CELLULAR MECHANISMS OF ACID/BASE TRANSPORT IN AN INSECT EXCRETORY EPITHELium

By

ROBERT BRENT THOMSON

B.Sc. (HONS), The University of Guelph, 1982

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in

THE FACULTY OF GRADUATE STUDIES
DEPARTMENT OF ZOOLOGY

We accept this thesis as conforming
to the required standard

THE UNIVERSITY OF BRITISH COLUMBIA
JANUARY 1990

© Robert Brent Thomson, 1990
In presenting this thesis in partial fulfilment of the requirements for an advanced degree at the University of British Columbia, I agree that the Library shall make it freely available for reference and study. I further agree that permission for extensive copying of this thesis for scholarly purposes may be granted by the head of my department or by his or her representatives. It is understood that copying or publication of this thesis for financial gain shall not be allowed without my written permission.

Department of ZOOLOGY

The University of British Columbia
Vancouver, Canada

Date JANUARY 7, 1990
ABSTRACT

The cellular mechanisms responsible for rectal acidification in the desert locust, Schistocerca gregaria, were investigated in isolated recta mounted as flat sheets in modified Ussing chambers. In the absence of exogenous CO₂, HCO₃⁻, and phosphate, the isolated rectum (under both open- and short-circuit current conditions) was capable of rates of net acid secretion (JₚH⁺) similar to those observed in vivo, demonstrating the viability of the preparation and suggesting that rectal acidification was due to proton secretion rather than selective movements of HCO₃⁻ or phosphate. The possibility that trace levels of metabolic CO₂ might be generating sufficient HCO₃⁻ to account for the observed rates of rectal acidification (via HCO₃⁻ reabsorption) was assessed by adding exogenous CO₂/HCO₃⁻ to the contraluminal bath. The small increases in JₚH⁺ observed after addition of 2% or 5% CO₂ were shown to be due to simple hydration of CO₂ which had diffused into the lumen (from the contraluminal bath), rather than changes in rates of HCO₃⁻ reabsorption. Since measurable quantities of luminal HCO₃⁻ did not directly affect the apical acid/base transport mechanism per se, it was concluded that metabolic CO₂ could not generate sufficient HCO₃⁻ in the lumen to account for the rates of rectal acidification observed under nominally CO₂/HCO₃⁻-free conditions and that JₚH⁺ must be due to a proton secretory rather than bicarbonate reabsorptive mechanism. Microelectrode measurements of intracellular pH (pHi) and apical and basolateral membrane potentials (Va and Vb respectively) indicated that luminal pH was not in equilibrium with either contraluminal pH or pHi and that the mechanism responsible for active luminal acid secretion resided on the apical membrane. Preliminary measurements of bath total ammonia (ie. NH₃ + NH₄⁺) levels in the previous experiments suggested that the rectum was actively secreting ammonia at significant rates across the apical membrane into the lumen. If the ammonia crossed the apical membrane as NH₃ rather than NH₄⁺, rates of luminal ammonia secretion (Jₐₐ₄) would have to be added to JₚH⁺ to obtain corrected values of luminal proton secretion. In the absence of exogenously
added ammonia and CO₂, ammonia was preferentially secreted into the lumen under both open- and short-circuit current conditions. \( J_{\text{Amn}} \) was dependent on the presence of luminal amino acids and was relatively unaffected by K⁺ removal or changes in luminal pH from 7.00 to 5.00. Bilateral Na⁺ substitution or luminal addition of 1mM amiloride reduced \( J_{\text{Amn}} \) by 63% and 65% respectively. The data consistently demonstrate that the rectum secretes significant quantities of endogenously produced ammonia preferentially into the lumen as \( \text{NH}_4^+ \) rather than \( \text{NH}_3 \) via an apical Na⁺/\( \text{NH}_4^+ \) exchange mechanism. Clearly, rates of net acid secretion estimated by titratable acidity do not have to include a correction for luminal ammonia secretion. Although \( J_{\text{H}^+} \) was completely unaffected by changes in contraluminal pH, it could be progressively reduced (and eventually abolished) by imposition of either transepithelial pH gradients (lumen acid) or transepithelial electrical gradients (lumen positive). Under short-circuit current conditions, the bulk of \( J_{\text{H}^+} \) was not dependent on Na⁺, K⁺, Cl⁻, Mg²⁺, or Ca²⁺ and was due to a primary electrogenic proton translocating mechanism located on the apical membrane. A small component (10-16%) of \( J_{\text{H}^+} \) measured under these conditions could be attributed to an apical amiloride-inhibitable Na⁺/H⁺ exchange mechanism. Inhibition of \( J_{\text{H}^+} \) by anoxia or reduction of luminal pH unmasked a significant proton diffusional pathway on the apical membrane in parallel with the active proton pump. The fact that \( J_{\text{H}^+} \) was significantly inhibited (42%-66%) by contraluminal addition of 1mM cAMP and relatively unaffected by changes in contraluminal pCO₂ or pH suggests that net acid secretion in the locust rectum \textit{in vivo} is modulated by circulating hormonal factors rather than haemolymph pH or pCO₂ \textit{per se}. 
# Table of Contents

Abstract .................................................. ii  
Table of Contents .......................................... iv  
List of Tables .............................................. vi  
List of Figures ............................................. viii  
List of Abbreviations ...................................... x  
Acknowledgements .......................................... xiii  

Chapter 1: General Introduction ............................................. 1  
  Locust Excretory System ............................................. 3  
  Ultrastructure of the Rectal Epithelium ................................. 5  
  Solute and Water Transport in the Locust Rectum ............................ 7  
  Preliminary Acid/Base Observations .................................... 10  
  General Acid/Base Transport Mechanisms .................................. 13  
  Transepithelial Acid/Base Transport .................................... 20  
  Intracellular pH Regulation .......................................... 21  
  Nature of Rectal Acidification in the Desert Locust ...................... 25

Chapter 2: Confirmation of Rectal Acidification .......................... 28  
  Introduction .................................................................. 28  
  Materials and Methods .............................................. 29  
  Results ...................................................................... 38  
    Confirmation of rectal acidification .................................. 38  
    Source of rectal acidification ...................................... 51  
    Acid secretion versus base reabsorption .............................. 58  
  Discussion .................................................................... 65  
    Proton secretion versus bicarbonate reabsorption .................... 67  
  Summary ..................................................................... 69

Chapter 3: Characterization of Rectal Ammonia Secretion ................ 73  
  Introduction .................................................................. 73  
  Materials and Methods .............................................. 75  
  Results ...................................................................... 76  
    Characterization of ammonia secretion ................................ 76  
    Effect of pH and $J_{H^+}$ on ammonia secretion ...................... 80  
  Discussion .................................................................... 91  
    Characterization of ammonia production and secretion ............. 91  
    Nature of secreted ammonia ....................................... 94  
    Mechanism of NH$_4^+$ transport .................................. 96  
  Summary ..................................................................... 98

Chapter 4: Characterization of Rectal Acid Secretion .................... 99  
  Introduction .................................................................. 99  
  Materials and Methods .............................................. 100  
  Results .................................................................... 101  
    Effect of transepithelial electrochemical gradients on rectal acid secretion 101  
    Ionic requirements of rectal acid secretion .......................... 110  
    Sodium .................................................................... 110
List of Tables

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Table 2.1</td>
<td><em>In vivo</em> and <em>in vitro</em> measurements of luminal acidification in the locust rectum.</td>
<td>41</td>
</tr>
<tr>
<td>Table 2.2</td>
<td>Effect of cAMP on luminal and contraluminal acid/base transport ($J_{H/\text{OH}}$) under Isc conditions when simple silver electrodes are used to pass the short-circuit current.</td>
<td>45</td>
</tr>
<tr>
<td>Table 2.3</td>
<td>Effect of current sending electrode configuration on Isc, Vt, and Rt when locust recta are stimulated with cAMP under CO$_2$/HCO$_3^-$-free conditions.</td>
<td>50</td>
</tr>
<tr>
<td>Table 2.4</td>
<td>Relative rates of luminal acidification ($J_{\text{H}^+}$) and contraluminal alkalinization ($J_{\text{OH}^-}$) by locust recta under CO$_2$/HCO$_3^-$-free conditions.</td>
<td>52</td>
</tr>
<tr>
<td>Table 2.5</td>
<td>Intracellular pH and calculated H$^+$ electrochemical potentials for apical and basolateral membranes of locust recta bathed bilaterally by phosphate- and CO$_2$/HCO$_3^-$-free saline.</td>
<td>56</td>
</tr>
<tr>
<td>Table 2.6</td>
<td>Changes in rates of rectal acidification ($\Delta J_{\text{H}^+}$) induced by contraluminal addition of CO$_2$/HCO$_3^-$-free saline.</td>
<td>61</td>
</tr>
<tr>
<td>Table 3.1</td>
<td>Amino acid precursors of luminal ammonia secretion.</td>
<td>79</td>
</tr>
<tr>
<td>Table 3.2</td>
<td>Effect of saline buffer concentration on luminal ammonia secretion rates.</td>
<td>86</td>
</tr>
<tr>
<td>Table 3.3</td>
<td>Effect of reversing transepithelial pH gradients on luminal and contraluminal ammonia secretion rates.</td>
<td>88</td>
</tr>
<tr>
<td>Table 3.4</td>
<td>Effect of amiloride on luminal ammonia secretion rates under short-circuit current conditions.</td>
<td>90</td>
</tr>
<tr>
<td>Table 3.5</td>
<td>Effect of contraluminal cAMP on luminal ammonia secretion rates under short-circuit current conditions.</td>
<td>92</td>
</tr>
<tr>
<td>Table 4.1</td>
<td>Effect of bilateral and luminal Na$^+$ removal on rectal acid secretion ($J_{\text{H}^+}$) under short-circuit current conditions.</td>
<td>112</td>
</tr>
<tr>
<td>Table 4.2</td>
<td>Effect of amiloride on rectal acid secretion ($J_{\text{H}^+}$) under short-circuit current conditions.</td>
<td>115</td>
</tr>
<tr>
<td>Table 4.3</td>
<td>Effect of bilateral and luminal K$^+$ removal on rectal acid secretion ($J_{\text{H}^+}$) under short-circuit current conditions.</td>
<td>117</td>
</tr>
<tr>
<td>Table 4.4</td>
<td>Effect of omeprazole on rectal acid secretion ($J_{\text{H}^+}$) under short-circuit current conditions.</td>
<td>120</td>
</tr>
<tr>
<td>Table</td>
<td>Description</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>-------------</td>
<td></td>
</tr>
<tr>
<td>4.5</td>
<td>Effect of bilateral Cl⁻ removal on rectal acid secretion ($J_{H^+}$) under open- and short-circuit current conditions. 122</td>
<td></td>
</tr>
<tr>
<td>4.6</td>
<td>Effect of bilateral removal of Ca²⁺ or Mg²⁺ on rectal acid secretion ($J_{H^+}$) under short-circuit current conditions. 124</td>
<td></td>
</tr>
<tr>
<td>4.7</td>
<td>Transepithelial pH gradients required to abolish net acid secretion after luminal ion substitution under open-circuit conditions. 129</td>
<td></td>
</tr>
<tr>
<td>4.8</td>
<td>Effect of transepithelial pH gradients and luminal ion substitutions on membrane potentials, $R_t$, and $I_{app}$. 130</td>
<td></td>
</tr>
<tr>
<td>4.9</td>
<td>Effect of acetazolamide on rectal acid secretion under short-circuit current conditions. 134</td>
<td></td>
</tr>
<tr>
<td>4.10</td>
<td>Effect of inhibitors on rectal acid secretion under short-circuit current conditions. 136</td>
<td></td>
</tr>
<tr>
<td>4.11</td>
<td>Effect of contraluminal cAMP on rectal acid secretion under open- and short-circuit current conditions. 143</td>
<td></td>
</tr>
<tr>
<td>4.12</td>
<td>The source of short-circuit current in unstimulated locust recta. 157</td>
<td></td>
</tr>
</tbody>
</table>
## List of Figures

