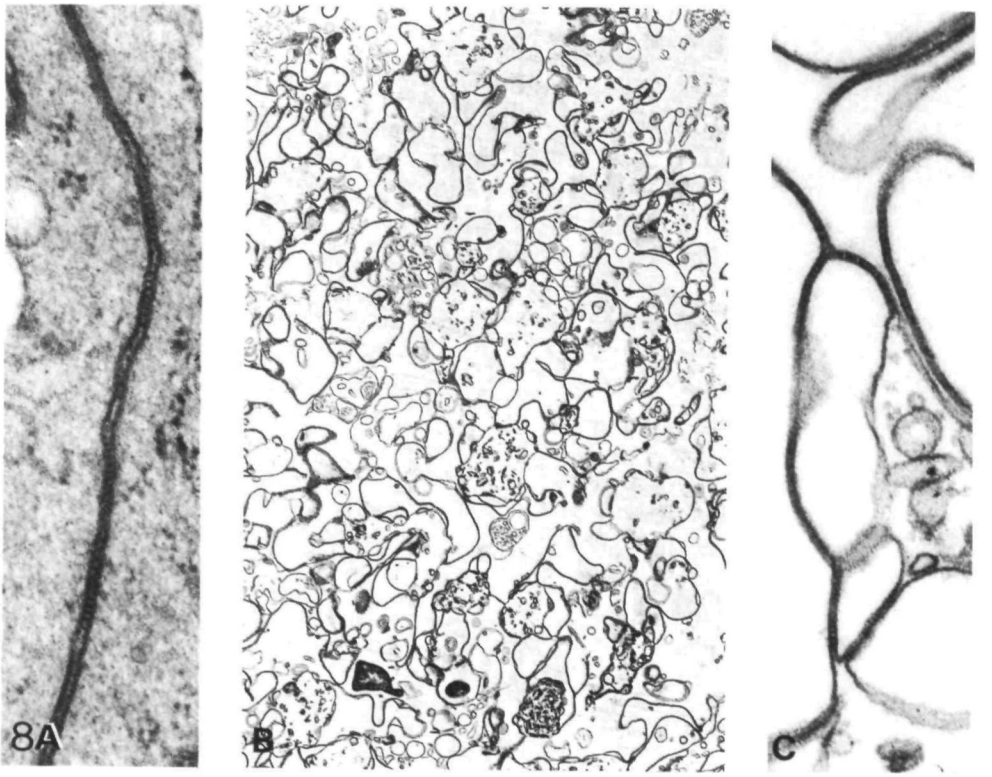


Fig. 8. Lateral membranes of *Manduca sexta* posterior midgut epithelial cells. (A) High magnification electron micrograph showing lateral membrane in intact epithelium; (B) low magnification electron micrograph of isolated and purified lateral membrane preparation; (C) high magnification electron micrograph of isolated lateral membranes shown in (B) (from Cioffi & Wolfersberger, 1983). Magnification: (A) ×64 000; (B) ×3600; (C) ×60 000.

e value measured with microelectrodes by Wood *et al.* (1969) and similar to the basal PD in the HPD impalements of Blankemeyer & Harvey (1978). The microprobe results are in excellent agreement with the findings of the recent K^+-selective microelectrode study by Moffett *et al.* (1982) mentioned above; they observed a mean cytoplasmic K^+ activity of 95 mM (for impalements with a basal PD step more negative than −20 mV). The conclusion from these new microprobe and microelectrode studies is that LPD impalements probably represent recordings from extracellular space or damaged cells, and that the HPD impalements are recordings from healthy columnar, goblet and regenerative cells.

The finding that the K^+ concentration in cells (over 100 mM) is substantially higher than that in blood (approximately 30 mM) demands explanation. Although K^+ is close to electrochemical equilibrium across the basal membrane, this finding does not imply that metabolic work is not expended in maintaining the concentration difference. Thus K^+ is close to equilibrium across almost all animal cell membranes; yet the concentration difference across cell membranes requires active maintenance by the Na^+,K^+ ATPase. Notwithstanding the arguments presented earlier, the microprobe determination that the cellular [Na] is less than 5 mM does not exclude the possibility that sufficient Na^+ leaks into the cells *in vivo* from the blood to allow a Na^+,K^+ ATPase (with a $K_{1/2}$ for Na^+ as low as 260 μM; Matsui & Homareda, 1979) to function. K^+ could be pumped into the cells from the blood in exchange for intracellular Na^+. The Na^+leaks back in (10 mM outside, <2 mM inside; inside 27 mV negative to outside) and is continuously cycled. M. Cioffi (unpublished observations) has found that ^{42}K loads rapidly only from the blood side and that this loading is abolished under anoxia. Measured K^+ loading times for the midgut (Cioffi & Harvey, 1981) are less than 10 min; since the rate of K^+ transport in midgut is one of the highest recorded for any tissue, it is hard to believe that the high cytoplasmic K^+ levels observed by the microprobe and microelectrodes are not metabolically maintained by active transport across the basal membrane. However, since the SCC agrees precisely with the net transepithelial K^+ flux (e.g. Cioffi & Harvey, 1981), the electrical signature of such basal movement would have to be insignificant compared with that across the goblet cell apical membrane. While anoxia might be expected to abolish both apical and basal K^+ movements, the action of *Bacillus thuringiensis* endotoxin is initially confined to the apical membranes; the residual SCC observed under Bt poisoning (Harvey & Wolfersberger, 1979) might represent an unmasked basal electrical signature.

A basal Na^+,K^+ ATPase is only one of several possible mechanisms consistent with the microprobe and microelectrode data; we must conclude that the basal potassium concentration step remains unexplained but that it is almost certainly maintained at metabolic expense.

