PROPERTIES OF ION AND FLUID TRANSPORT AND CONTROL IN HINDGUT OF THE DESERT LOCUST (*Schistocerca gregaria*)

by

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ABSTRACT

Previous studies of selective reabsorption in insect excretory system have concentrated almost exclusively on the rectum, while the role of the ileum has been assumed to be minor. The properties and control of solute and fluid transport in two segments of the hindgut, the ileum and rectum, from the desert locust (*Schistocerca gregaria*) have been studied and compared *in vitro* using everted sac and flat sheet preparations. Everted sacs of locust ileum transported fluid from the lumen side to hemocoel side over a 5 h period at near constant rates of 3.0 to 3.5 $\mu L \cdot h^{-1} \cdot ileum^{-1}$ and tissue volume did not change. Inhibition by azide indicated metabolic dependence of fluid transport. Fluid absorption occurred against osmotic concentration differences of up to 600 mosmol. Fluid transport was stimulated by cAMP, both nervous and glandular lobes of corpus cardiacum (CC), and fifth ventral ganglia (VG) in a dose-dependent manner. All stimulants caused ilea to absorb against larger osmotic concentration differences than unstimulated sacs. The ileal absorbate remained hyperosmotic to the luminal saline under all conditions and stimulants increased absorbate osmolarity. Unstimulated fluid transport was supported at 50% of control levels by any one of Na^+, K^+, or Cl^-. Stimulation of fluid transport by CC or VG was dependent on Cl^- and maximal stimulation occurred when the Na^+:K^+ ratio was 1:1. Cyclic AMP, CC and VG all stimulated Na^+,K^+ and Cl^- absorption across everted ileal sacs. This is the first direct demonstration that Na^+ reabsorption is controlled in insect excretory systems. Stimulation resulted in a decrease in absorbate HCO_3^- levels and pH concurrently with an increase in absorbate Cl^- levels. Stimulation of fluid transport was associated with a 3-fold increase in transepithelial potential (hemocoel negative) suggesting stimulation of electrogenic anion (Cl^-) movement to the hemocoel. Net Na^+ absorption occurs largely by electroneutral active transport. NH_4^+/Na^+ exchange may account for one-third of stimulated net Na^+ flux. Extracts from both CC and VG stimulated fluid, K^+, and Cl^-

transport across everted rectal sacs, but only a small stimulation of Na^+ flux was observed which was an order of magnitude less than that observed for stimulated ilea (0.4 versus 5.1 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$). Unlike the rectum, the ileum did not transport proline transepithelially and ileal fluid transport was not stimulated by increasing concentrations of proline in the bathing saline. Rectal fluid transport was stimulated 50% by increasing external proline concentration from 1 to 80 mM. Stimulation of rectal fluid transport by proline also occurred in the absence of Na^+, K^+, and Cl^- and occurred against larger osmotic concentration differences. These results are consistent with previous reports of a high capacity transport system for proline in locust rectum. The presence of anion-stimulated ATPase and Na^+,K^+-ATPase in locust hindgut was also investigated. Anion-stimulated ATPase activities were observed in microsomal fractions of both rectum and ileum. Microsomal fractions from both tissues had enriched specific activities of several plasma membrane marker enzymes and decreased activities of two mitochondrial markers as compared to homogenate enzyme activities. Na^+,K^+-ATPase activity was 20-fold higher in the rectum than in the ileum, associated with the greater development of the basolateral membrane in the rectum. Overall the results suggest that ion and fluid reabsorption in the locust ileum is much more important in the excretory process than previously supposed. Moreover, this reabsorption was shown to be under neuroendocrine control.

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I_{sc}	- short-circuit current
V_t	- transepithelial potential
R_t	- transepithelial resistance
SITS	- 4-acetamide-4'-isothiocyano-stilbene-2,2'-disulfonic acid
DIDS	- 4,4'-Diisothiocyanato-stilbene-2,2'-disulfonic acid
L	- lumen or lumen-facing side of the epithelium
H	- hemocoel or hemocoel-facing side of the epithelium
ATP	- adenosine 5'-triphosphate
ATPase	- adenosine 5'-triphosphatase
AMP	- adenosine 5'-monophosphate
cAMP	- adenosine 3':5'-cyclic monophosphoric acid
NADH	- β-nicotinamide adenine dinucleotide, reduced form
NADPH	- β-nicotinamide adenine dinucleotide phosphate, reduced form
$\mu equiv \cdot h^{-1} \cdot cm^{-2}$	- microequivalents per hour per square centimetre
CTSH	- chloride transport stimulating hormone
EDTA	- ethylenediamine tetraacetic acid
EGTA	- ethyleneglycol-bis-(B-aminoethyl ether) N,N,N',N'-tetraacetic acid
MOPS	- 3-(N-morpholino)propanesulfonic acid
cpm	- counts per minute
J_v	- transepithelial fluid transport
CC	- corpus cardiacum
NCC	- nervous (storage) lobe of corpus cardiacum
GCC	- glandular lobe of corpus cardiacum
SOG	- suboesophageal ganglion
VG	- fifth ventral ganglion

ADH	- antidiuretic hormone
DH	- diuretic hormone
VP	- vasopressin
IAA	- iodoacetic acid
P_i	- inorganic phosphate
osmol	- osmolar concentration
Δosmol	- transepithelial osmolar concentration difference
G_t	- transepithelial conductance
P_{osm}	- osmotic permeability
mV	- millivolts
$\mu L \cdot h^{-1} \cdot ileum^{-1}$	- microlitres per hour per ileum
$\mu L \cdot h^{-1} \cdot rectum^{-1}$	- microlitres per hour per rectum
$\mu L \cdot h^{-1} \cdot cm^{-2}$	- microlitres per hour per square centimetre
L	- litre (s)
mL	- millilitre (s)
μL	- microlitre (s)
μm	- micrometre (s)
nm	- nanometre (s)
Å	- Ångstrom (s)
h	- hour
min	- minute
kg	- kilogram (s)
mg	- milligram (s)
μg	- microgram (s)
M	- moles per litre (molar)
mM	- millimolar
nmole	- nanomole

mCi	- millicurie
SCR	- succinate cytochrome c reductase
R_a, R_b	- resistance of apical and basal membranes, respectively
V_a, V_b	- potential difference across apical and basal membranes, respectively
s. e.	- standard error
s. d.	- standard deviation

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CHAPTER 1: General Introduction

Control of insect hemolymph composition is accomplished by the excretory system which consists of the Malpighian tubules and the hindgut (ileum, colon and rectum; Fig. 1). A primary isosmotic urine rich in KCl and low in Na^+ is produced in the Malpighian tubules of most terrestrial insects. The exceptions are blood feeders, which ingest large meals of NaCl-rich blood and must excrete large volumes of water and NaCl while retaining metabolites from the meal (reviewed by Phillips 1981; Maddrell, 1978, 1980). Tubules remove excess ions and water and actively secrete harmful substances such as plant alkaloids. Most of the primary urine from the Malpighian tubules moves posteriorly into the hindgut while a small fraction moves anteriorly into the midgut where some fluid reabsorption occurs (Dow 1981). The final composition of the excreta is determined by selective reabsorption in the hindgut, particulary the rectum, from this primary urine. Hormonal control of tubule secretion and rectal reabsorption results in production of a very hyposmotic or hyperosmotic urine, or dry excreta, depending on the water and ion status of the insect (reviewed by Phillips *et al.* 1986; Phillips 1980, 1981, 1982, 1983a,b; Gee 1977; Wall & Oschman 1975, Bradley 1985). Desert locusts, *Schistocerca gregaria*, fed a hyperosmotic salt solution maintain hemolymph volume and ion levels at relatively constant levels (Phillips 1964a,b,c). However, dehydrated locusts undergo a substantial reduction in hemolymph volume (Hanrahan 1978; Chamberlin & Phillips 1982b). Feeding causes a rapid replenishment of hemolymph volume and only small changes ion concentrations (Hanrahan 1978; Phillips *et al.* 1980). Salt-water insects must conserve water and remove excess salt. This problem is solved by specialized hindgut segments which act as salt glands (Phillips *et al.* 1978, Strange *et al.* 1982, Strange & Phillips 1984, 1985). Some insects also have the ability to absorb water actively from the atmosphere in the hindgut (reviewed by Machin 1979, Machin

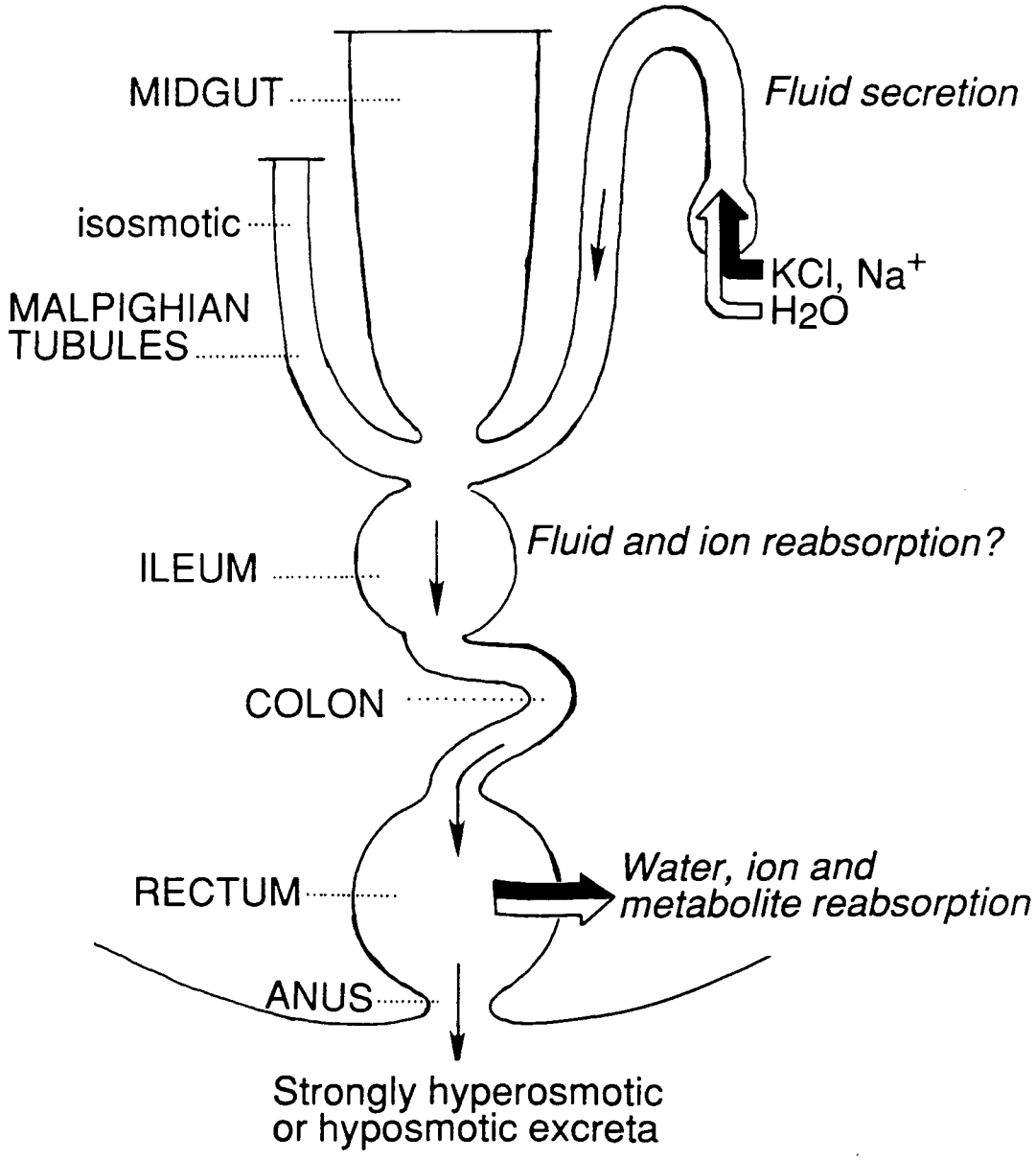


Figure 1. Diagram of a typical insect excretory system. The flow of urine is indicated by *thin arrows* and transfer across epithelia indicated by *thick arrows* (solid arrows, active transport; open arrows, passive transfer). The osmolarites of primary urine and final excreta are indicated. (Modified from Phillips 1981).

et al. 1982; Edney 1977; Rudolph & Knülle, 1978). This thesis investigates the selective reabsorption of the primary urine by *Schistocerca gregaria* (desert locust) hindgut and control of this process by neural factors.

Structure of the locust excretory system

The gross anatomy and histology of insect hindgut, including that of locusts, has been described by several investigators (reviewed by Wall & Oschman 1975). The locust excretory system consists of about 250 Malpighian tubules which insert between the midgut and hindgut (Fig. 1; Dow 1986; Garrett *et al.* 1988). The hindgut is divided into the anterior ileum and the posterior rectum by the "S" shaped colon, which acts to break the peritrophic membrane and gut contents into discrete fecal pellets (Goodhue 1963). The ileum is about 6 mm long by 2.5 mm outside diameter with a macroscopic surface area (0.4 cm^2) that is about two-thirds of the rectum (0.64 cm^2; Irvine *et al.* 1988). The hindgut is lined with a chitinous cuticle (2-10 μm thick), which in the ileum and rectum is highly permeable to small hydrophilic molecules, unlike the colon cuticle which has a much lower permeability to small molecules; therefore, the colon is not thought to be involved in absorption (Phillips & Dockrill 1968; Maddrell & Gardiner 1980). The cuticle of both the ileum and rectum has a reduced permeability to anions relative to cations at low salt concentrations, suggesting that the cuticle from both segments contain pores with fixed negative charges (pK of about 4; Lewis 1971; Maddrell & Gardiner 1980). The result is enhanced diffusion of Ca^{2+} and Mg^{2+}, which because of their large hydrated size would otherwise be excluded from the pores (6.5 Å radius) if they were uncharged (reviewed by Phillips *et al.* 1986). These pores allow for the reabsorption of major ions and basic metabolites from the primary urine, while excluding the larger and often toxic substances (eg. plant alkaloids) and causing them to accummulate in to the final excreta (Phillips & Dockrill 1968).

In many terrestrial insects, including the desert locust, ileal epithelium is generally

much thinner than that of the rectum. The locust rectum contains six radially arranged thickened rectal pads where the cuticle is often detacted (Martoja & Balan-Dufrancais 1984, Chapman 1985, Irvine *et al.* 1988). Each rectal pad in desert locusts is composed of columnar epithelial cells (17 by 100 μm) but within the pads there are occasional small secondary "Type B" cells with few mitochondria and lacking features of transporting epithelia and making contact with only the lumen side. The apical membranes of locust ileal and rectal pad epithelia are very similar, having closely packed infoldings (5-10 mm long) and abundant mitochondria (Fig. 2). The columnar epithelial cells of the rectal pads have highly folded lateral membranes with closely associated mitochondria. These membranes form complex intercellular channels of three types through which the absorbate must pass to reach the hemolymph. Ion recycling is believed to take place at these channels (Wall *et al.* 1970). In the first region of these intercellular channels it is hypothesized that an absorbate of high osmotic concentration is created by active K^+ or Na^+ transport (Fig. 3). The absorbate then flows into a second compartment of the channels where water moves into the channel from the cells by local osmosis. In the final region of the channel, solutes are actively reabsorbed across membranes having a low water permeablity thereby creating a hyposmotic absorbate which exits the the pads and flows into the hemocoel. A layer of basal (secondary) cells form a second thin epithelial layer on the hemocoel side and these cells may have a role in ion recycling. In contrast, the ileum consists of a single layer of epithelial cells of one cell type (40 by 20 μm) covered by a firmly attached apical cuticle with no expanded subcuticular space, as seen in the rectum (Fig. 2). The basal surface is covered by a thin basal lamina and unlike the rectum there is no elaborate intercellular lateral membrane system or secondary epithelial cell layer. However the ileal cells have elaborated narrow infoldings of the basal membrane, associated with numerous mitochondria, which may be analogous to lateral scalariform complexes of rectal pad cells (Irvine *et al.* 1988). Outside both hindgut epithelia are layers of longitudinal and

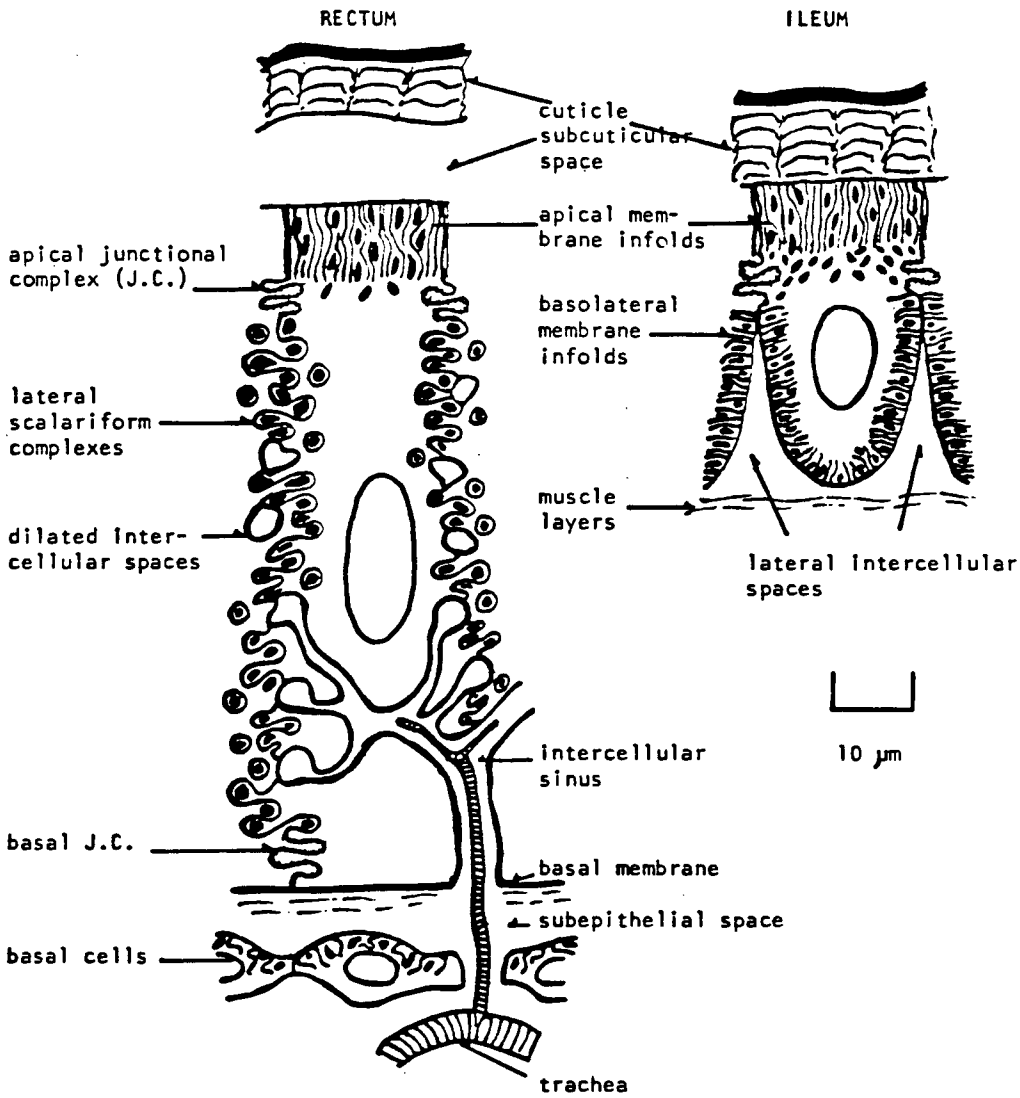


Figure 2. Comparison of ultrastructural organization and gross dimensions of locust rectal pad and ileal epithelium. (From Irvine *et al.* 1988).

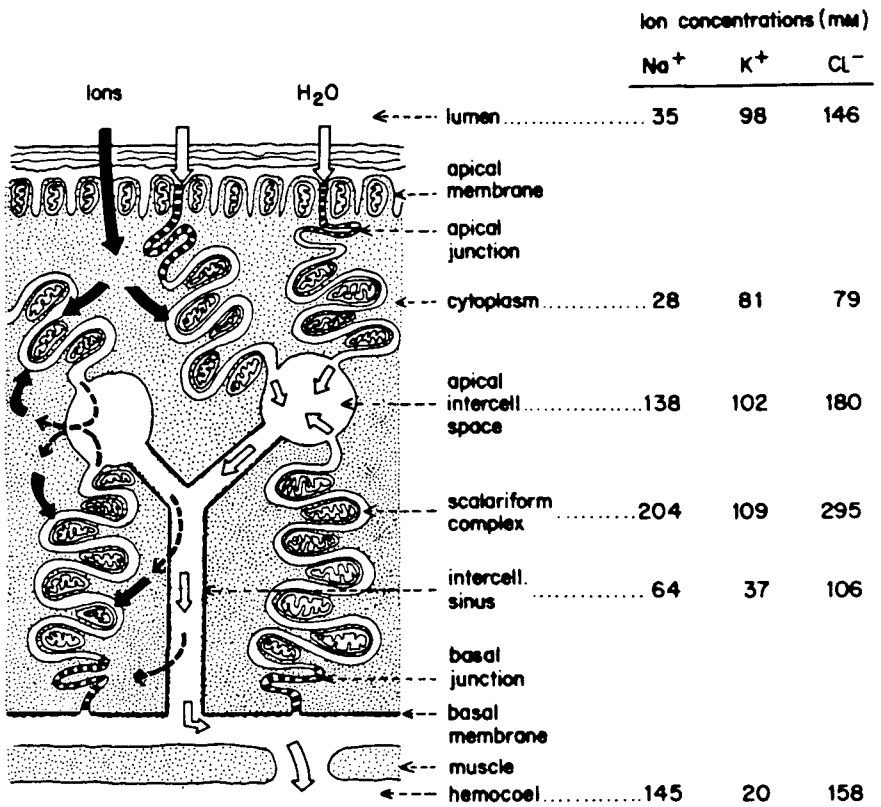


Figure 3. Diagram of principal cells of rectal pads in cockroaches and locusts. Local ion concentrations determined by electron-probe X-ray micro-analysis are shown for dehydrated blowfly, *Calliphora*, (which have a comparable arrangement of membranes) following injection of 50 mM NaCl + 100 mM KCl + 20% dextran into the rectal lumen (From Gupta *et al.* 1980 and Phillips *et al.* 1986).

circular muscles. Trachea and tracheoles penetrate both the muscle and epithelia layers. Wall *et al.* (1970) proposed that fluid leaves the rectal pads only at the points where larger trachea penetrate the muscle layer but this is still uncertain for locusts.

Fluid and ion transport

Active transport of ions across locust rectum was first demonstrated *in situ* by Phillips (1961, 1964a,b,c). He determined net fluid and ion transport by injecting electrolyte solutions containing an impermeant volume marker (^{131}I-albumin) into ligated recta and monitoring changes in ion concentrations and radioactivity with time. He demonstrated active Cl^- and fluid transport and partial active transport of K^+ and Na^+ from the lumen. Although these experiments describe the stituation in intact insects, there were limitations with this approach. First, absorption was studied under non-steady-state conditions since measurements relied on changes in luminal fluid composition. Second, neural or hormonal factors which controlled excretion in the locust were unknown and could not be controlled. Third, fluid in the luminal and hemolymph could not be stirred and only fluid in the lumen could be altered experimentally. For these reasons *in vitro* preparations for studying fluid and ion transport and their control have been developed and employed over the past 25 years.

An *in vitro* non-everted sac preparation of locust recta was initially used to determine ion and water transport (Irvine 1966; Irvine & Phillips 1971). However the luminal surface of the sacs were not adequately oxygenated so active transport of Cl^- and K^+ was not observed. Goh & Phillips (1978) used an everted rectal sac preparation which exhibited long-term steady-state fluid transport and net absorption of Na^+, K^+ and Cl^- for 6 h. The fluid transport was dependent on metabolism as demonstrated by inhibition with cyanide, anoxia and ouabian. In the absence of an initial osmotic concentration difference across the rectum, fluid transport to the hemocoel side (J_V) was 6 $\mu L \cdot h^{-1} \cdot rectum^{-1}$ *in vitro* (Goh & Phillips 1978) as compared with 17 $\mu L \cdot h^{-1} \cdot rectum^{-1}$ *in*

vivo, i.e. when hormonal influences were present (Phillips 1964a). The rectum was also shown to transport fluid against an osmotic gradient imposed across the rectal wall (Δmosmol). The equilibrium Δmosmol (J_V=0) for *in vitro* recta in the absence of natural stimulants was 380-400 mosmol (Goh & Phillips 1978; Balshin 1973), but natural high levels of K^+ on the lumen side increased this value to 586 mosmol (Andrusiak *et al.* 1980). The osmotic permeability (P_{osm}) of the rectal wall was determined from the relationship between steady-state J_V and Δmosmol to be 32 $\mu L \cdot h^{-1} \cdot \Delta osmol^{-1} \cdot cm^{-2}$ for absorption. However the locust rectal epithelium exhibited rectification so that, P_{osm} was only 11 $\mu L \cdot h^{-1} \cdot \Delta osmol^{-1} \cdot cm^{-2}$ when fluid flow was from the hemocoel to lumen. Using this same everted sac preparation, Phillips *et al.* (1982a) demonstrated that any one of Na^+, K^+, or Cl^- can support about 50% of the fluid transport (at Δosmol=0) observed when all three ions are present. When exposed to an isosmotic sucrose solution lacking these monovalent ions, fluid transport stops after 1.5 h, but addition of saline back to the luminal side restores J_V to normal steady-state rates (Phillips *et al.* 1982a). These results demonstrated the dependency of fluid transport on ion transport. Proux *et al.* (1984) observed that cAMP and aqueous extracts of corpus cardiacum (CC) stimulated fluid transport across everted rectal sacs *in vitro*. Because rectal fluid absorption is driven by salt transport, this antidiuretic action of CC extracts may be due to a stimulation of salt transport. A chloride transport stimulating hormone (CTSH), which stimulates active Cl^- transport and passive K^+ absorption across locust recta, has been demonstrated and is discussed later. No stimulation of fluid transport was observed by Proux *et al.* (1984) when recta were exposed to Cl^--free saline and readdition of luminal Cl^- restored cAMP-stimulation of fluid transport, as expected if the stimulation of J_V was due to CTSH. CC extracts also caused fluid absorption against larger initial osmotic gradients as expected if the antidiuretic factor acted on active transport of fluid (i.e. on ion transport) rather than osmotic permeability of the rectal wall.

In the vertebrate kidney, fluid reabsorption is controlled by ADH and aldosterone.

By analogy, one might predict that fluid reabsorption in locust recta would be controlled by at least two factors, one that regulates salt reabsorption and another which controls the P_{osm} of the rectal wall and hence the volume and osmolarity of the absorbed fluid (i.e. absorbate). The hormone, CTSH, appears to be responsible for regulation of salt reabsorption but factors which specifically change rectal P_{osm} have not been demonstrated to date. An unusual property of fluid transport in insect rectum compared to other epithelia is the ablility to concentrate the lumen contents by absorbing a fluid hyposmotic to the luminal fluid (Phillips *et al.* 1986).

Proposed mechanism of fluid transport across insect recta

Berridge and Gupta (1967) and Wall and Oschman (1970) proposed a model to explain absorption of hyposmotic urine in insect recta. They suggested that local high osmotic concentrations are created in the lateral scalariform spaces between the rectal epithelial cells by active K^+ (*Calliphora*) or Na^+ (*Periplaneta*) transport. Based on ultrastructure of the rectum and contemporary models for vertebrate epithelia they hypothesized that these osmotic gradients drive secondary fluid transport by local osmosis (Fig. 3). In support of this model the large intercellular spaces have been observed to expand during increased fluid transport (Berridge & Gupta 1967; Wall *et al.* 1970). More convincingly, the lateral intercellular spaces of dehydrated *Periplaneta* were shown to be hyperosmotic to the lumen contents by 30 to 300 mosmol (Wall & Oschman 1970; Wall *et al.* 1970). Solution collected by micropuncture of these lateral intercellular channels contained Na^+ and K^+ (1:2 ratio) and accompanying anions, but these inorganic ions only accounted for 50% of the total osmolarity measured (Wall 1971). This suggested that unknown organic solutes must also drive fluid transport. The absorbate in the subepithelial space was 350 mosmol hyposmotic to the luminal fluid, consistent with solute reabsorption within more distal regions of the intercellular lateral spaces (Wall 1971). It is this recycling of ions that distinguishes insect rectum

from other epithelia.

In support of this ion recycling hypothesis, electrolytes in the lateral scalariform complexes (membrane stacks) of *Calliphora* rectal papillae were measured by electron probe X-ray microanalysis of frozen hydrated sections and found to exceed values in other tissue compartments by 80 and 700 μequiv· L^{-1} in hydrated and dehydrated flies, respectively. The membrane stacks and lateral spaces had higher concentrations of Na^+, K^+ and Cl^- than the cytoplasm (Gupta *et al.* 1977, 1980; Gupta & Hall 1981). The final absorbate emerging from papillae was consistantly isosmotic to the hemolymph and generally had a higher Na^+:K^+ ratio than that of the fluid in lateral spaces at the scalariform complexes (Gupta *et al.* 1980). This could result from K^+ absorption by Na^+,K^+-ATPase in exchange for cytoplasmic Na^+ as fluid moved in the lateral channels toward the hemocoel side (Phillips *et al.* 1986). However, localization of Na^+,K^+-ATPase at this site has not been demonstrated in terrestrial insects, as it has been in a freshwater insect species which does not concentrate its urine (Komnick & Achenbach 1979).

Ion transport and control across recta

Until recently, reabsorption of Na^+, K^+, Cl^- and metabolites from the primary urine was thought to occur mostly in the rectum because this was where large changes in ionic and osmotic concentration occurred. The high concentration of K^+ in the primary urine relative to the hemolymph and the transepithelial potential (hemocoel side negative) both favor the passive reabsorption of K^+ across the rectal wall. The levels of Na^+ in the fluid entering the lumen are 15-30% of K^+ concentrations and therefore net absorption of Na^+ is normally much less important. Sodium uptake is active and probably driven by Na^+,K^+-ATPase, which is postulated to be located in the basolateral membrane of the rectal cells as in other epithelia (Phillips 1981; Komnick & Achenbach 1979). Reabsorption of the main anion, Cl^-, occurs against a large electrochemical gradient of 100

mV (Phillips *et al.* 1986). *In vitro* studies using short-circuited recta from the desert locust, *S. gregaria,* clearly demonstrated an electrogenic Cl^- pump of unknown nature (Williams *et al* . 1978).

This electrogenic Cl^- reabsorption from the lumen is under hormonal control (Spring *et al.* 1978). Homogenates of corpora cardiaca (CC) stimulate net Cl^- transport, short circuit current (I_{sc}), transepithelial potential (V_t, lumen positive) and transepithelial conductance (G_t) when applied to the hemocoel side of *in vitro* rectal preparations (Spring & Phillips 1980a,b). Measurable increases in I_{sc} are observed with as little as 0.005 CC in 5 mL external saline, while 0.1 CC causes maximal stimulation of I_{sc}. In contrast, large amounts of flight muscle, corpora allata, and various known or putative neural transmitters did not change I_{sc} (Spring & Phillips 1980a). A peptide hormone, chloride transport stimulating hormone (CTSH), has been partially purified from the corpus cardiacum (Phillips *et al.* 1980). CTSH has an approximate molecular weight of 8,000 Daltons and is sensitive to trypsin digestion (Phillips *et al.* 1980). A concentration of 7 nM of this purified CTSH is sufficient to cause maximal stimulation of I_{sc} (Phillips *et al.* 1980). A similar substance was observed in the hemolymph of fed locusts and cardiatectomy reduced this activity (Spring & Phillips 1980c). Addition of extracts containing CTSH to the hemocoel side of *in vitro* rectal preparations causes a 2- to 3-fold increase in intracellular levels of cAMP (Spring & Phillips 1980a,b; Chamberlin & Phillips 1988). Also, addition of extracellular cAMP to the hemocoel side causes similar changes in I_{sc}, V_t, and net active Cl^- transport as does CTSH (Spring & Phillips 1980a,b). Inhibitors of phosphodiesterases (i.e. theophylline) and stimulants of adenylate cyclase (1-100 μM forskolin) have similar action on locust recta (Phillips *et al.* 1986; Hanrahan 1982).

There are several reports of peptide antidiuretic (ADH) and diuretic factors (DH) which respectively increase or decrease short-term rectal fluid absorption *in vitro* from several insect species (reviewed by Phillips 1983b; Phillips *et al.* 1986). Proux and

Rougon-Rapuzzi (1980) have identified a vasopressin-like DH produced in the sub-esophageal ganglia (SOG) of *Locusta* acting on Malpighian tubule secretion but not on rectal fluid absorption (Proux *et al.* 1984). SOG does not inhibit CC-stimulated Cl^- transport in locust recta (Phillips *et al.* 1982a); therefore, VP-like DH probably does not reduce ion-dependent fluid transport. ADH factors in storage lobe and glandular lobes of CC of *Locusta* appear to be different (Herault *et al.* 1987). The ADH factor in the storage lobes may be CTSH because of similarities in responses of recta exposed to the fractions from the storage lobes and purified CTSH. Phillips *et al.* (1980) initially suggested that ADH and DH may be distinct from CTSH and may control the osmotic permeability of rectal epithelia and thereby determine the volume and osmolarity of the CTSH-stimulated, KCl-rich absorbate. Therefore, ultimate control of water and ion reabsorption by insect recta may be due to a combination of CTSH, ADH and DH.

Hanrahan and Phillips (1983, 1984a,b) have studied the cellular mechanisms of reabsorption and its control by CTSH and cAMP using locust recta mounted in Ussing chambers. They employed various techniques, including voltage-clamping, cable analysis, ion substitutions, radiotracer fluxes, inhibitor additions and intracellular recordings with double-barrelled ion-selective microelectrodes. Hanrahan and Phillips (1984a) observed that I_{sc} and net Cl^- flux declined exponentially after dissection and reached a pseudo-steady-state at about 3 hours. They observed a discrepency of about 1 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$ between the unstimulated I_{sc} and net Cl^- flux, due probably to H^+secretion, (Thomson, unpublished observation). Addition of 1mM cAMP to the hemocoel side caused a 10-fold increase in I_{sc} and net Cl^- flux (to about 10 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$), a 5-fold increase in V_t and a 50% reduction in transepithelial resistance (R_t). Cyclic AMP also increased the apical membrane potential (V_a) opposing Cl^- entry into the cell, thereby increasing the overall electrochemical gradient against which Cl^- must be transported. These results clearly indicated that the active step for Cl^- absorption occurred at the apical membrane (Hanrahan & Phillips 1984b). Under open-circuit conditions, the flux

ratios for Cl^- were an order of magnitude higher than those predicted for simple diffusion (Hanrahan & Phillips 1984b). They also determined that exchange diffusion was not a significant component of Cl^- fluxes under short-circuit conditions. Replacement of Cl^- with gluconate caused a 91% decrease in the cAMP-stimulated I_{sc}, confirming that stimulated I_{sc} was due almost entirely to stimulated active transport of Cl^- (Hanrahan & Phillips 1984a). This anion pump was highly specific for Cl^- and Br^- whereas cAMP caused only a slight or no increase in I_{sc} (0.5 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$) when Cl^- was replaced by phosphate, I^-, acetate, SCN^-, SO_4^{2-}, NO_3^-, or F^-.

Hanrahan and Phillips (1983, 1984a,b) showed that electrogenic Cl^- transport did not occur by NaCl co-transport. In nominally Na^+-free saline (1 to 200 μM Na^+) cAMP still stimulated I_{sc} and net Cl^- flux by 5-fold. Also, there was no correlation between the cAMP-stimulated I_{sc} and trace levels of external Na^+ remaining at the end of these experiments (Hanrahan & Phillips 1984a). Flux of $^{22}Na^+$ from the lumen into the rectal cells did not parallel $^{36}Cl^-$ accumulation during cAMP stimulation. Exposure to 1 mM ouabain at 22°C for 2 h had no effect on either net Cl^- flux in unstimulated recta or on the increase in I_{sc} in cAMP-stimulated recta (Hanrahan & Phillips 1984a). When mucosal Na^+ concentration was reduced to 49 μM, internal Na^+ activity (8mM) changed little and the net electrochemical potential for Na^+ across the apical membrane reversed (i.e. favoring Na^+ exit from the cell to the lumen). These changes had no effect on net active Cl^- flux, or on the net electrochemical gradient (38 mV) opposing Cl^- entry across the apical membrane or on intracellular Cl^- activity (Hanrahan & Phillips 1984b). Exposure to an inhibitor of NaCl co-transport, 1 mM furosemide, had no significant effect on cAMP-stimulated I_{sc} (Hanrahan & Phillips 1983). The above findings provide overwhelming evidence against secondary active transport of Cl^- by the Na^+ co-transport mechanism observed in many vertebrate epithelia (Frizzell *et al.* 1979).

To investigate whether net Cl^- flux across locust recta occurs by Cl^-/HCO_3^- exchange, Hanrahan and Phillips (1983, 1984a) exposed recta to nominally HCO_3^-- and

CO_2-free saline for up to 6 hours. They observed no change in I_{sc}, unidirectional Cl^- fluxes, transepithelial resistance (R_t) or V_t across locust recta. Although not all HCO_3^- can be removed from the test solutions due to metabolic production of CO_2, the contribution of metabolic CO_2 production by rectal tissue was calculated to be insufficient to drive transepithelial Cl^- fluxes (Hanrahan & Phillips 1984a). For example, they estimated that if all metabolically produced CO_2 was converted to HCO_3^- and was all exchanged across the apical membrane in a 1:1 fashion for Cl^-, then the maximum transport of Cl^- would be 3.2 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$ as compared to the measured unidirectional flux for Cl^- of 10 to 12 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$. Under nominally HCO_3^--free conditions some alkalinization of the luminal solution was observed but titratable base appeared at a much slower rate than Cl^- transport and was less than 39% of the I_{sc} (Hanrahan & Phillips 1984a). They suggested that this alkalinization might be due to ammonia production rather than HCO_3^- secretion. However, this possiblity has recently been eliminated by Thomson *et al.* (1988a). Inhibitors of Cl^-/HCO_3^- exchangers (1 mM SITS) or of carbonic anhydrase (1mM acetazolamide) had no effect on I_{sc} or net Cl^- flux (Hanrahan & Phillips 1983). Therefore, there is no evidence that Cl^-/HCO_3^- exchange constitutes a major mechanism for Cl^- transport in locust recta.

Hanrahan and Phillips (1983) also investigated whether Cl^- transport occurred by HCl co-transport or Cl^-/OH^- exchange. The cAMP-stimulated I_{sc} was independent of external pH over a wide range (pH 5.5-8.0), a change which should reduce the electrochemical gradient for protons across the apical membrane by two-fold (Hanrahan & Phillips 1983), and therefore change the hypothetical driving force for net Cl^- flux. More recently, Thomson and Phillips (1988) found that active proton secretion in this epithelium was not changed when all external Cl^- was replaced by gluconate: this finding provides further evidence against HCl co-transport.

The primary urine of Malpighian tubules contains high levels of K^+ (140 mM) as compared to hemolymph levels (10 mM; Hanrahan & Phillips 1984a). Phillips (1964b)

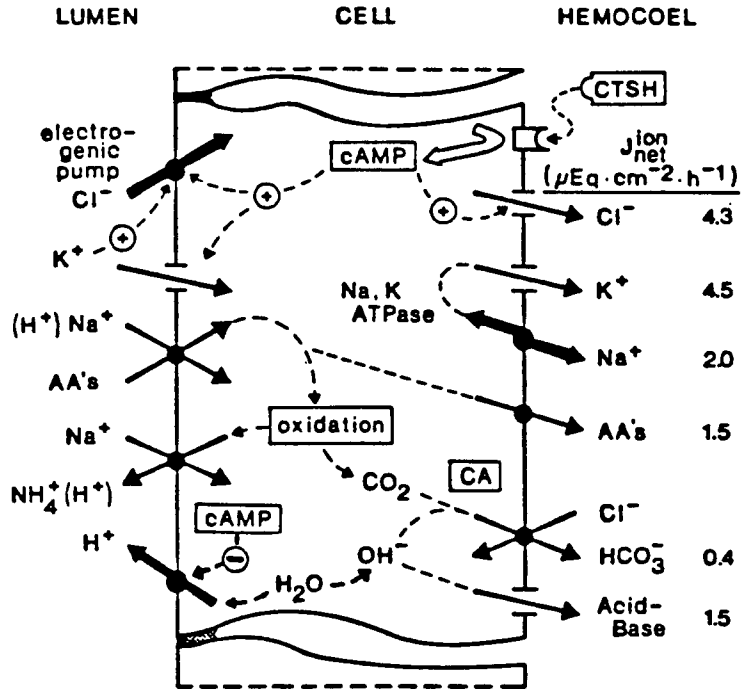
observed that net Cl^- absorption was 7.5 times faster *in vivo* if KCl, rather than NaCl, was injected into ligated recta. Hanrahan and Phillips (1984a) investigated the effects of K^+ replacement on cAMP-stimulated and unstimulated I_{sc}, net Cl^- flux, V_t, and R_t. Removal of K^+ caused no significant decrease in unstimulated I_{sc}. However, cAMP-stimulated I_{sc} was only 30% of control levels in K^+-free saline (Hanrahan & Phillips 1984a). Addition of cAMP to K^+-free saline increased the intracellular activity of Cl^- by 12 mM and elevated the net electochemical gradient opposing Cl^- entry by 12 mV. When 10 mM K^+ was restored to the saline, the I_{sc} increased to normal levels. The stepwise bilateral addition of K^+ resulted in a progressive increase in the cAMP-stimulated I_{sc} with an activation constant of 6 mM K^+ (Hanrahan & Phillips 1983). This also increased the net electrochemical gradient opposing Cl^- entry, which indicated that K^+ stimulation of Cl^- transport was not due to simple depolarization of the apical membrane (Hanrahan & Phillips 1984b). K^+ was shown to stimulate at the luminal surface of the apical membrane. Hanrahan and Phillips (1984b) concluded that Cl^-transport did not occur by KCl co-transport since: a) 35% of the cAMP-stimulated I_{sc} is K^+-independent; b) transepithelial $^{42}K^+$ fluxes are independent of external Cl^- levels; c) net electrochemical K^+ gradients across the apical membrane are not significantly different from zero under I_{sc} conditions when external K^+ concentrations range from 4 to 140 mM. Thus, there is no potential energy in a mucosal K^+ gradient to drive Cl^- entry into the cell.

The locust rectum appears to be a tight epithelium with 60% of transepithelial conductance (G_t) being transcellular under unstimulated conditions and 90% during cAMP stimulation (Hanrahan & Phillips 1984b) The addition of cAMP causes a 50% increase in G_t. This is a result of increased K^+ conductance in the apical membrane and an increased Cl^- conductance in the basolateral membrane. The back flux of K^+ (hemocoel to lumen) increases by 400% following cAMP exposure, consistant with an increase in K^+ conductance in the apical membrane (Hanrahan & Phillips 1984a).

In summary, Hanrahan and Phillips (1983, 1984a,b) found that entry of chloride into rectal tissue was active, electrogenic, and stimulated by both luminal K^+ and intracellular cAMP. Since they were unable to find evidence for secondary active transport of Cl^- coupled to apical Na^+, K^+, HCO_3^-, OH^- or H^+ gradients, they were forced to propose an epithelial model involving an electogenic Cl^- pump located in the apical membrane of rectal epithelia (Fig. 4). This primary pump was hypothesized to be a Cl^--stimulated ATPase activated by intracellular cAMP (cAMP levels elevated by CTSH *in vivo*) and by luminal K^+. In addition, they proposed that cAMP also increased passive absorption of K^+ by electrical coupling as a result of increased K^+ conductance in the apical membrane and Cl^-conductance (i.e. lower opposing V_b) in the basal membrane.

Anion-stimulated ATPases and Cl⁻ transport

Several investigators have reported the presence of anion-stimulated ATPase activity in invertebrate epithelia (Gerenscer & Lee 1983). DePew and Towle (1979) observed an anion-stimulated ATPase in gill plasma membrane of the fiddler crab (*Uca minax*). They demonstrated that anion-stimulated ATPase co-migrated with Na^+,K^+-ATPase during density gradient centrifugation while cytochrome oxidase eluted in different fractions. Lee (1982) observed a HCO_3^--stimulated ATPase in microsomal fractions from gills of blue crab (*Callinectes sapidus*). The enzyme had a K_m of 8.9 mM HCO_3^- and saturated at 20 mM HCO_3^-. Oligomycin (1-5 μg/mL) inhibited the enzyme in mitochondrial fractions to a greater extent than the enzyme in microsomal fractions although 68-70% of the ATPase activity in the microsomes was inhibited (Lee 1982). Density centrifugation of the microsomal pellet showed co-migration of 5'-nucleotidase and HCO_3^--stimulated ATPase with little cytochrome oxidase activity. Ouabain (5 mM) and vanadate (0.5 mM) had little effect on anion-stimulated ATPase activity while acetazolamide (5 mM), EDTA (5 mM) and p-chloromecuribenzene sulfonic acid (0.5 mM) all strongly inhibited ATPase activity. Thiocyanate also inhibited anion-stimulated



Ion Activities (mM) and PD (mV)			
Na^+	75	9	75
K^+	7.2	70	7.2
Cl^-	82	47	82
Proline	13	66	13
pH	7	7.36	7
mV	+64	0	+34

	Net Electrochemical PD (mV) (Apical)	(Basolateral)
Na^+	127 (favoring)	127 (opposing)
K^+	12 (favoring)	20 (favoring)
Cl^-	50 (opposing)	20 (favoring)
H^+	86 (opposing)	56 (favoring)

Figure 4. Model of transport mechanisms identified in locust rectal pad epithelium. The neuropeptide hormone, CTSH, acts via cAMP to stimulate ⊕ or inhibit ⊖ four mechanisms: *thick arrows*, major pumps; *thin arrows* through solid circles, carrier-mediated co- or counter-transport; arrows through gaps, ion channels. Steady-state values given for net transepithelial flux and electrochemical potential differences across the two cell borders are for stimulated recta in Ussing chambers and bathed bilaterally in control saline under open-circuit conditions, except short-circuited state for Na^+ and amino acids (AA). From Phillips *et al.* 1988.