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Figure 1.1</td>
<td>Schematic representation of the locust excretory system.</td>
<td>4</td>
</tr>
<tr>
<td>Figure 1.2</td>
<td>Diagrammatic representation of a transverse section through a locust rectal pad</td>
<td>6</td>
</tr>
<tr>
<td>Figure 1.3</td>
<td>Summary of ion transport processes in the locust rectum.</td>
<td>8</td>
</tr>
<tr>
<td>Figure 1.4</td>
<td><em>In vivo</em> pH profile of locust gut contents.</td>
<td>11</td>
</tr>
<tr>
<td>Figure 2.1</td>
<td>Standard Ussing chamber assembly used for measurement of $J_{H^+}$, $J_{OH^-}$, $\cdot V_t$, and $I_{sc}$.</td>
<td>30</td>
</tr>
<tr>
<td>Figure 2.2</td>
<td>Microelectrode preparation used for measurement of apical and basolateral membrane potentials and intracellular pH.</td>
<td>33</td>
</tr>
<tr>
<td>Figure 2.3</td>
<td>Time course of luminal acidification ($J_{H^+}$) and transepithelial potential ($V_t$) in isolated recta mounted as flat-sheets in Ussing chambers under open-circuit conditions.</td>
<td>39</td>
</tr>
<tr>
<td>Figure 2.4</td>
<td>Effect of contraluminal cAMP and bilateral Cl⁻ substitution on luminal acid/base transport, transepithelial net Cl⁻ flux, and short-circuit current.</td>
<td>44</td>
</tr>
<tr>
<td>Figure 2.5</td>
<td>Effect of electrode configuration and quantity of current passed on apparent rates of luminal alkalinization.</td>
<td>46</td>
</tr>
<tr>
<td>Figure 2.6</td>
<td>Typical trace obtained with double-barreled ion sensitive microelectrode under CO₂/HCO₃⁻-free conditions.</td>
<td>54</td>
</tr>
<tr>
<td>Figure 2.7</td>
<td>Distribution of intracellular pH values in rectal epithelial cells under open-circuit conditions.</td>
<td>57</td>
</tr>
<tr>
<td>Figure 2.8</td>
<td>Effect of 6h tissue exposure on buffering characteristics of CO₂/HCO₃⁻-free saline.</td>
<td>64</td>
</tr>
<tr>
<td>Figure 2.9</td>
<td>Proposed models for rectal acidification under bilateral CO₂/HCO₃⁻-free conditions.</td>
<td>71</td>
</tr>
<tr>
<td>Figure 3.1</td>
<td>Luminal and contraluminal ammonia secretion rates ($J_{Amm}$) measured under open- and short-circuit current conditions.</td>
<td>77</td>
</tr>
<tr>
<td>Figure 3.2</td>
<td>Effect of amino acid source on luminal ammonia secretion rates ($J_{Amm}$).</td>
<td>81</td>
</tr>
<tr>
<td>Figure 3.3</td>
<td>Effect of transepithelial pH gradients on luminal ammonia secretion rates and rates of luminal acidification.</td>
<td>83</td>
</tr>
<tr>
<td>Figure 3.4</td>
<td>Effect of transepithelial pH gradients on intracellular membrane potentials, transepithelial potential difference, and intracellular pH.</td>
<td>84</td>
</tr>
<tr>
<td>Figure 3.5</td>
<td>Effect of bilateral ion substitution on luminal ammonia secretion rates ($J_{Amm}$).</td>
<td></td>
</tr>
<tr>
<td>Figure 4.1</td>
<td>Effect of contraluminal pH on $J_{H^+}$, Vt, and $I_{app}$.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.2</td>
<td>Effect of luminal pH on $J_{H^+}$, Vt, and $I_{app}$.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.3</td>
<td>Effect of transepithelial membrane potential on net acid secretion.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.4</td>
<td>Typical recording of Va, Vb, and Vt under open-circuit conditions when luminal pH was decreased stepwise from 7.00 to the value required to abolish net acid secretion.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.5</td>
<td>Effect of anoxia on $J_{H^+}$, Vt, and Rt under open-circuit conditions.</td>
<td></td>
</tr>
<tr>
<td>Figure 4.6</td>
<td>Effect of transepithelial electrochemical proton gradients on rates of net acid secretion.</td>
<td></td>
</tr>
<tr>
<td>Figure 5.1</td>
<td>Proposed model for acid/base transport in the <em>in vivo</em> locust rectum.</td>
<td></td>
</tr>
</tbody>
</table>
List of Abbreviations

μm – micron  
μM – micromolar  
mM – millimolar  
h – hours  
min – minutes  
s – seconds  
Hz – Hertz  
mV – millivolts  
Ω cm² – ohms centimeter squared  
μA – microamps  
Ha – haemolymph  
Il – ileum  
Cn – colon  
Re – rectum  
CC – copora cardiaca  
VG – ventral ganglia  
μequiv cm⁻² h⁻¹ – microequivalents per square centimeter per hour  
Vt – transepithelial potential  
V_{CLAMP} – transepithelial potential set by voltage clamp  
V_a, V_b – apical and basolateral membrane potentials respectively  
V_i – electrical potential difference between the reference- and H⁺-sensitive barrels of a microelectrode  
I_{sc} – short-circuit current  
I_{app} – current required to clamp V_t at 0 mV  
R_a, R_b – resistance of the apical and basolateral membranes respectively
Rj - paracellular resistance
Rt - transepithelial resistance
$J_{H^+}$ - rate of luminal acidification
$J_{OH^-}$ - rate of contraluminal alkalinization
$J_{Amm}$ - rate of luminal ammonia secretion
$J_{HCO_3^-}$ - apparent rate of bicarbonate formation in the lumen
pHi - intracellular pH
PMF - proton motive force
cAMP - adenosine 3':5'-cyclic monophosphoric acid
CTSH - chloride transport stimulating hormone
ATP - adenosine 5'-triphosphate
ATPase - adenosine 5'-triphosphatase
NADH - $\beta$-nicotinamide adenine dinucleotide, reduced form
LDH - lactate dehydrogenase
GLDH - glutamate dehydrogenase
MES - 2(N-morpholino)ethanesulfonic acid
MOPS - 3-(N-morpholino)propanesulfonic acid
Glu - glutamine
Pro - proline
Ser - serine
Ala - alanine
DMSO - dimethylsulphoxide
ACTZ - acetazolamide
EGTA - ethyleneglycol-bis-(B-aminoethyl ether) N,N,N',N'-tetraacetic acid
SITS - 4-acetamide-4'isothiocyano-stilbene-2,2'-disulfonic acid
DIDS - 4,4'-Diisothiocyanato-stilbene-2,2'-disulfonic acid
SE — standard error

n — number

z — valence

R — gas constant

T — absolute temperature (°Kelvin)

F — Faraday’s number

\(a_i\) — intracellular activity of ion “i”

\(a_o\) — extracellular activity of ion “i”

\(r^2\) — coefficient of determination

\(\Delta\mu_{H}/F\) — electrochemical potential for protons
Acknowledgements

I would like to take this opportunity to sincerely thank Dr. John Phillips for his guidance, generosity, and unquenchable enthusiasm. It has truly been an enjoyable experience.

I am grateful to Drs. W.K. Milsom, V. Palaty, and P.W. Hochachka for comments on the manuscript.

I thank Joan Martin for helping me get things started when I first arrived and many stimulating and enjoyable conversations in the years since.

I thank Neil Audsley for making the lab such an enjoyable place to work and for providing countless hours of entertainment.

I am grateful to Dr. Jon Harrison for generous use of unpublished data, performing lactate measurements, and for enlightening me on “whole animal” acid/base physiology.

I especially wish to thank Joyce Thomson for technical assistance with the ammonia project, typing and illustrations in the “pre-computer” days, and patience and support over the past years.

Thank-you to Ben and Elise for the smiles which keep me going.
CHAPTER ONE: General Introduction

The mechanisms of pH regulation in terrestrial insects are virtually unknown. Previous studies have provided valuable measurements of pertinent acid/base parameters (e.g., haemolymph pH, pCO₂, and buffering capacity; Craig and Clark, 1938; Hastings and Pepper, 1943; Levenbook, 1950a,b), but generally very little insight into actual mechanisms of pH regulation. Respiration studies with *Nauphoeta cinerea* (Snyder *et al.*, 1980) and *Schistocerca nitens* (Harrison, 1987) suggest that like terrestrial vertebrates, terrestrial insects respond to acute haemolymph pH changes by varying rates of ventilation and presumably carbon dioxide excretion. It is not clear however, how, or to what extent, terrestrial insects regulate levels of non-volatile acids and bases (particularly HCO₃⁻) to maintain long-term homeostatic pH values.

In terrestrial vertebrates, long-term pH regulation is achieved by controlled excretion or reabsorption of titratable acids, ammonia, and bicarbonate in the kidney. In terrestrial insects most of the homeostatic functions performed by the vertebrate kidney are carried out in the Malpighian tubule-hindgut complex (reviewed by Phillips, 1981). A primary isosmotic urine containing most blood solutes is formed in the Malpighian tubules and selective reabsorption of water and solutes occurs in the ileum and rectum. Harrison (1988, 1989) has shown that *Schistocerca nitens* and *Melanopus bivittatus* can modulate haemolymph pH and bicarbonate concentrations without concomitant changes in haemolymph pCO₂. More recently, he has shown (Harrison, personal communication) that desert locusts (*Schistocerca gregaria*) made acidotic by injection with HCl recover completely from the pH insult (6-8 h recovery) almost entirely by modulating haemolymph HCO₃⁻ concentrations rather than pCO₂. In order to do this, some form of transmembrane transport of acid/base relevant ions clearly must occur. Given the general nature of the functions carried out by the excretory system, it is likely that regulation of non-volatile acids and bases occurs somewhere in the hindgut.
The terminal segment of the hindgut (ie. the rectum) is an important site for pH and bicarbonate regulation in mosquito larvae inhabiting alkaline salt lakes (Strange et al., 1982). In a number of terrestrial insects, the rectum has been indirectly implicated in pH regulation by virtue of acidic rectal contents (eg. Waterhouse, 1940; Ramsay, 1956; Phillips, 1961) and an ability to transport a variety of weak acids and bases (ammonia: Prusch, 1972; phosphate: Andrusiak et al., 1980; acetate: Baumeister et al., 1981).

Phillips (1961) and Speight (1967) conducted preliminary studies directly investigating the possibility of the rectum playing a role in haemolymph pH regulation in a representative terrestrial insect, the desert locust (Schistocerca gregaria). Phillips (1961) observed that the rectal contents were consistently acid (pH 5-6) even after the lumen had been rinsed out several times with alkaline buffer solutions. On the basis of haemolymph pH values (7.13) and rectal transepithelial potentials (20 mV lumen positive), he concluded that the low pH observed in the locust rectum in vivo (eg. 4.73) could not be the result of simple passive distribution of H⁺ ions across the rectal epithelium (ie. predicted Nernst pH for the lumen = 7.47). He proposed that the rectal epithelium per se actively maintained the low luminal pH observed in the rectum and that the rectum might be the site in the excretory system involved in haemolymph pH regulation. Speight (1967) confirmed Phillips’ observations and further showed that rates of rectal acidification were closely linked to the acid/base status of the animal. Clearly, locusts were able to regulate their haemolymph pH and the rectum appeared to play a role in that recovery.

Despite Phillips’ and Speight’s initial investigations, there was still some speculation as to whether rectal acidification was due to the epithelial cells per se, bacterial metabolism in the lumen, or some as yet unidentified source of acid equivalents. In addition, no one had been able to measure significant rates of luminal acidification in standard in vitro isolated rectal preparations (eg. Ussing chamber). These new findings casted doubt on the role of the rectum in pH regulation and emphasized the need for a serious assessment of the acid/base
transporting capabilities of the rectal epithelium. The present study addresses that need. Using a variety of in vitro electrophysiological techniques, the locust rectum is assayed for several acid/base transport parameters, relevant cellular mechanisms, their control, and their implications.

Locust Excretory System

The general form of the locust excretory system is typical of that found in many terrestrial insects (Fig. 1.1; see Phillips, 1981 for review). A primary isosmotic urine containing most blood solutes is formed in the Malpighian tubules and selective reabsorption of salts, water, and metabolites occurs in the ileum and rectum. The primary urine formed in the Malpighian tubules (high in K\(^+\), low in Na\(^+\)) is a result of active K\(^+\) transport rather than pressure driven filtration. An apical K\(^+\) pump sets up favourable concentration gradients for passive water movement and diffusion of small blood solutes into the lumen (reviewed by Phillips, 1981).