ISOLATION OF PLASMA MEMBRANE FRAGMENTS

Lateral membrane fragments (LM)

Conventional homogenization of *M. sexta* midgut followed by differential and density gradient centrifugation, enabled Cioffi & Wolfersberger (1981, 1983) to prepare a pure fraction of lateral membranes; the fractions being monitored by electron microscopy, (Fig. 8). To our knowledge this is the first time that epithelial

lateral membranes have been separated cleanly from basal membranes although Bo dan & Gilula (1982) have isolated gap junctions. The isolation of LM free of basal membranes (BM) is possible because insect epithelial cells characteristically are joined by septate junctions between long segments of adjacent LM; homogenization causes the cells to break into separate apical, lateral and basal fragments. By contrast the more familiar vertebrate epithelial cells are joined by continuous junctions (zonulae occludentes) only at their apical surface; homogenization causes the cells to break into apical and basolateral fragments.

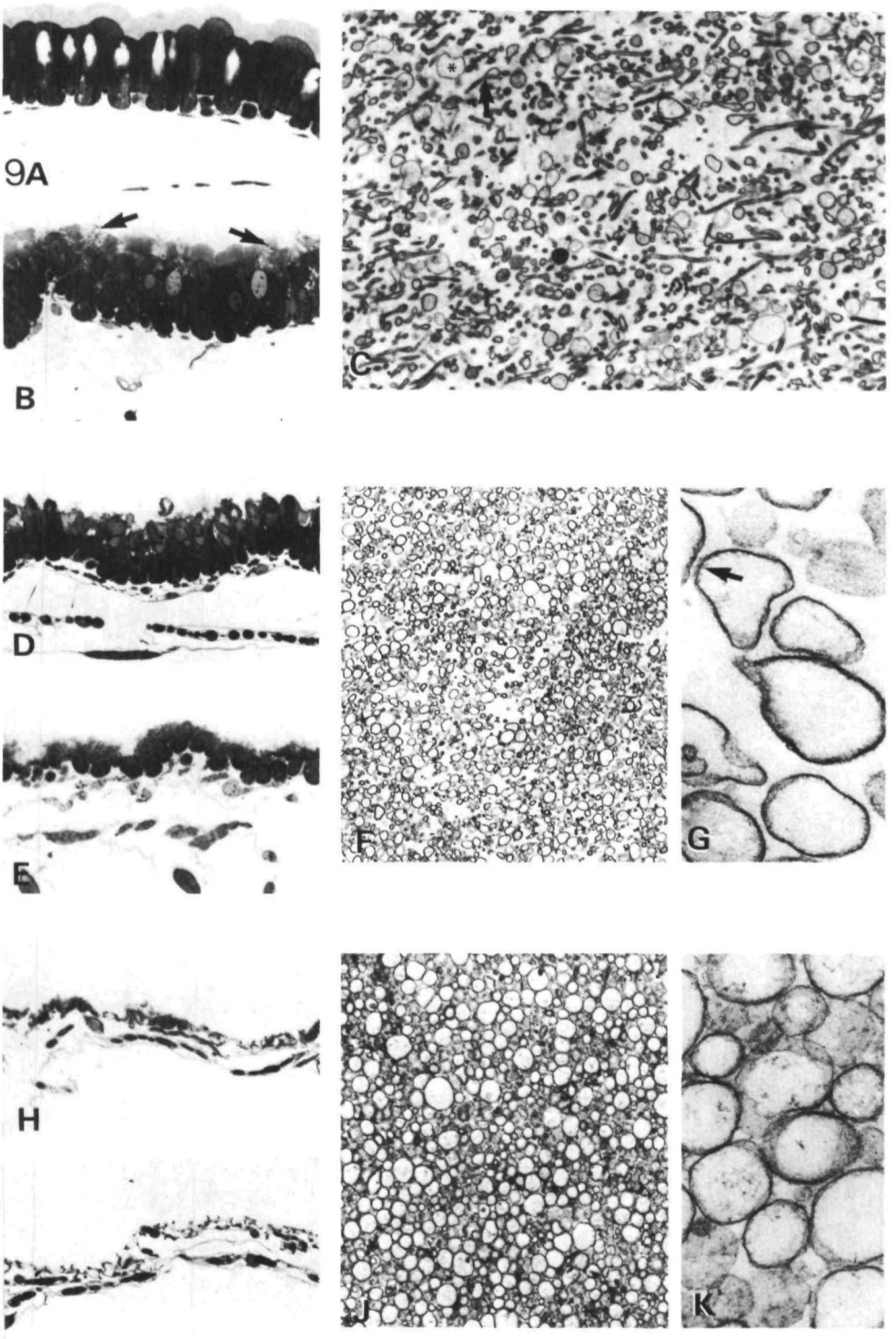
Fractionating insect plasma membranes by ultrasound

Conventional techniques were not effective in separating fragments of apical or basal plasma membranes. Therefore Cioffi & Wolfersberger (1983) developed a new technique based upon disrupting the epithelium with ultrasound and aspiration through a pipette followed by differential and density gradient centrifugation. Each cell was disrupted in layers starting at the apical surface. Thus the first layer consists of CCAM, the second layer consists of invaginated GCAM, and the third layer consists of BM. The process is illustrated by the photographs of the epithelium during progressive disruption on the left side of Fig. 9 (A, B; D, E; H, I). Plasma membrane fragments retained sufficient structure to enable their origin to be deduced by electron microscopy. Three clean fractions of midgut epithelial cell plasma membrane were prepared by this technique – columnar cell apical membranes (CCAM), goblet cell apical membranes (GCAM) and basal membranes derived from both columnar and goblet cells (BM).