ATPase activity with an I_{50} of 4.8 mM. Lee (1982) found that anion-stimulated ATPase activity increased in gills of crabs acclimated to low salinity water and suggested that the enzyme may be important in osmoregulation and/or acid-base homeostasis. Wheeler and Harrison (1982) observed a HCO_3^--stimulated ATPase in microsomes of the fresh-water clam, *Anodonta cataracta*, but were unable to completely eliminate mitochondrial contamination. Gerenscer and Lee (1983) isolated plasma membranes from enterocytes of *Aplysia californica* which contained both Cl^-- and HCO_3^--stimulated ATPase activities. The anion-stimulated ATPase activities were inhibited by thiocyanate but not by SITS, amiloride, or furosemide (Gerenscer 1983). Active Cl^-transport across *A. californica* gut was also inhibited by thiocyanate (Gerenscer 1983).

Anion-stimulated ATPase activity has been observed in several insect species. Turbeck *et al.* (1968) observed anion-stimulated ATPase activity in midgut from *Hyalophara cecropia* with a pH optimum of 8.7. The activity wasn't believed to be due to alkaline phosphatase which had a pH optimum of 10, and was not affected by the ATPase inhibitor, thiocyanate. Herrera *et al.* (1978) observed anion-stimulated ATPase activity in 14,000 g pellets of rectal tissue from *S. gregaria.* ATPase activity was stimulated by the addition of Cl^-, sulfate, and nitrite but no attempt was made to distinguish mitochondrial and microsomal enzyme activity. Anstee and Fathpour (1979, 1981) observed an anion-stimulated ATPase in Malpighian tubule microsomes from *Locusta migratoria.* ATPase activity was stimulated to the greatest extent by sulfite and was not stimulated by Cl^-. Microsomal fractions were relatively free of mitochondrial contamination and contained only 16% of the succinate dehydrogenase activity found in mitochondrial fractions (Anstee & Fathpour 1981). Oligomycin inhibited ATPase activity in both mitochondrial and microsomal fractions (pI_{50} 4.29 and 4.74 respectively). Komnick *et al.* (1980) observed a ouabain-insensitive anion-stimulated ATPase in the rectum of dragonfly nymphs (*Aeshna cyanea*). They observed maximal enzyme activity in 30 mM HCO_3^- with a K_m of 4.65 mM HCO_3^-. Chloride also stimulated ATPase ac-

tivity with a K_m of 10.25 mM Cl^-. Thiocyanate inhibited both anion-stimulated ATPase activity and uptake of Cl^- from hyposmotic external saline by the whole rectum (Komnick *et al.* 1980). Komnick (1978) also showed an increase in HCO_3^--stimulated ATPase activity in recta from nymphs exposed to low salinity water (0.05 mosmol) as compared to ATPase activities from control organisms in 5 mosmol water. Gassner and Komnick (1982) showed that furosemide inhibited anion-stimulated ATPase activity in homogenates of *A. cyanea* recta. Furosemide acted as a non-competitive inhibitor, with a K_i of 4.3 mM. They observed no effect of furosemide (at concentrations up to 10 mM) on Na^+-K^+ ATPase activity. Deaton (1984) observed a HCO_3^--stimulated ATPase in microsomes from the midgut and integument of *Manduca sexta.* Microsomal activities of succinate dehydrogenase were 12% of that of mitochondrial fractions in both tissues. There was no significant effect of 0.1 mM oligomycin or 1 mM carboxyatractyloside on microsomal HCO_3^- ATPase, while both substances inhibited ATPase activities in the mitochondrial fractions.

As discussed above, there are a number of fairly convincing studies which have demonstrated anion-stimulated ATPase activities in plasma membrane fractions from a variety of animals. Gerenscer and Lee (1985a,b) have demonstrated ATP-stimulated active Cl^- transport in isolated plasma membrane vesicles from enterocytes of *Aplysia* which also had anion-stimulated ATPase activity. Ultimately, the enzyme should be isolated and incorporated in artificial liposomes and transport of Cl^- demonstrated in the same way that K^+ and Na^+ have been shown to be transported in reconstituted vesicles containing Na^+,K^+-ATPase (Goldin 1977).

Na^+ transport

Net active flux of Na^+ across short-circuited locust recta ranges from 1.2 to 3.0 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$ (Phillips *et al.* 1986; Black *et al.* 1987; Spring & Phillips 1980b). Black *et al.* (1987) determined the kinetics of $^{22}Na^+$ fluxes across locust recta under short-cir-

cuit conditions. Net Na^+ flux exhibits Michaelis-Menten kinetics with a K_t of 17 mM Na^+ and a V_{max} of 1.5 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$. Black *et al.* (1987) found that 1mM amiloride inhibited 75% of the net Na^+ flux when saline Na^+ was 100 mM. The addition of 1 mM ouabain at 30°C caused a 37% inhibition of net Na^+ flux. These partial inhibitions of net Na^+ flux by amiloride and ouabain had no effect on the much larger Cl^--dependent I_{sc}. The addition of 5 mM vanadate did not inhibit net Na^+ flux (Phillips *et al.* 1986).

Net active flux of Na^+ across short-circuited locust recta was not affected by the addition of stimulants which increase KCl absorption across locust recta (i.e. cAMP, corpus cardiacum extracts) at either high or low levels of external Na^+ (Spring & Phillips 1980b; Black *et al.* 1987). Black *et al.* (1987) surveyed the the locust nervous system and were unable to find any evidence for a neurohormone which controlled Na^+ reabsorption in the rectum. Such a factor was suspected because Steele and Tolman (1980) had observed that extracts of retrocerebral complex of *Periplaneta americana* stimulated oxygen consumption and short-term (1 h) fluid absorption by rectal sacs only if Na^+ was present on the hemocoel side. However ion movements were not measured during these studies so there was no direct evidence that the factors from the retrocerebral complex acted directly on Na^+ transport. Indeed, there was no direct evidence for an insect hormone controlling Na^+ reabsorption in the excretory system of any insect.

Hanrahan and Phillips (1984b) used Na^+-selective double-barrelled microelectrodes to measure electrochemical gradients for Na^+ across the apical and basolateral membranes of locust rectal pads. They showed a very large driving force for passive Na^+ entry across the apical membrane, but found little evidence for conductive pathways (i.e. Na^+ channels) as a major mechanism for Na^+ entry. Recently, Black *et al.* (1987) used unstimulated preparations to show that a conductive pathway (channels) accounted for a third of Na^+ entry. Na^+-dependent glycine uptake into the rectal cells of locusts does occur at a rate of 0.13 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$. Active uptake of proline may also account some

of the Na^+ flux although this process is largely Na^+-independent (Meredith & Phillips 1988). Active proton secretion occurs in the locust rectum and 15% of this is dependent on Na^+(Thomson *et al.* 1988b). Thus Na^+/H^+ exchange may account for 0.2 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$ of Na^+ entry. Another mechanism of Na^+ influx, NH_4^+/Na^+ exchange, is apparently responsible for another small portion of the Na^+ transport (0.2 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$; Thomson *et al.* 1988a). The co-entry of Na^+ with organic acids (e.g. acetate; Baumiester *et al.* 1981), other metabolites and phosphate remains to be investigated (Phillips *et al.* 1986). Hanrahan and Phillips (1984b) showed that the active step for Na^+ flux is across the basolateral membrane where Na^+ is pumped against a very large electrochemical gradient of 127 mV under short-circuit conditions. In addition, Hanrahan and Phillips (1984b) showed active cellular accumulation of K^+ from the hemocoel side against large concentration differences (10-fold). These observations are consistent with a basolateral Na^+/K^+ exchange pump (Fig. 4).

Na^+,K^+- ATPase in insect excretory epithelia

In most animal cells, Na^+,K^+-ATPase controls intracellular Na^+ and K^+ levels, while in most epithelia, transport of ions, nonelectrolytes and fluid is driven by the Na^+ electrochemical gradients established by this enzyme located in the basolateral membrane. Rectal tissues from several insect species have been analyzed for Na^+,K^+-ATPase activity (reviewed by Anstee & Bowler 1984; Towle 1984). Peacock (1977) found Na^+,K^+-ATPase activity (75 nmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$) in microsomes from recta of *S. gregaria.* Rectal tissue had higher specific activities than did the ileum and colon (15 nmol $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$). Similar results were observed for recta from the cockroach *Blaberus croniifer* (Peacock 1977). Peacock (1976) investigated the distribution of Na^+,K^+-ATPase in the alimentary tract of *L. migratoria.* The highest activities were found in the rectum, with lower activities found in the foregut, midgut, ileum and colon. Attempts to localize Na^+,K^+-ATPase within the rectal pads using the Ernst method were

unsuccessful (Peacock 1976). Komnick and Achenbach (1979) localized Na^+,K^+-ATPase in the basolateral membrane of recta from a freshwater dragonfly nymph by ^3H-ouabain autoradiography. However this tissue has a different function from recta of terrestrial insects.

Na^+,K^+-ATPase from insects are inhibited by both ouabain and vanadate, inhibitors of vertebrate Na^+,K^+-ATPases. Peacock (1981) determined a pI_{50} for ouabain of 6.0 in recta from *L. migratoria.* He found that ouabain inhibition increased as temperature increased from 5 to 30°C. The Malpighian tubules of *L. migratoria* also have Na^+,K^+-ATPase, with specific activities ranging from 213 to 292 nmoles $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$ (Anstee & Bell 1978; Donkin & Anstee 1980). Inhibition of Na^+,K^+-ATPase in Malpighian tubule microsomes of *L. migratoria* by orthovanadate showed a pI_{50} of 6.0 (Anstee & Bowler 1984).

Metabolic dependence of ion transport and amino acid transport

Chloride transport in locust recta is supported by aerobic metabolism since I_{sc} and net Cl^- flux are completely and rapidly abolished by azide, cyanide and anoxia (Baumeister *et al.* 1981). Recta depleted of endogenous metabolites showed little cAMP stimulation of I_{sc} as compared to recta exposed to natural levels of sugars and amino acids (Chamberlin & Phillips 1982a). Proline is found at high concentrations in the rectal lumen and is actively reabsorbed by rectal epithelial cells (Meredith & Phillips 1988). Proline occurs at the highest levels in rectal cells (66 mM), followed by glutamine (44 mM), glycine (21 mM) and alanine (8.5 mM; Chamberlin & Phillips 1983). Mucosal addition of 50 mM proline to substrate-depleted recta *in vitro* caused a 5-fold increase in cAMP-stimulated I_{sc}, whereas mucosal glycine (50 mM) failed to stimulate I_{sc}. Rectal I_{sc} was sustained as well by proline alone as by the full complement of amino acids and sugars found in the hemolymph.

Balshin and Phillips (1971) and Balshin (1973) demonstrated that five neutral L-

amino acids (proline, glycine, serine, alanine, theronine) are all actively absorbed against large concentration gradients across everted rectal sacs when net fluid transport was prevented by an osmotic concentration difference. Only small fractions of the total proline, alanine, serine, and theronine which are transported across the locust rectum are metabolized by the tissue (Balshin 1973; Phillips *et al.* 1986). While glutamate enters the rectal cells from the lumen and acts as metabolic substrate it is not transported transepithelially (Chamberlin & Phillips 1983; Balshin 1973). The two major amino acids of the hemolymph and primary urine, proline and glycine, are transported at the highest rate and against the largest concentration gradients. At a natural luminal proline concentration of 15 mM, the net active flux of proline was 2 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$, and the flux ratio (forward flux to back flux) was 40:1 (Spring & Phillips 1984). Meredith & Phillips (1988) observed that 85% of ^{14}C-activity appearing on the hemocoel side was proline when either 2 or 80 mM proline was added to the lumen side. Only 10% of ^{14}C-proline was oxidized to $^{14}CO_2$ during net transport across short-circuited locust recta (Spring & Phillips 1984). Addition of cAMP caused a 45% increase in the oxidation of ^{14}C-proline to $^{14}CO_2$ and a 40% decrease in the net flux of proline across the tissue (Spring & Phillips 1984). Net flux of proline obeys Michaelis-Menten kinetics with a K_t of 10 mM and a V_{max} of 4.2 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$ (Meredith & Phillips 1988). Meredith & Phillips (1988) found that only a small fraction of this flux was Na^+ or K^+ dependent. They hypothesized that the major component of proline flux might be driven by the large proton gradients observed across the apical membrane (Thomson *et al.* 1988a,b). This is in contrast to the much smaller net glycine transport which was largely Na^+-dependent (Balshin 1973).

The high rates of net proline transport *in vitro* (2 to 4 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$) are ten times those needed to maintain rectal metabolism or to recover proline from the primary urine. To sustain cAMP-stimulated Cl^- transport only 1 mM luminal proline is required as a metabolic substrate (Meredith & Phillips 1988). Since unknown organic solutes

account for half of the measured osmolarities in the lateral spaces of cockroach pads (Wall 1971), possibly proline is the unknown organic solute that is believed to drive fluid transport (Phillips *et al.* 1986).

Ammonia secretion and acid-base transport

As stated above, locust Malpighian tubules actively secrete proline which constitutes 80% of the amino acids entering the hindgut where proline is actively reabsorbed. The metabolic pathway for proline oxidation in locust rectum has been determined. Complete oxidation is the predominate pathway in short-circuited recta *in vitro* and this results in substantial ammonia production (Chamberlin & Phillips 1982a,b). Amino acids absorbed from the lumen are the major source of ammonia production in locust rectum and 90% of this ammonia is secreted to the lumen side (0.6 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$; Thomson *et al.* 1988a). Ammonia secretion (cell to rectal lumen) apparently occurs largely by exchange of NH_4^+ for Na^+ since ammonia secretion is; a) not effected by changes in luminal pH from 7 to 5, b) inhibited 60% by either 1 mM amiloride or removing all luminal Na^+, c) not effected by changes in V_a or V_t, i.e. as expected for a neutral cation exchanger, and d) not reduced by the absence of luminal Cl^- or K^+ (Thomson *et al.* 1988a).

Phillips (1961) observed that rectal contents were consistently acidic (pH 5-6) *in situ* and he proposed that the rectum might be functioning in a pH regulatory role. *In vitro* experiments have demonstrated that protons are actively secreted across the apical membrane of the rectum against electrochemical gradients of at least 79 mV (Thomson *et al.* 1988b). This proton secretion is electrogenic, inhibited by 1 mM azide and is stimulated 50% by including HCO_3^-/CO_2 in the saline and is not changed by Cl^- or Na^+ removal or by addition of either 1 mM SITS or 1 mM acetazolamide (Thomson & Phillips 1985,1988). This proton pump has properties similar to the one described for turtle bladder (Al-Awqati *et al.* 1983).

Acid secretion in locust rectum is accompanied by an equal movement of base equivalents (OH^-, HCO_3^-) to the hemocoel side. In unstimulated everted rectal sacs bicarbonate absorption was 0.4 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$, which accounted for much of the anion deficit ($Na^+ + K^+ - Cl^-$) observed in rectal absorbate (Thomson & Phillips 1985; Phillips *et al.* 1988). This bicarbonate absorption was completely inhibited by hemocoel addition of DIDS or acetazolamide, suggesting that HCO_3^-/Cl^- exchange occurs at the basolateral membrane (Thomson & Phillps 1985). Cyclic AMP stimulation of locust recta under open-circuit conditions reduces active secretion of protons by 66% and a reduction in proton secretion is also observed in stimulated ileum (Phillips *et al.* 1988; Thomson unpublished observation). However, the effect of cAMP on bicarbonate absorption has not been determined in either hindgut segment.

There have been no studies of amino acid absorption and metabolic dependence of ion transport on specific substrates in the ileum. Prusch (1972) showed ammonia secretion in unsegmented hindgut of blowfly larvae but ammonia secretion in the ileum of locusts or other insects has not been investigated.

Comparison of fluid and ion transport in the two hindgut segments

This thesis compares the properties and control of solute and fluid transport in two segments of the locust hindgut, the ileum and rectum. Since work in our laboratory was previously restricted to studies of transport processes and their control in the locust rectum, the work described in this thesis deals largely with the ileum. However, there are no previous reports of changes in ion transport (e.g. absorbate composition) across everted rectal sacs after stimulation or on the effect of external proline on rectal fluid transport *in vitro*; therefore it was necessary to investigate these aspects of rectal transport to complete this comparison. Chapter 2 presents the first detailed description of the properties of fluid transport across an insect ileum, using everted sacs from locusts. Evidence is presented for: 1) metabolic dependence of fluid transport, 2) stimulation by

cAMP and neuroendocrine extracts, 3) the relationship between fluid transport and transpeithelial osmotic difference, and 4) osmotic concentration of ileal absorbate.

In chapter 3, I describe the effect of different luminal ion ratios and stimulants on the rate of fluid transport across locust ileal epithelia and on the ionic composition of the absorbed fluid (i.e. absorbate), in particular Na^+, K^+, Cl^- and total CO_2 levels with time, and the rates of transport of these ions. This study provided the first direct evidence for control of Na^+ reabsorption in an insect hindgut. Changes in transepithelial potential after exposure to neural extracts were measured to determine total electrochemical potential differences favoring or opposing these ion movements. In chapter 4 I report on the properties of net flux of Na^+ across stimulated and unstimulated short-circuited locust ilea in Ussing chambers to determine what component of the cAMP-stimulated net Na^+ absorption described in chapter 3 is due to active transport, as opposed to passive absortion by electrical coupling. I also consider whether CC and VG factors also have a similar action on active or passive components of Na^+ absorption.

No previous studies had examined the effect of stimulants (cAMP and neural extracts) on composition of rectal absorbate; therefore, chapter 5 is concerned with the changes in Na^+, K^+, Cl^- and osmotic concentrations of rectal absorbate following stimulation, when everted rectal sacs were exposed to salines with different Na^+:K^+ ratios. In addition, the effects of proline on fluid transport across everted rectal sacs and everted ileal sacs are compared in chapter 5. Because these studies revealed very different responses of fluid transport to increasing proline concentrations in the two hindgut segments, proline flux across flat sheet preparations of locust ilea in Ussing chambers was determined and compared to those reported previously for locust recta.

Given the previous evidence suggesting that Cl^- transport in locust rectum may involve a primary transport process, and given the very different ratios of Cl^- to Na^+ transport in locust ileum and rectum, chapter 6 investigates and compares activities of Na^+,K^+-ATPase and anion-stimulated ATPases in the two hindgut segments and their

localization in epithelial membranes. The properties of anion-stimulated ATPase activities in rectal tissue are investigated to determine if such a protein could account for part or all of the active electrogenic Cl^- transport observed in both segments of the locust hindgut. In the general discussion (chapter 7) the capacities, properties and control of fluid and solute transport in the two segments of the hindgut are compared.

The overall conclusion from this study is that reabsorption in the ileum and its hormonal control probably play a much greater role in the excretory process than previously anticipated.

CHAPTER 2: Actions of cAMP, Corpus Cardiacum and Ventral Ganglia on Fluid Absorption by the Ileum

INTRODUCTION

The excretory process in the desert locust, *Schistocerca gregaria,* involves secretion of a KCl-rich primary urine by the Malphigian tubules followed by selective reabsorption in the rectum (reviewed by Phillips *et al.* 1986). The contribution of the intermediate segment (i.e. the anterior hindgut or ileum) in insect renal function has been largely neglected by investigators. Recently, however, Irvine *et al.* (1988) have used short-circuited flat-sheet preparations of locust ileum to demonstrate salt transport at rates equal to or greater than those reported for the rectum. Moreover, Cl^-, Na^+ and K^+ absorption and OH^-secretion across locust ileum were all stimulated by 5 mM cAMP, which may act as second messenger for neuropeptide stimulants in the corpus cardiacum (CC) and ventral ganglia (VG). In support of this hypothesis, aqueous extracts of both these neuroendocrine tissues stimulated ileal short-circuit current (largely Cl^- transport), transepithelial potential (V_t) and conductance in a manner qualitatively and quantitatively similar to that reported for locust rectum (Irvine *et al.* 1988).

There is considerable evidence that fluid transport in the insect recta, leading to the formation of semi-dry hyperosmotic excreta in terrestrial species, is the result of active transport and recycling of monovalvent ions at elaborate intercellular lateral membranes within this epithelium (reviewed by Phillips *et al.* 1986). Evidence for control of rectal fluid absorption by putative antidiuretic (ADH) and diuretic (DH) factors in several insects has been reviewed recently by Phillips (1983b). Since active absorption of fluid across epithelia against (or in the absence of) osmotic concentration differences is invariably driven by solute transport, I postulate that ADH activity reported in insects is probably the result of factors which first stimulate salt transport. Indeed, a chloride

transport stimulating hormone (CTSH; a 8,000 Dalton neuropeptide) has been partially purified from locust CC (Phillips *et al.* 1980). Proux *et al.* (1984) have provided some evidence that CTSH may be responsible for ADH activity in the locust rectum (see also Chamberlin & Phillips 1988).

Given these recently demonstrated similarities between salt transport and its control in both locust ileum and rectum, in this chapter I consider for the first time in insects whether ileal fluid reabsorption is also substantial and is possibly under hormonal control by factors in CC and VG extracts, which act in a dose-dependent manner. Because the locust ileum lacks the elaborate lateral intercellular membrane system observed in the rectum (Irvine *et al.* 1988), I also investigated the ability of the ileal epithelium to transport fluid against osmotic concentration differences and measured the total osmolarity of the absorbed fluid. As predicted from the ultrastructural differences and from current models of fluid transport, I show that the ileum cannot produce an absorbate which is hyposmotic to the luminal fluid, as previously observed for the rectum (Goh & Phillps 1978). In chapter 3, I describe the influence of CC and VG extracts and of luminal ion ratios on the compostion of the fluid absorbed across everted ileal sacs from the desert locust.

MATERIALS AND METHODS

The experimental animals were adult female *Schistocerca gregaria*, two to four weeks past their final molt. The locusts were maintained on a 12 h light : 12 h dark cycle at 28° C and 60% relative humidity. Animals were fed a mixture of dried grass, bran, powdered milk and yeast with fresh lettuce supplied daily.

Methods for studying ileal fluid transport were generally similar to those successfully used in earlier studies of locust rectum (reviewed by Hanrahan *et al.* 1984). Everted ileal sacs were prepared by inserting a 3 cm length of PE 90 tubing with a slightly flared end into the ileum from the midgut until it passed the posterior border of

the ileum. The hindgut was raised slightly and ligated with surgical silk on to the flared end of the tubing at the posterior end of the ileum. The colon, rectum and connecting tracheae were cut away and the ileum was slowly everted by sliding it over the PE tubing. The ileum was rinsed with 1 mL of saline to remove any hemolymph and fecal material. A second ligature was tied at the anterior of the everted ileum to close the sac. Any remaining internal fluid was withdrawn completely with a 'Hamilton' syringe and the empty sac was weighed to an accuracy of ± 0.1 mg on an August Sauter balance. Sacs were filled hourly with 10 µL of fresh saline or in some experiments left empty and incubated in 25 mL of external saline bubbled with 95% O_2-5% CO_2 and maintained at 30°C. The bathing saline (Proux *et al.* 1984) was based on the measured composition of locust hemolymph (Hanrahan *et al.* 1984) and contained (mM): 100 NaCl, 5 K_2SO_4, 10 $MgSO_4$, 10 $NaHCO_3$, 5 $CaCl_2$, 10 glucose, 100 sucrose (to adjust total osmolarity to hemolymph value), 2.9 alanine, 1.3 asparagine, 1.0 arginine, 5.0 glutamine, 11.4 glycine, 1.4 histidine, 1.4 lysine, 13.1 proline, 6.5 serine, 1.0 tyrosine, 1.8 valine, with pH of 7.1. This saline and gas mixture was previously found to maintain steady rates of ion and fluid transport across locust recta for more than 8 h at high rates comparable to those observed *in situ* (reviewed by Hanrahan *et al.* 1984). This was also true for short-circuit current and NaCl transport across locust ilea maintained as flat sheets in Ussing chambers (Irvine *et al.* 1988). Sucrose was added to this saline to prepare solutions with higher osmolarities for experiments involving osmotic concentration differences across the ileum. At hourly intervals weight gain and tissue volume change were determined by weighing ilea before and after removal of the fluid in the sac. The true rate of transepithelial fluid movement was determined by correcting for tissue volume changes. Statistical difference between means were determined by Student's t-test.

Effects of cAMP on fluid transport were determined by adding this agent to physiological saline on the hemocoel side. Entire corpora cardiaca (CC), or separated

nervous (storage, NCC) and gandular (GCC) lobes, and fifth ventral ganglia (VG) were removed from adult males (two to four weeks past final molt) to avoid the cyclic changes associated with female reproduction. Each tissue was homogenized in physiological saline using a glass-Teflon homogenizer and was centrifuged at 12,000 g for 5 min at 4°C. The supernatants were stored at -20°C until used. Aliquotes (10 µL) of the supernatant were added to the hemocoel side of ileal sacs. As a control tissue, larger amounts of flight muscle were treated in the same manner as the CC and VG. Changes in osmolarity of the saline due to additions of tissue homogenates were monitored with a Westcor vapor pressure osmometer (Model 5500; Logen, Utah) and were found to be insignificant. Fresh CC, VG or muscle extracts were replaced hourly on the hemocoel side (inside) of the sacs. Osmotic concentrations of the absorbate collected at the end of each hour were determined using the Wescor osmometer. To test the metabolic dependence of fluid transport, KCN and iodoacetic acid (IAA) were added to the bathing saline at a concentration of 1mM, or 5mM NaN_3 was added to the bath. The effect of hemocoel addition of 5 mM ouabain on fluid transport was also determined.

To determine if the stimulants of fluid transport present in the CC or VG were peptides I pretreated the extracts with trypsin (Sigma type III). The extracts were exposed for two hours at a concentration of 1mg trypsin per mL saline at 30° C and pH 7.1. Trypsin inhibitor (Sigma type IS) was added to stop further digestion. The treated extracts were then added to the inside of everted ileal sacs.

Chemicals

Trypsin, trypsin inhibitor, ouabain, iodoacetic acid, NaN_3 and all amino acids were obtained from Sigma Chemical Co. All other chemicals were of reagent grade.

RESULTS

Time course, stimulation and metabolic dependence of fluid absorption

In the absence of an initial osmotic concentration difference across the ileum, rates of fluid absorption (J_V) and tissue volume remained reasonably constant or declined slightly over the 5 h experimental period (Fig. 5a). This indicates that the incubation conditions which I used sustain transport activities very well, as anticipated from earlier studies using flat-sheet preparations of locust ileum (Irvine *et al.* 1988). Control sacs which lack stimulants or contained muscle extracts both transported fluid at similar mean rates of 3.0-3.5 $\mu L^{-1} \cdot h^{-1} \cdot ileum^{-1}$ after an initial decline in the first hour. Using the average gross surface area for locust ilea (0.4 cm^2; Irvine *et al.* 1988), the transport rate per unit area (9 $\mu L \cdot h^{-1} \cdot cm^{-2}$) was slghtly lower than that for locust rectum (11 $\mu L \cdot h^{-1} \cdot cm^{-2}$) under comparable conditions (Goh & Phillips 1978).

High doses of either CC or VG (1 gland per 10μL;Fig. 5c and Fig. 6b) caused a 5-fold increase in ileal J_V to maximium sustained values of 15 to 17 $\mu L \cdot h^{-1} \cdot ileum^{-1}$ or 40 $\mu L \cdot h^{-1} \cdot cm^{-2}$. This value is higher than that obtained with 20 mM cAMP (12 $\mu L \cdot h^{-1} \cdot ileum^{-1}$;Fig. 5a;6a) or than the maximium stimulated rate for locust recta (J_V = 23 $\mu L \cdot h^{-1} \cdot cm^{-2}$; Proux *et al.* 1984).

The metabolic dependence of ileal fluid transport was confirmed using inhibitors applied unilaterally (Table 1). Long-term (5 h) J_V was reduced by 76% and 84% respectively when the respiratory inhibitors, 1 mM cyanide plus 1 mM iodoacetate or 5 mM azide were included in the saline. This confirms the strong dependence of hindgut transport on aerobic respiration, which was demonstrated using electrical measurements on ilea in Ussing chambers (Irvine *et al.* 1988; reviewed by Phillips *et al.* 1986). Ouabain, an inhibitor of Na^+,K^+-ATPase and hence active Na^+ transport in most animal tissues, only caused a 30% inhibition of ileal fluid transport. This is less than the 75% inhibition of of net $^{22}Na^+$ flux observed across short-circuited locust recta (Black *et al.*

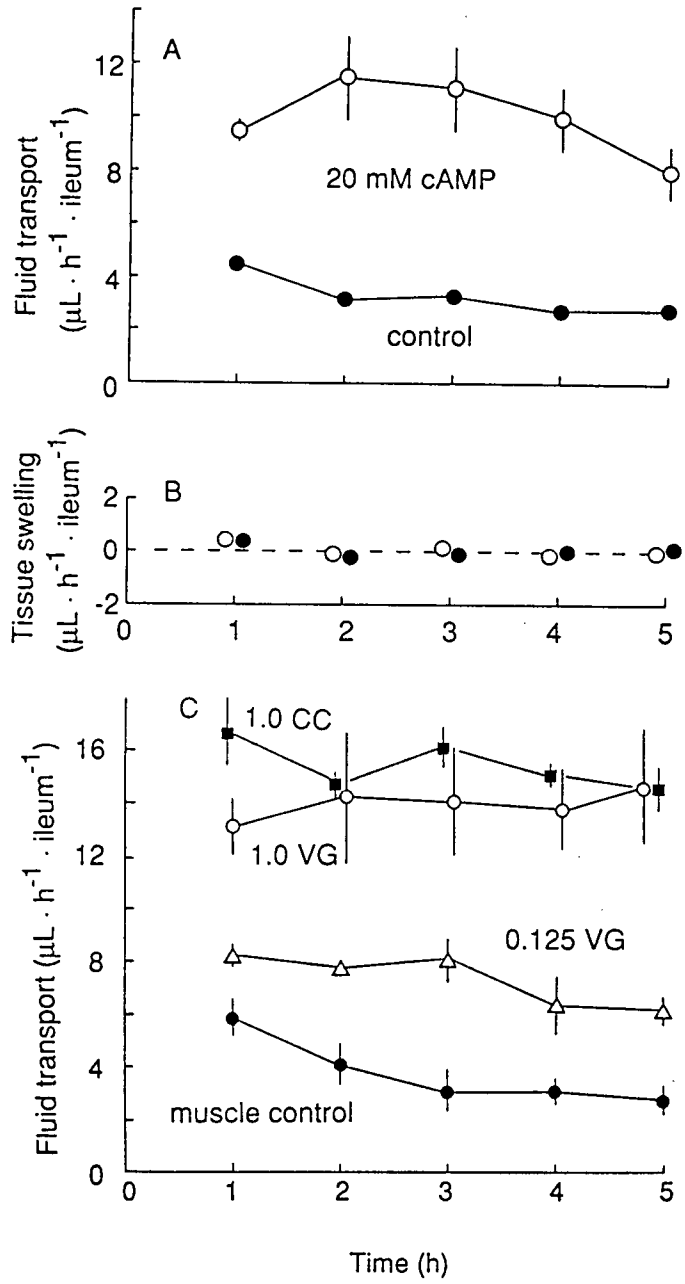


Figure 5. Rate of fluid absorption with time across everted ileal sacs bathed bilaterally in physiological saline (at 405 mosmol i.e. no initial osmotic difference): a) Effect of 20 mM cAMP on hemocoel side (○) compared with controls (●); b) associated changes in volume of ileal tissue (tissue swelling) over each 1 h incubation period (symbols as in a): c) Rate of fluid transport by sacs exposed to 1.0 CC/10 µL (■), 1.0 VG/10 µL (○), 0.125 VG/10 µL (Δ) or Muscle (control;●) on the hemocoel side. (mean ± s.e., n=4).

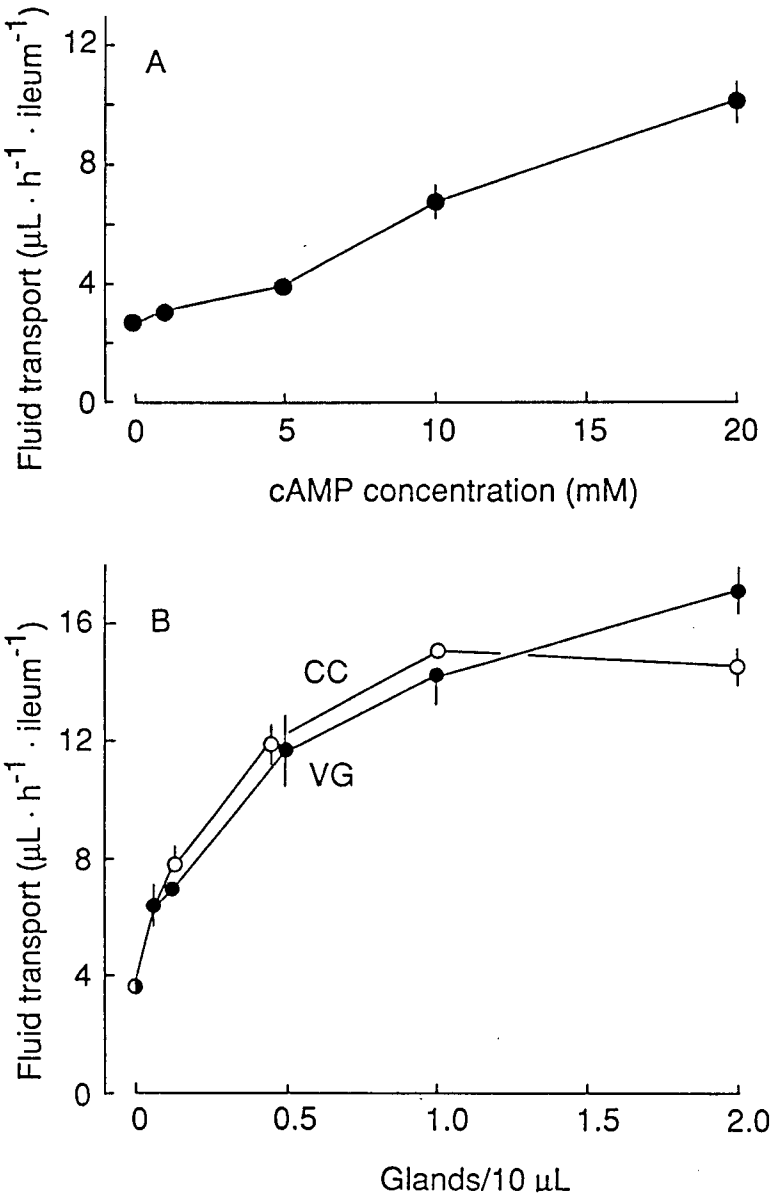


Figure 6. Dose-response relationships for agents which stimulate steady-state rates (2 to 5 h) of fluid absorption by everted ileal sacs bathed bilaterally in physiological saline at 405 mosmol: Effect of (a) cAMP and b) CC (○) and VG (●) on the hemocoel side. (mean ± s.e., n=16).

Table 1. Effect of metabolic inhibitors on steady state (2-5h) rates of fluid transport by everted ileal sacs at 30°C.

Treatment	Fluid transport ($\mu L \cdot h^{-1} \cdot ileum^{-1}$)	%Control
Control	3.02 ± 0.21	100%
1 mM KCN + 1 mM IAA	0.73 ± 0.08*	24%
5 mM NaN_3	0.49 ± 0.11*	16%
5 mM Ouabain	2.12 ± 0.16*	70%

The sacs were bathed in physiological saline bubbled with 95% O_2/ 5% CO_2 on lumen side. KCN + IAA and NaN_3 were applied to the lumen side only, and ouabain was added to the hemocoel side only (mean ± s.e., n=24-72).

* Significantly different ($P<0.05$) from control.

1987). This small effect of ouabain on ileal J_V is perhaps not suprising, because Na^+-independent Cl^- transport is a predominant mechanism in both segments of locust hindgut (Irvine *et al.* 1988; Phillips *et al.* 1986), and rectal J_V was previously shown to be dependent on the presence of Cl^- in the saline (Proux *et al.* 1984).

Dose-response relationships

I first established that everted ileal sacs exhibited near steady-state rates of fluid transport for long periods of time regardless of stimulant and stimulant concentration (Fig. 5c). I then determined the dose-dependency of J_V stimulation (Fig. 6a,b). When cAMP levels on the hemocoel side were increased from 1 to 20 mM, J_V increased linearly (Fig. 6a). But even at the highest concentrations of cAMP, rates were 70% of maximum values reached with CC or VG extracts (Fig. 6b). This may reflect a particularly low rate of cAMP diffusion across the plasma membrane of ileal cells or a rapid destruction of the small total amount of this cyclic nucleotide placed inside the ileal sacs. I did not test more hydrophobic analogues of cAMP because these were no more effective than cAMP in stimulating transport across locust recta (Phillips *et al.* 1986).

In contrast to cAMP, both CC and VG extracts stimulated J_V in a dose-dependent and saturable manner over the same range of 0.05 to 2.0 glands added to 10 µL of saline on the hemocoel side (Fig. 6b). This dose response range is similar to that previously reported for stimulation of rectal J_V by CC extracts (Proux *et al.* 1984). CTSH, the factor in CC which stimulates rectal Cl^- transport and a probable agent which increases rectal J_V, is a 8,000 Dalton neuropeptide (Phillips *et al.* 1980). To determine if the stimulants of ileal J_V were also proteinaceous, I exposed CC and VG extracts to trypsin at 30° C and pH 7.1 for 2 h prior to J_V assays (Table 2). This pretreatment destroyed all of the activity in VG extracts and 66% of that in CC compared with controls. Therefore the major active agents in CC and VG which stimulate ileal J_V are also apparently neuropeptides. Similar results were obtained using stimulation of ileal I_{SC} (i.e. Cl^-

Table 2. The effect of trypsin treatment on ability of CC and VG extracts to stimulate steady- state fluid transport across everted ileal sacs (mean ± s.e., n=8).

Treatment	Fluid transport experimental ($\mu L \cdot h^{-1} \cdot ileum^{-1}$)	Fluid transport experimental-control ($\mu L \cdot h^{-1} \cdot ileum^{-1}$)	% of untreated rate
No Extract (Control)	3.4 ± 0.2		
0.5VG/10 µL	10.1 ± 0.7	6.8	100%
0.5VG/10 µL +trypsin*	3.2 ± 0.5**	0	0%
0.5CC/10 µL	16.8 ± 1.8	13.0	100%
0.5CC/10 µL +trypsin*	7.8 ± 0.5**	4.4	34%

*Trypsin present at 1 mg/mL for 2 h at 30°C and pH 7.1.

** Significantly different ($P<0.05$) from comparable treatment without trypsin.

transport) as a bioassay of CC and VG (Audsley *et al.* 1988).

Activity in separated lobes of corpus cardiacum (CC)

Nervous (storage, NCC) and, to a much lesser extent, glandular lobes (GCC) of locust CC both contain stimulants of Cl^- transport in locust rectum (Phillips *et al.* 1980;reviewed by Phillips *et al.* 1986) and in locust ilea (Audsley *et al.* 1988). Using everted rectal sacs to study fluid transport, Proux *et al* . (1984) observed equal stimulatory activity in both locust NCC and GCC. In the present study, GCC stimulated J_V across everted ileal sacs 33% more than did NCC. Recall that unstimulated J_V was consistantly between 3.0 and 3.5 $\mu L \cdot h^{-1} \cdot ileum^{-1}$ (Fig.5a,c). Steady state J_V over the 2nd to 5th h was increased 3-fold (8.9 ± 0.3 $\mu L \cdot h^{-1} \cdot ileum^{-1}$) in the presence of 0.5 NCC and 4-fold (11.9 ± 0.7 $\mu L \cdot h^{-1} \cdot ileum^{-1}$) when 0.5 GCC was present (n=20; Fig. 7). The small difference in mean activity in the two lobes was significant ($P< 0.05$).

Fluid transport against osmotic concentration differences

Many epithelia are capable of secondary active transport of fluid by local osmosis, driven by salt transport, against small transepithelial osmotic concentration differences (Δosmol). Because a passive osmotic flux in the opposite direction increases with Δosmol, net fluid transport (J_V) typically falls with increasing Δosmol until an equilibrium point (i.e. Δosmol when $J_V=0$) is reached. This point reflects the strength of fluid transport. The slope of the line (J_V/Δosmol) indicates the apparent osmotic permeablity (P_{osm}) of the epithelium. Epithelia also commonly exhibit rectification of J_V; i.e. the apparent osmotic permeablity is much less above than below the equilibrium point. Hanrahan and Phillips (1985) observed a 40% decrease in transepithelial conductivity (G_t) when flat sheet locust recta were exposed to hyperosmotic saline (1220 mosmol). I investigated these relationships in locust ileum and studied the effect of stimulants on the osmotic permeablity (Fig. 8).

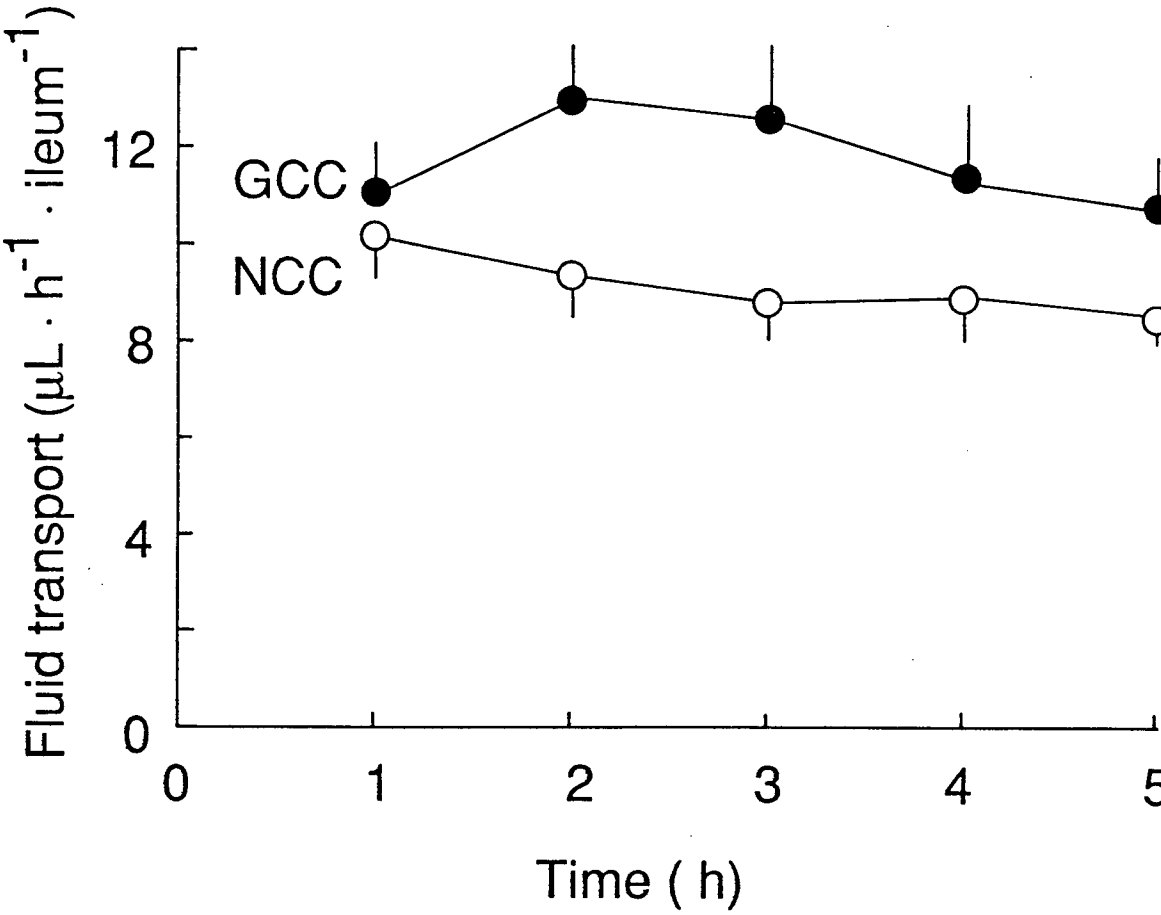


Figure 7. The effects of nervous (NCC) and glandular (GCC) lobes of corpora cardiacum (0.5 lobe/ 10 μL) on fluid absorption by everted ileal sacs bathed in physiological saline (mean ± s.e., n=4).