Both the ileum and rectum play major roles in solute and water recovery and together determine the final composition of the excreta. It appears that the primary function of the ileum is bulk recovery of desirable solutes and water while that of the rectum is adjusting the final osmotic and ionic content of the excreta. Although the two segments of the hindgut do perform many similar functions, there are several notable differences (see Lechleitner, 1988 for complete review). The ileum is only capable of isosmotic fluid reabsorption, whereas the rectum actively reabsorbs water from the lumen to create a strongly hyperosmotic excreta (Lechleitner and Phillips, 1989). Moreover, while fluid uptake in the ileum is primarily driven by ionic means, fluid movement in the rectum is driven by both ion and amino acid (eg. proline) uptake. The second major difference is that whereas only K\(^+\) and Cl\(^-\) uptake appear to be under hormonal control in the rectum, K\(^+\), Cl\(^-\), and Na\(^+\) uptake are under hormonal control in the ileum. On the basis of in vitro Na\(^+\) uptake rates, Lechleitner (1988)
Figure 1.1. Schematic representation of the locust excretory system. See text for complete details. Thick solid arrows indicate active transport processes; open arrows indicate passive transfer. Figure taken from Phillips (1981).
has suggested that most of the \( \text{Na}^{+} \) entering the hindgut \textit{in vivo} will be reabsorbed in the ileum before the gut contents reach the rectum.

**Ultrastructure of the Rectal Epithelium**

The rectum consists of 6 radially arranged longitudinal pads covered with a selectively permeable cuticular intima (Fig. 1.2; see Phillips \textit{et al.}, 1986 for review). The cuticle is attached to the epithelium at narrow junctional cell complexes between each pad, creating a subcuticular space above the apical border of the pad cells. The cuticle acts as a molecular sieve by virtue of water filled pores (6.5 Å radius; Phillips and Dockrill, 1968) lined with fixed negative charges (\( \text{pK} = 4 \); Lewis, 1971). This arrangement precludes uptake of large toxic substances while permitting reabsorption of water, salts, and desirable solutes.

The rectal pads are composed almost entirely of large columnar “Principal” cells (17 x 100 µm) and occasional small secondary “B” cells. The apical membrane of the principal cells is highly infolded and densely packed with mitochondria. The lateral membranes are also highly infolded (with large numbers of mitochondria) and interdigitate with the lateral membranes of adjacent cells. Extensive electrical coupling between cells has been demonstrated by dye injection and cable analysis (Hanrahan and Phillips, 1984). \( \text{B} \) cells typically have much fewer mitochondria and lack the elaborate membrane differentiation characteristic of most transporting epithelial cells (Peacock, 1979). Moreover, \( \text{B} \) cells only make contact with the luminal side of the epithelium.

Beneath the rectal pads is a distinct subepithelial space and a smaller secondary cell layer. The cells within this secondary layer show significant membrane differentiation and possess numerous mitochondria, suggesting probable water or solute transport capability. However, the size of the principal cells, degree of membrane differentiation, number of mitochondria, extent of tracheal development, and intracellular measurements, all suggest
Figure 1.2. Diagramatic representation of a transverse section through a locust rectal pad. See text for details. Figure taken from Irvine et al (1988).
that the principal cells of the primary cell layer are the main site for transepithelial transport of solutes and water in the rectum (Phillips et al., 1986).

**Solute and Water Transport in the Locust Rectum**

The rectal epithelium is a major site for solute and water recovery in the locust excretory system (for reviews see: Phillips 1977, 1981; Hanrahan and Phillips 1983; Phillips et al. 1986). Na⁺, K⁺, Cl⁻, H₂O, and basic metabolites are all reabsorbed from a KCl-rich fluid entering the lumen from the anterior hindgut (Fig. 1.3).

Na⁺ reabsorption is facilitated by an active transport step on the basolateral membrane putatively designated as an Na⁺/K⁺-ATPase. The apical membrane appears to have a low conductive permeability for Na⁺ and as such Na⁺ entry is thought to occur primarily by coupled transport with other ions or metabolites (Hanrahan, 1982; Black et al., 1987). Ouabain and amiloride had no effect on transepithelial potential but did inhibit net Na⁺ influx by 37% and 75% respectively (Black et al., 1987).

K⁺ is the primary cation reabsorbed from the lumen. The bulk of its net transport (>80%) occurs passively down an electrodiffusive pathway (haemocoel negative) established by electrogenic Cl⁻ transport (Hanrahan and Phillips, 1983). Addition of cAMP increases K⁺ reabsorption by increasing the K⁺ conductance of the apical membrane and the electrodiffusive potential generated by Cl⁻ transport (Hanrahan and Phillips, 1984).

Cl⁻ is the major anion reabsorbed from the lumen. Entry at the apical membrane occurs by an active, electrogenic process stimulated by cAMP and luminal K⁺ (Hanrahan, 1982). Cl⁻ exits the cell across the basolateral membrane passively down a favourable electrochemical gradient. Active net flux of Cl⁻ is independent of Na⁺ or HCO₃⁻ and is unaffected by conventional inhibitors of Cl⁻ transport (e.g. furosemide, ouabain, SITS, and acetazolamide). cAMP appears to act as a second messenger for a neuropeptide hormone (CTSH) isolated from the corpus cardiacum (Spring et al., 1978). The net effect is a direct
Figure 1.3. Summary of ion transport processes in the locust rectum. Upper diagram: proposed model for KCl absorption. Solid arrows indicate active and coupled transport; dashed arrows indicate dissipative net movements; dotted arrows indicate sites of control by cAMP and K⁺. Lower diagram: summary of net ³⁶Cl⁻ and ⁴²K⁺ fluxes, apical and basal cell membrane potentials (Vₐ, Vₖ) and resistances (Rₐ, Rₖ), and paracellular resistance (Rⱼ), before and after addition of 1 mM cAMP under open-circuit conditions. Figure taken from Hanrahan and Phillips (1983).
stimulation of the apical electrogenic Cl⁻ pump and an increase in the basolateral conductance to Cl⁻. The nature of the apical pump is presently unknown, but an anion ATPase has tentatively been proposed (Hanrahan and Phillips, 1983).

The rectum actively reabsorbs water from the lumen against significant transepithelial osmotic gradients. The ability to form a hyposmotic absorbate through an elaborate solute recycling mechanism facilitates the production of a strongly hyperosmotic excreta (see Phillips et al., 1986 and Lechleitner, 1988 for thorough reviews). Water reabsorption is apparently linked to both Cl⁻ and proline transport and is stimulated several fold by cAMP and aqueous extracts of both the corpus cardiacum and the ventral ganglia (Lechleitner and Phillips, 1989). Preliminary evidence obtained from ion substitution studies suggests that Cl⁻-dependent and proline-dependent fluid movements are the result of functionally separate transport mechanisms for Cl⁻ and proline.

Several neutral amino acids are actively reabsorbed from the rectal lumen against significant transepithelial concentration gradients (see Phillips et al., 1986 for review). Using an everted rectal sac preparation, Balshin and Phillips (1971) and Balshin (1973) clearly demonstrated active uptake of proline, glycine, serine, alanine, and threonine. Of these amino acids, only proline and glycine have been studied in any detail. Not surprisingly, glycine uptake conforms to the classic Na⁺-cotransport model proposed by Crane (1977) for a number of other amino acid transporting epithelia. Proline uptake, on the other hand, is largely Na⁺-independent and preliminary results suggest that a significant fraction of proline uptake may be proton-linked (Meredith and Phillips, 1988). Although most of these amino acids are largely transported across the epithelium in an unaltered state, a number of others (particularly glutamine) appear to be taken up into the rectum where they act as metabolic substrates (Chamberlin and Phillips, 1983). It should be noted that although a large component of proline uptake occurs transepithelially, Chamberlin and Phillips (1982) have
shown that luminal proline is one of the major metabolic substrates for ion transport (especially Cl⁻) in the rectum.

**Preliminary Acid/Base Observations**

*In vivo* pH measurements along the length of the locust excretory system (Speight, 1967) reveal that gut contents (pH 5-6 in the midgut) become first markedly alkaline (pH 7-8) in the region of the Malpighian tubules and then markedly acid (pH 5-6) by the time they reach the rectum (Fig. 1.4). Similar trends were reported by Waterhouse (1940) and Ramsay (1956) in *Lucilia* and *Dixippus*, suggesting that both the Malpighian tubules and the hindgut play a role in haemolymph pH regulation in a number of terrestrial insects. The dramatic increase in gut pH in the region of the Malpighian tubules (Fig. 1.4) is presumably due to secretion of an alkali-rich primary urine into the hindgut. Ramsay (1956) demonstrated that tubule secretions in *Dixippus* were always alkaline to the bathing medium and Cooper *et al.* (1987) showed that isolated corixid (*Cenocorixa blaisdelli*) tubules secrete a bicarbonate-rich alkaline urine when stimulated with cAMP. There are no direct measurements of pH or bicarbonate concentration in locust Malpighian tubule secretions, but the large anion deficit observed in the primary urine (40-60 mM; Hanrahan *et al.*, 1984) and the alkaline pH in the gut in the region of the tubules suggest that locust tubules also secrete significant quantities of bicarbonate.

The rectal pH values reported by Speight (pH 5.9-6.2; Fig. 1.4; 1967) are slightly higher than those originally observed by Phillips (pH 4.4-5.0; 1961), but the relative pH changes along the length of the gut are very similar. From Speight's *in vivo* pH profile (Fig. 1.4) it appears that the hindgut contents are largely acidified in the ileum (pH 7.8 → 6.2) before reaching the rectum (Fig. 1.4; pH 6.2 → 5.9). This figure is very misleading however, due to the logarithmic nature of the pH scale. If these pH values are converted to H⁺ ion activities, the respective increases in H⁺ are virtually identical in each segment.
Figure 1.4. *In vivo* pH profile of locust gut contents. Ha, haemolymph; II, ileum; Cn, colon; Re, rectum. *n* = 10 for each value; mean ± SE. Data from Speight (1967).
(ie. $6.2 \times 10^{-7}$ equiv/l H$^+$ increase in the ileum; $6.3 \times 10^{-7}$ equiv/l H$^+$ increase in the rectum). It is difficult to determine how much of the observed pH change in the ileum is actually due to the ileum \textit{per se} and how much is due to forward acid efflux from the rectum merely by measuring pH at discrete intervals along the gut. Moreover, the rectum must acidify its contents against significantly larger electrochemical gradients than those found in the ileum to maintain the observed transepithelial pH gradient.

To determine if the rectum itself was capable of generating the observed luminal pH changes, Speight (1967) performed injection-retrieval experiments with a ligated rectal preparation which isolated the rectal lumen from the rest of the gut while maintaining the integrity of the organ within the haemocoel. She showed that the rectum itself was capable of generating and maintaining the low pH values observed in the rectum of the intact animal and that the acidification was likely not due to selective secretion or reabsorption of phosphate into or out of the lumen. It was difficult, however, to completely rule out a role for phosphate (or any other acid/base equivalent) \textit{in vivo} when the composition of the fluid bathing the contraluminal surface of the epithelium could not be closely regulated.

In 1978 Williams \textit{et al.} developed an \textit{in vitro} preparation in which the rectum could be mounted as a flat sheet in a 'Ussing-type' chamber. This preparation gave the investigator complete control of the composition of the media bathing both sides of the epithelium while at the same time permitting measurement of transepithelial membrane potentials or application of short-circuit current (Isc). Concurrent measurements of Isc and ion fluxes ($^{22}$Na, $^{42}$K, and $^{36}$Cl) on unstimulated recta indicated that a large component of the observed Isc ($2.4 \mu$Equiv-cm$^{-2}$h$^{-1}$) must be due to some unidentified ion transport process. It was suggested that H$^+$/OH$^-$ movement might account for this unknown component. The direction of Isc was consistent with luminal acidification and was of similar magnitude to Speight's (1967) estimate for H$^+$/OH$^-$ transport in intact recta ($2.1 \mu$Equiv-cm$^{-2}$h$^{-1}$).