Columnar cell apical membrane fragments (CCAM)

To prepare the CCAM fragments, which are derived from a microvillar brush border, the midgut is cut into small pieces and suspended in buffer. The tissue pieces (Fig. 9A) are then subjected to a few seconds of ultrasound, which is sufficient to remove patches of microvilli while leaving the rest of the epithelium intact (Fig. 9B). Where the microvilli have been removed (arrows) mitochondria and other organelles can be seen spilling out of the cells. Following filtration through gauze, to remove the tissue pieces, the CCAM is separated from mitochondria and other contaminants in suspension by differential centrifugation. A section through the pellet of pure CCAM is shown in Fig. 9C. The orientation of microvilli in the pellet is random; some have

Fig. 9. Stepwise disruption of *Manduca sexta* posterior midgut epithelium and isolation of plasma membrane segments. (A) Light micrograph of intact epithelium; (B) light micrograph of epithelium after treatment to remove a portion of columnar cell microvilli; (C) electron micrograph of purified columnar cell microvilli (CCAM); swollen microvillus (arrow), irregular vesicle (*), see text. (D) Light micrograph of epithelium after treatment to remove essentially all columnar cell microvilli; (E) epithelium after treatment to remove goblet cell apical membranes (GCAM); (F) low magnification electron micrograph of purified GCAM preparation; (G) higher magnification electron micrograph of GCAM preparation showing portasomes (arrow). (H) Light micrograph of epithelium after treatment to remove all but the basal portion of epithelial cells; (I) light micrograph of epithelium after treatment to remove basal portion of epithelial cells; (J) low magnification electron micrograph of purified basal membrane (BM) preparation; (K) higher magnification electron micrograph of purified basal membrane preparation. (From Cioffi & Wolfersberger, 1983.) Magnification: A, B, D, E, H, I ×150; C ×1500; F ×2700; G ×37000; J ×6400; K ×41000.


9A
B
C
D
E
F
G
H
J
K

[illegible]n sectioned longitudinally and appear as long narrow filaments, whereas others have been sectioned transversely or obliquely and appear as circular or oval profiles. The procedure often causes the tips of the microvilli to swell (arrow) and they appear in section as large irregularly shaped vesicles (asterisk).

Goblet cell apical membrane fragments (GCAM)

To prepare GCAM fragments the intact tissue pieces are sonicated to release the entire brush border into suspension and to expose the invaginated GCAM (Fig. 9D). In this case the buffer is discarded and the tissue pieces are resuspended in fresh medium. Then by drawing the tissue pieces in and out of a Pasteur pipette several times the remaining apical part of the epithelial cells is broken off leaving the basal portion intact (Fig. 9E). The tissue pieces are removed by filtration through gauze and the GCAM, together with associated mitochondria and fragments of lateral membranes, are separated from any contaminants in suspension by sucrose density gradient centrifugation. The mitochondria are then dissociated from the GCAM by sonication, which also converts the GCAM into small vesicles. The mitochondria are removed by differential centrifugation. A section through the pellet of pure GCAM vesicles is shown at low (Fig. 9F) and high (Fig. 9G) magnification. Portasomes (arrow) can still be recognized on the inside of these GCAM vesicles.

Basal membrane fragments (BM)

To prepare BM fragments the tissue pieces are subjected to ultrasound and pipetting which removes the entire apical parts of the cells but leaves the basal part intact (Fig. 9H). The tissue pieces are suspended in fresh buffer, pipetted again to release the BM fragments into suspension, and the remaining basal lamina and muscle layer (Fig. 9I) are removed by filtration through gauze. The filtrate is sonicated briefly to convert the BM into small vesicles and to release their associated mitochondria, which are then removed by differential centrifugation. A section through a pellet of pure BM vesicles is shown at low (Fig. 9J) and high (Fig. 9K) magnification.

Properties of isolated membrane fragments

The approximate yield of the four membrane fragments was: LM, 8 μg/larva; CCAM, 200 μg/larva; GCAM, 10 μg/larva; and BM 20 μg/larva. There was no detectable succinate dehydrogenase activity in any of the purified preparations; in each case the enzyme activity declined during purification, as was expected since mitochondria were being removed as contaminants. Alkaline phosphatase activity was enriched in the CCAM and declined to near zero in the other fragments during purification. We had thought that 5'nucleotidase might be enriched in BM; however, it was present in but low activity in all four fragments and not enriched in any of them during purification.

Status of K^+ ATPase in insect plasma membrane preparations

Little of no ATPase activity was detected in our initial studies with GCAM fragments. In retrospect this result might have been expected: the fragments are prepared under gentle conditions to preserve their structure; they form vesicles from a membrane known to be impermeable to $MgATP^{2-}$ and to K^+. Since the K^+ portasomes,

thought to contain the K^+ ATPase are on the inside of the vesicles (Fig. 9G) MgATP and K^+ would have to penetrate them to reach the ATPase. Attempts to expose the ATPase and measure the effects of K^+ on V_{max} and K_m as well as the effects of Bt peptides, ouabain, oligomycin and other kinetic studies are in progress.

In the meantime we were able to deduce from partially purified preparations that the K^+ ATPase is localized in the plasma membrane fraction (Wolfersberger *et al.* 1982) and further restricted to the goblet cell apical membranes (Harvey *et al.* 1983). A pellet obtained from posterior midgut by homogenization followed by differential centrifugation contained all four plasma membrane fragments and had a K^+-stimulated MgATPase activity of 6·3 μmol mg protein^{-1} h^{-1}. The columnar cell apical membranes and lateral membranes along with all mitochondria, were removed by sucrose density gradient centrifugation; a band was recovered which contained only goblet cell apical membranes and basal membranes; its K^+-stimulated MgATPase activity was increased to 41·5 μmol mg protein^{-1} h^{-1}; hence the enzyme is not mitochondrial and is localized either in goblet cell apical membranes, basal membranes, or both. An entirely different plasma membrane fraction, prepared by sonication, was enriched in goblet cell apical membranes and heavily contaminated with mitochondria, but contained only traces of columnar cell microvilli, cellular fragments and basal membrane. This fraction had a high MgATPase activity, much of which was presumably mitochondrial. However, this activity was stimulated 35 % by K^+ whereas midgut mitochondrial preparations are not stimulated by K^+ (Wolfersberger *et al.* 1982). Taken together these results demonstrate that the midgut plasma membranes contain K^+ ATPase activity and are consistent with its localization in goblet cell apical membrane.

