CELLULAR MECHANISM AND REGULATION OF KCl TRANSPORT ACROSS AN INSECT EPITHELIUM

by

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ABSTRACT

The cellular mechanism and regulation of KCl reabsorption across the rectum of the desert locust *Schistocerca gregaria* has been studied using tracer fluxes, ion-sensitive microelectrodes, and electrophysiological techniques. Serosal addition of 1 mM cAMP stimulates transepithelial short-circuit current (I_{sc}) and net Cl absorption (J_{Cl}^{net}) 10-fold, increases transepithelial potential (V_{t}) 4-fold, and reduces transepithelial resistance (R_{t}) by 40-65%. The properties of locust Cl transport are not consistent with NaCl cotransport models proposed in other epithelia: i) Cl is absorbed from nominally Na-free saline, ii) there is no correlation between trace amounts of Na contamination and the rate of Cl transport, iii) exposure to cAMP increases ^36 Cl influx across the apical border into rectal tissue without affecting ^22 Na influx, iv) Cl-dependent I_{sc} is not inhibited by 1 mM ouabain (2 h) or 1 mM furosemide (1 h), v) J_{Cl}^{net} is not affected when the apical Na electrochemical gradient is reduced by 85%, and vi) there is no relationship between Na and Cl net electrochemical gradients across the apical membrane. Cl/HCO₃⁻ exchange is also unlikely since i) Cl-transport is electrogenic, ii) J_{Cl}^{net} is insensitive to CO₃⁻ and HCO₃⁻-removal, and iii) Cl-dependent I_{sc} is not inhibited by 1 mM SITS or 1 mM acetazolamide after 1 h exposure. The cAMP-stimulated system is Cl-selective: Cl >> Br >> I,F,SCN,PO₄,SO₄,C₂H₂O₂,urate. The halide sequence suggests a site having high field strength. Cl-dependent I_{sc} is inhibited by low mucosal pH and high osmotic pressure. J_{Cl}^{net} obeys Michaelis-Menten-type kinetics. Mucosal K increases both the K_m and V_{max} of transepithelial Cl absorption (K_a = 5.3 mM K). The active step in J_{Cl}^{net} is at the apical membrane because net entry of Cl occurs against a large, unfavourable
electrochemical gradient. Serosal cAMP and mucosal K directly stimulate the active step since both of these agents cause simultaneous increases in $J_{\text{Cl} \text{net}}$ and the electrochemical potential opposing Cl entry. Passive K transport in the mucosa-to-serosa direction is favoured across apical and basal membranes. Most K absorption (~84%) is electrically coupled to active Cl transport under open-circuit conditions, however a small active component is apparent during exposure to cAMP. The response of $V_t$ to transepithelial salt gradients depends strongly on the direction of the gradients, suggesting that locust rectum is a "tight" epithelium. Intracellular current and fluorescent dye injections reveal strong coupling between rectal cells. Flat-sheet cable analysis indicates that locust rectum becomes "tighter" during cAMP exposure, when transcellular conductance increases from 60 to 95% of the total tissue conductance. cAMP increases apical membrane K conductance and basal membrane Cl conductance. K permeability is inhibited by high (physiological) K and osmotic concentrations. The driving force of Cl transport is calculated by two independent methods and the results are interpreted in terms of an equivalent electrical circuit model for KCl reabsorption across locust rectum.
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<tr>
<td>K, Cl, Na, Ca, Mg, HCO$_3^-$</td>
<td>read as ions, valence not shown</td>
</tr>
<tr>
<td>H, etcetera</td>
<td></td>
</tr>
<tr>
<td>$\mu$A</td>
<td>microamp</td>
</tr>
<tr>
<td>$I_{sc}$</td>
<td>short-circuit current</td>
</tr>
<tr>
<td>$J_{net}^i$</td>
<td>net, transepithelial absorption of ion $&quot;i&quot;$</td>
</tr>
<tr>
<td>$V_t$</td>
<td>transepithelial potential ($\Psi$mucosa-$\Psi$serosa)</td>
</tr>
<tr>
<td>$R_t$</td>
<td>transepithelial resistance</td>
</tr>
<tr>
<td>SITS</td>
<td>4-acetamide-4'-isothiocyanostilbene-2,2'-disulfonic acid</td>
</tr>
<tr>
<td>M</td>
<td>mucosal or lumen-facing side of the epithelium</td>
</tr>
<tr>
<td>S</td>
<td>serosal or hemocoel-facing side of the epithelium</td>
</tr>
<tr>
<td>$\Delta\mu_a$, $\Delta\mu_b^i$</td>
<td>gradient of electrochemical potential of ion &quot;$i&quot;&quot; across the apical and basal membrane, respectively</td>
</tr>
<tr>
<td>ATP</td>
<td>adenosine 5'-triphosphate</td>
</tr>
<tr>
<td>cAMP</td>
<td>adenosine 3':5'-cyclic monophosphoric acid</td>
</tr>
<tr>
<td>$\mu$Eqcm$^{-2}h^{-1}$</td>
<td>microequivalents per square centimetre per hour</td>
</tr>
<tr>
<td>CTSH</td>
<td>chloride transport stimulating hormone</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>$xP_i^1$</td>
<td>transepithelial tracer permeability to &quot;$i&quot;$</td>
</tr>
<tr>
<td>$J_{ms}^i$, $J_{sm}^i$</td>
<td>unidirectional flux of &quot;$i&quot;&quot; from mucosa to serosa, and from serosa to mucosa, respectively</td>
</tr>
<tr>
<td>$\mu$M$^{-2}h^{-1}$</td>
<td>micromoles per square centimetre per hour</td>
</tr>
<tr>
<td>$[i]$</td>
<td>concentration of &quot;$i&quot;$</td>
</tr>
<tr>
<td>(i)</td>
<td>activity of &quot;$i&quot;$</td>
</tr>
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Z - valence
R - gas constant
T - absolute temperature (°Kelvin)
F - Faradays number
W - work (calories/equivalent)
\( \kappa_{\text{Cl}}^{\text{oc}}, \kappa_{\text{Cl}}^{\text{sc}} \) - transepithelial \( ^{36}\text{Cl} \) permeability under open- and short-circuit conditions, respectively
\( \Omega \) - ohms
EGTA - ethyleneglycol-bis(\( \beta \)-aminoethyl ether)N,N'-tetraacetic acid
\( G_t \) - transepithelial conductance
mS - millisiemens
Posm - osmotic permeability
mV - millivolts
\( K_t \) - \([\text{Cl}]\) at half maximal \( J_{\text{Cl}}^{\text{net}} \)
\( J_{\text{max}}^{\text{Cl}} \) - maximal \( J_{\text{net}}^{\text{Cl}} \)
\( n_{\text{Cl}} \) - Hill constant for active Cl transport
\( n_t^{\text{Cl}} \) - flux ratio exponent
\( a_i^{\text{m}} , a_i^{\text{c}} , a_s \) - activity of ion "i" in the mucosal, intracellular and serosal compartments, respectively
Hz - hertz
\( \alpha \) - voltage divider ratio, ratio of apical-to-basal membrane resistance, \( R_a / R_b \)
db - decibels
\( K_{i,j} \) - selectivity of microelectrode for j as compared to i
\( V_a \) - apical membrane potential (\( \Psi_{\text{cell}} - \Psi_{\text{mucosa}} \))
\( V_b \) - basal membrane potential (\( \Psi_{\text{cell}} - \Psi_{\text{serosa}} \))
\[ I_t \] - transepithelial square current pulse

\[ V_i \] - electrical potential difference between the reference- and ion-sensitive barrels of a microelectrode

NMDG - N-methyl-D-glucamine

\[ \gamma_i \] - activity coefficient of "i"

\[ R_a, R_b \] - resistance of apical and basal membranes, respectively

\[ \mu m \] - micrometre

\[ I_o \] - intracellularly injected current

nA - nanoamps

\[ R_z \] - resistance to intracellularly injected current

\[ R_j \] - transjunctional resistance (paracellular)

\[ G_a, G_b \] - conductance of apical and basal membranes, respectively \((1/R_a, 1/R_b)\)

\[ \underline{p_i}^a, \underline{p_i}^b \] - permeability of apical and basal membrane to "i" respectively

\[ \underline{a_i}, \underline{\hat{a_i}} \] - logarithmic mean activity of "i" in apical and basal membranes

cGMP - guanosine 3'5'-cyclic monophosphoric acid

\[ E_a, E_b \] - net electromotive force of apical and basal membranes as calculated from measured voltages and resistances

\[ E_i^a, E_i^b \] - electromotive force of "i" across apical and basal membranes due to a gradient of ion activity across the membrane

\[ E^*, E^*_a, E^*_b \] - total electromotive force of apical and basal membrane calculated from ion activity measurements and estimates of partial ionic conductance

EMF - electromotive force

\[ E_{Cl} \] - net driving force of transepithelial, active Cl transport
Acknowledgments

It is a pleasure to thank Dr. J. Phillips for encouragement, suggestions and financial support during the course of this study. Joan Martin contributed daily with helpful advice and expertise. I thank Dr. Mary Ella Chamberlin (28) for many discussions, for introducing me to margaritas and the sun, and for brightening the past five years with her refined sense of humour.

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CHAPTER 1: GENERAL INTRODUCTION

A. Ion transport

Epithelia are ultimately responsible for ionic and osmotic homeostasis in most animals. The regulation of transport is particularly important in these tissues since they must respond to the fluctuating requirements of the animal and at the same time maintain their own cellular composition. The regulatory features of epithelia are accentuated in freshwater and terrestrial habitats where ionic stress is greatest, especially for small animals such as insects, which have large surface-to-volume ratios. Both active (energy-requiring) and passive fluxes of water and solutes across the gut, integument, respiratory surface and excretory organs may be subject to control mechanisms.

Most insects which have been studied to date are capable of regulating hemolymph ion levels (for general literature see reviews by Wigglesworth, 1972; Stobbart and Shaw, 1974; Edney, 1977). In the desert locust, Schistocerca gregaria, hemolymph ion levels remain relatively constant in animals which are fed on hypertonic salt solutions (Phillips, 1964; Stobbart, 1968; Stobbart and Shaw, 1974) and also during the drastic reduction in hemolymph volume which accompanies dehydration (Hanrahan, 1978; Chamberlin and Phillips, 1979). As in the Australian locust (Djajakusumah and Miles, 1966) and the American cockroach (Wall, 1970), feeding leads to rapid restoration of hemolymph volume in locusts with only small, transient changes in ion levels (Hanrahan, 1978; Phillips et al., 1980).

The role of the insect excretory system in maintaining hemolymph composition is well established (reviewed by Phillips, 1970, 1977, 1980, 1981; Ramsay,
1971; Maddrell, 1971, 1980; Wall and Oschman, 1975). This system is composed of two principal epithelia, the Malpighian tubules and the rectum. First, Malpighian tubules secrete an isosmotic primary urine containing most small hemolymph solutes into the gut. Then, according to the "recycling hypothesis" of Ramsay (1958), this fluid passes to the rectum where selective reabsorption of ions, water and useful organic solutes occurs. Although the morphology of insect rectum was described in 1737 by Swammerdam, it was not until much later that evidence suggesting the reabsorption of water (Wigglesworth, 1931, 1932) and ions (Boné and Koch, 1942; Ramsay, 1953, 1955; Shaw, 1955) was obtained. However, early studies of rectal ion transport were not definitive because i) volume changes were not measured as fluid passed through the rectum, and ii) electrical potentials were not measured.

Previous studies on locust rectum

Active ion transport across insect rectum was first shown unequivocally in situ in the locust by Phillips (1961, 1964b). He injected electrolyte solutions containing an impermeant volume marker (\(^{131}\)I-albumin) into ligated recta and measured the ion concentrations and radioactivity of the fluid as a function of time. Net absorption rates of ions and water were then calculated. It was concluded that chloride and water are actively transported from the rectal lumen and that sodium and potassium are also absorbed, at least partially by active transport. Since the insect remained virtually intact during these experiments and displayed normal activities, it is probable that net uptake rates were close to those which normally occur in unperturbed insects. Nevertheless there were limitations to the approach: absorption could only be studied under non-steady-state conditions since measurements relied on changes in the luminal fluid, individual forward and back fluxes could not be measured.
accurately, fluid composition could only be altered on the lumen side, neither luminal nor hemolymph compartments were well stirred, and finally, neural or hormonal factors which normally control rectal reabsorption were unknown and could not be controlled by the investigator. For these reasons, a good in vitro preparation was required for more detailed studies of transport mechanisms and their regulation.

Ion and water transport was studied across isolated locust recta by Irvine (1966; Irvine and Phillips, 1971), using a non-everted sac preparation. Active transport of K and Cl was not observed, presumably because of inadequate oxygenation of the luminal (or mucosal) surface. Transport of these ions was maintained in later studies using everted sacs in which the mucosal side was vigorously aerated (Goh, 1971; Goh and Phillips, 1978). Goh measured transepithelial potential ($V_t$), absorbate composition, and net water flux with time over 5-6 h, and showed that either Na, K, or Cl ions could support prolonged water absorption in vitro. However, this everted rectal sac preparation was not amenable to the study of ion transport mechanisms per se, since unidirectional ion fluxes could not be easily measured nor could $V_t$ be controlled.

Herrera, Jordana and Ponz (1976; 1977) used a different sac preparation to study the effects of ion substitutions and inhibitors on $V_t$ and short-circuit current ($I_{sc}$; an indirect measure of net active ion transport) across locust rectum. These workers found that Cl removal results in a 49% reduction in $I_{sc}$ after 2 min, increasing to a 91% reduction after 12 min. However, all of their experiments were performed under non-steady-state conditions (i.e. during the first 20 min after dissection) when intracellular ions are still equilibrating with the external saline and when active transport is declining rapidly. Furthermore, non-steady-state ion substitutions may give misleading results.
For example, they observed large increases in $I_{sc}$ when Na and K were removed from both sides (22 and 28%, respectively). In chapters 2 and 3, I will show that steady-state K removal actually reduces $I_{sc}$ and that the effects reported by Herrera et al. probably result from passive diffusion transients rather than active transport. Also, in their study, $I_{sc}$ was often measured when ion replacements were made only to one side of the epithelium, so that "$I_{sc}$" was no longer a valid measure of active ion transport. Tracer fluxes, membrane potentials and intracellular ion activities were not measured. Initially, Herrera et al. made no serious errors in the interpretation of their results. They proposed a cellular model for ion transport across this epithelium which featured a Cl pump at the apical membrane (Herrera et al., 1976). However, the results in this paper did not provide any information regarding pump location, and this scheme was revised in a follow-up study in which a ouabain-sensitive Cl pump was proposed at the basal membrane (Herrera et al., 1977). Their revised model is not supported by the tracer and electro-physiological data which will be presented in this thesis.

Vietinghoff et al. (1969) made preliminary measurements of membrane potentials in recta of Carassius and Locusta using a sac preparation. In agreement with the present study on Schistocerca, they observed apical and basal membrane potentials of -59 and -47 mV, respectively (cell interior negative). However, in the absence of tracer flux and intracellular ion activity measurements, membrane potentials did not permit any new conclusions regarding the mechanism of ion transport.

Williams (1975; Williams et al., 1978) developed a flat-sheet preparation and short-circuiting device for locust rectum in order to measure transepithelial tracer fluxes under $I_{sc}$ conditions as first described by Ussing and Zerahn (1951). The chamber design was adapted from that of Wood (1972; Wood
and Moreton, 1978). Transrectal $I_{sc}$ declined exponentially during the first three hours although less rapidly than was observed by Herrera et al. (1976). This decline was paralleled by a decrease in net Cl flux. Replacement of Cl with other anions abolished the initial decay in $I_{sc}$, suggesting that electrogenic Cl transport generated most, if not all, of the initial short-circuit current. Properties of the transport mechanism such as possible ionic coupling, kinetics, selectivity, etc. were not investigated. Although a cellular model of ion transport was not warranted, Williams (1976) did demonstrate the viability of this in vitro preparation. Rates of active ion transport in the Ussing chamber were comparable to those observed in situ (Williams et al., 1978).

Phillips (1961, 1964) measured the rate of net ion absorption from the rectal lumen in situ as a function of concentration in locusts fed on either tap water or on hypertonic saline. In saline-fed locusts, salt absorption saturated at lower rates when compared with water-fed locusts. He suggested that this regulation could occur through changes in epithelial permeability without excluding the possibility that changes might also occur in the kinetics of active transport (Phillips, 1964).

Williams (1976; Williams et al., 1978) reported that electrogenic Cl transport declined by 80% over the first few hours in vitro and speculated that this occurred because of removal from some neural or hormonal agent which normally stimulates active transport in the intact locust. Using this preparation, Spring (1979; Spring et al., 1978; Spring and Phillips, 1980a, b) showed that Cl absorption and $I_{sc}$ could be restored to initial levels in vitro by adding small amounts of homogenized corpus cardiacum, a major neuroendocrine organ in insects.
Evidence suggesting that the factor is in fact a natural hormone came from observations by the author that initial transepithelial potential is significantly higher across recta from fed as compared to unfed locusts (Hanrahan, 1978; Spring et al., 1978). Also, hemolymph from recently fed locusts was found to increase Cl-dependent $I_{sc}$ and $V_t$ and was more stimulatory than hemolymph from unfed locusts (see Phillips et al., 1981). Although the source of this hemolymph factor was not established, it was proposed that feeding (which is known to cause massive neurosecretory release in many insects including locusts; Highnam et al., 1966; Mordue, 1969; Highnam and West, 1971; Highnam and Mordue (Luntz), 1974) might trigger release of the factor from the corpus cardiacum (Hanrahan, 1978; Spring et al., 1978). In support of this hypothesis, ablation of the corpus cardiacum reduced the hemolymph titre of this factor by 86%, and the stimulatory effect of hemolymph on Cl absorption was later confirmed using tracers (Spring and Phillips, 1980c).

**Mechanisms of active Cl transport in insects**

The results described above suggest that Cl absorption is the predominant ion transport process in locust rectum following hormonal stimulation. This raises the obvious question: How does Cl transport occur in this tissue? The cellular mechanism of active Cl transport has not yet been examined in insect epithelia despite its widespread occurrence. Previous studies have demonstrated Cl transport in the classical sense, but have not explored the membrane mechanisms using modern electrophysiological and tracer techniques. Evidence for net transport of Cl against a chemical gradient has been obtained in intact *Chironomus* larvae (Hers, 1942), in the anal papillae of mosquito larvae (Koch, 1938; Wigglesworth, 1938; Stobbart, 1967; Phillips and Meredith, 1969) and marsh beetles (Treherne, 1954), in the Malpighian tubules of *Rhodnius* (Maddrell, 1971), salivary glands of ticks (Kaufman and Phillips,
1973; Sauer et al., 1976), anal canal of *Drosophila* (Cloor and Chen, 1950),
midgut of aphids (Downing; 1980), in the recta of several larvae including the
caddis fly (Sutcliffe, 1961), mosquito (Bradley and Phillips, 1977) and blow-
fly (Prusch, 1974). In addition to locust rectum, active Cl transport has
been measured directly *in vitro* by tracer flux in dragonfly rectum (Leader and
Green, 1978) and the pupal integument of the hornworm (Cooper et al.,
1980). Histochemical evidence for chloride cells is also very suggestive in a
variety of aquatic insects (reviewed by Komnick, 1977).

Koch (1938) and Wigglesworth (1938) have shown that the anal papillae of
dipteran larvae absorb Cl from dilute NaCl although specific mechanisms were
not proposed. A Cl/HCO₃⁻ exchange mechanism was suggested by Stobbart (1967)
to explain how mosquito larvae absorb Cl from different solutions when they
contain an impermeant cation (i.e. from NaCl, KCl, CaCl₂ or NH₄Cl). Chloride/
bicarbonate exchange has not been tested directly in mosquito larvae, since
base efflux has not been measured or correlated with Cl uptake. However, the
results do clearly indicate that some Cl absorption is cation-independent.

Potassium has been found to stimulate Cl absorption in two insect recta,
although this effect may be explained by changes in electrical potential
rather than KCl cotransport. Phillips (1964b) found that net Cl absorption by
locust rectum was 7.5-fold faster when KCl rather than NaCl was injected into
ligated locust recta. The enhanced absorption of Cl following KCl injection
was attributed to a temporary reversal in $V_t$ (lumen becoming negative) which
would drive Cl out of the lumen. Prusch (1976) found that unidirectional Cl
flux into the lumen of isolated maggot hindgut declined when K was removed
from the hemolymph side. This might also be explained as an electrical effect
of K, since the lumen became strongly negative under these conditions (-60 mV).
In spite of the electrical effects of K on transepithelial potential, the fact that Cl is absorbed from KCl solutions when injected into the locust rectum in situ suggests that Cl transport does not require a high concentration of sodium in the lumen (Phillips, 1964b). This is not surprising as the rectum contains a KCl-rich rather than NaCl-rich fluid in vivo.

In conclusion, active Cl transport is common in insect epithelia, but it has not received detailed study at the cellular level. A Cl/HCO₃ exchange has been proposed in the anal papillae of mosquitoes and clear evidence for electrogenic Cl transport has been obtained in the locust rectum and in the pupal integument of the tobacco hornworm. However, there is no compelling evidence for any particular active Cl transport model or coupling mechanism in insect epithelia.

Models proposed for active Cl transport in other epithelia

To anticipate possible mechanisms of Cl transport in insects, it is useful to consider the various models which have been proposed for other epithelia.

Using tracers, Shaw (1960) showed that active absorption of Cl by intact crayfish is independent of external sodium. The uptake of external Cl is thought to occur by a countertransport mechanism which switches in chloride-depleted animals from Cl/Cl exchange to an exchange of external Cl with some endogenous anion, presumably HCO₃ (Fig. 1(a)). A similar switchover has been suggested for Cl/Cl exchange in brine shrimp (Smith, 1969). In contrast, Cl absorption by prawn intestine is Na-coupled (Fig. 1(b); Ahearn and Tornquist, 1977; Ahearn, 1978). Also, intestinal Cl absorption shows an "S-shaped" dependence on luminal Cl concentration, suggestive of cooperative binding to the carrier. It has not been shown that the apical entry step is an energy-
Figure 1.1  Cellular models which have been proposed for active transepithelial Cl transport in invertebrates. Apical (external or mucosal "M") surface is shown to the left in each model, basal (internal or serosal "S") surface is shown to the right. Large arrows indicate the direction of net Cl flux. Primary and secondary active transports are shown by smaller arrows attached to circles. Electrodiffusion and uncharacterized ion movements are represented as dashed arrows.

a) Cl/HCO₃ exchange entry

- nymphs of *Libbellula* and *Aeschna* (Krogh, 1939)
- crayfish (gill; Shaw, 1960)
- brine shrimp (gill; Smith, 1969)
- earthworm (integument; Dietz, 1974)
- freshwater mussel (gill; Dietz and Branton, 1975)
- mosquito larva (anal papilla; Stobbart, 1967)

b) Na,Cl coentry

- prawn (intestine; Ahearn and Tornquist, 1977)

c) Active Cl exit

- *Aplysia* (intestine; Gerenscer and White, 1980)
requiring process, but there is evidence that transepithelial absorption is active (Ahearn, 1980).

Active Cl absorption is also present in epithelia of molluscs and annelids. Electrogenic Cl absorption across *Aplysia* intestine has been demonstrated using tracers (Fig. 1(c); Gerenscer et al., 1977). Furthermore, microelectrode data suggest that the active step for Cl absorption is located at the basal membrane (Gerenscer and White, 1980; reviewed by Gerenscer, 1981). Freshwater mussels and annelids actively absorb Cl from dilute salt solutions independently of the cation and electroneutral exchange mechanisms have been proposed for Cl absorption by both of these animals (Fig. 1(a); Dietz and Branton, 1976; Dietz, 1974). Chloride absorption by earthworm integument is inhibited by injection of the carbonic anhydrase inhibitor acetazolamide, suggestive of a Cl/HCO₃⁻ exchange mechanism (Dietz, 1974).

Chloride/bicarbonate exchange was the first mechanism proposed for active Cl transport in vertebrates. It arose from the observation that Cl uptake by frogs and other aquatic animals could occur from dilute salt solutions irrespective of simultaneous cation absorption (Krogh, 1937). Krogh postulated that Cl must exchange with an internal anion (HCO₃⁻) in order to maintain charge balance when Cl is absorbed (see Fig. 2(a)). Direct chemical coupling of this type has been demonstrated rigorously with tracers in toad skin (Leslie et al., 1973) although a recent study using vesicles prepared from intestinal brush border suggests that Cl may be exchanged for hydroxyl ions (Liedtke and Hopfer, 1980). Nevertheless, the model which proposes a Cl/HCO₃⁻ exchange at the apical plasma membrane (or Cl entry step) is still widely held. The gastric mucosa model shown in Fig. 2(b) has been suggested by Machen and Forte (1979) from their review of the literature. There are at least two different mechanisms for gastric Cl transport, one which is acid-
coupled, and the other which is independent of proton secretion. The acid-
coupled mechanism is thought to involve a K-stimulated ATPase at the apical
membrane and a Cl/HCO$_3^-$ exchange at the basal membrane. The non-acidic compon­
ent of Cl secretion is known to have a lower affinity for Cl, although the
precise location of this pump is uncertain. For a discussion of the contro­
versial aspects of gastric Cl secretion, see the review by Machen and Forte
(1979).

Although chloride/bicarbonate exchange is usually considered electroneut­
ral, actual estimates of the coupling ratio (i.e. ratio of the number of Cl
and HCO$_3^-$ ions which are exchanged) vary somewhat. In skin of the frog
Calyptocephallela, the coupling ratio has been calculated as 2Cl/3HCO$_3^-$ (Garcia
Romeu et al., 1969) although ratios of 2Cl/1HCO$_3^-$ in the frog Rana esculenta
(Ehrenfeld and Garcia Romeu, 1978) and 4Cl/3HCO$_3^-$ in goldfish gills (DeRenzis
and Maetz, 1973) have also been reported. The energetics of epithelial Cl/HCO$_3^-$
exchange are not known; however, energy for "uphill" Cl absorption may be
supplied by an ATPase to be discussed later in this section, or by the efflux
of HCO$_3^-$ from the cell down its net electrochemical gradient. It has been
suggested that the mechanism of epithelial Cl/HCO$_3^-$ exchange may resemble that
of anion exchange across red blood cell membranes (reviewed by Gunn, 1979).

A second general category of Cl transport across epithelia requires
external sodium (Fig. 2(c-e); reviewed by Frizzell et al., 1979). In this
model, coentry of NaCl across the cell membrane is energized by inward
movement of Na down its net electrochemical gradient ($\Delta u_Na^-$) in a manner
analogous to Na-coupled amino acid and sugar absorption (reviewed by Crane,
1977). Obligatory $^{22}$Na, $^{36}$Cl coentry across the apical cell membrane has been
shown directly in rabbit gallbladder (Frizzell et al., 1975; Cremaschi and
Henin, 1975) and in fish intestine (Frizzell et al., 1979; Ramos and
Figure 1.2 Cellular models which have been proposed for active Cl transport across epithelia in vertebrate animals. See legend of Figure 1 for explanation.

a) Cl/HCO₃ exchange entry: exit unknown
   - fish gill
   - frog skin
   - turtle bladder
   - small intestine
   - colon

b) Cl/HCO₃ exchange entry: proton-coupled exit
   - gastric mucosa

c) Na,Cl coentry: KCl coexit
   - amphibian gall bladder

d) Na,Cl coentry: Cl/HCO₃ exchange exit
   - amphibian proximal tubule, intestine

e) Na,Cl coentry: Cl exit unknown, Na recycled paracellularly
   - fish intestine

f) Na,Cl coentry: exit by diffusion
   - cornea
   - shark rectal gland
   - fish operculum
   - small intestine (secreting)

g) Na,K,Cl coupled entry mechanism (?): exit by diffusion
   - MDCK cultured monolayers

h) Electrogenic entry: exit by Cl/HCO₃ exchange
   - amphibian small intestine
Ellory, 1981). Transepithelial Cl absorption would be electroneutral if Na ions entering the cell through the apical plasma membrane were pumped out across the basal plasma membrane by the Na/K exchange pump (Fig. 2(c) and Fig. 2(d)). Alternatively, transepithelial absorption of Cl would be electrogenic if some Na recycles back through Na-selective tight junctions from the lateral intercellular spaces as proposed for flounder intestine (Fig. 2(e); Field et al., 1978).

Chloride secretion is stimulated by cAMP in several vertebrate epithelia including rabbit ileum (Field, 1971), rabbit colon (Frizzell et al., 1976), cornea (Chalfie et al., 1972), shark rectal gland (Silva et al., 1977) and killifish operculum (Karnaky et al., 1977; Degnan et al., 1977). A widely accepted model for this process involves electroneutral NaCl coentry across the basal membrane (Fig. 2(f)). Sodium is returned to the serosal side via the ubiquitous Na/K exchange pump (i.e. "recycled") while Cl leaves the cell down its net electrochemical gradient across the apical membrane. Chloride secretion across cornea epithelium is stimulated by adrenaline, via an increase in the Cl conductance of the apical membrane (Klyce and Wong, 1977). A variation of NaCl coentry has recently been proposed in cultured monolayers from dog kidney (MDCK cells; Simmons, 1981). Exposure to ATP on the serosal side stimulates Cl secretion across the monolayer, as shown in Fig. 2(g). This secretion is inhibited by adding furosemide or ouabain to the serosal side and by elevating the K concentration on the serosal side. Chloride entry across the basal membrane is thought to occur by a NaK-coupled mechanism analogous to that found in Ehrlich ascites tumour cells (Geck et al., 1980) and in red blood cells of ducks (Kregenow and Caryk, 1979) and humans (Dunham et al., 1980). In Ehrlich cells, Cl entry is electroneutral (2Cl:1Na:1K) although some other stoichiometry must exist in MDCK cells. Geck and co-workers have
suggested that the NaCl coentry mechanism which is widespread in epithelia might actually be a Na,K,Cl-coupled system.

A very different interpretation of Na dependence has been suggested for amphibian small intestine (Fig. 2(h); White, 1980). Chloride absorption is dependent on both mucosal sodium and serosal bicarbonate in this tissue. Studies utilizing inhibitors, tracers and ion-sensitive microelectrodes suggest that Cl entry occurs by primary active transport, and that intracellular Cl is exchanged for serosal HCO$_3^-$ at the exit step. According to this model, HCO$_3^-$ is maintained at high levels in the cell through the actions of an apical Na/H exchange and intracellular carbonic anhydrase. Intracellular bicarbonate is hypothesized to reduce the backflux of Cl from cell to mucosal solution. However, at present the most widely held model for Na-dependent Cl transport involves an obligatory NaCl coentry into the epithelial cells. Like the Cl/HCO$_3^-$ exchange mechanism described earlier, this cotransport system appears to be widespread in vertebrate epithelia (see Frizzell et al., 1979; Ramos and Ellory, 1980).

Both Na- and HCO$_3^-$-coupled systems are examples of "secondary active transport" in which the uphill entry of Cl into the cells is coupled to the flow of a second solute down its net electrochemical gradient (reviewed by Aronson, 1981). Primary active transport of Cl has not yet been demonstrated in epithelia. Bicarbonate-stimulated ATPase activity has been reported in gastric mucosa (Kasbekar and Durbin, 1965), pancreas (Simon et al., 1972) and kidney (Kinne-Saffran and Kinne, 1974) although these early preparations may have been contaminated with mitochondrial anion-stimulated ATPase activity. Results of recent studies in rat intestinal brush border (Humphreys and Chou, 1979) and fish gill (Bornancin et al., 1980) strongly suggest the presence of non-mitochondrial ATPase activity which is stimulated by both HCO$_3^-$ and Cl,
however this conclusion has been disputed (Bonting, 1980). Anion-ATPase has been demonstrated in rectal tissue of dragonflies (Komnick et al., 1980) and HCO₃-stimulated ATPase activity has been reported in locust Malpighian tubules (Anstee and Fathpour, 1979) and rectum (Herrera et al., 1978) although mitochondrial contamination was not considered in the latter study.

In summary, little is known regarding the mechanism of Cl transport in insects. Based on studies of vertebrate epithelia, secondary transport of Cl coupled to HCO₃ or Na movements are most common although the possibility of an active pump cannot be ruled out. In locust rectum, there is little direct information regarding possible ionic dependency, coupling or the kinetics of active Cl absorption. Moreover, an understanding of Cl transport at the cellular level is not possible since the location of the active step is not known.

**Ionic permeability in epithelia**

Information regarding permeability is crucial to understanding ion transport across any epithelium. With the exception of blowfly salivary gland, where a Ca-stimulated increase in Cl conductance has been demonstrated (Berridge et al., 1975), studies of insect epithelia have generally focused on active transport, ignoring the equally important permeability properties. A basic question which must be answered is whether ions diffuse passively across the epithelium transcellularly or by a paracellular route.

The tight junctions were originally considered to be impermeable barriers which required ions to traverse the apical and basal cell membrane (see Ussing et al., 1974). While this is true of "tight" epithelia such as amphibian skin, the bladder and kidney distal tubule, it is now well known that in many "leaky" epithelia ions diffuse largely by a paracellular route (i.e. inter-
cellular spaces and tight junctions) rather than through individual cell membrane (early evidence reviewed by Frömter and Diamond, 1972).

The electrical potentials observed across the epithelium and across each membrane are dependent on both the paracellular and membrane conductances. Despite this importance, the relative contributions of cellular and paracellular routes to passive ion movements have not been directly measured in any invertebrate epithelium. The need for a study of epithelial tightness is indicated.

Towards a cellular model for insect Cl transport

This thesis examines some of the more important properties of Cl transport and its regulation in locust rectal epithelium. Chapter 2 describes the basic features of chloride absorption by measuring transepithelial tracer fluxes, short-circuit current and transepithelial potential under various conditions. Several predictions of the Na,Cl cotransport and Cl/HCO₃ exchange models proposed for vertebrates are tested using ion substitutions and well-known transport inhibitors. Also, several approaches are used: i) to establish whether Cl/Cl exchange diffusion is important in this epithelium as it is in other arthropods such as crayfish, brine shrimp and mosquito larvae, and ii) to examine the selectivity of transepithelial transport for Cl over other anions. Finally, the effects of local factors (i.e. calcium, variations in mucosal pH and osmotic pressure) which might regulate active Cl transport are studied in vitro. During these initial flux experiments, it was discovered that potassium stimulates active Cl transport several fold. Also, electrical resistance measurements indicated that K might be the main counter ion for active absorption. Thus the interrelationship between Cl transport and K is considered in more detail in Chapter 3. First, the effects of K on transepithelial Cl transport kinetics are measured under short-circuit condi-
tions using tracers. I also determine the activation constant for K stimulation of Cl absorption and the specificity of this stimulation for K as compared to other cations. The possible sidedness of K stimulation is also tested. In a second series of experiments, the effects of cAMP on transepithelial K transport are measured under open- and short-circuit conditions to determine whether K is indeed the counter ion for Cl transport and to find out whether cAMP might have some effect on K transport in addition to stimulating active Cl absorption.

In Chapter 4, the active step in transepithelial Cl transport is localized by using double-barrelled microelectrodes to measure steady-state intracellular ion activities and membrane potentials under open-circuit conditions. The effects of cAMP addition and K removal on transmembrane electrochemical gradients are also assessed under these conditions. Ion-sensitive microelectrodes are used to test further the possibility of Na-coupled Cl transport by measuring i) the relationship between the Na electrochemical gradient across the apical membrane and transepithelial \(^{36}\text{Cl}\) fluxes, and ii) steady-state Na and Cl electrochemical gradients between the mucosal solution and the cell.

After localizing the active step in Cl absorption, the permeability properties of the epithelium are studied in Chapter 5 using electrophysiological techniques. First, membrane and junctional resistances are measured i) to determine whether locust rectum is a "tight" or "leaky" epithelium, and ii) to calculate the equivalent electromotive forces of apical and basal membranes. The effects of cAMP during ion substitutions reveal specific permeability changes during hormone stimulation. Tracer and microelectrode results are used to construct an equivalent circuit model for KCl absorption across locust rectum. Finally, in order to test the validity of measured
tracer fluxes, electrical potentials, intracellular ion activities and mem-
brane resistance, all four types of data are combined to estimate the driving
force of Cl transport. This value is found to be in satisfactory agreement
with that calculated using a second, independent method. In the General
Discussion (Chapter 6) the main conclusions are assembled into a tentative
model for KCl transport and its regulation in locust rectum. A few specula-
tions regarding the physiological role and significance of this unusual
transport system are also discussed.

B. Organization of the locust excretory system

The gross anatomy and histology of the gut have been thoroughly described
in locusts and grasshoppers (Chauvin, 1938; Hodge, 1939; Marshall, 1945;
Albrecht, 1953; Phillips, 1961; Boccetti, 1962; Irvine, 1966; Phillips,
Jarial and Irvine, unpubl. man.; Jonas and Vietinghoff, 1975; reviewed by Wall
and Oschman, 1975). The purpose of this section is to outline those features
of the excretory system which are relevant to this thesis and to briefly
review some important aspects of rectal ultrastructure.

The locust excretory system consists of two transporting epithelia, the
Malpighian tubules and the rectum (Fig. 3). Approximately 250 tubules join
the alimentary canal at the junction between the midgut and ileum, and secrete
a fluid into the gut which is isosmotic to the hemolymph. Most of this
"primary urine" moves posteriorly to the rectum for selective reabsorption of
water and solutes (Phillips, 1961; 1964a-c). Some tubular fluid also moves
anteriorty to the midgut for fluid reabsorption (Dow, 1981).

The locust rectum is made up of six discrete pads which are connected by
narrow regions of reduced epithelium (Fig. 3b). The intima, a thin porous
layer of cuticle, 2-5 μm thick, is draped loosely over each pad. In cockroach
Figure 1.3  Diagram of the organization of the locust alimentary canal (a), histology of the rectum (b) and ultrastructure of rectal pad cells (c). Figure 3(c) incorporates features from Schistocerca rectum (Jarial et al., unpubl. obs.) and rectum of the cockroach Periplaneta americana (Oschman and Wall, 1969; Wall and Oschman, 1975; Lane, 1979).

a. 1) pharynx
   2) esophagus
   3) crop
   4) anterior midgut caeca
   5) posterior midgut caeca
   6) ventriculus
   7) proctodeal valve
   8) Malpighian tubule
   9) ileum
  10) rectum
b. 11) inter-pad epithelium
   12) rectal pad cell
   13) circular muscle
   14) rectal lumen
   15) sub-intimal space
   16) intima
   17) Type "B" cell
   18) nucleus
   19) lateral membrane
   20) trachea
   21) longitudinal muscle

c. 22) intima
   23) sub intimal space
   24) desmosome
   25) septate junction
   26) gap junction
   27) nucleus
   28) tracheole
   29) scalariform junction
   30) lateral intercellular sinus
   31) mitochondrion
   32) tracheal sinus
   33) lateral intercellular space
   34) tight, gap, and septate junctional complex
   35) sub-epithelial space
   36) sub-epithelial "secondary" cells
   37) circular muscle
rectum, which is structurally similar and has been studied in more detail, the intima attaches tightly to the apical membrane of specialized "sheath cells" which are located at the seams between each pad (Noirot et al., 1979). The intima allows passage of water, ions and most organic solutes from the rectal lumen into the sub-intimal space, but excludes solutes having a molecular weight greater than 400 (Phillips and Dockrill, 1968; Lewis, 1971).

The rectal pad consists mostly of large columnar "principal" cells 17 μm in diameter and 100 μm long. These cells are undoubtedly responsible for active reabsorption of water and solutes from the rectal fluid. Occasional small, goblet-shaped "Type B" cells are also scattered throughout the pad. These cells have few mitochondria and their function is unknown (Peacock, 1979). Electron micrographs indicate that recta of locusts (Irvine, 1966; Jarial et al., unpubl. obs.; Jonas and Vietinghoff, 1975) and of cockroaches (Oschman and Wall, 1969; reviewed by Wall and Oschman, 1975) have many structural similarities and it is reasonable to integrate findings from both tissues.

Both recta are richly supplied with trachea and have an extraordinary number of mitochondria as might be expected for an actively transporting epithelium. In cockroach rectum, freeze-fracture studies by Lane (1979) have shown the presence of many intramembranous particles in the lateral membrane and these may be involved in transport. The lateral membrane of adjacent cells is held 20-25 nm apart (except for occasional dilations), apparently by intercellular columns which insert on some of these particles. Gap junctions are numerous along the lateral cell border, suggestive of cell-cell coupling (Lane, 1979). Interestingly, the sheath cells seem to lack these gap junctions (Noirot et al., 1979) and may isolate the intracellular compartments of adjacent pads. Based on studies with ionic lanthanum, Lane (1979) has
suggested that occluding "tight" junctions are located at the basal ends of
the lateral intercellular spaces in cockroach rectum and that the more apical
septate junctions do not block off access to the lateral space from the lumen.
The exact location of the paracellular barrier to transepithelial solute and
water movement is not known, however fluid and solutes which have been absorb-
ed by the rectal epithelium are thought to pass to the hemolymph via the
lateral intercellular spaces and tracheal sinuses (Fig. 3c). A single layer
of secondary cells, a layer of circular muscle 2-4 fibers thick, and a bundle
of longitudinal muscle fibers lay between the epithelium and the hemolymph,
however these are penetrated by the trachea and do not form a continuous
barrier. Secondary cells are absent in cockroach rectum.

In summary, the epithelium has large transporting cells with numerous
mitochondria and extensively folded membranes. The serosal border (hemolymph-
facing side of the tissue) is extremely complex so that absorbate must pass
through lateral spaces, sinuses and tracheal sinuses where separate secretory
and reabsorptive processes have been proposed (i.e. solute recycling;
Phillips, 1970; Wall and Oschman, 1970). The problems which result from this
complexity will be noted in the appropriate chapters.
CHAPTER 2: PROPERTIES OF TRANSEPITHELIAL CHLORIDE TRANSPORT

Summary

The mechanism and properties of transepithelial chloride transport across the locust rectum were examined in vitro. Serosal addition of 1 mM cAMP, a known stimulant of Cl transport in this tissue, increased short-circuit current (I_sc) and net Cl transport (J_{Cl,net}) by 10-fold. Several predictions of Na- and HCO_3-coupled models for transepithelial Cl transport were tested: Cl-dependent I_{sc} was not affected by Na-removal (<0.05 mM) during the first 75 min. Also, a large stimulation of J_{Cl,net} was elicited by cAMP when recta were bathed for six hours in Na-free saline (<0.001 mM-0.2 mM). In these experiments, there was no correlation between Cl transport rate and the presence of micromolar quantities of Na contamination. Increased unidirectional influx of ^36 Cl into rectal tissue after cAMP-stimulation was not accompanied by a comparable uptake of ^22 Na. J_{Cl,net} was independent of exogenous CO_2 and HCO_3.

Rectal I_{sc} was not affected by long-term exposure to 1 mM ouabain, furosemide, SITS or acetazolamide. In short, there is no evidence that the major fraction of Cl transport across locust rectum occurs by the usual Na- or HCO_3-coupled systems. Cl transport did not exhibit an exchange diffusion component, and was highly selective for Cl over all anions tested except Br. cAMP-stimulated Cl transport was strongly dependent on K but was insensitive to removal of Ca or Mg during the first hour. Exposure to amino acid-free saline i) lowered I_{sc} from control levels, ii) reduced the stimulatory effects of cAMP, and iii) increased transepithelial Cl permeability. I_{sc} was not affected by serosal pH between 3.0 and 8.0 but was strongly inhibited by low mucosal pH within the physiological range (pH 7.0-4.5). Physiological hyperosmolarity also reduced I_{sc} and tissue conductance. The results in vitro suggest that rectal
Cl transport is subject to several modes of regulation in vivo. Further details of K-dependence, and a cellular model for KCl absorption are presented in subsequent chapters.
**Introduction**

Epithelial transport of chloride underlies many important regulatory processes in animals. Studies of various vertebrate epithelia have demonstrated two general mechanisms of secondary Cl transport: sodium-coupled systems, where Cl entry into the epithelial cells involves electroneutral cotransport with Na (see chapter 1; reviewed by Frizzell et al., 1979), and bicarbonate-coupled systems where Cl entry or exit involves an electroneutral (i.e. 1:1) exchange for HCO₃⁻ (Garcia Romeu et al., 1969; Leslie et al., 1973). Active Cl transport is also widespread in invertebrate epithelia, and has been identified in at least 15 species of insects. However, virtually nothing is known regarding the properties and underlying mechanisms of chloride transport in insects (see reviews by Harvey, 1981; Phillips, 1981).

As might be expected, many functions of gut epithelia in insects parallel those of vertebrate gastrointestinal and renal systems (e.g. absorption of salts, amino acids and water; reviewed by Phillips, 1980, 1981). Net active Cl absorption across locust rectum is well established in situ (Phillips, 1964b) and in vitro (Williams, 1976; Williams et al., 1978). Also, absorption of Cl is normally regulated in the intact locust in response to salt loading or depletion (Phillips, 1964b) and is stimulated in vitro by neuroendocrine gland homogenates (Spring et al., 1978; Spring and Phillips, 1980a,b), hemolymph from recently fed locusts (Hanrahan, 1978; Spring and Phillips, 1980c; Phillips et al., 1981), purified neuropeptide "chloride transport stimulating hormone" (CTSH; Phillips et al., 1980) and by cAMP (Spring et al., 1978; Hanrahan, 1978; Spring and Phillips, 1980b). After stimulation by CTSH or cAMP in saline containing 200 mM Cl, the rate of net Cl absorption is approximately 7 μEq cm⁻² h⁻¹ (Spring and Phillips, 1980b) under approximate
short-circuit conditions (i.e. no correction for saline resistance). This value is very similar to that observed in situ following injection of 300 mM KCl into the rectal lumen (Phillips, 1964b). Also, the spontaneous transepithelial potential in vitro (30 mV during stimulation; Spring et al., 1978; Hanrahan, 1978; Spring and Phillips, 1980a) is similar to that observed in situ (Phillips, 1964b). In summary, the locust rectum transports Cl at high rates in vivo and this ability is apparently preserved in vitro.

The rectal epithelium of the desert locust Schistocerca gregaria is particularly well suited for the detailed study of ion transport mechanisms. Locust rectum has a high rate of salt absorption in vivo (potassium chloride; 7.5 μEq cm⁻² h⁻¹; Phillips, 1964a-c), a large surface area when compared to most insect epithelia (60 mm² as a flat sheet), and is sufficiently robust to withstand handling in vitro. A chitinous cuticle which covers the mucosal surface acts as a natural support grid. The transporting columnar cells are large (17 x 100 μm), accept microelectrode impalement readily, and make up the great bulk of the epithelium.

The purpose of this chapter is to examine the properties of insect Cl transport using transepithelial tracer flux and short-circuit current methods. Steady-state rates of active Cl transport across locust rectum are measured under control conditions and during cAMP stimulation using a voltage clamp which accurately short-circuits the epithelium and a saline which is based on the composition of locust hemolymph. Ion substitutions and inhibitors are then used to test the various predictions of current models for Cl transport across epithelia. Possible exchange diffusion and anion selectivity are also examined. Finally, several factors which might regulate rectal ion transport including intra- and extracellular Ca, cGMP, external osmolarity and pH are investigated.
The major conclusions in this chapter and those which follow (chapters 3-5) are summarized diagrammatically in the General Discussion (chapter 6). Some of the findings reported in this chapter have appeared in abstracts (Hanrahan and Phillips, 1980a,b).

Materials and methods

Animals

Desert locusts, *Schistocerca gregaria* Forskål, were obtained from a colony maintained at U.B.C. in gregarious phase under a 15:9 hour light:dark cycle, at 50% relative humidity, and with temperatures oscillating between 40°C (light) and 26°C (dark). The colony was fed fresh lettuce daily and a dry mixture of alfalfa, bran, yeast and powdered milk. Unless otherwise indicated, adult female locusts 18-30 days beyond final moult were used in all experiments because of their larger size.