Hanrahan (1982) was unable to detect luminal acidification in the *in vitro* flat-sheet preparation under unstimulated, short-circuited conditions. He was, however, able to induce a luminal alkalinization under short-circuit current conditions by stimulating the tissue with cAMP. Consideration of the observed pH changes and the buffering capacity of the saline he used yields a first approximation of $8 \mu\text{Equiv cm}^{-2}\cdot\text{h}^{-1}$ in a direction opposite to all previous observations in the intact animal. Neither SITS nor acetazolamide inhibited the rate of alkalinization significantly. Hanrahan proposed that the alkalinization might be due to luminal accumulation of amino acid metabolites (ie. ammonia) during cAMP stimulation.

It is clear that there is very good circumstantial evidence to support a role for the rectum in haemolymph pH regulation in the locust. However, the failure to demonstrate acid/base transporting capabilities (in the proper direction) in an extremely viable, well characterized *in vitro* preparation is a serious problem which has to be addressed before further characterization of the epithelium is attempted. Before considering the resolution of this issue, a brief review of general acid/base transport mechanisms is presented to orient the reader and consolidate the underlying rationale for the experimental approaches followed in this study.

**General Acid/Base Transport Mechanisms**

The net transfer of acid/base equivalents across cell membranes can be achieved by a number of different mechanisms (for reviews see Rehm, 1972; Aronson, 1981; Steinmetz and Andersen, 1982; Boron, 1983; Steinmetz, 1986). Transport may be active or passive, and involves movements of weak acids and bases as well as direct transfer of $\text{H}^+$/OH$^-$ *per se*.

It is very difficult to distinguish between $\text{H}^+$ movements in one direction and OH$^-$ in the other. The net effect on pH is the same, but by convention the transport is usually referred to as $\text{H}^+$ flux. Implicit with the use of this convention is the recognition that $\text{H}^+$ movement in one direction and OH$^-$ movement in the other can be used interchangeably.
i. Passive transport

Direct proton permeation through the lipid bilayer is the simplest mechanism for achieving a net transfer of acid/base equivalents across cell membranes. Reported values for proton permeability are consistently larger than those for other monovalent cations (by $10^3$ to $10^6$ fold), but estimates have varied by several orders of magnitude ($10^{-3}$ to $10^{-9}$ cm sec$^{-1}$) depending on the techniques employed and the composition of the bilayer (Deamer, 1982; Gutknecht, 1984, 1987a). The mechanism(s) by which protons pass through the bilayer is largely unknown, but studies with phospholipid vesicles have suggested several intriguing possibilities (see Gutknecht, 1987b; Deamer and Barchfeld, 1984; Miller, 1987). The currently favoured model suggests that protons cross the bilayer by moving along hydrogen bonds between successive water molecules within the bilayer (the so called "water wire" hypothesis). This model requires a sufficiently high concentration of water in the hydrocarbon layer or some sort of hydrated defect in the bilayer to realize the observed rates of proton permeation.

Despite the relatively high proton permeability observed in the hydrophobic region of artificial bilayers, it is unclear whether or not this represents a physiologically significant diffusional pathway for protons in native bilayers. It is more likely that the bulk of uncoupled passive transport occurs through or around existing integral membrane proteins (see Boron, 1983; Gutknecht, 1984). Nagle and Tristram-Nagle (1983) have suggested that protons might be able to cross biological membranes by the successive hydrogen bonding of the side chains of these proteins. It is also not difficult to envisage significant leak pathways for protons through transport routes (eg. channels or carriers) specific for larger solutes or routes which facilitate any form of water movement (Deamer, 1982). Specific passive proton conducting pathways (ie. H$^+$ channels) have been demonstrated in several cell and organelle types (eg. snail neurons; Byerly et al., 1984). Perhaps the most thoroughly studied example is the $F_o$ component of the mitochondrial $F_o$$F_1$ H$^+$-translocating ATPase (see
Maloney, 1982 for review). When reconstituted into artificial bilayers, the $F_0$ component behaves as a completely reversible, selective protonophore (putatively designated as a channel).

Though there appear to be many routes for passive $H^+$ permeation and permeability values for $H^+$ are greater than those for other cations in artificial bilayers, biological membranes do pose significant diffusion barriers for passive proton movements (Nichols and Deamer, 1980; Sanders et al., 1985). The secretory membrane of the gastric mucosa, for example, must have effective proton permeabilities of less than $10^{-7}$ cm s$^{-1}$ to maintain the pH gradients observed *in situ* (Deamer and Nichols, 1983). Moreover, although the calculated permeability coefficients for protons are high relative to other monovalent cations, one must consider that the driving concentrations for passive proton flux are in the sub-micromolar range under most physiological conditions. Clearly it is questionable whether passive proton fluxes by themselves have significant effects on overall pH compared to the relative fluxes of the nonionic and ionic forms of weak acids and bases which are generally present in much higher concentrations.

Nonionic diffusion of weak acids and bases (particularly $CO_2$ and $NH_3$) accounts for a large portion of passive acid/base movements across biological membranes (Roos and Boron, 1981). This observation reflects the high lipid solubility of the neutral form relative to its charged conjugate. A number of transport epithelia utilize this property to facilitate transcellular acid/base movements and it is fundamental to intracellular pH regulation in many cell types. The vertebrate proximal tubule is an example of a transport epithelium which accomplishes net total $CO_2$ (ie. $CO_2$ and $HCO_3^-$) and net total ammonia (ie. $NH_3$ and $NH_4^+$) transfer by this mechanism (see Warnock and Rector, 1981; Good and Knepper, 1985; Giebisch and Aronson, 1987). In the proximal tubule, both $CO_2$ and $NH_3$ diffuse passively across the apical membrane down their respective concentration gradients. Once inside the cell, $CO_2$ is largely converted to its much less permeable charged conjugate, $HCO_3^-$, and the
inwardly directed concentration gradient driving CO₂ diffusion is maintained. Likewise, once in the lumen, NH₃ is protonated (ie. converted to NH₄⁺) and the outwardly directed concentration gradient for NH₃ diffusion is maintained. Although this particular example utilizes the same transport mechanisms for both CO₂ and NH₃, there is no reason to expect that all CO₂/HCO₃⁻ and NH₃/NH₄⁺ transporting epithelia do likewise.

There is a growing body of evidence to substantiate a significant role for passive ionic diffusion in acid/base transport (reviewed by Boron, 1983). This is particularly true for bicarbonate ions where a passive conductance step on one of the membranes of an epithelial cell is often implicated in transepithelial acid/base transfer (Knauf et al., 1982; Schulz and Ullrich, 1979; Burckhardt and Fromter, 1980; Sasaki and Berry, 1983; Simson et al., 1981; Berry et al., 1984). The driving force for this type of conductance is often established by a rapid inward diffusion of the uncharged conjugate and subsequent conversion within the cell to its less permeable ionic form (see above).

Co- or countertransport mechanisms also mediate passive movements of acid/base equivalents. This necessarily implies the involvement of a common transport protein and a secondary solute (or ion) to facilitate the diffusion step. In this case, the energy for the transfer is supplied by the electrochemical potential of the primary solute. Often this energy is used to drive the uphill movement of the secondary solute. Net transport can be electroneutral (eg. HCO₃⁻/Cl⁻ antiport; reviewed by Lowe and Lambert, 1983) or electrogenic (eg. lactose/proton symport; reviewed by Rosen, 1978).

**ii. Active transport**

Active transport can be operationally defined as the transmembrane movement of a solute against an electrochemical potential difference. The energy for this transport may be derived directly from cellular metabolic events (eg. ATP hydrolysis) or secondarily from
coupling to spontaneous fluxes of other solutes (eg. Na\textsuperscript{+} gradient hypothesis; reviewed by Crane, 1977).

\textit{a. Primary.} Primary active transport implicates a “pump” which directs the energy from cellular metabolism into the vectorial displacement of a specific solute. Two pump types have this far been described in the translocation of acid/base equivalents: redox pumps and ATPases.

Redox pumps have been characterized in detail in bacteria, chloroplasts, and mitochondria (see Harold and Altendorf, 1974, Dilley and Giaquinta, 1975, and Wikstrom, 1982, for respective reviews). The basic principles and underlying mechanisms are much the same for all the redox pumps characterized thus far. A series of reactions transfers electrons irreversibly down an energy gradient from a reduced metabolite to molecular oxygen (reviewed by Holum, 1982, and Becker, 1983). In each transfer sequence electron transport is coupled obligatorily with vectorial proton translocation. The net result is a difference in the electrochemical potential for H\textsuperscript{+} across the coupling membrane.

In the past, similar mechanisms have been proposed for various H\textsuperscript{+} transporting epithelia (Dies and Lotspeich, 1967; Rehm, 1972), but as of yet no unequivocal evidence has been provided (reviewed by Steinmetz and Andersen, 1982, and Aronson, 1983). The current view is that primary active transport of protons across eukaryote plasma membranes is facilitated largely by membrane bound ATPases.

Ion translocating ATPases function by directing the bond energy released in ATP hydrolysis into the vectorial displacement of the appropriate ion(s). The reaction sequence may be electrogenic (eg. turtle bladder; reviewed by Steinmetz and Andersen, 1982; Al-Awqati \textit{et al.}, 1983) or electroneutral (eg. Gastric parietal cells; Forte \textit{et al.}, 1980; Sachs \textit{et al.}, 1982; Rabon \textit{et al.}, 1983). Acid/base relevant ATPases have only been described in detail for protons (see reviews by Maloney, 1982, Steinmetz and Andersen, 1982, Senior and Wise, 1983; Serrano, 1988; Stone and Xie, 1988), but there have been many references to HCO\textsubscript{3}⁻
stimulated ATPase activity in vitro (Turbeck et al., 1968; Stekhoven and Bonting, 1981; Anstee and Fathpour, 1981; Deaton, 1984; Abdelkhalek et al., 1986). Failure to demonstrate HCO₃⁻ stimulation in intact preparations has caused a great deal of controversy and many are not as yet convinced of its existence. There has been some evidence for NH₄⁺ transport by the Na⁺/K⁺-ATPase in the absence of external K⁺ (Aickin and Thomas, 1977b; Towle and Holleland, 1987; Good, 1988), but how significant this transport is under physiological conditions remains to be seen.

It is extremely difficult to demonstrate conclusively the existence of proton or HCO₃⁻ ATPases in epithelial plasma membranes. This is largely due to the existence of similar transport proteins in organelle membranes which tend to disperse throughout other fractions during membrane isolation procedures. The problem is typically overcome using specific labels for mitochondrial and plasma membrane fractions (e.g. succinate dehydrogenase and Na⁺/K⁺ ATPase respectively) and inhibitors specific to the activity of each fraction. The K⁺/H⁺ ATPase of gastric parietal cells was somewhat simpler than other ATPases to isolate because of the lack of K⁺ stimulated activity in the mitochondrial fraction (Sachs et al., 1982).

A major problem when characterizing primary proton transport in intact preparations is distinguishing between redox pumps and proton ATPases. With cell types which continue to move acid under anoxic conditions (e.g. turtle bladder; Steinmetz and Andersen, 1982) the distinction is relatively straightforward. If anoxia does stop acid flux, however, it does not necessarily imply a plasma redox pump. The effect may be a secondary one manifested by mitochondrial inhibition. The gastric parietal cell, for example, under anoxic conditions could resume acid secretion only upon addition of exogenous ATP (Sachs et al., 1982).

In practice, isolated membrane fractions have been much better for determining the nature of the energy source driving proton pumps (see for example Gluck et al., 1982). The investigator no longer worries about maintaining secondary cell functions, can dictate the
precise chemical composition of the fluid bathing each side of the membrane, and can control
the orientation of the pump. Even so, it has historically been simpler to demonstrate the
existence of proton ATPases than to disprove the existence of redox pumps.

b. Secondary. Secondary active transport couples the uphill movement of an acid/base
equivalent with the spontaneous flux of another ion. Transport may occur via an antiport (eg.
$\text{Na}^+$/H$^+$ exchange) or symport (eg. $\text{Na}^+$/HCO$_3^-$ cotransport) and by-in-large does not promote
a net transfer of charge across the membrane (for a detailed review of secondary active
transport mechanisms see Aronson, 1981).