A non-mitochondrial, K^+-activated ATPase has been identified in plasma membrane fractions prepared by sucrose density gradient centrifugation from the labellum of the fly, *Protoformia terraenovae* (Wieczorek, 1982). The enzyme is found only in the labella, which are rich in sensilla, and not in the haustella, which contain few sensilla. Recall that electrochemical studies placed the K^+ pump in the sensilla (reviewed by Thurm & Küppers, 1980). The enzyme is not inhibited by sodium azide (a mitochondrial ATPase inhibitor) and is routinely studied in 10^{-3} M-ouabain. Its half maximal activation occurs at approximately 70 mM-K^+.

SDS-PAGE patterns of membrane fragments

In June 1981, lateral membrane fragments from *M. sexta* midgut were solubilized by suspension in Tris buffer containing sodium dodecyl sulphate (SDS) plus 2-mercaptoethanol and heating for 3 min in a boiling water bath. The resulting mixture was separated by electrophoresis on 10 % acrylamide gel slabs containing 0·1 % SDS (Laemmli, 1970). In January 1983, freshly prepared columnar cell apical membrane fragments, goblet cell apical membrane fragments, and basal membrane fragments were solubilized and electrophoretically separated under conditions similar to those used with lateral membrane fragments. The electrophoretic patterns of all of these membrane fragments are shown in Fig. 10. The most striking finding is that the major bands are different in each of the four different membrane samples. This finding confirms that serious cross contamination between membrane fragment preparations does not exist and provides direct evidence that the different portions of the plas

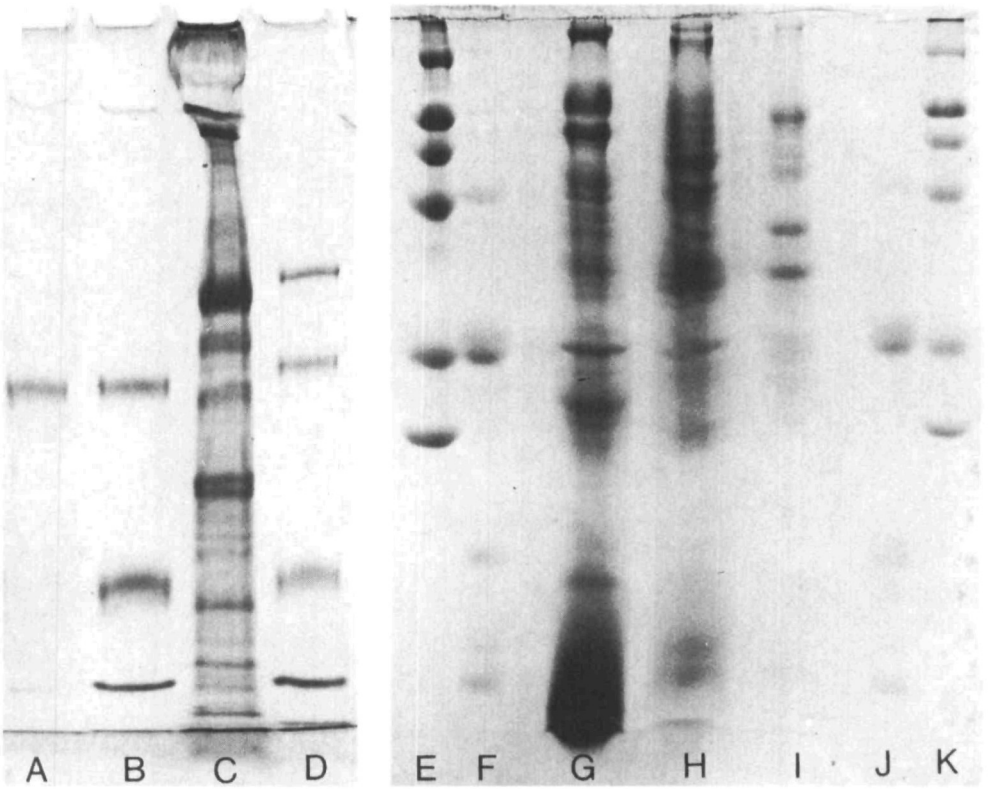


Fig. 10. Sodium dodecyl sulphate polyacrylamide gel electrophoretic separations of *Manduca sexta* midgut plasma membrane proteins. This figure is composed of photographs of portions of two SDS-PAGE slabs upon which samples of solubilized plasma membranes as well as various commercially purified standard polypeptides were electrophoretically separated. Samples and standard polypeptides (molecular weight) are coded as follows. Lane A; bovine serum albumin (66 200). Lane B; a mixture of carbonic anhydrase (31 000), ovalbumin (45 000) and bovine serum albumin. Lane C; solubilized lateral membrane fragments. Lane D; a mixture of lysozyme (14 400), soybean trypsin inhibitor (21 500), carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase B (92 500). Lane E; carbonic anhydrase, ovalbumin, bovine serum albumin, phosphorylase B, β-galactosidase (116 000) and myosin (200 000). Lane F; lysozyme, β-lactoglobulin (18 400), trypsinogen (24 000), ovalbumin and bovine serum albumin. Lane G; solubilized columnar cell apical membrane fragments. Lane H; solubilized basal membrane fragments. Lane I; solubilized goblet cell apical membrane fragments. Lane J; same polypeptides as lane F. Lane K; same polypeptides as lane E.

membrane have different chemical compositions. This result is most encouraging and suggests that it may be possible to identify specific proteins with specific membrane functions in the future.