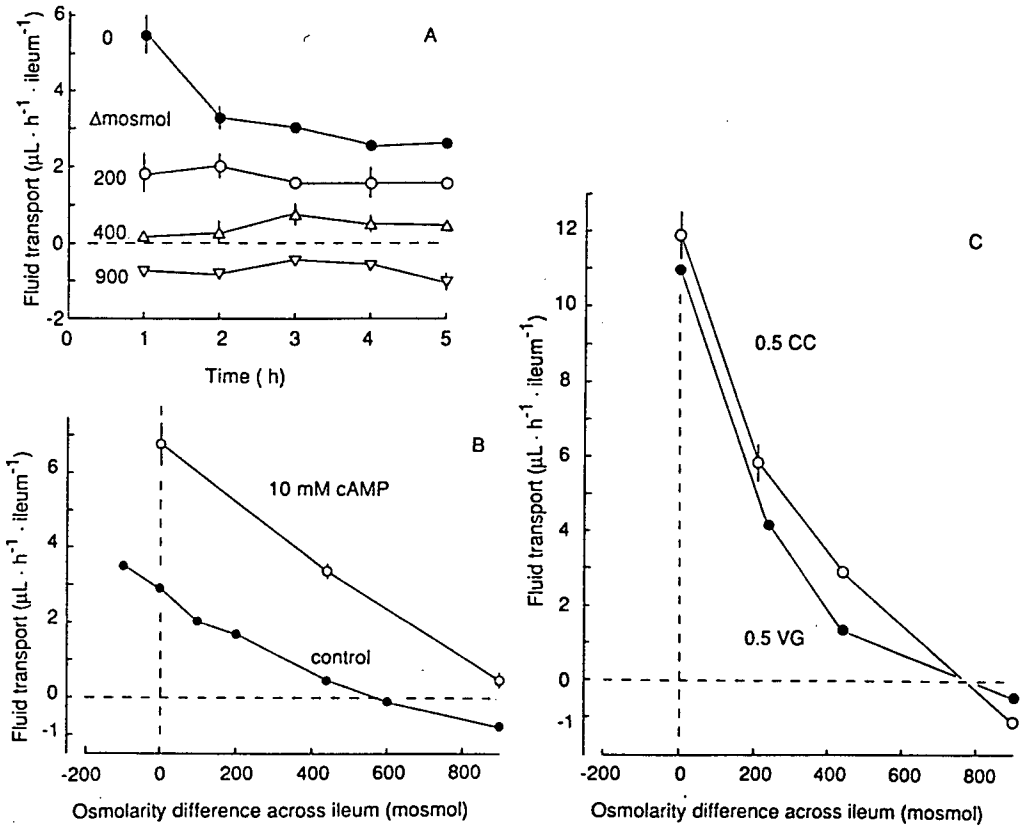


Figure 8. The effect of various osmotic concentration differences (lumen side made hyperosmotic with sucrose) on long-term absorption of fluid across everted ileal sacs. The sign refers to lumen osmotic concentration minus hemocoel concentration. a) The average rate of fluid absorption with time is shown for selected osmotic concentration differences to illustrate that uptake is nearly constant after the first hour, Δmosmol= 0 (●), 200 (○), 400 (Δ), 900 (∇); b) Steady-state rates of fluid absorption (i.e. average of second to fifth h) as a function of osmotic concentration differences for control sacs (●) and those exposed to 10 mM cAMP (○), or (c) to 0.5 CC/10 μL (○) and 0.5 VG/10 μL (●) on the hemocoel side. (mean ± s.e. n=16 on 4 preparations).

After the first h, rates of net fluid movement were nearly constant with time regardless of the osmotic concentration difference across the ileum (Fig. 8a) and tissue volume also did not change significantly after the first h during these studies (data not shown). Steady-state J_V (2nd to 5th h) is plotted as a function of Δosmol (lumen side hyperosmotic to hemocoel side) in Fig. 8b,c. Unstimulated ilea can transport fluid against unusually large osmotic concentration differences. The equlibrium Δmosmol value equals 600 mosmol as compared to a Δmosmol of 400 for everted rectal sacs under similar conditions (reviewed by Phillips *et al.* 1986), a Δmosmol of 80 for rabbit gallbladder (Diamond 1964) and a Δmosmol of 110 for rat ileum (Parsons & Wingate 1964). Above the equilibrium point, the apparent osmotic permeability of locust ileum is 13 $\mu L \cdot h^{-1} \cdot \Delta osmol^{-1} \cdot cm^{-2}$ compared to 33 $\mu L \cdot h^{-1} \cdot \Delta osmol^{-1} \cdot cm^{-2}$ for locust rectum (Goh & Phillips 1978). Below the equilibrium point, the apparent osmotic permeability of locust ileum is 6 $\mu L \cdot h^{-1} \cdot \Delta osmol^{-1} \cdot cm^{-2}$ compared to 12 $\mu L \cdot h^{-1} \cdot \Delta osmol^{-1} \cdot cm^{-2}$ for locust rectum. Cyclic AMP (10 mM) increases the J_V at all values of Δosmol and the equilibrium point is raised by more than 50% (to about 1000 mosmol) with a 50% increase in apparent osmotic permeablity (18 $\mu L \cdot h^{-1} \cdot \Delta osmol^{-1} \cdot cm^{-2}$ with cAMP).

Exposure to CC or VG extracts at a dosage of 0.5 glands also stimulated ileal J_V at all Δosmol values (Fig. 8c), but the relationship differed from that observed with 10 mM cAMP. Extracts of CC and VG caused a greater stimulation of J_V when Δosmol was small, whereas the equilibrium Δmosmol of 750 was actually slightly lower than that with cAMP (about 1000 mosmol). These differences reflect a marked curvilinear relationship of ileal $J_V/\Delta osmol$ (i.e. indicating much greater rectification) caused by the two glandular extracts. These results suggest that VG and CC cause much larger increases in passive osmotic permeability of locust ileum (i.e. 53 $\mu L \cdot h^{-1} \cdot \Delta osmol^{-1} \cdot cm^{-2}$) than does cAMP when the osmotic gradients are small (Δmosmol = 0-200). Moreover, VG and CC stimulate the solute-driven active component of J_V (i.e. values at Δosmol=0) much more strongly than cAMP. The two actions of the glandular extracts on ileal J_V

which are apparent from this analysis could reflect the presence of more than one stimulant or activation of more than one second messenger system (e.g. cAMP plus Ca^{2+}) by a single neuropeptide. Audsley *et al.* (1988) have shown that known or putative neurotransmitter substances do not stimulate Cl^--dependent I_{sc} in locust ileum, and hence these agents are unlikely to be involved in stimulation of the active component of J_v in this epithelium by CC and VG.

Absorbate osmolarity

Current models for epithelial fluid transport by solute-driven local osmosis predict that the transported fluid emerging on the blood side (i.e. absorbate) should be at least slightly hyperosmotic to the external saline (Wall & Oschman 1975). However, many insect recta have the unusual ability to transport an absorbate which is 20-30% hyposmotic to the external saline (under unstimulated conditions): This is believed to be due to ion recycling within the elaborate lateral intercellular channels and via a secondary epithelial layer (reviewed by Phillips *et al.* 1986). A more typical hyperosmotic absorbate might be anticipated for the locust ileum, which lacks the elaborate lateral membrane system and secondary cell layer present in the locust rectum (Irvine *et al.* 1988).

I first demonstrated that absorbate osmolarity was relatively constant with time under various experimental conditions (Fig. 9a). As predicted from ileal ultrastructure and from fluid transport models, the absorbate remained 5% hyperosmotic to the bathing saline for control ilea when the initial Δmosmol was zero. Addition of 0.5 VG or 0.5 CC increased absorbate osmotic concentration to a value 15% hyperosmotic to external saline. This is consistent with the slight decline in the osmotic concentration which has been observed *in situ* as the gut contents move through ilea of several insects (eg. 10% decline in desert locust; Dow 1981: reviewed by Phillips *et al.* 1986). My *in vitro* results indicate that CC and VG factors should cause the urine leaving the ileum *in situ* to become slightly more dilute. The increase in osmotic concentration of absor-

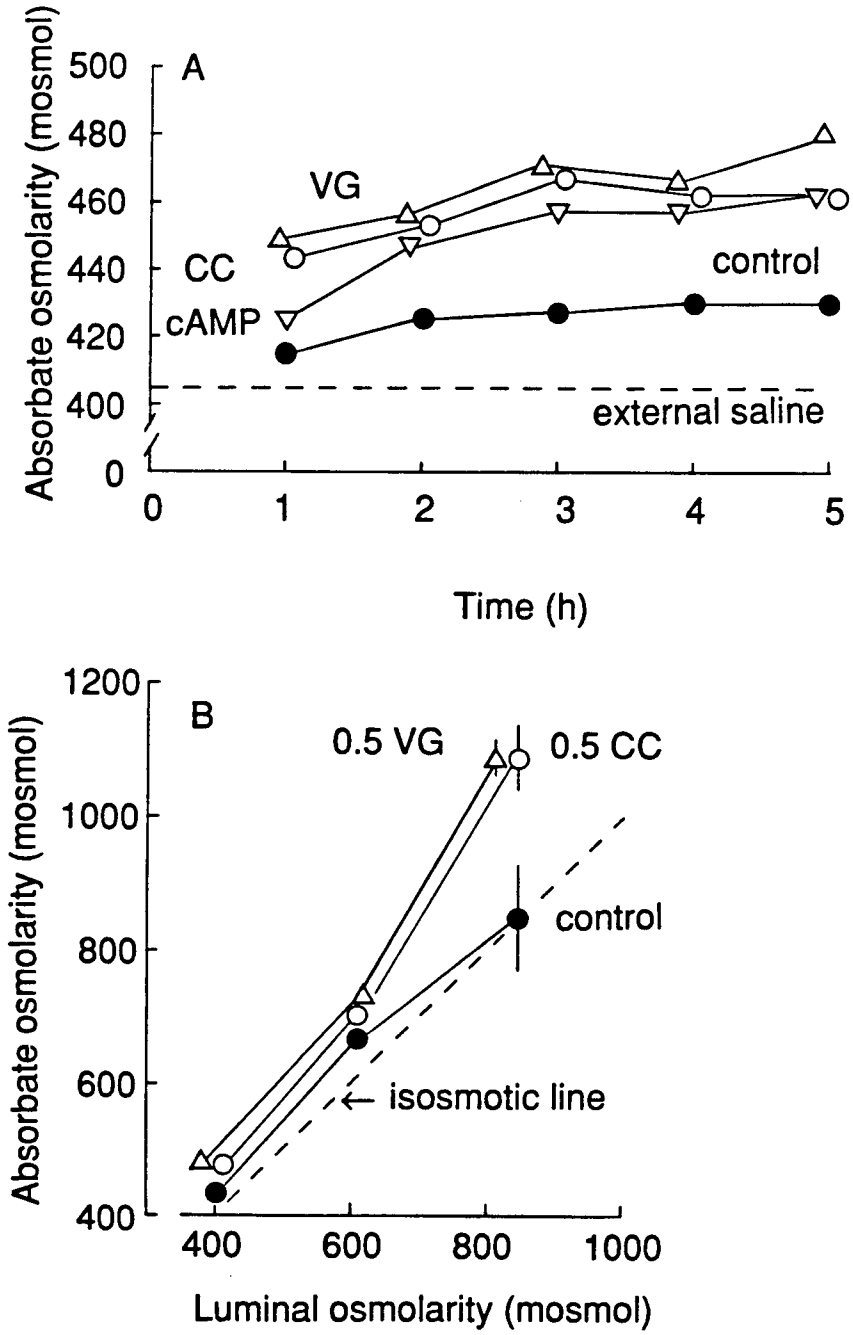


Figure 9. a) Osmolarity of absorbate with time for sacs exposed bilaterally to physiological saline (starting osmolarity = 405 mosmol): control (●), and with 10 mM cAMP (▽), 0.5 CC/10 µL (○), or 0.5 VG/10 µL (△) on the hemocoel side, (mean ± s.e., n=4): b) Absorbate osmolarity (average of 2-5 h) for ileal sacs exposed to physiological saline (control, ●), 0.5 CC/10 µL(○), or 0.5 VG/10 µL (△)on the hemocoel side (405 mosmol) when luminal osmolarity was varied from 405 to 880 mosmol; The broken line shows the isosmotic relationship between the two fluids (mean ± s.e. n= 16 on 4

bate caused by CC and VG extracts persisted when ileal J_v occurred against large osmotic gradients of up to 400 mosmol (Fig. 9b); indeed absorbate was in fact much more hyperosmotic, by 220 mosmol or 25% above saline level, when the opposing osmotic gradient was the greatest (i.e. near the equilbrium point; Fig. 8b).

DISCUSSION

I have established in this chapter that locust ileum can transport fluid against osmotic concentration gradients at very high rates which actually exceed those previously observed in the locust rectum (Goh & Phillips 1978). Likewise, the maximum factoral increase in fluid absorption caused by neuroendocrine factors from CC and VG was 5-fold for ileum (Fig. 6) compared to only a 2-fold increase in fluid absorption by rectal sacs exposed to CC extracts (Proux *et al.* 1984). Therefore regulation of fluid absorption in the ileum probably plays a much greater role in controlling water balance of locusts than previously supposed. Clearly measurements are required *in situ* to assess the ileal contribution to water balance more precisely.

My results establish the presence of proteinaceous stimulants in VG and CC which influence both the active component of fluid absorption and also the osmotic permeablity of the ileum, and these factors may act at least partly through cAMP as second messanger. However it must still be demonstrated that stimulants are normally released *in situ* from VG and CC into the hemolymph at levels sufficient to influence ileal J_v and that these factors do cause the elevation of cAMP levels in ileal tissue coincidental with J_v increases. There is some indirect support for this hypothesis. The release of a CTSH-like factor into the hemolymph has been observed from locust CC, which caused an elevation of cAMP levels in rectal cells concurrent with a rise in rectal I_{sc} (Spring & Phillips 1980c; Chamberlin & Phillips 1988). Since CC extracts and cAMP have the same broad range of effects (i.e. stimulation of Cl^- transport, J_v, I_{sc}, V_t, G_t, potassium permeablity and inhibition of acid secretion) on both locust ileum and rectum (Irvine *et*

al. 1988), it seems reasonable to propose that the same factors may stimulate salt and hence fluid transport in both hindgut segments. Moreover NCC and GCC have the same relative stimulatory effect on both gut segments using two assays (I_{sc} and J_v). In the next chapter I provide some evidence that at least one of the effects of CC and VG on J_v is through stimulation of Cl^- transport, as previously reported by Proux *et al.* (1984) for locust rectum. Currently there is no equilvalent circumstantial evidence that stimulants in VG normally control ileal absorption *in situ.*

I was unable to detect differences between VG and CC actions on fluid transport in locust ileum. Similarly Audsley *et al.* (1988) report that cAMP, CC, and VG extracts have the same board range of actions on transport processes across flat-sheet preparations of locust hindgut; however, active agents from the two glandular sources are apparently somewhat different. For example, the duration of I_{sc} stimulation is much shorter following VG stimulation as compared to that observed with CC extracts (Audsley *et al.* 1988). Secondly VG stimulatory activity was completely destroyed by boiling extracts for only 1 min, whereas boiling for 10 min was required to reduce activity in CC extracts. The structural differences between stimulants from NCC, GCC and VG will only be resolved by purification and determination of molecular structure of these factors.

This study demonstrates the expected correlation between absorbate osmotic concentrations and ultrastructural differences observed between locust ileum and rectum (see Fig. 2, chapter 1; Irvine *et al.* 1988). The lateral cell membranes in the rectum are highly infolded and closely associated with mitochondria. Consequently long intercellular channels are present. It is generally accepted that the unusual ability of insect recta to concentrate the urine by absorbing a fluid hyposmotic to the lumen contents is associated with ion recycling at the extensive lateral intercellular borders in this tissue (Phillips *et al.* 1986). A hyperosmotic absorbate is first formed within lateral spaces by local osmosis, driven by ion transport, and ions are subsequently reabsorbed into the rectal cells

as fluid flows in the lateral channels toward the hemocoel side; consequently the absorbate becomes hyposmotic relative to the luminal saline. Three distinct regions of lateral membrane have been demonstrated in the rectum. Na^+,K^+- ATPase which may be important in ion recycling has been localized at this site (Lechleitner & Phillips 1988; reviewed by Phillips *et al.* 1986; Gupta & Hall 1981). The locust ileum lacks extensive lateral membranes. The whole basolateral cell border appears homogeneous with short narrow infoldings found equally in all regions (Fig. 2, chapter 1; Irvine *et al.* 1988). As expected for simple local osmosis driven by salt transport (without ion recycling) at these basolateral infoldings, the absorbate was hyperosmotic to the luminal saline under all conditions (Fig. 9a,b). I suggest that local osmosis is driven by salt transport occurs across the short basalateral infoldings without extensive ion recycling.

SUMMARY

Ileal fluid transport (3.0 to 3.5 $\mu L \cdot h^{-1} \cdot ileum^{-1}$) and tissue volume were nearly constant after the first h of incubation in physiological saline. Inhibition of absorption by KCN + IAA, and by azide indicated metabolic dependence of fluid transport. Fluid absorption occurred against osmotic concentration differences of up to 600 mosmol (luminal osmolarity > hemocoel osmolarity). Fluid absorption is stimulated by cAMP, by both nervous (NCC) and glandular (GCC) lobes of corpus cardiacum (CC), and by fifth ventral ganglia (VG) in a dose-dependent manner. All stimulants caused ilea to absorb against larger osmotic gradients. Stimulants in CC and VG extracts increased the osmotic permeablity (P_{osm}) of the ileal wall at small Δosmol values while cAMP had a much smaller effect on P_{osm}. The absorbate remained hyperosmotic to the external saline under all conditions and stimulants caused an increase in absorbate osmolarity.

CHAPTER 3: Composition of Ileal Absorbate

INTRODUCTION

Irvine *et al.* (1988) used locust ilea mounted as flat sheets in Ussing chambers to observe rates of ion flux and short-circuit current across this tissue. Per gross surface area the rates observed were similar to or greater than those for the rectum. Cyclic AMP, the second messenger for many neuropeptide hormones, caused an increase in electrogenic transport of Cl^- by 10-fold, leading to a dramatic elevation of short-circuit current (I_{sc}), transepithelial potential (V_t, hemocoel negative) and transepithelial conductance (G_t). Cyclic AMP also increased passive permeablilty to K^+, and caused an abrupt switch from active secretion of H^+ to that of OH^- secretion into the hindgut lumen. These changes in transfer of acid-base equivilents are believed to be associated with regulation of hemolymph pH *in vivo* (Thomson & Phillips 1988). Audsley *et al.* (1988) surveyed the locust neuroendocrine system and found proteinaceous factors which stimulated ileal I_{sc} and V_t in extracts of corpus cardiacum (CC) and ventral abdominal ganglia 4 to 7, with maximium activity in number 5 (VG).

Fluid transport across most epithelia (J_v) against, or in the absence of, osmotic concentration differences (Δmosmol) is normally the result of ion transport (Spring 1983). In the previous chapter I have demonstrated that agents which stimulate ileal NaCl and KCl reabsorption across short-circuited ileal preparations (namely cAMP, CC and VG) also increased J_v across everted locust ileal sacs against larger Δmosmol values.

In all previous studies, locust ilea were exposed bilaterally to a NaCl-rich saline resembling hemolymph. While fluid entering the locust ileum *in situ* can be high in NaCl under unusual circumstances (Phillips 1964b,c), normally this fluid contains 100-140 mM K^+ and only 20-40 mM Na^+ (Hanrahan *et al.* 1984). Therefore in this chap-

ter I address three related questions: (a) what effect does luminal Na^+:K^+ ratio have on stimulation of ileal J_v by CC and VG, (b) do factors in these neuroendocrine extracts and cAMP stimulate ion transport processes similarly (i.e. how do these factors change the ionic composition of the absorbed fluid), (c) is ileal J_v dependent on (i.e. driven by) specific ion transport processes and does this specificity change after stimulation by CC and VG? For example, Proux *et al.* (1984) showed that stimulation of J_v across locust recta by CC specifically required Cl^-.

MATERIALS AND METHODS

Many of the methods used in this study were similar to those described in the preceding chapter, including maintenance of the *Schistocercia gregaria* colony, preparation of cannulated everted ileal sacs from female locusts, and determination of net transepithelial absorption of fluid (J_v) by weighing sacs at hourly intervals before and after completely removing the sac contents (i.e. hemocoel fluid). Sacs were filled hourly with 10 µL of fresh saline when the only determinations were of fluid absorption rate or transepithelial potential (V_t). Ileal sacs also transported fluid at comparable rates if saline was only initially placed on the outside of the sacs (3.0 ± 0.2 versus 2.8 ± 0.2 $\mu L \cdot h^{-1} \cdot ileum^{-1}$). I took advantage of this fact to collect absorbate directly from the sacs to make hourly determinations of ionic composition. Rates of ion absorption were calculated for each hour from changes in volume and ion concentrations of the internal fluid collected from the same preparations (Goh & Phillips 1978; Phillips *et al.* 1982).

Stimulation of J_v and V_t was achieved by including homogenate of whole corpora cardiaca (CC) and fifth ventral ganglia (VG) in the 10 µL of fresh saline placed inside the ileal sacs. The preparation of the CC and VG extracts were as described previously (chapter 2). The small amount of tissue added did not significantly alter the osmotic or ionic composition of the saline. Homogenates of CC and VG in 1 µL of fresh saline were placed hourly inside ileal sacs when ionic composition of the absorbate was deter-

mined; i.e. correction was made by subtracting the small quantities of ions present initially inside the sac from the large amount in the final contents. In all experiments, the 25 mL of external saline was bubbled with 95% O_2:5% CO_2 at pH 7.1 and 30°C.

Unless otherwise indicated, a control NaCl-rich saline (resembling locust hemolymph, Hanrahan *et al.* 1984) was placed on the hemocoel side in all experiments (mM):100 NaCl, 5 K_2SO_4, 10 $MgSO_4$, 10 $NaHCO_3$, 5 $CaCl_2$, 10 glucose, 100 sucrose (to adjust total osmolarity to hemolymph value), 2.9 alanine, 1.3 asparagine, 1.0 arginine, 5.0 glutamine, 11.4 glycine, 1.4 histidine, 1.4 lysine, 13.1 proline, 6.5 serine, 1.0 tyrosine, 1.8 valine, with pH of 7.1. The composition of saline placed on the lumen side was varied. KCl-rich salines with different K^+:Na^+ ratios were prepared by replacing all or a proportion of the Na^+ salts in the control saline with the equivalent K^+ salts. A chloride-free saline was prepared by replacing all Cl^- with nitrate. To further test ion dependence of J_v, salines were prepared which contained only one of Na^+, K^+ or Cl^- (Na^+ and K^+ were replaced with choline and Cl^- was replaced with gluconate in these salines). The total osmotic concentration was maintained at 405 mosmol in all salines by adjusting sucrose concentrations.

Electropotential difference (V_t) across the ileum was measured using two calomel electrodes in series with 3 M KCl-agar bridges. The electrodes were connected to a high input impedence differential amplifier (Beckman digital multimeter model HD110). The V_t was measured at the end of each hourly incubation period prior to replacing the saline within the ileal sacs.

Sodium and potassium concentrations in 1 µl samples were determined with a 'Techtron AA 120' flame spectrophotometer according to the method of Kaufman & Phillips (1973). Chloride concentration was determined by electrometric titration (Ramsay *et al.* 1955). Osmotic pressure was determined by a vapor pressure osmometer (Wescor model 5500, Logan, Utah). Total CO_2 in 10 µL samples of absorbate were estimated using a Carle gas analyser (model 101, Loveland, Colorado) as described by

Thomson and Phillips (1988) and mean HCO_3^- levels were calculated from the Henderson-Hasselbach equation according to Heisler (1986) using mean pH values measured in duplicate experiments with a pH microelectrode. Rates of ion absorption were calculated for each hour from the change in volume and concentration of the internal medium (Goh & Phillips 1978).

Statitical differences between means were determined by Student's t-test.

RESULTS

Ionic dependence of fluid tranport

Phillips *et al.* (1982a) found that fluid transport across unstimulated locust rectal sacs, when there was no intial osmotic gradient, required K^+, Na^+, and Cl^- for maximium transport rate. Rectal J_V was completely abolished after 1 h if all of these monovalent ions were absent, but any one of these three ions alone sustained J_V at near 50% of control rates. Repetition of these experiments (bilateral ion substitutions) on locust ileum yielded similar results (Fig. 10). The only difference was the small residual J_V across ileal sacs for 5 h when the bathing saline lacked bilaterally all but trace levels of Na^+, K^+ and Cl^-: conceivably tissue ions maintained the small J_V under this experimental condition.

I looked at ionic requirements for stimulation of ileal J_V by CC and VG extracts (Fig. 11). As previously observed for locust recta (Proux *et al.* 1984), ileal J_V was not significantly stimulated by CC or VG extracts, as compared to control sacs exposed to muscle homogenates, when all external Cl^- was replaced with nitrate (Fig 11a). Restoring 110 mM Cl^- to preparations after the first two hours under Cl^--free conditions lead to a 3- to 4-fold increase in J_V when either 0.5 VG or 0.5 CC was present on the hemocoel side. In contrast the addition of Cl^- had no effect on J_V across sacs exposed to muscle homogenates (Fig. 11b). These results are consistent with the hypothesis that CC and VG factors (eg. CTSH, Phillips *et al.* 1980) act on electrogenic Cl^- transport in

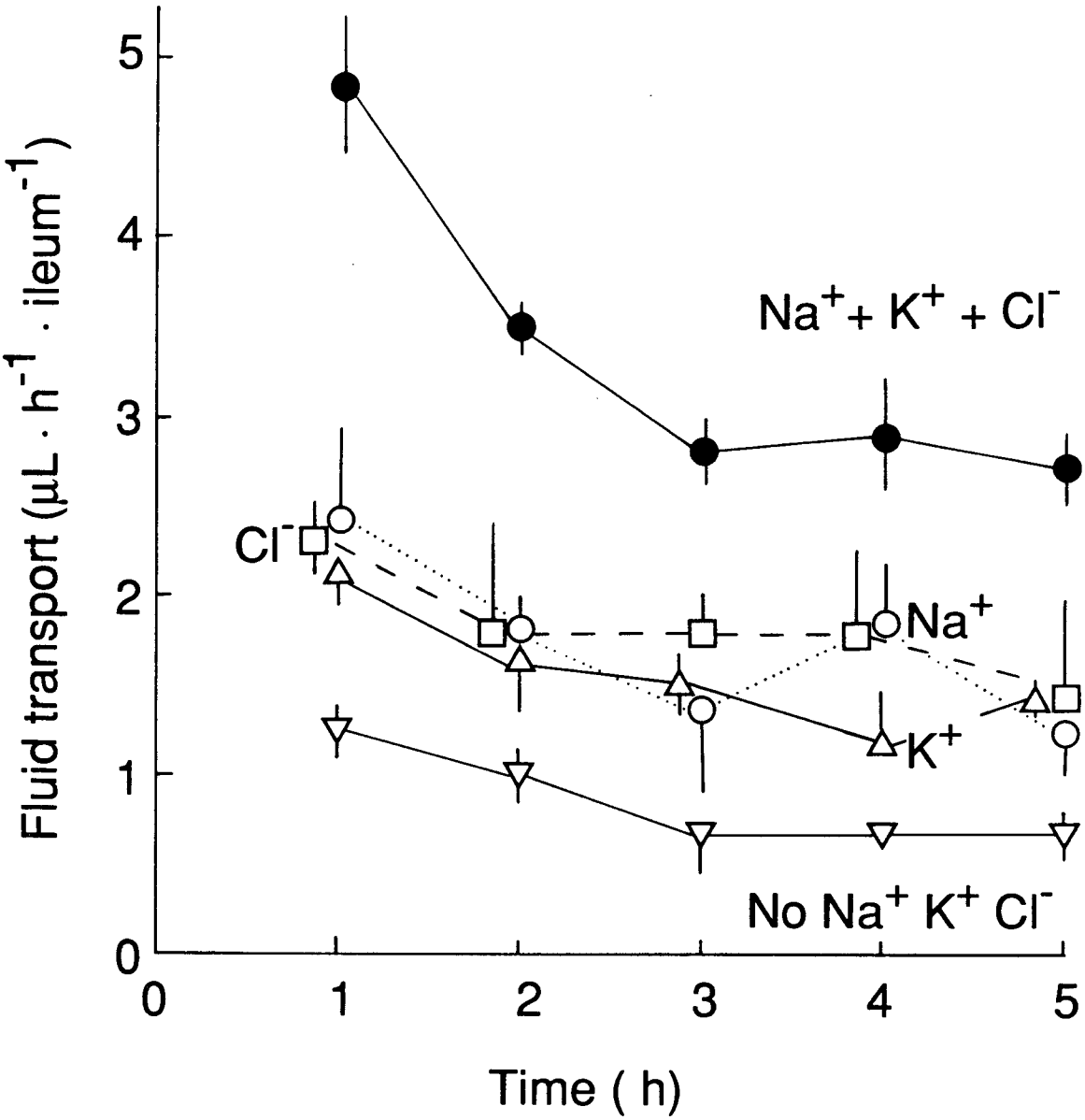


Figure 10. Fluid transport across everted ileal sacs with time after dissection in different salines. Ilea were incubated bilaterally with physiological saline (●) or experimental salines containing only one of the main monovalent ions (Na^+,○; K^+,△; Cl^-,□) or none of these (▽). All salines had the same osmotic concentration and the absorbate was collected hourly and new saline added to the hemocoel side (mean ± s.e., n=4-20).

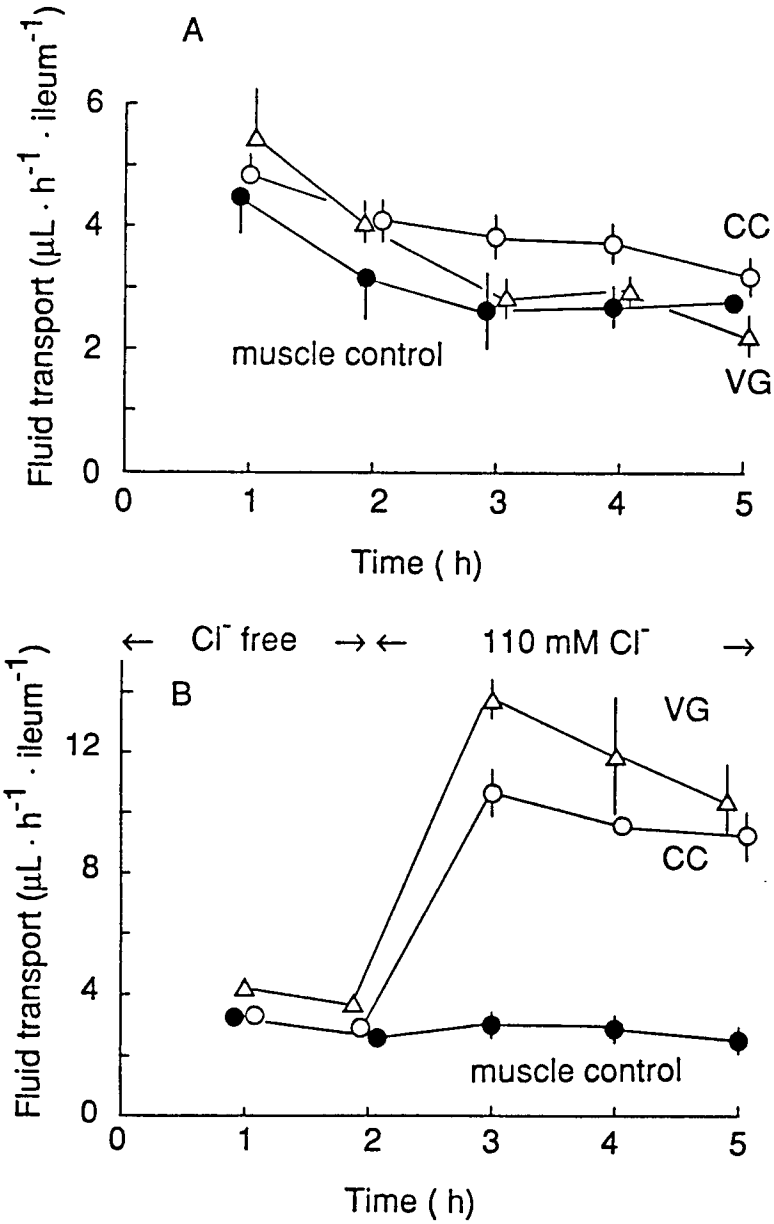


Figure 11. The Cl^--dependence of long-term fluid transport across everted ileal sacs. a) Sacs were exposed to Cl^--free saline over a 5-h period; Cl^--free saline was replaced hourly on the hemocoel side with muscle (●), with 0.5 CC extract (○), or with 0.5 VG extract (Δ): b)Cl^-free saline for the first 2 h and then placed in normal physiological saline (110 mM Cl^-). The hemocoel side was exposed to fresh saline hourly with muscle (●), with 0.5 CC (○), or 0.5 VG (Δ) in Cl^--free saline for the first 2 h and then in normal saline (110 mM Cl^-) during the third to fifth hours (mean ± s.e. n=4). Addition of Cl^- caused a significant ($P< 0.05$) increase in fluid transport in sacs exposed to 0.5CC/10 µL or 0.5 VG/10 µL; but not when exposed to muscle homogenates.

both locust ileum and rectum to drive secondary transport of fluid. In contrast to the absolute requirement for Cl^- to achieve stimulaton of J_v, a 2-fold increase in J_v still occurred after stimulation when either Na^+ or K^+ was absent on the luminal side (Fig. 12).

The effect of changing the Na^+:K^+ ratio on the lumen side from 0:110 to 110:0, while maintaining control saline resembling hemolymph on the inside of the sacs is summarized in Fig.12. Unstimulated ileal J_v was independent of luminal Na^+:K^+ ratio over a wide range of 110:10 to 10:110, the latter value reflecting the more common situation *in vivo*. However J_v decreased 50% if either Na^+ or K^+ was absent as discussed earlier. The pattern of stimulation by equal amounts of CC or VG extracts (0.5 glands in 10 μL) was similar. Increase in J_v was greatest (5-fold) when luminal concentrations of Na^+ and K^+ were equal (60 mM) but ΔJ_v was still substantial (3-fold stimulation) when the Na^+:K^+ ratio was either 110:10 or 10:110. Again, removing either Na^+ or K^+ completely from the lumen side only, drastically reduced stimulation by both CC and VG. Because large ion gradients were imposed across the ileal epithelium when high KCl saline was placed on the lumen side only, which might introduce a substantial diffusion driven J_v component, I carried out additional control experiments in which these ion gradients were abolished. When the same high KCl saline (Na^+:K^+ of 10:110) was placed hourly on both sides of the ileum, CC and VG caused equally large increases in J_v (4-fold) and unstimulated J_v was the same as J_v for sacs exposed to high NaCl saline on the hemocoel side (Fig. 12).

Ionic composition of ileal absorbate

Locust recta mounted in Ussing chambers preferentially absorb KCl rather than NaCl even when the luminal Na^+:K^+ ratio is 110:10 mM (Hanrahan & Phillips 1983). In contrast, locust ilea under similar conditions transported NaCl at much higher rates both before and after stimulation with cAMP (Irvine *et al.* 1988). However, it remained unclear (a) whether the ileum still transported a NaCl-rich absorbate when exposed to

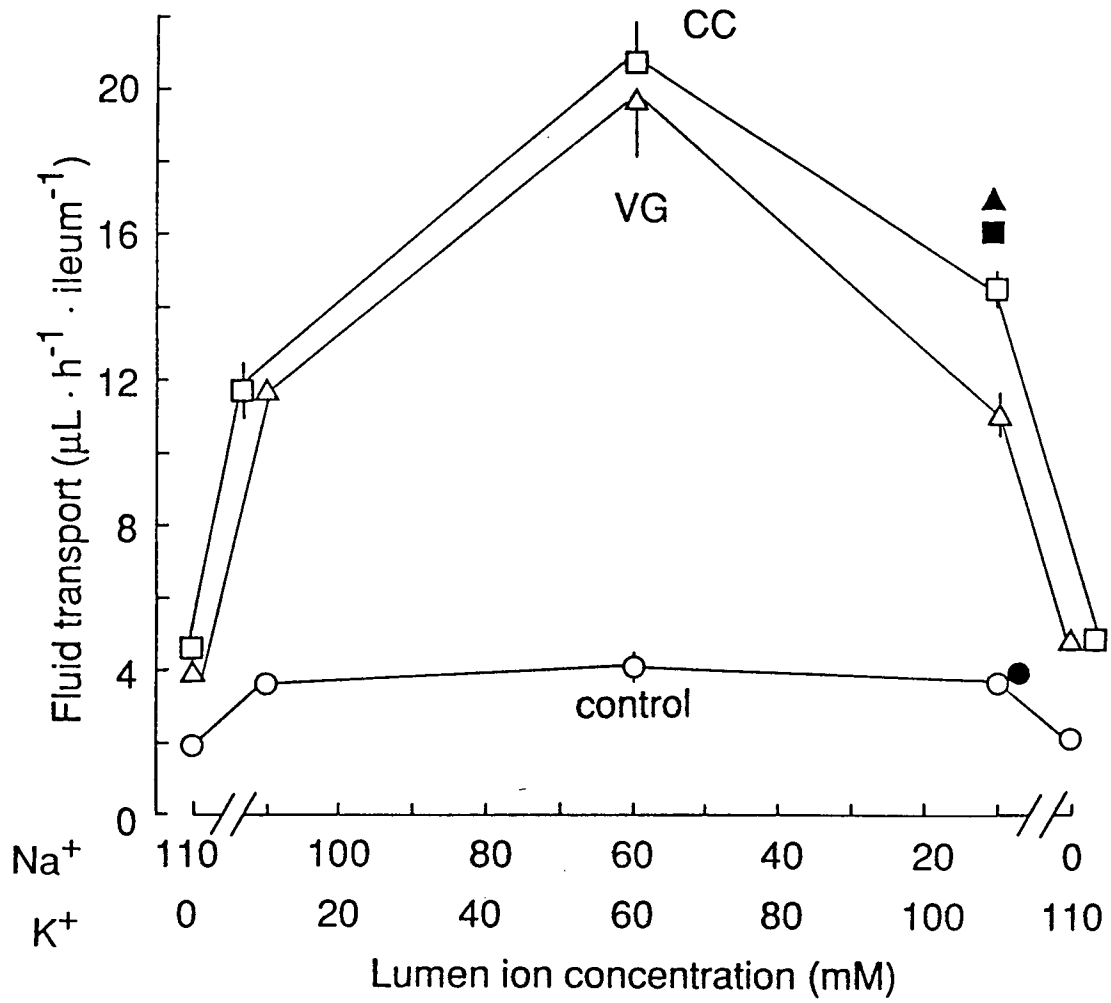


Figure 12. The effect of altering the ratio of Na^+:K^+ in the external saline on long-term (2 to 5 h) fluid transport across everted ileal sacs. Sacs were exposed to physiological saline (110 mM NaCl, 10 mM K^+) on the hemocoel side without extract (○), with 0.5 CC/10 µL (□) and with 0.5 VG/10 µL (Δ). Fluid transport for sacs exposed to high KCl bilaterally is also shown with sacs containing no extract (●), 0.5 CC/10 µL (■) or 0.5 VG/ 10µL (▲) (mean ± s.e., n=20).

more physiological high K^+:Na^+ ratios on the lumen side, and (b) whether CC and VG extracts might influence absorbate ionic composition somewhat differently than does cAMP.

As shown in Fig. 13 and 14, cAMP and both CC and VG homogenates changed ileal absorbate composition qualitatively in the same manner when high NaCl saline was present bilaterally. Absorbate Na^+ concentrations remained high (twice saline concentration) over the 5 h experimental period and were unaffected by CC and VG. In contrast, absorbate Cl^- levels declined with time when ilea were removed from natural stimulants presumably present *in situ*, suggesting that HCO_3^- and other anions were preferentially absorbed with Na^+ under unstimulated conditions. However, exposure to CC and VG homogenates completely reversed this situation so that absorbate Cl^- concentrations were elevated 4-fold to levels equal to those of Na^+. This change in anion accompanying Na^+ was the most dramatic change in absorbate composition caused by these stimulants. Absorbate K^+ concentrations were increased 3-fold by both CC and VG but the absorbate Na^+:K^+ ratio reamined high (8:1). Average rates of Na^+, K^+ and Cl^- absorption across ileal sacs over the 2nd to 5th h, calculated from absorbate concentrations and J_v values in Fig. 14 are presented in Table 3a. The addition of CC and VG caused an increase in the flux of all three ions with the flux of Cl^- equaling that of Na^+ under stimulated conditions. In summary while VG and CC caused quantitatively much larger increases in J_v and ion transport rates (Table 3a) than did high doses of cAMP (Fig. 13), the changes were not qualitatively different for all three stimulants.

The effect of low luminal Na^+:K^+ ratios (10:110 mM), which resembles those *in situ*, on ileal absorbate composition is shown in Fig. 15. Control saline resembling hemolymph was present initially inside ileal sacs in these experiments. Even when luminal Na^+ was low (10 mM) absorbate from unstimulated ilea contained largely Na^+ (160 mM) although K^+ concentrations were now considerably elevated (40 mM). High luminal K^+ was also associated with less decline in absorbate Cl^- with time than when

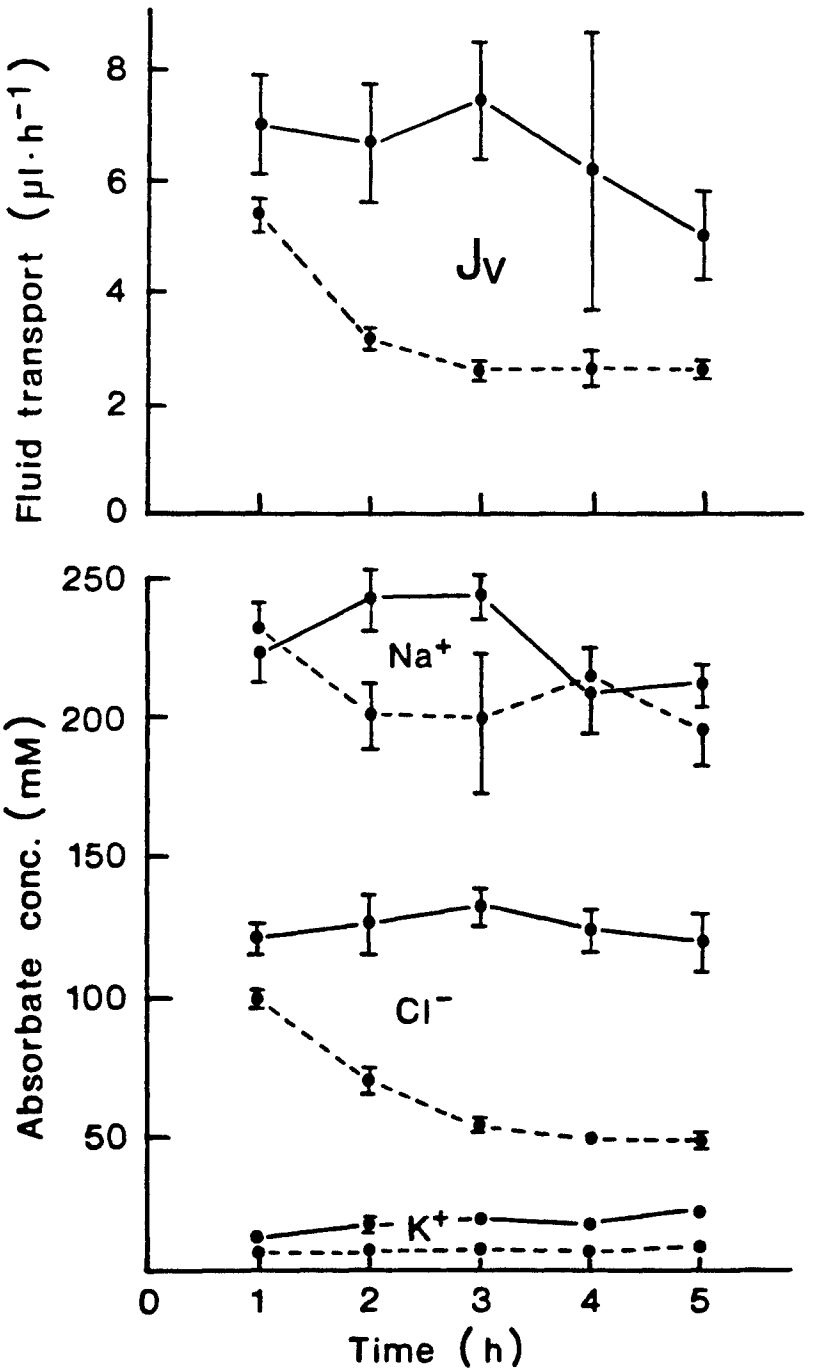


Figure 13. Influence of cAMP on fluid transport (J_V) and absorbate ion concentrations (Na^+, K^+, Cl^-) for ileal sacs exposed to high NaCl saline (110 mM Na^+, 10 mM K^+, 110 mM Cl^-) on lumen side. At the beginning of each h sacs contained no extract (●, solid lines), or contained 10 mM cAMP in NaCl saline (1 µL; ●, broken lines). Cyclic AMP caused a significant ($P<0.05$) increase in Cl^- and K^+ concentrations and in fluid transport (mean ± s.e., n=6).