Flux chambers

I used modified Ussing-type chambers (Williams et al., 1978) which had been adapted from a design by Wood (1972). Briefly, the tissue was mounted as a flat sheet over a collar-shaped opening and fastened on the outside by six small pins near the base of the collar. To form a seal, a rubber O-ring was placed around the tissue on the collar so that it fit snugly into a shallow groove above the pins. The area of the opening was 0.196 cm². This collar design ensured that no pressure was exerted on the tissue when the two "plexiglas" half-chambers were clamped together.

Solutions

Five ml of saline were circulated vigorously in both half-chambers by a gas-lift pump. Salines containing 10 mM HCO₃ were bubbled with 95% O₂:5% CO₂ and remained at pH 6.8-7.0 during experiments. When HCO₃-free saline was stirred with 100% O₂, pH increased on the mucosal side from 7.0-7.4. This
change was ignored since $I_{sc}$ is not affected by pH over this range (see Fig. 16). Experiments were performed at 22±1°C, a value which is within the daily range of temperatures normally experienced by locusts in the wild.

The composition of experimental salines was based on analyses of locust hemolymph and Malpighian tubule fluid using a flame spectrophotometer (AA 120, Varian Techtron, PTY, Ltd., Melbourne, Aust.) on emission (Na, K) or absorption mode (Mg, Ca). Samples of body fluids were analysed after dilution in distilled H$_2$O (for Na measurements), 500 mM NaCl (K), 1.5% EDTA (Mg) or 0.5% LaCl$_3$ (Ca). Chloride concentration was measured by titration with AgNO$_3$ according to Ramsay et al. (1955). The pH of fresh locust hemolymph was measured using a thermostated pH microelectrode (PHM 71, Radiometer, Copenhagen). Total CO$_2$ was estimated using a Micro-Van Slyke method (Micro-CO$_2$ device, Harleco, Gibbstown, N.J.). Bicarbonate was calculated from the Henderson-Hasselbach equation assuming negligible carbamino CO$_2$, equilibrium between the dissolved gas and HCO$_3^-$, and a pK in locust hemolymph of ~6.1 (see Davenport, 1974). Hemolymph samples (10 μl) were collected from the neck and dissolved in 0.5 ml of 3.75 (w/v) sulfasalicylic acid (pH 1.8, lithium citrate buffer) for analysis using an amino acid analyser (Model 118c, Beckman, Palo Alto, Ca.).

The ionic composition of physiological fluids and experimental salines are given in Table 1. All experimental salines used in this study contained (in mM): alanine (2.9), arginine (1.0), asparagine (1.3), glutamine (5.0), glycine (11.4), histidine (1.4), lysine (1.4), proline (13.1), serine (1.5), tyrosine (1.9), valine (1.8). Glucose (10 mM) was also included. Sodium was completely replaced with choline in Na-free saline, except where indicated otherwise. K- and HCO$_3^-$-free salines were prepared by omitting the normally
Table 2.1  Composition of physiological fluids and experimental salines used to study ion transport across locust rectum

<table>
<thead>
<tr>
<th>Body fluids (means ± s.e.)</th>
<th>Cl</th>
<th>K</th>
<th>Na</th>
<th>Mg</th>
<th>Ca</th>
<th>HCO₃⁻</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemolymph</td>
<td>106.7</td>
<td>12.2</td>
<td>103.0</td>
<td>24.4</td>
<td>18.4</td>
<td>13.0</td>
<td>7.1</td>
</tr>
<tr>
<td>Malpighian tubule fluid (obtained by ligation)</td>
<td>87.6</td>
<td>165.1</td>
<td>46.9</td>
<td>39.3</td>
<td>13.1</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Food (fresh lettuce)</td>
<td>34(2)</td>
<td>114(2)</td>
<td>14(2)</td>
<td>5.2(2)</td>
<td>14.0(2)</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Salines: Present study

<table>
<thead>
<tr>
<th>Salines: Present study</th>
<th>Cl</th>
<th>K</th>
<th>Na</th>
<th>Mg</th>
<th>Ca</th>
<th>HCO₃⁻</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal saline</td>
<td>110-114</td>
<td>10</td>
<td>110-114</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>7.1</td>
</tr>
<tr>
<td>'High K' saline (used in chapter 3)</td>
<td>50</td>
<td>140</td>
<td>50</td>
<td>20</td>
<td>10</td>
<td>10</td>
<td>7.1</td>
</tr>
</tbody>
</table>

Salines: Previous studies

<table>
<thead>
<tr>
<th>Salines: Previous studies</th>
<th>Cl</th>
<th>K</th>
<th>Na</th>
<th>Mg</th>
<th>Ca</th>
<th>HCO₃⁻</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Williams et al., 1978 (from Berridge, 1967)</td>
<td>63</td>
<td>9</td>
<td>55</td>
<td>26</td>
<td>4</td>
<td>10.5</td>
<td>6.7</td>
</tr>
<tr>
<td>Spring et al., 1978; Spring and Phillips, 1980a-c</td>
<td>201</td>
<td>11</td>
<td>209</td>
<td>5</td>
<td>5</td>
<td>24</td>
<td>7.0</td>
</tr>
</tbody>
</table>

1 See text (P.30) for organic constituents common to these salines. K-methylsulfate salt was used in "High K" saline.
2 For organic constituents, see original references.
small amounts of these ions and adjusting sulfate salts and sucrose to main-
tain constant Na, Mg, Ca, Cl and osmotic concentrations.

A large anion deficit was observed in Malpighian tubule fluid, perhaps
the result of dissolved urate or polyanions. Since the anion was not
identified, I arbitrarily used K-methylsulfate to prepare high K saline.
Methylsulfate had no deleterious effects on I_{sc} when added to normal saline in
preliminary experiments.

Osmotic concentration of salines was routinely checked using an osmometer
(Wide Range, Advanced Instr. Inc., Newton Highlands, Mass.) and for 15 differ-
ent bulk preparations was 444.2±9.18 mOsm/l. As discussed in later sections,
saline osmolarity was increased on both sides during some experiments by
additions of Na- or K-methylsulfate; however, these changes were well within
the normal range experienced by this tissue in vivo (400-1100 mOsm/l,

Prior to tracer flux measurements, tissues were exposed to Na, K or HCO_{3}−
free salines for 4 h and rinsed at least twice with fresh saline, except
during Na-free experiments when saline was replaced three times. After turn-
ing off the I_{sc}, solutions were drained by suction and replaced using a
syringe. I_{sc} was not affected artifactually by this method of changing
solutions.

To determine trace levels of contamination during flux studies using
Na- and K-free salines, these cations were routinely analysed by flame
spectrophotometry as described above at the beginning and at the end of
experiments. Maximal stimulation of rectal transport (1 mM cAMP, final concen-
tration) was achieved by adding 50 µl of a 101 mM cAMP solution to the serosal
half-chamber. The sodium salt of this cyclic nucleotide was used except in
Na-free saline, when acidic cAMP stock solution was titrated to neutrality
with KOH. A stock solution of calcium ionophore was prepared in absolute ethanol (400 \( \mu \)g/ml). In most experiments, A23187 was added to the chambers to a final concentration of 0.1-1.0 \( \mu \)g per ml saline so that the final ethanol concentration was less than 0.25% v/v. At the highest dose used (5 \( \mu \)g/ml), it was necessary to correct for a small increase in \( I_{sc} \) (1.12 \( \pm \) 0.08 \( \mu \)Eqcm\(^{-2}\)h\(^{-1}\)) elicited by ethanol.

Calcium ionophore A23187 and furosemide were gifts of R. Dolman, Eli-Lilly Canada, and J. Rees, Hoechst Pharm., respectively. SITS (4-acetamido-4'-isothiocyanostilbene-1,2'-disulfonic acid) was obtained from BDH Chem. Ltd., Poole, Eng. Amino acids and cAMP were obtained from Sigma. All salts were reagent grade.

**Electrical methods**

Transepithelial potential (\( V_t \)) was measured using a high input impedance differential amplifier (10\(^{12}\) \( \Omega \); 4253 Teledyne Philbrick, Dedham, Mass.) connected to the chambers via calomel electrodes and 3 M KCl agar bridges. Operational amplifiers (725, National Semi-conductor Corp., Santa Clara, Ca., and 308, Fairchild Inc., Mountain View, Ca.) were used for voltage clamping and to measure \( I_{sc} \) respectively. Both \( V_t \) and \( I_{sc} \) were recorded on a strip chart recorder (220, Soltec Corp., Sun Valley, Ca.). Corrections were made for asymmetries between voltage-sensing electrodes and for resistance of the bathing saline by the method of Rothe et al. (1969; also see appendix of this thesis). These corrections were essential for several reasons: i) the error in \( I_{sc} \) was \( \sim 10\% \) under control conditions if correction for saline resistance was not made; ii) this error changed unpredictably during K and Cl additions or substitutions since these ions affected tissue conductance more than the conductivity of the external saline; iii) cAMP greatly increased tissue conductance (\( \sim 2\)-fold) without changing saline conductivity, thereby increasing
the saline resistance error proportionally. A different non-compensating voltage clamp has been used in previous studies of locust rectum (Williams et al., 1978; Spring et al., 1978; Spring and Phillips, 1980a-c).

In this study, the normal protocol was to leave the rectum in the short-circuited state and to measure open-circuit transepithelial potential at 15 min intervals by briefly turning off $I_{sc}$. Both $I_{sc}$ and $V_t$ approached steady-state levels exponentially when the epithelium was voltage-clamped or unclamped ($t_{1/2} = 18$ sec). To obtain accurate measurements of $V_t$, the tissue was unclamped for 1.5 min. Open-circuit potential was then $96.2 \pm 0.63\%$ ($x \pm$ s.e., n=8) of its steady-state value under continuous open-circuit conditions without cAMP and $92.2 \pm 0.76\%$ during exposure to cAMP. $V_t$ measured after 1.5 min were similar to those obtained in later experiments under continuous open-circuit conditions using tracers (Fig. 5) and ion sensitive microelectrodes (Tables 2 and 3 in chapter 4).

**Transepithelial tracer fluxes**

$^{36}\text{Cl}$ (New England Nuclear, carrier-free, 5.9 mCi/g Cl) was added as $\text{H}^{36}\text{Cl}$ to control and Na-free salines, and as Na$^{36}\text{Cl}$ to HCO$_3$-free saline. These amounts were too small to cause significant changes in Cl concentration (3% error) or pH.

The protocol for measuring transepithelial tracer fluxes was as follows:

Aliquots of stock isotope (10-50 µl) were added to one half-chamber referred to as the "hot side". After 10 min of vigorous mixing, 1 µl samples were taken in duplicate from the hot side. These were placed into vials containing 1 ml of "cold" saline and 10 ml of scintillation fluid (ACS Amersham Corp., Oakville, Ont.) for counting with a liquid scintillation counter (Isocap, Nuclear Chicago). One ml samples were taken from the "cold side" at 15 or 20 minute intervals and these were replaced with cold saline.
activity of the hot side did not change measurably during flux experiments. The tracer activity of the cold side never exceeded 0.5% of that on the hot side, hence no correction was necessary for tracer backflux. Unidirectional flux was calculated using the formula (Williams et al., 1978):

$$J_{\text{1}\rightarrow\text{2}} = \frac{a_2 \cdot V \cdot C}{a_1 \cdot T \cdot A}$$

where \(J_{\text{1}\rightarrow\text{2}}\) is unidirectional flux (\(\mu\text{Eqcm}^{-2}\text{h}^{-1}\)), \(a_1\) is radioactivity of the hot side (cpm/ml), \(a_2\) is the increase in radioactivity of the cold side (\(\Delta\text{cpm/ml}\)), \(V\) is volume of the cold side (5 ml), \(C\) is total concentration of bulk and tracer isotopes (\(\mu\text{Eq/ml}\)), \(T\) is time between samples (h) and \(A\) is tissue surface area (0.196 cm\(^2\)). Appropriate corrections were made for dilution during sampling. Initial flux values (which included equilibration with the tissue pool) were not included in the calculations.

No attempt was made to measure both unidirectional fluxes on each tissue with \(^{36}\text{Cl}\) and \(^{77}\text{Br}\) since selectivity experiments showed that Br is transported at less than 1/2 the rate of Cl. Paired tissues were not available. Nevertheless, differences in \(I_{\text{sc}}\), \(V_t\) and transepithelial resistance (\(R_t\)) were negligible between tissues used for forward and back fluxes and these differences will be specified in the results section.

Although the method of mounting tissues used in this study has been described previously (Wood, 1972; Williams et al., 1978; Spring et al., 1978; Blankemeyer, 1978), no estimates of the permeability induced by edge damage have been published using this technique.

To estimate the magnitude of artifactual shunting due to edge damage, unidirectional fluxes of \(^{35}\text{SO}_4\) were measured across recta in "high SO\(_4\)" saline (207 mEq/l; see simple SO\(_4\) saline #1, Spring and Phillips, 1980b) under control
conditions and following sequential additions of 1 mM cAMP and 114 mM NaCl (Table 2). Forward and back fluxes of $^{35}$SO$_4$ are not significantly different ($P > 0.2$) suggesting that little if any active SO$_4$ transport occurs (see also Williams et al., 1978). If all $^{35}$SO$_4$ flux occurs through non-selective edge damage, then the calculated upper limit of SO$_4$ permeability for this pathway is $3.7 \times 10^{-7}$ cm sec$^{-1}$. An upper limit for $^{36}$Cl flux due to edge damage can then be calculated from this $^{35}$SO$_4$ flux data using the relative free-solution mobilities of Cl and SO$_4$. Such calculations reveal that the maximum Cl flux due to edge damage is <12% of the total passive Cl backflux from serosa-to-mucosa and is negligible (1.5%) compared to the rate of net Cl absorption.

In summary, I believe that $^{36}$Cl and $^{42}$K flux measurements with this chamber design are not significantly influenced by edge damage. Independent electrophysiological data supporting this view are presented in chapter 5.

**Tracer influxes across the apical cell border**

Everted rectal sacs cannulated with polyethylene tubing (PE 90, Parsippany, Dickinson and Co., N.J.) and containing control saline were prepared according to the method of Goh and Phillips (1978). This preparation was convenient for measuring $V_t$ and unidirectional fluxes of $^{22}$Na and $^{36}$Cl from the mucosal side into epithelial tissue under open-circuit conditions. The following protocol was used: everted sacs (mucosal surface facing outwards) were incubated with control saline on both sides for three hours. Some sacs were injected with 10 mM cAMP for the final hour. $V_t$ was measured using a high input impedance electrometer (602, Keithley Instr., Cleveland, Oh.) connected via calomel electrode and 3 M KCl agar bridges. Tissues were then placed in control saline containing $^3$H-mannitol for 20 min, followed by incubations in saline containing $^3$H-mannitol and $^{36}$Cl or $^{22}$Na for an additional 30-45 min at pH 4.5 to temporarily inhibit Cl transport.

After a timed exposure to the test solution (pH = 7.0,
Table 2.2  \(^{35}\)SO\(_4\) fluxes as an indication of maximum permeability through edge damage under control conditions, and after sequential cAMP and NaCl additions.  \(^1\)

<table>
<thead>
<tr>
<th>Condition</th>
<th>(J_{ms} ) J(^{-2})h(^{-1})</th>
<th>(J_{sm} ) J(^{-2})h(^{-1})</th>
<th>*(P_{SO_4}) cm sec(^{-1}) x 10(^7)</th>
<th>*(P_{Cl}) calculated from *(P_{SO_4}) cm sec(^{-1}) x 10(^7)</th>
<th>*(P_{Cl}) observed in normal saline cm sec(^{-1}) x 10(^7)</th>
<th>Maximum fraction of *(P_{Cl}) through edge damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0804</td>
<td>0.1019</td>
<td>2.74</td>
<td>5.23</td>
<td>50.0</td>
<td>10.5%</td>
</tr>
<tr>
<td>±0.011</td>
<td>±0.015</td>
<td>±0.4</td>
<td></td>
<td>±5.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(12;4)</td>
<td>(18;6)</td>
<td>(18;6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1mM cAMP added to</td>
<td>0.1241</td>
<td>0.1326</td>
<td>3.47</td>
<td>6.62</td>
<td>57.0</td>
<td>11.61%</td>
</tr>
<tr>
<td>serosal side</td>
<td>±0.012</td>
<td>±0.012</td>
<td>±0.54</td>
<td>±9.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(12;4)</td>
<td>(18;6)</td>
<td>(18;6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP + 114mM Cl</td>
<td>0.1182</td>
<td>0.1843</td>
<td>4.97</td>
<td>9.43</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cl added to both</td>
<td>±0.012</td>
<td>±0.030</td>
<td>±0.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>side</td>
<td>(6;4)</td>
<td>(12;6)</td>
<td>(12;6)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Parentheses show (number of flux periods; number of animals); \(J_{ms}\) and \(J_{sm}\) are sulfate fluxes from mucosa-to-serosa and serosa-to mucosa, respectively. Recta were bathed in sulfate saline #1 (Spring and Phillips, 1980a) under I\(_{sc}\) conditions.

\(^2\)Transepithelial \(^{35}\)SO\(_4\) permeability was calculated as \(J_{sm}/[SO_4]\)

\(^3\)"Calculated *\(P_{Cl}\)" was obtained from *\(P_{SO_4}\) by correcting for the difference between SO\(_4\) and Cl mobilities in free solution, and therefore represents an upper limit for Cl permeability through non-selective edge damage.
3H-mannitol plus 36Cl or 22Na, tissues were dissected from the cannula, blotted on bibulous paper, weighed, macerated in vials containing 1 N KOH, and digested at 80°C over night. After neutralizing with H₂SO₄, the vials were counted for 3H-mannitol and 36Cl as previously described. 22Na was counted using an automatic gamma counter (model 4253, Searle). Tritium counts were corrected for activity due to other tracers.

3H-mannitol was used to estimate extracellular tissue space which was in continuity with the mucosal side. To test whether mannitol is metabolised by locust rectum, 6 tissues were incubated separately in stoppered test tubes with 1 ml of control saline containing 14C-mannitol (0.12 mM, approx. 4.5 x 10⁶ cpm). The bathing saline was acidified after 1 h to drive off dissolved 14CO₂ which was collected on glass fiber filters soaked in hyamine hydroxide and counted by liquid scintillation. Since no 14C-activity was detectable (sensitivity of the method was estimated to be 5.4 x 10⁻¹⁰ moles 14CO₂/h) it was concluded that mannitol is not metabolised.

A layer of porous cuticle covers the mucosal surface of the epithelium, trapping a large unstirred layer external to the apical membrane. In order to equilibrate tracers with this dead space, tissues were preincubated at low pH as described above. Very low counts were observed in sac absorbate after 30 min exposure to pH 4.5. Ratios of 36Cl: 3H and 22Na: 3H were similar to those outside the sac indicating i) a non-selective shunt was probably responsible for these fluxes, ii) 3H-mannitol still provided a reasonable estimate of extracellular 36Cl and 22Na. In control experiments, 22Na and 36Cl did not accumulate measurably inside rectal sacs during 1-10 min exposures to tracers at pH 7.0. Finally, cAMP stimulates the influx of 36Cl into rectal tissue against an opposing electrical gradient, providing further evidence for the validity of the technique (Fig. 12a and discussion).
Calculations and statistics

In order to compare the instantaneous short-circuit current with tracer fluxes measured at intervals, $I_{sc}$ traces were integrated using a planimeter (model L30M, Lasico, Los Angeles, Ca.). Values are means ± standard errors unless stated otherwise; $n =$ number of recta.

Significant difference was determined using paired or unpaired t-tests.

Results

1) Verification of active Cl transport

The mucosal (luminal) solution was electrically positive with respect to the serosa at all times for locust recta exposed to normal saline in Ussing-type chambers under open-circuit conditions. Under control conditions, rectal $I_{sc}$ (indicating net transport of anions to the serosal side or cations to the mucosal side) declined exponentially from 6 to 1.1 $\mu$Eqcm$^{-2}$h$^{-1}$ ($t_{1/2} = 40$ min). $I_{sc}$ approached a steady-state condition after 3 h (Fig. 1a). After an initial increase, $R_t$ remained constant for several hours (Fig. 1d). These results agree with previous findings using everted sacs (Goh and Phillips, 1978) and flat sheet preparations of this tissue (Williams et al., 1978; Spring and Phillips, 1980b). Net Cl flux ($J_{Cl}^{net}$) parallels $I_{sc}$ during the nine hour experimental period. The discrepancy of 1 $\mu$Eqcm$^{-2}$h$^{-1}$ between $I_{sc}$ and $J_{Cl}^{net}$ in unstimulated recta may be due to some unidentified electrogenic process (Williams et al., 1978; Baumeister et al., 1980). Inter-tissue variability is probably not responsible for the discrepancy, since $I_{sc}$, $V_t$ and $R_t$ were virtually identical during forward and back flux experiments (i.e. difference was not significant at any sample time, $P >> 0.2$). $J_{Cl}^{net}$ was lower than previous steady-state Cl flux measurements (0.5 $\mu$Eqcm$^{-2}$h$^{-1}$ as compared to 3-4 $\mu$Eqcm$^{-2}$h$^{-1}$; Williams et al., 1978). Two major differences from previous methods might explain these different results: 1) compensation was made for
Figure 2.1  The approach of isolated, unstimulated rectal tissue to steady-state conditions. (a) short-circuit current ($I_{sc}$) and net Cl flux ($J^{Cl}_{net}$); (b) unidirectional Cl fluxes measured under $I_{sc}$ conditions, from mucosa to serosa, and from serosa to mucosa; (c) spontaneous transepithelial potential ($V_t$); and (d) transepithelial resistance ($R_t$). Experiments were performed during the time interval shown by the horizontal bracket as in (a). Recta were bathed in normal saline (Table 2.1) and were left short-circuited except for 90 second intervals when spontaneous $V_t$ was measured. Means ± s.e.; $n = 6$ ($J^{Cl}_{ms}$, $J^{Cl}_{ms}$$)$; $n = 12$ ($I_{sc}$, $V_t$, $R_t$).
Figure 2.2  Effects of cAMP on Cl fluxes and electrical parameters under $I_{sc}$ conditions in normal saline. Cyclic-AMP (1 mM) was added at the arrow to the serosal side. Time 0 was preceded by 4 h equilibration in order to achieve a steady-state. Other conditions and definitions as in Figure 2.1.
Figure 2.3 Responsiveness of $I_{sc}$ to cAMP as a function of time after dissection of recta from locusts. Cyclic-AMP (1 mM) was added at the arrows to the serosal side of two recta bathed in normal saline (Table 2.1).
Figure 2.4  Chloride dependence of cAMP-stimulated $I_{sc}$. Time 0 indicates time after dissection. At the first arrow, 1 mM cAMP was added to the serosal side while the rectum was bathed in normal saline. All Cl was replaced bilaterally with gluconate at the second arrow; 1 mM cAMP was still present on the serosal side.
the series resistance of the saline, ii) a complex saline based on normal
hemolymph ion and amino acid levels was used in flux experiments. For the
purposes of this study, the important conclusion is that no spontaneous
increases in $I_{sc}$, Cl fluxes, $V_t$, or $R_t$ occur in vitro after the initial decay
in Cl transport. An approximate steady-state condition is maintained between
4-8 hours after dissecting recta from locusts. Experiments were performed
during this period unless indicated otherwise.

Figure 2 shows the effects of adding 1 mM cAMP to the serosal (hemocoel)
side on Cl fluxes under $I_{sc}$ conditions, $V_t$ and $R_t$. Zero time shown on this
and subsequent figures is preceded by a four-hour equilibration period under
$I_{sc}$ conditions to ensure a steady-state; i.e. experiments were conducted
between the 4th and 8th hour after dissection. Cyclic-AMP caused very large
increases in $I_{sc}$, $V_t$ and $J_{Cl}$, reduced $R_t$ by $\sim$50%, and caused a slight elevation
in $J_{sm}$ (from $1.47 \pm 0.14$ to $1.68 \pm 0.26 \text{ mEq cm}^{-2} \text{ h}^{-1}$). Maximal stimulation was
obtained with 1 mM cAMP, a higher dose than previously reported (0.3 mM) by
Spring (1979), who conducted studies between the second and fourth hour after
dissection.

The effects of cAMP are independent of sex since similar stimulations of
$I_{sc}$ (from $1.9 \pm 0.12$ to $8.8 \pm 0.53 \text{ mEq cm}^{-2} \text{ h}^{-1}$; $n = 6$) were obtained using
recta from male locusts. Responsiveness to cAMP is also independent of time
following dissection. This is illustrated by the traces in Figure 3. Even
after 6 hours, the $I_{sc}$ across in vitro recta increased after cAMP addition to
the original high values of $10 \text{ mEq cm}^{-2} \text{ h}^{-1}$.

Active Cl transport during cAMP stimulation was further confirmed by
replacing Cl with anions which are not known to be transported in other
tissues. Figure 4 shows that cAMP-stimulated $I_{sc}$ is reduced by 91% when Cl is
replaced with gluconate. This result is consistent with those results obtain-
ed by other workers using different salines and voltage clamps: Williams et al. (1978) and Herrera et al. (1976) found that high initial $I_{sc}$ after dissection is abolished by Cl replacement. Corpus cardiacum extracts did not stimulate transrectal $I_{sc}$ when bathed in salines containing sulfate or nitrate instead of Cl (Spring and Phillips, 1980). As previously shown for unstimulated recta (Fig. 1), a small residual $I_{sc}$ (1 μEq cm$^{-2}$ h$^{-1}$ in these experiments) was insensitive to Cl replacement after cAMP stimulation (Fig. 2). However, all $\Delta I_{sc}$ after stimulation could be accounted for by $\Delta J_{net}^{Cl}$ and this is true of all subsequent experiments in this study.

Finally in a third series of experiments, $V_t$ and unidirectional Cl fluxes were measured under open-circuit conditions, and the observed flux ratios were compared with those values calculated from $V_t$ using the Ussing flux ratio equation and assuming that Cl crosses the rectal wall only by simple diffusion (Fig. 5). As in previous experiments, cAMP caused a large increase in $V_t$ and $J_{ms}^{Cl}$. After adding cAMP, $J_{ms}^{Cl}$ was similar under open-circuit and short-circuit conditions (1.34 ± 0.06 μEq cm$^{-2}$ h$^{-1}$ and 1.71 ± 0.12 μEq cm$^{-2}$ h$^{-1}$, respectively), however $J_{ms}^{Cl}$ was significantly lower (4.75 ± 0.7 μEq cm$^{-2}$ h$^{-1}$ and 11.04 ± 0.64 μEq cm$^{-2}$ h$^{-1}$; $P < 0.001$). When K methylsulfate was added to the mucosal side to raise [K] from 10 to 100 mM and thereby mimic in vivo conditions, $V_t$ decreased by 92% (23 mV), $J_{ms}^{Cl}$ increased by 38% but $J_{sm}^{Cl}$ remained unchanged ($P > 0.2$). Observed flux ratios were higher than those predicted from the flux ratio equation (Fig. 5).

Equations derived by Ussing (see Zerahn, 1956) may be used to calculate the minimal metabolic energy requirement for Cl transport under each of these experimental conditions. The minimum work per equivalent of Cl transported is:

$$W = \left( V_t F + RT \ln \frac{J_{ms}^{Cl}}{J_{sm}^{Cl}} \right) 0.239$$
Figure 2.5  Effects of sequential addition of 1 mM cAMP to the serosal side and mucosal addition of potassium on Cl fluxes under open-circuit conditions. Potassium concentration was increased from 10 to 100 mM by adding K-methylsulfate to the mucosal side. (a) Transepithelial potential (● $V_t$); (b) unidirectional $^{36}$Cl fluxes from mucosa to serosa (▲ m→s), and serosa to mucosa (▲ s→m; n = 6); and (c) Cl flux ratios (■) observed, and (□) predicted from $V_t$ by the Ussing flux ratio equation. Means ± s.e.; n = 12 ($V_t$), n = 6 ($J_{ms}^{Cl}$), n = 6 ($J_{sm}^{Cl}$).
where $W$ is work (calories/equiv.), $V_t$ is transepithelial potential (volts), and $R$, $T$ and $F$ have their usual meanings. Under open-circuit conditions, unstimulated recta in normal saline must expend $>3.1 \times 10^{-4}$ calories cm$^{-2}$h$^{-1}$ in Cl transport work (taking $V_t = 0.008$ V, $J_{ms}^{Cl}/J_{sm}^{Cl} = 2.1$, and $J_{net}^{Cl} = 0.5 \times 10^{-6}$ Eq cm$^{-2}$h$^{-1}$). During cAMP stimulation, Cl transport work increased by 20-fold to $6.0 \times 10^{-3}$ calories cm$^{-2}$h$^{-1}$ ($V_t = 0.025$ V, $J_{ms}^{Cl}/J_{sm}^{Cl} = 4.1$, $J_{net}^{Cl} = 4.3 \times 10^{-6}$ Eq cm$^{-2}$h$^{-1}$). These calculations in 10 mM K saline are not representative of the Cl transport work in vivo where the lumen contains $>100$ mM K. Although addition of mucosal K increased both the flux ratio and net Cl flux 42%, transport work rose only 5% to $6.3 \times 10^{-3}$ calories cm$^{-2}$h$^{-1}$ due to the reduced transepithelial potential ($V_t = 0.002$ V, $J_{ms}^{Cl}/J_{sm}^{Cl} = 5.4$, $J = 6.1$ μEq cm$^{-2}$h$^{-1}$).

One implication of the effects of high mucosal K on rectal Cl transport is that energy stored in the K gradient between Malpighian tubule fluid and hemolymph is partially utilized during Cl reabsorption in the rectum. In effect, the K pump in the Malpighian tubules and the Cl pump in the rectum exhibit a form of energetic coupling.

In summary, Cl absorption across locust rectum fulfills classical criteria for active transport reviewed by Koch, 1970): $J_{net}^{Cl}$ is maintained in the absence of electrical and chemical gradients (i.e. under $I_{sc}$ conditions) and flux ratios at open-circuit are an order of magnitude higher than those predicted for simple diffusion. The sensitivity of cAMP stimulated $I_{sc}$ to anion substitutions and good agreement between $I_{sc}$ and $J_{net}^{Cl}$ after cAMP addition in the present and previous studies (Williams et al., 1978; Spring and Phillips, 1980b) indicate that Cl transport is the predominant electrogenic process in this epithelium. Also, 1 mM cAMP causes a very large (~50%) decrease in transepithelial resistance. We shall see in chapter 5 that this stimulation of transepithelial conductance has two components: an enhanced K permeability and an "active Cl transport" conductance.
2) **Exchange diffusion**

The presence of a large, electrogenic Cl flux across locust rectum (Fig. 2) does not preclude an exchange diffusion component. Exchange diffusion has been suggested in other invertebrate epithelia (see Krogh, 1939; Shaw, 1960; Stobart, 1965; Smith, 1969; Dietz, 1974; Dietz and Branton, 1975). If \( J_{Cl}^{sm} \) occurs partly by exchange with mucosal Cl, then the following behaviour is predicted: i) reduction of mucosal \([Cl]_m\) should decrease \( J_{Cl}^{sm} \) (e.g. Kristensen and Larsen, 1978; Ques-von Petery et al., 1978; Biber et al., 1980), and ii) estimates of transepithelial Cl permeability calculated from \( J_{Cl}^{sm} \) under \( I_{sc} \) conditions should be higher than when calculated under open-circuit conditions, since electroneutral exchange flux would be insensitive to \( V_t \) (Lewis and Diamond, 1976). These predictions were not observed in locust rectum. \( J_{Cl}^{sm} \) was not reduced, but increased slightly from 0.62 ± 0.1 to 0.83 ± 0.15 \( \mu \text{Eqcm}^{-2}\text{h}^{-1} \) (\( \bar{X} \pm \text{s.e., n} = 6 \text{ diff. not significant at P} > 0.1 \) under \( I_{sc} \) conditions when mucosal Cl was replaced with methylsulfate, indicating that \( J_{Cl}^{sm} \) was not stimulated by Cl on the "trans" side (Table 3). Secondly, the apparent transepithelial Cl permeability was similar under short-circuit (\( *P_{SC}^{Cl} \)) and open-circuit current (\( *P_{OC}^{Cl} \)) conditions. As a first approximation, \( *P_{SC}^{Cl} \) is given by the equation \( *P_{SC}^{Cl} = J_{Cl}^{sm}/[Cl] \) where \( J_{Cl}^{sm} \) is 4.7 x 10^{-10} \( \mu \text{Eqcm}^{-2}\text{h}^{-1} \) (from Fig. 2) and \([Cl] \) is 114 x 10^{-6} \( \text{Eqcm}^{-3} \). To estimate Cl permeability from the effects of transepithelial potential, the equation \( *P_{OC}^{Cl} = -RT J_{Cl}^{passive}/[Cl] V_t z F \), where \( V_t \) is 24 mV (Fig. 5), and net passive flux (\( J_{Cl}^{passive} \)) was estimated as \( 2(J_{Cl}^{sm} - J_{Cl}^{sm}) = 2.78 x 10^{-4} \mu \text{Eqcm}^{-2}\text{h}^{-1} \) (Figs. 2 and 5).

The value of \( *P_{OC}^{Cl} \) (6.0 x 10^{-6} \( \text{cm sec}^{-1} \)) is actually higher than \( *P_{SC}^{Cl} \) (4.1 x 10^{-6} \( \text{cm sec}^{-1} \)), a result which is not consistent with exchange diffusion. Both of these calculations assume that \( J_{Cl}^{sm} \) is passive, independent, and that
Table 2.3 Effect of removing mucosal Cl on serosa-to-mucosa flux of Cl under \( I_{sc} \) conditions.

<table>
<thead>
<tr>
<th></th>
<th>Serosa: 114 mM Cl</th>
<th>Serosa: 114 mM Cl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mucosa:</td>
<td>114 mM Cl</td>
<td>Mucosa: 0 mM Cl</td>
</tr>
</tbody>
</table>

\[ J_{sm}^{Cl} \left( \mu \text{Eq cm}^{-2} \text{h}^{-1} \right) \]

|          | ±0.10             | ±0.15             |

Both sides were initially bathed in normal saline (114 mM Cl; Table 2.1). Cl was replaced on the mucosal side with methyl sulfate and a small amount of sulfate. Sucrose was adjusted to maintain isosmocity. Means ± s.e., \( n = 6 \); not significantly different (\( p > 0.1 \)).
the epithelium behaves as if it were a single barrier to Cl movement. The validity of these assumptions is discussed later in this chapter and in chapter 5.

In summary, if Cl exchange diffusion is present in locust rectal epithelium, it must occur at an extremely low rate which does not contribute significantly to $^{36}$Cl fluxes under $I_{sc}$ conditions.

3) **Selectivity of Cl transport**

Two methods were used to examine the selectivity of Cl transport. The first method was semi-quantitative, but permitted anions to be tested in the presence of 20 mM Cl on both sides of the epithelium. $J_{Cl}^{Cl\text{-ms}}$ was measured under $I_{sc}$ conditions during cAMP stimulation. After three control flux periods, various unlabelled test anions (A) were added to both sides as Na salts (final concentration of A was 20 or 50 mM). $J_{ms}$ and $I_{sc}$ were measured for a further three flux periods ($3 \times 15$ minutes). If A (the added, unlabelled anion) is transported electrogenically, then it should increase the difference between $I_{sc}$ and $J_{ms}^{Cl}$ since this unmarked anion transport would contribute to $I_{sc}$ but not to the mucosa-to-serosa flux of $^{36}$Cl. Moreover, the foreign anions should depress $J_{ms}^{Cl}$ if the transport sites are nearly saturated with "substrate". Parallel decreases of similar magnitude in $I_{sc}$ and $J_{ms}^{Cl}$ would suggest i) competition between test anion and Cl without A translocation or ii) metabolic inhibition. Addition of unlabelled Cl under identical conditions was used as a standard against which the effects of other anions were compared.

Figure 6 shows the effects of nine test anions on the difference between short-circuit current and $^{36}$Cl forward flux ($I_{sc}^{Cl} - J_{ms}^{Cl}$). As expected, the addition of 50 mM unlabelled Cl (raising total [Cl] to 70 mM) increased ($I_{sc}^{Cl} - J_{ms}^{Cl}$) by 4.3 and 4.5 $\mu$Eqcm$^{-2}$h$^{-1}$ in two preparations. Increases were partly due to reductions in $J_{ms}^{Cl}$ ($-1.6$ and $-2.1$ $\mu$Eqcm$^{-2}$h$^{-1}$, respectively). These tissues had normal values of $I_{sc}$ ($7.1$ and $8.5$ $\mu$Eqcm$^{-2}$h$^{-1}$), consistent with
Figure 2.6  Effect of adding anions on the difference between $I_{sc}$ and the unidirectional flux of Cl from mucosa to serosa. Anions (20–50 mM) were added at the arrow to both sides of cAMP-stimulated recta bathed in normal saline (Table 2.) in which 90 mM Cl had been replaced by gluconate. The shaded area is $(I_{sc} - J_{ms}^{Cl})$ for individual preparations. As a control (upper left-hand panel), 50 mM unlabelled Cl was added at the arrow.
results which will be reported in chapter 3 at a [Cl] of 70 mM (7.3 μEqcm⁻²h⁻¹). Bromide addition caused smaller increases in \( I_{sc} - J_{ms}^{Cl} \) than did Cl addition (3.8 and 2.7 μEqcm⁻²h⁻¹ in two preparations). Fluoride addition produced a gradual decline in both \( I_{sc} \) and \( J_{ms}^{Cl} \), suggesting a metabolic inhibition since this ion is often toxic to tissues (see Diamond and Wright, 1969). No other anions had sizable effects on \( I_{sc} \) or \( J_{ms}^{Cl} \) (n = 2, for each anion), suggesting little competition for Cl binding or translocation.

In a second series of experiments, recta were equilibrated under \( I_{sc} \) conditions and rinsed for 4 hours with salines in which Cl was completely replaced by other anions. \( I_{sc} \) was measured before and after addition of 1 mM cAMP to the serosal side.

Cyclic-AMP stimulated \( I_{sc} \) significantly only when Cl or Br was present (Fig. 7). The selectivity sequence, in order of decreasing \( \Delta I_{sc} \) (μEqcm⁻²h⁻¹), was Cl (5.4) > Br (2.7) > PO₄⁻HPO₄⁻ (0.5) > I, acetate, thiocyanate, SO₄²⁻, NO₃⁻, F (all less than 0.2, i.e. not significantly different from unstimulated recta). Urate was also unable to sustain the \( I_{sc} \). These data strongly suggest that Cl is the principal anion which is transported by this system in vitro. Overall selectivity could be determined at the Cl translocation step or at the binding site. The latter is suggested by the lack of inhibitory effect of most anions on \( J_{ms}^{Cl} \).

4) Ionic dependencies of Cl transport

a) Sodium dependence:

Several experiments were performed to test whether Cl absorption requires external Na as predicted by the NaCl cotransport model (reviewed by Frizzell et al., 1979).
Figure 2.7 Effect of 1 mM cAMP on $I_{sc}$ in anion-substituted salines. Tissues were pre-equilibrated for 4 h under $I_{sc}$ conditions in saline in which all Cl was substituted with a test anion (see Table 1). Means ± s.e.; $n = 5-6$. 
i) **Prolonged removal of external Na**

Recta were equilibrated for 4 h in nominally Na-free saline and were rinsed at least three times with fresh saline to remove Na which had leaked from the tissue. Final external Na concentration ranged from <1-200 µM.

In Na-free saline, addition of 1 mM cAMP increased $I_{sc}$ (1.9 ± 0.44 to 8.8 ± 1.0 µEqcm⁻²h⁻¹) and $J_{Cl}^{Clms}$ (0.1 ± 0.68 to 5.3 ± 2.8 µEqcm⁻²h⁻¹, ±95% confidence interval) and Δ$J_{Cl}^{Clsm}$ still accounted for >75% of Δ$I_{sc}$ (Fig. 8). Stimulated values of $J_{Cl}^{Clnet}$ and $I_{sc}$ were smaller than controls, but this was not statistically significant at P > 0.1 (compare Figs. 2 and 8). Electrical parameters were similar in the groups of locusts used for forward and backfluxes. After 1 h of exposure to cAMP, $I_{sc}$ was 7.62 ± 0.47 vs 8.86 ± 0.8 µEqcm⁻²h⁻¹, $V_t$ was 27.93 ± 0.23 vs 31.29 ± 3.01 mV and $R_t$ was 136.78 ± 7.89 vs 135.51 ± 11.81 Ω cm⁻² for m→s vs s→m flux measurements, respectively.

It was important to test whether Na-coupled Cl flux might persist because of the presence of minute quantities of Na in nominally Na-free saline. However, it should be noted that such a mechanism would require an extremely high Na affinity for a NaCl cotransport mechanism. Sodium contamination was measured at the beginning and at the end of most experiments as described in the methods except that samples were not diluted in distilled water. Figure 9 shows that there was no correlation between cAMP-stimulated $I_{sc}$ (which remained high) and trace levels of Na. In several preparations, Na could not be detected (<1 µM) at the end of the experiment when [Na] is highest. Less than 3% of the variation in $I_{sc}$ may be attributed to [Na] ($r^2 = 0.0288$).

The 40% reduction in average $J_{Cl}^{Clnet}$ after long exposures (>5.5 h) to Na-free saline containing cAMP requires some comment. Much of this decline may be due to the absence of Na-coupled amino acid absorption which has been demonstrated in locust rectum (Balshin and Phillips, 1971). Consistent with
Figure 2.8  Effect of cAMP on Cl fluxes and electrical parameters in Na-free and HCO₃-free salines. Cyclic-AMP (1 mM) was added to the serosal side at the arrow. (a) Short-circuit current (Iₛₗ𝒄); (b) unidirectional Cl fluxes (Jₘₛ and Jₛₘ); (c) transepithelial resistance (Rₜ); and (d) spontaneous transepithelial potential (Vₜ). Means ± s.e.; n = 6 (HCO₃-free Jₘₛ, Jₛₘ), n = 10 (Na-free Jₘₛ, Jₛₘ), n = 12 (HCO₃-free Iₛₗ𝒄, Vₜ, Rₜ), n = 20 (Na-free Iₛₗ𝒄, Vₜ, Rₜ).
Figure 2.9  Relationship between $I_{sc}$ and trace level of Na in the mucosal half-chamber during Na-free experiments. Recta were exposed to nominally Na-free saline for 7-8 h. Cyclic-AMP (1 mM) was present on the serosal side during the last 2 h. Each value is for a different rectal preparation.
this suggestion, saline containing 10 mM glucose and normal sodium levels (114 mM), but lacking amino acids, does not maintain the short-circuit current or the stimulation produced by 1 mM cAMP (Fig. 10). Moreover, both Na-free and amino acid-free salines produced significantly higher serosa-to-mucosa $^{36}\text{Cl}$ flux and lower transepithelial resistance (compare Figs. 2, 8 and 10). It will be shown later that amino acids are necessary to maintain normal transport capability. Chamberlin (1981) has shown that proline is metabolised by this tissue and restores $I_{sc}$ most effectively when added to the mucosal side. In view of the importance of amino acids in maintaining Cl transport and also the presence of Na-coupled amino acid absorption, some reduction in active Cl transport is to be expected. The important finding is that a large cAMP-stimulated Cl flux (6.0 $\mu \text{Eqcm}^{-2}\text{h}^{-1}$) is observed after 5.5 h exposure to nominally Na-free saline, conditions which abolish $J_{\text{net}}^{\text{Cl}}$ and the "uphill" Cl electrochemical gradient in epithelia where NaCl coentry are well established (Nellans et al., 1973; Frizzell et al., 1975; Cremaschi and Henin, 1975; Frizzell et al., 1979; Duffey et al., 1978; Reuss and Grady, 1979; Garcia-Diaz and Armstrong, 1980). To avoid the possible metabolic side-effects of prolonged exposure to Na-free saline, results of short-term removal are examined in the following section.

ii) **Short-term removal of external Na**

The results in Figure 8 suggested that some Na might be needed with amino acids to maintain normal Cl permeability and high transport rates. During the first 4 hours, $I_{sc}$ in amino acid-free saline ($1.97 \pm 0.36 \mu \text{Eqcm}^{-2}\text{h}^{-1}$) is not different from controls ($1.62 \pm 0.15 \mu \text{Eqcm}^{-2}\text{h}^{-1}$). Also, Chamberlin (1981) has found that non-cAMP-stimulated $I_{sc}$ is maintained near control levels for 2 h when only endogenous substrates are available. To examine Na-dependency of locust Cl
Figure 2.10  Effect of cAMP after prolonged exposure to amino acid-free saline containing normal ion levels and 10 mM glucose. See Table for ion levels and Figure 2.1 for details and definitions. Cyclic-AMP was added to the serosal side at the arrow. Dotted line shows data obtained in normal saline containing amino acids (from Fig. 2.2). Means ± s.e., n = 6.
transport without substrate limitation, the short-term effects of Na-removal on Cl-dependent $I_{sc}$ were also measured.

Chambers were rinsed thoroughly with Na-free saline so that $[\text{Na}]$ dropped from 114 mM to less than 0.05 mM. There was no change ($P >> 0.2$) in Cl-dependent $I_{sc}$ within the first 75 minutes (Fig. 11). This result is consistent with the notion that any inhibition during Na-removal is slow, and is probably not a direct action on the transport mechanism per se.

iii) Unidirectional influxes of $^{36}\text{Cl}$ and $^{22}\text{Na}$ across the apical border

Figure 12 shows the accumulation of $^{36}\text{Cl}$ by rectal tissue at pH 7.0 after pre-equilibration with tracers at pH 4.5. Variation between preparations was reduced, but not eliminated by correcting for extracellular tracers with $^3\text{H}$-mannitol as described in the methods. Control experiments showed that no time-dependent changes in extracellular volume occurred. In some experiments influx data had y-intercepts which were less than zero, suggesting a systematic overestimate of extracellular space. It was not possible to use inulin as a space marker since this polysaccharide does not penetrate the layer of cuticle which lies on the mucosal surface of the epithelium. Only the rates of tracer influx (i.e. the number of counts as a function of time) are used in this study.

Injection of 10 mM cAMP into sacs resulted in significantly higher Cl unidirectional influxes than for unstimulated controls ($4.92 \pm 1.57$ vs $0.83 \pm 1.01$ nEq/mg/min; $P < 0.05$). $V_t$ also increased from $15.5 \pm 1.1$ to $58.9 \pm 2.1$ mV ($\bar{X} \pm s.e., n = 18-23$). A higher concentration of cAMP was required to stimulate $V_t$ maximally in this sac preparation than was necessary using the flat sheet preparation. This may reflect more rapid degradation of the nucleotide by rectal tissue since the sac contains only a small volume of fluid (20 μl) as compared to 5 ml in the flat sheet preparation. For comparison with
Figure 2.11 Effects of bilateral Na removal on electrical parameters across cAMP-stimulated locust rectum. Normal saline (114 mM Na) was replaced with nominally Na-free N-methyl-D-glucamine saline at constant Cl concentration (114 mM). Means ± s.e., n = 6.
Figure 2.12  Effects of cAMP on unidirectional influxes of $^{36}$Cl and $^{22}$Na into rectal tissue from the mucosal side. Initial unidirectional influx of $^{36}$Cl (a) and $^{22}$Na (b) into tissue of everted rectal sacs was measured under control conditions (○□) and after injection with 10 mM cAMP (■■). Each point is the result obtained from one animal. Tissue wet weight: 3-6 mg each. Non-zero Y-intercepts in (b) indicate some overestimate of extracellular space. The difference between control and cAMP-stimulated influx of $^{36}$Cl (i.e. slopes of regression lines) is significant at $P < 0.05$. In contrast, $^{22}$Na influx is not different after exposure to cAMP ($P >> 0.2$).
transepithelial flux measurements, 1 mg tissue (wet weight) = 7 mm² macroscopic tissue area.