The solubilized lateral membranes were separated into over 20 components that stained with Coomassie brilliant blue (Fig. 10, lane C). On the basis of their migration relative to that of the standard proteins on the gel slabs we estimate the molecular weight of the four most heavily stained bands to be 87 000, 77 000, 68 000 and 54 000 Da. The 87 000 Da band is much more heavily stained than any of the others and it is tempting to speculate that this polypeptide may be characteristic of septate desmosomes.

The solubilized columnar cell apical membrane fragments were also separated into at least 20 components that stained with Coomassie blue (Fig. 10, lane G). On the basis of their migration relative to that of the standard proteins we estimate the molecular weights of the five most heavily stained microvilli protein bands to be approximately 100 000, 92 000, 45 000, 37 000 and 20 000 Da. In addition to the resolved bands there was a considerable amount of unresolved material at the low molecular weight end of the microvilli fragment lane. Similarly large amounts of low molecular weight material were not detected in any of the other solubilized plasma membrane samples (Fig. 10).

The solubilized basal membrane fragments were separated into 18 or more bands that stained with Coomassie brilliant blue (Fig. 10, lane H). Almost all of these bands were in the same portion of the gel as standard proteins with molecular weights between 116 000 and 29 000 Da. The estimated molecular weights of the four most heavily stained basal membrane proteins are approximately 82 000, 77 000, 60 000 and 54 000 Da.

Due to the limited amount of sample available for electrophoresis, there are only about 12 bands discernible in the goblet cell apical membrane lane of the gel (Fig. 10, lane I). The three bands that stained most heavily with Coomassie blue are at positions on the gel corresponding to proteins with molecular weights of 96 000, 66 000 and 57 000 Da. One or more of these bands or the minor bands may represent subunits of the K^+ ATPase.

CONCLUSION

The coupling coefficient, R_{kr}, in non-equilibrium thermodynamic equation (1) is a formal expression for an ion transport ATPase. The midgut K^+ ATPase appears to be more like the H^+ ATPases of phosphorylating membranes than like the nearly ubiquitous Na^+,K^+ ATPase of animal cell plasma membranes. The K^+ pump is present in the apical plasma membrane of certain K^+-transporting epithelia including midgut, salivary glands, Malpighian tubules, sensory sensilla, and perhaps rectum. Although Na^+,K^+ ATPases are well established in many insect cells it is clear that the K^+ ATPase cannot be explained as a disguised form of this enzyme but appears to be a unique transport ATPase. The δ-endotoxin from *Bacillus thuringiensis* inhibits the K^+ transport in midgut and, along with small Bt peptides, has promise as an apical membrane and K^+ pump probe. New microprobe studies of lepidopteran midgut, like earlier studies of the dipteran salivary glands, reveal cation concentration profiles

which confirm the apical location of the electrogenic K^+ pump; in addition they suggest that an electrically silent K^+ pump may be present on the basal membranes in midgut. We have now isolated plasma membrane fragments from four specific regions of midgut epithelial cells. The goblet cell apical membrane of lepidopteran midgut and plasma membrane fractions of fly sensilla appear to have K^+ ATPase activity. Ion transport and its enzymology can now be studied in membrane vesicles; there is reasonable hope that specific functional membrane proteins can soon be isolated.

The experimental work from this laboratory cited in this paper was supported in part by Research Grant AI-09503 from the National Institute of Allergy and Infectious Diseases, by Research Incentive Fund awards 700 950-15 and 950-82 and Biomedical Research Support Grant 501-902-15 from Temple University. Dr J. A. T. Dow is a Harkness Fellow of the Commonwealth Fund. We thank the Company of Biologists for this discussion meeting.

REFERENCES

AHMED, K. & JUDAH, J. D. (1965). On the action of Strophanthin-G. *Can. J. Biochem.* **43**, 877–880.

ANSTEE, J. H. & BELL, D. M. (1975). Relationship of Na^+-K^+-activated ATPase to fluid production by Malpighian tubules of *Locusta migratoria*. *J. Insect Physiol.* **21**, 1779–1784.

ANSTEE, J. H. & BOWLER, K. (1979). Ouabain-sensitivity of insect epithelial tissues. *Comp. Biochem. Physiol.* **62A**, 763–769.

BERDAN, R. & GILULA, N. B. (1982). Isolation and preliminary biochemical analysis of invertebrate gap junctions. *J. Cell Biol.* **95**, 94a.

BERRIDGE, M. J. (1980). The role of cyclic nucleotides and calcium in the regulation of chloride transport. *Ann. N.Y. Acad. Sci.* **341**, 156–171.

BERRIDGE, M. J., LINDLEY, B. D. & PRINCE, W. T. (1975). Membrane permeability changes during stimulation of isolated salivary glands of *Calliphora* by 5-hydroxytryptamine. *J. Physiol., Lond.* **244**, 549–567.

BERRIDGE, M. J. & OSCHMAN, J. L. (1969). A structural basis for fluid secretion by Malpighian tubules. *Tissue and Cell* **1**, 247–272.

BERRIDGE, M. J. & OSCHMAN, J. L. (1972). *Transporting Epithelia*. New York: Academic Press.

BLANKEMEYER, J. T. (1981). Association of microelectrode impalement potentials with morphologically distinguishable cell types in the insect midgut. *The Physiologist* **24**, 57.

BLANKEMEYER, J. T. & HARVEY, W. R. (1977). Insect midgut as a model epithelium. In *Water Relations in Membranes in Plants and Animals*, (eds A. M. Jungreis, T. Hodges, A. M. Kleinzeller & S. G. Schultz), pp. 161–182. New York: Academic Press.

BLANKEMEYER, J. T. & HARVEY, W. R. (1978). Identification of active cell in potassium transporting epithelium. *J. exp. Biol.* **77**, 1–13.

BROWER, L. P. & GLAZIER, S. C. (1975). Localization of heart poisons in the monarch butterfly. *Science, N.Y.* **188**, 19–25.