It is important to bear in mind that actions which influence the electrochemical
potential of the primary solute indirectly influence the active transport of the acid/base
equivalent. A prime example is the use of ouabain to block active proton extrusion in cells
where $\text{Na}^+/\text{H}^+$ exchange is known to occur. The effect is not due to the direct action of
ouabain on the $\text{Na}^+/\text{H}^+$ exchanger per se, but rather to the dissipation of the electrochemical
potential driving the spontaneous influx of $\text{Na}^+$.

iii. Vesicle mediated transport

Vesicular mediated transport events are now well documented phenomena in cell
biology. Their putative role as vehicles for endocytic and exocytic transfer of
macromolecules has been supported by numerous morphological and biochemical
investigations (for reviews see Orci et al., 1981; Meldolesi and Ceccarelli, 1981; Herzog,
1981; Steinman et al., 1983; Madsen and Tisher, 1985; Brown, 1989). Involvement in ion
transport, however, is only a very recent discovery (for examples see Forte et al., 1977;
Wade, 1980; Gluck et al., 1982; Karniele et al., 1982; Lewis and de Moura, 1982; Spring and
Ericson, 1982; Schwartz and Al-Awqati, 1985; Dixon et al., 1986).
Al-Awqati et al. (1983) have isolated cytoplasmic vesicles from turtle bladder epithelia and beef kidney medulla which actively transport proton equivalents intravesicularly. They have characterized the H⁺ pump as a proton translocating ATPase unlike the mitochondrial F₀F₁ ATPase, yeast plasma membrane ATPase, or the gastric H⁺/K⁺ ATPase. Vesicle labelling experiments (Gluck et al., 1982) in turtle bladder epithelial cells and concomitant measurements of epithelial H⁺ transport (J_{H⁺}) and apical membrane surface area (Stetson and Steinmetz, 1983) indicate that vesicle fusion with the apical membrane is essential for the rapid increases in J_{H⁺} induced by addition of exogenous CO₂. When the vesicles fused with the apical membrane, the vesicle label (fluorescent dextran) was released from the cell into the bladder lumen. The dextran release proved to be transient, but the elevated J_{H⁺} persisted, suggesting the incorporation of proton pumps into the apical membrane rather than a simple "unloading of acid" from the vesicles. Decreasing J_{H⁺} by reducing exogenous CO₂ enhanced endocytosis and net removal of luminal H⁺ pumps from the apical membrane (Reeves et al., 1983).

Clearly, these findings have suggested an entirely new direction for studying acid/base transport mechanisms and their controls. If the widespread nature of other vesicle-mediated events is any indication, it should come as no surprise to find similar mechanisms in other cell types.

**Transepithelial Acid/Base Transport**

Epithelial cells which function in secretion or reabsorption of pH relevant ions are capable of promoting net active transport of acid/base equivalents across the epithelia as a whole. There is no reason, however, to presuppose active transport steps on both apical and basolateral membranes to achieve this end. A common configuration observed in many acid/base transporting epithelia is an active step at the luminal membrane and a passive

As mentioned earlier, a major difficulty with acid/base transport studies is distinguishing between acid secretion and base reabsorption *per se*. The net effect on extracellular pH is the same, but the actual transport mechanisms and their implications for intracellular pH regulation can be markedly different. It has been particularly difficult to determine the role played by HCO$_3^-$ in luminal acidification in many epithelia. In mammalian proximal tubules, for example, luminal acidification occurs concomitantly with a decline in luminal HCO$_3^-$ to levels well below plasma concentrations (reviewed by Warnock and Rector, 1981). This and consideration of the mass equilibrium equation for CO$_2$/HCO$_3^-$ systems, led many to believe that the observed net effect was the result of active reabsorption of bicarbonate ions *per se*. Pitts and Alexander (1945) were the first to propose that these events were not the result of ionic HCO$_3^-$ reabsorption, but rather active proton secretion. They reasoned that acid secretion would titrate filtered HCO$_3^-$ to H$_2$CO$_3$ and that base equivalents would cross the apical membrane as CO$_2$. This explained the drop in luminal HCO$_3^-$ concentration and their own observation of net acid addition to glomerular filtrate. It was not until Rector *et al.*, (1965) were able to induce a disequilibrium pH in the tubules by infusing with benzolamide (a potent carbonic anhydrase inhibitor) that this theory became widely accepted (see Walser and Mudge, 1960, and Dubose, 1983, for reviews of disequilibrium pH and its implication for CO$_2$/HCO$_3^-$ transport).
Intracellular pH Regulation

All acid/base transport studies conducted at the cellular level must consider the homeostatic implications of moving acid/base equivalents into or out of the cytoplasm. How is intracellular pH (pHi) regulated and how closely is it regulated during transepithelial acid/base transport? Are there separate mechanisms for transepithelial acid/base transport and pHi regulation? Does pHi regulation modulate transepithelial acid/base transport or vice versa, or are the two mechanisms completely independent? Each of these questions must eventually be addressed before a complete understanding of cellular acid/base dynamics can be achieved. For a detailed review of intracellular pH regulation in eukaryotic cells and its historical development see Caldwell (1956), Robson et al. (1968), Waddell and Bates (1969), Cohen and Iles (1975), Roos and Boron (1981), and Boron (1987).

At physiological pH values the relative concentrations of weak acids and bases are much larger than those of free protons or hydroxyl ions. As such, their attendant fluxes and chemical equilibria will have substantially larger effects on measured pHi (Robson et al., 1968). The bulk of intracellular acid/base perturbations (greater than 99% by Roos’ and Boron’s 1981 estimate) are neutralized by direct physiochemical buffering of the cytoplasm and cellular mechanisms (predominantly metabolic) which consume excess free H⁺/OH⁻. These cellular mechanisms can take the form of biochemical reactions which convert organic acids or bases into neutral end products (eg. oxidation of lactate; Cohen and Iles, 1975) or the sequestering of weak acids or bases into intracellular organelles (Roos and Boron, 1981). It is evident that these mechanisms do have finite capacities and can only provide short-term solutions to chronic acid/base loads. If long term pHi stability is to be maintained, acid or alkali equivalents must be actively transported into or out of the cell.

On strictly conceptual grounds this notion is readily acceptable. It was not until the last decade, however, that experimental evidence correlated active acid extrusion with imposed acid loads; see for example Messeter and Siesjo (1971), Thomas (1974), Roos
Compensatory responses to intracellular alkaline loads have also been observed, but pH recovery is often slower and the actual mechanisms are frequently unclear (Aickin and Thomas, 1977; Boron, 1977; Aickin, 1984; Evans and Thomas, 1984; Zeidel et al., 1986; Simchowitz and Roos, 1985; Keller et al., 1988).

Most cell types examined thus far respond to imposed acid loads by one or more of the following mechanisms: coupled transport of Na\(^+\), HCO\(_3\)^-, Cl\(^-\), and possibly H\(^+\), Na\(^+\)/H\(^+\) exchange, or parallel Na\(^+\)/H\(^+\) and Cl\(^-\)/HCO\(_3\)^- exchangers.

The squid axon (Boron and De Weer, 1976; Russell and Boron, 1979), snail neuron (Thomas, 1982; Evans and Thomas, 1984) and giant barnacle muscle (Boron, 1977; Boron et al., 1981) all exhibit low rates of acid extrusion at physiological pH values. The rate is significantly enhanced when pH is lowered or extracellular pH is raised. pH recovery requires the simultaneous presence of external Na\(^+\) and HCO\(_3\)^- and internal Cl\(^-\) and is blocked by inhibitors of anion transport (e.g. SITS and DIDS). Thomas (1982) has proposed two models for the initial recovery step: 1) external Na\(^+\) and HCO\(_3\)^- exchanging for internal Cl\(^-\) and H\(^+\), or 2) one external Na\(^+\) and two external HCO\(_3\)^- exchanging for one internal Cl\(^-\). Net acid extrusion is thought to be mediated by HCO\(_3\)^- entry titrating excess free protons to CO\(_2\) (Thomas, 1977; Roos and Boron, 1981).

In crayfish neurons (Moody, 1981), mouse soleus muscle (Aickin and Thomas, 1977a), cardiac muscle (Deitmer and Ellis, 1980; Vaughan-Jones, 1979), rat diaphragm (Roos, 1975), and amphibian proximal tubule cells (Boron and Boulpaep, 1983a,b), pH recovery from an intracellular acid load is accomplished primarily by electroneutral Na\(^+\)/H\(^+\) exchange. Recovery is directly dependent on external Na\(^+\) and can be largely inhibited by amiloride. Crayfish neurons and mouse soleus cells recovered from acid loads much quicker (ca. 45%) if external HCO\(_3\)^- and internal Cl\(^-\) were present. This effect could be inhibited with stilbene derivatives. Cl\(^-\)/HCO\(_3\)^- exchangers are present in the other cell types as well, but they do not appear to participate to a significant extent in pH recovery from imposed acid loads.
Cl⁻/HCO₃⁻ exchangers also play a major role in pH regulation in response to alkaline loads in a number of different cell types (see Vaughan-Jones, 1979, Keller et al., 1988, Simchowitz and Roos, 1985, Tonnessen et al., 1987, Olsnes et al., 1987, Reid et al., 1987, Zeidel et al., 1986, and Machen and Paradiso, 1987). This exchanger is typically located on the basolateral membrane (of the epithelial cells studied) and inhibited by stilbene derivatives. In many cases, the mechanisms for recovery from acid or alkali loading are completely separate and function independently of one another.

Of particular interest to this dissertation is the effect that transepithelial acid/base fluxes have on pH. Boron and Boulpaep (1983a,b) proposed that proximal tubule acid secretion in *Ambystoma* is a consequence of active pH regulation. They identified Na⁺/H⁺ antiporters on apical and basolateral membranes as the principle ionic means of active pH regulation. They suggest that acid secretion in proximal tubules in general is a function of electrodiffusive HCO₃⁻ pathways limited to the basolateral membrane (ie. net acid movement only across the apical membrane). This has since proven not to be a universal mechanism; in rabbit proximal tubules Na⁺/H⁺ exchange sites are found only on the apical membrane (Ives et al., 1983). Rabbit proximal tubules (S₃ segment) also possess an apical H⁺-ATPase which appears to play a prominent role in both transepithelial acid/base flux and intracellular pH regulation (Kurtz, 1987; see Zeidel et al., 1986, for a similar mechanism in rabbit medullary collecting ducts). In a variety of other cell types, intracellular pH (and by implication, its regulatory mechanisms) has been found to modulate rates of transepithelial acid/base transport (see for examples: Olsnes et al., 1987; Machen and Paradiso, 1987; Mason et al., 1989). Interestingly, changes in net acid/base flux are not always accompanied by concomitant changes in pH (eg. gastric oxyntic cells; Machen and Paradiso, 1987). Clearly, as one would expect, transepithelial acid/base movements and intracellular pH regulation are tightly linked. Moreover, the preliminary evidence seems to indicate that although net acid/base flux may be a result of pH regulation, the opposite is rarely true.
Nature of rectal acidification in the desert locust

The principal aim of the present study was to characterize the cellular mechanisms responsible for the rectal acidification reported by Phillips (1961) and Speight (1967). Before this objective could be achieved, it was necessary to develop an in vitro isolated rectal preparation which maintained acid/base transport properties similar to those observed in vivo. Chapter Two outlines the development and testing of this preparation and presents the first unambiguous evidence for actively maintained rectal acidification in the desert locust. Intracellular double-barreled microelectrodes were used to confirm that rectal acidification was mediated by an active process and that the active step was located at the apical membrane. Evidence is presented to demonstrate that rectal acidification is due to active proton secretion rather than selective movements of HCO$_3^-$, phosphate, or other unidentified buffer components.