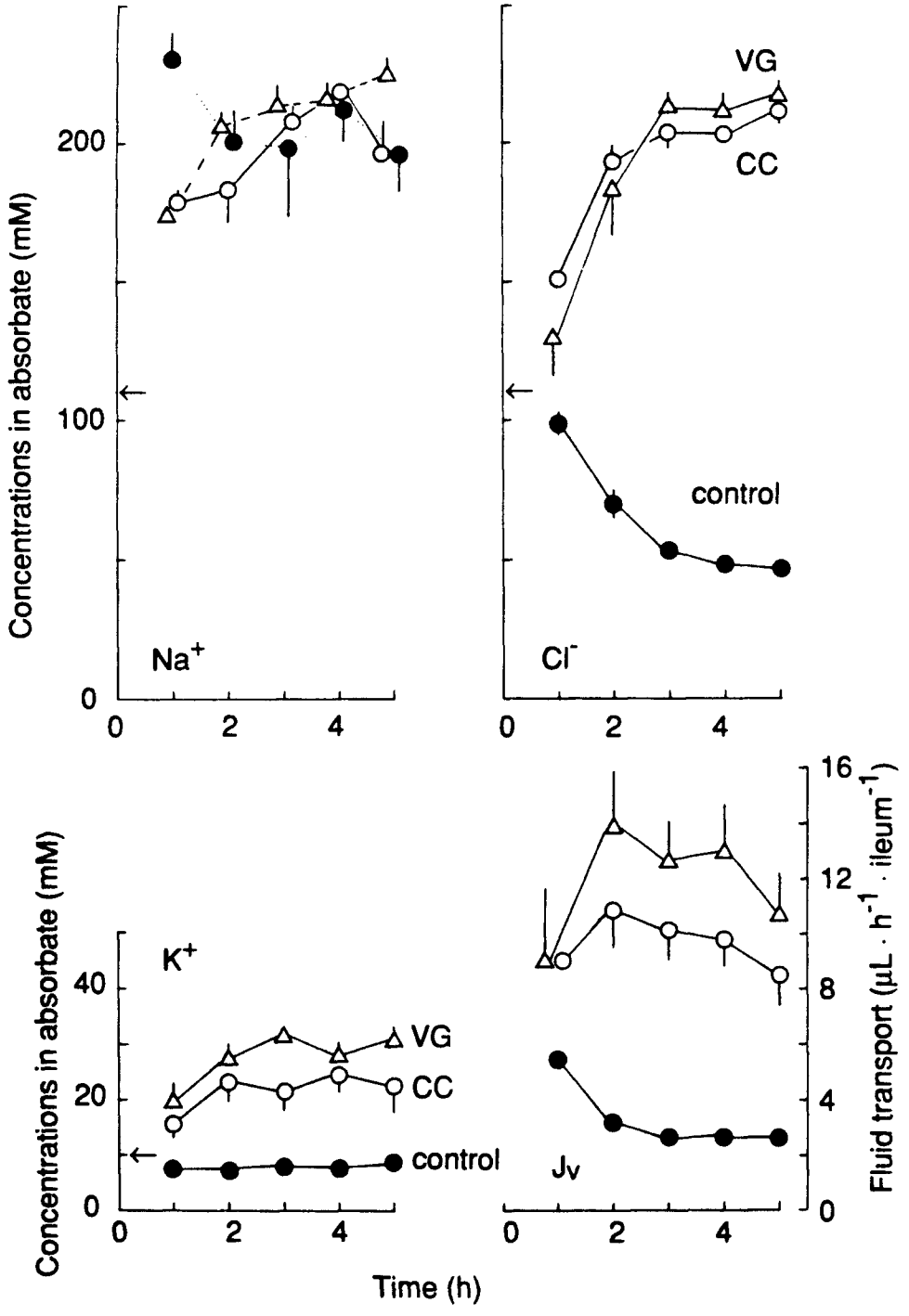


Figure 14. Influence of CC and VG on fluid transport (J_V) and absorbate ion concentrations (Na^+, K^+, Cl^-) for ileal sacs exposed to high NaCl saline (110 mM Na^+, 10 mM K^+, 110 mM Cl^-) on lumen side. At the beginning of each h sacs contained no extract (●), or contained NaCl saline (1 µL) with 0.05 CC extract (○), or 0.05 VG extract (Δ) . The addition of CC or VG caused a significant ($P<0.05$) increases in Cl^- and K^+ concentrations and in fluid transport. The horizontal arrow on each graph indicates the concentration in the luminal saline. (mean ± s.e., n=6).

Table 3. Long-term (2-5 h) rates of ion absorption ($\mu equiv \cdot h^{-1} \cdot ileum^{-1}$) across everted ileal sacs exposed to (a) high NaCl physiological saline (110 mM NaCl, 10 mM K^+, 405 mosmol) on the luminal side or (b) to high KCl physiological saline (110 mM KCl, 10 mM Na^+, 405 mosmol) on the luminal side and either no saline (control) or high NaCl physiological saline containing neural extracts on the hemocoel side (mean ± s.e., n=20).

a) NaCl saline

Ion	Control	0.05VG/1 µL	0.05CC/1 µL
Cl^-	0.18 ± 0.02	2.51 ± 0.20	2.01 ± 0.12
Na^+	0.46 ± 0.03	2.47 ± 0.20	1.96 ± 0.11
K^+	0.02 ± 0.01	0.35 ± 0.04	0.24 ± 0.03
Total CO_2	0.16 ± 0.02	0.12 ± 0.04	0.12 ± 0.02

b) KCl saline

Ion	Control	0.05VG/1 µL	0.05CC/1 µL
Cl^-	0.30 ± 0.02	2.38 ± 0.25	2.82 ± 0.12
Na^+	0.75 ± 0.05	2.25 ± 0.19	2.30 ± 0.09
K^+	0.15 ± 0.01	0.81 ± 0.10	0.99 ± 0.07

All VG and CC values are significantly different ($P<0.05$) from controls

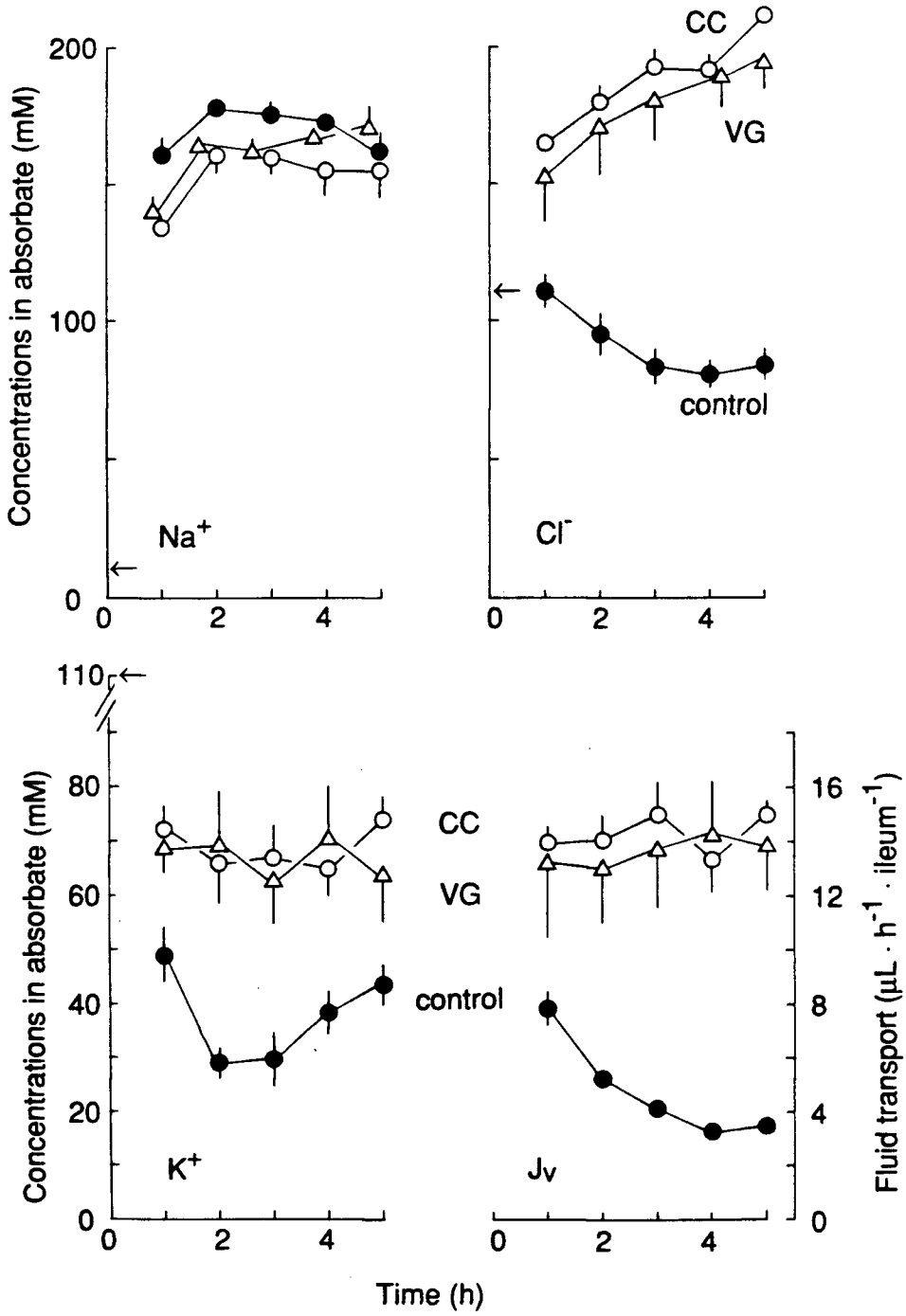


Figure 15. Influence of CC and VG on fluid transport (J_V) and absorbate ion concentrations (Na^+, K^+, Cl^-) for ileal sacs when exposed to high KCl saline (110 mM KCl, 10 mM Na^+) on the lumen side. At the beginning of each h sacs contained no extract (●), or contained NaCl saline (1 µL) with 0.05 CC extract (○), or 0.05 VG extract (Δ). The addition of CC or VG caused a significant ($P<0.05$) increases in Cl^- and K^+ concentrations and in fluid transport. The horizontal arrow on each graph indicates the concentration in the luminal saline (mean ± s.e., n=6).

Na^+ was the predominant external cation. Stimulation with CC and VG caused qualitatively similar effects whether the luminal Na^+:K^+ ratio was low (Fig. 15) or high (Fig. 14); i.e. absorbate concentrations of Cl^- and K^+ were elevated, Na^+ levels did not change, and the anion deficit (Na^++K^+-Cl^-) was decreased. Average rates of ion absorption from KCl-rich luminal fluid over the 2nd to 5th h, calculated from data in Fig. 15, are presented in Table 3b for comparison with values when sacs are exposed to a NaCl-rich saline (Table 3a). To summarize, both CC and VG factors stimulate K^+ absorption more when luminal K^+ levels are at high levels observed *in situ*, but the principal effect of all stimulants is to preferentially increase rates of ileal NaCl absorption regardless of luminal cation ratios.

Effect of stimulants on absorbate bicarbonate and pH

There was a large anion deficit in absorbate from unstimulated ileal sacs which was greatly reduced by stimulation with CC and VG (Figs. 14,15; Table 3) and cAMP (Fig. 13). Other observations suggested that part of the anion deficit may be due to HCO_3^- absorption under unstimulated conditions. To explain, locust recta contain a powerful electrogenic proton pump in the apical membrane which causes acidification of luminal contents and net transfer of base equivalents (OH^-/HCO_3^-) to the hemocoel side (Thomson *et al.* 1988b; Thomson and Phillips 1988; Phillips *et al.* 1986). Preliminary results indicate a similar situation for the locust ileum. Irvine *et al.* (1988) found that cAMP caused an abrupt change from H^+ to OH^- secretion at high rates across short-cicuited locust ilea in Ussing chambers and bathed in HCO_3^-/CO_2-free saline. Assuming that OH^- should form HCO_3^- when external CO_2 is present, the results of Irvine *et al.* (1988) predict that stimulants should decrease HCO_3^- levels in ileal absorbate.

I tested this prediction by measuring total CO_2 content and pH of absorbate in the presence and absence of stimulants (Fig. 16). Total CO_2 levels were high (45 mM) and constant with time in absorbate from unstimulated ileal sacs as predicted by earlier

studies. As also predicted by Irvine *et al.* (1988), CC and VG both dramatically decreased total CO_2 concentrations in the absorbate to less than 10 mM (Fig. 16). However, this decline was largely the consquence of greatly increased J_v, because calculated rates of total CO_2 transport (Table 3a) declined only slightly as compared to control. The transported CO_2 was largely in the form of HCO_3^- because the measured pH of the ileal absorbate in duplicate experiments was 7.75 ± 0.08 for controls and 7.00 ± 0.26 and 7.20 ± 0.15 respectively when CC and VG were present (n=4). Using the Henderson-Hasselbach equation, HCO_3^- constituted 88-98% of the total absorbate CO_2.

Transepithelial potentials

The transepithelial electropotential (V_t) across the ileal epithelia could drive a passive component of ion absorption. I therefore determined V_t hourly across ileal sacs bathed bilaterally in control saline with and without CC, VG and muscle extracts (Fig. 17). As previously observed for rectal sacs (Proux *et al.* 1984) and for both locust hindgut segments in Ussing chambers (Phillips *et al.* 1986; Irvine *et al.* 1988), V_t across unstimulated ileal sacs was 15-18 mV, hemocoel side negative. This is consistent with the observation that net active flux of $^{36}Cl^-$ is electrogenic and exceeds that of $^{22}Na^+$ across both hindgut segments under short-circuit conditions. Net Na^+ absorption rates are much greater in locust ileum compared to rectum and Na^+ transport in ileum is largely electroneutral (chapter 4). Since earlier experiments indicated that CC and VG stimulate Cl^- transport proportionately more than that of cation transport, it was not suprising that these agents caused V_t to increase 3-fold to 45 mV across the everted sacs. In contrast, the addition of muscle extract had no effect on V_t (Fig. 17). While these results indicated direct stimulation of electrogenic Cl^- transport by factors in CC and VG, it also follows that at least some of the corresponding increase in Na^+ and K^+ absorption could be achieved indirectly through electrical coupling caused by the larger V_t. An ileal V_t of 45 mV is more than enough to permit K^+ absorption by net diffusion against the 2-

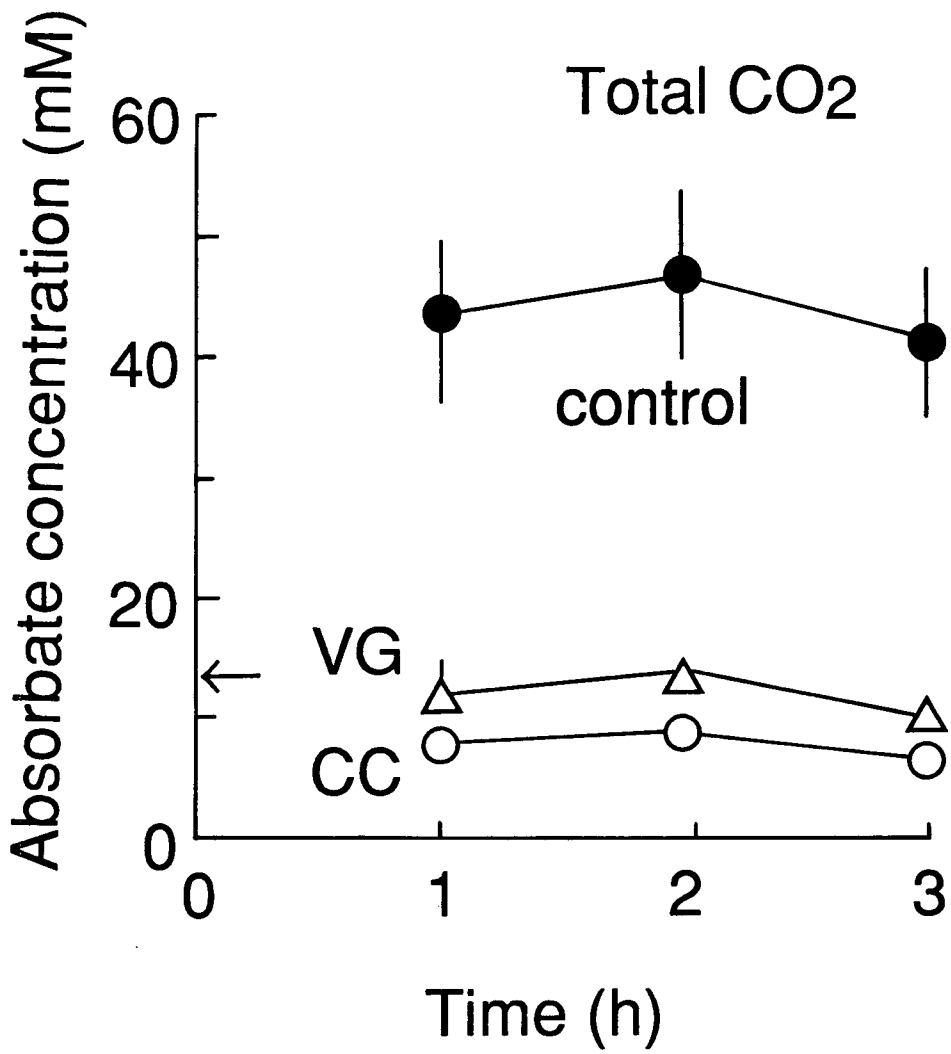


Figure 16. The effect of CC and VG on total CO_2 in absorbate with time for ileal sacs exposed to high NaCl saline (110 mM NaCl, 10 mM K^+) on the lumen side. High NaCl (10 µL) containing no extract (●), 0.05 CC extract (○), or 0.05 VG extract (Δ) was placed in the hemocoel compartment hourly. Both CC and VG caused a significant ($P< 0.05$) decrease in absorbate total CO_2. The horizontal arrow on the graph indicates the concentration in the external saline (mean ± s.e., n=6).

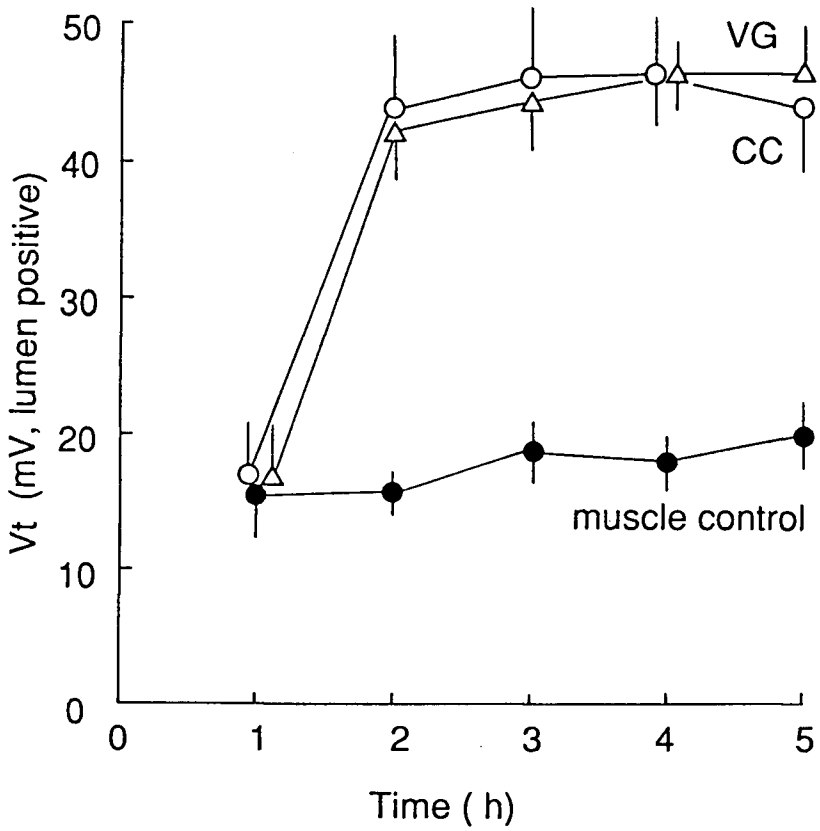


Figure 17. Effect of CC and VG extracts on potential difference (V_t) across everted ileal sacs. Saline containing 0.5 CC (○), 0.5 VG (Δ) or muscle (●) was added hourly after the first hour. V_t was measured hourly prior to replacing the saline (mean ± s.e., n=4). CC extracts and VG extracts caused a significant increase in the transepithelial V_t ($P< 0.001$).

to 3-fold concentration differences (absorbate relative to luminal levels) observed under these conditions (Fig. 14).

DISCUSSION

The homogenates of CC and VG have now both been shown to have the same broad range of actions as does cAMP on the ileum, including: 1) increases in electrical parameters (I_{sc}, V_t, and G_t), 2) increases in fluid transport against greater concentration gradients, and 3) changes in transfer of salts and acid-base equivalents (Irvine *et al.* 1988 and this chapter). The neuropeptide factors in CC and VG which stimulate salt and water transport across locust ileum are apparently somewhat different molecules based on preliminary studies by Audsley *et al.* (1988) using I_{sc} as a bioassay. Therefore the simplest working hypothesis is that CC and VG stimulants both act through cAMP as a second messenger to directly stimulate salt transport and consequently also fluid transport. In support of this interpretation, direct elevation of intracellular cAMP levels by stimulation of adenylate cyclase with forskolin (μM) or by inhibition of phosphodiesterase with theophylline (mM), both mimick the actions of exogenous stimulants (cAMP, CC, VG) on ileal I_{sc}, V_t and G_t (Audsley *et al.* 1988). One possibility is that the more heat-labile VG factor is simply a larger precursor of a CC stimulant, possibly CTSH which has been partially purified (Phillips *et al.* 1980).

The range of actions of cAMP and CC homogenates on salt, fluid and acid-base transfer across the locust ileum (Irvine *et al.* 1988 and this chapter) are very similar to those previously shown for locust rectum (reviewed by Phillips *et al.* 1986). Given the similar properties of ion and fluid transport in these two hindgut segments and their responses to CC and cAMP, the simplest working hypothesis is that the same neuropeptides stimulate both parts of the gut. However, the two locust hindgut segments do differ in some respects. First, stimulants increase Na^+ absorption across the ileum (i.e. both flat-sheet and sac preparations; Fig. 14 & 15; chapter 4) but not across recta under

short-circuit conditions (i.e. flat sheets; Black *et al.* 1987). Likewise, rectal Na^+ absorption across flat-sheet preparations under open-circuit conditions was not stimulated by the addition of 1 mM cAMP (Lechleitner, unpublished observation). Purification of the active factors in CC and VG are necessary to determine whether ileal Na^+ absorption is stimulated by different agents from those acting on KCl and fluid absorption in locust ileum and rectum.

A second possible difference concerns the action of VG homogenates on the two locust hindgut segments. Proux *et al.* (1985) observed negligible stimulation of rectal I_{sc} when VG (0.5 gland) was added to 5 mL of saline in Ussing chambers. Audsley *et al.* (1988) observed stimulation of I_{sc} across ilea exposed to VG at the same concentration. I suspect that the stimulatory activity in VG extracts used by Proux *et al.* (1984) was lost during storage because Thomson and Audsley (unpublished observation) recently observed substantial stimulation of rectal I_{sc} by VG and I report equal stimulation of rectal fluid transport by CC and VG in chapter 5.

Finally I considered whether I had measured all the major solutes associated with stimulation of ileal fluid transport. Using typical values of 215 mM NaCl, 25 mM K^+ and 10 mM HCO_3^- measured in ileal absorbate after stimulation (Fig. 14 & 16), these four inorganic ions (total of 425 mosmol) can account for 92% of the measure absorbate osmolarity (i.e. 460 mosmol; chapter 2). The small anion deficit of 15 mM could be explained by movement of phosphate to the hemocoel side or NH_4^+ to the lumen side. Both of these processes have been demonstrated for locust rectum (Andrusiak *et al.* 1980; Thomson *et al.* 1988a). These calculations suggest that absorption of amino acids and other solutes in the saline is probably negligible in locust ileum, in contrast to the high rates of proline transport across locust recta (Meredith & Phillips 1988).

SUMMARY

Unstimulated fluid transport was supported at 50% of control levels by the presence of any one of Na^+, K^+, or Cl^-, while removal of all but trace levels of these ions reduced fluid transport to 25% of control transport rates. Stimulation of fluid transport by CC or VG extracts did not occur unless Cl^- was present. The presence of Na^+ or K^+ was also required for maximum stimulation of fluid transport by these factors with greatest stimulation occurring when the Na^+:K^+ ratio was 1:1. Cyclic AMP, and CC and VG extracts all stimulated Na^+, Cl^- and K^+ absorption across everted ileal sacs. Stimulation of fluid transport by these factors largely eliminated the anion deficit ($Na^+ + K^+ - Cl^-$) observed under unstimulated conditions. Stimulation caused decreases in absorbate HCO_3^- concentrations concurrent with the increased absorbate Cl^- levels. These results indicate a switch from of low capacity $NaHCO_3$ transport under unstimulated conditions and to high capacity NaCl transport under stimulated conditions. Stimulation of fluid transport also causes a 3-fold increase in transepithelial potential (hemocoel negative) indicating stimulation of electrogenic anion (Cl^-) movement to the hemocoel. These results provide the first direct evidence for hormonal control of Na^+ reabsorption in insect excretory systems.

CHAPTER 4: $^{22}Na^+$ Flux Across Locust Ileum

INTRODUCTION

In chapter 3, I reported that K^+, Cl^- and Na^+ absorption across everted ileal sacs were stimulated by CC, VG and cAMP (Table 3). Previous investigators had observed stimulation of K^+ and Cl^- fluxes across flat sheet preparations of locust recta by cAMP and CC but Na^+ flux was not affected by either of these stimulants (Phillips *et al.* 1986; Black *et al.* 1987). Black *et al.* (1987) were unable to find any evidence for a neurohormone in the locust nervous system which controlled Na^+ reabsorption in the rectum. The stimulation of Na^+ absorption across locust ileum observed in chapter 3 is the first direct evidence for control of Na^+ reabsorption in an insect excretory system. The only other evidence for hormonal control of Na^+ reabsorption was that retrocerebral complex of *Periplaneta americana* stimulated short-term fluid transport by rectal sacs only if Na^+ was present (Steele & Tolman 1980). However these investigators did not measure Na^+ fluxes.

Since my observations were made using everted sacs rather than with flat sheet preparations mounted in Ussing chambers (as used in previous investigations), there was a possiblity that my observations on stimulation of ileal Na^+ absorption were due to the method used. It was possible that the observed increase in Na^+ absorption across everted ileal sacs was due to increased passive diffusion and electrical coupling to Cl^- transport. In this chapter I investigate the effects of cAMP, CC and VG on $^{22}Na^+$ flux across short-circuit and open-circuit locust ilea mounted as flat sheets in modified Ussing chambers. In the short-circuit state, electrical and chemical gradients across the epithelium are abolished by clamping the transepithelial potential at 0 mV and using identical solutions on each side. Under these conditions net flux of Na^+ is completely due to active

transport (Hanrahan *et al.* 1984).

Amiloride blocks epithelial Na^+ channels at μM levels and also Na^+/H^+ and Na^+/NH_4^+ exchangers at mM levels. Black *et al.* (1987) observed a 75% inhibition of net Na^+ flux across locust recta exposed to 1 mM amiloride; therefore, the above mentioned processes are likely mechanisms whereby Na^+ might enter locust hindgut epithelia from the lumen. The effect of this agent was tested on short-circuited ilea.

MATERIALS AND METHODS

Short-circuit current and $^{22}Na^+$ flux measurements

Locust ilea were mounted as flat sheets in modified Ussing chambers, bathed bilaterally with 5 mL of high NaCl physiological saline, and short-circuited as previously described for recta by Hanrahan *et al.* (1984). Ilea were mounted as a sheet and secured over a 0.196 cm^2-opening by means of tungsten pins and an overlaying neoprene O-ring. Edge-damage to tissue was negligible with this technique. The half-chambers were clamped together in a vice-like frame. To measure the transepithelial potential (V_t), 3 M KCl agar bridges (size PE 90) were placed near the tissue through ports on the side of the chambers. Each agar bridge connected with a reservior of KCl and standard calomel electrodes which were connected to a high input impedance differental amplifier (4253, Teledyne Philbrick, Dedham, Mass.) which continuosly monitored V_t. Under short-circuit conditions V_t was maintainted at 0 mV by a second operational amplifier (725, National Semi-conductor Corp. Santa Clara, Calif.) which passed current (I_{sc}) between two silver electrodes mounted at either end of the chambers. A third amplifier (308, Fairchild, Mountain Veiw, Calif.) was used to measure I_{sc}. Both I_{sc} and V_t were monitored on a strip chart recorder (220, Soltec Corp., Sun Valley, Calif.). Corrections were made for series resistance of the external saline and asymetries between voltage-sensing electrodes (Hanrahan *et al.* 1984). During measurement of $^{22}Na^+$ fluxes under short-circuit conditions, the short-circuit current was occasionally and briefly

(< 1 min) interrupted to determine V_t and resistance (R_t) as previously described (Hanrahan *et al.* 1984). Under open-circuit conditions V_t was monitored continuously during flux measurements. The external physiological saline resembled locust hemolymph as described previously in chapters 2 and 3. The saline was vigorously circulated by bubbling with a 95% O_2 / 5% CO_2 gas mixture. Because of solubility problems, amiloride was dissolved in a sulphate-free control saline in which KCl and $Mg(NO_3)_2$ replaced the equivalent sulphate salts. Since amiloride is a competitive inhibitor of NH_4^+/Na^+ exchange, amiloride experiments were run at both 110 mM Na^+ and 20 mM $Na^+$to insure that the high Na^+ concentration was not masking the action of amiloride. Amiloride was a gift from W. D. Dorian of Merck Frosst Laboratories. Amino acids and cAMP were obtained from Sigma Corp. All other chemicals were of reagent grade. All experiments were conducted at room temperature (21-25° C).

Flux of $^{22}Na^+$ across ilea in the steady-state phase (2 h after dissection) under short-circuited and open-circuit conditions were determined at 15 min intervals for 2 h before and after stimulation with 10 cAMP, 0.1 CC/mL or 1 VG/mL on the hemocoel side, as previously described by Hanrahan *et al.* (1984). Extracts of entire corpora cardiaca (CC) and fifth ventral ganglia (VG) were prepared as described in chapter 2. Aliquotes (1 mL) of the extracts were added to the hemocoel side of the Ussing chambers. Additional fresh CC or VG extracts were replaced after each 1 mL saline sample was collected from the hemocoel side to insure that the concentration of extract remained the same. The $^{22}Na^+$ was obtained as NaCl from New England Nuclear Corporation. Aliquots (5 µL) of stock ^{22}NaCl solution were added to one chamber, which was referred to as the "hot" side. After allowing 30 min for mixing, duplicate 50 µL samples were taken from the "hot" side and placed in 1 mL of "cold" saline. To determine increase in radioactivity of the "cold" side, 1 mL samples were taken at intervals of 15 minutes and were replaced in the chamber with equal amounts of "cold" saline. Samples of saline (1 mL) were counted using an automatic well-type gamma counter (Nuclear

Chicago Model 1058). Unidirectional flux was calculated using the following formula (Williams *et al.* 1978):

$$J_{1\text{-}2} = a_2VC/a_1TA$$

Where: $J_{1\text{-}2}$ is the unidirectional flux (μequiv$\cdot$ $h^{-1}\cdot$ cm^{-2}),

a_1 is the radioactivity of the "hot side" (cpm$\cdot$ mL^{-1})

a_2 is the increase in radioactivity of the "cold side" (cpm$\cdot$ mL^{-1})

C is the concentration of the unlabelled Na^+ in solution (mM)

V is the volume of the solution in the chambers (5 mL)

A is the tissue surface area (0.196 cm^2)

T is the time interval between sample (h).

Forward flux is from lumen (L) to hemocoel (H)

Back flux is from hemocoel (H) to lumen (L)

Mean fluxes reported are steady-state values averaged from at least 3 sequential determinations starting 0.5 h after adding radiotracer or adding hormonal extracts. Control and experimental treatments were compared on the same preparations and the statistical significance was determined by paired t-test.

Ammonia secretion

Ilea were mounted as flat sheets in miniaturized versions of the Ussing chambers with 2 mL rather than 5 mL of saline per chamber. Chambers were perfused (4-5 mL/min) with saline on both sides of the tissue and saline on both sides of the chamber was mixed by bubbling with 100% O_2. Typically, ilea were brought to steady-state conditions (as defined by stable short-circuit current (I_{sc}); $\cong$ 2 h) under bilateral perfusion, and then flow was stopped on the lumen side for the experimental period (1 h) but mixing by bubbling was continued. Samples (1 mL) were then collected from the lumen side and assayed for ammonia as described below. Salines were CO_2-HCO_3^- free to facilitate comparisons with pervious work on the rectum (Thomson *et al.* 1988a,b)

and remove the effects of volatile buffer components other than NH_3. Saline composition was based on locust hemolymph except for the absence of CO_2/HCO_3^- and contained the following (in mM): 100 NaCl, 5 K_2SO_4, 10 $MgSO_4$, 10 Na^+-isethionate, 10 glucose, 100 sucrose, 5 $CaCl_2$, 10 3-(N-morpholino)propanesulfonic acid (MOPS; pK_a 7.2 at 25° C), 2.9 alanine, 1.0 arginine, 1.3 asparagine, 5.0 glutamine, 11.4 glycine, 1.4 histidine, 1.4 lysine, 13.1 proline, 1.5 serine, 1.0 tyrosine and 1.8 valine. All salines were also initially ammonia free (< 20 μM) to confine the scope of the investigation to endogenously produced ammonia. Salines were aerated with 100% O_2 for at least 2 h prior to use, and the prefusion reservoirs were continously aerated throughout the experiments. The saline pH was manually titrated to 7 with concentrated HNO_3 or NaOH pellets using a Radiometer PHM 84 pH meter (Copenhagen) before each experiment.

The rate of ammonia secretion was determined as (total final ammonia) - (total initial ammona) and expressed as a flux rate per square centimetre per hour. Ammonia concentrations were determined by enzymatic assay using an ammonia assay kit from Sigma (Procedure No. 170-UV), which utilizes the reductive amination of 2-oxoglutarate by glutamate dehydrogenase to bring about a change in extinction (at 340 nm) proportional to the ammonia content of the sample: NADH + NH_4^+ + 2-oxoglutarate + glutamate dehydrogenase → L-glutamate + NAD^+ + H_2O + glutamate dehydrogenase.

RESULTS

Effect of cAMP on $^{22}Na^+$ fluxes

The effect of 5 mM cAMP on unidirectional and net Na^+ fluxes under short-circuit conditions is shown in Fig. 18. The short-circuit current values indicated anion movement from lumen to hemocoel at of 0.14 ± 0.05 μequiv·h^{-1}·cm^{-2} under unstimulated conditions and 7.5 ± 0.15 μequiv·h^{-1}·cm^{-2} after the addition of 5 mM cAMP (Fig. 18c). $^{22}Na^+$ fluxes (μequiv·h^{-1}·cm^{-2}) remained steady over a 4 h period and averaged : forward flux (lumen to hemocoel)= 4.8 ± 0.09, back flux (hemocoel to lumen) = 0.68

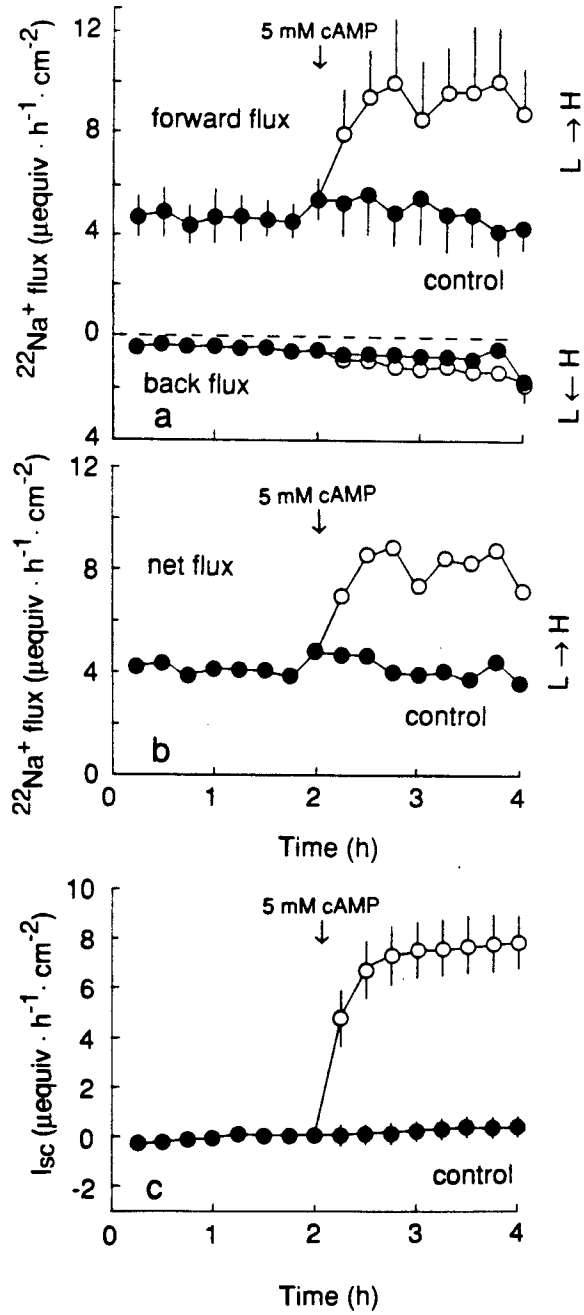


Figure 18. a) The time course of unidirectional sodium fluxes across short-circuited ilea under control and cAMP-stimulated conditions (forward flux is from lumen (L) to hemocoel (H) and back flux is from H to L). Time 0 was 2 h after dissection. Control preparations (●) were bathed in normal saline throughout. Expermental preparations (○) were the same as controls except that 5 mM cAMP was added to the hemocoel side after 2 h. b) Net Na^+ fluxes to hemocoel side was calculated from unidirectional fluxes in (a). c) The short-circuit current (I_{sc}) across the locust ileum measured during the flux determinations indicated net anion flux from L to H, (mean ± s.e., n=3-15).

± 0.09, with net flux to the hemocoel side of 4.2 ± 0.18 and a flux ratio (forward flux:back flux) of 7.1:1. Addition of 5mM cAMP caused a significant ($P < 0.05$) increase in forward flux to 9.4 ± 4.4, back flux to 1.3 ± 0.2 giving a net flux of 8.1 ± 4.4, while the flux ratio remained at 7.2:1 (Fig. 18).

The effect of 5 mM cAMP on unidirectional and net Na^+ fluxes under open-circuit conditions are shown in Fig. 19. The steady-state transepithelial potential difference (V_t) was -5.7 ± 1.4 mV (lumen positive relative to hemocoel) before stimulation. Upon stimulation V_t increased to 12.0 ± 2.4 mV at 0.5 h after cAMP addition and to 17.2 ± 3.2 mV after 2 h (Fig. 19b). Steady-state unidirectional and net fluxes ($\mu equiv \cdot h^{-1} \cdot cm^{-2}$) all increased significantly ($P<0.05$) after adding 5 mM cAMP, with forward flux rising from 3.4 ± 0.5 to 7.6 ± 1.0, back flux going from 0.3 ± 0.1 to 1.3 ± 0.4 and net flux doubling from 3.1 ± 0.1 to 6.2 ± 0.1 while the flux ratio dropped from 11.6:1 to 5.8:1 (Fig. 19). Net $^{22}Na^+$ flux was doubled by adding cAMP regardless of whether ilea were in the open-circuit (Fig. 19) or short-circuit state (Fig. 18). There was no evidence of a significant additional passive net flux of Na^+ resulting from increased V_t after stimulation (compare Figs. 18 and 19).

Effect of CC and VG extracts on $^{22}Na^+$ fluxes

Unidirectional and net Na^+ fluxes before and after addition of VG (1 gland/1 mL saline) under short-circuit conditions are shown in Fig. 20. Steady-state fluxes ($\mu equiv \cdot h^{-1} \cdot cm^{-2}$ over 2 h) before VG addition were: forward flux=3.1 ± 0.3, back flux=0.56 ± 0.16 with net flux to the hemocoel side of 2.6 ± 0.13 and a flux ratio of 5.5:1. The addition of VG caused a significant ($P < 0.05$) increase in forward flux to 5.9 ± 0.5, in back flux to 1.88 ± 0.2, so that net flux increased to 4.0 ± 0.1 and the flux ratio decreased slightly to 3.1:1. A similar effect was observed upon the addition of CC (0.1gland/1 mL) where net flux increased from 3.6 ± 0.1 to 5.7 ± 0.1 (Fig. 21). Both extracts increased the I_{sc} across the ileum and the low dose of CC used in this ex-

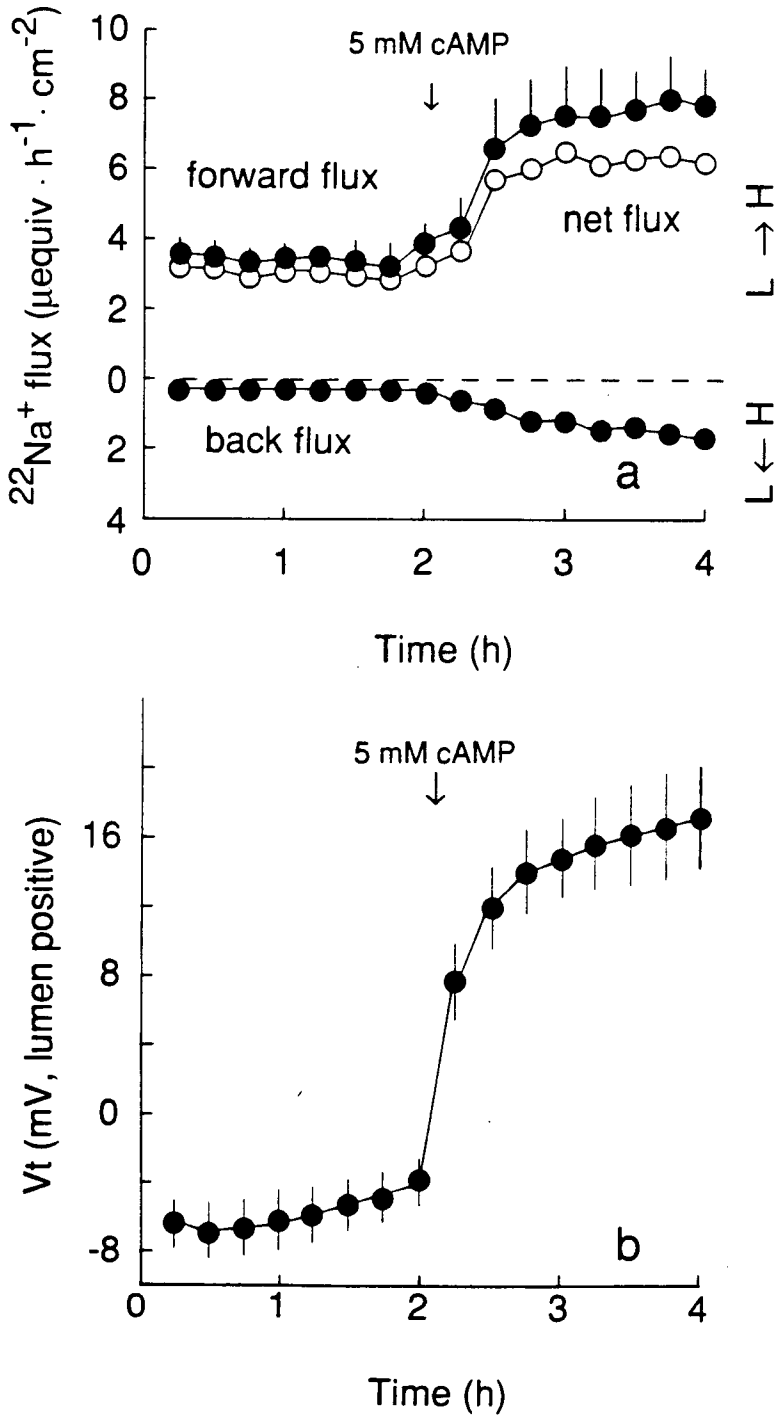


Figure 19. a) The time course of unidirectional (●) and calculated net (○) sodium fluxes across ilea in the open-circuited state (forward flux is from lumen (L) to hemocoel (H) and back flux is from H to L) under cAMP-stimulated conditions. Time 0 was 2 h after dissection. Preparations were bathed bilaterally in normal saline and 5 mM cAMP was added to the hemocoel side after 2 h. b) Tranepithelial potential across locust ilea during these flux determinations. (mean ± s.e. where larger than symbol, n=6).