Cyclic-AMP stimulation caused a 6-fold increase in \( {^{36}}Cl \) influx into rectal sacs and a 4-fold increase in \( J_{ms}^{Cl} \) in Ussing-type chambers. Absolute \( {^{36}}Cl \) influx and \( J_{ms}^{Cl} \) during cAMP exposure were also comparable (0.71 to 4.22 µEq cm⁻² h⁻¹ using sac preparations and from 1.52 ± 0.16 to 5.65 ± 0.77 µEq cm⁻² h⁻¹, respectively). Na influxes were low under these same conditions (Fig. 12b) and were not affected by exposure to 10 mM cAMP (\( P >> 0.2 \)). In contrast to cAMP-stimulated \( {^{36}}Cl \) influx, \( ^{22}Na \) influx was not significantly different from zero (\( P > 0.2 \)). These lumen-to-tissue fluxes are not consistent with NaCl coentry across the apical membrane, and furthermore, they suggest that electrical coupling between active Cl absorption and Na influx is minimal since \( ^{22}Na \) influx was not enhanced by the favourable \( V_t \) during cAMP stimulation.

In the following chapter, I report that net K transport is nearly identical to \( J_{net}^{Cl} \) under open-circuit conditions. In other words, K is the principal counter-ion accompanying electrogenic Cl transport across locust rectum, even when the Na concentration is much higher than that of K (114 mM Na, 10 mM K).

iv) Effects of inhibitors of Na-coupled Cl transport

I examined the sensitivity of Cl transport to agents which inhibit Na-coupled Cl transport in other epithelia. Locust and other insect rectal tissues contain a typical ouabain-sensitive Na/K ATPase (Peacock, 1979; 1981) which has been localized by \( ^3H \)-ouabain binding and autoradiography at the basolateral cell border in dragonfly recta (Kommick and Achenbach, 1979).

Table 4 shows that the addition of 1 mM ouabain to both sides for 1 h did not affect \( J_{ms}^{Cl} \) in unstimulated recta or the stimulation of Cl transport produced by sequential addition of 1 mM cAMP (total exposure time = 2 h; \( P > 0.2 \); compare Fig. 2). In fact, 1 mM ouabain increased \( I_{sc} \) significantly (\( P < 0.01 \)) from
Table 2.4  Effect of sequential addition of 1 mM ouabain and 1 mM cAMP on transepithelial Cl fluxes and electrical parameters.

<table>
<thead>
<tr>
<th></th>
<th>$I_{sc}$ (μEq cm$^{-2}$ h$^{-1}$)</th>
<th>$J_{Cl}^{ms}$ (μEq cm$^{-2}$ h$^{-1}$)</th>
<th>$J_{Cl}^{sm}$ (μEq cm$^{-2}$ h$^{-1}$)</th>
<th>$J_{Cl}^{net}$ (μEq cm$^{-2}$ h$^{-1}$)</th>
<th>$V_t$ (mV)</th>
<th>$R_t$ (Ω cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.51 ±0.13 (48;12)</td>
<td>1.65 ±0.08 (24;6)</td>
<td>1.10 ±0.07 (24;6)</td>
<td>7.75 ±0.55 (48;12)</td>
<td>266.1 ±16.1 (48;12)</td>
<td></td>
</tr>
<tr>
<td>1 mM ouabain</td>
<td>1.84 ±0.21 (36;12)</td>
<td>1.73 ±0.08 (18;6)</td>
<td>1.32 ±0.07 (18;6)</td>
<td>9.01 ±0.55 (36;12)</td>
<td>241.0 ±17.1 (36;12)</td>
<td></td>
</tr>
<tr>
<td>ouabain + 1 mM cAMP</td>
<td>9.50 ±0.54 (36;12)</td>
<td>9.1 ±0.51 (18;6)</td>
<td>1.73 ±0.12 (18;6)</td>
<td>7.37 ±0.79 (36;12)</td>
<td>134.5 ±10.0 (36;12)</td>
<td></td>
</tr>
</tbody>
</table>

Recta were bathed in normal saline containing 10 mM K (see Table 1) and ouabain at 22°C for 1 hour prior to addition of 1 mM cAMP. Tissues were pre-equilibrated under $I_{sc}$ conditions as described in the text. Quasi-steady-state values are calculated from measurements between 15 minutes and 60 minutes under each condition. Means ± s.e.; (number of flux periods; number of animals).
1.5 ± 0.3 to 1.84 ± 0.21 μEq cm⁻²h⁻¹, and \( J_{\text{Cl}}^{\text{ms}} \) showed a corresponding increase from 1.65 to 1.93 μEq cm⁻²h⁻¹ (P < 0.1). In two preparations exposed to 1 mM ouabain for 5 h at 22°C and \([K] = 10 \text{ mM}\), addition of 1 mM cAMP produced stimulations of 8.33 and 4.83 μEq cm⁻²h⁻¹. However exposure to 10 mM ouabain under these conditions may reduce the stimulatory effect of cAMP nonspecifically.

In summary, Cl transport in locust rectum is not sensitive to high concentrations of ouabain, in contrast to those vertebrate epithelia where Na-dependent Cl transport has been demonstrated (reviewed by Frizzell et al., 1979). Na/K ATPase from locust rectum exhibits normal sensitivity to ouabain (\( K_i = 10^{-6} \text{ M} \); Peacock, 1981) although the potency of this inhibitor in intact insect tissues is controversial (reviewed by Anstee and Bowler, 1979). It was therefore desirable to test the effects of another, more specific inhibitor of Na-coupled Cl transport.

The diuretic furosemide is an effective inhibitor of Na-coupled Cl transport in a variety of epithelia (see Frizzell et al., 1979; Ramos and Ellory, 1981). Furosemide (10 mM) inhibits electrogenic Cl secretion across rabbit colon in less than 10 min (Frizzell and Heintze, 1979). In contrast, Cl-dependent \( I_{\text{sc}} \) in locust rectum is clearly insensitive to 1 mM furosemide when added to both sides, even after a 75 min exposure (Fig. 13). There is no obvious barrier which would limit access of this agent to the apical plasma membrane because the rectal cuticle was cut. This insensitivity to furosemide is in marked contrast to all Na-coupled systems which have been examined to date.

b) Bicarbonate dependence:

At present, it is practically impossible to exclude the possibility of some Cl/HCO₃ exchange in intact tissue since bicarbonate is produced intracellularly. Although perfectly HCO₃⁻-free conditions cannot be ensured,
several different experiments provide strong indirect evidence against such an exchange system as the major mechanism of locust Cl transport.

1) Cl fluxes in HCO$_3^-$, CO$_2$-free saline

Since cell membranes are permeable to CO$_2$ (Jacobs, 1940; see Gutknecht et al., 1977), removal of all exogenous HCO$_3^-$ and CO$_2$ should deplete cells of HCO$_3^-$, H$_2$CO$_3$ and CO$_2$ via efflux of these species down their enlarged electrochemical gradients. Any exchange process which involves intracellular HCO$_3^-$ should be inhibited under these conditions. In skeletal muscle fibers, HCO$_3^-$ "washout" occurs within 5 min (Bolton and Vaughan-Jones, 1977). When salines are stirred with 100% O$_2$, any bicarbonate for a potential Cl/HCO$_3^-$ exchange process must be derived from aerobic metabolism. Removal of all external HCO$_3^-$ and CO$_2$ had no effect on I$_{sc}$, unidirectional Cl fluxes, R$_t$, or V$_t$ across stimulated locust recta (Fig. 8, compare with Fig. 2). Evidence that cytoplasmic levels of HCO$_3^-$ are indeed low during perfusion of the epithelium with CO$_2$, HCO$_3^-$-free saline comes from measuring intracellular Cl activity with double-barrelled ion-sensitive microelectrodes under these conditions. These experiments will be described in chapter 4, but some conclusions are relevant at this point. After 3 h, apparent intracellular Cl activity was 4.84 ± 0.38 mM, not significantly different from the apparent Cl activity in saline when gluconate and SO$_4^-$ are used as replacement anions (5.2 mM). In addition to residual Cl, some replacement anions are also probably sensed intracellularly. Therefore intracellular HCO$_3^-$ must be lower than 4.84 mM and is probably close to zero. A similar argument has been used by Garcia-Diaz and Armstrong (1980), who suggested that HCO$_3^-$ in cells of Necturus gallbladder is <1.0 mM in CO$_2$, HCO$_3^-$-free salines. This view has been expressed by others using Cl-sensitive liquid-ion exchanger microelectrodes and HCO$_3^-$-free salines (Brown, 1976; Bolton and Vaughan-Jones, 1977; Saunders and Brown, 1977).
Figure 2.13 Effects of furosemide and SITS addition on $I_{sc}$ across locust rectum stimulated with cAMP. Furosemide (1 mM) and 1 mM SITS were added to both sides of locust recta in separate experiments. Tissues were bathed in normal saline (see Table 2.1). The cuticle was removed from the mucosal surface of recta to allow access of the apical cell membrane to inhibitors. Means ± s.e.; $n = 8$. 
ii) Changes in external pH induced by active Cl transport

Exchange of intracellular HCO$_3^-$ for external Cl should result in alkalinization of the mucosal saline at rates commensurate with the rate of Cl entry (i.e. $J_{ms}^{Cl}$). Figure 14 shows continuous traces of mucosal and serosal pH (HCO$_3^-$-free). In four recta, average alkalinization of the mucosal side was $4.5 \pm 0.5 \mu$Eq cm$^{-2}$ h$^{-1}$ or 66% of the simultaneous $I_{sc}$. By comparison with Figure 2, this rate of alkalinization is only 39% of $J_{ms}^{Cl}$. Exposure to acetazolamide (1 mM) on both sides did not affect $I_{sc}$ but reduced the rate of mucosal alkalinization by 10 and 30% in two preparations. Addition of 1 mM SITS (4-acetamido-4'-isothiocyanostilbene-2,2'-disulfonic acid) to both sides did not block mucosal alkalinization (Fig. 14). Also, Cl-dependent $I_{sc}$ increased with a much more rapid time course than did alkalinization.

One might postulate a coupling ratio of 3Cl to 1HCO$_3^-$ to account for the observed stoichiometry, however to my knowledge no such exchange has been reported and it is doubtful that such an exchange would be thermodynamically feasible given the large electrochemical gradient opposing Cl entry (46 mV, for each Cl) and the presumed gradient favouring HCO$_3^-$ efflux in normal saline (maximum 76 mV; see discussion).

There is also the problem of excess protons produced in the reaction $CO_2 + H_2O \Rightleftharpoons H_2CO_3 \Rightleftharpoons H + HCO_3^-$. Acetazolamide (1 mM) is thought to inhibit epithelial Cl/HCO$_3^-$ exchange by its effects on carbonic anhydrase (Maetz and Garcia Romeu, 1964; Maetz, 1971; Garcia Romeu et al., 1969; Erlij, 1971) although other actions of this drug are indicated at concentrations >1 mM (see Hogben and Karal, 1973; Radtke et al., 1972; Cousin and Motais, 1976; Bruus et al., 1976; reviewed by Maren, 1977). Mucosal pH was observed to increase in 22 preparations, however serosal pH never decreased (Fig. 14c).

A significant fraction of mucosal alkalinization in CO$_2$/HCO$_3^-$-free saline may be due to rectal NH$_3$ production. Faecal NH$_3$ is a major route of
Figure 2.14 Continuous recordings of $I_{sc}$ and external pH during exposure of recta to cAMP in HCO$_3$-free saline. Cyclic-AMP (1 mM), SITS (1 mM), acetazolamide (1 mM), and azide (1 mM) were added bilaterally after the cuticular intima had been removed from the mucosal surface of the tissue. See text for details and Table 2.1 for composition of the saline.
nitrogen secretion in cockroaches (Mullins and Cochran, 1972) and both locusts
and grasshoppers are known to excrete ammonia in the faeces (see Bursell,
1967). Volatile ammonia and other nitrogenous bases are produced by laboratory
locust colonies (Blight, 1969) and a detailed review by Cochran (1975) has
emphasized the importance of ammonia excretion in insects. Chamberlin (1981)
showed that amino acids are rapidly metabolized by rectal tissue.

To test whether NH₃ might be produced by locust rectum in vitro, accumu-
lation of total NH₄ was measured on both sides under I_sc conditions in HCO₃-
free saline (containing the usual 11 amino acids). Before addition of cAMP,
mucosal ammonium accumulated at a rate of 3.0 and 4.5 µMcm⁻²h⁻¹, increasing to
6 and 12 µMcm⁻²h⁻¹ during cAMP exposure. Chamberlin (1981) has measured very
low rates of NH₄ accumulation when proline is the only substrate available to
suspended recta. However, glutamine is the normal source of renal ammonia in
vertebrates and this could also be true of locust rectum, particularly in view
of the high glutamine concentration in rectal tissue (44.5 mM; Chamberlin,

iii) Effects of inhibitors of HCO₃-coupled Cl transport

Table 5 summarizes the effects of exposing recta to 1 mM SITS, acetazolamide
and azide for 1 h on Cl-dependent I_sc. Control periods for each
preparation preceded addition of the inhibitor. SITS and acetazolamide
exposure did not change I_sc (P > 0.2). In contrast, azide virtually abolished
I_sc within 10-15 min (see also Fig. 14).

c) Potassium dependence:

The effects of K were of particular interest since most terrestrial
insect recta normally absorb ions and water from a KCl-rich fluid in vivo.
For example, the primary urine entering the lumen of locust rectum from the
Malpighian tubule contains 140 mM K as compared to 10 mM K in the hemolymph.
Table 2.5  Effect of inhibitors after 1-2 h exposure on Cl-dependent $I_{sc}$ across cAMP-stimulated recta *P < 0.001

<table>
<thead>
<tr>
<th>Inhibitor (n)</th>
<th>$I_{sc}$ (µeq cm$^{-2}$ h$^{-1}$)</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>5.6±0.5</td>
<td></td>
</tr>
<tr>
<td>Inhibitor</td>
<td>5.6±0.5</td>
<td></td>
</tr>
<tr>
<td>SITS (8)</td>
<td>5.6±0.5</td>
<td>0</td>
</tr>
<tr>
<td>Acetazolamide (4)</td>
<td>9.1±1.0</td>
<td>0</td>
</tr>
<tr>
<td>Azide (5)</td>
<td>7.5±1.1</td>
<td>0.29±0.1*</td>
</tr>
</tbody>
</table>
i) Effects of K-free saline

Lowering saline K from 10 mM (control) to 0 mM on both sides had no significant effect on $I_{sc}$, $J_{ms}^{Cl}$ and $V_t$ across unstimulated recta, although $R_t$ was 25% higher under K-free conditions (Fig. 15, compare with Fig. 2). Importantly, 1 mM cAMP produced only small increases in $J_{net}^{Cl}$, $I_{sc}$, $V_t$ and $R_t$. During exposure to 1 mM cAMP, restoring 10 mM K to both sides (same as in normal saline) greatly enhanced $I_{sc}$, $J_{ms}^{Cl}$, $V_t$ and reduced $R_t$ to control levels (Fig. 15).

There is clearly a strong dependence of active Cl transport on exogenous K. Approximately 70% of the net $^{36}$Cl absorption is K-dependent while the remainder is not affected by removing external K. The nature of this K-dependence is examined in chapters 3 and 4.

d) Dependence on divalent cations:

Omitting Mg from control saline did not alter Cl-dependent $I_{sc}$ significantly during the first hour (Table 6). Similarly, when external Ca was removed for 1 h there was no detectable change in cAMP-stimulated $I_{sc}$. To ensure that external Ca levels were $<10^{-7}$ M, 5 mM ECTA was also included in nominally Ca-free saline. Exposure to Ca-free conditions for longer periods (>3 h) may reduce transport capability, however this was not investigated. The lack of short-term inhibition of $I_{sc}$ suggests that Cl transport by rectal tissue per se does not directly require exogenous Ca or Mg.

Regulation of Cl transport by low pH, hyperosmocity and "second messengers"

i) Effects of pH

The pH of luminal contents can be as low as 4.5 in the locust rectum in situ (Phillips, 1964b; Speight, 1968). Figure 16 shows the effects of varying mucosal pH on cAMP-stimulated $I_{sc}$ and $R_t$. Abrupt reduction of mucosal pH over the range 7.0 to 4.0 reversibly reduced $I_{sc}$ and $V_t$, and increased $R_t$. In
Figure 2.15  Effects of cAMP on electrical parameters and Cl fluxes under K-free conditions. Cyclic-AMP (1 mM) was added to the serosal side at the first arrow. Potassium methylsulfate (10 mM) was added to both sides at the second arrow. See Figure 2.1 for definitions.
Table 2.6  Effects of exposure to Ca-free or Mg-free Saline on electrical parameters.$^1$

<table>
<thead>
<tr>
<th>Sequential Condition</th>
<th>$I_{sc}$ (μEq cm$^{-2}$ h$^{-1}$)</th>
<th>$V_t$ (mV)</th>
<th>$R_t$ (ohms cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>9.44 ± 0.28</td>
<td>29.16 ± 0.85</td>
<td>116.1 ± 3.1</td>
</tr>
<tr>
<td>(24;6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca-free (1h)</td>
<td>10.13 ± 0.21</td>
<td>31.25 ± 0.76</td>
<td>114.9 ± 12.7</td>
</tr>
<tr>
<td>(24;6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ca-free + 5 mM EGTA</td>
<td>9.89 ± 0.38</td>
<td>30.36 ± 1.14</td>
<td>115.3 ± 4.3</td>
</tr>
<tr>
<td>(0.5h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(12;6)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Magnesium</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.67 ± 0.45</td>
<td>29.98 ± 0.84</td>
<td>131.9 ± 4.6</td>
</tr>
<tr>
<td>(20;5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mg-free (1h)</td>
<td>9.01 ± 0.46</td>
<td>28.53 ± 0.96</td>
<td>121.4 ± 4.5</td>
</tr>
<tr>
<td>(20;5)</td>
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</table>

$^1$Calcium-free and magnesium-free experiments were performed on different tissues. Means ± s.e., (number of observations; number of tissues). Differences between controls and experimentals were not significant (p < .05).
Figure 2.16  Effects of external pH on transepithelial electrical parameters in cAMP-stimulated recta. Mucosal (m) and serosal (s) pH were varied in separate experiments. $I_{sc}$, $V_t$ and $R_t$ were determined after 30 min exposure to each external pH. Tissues were bathed bilaterally in normal saline containing 20 mM phosphate. Means ± s.e.; $n = 8$ ($\Delta p$H on mucosal side), $n = 6-8$ ($\Delta p$H on serosal side).
contrast, I_{sc} was surprisingly insensitive to changes in pH over a wide range (3.0-8.0) on the serosal side, even though hemolymph pH is relatively constant in vivo (7.1 ± 0.04, \bar{x} ± s.e., n = 6). Possible explanations for the effects of mucosal pH on I_{sc} include: i) A passive net flux of protons from the acidified mucosal to more alkaline serosal side. This could cancel out some of the I_{sc} which results from active Cl transport. ii) High mucosal levels of protons might competitively inhibit the K-activation sites on a Cl transport carrier (see chapters 3 and 4 for evidence regarding direct "enzyme-like" activation of a Cl-transport mechanism by K). Some other direct effect of low pH on the Cl transporter remains possible. iii) The effects may be indirect and mediated by changes in intracellular pH.

The first of these possibilities seems unlikely since reversal of the pH gradient (serosal side acidified; Fig. 16) did not have opposite effects on I_{sc}. According to the hypothesis (i) a very large increase in I_{sc} is predicted, comparable to the decrease in I_{sc} observed during mucosal acidification. Competition between protons and potassium ions (possibility ii) cannot alone account for the inhibition by low pH since mucosal acidity reduced I_{sc} to a value which is lower than the rate of Cl transport under K-free conditions (compare I_{sc} in Fig. 16 and Fig. 15). Low intracellular pH (i.e. hypothesis iii) appears to mediate the inhibitory effects of low external pH on active Na transport across frog skin. In support of this view, highly permeant acidic buffer systems are more effective in inhibiting transport than are less permeant buffers (Funder et al., 1967; Mandel, 1978).

The increase in tissue resistance during exposure of locust recta to mucosal acidity could be due to two different processes: i) a reduction in conductance associated with the active Cl transport pathway, or ii) a reduction in passive K permeability. In chapters 4 and 5, evidence based on the
relationship between $I_{sc}$ and active conductance will suggest that most of the passive or "shunt conductance" in locust rectum is due to K permeability. The values of $I_{sc}$ (32.2 μA cm$^{-2}$) and $G_t$ (or $1/R_t = 5.9$ mS cm$^{-2}$) observed during exposure to low pH are those predicted from the normal $I_{sc}/G_t$ relation (see chapter 5); therefore, no effect of low pH on passive K permeability is required in order to explain $\Delta G_t$.

ii) Effects of high osmotic concentration

The locust rectum is exposed to extraordinary fluctuations in osmotic concentration on the lumen side during the feeding-dehydration cycle. Rectal contents oscillate between 0.4 Osmoles/l and >1.1 Osmoles/l depending on the state of hydration of locusts. It seemed possible that active Cl transport or passive permeability to ions might be altered by these large changes in luminal osmolarity. Figure 17 shows the response of $I_{sc}$ and $R_t$ when saline osmolarity was adjusted by varying the sucrose concentration on both sides of the rectum or on the mucosal side only.

Perfusion with hyposmotic saline on both sides (364 mOsm/l, measured) had no significant effect on $I_{sc}$ and $R_t$; however, both $I_{sc}$ and $R_t$ showed a 40% decline when hyperosmotic saline (1,220 mOsm/l, measured) was present on both sides. In normal saline, when $I_{sc}$ was $11.2 \pm 1.5 \mu$Eq cm$^{-2}$ h$^{-1}$, chloride activity was 81.9 mM as measured using an ion-sensitive microelectrode (see chapter 4 for methods). When osmotic concentration was increased to 1,220 mOsm/l with sucrose, Cl activity decreased to 75.4 mM and $I_{sc}$ dropped from $11.2 \pm 1.5$ to $7.7 \pm 0.2 \mu$Eq cm$^{-2}$ h$^{-1}$. Based on the kinetics of Cl transport across locust rectum (chapter 3), this 30% inhibition of $I_{sc}$ cannot be attributed simply to a reduction in Cl activity coefficient from 0.718 to 0.661. Such a change should decrease $I_{sc} \approx 5\%$. Similarly, additional passive backflux of Cl due to small differences in ion activities across the rectal
Figure 2.17 Influence of mucosal and serosal osmotic pressure on $I_{sc}$ and transepithelial conductance ($G_t$). Mucosal and/or serosal osmotic pressure was elevated by adding sucrose. Dotted line shows values of $G_t$ which are predicted from the normal relationship between $I_{sc}$ and $G_t$ as described in chapter 5 (Fig. 5.21). The much lower values of $G_t$ observed at high osmotic pressures indicate that passive permeability of the epithelium is reduced under these conditions. Means ± s.e.; n = 7.
The wall under these conditions was too small to alter $I_{sc}$ (see chapter 5). These results suggest that Cl is actively transported at a reduced rate during exposure to high osmolarity.

A large decrease in leak or "shunt" conductance must also occur under these conditions since $R_t$ is 70% higher than the value predicted by the relationship between $I_{sc}$ and $G_t$ during cAMP stimulation (chapter 5). This effect is not due simply to a decrease in saline conductivity, since compensation for saline resistance was made during all experiments involving short-circuit current. In vivo, the osmotic concentration of rectal contents gradually increases towards the end of the reabsorptive cycle. A decrease in ionic permeability due to local hypertonicity would greatly reduce the work necessary to maintain ionic gradients across the epithelium. An analogous situation exists with respect to osmotic permeability ($P_{osm}$), which is lower in the serosa-to-mucosa direction than in the mucosa-to-serosa direction (Goh and Phillips, 1978).

### iii) Second messengers and hormonal stimulation

#### a) Cyclic nucleotides: Cyclic-AMP has been used in the present study to mimic in vivo hormonal control of Cl transport. Corpus cardiacum extracts, cAMP (Spring et al., 1978; Spring and Phillips, 1980a,b) and hemolymph from fed locusts (Hanrahan, 1978; Spring et al., 1978; Spring and Phillips, 1980c) all produce comparable stimulations of $I_{sc}$, $V_t$ and net Cl transport. However, adding cAMP produces a more immediate stimulation of $I_{sc}$, and more importantly, results in a much larger decrease in transepithelial resistance (25% according to Spring and Phillips, 1980b; 40-65%, this study) than has been reported using natural stimulants (4%; Spring and Phillips, 1980a).

In view of the large resistance change produced with cAMP, I re-examined the effects of corpus cardiacum at higher doses than previously tested.
Exposure of locust recta to 0.08 gL pair/5 ml (a maximal or near-maximal dose according to Spring and Phillips, 1980) resulted in a small drop in $R_t$ which was comparable to that observed previously (8%, not significant at $P > 0.1$; Table 7). However, when the dose was increased approximately 10-fold to 1 gL pair/5 ml, $R_t$ decreased significantly (24%, $P < 0.01$) and $I_{sc}$ increased further (35%, $P < 0.02$; Table 7). No significant change in $I_{sc}$ ($P > 0.2$) was observed when the concentration of extract was increased from 1 gland pair/5 ml to 2 gland pair/5 ml although $R_t$ declined further to 70% of controls.

In summary, large resistance changes do occur during exposure to corpus cardiacum extract, but only at higher concentrations. The dose (~0.08 gland pair/5 ml) which was previously reported to cause a maximum $\Delta I_{sc}$ (but only 4% reduction in $R_t$) did not produce maximal stimulation in this study (Table 7). It should be noted that the levels of corpus cardiacum required to decrease $R_t$ are not "unphysiological". For example, only 6% of the total active peptide in the corpora cardiaca must be released into the hemolymph (total volume 200-300 µl) in order to achieve the maximal doses used in my experiments. Highnam et al. (1974) have calculated that 70% of the stored neurosecretory material in these glands is released within 10 min of feeding. Therefore, use of cAMP to study the details of Cl transport in vitro seems justified.

Relative effectiveness of cAMP and cGMP in stimulating $I_{sc}$ across locust rectum was tested using control saline and conditions using the saline of Spring et al. (see Table 1 of this chapter). Exposure to 1 mM cAMP caused an $\Delta I_{sc}$ of $8.8 \pm 1.4 \mu$Eqcm$^{-2}$h$^{-1}$ ($\bar{x} \pm$ s.d., $n = 5$) while addition of 1 mM cGMP under identical conditions increased $I_{sc}$ by $9.9 \pm 0.71 \mu$Eqcm$^{-2}$h$^{-1}$. The difference in $\Delta I_{sc}$ caused by these two stimulants was not statistically significant ($P > 0.2$) in marked contrast to blowfly salivary gland (Berridge, 1973).
Table 2.7 Influence of corpora cardiaca on $I_{sc}$ across recta in normal and Ca-free saline

<table>
<thead>
<tr>
<th></th>
<th>$I_{sc}$ (µEqcm$^{-2}$h$^{-1}$)</th>
<th>$V_t$ (mV)</th>
<th>$R_t$ (ohms cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(a) Normal saline</td>
<td></td>
<td></td>
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<tr>
<td>control</td>
<td>1.9±0.4</td>
<td>8.9±2.0</td>
<td>176.9±9.8</td>
</tr>
<tr>
<td></td>
<td>(7.7)</td>
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<td></td>
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<tr>
<td>0.08 gl pair/5ml</td>
<td>7.1±2.0</td>
<td>29.3±7.0</td>
<td>162.7±14.4</td>
</tr>
<tr>
<td></td>
<td>(7;7)</td>
<td></td>
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</tr>
<tr>
<td>1 gl pair/5ml</td>
<td>9.6±2.2</td>
<td>33.9±7.1</td>
<td>132.8±13.0</td>
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<td></td>
<td>(7;7)</td>
<td></td>
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</tr>
<tr>
<td>2 gl pairs/5ml</td>
<td>9.3±2.0</td>
<td>30.9±6.6</td>
<td>124.1±11.7</td>
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<td></td>
<td>(7;7)</td>
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<tr>
<td>(b) Ca-free saline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+EGTA(2.5 mM)</td>
<td>2.0</td>
<td>8.6</td>
<td>145.7</td>
</tr>
<tr>
<td>+A23187(1µg/ml)</td>
<td>±0.1</td>
<td>±1.6</td>
<td>±10.8</td>
</tr>
<tr>
<td></td>
<td>(20;4)</td>
<td>(4;4)</td>
<td>(4;4)</td>
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<tr>
<td>as above</td>
<td>10.0</td>
<td>26.7</td>
<td>100.2</td>
</tr>
<tr>
<td>+1 gl pair/5ml</td>
<td>±0.5</td>
<td>±1.9</td>
<td>±8.1</td>
</tr>
<tr>
<td></td>
<td>(20;4)</td>
<td>(4;4)</td>
<td>(4;4)</td>
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Means ± s.e., (n = number of observations; animals).
Tissue cAMP levels increase ~2.6-fold when isolated locust recta are exposed to extracts of corpus cardiacum (Spring et al., 1978; Spring and Phillips, 1980a) possibly due to cyclic-nucleotide synthesis, although the results could also be explained by a reduction in the rate of cAMP degradation (Wells and Hardman, 1977). Moreover, if cAMP is normally produced at a low rate in the absence of hormonal stimulation, intracellular levels should increase at a rate which is proportional to the rate of synthesis when degradation of the cyclic-nucleotide is blocked. The relative rates of cAMP synthesis under control conditions and during hormonal stimulation were estimated semi-quantitatively by comparing the initial rise in I_{sc} (dI_{sc}/dt) after addition of the phosphodiesterase inhibitor theophylline. The initial increase in I_{sc} in the absence of corpus cardiacum extract must be due to the accumulation of cAMP from "basal" cAMP synthesis (Fig. 18). After addition of corpus cardiacum extract, dI_{sc}/dt increased 5-fold from 448.4 ± 56.7 nAcm^{-2} min^{-1} to 2640.8 ± 373.5 nAcm^{-2} min^{-1} (x ± s.e., n = 4). Note that complete inhibition of phosphodiesterase is not necessary for this calculation, only that the fractional inhibition of the enzyme remains constant during exposure to corpus cardiacum extract. This result provides further evidence that the rate of cAMP synthesis increases during hormonal stimulation.

b) Intracellular calcium: Intracellular Ca is known to regulate Cl permeability and active Cl transport in epithelia (Prince and Berridge, 1973; Candia et al., 1977; Frizzell, 1977; Bolton and Field, 1979). Although the Cl transport system has no direct and immediate requirement for external Ca (Table 6), it is possible that following hormone binding, an increase in cytosolic Ca released from intracellular stores accompanies the rise in intra-
Figure 2.18 Effects of sequential addition of theophylline (4 mM) and corpus cardiacum homogenate (1 gland pair/5 ml) on $I_{sc}$ on $I_{sc}$. Both agents were to the serosal side. Initial $dI_{sc}/dt$ is indicated by straight lines.
cellular cAMP. If this were true, in vitro addition of cAMP might bypass normal calcium requirements. To further test whether intracellular Ca is involved in the hormone response, recta were placed in Ca-free saline containing 2.5 mM EGTA (ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetra-acetic acid; a Ca-chelating agent) and also containing the Ca ionophore A23187 (1 µg/ml). After 1 h exposure to both ionophore and EGTA, corpus cardiacum extract (1 gland pair/5 ml) was added to the bath. The ΔI_{sc} elicited by hormone extract under Ca-free conditions was identical to that in normal saline (compare Tables 7a and 7b; 1 gland pair/5 ml).

Finally, if cytoplasmic Ca activity is maintained at low levels (10^{-7} M) as in most cells, increasing membrane Ca permeability in the presence of normal external Ca levels (5 mM) should have the effect of increasing intracellular Ca levels, depending on the Ca buffering capacity of the cells. However, there was no change in I_{sc} when A23187 was added to both sides of locust recta (0.1, 1.0 and 5.0 µg/ml) bathed in normal saline (5 mM Ca).

These results provide no evidence that intracellular Ca is involved in regulating Cl transport across locust rectum although intracellular calcium measurements are needed to exclude this possibility definitively.

Discussion

Comparison with vertebrate systems

Results presented in this chapter indicate that chloride absorption by locust rectal epithelium is substantially different from mechanisms previously reported for several well-studied systems. Two general mechanisms of ionic coupling have been proposed for active Cl transport across these epithelia: one involves NaCl coentry into the cells in a manner analogous to Na-coupled amino acid and sugar transport (reviewed by Frizzell et al., 1979).
Sodium-coupled models:

The Na-coupled coentry model allows several testable predictions:

1) removal of external Na should inhibit Cl entry and transepithelial Cl transport and there should be a positive correlation between the amount of Na contamination and the rate of Cl transport; 2) alterations in the rate of Cl influx across the apical membrane should be paralleled by changes in the rate of Na influx; 3) serosal addition of ouabain should reduce Cl transport indirectly through elimination of the favourable Na electrochemical gradient across the apical membrane; 4) mucosal addition of furosemide should block coentry of Na and Cl as in other cells (see Frizzell et al., 1979).

The results in locust rectum are not consistent with any of these predictions. No significant inhibition of Cl-dependent $I_{sc}$ occurred during the first 75 min after replacing control saline (≈114 mM Na) with nominally Na-free saline. Also, large short-circuit currents and net Cl fluxes (60% of controls) were measured during prolonged exposure to nominally Na-free saline. Although incubation in Na-free saline for periods exceeding 6 hours depressed $J_{net}^{\text{Cl}}$ by approximately 40%, this effect was highly variable and hence was not statistically significant, even at the P < 0.1 level. As described in the results section, some reduction in transport is expected in Na-free saline since

1) Na-coupled amino acid absorption has been demonstrated in locust rectum (Balshin and Phillips, 1971), ii) amino acids are necessary to maintain active Cl transport at normal levels (Fig. 10), and iii) amino acids (particularly proline) are preferred metabolic substrates for supporting Cl transport (Chamberlin, 1981). Na removal could also have other side-effects: 1) Na/H exchange may be important in maintaining intracellular pH about one pH unit higher than expected for the passive distribution of protons across the cell membrane (reviewed by Roos and Boron, 1981). 2) There is evidence for Na/Ca...
exchange at the basal membrane of at least three epithelia (Grinstein and Erlij, 1978; Lee et al., 1980; Chase and Al-Awqati, 1981) and a rise in intracellular Ca activity might depress Cl transport after extended periods. In summary, the finding that $J_{\text{Cl}}^{\text{net}}$ was partially reduced by the rather drastic removal of Na for >6 h is not surprising. The more important observation is that a large net flux of Cl (6 μEq cm⁻² h⁻¹) persists under these conditions.

During tracer flux experiments, some sodium leached from the tissue, increasing the average Na contamination of the mucosal half chamber from 53.9 ± 12.2 μM at the start of $^{36}\text{Cl}$ flux measurements to 94.6 ± 47.3 μM after 3.5 h. At these levels, NaCl coentry is unlikely due to the "turnover" rate of Na that would be required, particularly in those preparations where Na contamination was not detected (<1 μM). For example, based on average Na contamination, all mucosal Na ions would need to recycle through the apical plasma membrane >200,000 times per second to sustain 1:1 NaCl coentry during some experiments. This frequency is higher than expected for carrier-mediated translocations (∼10⁴ ions sec⁻¹; Armstrong, 1975; Lindemann and Van Dreissche, 1977). Turnover numbers of anion carriers in erythrocyte membrane, for example, have been estimated at $2 \times 10^4$ ions sec⁻¹ (H. Passow, cited by Lindemann and Van Dreissche, 1977) similar to that of the mobile K⁺ "carrier" valinomycin in artificial systems (Läuger, 1972). Such carrier mechanisms would be too slow to permit Na recycling in the present system. Sodium/potassium ATPase is seen only on the basal membrane in dragonfly recta and there is considerable evidence for this location in locust rectum (Phillips, 1980, and chapter 4 of this thesis). To account for the equivalence of $I_{\text{sc}}$ and $J_{\text{Cl}}^{\text{net}}$ in the absence of an apical Na pumping from cell to mucosa, Na entering the cells with Cl would have to "recycle" back to the mucosal side by a mechanism analogous to that proposed by Field et al. (1978) for flounder intestine. In this model,
Na ions which enter the cells with Cl are pumped into the lateral intercellular spaces by a basolateral Na/K pump and then leak back to the lumen paracellularly through permselective junctions (see chapter 1). However, in locust rectum the back-diffusion of Na via paracellular shunts to the mucosal side would be rate-limiting during experiments in which tissues were exposed to nominally Na-free saline. From known dimensions of locust rectal cells (see chapters 1 and 5) the diffusion distance for Na ions would, on average, exceed 5 μm. Assuming the minimum recycling frequency of 1000 Hz for Na (based on an average $J_{\text{net}}^{\text{Cl}}$ of $2.89 \times 10^{20}$ Cl ions/0.196 cm sec) to be matched by a total of $2.85 \times 10^{17}$ Na ions in bulk solution, Na ions moving paracellularly would need a mean velocity of 0.5 cm sec$^{-1}$. Since electrical gradients of 1 volt cm$^{-1}$ result in mean velocities of only $10^{-3}$-$10^{-4}$ cm sec$^{-1}$ (Robinson and Stokes, 1959), an absurdly high driving force equivalent to 2.5-25 volts would be required between the lateral intercellular space and the mucosal saline for this mechanism to operate in locust rectum. Furthermore, at low levels of Na contamination, there was no correlation between trace amounts of Na and $I_{\text{sc}}$ (Fig. 9); short-circuit currents of $\approx 10 \ \mu\text{Eq cm}^{-2}\ \text{h}^{-1}$ were observed when no sodium was detected in the bath. Most contamination resulted from slow leakage of Na from tissue to saline after several hours of exposure under nominally Na-free conditions. This observation alone suggests that the net electrochemical gradient for sodium under these conditions must favour Na efflux from the cells rather than coentry with Cl. In chapter 4 we will see that when the Na electrochemical gradient across the apical membrane is reduced to near zero (as measured using Na-selective microelectrodes), net $^{36}$Cl flux and transmembrane Cl electrochemical gradients are not affected.

There is also no correlation between unidirectional influx of $^{22}$Na and $^{36}$Cl from the lumen into rectal tissue using an everted sac preparation.
(Fig. 12). Technically, the experiments were difficult because of small tissue size and the presence of a large unstirred compartment on the lumen side between the apical membrane and cuticle. However, since Cl transport was found to be quickly and reversibly blocked by lowering mucosal pH within the physiological range (Fig. 16), tissues were pre-incubated at low pH so as to allow $^{36}$Cl and $^{22}$Na to equilibrate in the subcuticular compartment prior to starting Cl transport by raising pH. Most of the fluid in this external compartment was quickly removed at the end of experiments. $^3$H-mannitol space was measured in order to correct for any extracellular $^{36}$Cl and $^{22}$Na, although this correction met only limited success. Nevertheless, the stimulatory effect of cAMP on $^{36}$Cl uptake by this preparation suggests that it is a reasonable estimate of unidirectional influx. Paracellular fluxes are probably low, due to high junctional resistance (see chapter 5). When 10 mM cAMP was injected into the sac, the influx of $^{36}$Cl increased significantly by 6-fold ($P < 0.01$) while that of $^{22}$Na did not change ($P > 0.2$; Fig. 12). The results are very different from those of similar experiments on mammalian intestine (Nellans et al., 1973), gallbladder (Frizzell et al., 1975; Cremaschi and Hénin, 1975) and fish intestine (Frizzell et al., 1979; Ramos and Ellory, 1980), where interdependent NaCl influxes have been observed. The lack of correlation between $^{22}$Na and $^{36}$Cl influxes suggests that their movements are neither chemically nor electrically coupled. In chapter 3 I show that K is normally the principal counter ion for active Cl transport across locust rectum.

Further evidence for Na-independence is provided by insensitivity of locust Cl transport to known inhibitors. Furosemide, which blocks Na-dependent Cl transport in other tissues (Candia, 1973; Humphreys, 1976; Degnan et al., 1977; Silva et al., 1977; Frizzell et al., 1979), has no effect on locust
rectal Cl transport. Since the rectal cuticle was cut in my experiments, there was no obvious barrier to furosemide diffusion to a postulated NaCl carrier on the 10 um-long folds in the apical membrane.

Locust rectum was also insensitive to ouabain, a known inhibitor of Na-coupled Cl transport in other tissues. Although ouabain sensitivity in intact insect tissues is controversial, Na-K ATPase isolated from locust rectum is sensitive to ouabain \((K_i = 10^{-6} \text{ M}; \text{ Peacock, 1981})\). In principle, the effects of inhibitors alone do not establish the presence or absence of a particular transport mechanism, since the same mechanism might vary in sensitivity between tissues. For example, it could be argued that an unusual furosemide-insensitive NaCl coentry process may exist in locust rectum. Nevertheless, when combined with tracer flux results (this chapter) and ion-sensitive microelectrode data (chapter 4), results of inhibitor studies do provide circumstantial evidence against known mechanisms.

Bicarbonate-coupled models:

A second type of active chloride transport, Cl/HCO_3 exchange, has been demonstrated or inferred in a variety of tissues (see chapter 1). In most of these cases there is some requirement for exogenous CO_2 and HCO_3. Presumably, intracellular HCO_3 production is inadequate to sustain the exchanger. In locust rectum, net Cl absorption has no requirement for exogenous CO_2 or HCO_3 (Fig. 8). Therefore, under CO_2,HCO_3-free conditions, intracellular HCO_3 for a possible Cl/HCO_3 exchange must necessarily originate from metabolic CO_2. However, it appears that aerobic metabolism by locust rectum is too low to produce sufficient CO_2. When recta are freely suspended in saline, it may be calculated that \(V_t\) must be less than 3 mV due to the small tissue dimensions and saline conductivity. Oxygen consumption by recta under these approximate short-circuit conditions has been measured by Chamberlin (1981) and was found
to be (2.8 μmoles/rectum/h). Assuming that the tissue has an RQ of 1 (maximum), that 100% of the metabolically produced CO₂ is converted to HCO₃⁻, then the maximum bicarbonate which could possibly be supplied for 1:1 exchange with Cl is 3.2 μEq cm⁻² h⁻¹. However, measured Cl transport is 3-4 times greater than this value under Iₛc conditions. This scenario requires perfectly efficient CO₂ hydration and direction of HCO₃⁻ to the apical side of the cell and would probably require the enzyme carbonic anhydrase. The presence of this enzyme has been shown in locust rectum (Hanrahan, unpubl. obs.), however acetazolamide had no effect on rectal Cl transport as indicated by Iₛc after cAMP stimulation (Fig. 14).

If mucosal Cl is exchanged for cytoplasmic bicarbonate, the lumen should become alkaline at a rate commensurate with Jₘₛ Cl. Some mucosal alkalinization was observed but the rate was only 39% of the unidirectional mucosa-to-serosa Cl flux measured under the same conditions (Fig. 14). In fact, there is no evidence that this alkalinization is due to HCO₃⁻ efflux. The anion exchange inhibitor SITS (1 mM) had no effect on Cl-dependent Iₛc or on the rate of base efflux (Fig. 14). Acetazolamide (1 mM) caused only small reductions in the rate of alkalinization (10-30%) and did not affect Iₛc. These results are not consistent with the usual one-for-one Cl/HCO₃⁻ exchange. A stoichiometry of 3Cl moving into the cell for each HCO₃⁻ moving out would not seem to be energetically feasible in normal saline given the normal range of intracellular HCO₃⁻ concentrations which have been measured or calculated in other cells in bicarbonate salines (7.6-20.2 mM; Khuri et al., 1974; Fujimoto et al., 1980; see Roos and Boron, 1981). Assuming a concentration gradient of 20 mM cell [HCO₃⁻] to 10 mM saline [HCO₃⁻] and membrane potential of -58 mV under Iₛc conditions (measured in chapter 4), HCO₃⁻ efflux would provide an electrochemical driving force of 76 mV as compared to a total of 138 mV opposing entry of
Moreover, hydration of $\text{CO}_2$ produces both $\text{HCO}_3^-$ and $\text{H}$. At steady-state, the flux of $\text{HCO}_3^-$ into the lumen of short-circuited recta would require a similar flux of protons to the hemolymph side. In reality, hemolymph side pH does not change measurably, in fact there is a slight alkalinization rather than acidification of the serosal side (Fig. 14).

Apical Na/H or K/H exchanges might also explain luminal alkalinization, however these mechanisms seem inappropriate since they should ultimately acidify the serosal side as well. Moreover, in preliminary experiments, alkalinization continued after removal of all K from the bathing saline, further suggesting that K/H exchange is not responsible for mucosal alkalinization.

Ammonia production by locust rectum cells is one plausible explanation for mucosal alkalinization (Fig. 14). $\text{NH}_3$ is excreted in the faeces of many insects including *Schistocerca* (reviewed by Bursell, 1967). Deamination of amino acids is the most likely source of rectal ammonia. Proline and glycine are actively accumulated from the mucosal side (Balshin, 1973) and both proline (Balshin, 1973) and glutamine (Chamberlin, 1981) are found at high concentrations in rectal tissue (65 and 45 mM, respectively). I found that amino acids are necessary to maintain $I_{sc}$ across locust rectum (Fig. 10), and oxidation of proline has been shown directly by Chamberlin (1981). Ammonia could be generated by deamination of glutamine as is the case in vertebrate kidney. Balshin (1973) measured a significant level of $\text{NH}_3$ in locust rectal tissue (10 mM). Proximity of mitochondria to the apical membrane would cause the $\text{NH}_3$ which is produced to diffuse preferentially to the lumen, raising the pH on the mucosal side, as shown in Figure 14. Further studies are required to support this hypothesis.
In summary, I tested the major predictions which have been used to substantiate NaCl cotransport and Cl/HCO$_3^-$ exchange systems in other epithelia and found that neither mechanism could adequately explain Cl transport by this insect epithelium. It should also be emphasized that both these entry mechanisms (by themselves) are electrically silent whereas there is strong evidence that Cl entry in locust rectum is a "rheogenic" process (i.e. it directly separates charge; see chapter 4).

**Potassium dependence of Cl transport**

Chloride absorption by locust rectum is strongly dependent on K. After prolonged exposure to K-free saline, 1 mM cAMP stimulated $J_{Cl}^{net}$ to only 1/3 of control rates. This "K-insensitive" component is not due to imperfect K selectivity (i.e. Na taking the place of K ions in K-free saline) because in control experiments, addition of cAMP stimulated $I_{sc}^K$ when both Na and K were substituted with N-methyl-D-glucamine, choline or tetramethyl ammonium ions. In other words, the K-independent component of $\Delta I_{sc}$ (and $J_{Cl}^{net}$) is completely independent of cations. This finding has been confirmed using ion-sensitive microelectrodes.