Preliminary measurements of bath total ammonia (ie. NH$_3$ + NH$_4^+$) levels in the in vitro preparation developed in Chapter Two suggested that the rectum was actively secreting ammonia at significant rates across the apical membrane. Since NH$_3$ secretion can reduce apparent titratable acidity to the point at which it no longer accurately estimates net proton secretion, it was important to determine whether the ammonia crossed the apical membrane of the locust rectum as NH$_3$ or NH$_4^+$. In Chapter Three, significant rates of luminal ammonia secretion in the locust rectum are confirmed under both open- and short-circuit current conditions. The secreted ammonia is shown to be derived almost exclusively from amino acids present in the luminal bath. The experimental evidence presented in this chapter clearly shows that ammonia crosses the apical membrane as NH$_4^+$ rather than NH$_3$ (via Na$^+$/NH$_4^+$ exchange) and that rates of net acid secretion estimated by titratable acidity do not have to include a correction for luminal ammonia secretion.
The cellular mechanisms responsible for net acid secretion in the locust rectum are examined in Chapter Four using the electrophysiological techniques established in Chapters Two and Three. The validity of the apparent transepithelial proton motive force (PMF) estimated by applying either a transepithelial electrical or chemical gradient and its implications are discussed with respect to the true PMF of the apical proton secretory mechanism per se. The modulation of net acid secretion by application of transepithelial electrical gradients suggested that active proton secretion might occur by an electrogenic mechanism located on the apical membrane. The results of a series of ion-substitution experiments performed under a variety of conditions consistently support a model in which the bulk (80%-90%) of net acid secretion occurs by an apical electrogenic proton secretory mechanism and the remainder by electroneutral Na\(^+\)/H\(^+\) exchange. The apparent proton permeability of the apical membrane was assessed both in the presence of a transepithelial pH gradient and when active proton secretion was abolished by exposure to anoxia. The results of these experiments imply that there is a significant proton diffusional pathway in parallel with the active proton secretory mechanisms on the apical membrane. The ambiguous effects of putative inhibitors of acid/base transport on luminal acid secretion under CO\(_2\)/HCO\(_3\)-free conditions are compared with results obtained on HCO\(_3\) reabsorption in rectal sacs incubated in exogenous CO\(_2\) and HCO\(_3\)- and collectively discussed with reference to a model relating active proton secretion to net bicarbonate reabsorption. Given the previous evidence demonstrating cAMP stimulation of net Cl\(^-\) and net K\(^+\) flux in the locust rectum (reviewed by Phillips et al., 1986), the effect of contraluminal cAMP on rates of rectal acid secretion was tested under both open- and short-circuit current conditions. Although net acid secretion was reduced by up to 66% by this treatment, it is not clear whether cAMP inhibition is the result of a direct interaction with the apical proton pump per se or an indirect effect of changes in the electrochemical gradient for protons across the apical membrane.
In the final chapter (Chapter Five: General Discussion), the observations reported throughout the thesis are collectively integrated into a tentative model for acid/base transport in the locust rectum. The physiological implications of this model and the role played by the rectum in haemolymph pH regulation are discussed with reference to observations reported for the intact animal.
CHAPTER TWO: Confirmation of Rectal Acidification

INTRODUCTION

As outlined in the previous chapter, the pH of the gut contents of the Desert Locust decreases dramatically between the anterior hindgut (pH 8; Fig. 1.4) and the rectum (pH 6; Fig. 1.4). Preliminary studies by Phillips (1961) and Speight (1967) using a standard in vivo ligated rectal preparation (see Hanrahan et al., 1984) led them to propose that the rectal epithelium per se was the principal source of this acidification and that the rectum might be an important pH regulatory organ functionally analogous to the vertebrate kidney.

It was clear from Phillips' and Speight's initial observations, that acidification in the rectum was not due to acid efflux from other regions of the gut. Unfortunately, due to the technical limitations of the preparation used, they were unable to conclusively identify either the nature (ie. acid secretion or base reabsorption) or the source (ie. epithelial or microbial) of the acidification within the rectum. To complicate matters further, subsequent attempts at duplicating Phillips' and Speight's original observations in vitro were completely unsuccessful. In 1982, Hanrahan reported that although he could not detect significant levels of luminal acidification with a classical "Ussing"-type in vitro preparation, he did observe considerable rates of luminal alkalinization when the rectum was placed under short-circuit current conditions and 1 mM cAMP was added to the contraluminal bath. It was obvious from these conflicting results that a reliable in vitro rectal preparation had to be developed and serious assessment of the general acid/base transporting characteristics of the rectum undertaken.

The present chapter describes the development of an in vitro flat-sheet preparation which can be routinely used to study acid/base transport in the locust rectum or other similar epithelia. Using this preparation I was able to clearly demonstrate rates of luminal acidification similar to those observed in vivo and to confirm Phillips' original hypothesis that
the rectal epithelial cells per se were responsible for the observed rates of luminal acidification. Using double-barreled microelectrodes, I further showed that luminal pH was not in equilibrium with either contraluminal pH or intracellular pH and that the active mechanism responsible for the luminal acidification must reside in the apical membrane. A series of experiments performed in the absence of exogenous phosphate and CO$_2$/HCO$_3^-$ also suggest that the acidification is due to active H$^+$ secretion rather than selective reabsorption of either phosphate or bicarbonate.

MATERIALS AND METHODS

Animals

Adult female desert locusts (Schistocerca gregaria Forskal) 14-22 days beyond their final moult were used for all experiments. They were maintained at 28°C and 60% r.h. on a 12:12 h light:dark cycle, and fed daily on fresh lettuce and a dried mixture of bran, alfalfa, and powdered milk.

In vitro rectal preparation

Isolated recta were mounted as flat-sheets in miniaturized versions of the Ussing-style chambers described by Williams et al. (1977), with 2.0 rather than 5.0 ml of solution per chamber (Fig. 2.1). Recta were removed from the animals after careful detachment of all connecting tissue with fine dissecting scissors. The tubular rectum was cut longitudinally between adjacent rectal pads to produce a flat sheet. The epithelial sheet was then secured over a 0.196 cm$^2$ opening by means of tungsten pins and an overlaying neoprene O-ring. Edge damage does not appear to be a significant factor with this method of tissue attachment (Hanrahan and Phillips, 1984). Saline was constantly circulated and oxygenated in each chamber by means of a gas lift pump which maintained constant gas tension and circulation.
Figure 2.1. Standard Ussing chamber assembly used for measurement of $J_{H^+}$, $J_{OH^-}$, $V_t$, and Isc. (1) flat-sheet rectal preparation, (2) plexiglass collar over which rectum is mounted, (3) neoprene O-ring for securing rectal attachment to collar, (4) neoprene chamber seal, (5) agar bridge port for measurement of $V_t$, (6) gas inlet for saline aeration and mixing, (7) current sending electrodes, (8) rear chamber seal, (9) tungsten pins for attachment of rectum to collar (see text for complete details; figure taken from Hanrahan et al, 1984).
regardless of perfusion flow rates. Provision was made for gravity-fed perfusion (4-5 ml/min) of each chamber as protocol dictated. Typically, recta were brought to steady-state conditions (as defined by stable short-circuit current or transepithelial potential; approximately 2h) under bilateral perfusion, and then perfusion was stopped unilaterally for the experimental period.

Transepithelial potential (Vt) and short-circuit current (Isc) were determined as described by Hanrahan et al. (1984). Briefly, Vt was measured as the potential difference between luminal and contraluminal 3M KCl agar bridges. Short-circuit current was applied with a dual channel automatic voltage clamp which allowed for compensation of saline resistance and variable voltage clamp settings (see Hanrahan et al., 1984 for complete circuit description). Vt and Isc were monitored and recorded on dual channel strip-chart recorders (1242; Soltec, Sun Valley, CA.).

Measurement of rectal acidification

Recta were mounted and perfused as above. Rates of luminal acidification ($J_{H^+}$) and contraluminal alkalinization ($J_{OH^-}$) were determined with a pH-stat technique (PHM 84 research pH meter, TTT 80 titrator, ABU 80 autoburette; Radiometer, Copenhagen, Denmark). $J_{H^+}$ and $J_{OH^-}$ were calculated as the rate of titrant addition (0.01N NaOH and 0.01N HNO$_3$ respectively) required to maintain the initial pH.

Total CO$_2$ measurement

Saline samples (100 µl) were acidified in gas-tight Hamilton syringes containing 2 ml of 0.1N HCl and 2 ml of nitrogen gas (Sample size and relative quantities of HCl and N$_2$ were varied to give optimal detection at extremely low CO$_2$ concentrations). After 5 min the gas phase was assayed for CO$_2$ content by standard gas chromatography using a Carle 1111 gas chromatogram (Hach Corp., Loveland, CO). CO$_2$ standards were prepared immediately
before use with NaHCO₃ and N₂ saturated distilled water. With very careful sample handling and CO₂ standard preparation, CO₂ concentrations could be determined repeatedly within 1-2 µM at low CO₂ concentrations. The practical detection limit for this method was approximately 10 µM, below which repeatability and accuracy deteriorated rapidly. This detection limit appeared to be more a function of standard preparation and sample handling than a performance limitation imposed by the gas chromatograph per se.

**Intracellular pH measurement**

Intracellular pH was measured with ion-sensitive double-barreled microelectrodes using a H⁺ exchange resin (IE-010; WPI, New Haven, CT) based on that developed by Amman et al. (1981). Microelectrodes with tip diameters of ≤ 1 µm were fabricated with a vertical electrode puller (PE-2; Narashige, Tokyo, Japan). Electrodes were silanized by exposing the ion-sensitive barrel to dimethyldichlorosilane vapours for 30 sec and then baking the electrodes at 150° C for 30 min. A 2-4 mm column of H⁺-sensitive resin was injected into the silanized barrel and left overnight in a dessicator under 100% CO₂. Prior to use the ion-sensitive barrel was backfilled with a pH 7 phosphate buffer (see Amman et al., 1981) and the reference barrel backfilled with 0.5M KCl.

Electrodes were calibrated between each impalement in three buffers of constant ionic strength encompassing the pH range that would be encountered during the experimental period. Calibration buffer pH was determined before each experiment with a Radiometer PHM 84 pH meter and Radiometer precision buffer solutions (pH±0.005). Electrode resistances were typically on the order of 10¹¹ Ω, and electrodes demonstrated a >90% response in <10 sec. There was essentially no interference from Na⁺ or K⁺ at concentrations up to 100 mM over the pH range of 5-8. Electrodes were rejected if electromotive force slopes were not in the range of 55-65 mV/pH unit or 90% response times were >10 sec.
Figure 2.2. Microelectrode preparation used for measurement of apical and basolateral membrane potentials and intracellular pH. (a) Recta were mounted as flat sheets in modified Ussing chambers which facilitated impalement with ion-sensitive double-barreled microelectrodes: (1) perfusion inlet, (2) perfusion drain, (3) double-barreled microelectrode assembly, (4) removable glass coverslip for axial positioning of dissecting microscope, (5) fiber optic light source, (6) current sending electrodes. (b) Vt was measured as the potential difference between luminal and contraluminal 3M KCl agar bridges. Va and Vb were measured as the potential difference between the reference barrel of the microelectrode and the luminal or contraluminal 3M KCl agar bridge respectively. The intracellular ion activity (Vi) was determined as the differential voltage between the ion-sensitive barrel referenced to the luminal bath and the apical membrane potential (Va). Transepithelial resistance and voltage divider ratios were calculated from deflections in Vt, Va, and Vb produced by transepithelial current pulses. The magnitude of the current pulses (It) was monitored as the voltage drop across a 1K \( \Omega \) resistor in series. (see text for complete details; figure taken from Hanrahan et al., 1984).
Recta were mounted as flat sheets in specially designed microelectrode chambers (Fig. 2.2) which required continuous bilateral perfusion (8-10 ml/min; gravity fed) to maintain the required gas tensions. To facilitate apical impalement, the cuticular intima was removed from the rectal pad with fine forceps and iridectomy scissors. This treatment does not affect epithelial transport as judged by Isc, Vt, and transepithelial resistance (see Hanrahan et al., 1984). Microelectrodes were advanced manually via hydraulic microdrive (MO-8; Narashige, Tokyo, Japan) at an angle of 30-40° to the plane of the epithelium.

The potential differences between the reference and pH-sensitive barrels (Vi) and the apical (Va) and basolateral (Vb) membrane potentials were measured with a high-input impedance differential electrometer ($10^{15}$ Ω; FD 223; WPI, New Haven, CT). Va and Vb were measured as the potential difference between the reference barrel and a luminal or contraluminal 3M KCl agar bridge, respectively. Va, Vb, and Vi were filtered (-36 dB at 15 Hz) with 3 operational amplifiers in a six-pole butterworth configuration (LF 356, National Semiconductor, Santa Clara, CA) before recording on a three-channel strip-chart recorder (1243; Soltec, Sun Valley, CA). Vt was monitored and recorded as above. Signals were also monitored on a storage oscilloscope (D15 Tektronics, Beaverton, OR).