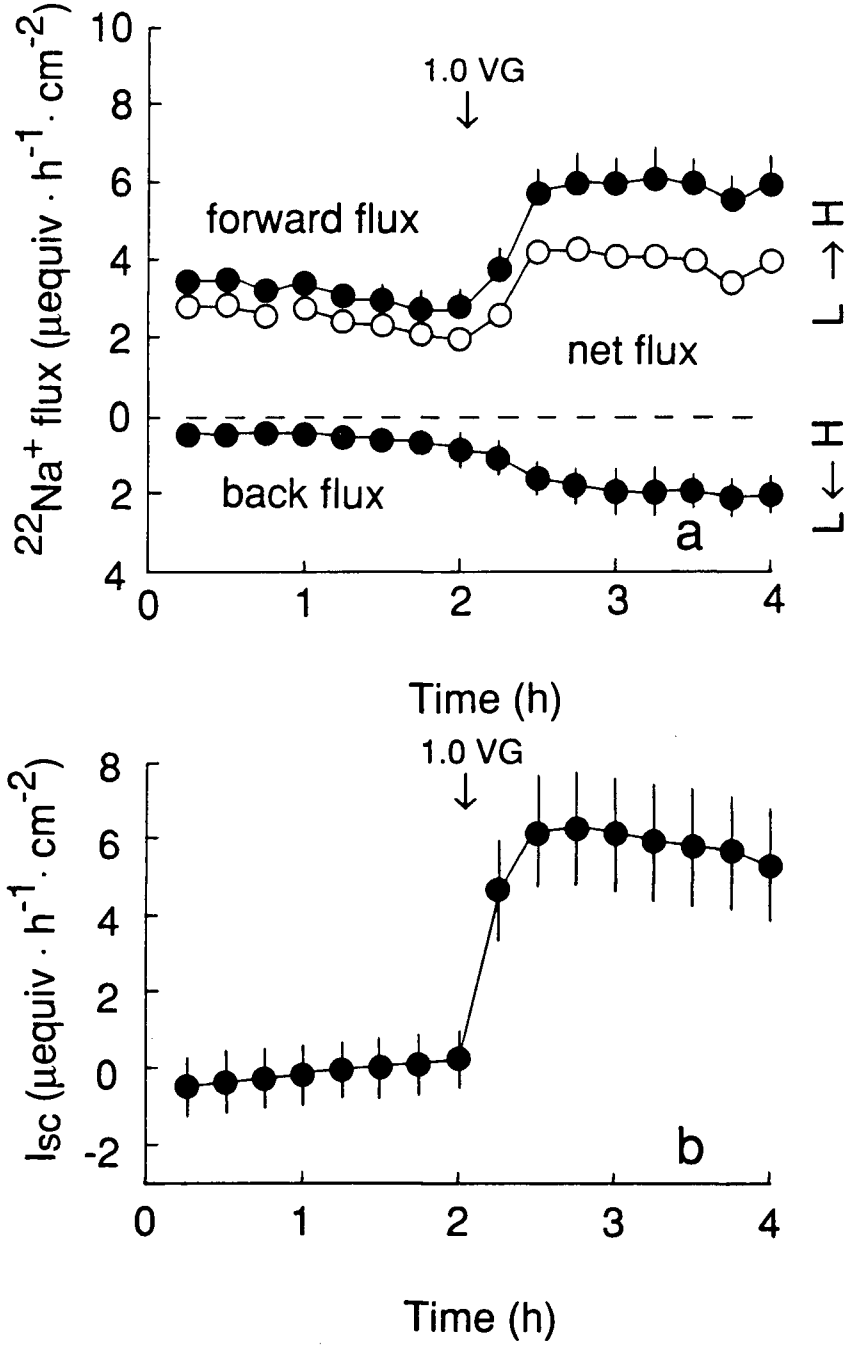


Figure 20. The time course of unidirectional (●) and calculated net (○) sodium fluxes across short-circuited ilea under VG-stimulated conditions (forward flux is flux from lumen (L) to hemocoel (H) and back flux is from H to L). Time 0 was 2 h after dissection. Preparations were bathed bilaterally in normal saline with 1 VG/mL added to the hemocoel side after 2 h. b) The short-circuit current (I_{sc}) across the locust ileum during these experiments indicated net anion flux from lumen(L) to hemocoel (H), (mean ± s.e. where larger than symbol, n=6).

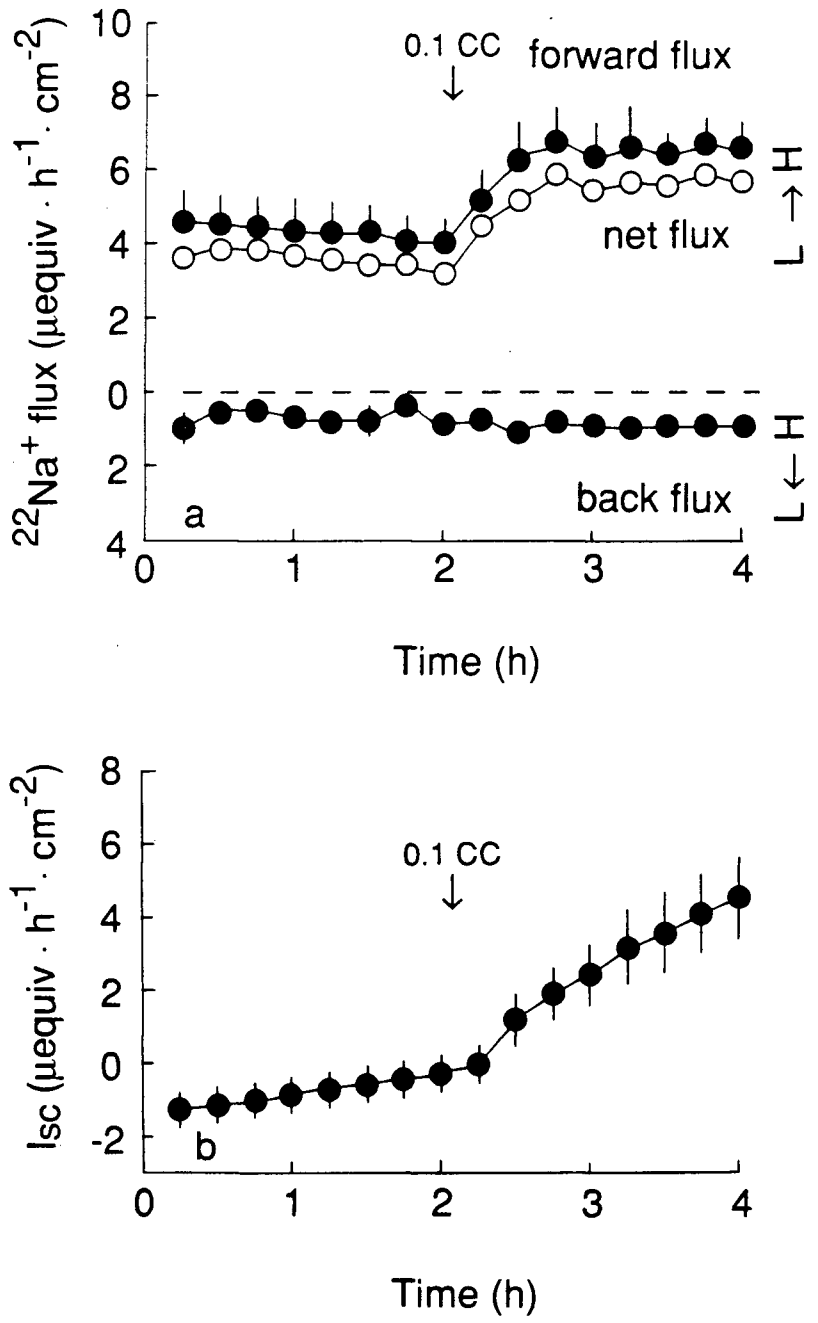


Figure 21. The time course of unidirectional (●) and calculated net (○) sodium fluxes across short-circuited ilea under CC-stimulated conditions (forward flux is flux from lumen (L) to hemocoel (H) and back flux is from H to L). Time 0 was 2 h after dissection. Preparations were bathed bilaterally in normal saline with 0.1 CC/mL added to the hemocoel side after 2 h. b) The short-circuit current (I_{sc}) across the locust ileum during these experiments indicated net anion flux from lumen(L) to hemocoel (H), (mean ± s.e. where larger than symbol, n=6).

periment resulted in a slow rate of rise in I_{sc} (Fig. 20 & 21). In summary extracts of both of these neuroendocrine tissues had the same effect as cAMP; i.e. they doubled active transport of Na^+ with relatively little change in Na^+ permeability.

Effect of amiloride on $^{22}Na^+$ flux

Cyclic AMP-stimulated unidirectional $^{22}Na^+$ flux to the hemocoel side was not inhibited by luminal addition of 1 mM amiloride when the external Na^+ concentration was 110 mM (Table 4). When Na^+ level was lowered to 20 mM stimulated $^{22}Na^+$ flux to the hemocoel side fell slightly but not significantly ($P > 0.5$) from 6.5 ± 2.0 before to 5.7 ± 1.0 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$ after adding amiloride (Table 4). Possibly amiloride was unable to penetrate the chitinous cuticle on the lumen side.

Ammonia Secretion

I observed a large ammonia secretion to the lumen side of ilea (1.3 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$, Table 5) under control conditions. Ammonia secretion rate increased 2-fold when ilea were exposed to 5 mM cAMP. I have not investigated yet whether this ammonia secretion is Na^+-dependent. This is an area for future study.

DISCUSSION

Active transport of Na^+ across short-circuited ileum (Fig. 18-21) was 3 to 4 times greater than that across similar preparations of rectum (Black *et al.* 1987). These results confirm those obtained with everted sacs (chapter 3). Net absorption of Na^+ across the ileum was stimulated by cAMP and extracts of CC and VG whether everted sacs or flat sheets are used to determine net Na^+ movement (Table 3; Fig. 18-21). The results in this chapter clearly demonstrate that these stimulants increase active transport of Na^+ rather than simply increasing passive permeablity and absorption by electrical coupling to Cl^- transport. These results provide the first direct evidence for hormonal control of active Na^+ reabsorption in any insect excretory system. However, the small increase in

Table 4. Effect of amiloride on unidirectional $^{22}Na^+$ flux to hemocoel side across stimulated (5 mM cAMP) short-circuited locust ilea. (mean ± s.e.,n=24-32, on 3-4 preparations).

External Na^+ * (mM)	$^{22}Na^+$ flux ($\mu equiv \cdot cm^{-2} \cdot h^{-1}$)** Control	1mM Amiloride
110	8.1 ± 1.3	8.4 ± 0.4
20	6.5 ± 2.0	5.7 ± 1.0

*Identical salines on both sides

**Averaged of 8 determinations over 2 h on 3-4 preparations.

Control $^{22}Na^+$ flux measurements were made every 15 min on preparations for 2 h prior to addition of 1 mM amiloride to the lumen side. The effect of amiloride on $^{22}Na^+$ flux was measured every 15 min over the next 2 h.

Table 5. Effect of 5 mM cAMP on lumen ammonia secretion rates across short-circuited locust ilea.

	Ammonia Secretion $\mu mol \cdot cm^{-2} \cdot h^{-1}$	I_{sc} $\mu equiv \cdot cm^{-2} \cdot h^{-1}$
Control saline	1.3 ± 0.2	2.2 ± 0.3
+ 5 mM cAMP	2.7 ± 0.3	8.5 ± 1.9

Values are means ± s.e., n=4. Addition of cAMP to the hemocoel caused a significant ($P < 0.05$) increase in both parameters.

back flux of $^{22}Na^+$ to the lumen side after adding VG (Fig. 20) or cAMP (Fig. 19) does suggest that a small increase in passive permeability of the ileum to Na^+ also occurred.

The mechanism of Na^+ transport across the ileum was not investigated but Irvine *et al.* (1988) has reported some information on its characteristics. Net transepithelia Na^+ flux appears to be electroneutral. Two observations support this hypothesis. First, replacing all external Na^+ with other cations has a relatively small effect on ileal I_{sc}, V_t, or Cl^- transport (Irvine *et al.* 1988). Second, there was no significant difference in net Na^+ flux under open-circuit and short-circuit conditions (Fig. 14,15) indicating that this process is not greatly influenced by changes in V_t. This electroneutrality could be explained if apical entry of Na^+ occurred largely by a 1-to-1 exchange for NH_4^+, H^+ or other cations produced by oxidation of amino acids within the hindgut. Such an amiloride-sensitive cation exchanger accounts for about one-third of Na^+ entry into locust rectal epithelium (Black *et al.* 1987; Thomson *et al.* 1988a). When locust recta were bathed bilaterally in saline containing a full complement of hemolymph amino acids but only traces of NH_4^+, 95% of ammonia produced by the tissue was transported to the lumen at a rate of 0.6 $\mu equiv \cdot h^{-1}\ cm^{-2}$ largely as ammonium ions (Thomson *et al.* 1988a). Addition of 1 mM amiloride inhibited 60% of the ammonia flux and removal of luminal Na^+ caused a 60% decrease in ammonia flux whereas removal of K^+ had no effect (Thompson *et al.* 1988a). Black *et al.* (1987) also observed a partial inhibition of Na^+ flux upon addition of 1 mM amiloride. However, I did not observe inhibition of Na^+ flux from lumen to hemocoel when 1 mM amiloride was applied to the lumen side of stimulated ilea (Table 4). Therefore, either this pathway is not important in the ileum or possibly amiloride did not reach the ileal cells. The rate of ammonia secretion in unstimulated ileum was 2-fold higher than that in unstimulated recta. Ileal ammonia secretion rate was stimulated 2-fold by the addition of 5 mM cAMP while rectal ammonia secretion was unaffected by cAMP. Therefore stimulated ammonia secretion rate in the ileum was 4 times that observed in the rectum. Ammonia secretion in the ileum

was stimulated by cAMP in a similar manner to $^{22}Na^+$ flux. Ammonia secretion would account for about one-third of the stimulated net Na^+ flux across the ileum (8.1 ± 4.4 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$, Fig. 18). The possible basis for electroneutrality of the remaining two-thirds of Na^+ absorption has not been investigated, but cotransport of Na^+ with anions such as phosphate and acetate have been proposed for locust rectum (Phillips *et al.* 1986).

Active absorption of Na^+ is usually driven by a ouabain-sensitive Na^+,K^+-ATPase located in the basolateral membrane of epithelia. Black *et al.* (1987) observed 37% inhibition of of net Na^+ flux across locust recta by ouabain at 30°C. I did not determine the effect of ouabain on Na^+ flux across the ileum although I did observe a 30% inhibiton of fluid transport across everted ileal sacs when 5 mM ouabain was applied to the hemocoel side (Table 1). I also report in chapter 6 the presence of ouabain-sensitive Na^+,K^+-ATPase in the homogenates and membrane fractions of ileal cells (Table 9), although the specific activity was much lower than that observed in rectal cells (Table 8). The pattern of ion transport in locust ileum is very similar to that in locust rectum, where ion-sensitive electrodes have been used by Hanrahan and Phillips (1984b) to localize the active step for Na^+ transport at the basolateral border (see Fig. 4). From these results it seems reasonable to assume that Na^+,K^+-ATPase is at least partially involved in Na^+ flux across ilea.

Other major mechanisms for electroneutral Na^+ transport in various epithelia include NaCl or Na^+, K^+, $2Cl^-$ cotransporters which are sensitive to diuretics such as furosemide (Greger 1985). In both segments of the locust hindgut such cotransport mechanisms are clearly not an important mechanism of Na^+ transport. For example, active net Na^+ flux across locust recta in Cl^--free saline was similar to that observed in normal NaCl saline (Black *et al.* 1987). Likewise in locust rectum, Black *et al.* (1987) observed that Cl^--dependent I_{sc} remained constant at 10 to 14 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$ when external Na^+ was varied between 0 to 140 mM. Also $^{36}Cl^-$ flux was relatively unchanged

when the Na^+ electrochemical gradient across the apical membrane is decreased from 118 to 16 mV (Hanrahan & Phillips 1983, 1984a). Hanrahan and Phillips (1983) observed no effect of 1 mM furosemide on $^{36}Cl^-$ fluxes and I_{sc}. The more limited results for locust ileum are similar: Replacing all external Na^+ also has no effect on the Cl^--dependent I_{sc} across locust ileum (Irvine *et al.* 1988). From these observations and those outlined by Hanrahan and Phillips (1983, 1984a,b) the pathways of Na^+ and Cl^- transport across locust hindgut appear to be largely independent of each other.

SUMMARY

The results of this chapter confirm that net absorption of Na^+ observed across everted ileal sacs in chapter 3 was largely by active transport rather than passive diffusion and electrical coupling to Cl^- transport. This active Na^+ flux was stimulated to a similar degree by cAMP, and by CC or VG extracts. I confirmed earlier evidence (Irvine *et al.* 1988) that Na^+ flux was largely electroneutral, since cAMP-stimulated net Na^+ flux was similar under open-circuit and short-circuit conditions. Rates of ammonia secretion suggest that only one third of the Na^+ flux may occur by a 1-to-1 exchange for NH_4^+ produced by oxidation of amino acids across the locust ileum. Failure of amiloride to inhibit Na^+ flux does not support the hypothesis of a Na^+/NH_4^+ exchanger in the ileum, although other explanations of this result have not been eliminated.

CHAPTER 5: Composition of Stimulated Rectal Absorbate and the Effect of Proline on Hindgut Fluid Absorption

INTRODUCTION

Results in chapters 2, 3 and 4 and Irvine *et al.* (1988) have demonstrated that active transport processes in the ileum are similar to those described in detail for the rectum (Phillips *et al.* 1986), namely absorption of Cl^- (the major electrogenic process), Na^+ (largely electroneutral) and HCO_3^-, while both H^+ and NH_4^+ are actively secreted into the lumen. Homogenates of corpus cardiacum (CC, both lobes), ventral ganglia 4 to 7 (VG), cAMP, forskolin and theophylline all stimulate Cl^- transport and inhibit or reverses H^+ and HCO_3^- movements in both hindgut segments mounted in Ussing chambers (Irvine *et al.* 1988). However, one major difference has been observed between the two segments in that Na^+ transport is greater and is stimulated 2-fold by cAMP, CC and VG in the ileum. Given that all stimulants otherwise had the same qualitative actions on both locust hindgut segments, I wondered whether experimental artifacts might account for the lack of effect of cAMP and CC on net Na^+ flux across short-circuited recta mounted in Ussing chambers. To explain, exit of absorbate from rectal pads might occur only at the anterior end of this organ as suggested by Wall and Oschman (1970) and this natural pathway might be obstructed by the O-ring applied to flat sheet preparations. Consequently cation preference of the alternative pathway across the secondary epithelial layer might have been measured using short-circuited recta in earlier studies, whereas the ileum lacks a comparable ultrastructural pathway. This uncertainty can be eliminated by using everted cannulated sacs of whole gut segments. In chapter 3, I described changes in absorbate composition caused by cAMP, CC and VG for locust ileum. However, comparable data is not available for everted rectal sacs.

The influence of luminal ion and osmotic concentrations on J_V across unstimulated

everted rectal sacs has been reported (Goh & Phillips 1978; Phillips *et al.* 1982a) and Proux *et al.* (1984) described Cl^--dependence of rectal J_V after stimulation by cAMP and CC when NaCl-rich saline was present bilaterally. These workers did not investigate changes in composition of rectal absorbate after stimulation, in the presence of either high luminal KCl or NaCl, nor did they test the effect of VG on rectal J_V. These relationships are described in this chapter in order to compare fully the actions of stimulants on everted sacs of both locust hindgut segments exposed to either NaCl-rich or KCl-rich salines luminally.

I also address a second aspect of fluid transport across locust hindgut. Wall *et al.* (1970) reported that unknown organic substances must account for half of the high osmotic concentrations measured in the lateral intercellular spaces of cockroach rectal pads, suggesting that active recycling of such substances drives a component of J_V. Meredith and Phillips (1988) have characterized a Na^+, K^+ and Cl^--independent proline transport system which at *in situ* concentrations transports proline at rates (V_{max}=4.2 μequil·cm^{-2}·h^{-1}) which are second in magnitude only to stimulated Cl^- flux (Phillips *et al.* 1986). The capacity of this proline transport system is orders of magnitude greater than that required to provide metabolic substrate to this tissue. I therefore tested the dependence of hindgut J_V on luminal proline, both in the presence and absence of Na^+, $K^+$and Cl^-.

MATERIALS AND METHODS

The experimental animals were adult female *Schistocerca gregaria* 2-4 weeks past their final molt and maintained as described in chapter 2.

Locusts were dissected, the midgut and Malpighian tubules removed. Everted rectal sacs were prepared by inserting a 3 cm length of PE 90 tubing with a slightly flared end into the rectum until it passed the anterior of the rectum. The hindgut was raised slightly and a piece of surgical silk was passed under it and a ligature was tied between

the flared end of the tubing and the anterior end of the rectum. The colon and connecting tracheae were cut away and the rectum was slowly everted by sliding it over the PE tubing. The rectum was rinsed with 1 mL of saline to remove any hemolymph and fecal material. A second ligature was tied just posterior to the rectum to close the sacs. Ileal sacs were also prepared as descrbed in chapter 2. Any remaining internal fluid was withdrawn completely with a 'Hamilton' syringe and the empty sac was weighed to ± 0.1 mg on a August Sauter balance. Sacs were filled hourly with 1 μL of fresh saline or in some experiments left empty and incubated in 25 mL of saline bubbled with 95% O_2 / 5% CO_2 and maintained at 30°C. At hourly intervals weight gain and tissue volume change were determined by weighing sacs before and after removal of fluid. The true rate of transepithelial fluid movement was determined by correcting for tissue volume changes. Statistical difference between means were determined by Student's t-test.

The NaCl saline (control) was the same as that described in chapter 3 and was based on ion, sugar and amino acid concentrations measured in locust hemolymph. The only difference in the high KCl saline was that the K^+:Na^+ ratio was reversed to mimic that in the primary urine (110 mM KCl and 10 mM Na^+ ;Chamberlin, 1981). Effects of cAMP on fluid transport were determined by adding this agent to high NaCl saline on the hemocoel side. Extracts of entire corpora cardiaca (CC), or fifth ventral ganglia (VG) were prepared as described in chapter 2.

Sodium, chloride, potassium and osmotic concentrations of the rectal absorbate were determined as described previously in chapter 3. Rates of ion absorption were calculated for each hour from the change in volume and concentration of the internal medium (i.e. absorbate; Goh & Phillips 1978).

The effect of proline on fluid transport across everted rectal and everted ileal sacs was determined using high NaCl saline but with no amino acids except proline on both sides of the sac. Chamberlin and Phillips (1982a) have shown that proline alone can

support the metabolic needs of this tissue. In another saline all Na^+, K^+, and Cl^- were replaced with choline and gluconate salts, and contained either 1 mM or 80 mM proline. All salines were adjusted to a constant osmolarity with sucrose. Osmotic concentration gradients across the everted sacs were established by adding sucrose to the bathing saline. The lowest proline concentration used was 1 mM since this was found to support cAMP-stimulated short-circuit current (I_{sc}) across the rectum at maximum levels (Meredith & Phillips 1988). A proline concentration of 80 mM was chosen because Meredith and Phillips (1988) observed maximum proline flux at this proline concentration, yet cAMP-stimulated I_{sc} was still at the same level observed with 1 mM proline. Recall that proline levels in primary urine *in situ* are about 40 mM.

Proline flux across ileum

Ilea were dissected and mounted as flat sheets in modified Ussing chambers and bathed bilaterally in high NaCl saline (13.1 mM proline). Transepithelial potential (V_t) was clamped at 0 mV (i.e. short-circuited) as described previously (chapter 4; Hanrahan *et al.* 1984). Steady-state was established under short-circuit conditions after about 2 h. Small amounts (20 µL) of (^{14}C)proline (specific activity >> 250 mCi/mmol; ICN Biochemicals) were added to one half-chamber, i.e. the "hot" side. At 15 min intervals, 1 mL samples were collected from the other half or "cold" side and volume was replaced with "cold" saline. Radioactive samples were added to 10 mL of scintillation fluid (ACS II; Amersham, USA) and counted using a Beckman LS 9000 liquid scintillation counter. Correction for sample quenching was made by the "H number" method. Short-circuit current (I_{sc}) was monitored continously and transepithelial potential (V_t) measured periodically on a strip chart recorder (220 Soltec Corp., Sun Valley, Calif.) Proline fluxes were calculated as described in chapter 4 for Na^+ flux. The values reported are means ± standard errors over a 4 h period, uncorrected for proline metabolism.

RESULTS

Stimulation of fluid transport by cAMP, CC extract and VG extract

The addition of cAMP, VG or CC extracts to the inside of everted rectal sacs bathed in physiological saline caused a small significant ($P<0.05$) increase in fluid transport (Fig. 22). CC or VG extracts caused the greatest increase in J_v (3.9 $\mu L \cdot h^{-1} \cdot rectum^{-1}$) above control values (10.3 ± 0.4 $\mu L \cdot h^{-1} \cdot rectum^{-1}$, 2-5 h) while 10 mM cAMP increased fluid transport to a lesser degree (2.3 $\mu L \cdot h^{-1} \cdot rectum^{-1}$, data not shown).

The physiological saline used in the above (Fig. 22) and in all previous experiments by Proux *et al.* (1984) were NaCl-rich salines (110 mM NaCl, 10 mM K^+). These salines are similar to the hemolymph of the desert locust (Chamberlin 1981). However the primary urine of the locust is KCl-rich and low in Na^+ (Chamberlin 1981). The effects of CC or VG on fluid transport when rectal sacs were exposed to a high KCl saline (110 mM KCl, 10 mM Na^+) on the lumen side are shown in Fig. 23 . These rectal sacs had similar unstimulated rates of fluid transport to those exposed to NaCl-rich saline under unstimulated conditions(10.4 ± 0.8 versus 10.3 ± 0.4 $\mu L \cdot h^{-1} \cdot rectum^{-1}$, respectively). The addition of 0.05 CC/1 µL or 0.05 VG/1 µL caused significant increases in long-term (2-5 h) fluid uptake in sacs exposed to high KCl saline to 16.5 ± 0.9 and 16.3 ± 1.6 $\mu L \cdot h^{-1} \cdot rectum^{-1}$, respectively, which was greater than the corresponding fluid transport rates for sacs in high NaCl saline (14.2 ± 0.7 and 14.2 ± 0.5 $\mu L \cdot h^{-1} \cdot rectum^{-1}$, respectively; Fig. 23). However, the fluid transport was not stimulated as much by 10mM cAMP and there was no significant difference in transport between recta in the two salines (NaCl, 12.6 ± 0.6 $\mu L \cdot h^{-1} \cdot rectum^{-1}$; KCl, 12.4 ± 0.7 $\mu L \cdot h^{-1} \cdot rectum^{-1}$; 2-5 h, n= 16).

Stimulation of ion transport by CC and VG

The osmotic concentration of absorbate from unstimulated sacs remained about 5-

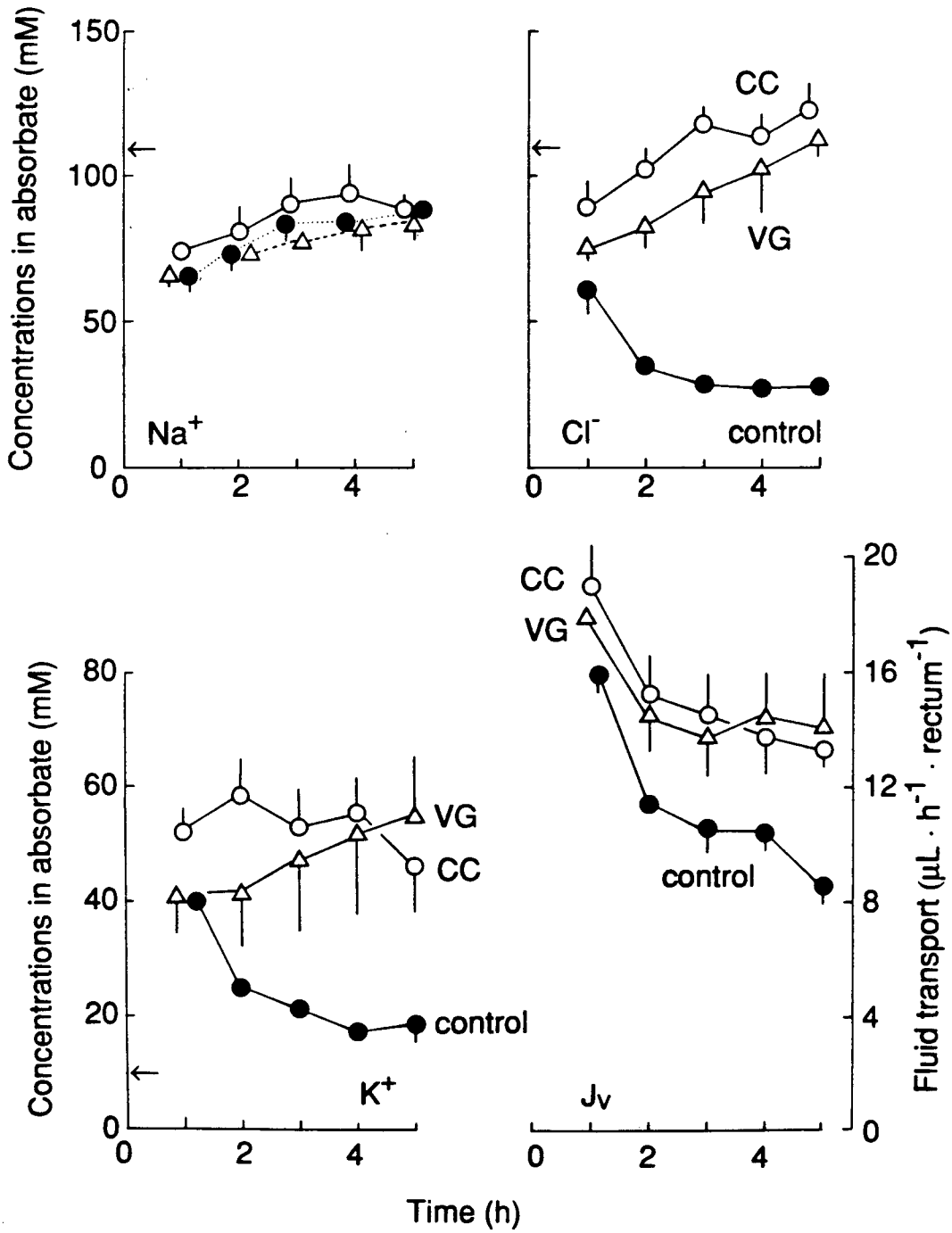


Figure 22. Influence of CC and VG on fluid transport (J_V) and absorbate ion concentrations (Na^+, K^+, Cl^-) for rectal sacs exposed to high NaCl saline (110 mM Na^+, 10 mM K^+, 110 mM Cl^-) on lumen side. Sacs initially contained no extract (●), or NaCl saline (1 µL) containing either 0.05 CC extract (○), or 0.05 VG extract (Δ) and sac contents (i.e. in the hemocoel compartment) were replaced hourly. The addition of CC or VG caused a significant ($P<0.05$) increase in Cl^- and K^+ concentrations and in fluid transport. The horizontal arrow on each graph indicates the concentration in the luminal saline. (mean ± s.e., n=5)

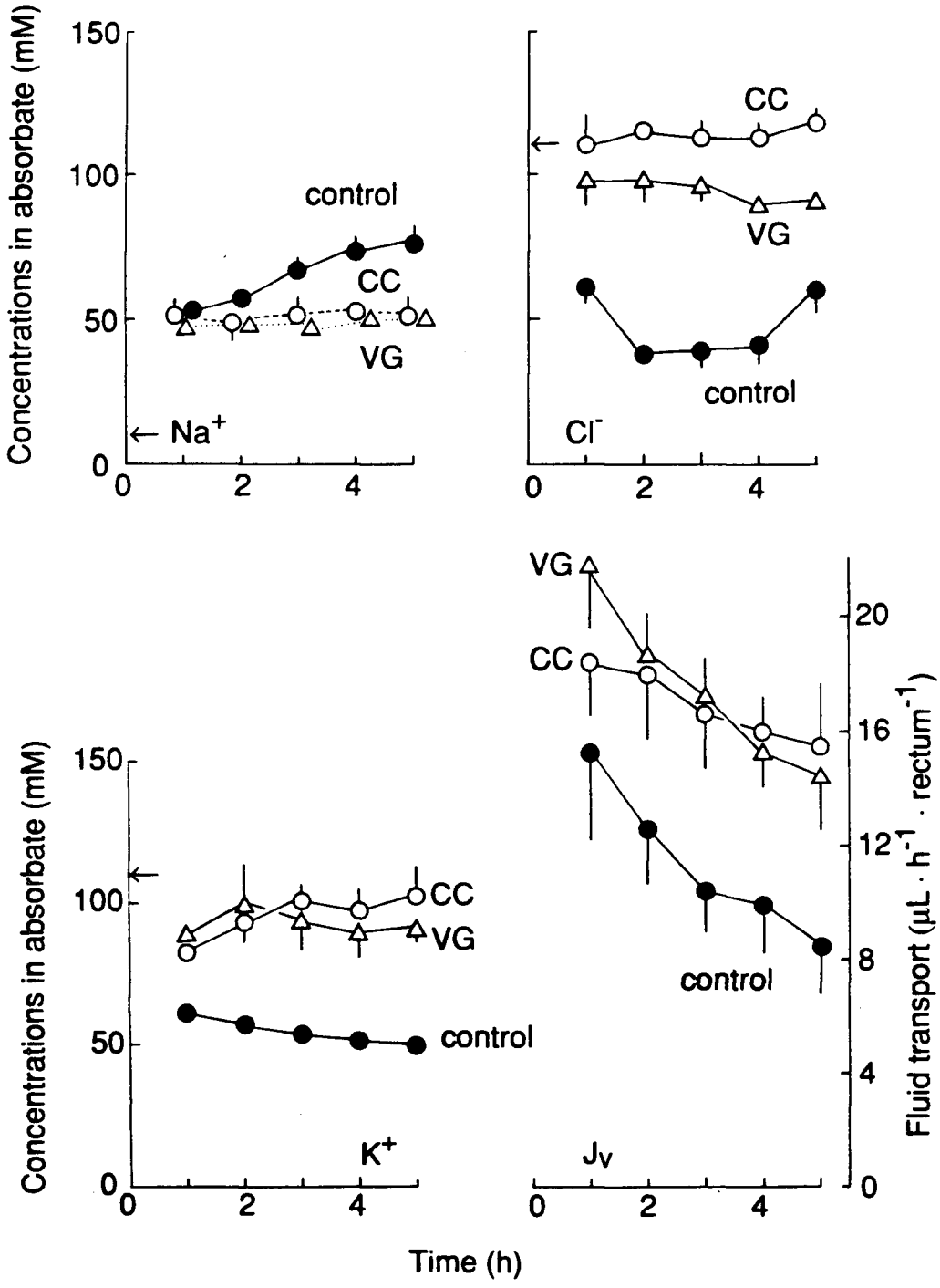


Figure 23. Influence of CC and VG on fluid transport (J_v) and absorbate ion concentrations (Na^+, K^+, Cl^-) for rectal sacs exposed to high KCl saline (110 mM KCl, 10 mM Na^+) on the lumen side. Sacs initially contained no extract (●), or high NaCl saline (1 µL) containing either 0.05 CC extract (○), or 0.05 VG extract (Δ) and sac contents (i.e. the hemocoel compartment) were replaced hourly. The addition of CC or VG caused a significant ($P<0.05$) increase in Cl^- and K^+ concentrations and in fluid transport. The horizontal arrow on each graph indicates the concentration in the luminal saline (mean ± s.e., n=5).

10% below that of the external saline throughout the experiment (Fig. 24). The addition of 10 mM cAMP, 0.05 CC or 0.05 VG/1 μL caused a significant increase in absorbate osmolarity to values approximately isosmotic with the external saline (405 mosmol). The osmolarity of the absorbate from sacs exposed to high KCl saline was similar to that observed in the NaCl saline (Fig. 24).

The ionic composition of the absorbate from everted rectal sacs exposed to a high NaCl physiological saline is shown in Fig. 22. When unstimulated recta were bathed in NaCl saline, absorbate Cl^- concentration initially fell from 60 mM at 1 h to a steady value of 30 mM at 3-5 h (Fig 22). Absorbate Cl^- concentrations in rectal sacs stimulated by the addition of 10 mM cAMP, CC or VG increased from 70 to 90 mM at 1 h to a steady state of 90 to 110 mM at 3-5 h (Fig. 22). This stimulation of absorbate Cl^- concentration along with an increase in fluid transport resulted in a 5-fold (VG and CC) or a 3-fold (cAMP) increase in Cl^-absorption across the sacs (Table 6a). When sacs were exposed to high KCl saline, the Cl^-concentration under unstimulated (control) conditions did not fall as much (45 to 55 mM over 3-5 h; Fig.23) as when preparations were exposed to NaCl saline. Absorbate Cl^-concentrations under stimulated conditions were similar whether KCl or NaCl saline was present luminally. The Cl^- absorption across sacs exposed to KCl saline was slightly higher under both control and stimulated conditions as compared to the sacs exposed to high NaCl saline (Table 6).

Absorbate Na^+ concentrations from sacs exposed to high NaCl saline were not changed by addition of stimulants and increased steadily with time from 70 mM at 1 h to 90 mM at 5 h. These absorbate values were significantly below the external Na^+ concentration (110 mM; Fig. 22). The rate of Na^+ absorption increased slightly after stimulation associated with the increased fluid transport (Table 6a). Sacs exposed to high KCl saline (10 mM Na^+) had absorbate Na^+ concentrations under unstimulated conditions ranging from 50 mM at 1 h to 75 mM at 5 h (Fig. 23). These concentrations were about 5- to 7-fold higher than the luminal Na^+ concentration of 10 mM (Fig. 23).

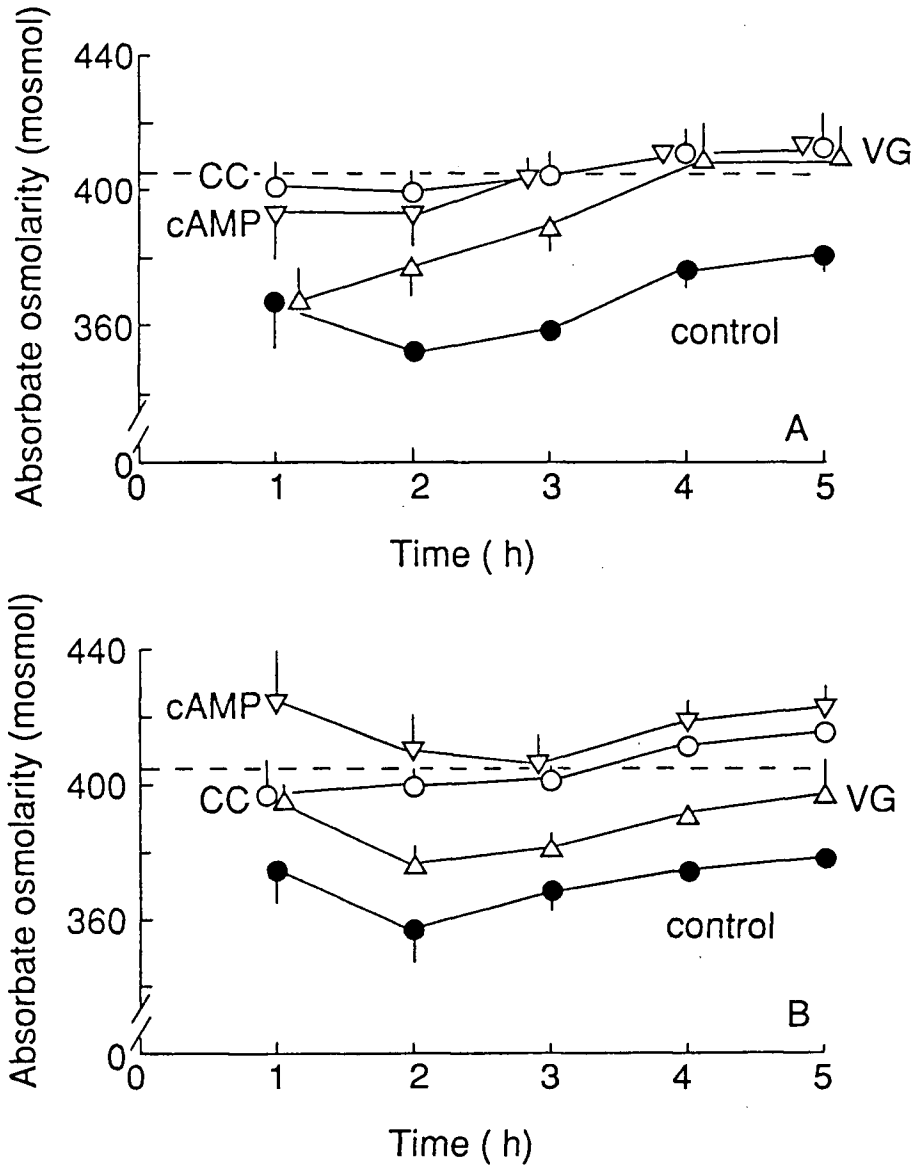


Figure 24. Osmolarity of absorbate from everted rectal sacs. Sacs initially contained no extract (●), high NaCl saline (1 μL) containing 10 mM cAMP (▽), 0.05 CC extract (○), or 0.05 VG extract (△) and were exposed to either a) high NaCl saline (110 mM Na^+, 10 mM K^+, 110 mM Cl^-) or b) high KCl saline (110 mM KCl, 10 mM Na^+) on the lumen side (mean ± s.e., n=5). Broken lines indicate osmolarity of the luminal salines.

Table 6. Long-term (2-5 h) rates of ion absorption ($\mu equiv \cdot h^{-1} \cdot rectum^{-1}$) across everted rectal sacs exposed to (a) high NaCl saline (110 mM NaCl, 10 mM K^+, 405 mosmol) on the luminal side or (b) to high KCl saline (110 mM KCl, 10 mM Na^+, 405 mosmol) on the luminal side and either no saline (control) initially or high NaCl saline (1 µL) containing cAMP or neural extracts on the hemocoel side (mean ± s.e., n=20).

a) NaCl saline

Ion	Control	0.05VG/1 µL	0.05CC/1 µL	10 mM cAMP
Cl^-	0.32 ± 0.03	1.45 ± 0.13*	1.63 ± 0.08*	1.07 ± 0.07*
Na^+	0.83 ± 0.04	1.11 ± 0.04*	1.26 ± 0.08*	1.10 ± 0.04*
K^+	0.22 ± 0.04	0.73 ± 0.10*	0.77 ± 0.07*	0.52 ± 0.04*

b) KCl saline

Ion	Control	0.05VG/1 µL	0.05CC/1 µL	10 mM cAMP
Cl^-	0.51 ± 0.04	1.59 ± 0.08*	1.89 ± 0.19*	1.29 ± 0.08*
Na^+	0.68 ± 0.04	0.77 ± 0.07	0.81 ± 0.04	0.65 ± 0.06
K^+	0.54 ± 0.04	1.54 ± 0.10*	1.67 ± 0.15*	1.18 ± 0.09*

*significantly ($P<0.05$) different from control rates

After stimulation, absorbate Na^+ concentration remained constant at 50 mM (Fig. 23), and stimulation did not increase the Na^+ absorption rate significantly above control levels (Table 6b).

Under unstimulated conditions, absorbate K^+ concentrations for sacs exposed to high NaCl saline (10mM K^+) fell from 40 mM at 1 h to 20 mM at 3-5 h (Fig.22). The addition of CC or VG caused a 2- to 3-fold increase in absorbate K^+ levels (Fig. 23), and hence a 2- to 5-fold increase in K^+ absorption rates across rectal sacs (Table 6b). The absorbate K^+ levels (55 to 60 mM; Fig. 23) were always lower than the luminal K^+ concentrations for unstimulated sacs exposed to high KCl saline. However, the addition of CC or VG did cause a nearly 2-fold increase in absorbate K^+ concentration to 100 mM, while increasing K^+ absorption rate by 2-to 3-fold (Fig. 23, Table 6b).

Effect of proline on fluid transport in recta and ilea

The effect of external proline at 80 or 1 mM on fluid transport by everted rectal and everted ileal sacs is shown in Fig. 25a. Fluid transport by rectal sacs in the presence of 80 mM proline was significantly ($P<0.05$) greater than that of recta exposed to 1 mM proline (steady-state J_v of 9.7 ± 0.7 $\mu L \cdot h^{-1} \cdot rectum^{-1}$ versus 5.1 ± 0.2 $\mu L \cdot h^{-1} \cdot rectum^{-1}$). When an everted rectal sac was exposed to 80 mM proline for 5 h and then switched to 1 mM proline, fluid transport rate fell from 13.2 to 2.5 $\mu L \cdot h^{-1} \cdot rectum^{-1}$ (Fig. 25b). When a rectal sac exposed to 1 mM proline for 5 h was switched to 80 mM proline, fluid transport rate increased from 4 to 11 $\mu L \cdot h^{-1} \cdot rectum^{-1}$ (Fig. 25b). The relationship between external proline concentration and long-term fluid transport rates is shown in Fig. 26. Stimulation of J_v was substantial at physiological levels (40 mM) of proline.