K-dependent Cl movements have been demonstrated in Ehrlich tumour cells (Geck et al., 1980), red blood cells (Kregenow and Caryk, 1979; Dunham et al., 1980), and are thought to function in cell volume regulation (reviewed by Kregenow, 1981). Also, an electroneutral KCl transport mechanism has been demonstrated in oxyntic cell apical membrane (Wolosin and Forte, 1981). Sodium-K-Cl coupling has been suggested at the basal membrane of cultured MDCK cell monolayers (Simmons, 1981). However the results presented in chapters 3 and 4 of this thesis will suggest yet another K-dependent Cl transport system is present in the locust rectum; potassium is proposed to have a direct, stimulatory effect on the Cl pump at the external surface of the apical membrane.
Exchange diffusion

A Cl/C1 exchange diffusion process which is similar to that found in red blood cell membranes (reviewed by Gunn, 1979) has been suggested in a variety of epithelia including gastric mucosa (Heinz and Durbin, 1958), frog skin (Bruus et al., 1976; Ques-von Petery et al., 1978), across the gills of crayfish (Shaw, 1960), and brine shrimp (Smith, 1969) and the anal papillae of mosquitoes (Stobbart, 1967). Cl flux via an exchange process should 1) require the presence of Cl on the opposite or "trans" side, and 2) reduce apparent Cl permeability at open-circuit because of its insensitivity to electrical gradients. Neither of these properties were exhibited by J in locust rectum. In fact, J increased slightly when mucosal Cl was replaced with methyl sulfate under I conditions, although this change was not statistically significant (P > 0.1). Also, the apparent transepithelial Cl permeability was enhanced rather than depressed in the presence of a spontaneous transepithelial potential (see p. 49). Finally, if Cl/Cl exchange is a carrier-mediated process, one might expect a saturation effect when chloride concentration is elevated on mucosal and serosal side. In chapter 3, it will be shown that J is linear over the range of 2-114 mM. In summary, exchange diffusion is not apparent in locust rectum, and J must occur via some other pathway.

Anion selectivity

Chloride is probably the only anion transported by the cAMP-stimulated mechanism in locust rectum. Of the 9 anions tested, only Br addition increased (I - J) by simultaneously increasing I and decreasing J , suggestive of competition with Cl (Fig. 6). This increase in (I - J) was ~50% of that obtained by adding the same amount of Cl and is consistent with active Br transport. This interpretation is supported by results of a second series of experiments in which the Cl in normal saline was replaced with various test
anions. In Br saline, the stimulation elicited by cAMP was 49% of that in normal Cl saline. Significant stimulations were not observed when other anions were used to replace Cl.

Neither of these experiments can distinguish between the selectivities of the binding and translocation steps. For example, although fluoride is not transported, F inhibition of $J_{\text{Cl}}^{\text{Cl}}$ in Figure 7 could result from competitive or noncompetitive interactions with the binding site or translocation mechanism, or from inhibition of Cl translocation indirectly via metabolic effects. Further investigations are required to distinguish between these possibilities.

Whether determined by Cl binding, or by some step in translocation, selectivity will ultimately depend on similar types of interatomic forces (Wright and Diamond, 1977). Some properties of the sites may be inferred from the anion "selectivity sequence". According to the theory of Eisenman (1961), the selectivity sequence results from the relative energies of hydration and of electrostatic interactions of the ion with selectivity sites. The anion sequence obtained for locust rectum suggests selectivity sites with high field strength. If F is omitted on the basis of its being a respiratory inhibitor, selectivity ratios may be fit quantitatively to the isotherms of Wright and Diamond to give sequence VI for a site having very high field strength. If F is not omitted, then the Cl > Br > I,F is "sequence IV", expected for a site of moderately high field strength. In the latter case, the relative anion selectivities are not in quantitative agreement with those calculated by Diamond and Wright (1969).

The selectivity sequence Cl > Br > I is not unusual for membrane Cl transfer in animals; some examples include frog skin (Kristensen, 1972; Kristensen and Larsen, 1978), stomach (Durbin, 1964), rabbit ileum
Frizzell et al., 1973), red blood cell anion exchange (Dalmark, 1973a,b) and cation+ Cl-cotransport (Kregenow and Caryk, 1979), in dog trachea (Widdicombe et al., 1979), frog cornea (Zadunaisky et al., 1971), elasmobranch electric organ (Miller and White, 1980).

Selectivity sites may be very different for active and passive anion transport in insect epithelia. Koch (1938) demonstrated that Cl uptake in vivo by anal papillae of mosquito larvae was 2.5 times faster than Br uptake, in satisfactory agreement with the present study which reports a Cl:Br selectivity of two. In contrast, Berridge (1969) found that anions passively support fluid secretion by blowfly Malpighian tubules according to sequence I of Wright and Diamond (I > Br > Cl > F), a finding which implies selectivity sites having a weak field strength.

Regulation of Cl transport

Results described in this chapter suggest that Cl transport by locust rectum is probably regulated at several levels. Overall control of rectal absorption is hormonal (Spring et al., 1978; Hanrahan, 1978; Spring and Phillips, 1980a-c; Phillips et al., 1980; and this chapter). At present there is no evidence to implicate Ca in this response although data are still very limited. When stimulated hormonally, Cl transport is also probably modified by local factors, particularly luminal K concentration (Fig. 15), pH (Fig. 16) and osmotic pressure (Fig. 17), which are known to vary over a wide range in vivo. The relationship between K and active Cl transport is examined in more detail in the following chapter.
CHAPTER 3: INTERRELATIONSHIP BETWEEN ACTIVE CHLORIDE TRANSPORT AND POTASSIUM

Summary

The relationship between transmural chloride and potassium transport has been examined in an insect epithelium. Unidirectional Cl flux from mucosa-to-serosa ($J^{\text{Cl}}_{\text{ms}}$) and short-circuit current ($I_{\text{sc}}$) both increase hyperbolically as chloride concentration is raised bilaterally from 0 to 114 mM. In contrast, the serosa-to-mucosa flux ($J^{\text{Cl}}_{\text{sm}}$) is a linear function of $[\text{Cl}]$. When $[\text{K}]$ is raised bilaterally from 0 to 100 mM, both the Cl concentration required for half maximal Cl absorption ($K_{\text{Cl}}$) and the maximum rate of Cl absorption ($J_{\text{Cl}}^{\text{max}}$) increase dramatically due to stimulation of $J^{\text{Cl}}_{\text{ms}}$. Hill plots of $J^{\text{Cl}}_{\text{net}}$ data are linear and have slopes near one, indicating non-cooperative Cl interactions. Increases in $J^{\text{Cl}}_{\text{net}}$ account for $\Delta I_{\text{sc}}$ at all K concentrations after cAMP stimulation. By analogy with enzyme-catalyzed reactions, the external potassium concentration required for half-maximal stimulation of $J^{\text{Cl}}_{\text{net}}$ is 5.3 mM. The selectivity sequence for cation activation of $J^{\text{Cl}}_{\text{net}}$ is $\text{K} > \text{Rb} > \text{Cs} > \text{Na}$, which is consistent with a site having moderately weak field strength. Addition of external Na has little effect on Cl-dependent $I_{\text{sc}}$. Low concentrations of K are stimulatory only when present on the mucosal side. In the absence of cAMP there is no $J^{\text{K}}_{\text{net}}$ under $I_{\text{sc}}$ conditions, suggesting that active K transport is negligible under control conditions. However, the ratio of $J^{\text{K}}_{\text{ms}}/J^{\text{K}}_{\text{sm}}$ was higher than that predicted from the Ussing flux ratio equation for passive, independent ion transport suggesting the presence of non-independent ion diffusion in the mucosal or serosal membrane. Transepithelial potassium permeability is reduced by high K levels in the saline and enhanced >4-fold.
during exposure to 1 mM cAMP under $I_{sc}$ conditions. $J_{net}^K$ equals $J_{net}^{Cl}$ during cAMP exposure under open-circuit conditions, however under $I_{sc}$ conditions $J_{net}^K$ is less than 10% of $J_{net}^{Cl}$. These observations indicate that transepithelial K absorption is electrically coupled to Cl transport. Cotransport of K and Cl by a common carrier is unlikely because transepithelial K fluxes are not affected by Cl removal under $I_{sc}$ conditions. The results are consistent with the stimulation of an electrogenic Cl pump by mucosal K ions.
Introduction

In the previous chapter, it was shown that Cl is actively transported across locust rectum by an unusual mechanism which apparently does not require sodium or bicarbonate and which is strongly stimulated by K. In this chapter, the relationship between Cl transport and K absorption is studied in more detail.

Interdependence between transepithelial Cl and K movements has been suggested in the rectal epithelium of the desert locust, *Schistocerca gregaria* (Phillips, 1964b) and the blowfly, *Sarcophaga bullata* (Prusch, 1976); however, no evidence for specific chemical or electrical interactions between these ion movements has been reported. In the Malpighian tubule of the blowfly, *Calliphora erythrocephala*, electrogenic K secretion is dependent on the presence of permeant anions bathing the basal membrane and varies inversely with the hydrated radius of the anion (Berridge, 1969). In Malpighian tubules, and salivary glands, an indirect dependent of fluid secretion (driven by active K secretion) on passive Cl movements has been demonstrated (Berridge, 1969, 1980).

In light of the K stimulation of active Cl transport in locust rectum, I undertook a study of K absorption and its relationship to Cl transport in order to better understand this interaction between K and Cl movements. Moreover, the rate of K absorption and the dependence of Cl transport on Cl and K concentrations are important physiologically since both [Cl] and [K] in rectal fluid decrease drastically during the reabsorptive cycle (Phillips, 1964b).

Previous studies suggested that K transport across this tissue might be active since luminal K reached a final concentration of only 0.2 mM (Phillips, 1964b). While approaching this final concentration, net K absorption would have to occur against a net electrochemical gradient of 72 ± 9.39 mV (X ± s.e., n = 5;
calculated from Phillips, 1964b). However, Williams et al. (1978) reported only low rates of K transport under control \( I_{sc} \) conditions. No evidence for cAMP stimulation of active K transport was obtained by Spring and Phillips (1980b) who found that \( \Delta I_{sc} \) could be accounted for by an increase in Cl absorption alone. Nevertheless, the effects of cAMP on K transport were not directly measured and a small electrogenic K flux or a large electroneutral absorption could be of great importance in understanding the mechanism of K-stimulated Cl transport.

In this chapter, I i) investigate the kinetics of transepithelial Cl absorption and determine whether K stimulates Cl absorption by altering the maximal rate of transport (\( J_{Cl}^{\text{max}} \)) or the apparent affinity of the mechanism for Cl (\( K_{L}^{Cl} \)), ii) quantify K requirements of Cl transport and determine the sidedness and K selectivity of the stimulation, and iii) test whether the active components of net Cl and K absorptions are interdependent by measuring the effects of cAMP and Cl omission on transepithelial \(^{42}\text{K} \) fluxes. These studies also provide the background information which is needed to interpret the electrochemical potential profiles and conductance data presented in the following two chapters.

**Materials and methods**

Experimental animals and methods were similar to those described in the preceding chapter and only additional experimental protocols used in this study are described below.

**Kinetics of transepithelial \(^{36}\text{Cl} \) fluxes**

Tissues were equilibrated in normal saline (Table 1 of chapter 1) under \( I_{sc} \) conditions. After 3 h, the external medium was replaced bilaterally with chloride-free saline which was prepared by replacing Cl with methylsulfate.
I\textsubscript{sc} was near zero under these conditions (Fig. 1). Approximately 0.5 h later, the tissue was rinsed three times with fresh Cl-free saline and cAMP was added to the serosal side at a final concentration of 1 mM. Small aliquots of 2 M NaCl were added to both sides, raising [Cl] stepwise from 0 to 2, 4, 10, 40 and 114 mM. \textsuperscript{36}Cl fluxes were measured during each step as described in chapter 1. As will be shown in the results section, a new steady-state flux from mucosa-to-serosa (J\textsubscript{ms}^\text{Cl}) is reached between 15 and 30 min after a change in [Cl], although tracer mixing results in lower fluxes during the first 15 min. Only data from the second flux period were used in calculations.

The dependence of J\textsubscript{ms}^\text{Cl} on Cl concentration was determined using salines containing 0, 10 and 100 mM potassium in separate experiments using different animals. [K] was adjusted with K-methylsulfate. Corrections were made in all experiments for the asymmetry between voltage-measuring electrodes and for series resistance of the saline, which changes significantly during ion additions.

**Measurement of potassium dependence of chloride transport**

The increase in I\textsubscript{sc} was used as a measure of \Delta I\textsubscript{net}^\text{Cl} while adding K ions bilaterally as methylsulfate salt. The equivalence of these two parameters in cAMP-stimulated tissues has been established for K concentrations between 0 and 140 mM (Fig. 15 of chapter 2; Fig. 7, this chapter). That is, elevating saline [K] did not result in electrogenic transport of ions other than Cl.

Tissues were equilibrated for 3-4 h in K-free saline (see methods in chapter 2) under I\textsubscript{sc} conditions, and rinsed periodically with fresh K-free saline. When tissues were judged to be in a steady-state condition, 1 mM cAMP was added to the serosal side. I\textsubscript{sc} increased to a new steady-state level within 1 h. Aliquots of K-methylsulfate (2 M) were then added to both sides.
Figure 3.1  Representative trace of $I_{sc}$ and transepithelial potential ($V_t$) during measurement of transepithelial $^{36}$Cl flux kinetics. After equilibrating recta in normal saline (114 mM Cl, 10 mM K) under $I_{sc}$ conditions for >3 h, the tissue was rinsed twice on both sides with Cl-free (methylsulfate) saline. After a third rinse, 1 mM cAMP was added to the serosal side. Chloride was added as NaCl stepwise every 30 min under $I_{sc}$ conditions. $V_t$ and $^{36}$Cl fluxes were measured at 15 min intervals as in Figure 2.1. $I_{sc}$ traces were integrated by planimetry over each 15 min interval for comparison with the corresponding tracer flux.
at 0.5 h intervals, to final K concentrations of 0, 2, 4, 10, 40, 100, 140 and 200 mM. An identical protocol was followed during control experiments in which Na-methylsulfate (2 M) was added. Transepithelial potential and resistance were determined at 15 min intervals as described previously in the methods section of chapter 2.

**Transepithelial $^{42}\text{K}$ fluxes**

$^{42}\text{K}$ (New England Nuclear Corp., Lachine, P.Q.; 0.13-0.15 Ci/g) was added as $^{42}\text{KCl}$ to normal saline or as $^{42}\text{KCO}_3$ to Cl-free saline. Samples were taken at intervals as previously described and counted using an automatic gamma counter (1085, Nuclear Chicago; see chapter 2, methods). Initial radioactivity of the labelled side served as a reference in order to correct for tracer decay during experiments (total sampling and counting time: 11 hours; $^{42}\text{K}$ half-life: 12.42 hours). Fluxes were calculated as described in the preceding chapter.

**Results**

1) **Kinetics of chloride absorption**

Transepithelial $^{36}\text{Cl}$ fluxes were measured under $I_{\text{sc}}$ conditions over the range of chloride concentrations which occur in vivo. Figure 1 is a representative trace of instantaneous $I_{\text{sc}}$ and $V_t$ obtained during these experiments with 10 mM K. $I_{\text{sc}}$ usually decayed over the first 2-4 h in normal saline, reaching a value which includes baseline Cl transport and any other electrogenic transport processes. $I_{\text{sc}}$ decays further during exposure to Cl-free saline (methylsulfate substitution). Addition of 1 mM cAMP under these conditions usually increased $I_{\text{sc}}$ very slightly ($\Delta I_{\text{sc}} = 0.15 \pm 0.02 \, \mu\text{Eqcm}^{-2}\cdot\text{h}^{-1}; \bar{x} \pm \text{s.e.}, n = 28; P << 0.01$).

It is not clear whether this stimulation results from trace amounts of Cl contamination or to some minor cAMP-sensitive transport system. These small
currents under Cl-free conditions were ignored since they averaged only 1.5% of the I_{sc} in normal (114 mM Cl) saline. Figure 2 shows that J_{Cl}^{\text{ms}} reached new steady-state levels within 30 min after increasing chloride concentration. Therefore only two 15 min flux periods were required at each Cl concentration to measure approximate steady-state flux rates and only the second of these was used in these calculations. Short-circuit current, J_{Cl}^{\text{ms}} and J_{Cl}^{\text{net}} increased hyperbolically as the Cl concentration was raised on both sides (Fig. 3). In contrast, J_{Cl}^{\text{sm}} increased linearly, and showed no evidence of saturation. Since J_{Cl}^{\text{ms}} and J_{Cl}^{\text{sm}} could not be measured on the same tissues, J_{Cl}^{\text{net}} was calculated by subtracting the mean J_{Cl}^{\text{sm}} (n = 6 animals) from J_{Cl}^{\text{ms}} for individual tissues. As expected, J_{Cl}^{\text{net}} and I_{sc} were nearly identical at all Cl concentrations (Fig. 3).

Forward fluxes of $^{36}$Cl were significantly lower in K-free salines (P << 0.01) and higher in 100 mM K saline (P << 0.01) when compared to values in normal saline (10 mM K). Figure 4 shows that J_{Cl}^{\text{ms}} approaches saturation at lower rates in K-free saline as compared to normal saline. I will show that J_{Cl}^{\text{sm}} is similar whether tissues are bathed in saline containing 10 or 140 mM K, justifying the use of J_{Cl}^{\text{sm}} obtained in normal saline to calculate J_{Cl}^{\text{net}} (i.e. J_{Cl}^{\text{net}} = \bar{X} J_{Cl}^{\text{sm}}) at other K levels. Data from each preparation, as exemplified by Figure 4 and corrected for J_{Cl}^{\text{sm}} to obtain J_{Cl}^{\text{net}}, were fitted to the Michaelis-Menten (MM) equation using standard linear regression.

The Woolf transformation (Haldane, 1957) was used:

$$\frac{[\text{Cl}]}{J_{\text{net}}} = \frac{K_t}{J_{\text{max}}} + \frac{1}{J_{\text{max}}} [\text{Cl}]$$

(1)
Figure 3.2  Histogram of $J_{ms}^{Cl}$ measured at 15 min intervals under $I_{sc}$ conditions. Chloride concentration of the saline was abruptly increased every 45 minutes to the values shown. Since $J_{ms}^{Cl}$ approached new steady-state levels after the first flux period, only 2 flux intervals were used at each [Cl] in subsequent experiments and the first of these was disregarded in calculations.
Figure 3.3 The dependence of Cl fluxes and $I_{sc}$ on Cl concentration.

- unidirectional Cl flux from mucosa to serosa ($J_{ms}^{Cl}$);
- unidirectional Cl flux from serosa to mucosa ($J_{sm}^{Cl}$);
- calculated net Cl flux ($J_{net}^{Cl}$);
- short-circuit current ($I_{sc}$). Experimental protocol as in Figure 3.1. Potassium concentration = 10 mM at all times. Means ± s.e.; n = 10.
Figure 3.4  The influence of external K concentration on the relationship between $J_{Cl}^{ms}$ and Cl concentration of the saline. Tissues were stimulated by 1 mM cAMP on the serosal side under $I_{sc}$ conditions. Potassium concentration was increased bilaterally from 0 mM K (□) to •10 mM K, or △100 mM K by adding K methylsulfate. Means ± s.e.; n = 6-10.
where,

\[ [Cl] = \text{chloride concentration} \]

\[ J_{\text{net}}^{Cl} = \text{net Cl flux} \]

\[ K_t = [Cl] \text{ at half maximal } J_{\text{net}}^{Cl} \]

\[ J_{\text{max}}^{Cl} = \text{maximum } J_{\text{net}}^{Cl} \]

This method is considered to be less sensitive to measurement errors than other methods (Blunck and Mommsen, 1978). Non-trivial weighting was not used since the type of error (absolute vs relative) was unknown. Woolf plots had linear relationships as indicated in Figure 5. For the ten preparations used to calculate \( K_t \) and \( J_{\text{max}}^{Cl} \) at 10 mM K, 98.2 ± 0.65% of the variation in \( J_{\text{net}}^{Cl} \) was attributable to the relationship with \([Cl]\). Table 1 summarizes the values of \( K_t \) and \( J_{\text{net}}^{Cl} \) at each concentration of K for 6-10 preparations. It is clear that both \( K_t \) and \( J_{\text{max}}^{Cl} \) increase significantly (P << 0.001) as the K concentration is raised from 0 to 100 mM. The effects of K addition are reminiscent of enzyme activation. In the following chapter, I report that K effects are probably due to direct stimulation of the Cl pump rather than to an indirect electrical effect on the potential opposing Cl transport.

Finally, cooperative interactions between Cl binding sites have previously been demonstrated for the transporting epithelium of prawn intestine (Ahearn, 1979) and the mosquito posterior rectum (Bradley and Phillips, 1977). In contrast, Cl absorption by locust rectum increases hyperbolically with increasing \([Cl]\) (Fig. 4) and is describable by Michaelis-Menten kinetics. Figure 6 shows that Hill plots of mean \( J_{\text{net}}^{Cl} \) had slopes near 1 at all concentrations of K (e.g. 1.09 for 0mM K; 0.91 for 10 mM K; and 0.99 for 100 mM K). The slope is a measure of the Hill constant or the number of interacting sites (see Segel, 1975). These results suggest that the rate-limiting
Table 3.1 Influence of external $K^+$ on kinetics of net $^{36}$Cl flux across cAMP-stimulated short-circuited recta $^a$

<table>
<thead>
<tr>
<th>K conc. (mM)</th>
<th>$K_t$ (mM Cl)</th>
<th>$J_{Cl}^{max}$ (n) (peq Cl cm$^{-2}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>22.7 ±4.0</td>
<td>3.5 ±0.7</td>
</tr>
<tr>
<td>10</td>
<td>60.2 ±8.7</td>
<td>14.9 ±1.9</td>
</tr>
<tr>
<td>100</td>
<td>99.6 ±13.4</td>
<td>23.1 ±5.3</td>
</tr>
</tbody>
</table>

$^a$Mean ± s.e.m.

Note: $K_t$ and $J_{Cl}^{max}$ were determined for individual recta using Woolf plots ([Cl$^-$]/$J_{Cl}^{net}$ vs. [Cl$^-$]). The mean $J_{Cl}^{sm}$ was subtracted from $J_{ms}^{Cl}$ at each chloride concentration to calculate $J_{net}^{Cl}$. 


Figure 3.5 Representative plot of data used in calculating kinetic constants of rectal Cl transport. Net $^{36}\text{Cl}$ fluxes (at 10 mM K) were calculated for each Cl concentration by subtracting mean $J_{\text{sm}}^{\text{Cl}}$ (n = 6 recta, see Fig. 3.3) from individually measured forward fluxes. Data were fitted by linear regression to the Michaelis-Menten equation according to the Woolf transformation.

$$r^2 = 0.9980$$

$$y = 0.0754x + 4.766$$

$J_{\text{max}}^{\text{Cl}} = 13.3 \mu\text{Eq. cm}^{-2}\text{ h}^{-1}$

$K_t = 63.2 \text{ mM}$
Figure 3.6  Hill plots of $J_{\text{Cl}}^{\text{ms}}$ at external K concentrations of 0, 10 and 100 mM. Net fluxes were calculated under $I_{\text{sc}}$ conditions in the presence of 1 mM cAMP on the serosal side, and plotted according to the Hill equation for homotropic cooperativity: $\log(J_{\text{max}}^{\text{Cl}} / J_{\text{net}}^{\text{Cl}} - J_{\text{Cl}}^{\text{Cl}}) = n_{\text{Cl}} \log [\text{Cl}] - \log K_{c}$. Symbols indicate mean values obtained from Figure 3.4 as described in the text.
step in transepithelial Cl absorption does not involve cooperative interaction between sites.

2) $I_{sc}$ and $^{36}\text{Cl}$ fluxes in "high K" saline

Normally in vivo, the rectum contains a K-rich fluid secreted by the Malpighian tubules (140 mM; Phillips, 1964b; see also Table 1 of chapter 2 of this thesis). Nevertheless, all previous studies of active transport across the tissue and other insect recta have employed high Na, low K salines.

Transepithelial $^{36}\text{Cl}$ fluxes were measured in saline containing 140 mM K a) to determine if $\Delta I_{sc}$ equals $\Delta J_{net}$, as it does in normal saline, b) to measure the possible effects of high [K] on $J_{sc}^{Cl}$, and c) to examine whether responsiveness to cAMP is altered by this physiological level of K.

Both $J_{ms}^{Cl}$ and $I_{sc}$ increased during exposure to 1 mM cAMP from 1.55 ± 0.2 to 10.28 ± 0.99 $\mu$Eqcm$^{-2}$h$^{-1}$ and from 2.59 ± 0.36 to 8.53 ± 1.07 $\mu$Eqcm$^{-2}$h$^{-1}$, respectively (Fig. 7). Mean transepithelial resistance was 40 $\Omega$cm$^2$ higher in high K saline (140 mM K) than in normal saline (10 mM K) during cAMP stimulation (P << 0.01). Results of subsequent tracer and microelectrode experiments (chapters 4 and 5) also suggest that K permeability of the epithelium varies inversely with saline [K].

The passive backflux of chloride in high K saline (140 mM K, 50 mM Cl) may be compared with $J_{sm}^{Cl}$ at normal K levels (10 mM K, 50 mM Cl). From Figure 3 it may be seen that at [Cl] = 50 mM, $J_{sm}^{Cl}$ was 1.3 $\mu$Eqcm$^{-2}$h$^{-1}$, in good agreement with values observed with 140 mM K and 50 mM Cl (0.9-1.3 $\mu$Eqcm$^{-2}$h$^{-1}$). Transepithelial Cl permeability (as indicated by $^{36}\text{Cl}$ backflux) is apparently not affected by external [K] over this range, since $J_{sm}^{Cl}$ was identical in 10 and 140 mM K saline. Therefore it was necessary to measure $J_{sm}^{Cl}$ at only one external concentration of K. In summary, $\Delta I_{sc}$ equals $\Delta J_{net}$ when external [K]
Figure 3.7  Effects of 1 mM cAMP on unidirectional Cl fluxes and electrical parameters in "high-K" saline. (a) $I_{sc}$; (b) transepithelial $^{36}$Cl fluxes from mucosa to serosa (m→s), and in the reverse direction (s→m); (c) transepithelial resistance $R_t$; and (d) transepithelial potential difference $V_t$. See Table 2.1 for composition of "high-K" saline. Means ± s.e.; $n = 12 (V_t, R_t), n = 6 (J_{Cl}^{ms}, J_{Cl}^{sm})$. 
is 140 mM (Fig. 7) or 0 or 10 mM (Fig. 15 of chapter 2) and active Cl transport is the major electrogenic process in locust rectum under all these conditions.

3) **Apparent potassium activation constant (Kₐ) of Cl transport**

Since ΔIₛₑₖ is a valid measure of active Cl transport regardless of the concentration of K in the saline, the apparent Kₐ of K stimulation can be estimated from the effects of K on ΔIₛₑₖ in the presence of cAMP, as described in the methods section. To ensure that the K-independent component of ΔIₛₑₖ (see Fig. 15 of chapter 2) was not partly the result of increases in osmotic pressure and ionic strength, Na methylsulfate was added in parallel experiments under identical conditions. Figure 8a shows that addition of cAMP caused Iₛₑₖ to increase from 0.86 ± 0.09 μEq cm⁻² h⁻¹ to 2.56 ± 0.38 μEq cm⁻² h⁻¹, in excellent agreement with previous results in K-free saline (Fig. 14 of chapter 2). As external [K] was then elevated by stepwise addition of KCH₃SO₄ to both sides, Iₛₑₖ increased similarly to a maximum of 12.68 ± 0.67 μEq cm⁻² h⁻¹ at 100 mM K. Above this level, Iₛₑₖ decreased reversibly (Fig. 8a). Vₑ increased from 7.2 ± 0.5 to 16.8 ± 1.8 mV when 1 mM cAMP was added to K-free saline in close agreement with values obtained during ³⁶Cl flux experiments (8.1 ± 2.0 to 16.8 ± 3.0 mV, Fig. 14 of chapter 2). Rₑ remained constant when [K] was raised from 10 to 200 mM (Fig. 8c), a surprising result considering the normally high K permeability of this epithelium at low external K levels. As argued in the preceding section, transepithelial K permeability must decrease at high K levels in order for electrical conductance to remain constant as external K concentration is raised.

Results obtained during NaCH₃SO₄ addition were very different (Fig. 9a-c). Na addition did not produce large step-like increases in Iₛₑₖ. Iₛₑₖ reached a maximum at ~5 μEq cm⁻² h⁻¹ as compared to 13 μEq cm⁻² h⁻¹ when K was added.
Figure 3.8  Effects of stepwise bilateral K additions on electrical parameters under $I_{sc}$ conditions. Recta were equilibrated in K-free saline for 4 hours. Cyclic-AMP (1 mM) was added to the serosal side at the arrows. After 1 h of cAMP exposure, K-methylsulfate was added to both sides to give the final concentrations shown. Means ± s.e.; n = 9-10.
Figure 3.9  Effects of stepwise bilateral Na additions on electrical parameters under Isc conditions. Experimental protocol was identical to that described in Figure 3.8 except that Na-methylsulfate was added to both sides instead of K-methylsulfate. Means ± s.e.; n = 6.
(P << 0.01). Also, high Na levels (>100 mM) did not inhibit $I_{sc}$, in marked contrast to the effects of elevated K levels. Transepithelial resistance declined in a predictable manner when saline [Na] was increased above 10 mM (Fig. 9c), in contrast to the relatively constant $R_t$ observed during K addition over the same range.

The difference between mean $I_{sc}$ obtained during K and Na additions was used as a measured of K-dependent Cl transport, since i) $\Delta I_{sc}$ equals $\Delta I_{net}$ at all K concentrations (previous section and chapter 2), and ii) $I_{sc}$ is not strongly Na-dependent (Fig. 8 of chapter 2).

Figure 10 shows a conventional Lineweaver-Burke plot of $I_{sc}$ stimulation which was used to determine the maximal K-dependent $I_{sc}$ (K-dependent Cl transport) and the K concentration giving half maximal stimulation ($K_a$).

Potassium activity of the salines was also measured using a liquid ion exchanger-type K-sensitive electrode (see chapter 4). A linear relationship was obtained between $[K^+]^{-1}$ and $(K-dependent-I_{sc})^{-1}$ when $[K^+]$ was greater than 2 mM ($r^2 = 0.9992$). $K_a$ was calculated to be 5.3 mM K concentration or 3.6 mM K activity. Maximum K-dependent $I_{sc}$ was 8.3 $\mu$Eqcm$^{-2}$h$^{-1}$.

These calculations rely on the assumption that Na does not stimulate Cl transport significantly since 1) Na (110 mM) was present when the effects of K addition were tested, and 2) NaCH$_3$SO$_4$ addition was used as a control for osmotic and ionic effects on the K-dependent component of $I_{sc}$. In the next section the selectivity of K activation is examined.

4) **Selectivity of K activation of Cl transport**

Malpighian tubule fluid entering the locust rectal lumen in vivo contains Na and probably NH$_4$ ions in addition to the much higher levels of K (Phillips, 1964b; Maddrell and Klunsuwan, 1973; see also Table 1 of chapter 2). The cation selectivity of K-stimulation of active Cl transport was determined in
Figure 3.10 Lineweaver-Burke plot of the relationship between external K concentration and Cl-dependent $I_{sc}$. Recta were exposed to 1 mM cAMP on the serosal side throughout this experiment. The difference between $I_{sc}$ in Figures 3.8 and 3.9 was used as a measure of K-stimulated Cl transport. The K concentration resulting in half-maximal stimulation of $I_{sc}$ is 5.3 mM. External [Cl] was constant at 114 mM.
order to find out whether other cations, particularly NH$_4^+$, might substitute for K in enhancing active Cl transport. It was also important to test whether the K-dependent component of $I_{sc}$ during cAMP stimulation results from the presence of Na (110 mM) in K-free saline. If this were so, lack of selectivity for K would result in a large error in my estimate of the $K_a$ for potassium "activation" of Cl transport.

Tissues were equilibrated for 3-4 h under $I_{sc}$ conditions in K-free saline and were then exposed to 1 mM cAMP. After 2-3 h exposure to cAMP, various test cations were added bilaterally so that the concentration of cation was raised by 40 mM (Fig. 1la-e). A selectivity sequence was determined by comparing $\Delta I_{sc}$ after 1 h of exposure to the test cation. Arranged in order to decreasing potency, the sequence was: 1.0 K > 0.58 Rb > 0.49 Cs > 0.08 NH$_4^+$, 0.03 Na. During addition of K, Rb, Cs and NH$_4^+$, the concentration of these cations increased from 0 to 40 mM whereas the Na level increased from 114 to 154 mM during the experiment. Therefore, results do not include the possibility that low levels of Na stimulate Cl transport during exposure to cAMP, i.e. the possible stimulating effect of Na ions might be maximal at <110 mM and might not be enhanced further by addition of 40 mM Na. To test this hypothesis, the effect of 1 mM cAMP on $I_{sc}$ was measured in two tissues when choline was the only monovalent cation present for 3 h. The stimulations obtained in the absence of both Na and K were 4.0 and 4.5 µEq cm$^{-2}$ h$^{-1}$, identical to those observed with ~200 mM Na present (Fig. 9). Moreover, cAMP stimulations of this magnitude were also observed when Na and K were replaced with tetramethyl ammonium. Even when Na, K, Ca and Mg in normal saline (Table 1 of chapter 2) were completely replaced by choline for 3 h so that choline was the only cation present above 1 mM, cAMP stimulated $I_{sc}$ from 1.33 to 2.62 and 0.91 to 2.66 µEq cm$^{-2}$ h$^{-1}$ in two recta. These results suggest that the
Specificity of cation stimulation of Cl transport. Cyclic-AMP-stimulated recta were equilibrated under \( I_{sc} \) conditions for 2-4 h in K-free saline (Table 2.1). When \( I_{sc} \) reached an approximate steady-state value, concentrated solutions of various cations were added bilaterally as sulfate salts in order to achieve a final test cation concentration of 40 mM (K, Rb, Cs, NH\(_4\)) or 150 mM (Na). Means ± s.e.; \( n = 5-6 \).
K-independent component of cAMP-stimulated $I_{sc}$ is independent of cations in the external saline.

Sequence I (K > Rb > Cs > Na) observed for cation stimulation of locust Cl transport is the series expected for selectivity sites having moderately weak field strength (Eisenman, 1961). A similar sequence is observed in the serosal membrane of frog skin (Lindley and Hoshiko, 1964) and Malpighian tubules of the blowfly (Berridge, 1969; for reviews see Diamond and Wright, 1969).

5) **Sidedness of K activation**

To determine whether K "activation" of Cl transport occurred specifically at one side of the tissue, $I_{sc}$ was measured during stepwise addition of K methylsulfate to either mucosal or serosal side. Recta were first equilibrated under $I_{sc}$ conditions in K-free saline for 3-4 h and then exposed to 1 mM cAMP. After $I_{sc}$ reached a new steady-state, low concentrations (2-10 mM) of K methylsulfate were added to the mucosal or serosal side. Only low concentrations of K were used in order to minimize the K diffusion current caused by a transepithelial K gradient and also the rate of contamination of the K-free side. To measure passive K currents, $I_{sc}$ was recorded during asymmetrical K additions using recta poisoned with 1 mM azide. Currents produced by K gradients in Cl-free saline were similar to those observed in azide-poisoned tissues. Correction of $I_{sc}$ for K diffusion ranged from 0% to 21% in the presence of a 10 mM → 0 mM (mucosa-to-serosa) K gradient. The mean K diffusion currents measured in this way were subtracted from the $I_{sc}$ measured in unpoisoned tissues in order to calculate true Cl-dependent $I_{sc}$ in the presence of a K gradient.

Figure 12 shows the effects of adding K methylsulfate to either side of the epithelium. After corrections, $I_{sc}$ attributable to active Cl transport
Figure 3.12  Effect of adding K-methylsulfate stepwise to one side only on Cl-dependent $I_{sc}$. After equilibrating recta 3 h under $I_{sc}$ conditions in K-free saline, 1 mM cAMP was added to the serosal side and $I_{sc}$ was monitored for 1 hour. Aliquots of K-methylsulfate were then added to the mucosal or serosal chambers to give the concentrations indicated. The apparent $\Delta I_{sc}$ produced by asymmetrical K addition in azide/H$_2$ saline was subtracted to correct for K diffusional current as described in the text. Means ± s.e.; n = 7.
increased from $1.55 \mu \text{Eq cm}^{-2} \text{h}^{-1}$ to $6.85 \mu \text{Eq cm}^{-2} \text{h}^{-1}$ when 10 mM K was added to the mucosa. In contrast, $I_{sc}$ was not changed significantly by serosal addition of K ($P > 0.2$). In chapter 4, I will show that K enters K-depleted rectal cells much more effectively from the serosal side than from the lumen, presumably because of a Na/K exchange pump at the basal cell border. Potassium must act at the external surface of the mucosal membrane rather than at some intracellular site, because serosal K addition fails to stimulate Cl transport in spite of the fact that intracellular K concentration is maintained at high levels (~60 mM) when mucosal [K] is 0 mM and serosal [K] is 10 mM (see Fig. 6 of chapter 4).

6) **Transepithelial $^{42}$K fluxes (short-circuit conditions)**

i) **Normal saline**

Active K absorption has been reported across locust rectum *in vivo* (Phillips, 1964b) and *in vitro* (Williams et al., 1978), however little is known regarding its ionic requirements or hormonal regulation or the relative size of active and passive components of K absorption. Considering the dependence of active Cl transport on external K, it was important to study the properties of active K absorption, particularly the effects of cAMP and possible Cl dependence. For example, does KCl cotransport occur in this epithelium as in red blood cells (Kregenow and Caryk, 1979) and Ehrlich tumour cells (Geck et al., 1980)?

Figure 13 shows the effects of 1 mM cAMP on a) transepithelial fluxes of $^{42}$K and b) $J^K_{net}$ and across recta bathed in normal saline (114 mM Cl, 10 mM K). Values of $R_t$ and $V_t$ were identical to those shown in Figure 2 of chapter 2 and therefore are not shown. Upon adding 1 mM cAMP, both $J^K_{ms}$ and $J^{K}_{sm}$ increased from about 0.35 to $2.08 \pm 0.22$ and $1.56 \pm 0.18 \mu \text{Eq cm}^{-2} \text{h}^{-1}$, respectively. Under steady-state, cAMP-stimulated conditions, $J^K_{net}$ ($0.632 \pm 0.26$) was
Figure 3.13  Effects of cAMP on $I_{sc}$ and $K$ fluxes measured under $I_{sc}$ conditions in normal saline. (a) Unidirectional $^{42}$K flux from mucosal-to-serosal side (■, m→s) and in the reverse direction (□, s→m), (b) $J^{K}_{net}$ (○) and $I_{sc}$ (▲) across short-circuited recta bathed in normal saline (114 mM Cl, 10 mM K). Tissues were pre-equilibrated 4 h under $I_{sc}$ conditions. Means ± s.e.; n = 6 ($J^{K}_{ms}$, $J^{K}_{sm}$), n = 12 ($I_{sc}$).
Figure 3.14  Effects of 1 mM cAMP on K fluxes and $I_{sc}$ in Cl-free saline. Cl was replaced with methylsulfate; basic saline as described in Table 2.1 (10 mM K, 110 mM Na). NaCl was added to both sides in order to restore normal (114 mM) Cl concentration. Means ± s.e.; $n = 6 (J_{\text{ms}}^K, J_{\text{sm}}^K), n = 12 (I_{sc})$. 
significantly different from zero ($P << 0.01$) but was less than 7% of $J_{\text{net}}^{\text{Cl}}$ measured under these conditions.

The large (400%) stimulation of both $J_{\text{ms}}^K$ and $J_{\text{sm}}^K$ suggests that cAMP increases transepithelial permeability to potassium ($P_K$). Using the approximation, $P_K = J_{\text{sm}}^K / [K]$, where $[K]$ is 10 mM and $J_{\text{sm}}^K$ is assumed to be passive, $P_K$ increases from $0.98 \pm 0.14$ to $4.0 \pm 0.62 \times 10^{-5}$ cm sec$^{-1}$ ($\bar{x} \pm$ s.e., $n = 6$) after adding cAMP. Quantitatively, this method of calculating transepithelial $P_K$ may lead to some error because $^{42}K$ must penetrate two membranes in series whereas this approach assumes the tissue is a single barrier (see chapter 5). Nevertheless, these results clearly indicate a large increase in $P_K$ during cAMP stimulation and this interpretation is supported by electrophysiological results described in chapter 5.

In summary, cAMP has two major effects on transepithelial K movements under $I_{\text{sc}}$ conditions: It causes i) a very large ($\sim$4-fold) increase in transepithelial permeability to $^{42}K$, and ii) the appearance of a small net K absorption of approximately 0.8 µEq cm$^{-2}$ h$^{-1}$ (i.e. a small active K transport component).

ii) Cl-free saline

Figure 14a shows the effects of 1 mM cAMP on transepithelial $^{42}K$ fluxes before and after restoring normal chloride levels to Cl-free saline. After 1 h of exposure to cAMP both unidirectional fluxes increased by about 4-fold, $J_{\text{ms}}^K$ from $0.45 \pm 0.13$ to $1.91 \pm 0.2$ µEq cm$^{-2}$ h$^{-1}$ and $J_{\text{sm}}^K$ from $0.31 \pm 0.06$ to $1.25 \pm 0.19$ µEq cm$^{-2}$ h$^{-1}$. $J_{\text{net}}^K$ (0.66 \pm 0.39 µEq cm$^{-2}$ h$^{-1}$) was significantly greater than zero ($P < 0.01$). When Cl was restored to normal levels on both sides (114 mM), there was no change in $J_{\text{sm}}^K$ or $J_{\text{ms}}^K$ ($P > 0.2$) although $I_{\text{sc}}$ increased 6-fold following Cl addition to values not different from those obtained in experiments with normal saline (compare Figs. 14 and 13).
The present results show that neither the K permeability increase caused by cAMP nor the small net K transport are affected by Cl removal. In view of this independence, it seems unlikely that there is strict chemical coupling between Cl and K movements at the apical or basal membranes. This conclusion is supported by the previous observation that $J_{\text{net}}^K$ is less than 8% of $J_{\text{net}}^{\text{Cl}}$ under $I_{sc}$ conditions (Fig. 13) and approximately 35% of $J_{\text{net}}^{\text{Cl}}$ is cation-independent.

7) Transepithelial $^{42}\text{K}$ fluxes (open-circuit conditions)

Chloride absorption across locust rectum is electrogenic and must, under normal open-circuit conditions, be matched by a similar flow of cations from mucosa-to-serosa or alternatively, a flow of anions in the reverse direction. To determine whether K moves to maintain electroneutrality during Cl transport, open-circuit transepithelial $^{42}\text{K}$ fluxes were measured sequentially; first in normal saline (i.e. under control conditions) and then during cAMP-stimulation (10 mM K bilaterally). Finally, mucosal [K] was raised to 100 mM in the presence of 1 mM cAMP to mimic normal in vivo K gradients (10:1) across the locust rectum (Fig. 15).

In control (10 mM K) saline before stimulation, $V_t$ ranged between 8-10 mV, in close agreement with previous results (Fig. 5, chapter 2). Unidirectional $^{42}\text{K}$ fluxes were less than 1 $\mu$Eq cm$^{-2}$ h$^{-1}$ (Fig. 15a,b). Serosal addition of cAMP (1 mM) increased $V_t$ from 8 to 28 mV (see also Fig. 5, chapter 2), enhanced $J_{\text{ms}}^K$ 5-fold, and produced a small but significant increase in $J_{\text{sm}}^K$ (significant $P < 0.01$). Net K flux ranged from 4.5 to 5.0 $\mu$Eq cm$^{-2}$ h$^{-1}$. It is noteworthy that $J_{\text{net}}^{\text{Cl}}$ equals $J_{\text{net}}^K$ at open-circuit before and during cAMP exposure (compare Fig. 15b of this chapter with Fig. 5 of chapter 2). This result indicates that K is the main counter ion during active Cl transport even when the ratio of [Na]/[K] is 11/1 in the external saline.
Figure 3.15  Effects of sequential addition of 1 mM cAMP to the serosal side and mucosal addition of potassium on K fluxes under open-circuit conditions. Recta were initially bathed in normal saline (10 mM K, 110 mM Na, 110 mM Cl). Time "0" was preceded by 4 h equilibration under open-circuit conditions. After 75 min exposure to cAMP, mucosal [K] was increased from 10 to 100 mM by adding concentrated K-methylsulfate solution. Means ± s.e.; n = 16 (Vt), n = 8 (Jms, sm).
\[ V_t \text{ declined from 28 mV to about 5 mV when mucosal } [K] \text{ was raised from 10 to 100 mM (Fig. 15a,b). } J_{\text{net}}^K \text{ increased concurrently by more than 3-fold to about 16 } \mu\text{Eqcm}^{-2}\text{h}^{-1}. \text{ This transepithelial flux rate is twice that of } J_{\text{net}}^{\text{Cl}} \text{ observed under these conditions (8 } \mu\text{Eqcm}^{-2}\text{h}^{-1}; \text{ Fig. 5, chapter 2). It is not yet known which ion(s) maintain charge balance when mucosal } [K] \text{ is elevated. Preliminary experiments suggest that } K/H \text{ exchange is not involved:}

Mucosal pH was monitored using phenol red as mucosal } [K] \text{ was raised from 10 to 100 mM in } HCO_3^-/CO_2^-\text{-free saline. From titration curves it was calculated that mucosal pH would decrease 0.3-0.4 pH units within 1 h if protons were exchanged 1:1 with K across the mucosal membrane during cAMP stimulation under } I_{sc} \text{ conditions. Contrary to this prediction, mucosal pH increased during exposure to high mucosal } [K]. \text{ Other fluxes which might balance } J_{\text{ms}}^K \text{ under these conditions of very high mucosal } [K] \text{ include serosa-to-mucosa fluxes of some other cation such as Ca, Mg or perhaps NH}_4^. \text{ These possibilities were not tested.}

According to the flux ratio equation (Ussing, 1949) for passive, independent movements of an ion such as K,

\[ \frac{J_{\text{ms}}^K}{J_{\text{sm}}^K} = \frac{(K)_m}{(K)_s} \exp \left( \frac{V_t}{F/RT} \right) \]  

(2)

where \( J_{\text{ms}}^K \) and \( J_{\text{sm}}^K \) are the forward and back fluxes measured with tracers, \( (K)_m \) and \( (K)_s \) are mucosal and serosal K activities, \( V_t \) is the transepithelial potential, and F, R and T have their usual meanings.

In normal saline, ratios of unidirectional \( ^{42}K \) fluxes at open-circuit are much higher than those predicted from the flux ratio equation (Fig. 16). Under control conditions (normal saline, no cAMP) the \( ^{42}K \) flux ratios were between 4 and 6, whereas the predicted ratio was only 1.4. It is unlikely that this discrepancy could arise from active transport since no net flux of
Figure 3.16  Comparison of K flux ratios $\frac{J^K_{ms}}{J^K_{sm}}$ under open-circuit conditions with those predicted from the Ussing flux-ratio equation. See Figure 3.15 for original flux data and experimental protocol. Note log scale. Also shown are values of the transepithelial flux ratio exponent ($n'$) calculated as

$$n' = \frac{RT}{F} \ln \left( \frac{(K)^m \cdot J^K_{ms}}{(K)^s \cdot J^K_{sm}} \right)$$

where $(K)^m$, $(K)^s$ are K activities on the mucosal and serosal sides, respectively; $R$, $T$, $F$, $V_t$, $J^K_{sm}$ and $J^K_{ms}$ have their usual meanings.
potassium was observed when tissues were short-circuited in the absence of cAMP. Rather, the discrepancy is suggestive evidence for non-independence of K movements across the tissue as discussed in a later section.

As expected, flux ratios increased further after addition of cAMP, a manoeuvre which resulted in a small active flux under $I_{sc}$ conditions. However, a step increase in mucosal [K] from 10 to 100 mM increased the steady-state flux ratio to >100:1, much larger than the predicted value of 10.4. This larger discrepancy is not due to enhanced active transport because it was later found that $J_{net}^K$ under $I_{sc}$ conditions is similar whether saline contains 10 mM or 140 mM K. These results are again consistent with non-independence between transmural $^{42}\text{K}$ fluxes.