CHAN, G. L. & LITTLE, J. B. (1978). Induction of ouabain-resistant mutations in C3H10T1/2 mouse cells by ultraviolet light. *Proc. Natn Acad. Sci. U.S.A.* **75**, 3363–3366.

CHANG, T. & PENEFSKY, H. S. (1974). Energy-dependent enhancement of aurovertin fluorescence. *J. biol. Chem.* **249**, 1090–1098.

CIOFFI, M. (1979). The morphology and fine structure of the larval midgut of a moth (*Manduca sexta*) in relation to active ion transport. *Tissue and Cell* **11**, 467–479.

CIOFFI, M. & HARVEY, W. R. (1981). Comparison of potassium transport in three structurally distinct regions of the insect midgut. *J. exp. Biol.* **91**, 103–106.

CIOFFI, M. & WOLFERSBERGER, M. G. (1981). Isolation of separate apical, lateral and basal membrane from cells of tobacco hornworm larval midgut. *Am. Zool.* **21**, 997.

CIOFFI, M. & WOLFERSBERGER, M. G. (1983). Isolation of separate apical, lateral and basal plasma membrane from cells of an insect epithelium. A procedure based on tissue organization and ultrastructure. *Tissue and Cell* **15** (in press).

DOW, J. A. T. (1981). Localization and characterization of water uptake in the midgut of the locust, *Schistocerca gregaria* Forsk. *J. exp. Biol.* **93**, 269–281.

[redacted]MER, J., MADDRELL, S. H. P. & SPRING, J. H. (1981). Absorption of fluid by the midgut of *Rhodnius*. *J. exp. Biol.* **94**, 301–316.

FAST, P. G. (1981). In *Microbial Control of Pests and Plant Diseases 1970–80*, (ed. H. D. Burges), pp. 223–248. New York: Academic Press.

FAST, P. G. & DONAGHUE, T. P. (1971). The delta-endotoxin of *Bacillus thuringiensis* II. On the mode of action. *J. Invertebr. Pathol.* **18**, 135–138.

FAST, P. G. & MARTIN, W. G. (1980). *Bacillus thuringiensis* parasporal crystal toxin: dissociation into toxic low molecular weight peptides. *Biochem. biophys. Res. Commun.* **95**, 1314–1320.

FAST, P. G., MURPHY, D. W. & SOHI, S. S. (1978). *Bacillus thuringiensis* δ-endotoxin: Evidence that toxin acts at the surface of susceptible cells. *Experientia* **34**, 762–763.

FILLINGAME, R. H. (1980). The proton-translocating pumps of oxidative phosphorylation. *Ann. Rev. Biochem.* **49**, 1079–1113.

GIORDANO, B., SACCHI, V. F. & HANOZET, G. M. (1982). Intentinal amino acid absorption in lepidopteran larvae. *Biochim. biophys. Acta* **692**, 81–88.

GUPTA, B. L., BERRIDGE, M. J., HALL, T. A. & MORETON, R. B. (1978). Electron microprobe and ion-selective microelectrode studies of fluid secretion in the salivary glands of *Calliphora*. *J. exp. Biol.* **72**, 261–284.

HAKIM, R. S. & KAFATOS, F. C. (1974). The structure and salivary function of the labial gland in adult *Manduca sexta*. *Tissue and Cell* **6**, 729–750.

HANRAHAN, J. W. & PHILLIPS, J. E. (1983). Cellular mechanism and control of KCl absorption in insect midgut. *J. exp. Biol.* **106**, 71–89.

HARVEY, W. R. (1980). Water and ions in the gut. In *Insect Biology in the Future 'VBW 80'*, (eds M. Locke & D. S. Smith), pp. 105–124. London, New York: Academic Press.

HARVEY, W. R. (1982). Membrane physiology of insects. In *Membrane Physiology of Invertebrates*, (eds R. Podesta, L. L. Dean, S. S. McDiarmid, S. F. Timmers & B. W. Young), pp. 496–566. New York: Marcell Dekker.

HARVEY, W. R., CIOFFI, M. & WOLFERSBERGER, M. G. (1981). Portasomes as coupling factors in active ion transport and oxidative phosphorylation. *Am. Zool.* **21**, 775–791.

HARVEY, W. R., CIOFFI, M. & WOLFERSBERGER, M. G. (1983). Chemiosmotic potassium ion pump of insect epithelia. *Am. J. Physiol.* **244**, R163–R175.

HARVEY, W. R. & NEDERGAARD, S. (1964). Sodium independent active transport of potassium in the isolated midgut of the Cecropia silkworm. *Proc. Natn Acad. Sci. U.S.A.* **51**, 757–765.

HARVEY, W. R. & WOLFERSBERGER, M. G. (1979). Mechanism of inhibition of active potassium transport in isolated midgut of *Manduca sexta* by *Bacillus thuringiensis* endotoxin. *J. exp. Biol.* **83**, 293–304.

HARVEY, W. R. & ZERAHN, K. (1969). Kinetics and route of active K-transport in the isolated midgut of *Hyalophora cecropia*. *J. exp. Biol.* **50**, 297–306.

HARVEY, W. R. & ZERAHN, K. (1972). Active transport of potassium and other alkali metals by the isolated midgut of the silkworm. In *Current Topics in Membranes and Transport*, Vol. III, (eds F. Bronner & A. Kleinzeller), pp. 367–410. New York: Academic Press.

HEIMPEL, A. M. & ANGUS, T. A. (1959). The site of action of crystalliferous bacteria in lepidopteran larvae. *J. Insect Pathol.* **1**, 152–170.

HUBER, H. E. & LÜTHY, P. (1982). *Bacillus thuringiensis* delta-endotoxin: composition and activation. In *Pathogenesis of Invertebrate Microbial Diseases*, (ed. B. Davidson), pp. 209–234. Totowa, NJ: Allanheld & Osmun.