When proline was the only exogenous amino acid in the saline, fluid transport by everted ileal sacs was low and unaffected by increasing proline levels from 1 to 80 mM (Fig. 25a). Proline fluxes across ilea mounted as flat sheets in Ussing chambers under short-circuit conditions were measured to see if proline flux was substantially different

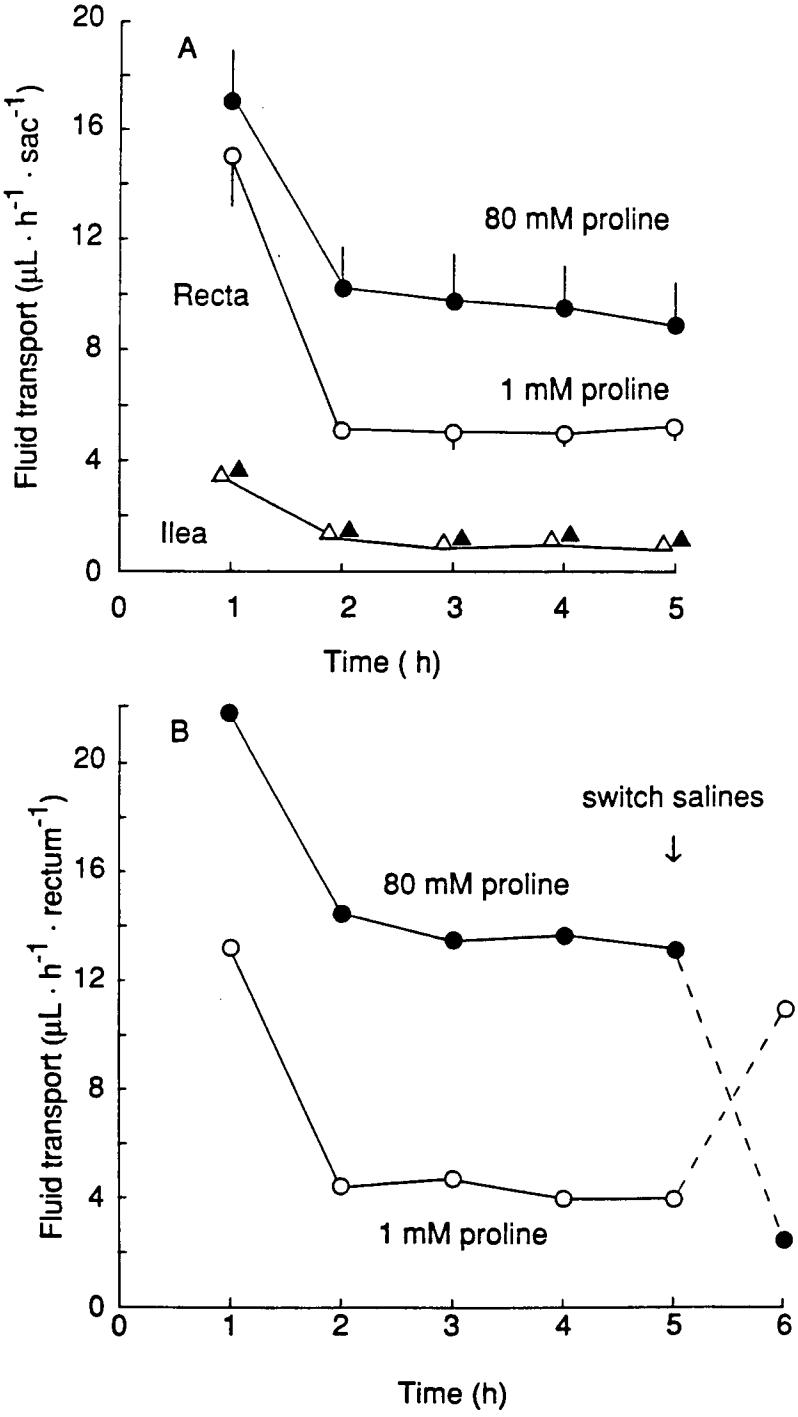


Figure 25. a)The effect of 1 mM (○) and 80 mM (●) proline in high NaCl saline on long-term fluid transport across everted rectal sacs, and on everted ileal sacs (Δ, 1 mM proline; ▲, 80 mM proline; mean ± s.e.,n=4). b) The effect of switching an everted rectal sac from 80 mM proline to 1 mM proline after 5 h (●) or switching a sac from 1 mM proline to 80 mM proline after 5 h (○).

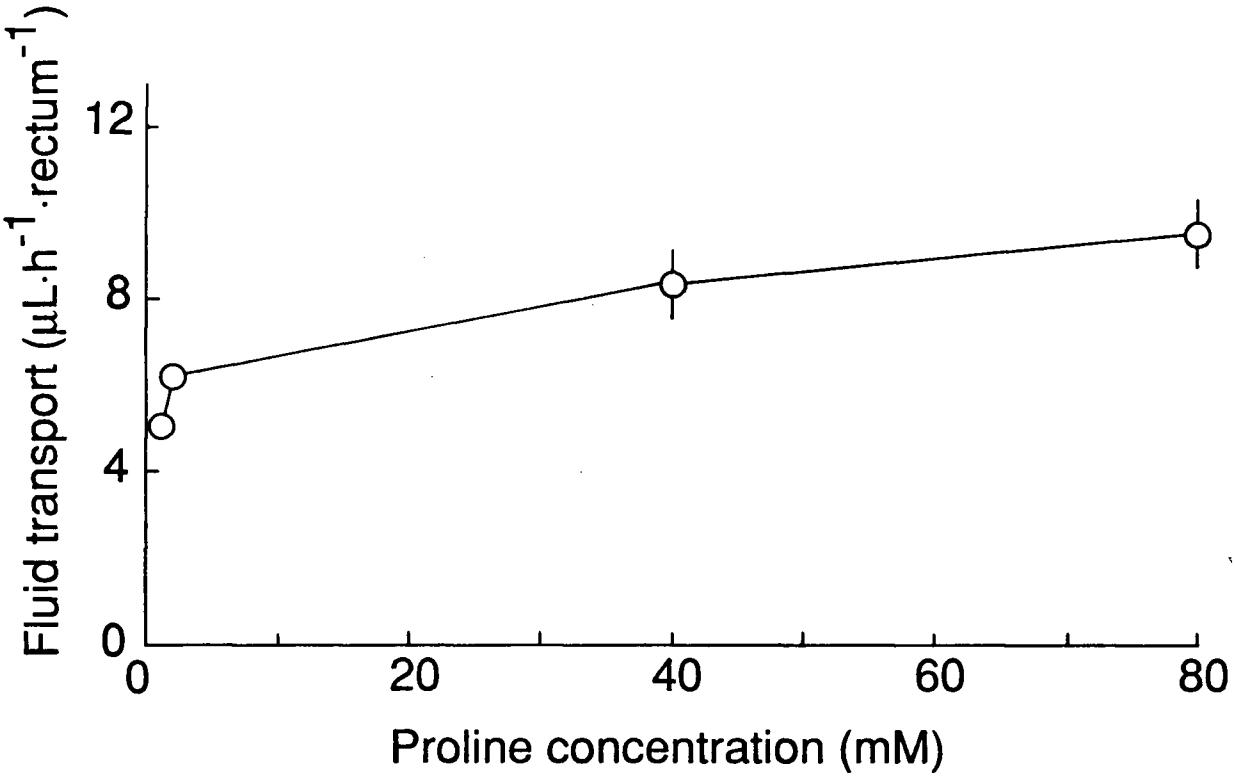


Figure 26. The effect of bilateral proline concentration in high NaCl saline on long-term (2 -5 h) fluid transport across everted rectal sacs. (mean ± s.e., n=16)

from that across locust recta. Opposing unidirectional fluxes of proline were equal when ilea were exposed to 13.1 mM proline (i.e. hemolymph levels) bilaterally (Table 7). Therefore there was no active net absorption of proline across locust ilea in contrast to the exceptionally large net flux of proline (2.34 ± 0.26 $\mu equil \cdot h^{-1} \cdot cm^{-2}$) across locust rectum (Meredith & Phillips 1988).

Everted rectal sacs exposed to saline lacking Na^+, K^+, and Cl^- but containing 80 mM proline were able to maintain fluid transport between 3.1 ± 0.3 $\mu L \cdot h^{-1} \cdot rectum^{-1}$ at 2 h and 2.1 ± 0.1 $\mu L \cdot h^{-1} \cdot rectum^{-1}$ at 5 h (Fig. 27). Fluid transport was completely inhibited when rectal sacs were exposed to 1 mM proline in the absence of Na^+, K^+, and Cl^- (Fig. 27). Rectal sacs exposed to 80 mM proline were able to transport against a larger osmotic concentration difference than sacs exposed to 1 mM proline (Fig. 28). The increases in fluid transport caused by high proline levels were similar when osmotic concentration differences across recta were increased from 0 to 820 mosmol (3.5-4.5 $\mu L \cdot h^{-1} \cdot rectum^{-1}$). This constant increase in fluid transport over the range of osmotic changes suggests that increased fluid flow is due to an increase in net solute flux in the 80 mM proline saline and not due to changes in osmotic permeability.

DISCUSSION

The response of locust rectum to cAMP, CC and VG was somewhat different from what was observed in chapter 3 for locust ileum. All three stimulants caused an increase in fluid tranport in both tissues; however, the amount of stimulation was much greater in the ileum. Rectal fluid transport was stimulated 6 $\mu L \cdot h^{-1} \cdot cm^{-2}$ by CC or VG while the same concentration of stimulants caused a 22 $\mu L \cdot h^{-1} \cdot cm^{-2}$ increase in ileal fluid transport. The stimulants caused an increase in K^+ and Cl^- absorption and decreased the anion deficit in both hindgut segments. However, the stimulation of Cl^- was much greater in the ileum as compared to the rectum. Proux *et al.* (1985) observed a negligible stimulation of rectal I_{sc} when VG (0.5 gland) was added to 5 mL of saline

Table 7. A comparison of proline fluxes across locust ilea and recta under short-circuit conditions.

Flux direction	Rectal proline fluxes[a] ($\mu equiv \cdot cm^{-2} \cdot h^{-1}$)	Ileal proline fluxes[b] ($\mu equiv \cdot cm^{-2} \cdot h^{-1}$)
Lumen to hemocoel	2.41 ± 0.43	0.154 ± 0.003
Hemocoel to lumen	0.06 ± 0.03	0.153 ± 0.004
Net flux to hemocoel	2.34 ± 0.26	0.001 ± 0.007

[a]Data from Meredith and Phillips (1988) at a bilateral external proline concentration of 12 mM; means ± s.e., n=5 .

[b]Average proline flux over a 4 h period at a bilateral external proline concentration of 13.1 mM; means ± s.e.; n=6.

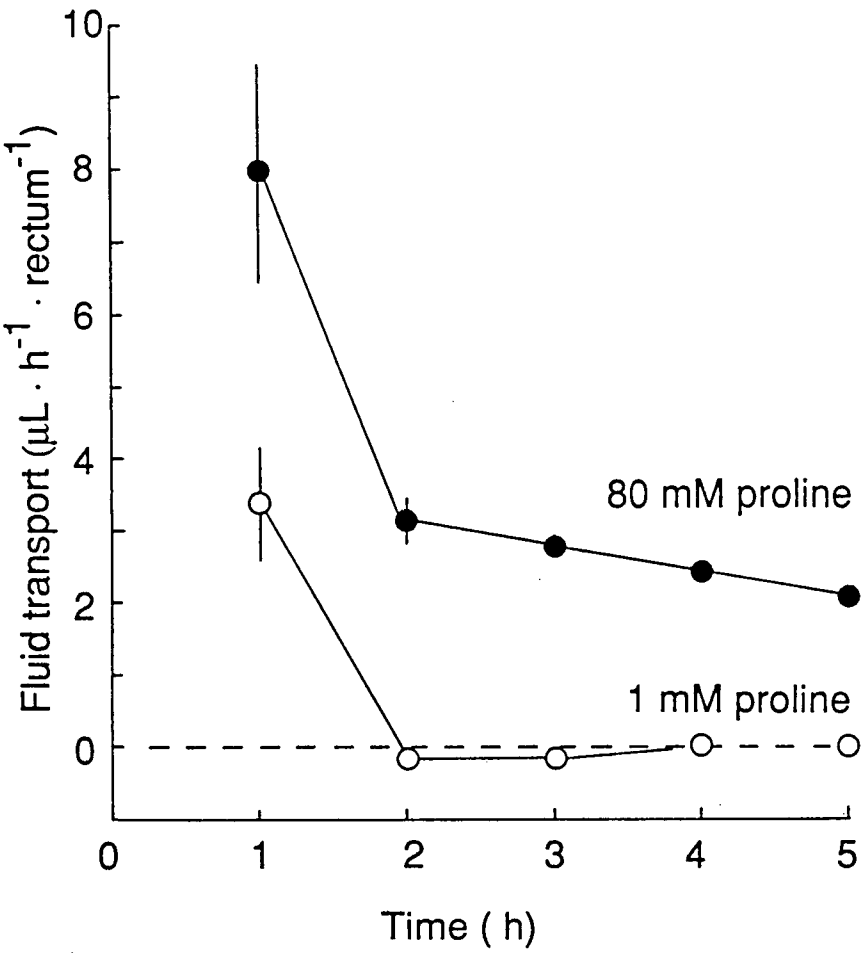


Figure 27. The effect of 1 mM (○) and 80 mM (●) proline in saline lacking Na^+, K^+, and Cl^- on fluid transport across everted rectal sacs. (mean ± s.e., n=4).

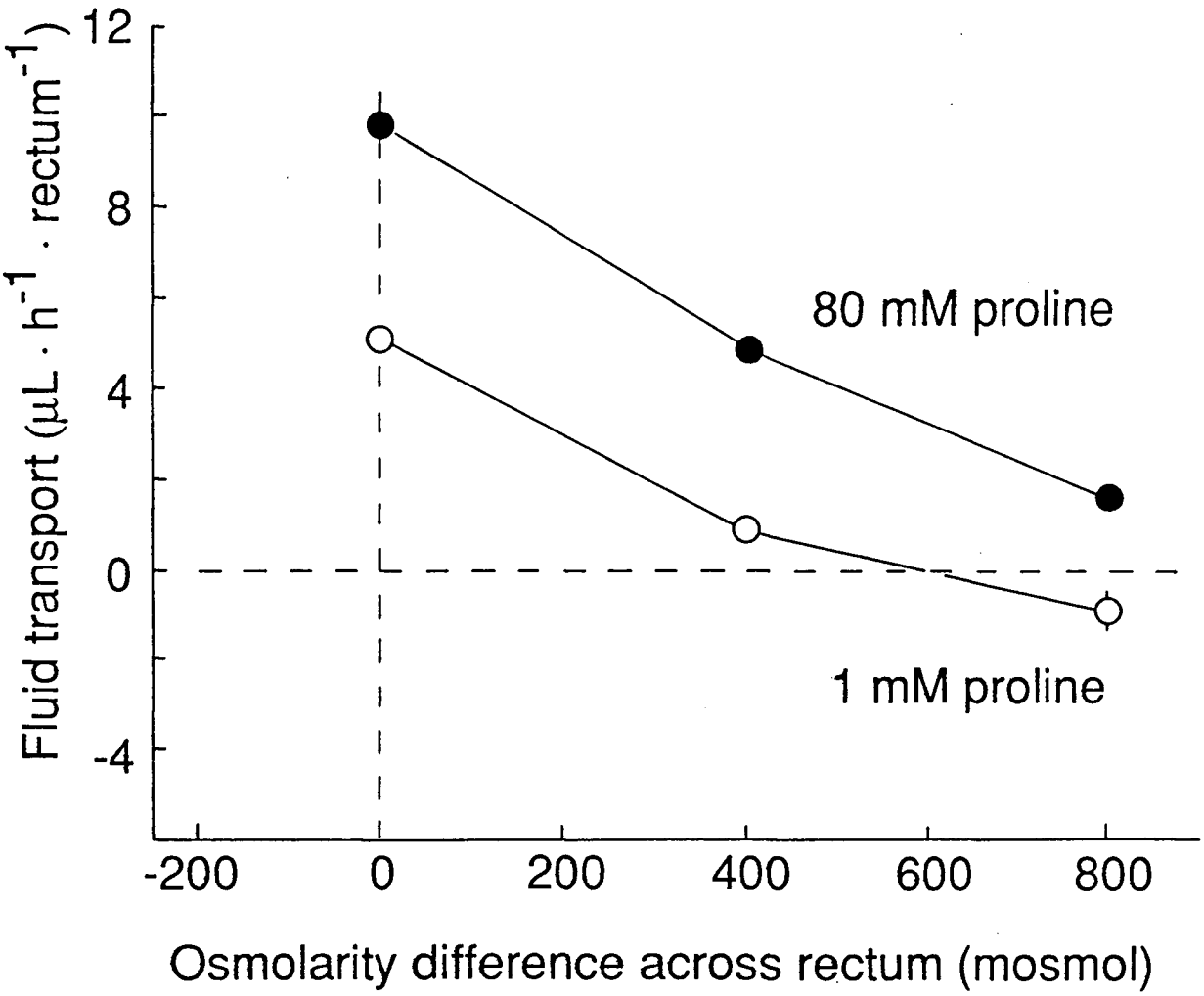


Figure 28. The effect of proline on long-term (2-5 h) fluid absorption when various osmotic concentration differences were created across everted rectal sacs. Preparations were bathed bilaterally in saline containing 1 mM proline (○) or 80 mM proline (●) and the luminal saline was made hyperosmotic by adding sucrose. The sign refers to lumen osmotic concentration minus hemocoel concentration. The saline on the hemocoel side was replaced hourly. (mean ± s.e., where larger than symbol, n=16 ; on 4 preparations).

in Ussing chambers. However recent observations by Thomson (unpublished observation) indicate that VG does increase rectal I_{sc}.. Thomson's results are more consistent with the results I obtained; i.e.VG stimulated Cl^- and K^+ absorption across everted rectal sacs to the same extent as CC did (Table 6) and therefore these stimulants should increase I_{sc}. The K^+ absorption across rectal sacs was greater than that observed for ileal sacs whether the sacs were stimulated or not. The difference in K^+ absorption between unstimulated and stimulated sacs was about the same in the two hindgut segments (0.9 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$).

The three stimulants did not affect Na^+ absorption in the everted rectal sacs to the extent observed for the ileum. I observed much lower Na^+ concentrations (80-90 mM) in the absorbate from everted rectal sacs as compared to 215 mM Na^+ observed in absorbate of both stimulated and unstimulated ileal sacs (Fig. 14). The Na^+ transport across everted rectal sacs exposed to high NaCl saline did increase slightly by 0.4 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$ when exposed to the three stimulants, mainly as the result of increased water flow; however the magnitude of this increase was only 10% of that observed in the ileum (5.1 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$). The Na^+ absorption across everted rectal sacs exposed to high KCl saline (10 mM Na^+) was not significantly increased by stimulants, while both Cl^- and K^+ fluxes were increased 3-fold. (Table 6). Again the ileum showed a large increase in Na^+ flux under similar conditions (Table 3). Black *et al.* (1987) did not observe any change in net $^{22}Na^+$ absorption across short-circuited locust recta mounted in Ussing chambers when cAMP or CC were added.. The observations presented in this chapter demonstrate that results of Black *et al.* (1987) were not due to obstruction of natural pathways of Na^+ exit from recta mounted in Ussing chambers.

The second difference between the locust ileum and rectum is in proline transport across the two tissues (Table 7). In the rectum, Meredith and Phillips (1988) observed a large net flux of proline which at physiological levels was second only to stimulated Cl^- flux. I observed small and equal unidirectional fluxes of proline indicating no net

transport of proline across the ileum. The transport of other amino acids across the ileum has not been investigated but seems likely to occur given the greater production of ammonia in this segment (see Table 5).

There was no effect of proline on the fluid transport rate across everted ileal sacs, consistant with the observation that there was no net proline flux. In the rectum however, increasing the bathing saline proline concentration increased the fluid transport rate by 50% at physiological levels (40 mM; Fig.26). This effect of proline was seen in the present and absence of Na^+, K^+ and Cl^-, the other major solutes transported by the rectum. These results suggest that proline may be one of the unknown organic substances postulated by Wall *et al.* (1971) to account for over half of the high osmotic concentration observed in the lateral spaces of cockroach recta. Wall and Oschman (1970) found 20% of the total osmotic concentration in the subepithelial sinus of the cockroach could be attributed to a ninhydrin-positive substance. Part of the increase in J_V caused by proline must be due to proline flux itself and not just increased flux of Na^+, K^+ or Cl^- since the increased J_V caused by 80 mM proline persisted for several h in the absence of Na^+, K^+ and Cl^-. Meredith and Phillips (1988) have demonstrated Na^+,K^+,Cl^--independent proline transport across locust recta which is consistent with the observed effect of proline on J_V. They suggest that this proline transport is driven at least partially by proton gradients. The effect of other amino acids on J_V was not determined but should be small. For example, the other major amino acid in the primary urine of locusts is glycine. However glycine transport (0.13 $\mu equiv \cdot h^{-1} \cdot cm^{-2}$) is 20-fold lower than proline transport and is dependent on Na^+ (Balshin 1973). Glycine should have a lesser effect on J_V and any effect it has should be Na^+-dependent.

Another indication that solutes other than Na^+, K^+ and Cl^- are important in fluid transport across the rectum is the contribution of these three ions to the total osmotic concentration of absorbate. Typical ion concentrations for absorbate from stimulated rectal sacs are 80 mM Na^+, 100 mM Cl^- and 50 mM K^+ (Fig. 18). These three inor-

ganic ions (total of 210 mosmol) can account for only 50% of the measured osmolarity of rectal absorbate (i.e. 400 mosmol;Fig. 24). Assuming that bicarbonate levels in stimulated rectal absorbate are similar to those observed in the ileum (10 mM) then a substantial fraction of the total absorbate osmolarity is still not accounted for by these four major inorganic ions. In contrast, 92% of the total absorbate osmolarity can be accounted for by these four ions in the ileum. These results suggest that organic solutes make up a large proportion of the total osmolarity in rectal absorbate. From my results on proline-stimulated J_V and the large proline fluxes observed by Meredith and Phillips (1988) proline is the likely solute.

SUMMARY

Extracts from both CC and VG stimulated transport of K^+, Cl^- and fluid across everted rectal sacs exposed luminally to either a high NaCl or high KCl salines, whereas there was little or no increase in Na^+ absorption. These results contrast with the large increase in Na^+ absorption across the ileum caused by the same stimulants. Proline transport across the two segments of the hindgut differ. Meredith and Phillips (1988) have reported a large net flux across the rectum at physiological concentrations; however, no net flux of proline was observed across the ileum under similar conditions. Increasing proline concentrations in the bathing saline had no effect on ileal fluid transport probably because of the lack of proline transport across the ileum. However, changing the external proline concentration from 1 mM to 80 mM proline caused a significant increase in fluid transport (4.6 $\mu L \cdot h^{-1} \cdot rectum^{-1}$) across everted rectal sacs. This stimulation of fluid transport was also observed in the absence of external Na^+, K^+, and Cl^-, and when the osmolarity of the luminal saline was increased.

CHAPTER 6:Anion-stimulated ATPase and Na^+,K^+-ATPase in Locust Hindgut

INTRODUCTION

In the rectum the predominant active transport process is an electrogenic Cl^- pump located in the apical membrane, while K^+ is largely absorbed passively through membrane channels by electrical coupling (Hanrahan & Phillips 1984a,b). A Na^+ pump has been demonstrated in the basolateral membrane but absorption of this cation is quantitatively much less important in the rectum (Phillips *et al.* 1986), while in the ileum it appears to be more important than K^+ absorption (chapters 3,4,5). In an extensive series of experiments, Hanrahan and Phillips (1984a,b; reviewed by Phillips *et al.* 1986) could find no evidence that Cl^- transport in locust rectum was coupled to and driven by Na^+, K^+, HCO_3^-, OH^-, or H^+ electrochemical gradients across the apical membrane (i.e. secondary transport). They were forced to propose that the apical Cl^- pump in this epithelium is probably directly coupled to cell metabolism (i.e. primary transport), possibly involving a membrane-bound anion-stimulated ATPase. More recent observations indicate similar transport mechanisms in locust ileum (Irvine *et al.* 1988; chapters 2 and 3).

Evidence for primary transport of anions across plasma membranes remains controversal (reviewed by Gerenscer & Lee 1983). Anion-stimulated ATPase activity has been described in many epithelia, including *Locusta migratoria* Malpighian tublues (Anstee & Fathpour 1981, 1979) and dragonfly recta (Komnick *et al.* 1980) but may be associated with mitochondrial contamination of the plasma membrane fraction (Van Amelsvoort *et al.* 1977a,b; DePont & Bonting 1981). Recently, Gerenscer and Lee (1985a,b) have demonstrated Cl^--HCO_3^--stimulated ATPase activity in a plasma membrane fraction of *Aplysia* intestine and ATP-dependent Cl^- uptake into *Aplysia* plasma membrane vesicles. These results provide strong evidence for an anion-stimulated

ATPase of extra-mitochondrial origin in an invertebrate epithelium.

In this chapter I test the hypothesis that the apical plasma membrane of locust rectum and presumably also the ileum might contain an anion-stimulated ATPase, while the basolateral membrane is the location of a typical Na^+,K^+-ATPase which is responsible for active reabsorption of Na^+. The Na^+,K^+-ATPase from insect hindgut-rectum has been extensively studied; however, the location of this enzyme within this tissue has only been determined in dragonfly larvae and not in papillate recta of terrestrial insects (reviewed by Anstee & Bowler 1984). In this chapter, I used specific activities of marker enzymes in cell fractions from homogenized locust recta and ilea to determine the presence and location of anion-stimulated ATPase and Na^+,K^+-ATPase in locust hindgut. I also examined the subcellular preparations of locust rectum by electron microscopy to assess various methods of separating plasma membrane fractions from mitochondria (ultrastructure reviewed by Wall & Oschman 1975; Martoja & Ballan-Dufrancais 1984; Chapman 1985).

MATERIALS AND METHODS

Experimental animals were sexually mature male and female desert locusts (*Schistocerca gregaria*) and maintained as described in chapter 2.

Preparation of membrane fractions

Recta or ilea were removed from 50-100 locusts and placed in ice cold 250 mM sucrose and 5 mM HEPES adjusted to pH 8.3 with Tris base. Recta were either homogenized whole or mechanically divided into two fractions and homogenized separately. In the latter procedure, the muscle and tracheae were stripped away from the epithelial layer with forceps. One fraction consisted of the tracheae and muscles while the other contained epithelial cells and cuticle, as confirmed microspically. Homogenization of entire ilea, entire recta or rectal fractions was performed using a

glass-Teflon homogenizer (15 strokes at 1,000 rpm) at 0°C. The homogenate was centrifuged at 1,000 g for 10 min at 5°C in a Sorval SS34 rotor yielding a nuclear pellet. The supernatant was centrifuged at 20,000 g for 20 min yielding a mitochondrial pellet. The supernatant was then centrifuged in a Beckman Ti50 rotor at 100,000 g for 60 min yielding a microsomal pellet and cytosol. The pellets were resuspended and assayed for enzyme activity.

Na^+,K^+-ATPase Activity

The ATPase activities of the different fractions were assayed in the following media: 1) 4 mM $MgCl_2$; 2) 4 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl; 3) 4 mM $MgCl_2$, 100 mM NaCl, 20 mM KCl, 0.6 mM ouabain. Na^+,K^+-ATPase was the average of both mean differences of phosphate production between 2 and 1 and between 2 and 3 (i.e. average of the two differences, which were usually similar). Each medium contained 3 mM ATP (Tris salt) and 50 mM imidazole/HCl (pH 7.4) to final volume of 1 mL (Peacock 1981). All tubes were equilibrated at 30 ± 0.5°C for 10 min before starting the reaction with the addition cell fractions containing 25-50 µg protein. The reaction was stopped at 15 min by adding 250 µL of 30% TCA. The protein precipitate was removed by centrifugation and the amount of inorganic phosphate released was measured by the method of Chen *et al.* (1956).

Anion-stimulated ATPase

Anion-stimulated ATPase activities were measured in the following basic media: 0.5 MgAcetate, 0.2 mM ouabain, 0.5 mM ATP (Tris salt) in 20 mM histidine adjusted to pH 8.3 with Tris in a final volume of 1 mL (Gassner & Komnick 1982). To test for activity due to Cl^-, three chloride salts were tested separately: choline Cl, KCl, or NaCl were added to the basic media to a final concnetration of 25 mM. To determine the kinetics of Cl^--stimulated ATPase activity, varying concentrations of choline Cl were

added. Other anions tested for their effects on ATPase activity were sulphite (as Na_2SO_3), bicarbonate (as $NaHCO_3$) and thiocyanate (as NaSCN). Incubations were started by the addition of cell fractions containing 10-25 μg protein and were carried out at 30 ± 0.5°C for 15 min. The reaction was stopped with 30% TCA and supernatant was assayed for PO_4^{3-} as previously stated.

Inhibitors

Various inhibitors of ATPase activity were added to determine the effect on ATPase activity in the locust rectum. Ortho-vanadate was added as Na_3VO_4 to a final concentration from 0.1 μM to 1.0 mM. Oligomycin, dissolved in 95% ethanol, was added to the reaction media and controls were run with an equal quantity of ethanol in the media. Other inhibitors used included the mitochondrial ATPase inhibitor, efrapeptin, and the alkaline phosphatase inhibitor, levamisole.

Other Enzyme Assays

The activity of the inner mitochondrial membrane marker enzyme, succinate cytochrome c reductase, was measured by a modification of the method of Ives *et al.* (1980). The reduction of cytochrome c was determined by the increase in absorption at 550 nm. The 1 mL reaction mixture contained 40 mM sodium phosphate buffer (pH 7.4), 20 mM sodium succinate, 6 mM potassium cyanide and 0.8 mg of cytochrome c. The reaction was started by the addition of 10-30 μg protein and the change in absorption was followed using a Perkin Elmer recording spectrophotometer. Succinate cytochrome c reductase activity was expressed as nmoles cytochrome c reduced per mg protein per min using a millimolar extinction coefficient of 18.5. The activity of another inner mitochondrial membrane marker enzyme, cytochrome oxidase, was measured by the method of Cooperstein and Lazarow (1951). The endoplasmic reticulum marker enzyme, NADPH-cytochrome c reductase, was measured in a similar manner to succinate

cytochrome c reductase by substituting NADPH for succinate.

Protein concentrations were determined by the method of Lowry *et al.* (1951) using BioRad γ-globulin as a protein standard. The plasma membrane markers, 5'-nucleotidase and alkaline phosphatase, were assayed by the methods of Aronson and Touster (1974) and Bowers and McComb (1966), respectively. Two additional apical membrane markers of other epithelia were also measured. Gamma-glutamyltranspeptidase was assayed with L-glutamyl-p-nitroanilide (5 mM) serving as the substrate and 10 mM glycylglycine as the acceptor in 100 mM Tris-HCl, pH 8.8 (Bodnaryk & Skilling 1971). Leucyl aminopeptidase was assayed in 50 mM mannitol, 2 mM Tris-HCl, pH 7.5 with 2 mM leucine-p-nitroanilide as the substrate.

Electron Microscopy

The 20,000 and 100,000 g pellets from differential centrifugation were fixed for 90 min in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.5) containing 0.25 M sucrose. The pellets were post-fixed in 2% osmium tetroxide in 0.1 sodium cacodylate buffer. Pellets were block stained in 1% uranyl acetate for 1 h, dehydrated in an ethanol series, mounted on copper grids, stained with lead citrate and viewed with a Zeiss Model EM10A transmission electron microscope.

Chemicals

Ouabain, oligomycin, tris(hydroxymethyl)aminomethane, Tris-ATP, p-nitrophenol phosphate, sodium AMP, cytochrome c, levamisole, leucine-p-nitroanilide, and sodium azide were purchased from Sigma Chemical Co. Gamma-glutamyl-p-nitroanilde was purchased from Boehringer Ltd. Sodium ortho-vanadate was purchased from Fisher Scientific. Efrapeptin was a gift from Dr. W. R. Fields of Lilly Research Laboratories, Indianapolis, IN. All other chemicals were of reagent grade purity.

RESULTS

Na^+,K^+-ATPase Activity in whole recta

Activities of Mg^{2+}-ATPase and Na^+,K^+-ATPase in crude homogenate, mitochondrial and microsomal fractions of whole locust recta are shown in Fig. 29. The specific activity of Na^+,K^+-ATPase was five times higher in the mitochondrial fraction (20,000 g pellet) than in the crude homogenate. The activity of succinate cytochrome c reductase was also the greatest in the mitochondrial fractions, with a specific activity five times that in the microsomal fraction (Fig. 29). Ultrastructural studies (reviewed by Wall & Oschman 1975) have shown that lateral membranes of locust are closely apposed to and nearly encapsulate a majority of mitochondria in this tissue. In Fig. 30a there are double-membrane vesicles surrounding many of the mitochondria and septate desmosome (scalariform junctions) between the two encapsulating membranes present in this fraction, indicating that these membranes are clearly basolateral in origin. When the cells were disrupted by homogenation, lateral membranes containing Na^+,K^+-ATPase formed vesicles around mitochondria and therefore these elements moved together when centrifuged. Together these observations suggest Na^+,K^+-ATPase is concentrated in the lateral membranes of locust rectal pad epithelium, as predicted by the electrophysiological studies of Hanrahan and Phillips (1984a,b).

The Na^+,K^+-ATPase activity of the homogenate was strongly inhibited by orthovanadate (Fig. 31). Activity was inhibited 50% by 1 μM vanadate and 98% by 100 μM vanadate.

Anion-stimulated ATPase Activity in whole recta

The anion-stimulated ATPase activity of microsomal, mitochondrial and homogenate fractions from whole rectal tissues in the presence of various salts are shown in Fig. 32. In the microsomal fraction, the addition of 25 mM KCl, NaCl, or choline

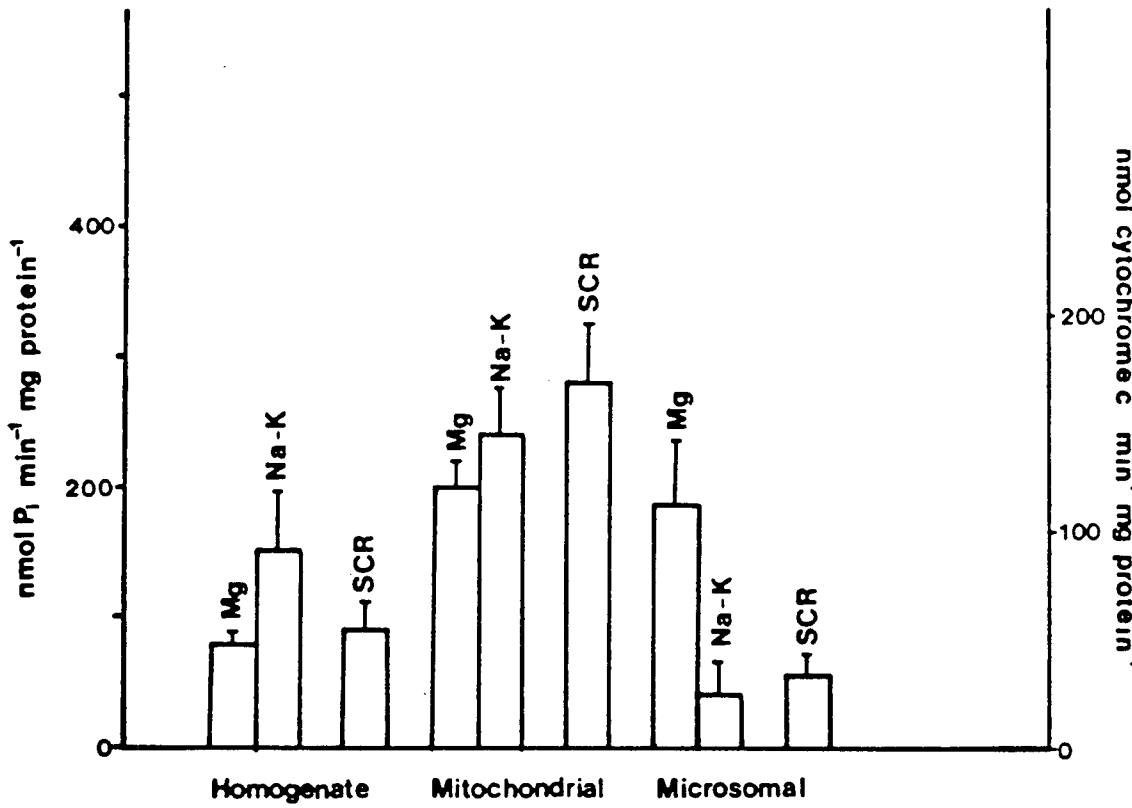


Figure 29. Residual ATPase (Mg), Na^+,K^+-ATPase (Na-K), and succinate cytochrome c reductase (SCR) in homogenate, mitochondrial (20,000 g pellet) and microsomal (100,000 g pellet) fractions from whole locust recta. Residual ATPase activities were those in the presence of 4 mM $MgCl_2$ alone. Succinate cytochrome c reductase and Na^+,K^+-ATPase activities were calculated as stated in the Methods section (mean ± s.d.,n=4).

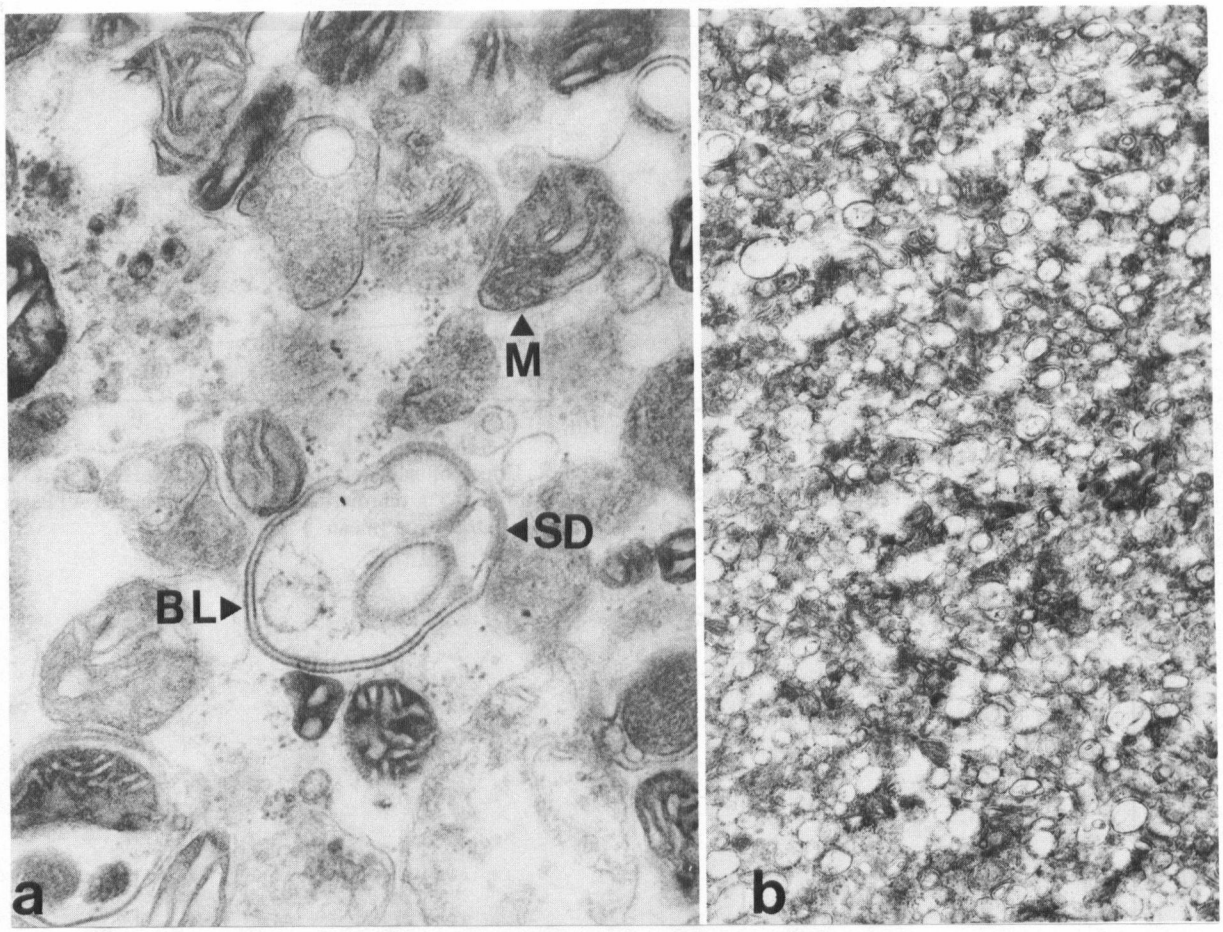


Figure 30. a) Electron micrograph of 20,000 pellet from whole locust recta, showing numerous mitochondria (M), and paired basolateral membranes (BL) of adjacent cells with septate desmosomes (SD) (31,000X). b) Electron micrograph of 100,000 g pellet from whole locust recta showing numerous membrane vesicles (21,000X).

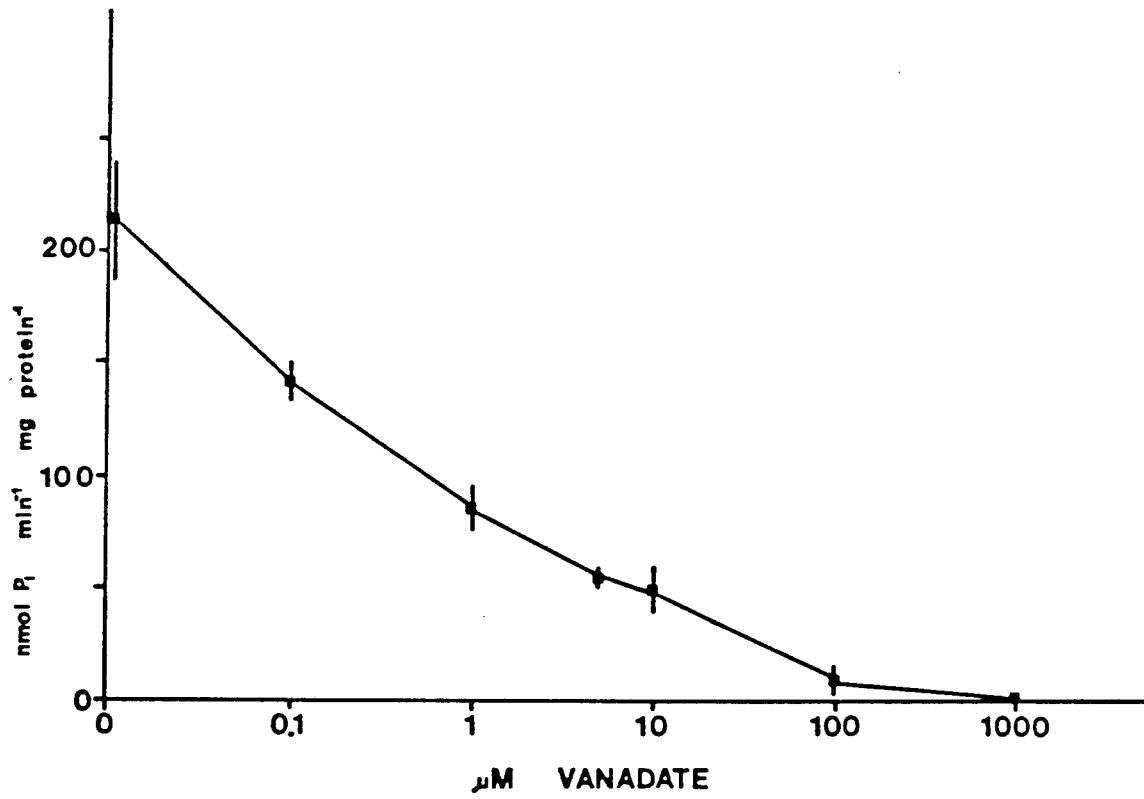


Figure 31. Inhibition of homogenate Na^+,K^+-ATPase in locust recta by Na_3VO_4 (mean ± s.d.,n=4).