A measure of non-independence may be obtained by raising the right-hand side of equation 2 to some power $n'$, the flux ratio exponent. According to the "single-file theory" $n'$ is related to the number of sites within a channel (Hodgkin and Keynes, 1955). Values of $n'$ for transepithelial fluxes ($n'^t$) were calculated after correcting the flux ratio for the small $J_{net}^K$ measured under $I_{sc}$ conditions as,

$$ n'^t = \frac{RT}{V_T} F \ln \left( \frac{(K)_m \cdot J_{sm}^K}{(K)_s \cdot J_{ms}^K} \right) $$

Values of $n'$ are shown in Figure 16. Under control conditions, $n'^t$ ranged between 4.0 and 5.10 and decreased to 1.97-2.30 during exposure to 1 nM cAMP. When [K] was elevated on the mucosal side, $n'^t$ increased to 10.9-13.2. Although high values of $n'^t$ do indicate non-independence in transepithelial K fluxes, interpretation of $n'^t$ is complicated in epithelia by the presence of two barriers in series since the cellular compartment contains tracer at some unknown activity. This will be discussed in a later section.
8) **Transepithelial $^{42}\text{K}$ fluxes in "high-K" saline**

Figure 17 shows the effects of 1 mM cAMP on unidirectional $^{42}\text{K}$ fluxes under I$_{sc}$ conditions when recta were bathed bilaterally in "high-K" saline (140 mM K, 50 mM Cl; see Table 1 of chapter 2). Unidirectional $^{42}\text{K}$ fluxes (1.0-1.7 uEq cm$^{-2}$ h$^{-1}$) and net $^{42}\text{K}$ fluxes (-0.1 to +0.5 uEq cm$^{-2}$ h$^{-1}$) were surprisingly similar to those observed in normal saline in which [K] was 14-fold lower. As before, $J_{ms}^K$ increased significantly during cAMP exposure (P < 0.05). Although $J_{sm}^K$ increased steadily over the course of the experiment, addition of 1 mM cAMP did not increase $J_{sm}^K$ (P >> 0.2), in marked contrast to the effects observed in normal (10 mM K) saline.

Several conclusions regarding active K transport may be drawn from these data. First, active K transport saturates at low concentrations of K ($K_t < 10$ mM) since $J_{net}^K$ was 0.8-1.0 uEq cm$^{-2}$ h$^{-1}$ under I$_{sc}$ conditions whether [K] of the saline was 10 mM (normal saline) or 140 mM (high-K saline).

Second, when transepithelial K permeability ($*P_K$) is calculated from $J_{sm}^K$ (see p.295), $*P_K$ must decrease several-fold at high [K]: $J_{sm}^K$ does not increase proportionately at higher K levels, as shown by comparing $J_{sm}^K$ in Figure 13a and Figure 16a. Rather, $*P_K$ is apparently 3- to 4-fold higher when tissues are bathed in normal saline (10 mM K, 114 mM Cl) than in "high-K" saline (140 mM K, 50 mM Cl).

Chloride concentration was lower in "high-K" saline than in normal saline (50 mM and 110 mM, respectively); however, this could not account for differences in potassium permeability because $*P_K$ in Cl-free saline (10 mM K) is also 4-fold higher than in "high-K" saline; i.e. $J_{sm}^K$ is not proportionally higher in Figure 17 (140 mM K, 50 mM Cl) as compared to $J_{sm}^K$ in Figure 14 (10 mM K, 0 mM Cl). Moreover, addition of 1 mM cAMP does not increase $*P_K$ 4-fold as it does
Figure 3.17  Effects of cAMP on K fluxes and \( I_{sc} \) across recta bathed in "high-K" saline on both sides. High-K saline contained 140 mM and 50 mM Cl (see Table 2.1 for details). Tissues were equilibrated for 3-4 h under \( I_{sc} \) conditions prior to adding 1 mM cAMP to the serosal side. Means ± s.e.; \( n = 12 \) (\( I_{sc} \)), \( n = 6 \) (\( J^K_{ms} \), \( J^K_{sm} \)).
in normal or Cl-free salines containing 10 mM K. This is consistent with the earlier unexpected observation that transepithelial electrical resistance was higher in "high-K" saline than in normal saline (compare present Fig. 8c and Fig. 2d of Chapter 2) and also with the finding that $R_e$ did not decrease as [K] was increased stepwise from 10 mM to 200 mM (Fig. 9c). The simplest explanation for all these results is that high external [K] lowers transepithelial K permeability. Electrophysiological results to be described in Chapter 5 support this conclusion.

**Discussion**

The data in this paper provide evidence for an unusual interrelationship between K and Cl during KCl reabsorption in locust rectum. Although the largest component of active Cl absorption requires potassium in the mucosa, K does not act simply as a permeant counterion because most active Cl transport (70%) is stimulated by external K even when recta are short-circuited by externally applied current. On the other hand, transepithelial K absorption under open-circuit conditions is mostly passive (>80%), and electrically coupled to active Cl absorption. The much smaller active flux of K to the serosal side during cAMP stimulation is not Cl-dependent.

**Active Cl transport: the effects of K**

The effects of K on transepithelial Cl fluxes were examined over the normal physiological range of Cl concentrations. The relationship between steady-state $J_{net}^{Cl}$ and external [Cl] is well described by the Michaelis-Menten relationship for enzyme-catalysed reactions, although other hyperbolic functions could also fit the data. Potassium addition had the effect of increasing both the Cl concentration causing half maximal $J_{net}^{Cl} (K_e)$ and also the maximal rate of active Cl transport ($J_{max}^{Cl}$). These parameters could depend
on the properties of the transfer process for Cl across the basal membrane (localized using ion-sensitive microelectrodes, in the following chapter), but they are more likely to reflect the properties of the Cl pump in the apical membrane. There is some indirect evidence that $K_t$ and $J_{\text{max}}^{Cl}$ are properties of the apical entry step: The net electrochemical gradient opposing Cl entry across the mucosal membrane varies with transport rate while the gradient favouring Cl exit across the serosal membrane remains constant. This finding suggests that the pump at the apical membrane is rate-limiting for Cl transport; however, more direct evidence for this interpretation is required. If the apical Cl pump is an ATPase, one convincing approach would be to compare the properties of isolated enzyme ($K_m$ and potassium $K_a$) with the $K_t$ and $K_a$ for K-stimulation of transepithelial Cl transport. Mucosal K might depolarize the mucosal membrane, thereby reducing the local electrochemical gradient against which the apical Cl pump must work. This type of electrical coupling would not be obvious from transepithelial flux measurements which treat the epithelium as a black box. Nevertheless, in order to explain the 10-fold difference between $J_{\text{net}}^{Cl}$ and $J_{\text{net}}^{K}$ under $I_{sc}$ conditions, electrical coupling across the apical membrane between K and active Cl transport would require that K is actively pumped from the cell to mucosal side (i.e. recycled). No evidence was found for K secretion or recycling at the mucosal cell border by using tracer fluxes (this chapter) or by ion-sensitive microelectrode techniques (chapter 4). A similar argument may be made against carrier-mediated coentry of K and Cl under $I_{sc}$ conditions. This will be discussed further in chapter 4.

By what mechanism does K stimulate net Cl transport under $I_{sc}$ conditions? In the absence of KCl coentry, I propose that mucosal K at the external surface of the apical membrane enhances Cl transport by stimulating the Cl
pump without actually being transported, analogous to an enzyme-activator which accelerates a reaction but is not altered (or transported) by it. To explain the K-insensitive component of cAMP-stimulated $J_{\text{Cl}}^{\text{net}}$, the apical membrane may contain a single population of Cl pump sites having a graded response to mucosal K or alternatively two populations of Cl pumps may exist; one which operates without K and the other which is stimulated as [K] is increased. Further studies are required to distinguish between these alternative hypotheses.

**Passive K transport**

Most transrectal K flux is passive under open-circuit conditions. Net K flux ($J_{\text{K}}^{\text{net}}$) is only 8% of $J_{\text{Cl}}^{\text{net}}$ when locust rectum is short-circuited, however under open-circuit conditions $J_{\text{K}}^{\text{net}}$ equals $J_{\text{Cl}}^{\text{net}}$. K acts as the major counter ion for electrogenic Cl transport even when much higher concentrations of Na are present in the experimental saline bathing the mucosal side (114 mM Na, 10 mM K). Moreover, the predominance of K as a counter ion is ensured in vivo for two reasons: i) normal K levels (140 mM) are much higher than Na (20-40 mM) in the rectal lumen; ii) cAMP increases apparent transepithelial K permeability by more than 4-fold.

When high concentrations of K are added to the mucosal side under open-circuit conditions to simulate natural in vivo K gradients, the mucosal side should become electrically negative with respect to the serosal side if tissue potassium conductance is high. However, no reversal of $V_t$ was observed. Stimulation of electrogenic Cl absorption by K and cAMP, as described previously, tends to balance the reduction in $V_t$ by K diffusion from mucosal-to-serosal side. It will be shown in appendix 3 that $V_t$ reverses (i.e. lumen becomes negative with respect to hemolymph) in the presence of a normal mucosa-to-serosa K gradient under Cl-free conditions both in situ and in vitro.
Several types of experiments reported in this chapter suggest that trans-
epithelial $P_K$ is lowered by high levels of $K$. First, when Na methylsulfate
was added stepwise to both sides of the epithelium, $R_t$ decreased in a
predictable manner as salt concentration was increased to 200 mM. In
contrast, $R_t$ remained constant when $K$ methylsulfate concentration was raised
under identical conditions. Second, the observation that $K$ is the counter ion
for electrogenic Cl transport indicates that $K$ conductance of the rectum is
high relative to other ions. If $P_K$ remained constant in the presence of high
$[K]$, increasing $K$ methylsulfate concentration from 10 to 200 mM should have
reduced tissue resistance ($R_t$) drastically. Since $R_t$ did not decrease when
$[K]$ was elevated, this result indicates that $P_K$ declines as $[K]$ increases.
Concentration-dependent permeability is to be expected whether ion permeation
occurs via carriers or channels (reviewed by Heckmann, 1973; Hille, 1979;
Lauger, 1980) and an inverse relationship between mucosal $[Na]$ and the rate of
Na entry at the apical membrane has been reported in frog skin (Biber and
Curran, 1970; Rotunno et al., 1970; Erlij and Smith, 1971; Moreno et al., 1973;
Rick et al., 1975; Lindemann, 1977; Mandel, 1978) and in proximal tubule
(Spring and Giebisch, 1977).

Alternatively, elevated intracellular $K$ activity during exposure to high
external $[K]$ may exert a negative feedback effect on apical $P_K$. There is
evidence for such a feedback system regulating Na entry in frog skin
(MacRobbie and Ussing, 1961; Biber, 1971; Erlij and Smith, 1973; Rick et al.,
1975), toad bladder (Finn, 1975), rabbit bladder (Lewis et al., 1976) and
rabbit colon (Turnheim et al., 1978; Frizzell, 1979).

Inhibition of $P_K$ by external $[K]$ seems more likely than negative feedback
exerted by intracellular $K$, since raising external $[K]$ bilaterally from 10 to
40 mM under $I_{sc}$ conditions produces only a small increase in intracellular $K$
activity (≈7 mM; Fig. 6 of chapter 4), whereas $^\star P_K$ declines more than 70% under these conditions.

Calculating $^\star P_K$ from $J_{sm}^K$ under $I_{sc}$ conditions should result in an overestimate of transepithelial potassium permeability (see equation 14 of Schultz and Frizzell, 1976). This prediction is confirmed in chapter 5; however, it will also be shown in chapter 5 that $^\star P_K$ is a reasonable indicator of changes in transepithelial $P_K$ during cAMP stimulation. Although $^\star P_K$ is dependent on membrane potentials, the $\Delta^\star P_K$ observed at high [K] can not be accounted for by membrane depolarization. Intracellular potential has been measured as a function of external [K] under $I_{sc}$ conditions (chapter 4). Using apical and basal membrane K permeabilities (estimated from cable analysis; chapter 5), the error which results from considering the epithelium as a single barrier was calculated to be <40% (see Schultz and Frizzell, 1976). In other words, 60% of the observed $\Delta^\star P_K$ must be due to a real decline in membrane K permeability. Also, an error in $^\star P_K$ would not explain why cAMP stimulates $J_{sm}^K$ 4-fold in normal saline (10 mM K) but not in high-K saline (140 mM K).

Electrophysiological results described in chapter 5 will confirm that potassium permeability is influenced by cAMP and external [K] and that changes in $P_K$ occur mainly at the mucosal cell border.

When electrogenic Cl transport and counterion permeability ($P_K$) are enhanced simultaneously in normal saline, less work must be done by the Cl pump per mole of Cl absorbed (see chapter 2 for calculations). What advantages might arise from K-sensitive $P_K$? When the rectum contains unmodified Malpighian tubule fluid, a very large concentration gradient (140:12 mM K) favours movement of this cation from mucosal to serosal side. In spite of this, the lumen does not become electrically negative to the serosal side, presumably due to the decline in $P_K$ as mentioned above. Maintenance of a mucosal-
positive $V_t$ would be advantageous in that it would minimize Na loss from the hemolymph (110 mM Na) into the lumen (40 mM) and hence the work of Na transport. Active Na transport is low (20%) compared to Cl absorption even when mucosal Na levels are high (90-200 mM; Williams et al., 1978; Spring and Phillips, 1980b). The maximum transepithelial electrochemical gradients for Na developed across the rectum of hydrated locusts in vivo are also smaller than for Cl or K (Phillips, 1964b). A large net potassium flux from K-rich rectal fluid would result in a flow of anions from mucosal-to-serosal side and/or a flow of cations (Na) in the reverse direction under open-circuit conditions. Conserving hemolymph Na in this way may be significant for an insect which feeds on fresh plant matter which is low in Na (14 mM) versus K (114 mM; for lettuce).

Prior to stimulation by cAMP, open-circuit $^{42}$K fluxes deviated from the using flux ratio equation for simple diffusion. Deviation from the predicted flux ratios in control recta is probably not the result of active transport, since $J^K_{net}$ was zero under $I_{sc}$ conditions.

Passive transepithelial K fluxes apparently do not occur independently across locust rectum although non-independence can not be localized to a specific cell membrane since transepithelial K fluxes depend on steady-state tracer activity within the epithelium, which is determined by K permeabilities of both apical and basal membranes. If intracellular $^{42}$K activity is identical to that of the serosal chamber, then the flux ratio exponent ($n'$) will be a measure of interactions between K ions as they move through the mucosal membrane. Conversely, if $^{42}$K levels are similar in mucosal and intracellular compartments, $n'$ will be determined by the properties of the serosal membrane. According to the single-file diffusion model with "knock-on" collisions
(Hodgkin and Keynes, 1955; Hladky and Harris, 1967), \( n' \) is the number of single-file sites in the membrane channel or one plus the number of sites depending on whether all sites are filled. The maximum value of \( n' \) places an upper limit on the number of ions permitted within the channel (Hille and Schwarz, 1978). In the case of 2 series membranes, transepithelial values of \( n' \) will be determined by mucosal, serosal, or both membranes according to the relative permeabilities of musocal and serosal membranes.

The present data suggest that either mucosal or serosal membrane may have a K flux ratio exponent indicative of single-filing through channels with at least 5 sites. Further studies of \( n' \) value in this tissue might involve measurement of steady-state tracer activity within the epithelial cells, of the use of ionophores to greatly increase the permeability of one membrane so that the properties of the other might be studied with voltage clamping techniques. The first evidence for single-filing in epithelial K channels was obtained recently for the Ba-sensitive K channels in the basolateral membrane of turtle bladder (Kirk and Dawson, 1981). Concentration-dependent K permeability has also been shown in the paracellular pathway of rabbit colon (Fromm and Schultz, 1981).

**Properties of active K transport**

A net flux of \(^{42}\text{K}\) from mucosal-to-serosal side of locust rectum was measured under \( I_{sc} \) conditions during cAMP stimulation. The presence of a small active absorption of K across the rectal epithelium is consistant with earlier findings that K concentration in the rectal lumen is maintained far from electrochemical equilibrium in salt-depleted (hydrated) locusts (Phillips, 1964b). In the present study, no net flux of K was observed until cAMP was added. This differs from the results obtained by Williams et al. (1978). They reported a small net K absorption by locust rectum using the blowfly
saline of Berridge (1966) and a voltage clamp which did not correct for series resistance.

Two characteristics of active K absorption are apparent from this study. First, the mechanism for active K absorption must have a $K_t$ of less than 10 mM K, because $J^K_{\text{net}}$ was similar whether the bathing saline contained either 10 or 140 mM K. This low rate of active K absorption is probably responsible for reducing $[K]$ in the rectal fluid to low levels in vivo (<0.5 mM; Phillips, 1964). Second, when Cl was omitted from the saline, there was no reduction in the active net $^{42}K$ flux elicited by cAMP. Whereas the major component of active Cl transport is K-dependent, all of the active K transport is Cl-independent. The latter finding strongly suggests that obligatory KCl cotransport entry is not involved in transepithelial K absorption and by implication K must enhance Cl absorption by some other means. Further evidence against KCl cotransport was obtained using ion-sensitive microelectrodes and is reported in the following chapter.
Summary

Double-barrelled liquid ion-exchanger electrodes were used to measure intracellular K, Cl and Na activities (\(a^c_K\), \(a^c_{Cl}\) and \(a^c_{Na}\), respectively) and membrane potentials in locust rectal epithelium. Steady-state net electrochemical gradients for Cl and K across apical and basal membranes were calculated during exposure to 1 mM cAMP, and during ion substitutions under open-circuit conditions. Under control conditions, \(a^c_{Cl}\) (30.7 ± 1.1 mM) was 3.5 times higher than that predicted for passive equilibrium across the mucosal membrane.

Serosal addition of 1 mM cAMP resulted in i) hyperpolarization of the mucosal membrane (\(V^m_a\)), ii) depolarization of the serosal membrane (\(V^m_b\)), and iii) a 50% increase in \(a^c_{Cl}\). The net electrochemical gradient (\(\Delta\mu^a_{Cl}/F\)) opposing Cl entry from the mucosal side increased from -32.0 ± 1.2 mV (unstimulated control) to -49.8 ± 0.5 mV during cAMP stimulation, whereas the net gradient favouring Cl exit across the basal membrane \(\Delta\mu^b_{Cl}/F\) did not change. When cAMP-stimulated recta were exposed to K-free conditions for 1 hour, \(\Delta\mu^a_{Cl}/F\) declined to control levels while \(\Delta\mu^b_{Cl}/F\) again was unchanged. Measurements of \(a^c_{Cl}\), \(a^c_K\) and membrane potentials as a function of external [K] under short-circuit current conditions indicate that cAMP and low concentrations of K directly stimulate active Cl entry across the apical membrane. The hyperpolarization of \(V^m_a\) is consistent with electrogenic Cl entry. No correlation exists between \(\Delta\mu^a_{Cl}/F\) and the net electrochemical gradient across the apical membrane for sodium (\(\Delta\mu^a_{Na}/F\)), or between \(\Delta\mu^a_{Na}/F\) and transepithelial net flux of \(^{36}\text{Cl}\) measured under short-circuit (\(I_{sc}\)) conditions. The net electrochemical gradient favouring K entry across the apical membrane (\(\Delta\mu^a_{K}/F\)) is only 25% of
$\Delta\mu_{Cl}^-/F$ at open-circuit, and is negligible under $I_{sc}$ conditions when $Cl$ transport is high ($10 \mu\text{Eqcm}^{-2}\text{h}^{-1}$). In summary, net absorption involves an active entry step at the apical membrane which is stimulated by cAMP, and by low levels of $K$ on the mucosal side, but is not energized by $\Delta\mu_{Na}^-/F$ or $\Delta\mu_{K}^+/F$. 
Introduction

In the preceding chapter, transepithelial flux experiments revealed that active chloride transport across locust rectum occurs by a mechanism which differs from the well established Na- and HCO₃-coupled systems found in vertebrate epithelia.

Transepithelial Cl absorption in locust rectum is electrogenic, is stimulated by a neuropeptide from the corpus cardiacum and by cAMP (Spring et al., 1978; Spring and Phillips, 1980a,b; Phillips et al., 1980; and chapter 2 of this thesis), and is greatly enhanced by addition of K on the mucosal side (chapter 3). Furthermore, addition of 1 mM cAMP to the serosal side under Iₜ conditions results in a very small net flux of K from mucosal to serosal side and a large increase in transepithelial K permeability.

In this chapter, the cellular mechanism of Cl absorption is examined using double-barrelled ion-sensitive microelectrodes.

Specifically, I attempt i) to establish whether the active step for transrectal Cl transport is located at the mucosal or the serosal cell border, ii) to determine whether K stimulates active Cl transport directly, or indirectly through changes in membrane potential, and iii) to examine whether active Cl absorption might be driven by transmembrane electrochemical gradients for Na or K through coupled ion movements (i.e. "secondary" active transport). Answers to these questions are obtained by comparing previous tracer flux results in chapters 2 and 3 with ion-sensitive microelectrode measurements made under identical conditions.

The results suggest that the entry mechanism for Cl across the apical membrane is active, electrogenic, stimulated directly by cAMP and also by mucosal K, but is not energized by Na or K net electrochemical gradients across the apical membrane.
The electrochemical potential profiles observed in this study and electrophysiological experiments to be described in chapter 5 allow estimation of membrane permeability properties and thus driving force of active chloride transport in locust rectum.

Materials and methods

The rectal preparation

Locust recta were dissected and mounted as flat sheets in a plexiglass perfusion chamber (shown in Fig. 1) in the same manner as previously described for tracer flux experiments (see chapter 2). The chamber design permitted independent perfusion of both sides of the tissue and allowed microelectrodes to be positioned on the mucosal side. The preparation was observed through a glass window at the end of the chamber, using a dissecting microscope (Zeiss, Jena, GDR) at 25-100x magnification, and was illuminated front and rear by fiber optics (Intralux 150H, Volpi AG, Urdorf, Switz.). Electrodes were advanced manually at an angle of 30°-40° to the plane of the epithelium using Leitz micromanipulators (Wetzlar, F.R.G.). The tissue was continuously perfused by gravity-feed from well-gassed reservoirs.

The basic saline is described in chapter 2 (Table 1) and was modified as follows in this study: i) N-methyl D glucamine was substituted for Na, and gluconate for Cl, during microelectrode experiments because choline and methylsulfate are sensed by K and Cl liquid ion-exchangers, respectively; ii) all salines were HCO₃⁻-free and were oxygenated with 100% O₂ in order to minimize HCO₃⁻ interference during intracellular Cl measurements. Previous tracer flux measurements showed that Cl transport and transepithelial electrical properties are not affected by removal of all external CO₂ and HCO₃⁻ (chapter 2).
Figure 4.1  Cross section of the chamber used in microelectrode experiments. A, locust rectum with cuticle removed; B, "O"-ring; C, pin; D, inlet for potential sensing agar bridge; E, suction outlet; F, current-passing silver foil electrode; G, support stand; H, fiber optic light; I, perfusate inlet; J, glass window; K, dissecting microscope; L, double-barrelled microelectrode.
Electrical measurements

Transepithelial potential \((V_t)\) was measured as in chapter 2, with the exception that calomel half-cells were replaced with Ag/AgCl wires. The potential difference between reference and ion-sensitive barrels of the double-barrelled microelectrode \((V_i)\) was measured using a differential electrometer with very high input impedance \((>10^{15}\ \Omega);\) FD223, WP Instruments, New Haven, Conn.). The potential difference between the reference barrel within the cell and external mucosal and serosal agar bridges \((V_a\) and \(V_b,\) respectively) were measured individually using high input impedance operational amplifiers at unity gain \((10^{12}\ \Omega);\) 4253, Teledyne Philbrick, Dedham, MA). Transepithelial resistance \((R_t)\) was monitored as the deflections in \(V_t\) produced by transepithelial current pulses to indicate possible tissue damage during mounting and also tissue viability. The ratio of voltage deflections across apical and basal membranes resulting from transepithelial current pulses (i.e. voltage-divider ratio "\(a\)") was measured during measurements under open-circuit conditions as a test for possible impalement damage. Constant current pulses \((\sim 1\ \text{sec duration, } 0.3\ \text{Hz})\) were supplied by waveform/pulse generators (Type 160 Series, Tektronix, Beaverton, Ore.). Current was measured as the voltage drop across a series resistor.

After filtering \((3\ \text{dB at } 5\ \text{Hz}),\) \(V_t,\) \(V_a,\) \(V_b,\) \(V_i\) and \(I_t\) were recorded simultaneously using a 6-channel pen recorder (Brush 260, Gould Inc., St. Louis, Mo.) or, when fewer parameters were required, on a 2-channel recorder (7402A, Hewlett-Packard, San Diego, Calif.). Signals were also monitored using a storage oscilloscope (DL15, Tektronix) and \(V_t\) was always displayed digitally (616, Keithley Instr. Inc., Cleveland, OH).
Fabrication and calibration of ion-sensitive microelectrodes

The techniques used for constructing double-barrelled microelectrodes were similar to those of Fujimoto and Kubota (1976). Capillary glass (1.0 mm O.D., Frederick Haer and Co., Brunswick, Maine) was cleaned for at least 2 h in conc. HNO₃, thoroughly rinsed with distilled water, dried in an oven, and stored dust-free at 0% relative humidity. Two glass capillaries were heated in a vertical puller (PE 2, Narishige Scientific Instr. Lab., Tokyo), rotated one-half turn, and drawn to a final tip diameter of less than 1 μm.

After back-filling the reference barrel with acetone (ACS, Eastman Kodak Co., Rochester, N.Y.) electrodes were dipped into a 0.1% solution v/v of Dow Corning 1107 silicone oil in acetone (1107 was a generous gift of D. Jenkins, Dow Corning, Vancouver) for approximately 10 sec in order to silanize the ion-sensitive barrel. Electrodes were then cured on a hot plate at ~300°C for 15 min. After the electrode shaft was reinforced with fast-drying epoxy, a 2-4 mm column of liquid-ion exchange resin was injected into the silanized barrel from a syringe through fine polyethylene tubing and coaxed to the tip using a cat whisker. When resin reached the tip, the electrode was back-filled with one of the solutions listed in Table 1.

The resistance of the ion-sensitive electrodes ranged between $2 \times 10^9$ and $5 \times 10^{10}$ Ω.

Electrodes were calibrated frequently during the course of experiments in electrolyte solutions encompassing the entire range of intra- and extracellular ion activities. In most experiments 5, 50, 120, 500 mM KCl (conc.) solutions were used for calibrating K and Cl electrodes except during perfusion with nominally K- or Cl-free salines, when 1 mM KCl was also included in the calibration series. Sodium electrodes were calibrated in pure solutions containing 1, 10, 100, 120 mM NaCl and mixed solutions containing 10 mM NaCl and 110 mM KCl. Ion
Table 4.1  Liquid ion exchangers and solutions used in fabricating double-barrelled ion-sensitive microelectrodes.

<table>
<thead>
<tr>
<th>Electrode</th>
<th>Resin</th>
<th>Backing electrolyte</th>
<th>Reference Barrel</th>
</tr>
</thead>
<tbody>
<tr>
<td>K</td>
<td>Corning 477317</td>
<td>0.5M KCl</td>
<td>1.0M Na acetate</td>
</tr>
<tr>
<td>Cl</td>
<td>Orion 92-17102</td>
<td>0.5M KCl</td>
<td>1.0M Na acetate</td>
</tr>
<tr>
<td>Na</td>
<td>Monensin ~10% w/w in Corning 477317 (see Koter et al., Membr. Biochem. vol.2:323-338,1979)</td>
<td>0.49M NaCl at pH=3.0 (0.1M citrate buffer)</td>
<td>0.5M KCl</td>
</tr>
</tbody>
</table>
activities in calibrating solutions were calculated using the modified Debye–Hückel equation (Robinson and Stokes, 1970).

Plots of electrode electromotive force (mV) against the logarithm of the ion activity (mM) yielded the following slopes (s) and r² values (± s.e.): Cl, s = 57.77 ± 0.87 mV, r² = 0.9950 ± 0.0013, 29 electrodes; K, s = 52.44 ± 0.62 mV, r² = 0.9961 ± 0.0008, 26 electrodes; Na, s = 54.08 ± 1.4 mV, r² = 0.9988 ± 0.0007, 11 electrodes. These values compare well with those obtained in previous studies (Walker, 1971; Fujimoto and Kubota, 1976; Spring and Kimura, 1978; Reuss and Weinman, 1979; Duffey et al., 1979; Fujimoto et al., 1980; and García-Díaz and Armstrong, 1980). Response time of the ion-sensitive barrel was usually about 1-3 seconds except for Na-sensitive electrodes which occasionally required 15-20 seconds to reach a stable value.

Selectivity of Cl electrodes over gluconate using the separate solution method of Moody and Thomas (1971) gave values for gluconate-to-Cl selectivity (K_{Cl,gluconate}) of 0.039 ± 0.006 and K_{Cl,SO₄} of 0.1324 ± 0.0215. The anion interference observed during perfusion with nominally Cl-free saline (i.e. "apparent" Cl activity after gluconate + sulfate substitution) was 5.2 mM, as predicted using the microelectrode selectivity coefficients. Perfusion for 3 h with Cl-free saline resulted in apparent a_{Cl} = 4.84 ± 0.38. Under this unnatural condition, the cells may contain some residual Cl, replacement ions (i.e. gluconate, SO₄) in addition to the interfering anions which are normally present.

The validity of subtracting "apparent a_{Cl}" observed in nominally Cl-free saline is debatable when the identity of the intracellular anion is not known under normal conditions. An identical "apparent Cl activity" has recently been reported in heart muscle after prolonged exposure to methylsulfonate
saline in the absence of HCO$_3^-$ (4.8 ± 0.6 mM, Baumgarten and Fozzard, 1981). Measurement of residual intracellular Cl was not attempted since no method having the required sensitivity was available (total non-mannitol space of the tissue is 3.61 ± 0.24 μl). It is unlikely that intracellular HCO$_3^-$ ($K_{Cl,HCO_3^-} = 0.2-0.09$) accounts for all of the "apparent Cl activity" since salines were HCO$_3^-$-free and vigorously stirred with 100% O$_2$. The values reported in this thesis are not "corrected" for possible anion interference. Regardless, since $a^c_{Cl}$ normally ranged between 30-60 mM, such interference would not be sufficient to change any of the conclusions in this study.

It is also unlikely that Na interference was serious during intracellular K measurements since 1) electrodes showed high selectivity ($K_{Na,K} < 0.02$) and measured intracellular Na activities were low ($a^c_{Na} = 8.0 ± 0.41$ mM, 125 cells in 11 recta under I$_{sc}$ conditions), and 2) intracellular K was reduced to approximately 1 mM after prolonged perfusion with K-free saline (time course shown in Fig. 5). Results obtained with Na-sensitive microelectrodes required corrections for K interference. Selectivity for Na over K in eleven electrodes was 11.2 ± 1.1. Double-barrelled microelectrodes were used to measure $a^c_{K}$ immediately before or after Na measurements on the same tissue under each experimental condition.

Successful impalements were characterized by i) abrupt monotonic deflections in voltage, ii) stable intracellular potential which remained within ± 1 mV, iii) constant voltage divider ratios, and iv) return to the original baseline potentials upon retraction of the electrodes. No evidence of impalement damage was obtained using double-barrelled as compared to single-barrelled microelectrodes, probably due to the large size of the columnar epithelial cells (~17 x 90 μm) and extensive infolding of the cell membrane which, judging from electron micrographs, results in a 9- to 200-fold increase in membrane area in various regions of the cell.
Mannitol space

Since changes in ion activity during cAMP stimulation might result from alterations in cell volume rather than ionic fluxes, mannitol space of recta was measured in normal saline, with or without 1 mM cAMP. Tissues were placed in small vials of vigorously oxygenated saline containing $^3$H-mannitol. After 1 h of incubation, three 1 µl samples of saline were counted by liquid scintillation to estimate external $^3$H activity. Tissues were blotted dry on bibulous paper and wet weight was determined to within ± 0.1 mg. After drying to constant weight in a desiccating oven at 60°C, tissues were digested in 1 N KOH at 60°C overnight, neutralized with conc. $H_2SO_4$, and counted as described in chapter 2. The difference between wet and dry weights was used as an estimate of total tissue water, and intracellular volume was calculated as the difference between total tissue water and mannitol space, assuming that mannitol distributes homogeneously throughout the extracellular space, does not enter the cells, and does not adsorb to the tissue surface. Similar estimates of mannitol were obtained after 1 h or 1.5 h, suggesting that 1 h was adequate for mannitol distribution to reach a steady-state. $^3$H-mannitol space could not be checked using $^{14}C$-inulin because the cuticular intima is virtually impermeable to this polysaccharide (Phillips and Dockerill, 1968). Mannitol is not metabolized by locust rectum.

Calculation and statistics

Voltage divider ratios, transmembrane electrical potential differences, and equilibrium ion activities were calculated with corrections for saline, resistance and ionic activities by computer (PDP-11). Calibration curves for ion-sensitive microelectrodes were determined by linear regression analysis. Net electrochemical gradients were calculated as:
\[ \Delta \mu_i/F = RT\ln \frac{a_i^c}{a_i^{m,s}}/F + zV_{a,b} \]

where \(a_i^c\) is the activity of ion "i" in the cell, \(a_i^{m,s}\) is the activity of ion "i" in mucosal or serosal solution, \(V_a\) and \(V_b\) are apical or basal membrane potentials, respectively, and \(z, F, R, T\) have their usual meanings.

"Uphill" net electrochemical gradients are given as negative values; favourable gradients are positive in relation to the direction of net transport of the ion in question.

Statistical comparisons were made using standard paired or unpaired t-tests.

Results

1) **Steady-state measurements of intracellular chloride and potassium activities**

Figure 2 shows original traces of \(V_t\), \(V_a\), \(V_b\) and intracellular ion activity obtained during impalments with double-barrelled K- and Cl-sensitive microelectrodes. Values were usually stable and showed little variability between different cells in any particular tissue. Apical, basal and trans-epithelial potential changes due to applied current pulses are also shown. After correcting for series resistance, the deflections in \(V_a\) and \(V_b\) were used to calculate the voltage divider ratio (\(R_a/R_b\) or "a"), and served as an indicator of impalement damage. Values of \(a\) will be discussed in more detail in chapter 5, which deals with membrane permeability changes. Transient deflections in the Cl activity trace are artifacts resulting from the different electrical time constants of ion-sensitive and reference barrels. K-sensitive microelectrodes usually had resistances that were one order of magnitude lower than did Na or Cl electrodes, and generally did not show these transient deflections.
Figure 4.2 Representative traces obtained using double-barrelled ion-sensitive microelectrodes. Simultaneous recording of transepithelial potential ($V_t$), apical membrane potential ($V_a$), basal membrane potential ($V_b$; also shows $V_t$ when the electrode is withdrawn into the mucosal half-chamber), potential difference between reference and ion-sensitive barrels of double-barrelled ion-sensitive microelectrodes ($V_i$) under open-circuit conditions in normal saline (Table 2.1). Transepithelial current pulses are shown at bottom ($I_t$).
$V_t$ was monitored continuously after mounting tissues in the perfusion chamber. As in previous open-circuit flux experiments (Figures and chapters 1 and 2, respectively), $V_t$ was initially 25-40 mV mucosal-side positive, but decayed to approximately 8 mV after 2-3 hours. Control intracellular measurements were made when $V_t$ had declined to this level (Table 2). Approximately 30 minutes was required for measured parameters across recta to reach new steady-state following addition of 1 mM cAMP or external K (indicated by transepithelial resistance and potential, Fig. 3). Therefore, steady-state intracellular measurements were made following 30-60 min equilibration under each new condition. Figure 4 shows histograms of $a^{C}_{Cl}$ and $a^{C}_{K}$ in control preparations (unstimulated, 10 mM K), and during exposure to 1 mM cAMP. Both Cl and K activities appear normally distributed about their mean values, suggesting that a single cell population was sampled or that there is effective cell-cell coupling if different populations do exist (see chapter 5).

a) Chloride

Table 2 summarizes results obtained in one series of experiments using double-barreled Cl-sensitive microelectrodes. Under control (open-circuit) conditions, $a^{C}_{Cl}$ was 30.7 ± 1.1 mM, approximately 3.5-fold higher than the value predicted for passive distribution across the apical membrane (8-9 mM).

As shown previously (chapter 2), there is a net flux of $^{36}$Cl from mucosa to serosa under these conditions; therefore an active step or "pump" must be postulated at the apical membrane while exit of Cl across the basal membrane may occur passively down a favourable electrochemical gradient ($\Delta i^{b}_{Cl}$).

The effects of cAMP exposure provide further evidence for an apical Cl pump. It was shown previously (chapters 2 and 3) that the mucosal side becomes ~20 mV more positive with respect to the serosal side and net Cl
Figure 4.3  Time course of the effects of cAMP on transepithelial potential ($V_t$), apical membrane potential ($V_a$) and Cl-sensitive potential ($V_i$, differential). See legend of Figure 4.2 for definitions. Deflections in $V_t$ and $V_a$ are due to transepithelial current pulses (20 $\mu$A/0.1962 cm$^2$), and indicate transepithelial resistance. The regular deflections in $V_i$ are artifacts which result from the large difference between time constants of ion-sensitive and reference barrels. As indicated by $V_a$ and $V_i$, the electrode became dislodged after 8 min (at *) and a second cell was impaled.
Figure 4.4  Distribution of intracellular Cl and K activities in rectal pad epithelial cells under control conditions, and after sequential exposure to 1 mM cAMP, and to K-free saline with cAMP present. Tissues were in open-circuited state. Chloride and potassium measurements were made on recta from different animals (n = 6-10).
Table 4.2  Effects of adding cAMP and removing external K on electrical potentials, intracellular Cl activity, and calculated Cl electrochemical gradients.

<table>
<thead>
<tr>
<th>Sequential Condition</th>
<th>$V_t$ (mV)</th>
<th>$V_a$ (mV)</th>
<th>$V_b$ (mV)</th>
<th>$a^c_{Cl}$ (mV)</th>
<th>$\Delta\mu^a_{Cl}/F$ (mV)</th>
<th>$\Delta\mu^b_{Cl}/F$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+8.6 ±0.3</td>
<td>-57.0 ±0.8</td>
<td>-48.7 ±0.9</td>
<td>30.7 ±1.1</td>
<td>32.0 ±1.2</td>
<td>23.3 ±1.4</td>
</tr>
<tr>
<td>(6;40)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 1mM cAMP</td>
<td>+29.8 ±0.5</td>
<td>-63.8 ±0.6</td>
<td>-34.0 ±0.6</td>
<td>46.6 ±0.8</td>
<td>49.8 ±0.5</td>
<td>20.1 ±0.6</td>
</tr>
<tr>
<td>(6;42)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ cAMP, K-free,</td>
<td>+4.8 ±0.4</td>
<td>-79.6 ±0.8</td>
<td>-74.8 ±0.8</td>
<td>11.8 ±0.9</td>
<td>-23.8 ±4.2</td>
<td>23.5 ±4.4</td>
</tr>
<tr>
<td>after 1 hour</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(6;42)</td>
<td></td>
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</tbody>
</table>

$V_t$, transepithelial potential; $V_a$, apical membrane potential; $V_b$, basal membrane potential; $a^c_{Cl}$, intracellular Cl activity; $\Delta\mu^a_{Cl}/F$, $\Delta\mu^b_{Cl}/F$, Cl net electrochemical gradients calculated for apical and basal membranes. Sign convention: $V_t$, mucosal side relative to serosal; $V_a$ and $V_b$, transepertimental potential relative to mucosal and serosal side, respectively; $\Delta\mu^a_{Cl}/F$ and $\Delta\mu^b_{Cl}/F$, a positive sign indicates gradients favouring passive net Cl movement in the mucosal-to-serosal direction, negative sign indicates uphill gradient. Means ± s.e., (number of recta; number of cells) for intracellular measurements; (number of recta; number of observations) for measurements of $V_t$. 
absorption \( J_{\text{Cl}}^{\text{net}} \) increases 9-fold when 1 mM cAMP is added to the serosal side under open-circuit conditions. Similar changes in transepithelial potential were observed in the present microelectrode experiments (Table 2).

Serosal addition of 1 mM cAMP results in both hyperpolarization of \( V_a \) by \( \sim 6 \) mV, and a 50% elevation of \( a_{\text{Cl}}^c \). If a cAMP-stimulated Cl pump were located at the serosal cell border, one would expect \( a_{\text{Cl}}^c \) to either decrease or remain constant during cAMP exposure: an increase in \( a_{\text{Cl}}^c \) is not consistent with a pump at the basal membrane. When combined with the earlier finding that steady-state \( J_{\text{Cl}}^{\text{net}} \) is elevated 9-fold under these conditions (chapter 2), these data indicate that a cAMP-stimulated Cl pump is located at the apical membrane.

Finally it is interesting that \( \Delta V_{\text{Cl}} \) did not change significantly following cAMP addition. This suggests that conductance of the basal membrane may also be stimulated by cAMP if Cl exits by electrodiffusion, since the net electrochemical gradient for Cl across the serosal cell border is not changed despite a 9-fold increase in net Cl flux through the membrane. This prediction is confirmed in chapter 5 using flat sheet cable analysis. It will be shown that the increase in basal membrane conductance is larger than is needed to account for the observed flux of Cl by electrodiffusion. No electroneutral exchange or cotransport mechanisms need be postulated at the Cl exit step.

Following K-removal from both sides, \( V_a \) and \( V_b \) both hyperpolarized while \( V_t \) increased temporarily from 29.8 ± 0.5 mV to \( \sim 45 \) mV and then declined to 4.8 ± 0.4 mV, consistent with the reduced \( J_{\text{Cl}}^{\text{net}} \) shown previously (Fig. 15 of chapter 2). Membrane potentials immediately hyperpolarized to -130 mV \( (V_a) \) and -85 mV \( (V_b) \) and then declined exponentially to \( \sim -80 \) mV after 1 hour. Most importantly, \( \Delta V_{\text{Cl}}^{\Delta a} \) decreased in K-free saline from -49.8 ± 0.5 to -28.3 ± 4.2 mV, indicating that K directly stimulated Cl entry. Note
that if potassium-stimulated $\text{Cl}_{\text{net}}$ resulted from K "short-circuiting" $V_a$ (thereby reducing the work required for Cl entry), then the uphill step for Cl entry would have increased following K removal.

Finally, it is worth pointing out that the net electrochemical gradient for Cl across the apical membrane is at least partly determined by processes at the serosal cell border. For example, both enhancing Cl entry and blocking Cl exit would both increase $\Delta\mu_{\text{Cl}}$. However a decrease in Cl exit would also increase the favourable Cl electrochemical gradient for exit across the basal membrane. Contrary to this prediction, perfusion with K-free saline has no effect on the Cl electrochemical gradient across the serosal cell border (Table 2).

Taken together, these results indicate that Cl is pumped into the epithelial cells across the apical membrane by an active mechanism which is stimulated by both cAMP and potassium ions.

b) Potassium

Table 3 shows results obtained using double-barrelled K-sensitive microelectrodes during experiments similar to those just described for Cl: control unstimulated, and cAMP stimulated, in the presence and then absence of external K for 1 hour. Values of $V_t$, $V_a$, and $V_b$ were similar to those obtained with Cl-sensitive microelectrodes (compare Tables 2 and 3). The mean intracellular K activity ($a_K^c$) was slightly below that predicted for equilibrium across the apical membrane; however, this was not significant ($P < 0.05$). In order for $V_a$ to be attributed solely to a K diffusion potential, $a_K^c$ would have to be above equilibrium across the apical membrane. Therefore some additional electromotive force must be proposed at the apical membrane (such as an inwards-directed, electrogenic Cl pump) in order to explain $V_a$. 
Table 4.3  Effects of adding cAMP and removing external K on electrical potentials, intracellular K activity and calculated K electrochemical gradients

<table>
<thead>
<tr>
<th>Condition</th>
<th>$V_t$</th>
<th>$V_a$</th>
<th>$V_b$</th>
<th>$a_{a K}^{c}$</th>
<th>$\Delta V_{K/F}^{a}$</th>
<th>$\Delta V_{K/F}^{b}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+7.2</td>
<td>-57.8</td>
<td>-50.7</td>
<td>61.4</td>
<td>+3.3</td>
<td>+3.9</td>
</tr>
<tr>
<td>[10;97]</td>
<td>±0.3</td>
<td>±0.5</td>
<td>±0.3</td>
<td>±0.7</td>
<td>±0.5</td>
<td>±0.3</td>
</tr>
<tr>
<td>+ 1mM cAMP</td>
<td>+32.2</td>
<td>-70.2</td>
<td>38.0</td>
<td>70.3</td>
<td>+12.4</td>
<td>+19.8</td>
</tr>
<tr>
<td>[9;81]</td>
<td>±0.8</td>
<td>±0.6</td>
<td>±1.0</td>
<td>±1.1</td>
<td>±0.8</td>
<td>±1.2</td>
</tr>
<tr>
<td>+ cAMP</td>
<td>+9.8</td>
<td>-84.1</td>
<td>-74.3</td>
<td>13.9</td>
<td>&lt; -55.2</td>
<td>&gt; +65.0</td>
</tr>
<tr>
<td>K-free, after 1 hour</td>
<td>±0.7</td>
<td>±3.4</td>
<td>±3.3</td>
<td>±1.2</td>
<td>±2.0</td>
<td>±1.9</td>
</tr>
</tbody>
</table>

See footnote to Table 4.2 for explanations
Small electrochemical gradients (<5 mV) favouring K net absorption from mucosa to serosa were observed across mucosal $\Delta \mu_{K}^{a}/F$ and serosal $\Delta \mu_{K}^{b}/F$ membrane under control conditions. This finding is consistent with passive transepithelial K absorption, but alone does not exclude the presence of an additional active flux. In chapter 3 evidence was presented that movements are largely passive in the absence of cAMP; i.e. there was no $J_{\text{net}}^{K}$ in unstimulated recta under short-circuit conditions (Fig. 13 of chapter 3).

Serosal addition of cAMP dramatically increased both $\Delta \mu_{K}^{a}$ and $\Delta \mu_{K}^{b}$, mostly through changes in membrane potentials. The active step in K transport is difficult to localize under these conditions.

It may be noted that $\Delta \mu_{K}^{a}/F$ increased significantly less than $\Delta \mu_{K}^{b}/F$ during cAMP exposure ($P < 0.05$), however this may simply reflect a higher K conductance at the apical membrane. Regardless, under open-circuit conditions, active K transport is very small compared to the net passive flux which is electrically coupled to active Cl absorption (<20%, chapter 3). Locust rectal cells contained some potassium after perfusion with K-free saline for 1 hour (Table 3): $a_{K}^{c}$ continues to decline for several hours (see Fig. 5). Although the conditions are not strictly steady-state with respect to $a_{K}^{c}$ after 1 h, this does not influence my conclusions regarding the mode of action of K on chloride transport, because these are based on Cl net fluxes and net electrochemical gradients when transepithelial Cl transport is approximately steady-state as judged by $V_{t}$ and $R_{t}$. Moreover, in the next section, very similar results are reported for experiments which begin after recta were more nearly depleted of intracellular K (<5 mM).

2) Relationship between chloride and potassium electrochemical potentials

Chloride-dependent $I_{SC}$ increases dramatically when K is added stepwise to both sides of K-depleted tissues (Fig. 8 of chapter 3). From the transepithel-
Figure 4.5  Time course of the decline in intracellular K activity ($a^c_K$) and $V_a$ during bilateral perfusion with K-free saline under open-circuit conditions. Each point is the mean ± s.e. of 6 measurements using a double-barrelled microelectrode in one tissue. Compare with $a^c_K = 61.4 ± 0.7$ under control conditions.
ial measurements above, it is not possible to determine whether this is due to indirect electrical effects (i.e. changes in membrane potentials), to direct stimulation of a Cl pump, or to both. Direct stimulation of the Cl pump is indicated in the preceding section because removal of 10 mM external K under open-circuit conditions reduces the net electrochemical gradient opposing Cl entry. Nevertheless, increasing the K activity on the mucosal side should depolarize the apical membrane and reduce the work required for Cl entry. I attempted to evaluate this effect. Also, it is of some interest to compare apical and basal membrane $\Delta \nu_K/F$ and $\Delta \nu_{Cl}/F$ under $I_{sc}$ conditions. Such a comparison would indicate whether Cl movements could be energized by coupled movements with K (i.e. cotransport).