JUNGREIS, A. M. (1977). Comparative aspects of invertebrate epithelial transport. In *Water Relations in Membrane Transport in Plants and Animals*, (eds A. M. Jungreis, T. K. Hodges, A. Kleinzeller & S. G. Schultz), pp. 89–96. New York: Academic Press.

JUNGREIS, A. M. & HARVEY, W. R. (1975). Role of active potassium transport by integumentary epithelium in secretion of larval-pupal moulting fluid during silk moth development. *J. exp. Biol.* **62**, 357–366.

JUNGREIS, A. & VAUGHAN, G. L. (1977). Insensitivity of Lepidopteran tissues to ouabain: Absence of ouabain binding and Na^+-K^+ ATPases in larval and adult midgut. *J. Insect Physiol.* **23**, 503–509.

KAFATOS, F. C. (1968). The labial gland: A salt-secreting organ of saturniid moths. *J. exp. Biol.* **48**, 435–453.

KAGAWA, Y., RACKER, E. & HAUSER, R. E. (1966). Partial resolution of the enzymes catalizing oxidative phosphorylation, X: correlation of morphology and function in submitochondrial particles. *J. biol. Chem.* **241**, 2475–2484.

KEDEM, O. & KATCHALSKY, A. (1961). A physical interpretation of the phenomenological coefficients of membrane permeability. *J. gen. Physiol.* **45**, 143–179.

KEYNES, R. D. (1969). From frog skin to sheep rumen: A survey of transport of salts and water across multicellular structures. *Quart. Rev. Biophys.* **2**, 177–281.

KÜPPERS, J. & THURM, U. (1980). Water transport by electroosmosis. In *Insect Biology in the Future 'VBW 80'*, (eds. M. Locke & D. S. Smith), pp. 125–144. London, New York: Academic Press.

KÜPPERS, J. & THURM, W. (1982). On the functional significance of ion circulation induced by electrogenic transport. In *Exogenous and Endogenous Influences on Metabolic and Neural Control*, (eds A. D. F. Addink & N. Spronk), pp. 313–327. Oxford, New York: Pergamon.

LAEMMLI, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *[redacted]ature, Lond.* **227**, 680–685.

LINDERHOLM, H. (1952). Active transport of ions through frog skin with special reference to the action of cer[redacted] diuretics. *Acta physiol. scand.* **27**, (Suppl. 97) 1–144.

MADDRELL, S. H. P. (1969). Secretion by the Malpighian tubules of *Rhodnius*. The movements of ions and water. *J. exp. Biol.* **51**, 71–97.

MADDRELL, S. H. P. (1971). The mechanisms of insect excretory systems. In *Advances in Insect Physiology*, Vol. 8, (eds J. W. L. Beament, J. E. Treherne & V. B. Wigglesworth), pp. 199–331. New York: Academic Press.

MADDRELL, S. H. P. (1972). The functioning of insect Malpighian tubules. In *Role of Membranes in Secretory Processes*, (eds L. Bolis, R. D. Keynes & W. Willbrandt), pp. 338–356. Amsterdam: North Holland.

MADDRELL, S. H. P. (1977*a*). Insect Malpighian tubules. In *Transport of Ions and Water in Animals*, (eds B. L. Gupta, R. B. Moreton, J. L. Oschman & B. J. Wall), pp. 541–569. New York: Academic Press.

MADDRELL, S. H. P. (1977*b*). Hormonal action in the control of fluid and salt transporting epithelia. In *Water Relations in Membrane Transport in Plants and Animals*, (eds A. M. Jungreis, T. K. Hodges, A. Kleinzeller & S. G. Schultz), pp. 303–313. New York: Academic Press.

MADDRELL, S. H. P. (1978). Transport across insect excretory epithelia. In *Membrane Transport in Biology III, Transport across Multimembrane Systems*, (eds G. Giebisch, D. C. Tosteson & H. H. Ussing), pp. 239–271. New York: Springer-Verlag.

MALONEY, P. C. (1982). Energy coupling to ATP synthesis by the proton-translocating ATPase. *J. Membr. Biol.* **67**, 1–12.

MATSUI, H. & HOMAREDA, H. (1979). Ouabain-sensitive and stoichiometrical binding of sodium and potassium ions to Na^+-, K^+-ATPase. In *Cation Flux across Biomembranes*, (eds Y. Mukohata & L. Packer), pp. 29–39. New York and London: Academic Press.

MITCHELL, P. (1961). Coupling of phosphorylation of electron and hydrogen transfer by a chemi-osmotic type of mechanism. *Nature, Lond.* **191**, 144–148.

MITCHELL, P. (1979). *The ninth Sir Hans Krebs Lecture*: Compartmentation and Communication in living systems. Ligand conduction: a general catalytic principle in chemical, osmotic and chemiosmotic reaction systems. *Eur. J. Biochem.* **95**, 1–20.

MOFFETT, D. F. (1979). Potassium activity of single insect midgut cells. *Am. Zool.* **19**, 996.

MOFFETT, D. F., HUDSON, R. L., MOFFETT, S. B. & RIDGWAY, R. L. (1982). Intracellular K^+ activities and cell membrane potentials in a K^+-transporting epithelium, the midgut of tobacco hornworm (*Manduca sexta*). *J. Memb. Biol.* **70**, 59–68.

MURPHY, D. W., SOHI, S. S. & FAST, P. G. (1976). *Bacillus thuringiensis* enzyme-digested delta endotoxin: effect on cultured insect cells. *Science, N.Y.* **194**, 954–956.

NOIROT, C. & NOIROT-TIMOTHÉE, C. (1971). Ultrastructure du proctodeum chez le thysanoure *Lepismodes inquillinus* Newman (*Thermobia domestica* Packard) II. Le sac anal. *J. ultrastruct. Res.* **37**, 335–350.

OSCHMAN, J. L. & BERRIDGE, M. J. (1970). Structural and functional aspects of salivary fluid secretion in *Calliphora*. *Tissue and Cell* **2**, 281–310.