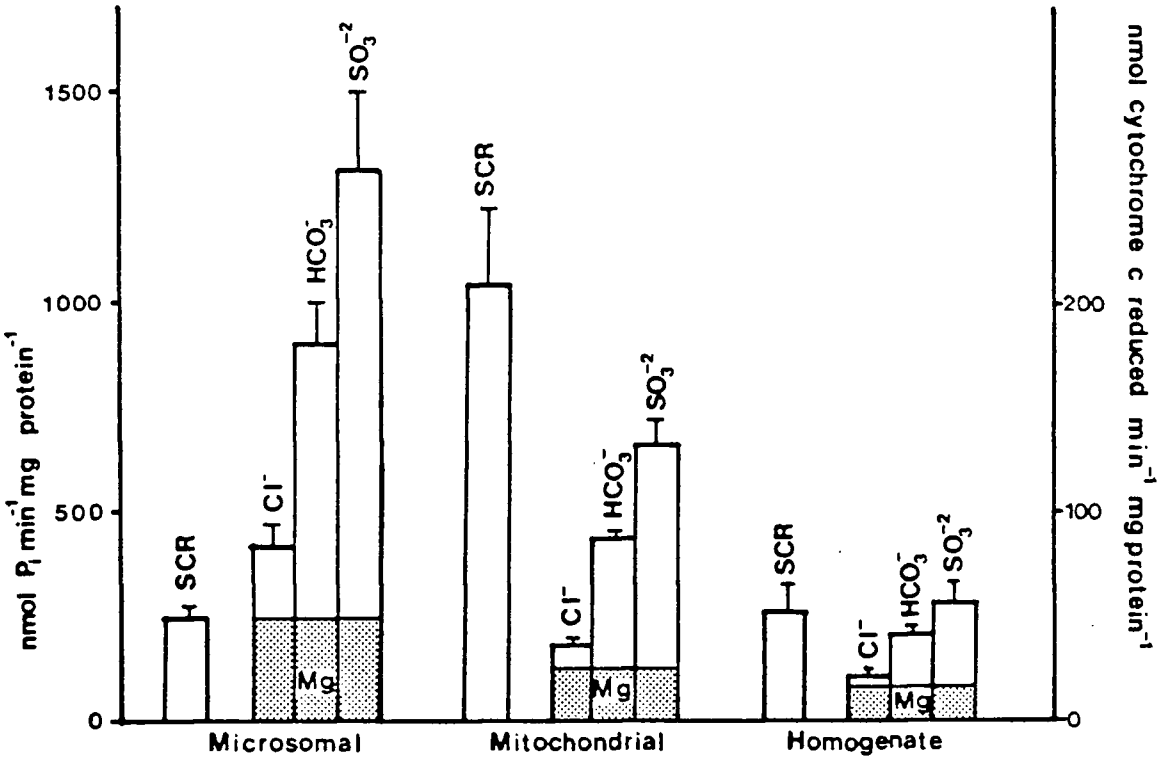


Figure 32. Succinate cytochrome c reductase (SCR), anion-stimulated ATPase activity in 25 mM choline Cl (Cl^-), 25 mM $NaHCO_3$ (HCO_3^-) or 25 mM Na_2SO_3 (SO_3^{-2}) and residual ATPase (Mg) in microsomal (100,000 g pellet), mitochondrial (20,000 g pellet) fractions and homogenate from whole locust recta (mean ± s.d.,n=4).

Cl, each caused a 2-fold stimulation of ATPase activity when compared to the 0.5 mM MgAcetate control. No significant difference was observed between the three chloride salts, so only ATPase activities from 25 mM choline Cl are shown in Fig. 32. The additon of 25 mM sulphite caused a 5-fold increase and 25 mM bicarbonate caused a 3-fold increase in ATPase activity. A similar distribution of anion-stimulated ATPase activity was observed in homogenate and mitochondrial fractions but specific activities were much higher in the microsomal fractions (Fig. 32). The specific activity of succinate cytochrome c reductase in the microsomal fraction was 20% of that observed in mitochondrial fractions and 80% of the activity in crude homogenate (Fig. 32). The microsomal pellets contained little mitochondrial contamination as seen by electron microscopy (Fig. 30b).

The effect of substrate concentration on Cl^--stimulated and HCO_3^--stimulated ATPase activities of microsomal fractions are shown in Fig. 33 & 34. The activities saturate between 25 and 50 mM for both substrates, with substrate inhibtion at 100 mM. Reciprocal plots of reaction rates versus substrate concentration gave K_m values of 7.2 and 8.9 mM and V_{max} of 187 and 865 nmoles Pi·mg protein^{-1}·min^{-1}, for Cl^- and HCO_3^- respectively (Fig. 34).

Although specific activities suggested Cl^--stimulated ATPase activities increase in microsomal fractions inhibitor studies indicated that microsomal anion-stimulated ATPase activity may be due to mitochondrial contamination. Thiocyanate (10 mM) strongly inhibited microsomal Cl^--stimulated ATPase activities (Fig. 35). Vanadate (1 mM) and alkaline phosphatase inhibitor, 1 mM levamisole, had little effect on Cl^--stimulated ATPase activity (Fig. 35). The mitochondrial ATPase inhibtors, oligomycin and sodium azide, caused 82% and 69% inhibition respectively of microsomal Cl^--stimulated ATPase (Fig. 35). Possibly the plasma membrane anion-stimulated ATPase is sensitive to these mitochondrial inhibitors, i.e. given the high ratio of plasma membrane to mitochondrial marker enzymes demonstrated in the microsomal fraction and the lack of

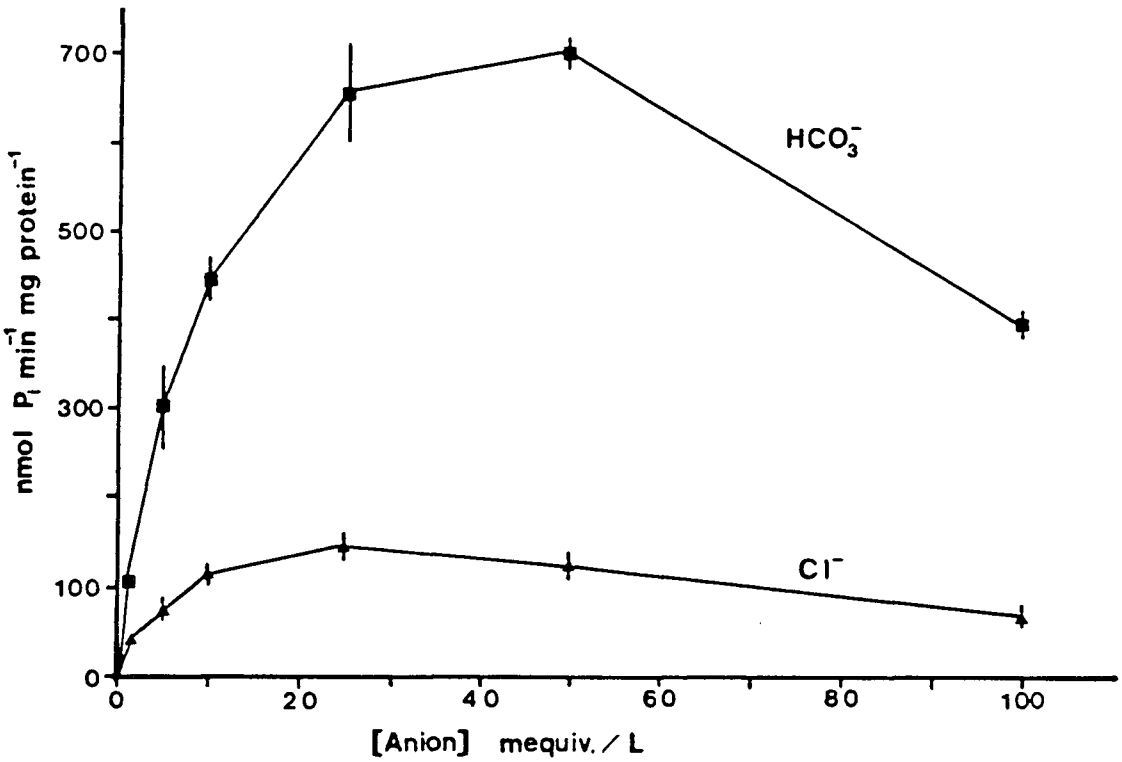


Figure 33. Effect of substrate concentration, $NaHCO_3$ (HCO_3^-) or choline Cl (Cl^-), on microsomal anion-stimulated ATPase activity in whole locust recta (mean ± s.d.,n=4).

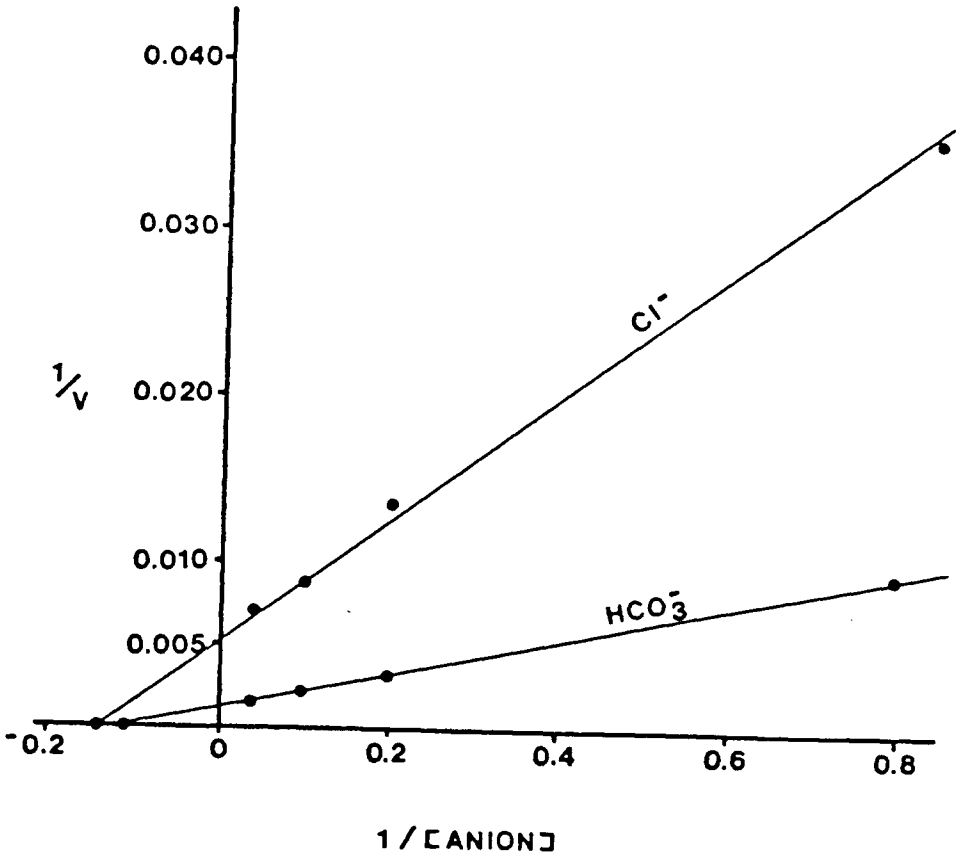


Figure 34. Lineweaver-Burk plot for effect of Cl^- and HCO_3^- on microsomal anion-stimulated ATPase activity in whole locust recta. Lines were determined by linear regression (r^2=0.99).

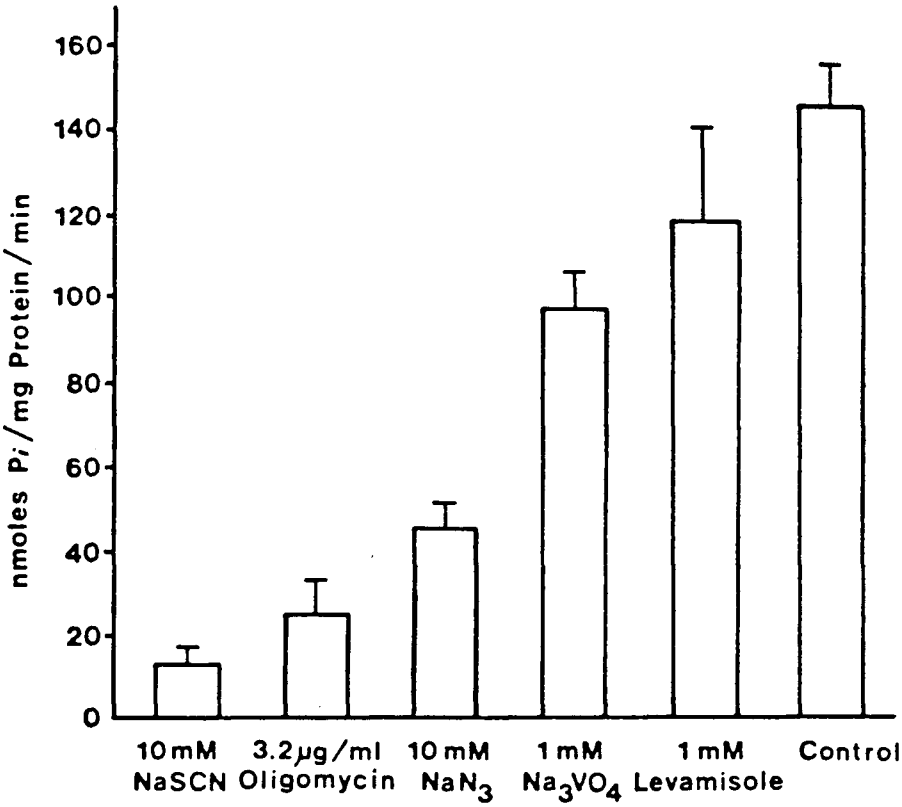


Figure 35. Inhibition of microsomal (100,000 g pellet) Cl^--stimulated ATPase activity from whole locust recta by NaSCN, oligomycin, NaN_3, Na_3VO_4, and levamisole compared with control Cl^--stimulated ATPase activity (mean ± s.d.,n=3).

mitochondria observed in the microsomal fraction (Fig. 30b).

Membrane marker enzymes in rectal cells and whole ilea

To obtain a purer preparation of epithelial cell plasma membranes, the tracheae and muscle layers of recta were mechanically separated from epithelial cells and cuticle before cell fractionation was conducted. These fractions were assayed for several plasma membrane and organelle marker enzymes. Considering first the rectal epithelial cell/cuticle layer, the microsomal fraction (100,000 g pellet) was enriched in the plasma membrane markers, γ-glutamyltranspeptidase, leucyl aminopeptidase, 5'-nucleotidase and alkaline phosphatase (Table 8). There was a decrease in specific activity of the mitochondrial marker enzymes (succinate cytochrome c reductase and cytochrome oxidase) and in the basolateral membrane marker (Na^+,K^+-ATPase) in the microsomal fraction. The 100,000 g pellet was also enriched in the endoplasmic reticulum marker, NADPH cytochrome c reductase. These results indicate that this fraction contained mainly a mixture of apical plasma membrane and endoplasmic reticulum.

The distribution of various marker enzymes and Cl^--stimulated ATPase from locust ileum was also determined (Table 9). These results were similar to those with the separated rectal cells in that the 100,000 g pellet was enriched in the plasma membrane markers, γ-glutamyltranspeptidase, leucyl aminopeptidase and alkaline phosphatase and the specific activities of the mitochondrial markers were decreased in the 100,000 g pellet as compared to the 20,000 g pellet (Table 9). The main differences between the ileal preparation and the rectal preparation were that the ileal preparation had much lower specific activities of Na^+,K^+-ATPase, Cl^--stimulated ATPase and alkaline phosphatase (Tables 8 & 9).

Table 8. Enzyme activities in homogenate and subcellular fractions of locust rectal epithelial cells[a]. Activities are expressed as nmole product per mg protein per minute (mean ± s.e., n=3-5). Relative activity as compared with homogenate is shown in parentheses.

Enzyme	Homogenate	20,000 g Pellet	100,000 g Pellet
γ-Glutamyl Transpeptidase	4.24 ± 0.62 (1.0)	8.30 ± 0.91 (2.0)	12.50 ± 1.63 (3.0)
Leucyl Amino-Peptidase	14.90 ± 3.88 (1.0)	26.20 ± 3.36 (1.8)	58.10 ± 9.58 (3.9)
Alkaline Phosphatase	1.79 ± 0.38 (1.0)	2.74 ± 0.47 (1.5)	5.42 ± 0.93 (3.1)
5'-Nucleotidase	0.45 ± 0.07 (1.0)	1.33 ± 0.17 (3.0)	1.85 ± 0.28 (4.1)
Succinate Cyto-chrome c Reductase	44.7 ± 2.29 (1.0)	153.5 ± 8.20 (3.4)	17.50 ± 2.81 (0.4)
Cytochrome c Oxidase*	15.50 ± 0.79 (1.0)	57.70 ± 5.13 (3.7)	11.23 ± 1.84 (0.7)
NADPH Cyto-chrome c Reductase	18.6 ± 2.37 (1.0)	30.00 ± 2.37 (1.6)	100.2 ± 6.70 (5.4)
Na^+,K^+-ATPase	262.5 ± 54.3 (1.0)	372.1 ± 7.51 (1.4)	137.1 ± 29.6 (0.5)
Cl^--stimulated ATPase	59.02 ± 12.1 (1.0)	143.0 ± 7.20 (2.4)	201.0 ± 21.0 (3.4)

[a]Epithelium mechanically separated from muscle/trachea layers.

*Cytochrome c Oxidase is expressed as Δlog (ferrocytochrome c)·mg protein^{-1} ·min^{-1}

Table 9. Enzyme activities in homogenate and subcellular fractions of locust ilea. Activities are expressed as nmole product per mg protein per minute (mean ± s.e., n=3). Relative activity as compared with homogenate is shown in parentheses.

Enzyme	Homogenate	20,000 g Pellet	100,000 g Pellet
γ-Glutamyl Transpeptidase	5.46 ± 1.01 (1.0)	13.81 ± 3.10 (2.5)	20.08 ± 3.82 (3.7)
Leucyl Amino-Peptidase	15.94 ± 2.46 (1.0)	44.72 ± 3.48 (2.8)	61.85 ± 5.97 (3.9)
Alkaline Phosphatase	0.91 ± 0.14 (1.0)	1.79 ± 0.39 (2.0)	1.51 ± 0.39 (1.7)
Acid Phosphatase	18.64 ± 0.06 (1.0)	39.64 ± 1.11 (2.1)	11.49 ± 0.54 (0.6)
Succinate Cyto-chrome c Reductase	104.2 ± 34.1 (1.0)	366.8 ± 35.3 (3.5)	61.90 ± 9.97 (0.6)
Cytochrome c Oxidase*	17.14 ± 1.18 (1.0)	65.03 ± 9.75 (3.8)	21.29 ± 1.16 (1.2)
NADPH Cyto-chrome c Reductase	21.80 ± 5.31 (1.0)	40.10 ± 5.52 (1.8)	171.5 ± 12.3 (7.9)
Na^+,K^+-ATPase	13.75 ± 1.52 (1.0)	36.47 ± 8.19 (2.7)	25.17 ± 5.35 (1.8)
Cl^--stimulated ATPase	29.02 ± 3.81 (1.0)	42.65 ± 8.04 (1.5)	39.23 ± 3.80 (1.4)

*Cytochrome c Oxidase is expressed as Δlog (ferrocytochrome c)·mg protein^{-1} ·min^{-1}

Anion-stimulated ATPase in rectal cells

Anion-stimulated ATPase activities in the muscle and tracheal fractions were only 15% of the specific activities in the epithelial cell preparation (data not shown). The anion-stimualted ATPase activities in the epithelial cell preparation are shown in Table 10. There were no significant differences in specific activities between the three chloride salts in either the microsomal or mitochondrial fractions; therefore the Cl^--stimulated ATPase was determined using choline Cl. The ATPase activities were the greatest in the microsomal fractions, while activities of succinate cytochrome c reductase (SCR) and cytochrome oxidase were the greatest in mitochondrial fractions. The activity ratios (mitochondrial:microsomal) were 8.79 for SCR, 5.16 for cytochrome oxidase and between 0.70 and 0.72 for anion-stimulated ATPases. These results indicated that anion-stimulated ATPase had a different distribution than the mitochondrial markers in cell fractions isolated from rectal epithelia.

The effect of efrapeptin, an inhibitor of mitochondrial F_1-ATPase (Cross & Kohlbrenner 1978), on Mg^{2+}-ATPase and Cl^--ATPase from the isolated rectal cells was determined on 20,000 g and 100,000 g pellets (Table 11). Efrapeptin at concentrations from 0.5 μg/mL to 0.05 μg/mL strongly inhibited both of these ATPase acitivities in both fractions.

DISCUSSION

There is considerable physiological evidence that electrogenic Cl^- transport across the apical membrane of locust rectum is not driven by cotransport using Na^+, K^+, H^+, OH^- or HCO_3^- gradients (Hanrahan 1982; Hanrahan & Phillips 1983; Phillips *et al.* 1986); therefore, Hanrahan and Phillips (1983) have suggested that active Cl^- transport in locust rectum might be a primary transport process, possibly involving an apical anion-stimulated ATPase. Herrera *et al.* (1978) observed anion-stimulated ATPase activity in

Table 10. Comparison of anion-stimulated ATPase, succinate cytochrome c reductase, and cytochrome c oxidase activities in the 20,000 g and 100,000 g pellets from locust rectal epithelial cells[a]. Activities are expressed as nmole product per mg protein per minute (mean ± s.e., n=5).

Enzyme	Activities		Ratio
	20,000 g Pellet	100,000 g Pellet	20K/100K
Succinate Cytochrome c Reductase	154 ± 8.2	17 ± 2.8	8.8
Cytochrome c Oxidase *	58 ± 5.1	11 ± 2.8	5.2
Cl^--ATPase[b]	143 ± 7.2	201 ± 21	0.71
SO_3^{2-}-ATPase	961 ± 65	1338 ± 107	0.72
HCO_3^--ATPase	642 ± 54	921 ± 89	0.70

[a]Epithelium mechanically separated from muscle/trachea layers.

[b]Choline Chloride

*Cytochrome c Oxidase is expressed as Δlog (ferrocytochrome c)·mg protein^{-1} ·min^{-1}

Table 11. Effect of efrapeptin on Mg^{2+}-stimulated and Cl^--stimulated ATPase activities in mitochondrial (20,000 g pellet) and microsomal (100,000 g pellet) fractions of locust rectal epithelial cells[a]. Activities are expressed as nmole product per mg protein per minute (mean ± s.e., n=3). Percentage of control activity is shown in parentheses.

Efrapeptin concentration (μg/mL)	Mg^{2+}-ATPase		Cl^--ATPase	
	20,000 g Pellet	100,000 g Pellet	20,000 g Pellet	100,000 g Pellet
Control	197 ± 33	205 ± 38	72 ± 17	58 ± 5.2
0.5	6.0 ± 3.2 (3%)	21 ± 4.6 (10%)	17 ± 6.9 (24%)	17 ± 8.7 (29%)
0.1	25 ± 0.5 (13%)	46 ± 19 (22%)	45 ± 18 (62%)	22 ± 12 (39%)
0.05	136 ± 50 (69%)	83 ± 43 (41%)	60 ± 27 (83%)	38 ± 11 (65%)
0.01	205 ± 43 (104%)	165 ± 42 (80%)	57 ± 20 (78%)	66 ± 13 (113%)

[a]Epithelium mechanically separated from muscle/trachea layers.

14,000 g pellets of rectal tissue of *S. gregaria*. This ATPase activity was stimulated by the addition of Cl^-, sulphate, and nitrite. However, these authors made no attempt to determine the subcellular source of this anion-stimulated ATPase activity. Komnick *et al.* (1980) reported the presence of an anion-stimulated ATPase in rectal plasma membranes of larval dragonfly (*Aeshna sp.*). These and several authors have proposed that active anion transport is linked to anion-stimulated ATPase activity (Komnick *et al.* 1980; Bornancin *et al.* 1980; Gerenscer & Lee 1985a, 1983).

Many investigators have demonstrated anion-stimulated ATPase activity in microsomal and plasma membrane fractions of various animal tissues (reviewed by Gerenscer & Lee 1983). The results I obtained are similar to those observed by Gerenscer and Lee (1985a) for *Aplysia* intestine. In microsomal fractions either from whole locust recta (i.e. including muscle and trachea) or from isolated rectal epithelial layer, there was an enrichment in specific activity of anion-stimulated ATPase with respect to the whole homogenate (Fig. 32;Table 10). The rectal and ileal microsomal fractions had much lower specific activities of mitochondrial markers, succinate cytochrome c reductase and cytochrome oxidase, in comparison with either the homogenate or mitochondrial fractions (Fig. 32;Tables 8 & 9). There was an enrichment of plasma membrane markers, γ-glutamyltranspeptidase, leucine aminopeptidase, 5'-nucleotidase, and alkaline phosphatase in the microsomal pellet from isolated rectal cells (Table 8) and a similar distribution was observed for preparations of locust ileum (Table 9). Micrographs of rectal microsomal pellets reveals little mitochondrial contamination (Fig. 30b). These results suggest that there is an anion-stimulated ATPase of extramitochondrial origin associated with plasma membranes other than those at the basolateral border, which are rich in Na^+,K^+-ATPase. Attempts to further purify the rectal microsomal fractions on continous sucrose or sorbitol gradients, or by pretreatment with deoxycholate or Triton X-100 at several concentrations, were unsuccessful (data not shown).

Several invertebrate epithelia contain anion-stimulated ATPase in membrane fractions with low mitochondrial contamination. These tissues include fiddler crab gill (DePew & Towle 1979), blue crab gill (Lee 1982), oyster mantle epithelium (Wheeler & Harrison 1982), and *Aplysia* intestine (Gerenscer & Lee 1985a). Deaton (1984) observed HCO_3^--stimulated ATPase activity in microsomes from midgut and integument of *Manduca sexta*. Microsomal activities of succinate dehydrogenase were 12% of those found in mitochondrial fractions (Deaton 1984). Turbeck *et al.* (1968) observed anion-stimulated ATPase activity in midgut from *Hyalophora cecropia* with a pH optimium of 8.7. Anstee and Fathpour (1979,1981) observed an anion-stimulated ATPase in Malphigian tubule microsomes from *Locusta migratoria*. ATPase activity was stimulated to the greatest extent by sulphite and was not stimulated by chloride. Microsomal fractions were relatively free of mitochondrial contamination and contained only 16% of the succinate dehydrogenase activity in mitochondrial fractions (Anstee & Fathpour 1981).

Efrapeptin, an inhibitor of mitochondrial F_1-ATPase (Cross & Kohlbrenner 1978), inhibited anion-stimulated ATPase activities from both mitochondrial and microsomal fractions from locust recta (Table 11). These results could indicate that either microsomal ATPase may be mitochondrial contamination or that plasma membrane anion-stimulated ATPase from insect tissue is also sensitive to efrapeptin. Anion-stimulated ATPase activity in plasma membrane of *Aplysia* intestine was not inhibited by efrapeptin to the same extent as ATPase activity in the mitochondrial fraction, but there was some inhibition of ATPases from both fractions (Gerenscer & Lee 1985a). Anion-stimulated ATPase activity in locust recta was also inhibited by azide and oligomycin (Fig. 35). Deaton (1984) observed no significant inhibition of microsomal HCO_3^--stimulated ATPase activity by 0.1 mM oligomycin, which did inhibit ATPase activities in mitochondrial fractions of *Manduca sexta*. Oligomycin inhibited anion-stimulated ATPase activity in both microsomal and mitochondrial fractions of *Locusta migratoria* Malpighian tubules (pI_{50} values of 4.29 and 4.74, respectively; Anstee & Fathpour 1981).

Vanadate only inhibited locust rectal anion-stimulated ATPase by 27% at a high concentration of 1 mM (Fig. 35). This is in contrast to the strong inhibition of locust Na^+,K^+-ATPase at concentrations between 0.1 μM and 100 μM vanadate (Fig. 31). Vanadate inhibits vertebrate Na^+,K^+-ATPase at the same levels (Cantley *et al.* 1978; Grantham & Glynn 1979). Gerenscer and Lee (1985a) observed 63% and 50% inhibition of Cl^-- and HCO_3^--stimulated ATPases respectively by 1 mM vanadate in *Aplysia.*

For locust rectum, the maximal Cl^-- or HCO_3^--stimulated ATPase activity was observed between 25 and 50 mM for Cl^- and HCO_3^- (Fig. 33). These were the same substrate concentrations which stimulated maximal ATPase activities in *Aplysia* intestine (Gerenscer & Lee 1985a). Komnick *et al.* (1980) observed maximal anion-stimulated ATPase activity at 30 mM HCO_3^- with a K_m of 4.65 mM HCO_3^-, while Cl^- caused maximal stimulation at a concentration of 20 mM with a K_m of 10.25 mM. In *S. gregaria*, the apparent K_m of 7.2 and 8.9 for Cl^- and HCO_3^- stimulation, respectively, were similar to values reported for *Aplysia* (Gerenscer & Lee 1985a), blue crab (Lee 1982), oyster mantle epithelium (Wheeler & Harrison 1982) and freshwater eel (Ho & Chan 1981).

Anion-stimulated ATPase activity from locust rectal epithelia was stimulated to the greatest extent by sulphite followed by HCO_3^- and finally Cl^- (Fig. 32). This sequence of anion stimulation has been observed by many other investigators in a variety of tissues (reviewed by Gerenscer & Lee 1983). Thiocyanate strongly inhibited anion-stimulated ATPase activity in rectal epithelia (Fig. 35). Thiocyanate has been shown to inhibit both Cl^--stimulated ATPase activities and Cl^- transport in *Aplysia* intestine (Gerenscer & Lee 1985a,b) and larval dragonfly rectal epithelium (Komnick *et al.* 1980). However, 10 mM thiocyanate had no effect on short-circuit current or potential difference across unstimulated or stimulated locust recta *in vitro* (Spring & Phillips 1980c).

The preparations of rectal and ileal tissues had similar specific activties of most of the marker enzymes tested (Tables 8 & 9). The first major difference between the two

tissues was the 20-fold higher specific activities of Na^+,K^+-ATPase found in the rectal tissue (homogenate activity: 262 versus 14 nmole $P_i \cdot mg\ protein^{-1} \cdot min^{-1}$). This difference may be a due to a greater amount of Na^+,K^+-ATPase found on the elaborate basolateral membranes found in the rectum while the ileum has a much simpler and less extensive basolateral membrane complex (Irvine *et al.* 1988; Wall & Oschman 1975). Further studies are needed to localize Na^+,K^+-ATPase in the locust hindgut. The second major difference was the lower specific activities of Cl^--stimulated ATPase found in the ileal preparations. The rectum would appear to have a greater concentration of this enzyme.

SUMMARY

Both Na^+,K^+-ATPase and mitochondrial marker enzymes were concentrated in the 20,000 g mitochondrial fraction of rectal tissue. Electron microscopic examination of this fraction indicated that it contained many mitochondria surrounded by basolateral membranes with scalariform junctions. This localizes most of this cation ATPase at the basolateral membrane. Anion-stimulated ATPase activities were observed in locust rectal epithelial cells and ileum, with the highest specific activities concentrated in the 100,000 g (microsomal) fractions. The distribution of anion-stimulated ATPase activity was different from that of the mitochondrial markers, succinate cytochrome c reductase and cytochrome oxidase. The microsomal fraction of the rectal epithelial cells and ileum had enriched activities of the plasma membrane markers, γ-glutamyltranspeptidase, leucyl aminopeptidase, 5'-nucleotidase, and alkaline phosphatase. These observations provide some evidence that there is an apical plasma membrane anion-stimulated ATPase, which may be responsible for active Cl^- transport in locust rectum and ileum. However direct experimental evidence is required to confirm whether this ATPase is indeed responsible for Cl^- transport. To obtain more direct proof, it will be necessary to show ATP stimulation of active Cl^- uptake by apical membrane vesicles from locust hindgut and also to demonstrate synthesis of ATP driven by large Cl^- gradients across vesicular membranes.

CHAPTER 7: General Discussion

The overall objectives of this thesis and a related study from our laboratory (Irvine *et al.* 1988) were to determine, using *in vitro* preparations, (a) which epithelial transport processes are present in locust ileum, (b) their magnitude relative to the processes in the rectum, and (c) whether their rates are potentially under neuroendocrine control. The results (Tables 12 & 13) strongly suggest that the ileum has a much greater role in regulating hemolymph volume and composition than previously supposed. Irvine *et al.* (1988) measured electrical parameters, including intracellular recordings, and also net ion fluxes under short-circuit conditions (i.e. active transport rates) using flat sheet preparations of ilea. As shown in Table 12, electrical parameters of locust rectum and ileum are remarkably similar both before and after stimulation. Both hindgut segments are tight epithelia with low transcellular resistance. This has been confirmed by cable analysis of locust rectum (Hanrahan & Phillips 1984b). Not surprisingly, given the lesser development of the basolateral cell border in the ileum (Fig. 2), the apical membrane constitutes the major resistance to ion diffusion (see voltage divider ratio, R_a/R_b, Table 12) in the ileum , whereas the two epithelial cell borders have more equal resistances in the rectum. Locust ileum and rectum both actively reabsorb Na^+, Cl^-, K^+ and HCO_3^- to the hemocoel side and actively secrete into the lumen ammonia, H^+, or OH^- in stimulated short-circuited state. Given that the rectal epithelial cells are 2 to 3 times longer than the ileum (Fig. 2), it is at first surprising that stimulated rates of active Na^+ and Cl^- absorption in the ileum per macroscopic surface area actually exceed those in the rectum. This is less of a paradox on closer inspection. For example, electrogenic Cl^-transport has been localized at the apical membrane in the rectum (Hanrahan & Phillips 1984b) and the apical membrane development (i.e. degree of infolding and associated mitochondria, Fig. 2) in the two segments is very similar. There is thus

Table 12. A comparasion of locust ileal and rectal transport capacities (mean value per cm^2 macroscopic surface area) at steady state with and without stimulants across flat sheet preparations.

Parameters*	Rectum		Ileum	
	Unstimulated	Stimulated	Unstimulated	Stimulated
I_{sc} ($\mu equiv \cdot h^{-1}$)	1	10	±1	10
V_t (mV, lumen)	7	32	-1	46
R_t ($\Omega \cdot cm^{-2}$)	280	160	240	98
R_a/R_b	1	1	14	6
		NET FLUXES ($\mu equil \cdot h^{-1}$)		
Na^+ absorption (short-circuit)	2	2	4	8
Cl^- absorption (short-circuit)	1	10	2	15
H^+ secretion (open-circuit)	1.8	0.6	0.4	—
NH_4^+ secretion (short-circuit)	0.6	0.6	1.2	2.7
OH^- secretion (open-circuit)	0	10	0	8
Proline absorption (short-circuit)	2.3	1.7	0	—

*Saline resembling hemolymph intially present bilaterally unless indicated otherwise Data from Irvine *et al.* (1988) and this thesis. The stimulated mean value is the highest observed stimulation with VG, CC or cAMP present.

Table 13. Comparison of locust ileal and rectal transport capacities (mean value per h per tissue) at steady-state with and without stimulants.

a) Everted sacs

Parameters*	Rectum		Ileum	
	Unstimulated	Stimulated	Unstimulated	Stimulated
J_V (Δosmol =0, NaCl saline)	10	14	3	16
J_V (Δosmol =0, KCl saline)	10	16	3	16
Δmosmol at J_V=0 (NaCl saline)	600	—	600	1000
Absorbate osmolarity (NaCl saline) (% difference from L saline)	-25	0	+6	+18
	NET FLUXES (μequil·h^{-1})			
Na^+absorption (NaCl saline)	0.8	1.2	0.5	2.5
Na^+absorption (KCl saline)	0.7	0.8	0.7	2.3
Cl^- absorption (NaCl saline)	0.3	1.6	0.2	2.5
Cl^-absorption (KCl saline)	0.5	1.9	0.3	2.8
K^+absorption (NaCl saline)	0.2	0.8	0.02	0.4
K^+absorption (KCl saline)	0.5	1.7	0.15	1.0
V_t (mV, lumen)	15	35	15	45

b) Flat sheet preparations

	OPEN-CIRCUIT NET FLUXES (μequil·h^{-1})			
Na^+absorption (NaCl saline)	—	—	1.2	2.5
Cl^- absorption (NaCl saline)	0.3	2.2	—	—
Cl^- absorption (KCl saline)	0.4	4.2	—	—
K^+absorption (NaCl saline)	0.4	2.9	—	—
K^+absorption (KCl saline)	0.5	10	—	—

*Saline resembling hemolymph intially present bilaterally (NaCl saline) or only on hemocoel side with high KCl saline on the lumen side (KCl saline). The highest stimulation of the process is shown with VG, CC or cAMP.

a good structure-function correlation in the case of Cl^- transport across the two hindgut segments.

My thesis reveals major differences between the two hindgut segments in that the ileum preferentially absorbs Na^+ actively at much higher rates than does the rectum and perhaps more significantly the rate of ileal Na^+ reabsorption can be controlled. The Na^+,K^+-ATPase responsible for Na^+ transport is normally localized in the basolateral membrane of most epithelia and I obtained some confirmatory evidence for this location (chapter 6) in locust rectum, which correlates with earlier electrophysiological evidence by Hanrahan and Phillips (1984b). The 20-fold greater specific activity of Na^+,K^+-ATPase in rectal tissue (Table 8) as compared to ileal tissue (Table 9) correlates with the much more extensive development of lateral cell membrane in the rectum (Fig. 2). Moveover, O_2 consumption studies by Chamberlin (reported in Phillips *et al.* 1986) indicated that Na^+ transport by Na^+,K^+-ATPase rather than KCl transport is the major determinant of metabolic rate in locust rectum. All of these observations which predict high rates of Na^+ transport in locust rectum can be reconciled with the low measured rates of transepithelial net fluxes (Table 12) if Na^+ is largely recycled at the extensive lateral border of the rectum as proposed by earlier authors (Wall 1971; Gupta *et al.* 1980; Fig. 4). This Na^+ recycling is necessary to extract a hyposmotic absorbate and thereby concentrate the rectal contents.

The second major qualitative difference which I observed was that the ileum does not actively absorb proline in contrast to the high rate transepithelial proline transport across the rectum. Again this may be attributed to solute recycling and extraction of hyposmotic fluid in the rectum. In support of this suggestion, I found that proline can drive active fluid absorption in locust rectum but not in the ileum (Fig. 25).

Since the ileum actually secretes ammonia four times faster than the rectum presumably the ileum oxidizes amino acids to form ammonia for secretion. In the rectum, luminal amino acids (mainly glutamine, proline, alanine and serine) are the major

sources of ammonia substrate (Thomson *et al.* 1988a). The ileum may not use proline as its major metabolic substrate since unidirectional proline fluxes across the ileum are equal and much lower than those across the rectum. Meredith (unpublished observations) has recently shown that several amino acids from either the lumen or hemocoel sides can indeed act as respiratory substrates in locust ileum. The metabolic substrates and pathways of metabolism in the ileum require further investigation.

The greater secretion of ammonia correlates with the greater absorption of Na^+ in the ileum, as compared to the rectum (Table 12). Ammonia secretion was shown by Thomson *et al.* (1988a) to occur by amiloride-sensitive, electroneutral exchange for Na^+ in the rectum. A similar exchanger in the ileum would help to explain why Na^+ transport is largely electroneutral (Irvine *et al.* 1988). In support of this hypothesis, cAMP increased both Na^+ absorption and also ammonia secretion in the ileum but not in the rectum (Table 12). The hindgut (ileum or colon segments) of some insects is enlarged into a fermentation chamber and even in locusts which lack such structures volatile fatty acids such as acetate are actively reabsorbed in the rectum. Secretion of ammonia in the hindgut of such insects might provide the nitrogen source required by microorganisms responsible for gut fermentation and thereby enhance production of metabolic substrates for such insects.

In summary, while there are some quantitative and qualitative differences between the two locust hindgut segments, results to date for the locust ileum are generally consistent with the epithelial transport model (Fig. 4) proposed for the rectum. A necessary major addition to this model is control of Na^+ entry mechanisms by cAMP in the ileum, which probably occurs at the apical membrane of the ileum. This can only be confirmed by more extensive experiments using intracellular electrodes to localize changes in specific Na^+ transport processes at the apical and basolateral membranes after stimulation.

The complex lateral membranes of the rectum make it difficult to study specific

mechanisms of transport across the basolateral border of the rectum because net fluxes are probably the result of several processes in series. However the simpler basal membranes of the ileum appear homogenous and much more accessible (Irvine *et al.* 1988). Unlike the rectum, it should be easier to study specific mechanisms of ion transport across ileal basal membranes using techniques such as intracellular ion-selective microelectrodes and patch clamping. Using these techniques the properties of K^+, Na^+, and Cl^- transport across the basal membrane may be elucidated. Earlier studies with ion-selective microelectrodes provided evidence for a Cl^- channel which is opened by the addition of cAMP and a K^+ channel which is inhibited Ba^{2+} in the basolateral membranes of the rectum (Phillips *et al.* 1986). There also is evidence that rectal basolateral membranes may contain a Cl^-/HCO_3^- exchanger possibly similar to the one conclusively demonstrated in the basal membrane of the anterior rectum of *Aedes dorsalis* larvae (Strange & Phillips 1985). Because of the similarities of ion transport in the two segments of the hindgut one might expect that similar mechanisms are present in the ileum although those have not been investigated to date. The most reasonable hypothesis is that ileal basolateral membrane should be similar to the scalariform complexes of the rectum where a primary hyperosmotic absorbate forms. Study of the ion transport mechanisms across ileal basal membrane might give more insight into these processes already hypothesized to be present in the rectum.

Earlier studies from our laboratory (Proux *et al.* 1984; Irvine *et al.* 1988; Phillips *et al.* 1988) suggested another major difference in control of absorption in the two hindgut segments. While both lobes of CC stimulated Cl^--dependent I_{sc} in both ileum and rectum, ventral ganglia was only observed to increase I_{sc} in the ileum. My results on fluid absorption and those of Audsley and Thomson (unpublished observation) on I_{sc} suggest that this apparent difference was probably an experimental artifact, namely loss of VG activity during storage. Our recent results reveal that CC and VG increase both I_{sc} and J_v in both locust rectum and ileum. For both segments, NCC is about four times

more potent stimulant of I_{sc} than GCC using flat sheet preparations as an assay, but the two lobes of CC have more equal effect in stimulating fluid absorption by everted sacs. These differences in relative potencies may result from the different external fluid volumes (hence concentration of stimulants) and in foreign surface areas (e.g. Ussing chambers) in the two assay methods. Such differences could also explain why ventral ganglia and corpus cardiacum are equally effective in stimulating fluid absorption in both rectum and ileum, whereas CC is a much more effective stimulant of I_{sc} across flat sheet preparations than VG. While there are other interpretations of these differences as discussed below, the relative responses of both locust hindgut segments to these different sources of stimulants now appear to be similar. There is therefore no compelling evidence to date which indicates that ileal and rectal reabsorption are controlled by different neuropeptide hormones. Moreover, since all of the demonstrated actions of VG and CC extracts on both hindgut segments (namely increases in Cl^-, K^+, Na^+, HCO_3^- and fluid absorption and changes in secretion of acid-base equivalents) are mimicked qualitatively by cAMP, there is no evidence at present to postulate the presence of more than one active factor per glandular source. Indeed it is difficult to envision how different neuropeptides could mediate separate actions commonly through intracellular cAMP unless other second messengers are also differentially involved, or unless specific transport events occur in different cell types. These questions will only be answered by purifying the active agents from each glandular source, testing their actions on each transport process in both hindgut segments, and observing their effects on second messenger systems in hindgut epithelia. N. Audsley is currently attempting to purify active factors in CC and VG using ileal I_{sc} as a bioassay.

Earlier work on control of ileal transport from our laboratory concentrated on solute transport across flat sheet preparations of ileum under short-circuit conditions when the saline on the lumen side had a high Na^+:K^+ ratio, unlike the situation *in vivo*. A major objective of my thesis was to characterize and quantify fluid transport in the ileum under

open-circuit conditions (i.e. as *in vivo*), and to investigate how stimulants and luminal cation ratios influence the rate of fluid absorption and absorbate composition. Fluid transport by everted sacs of locust ileum and rectum is compared in Table 13. Table 13 also compares estimates for net absorption by two *in vitro* preparations (everted sacs and flat sheet preparations (everted sacs and flat sheet preparations) under similar conditions. Williams *et al.* (1978) have discussed the problem of comparing the effective surface area of flat sheet and everted sac preparations because of the manner in which epithelia are mounted in Ussing chambers to avoid edge damage. Vigorous mixing and oxygenation is bilateral for flat sheets but unilateral (luminal side only) for everted sacs, resulting in different unstirred layers in the two methods. Finally, the composition of fluid inside everted sacs differs from the external saline whereas the composition is identical and held constant on both sides of flat sheet preparations. Consequently V_t is somewhat different in the two preparations as is the experimental temperature. Despite these differences, results with everted sacs generally confirmed results obtained with flat sheet preparations and provide considerable new information (Table 13).

There are several quantitative differences between the ileum and rectum. First, there is greater control of fluid reabsorption by neural extracts in the ileum (5-fold change) than the rectum (2-fold or less). After maximum stimulation, both organs have similar total fluid transport capacities even though the surface area of the ileum is only two-thirds that of the rectum. Luminal fluid entering the ileum is normally near isosmotic to hemolymph and even though the ileum can absorb fluid against exceptionally large osmotic gradients, absorption should only cause a small decrease in osmotic concentration of lumen contents *in vivo* because the absorbate is always slightly hyperosmotic. This agrees with measurements *in situ* which demonstrate that the urine becomes slightly hyposmotic as it passes through the ilea of several terrestrial insects (reviewed by Phillips 1981). The substantial fluid reabsorption revealed by this study suggests that considerable concentration of waste (i.e. unabsorbed) substances should occur.