Figure 6 illustrates intracellular potassium and chloride activities and potential differences in one rectal preparation (means ± s.e.; $V_a = V_b$ under $I_{sc}$ conditions; ionic activities and electrochemical gradients, $n = 9-10$; intracellular potentials, $n = 18-20$). Table 4 summarizes values from 5 preparations. Data were obtained sequentially under the following conditions: unstimulated (K-free saline), cAMP-stimulated (K-free saline), cAMP-stimulated (with stepwise increases in K concentration on both sides; $[K] = 0, 2, 4, 10, 40, 100$ and 140 mM). Measurements were made at least 0.5 h after exposure to cAMP and after each change in $[K]$ (i.e. steady-state conditions were approximated during measurements). Also shown are the K and Cl net electrochemical gradients during these experiments, calculated from the data in the upper panels. Several points should be noted:

1) Prolonged exposure (>2.5 h) to K-free saline resulted in reduction of $a_K^C$ to <5 mM and mucosal and serosal membrane depolarization to 23 mV (compare with Fig. 5).
**Figure 4.6** Effects of 1 mM cAMP and bilateral K additions on intracellular potential, intracellular activities of K and Cl, and calculated electrochemical potentials for K ($\Delta\mu_K^a/F$) and Cl ($\Delta\mu_{Cl}^a/F$) across the apical membrane under $I_{sc}$ conditions. Cyclic-AMP was added at the arrows under K-free conditions. Note that i) intracellular potential changes about 50 mV/decade change in external K activity, ii) $a_K^c$ in K-depleted tissue increases from 3 mM to near control levels (65 mM) when recta are re-exposed to 2 mM K, iii) $a_{Cl}^c$ decreases during hyperpolarization of $V_{a,b}$, iv) $\Delta\mu_K^a/F$ is approximately 0 mV at all external K activities indicating that little energy is available in the K gradient for KCl coentry; $\Delta\mu_{Cl}^a/F$ becomes more negative when external K is increased. When combined with $^{36}$Cl flux and $I_{sc}$ measurements under comparable conditions (chapters 2 and 3), this latter observation suggests a direct stimulation by K of active Cl entry. Results shown are from one preparation. Means ± s.e.; $n = 20$ obs. ($V_{a,b}$), $n = 10$ obs. ($a_K^c$, $a_{Cl}^c$, $\Delta\mu_K^a/F$, $\Delta\mu_{Cl}^a/F$). See Table 4.4 for complete data.
1mM cAMP

$V_{a,b}$ (mV)

$\alpha_{K}$ (mM)

$\alpha_{Cl}$ (mM)

$\Delta P_{K}^{0}/F$ (mV)

$\Delta \bar{V}_{a}^{0}/F$ (mV)

Bilateral K activity (mM)
Table 4.4  Effects of sequential cAMP addition and increased [K] bilaterally on intracellular potential, ion activities and net electrochemical gradients measured with double-barrelled micro-electrodes under $I_{sc}$ conditions

<table>
<thead>
<tr>
<th>Sequential Condition</th>
<th>$V_{a,b}$ (mV)</th>
<th>$a_{Cl}^c$ (mM)</th>
<th>$a_K^c$ (mM)</th>
<th>$\Delta\mu_{Cl}^a/F$ (mV)</th>
<th>$\Delta\mu_{K}^a/F$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM K (30-60;3)</td>
<td>-40.1 ±0.4</td>
<td>37.8 ±3.1</td>
<td>1.55 ±0.1</td>
<td>-18.11 ±2.0</td>
<td>-31.89 ±3.3</td>
</tr>
<tr>
<td>+ 1mM cAMP (40-90;5)</td>
<td>-54.7 ±2.5</td>
<td>50.0 ±2.8</td>
<td>1.2 ±0.1</td>
<td>-40.6 ±1.8</td>
<td>-18.2 ±2.8</td>
</tr>
<tr>
<td>2mM K (30-70;4)</td>
<td>-89.5 ±1.3</td>
<td>20.8 ±0.7</td>
<td>52.0 ±1.3</td>
<td>-55.3 ±6.2</td>
<td>-5.4 ±1.2</td>
</tr>
<tr>
<td>4mM K (38-87;5)</td>
<td>-76.2 ±0.5</td>
<td>30.7 ±1.1</td>
<td>59.1 ±2.0</td>
<td>-48.3 ±1.5</td>
<td>-0.9 ±0.6</td>
</tr>
<tr>
<td>10mM K (26-66;4)</td>
<td>-57.5 ±0.4</td>
<td>40.3 ±0.9</td>
<td>74.8 ±1.2</td>
<td>-39.5 ±0.9</td>
<td>-0.1 ±0.6</td>
</tr>
<tr>
<td>40mM K (30-70;4)</td>
<td>-29.8 ±0.53</td>
<td>58.5 ±1.2</td>
<td>90.6 ±1.6</td>
<td>-19.2 ±1.3</td>
<td>+1.5 ±0.6</td>
</tr>
<tr>
<td>100mM K (30-70;4)</td>
<td>-10.6 ±0.4</td>
<td>74.4 ±1.2</td>
<td>101.3 ±2.3</td>
<td>-7.9 ±1.1</td>
<td>+0.9 ±0.2</td>
</tr>
<tr>
<td>140mM K (34-64;4)</td>
<td>-10.2 ±0.6</td>
<td>84.2 ±2.7</td>
<td>117.8 ±1.1</td>
<td>-3.62 ±1.3</td>
<td>+0.4 ±0.4</td>
</tr>
</tbody>
</table>

Means ± s.e., (number of observations; number of tissues). See legend of Fig. 4.6 and text for further explanation.
ii) Addition of 1 mM cAMP to the serosal side of K-depleted tissues increased $a^c_{Cl}$ by 12 mM, hyperpolarized the mucosal and serosal membranes by 15 mV (both changes significant at $P < 0.05$), and elevated the net electrochemical gradient opposing Cl entry across the mucosal membrane by 12 mV. Each of these observations is consistent with an apical electrogenic Cl pump and with the previous observation that some Cl transport is stimulated by cAMP in K-free saline ($\sim$35%; Fig. 15 of chapter 2).

iii) Addition of K (2 or 4 mM) to both sides caused recovery of $a^c_K$ to control levels and membrane repolarization within 0.5 h (see Fig. 6, K activities of 1.44 and 2.89 mM). Under these $I_{sc}$ conditions, both $V_a$ and $V_b$ were virtually identical to $E_K$, the equilibrium potential for K. This is not surprising since the top panel shows that when saline [K] is increased, $V_a$ and $V_b$ vary approximately 50 mV per decade change in external K activity, indicating extremely high selectivity of both cell borders to K (note that cell borders are essentially studied in parallel under these $I_{sc}$ conditions).

iv) Potassium (2-4 mM conc.) on both sides of the epithelium increased the electrochemical gradient opposing Cl entry ($\Delta u^a_{Cl}/F$), as shown previously by removal of 10 mM K from both sides (Table 2). As explained earlier, this particular result cannot be explained as a simple "short-circuiting" of the apical membrane potential by external K; thus a more direct action of K on Cl entry must be postulated (i.e. direct pump stimulation). However, when [K] is increased further (from 10 to 100 mM conc. or 7.2 to 66.7 mM activity), uphill $\Delta u_{Cl}/F$ varies directly with intracellular potential, suggesting that at high K concentrations bilaterally under $I_{sc}$ conditions, simple membrane depolarization may contribute to the enhanced rate of Cl transport observed at these high levels (Fig. 8 of chapter 3).
v) There is no relationship between $\Delta \mu_{K}^{a}/F$ and $\Delta \mu_{Cl}^{a}/F$ when external $K$ is varied, or between $\Delta \mu_{K}^{b}/F$ and Cl-dependent $I_{sc}$ (compare Fig. 6 of this chapter with Fig. 9 of chapter 3). Clearly Cl entry cannot be energized by cotransport with $K$, since $\Delta \mu_{K}^{b}/F$ is not significantly different from zero under $I_{sc}$ conditions ($P > 0.2$), over the range 4-140 mM $K$ concentration.

Although useful for examining mechanisms, the above experiment was performed under highly unnatural conditions; i.e. transepithelial potential ($V_{T}$) was clamped at zero and $[K]$ was varied over a wide range on both sides. In vivo, the lumen is normally positive with respect to the hemolymph and $[K]$ fluctuates only in the lumen. The experiment described above was repeated under more physiological conditions: i) tissues were left open-circuited, ii) serosal $[K]$ was 10 mM throughout the experiment while $[K]$ was increased from 0 to 140 mM on the mucosal side only. Table 5 summarizes data from 5 preparations and Figure 7 illustrates results obtained from one rectum.

The top panel of Figure 7 shows apical ($V_{a}$) and basal ($V_{b}$) membrane potentials (note that $V_{a}$ no longer equals $V_{b}$). Transepithelial potential is simply the difference between $V_{a}$ and $V_{b}$. The following observations are of interest:

i) When mucosal $[K]$ equaled zero, addition of 1 mM cAMP to the serosal side caused a 20 mV hyperpolarization of $V_{a}$ and an increase in $a_{Cl}^{c}$, consistent with the stimulation of an electrogenic Cl pump in the apical membrane.

ii) $a_{K}^{c}$ is near control levels ($\approx 60$ mM) when the mucosal side is K-free for >2.5 h. Under these conditions intracellular $K$ must have been acquired from the serosal side, perhaps by the ubiquitous Na/K exchange pump. Na/K ATPase activity has been demonstrated in this tissue (see Peacock, 1981).

iii) $K$ diffusion from the cell across the mucosal membrane hyperpolarizes $V_{a}$, thereby increasing the electrochemical gradient opposing Cl entry (compare $\Delta \mu_{Cl}^{a}/F$ in Figs. 6 and 7). Also, since apical and basal membranes are not
Table 4.5  Effects of cAMP and mucosal [K] on apical membrane potential, intracellular K and Cl activities, and net electrochemical gradients across the apical membrane under open circuit conditions*

<table>
<thead>
<tr>
<th>Condition</th>
<th>$V_a$</th>
<th>$a_{Cl}^c$</th>
<th>$a_K^c$</th>
<th>$\Delta\mu_{Cl}^a/F$</th>
<th>$\Delta\mu_K^a/F$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0mM K</td>
<td>-91.1</td>
<td>21.1</td>
<td>39.4</td>
<td>-56.9</td>
<td>-63.1</td>
</tr>
<tr>
<td></td>
<td>±2.8</td>
<td>±0.6</td>
<td>±2.6</td>
<td>±4.4</td>
<td>±3.8</td>
</tr>
<tr>
<td>+ cAMP</td>
<td>-110.8</td>
<td>32.4</td>
<td>56.1</td>
<td>-86.4</td>
<td>-51.1</td>
</tr>
<tr>
<td></td>
<td>±1.2</td>
<td>±1.2</td>
<td>±1.3</td>
<td>±2.1</td>
<td>±1.1</td>
</tr>
<tr>
<td>2mM K</td>
<td>-94.2</td>
<td>41.8</td>
<td>51.5</td>
<td>-77.4</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>±1.0</td>
<td>±1.3</td>
<td>±1.8</td>
<td>±1.8</td>
<td>±0.5</td>
</tr>
<tr>
<td>4mM K</td>
<td>-86.4</td>
<td>40.9</td>
<td>57.0</td>
<td>-68.8</td>
<td>5.0</td>
</tr>
<tr>
<td></td>
<td>±0.9</td>
<td>±1.0</td>
<td>±1.6</td>
<td>±1.5</td>
<td>±1.3</td>
</tr>
<tr>
<td>10mM K</td>
<td>-70.0</td>
<td>46.2</td>
<td>66.9</td>
<td>-57.1</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>±1.1</td>
<td>±1.3</td>
<td>±1.7</td>
<td>±1.5</td>
<td>±1.1</td>
</tr>
<tr>
<td>40mM K</td>
<td>-47.7</td>
<td>48.5</td>
<td>83.4</td>
<td>-37.5</td>
<td>15.5</td>
</tr>
<tr>
<td></td>
<td>±0.7</td>
<td>±2.4</td>
<td>±2.6</td>
<td>±1.3</td>
<td>±0.6</td>
</tr>
<tr>
<td>100mM K</td>
<td>-40.5</td>
<td>58.2</td>
<td>84.0</td>
<td>-32.0</td>
<td>26.8</td>
</tr>
<tr>
<td></td>
<td>±0.8</td>
<td>±1.6</td>
<td>±1.6</td>
<td>±1.5</td>
<td>±0.9</td>
</tr>
<tr>
<td>140mM K</td>
<td>-41.8</td>
<td>59.8</td>
<td>82.3</td>
<td>-33.3</td>
<td>35.2</td>
</tr>
<tr>
<td></td>
<td>±0.9</td>
<td>±1.8</td>
<td>±4.6</td>
<td>±1.4</td>
<td>±1.0</td>
</tr>
</tbody>
</table>

* serosal side contained 10mM under all conditions

$x \pm$ s.e. (number of observations; number of tissues), see text.
Figure 4.7  Effects of sequential cAMP and mucosal K additions on membrane potentials, intracellular K and Cl activities, $a_K^a$, $\Delta \mu_K^a/F$ and $a_{Cl}^{-a}$, $\Delta \mu_{Cl}^{-a}/F$ under open-circuit conditions during serosal perfusion with normal saline (10 mM K). For definitions see legends of Figures 4.2 and 4.6. Measurements were made 30-60 min after exposure to each condition. Note that $a_K^c$ is near normal levels when the mucosal side is K-free and serosal $[K] = 10$ mM. Also, the electrochemical gradient opposing Cl entry declines progressively as mucosal $[K]$ is elevated (compare Fig. 4.6). Results are from one preparation. Means $\pm$ s.e.; $n = 20$ obs. ($V_a$, $V_b$), $n = 10$ obs. ($a_K^c$, $a_{Cl}^c$, $\Delta \mu_K^{a}/F$, $\Delta \mu_{Cl}^{a}/F$). See Table 4.5 for complete data.
clamped at the same potential, $V_b$ is lower at open-circuit than under $I_{sc}$ conditions (compare Figs. 6 and 7 when mucosal K activity is 7.2 mM; at short-circuit $V_b$ equals -58 mV, but at open-circuit $V_b$ equals -34 mV).

It will be shown in the following chapter that depolarization of $V_b$ alone would cause some increase in $a_{cl}^c$, since basal membrane Cl conductance is greater than that of the apical membrane. Since any increase in $a_{cl}^c$ which might be induced by depolarization of $V_b$ would also increase $\Delta\mu_{cl}^a/F$, it is clear that unknown active or passive processes at the basal membrane may, under certain conditions, affect the electrochemical gradient across the apical membrane and complicate the interpretation of the electrochemical potential profile (see discussion).

iv) When 10 mM K (concentration) is present on the serosal side as in the present experiment, increasing mucosal [K] from 0 to 10 mM causes a decrease in $\Delta\mu_{cl}^a/F$ rather than an increase as was observed when [K] was raised symmetrically on both sides of the epithium.

In summary, the results of varying [K] symmetrically under $I_{sc}$ conditions or asymmetrically under open-circuit conditions show that 1) both mucosal and serosal cell borders are highly K-selective, 2) K is always near electrochemical equilibrium across both membranes under $I_{sc}$ conditions and therefore $\Delta\mu_{K}^a/F$ could not energize Cl entry, and 3) high concentrations of potassium reduce $\Delta\mu_{cl}^a/F$ by depolarizing apical and basal membranes. Under $I_{sc}$ conditions, addition of low levels of K (2-4 mM) increases the unfavourable $\Delta\mu_{cl}^a$ across the apical membrane while stimulating net Cl transport, indicating that K directly stimulates the active mechanism. However, under open-circuit conditions in which serosal [K] is "physiological" (i.e. 10 mM conc.), this effect on $\Delta\mu_{cl}^a$ is obscured, presumably by a large (hyperpolarizing) K diffusion potential at the apical membrane which results from the high level of K in the cell and K-free mucosal solution.
3) **Effects of altering the sodium electrochemical gradient across the apical membrane**

Previous results indicated that Cl transport across locust rectum is relatively insensitive to Na removal (chapter 2). Nevertheless, even trace quantities of sodium might sustain NaCl coentry in the presence of a favourable Na electrochemical gradient across the apical membrane ($\Delta \mu^a_{Na}$) provided that the Na affinity of the carrier is exceptionally high and Na is recycled. Therefore the relationship between transepithelial Cl fluxes and $\Delta \mu^a_{Na}/F$ was directly examined during cAMP exposure (see chapter 2 for flux methods). $^{36}$Cl fluxes were measured while mucosal [Na] was varied and $V_t$ was clamped at zero (mV). Corrections were made for saline resistance and for asymmetrical liquid junctions at the potential-sensing agar bridges. The serosal side contained normal saline throughout the experiment and Na levels were measured to ensure no significant changes occurred ([Na] = 115.3 ± 0.9 mM). Transepithelial $^{36}$Cl fluxes in both directions (n = 7) and Na contamination on the mucosal side (n = 14) were measured before and after the mucosal side was rinsed with N-methyl-D-glucamine saline. The measured Na level was 48.8 ± 0.32 µM in nominally Na-free saline. Thirty minutes after the first wash, mucosal [Na] was 6.81 ± 0.98 mM (n = 14). This contamination level was due to i) residual Na which had not been completely out of the chamber and ii) Na which had been leached from the serosal chamber (115 mM [Na]), and represents the maximum Na contamination during the first two "low-Na" flux periods.

Thirty minutes after the second rinse with nominally Na-free saline on the mucosal side, Na contamination was 2.18 ± 0.74 mM (n = 14). Again this value represents the maximum Na level on the mucosal side during the third and fourth "low-Na" $^{36}$Cl flux periods.
Figure 4.8  Representative traces obtained with double-barrelled Na-sensitive microelectrode under $I_{sc}$ conditions during perfusion of mucosal side with normal saline (115 mM Na) and nominally Na-free saline (49 μM Na). Tissue was bathed in normal saline containing 1 mM cAMP on the serosal side throughout the experiment. $V_e$ was clamped at 0 mV. Values of intracellular potential ($V_{a,b}$) are shown after correction for series resistance. Intracellular Na activity ($a_{Na}^c$) is corrected for electrode selectivity using K activities measured in the same tissue under both conditions. As shown at lower right, the differential Na-sensitive trace goes off-scale when the microelectrode is retracted into nominally Na-free saline (N-methyl-D-glucamine substituted). Arrows indicate impalement (‡) and retraction (‡) from several cells of one rectal preparation.
Double-barrelled Na-sensitive microelectrodes were then used to measure $a_{Na}^c$ and intracellular potential under $I_{sc}$ conditions when the mucosal side was perfused with Na levels identical to those measured in flux experiments. It is important to note that by using the final Na contamination during each "low sodium" flux period, we undoubtedly overestimate the $\Delta\mu_{Na}^a$ favouring inward movement during the flux period. Correcting for this error, if such correction was possible, would further strengthen the conclusion that inward Na gradient does not drive Cl entry. Also, $a_K^c$ was measured immediately before or after Na measurements in every tissue and at each mucosal [Na]. The mean $a_K^c$ from at least 3 impalements was used to correct for the selectivity properties of the sodium electrodes as determined with each calibration (mean $K_{NaK} = 0.089$, see methods).

Figure 8 shows typical recordings of impalements made using double-barrelled Na-sensitive microelectrodes under $I_{sc}$ conditions. Tissues were perfused with normal saline and were exposed to 1 mM cAMP on the serosal side. Under these conditions, $a_{Na}^c$ averaged 8.01 ± 0.41 mM and intracellular potential was 58.06 ± 0.54 ($\bar{x} \pm$ s.e., 125 cells, 11 tissues). Large net electrochemical gradients favour movement of Na into the cells across both cell membranes. Also shown are measurements of $a_{Na}^c$ obtained when mucosal [Na] was 49 µM.

Figure 9 shows $^{36}$Cl fluxes and intracellular measurements made under the same conditions in parallel experiments. Not shown are periods (~5 min) when $a_K^c$ was measured, electrodes were calibrated and solutions were changed. Replacement of mucosal Na with N-methyl-D-glucamine had no detectable effect on $V_a$ or on $a_{Na}^c$, suggesting that Na permeability of the mucosal membrane is low. Importantly, reducing $\Delta\mu_{Na}^a / F$ from 127.6 ± 3.2 mV ($\bar{x} \pm$ s.e., 59 cells, 6 animals) to 22.3 ± 1.55 ($\bar{x} \pm$ s.e., 60 cells, 5 animals) had no effect on
Figure 4.9  Relationship between Na electrochemical gradient ($\Delta \mu_{Na}^{a}/F$) across the apical membrane and Cl fluxes across cAMP-stimulated locust recta when $V_{t}$ was clamped at 0 mV. Na in normal saline (bathing both sides initially) was replaced stepwise on the mucosal side with N-methyl-D-glucamine, and external Na levels were measured. Recta were repeatedly impaled with doubled-barreled microelectrodes to measure apical membrane potential ($V_{a}$) and intracellular Na activity ($a_{Na}^{c}$, corrected for measured $a_{K}^{c}$) under these conditions. Values of $\Delta \mu_{Na}^{a}/F$ were calculated from these data. A typical experiment is shown where each point (◊, □) represents one cell. $^{36}$Cl fluxes were measured in identical experiments and were independent of the inward sodium gradient across the apical membrane.
transepithelial $^{36}$Cl fluxes. This result is not consistent with predictions of NaCl cotransport if energized by the flow of Na down its electrochemical potential gradient across the mucosal membrane. The effects of $\Delta\mu_{\text{Na}}^{a}/F$ on transepithelial $^{36}$Cl fluxes have not yet been directly measured in vertebrate epithelia; however, based on comparisons of $\Delta\mu_{\text{Cl}}^{a}/F$ and $\Delta\mu_{\text{Na}}^{a}/F$, $J_{\text{Cl}}^{\text{net}}$ should decrease linearly as $\Delta\mu_{\text{Na}}^{a}/F$ is reduced (Garcia-Diaz and Armstrong, 1980).

Since transepithelial Cl transport is not inhibited by drastic reduction (i.e. 83%) in $\Delta\mu_{\text{Na}}^{a}/F$, it follows that the active Cl entry and thus $\Delta\mu_{\text{Cl}}^{a}/F$ should also be insensitive to $\Delta\mu_{\text{Na}}^{a}$. This suggestion is confirmed in Figure 10, which shows the relationship between mucosal [Na], $\Delta\mu_{\text{Na}}^{a}/F$ and $\Delta\mu_{\text{Cl}}^{a}/F$ measured under $I_{\text{sc}}$ conditions. The sodium gradient across the apical membrane varied directly with mucosal [Na], as would be expected if the apical membrane had low Na permeability. In contrast, $\Delta\mu_{\text{Cl}}^{a}/F$ did not change significantly (P > 0.05) when mucosal [Na] was decreased from 115 mM to 49 $\mu$M. Chloride was above electrochemical equilibrium by more than 38 mV, even when $\Delta\mu_{\text{Na}}^{a}$ was reversed. The "uphill" $\Delta\mu_{\text{Cl}}^{a}/F$ observed in these and in previous experiments is probably not due to artificially high estimates of intracellular Cl activity since less than 3.7% of $\Delta\mu_{\text{Cl}}^{a}/F$ may be accounted for by assuming normal intracellular anion interference (≈5 mM) and less than 14% by assuming very high (10 mM) interference during impalements.

4) Mannitol space

Although it is clear that cAMP increases $\alpha_{\text{Cl}}^{c}$ and the electrochemical gradient opposing Cl entry, both of these effects might be simply explained without postulating effects on the Cl pump if cAMP exposure results in tissue shrinkage. To examine this possibility, intracellular volume was estimated in controls and during cAMP exposure by comparing total tissue water with tissue radioactivity after equilibrating recta for 1.5 h in saline containing
Figure 4.10 Relationship between $\Delta \mu_{\text{Na}}^a / F$ and $\Delta \mu_{\text{Cl}}^a / F$ in cAMP-stimulated recta with $V_t$ clamped at 0 mV. The value of $\Delta \mu_{\text{Na}}^a$ declined linearly 58 mV/decade change in mucosal Na concentration and reversed when mucosal $[\text{Na}] \leq 1$ mM. In contrast, $\Delta \mu_{\text{Cl}}^a / F$ changed little when mucosal $[\text{Na}]$ was varied. The shaded area shows energetically feasible trajectories for $\Delta \mu_{\text{Cl}}^a / F$ if Cl were to enter across the apical membrane by "secondary" active transport energized by $\Delta \mu_{\text{Na}}^a / F$. The dashed line shows the predicted values of $\Delta \mu_{\text{Cl}}^a / F$ if Cl influx is coupled 1:1 with that of Na. A depolarizing entry stoichiometry (>1 Na per Cl) would be indicated by the shaded area below the dashed line. A hyperpolarizing entry process (i.e. less than 1 Na:Cl) would lie in the shaded region above the dashed line. Note that $\Delta \mu_{\text{Cl}}^a / F$ falls completely outside the shaded region. Mean ± s.e.; 58-125 impalements, 6-11 recta.
$^3$H-mannitol (see methods for justification). Results are summarized in Table 6. There was no significant difference in $^3$H-mannitol space or cell volume between controls (cell volume: $3.61 \pm 0.24 \mu l$; and cAMP-treated tissues: $3.79 \pm 0.24 \mu l$). $^3$H-inulin space was not determined because it does not penetrate the cuticular intima which covers the mucosal surface of the epithelium (Phillips and Dockerill, 1968). However, the estimates of cell volume from "non-mannitol space" agree well with those predicted from the dimensions of the cells measured after injection with the fluorescent dye Lucifer Yellow CH (chapter 5) and from electron micrographs (Jarial et al., unpublished).

**Discussion**

Several observations confirm the viability of the preparation used in this study as well as the validity of the microelectrode techniques. Transepithelial potential across recta in the microelectrode chamber was initially high (25-40 mV, lumen positive) and declined exponentially to reach an approximate steady-state transepithelial potential of about +8 mV. These changes in $V_e$ are similar to those observed in previous flux experiments where long-term viability of the tissue is well established (see chapter 2). The salines and the method of mounting the tissue onto the chamber were identical in flux and microelectrode experiments. In microelectrode experiments in which tissues were short-circuited, currents of 250-300 $\mu A \ cm^{-2}$ were not unusual. Stimulation of $V_e$ from ~8 to ~30 mV and reduction of $R_e$ during cAMP exposure provides further evidence that tissue properties were similar during microelectrode and tracer flux experiments. Evidence that little tissue damage is caused by mounting the preparation is provided by measurements of very low $^{35}$SO$_4$ permeability (chapter 2) and also
Table 4.6 Effect of cAMP and high osmotic pressure on total tissue water and non-mannitol space in suspended recta ¹

<table>
<thead>
<tr>
<th>Condition (n)</th>
<th>Wet weight (mg)</th>
<th>Total Tissue Water (μl)</th>
<th>³H-mannitol space (μl)</th>
<th>Calculated Non-Mannitol space (μl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (6)</td>
<td>7.07 ±0.20</td>
<td>4.87 ±0.22</td>
<td>1.26 ±0.05</td>
<td>3.61 ±0.24</td>
</tr>
<tr>
<td>1mM cAMP (7)</td>
<td>7.10 ±0.27</td>
<td>5.07 ±0.21</td>
<td>1.28 ±0.07</td>
<td>3.79 ±0.24</td>
</tr>
<tr>
<td>High osmotic pressure</td>
<td>5.60 ±0.30</td>
<td>2.80 ±0.23</td>
<td>1.97 ±0.04</td>
<td>0.43 ±0.11</td>
</tr>
</tbody>
</table>

¹Tissues were incubated for 1-1.5h suspended in vials containing ³H-mannitol-labelled normal saline, or normal saline + 1mM cAMP, or normal saline containing 600mM sucrose.

²Taken as the difference between wet weight and dry weight.

³Used as an estimate of extracellular volume.

⁴Used as an estimate of the intracellular volume.
from electrophysiological data (cable analysis and voltage scanning) to be presented in chapter 5.

In any one preparation, membrane potentials and intracellular ion activities showed little variation between cells. Potential changes were abrupt after impalement, and potentials and ion activities rarely changed during the course of measurements. Recordings were occasionally made from one cell for more than 30 min without detectable changes in membrane potentials, ion activities, or voltage divider ratios. No consistent differences were observed between results obtained with single- or double-barrelled electrodes. Double-barrelled microelectrodes are thought to cause serious impalement damage artifacts in some epithelia (see Lewis and Graf, 1979; Delong and Civan, 1978; Reuss and Weinman, 1979). The apparent lack of damage in the present study is probably due i) to the large size of locust rectal cells, and ii) to the high degree of membrane infolding which greatly increases cell membrane area, and probably reduces the importance of shunting caused by leaky impalements.

No gradients were observed when electrodes were advanced deep into the epithelium. Only one electrical potential "well" was observed when electrodes were pushed through the tissue and only one population of cells was evident from the distribution of intracellular ion activities (Fig. 4). The underlying secondary cells do not constitute a continuous layer (they are penetrated by tracheoles) and therefore they probably do not contribute greatly to $R_t$.

Measurements of membrane potentials and intracellular ion activities

Membrane potentials observed in this study are "typical" for many vertebrate and invertebrate cells, and agree well with values reported for isolated recta of Locusta migratoria ($V_a = -65$ to $-59$ mV, $V_b = -45$ to $-47$ mV over the first 4 h; Vietinghoff et al., 1969). Lower membrane potentials in
Schistocerca recta were observed by Phillips (1964b; $V_a = -50$, $V_b = -37$), however his measurements were made in situ using microelectrodes with large tip diameters, thus differences might be due to the different experimental conditions used, or more probably, to impalement damage.

The structural complexity of the serosal cell border makes it difficult to interpret results strictly in terms of the basal membrane (see chapter 1). Localized regions of ion recycling at the lateral membrane would not be detected using these microelectrode techniques. It is noteworthy that membrane potentials in insect epithelia are not always similar to those in vertebrate preparations; basal membrane potentials of $\sim 1$ mV have been described in K-transporting cells of silkworm midgut epithelium (Blankemeyer and Harvey, 1978).

Ion activities have not previously been measured in locust rectal cells, however, intracellular activities of Cl (30-46 mM), K (60-70 mM) and Na (8-10 mM) are similar to most vertebrate epithelia, with the possible exception of $a^C_K$ which is perhaps slightly lower than average. Somewhat higher $a^C_K$ (133 mM) and lower $a^C_{Cl}$ (9-10 mM) have been reported in blowfly salivary gland, although in this preparation, the mucosal surface is normally bathed with KCl-rich fluid secreted by the gland rather than normal "low-K" saline (Berridge and Schlue, 1978; Berridge, 1980). In the lepidoteran midgut, which has 2 major cell types, Blankemeyer and Duncan (1980) reported that the "low PD" cells ($V_b = 0$) have $a^C_K$ of 36.2 mM (approximately equal to that of the bathing saline) whereas in "high PD" cells ($V_b = -25$ mV), have $a^C_K$ of 75.7 mM. The "low PD" cells are thought to secrete K from serosa to mucosa. Intracellular ion activities in the present study are in reasonable agreement with concentrations measured chemically in homogenates of whole rectal tissue (55 mM Na, 88 mM K, 39 mM Cl; recta from water-fed locusts; Phillips, 1964b)
and in very good agreement with concentrations measured by electron microprobe in blowfly rectal papillae (23-47 mM Na, 65-85 mM K, 23-43 mM Cl; Gupta et al., 1980). Comparison of ion concentrations measured in homogenates of whole tissue with intracellular ion activities obtained using microelectrodes is complicated by the presence of ions in the extracellular space. This could explain why [Na] is so much higher in rectal homogenates compared to microelectrode measurements. Differences in concentrations (electron microprobe) and ion activities (this study) are well within the range which can be expected based on wide range of activity coefficients which have been reported in other tissues (see reviews by Edzes and Berendsen, 1975; Lev and Armstrong, 1975; Civan, 1978, 1980). The data available for locust rectum do not allow reliable calculations of intracellular activity coefficients. However, microelectrode and electron microprobe measurements have been made in blowfly salivary glands under similar conditions (i.e. during peritubular perfusion of tubules with saline and in tubules suspended in saline (Gupta et al., 1978). The results indicate that the K activity coefficient is near one as in amphibian oocytes (Palmer et al., 1978; Horowitz et al., 1979), however approximately 30-40% of intracellular Cl may be bound. Low activity coefficients have been reported in amphibian oocytes using both microelectrode (γNa = 0.08 ± 0.02; Palmer et al., 1978) and reference phase analysis (γNa = 0.22; Horowitz et al., 1979); however, NMR data are not consistent with intracellular Na binding (evidence reviewed by Civan, 1978).

**Location and mechanism of active chloride transport**

Chloride entry across the apical membrane occurs against a net electrochemical gradient under both open- and short-circuit conditions. Net Cl flux across both membranes is in the mucosa-to-serosa direction as judged by previous tracer measurements under both open- and short-circuit conditions.
Consequently, Cl entry is an active process for which energy could be obtained through coupled ion movements such as NaCl cotransport or alternatively, from an exergonic reaction such as ATP hydrolysis. In vertebrates, microelectrode studies of Cl-absorbing epithelia also indicate an apical active step (Duffey et al., 1978; Spring and Kimura, 1978; Reuss and Weinman, 1979; Reuss and Grady, 1979; Duffey et al., 1979; Garcia-Diaz and Armstrong, 1980). Results from this wide variety of vertebrate epithelia are consistent with a model involving sodium-coupled Cl entry energized by the inward flux of Na down its net electrochemical gradient (Δμ^a_{Na}), as suggested by Quay and Armstrong (1969), Nellans et al. (1973) and by Frizzell and coworkers, and reviewed by Frizzell et al. (1979). In contrast, the insensitivity of J^Cl^ to a very large reduction in Δμ^a_{Na} in locust rectum, and the lack of correlation between Δμ^a_{Cl} and Δμ^a_{Na} provides strong evidence that Cl does not enter by this mechanism, as indicated previously by other observations (see chapter 2).

Both 1 mM cAMP and low concentrations of K stimulate steady-state J_Cl^net, Cl-dependent I_sc, and the Δμ^a_{Cl} opposing Cl entry across the apical membrane. The effects of K on Δμ^a_{Cl} are not compatible with stimulation of Cl transport solely by electrical coupling across either the apical membrane or the entire rectal wall. Instead, K must stimulate the active entry mechanism. Also, small quantities of K stimulate I_sc only when added to the mucosal side.

In the presence of 10 mM K on the serosal side of locust rectum, Cl-dependent I_sc remains very low (Fig. 13 of chapter 3), a^c_K is maintained at control levels (74.8 mM) under these conditions (Table 4). This result strongly suggests that mucosal potassium acts at the external surface of the apical membrane when stimulating active Cl transport, and not through some action on a^c_K.
Intracellular ion activities are determined by the properties of both cell membranes. For example, if the Cl conductance of the serosal border is much higher than that of the apical membrane (as will be shown in chapter 5), changes in $a_{Cl}^c$ will largely depend on changes in $V_b$, even when $a_{Cl}^c$ is maintained above equilibrium across the serosal membrane by the action of an apical Cl pump. Short-circuit current, which in this tissue hyperpolarizes $V_b$ and depolarizes $V_a$ by approximately equal amounts, will therefore tend to reduce $a_{Cl}^c$; i.e., more Cl will be driven out of the cell by hyperpolarization of $V_b$ than will be drawn in by depolarization of $V_a$. In fact, significantly higher levels of $a_{Cl}^c$ were observed under open-circuit conditions ($46.2 \pm 1.3$, 49 cells, 5 tissues) than under $I_{sc}$ conditions ($40.3 \pm 0.9$, 40 cells, 4 tissues; $P < 0.01$).

Changes in cell volume may alter ion activities and electrochemical gradients independently of ion transport. If cAMP causes a 50% reduction in cell volume, this might account for the changes in $a_{Cl}^c$ and $\Delta u_{Cl}^a$ observed in this study. The results shown in Table 6 suggest that cell volume is not greatly affected by cAMP. Total tissue water, extracellular volume (space labelled by $^3$H-mannitol) and intracellular volume (non-mannitol space, NMS) were not different in controls and cAMP-treated tissues ($P > 0.2$). Two observations suggest that NMS provides a reasonable estimate of cell volume. First, exposing tissues to high osmotic pressure (1.2 osm/l) drastically reduced NMS and increased mannitol space as expected if water were drawn out of the cells. Second, we may predict approximately what the intracellular volume of the epithelium should be, based on cell dimensions. As described in chapter 1, the columnar epithelial cells are approximately 17 μm by 80-100 μm (judging from cells filled with fluorescent dye and from photomicrographs of rectal tissue). Assuming a cylinder with a 20 μm diameter base and height of 80 μm, I estimate the volume of one cell to be $2.5 \times 10^{-5}$ mm$^3$ and the number
of cells/tissue area to be $\sim 2 \times 10^5$ cells/cm$^2$. Since the area of one stretched male locust rectum is $0.66 \pm 0.02$ cm$^2$ ($\bar{x} \pm$ s.e., n = 6) and $(2.5 \times 10^{-5}) \times (2 \times 10^5) = 5 \mu l/cm^2$, then the total cell volume is calculated to be 3.3 $\mu l$, in good agreement with the measured NMS of 3.61 $\pm$ 0.24 $\mu l$. The slightly larger value of NMS may be due to other cell types such as muscles and "secondary" cells which would also exclude the $^3$H-mannitol. In summary, NMS appears to give reasonable estimates of cell volume. Since no changes in NMS were measurable during cAMP exposure, it is unlikely that the changes in $\Delta v_{Cl}^c$ and $\Delta u_{Cl}^a$ observed in this study are due to cell shrinkage but instead are due to ion transport processes. Also, one might expect volume changes to be temporary as the ions equilibrate, or perhaps were corrected by active cell volume regulation.

It must be emphasized that events at the serosal border do not affect conclusions regarding the location of the active step or the stimulatory effect of cAMP and K on the active process; these are based on measurements of both $\Delta u_{Cl}^a$ and $J_{Cl}$ measured in tissues under the same steady-state conditions. By definition of the steady-state, the net flux of Cl across apical and basal membranes and the entire epithelium are equal. Consequently, measurements of transepithelial Cl flux and $\Delta u_{Cl}^a$ are sufficient to calculate the energetic requirements of Cl entry across the apical membrane. A potassium-coupled influx could supply little if any energy for Cl transport across the apical membrane. In contrast, sodium entry is strongly favoured when mucosal Na is artificially high (115 mM, normal saline; $\Delta u_{Na}^a/F = 122$ mV; Fig. 9) and is more than adequate to energize Cl entry with 1:1 stoichiometry ($\Delta u_{Cl}^a/F = -45$ mV). However, no interdependences between $\Delta u_{Na}^a/F$ and $J_{Cl}$ (Fig. 9) or $\Delta u_{Na}^a/F$ and $\Delta u_{Cl}^a/F$ (Fig. 10) were observed and there is much evidence that Cl fluxes and Cl-dependent $I_{sc}$ are insensitive to sodium removal (chapter 2). Alternatively, Cl entry might be coupled to that of H and energized by an inward electrochemi-
cal gradient for protons ($\Delta u_H^{\text{H}} \approx 60 \text{ mV in most cells}$). This seems very unlikely since $\text{Cl}$-dependent $I_{\text{sc}}$ is not affected by raising saline pH from 6.2 to 8.0 (Fig. 16 of chapter 2), a manoeuvre which should reverse or at least greatly diminish $\Delta u_H^{\text{a}}$ since the pH in most cells is in the range 7.1-7.4 (reviewed by Roos and Boron, 1981). Furthermore, reducing mucosal pH inhibits rather than stimulates $I_{\text{sc}}$ as would be predicted for $\text{HCl}$ coentry (half maximal inhibition at approximately pH 4.7).

Finally, any coupled entry mechanism must involve active recycling of the unknown ion back to the same side of the membrane in order to explain the equivalence of $I_{\text{sc}}$ and $J_{\text{Cl}}^\text{net}$. There is much evidence that $\text{Cl}$ transport across the locust rectum is dependent on aerobic metabolism; extensive tracheation of the tissue, very abundant mitochondria, sensitivity of $I_{\text{sc}}$ to inhibitors of aerobic respiration (Williams et al., 1978; Baumeister et al., 1981; Chamberlin, 1981; this thesis, chapter 1). A $\text{Cl}$-ATPase pump should be considered in locust rectum. Anion-stimulated ATPase activity has been reported in epithelial plasma membrane preparations in which great care was taken to remove mitochondrial contamination (Humphreys and Chow, 1978; Bornancin et al., 1980) although even recent reports have been criticized (Bonting et al., 1980). Plants from salt marshes which actively secrete $\text{Cl}$ are known to have a $\text{Cl}$-stimulated ATPase (Hill and Hill, 1973; Hill and Hanke, 1979). There is some recent (but less convincing) evidence for anion-stimulated ATPases in recta of locusts (Herrera et al., 1978) and dragonfly larvae (Komnick et al., 1978). In these reports, no marker enzymes were assayed to check against mitochondrial contamination, although electron microscopic examination and parallel Na/K ATPase assays were employed in the latter study.

Interpretation of potassium and sodium electrochemical potential profiles

Transepithelial absorption of K across locust rectum under open-circuit conditions is largely passive, and electrically coupled to active $\text{Cl}$ absorption
(≈80%, chapter 3). It will be shown in chapter 5 that ions move mainly through the cells rather than by a paracellular route. It is clear from Table 3 that both apical and basal membrane net electrochemical gradients favour passive K movement in the mucosa-to-serosa direction. A small $J_{net}^K$ was observed previously under $I_{sc}$ conditions; however, it is difficult to localize the active step in the present experiments because $\Delta \mu^a_K/F$ and $\Delta \mu^b_K/F$ are extremely small when recta are short-circuited (Fig. 6).

High selectivity of the membranes for K presumably causes intracellular potentials to remain very close to the equilibrium potential for K when recta are short-circuited. Finally, a small $J_{net}^K$ under $I_{sc}$ conditions (0.8 μEqcm⁻² h⁻¹) could result from solvent drag since water is actively absorbed with ion by this tissue. If a "solvent-drag" K flux exists and is paracellular, no "uphill" step would be observed across either cell membrane using ion-sensitive microelectrodes. Further experiments are required to characterize the active step for the small K transport component.

There is evidence for active sodium absorption across locust rectum (Phillips, 1964b; Williams et al., 1978; Spring and Phillips, 1980), however this net flux must be electrically silent since Na removal does not affect $I_{sc}$ and $J_{net}^Cl$ equals $I_{sc}$. Some Na is taken up at the apical membrane with amino acids (Balshin and Phillips, 1971). The present study suggests two similarities between locust rectal epithelium and typical Na-transporting epithelial in vertebrates.

Firstly, passive Na entry across the apical membrane is favoured by a large net electrochemical gradient of 120 mV when a high NaCl saline is present on both sides. Secondly, ion-sensitive microelectrode data are consistent with a basal Na/K exchange since i) for transepithelial Na absorption, any Na entering the cells from the mucosal sides must be actively pumped across
the basal membrane against a large $\Delta_{Na}^{b}$/F (≈120 mV) as in vertebrates, and
ii) K-depleted rectal cells rapidly accumulate K from the serosal, but not from the mucosal side.

This serosal Na/K exchange, if it exists, may be relatively insensitive to ouabain. Membrane potentials and $a_{Na}^{c}$ were measured ($n = 10$ cells) at 1 h intervals for 3 h during perfusion of the serosal side (temperature = 22°C) with 1 mM ouabain and no changes were observed (Hanrahan and Black, unpubl. obs.). There is some controversy regarding the effects of ouabain in insect tissues, although many epithelia do appear to be insensitive (see reviews by Jungreis, 1977; Anstee and Bowler, 1979; Harvey, 1981). However ouabain may not reach the Na/K pump sites in the lateral spaces because of the "sweeping away" effect of net water absorption, the complexity of the basal cell border, and the presence of sub-epithelial tissue (Irvine and Phillips, 1969). It may be argued that a "sweeping out" effect due to fluid transport through limited exit points impedes ouabain penetration of the serosal border. However, the fact that cAMP which is similar in size to ouabain stimulates recta within minutes, makes this interpretation unlikely unless cAMP acts more externally in the tissue. Diffusion of ouabain to sites in the lateral intercellular spaces may be more difficult than movement of cAMP into the cells across the basal borders of the epithelial cells. Access of labelled ouabain to the pump sites must be demonstrated in this tissue before physiological sensitivity to ouabain can be ruled out, particularly since Na/K-dependent ATPase in homogenates of locust rectum is highly sensitive to ouabain (Peacock, 1981). It is still unclear whether this Na/K ATPase is present in muscle or epithelial layers or both.

The presence of a K-stimulated, rather than a Na-coupled mechanism for Cl transport may be advantageous given that the fluid entering the rectal lumen is
K-rich (140 mM) but low in Na (20-40 mM). Since [Cl] in this fluid is high (≈88 mM), coupled NaCl cotransport could recover only 25-50% of the total Cl present in the rectal lumen. Moreover, Na-coupled amino acid absorption would further reduce the amount of Na available for NaCl cotransport. For example, proline is actively secreted by the Malpighian tubules at high concentrations (38 mM) and is reabsorbed in the rectum where it serves as the main respiratory substrate to sustain Cl transport in the rectum (Chamberlin, 1981). It has been suggested that most of the Na in Malpighian tubule fluid is reabsorbed by a coupled process with organic substrates in the primary urine (Phillips, 1981).

It is clearly more efficient to absorb most K from the rectal lumen by a passive process since potential energy is available in the K electrochemical gradient created by tubular secretion (see chapter 3 for calculations). Because the rectal wall is highly K-selective, $V_t$ immediately reverses polarity if Cl is removed from the mucosal side. This result has been obtained in vitro and also during perfusion of recta in vivo (unpubl. obs.). No net K absorption can occur in the absence of electrogenic Cl transport since $V_t$ under these conditions is exactly equal to the transepithelial equilibrium potential for K (−67.4 mV). The function of active Cl transport may therefore be to cause passive K reabsorption by making $V_t$ more positive than the equilibrium potential for K. Direct modulation of the Cl pump by K would then represent an interesting control mechanism for passive K absorption analogous to enzyme-catalysed reactions which have substrate "feed-forward" activation.
Summary

Membrane permeability and its regulation by cAMP has been studied in locust rectum using electrophysiological and tracer methods. Transepithelial resistance ($R_t$) is $220 \ \Omega\text{cm}^2$ under control conditions, typical of moderately "leaky" epithelia. However, the mucosal and serosal surfaces have very different permeability properties as judged by the response of transepithelial potential to salt gradients. This asymmetry is suggestive of a transcellular route for passive ion movements. Voltage scanning did not reveal current leaks between the cells, between the rectal "pads" or at the edge of the tissue. Extensive cell-cell coupling was demonstrated by dye iontophoresis and by measuring voltage deflections in neighbouring cells during intracellular current injection. Resistances of the apical and basal membranes ($R_a$ and $R_b$) and the paracellular pathway ($R_j$) were obtained by flat-sheet cable analysis. "Tightness" of the epithelium increased during exposure to cAMP. Both $R_a$ and $R_b$ decreased by 80%, $R_t$ declined by more than 50%, and $R_j$ was not changed. The cAMP-induced $\Delta R_b$ was abolished in Cl-free saline, suggesting that $\Delta R_b$ results from a massive increase in basal membrane Cl conductance. When compared with previous measurements of the Cl electrochemical gradient, this increase in conductance is more than adequate to allow Cl exit from the cell by electrodiffusion when transepithelial flux is enhanced 10-fold. In contrast, cAMP-stimulated $\Delta R_a$ is insensitive to Cl removal, but is abolished under K-free conditions. Therefore, $\Delta R_a$ is interpreted as a cAMP-induced K conductance in the apical membrane, a conclusion which is consistent with the previous finding that cAMP stimulates transepithelial $^{42}\text{K}$ permeability.
Finally, an inverse relationship between apical membrane K permeability and external [K] was observed:

i) transepithelial $P_K$ calculated from $^{42}K$ backflux declined as [K] was elevated on both sides of the epithelium;

ii) $^{42}K$ fluxes in both mucosa-to-serosa, and serosa-to-mucosa, directions were stimulated 4-fold by cAMP in normal saline, but not in "high-K" saline;

iii) when mucosal [K] was elevated from 40 to 140 mM, $R_t$ remained constant while $R_a/R_b$ increased 5-fold, indicating a decrease in apical membrane conductance.