PEACOCK, A. J. (1981). Distribution of ($Na^+ + K^+$) ATPase activity in the mid- and hind-guts of adult *Glossina morsitans* and *Sarcophaga nodosa* and the hindgut of *Bombyx mori* larvae. *Comp. Biochem. Physiol.* **69**A, 133–136.

PEACOCK, A. J. (1982). Effects of sodium-transport inhibitors on diuresis and midgut ($Na^+ + K^+$) ATPase in the tsetse fly *Glossina morsitans*. *J. Insect Physiol.* **28**, 553–558.

PEACOCK, A. J., BOWLER, K. & ANSTEE, J. H. (1972). Demonstration of a Na^+-K^+-Mg^{2+} dependent ATPase in a preparation from hindgut and Malpighian tubules of two species of insect. *Experientia* **28**, 901–902.

PEACOCK, A. J., BOWLER, K. & ANSTEE, J. H. (1976). Properties of Na^+, K^+-dependent ATPase from Malpighian tubules and hindgut of *Homorocoryphus nitulidus vicinus*. *Insect Biochem.* **6**, 281–288.

PENEFSKY, H. S. (1979). Mitochondrial ATPase. In *Advances in Enzymology*, (ed. A. Meister), pp. 223–280. New York: John Wiley & Sons.

PERCY, J. & FAST, P. G. (1983). *Bacillus thuringiensis* crystal toxin: ultrastructural studies of its effect on silkworm midgut cells. *J. Invertebr. Pathol.* **41**, 86–98.

POST, R. L. (1979). A perspective on sodium and potassium ion transport adenosine triphosphatase. In *Cation Flux Across Biomembranes*, (eds Y. Mukohata & L. Packer), pp. 3–19. New York: Academic Press.

PRINCE, W. T. & BERRIDGE, M. J. (1972). The effects of 5-hydroxytryptamine and cyclic AMP on the potential profile across isolated salivary glands. *J. exp. Biol.* **56**, 323–333.

RABON, E., CUPPOLETTI, J., MALINOWSKA, D., SMOLKA, A., HELANDER, H. F., MENDLEIN, J. & SACHS, G. Proton secretion by the gastric parietal cell. *J. exp. Biol.* **106**, 119–133.

RAMSAY, J. A. (1953). Active transport of potassium by the Malpighian tubules of insects. *J. exp. Biol.* **30**, 358–369.

ROBBINS, A. R. & BAKER, R. M. (1977). (Na,K) ATPase activity in membrane preparations of ouabain resistant HeLa cells. *Biochemistry, N.Y.* **16**, 5163–5168.

SACHS, G., CHANG, H. H., RABON, E., SCHACKMAN, R., LEWIN, M. & SACCOMANI, G. (1976). A nonelectrogenic H^+ pump in plasma membranes of hog stomach. *J. biol. Chem.* **251**, 7690–7698.

SCHUURMANS STEKHOVEN, F. & BONTING, S. L. (1981). Transport adenosine triphosphatases: properties and functions. *Physiol. Rev.* **61**, 1–76.

[redacted], J. C. (1965). Enzymatic bases for active transport of Na^+ and K^+ across the cell membrane. *Physiol. Rev.* **45**, 596–617.

THURM, U. (1974). Basics of the generation of receptor potentials in epidermal mechanoreceptors of insects. *Rheinisch-Westfalische Akademie der Wissenschaften* **53**, 355–385.

THURM, V. & KÜPPERS, J. (1980). Epithelial physiology of insect sensilla. In *Insect Biology in the Future, 'VBW 80'*, (eds M. Locke & D. S. Smith), pp. 735–764. London, New York: Academic Press.

TOWLE, D. (1983). Membrane-bound ATPases in arthropod ion-transporting epithelia. *Am. Zool.* **23** (in press).

USSING, H. H. (1949). The distinction by means of tracers between active transport and diffusion. The transfer of iodide across the isolated frog skin. *Acta physiol. scand.* **19**, 43–56.

VAUGHAN, F. A. (1979). Effect of gross cardiac glycoside content of seeds of common milkweed, *Asclepias syriaca*, on cardiac glycoside uptake by the milkweed bug *Oncopeltus fasciatus*. *J. chem. Ecol.* **5**, 89–100.

WIECZOREK, H. (1982). A biochemical approach to the electrogenic potassium pump of insect sensilla: potassium sensitive ATPases in the labellum of the fly. *J. comp. Physiol.* **148**, 303–311.

WOLFERSBERGER, M. G., HARVEY, W. R. & CIOFFI, M. (1982). Transepithelial potassium transport in insect midgut by an electrogenic alkali metal ion pump. In *Current Topics in Membranes and Transport*, Vol. 16, (eds F. Bronner & A. Kleinzeller; guest ed. C. L. Slayman), pp. 109–133. New York: Academic Press.

WOLFF, J. (1964). Transport of iodide and other anions in the thyroid gland. *Physiol. Rev.* **44**, 45–90.

WOOD, J. L., FARRAND, P. S. & HARVEY, W. R. (1969). Active transport of potassium by the *Cecropia* midgut. VI. Microelectrode potential profile. *J. exp. Biol.* **50**, 169–178.

ZERAHN, K. (1956). Oxygen consumption and active sodium transport in the isolated and short-circuited frog skin. *Acta physiol. scand.* **36**, 300–318.

ZERAHN, K. (1977). Potassium transport in insect midgut. In *Transport of Ions and Water in Animals*, (eds B. L. Gupta, R. B. Moreton, J. L. Oschman & B. J. Wall), pp. 381–401. New York: Academic Press.

ZERAHN, K. (1978). Transport across insect gut epithelium. In *Membrane Transport in Biology, III: Transport Across Multimembrane Systems*, (eds G. Giebisch, D. C. Tosteson & H. H. Ussing), pp. 273–306. Berlin, Heidelberg, New York: Springer-Verlag.