Transepithelial resistance (Rt) and voltage-divider ratios ($\Delta R_a/\Delta R_b$) were calculated from deflections in Vt, Va, and Vb produced by transepithelial current pulses. Voltage deflections were corrected for saline resistance by subtracting background deflection values from experimental values. Background values were determined in chambers with saline only (ie. no tissue) by positioning the microelectrode tip in the same plane of focus as when the tissue was present and passing a train of current pulses. Constant current pulses (13 μA, 1.5 sec duration, 0.2 Hz) were generated by two operational amplifiers configured as a voltage controlled constant current source (LF 356, National Semiconductor, Santa Clara, CA). The pulses were triggered with an LM 555 timer (National Semiconductor). Current pulses were measured as the voltage drop across a 1K Ω resistor in series.
Acceptable impalements were characterized by 1) abrupt monotonic deflections in membrane potentials, 2) stable membrane potentials (± 1 mV) for at least 60 sec, 3) constant voltage divider ratios, 4) constant Rt, and 5) immediate return to baseline potential after electrode withdrawal (± 2 mV).

**Lactate measurement**

Lactate measurements were made with a spectrophotometric assay based on that described by Gleeson (1985) and Lowry and Passonneau (1972). This assay utilizes conversion of lactate to pyruvate in the presence of excess NAD, lactate dehydrogenase (LDH), and hydrazine (see eq. 1 and eq. 2).

\[
\begin{align*}
\text{Lactate} + \text{NAD} & \xrightarrow{\text{LDH}} \text{Pyruvate} + \text{NADH} \\
\text{Pyruvate} + \text{Hydrazine} & \xrightarrow{} \text{Pyruvate hydrazone}
\end{align*}
\]

(1)

(2)

The increased absorbance at 340 nm due to NADH formation is a measure of the lactate present in the sample. To force the reaction to completion, formed pyruvate is trapped with hydrazine. The practical detection limit with the sample size (100 µl) and assay conditions in this study used was approximately 40 µM.

**Solutions**

The composition of experimental salines was based on the content of locust haemolymph and Malpighian tubule fluid (Chamberlin and Phillips, 1982; Hanrahan, 1982) and, unless otherwise stated, contained (in mM): 100 NaCl, 5 K₂SO₄, 10 MgSO₄, 10 Na⁺-isethionate, 10 glucose, 100 sucrose (to adjust osmolarity), 5 CaCl₂, 2 MOPS (3-[N-morpholino]-propanesulphonic acid; pKa = 7.20 at 20° C), 1.0 arginine, 1.5 serine, 13.1 proline, 1.3 asparagine, 11.4 glycine, 2.9 alanine, 1.8 valine, 5.0 glutamine, 1.0 tyrosine, 1.4 lysine, and 1.4 histidine. Solutions were vigorously aerated with 100% O₂ for at least 2 h
prior to use, and likewise, perfusion reservoirs were continuously aerated throughout the entire experimental period. pH electrodes were calibrated with Radiometer Precision Buffer Solutions (pH ± 0.005) and experimental salines were manually titrated to the desired pH using concentrated nitric acid or sodium hydroxide crystals. All experiments were performed at 23 ± 1°C.

**Calculations and statistics**

Acid ($J_{H^+}$) secretion rates were calculated as apparent flux rates per rectum (average area = 0.64 cm$^2$) or per cm$^2$ of tissue per hour.

Net electrochemical potentials were calculated as:

$$ \Delta \mu_i / F = (RT \ln(a_o / a_i))/F + zV_{a,b} $$  \hspace{1cm} (3)

where $a_o$ is the activity of ion ‘i’ in the outside compartment, $a_i$ is the activity of ion ‘i’ in the inside compartment, $V_{a,b}$ are the apical and basolateral membrane potentials respectively, and $F$, $R$, $T$ and $z$ have their usual meanings. Negative values imply favourable electrochemical potentials for passive net flux, whereas positive values imply opposing electrochemical potentials (i.e. active net flux).

Bicarbonate concentrations were calculated from total CO$_2$ measurements using the Henderson-Hasselbach equation ($pK = 6.1695$). Saline pK values were calculated from Siggaard-Andersen (1976) for a salt solution at 22°C with an ionic strength of 0.18 mol·kg$^{-1}$.

All values are reported as means ± standard errors. Statistical significance was determined using paired or nonpaired $t$-tests. Differences were considered statistically significant if $P < 0.05$. 
RESULTS

Confirmation of rectal acidification

One of the primary objectives of this study was to develop an in vitro preparation which could be routinely used for studying acid/base transport in the rectum. Although previous attempts at detecting rectal acidification in standard Ussing chambers had been unsuccessful, it was felt that this method offered enough promise to be further pursued. The standard Ussing chambers used by Hanrahan (1982; 5.0 ml/chamber) were scaled down in size (2.0 ml/chamber) and saline composition and gas tension (ie. CO₂/HCO₃⁻-free) were modified to minimize buffering capacity and to duplicate as closely as possible the original conditions used by Phillips (1961) and Speight (1967). The voltage clamp arrangement and type and orientation of both current sending and voltage sensing electrodes were identical to those used by Hanrahan (1982; see also Hanrahan et al., 1984). The major advantage of this type of preparation is that it allows complete control of the composition of the media bathing both sides of the epithelium while at the same time permitting measurement of transepithelial membrane potentials or application of short-circuit current. This preparation has been used extensively in this laboratory and consistently yields long-term stable rates of solute transport similar to those observed in vivo (see Phillips et al., 1986).

Recta mounted in this manner and bathed bilaterally in a phosphate- and CO₂/HCO₃⁻-free saline (pH 7.00; see methods for full composition) under open-circuit conditions maintained constant rates of luminal acidification for at least 8h against transepithelial potentials of 6-8 mV (Fig. 2.3). Clearly, this is an extremely viable preparation capable of actively maintaining stable rates of acidification similar to those observed in vivo (see Table 2.1). The rapid decline in J_{H⁺} and Vt with bilateral addition of 1 mM KCN strongly suggests that rectal acidification is tightly linked to metabolic events in the rectal epithelial cells. The dramatic decrease in Vt immediately following extirpation of the rectum (Fig. 2.3) is well
Figure 2.3. Time-course of luminal acidification ($J_{H^+}$: closed circles) and transepithelial potential ($V_t$: open circles; luminal bath relative to contraluminal bath) in isolated recta mounted as flat-sheets in Ussing chambers under open-circuit conditions. Recta were bathed bilaterally with a phosphate- and CO$_2$/HCO$_3^-$-free saline (see text for details); luminal and contraluminal pH = 7.00. 1mM KCN was added bilaterally at the arrows. Values are means ± SE; $n=6$ for each value.
Table 2.1 In vivo and in vitro measurements of luminal acidification in the locust rectum.

<table>
<thead>
<tr>
<th>Condition</th>
<th>$J_{H^+}$ ($\mu$equiv cm$^{-2}$ h$^{-1}$)</th>
<th>$V_t$ (mV)</th>
<th>$I_{sc}$ ($\mu$equiv cm$^{-2}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>In vivo ligated rectum †</td>
<td>2.15 ± 0.22</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Ussing chamber; open circuit</td>
<td>1.54 ± 0.10 $^a$</td>
<td>7.8 ± 0.8</td>
<td>–</td>
</tr>
<tr>
<td>Ussing chamber; $I_{sc}$ (simple Ag electrodes) *</td>
<td>0.93 ± 0.09 $^b$</td>
<td>6.3 ± 1.1†</td>
<td>1.25 ± 0.27</td>
</tr>
<tr>
<td>Ussing chamber; $I_{sc}$ (Ag-AgCl electrodes: agar bridges) *</td>
<td>1.51 ± 0.10 $^{a,b}$</td>
<td>6.9 ± 1.3†</td>
<td>1.30 ± 0.21</td>
</tr>
</tbody>
</table>

$J_{H^+}$, rate of rectal acidification. $V_t$, transepithelial potential (lumen relative to haemocoel). $I_{sc}$, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). Recta were bathed bilaterally with phosphate- and CO$_2$/HCO$_3$-free salines in the in vitro preparations (see text for details on saline composition). Values are means ± SE; $n=6$ for each value. * see text for details on current sending electrode construction and configuration. † from Speight (1967). ‡ measured at end of short-circuit current experiments.

$^a$ values marked with common symbol not significantly different by Student’s $t$-test ($P > 0.80$).

$^b$ values marked with common symbol significantly different by Student’s $t$-test ($P < 0.005$).
documented and has been attributed to a decrease in electrogenic Cl⁻ transport from lumen to haemocoel (Phillips et al., 1986). The initial decay in Cl⁻ transport is thought to be due to a decrease in the titre of a chloride transport stimulating hormone (CTSH) and a concomitant decrease in intracellular cAMP levels (Chamberlin and Phillips, 1988) rather than an overall decrease in the viability of the preparation.

Although this chamber design was more than adequate for measurement of $J_{H^+}$ under CO₂/HCO₃⁻-free conditions, it did not permit reliable estimates of luminal acidification in the presence of bilateral CO₂ and HCO₃⁻ (concentrations ranging from 1-5% CO₂ and 1.5-10 mM HCO₃⁻). The volatile nature of the CO₂/HCO₃⁻ buffer pair and the much increased saline buffering capacity associated with CO₂/HCO₃⁻ addition made accurate detection of rectal acidification very difficult. The current chamber design (or size) clearly must be modified before this line of experimentation (ie. effect of luminal CO₂/HCO₃⁻ on $J_{H^+}$) can be pursued.

Although Hanrahan (1982) was unable to detect luminal acidification with the larger Ussing chambers (5.0 ml/chamber) under CO₂/HCO₃⁻-free conditions, he did observe high rates of luminal alkalinization (4.5 ± 0.5 μequiv·cm⁻²·h⁻¹) under short-circuit current conditions when the tissue was stimulated with cAMP. Since alkalinization had never been observed in vivo, it was important to know whether this alkalinization was a response to the addition of cAMP, the application of short-circuit current, or both. When unstimulated recta were short-circuiting in the present study (under the same conditions as the open-circuit experiment above), rates of rectal acidification (0.93 ± 0.09 μequiv·cm⁻²·h⁻¹) were significantly lower than those observed under open-circuit conditions (1.54 ± 0.10 μequiv·cm⁻²·h⁻¹). This is somewhat puzzling, since short-circuit current should have slightly enhanced net cation (eg. H⁺) movement into or net anion (eg. OH⁻ or HCO₃⁻) movement out of the lumen (and therefore enhanced rectal acidification) by abolishing the small 6-8 mV lumen positive transepithelial potential. Nevertheless, the results supported the existence of
an active luminal acidification mechanism and suggested that it was the addition of cAMP and not the application of short-circuit current which initiated luminal alkalinization.

When Hanrahan's (1982) conditions were duplicated exactly (apart from chamber size), high rates of luminal alkalinization were observed after contraluminal addition of 1mM cAMP (Fig. 2.4). Although Hanrahan reported that average rates of alkalinization were only 66% of simultaneous Isc and only 39% of unidirectional Cl⁻ flux, I found that the alkalinization was approximately 80% of the simultaneous Isc and that rates of alkalinization were virtually identical to the Cl⁻ fluxes reported by him (Hanrahan, 1982) under similar conditions. Moreover, when luminal Cl⁻ was removed from the bath, Isc, Cl⁻ flux, and alkalinization were all abolished (Fig. 2.4).