The results are interpreted using an equivalent electrical circuit model for transepithelial KCl transport.
Introduction

The preceding chapters provide evidence for an unusual chloride transport mechanism in the rectum of the desert locust. In order to understand ion transport across epithelia, it is also essential to know the permeability properties of the cell membranes and tight junctions. Epithelia are classified into two categories according to the relative importance of transcellular and paracellular routes for passive ion movements. "Tight" epithelia are those in which the cell junctions effectively seal off the paracellular pathway so that fluxes occur predominantly through the cells. In contrast, junctions in "leaky" epithelia have low electrical resistance and cause most passive fluxes to occur paracellularly (see Frömter and Diamond, 1972). Locust rectal epithelium has some properties associated with both types of junctions. This epithelium generates large electrical (35 mV), chemical (20-fold), and osmotic (600 mOsm/l) gradients indicative of "tightness", but has low electrical resistance consistent with moderately "leaky" epithelia. Since the relative resistances of transcellular and junctional (paracellular) routes have not been directly measured in locust rectum, the first part of this chapter investigates the passive permeability of locust rectum using electrophysiological methods. The effects of cAMP on membrane conductances are then measured during ion substitutions in order to identify which ion permeabilities are stimulated. Details of active Cl absorption, including the driving force and internal resistance of the transport mechanism, are deduced from membrane resistances and intracellular ion activities using a simple equivalent circuit model, and compared to independent estimates based on the relationship between short-circuit ($I_{sc}$) and transepithelial conductance ($G_t$). The results in this and preceding chapters are incorporated into a model for transepithelial ion transport and its regulation in locust rectum. In addition, this study
provides an opportunity to test several electrophysiological methods and their underlying assumptions on a very unusual epithelium.

Materials and methods

Details of the dissection and chambers are given in chapters 2 and 4. The microelectrode chamber (chapter 4) was used only when intracellular measurements were required. All other experiments were performed with the flux chambers of Williams et al. (1978; chapter 2). Bathing salines were identical to those described in chapter 2, except when the effects of single salt gradients were measured (see next section). Transepithelial potential ($V_t$), resistance ($R_t$) and short-circuit current ($I_{sc}$) were measured as previously described (chapters 2 and 4).

Effects of transepithelial salt gradients

$V_t$ was measured during exposure of the tissue to transepithelial concentration differences of various salts. Salines containing 200 mM NaCl, KCl, choline Cl, or K-methylsulfate were mixed separately with sucrose (400 mM) in order to give intermediate concentrations of each salt (2, 8, 40 and 120 mM). All solutions also contained the normal eleven amino acids as listed in chapter 2 as energy sources. After tissues had equilibrated in HCO$_3$-free saline for 3 hours, both sides were exposed to the full-strength salt solutions described above and re-equilibrated for a further 30 minutes. The mucosal or serosal side was then rapidly rinsed with lower concentrations of the same saline, alternating with the 200 mM full-strength solutions (e.g. sequence: 200, 2, 200, 8, 200, 40, 200, 120, 200 or reverse order). A vacuum pump was used to drain the chambers as fresh saline was injected by syringe. Solutions changes were completed within 10 seconds. Control experiments showed that neither $V_t$ nor $R_t$ were altered artifactually by the technique of changing solution. Also, $V_t$ returned to approximately the same value.
between each test solution. The average difference before and after was
+0.32 ± 0.32 mV following mucosal substitutions and +0.34 ± 0.12 mV after
serosal substitutions (x ± s.e., n = 214). Net liquid junction potentials
were measured by replacing the rectum with a short 3 M KCl agar bridge. It is
clear that correcting for such errors will strengthen our conclusion that
transepithelial permeability properties are asymmetrical, since liquid junc-
tion potentials at the agar bridges would be of similar magnitude when
testing gradients in opposite directions, and would therefore tend to increase
the symmetry of \( V_t \) responses.

When solutions were changed on the mucosal side, new quasi-steady-state
transepithelial potentials were observed within 10 sec. Responses were
slower when solutions were changed on the serosal side, often requiring several
minutes to reach a stable value when the largest gradients were tested. This
delay is probably due to subepithelial diffusion barriers (secondary cells,
and muscle bands) and the unstirred layer associated with them; however, the
possibility of changes in intracellular composition cannot be ruled out during
these slow transients at the serosal side.

**Voltage scanning**

This technique was used to explore the surface of the epithelium for
regions of high conductance (see Fromter, 1972; Higgins et al., 1975; Lewis et
al., 1976). Current (250 \( \mu \text{Acm}^{-2} \)) was passed transepithelially while the
potential difference between a "scanning" microelectrode (which was moved over
the tissue surface) and the mucosal 3 M KCl agar bridge was monitored in order
to determine the uniformity of the electrical field. The voltage between
scanning electrode and agar bridge was measured using a differential electromet-
er (FD 223, W.P. Instr., New Haven, Conn.). This signal was filtered,
displayed on a storage oscilloscope, and recorded using a pen recorder as
described in chapter 4.
Intracellular injection of fluorescent dye

The fluorescent dye Lucifer Yellow CH was injected intracellularly by iontophoresis. A 3-5 mm column of dye was placed into the tip of a single-barrelled microelectrode by immersing the blunt end of the electrode into a 5% solution of Lucifer Yellow CH in distilled water (approx. 0.1 M). Electrode tips were less than 1.0 μm in diameter and had resistances of 25-40 MΩ or 10-15 MΩ after bevelling in a stream of abrasive according to the method of Ogden et al. (1978). Cells were impaled at an angle of 30° to the plane of the epithelium as described previously. Hyperpolarizing current was passed through the microelectrode by a constant current source (M 701, W.P. Instr., New Haven, Conn.), driven by waveform and pulse generators (Type 160 series, Tektronix, Beaverton, Ore.). A switch allowed passage of direct current or pulses. Current was monitored continuously using a storage oscilloscope. As a precaution, a second electrometer (616, Keithley Instr., Cleveland, Ohio) was used to measure current flowing through the microelectrode during dye injection and this was displayed digitally. Continuous impalement during dye injections was ensured by switching to pulse mode and observing the membrane potential between pulses. Also, a standard bridge was occasionally used to inject current and measure membrane potential simultaneously through the same electrode. Sudden increases in "apparent" input resistance during Lucifer Yellow CH injection indicated tip blockage by dye particles. This problem was usually remedied by switching from direct current to pulse mode (1 sec duration, 0.5 Hz) for 0.5-1.0 min. Currents ranged between -5 and -50 nA during dye iontophoresis in different experiments. Two cells, at least 1 mm apart, were each injected for 30-45 min. This protocol allowed dye from the first injection to continue diffusing as the second cell was being filled; however, this should not affect the results noticeably since the dye spreads within seconds by diffusion (~200 μm/sec estimated by Stewart,
1978) and then reacts preferentially with the nuclei in locust rectal cells. The preparation was perfused on both sides with oxygenated normal saline during injections. For processing, recta were pinned onto thin wafers of polymerized Sylgard 184 (Dow Corning Corp., Midland, Mich.; 2.3 cm$^2$ with 0.2 cm$^2$ hole cut in the centre) and fixed in 4% paraformaldehyde buffered with 0.1 M phosphate at pH 7.2. After at least 1 h of fixation, recta were dehydrated in ethanol (50, 80, 100% for 5, 5, and 10 min, respectively) and then cleared for 5 min in methyl salicylate. Fluorescent cells were observed microscopically in whole mounts with incident light excitation at 125x or 500x magnification (Orthoplan, Leitz Wetzlar, W. Germany). Photomicrographs were taken using an automatic camera attachment (Orthomat W, Leitz Wetzlar) and 35 mm Kodak Ektachrome 160 (Tungsten) film.

**Electrical coupling and flat-sheet cable analysis**

Two single-barrelled microelectrodes were used to study cell-cell electrical coupling and to perform cable analysis. Current pulses (200 nA, frequency = 0.3 Hz, duration = 1 sec) were passed intracellularly through one microelectrode, and the resulting voltage deflections were measured in other cells using the second microelectrode (Fig. 1). Hyperpolarizing currents were usually used although depolarizing pulses gave similar results. The distance between current-injecting and voltage-sensing microelectrodes was measured using a calibrated eyepiece micrometer. Voltage responses were displayed on a storage oscilloscope after filtering (3 db at 5 Hz) and were measurable when greater than $\sim$0.3 mV. The mucosal and serosal sides are effectively short-circuited with respect to intracellular current in these experiments since the total resistance of the epithelium is much less than the resistance of the basal membrane in the region of current spread ($<0.5\%$ in locust rectum; see also Fromter, 1972; Lewis et al., 1976). The voltage spread adjacent to the
Figure 5.1  Method used for measuring the radial spread of current in rectal epithelium. Deflections in apical membrane potential ($V_a$) were measured by microelectrode "a" while current pulses were injected intracellularly through microelectrode "b". See text for details.
site of current injection is described by the differential equation (see Eisenberg and Johnson, 1970; Shiba, 1971; Frömter, 1972; Reuss and Finn, 1974, 1975; Lewis et al., 1976):

\[
\frac{d^2V}{dx^2} + \frac{1}{x} \frac{dV}{dx} - \frac{V}{\lambda^2} = 0 \tag{1}
\]

where \( V \) is the voltage deflection at some distance \( x \), and \( \lambda \) is the space constant defined as \( \sqrt{\frac{R_z}{R_x}} \). \( R_z \) is the effective input resistance (resistance to ground: \( R_a R_b / (R_a + R_b) \) in \( \Omega \text{cm}^2 \) and \( R_x \) is the resistance to current flow within the epithelial sheet in \( \Omega \). Under the condition \( V \to 0 \) at \( x = \infty \), the solution of Eq. (1) is \( V = A K_0(x/\lambda) \), where \( K_0 \) is the zero-order modified Bessel function, \( A \) is an integration constant (mV), and \( x \) is distance (\( \mu \text{m} \)).

Deflections in apical membrane potential were measured as a function of distance and compared to a set of curves obtained by drawing the Bessel function at 14 values of \( \lambda \) between 50 and 800 \( \mu \text{m} \) from published tables (Olver, 1967). Data were fitted by inspection to give values of \( A \) and \( \lambda \) (see Figs. 10 and 13) which were then used to calculate \( R_z \) according to

\[
R_z = 2 \pi A \lambda^2 / I_o
\]

where \( I_o \) is the current injected intracellularly (\( \mu \text{A} \)) and symbols are described as above.

The ratio of apical to basal membrane resistances (\( \alpha \)) was calculated, after corrections for series resistance, from the deflections in apical and basal membrane potentials produced by transepithelial current pulses (\( I_t = 20 \mu \text{A/} \text{cm}^2 \), frequency = 0.3 Hz, duration = 1 sec) as

\[
\alpha = \frac{\Delta V_a}{\Delta V_b} = \frac{R_a}{R_b} \tag{2}
\]
Resistance of the lateral intercellular space should not cause a significant underestimate of $R_a/R_b$ in this tissue since $\Delta V_a/\Delta V_b \leq 2$ and since the ratio of apical membrane:paracellular resistance is less than 4 (see Boulpaep and Sackin, 1980).

Transepithelial resistance was determined as

$$R_t = (\Delta V_a + \Delta V_b)/I_t$$

where $I_t$ is transepithelial current as described above.

Apical membrane resistance ($R_a$), basal membrane resistance ($R_b$), and junctional resistance ($R_j$) were then determined using the standard equations:

$$R_a = (1 + \alpha)R_z$$

$$R_b = (1 + \alpha)R_z/\alpha$$

$$R_j = (R_t^a R_a + R_t^b R_b)/(R_a + R_b - R_t)$$

which follow from the "lumped" equivalent electrical circuit model usually applied to epithelial tissues (Fig. 2). For further details regarding flat-sheet cable analysis, see references for equation (1). Equivalent electromotive forces for apical and basal membranes are calculated from mean resistances and membrane potentials by a circuit analysis described in the discussion section.

Transepithelial $^{42}$K fluxes

The unidirectional flux of $^{42}$K from serosa to mucosa ($J_{sm}^{K}$) may be used as a measured of transepithelial K permeability (see chapter 2 for assumptions). In order to quantify the effects of $[K]$ on $P_{K}^*$, the following protocol was used: Recta were equilibrated under $I_{sc}$ conditions for 3-4 hours in K-free saline and then exposed to 1 mM cAMP on the serosal side. After a new steady-state $I_{sc}$ was obtained, K-methylsulfate was added to both sides of the epithelium so that $K$ conc. increased stepwise (0, 2, 4, 10, 40, 100, 140, 200 mM). The serosa-
Figure 5.2  Equivalent circuit model of locust rectum. Shown are resistances of the apical and basal membranes ($R_a$, $R_b$), and tight junctions ($R_j$), and net electromotive forces at each of these barriers ($E_a$, $E_b$ and $E_j$). M, S and C indicate mucosal, serosal and intracellular compartments, respectively. The resistance of the cytoplasm and intercellular junctions to current within the epithelium ($R_x$) is also indicated diagrammatically. Note that unlike epithelia in vertebrates, gap junctions between insect recta cells are distributed along the entire intercellular space; see Lane, 1979, 1981).
to-mucosa flux of $^{42}$K was measured at each concentration for two 15-min periods. Previous experiments showed that the tracer flux measured during the first period included tracer equilibration and was therefore not used in calculations. Sampling and counting methods were identical to those described in chapter 3.

Results

1) Effects of salt gradients on transepithelial potential

Junctional complexes form passive barriers to diffusion across epithelia and probably do not have intrinsic rectifying properties. If the paracellular pathway is "leaky" and constitutes the major route for transepithelial ion diffusion, the effects of salt gradients on $V_t$ should be symmetrical and determined primarily by the properties of this barrier rather than by the permeability of apical and basal cell membranes. Conversely, if the junctions are in fact "tight", then $V_t$ responses may be very different depending on the direction of the salt gradient.

Figure 3 shows representative traces of transepithelial potential obtained in the presence of salt gradients. In this case, the mucosal side was exposed to various concentrations of NaCl while serosal [NaCl] remains constant at 200 mM. When NaCl and KCl solutions were used, $V_t$ reached a maximum within seconds and then declined. When choline solutions were used, the initial spike did not occur although $V_t$ still declined in the same manner. Only the initial deflections were used for calculating permeability properties. As may be seen in Figure 3b, deflections in $V_t$ were less abrupt when solutions were changed on the serosal side. Transients which sometimes occur at the serosal border were not studied in detail; nevertheless $\Delta V_t$ during mucosal solution changes are considered more reliable simply because they occurred instantly, with no opportunity for alterations in intracellular ion levels.
Figure 5.3 Representative recordings of $V_e$ during exposure to transepithelial NaCl or KCl gradients. (a) Serosal (S) composition held constant (200 mM NaCl) and the mucosal side (M) was exposed to solution of increasing [NaCl] as indicated. (b) Mucosal [KCl] was maintained at 200 mM while the serosal side was exposed to various concentrations of KCl. Sucrose was added to solutions having low salt concentration in order to minimize streaming potentials.
Figure 4 summarizes the relationship between the logarithm of the imposed gradient, and the change in $V_t$ produced across locust rectum by each salt solution. The effects of choline chloride, NaCl and KCl gradients are clearly asymmetrical, i.e. changes in mucosal or serosal concentrations produce different changes in $\Delta V_t$. This result implies that transepithelial properties are determined by two barriers with different properties (i.e. cell membranes) rather than by a single, symmetrical barrier (the junctions). The selectivity of mucosal and serosal surfaces may be assessed semi-quantitatively by treating $\Delta V_t$ as a liquid junction potential in free solution and calculating the apparent transference numbers for cation and anion using the modified Nernst-Planck-Henderson equation:

$$\Delta V_t = \left(\frac{t_+}{Z_+} + \frac{t_-}{Z_-}\right) \frac{RT}{F} \ln \frac{a_1}{a_2}$$

where $t_+$ and $t_-$ are the transference numbers of the cation and anion, $a_1$ and $a_2$ are the ionic activities on either side of the epithelium, and $\Delta V_t$, $R$, $T$ and $F$ have their usual meanings. Transference numbers were calculated as described in the methods section and are also shown in Figure 4. It is clear that the mucosal surface is selective for cations over Cl, contrary to predictions based on limiting equivalent conductances of Cl, K and Na (Weast, 1978). Assuming that Cl permeability is equal under each condition, the permeability ratios based on mucosal solution changes are Na = choline = K > MeSO$_4$ > Cl in cAMP-stimulated recta. One striking observation is that apical Cl permeability is extremely low, even when compared to the large cation choline. Also, apical membrane K permeability also seems low relative to Na although it will be shown later that high external [K] inhibits K permeability of the apical membrane. In contrast to the high cation selectivity of the mucosal border, the serosal border is only slightly cation-selective ($K > Na = Cl > MeSO_4 >>$ choline). The symmetry in $\Delta V_t$ produced by K-methylsulfate gradients is interpreted as low
Figure 5.4  Deflections in $V_t$ resulting from exposure of cAMP-stimulated recta to transepithelial salt gradients. (a) sodium chloride, (b) potassium chloride, (c) choline chloride, (d) potassium methylsulfate. See legend of Figure 3 and text for details of the method. Salt concentration was reduced either on the mucosal side (to the left of the ordinate) or on the serosal side (to the right of the ordinate). Sucrose was added to maintain approximately isosmotic conditions at low-salt concentrations. All solutions contained the standard amino acids listed in Table 2.1. Similar results were obtained using unstimulated recta and azide-poisoned tissues. Means ± s.e.; $n = 10$ recta (NaCl), $n = 6$ (KCl), $n = 6$ (choline Cl), $n = 8$ (K-methylsulfate). Apparent transference numbers were calculated by linear regression as described in the text.
methylsulfate permeability since all chloride salts gave asymmetrical results. Asymmetry implies that $\Delta V_t$ is determined by two barriers in series, each having different permeability properties. These barriers are almost certainly the apical and basal membranes; underlying muscle and secondary cells do not form a continuous layer due to penetration by tracheoles.

2) **Effects of cAMP exposure and Cl-removal on the voltage-divider ratio**

Results in the previous section suggested that the mucosal surface has very low Cl permeability compared to cations whereas Cl permeability is relatively high at the serosal border. If this interpretation is correct, then the ratio of apical-to-basal membrane resistance (voltage-divider ratio or "$\alpha$") should decrease when Cl is replaced by an impermeant anion.

Table 1 shows the effects on $\alpha$ of sequential cAMP additions (1 mM to serosal side) and gluconate substitution. Double-barrelled microelectrodes were used in this particular experiment. Addition of cAMP had no significant effect on $\alpha$ ($P > 0.2$) although tissue conductance increases dramatically during cAMP exposure (chapters 2 and 3). It will be shown that both apical and basal membrane resistances decrease by similar amounts under these conditions so that the ratio $R_a/R_b$ remains constant.

When Cl was removed from both sides, $\alpha$ decreased from 2.01 ± 0.34 to 0.52 ± 0.09. This four-fold increase in $R_a/R_b$ is consistent with low apical membrane Cl permeability and strongly suggests that the effects of transepithelial salt gradients on $\Delta V_t$ observed in the preceding section were determined by the selective permeability of the cell membranes rather than by the tight junctions.

3) **Voltage scanning**

When current was passed through the epithelium by silver foil electrodes at opposite ends of the chamber, a "scanning" microelectrode was used
Table 5.1  Relative resistance of apical and basal membranes ($\alpha$) as calculated from deflections in apical and basal membrane potentials during transepithelial constant-current pulses

<table>
<thead>
<tr>
<th>Normal saline (control)</th>
<th>Normal saline + 1mM cAMP$^1$</th>
<th>Cl-free saline + 1mM cAMP$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\alpha$</td>
<td>2.48 (137)</td>
<td>2.01 (123)</td>
</tr>
<tr>
<td>±0.35</td>
<td>±0.34</td>
<td>±0.09</td>
</tr>
</tbody>
</table>

1 mM cAMP added to serosal side only

Means ± 95% confidence interval; (number of cells)
to sense variations in the electrical field due to localized regions of current flow through the epithelium.

No current leaks were observed when the microelectrodes were moved over the epithelium proper, over the reduced epithelium between each rectal pad (see chapter 1) or at the edge of the tissue where it was fastened to the chamber (Fig. 5a-c). However, experimentally damaged areas (Fig. 5d) were easily detected because of the large sizes of the hole (∼50 μm diameter). The deflection in scanning voltage was also very large (13 mV, as compared to a limit of sensitivity = 0.3 mV), indicating that a 7.6 μm hole would have been detectable (compare with cell diameter of ∼15 μm). Finally, the absence of low-resistance shunts is consistent with previous $^{35}$SO$_4$ flux studies (chapter 2); these measurements showed that little, if any, transepithelial permeability is attributable to nonselective "leak" pathways.

4) Cell-cell interconnections

Since epithelial cell-cell coupling was demonstrated by Loewenstein and coworkers (1965; see review by Loewenstein, 1981), cellular interconnections (i.e. gap junctions) have been found in most epithelia. To study locust rectum with electrophysiological methods, it is important to know whether current injected into one cell flows mostly into adjacent cells or passes directly to the external solutions through the membranes of the injected cell. Both visual and electrical methods were used to examine the possibility of cell-cell coupling; first, by observing the lateral diffusion of fluorescent dye after injecting it into an epithelial cell and second, by measuring voltage deflections in neighbouring cells during intracellular current injection.

i) Dye coupling

Figure 6 shows the effect of Lucifer Yellow CH injection into locust rectal cells as observed by fluorescence microscopy. In recta from eleven locusts, eight out of thirteen experiments showed extensive diffusion of dye
Figure 5.5 Voltage scans of the epithelial surface. Current was passed transepithelially from serosa to mucosa (250 μA cm$^2$) while a blunt microelectrode was dragged across the epithelial surface to record inhomogeneities in the electrical field. Traces (a)-(c) show the potential difference between the microelectrode and a mucosal agar bridge, as the electrode was located in the region indicated in the photograph (different tissue was photographed). Trace (d) was obtained by moving the electrode over a hole (∼50 μm diameter) which had been made by deliberately damaging the tissue with the microelectrode.
Figure 5.6  Rectal cells following injection with the fluorescent dye Lucifer Yellow CH.  (a) Broken microelectrode tip remains in cell during processing of the tissue.  (b) The nuclei of 20-30 cells stain intensely after injection of dye into one cell.  (c) Cell margins are clearly visible in the region of dye injection when the surface of the tissue is in focus. Calibration lines:  50 μm.
from the injected cell into adjacent cells. The central cell was usually the brightest, and was presumed to be the one in which the microelectrode was situated. Cell staining became progressively fainter with distance away from the centre cell. No preferential routes of coupling were observed, i.e. the stained area was circular and did not stain particular pathways or clusters of cells. The average distance of dye spread (i.e. radius of stained area) was $78.9 \pm 13.8\ \mu m (\bar{x} \pm s.e.)$. Overall, an average of $28 \pm 4$ cells were stained when "coupled" cells were injected.

Cell dimensions were easily measured after dye injection. When the microscope was focused on the surface of the tissue, a fluorescent "fishnet" pattern corresponding to cell margins was observed (see Fig. 6c). The base of the columnar cell was $15.2 \pm 1.3\ \mu m (\bar{x} \pm s.e., n = 6$ locusts). When the plane of focus was lowered into the tissue, nuclei were observed at a depth of $53.0 \pm 7.5\ \mu m$ from the mucosal surface. These values agree with those obtained from electron micrographs (Jarial et al., unpubl. obs.) and from observations on unfixed tissues under a dissecting microscope (Hanrahan, unpubl.). Serious fixation artifacts are unlikely; a recent study in which gallbladders were observed continuously during fixation in $OsO_4$ and dehydration in alcohol showed little change in cell dimensions during these procedures (Rostgaard and Frederiksen, 1981).

Out of thirteen cells injected with dye, five appeared to be uncoupled. However, of these uncoupled cells, four were within $200\ \mu m$ of the edge of rectal pads, the site where the cuticular intima attaches to the rectum. It is possible that cells near the edge of the pad were damaged and became uncoupled during dissection of the intima.

In summary, under normal conditions, most (if not all) of the epithelial cells are probably interconnected by low resistance pathways. This is confirmed in the next section using electrical methods.


ii) Electrical coupling

Figure 7 shows the effects of current injection into one cell on the apical membrane potential of a second cell located 42 μ from the point of injection. In order to produce these deflections, current must flow intracellularly between the two cells, i.e. the cells must be electrically coupled (Fig. 1). It may be noted that cell-cell coupling in locust rectum is independent of the direction of current flow, since voltage responses were identical when negative (hyperpolarizing) or positive (depolarizing) currents were injected. The current-voltage relation of intracellularly injected current measured in different cells was reasonably linear under various conditions (Fig. 8), in contrast to many epithelia in which cells are uncoupled by depolarization (Socolar and Politoff, 1971; Lewis et al., 1976; reviewed by Loewenstein, 1981).

To test whether very large current pulses might cause time-dependent uncoupling or deterioration of membrane potential, very large depolarizing current pulses (300 nA) were injected into an epithelial cell for 20 minutes (Fig. 9). Membrane potential and voltage deflections recorded in a second cell were not altered (inter electrode distance = 42 μ). This insensitivity to large intracellular current injections made flat-sheet cable analysis of the epithelium feasible despite very low membrane resistances. Currents of 50 and 100 nA have been injected in studies of Chironomus Malpighian tubules and salivary glands (Loewenstein et al., 1965).

5) Cell membrane and paracellular resistance

If cells within the epithelium are electrically coupled, then it is not possible to estimate membrane resistances by injecting current and observing the voltage response in the same cell. Instead, voltage deflections must be measured as a function of distance from the point of current injection in
Figure 5.7  Electrical coupling between cells in the rectal epithelium. Hyperpolarizing ($-I_o$, upper traces) and depolarizing currents ($+I_o$, lower traces) were passed from an adjustable constant-current source (10-100 nA, 1 sec duration, square pulses) into a cell through one microelectrode, and the resulting deflections in apical membrane potential ($\Delta V_a$) were measured using a second microelectrode located 42 $\mu$m from the point of current injection. This distance represents 2- to 3-cell separation between electrodes (cell diameter =17 $\mu$m). Note that similar results were obtained with hyper- and depolarizing currents.
Figure 5.8  Current-voltage relation of intracellularly injected current as measured in a different cell. Recta were perfused with (△) normal saline, (▲) normal saline containing 1 mM cAMP on the serosal side, (〇) Cl-free saline, (●) Cl-free + cAMP. Results from different tissues are shown under each condition.
Figure 5.9  Effect of prolonged intracellular injection of large depolarizing current pulses on the voltage response measured in a different cell. The distance between current injecting and voltage sensing electrodes was 42 μm. See Figure 5.7 for definitions and explanation.
order to calculate effective input resistance \( R_z \) as described in the methods section and Figure 1. Transepithelial resistance \( R_t \) and the relative resistances of apical and basal membranes \( \alpha \) were measured as described previously. Once \( R_z \), \( R_t \) and \( \alpha \) were obtained, apical and basal membrane resistances \( R_a \) and \( R_b \) and junctional resistance \( R_j \) could be calculated (Fromter, 1972; Spenney et al., 1974; Reuss and Finn, 1974, 1975; Lewis et al., 1976; methods of this chapter).

Figure 10 shows deflections in apical membrane potential as a function of distance from the current injecting microelectrode before and after serosal addition of 1 mM cAMP in normal saline. The best-fit Bessel functions are also shown as solid lines. Table 2 summarizes the results of cable analysis under control conditions, and during cAMP exposure. Also shown are the calculated resistances of apical and basal membranes \( R_a \) and \( R_b \) and the paracellular pathway \( R_j \). The results suggest that locust rectum is a "tight" epithelium, particularly during cAMP exposure, when approximately 90-95% of passive ion movements across the tissue occur transcellularly rather than around the cells. Compared to other epithelial membranes, the electrical resistance of locust rectal cell membranes is extremely low (36-40 \( \Omega \)cm\(^2\) during cAMP stimulation). Typical values for epithelial membrane resistance are 3,000-5,000 \( \Omega \)cm\(^2\) based on macroscopic area (Fromter, 1972; Reuss and Finn, 1974, 1975) or 7,000-23,000 \( \Omega \)cm\(^2\) when normalized to true membrane area using capacitance measurements (Lewis and Diamond, 1976). This discrepancy will be discussed later in this chapter.

Addition of 1 mM cAMP causes a large decline in transepithelial resistance (65%) and 80% decreases in both apical and basal membrane resistance. The space constant decreased significantly after cAMP addition from 420.0 ± 40.6 to 218.8 ± 27.7 \( \mu \)m (\( P < 0.01 \)). Junctional resistance was difficult to measure in
Figure 5.10  Deflections in $V_a$ as a function of distance from the point of intracellular current injection before and after addition of cAMP. Measurements during cAMP exposure were made at least 30 min after addition of 1 mM cAMP to the serosal perfusate. The solid line indicates the best fitting Bessel function, constants "A" and "$\lambda$" were obtained by fitting the data as described in the text.
Table 5.2 Electrical parameters estimated by cable analysis in normal saline

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$R_t$ (Ω cm²)</th>
<th>$\alpha$</th>
<th>$R_z$ (Ω cm²)</th>
<th>$R_x$ (KΩ)</th>
<th>$A$ (mV)</th>
<th>$\lambda$ (µm)</th>
<th>$R_a$ (Ω cm²)</th>
<th>$R_b$ (Ω cm²)</th>
<th>$R_J$ (Ω cm²)</th>
<th>Current Direction</th>
<th>number of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>213.9</td>
<td>0.80</td>
<td>70.0</td>
<td>34.6</td>
<td>1.10</td>
<td>450</td>
<td>126.0</td>
<td>184.6</td>
<td>687.0</td>
<td>-</td>
<td>14</td>
</tr>
<tr>
<td>2</td>
<td>280.4</td>
<td>0.91</td>
<td>90.3</td>
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<td>1252.0</td>
<td>-</td>
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<tr>
<td>3</td>
<td>213.0</td>
<td>1.44</td>
<td>95.0</td>
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<td>1.00</td>
<td>550</td>
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<td>160.8</td>
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<td>118.8</td>
<td>131.9</td>
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<td>300</td>
<td>257.8</td>
<td>220.3</td>
<td>801.0</td>
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<td>28</td>
</tr>
<tr>
<td>6a</td>
<td>256.6</td>
<td>1.35</td>
<td>128.2</td>
<td>8.0</td>
<td>2.55</td>
<td>400</td>
<td>301.3</td>
<td>223.2</td>
<td>502.4</td>
<td>+</td>
<td>20</td>
</tr>
<tr>
<td>6b</td>
<td>256.6</td>
<td>1.35</td>
<td>115.6</td>
<td>7.2</td>
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<td>400</td>
<td>271.7</td>
<td>201.3</td>
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<td>21</td>
</tr>
<tr>
<td>$\bar{x} \pm \text{s.e.}$</td>
<td>252.7</td>
<td>1.13</td>
<td>100.5</td>
<td>50.3</td>
<td>2.05</td>
<td>420.0</td>
<td>220.1</td>
<td>193.4</td>
<td>742.4</td>
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</tr>
<tr>
<td>$n = 5$</td>
<td>±17.4</td>
<td>±0.12</td>
<td>±10.4</td>
<td>±21.2</td>
<td>±0.60</td>
<td>±40.6</td>
<td>±30.3</td>
<td>±12.2</td>
<td>±141.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>+ 1mM cAMP</td>
<td></td>
<td></td>
<td></td>
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<td>±244.9</td>
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</table>

Cont.
230

Table 5.2 (cont.)

1 cable analysis was repeated in order to test the reproducibility of measurements.

2 and 3 $R_z$ was extremely low during cAMP exposure ($15.4, 12.6 \, \Omega \, \text{cm}^2$), and approached the limits of sensitivity of the method. Since small errors resulted in non-sensical values of $R_j$, $R_a$, and $R_b$, it was necessary to calculate $R_a$ and $R_b$ in two recta from $R_t$ and $\alpha$ measured during cAMP stimulation and $R_j$ (measured under control conditions), assuming that $R_j$ was not affected by cAMP. This assumption seems justified since no consistent effect of cAMP was observed in preparations in which $R_z$ was high enough to allow direct measurement of $R_j$ (see Table 5.3).
cAMP-treated tissue in normal saline, however no large changes in $R_j$ were observed in Table 2 or in Cl-free saline when $R_j$ was easily measured.

Figure 11 shows the deflections in apical ($\Delta V_a$) and basal ($\Delta V_b$) membrane potentials during transepithelial current pulses before and after adding 1 mM cAMP to the serosal side. Both $\Delta V_a$ and $\Delta V_b$ were reduced during cAMP exposure.

In order to identify which ions are involved in these resistance changes, the effect of cAMP on $\alpha$ was determined in Cl-free (gluconate) saline (Fig. 12). Only the apical membrane resistance decreased during cAMP exposure under these conditions, indicating that resistance changes at the serosal border are Cl-dependent. It may be noted that $\Delta V_b$ actually increased during cAMP exposure. However, this is to be expected because more current flows transcellularly when $R_a$ declines and the increase in current should cause larger deflections in the basal membrane potential even though $R_b$ remains constant.

Table 3 shows the effects of cAMP on resistances under Cl-free conditions as determined by cable analysis. As in normal saline, apical membrane resistance declined by 80% during cAMP exposure. Obviously this change must be due to an increase in the permeability of the apical membrane to some ion other than Cl. Basal membrane resistance also decreased slightly. When expressed as a change in conductance ($\Delta G_b$),

$$\Delta G_b = \frac{1}{R_b^{\text{control}}} - \frac{1}{R_b^{\text{cAMP}}} \quad (8)$$

where $R_b$ is basal membrane resistance before and after cAMP addition, $G_b$ of the basal membrane increases 22.6 mmhos cm$^{-2}$ in normal saline (114 mM Cl) and only 0.4 mmhos cm$^{-2}$ in Cl-free saline. Since more than 98% of the cAMP-induced conductance of the basal membrane is abolished by Cl-removal, the cAMP-induced conductance of the basal membrane probably represents an increase in Cl permeability.
Figure 5.11  Effects of cAMP on the deflections in apical and basal membrane potentials produced by transepithelial current pulses (voltage divider ratio). Control conditions are shown at left. Results shown at the right were obtained 30-60 min after adding cAMP to the serosal side. Voltage deflections were produced by transepithelial constant-current pulses (20 μA/0.196 cm²). The numbers shown at left identify the preparation.
Figure 5.12 Effects of cAMP on the voltage divider ratio in Cl-free saline.

See legend of Figure 5.11 for definitions and conditions. 1 mM cAMP was added to the serosal side at the arrow.
Figure 5.13  Effects of cAMP on deflections in $V_a$ as a function of distance from the point of intracellular current injection under Cl-free conditions. Solid line indicates the best-fitting Bessel function calculated as described in the text.
Table 5.3  Results of cable analysis in Cl-free saline before and after adding 1mM cAMP

<table>
<thead>
<tr>
<th>Preparation</th>
<th>$R_t$ (Ω cm$^2$)</th>
<th>$\alpha$</th>
<th>$R_z$ (Ω cm$^2$)</th>
<th>$R_x$ (KΩ)</th>
<th>$A$ (mV)</th>
<th>$\lambda$ (μm)</th>
<th>$R_a$ (Ω cm$^2$)</th>
<th>$R_b$ (Ω cm$^2$)</th>
<th>$R_j$ (Ω cm$^2$)</th>
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<th>number of cells</th>
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<td></td>
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<td>±164.7</td>
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<td>±84.3</td>
<td>±507.4</td>
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Which ionic conductance increases in the apical membrane during cAMP stimulation? From measurements of $^{42}$K backflux, it was concluded in chapter 3 that transepithelial potassium permeability ($P_{K}$) increases during cAMP exposure. It seemed reasonable that apical membrane K permeability ($P_{K}^a$) might increase during cAMP exposure, and that this might account for the increase in both transepithelial $^{42}$K permeability and $\Delta R_a$ under these conditions.

Figure 14 shows the effects of cAMP when both K and Cl are removed from the saline. In contrast to the results in normal and Cl-free saline (compare Figs. 12 and 13) neither $\Delta V_a$ nor $\Delta V_b$ change following cAMP addition. Table 4 summarizes the results of cable analysis under these conditions. Only one complete cable analysis was successful during cAMP stimulation, although effects of cAMP on $a$ were measured in two preparations. cAMP-induced $\Delta R_a$ was abolished under K-free conditions, suggesting that i) the stimulation of apical membrane conductance is due to increased K permeability, ii) cAMP-induced transepithelial $^{42}$K permeability probably results from this apical K conductance. It may be noted that $R_a$ measured under KCl-free conditions is lower than when the saline is only Cl-free (107 versus 304 $\Omega\text{cm}^2$). The reason for this inconsistency is that $R_a$ and $R_t$ are underestimated under KCl-free conditions because $V_a$ does not reach steady-state values during 1 sec current pulses. This effect cannot be attributed to membrane capacitance since apical membrane area would have to be 50,000 times greater than the macroscopic tissue area (assuming 1 $\mu\text{F/cm}^2$ of membrane) in order to explain the observed time constant. A more detailed analysis will be required to determine whether the transient is due to membrane polarization, voltage-dependent conductance or some other mechanism (see Reuss and Finn, 1977). At present, the important finding is that K-removal blocks $\Delta R_a$ during cAMP-exposure.

In summary, the locust rectum is a tight epithelium with low transepithelial resistance. The results show that cAMP exposure produces a K-dependent
Figure 5.14  Effects of cAMP on the voltage divider ratio under KCl-free conditions. See legend of Figure 5.11 for definitions and conditions.
Table 5.4  Electrical parameters estimated by cable analysis in KCl-free saline

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<th>$R_x$ (KΩ)</th>
<th>$A$ (mV)</th>
<th>$\lambda$ (μm)</th>
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<td>±1085.6</td>
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See text for definitions.
increase in apical membrane conductance and a Cl-dependent increase in basal membrane conductance.

6) Effects of potassium concentration on the transepithelial permeability to $^{42}\text{K}$

The concentration-dependence of apparent potassium permeability ($^{*}\text{P}_k$) was determined from the mucosa-to-serosa flux under $I_\text{sc}$ conditions (Fig. 15). The calculated $^{*}\text{P}_k$ was approximately $5 \times 10^{-5}$ cm sec$^{-1}$ in the range 0-10 mM $\text{[K]}$, however $\text{P}_k$ declined drastically when $\text{[K]}$ was raised higher. The decrease in $^{*}\text{P}_k$ was half-maximal between 10 and 40 mM $\text{[K]}$, and was maximal at 100 mM $\text{[K]}$ (Fig. 15). This tracer method of calculating K permeability overestimates $\text{P}_k$ due to the effects of 2 barriers in series (see discussion and Schultz and Frizzell, 1976). Nevertheless, such error could not account for the drastic decline observed in $^{*}\text{P}_k$. The present results are consistent with previous findings that transepithelial K backflux is lower than expected in "high K" saline (chapter 3), and that K addition does not reduce $\text{R}_L$ as anticipated (chapter 2). A decline in $^{*}\text{P}_k$ could occur at the apical or basal membrane or both, since K was added to both sides of the tissue in the present experiments.

In the next section, results of microelectrode experiments indicate that the apical membrane is the site of concentration-dependent potassium permeability, and that high $\text{[K]}$ may exert its inhibitory effects at the luminal surface of the membrane.

7) Effects of mucosal potassium concentration on voltage divider ratio

The effects of mucosal $\text{[K]}$ on voltage divider ratios, membrane potentials and intracellular Cl and K activities (a$^c_{\text{Cl}}$ and a$^c_k$) were determined using an ion-sensitive microelectrode (chapter 4). Although not pertinent in chapter 4, the voltage divider ratio ($\alpha$, or ratio of apical to basal membrane resistance) was measured during these experiments to give insight into the $\text{[K]}$-dependent changes in potassium permeability. Intuitively, one would expect apical
Figure 5.15  Apparent transepithelial potassium permeability $P_K$ and K backflux under $I_{sc}$ conditions as a function of bilateral concentration. $P_K$ was calculated using equation 13 in text. Tissues were equilibrated in K-free saline and exposed to 1 mM cAMP on the serosal side. Serosa-to-mucosa flux of $^{42}$K was measured during two 15-min intervals under $I_{sc}$ conditions, however only the second flux period at each K concentration was used in calculations. Potassium concentration was elevated by adding K-methylsulfate bilaterally under $I_{sc}$ conditions to give the concentrations shown on the abscissa. Means ± s.e.; n = 6.
membrane resistance (and α) to decline when [K] is elevated on the mucosal side. In this section, a simple equation is derived to allow comparison of the measured values of α with those predicted if K and Cl permeability coefficients remained constant. Deviations from the predicted behaviour is then used as an indication of changes in ionic permeability. Sodium was not included in these calculations since Na conductance is a minor fraction of apical and basal membrane conductance (less than 15% and 7%, respectively, according to cable analysis). Further evidence for high K permeability comes from the observation that intracellular potential varies approximately 50 mV/decade change in saline K activity under I_sc conditions, close to the value predicted for a perfect K electrode (Table 5 of chapter 4). As a result of low intracellular Na activity and membrane permeability, sodium would not be expected to affect α significantly.

If K and Cl account for most of the apical membrane conductance, then that fraction due to K is

$$\frac{P^a_K}{(P^a_K + P^a_{Cl})} = 1 + \frac{P^a_K}{P^a_{Cl}}$$  \hspace{1cm} (9)

where $P^a_K$, $P^a_{Cl}$ are K and Cl permeabilities of the apical membranes (cm sec^{-1}). Similar expressions are obtained for both ions at mucosal and serosal membranes. During cAMP exposure, $P^a_K = 16 P^a_{Cl}$ and $P^b_K = 9 P^b_{Cl}$, according to the results of cable analysis (Tables 2 and 3). Values of α are then predicted as

$$\alpha = \left[ (1 + \frac{P^a_K}{P^a_{Cl}})a^a_K + (1 + \frac{P^a_K}{P^a_{Cl}})a^a_{Cl} \right] / \left[ (1 + \frac{P^b_K}{P^b_{Cl}})a^b_K + (1 + \frac{P^b_K}{P^b_{Cl}})a^b_{Cl} \right]$$  \hspace{1cm} (10)

where $P^a_{i}$, $P^b_{i}$ are the permeabilities of the apical and basal membranes to ion "i"; $a^a_i$, $a^b_i$ are the logarithmic mean activities of ion "i" in the apical and basal membranes, calculated as

$$\frac{a^c_i - a^a_i}{\ln \frac{a^c_i}{a^a_i}}$$

in place of the usual expression.
where $a_i^c$ and $a_i$ are intracellular and extracellular activities of ion "i", respectively. This relationship predicts $\alpha$ if i) most of the apical and basal membrane conductance is due to $K$ and $Cl$, and ii) permeability coefficients do not change during addition of $K$ to the mucosal side.

Figure 16 shows the effects of mucosal $K$ addition on $\alpha$ and the values of $\alpha$ predicted using equation (10). The values of $\alpha$ and intracellular ion activities used in the calculation are those in Figure 6 of chapter 4. When mucosal $K$ activity was elevated, $\alpha$ increased 5-fold instead of decreasing as predicted if permeability coefficients remained constant. It might be argued that higher values of $\alpha$ could result from a decline in $R_b$ rather than an increase in $R_a$. Certainly some decline in $R_b$ is expected since intracellular ion activities increase when mucosal $[K]$ is elevated. Regardless, there must still be an increase in $R_a$ when $K$ is added, even if $R_b$ does decline, because $R_t$ remains constant at 110-120 $\Omega$cm$^2$ under these conditions when $[K]$ is between 6 and 100 mM (Fig. 16). Since virtually all of the apical membrane conductance is due to $K$ (~96% during cAMP stimulation, from Table 2), the increase in $R_a$ during mucosal $K$ addition indicates a decline in $K$ permeability of the apical membrane.

8) Effects of cAMP concentration on short-circuit current and transepithelial conductance

Short-circuit current ($I_{sc}$) and transepithelial conductance ($G_t$) were measured as a function of cAMP concentration for several reasons. First, if stimulations of $G_t$ and $I_{sc}$ by cAMP reflect two different processes, this might become obvious if $G_t$ and $I_{sc}$ had different dose-response curves. Second, a dose-response relationship for the $Cl$-dependent effects could be calculated by comparing the results in normal and $Cl$-free saline. Finally, the relationship between $I_{sc}$ and $G_t$ may be used to determine the driving force of $Cl$ transport and the "shunt" conductance under certain conditions.
Figure 5.16 Relationship between mucosal K concentration and the voltage divider ratio in cAMP-stimulated recta during serosal perfusion of recta with normal saline. Voltage divider ratios were predicted using equation (10) of the text and i) measured values of intracellular ion activities, and ii) partial ionic conductances estimated by cable analysis during ion substitutions. Results are from one typical preparation; see text for full details. Means ± s.e.; n = 20 observations.
Figure 17 shows the effects of cAMP concentration on $G_t$ and $I_{sc}$ in normal and Cl-free saline. Tissues were kept under short-circuit conditions except at 15 min intervals, when $I_{sc}$ was turned off for 1.5 min to allow measurement of transepithelial potential ($V_t$). Conductance was calculated as $I_{sc}/V_t$. Both $I_{sc}$ and $G_t$ had "S" shaped dose-response curves with similar threshold doses ($5 \times 10^{-5}$ M cAMP) and maximal doses of approximately 1 mM cAMP. These results indicate that Cl-dependent and Cl-independent conductances have similar cAMP sensitivity (with the reasonable assumption that membrane permeability to cAMP is not affected by Cl removal). The value of $R_t$ obtained under Cl-free conditions in this experiment compares well with that obtained by cable analysis (see Table 3). In summary, these data indicate that $\Delta R_t$ during cAMP exposure results from an increase in apical membrane K conductance, a finding which is consistent with the cAMP-induced increase in transepithelial potassium permeability observed in chapter 3 using $^{42}$K fluxes.

Discussion

We begin this section by discussing whether locust rectum is a "tight" or "leaky" epithelium. Three factors which have been found to regulate permeability in this tissue are described: cAMP, K concentration, and osmotic pressure. An equivalent electrical circuit model is then derived from measurements of fluxes, intracellular ions and membrane potentials (chapter 4) and cable analysis (this chapter), and the model is used to predict the driving force of Cl transport ($E^{Cl}$). This prediction is then tested by an independent method. 