Second, varying luminal cation ratios (Na^+:K^+) from 10:1 (all previous studies) to 1:10 (typical *in vivo* situation) had little effect on fluid or Cl^- transport rates or their response to stimulants in either hindgut segment (Table 13). This apparently reflects the fact that Cl^- transport rate is the major determinant of total cation (counter-ion) reabsorption and fluid transport rates in both segments, as revealed by ion substitution studies (chapter 3). Only when either Na^+ or K^+ levels are exceptionally low, do these ions have a major influence on fluid transport rate in the ileum (Fig. 12). Over a ten-fold change in luminal Na^+:K^+ concentration (mM) ratio from 110:10 to 10:110, the ileum preferentially reabsorbs Na^+ (rather than K^+) at nearly a constant rate and response to stimulants is quantitatively the same. This could be explained if the Na^+ reabsorption is close to saturation at these physiological concentrations. While Na^+ reabsorption in the rectum is also relatively independent of luminal cation ratios (Table 13), the major effect of stimulation is to increase KCl rather than NaCl reabsorption. Overall my results suggest that most of the luminal Na^+ entering the hindgut is probably reabsorbed in the ileum before excreta enter the rectum (see Fig. 36).

A third major new observation from my studies with ileal sacs is that stimulation causes a major change in composition (particularly of anions) of the absorbate and this has particular consequences for hemolymph pH regulation. Before stimulation the ileum absorbs an alkaline fluid (pH 7.8) rich in bicarbonates (45 mM) and suitable to counteract acidosis, possibly associated with periods of starvation between meals. After stimulation, the ileum switches to absorption of a fluid which more closely resembles ion ratios (Na^+, K^+, Cl^- and HCO_3^-) and pH (7.1) in hemolymph. Assuming release of ileal stimulants after feeding (see below) ileal absorption thereby helps to increase hemolymph volume, which commonly occurs at this time in previously starved insects, without changing hemolymph pH.

This raises the question concerning the contribution of ileal reabsorption and its hormonal control *in vivo*. My studies only demonstrate transport capacities *in vitro*.

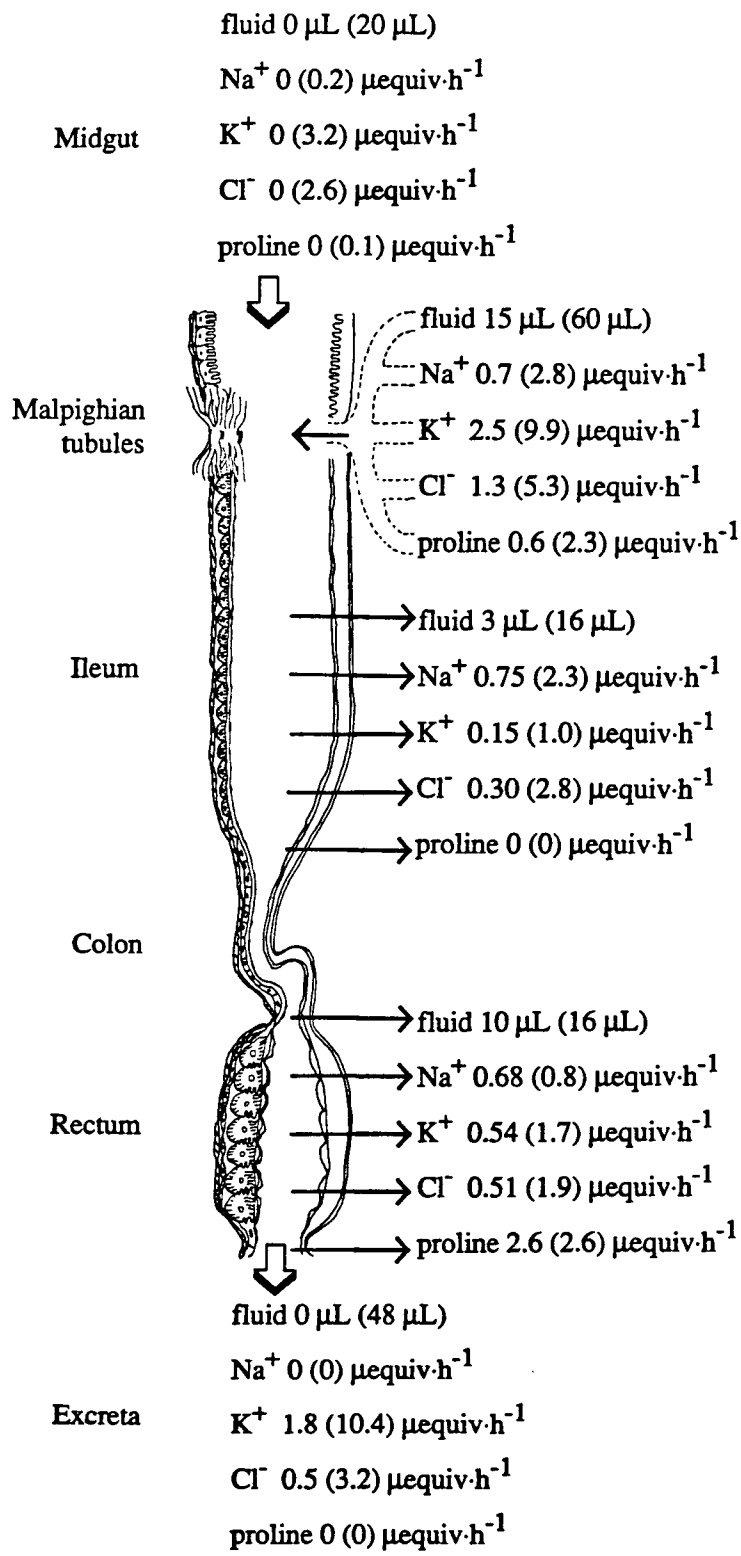


Figure. 36. Diagram of locust excretory system showing maximum *in vitro* transport rates of fluid, Na^+, K^+, Cl^- and proline. Unstimulated transport rates (unfed animals *in vivo)* are shown with stimulated rates (fed animals) in parentheses. Inputs to the hindgut are from the midgut and Malpighian tubules with output of fluid and solutes in the excreta.

Clearly some quantitative differences in ileal function may occur *in vivo* even though earlier studies on the rectum suggest that my incubation conditions should sustain ileal function close to that *in situ*. Even so, transit times for urine in the ileum are unknown. Indeed the most serious current limitation to our understanding of excretory function in insects is the lack of a method comparable to the inulin clearance method of the vertebrate renal physiologist, whereby quantitative changes can be determined in each nephron segment. Unfortunately formation of the primary urine by active secretion rather than filtration and mixing of the fluid with material from the midgut has to date precluded similar studies in intact insects. Development of a method to study renal function in whole insects should be urgently pursued. Moreover, until radioimmunoassays for specific neuropeptide stimulants acting on insect hindgut are developed, we can only presume that the active factors detected in VG and CC are actually released into the hemolymph, and we can only speculate as to when this occurs, what functions these factors normally serve, and what are the sensory mechanisms that initiate this hormonal release.

Having acknowledged these major limitations, I will attempt (Fig. 36) to integrate my observations with similar *in vitro* studies on other parts of the locust excretory system and midgut. I will now consider whether the observed rates of fluid and ion transport could remove ions and fluid that would be required to produce either moist excreta when insects are fed or dry excreta when the insect is dehydrated. The inputs of fluid and ions into the hindgut are from the midgut and Malpighian tubules. The fluid secretion in the Malpighian tubules is controlled by diuretic factors which are released in response to feeding (reviewed by Phillips 1981). The amount of fluid coming from the midgut is greatly influenced by the feeding status of the insect. Immediately following a meal (1-2 h) there is a large input (10-20 $\mu L \cdot h^{-1}$) of fluid into the ileum from the midgut (Dow 1981). Locusts which have not been fed for 6 h have little or no fluid flow from the midgut to the ileum (Dow 1981). Dow (1981) observed that in locusts deprived of food

for more than 2-4 h, fluid flow actually reversed and fluid secreted by the Malpighian tubules flowed anteriorly toward the anterior midgut and gastric caeca. Dow (1981) found that 8 $\mu L \cdot h^{-1}$ of fluid was absorbed by the anterior gastric caeca while the rest of the midgut had little effect on fluid reabsorption. Therefore when animals are unfed, the sources of fluid input are the Malpighian tubules, while the areas of fluid uptake are the ileum, rectum and anterior gastric caeca. Using *in vitro* estimates of fluid reabsorption these three tissues could absorb 21 $\mu L \cdot h^{-1}$. This is more than the estimated output of 15 $\mu L \cdot h^{-1}$ from the Malpighian tubules (Maddrell & Klunsuwan 1973) The ileum and rectum together having the capacity to remove 85% (13 $\mu L \cdot h^{-1}$) of the secreted primary urine.

After feeding, the countercurrent flow of fluid from the Malpighian tubules forward to the midgut ceases and there is an input of fluid into the hindgut from the midgut of up to 20 $\mu L \cdot h^{-1}$ (Dow 1981). After feeding, tubular secretion rates also increase due to stimulation by diuretic factors released to the hemolymph. *In vitro* tubular secretion rates can increase 4-fold upon stimulation which would increase the fluid input from the Malpighian tubules to 60 $\mu L \cdot h^{-1}$. The two inputs of fluid could therefore rise to 80 $\mu L \cdot h^{-1}$. After feeding previously starved locusts contain increased amounts of a CTSH-like substance in the hemolymph. When hemolymph from these animals was applied to *in vitro* rectal preparations there was an increased rectal fluid transport and transepithelial potential difference (Phillips *et al.* 1982b). Assuming that factors which stimulate ileal absorption are also increased at the same time hindgut fluid reabsorption could increase up to 32 $\mu L \cdot h^{-1}$. Nevertheless there would still be a large loss of fluid (48 $\mu L \cdot h^{-1}$) and therefore a very moist excreta, as has been observed after feeding *in vivo*. This post-prandial increase in both tubular secretion and hindgut reabsorption would result in increased fluid recycling through the excretory system and should help to clear the body of waste products either ingested or produced through metabolism of the meal (Phillips 1982). From the above calculations, the production of a very dry or

a very moist excreta would result from control of both the fluid secreted by the Malpighian tubules and also fluid reabsorption by the hindgut.

Factors controlling ileal reabsorption may also be concerned with conservation of specific ions and maintaining hemolymph pH and ion ratios. Fig. 36 shows the transport rates for fluid, ions and metabolites in the Malpighian tubules and hindgut of the locust under unstimulated and stimulated conditions using *in vitro* transport rates with a high KCl, low Na^+ primary urine (Maddrell & Klunsuwan 1973). Using *in situ* ionic composition for Malpighian tubular fluid (Phillips 1964b) the unstimulated tubular secretion of Na^+ would be 0.7 $\mu equiv \cdot h^{-1}$. The ileum has the capability to reabsorb all Na^+ from this fluid. Maximum stimulation of tubular secretion would increase the Na^+ output to 2.8 $\mu equiv \cdot h^{-1}$ and the midgut fluid would add another 0.2 $\mu equiv \cdot h^{-1}$. The ileum has the capability to reabsorb 80% of the Na^+ under stimulated conditions and the rectum would be able to remove the remaining Na^+. The other solute which should be completely reabsorbed is proline. The ileum should cause luminal proline concentrations to increase due to extensive fluid reabsorption without net absorption of proline. Further studies on proline uptake and metabolism by this tissue are needed to determine the influence of the ileum on proline levels in the urine. Nevertheless, the rectum has the capability to reabsorb all of the proline present in either unstimulated or stimulated primary urine, especially since fluid reabsorption in the ileum should raise its concentration and hence increase the reabsorption rate.

The calculations (Fig. 36) using my *in vitro* data from everted sacs show that there could be a loss of both K^+ and Cl^- from the animal under either unstimulated or stimulated conditions. When both tubular secretion and hindgut reabsorption are unstimulated, the calculated loss of K^+ and Cl^- in the excreta could be up to 1.8 and 0.5 $\mu equiv \cdot h^{-1}$, respectively. However, since tubular fluid would also flow forward to the anterior gastric caeca under these conditions some of the Cl^- and K^+ ions are undoubtedly reabsorbed in this tissue. If hindgut reabsorption is stimulated while tubular secretion is at

basal rates, then all K^+ and Cl^- would be reclaimed in the hindgut and this would enhance fluid reabsorption to produce a very dry feces. There is evidence that both the rectum and ileum are at least partially stimulated well after feeding has occurred (when tubular secretion is low): For example, hemolymph from locusts which have been starved and dehydrated for long periods (48 h) stimulates transepithelial potential across *in vitro* recta (Phillips *et al.* 1982b); however, this stimulation was only 50% of that caused by hemolymph from fed locusts. Moreover, all flat sheet preparations of ileum and rectum show high I_{sc} and V_t when first mounted in Ussing chambers regardless of previous hydration state and both parameters gradually decrease to steady-state levels in 2 h (Phillips *et al.* 1986; Irvine *et al.* 1988). Possibly the hindgut is stimulated (at least partially) in both starved and fed animals and the main function of these stimulants is to conserve K^+ and Cl^- and concentrate waste by fluid recycling. Alternatively, the act of handling and dissecting unanesthetized animals may simply cause release of stimulants (i.e. an experimental artifact of the preparation).

Following a meal there is a large influx of K^+ and Cl^- into the hindgut both from tubular secretion and midgut fluid. The *in vitro* estimates of K^+ and Cl^- reabsorption by the two hindgut segments using everted sacs indicate that large quantities of both ions would be lost in the excreta (Fig. 36). This is logical since lettuce contains (mmol·kg water^{-1}) K^+ 110, Cl^- 35 and Na^+ 14 and there would be a need to excrete excess K^+ and conserve Na^+ (Phillips 1981). Other plant materials probably have similar ion ratios. However, the rates of both $Cl^-$and K^+ reabsorption measured across stimulated everted rectal sacs exposed to high KCl saline were lower than those observed under similar conditions across flat sheet rectal preparations. Hanrahan (1982) observed 4.2 μequiv·h^{-1} of Cl^- transported to the hemocoel side of cAMP-stimulated open-circuit recta exposed to 100 mM K^+ as compared to 1.9 μequiv·h^{-1} across everted sac preparations (Table 12). Flat sheet recta had even larger net fluxes of K^+ under open-circuit conditions at luminal K^+ concentrations of 100 mM (10.2 μequiv·h^{-1}). If these values

for K^+ and Cl^- transport are used in Fig. 36 then there would be losses of 2.1 and 1.4 $\mu equiv \cdot h^{-1}$ for K^+ and Cl^- respectively in the feces.

The major process which is stimulated by cAMP and neural extracts in both hindgut segments is electrogenic Cl^- transport and stimulation of fluid transport depends largely on this process. The nature of the active Cl^- pump, hypothesized to be in the apical membrane of both hindgut segments, is still unclear. The active Cl^- transport across the rectum could not be explained by any models of secondary anion transport (Hanrahan & Phillips 1984b), therefore I investigated the possibility that this active Cl^- transport may be due to an anion-stimulated ATPase in the apical membrane. I demonstrated anion-stimulated ATPase activities in microsomal fractions from both hindgut segments, with the highest specific activities in rectal tissue, but I was unable to totally eliminate mitochondrial contaminants from these preparations. Anion-stimulated ATPase activities in microsomal fractions from rectal epithelia were inhibited by a number of mitochondrial ATPase inhibitors. These results are inconclusive as to whether primary active Cl^- transport involving an ATPase is the mechanism for the large Cl^- absorption observed across the locust hindgut. A vesicle preparation of purified apical plasma membrane is essential in order to demonstrate primary Cl^- transport across apical membranes of locust hindgut. Such a preparation from intestine of *Apylsia californica* was used to show ATP-dependent Cl^- uptake into apical membrane vesicles (Gerensecer & Lee 1985b). The crude microsomal fraction which I obtained from locust hindgut did not form sealed vesicles (data not shown) and were therefore unsuitable for such studies.

Some recent studies by Thomson (see Irvine *et al.* 1988) have demonstrated a powerful proton pump and also a OH^-/Cl^- antiport in the apical membrane of both hindgut segments. These recent results suggest that the role of proton gradients in driving electrogenic Cl^- transport in insect hindgut should be reassessed.

Comparison of vertebrate nephron and insect excretory system

The excretory system of insects has a number of analogies to the vertebrate nephron. The Malpighian tubules act much like the glomerulus to create an isosmotic primary urine containing metabolites. The Malpighian tubules also secrete harmful substances (i.e. plant alkaloids) into the urine, a function performed by the proximal tubules of the vertebrate kidney. The insect primary urine then flows into the ileum where Na^+, K^+, Cl^-, HCO_3^- and fluid are reabsorbed creating a urine which is hyposmotic to the original urine. The ileum has a large capacity for transport of these ions and fluid and under stimulated conditions may remove large quantities of fluid and ions. In the nephron from most vertebrates the proximal tubules remove 60-80% of the glomerular filtrate while having no effect on the osmolarity of the urine. Therefore the vertebrate proximal tubules and ileum have similar functions in the two systems. In the locust the rectum acts to determine the final levels of ions and fluid in the excreta to form srongly hyperosmotic or hyposmotic urine. In the nephron the distal tubules, loop of Henle and collecting ducts are required to create a hyperosmotic or hyposmotic urine. In both systems solute recycling is used to create a hyperosmotic urine but this recycling occurs at a different structural level in the two systems. In the vertebrate kidney the recycling involves the loop of Henle and collecting ducts (i.e. several epithelial types). Insects recycle solutes in the lateral channels between the principal cells of the rectal epithelium. The recycling solutes in both systems appears to be NaCl and organic solutes. In insects the organic solute may be proline while vertebrates commonly use urea. In a sense my thesis and the companion study by Irvine *et al.* (1988) have identified the ileum as the "proximal tubule" of the locust excretory system.

REFERENCES

AL-AWQATI, Q. S. GLUCK, W. REEVES and C. CANNON. 1983. Regulation of proton transport in urinary epithelia. J. exp. Biol. **106**:135-141.

ANDRUSIAK, E.W., J.E. PHILLIPS AND J. SPEIGHT. 1980. Phosphate transport by locust rectum *in vitro*. Can. J. Zool. **58**:1518-1523

ANSTEE, J.H., and D.M. BELL. 1978. Properties of Na^+, K^+-activated ATPase from the excretory system of *Locusta*. Insect Biochem. **8**:3-9.

ANSTEE, J.H., and K. BOWLER. 1984. Techniques for studying (Na^+-K^+) ATPase. *In* Measurement of Ion Transport and Metabolic Rate in Insects. *Edited by* T.J. Bradley and T.A. Miller. Springer-Verlag. New York. pp. 187-220.

ANSTEE, J.H., and H. FATHPOUR. 1979. The presence and properties of a Mg^{2+} dependent HCO_3^--stimulated ATPase in the Malphigian tubules of *Locusta migratoria*. Insect Biochem. **9**:383-388.

ANSTEE, J.H., and H. FATHPOUR. 1981. Studies on the anion-sensitivity, oligomycin-sensitivity and subcellular localization of adenosine triphosphatase activity in Malpighian tubules of *Locusta*. Insect Biochem. **11**:103-115.

ARONSON, N.N., and O. TOUSTER. 1974. Isolation of rat liver plasma membrane fragments in isotonic sucrose. *In* Methods of Enzymology. *Edited by* S.F. Fleischer and L. Packer. Acedemic Press, New York, Vol. 31, pp. 167-181.

AUDSLEY, N., R.A. LECHLEITNER, J. MEREDITH and J.E. PHILLIPS. 1988. Transport properties of locust ileum *in vitro*: effects of corpus cardiacum and ventral ganglia. submitted to J. Comp. Physiology

BALSHIN, M. 1973. Absorption of amino acids *in vitro* by the rectum of the desert locust (*Schistocerca gregaria*). Ph.D. thesis. University of British Columbia, Vancouver.

BALSHIN, M. and J.E. PHILLIPS.1971. Active absorption of amino acids in the rectum of the desert locust (*Schistocerca gregaria*). Nature, Lond. **233**:53-55.

BAUMEISTER, T., J. MEREDITH, W. JULIEN and J.E. PHILLIPS. 1981. Acetate transport by locust rectum *in vitro*. J. Insect Physiol. **27**:195-201.

BERRIDGE, M.J. and B.L. GUPTA. 1967. Fine-structural changes in relation to ion and water transport in rectal papillae of the blowfly, *Calliphora*. J. Cell Sci. **2**:89-112.

BLACK, K., J. MEREDITH, B. THOMSON, J. PHILLIPS, and T. DIETZ. 1987. Mechanisms and properties of sodium transport in locust rectum. Can. J. Zool. **65**:3064-3092.

BODNARYK, R.P. and J.R. SKILLINGS 1971. γ-Glutamyltranspeptidase catalyses the synthesis of γ-glutamylphenylalanine in the larva of the housefly *Musca domestica*. Insect Biochem. **1**:467-479.

BORNANCIN, M., G. DE RENZIS and R. NAON. 1980. Cl^--HCO_3^--ATPase in gills of the rainbow trout:evidence for its microsomal localization. Am. J. Physiol. **238**:R251-259.

BOWERS, G.N. Jr., and R.B. McCOMB. 1966. A continuous spectrophotometric method for measuring the activity of serum alkaline phosphatase. Clin. Chem. **12**:70-89.

BRADLEY, T.J. 1985. The excretory system: structure and physiology. *In* Comprehensive Insect Physiology, Biochemsitry and Pharmacology. *Edited by* G.A. Kerkut and L.I. Gilbert. Vol. 4, pp. 421-465. Pergamon, Oxford.

CANTLEY, L.C., Jr., L.G. GANTLEY and L. JOSEPHSON. 1978. A characterization of vanadate interactions with the (Na,K) ATPase. J. biol. Chem. **253**:7361-7368.

CHAMBERLIN, M. 1981. Metabolic studies on the locust rectum. Ph.D. thesis, University of British Columbia, Vancouver.

CHAMBERLIN, M.E. and J.E. PHILLIPS. 1982a. Metabolic support of chloride-dependent short-circuit current across locust rectum. J. exp. Biol. **99**:349-361.

CHAMBERLIN, M.E. and J.E. PHILLIPS. 1982b. Regulation of hemolymph amino acid levels and active secretion of proline by Malpighian tubules of locusts. Can. J. Zool. **60**:2745-2752.

CHAMBERLIN, M.E. and J.E. PHILLIPS. 1983. Oxidative metabolism in the locust rectum. J. Comp. Physiol. **151**:191-198.

CHAMBERLIN, M.E. and J.E. PHILLIPS. 1988. Effects of stimulants of electrogenic ion transport on cyclic AMP and cyclic GMP levels in locust rectum. J. exp. Zool. **245**:9-16.

CHAPMAN, R. F. 1985. Structure of the digestive system. *In* Comprehensive Insect Physiology, Biochemsitry and Pharmacology. *Edited by* G.A.Kerkut and L.I. Gilbert. Vol. 4, pp. 165-211. Pergamon, Oxford.

CHEN, P.S. Jr., TORIBARA, T.Y. and H. WARNER. 1956. Microdetermination of phosphorus. Anal. Chem. **28**:1756-1758.

COOPERSTEIN, S.J. and A. LAZAROW. 1951. A microspectrophotometric method for the determination of cytochrome oxidase. J. biol. Chem. **189**:665-670.

CROSS, R.L. and W.E. KOHLBRENNER. 1978. The mode of inhibition of oxidative phosphorylation of efrapeptin (A23871). J. biol. Chem. **253**:4865-4873.

DEATON, L.E. 1984. Tissue K^+-stimulated ATPase and HCO_3^--stimulated ATPase in the tobaco hornworm *Manduca sexta*. Insect Biochem. **14**:109-114.

DEPEW, E.F. and D.W. TOWLE. 1979. Bicarbonate-stimulated ATPase in plasma membrane fractions of fiddler crab (*Uca minax*) gill. Mar. Biol. Lett. **1**:59-67.

DEPONT, J.J.H.H.M. and S.L. BONTING. 1981. Anion-sensitive ATPase and (K^+-H^+) ATPase. *In* Membrane Transport. *Edited by* S.L. Bonting and J.J.H.H.M. DePont. Elsevier/North Holland Biomedical Press, Amsterdam, pp. 209-234.

DIAMOND, J.M. 1964. Transport of salt and water in rabbit and guinea pig gallbladder. J. gen. Physiol. **48**:1-14.

DONKIN, J.E. and J.H. ANSTEE. 1980. The effect of temperature on the ouabain-sensitivity of Na^+-K^+-activated ATPase and fluid secretion by the Malpighian tubules of *Locusta*. Experientia **36**:986-987.

DOW, J.A.T. 1986. Insect midgut function. Adv. Insect Physiol. **19**:187-328.

DOW, J.A.T. 1981. Localization and characterization of water uptake from the midgut of the locust *Schistocerca gregaria*. J. exp. Biol. **93**:269-281.

EDNEY, E.B. 1977. Water Balance in Land Arthropods. Springer-Verlag, New York.

FRIZZELL, R.A., M. FIELD and S.G. SCHULTZ. 1979. Sodium-coupled chloride transport by epithelial tissues. Am. J. Physiol. **236**:F1-F8.

GARRETT, M.A., T.J. BRADLEY, J.E. MEREDITH and J.E. PHILLIPS. 1988. Ultrastructure of the Malpighian tubules of *Schistocerca gregaria*. J. Morph. **195**:313-325.

GASSNER, D. and H. KOMNICK. 1982. The loop diuretic furosemide as a non-competitive inhibitor of Cl^-/HCO_3^- ATPases of vertebrate kidneys and insect rectum. Comp. Biochem. Physiol. **71C**:43-48.

GEE, J.D. 1977. The hormonal control of excretion. *In* Transport of Ions and Water in Animals. *Edited by* B.L.Gupta, R.B. Morton, J.L. Oschman and B.L. Wall. pp. 265-284. Academic, New York.

GERENSCER, G.A. 1983. Electrophysiology of chloride transport in *Aplysia* (mollusk) intestine. Am. J. Physiol. **244**:R143-R149.

GERENSCER, G.A. and S.H. LEE. 1983. Cl^--stimulated adenosine triphosphatase: Existance, location and function. J. exp. Biol. **106**:143-162.

GERENSCER, G.A. and S.H. LEE. 1985a. Cl^--HCO_3^--stimulated ATPase in intestinal mucosa of *Aplysia*. Am. J. Physiol. **248**:R241-R248.

GERENSCER, G.A. and S.H. LEE. 1985b. ATP-dependent chloride transport in plasma membrane vesicles from *Aplysia* intestine. Biochim. biophys. Acta **816**:415-417.

GOH, S.L. and J.E. PHILLIPS. 1978. Dependence of prolonged water absorption by *in vitro* locust rectum on ion transport. J. exp. Biol. **72**: 25-41.

GOLDIN, S.M. 1977. Active transport of sodium and potassium by the sodium and potassium ion-activated adenosine triphosphatese from renal medulla. J. Biol. Chem. **252**:5630-5642.

GOODHUE, D. 1963. Some differences in the passage of food through the intestines of the desert and migratory locusts. Nature **200**:288-289.

GRANTHAM, J.J. and I.M. GLYNN. 1979. Renal Na, K ATPase: Determination of inhibition by vanadium. Am. J. Physiol. **240**:F530-F535.

GREGER, R. 1985. Ion transport mechanisms in thick ascending limb of Henle's loop of mammalian nephron. Physiol. Rev. **65**:760-797.

GUPTA, B.L., T.A. HALL and R.B. MORETON. 1977. Electron probe x-ray microanalysis. *In* Transport of Ions and Water in Animals. *Edited by* B.L.Gupta, R.B. Morton, J.L. Oschman and B.L. Wall. pp. 83-143. Academic, New York.

GUPTA, B.L., B.J. WALL, J.L. OSCHMAN and T.A. HALL. 1980. Direct microprobe evidence of local concentration gradients and recycling of electrolytes during fluid absorption in the rectal papillae of *Calliphora.* J. exp. Biol. **88**: 21-48.

GUPTA, B.L. and T.A. HALL. 1981. Microprobe analysis of fluid-transporting epithelia: evidence for local osmosis and solute recycling. *In* Water Transport Across Epithelia. *Edited by* H.H. Ussing, N. Bindslev, N.A. Lassen and O. Sten-Knudsen. Munksgaard,Cophenhagen. pp. 17-35.

HANRAHAN, J.W. 1978. Hormonal regulation of chloride in locusts. The Physiologist. **21**:50.

HANRAHAN, J.W. 1982. Cellular mechanisms and regulation of KCl transport across an insect epithelium. Ph.D. thesis, University of British Columbia, Vancouver.

HANRAHAN, J.W. and J.E. PHILLIPS. 1983. Mechanism and control of salt absorption in locust rectum. Am. J. Physiol. **244**: R131-R142.

HANRAHAN, J.W. and J.E. PHILLIPS. 1984a. KCl transport across an insect epithelium: I. Tracer fluxes and the effects of ion substitutions. J. Membrane Biol. **80**:15-26

HANRAHAN, J.W. and J.E. PHILLIPS. 1984b. KCl transport across an insect epithelium: II. Electrochemical potentials and electrophysiology. J. Membrane Biol. **80**:27-47.

HANRAHAN, J.W. and J.E. PHILLIPS. 1985. Further observations on the regulation of KCl absorption across locust rectum. J. exp. Biol. **116**:153-167.

HANRAHAN, J.W., J. MEREDITH, J.E. PHILLIPS and D. BRANDYS. 1984. Methods for study of transport and control in insect hindgut. *In* Measurement of Ion Transport and Metabolic Rate in Insects. *Edited by* T.J. Bradley and T.A. Miller. Springer-Verlag. New York. pp. 19-67.

HEISLER, N. 1986. Buffering and transmembrane ion transfer processes. *In* Acid-Base Regulation in Animals. *Edited by* N. Heisler. Elsevier Sci. Pub. pp 3-47.

HERAULT, J.P., J. GIRARDIE and J. PROUX. 1987. Separation and characteristics of antidiuretic factors from the corpora cardiaca of the migratory locust. J. Insect Physiol. **33**:487-491.

HERRERA, L., N. LOPEZ-MORTALLA, E. SANTIAGO, F. PONZ and R. JORDANA. 1978. Effect of bicarbonate on chloride dependent transmural potential and ATPase activity in the rectal wall of *Schistocerca gregaria.* Revta esp. Fisiol. **34**:219-224.

HO, S. and D.K.O. CHAN. 1981. Branchial ATPases and ionic transport in the eel, *Anguilla japonica*. III. HCO_3^--stimulated SCN^- inhibited Mg^{+2}-ATPase. Comp. Biochem. Physiol. **68B**:113-117.

IRVINE, H.B. 1966. *In vitro* rectal transport and rectal ultrastructure in the desert locust, *Schistocerca gregaria*. M.Sc. thesis. University of British Columbia, Vancouver.

IRVINE, H.B. and J. E. PHILLIPS. 1970. Effects of respiratory inhibitors and ouabain on water transport by isolated locust rectum. J. Insect Physiol. **17**:381-393.

IRVINE, B., N. AUDSLEY, R. LECHLEITNER, J. MEREDITH, B. THOMSON and J. PHILLIPS. 1988. Transport properties of locust ileum *in vitro*:effects of cAMP. J. exp.Biol. (in press).

IVES, H.E., J.E. CASNELLE, P. GREENGARD and J.D. JAMIESON. 1980. Subcellular localization of cyclic GMP-dependent protein kinase and its substrates in vascular smooth muscle. J. biol. Chem. **255**:3777-3785.

KAUFMAN, W. and J.E. PHILLIPS. 1973. Ion and water balance in the ixodid tick *Dermacentor andersoni* I. Routes of ion and water excretion. J. exp. Biol. **58**:523-536.

KOMNICK, H 1978. Osmoregulatory role and transport ATPase of the rectum of dragonfly larvae. Odonatologica **7**:247-262.

KOMNICK, H, and U. ACHENBACH. 1979. Comparative biochemical, histochemical and autoradiographic studies of Na^+/K^+ ATPase in the rectum of dragonfly larvae (Odonata, Aeshnidae). Eur. J. Cell Biol. **20**:92-100.

KOMNICK, H., M. SCHMITZ and H. HINSEN. 1980. Biochemical demonstration of HCO_3^--and Cl^--dependent ATPase activities in the rectum of larval dragonflies and inhibition of rectal chloride uptake by thiocyanate. Eurp. J. Cell Biol. **20**:217-227.

LECHLEITNER, R.A. and J. E. PHILLIPS. 1988. Anion-stimulated ATPase in locust rectal epithelium. Can. J. Zool. **66**:431-438.

LEE, S.H. 1982. Salinity adaptation of HCO_3^--dependent ATPase activity in the gills of blue crab (*Callinetes sapidus*). Biochim. Biophys. Acta **689**:143-154.

LEWIS, S. 1971. Charge properties and ion selectivity of rectal intima of the desert locust. M.Sc. thesis. University of British Columbia, Vancouver.

LOWRY, O.H., N. H. ROSEBROUGH, R.J.FARR and R.J. RANDALL. 1951. Protein determination with the Folin phenol reagent. J. biol. Chem. **193**:265-275.

MACHIN, J. 1979. Atmospheric water absorption in arthropods. Adv. Insect Physiol. **8**: 199-331.

MACHIN, J., M.J. O'DONNELL and P.A. COUTCHIE. 1982. Mechanisms of water vapor absorption in insects. J. exp. Zool. **222**:309-320.

MADDRELL, S.H.P. 1978. Transport across insect excretory epithelia. *In* Membrane Transport in Biology. *Edited by* G. Giebisch, D.C. Tosteon and H.H. Ussing. pp. 239-272. Springer-Verlag, New York.

MADDRELL, S.H.P. 1980. Characteristics of epithelial transport in insect Malpighian tubules. *In* Current Topics in Membranes and Transport. *Edited by* G. Giebisch, D.C. Tosteon and H.H. Ussing. pp. 427-463. Acedemic, New York.

MADDRELL, S.H.P. and S. KLUNSUWAN. 1973. Fluid secretion by *in vitro* preparations of the Malpighian tubules of the desert locust *Schistocerca gregaria.* J. Insect Physiol. **19**:1369-1376

MADDRELL, S.H.P. and B.O.C. GARDINER. 1980. The permeability of the cuticular lining of the insect alimentary canal. J. exp. Biol. **87**:227-237.

MARTOJA, R. and C. BALAN-DUFRANCAIS. 1984. The ultrastructure of the digestive and excretory organs. *In* Insect Ultrastructure. *Edited by* R.C. King and H. Akai. Vol. 2, pp. 199-268.

MEREDITH, J. and J.E. PHILLIPS. 1988. Na-independent proline transport in the locust rectum. J. exp. Biol. (in press).

OSCHMAN, J.L. and B.J. WALL. 1969. The structure of rectal pads of *Periplaneta americana L.* with regard to fluid transport. J. Morph. **127**:475-510.

PARSONS, D.S. and D.L. WINGATE. 1961. The effect of osmotic gradients on fluid transfer across rat intestine *in vitro.* Biochim. Biophys. Acta. **46**:170-183.

PEACOCK, A.J. 1976. Distribution of Na^+-K^+ activated ATPase in the alimentary tract of *Locusta migratoria.* Insect Biochem. **6**:529-533.

PEACOCK, A.J. 1977. Distribution of Na^+-K^+ activated ATPase in the hindgut of two insects *Schistocerca* and *Blaberus.* Insect Biochem. **7**:393-395.

PEACOCK, A.J. 1981. Further studies of the properties of locust rectal Na^+-K^+ ATPase, with particular reference to ouabain sensitivity to the enzyme. Comp. Biochem. Physiol. **68C**:29-34.

PHILLIPS, J.E. 1961. Studies on the rectal absorption of water and salts in the locust, *Schistocerca gregaria,* and the blowfly, *Calliphora erythrocephala.* Ph.D. thesis. University of Cambridge.

PHILLIPS, J.E. 1964a. Rectal absorption in the desert locust, *Schistocerca gregaria* Forskal.I. Water. J. exp. Biol. **41**:15-38.

PHILLIPS, J.E. 1964b. Rectal absorption in the desert locust, *Schistocerca gregaria* Forskal.II. Sodium, potassium and chloride. J. exp. Biol. **41**:39-67.

PHILLIPS, J.E. 1964c. Rectal absorption in the desert locust, *Schistocerca gregaria* Forskal. III. The nature of the excretory process. J. exp. Biol. **41**:68-80.

PHILLIPS, J.E. 1970. Apparant transport of water by insect excretory systems. Am. Zool. **10**: 413-436.

PHILLIPS, J.E. 1980. Epithelial transport and control in recta of terrestrial insects. *In* Insect Biology in the Future. *Edited by* M.L. Locke and D.S. Smith. Acedemic, New York. pp. 145-177.

PHILLIPS, J.E. 1981. Comparative physiology of insect renal function. Am. J. Physiol. **241**: R241-R257.

PHILLIPS, J.E. 1982. Hormonal control of renal function in insects. Federation Proc. **41**:2348-2354.

PHILLIPS, J.E. 1983a. Cellular mechanisms and control of excretion in insects: recent advances. Verh. Dtsch. Zool. Ges. **1983**:55-68.

PHILLIPS, J.E. 1983b. Endocrine control of salt and water balance: excretion. *In* Endocrinology of Insects. *Edited by* H. Laufer and R. Downer. pp. 411-425. Alan R. Liss, New York.

PHILLIPS, J.E. and A.A. DOCKRILL. 1968. Molecular sieving of hydrophilic molecules by the rectal intima of the desert locust (*Schistocerca gregaria*). J. exp. Biol. **48**:521-532.

PHILLIPS, J.E., T.J. BRADLEY, and S.H.P. MADDRELL. 1978. Mechanisms of ionic and osmotic regulation in saline-water mosquito larvae. *In* Comparative Physiology-Water, Ions and Fluid Mechanisms. *Edited by* K. Schmidt-Nielsen, L. Bolis and S.H.P. Maddrell. pp. 151-171. Cambrdge University Press, Cambrdge.

PHILLIPS, J.E., W. MORDUE, J. MEREDITH, and J. SPRING. 1980. Purification and characteristics of the chloride transport stimulating factor from locust corpora cardiaca: a newpeptide. Can. J. Zool. **58**:1851-1860.

PHILLIPS, J.E., J. MEREDITH, J. SPRING and M. CHAMBERLIN. 1982a. Control of ion reabsorption in locust rectum: implications for fluid transport. J. exp. Zool. **222**:297-308.

PHILLIPS, J.E., J. SPRING, J. HANRAHAN, W. MORDUE and J. MEREDITH. 1982b Hormonal control of salt reabsorption by the excretory system of an insect. *In* Neurosecretion:Molecules, Cells, Systems *Edited by* D.S. Farner and K. Lederis Plenum Publishing. pp. 371-380.

PHILLIPS, J.E., J. HANRAHAN, M. E. CHAMBERLIN and B. THOMSON. 1986. Mechanism and control of reabsorption in insect hindgut. Adv. Insect Physiol. **19**: 329-422.

PHILLIPS, J.E., N. AUDSLEY, R. LECHLEITNER, B. THOMSON and J. MEREDITH. 1988. Some major transport mechanisms of insect absorptive epithelia. Comp. Biochem. Physiol.(in press)

PROUX, B., J. PROUX, and J.E. PHILLIPS. 1984. Antidiuretic action of corpus cardiacum (CTSH) on long-term fluid absorption across locust recta *in vitro*. J. exp. Biol. **113**: 409-421.

PROUX, J., B. PROUX, and J.E. PHILLIPS. 1985. Source and distribution of factors in locust nervous system which stimulate rectal Cl^- transport. Can. J. Zool. **63**:37-41.

PROUX, J. 1981. Regulation neutroendocrine de la diurese chez le criquet migrateur. Doctral thesis. L'Universite de Bordeaux, France.

PROUX, J. and G. ROUGON-RAPUZZI. 1980. Evidence for vasopressin-like molecule in migratory locust: Radioimmunilogical measurements in different tissues: Correlation with various states of hydration. Gen. Comp. Endo. **42**:378-383.

PRUSCH, R.D. 1972. Secretion of NH_4Cl by the hindgut of *Sarcophaga bullata* larvae Comp. Biochem. Physiol. **41**:215-223.

RAMSAY, J.A. 1955. The excretory system of the stick insect *Dixippus morosus* (Orthoptera, Phasmidae). J. exp. Biol. **32**:183-199.

RAMSAY, J.A., R.H.J. BROWN and P.C. CROGHAN. 1955. Electrometric titration of chloride in small volumes. J. exp. Biol. **32**:822-829.

RUDOLPH, D. and W. KNÜLLE. 1978. Uptake of water vapor from the air:process, site and mechanism in ticks. *In* Comparative Physiology-Water, Ions and Fluid Mechanisms. *Edited by* K. Schmidt-Nielsen, L. Bolis and S.H.P. Maddrell. pp. 97-113. Cambrdge University Press, Cambrdge.

SPRING, J.H. and J.E. PHILLIPS. 1980a. Studies on locust rectum. I. Stimulants of electrogenic ion transport. J. exp. Biol. **86**:211-223.

SPRING, J.H. and J.E. PHILLIPS . 1980b. Studies on locust rectum. II. Identification of specific ion transport processes regulation by corpora cardiaca and cyclic-AMP. J. exp. Biol. **86**:225-236.

SPRING, J.H. and J.E. PHILLIPS. 1980c. Studies on locust rectum. III. Stimulation of electrogenic chloride transport by hemolymph. Can. J. Zool. **58**:1933-1939.

SPRING, J.H. and J.E. PHILLIPS. 1984. Proline transport and oxidation in short-circuited locust rectum. Can. J. Zool. **62**:1732-1736.

SPRING, J.H., J. HANRAHAN and J.E. PHILLIPS. 1978. Hormonal control of chloride transport across locust rectum. Can. J. Zool. **56**:1879-1882.

SPRING, K.R. 1983. Fluid transport by gallbladder epithelium. J.exp. Biol. **106**:181-194.

STEELE, J.E. and J.H. TOLMAN. 1980. Regulation of water transport in cockroach rectum by the corpora cardiaca-corpora allata system: The requirement for Na^+. J. Comp. Physiol. **B138**:357-366.

STRANGE, K. and J.E. PHILLIPS. 1984. Mechanisms of CO_2 transport in the rectal salt glands of *Aedes*. I. Ionic requirements for CO_2 secretion. Am. J. Physiol. **246**:R727-R734.

STRANGE, K. and J.E. PHILLIPS. 1985. Cellular mechanism of HCO_3^- and Cl^- transport in insect salt gland. J. Membrane Biol. **83**:25-37.

STRANGE, K., J.E. PHILLIPS and G.A. QUAMME. 1982. Active HCO_3^- secretion in the rectal salt gland of a mosquito larva inhabiting $NaHCO_3$-CO_2 lakes. J. exp. Biol. **101**:171-186.

THOMSON, R.B. and J.E. PHILLIPS. 1985. Characterization of acid/base transport in an insect epithelium. Federation Proc. **44**:643.

THOMSON, R.B. and J.E. PHILLIPS. 1988. Regulation of acid/base transport in an insect excretory epithelium. FASEB. J. **2**:A941.

THOMSON, R.B., J.M. THOMSON and J.E. PHILLIPS. 1988a. NH_4^+ transport in an acid secreting epithelium. Am. J. Physiol. **254**:R348-R356.

THOMSON, R.B., J.D. SPEIGHT and J.E. PHILLIPS. 1988b. Rectal acid secretion in the desert locust, *Schistocerca gregaria*. J. Insect Physiol. (in press)

TOLMAN, J.H. and J.E. STEELE. 1980. The effect corpora cardiaca-corpora allata system on oxygen consumption in the cockroach rectum: The role of Na^+ and K^+. J. Comp. Physiol. **B138**:347-355.

TOWLE, D.W. 1984. Membrane-bound ATPases in arthropod ion-transporting tissues. Am. Zool. **24**:177-184.

TURBECK, B.O., S. NEEDERGAARD and H. KRUSE. 1968. An anion-stimulated adenosine triphosphatase from the potassium-transporting midgut of the larva of *Hyalophora cecropia*. Biochim. biophys. Acta **103**:354-361.

VAN AMELSVOORT, J.M.M., J.J.H.H.M. and S.L. BONTING. 1977a. Is there a plasma membrane-located anion-sensitive ATPase? Biochim. biophys. Acta **466**:283-301.

VAN AMELSVOORT, J.M.M., J.J.H.H.M. and S.L. BONTING. 1977b. Is there a plasmamembrane-located anion-sensitive ATPase? II. Further studies on rabbit kidney. Biochim. biophys. Acta **471**:78-91.

WALL, B.J. 1971. Local osmotic gradients in rectal pads of an insect. Federation Proc. **30**:42-48.

WALL, B.J. and J.L. OSCHMAN. 1970. Water and solute uptake by the rectal pads of *Periplaneta americana* L. Am. J. Physiol. **218**:1208-1215.

WALL, B.J. and J.L. OSCHMAN. 1975. Structure and function of the rectum in insects. Fortchr. Zool. **23**: 192-222.

WALL, B.J., and J.L. OSCHMAN and B. SCHMIDT-NIELSEN. 1970. Fluid transport: concentation of the intercellular compartments. Science **167**:1497-1498.

WHEELER, A.P. and E.W. HARRISON. 1982. Subcellular localization and characterization of HCO_3^--ATPase from the mantle of the freshwater clam, *Anodonta cataracta*. Comp. Biochem. Physiol. **71B**:629-636.

WILLIAMS, D., J.E. PHILLIPS, W.T. PRINCE and J. MEREDITH. 1978. The source of short-circuit current across locust rectum. J. exp. Biol. **77**:107-121.