Locust rectum: a "tight" epithelium with low electrical resistance

Several observations suggest that locust rectum is a "tight" epithelium. First, deflections in $V_t$ produced by transepithelial salt gradients were strongly dependent on the direction of the gradient, indicative of two barriers in series having different properties. When salt concentration was lowered on
Figure 5.17  Log dose-response curve showing the relationship between cAMP concentration and both transepithelial conductance ($G_t$) and short-circuit current ($I_{sc}$). Tissues were bathed in normal and Cl-free salines (Table 2.1 and gluconate-substituted). Means ± s.e.; n = 6 recta in normal saline, 4 recta in Cl-free saline.
the mucosal side, $\Delta V_t$ indicated high cation selectivity over Cl. However, the effects of lower serosal salt concentration suggest that the permeability of the basal border is more Cl-selective. Such asymmetry is not expected if ion diffusion occurs through a single (paracellular) barrier, since tight junctions are not known to "rectify" (e.g. proximal tubule; Boulpaep and Seely, 1971; Frömter et al., 1971). In contrast, epithelia which are known to be tight invariably have asymmetrical responses to transepithelial salt gradients (for references see Frömter and Diamond, 1972). In order to minimize possible streaming potentials, sucrose was added to maintain isosmocity when the concentration of salt was reduced on one side. The effective osmotic pressure in mixed solutions is not known with certainty since the osmotic reflection coefficient at the tight junction probably varies for each solute. Also, some active water absorption might entrain K even if mucosal and serosal sides were isosmotic. However, the fact that nearly symmetrical results were obtained with K-methylsulfate gradients, which also employed sucrose as an osmotic effector, suggests that the asymmetry is not some artifact of using sucrose. No attempt was made to measure streaming potentials due to active water uptake which is thought to occur by localized recycling of solutes in the lateral intercellular spaces (Phillips, 1970; Wall and Oschman, 1970; Coh and Phillips, 1978).

Permeability ratios calculated from Figure 4 probably underestimate the selectivity of the cell membranes, since even slight paracellular shunting would place an upper limit on transference numbers. For example, the mucosal ratio of $t_K/t_{Cl}$ (3.0) is lower than $t_{Na}/t_{Cl}$ (4.7), which would suggest that Na permeability of the apical membrane is greater than that of K. However, cable analysis (Tables 2-4) and the effects of K on membrane potentials, indicate that the opposite is true ($P_{Na}/P_K < 0.06$). In summary, transference
numbers in these experiments are not quantitative measures of permeability because of finite paracellular shunting, however, they do suggest that a significant fraction of transepithelial ion diffusion occurs through the cells.

Voltage scanning did not detect any low-resistance regions (Fig. 5). The same negative result has been obtained in tight epithelia including urinary bladders of *Necturus* (Higgins et al., 1975) and rabbit (Lewis and Diamond, 1976). This technique is qualitative in nature, and there is the possibility that small leaks could go undetected (see results). However, paracellular shunting is detectable in at least one leaky epithelium, *Necturus* gallbladder (Fromter and Diamond, 1972; Frömter, 1972).

Cable analysis of the epithelium indicates that locust rectum is moderately "tight" under control, unstimulated conditions, when ~60% of the transepithelial conductance is transcellular. The epithelium becomes "tighter" during cAMP stimulation, when transcellular conductance ranges between 89 and 96% of the total. The fractional conductance of the transcellular pathway of locust rectum under these conditions resembles that of other tight epithelia (53%, toad urinary bladder, Reuss and Finn, 1975; 65-96%, rabbit urinary bladder, Lewis and Diamond, 1976; 80%, *Necturus* stomach, Spenney et al., 1974; 31%, rabbit cornea, Marshall and Klyce, 1981) but is much higher than that of leaky epithelia (~4%, *Necturus* gallbladder, Fromter, 1971; ~1%, *Necturus* proximal tubule, Asterita and Boupaep, 1978; see Boupaep, 1979, for references).

Further evidence that locust rectum is "tight" comes from the large decrease in transepithelial resistance during cAMP stimulation (50-65%). cAMP is not known to increase tight junctional conductance so drastically in leaky epithelia, although a small, cAMP-dependent reduction of tight-junctional conductance has been reported in *Necturus* gallbladder (Duffey et al., 1981; see review by Powell, 1981). Finally, transepithelial $^{35}$SO$_4$ permeability is extremely low
both in unstimulated recta, and after exposure to 1 mM cAMP, a finding which is inconsistent with diffusion through water-filled channels of low or moderate selectivity (i.e. tight junctions). Results of early studies suggested that septate and gap junctions occlude the lateral intercellular space in insect rectal epithelia (Gupta and Berridge, 1966; Noirot-Timothee and Noirot, 1967; Oschman and Wall, 1969), although more recent work suggests that these junctions are patent to ionic lanthanum in cockroach and blowfly rectum (Lane, 1979). True tight junctions have also been reported at the basal ends of the lateral intercellular spaces in these tissues (Lane, 1979).

Most passive ion fluxes are believed to occur transcellularly in insect epithelia, although this has not been measured directly. Berridge et al. (1975) found the transepithelial resistance of blowfly salivary gland to be approximately 80 Ωcm² at rest, declining to 5.5 Ωcm² during stimulation with 5-HT. Such enormous resistance changes would not be expected if paracellular shunting was significant. Furthermore, if blowfly salivary glands have specific membrane resistances on the same order as calculated for basal membranes of other insect salivary glands (Drosophila, \( \sim 10,000 \frac{Ω}{cm^2} \), normalized to membrane area as estimated from electromicrographs, Loewenstein and Kanno, 1964; and Chironomus, \( \sim 9,000 \frac{Ω}{cm^2} \), normalized, Loewenstein et al., 1965), then most conductance would be paracellular. Exposure of blowfly glands to 5-HT results in a 20 mV depolarization of \( V_a \) but only a slight depolarization of \( V_b \) (2-3 mV) (Berridge and Prince, 1972). This observation suggests that the epithelium is reasonably tight, since large paracellular currents would be expected to depolarize \( V_b \) more dramatically, although the small change in basal membrane potential is in the right direction to be explained by paracellular current. Furthermore, the small \( \Delta V_b \) does not necessarily show high junctional resistance since 5 HT might alter the equivalent EMF at the basal membrane so as to counteract a depolarizing paracellular current.
In short, these observations provide indirect evidence that the paracellular pathway is "tight" in insect salivary glands. Malpighian tubules are also thought to have low junctional permeability (see Maddrell, 1980) and Loewenstein et al. (1965) have measured high basal membrane resistance in Chironomus Malpighian tubules (3,000 Ωcm², normalized to membrane area).

Lepidopteran midgut is also presumed to be a tight epithelium with low electrical resistance (~150 Ωcm²; Wood and Moreton, 1978). Also, from Blankemeyer's data (1978), one may calculate a flux ratio of ~50 in this tissue, a value which is similar to other tight epithelia when mounted without edge damage (e.g. >100, frog skin, O'Neil and Helman, 1976; 30, rabbit bladder, Lewis and Diamond, 1976a). Finally, there is one other report of epithelial cable analysis in the sensilla of the waxmoth caterpillar, although the data have not yet been published (Erler and Thurm, in preparation, cited in Thurm and Kuppers, 1980). Because of its sensory function the sensillum might not be typical of insect transporting epithelia.

Tightness may be a general characteristic of insect epithelia, which generally have high ratios of membrane/junctional area due to large transporting cells and microscopic infolding of membrane, particularly of the lateral cell border which is usually very complex and associated with mitochondria. It must be re-emphasized that the present study cannot distinguish local secretion and absorption processes at the lateral border. Further studies involving lanthanum tracer/electron microscope and electron microprobe are needed to answer these questions in locust rectal epithelium.

Regulation of passive permeability

1) cAMP

Addition of cAMP, cGMP or theophylline stimulates I_{sc} (10-fold) and trans-epithelial conductance (>60%). When the voltage deflections across apical and basal membranes during transepithelial current pulses are monitored before and
after cAMP addition, both apical and basal voltage deflections are greatly reduced, suggesting a decline in apical and basal membrane resistances. This conclusion was confirmed by measuring the radial spread of intracellular current. Since $\Delta R_d$ was nearly abolished in Cl-free saline whereas $\Delta R_a$ was K-dependent, the simplest explanation for these observations is that cAMP increases apical membrane conductance to K by 18.1 mmhos cm$^{-2}$, and increases basal membrane Cl-conductance by approximately 19.3 mmhos cm$^{-2}$.

How do these cAMP stimulations of membrane conductance compare with membrane and transepithelial permeabilities calculated using tracer flux measurements? Table 5 shows rough estimates of membrane and transepithelial permeability to K and Cl, before and during exposure to 1 mM cAMP. Three methods were used to calculate membrane permeabilities. These are illustrated using potassium conductance of the apical membrane as an example:

Method 1:

$$P_K = \frac{J_K^{\text{net}}}{V_{a_F}^c} \frac{RT}{a_K^c} \frac{\exp(V_{a_F}^c/RT) - 1}{\exp(V_{a_F}^m/RT) - a_K^m}$$

(11)

where $J_K^{\text{net}}$ is the net flux of K under open-circuit conditions, $V_a$ is the apical membrane potential, $a_K^c$ and $a_K^m$ are potassium activities in the cell and mucosal solutions, respectively, and $R$, $T$, and $F$ have their usual meanings. Activities of K and Cl in the saline were 7.2 and 82 mM, respectively, as measured using ion-sensitive microelectrodes (chapter 4). All other parameters used in the calculation are listed in Table 6a.

Method 2:

$$P_K = \frac{RT}{Z^2F^2} \frac{c_K^a}{a_K^a}$$

(12)

where $c_K^a$ is the potassium conductance of the membrane as estimated from cable analysis, and $a_K^a$ is the logarithmic mean activity of K in the apical membrane calculated as $(a_K^c - a_K^m)/(\ln a_K^c - \ln a_K^m)$.
Table 5.5(a) Summary of previous tracer and microelectrode results used in calculating permeabilities

<table>
<thead>
<tr>
<th>Condition</th>
<th>J_{sm}^k</th>
<th>J_{sm}^{Cl}</th>
<th>V_a</th>
<th>V_b</th>
<th>a_c^{-}</th>
<th>a_c^{Cl}</th>
<th>J_{net}^k</th>
<th>J_{net}^{Cl}</th>
<th>G_a</th>
<th>G_a^{Cl}</th>
<th>G_b</th>
<th>G_b^{Cl}</th>
<th>G_t</th>
<th>G_t^{Cl}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.351</td>
<td>1.47</td>
<td>-58</td>
<td>+51</td>
<td>31</td>
<td>52.4</td>
<td>131</td>
<td>1.2</td>
<td>1.9</td>
<td>3.3</td>
<td>1.19</td>
<td>0.88</td>
<td></td>
<td></td>
</tr>
<tr>
<td>cAMP added</td>
<td>1.564</td>
<td>1.67</td>
<td>-70</td>
<td>+38</td>
<td>70</td>
<td>62.9</td>
<td>1250</td>
<td>1194</td>
<td>21.3</td>
<td>1.4</td>
<td>5.2</td>
<td>22.6</td>
<td>4.2</td>
<td>1.3</td>
</tr>
</tbody>
</table>

(b) Permeabilities (P x 10^5 cm sec^-1) calculated by various methods

<table>
<thead>
<tr>
<th>Method</th>
<th>P_{K}^{a}</th>
<th>P_{Cl}^{a}</th>
<th>P_{K}^{b}</th>
<th>P_{Cl}^{b}</th>
<th>P_{K}</th>
<th>P_{Cl}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.85</td>
<td>3.0</td>
<td>-0.6</td>
<td>8.7</td>
<td>0.29</td>
<td>1.64</td>
</tr>
<tr>
<td>cAMP added</td>
<td>0.43</td>
<td>0.439</td>
<td>0.546</td>
<td>0.69</td>
<td>±0.19</td>
<td>±0.09</td>
</tr>
</tbody>
</table>
1
Method 1: Permeabilities were calculated from the Goldman constant field equation
for net flux using membrane potentials ($V_a$, $V_b$), ion activities ($a^c_i$), and net tracer
fluxes (open-circuit conditions, $J_{\text{net}}^i$) in Table 5.6(a)
Method 2: calculated according to the conductance equation using logarithmic mean
activities ($\bar{a}$) and partial ionic conductances ($G^i_a$, $G^i_b$), in Table 5.6(a)
Method 3: calculated from transepithelial backflux under short-circuit conditions
($J^K_{\text{sm}}$, $J^{Cl}_{\text{sm}}$) and activities of each ion in the saline, $\bar{x} \pm \text{s.e.}, n=6 \text{ recta}$

See text for further explanation.
**Method 3:** The simplest (but least accurate) approach allows calculation of transepithelial permeability only:

\[ P_K^{\text{a}} = J_{\text{sm}}^K / a_K^S \]  

(13)

where \( J_{\text{sm}}^K \) is the serosa-to-mucosa flux under \( I_{\text{sc}} \) conditions and \( a_K^S \) is the activity of K on the serosal side.

Application of method 1 (M1) assumes a constant field in the membrane (Goldman, 1943; Hodgkin and Katz, 1949), method 3 (M3) assumes a lack of isotope interactions (Essig and Li, 1975), and all three methods require ion independence, i.e. a lack of coupling to the flows of solvent or to other solutes and also the absence of significant active transport.

Apical membrane K permeability increases dramatically after cAMP addition according to methods 1 and 2 (Table 5b). \( P_C^{\text{a}} \) changes very little although it was not possible to use method 1 to calculate \( P_K^{\text{a}} \) since the net flux across this membrane is due to an active pump (shown in chapter 4). In contrast to the apical membrane, \( P_C^{\text{b}} \) changed little during cAMP exposure (decreasing somewhat according to M1, increasing somewhat according to M2), whereas both methods revealed a 6-fold increase in basal membrane Cl permeability. More experiments are needed to explain why \( P_C^{\text{b}} \) is 4-fold higher when calculated from the conductance equation (12; M2) than when the Goldman equation is used (11; M1) before and after addition of cAMP.

The important finding is that Cl conductance of the basal membrane increases \( \approx 21 \text{ mS cm}^{-2} \). Net Cl flux increases by approximately 4-5 \( \text{\mu Eq cm}^{-2}\text{h}^{-1} \) during cAMP exposure under open-circuit conditions (chapter 2), while the net electrochemical gradient favouring Cl exit across the basal membrane \( \Delta \mu_{\text{Cl}}^{\text{b}} \) is \( \approx 20 \text{ mV} \) (chapter 4). Converting the net Cl flux to current, 5 \( \text{\mu Eq cm}^{-2}\text{h}^{-1} \) becomes 134 \( \text{\mu A cm}^{-2} \). The minimum increase in basal membrane Cl conductance which would be required for passive Cl efflux by electrodiffusion is
(134.1 \times 10^{-6} \text{ A cm}^{-2}) / (20 \times 10^{-3} \text{ V}) = 6.7 \text{ mS cm}^{-2} \text{ or only 30\% of the observed increase in basal membrane conductance during cAMP exposure. Even after subtracting the possible error caused by interfering intracellular anions on } a_{\text{Cl}}^c (4.9 \text{ mM}) \text{ and allowing for a 36\% higher rate of transepithelial Cl transport which is indicated by the low value of } R_t \text{ in these experiments (from Fig. 21, } I_{\text{sc}} \text{ would be } 13.6 \mu \text{Eq cm}^{-2} \text{ h}^{-1} \text{ at } G_t = 13.0 \text{ mS cm}^{-2} \text{ as compared to } 10 \mu \text{Eq cm}^{-2} \text{ h}^{-1} \text{ obtained previously in chapter 2), the increase in basal membrane conductance according to cable analysis is still more than twice that required for passive Cl exit by electrodiffusion.}

During cAMP exposure, estimates of } P_K^a \text{ and } P_K^b \text{ from application of M1 and M2 agree more closely than under control conditions. In using method 1, the assumption was made that all of the net flux of } ^{42}\text{K} \text{ and } ^{36}\text{Cl} \text{ under open-circuit conditions is tranacellular. Ignoring the transjunctional fluxes should cause some error in unstimulated recta since 35-40\% of all passive ion movements occur paracellularly. However, during cAMP stimulation this junctional component of transepithelial permeability drops to } \sim 5\% \text{ of the total, and the assumption that net K flux is tranacellular is then realistic. In summary, permeability estimates based on net fluxes (method 1, the Goldman equation) will improve as the epithelium effectively becomes "tighter" during cAMP stimulation.}

Transepithelial K permeabilities compare well when determined using different methods (Table 5b). Transepithelial } P_K \text{ was calculated from

\[ P_K = \frac{P_K^a P_K^b}{P_K^a + P_K^b} \]  

using method 1 and method 2. As expected, } P_K \text{ is higher according to M3 than by methods 1 and 2. Schultz and Frizzell (1976) have predicted that an overestimate of transepithelial permeability should occur using M3 because the method
Effect of cAMP on apparent transepithelial potassium permeability ($\*P_K$) in various salines. All salines were present bilaterally; high-K (140 mM K), Cl-free (10 mM K) and normal (10 mM K). Note that the effect of cAMP on $\*P_K$ is blocked in high-K saline. Original $^{42}$K flux data are shown in Figures 3.13, 3.14 and 3.17. Means ± s.e.; n = 6.
considers the epithelium as a single diffusion barrier rather than two membranes in series. Nevertheless, by comparison with results obtained using M1 and M2, fluxes under short-circuit conditions do provide a good indication of the effects of cAMP and an excellent estimate of $P_{Cl}$. Also, the fact that transepithelial $K$ and $Cl$ permeabilities can be estimated closely by the conductance equation strongly suggests that electrically "silent" pathways such as $Cl/HCO_3$ exchange and NaCl cotransport are not significant. Further evidence that tracer backflux under $I_{sc}$ conditions is a reasonable measure of transepithelial permeability will be described in the next section.

ii) Inhibition of $P_K$ by high [K]

Several observations are consistent with the idea that transepithelial $K$ permeability is reduced when $K$ concentration is increased to normal physiological levels (140 mM in primary urine secreted by Malpighian tubules Chapter 2). First, transepithelial resistance remains constant when $[K]$ is raised on both sides from 40 to 200 mM, although $R_t$ would be expected to decline as ionic strength increases. Second, $^{42}K$ backflux in high $K$ (140 mM) saline does not increase during cAMP stimulation as observed in normal (10 mM) saline. This observation suggests that cAMP-induced $AP_K$ is blocked by high $[K]$. Third, $P_K$ (as estimated from the serosa-to-mucosa flux of $^{22}K$) is much lower in "high-K" saline than in normal (10 mM $K$) saline. Figure 18 shows the effects of cAMP on $P_K$ as calculated from flux data (see Figs. 14 and 18 in chapter 3). When compared to normal saline, $P_K$ is 70% lower in "high-K" saline, and is not stimulated by cAMP. When the error due to treating the epithelium as a single barrier is incorporated into $P_K$, this apparent drop in permeability might be diminished by $\approx 40\%$ as a result of membrane depolarization, but this would still not account for the drastic reduction in $P_K$ observed in high-K saline. Finally, an indirect inhibitory
effect on metabolism by high $[K]$ is unlikely since active Cl transport responds normally to cAMP in "high-K" saline (see Fig. 7 in chapter 2). The fact that cAMP-stimulated $P_K$ is blocked at high $[K]$ whereas cAMP-stimulated Cl transport is not affected implies that $\Delta P_K$ and $\Delta J_{Cl}^{\text{net}}$ are independent processes which are turned on by cAMP.

Microelectrode data also indicate that concentration-dependent $P_K$ occurs at the apical membrane. The effects of mucosal K addition on transepithelial resistance ($R_t$) and $\alpha$ may be calculated from equation (12) if intracellular ion activities are measured and membrane permeabilities remain constant. Surprisingly, when K was added to the mucosal side, $\alpha$ increased (at external $[K] = 40-140$ mM) while $R_t$ remained constant. This shows that apical membrane conductance declined in the presence of high mucosal $[K]$. Since potassium conductance is $\approx 96\%$ of the total apical membrane conductance, this decline in membrane conductance must result from a decrease in K conductance at the apical membrane. As explained in chapter 3, this regulatory mechanism would prevent the rectal lumen from becoming electrically negative to the hemolymph. One possible advantage of maintaining a positive lumen could be to reduce Na loss from the hemolymph to the faeces. Na is low in dietary plant matter (see chapter 2). Finally, any change in K permeability under open-circuit conditions will alter the rate of Cl absorption since K is probably the only significant counter-ion for Cl transport in vivo.

These results together indicate that $P_K$ is "up-regulated" by cAMP and "down-regulated" by high mucosal $[K]$. By varying the conductance of the apical membrane, these factors would provide a third, indirect mechanism for regulating active Cl transport in addition to direct stimulation by cAMP and mucosal K as described in chapters 2 and 3.
Equivalent electrical circuit model

The electrical properties of epithelia are usefully described using an equivalent electrical circuit model. Figure 2 shows the simplest model applicable to epithelia. The contribution of the cuticular intima to transepithelial resistance is low in intact tissue ($\approx 8 \, \Omega \text{cm}^2$; Hanrahan, unpub. obs.). Since the layer was dissected off during cable analysis, it is not included in the following calculations. Similarly, muscle and secondary cell layers were also not included since the series resistance of this layer is probably low because i) they are penetrated by tracheoles and are not continuous, ii) they allow cAMP to penetrate within seconds as indicated by the almost instantaneous response of $I_{sc}$ after cAMP addition. Each of the three major barriers (apical and basal membranes and tight junctions) are replaced with a resistor and an electromotive force, or "Thévenin equivalent". The apical and basal membrane potentials ($V_a$ and $V_b$) and transepithelial potential ($V_t$) are:

\[
V_a = \frac{E_a (R_b + R_j) + R_a (E_b + E_j)}{R_a + R_b + R_j} \tag{15}
\]

\[
V_b = \frac{E_b (R_a + R_j) + R_b (E_a + E_j)}{R_a + R_b + R_j} \tag{16}
\]

\[
V_t = \frac{(V_a - V_b) R_j}{R_a + R_b + R_j} \tag{17}
\]

with polarities of $V_a$, $E_a$, $V_b$ and $E_b$ relative to the intracellular compartment, and $V_t$ and $E_j$ relative to the serosal side. With these polarities, the equivalent electromotive force across apical ($E_a$) and basal ($E_b$) membranes is

\[
E_a = V_a - V_t \frac{R_a}{R_j} \tag{18}
\]

\[
E_b = V_b + V_t \frac{R_b}{R_j} \tag{19}
\]

It is assumed in the following calculations that $E_j = 0$, which is reasonable because identical solutions were used on both sides of the epithelium. However, a small $E_j$ would exist if ions are more concentrated in the lateral
space. There are several reports of elevated $[K]$ in the lateral spaces of insect recta. Wall and Oschman (1970) found higher $[K]$ in the intercellular sinuses of cockroach rectum than in hemolymph, probably the result of local $K^+$ secretion into the space as part of a solute recycling mechanism or alternatively, equilibration with $K^+$-rich fluid in the lumen through imperfectly tight junctions. Also, in rectal papillae of hydrated blowfly, $[K]$ of the intercellular sinus is higher than both lumen and hemolymph according to electron microprobe results (animal I; Gupta et al., 1980), again suggesting a local secretion of $K$. These studies used freshly dissected tissues whereas the present microelectrode study was performed \textit{in vitro} using vigorously perfused locust recta; i.e. conditions which might dissipate normal \textit{in vivo} gradients. No unusually high ion activities indicative of lateral spaces were observed in thousands of impalements. One might expect to impale the sinuses occasionally despite their small size ($<5 \mu m$). Nevertheless the results in this study provide no direct evidence for or against the existence of high concentrations of salt in the lateral spaces.

If concentration gradients between lateral spaces produce a significant $E_j$, this EMF might be expected to "run down" gradually during inhibition of metabolism by azide. The component of $V_t$ and $I_{sc}$ which is due to $E_j$ would also be expected to decay more gradually than an active pump mechanism \textit{per se}. However, azide rapidly and completely abolishes $V_t$ and $I_{sc}$ (see chapter 2), which suggests that any $E_j$ which does exist is either very small, or extremely sensitive to metabolic inhibitors.

Table 6 shows the equivalent EMF's for apical and basal membranes under control conditions, during exposure to 1 mM cAMP, and cAMP-stimulation in Cl-free saline. Membrane and junctional resistances from Table 2 and Table 3 were used to calculate $R_a/R_j$ and $R_b/R_j$. Since slightly different values of $V_a$
Table 5.6. Calculation of steady-state equivalent electromotive forces ($E_a$ and $E_b$, respectively) across apical and basal membranes

<table>
<thead>
<tr>
<th>Saline</th>
<th>$V_a$ (mV)</th>
<th>$V_b$ (mV)</th>
<th>$V_t$ (mV)</th>
<th>$R_a/R_j$</th>
<th>$R_b/R_j$</th>
<th>$E_a$ (mV)</th>
<th>$E_b$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>a)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>57.0</td>
<td>48.4</td>
<td>8.6</td>
<td>0.2965</td>
<td>0.2605</td>
<td>54.5</td>
<td>50.6</td>
</tr>
<tr>
<td>+cAMP</td>
<td>63.8</td>
<td>34.0</td>
<td>29.8</td>
<td>0.0711</td>
<td>0.0581</td>
<td>61.7</td>
<td>32.3</td>
</tr>
<tr>
<td>Cl-free</td>
<td>45.7</td>
<td>41.0</td>
<td>4.7</td>
<td>0.0633</td>
<td>0.4100</td>
<td>45.4</td>
<td>42.9</td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>b)</td>
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<tr>
<td>Control</td>
<td>57.8</td>
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<td>Cl-free</td>
<td>56.7</td>
<td>50.4</td>
<td>6.3</td>
<td></td>
<td></td>
<td>56.3</td>
<td>53.0</td>
</tr>
<tr>
<td>+cAMP</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
</tbody>
</table>

a) voltages from Table 4.2, resistance ratios calculated from Table 5.2
b) voltages from Table 4.3, resistance ratios from Table 5.3
1 Recta bathed in normal saline (see Table 2.1) and Cl-free saline in which all Cl was replaced with gluconate.

$V_a$, $V_b$, apical and basal membrane potential respectively, (with respect to the intracellular compartment), $V_t$ (with respect to the serosal side).
and \( V_b \) were obtained during Cl and K microelectrode experiments (chapter 4, Tables 2 and 3), membrane, \( E_a \), \( E_b \) and EMF's were calculated separately for the two sets of data using equations (18) and (19). In both cases, addition of 1 mM cAMP increased \( E_a \) by 7-12 mV (i.e. intracellular side of the apical membrane became more negative) as would be expected if an absorptive Cl pump was stimulated. Upon removing Cl from cAMP-stimulated recta \( E_a \) declined by 16 mV (Table 6a) or 12 mV (Table 6b). These values are similar to those obtained under control conditions. In normal saline, exposure to cAMP resulted in a decline in \( E_b \), consistent with enhanced Cl exit out of the cells across the basal membrane. This could be explained simply by an increase in conductance; i.e. intracellular Cl is above electrochemical equilibrium across the basal membrane (chapter 4) so that any increase in Cl conductance would enhance Cl diffusion from the cell and lower \( E_b \) as observed in Table 6. This explanation for cAMP-induced \( \Delta E_b \) is feasible since \( -\Delta E_b \) is less than the electrochemical gradient driving Cl out of the cell (i.e. \( -\Delta E_b \) ranges between -18.3 and -12.6 mV in Table 6 as compared to a \( \Delta \mu_{Cl}^b / F = 23.3 \pm 1.4 \) mV; chapter 4).

Finally, \( \Delta \mu_{Cl}^b / F \) does not decline when Cl conductance of the basal membrane increases, presumably because Cl is pumped into the cell across the opposite (apical) membrane 10 times faster during cAMP exposure. The Cl pump would maintain \( \Delta \mu_{Cl}^b \) at a constant level (20.1 ± 0.6 mV, chapter 4) as \( E_b \) declined (chapters 2 and 4).

It is now possible to derive an equivalent electrical circuit model for cAMP-stimulated Cl transport across locust rectum. This exercise will also test the validity of most of the methods used in this thesis since it relies on data from ion-sensitive microelectrode experiments, flat-sheet cable analysis, and tracer flux measurements.
Figure 5.19  Equivalent circuit model of rectal epithelium showing electromotive force (EMF) and conductance for each ion at the mucosal and serosal cell borders. EMF's were calculated from intracellular ion activity measurements described in chapter 4. $E_{Cl}^C$ (25.1 mV), $E_{K}^C$ (54.7 mV), $E_{Na}^C$ (57.2 mV) under control conditions and $E_{Cl}^C$ (14.4 mV), $E_{K}^C$ (58.2 mV), and $E_{Na}^C$ (57.2 mV) during exposure to 1 mM cAMP. See Table 5.5a for summary of conductances used in calculating membrane equivalent EMF's.
Figure 19 shows the relevant circuit parameters. The electromotive force for each ion was calculated from intracellular ion activities measured in chapter 4 using the Nernst equation. For example, under control conditions:

\[ E_K = \frac{RT}{F} \ln \left( \frac{61.4}{7.2} \right) = 54.7 \text{ mV} \]  \hspace{1cm} (20)

Intracellular ion activities used in calculating EMF's under control conditions were (in mM): Cl (30.7), K (61.4), Na (8.0), and with cAMP: Cl (46.6), K (70.3) and Na (8.0). The ion activities in saline were Cl (82), K (7.2) and Na (75.2). The EMF driving Cl entry also includes an active pump EMF, which will be estimated later in this discussion. Since apical and basal membranes have very low sodium conductance, Na was not included in calculations of the net equivalent EMF's of the apical and basal membranes. Using ion-sensitive microelectrode data and the results of ion substitution during cable analysis, membrane EMF's are obtained:

\[ E^*_a = \frac{E_{a}^K K_{a} G_{a} + E_{a}^C I_{a} C_{a}}{G_{a}} \]  \hspace{1cm} (21)

\[ E^*_b = \frac{E_{b}^K K_{b} G_{b} + E_{b}^C I_{b} C_{b}}{G_{b}} \]  \hspace{1cm} (22)

where \( E^*_a \) and \( E^*_b \) are the total electromotive forces of apical and basal cell borders as calculated from the Nernst potentials for K and Cl. Since the net EMF of the epithelium is \( E^*_a - E^*_b \), then transepithelial potential \( (V_t) \) may be calculated as

\[ V_t = \frac{(E^*_a - E^*_b)R_j}{R_a + R_b + R_j} \]  \hspace{1cm} (23)

Using \( E^*_a \) and \( E^*_b \) calculated before and during exposure to cAMP in conjunction with ionic conductances from Table 2, we predict that under control conditions,
and during cAMP exposure:

\[ V_t = (55.5 - 22.6) \frac{620}{44 + 36 + 620} = 29.1 \text{ mV} \]

These predictions are in agreement with experimentally observed values, 8.6 ± 0.3 mV and 29.8 ± 0.5 mV (Table 2 of chapter 4). This provides indirect evidence for the validity of the ion-sensitive microelectrode data, for the partial ionic conductances, and also for the assumption that sodium contributes little towards \( E^*_a \) or \( E^*_b \) and may be safely ignored. Also, values of \( E^*_a \) and \( E^*_b \) calculated in control and cAMP-stimulated tissues also compare well with values of \( E_a \) and \( E_b \) calculated by circuit analysis in Table 6, although this is less surprising since both methods rely partially on measurements of membrane resistance (equations 18, 19, 21 and 22).

What is the EMF of active Cl transport in locust rectum? After converting net transepithelial Cl flux to current (\( J^{\text{Cl}}_{\text{net}} = 4.3 \mu\text{Eqcm}^{-2}\text{h}^{-1} \) under open-circuit conditions during cAMP exposure), the total driving force required for Cl entry is calculated from the Cl conductance of the apical membrane as

\[
E_a^{\text{pump}} = \frac{J^{\text{Cl}}_{\text{net}} F}{G_a^{\text{Cl}} 3.6 \times 10^3} - (E_a^{\text{Cl}} - V_a)
\]

\[ = 81.1 - (14.4 - 63.8) \]

\[ = 130.5 \text{ mV} \]

By assuming that all of the Cl conductance of the apical membrane resides in the pump pathway, the overall driving force of Cl transport is

\[
E^{\text{Cl}} = E_a^{\text{pump}} + E_a^{\text{Cl}} - V_a - E_b^{\text{Cl}} + V_b
\]

\[ = 130.5 + 14.4 - 63.8 - 14.4 + 34.0 \]

\[ = 100.7 \text{ mV} \]
This EMF is slightly lower than that of active Na transport in most vertebrate epithelia (105-158 mV: Ussing and Zerahn, 1951; Civan et al., 1966; Civan, 1970; Hong and Essig, 1976; Feig et al., 1977; Yonath and Civan, 1971; Lewis et al., 1978; Saito et al., 1974; Chen and Walser, 1975; Schultz et al., 1977; Canessa et al., 1978), and is considerably lower than the 200 mV calculated for active K transport in lepidopteran midgut by three methods (reviewed by Harvey, 1981). $E_{Cl}$ is probably an underestimate, since the true EMF can be measured accurately only when the transport system does not work, i.e. when no current is drawn from the $E_{Cl}$ "battery". However, assuming that the true $E_{Cl}$ is near 100.7 mV, then the energy required for Cl transport is $FE_{Cl} = 9.72$ kJ/mole or 2.32 kcal/mole Cl transported. It has been estimated that $\sim$20 Cl ions are transported per mole of O$_2$ consumed based on the increase in O$_2$ consumption when Cl is added to cAMP-stimulated recta (Chamberlin, 1981). If the Cl pump is an ATPase, this means that $\sim$3.3 Cl are transported per ATP and the energy required per mole of ATP to transport 3.3 Cl ions is 7.7 kcal. This estimate is lower than the energetic efficiency of active Na transport across toad bladder (via a known ATPase pump), which requires 11.7 kcal/mole of ATP (Canessa et al., 1978). Therefore, the energetics of locust Cl transport would not require an unusually efficient coupling between metabolism and an ATPase pump. The present estimate of $E_{Cl}$ is subject to error since it relies on data obtained by a variety of different experimental techniques. An independent method of calculating $E_{Cl}$ is used in the next section which confirms the above calculations.

Relationship between "active" and "passive" conductance

The relationship between short-circuit current ($I_{sc}$) and tissue conductance ($G_{t}$) may be used to calculate both the driving force of active ion transport and also the shunt conductance. (In this context, shunt conductance includes
cellular and paracellular "leak" pathways.) Representing active Na transport across toad bladder by the equivalent circuit shown in Figure 20, Yonath and Civan (1971) found that a plot of $G_t$ versus $I_{sc}$ during vasopressin exposure gave a straight line with slope $= 1/E^{Na}$ and a "y intercept" equal to the shunt conductance "$k_L"$. The rationale of the method is as follows. Under $I_{sc}$ conditions, the voltage between mucosal and serosal sides is 0 mV, so there is no net current flow through $k_L$ (see Fig. 20). By analogy with Ohm's Law, $I_{sc} = E^{Na} k_A$. Further, tissue conductance is given by $G_t = k_A + k_L$. Substituting $I_{sc}/E^{Na}$ for $k_A$ yields the equation for a straight line $G_t = k_L + (I_{sc}/E^{Na})$ having slope $= 1/E^{Na}$, which is the inverse driving force of active sodium transport and an intercept of $k_L$, the shunt conductance (Yonath and Civan, 1971).

This method is applicable to electrogenic ion transport across any epithelium provided that i) $I_{sc}$ and $G_t$ can be made to vary over a large range, and ii) the rate of active transport of ion "i" is varied by changing $k_A$ rather than $E_i$ or $k_L$. The cAMP dose-response curves shown in Figure 17 indicate that both $I_{sc}$ and $G_t$ are strongly dependent on [cAMP], increasing seven-fold and three-fold respectively when maximal doses are added. However, from chapter 4 it is clear that active entry of Cl across the apical membrane is directly stimulated by cAMP, suggesting that both $E^{Cl}$ and $G_t$ increase during cAMP exposure. Also, tracer fluxes and cable analysis experiments have shown that cAMP stimulates passive permeability of the rectum to potassium. In view of these complications, the method of Yonath and Civan seems inappropriate since it requires that $E^{Cl}$ and $k_L$ remain constant during exposure to cAMP. Nevertheless, if cAMP stimulates $k_A$ much more than $E^{Cl}$, then the $I_{sc}/G_t$ relation should still be linear and approximate $(E^{Cl})^{-1}$. Similarly, if the effects of cAMP on potassium permeability can be measured and subtracted so that changes
Figure 5.20  Equivalent circuit model used by Yonath and Civan (1971) to calculate the electromotive force of active Na transport ($E_{Na}$) and shunt conductance ($k_L$) in toad bladder epithelium. Conductance of the active transport pathway ($k_A$) was varied by exposing the bladder to vasopressin.
in $k_L$ do not influence the calculations, then the Yonath and Civan method should be valid.

Figure 21 shows the relationship between transepithelial conductance and $I_{sc}$. Also shown is the "corrected" or "Cl-dependent" $G_t/I_{sc}$ relation which was calculated by subtracting the $\Delta G_t$ produced by cAMP in Cl-free saline. $I_{sc}$ was taken from that in normal saline (Fig. 17) for both regressions. Prior to correcting for Cl-independent conductance, the apparent driving force of Cl ($E^{Cl}$) was 43.4 mV, much lower than calculated previously from intracellular measurements (100.7 mV). However, the shunt conductance ($k_L$) was 4.55 mS cm$^{-2}$ (resistance = 219.8 $\Omega$cm$^2$), in close agreement with the transepithelial resistance observed under control conditions (200-250 $\Omega$cm$^2$ without cAMP). After subtracting the cAMP-induced conductance measured in Cl-free saline (presumably resulting from an increase in $P_K$) the estimated EMF increased from 43.4 to 92.6 mV and the shunt conductance $k_L$ decreased from 4.55 to 0.88 mS cm$^{-2}$ (resistance = 1,138.0 $\Omega$sm$^2$). This "corrected" estimate of $E^{Cl}$ (92.6 mV) is in reasonable agreement with the value (100.7 mV) obtained independently from intracellular ion activities and cable analysis as described above. From the linearity of the $I_{sc}/G_t$ relation, it is likely that changes in $E^{Cl}$ during cAMP stimulation are small when compared to the stimulation of active conductance. This is not surprising since cable analysis showed that cAMP increases the Cl conductance of the basal membrane by approximately sevenfold. A small increase in $E^{Cl}$ (<50%) would presumably be obscured by such a large increase in conductance.

Finally, comparison of the shunt conductances calculated from the two regressions shown in Figure 21 reveals that Cl-independent shunt conductance $(k_L^{total} - k_L^{Cl})/(k_L^{total} \times 100\%)$ is 81% of the total tissue conductance. This finding is consistent with high potassium permeability of the epithelium.
Figure 5.21  Relationship between $I_{sc}$ and transepithelial conductance ($G_t$) calculated from mean cAMP dose-response curves. See Figure 5.17 for original data and variability. The upper line shows the relationship between $G_t$ and $I_{sc}$ in normal saline but includes the stimulatory effect of cAMP on K conductance. The lower line is the corrected, Cl-dependent $G_t/I_{sc}$ relation obtained by subtracting the cAMP-induced $\Delta G_t$ observed in Cl-free saline from that observed in normal saline.
as indicated by 1) effects of external potassium on membrane potentials (≈50 mV/decade change in $[K]$, chapter 4), and 2) cable analysis (≈76% of transcellular conductance due to K).

In summary, cAMP may cause some increase in $E^{Cl}$, but this is small in comparison to the increase in Cl conductance of the basal membrane. As explained previously, depolarization of $V_b$ during cAMP-stimulation may be attributed to the diffusional exit of Cl from the cell across the basal membrane when Cl conductance of this membrane is increased by cAMP. The overall EMF driving transepithelial Cl transport is probably between 92.6 and 100.7 mV. The first of these two estimates is derived from i) intracellular ion activities measured using ion-sensitive microelectrodes, ii) membrane partial ionic conductances from cable analyses during ion substitutions, and iii) net Cl absorption rate from tracer flux measurements under open-circuit conditions. In contrast, the second estimate of $E^{Cl}$ is based simply on measurements of short-circuit current and transepithelial resistance. Agreement between these two independent estimates of $E^{Cl}$ provides circumstantial evidence for the results obtained in this thesis.
The properties of active Cl absorption across locust rectum are not consistent with models which have been proposed for Cl transport across other epithelia. In this chapter I briefly summarize the cellular mechanism of KCl transport and its regulation in locust rectum as deduced from the work in this thesis. The experimental results have already been discussed and integrated. The reader is referred to the appropriate chapters for details and supporting evidence.

Electrogenic absorption of Cl by in vitro locust rectum is now firmly established from i) $J_{\text{net}}^{\text{Cl}}$ measured under true $I_{\text{sc}}$ conditions, ii) unidirectional flux ratios measured under open-circuit conditions, and iii) the effects of Cl removal on $I_{\text{sc}}$ during cAMP stimulation (chapter 2; see also Williams et al., 1978, and Spring and Phillips, 1980b). Chloride entry into the cells is active (chapter 4), but is not driven by the Na-coupled mechanism found in vertebrate epithelia: i) $J_{\text{net}}^{\text{Cl}}$ and $I_{\text{sc}}$ both exceed 5.5 μEq cm$^{-2}$ h$^{-1}$ during cAMP stimulation after 6 h exposure to nominally Na-free saline (chapter 2); ii) there is no correlation between Cl-dependent $I_{\text{sc}}$ and mucosal Na concentration (chapter 2); iii) cAMP stimulates unidirectional $^{36}$Cl influx into rectal tissue seven-fold but does not increase the influx of $^{22}$Na (chapter 2); iv) $I_{\text{sc}}$ is not inhibited by 1 mM furosemide, a potent inhibitor of NaCl cotransport in other cells (chapter 2). $J_{\text{net}}^{\text{Cl}}$ and $I_{\text{sc}}$ are also insensitive to 1 mM ouabain after 2-5 h exposure (chapter 2) and Na/K ATPase isolated from locust rectum has normal sensitivity to this inhibitor (Peacock, 1981); v) $J_{\text{net}}^{\text{Cl}}$ is not affected by reducing the Na electrochemical gradient across the apical membrane by 85% from 128 mV to 22 mV (chapter 4); vi) there is no dependence of the Cl electrochemical gradient across the apical membrane ($\Delta u_{\text{Cl}}$) on the parallel Na gradient,
contrary to predictions of NaCl cotransport models in which $\Delta V_{cl}^{a}$ is maintained via Na-coupled influx (i.e., secondary active transport; chapter 4). There is strong indirect evidence that Cl does not enter the cells in exchange for HCO$_3^{-}$: i) transepithelial Cl transport is definitely electrogenic (chapter 2), and Cl entry may also be rheogenic (charge-separating) since cAMP produces a marked hyperpolarization of $V_a$, even in K,HCO$_3$,CO$_2$-free saline (chapter 4); ii) metabolic CO$_2$ production would be inadequate to provide HCO$_3^{-}$ for 1:1 exchange with Cl at the rates of $J_{ms}^{Cl}$ observed under HCO$_3$,CO$_2$-free conditions (calculated in chapter 2 from data of M. Chamberlin); iii) the rate of alkalinization of the mucosal side is too low under HCO$_3$,CO$_2$-free conditions to account for $J_{net}^{Cl}$ and may be explained by NH$_4$ secretion (chapter 2); iv) Cl-dependent $I_{sc}$ is not inhibited by exposure to the potent anion exchange inhibitor SITS (1 mM) or to 1 mM acetazolamide for 1 h. Cotransport of Cl with protons is also unlikely since i) Cl-dependent $I_{sc}$ is not inhibited by raising mucosal pH from 7.0 to 8.0, a manoeuvre which should reduce or abolish the proton electrochemical gradient across the apical membrane if the intracellular pH in locust rectum is similar to that in other tissues (chapter 2); ii) low mucosal pH inhibits rather than stimulates $I_{sc}$, contrary to expectations for a HCl coentry process.

Figure 6.1 summarizes my interpretation of the data in this thesis and is the simplest model which is consistent with all of the experimental results. Cl entry is active (chapter 4) and is stimulated by mucosal K (chapters 3, 4). Cl is probably not cotransported with K since: i) 35% of $I_{sc}$ during cAMP stimulation is independent of potassium (chapter 2); ii) the apical membrane hyperpolarizes during exposure to cAMP after prolonged exposure to K-free saline, indicating electrogenic Cl entry (chapter 4); iii) transepithelial K fluxes are completely independent of Cl.
Figure 6.1 Summary diagram of the cellular mechanism and regulation of KCl transport across locust rectum (π high osmotic concentration).
and provide no indication of rigid KCl cotransport; iv) Cl entry is definitely not energized by coentry with K, since $\Delta u^a_K = 0$ under $I_{sc}$ conditions when $J^C{l}_n$ is 10 $\mu$Eq cm$^{-2}$ h$^{-1}$, and is also low (<15 mV) under open-circuit conditions.

The simplest explanation for the effects of K on cAMP-stimulated Cl transport is that external K interacts in some manner with a rheogenic Cl pump in the apical membrane, stimulating Cl entry without actually being cotransported. Available data do not exclude the possibility that two "primary" Cl pumps are present, one which is K-independent and electrogenic, and a second KCl cotransport system. Although this hypothesis would explain the K-dependent and independent components, it requires that K be actively "recycled" at the apical membrane to account for the small $J^K_{net}$ observed under $I_{sc}$ conditions. Also, it is not consistent with the observation that $J^K_{ms}$ is Cl-independent (chapter 3). Further studies will be necessary to understand the details of K-stimulation. For example, isolation of a K + Cl-stimulated ATPase from the apical membrane would provide strong indirect evidence for this hypothesis.

Balshin (1971) showed that glycine uptake into rectal tissue is Na-dependent. Although Na-cotransport with amino acids was not directly demonstrated, coupled transport with organic solutes may be an important entry mechanism for Na into rectal cells. Na conductance of the apical membrane is probably low compared to K since: i) $V_a$ varies approximately 50 mV/decade external K activity (chapter 4); ii) $V_a$ is not affected by replacing mucosal Na with N-methyl-D-glucamine. Intracellular levels of Na and K are consistent with the presence of a Na/K ATPase pump in the basal membrane. Net Na absorption under $I_{sc}$ conditions would involve Na entry across the apical membrane down a large (>120 mV) electrochemical gradient and require exit across the basal membrane against the same gradient.
In addition to stimulating the Cl pump, cAMP reduces $R_t$. Results in chapter 5 suggest that locust rectum becomes a very "tight" epithelium due to cAMP-induced K conductance at the apical membrane and cAMP-induced Cl conductance at the basal membrane. These cAMP-stimulated conductances may be channels as shown in Figure 6. The increase in basal membrane Cl conductance is more than adequate to allow Cl exit from the cell by electrodiffusion down its measured electrochemical gradient (chapters 4 and 5). Most K may exit the cells by electrodiffusion during cAMP exposure, although some other electroneutral mechanism might also be involved since both basal membrane K electrochemical gradient and K conductance are lower than those for Cl. K entry is almost certainly by electrodiffusion as a counter ion for Cl transport (chapter 3). This electrical coupling is greatly enhanced by the high K selectivity of the apical membrane and the large cAMP-induced K conductance in the apical membrane (chapters 4 and 5). There is a small active flux of K (17% of the total under open-circuit conditions). This active component has high affinity for K ($K_c < 10 \text{ mM}$) and is probably responsible for reducing luminal K to very low concentrations in vivo in salt-depleted animals.

Finally, in addition to "up-regulation" of Cl absorption by CTSH and by mucosal K ions, "down-regulation" is exerted by high osmotic pressure and low mucosal pH (chapter 2), which are observed in vivo near the end of the normal reabsorptive cycle. The results also indicate that apical membrane K conductance is "down-regulated" by high mucosal K concentration and osmotic pressure (chapters 3 and 5). In summary, the mechanism of KCl reabsorption is unlike any system which has been described previously. Although KCl reabsorption is stimulated by a neuropeptide hormone (on-off control), the absorption rate may be controlled locally in vivo by several interacting factors including mucosal K concentration, pH and osmotic pressure.
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Figure A.1  Electrical circuit used to voltage-clamp locust rectum during tracer and microelectrode experiments.