Superficially, this data appeared to provide very convincing evidence for a Cl⁻-dependent alkalinization mechanism (eg. Cl⁻/OH⁻ or Cl⁻/HCO₃⁻ exchange), implying that active Cl⁻ transport was not the source of the cAMP-stimulated Isc as previously proposed by Hanrahan and Phillips (1984a). Upon closer inspection, however, this proved not to be the case. If base equivalents were being transported transepithelially, the increase in luminal pH should have been accompanied by a concomitant decrease in contraluminal pH (ie. under steady-state conditions, the changes in H⁺/OH⁻ activity on both sides of the epithelium should have been of similar magnitude but opposite direction). When the above experiments were repeated, significant decreases in rates of contraluminal alkalinization were observed, but they were several orders of magnitude less than the rate changes observed in the lumen (Table 2.2). This suggested that hydroxyl equivalents might have been added to the lumen from an external source rather than the epithelium itself. To test this hypothesis, Ussing chambers were set up as above without recta and 80 mAmps of current (a typical current under cAMP stimulated conditions) were passed across the current sending electrodes. As predicted from the previous experiment, the luminal bathing saline alkalinized at the same rate whether a tissue was present or not (eg. Fig. 2.5B, Ag-electrode-closed circles).
Figure 2.4. Effect of contraluminal cAMP and bilateral Cl⁻ substitution on luminal acid/base transport ($J_{\text{H/OH}}$; open circles), transepithelial net Cl⁻ flux ($J_{\text{CL}}$; dashed line), and short-circuit current (Isc; closed circles). Positive values of $J_{\text{CL}}$ indicate Cl⁻ movement from lumen to haemocoel (Cl⁻ fluxes were taken from Hanrahan, 1982). Positive values of $J_{\text{H/OH}}$ indicate luminal acidification whereas negative values indicate luminal alkalinization. Positive values of Isc indicate net cation movement into or net anion movement out of the lumen. Salines were CO₂/HCO₃⁻-free (see text for details). Contraluminal pH was maintained at 7.00 by continuous perfusion; luminal pH was maintained at 7.00 by pH-stat. values are means ± SE; $n=6$ for each value.
Table 2.2  **Effect of cAMP on luminal and contraluminal acid/base transport (J_{H/OH}) under Isc conditions when simple silver electrodes are used to pass the short-circuit current.**

<table>
<thead>
<tr>
<th></th>
<th>Luminal (μequiv cm⁻² h⁻¹)</th>
<th>Contraluminal (μequiv cm⁻² h⁻¹)</th>
<th>Isc (μequiv cm⁻² h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.93 ± 0.09ᵃ</td>
<td>1.47 ± 0.10ᵇ</td>
<td>1.25 ± 0.27ᶜ</td>
</tr>
<tr>
<td>+ 1mM cAMP</td>
<td>-13.7 ± 0.91ᵃ</td>
<td>0.98 ± 0.11ᵇ</td>
<td>16.40 ± 1.20ᶜ</td>
</tr>
</tbody>
</table>

Luminal $J_{H/OH}$, positive values indicate net acid addition to the lumen; negative values indicate net alkali addition to the lumen. Contraluminal $J_{H/OH}$, positive values indicate net alkali addition to the contraluminal bath. Isc, short-circuit current (positive values indicate net cation movement into or net anion movement out of the lumen). Recta were bathed bilaterally with phosphate- and CO₂/HCO₃⁻-free salines; pH = 7.00. Values are means ± SE; $n$=6.ᵃᵇᶜ values with common symbols are significantly different by paired t-test ($P < 0.001$).ᵇ values with common symbols are significantly different by paired t-test ($P < 0.03$).
Figure 2.5. Effect of electrode configuration and quantity of current passed on apparent rates of luminal alkalinization. See text for details on electrode construction. A,B, 10 or 80 μA of current passed across the current sending electrodes respectively. Experimental chambers were set up without recta and filled with the standard CO₂/HCO₃⁻-free saline used throughout this study. Rates of alkalinization were measured by pH-stat (saline pH maintained at 7.00). Values are means ± SE (where SE is not shown, it is within the size of the symbol); n = 6 for each value.
A. 10 μA current

B. 80 μA current
The observed luminal alkalinization reported by Hanrahan was clearly an experimental artifact.

The source of the alkalinization was the pair of silver electrodes used to pass the short-circuit current. When a simple silver electrode is used as a cathode (luminal electrode), bubbles of hydrogen gas are formed at the electrode surface and the solution surrounding it alkalinizes in proportion to the amount of current passed (eq. 4).

\[ 2H_2O + 2e^- \rightarrow H_2(g) + 2OH(aq) \] (4)

At the anode (contraluminal electrode), Cl\(^-\) ions from the bath combine with Ag to form AgCl (eq. 5).

\[ Ag^0 + Cl^- \rightarrow AgCl + 1e^- \] (5)

The combined effect of these reaction sequences is the replacement of bath Cl\(^-\) ions by an equal number of OH\(^-\) ions, thus explaining the similarity between rates of Cl\(^-\) flux, Isc, and alkalinization. The alkalinization appeared to be a cellular event modulated by cAMP simply because cAMP stimulated active electrogenic Cl\(^-\) transport which in turn was accompanied by concomitant increases in the amount of current passed between the silver electrodes (and the amount of OH\(^-\) formed).

This is not a new or novel observation. The problems associated with passing currents across bare silver electrodes have been known to electrophysiologists for decades. Under most circumstances, problems with unwanted products of electrolysis can largely be eliminated by simply coating the silver electrodes with AgCl (either electrolytically or by dipping in molten AgCl). If Ag-AgCl electrodes are used, the reaction at the anode is basically the same as above (see eq. 5), but the reaction at the cathode and its implications are markedly different. With the passage of current, Cl\(^-\) ions are electrolytically released from the AgCl and Ag\(^0\) is deposited on the electrode (eq. 6).
\[ \text{le}^- + \text{AgCl} \rightarrow \text{Ag}^0 + \text{Cl}^- \quad (6) \]

With Ag-AgCl electrodes, the quantity of Cl\(^-\) removed from the bath at the anode will be exactly matched by the quantity of Cl\(^-\) (rather than OH\(^-\)) released at the cathode.

The problem with Ag-AgCl electrodes, however, is that the formation of unwanted electrolytic by-products is directly related to the quality (and quantity) of the AgCl coating on the electrode. Intuitively this is obvious, but empirically the quality of the coating is often very difficult to judge. Protection from electrolytic by-products (as judged by effects on bath pH) varied significantly among Ag-AgCl electrodes which visually appeared very similar (data not shown). Moreover, the rate of pH change varied significantly (and often dramatically) with the intensity and length of time that the current was passed (Fig. 2.5B, Ag-AgCl electrode-open circles). Although freshly prepared Ag-AgCl electrodes are adequate at low current levels (similar to unstimulated, steady-state levels in the rectum) for prolonged periods (Fig. 2.5A), at high currents (similar to cAMP stimulated levels in the rectum) the degree of protection from electrolytic by-products (specifically OH\(^-\)) is very limited and extremely variable. Clearly, Ag-AgCl electrodes by themselves are not suitable for passing large amounts of current in weakly buffered solutions where exogenously induced changes in bath pH can not be tolerated. Adequate protection can be obtained, however, by isolating the current passing Ag-AgCl electrodes from the bath with agar bridges. When the chambers were modified to allow this configuration there was no effect on bath pH regardless of the duration or intensity of the current passed (Fig. 2.5).

When Hanrahan’s (1982) alkalinization experiments were repeated with Ag-AgCl electrodes isolated from the bath with 100 mM NaCl agar bridges, luminal alkalinization was abolished (see Chapter Four, Table 4.11); Vt, Isc, and Rt, however, were completely unaffected by the change in electrode configuration (Table 2.3). When my initial luminal acidification experiments were repeated with the Ag-AgCl:agar bridge configuration, there
Table 2.3  Effect of current sending electrode configuration on Isc, Vt, and Rt when locust recta are stimulated with cAMP under CO$_2$/HCO$_3^-$-free conditions.

<table>
<thead>
<tr>
<th>Electrode Configuration</th>
<th>Isc (μequiv cm$^{-2}$h$^{-1}$)</th>
<th>Vt (mV)</th>
<th>Rt (Ω cm$^2$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Simple Ag electrodes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control saline</td>
<td>1.25 ± 0.27 $^a$</td>
<td>6.3 ± 1.1 $^c$</td>
<td>188.1 ± 15.2 $^e$</td>
</tr>
<tr>
<td>+ 1mM contraluminal cAMP</td>
<td>17.95 ± 1.31 $^b$</td>
<td>41.5 ± 2.9 $^d$</td>
<td>86.2 ± 20.8 $^f$</td>
</tr>
<tr>
<td>Ag-AgCl electrodes: agar bridges</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>control saline</td>
<td>1.30 ± 0.21 $^a$</td>
<td>6.9 ± 1.3 $^c$</td>
<td>198.0 ± 14.0 $^e$</td>
</tr>
<tr>
<td>+ 1mM contraluminal cAMP</td>
<td>16.10 ± 1.69 $^b$</td>
<td>43.0 ± 2.1 $^d$</td>
<td>99.6 ± 18.6 $^f$</td>
</tr>
</tbody>
</table>

Isc, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). Vt, transepithelial potential (lumen relative to haemocoel); Vt measured at end of each treatment period. Rt, transepithelial resistance (calculated from Vt and Isc by Ohm’s Law). Bath pH was maintained at 7.00 by bilateral perfusion (8 ml/min). Values are means ± SE; $n=6$ for each electrode configuration. See text for details on current sending electrode construction and configuration and control saline composition. $^a,b,c,d,e,f$ values with common symbols not significantly different by Student’s t-test ($P > 0.40$).
was no longer a significant difference between open and short-circuit current conditions (Table 2.1). Unless otherwise specified, all future experiments described in this thesis conducted under short-circuit current conditions will have had the current sending electrodes (Ag-AgCl) isolated from the bathing media with 100 mM NaCl agar bridges.

**Source of rectal acidification**

One of the major concerns with the original studies done by Phillips (1961) and Speight (1967) was that a significant fraction of the rectal acidification observed *in vivo* might have been due to bacterial metabolism in the lumen. To guard against this possibility in the present study, recta were bilaterally perfused (4 ml/min) for 2h prior to experimentation to flush away any remaining rectal contaminants. To confirm that the epithelium itself was responsible for the pH changes, rates of luminal acidification ($J_{\text{H}^+}$) were compared with rates of contraluminal alkalinization ($J_{\text{OH}^-}$) under open-circuit ($\text{CO}_2/\text{HCO}_3^-$-free) conditions. By the rationale used above, $J_{\text{H}^+}$ should be of similar magnitude to $J_{\text{OH}^-}$ if the rectal epithelium *per se* is responsible for luminal acidification. If, on the other hand, acidification is due to bacterial activity in the lumen, one would not expect to see contraluminal alkalinization. Under the above conditions $J_{\text{OH}^-}$ was virtually identical to $J_{\text{H}^+}$ (Table 2.4), demonstrating very clearly that the epithelium itself was the source of the acidification.

The apparent rates of contraluminal alkalinization determined by the pH-stat technique were significantly influenced by the rate of bubbling (with 100% $\text{O}_2$) in the contraluminal chamber (this did not appear to be a factor for measurements of $J_{\text{H}^+}$). When low to moderate bubbling rates (15-30 ml $\text{O}_2$/min) were used, $J_{\text{OH}^-}$ was consistently 10-15% lower than corresponding rates of $J_{\text{H}^+}$ (eg. $J_{\text{H}^+} = 1.41 \pm 0.08 \text{mEq/cm}^2 \cdot \text{h}^{-1}$; $J_{\text{OH}^-} = 1.24 \pm 0.08 \text{mEq/cm}^2 \cdot \text{h}^{-1}$; $n = 6$; significantly different by paired $t$-test at $P < 0.05$). When bubbling rates were high (90-100 ml $\text{O}_2$/min), rates of contraluminal alkalinization and luminal
Table 2.4  Relative rates of luminal acidification ($J_{H^+}$) and contraluminal alkalinization ($J_{OH^-}$) by locust recta under CO$_2$/HCO$_3^-$-free conditions.

<table>
<thead>
<tr>
<th></th>
<th>$J_{H^+}$ (µequiv/cm$^2$·h$^{-1}$)</th>
<th>$J_{OH^-}$ (µequiv/cm$^2$·h$^{-1}$)</th>
<th>$V_t$ (mV)</th>
<th>$I_{sc}$ (µequiv/cm$^2$·h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Open-circuit conditions</td>
<td>1.47 ± 0.08 $^a$</td>
<td>1.44 ± 0.10 $^a$</td>
<td>6.1 ± 1.1</td>
<td>–</td>
</tr>
<tr>
<td>Isc conditions</td>
<td>1.55 ± 0.10 $^b$</td>
<td>1.57 ± 0.11 $^b$</td>
<td>7.3 ± 1.4 $^\dagger$</td>
<td>1.32 ± 0.22</td>
</tr>
</tbody>
</table>

$V_t$, transepithelial potential (luminal bath relative to contraluminal bath). $I_{sc}$, short-circuit current (positive values indicate net cation movement into or net anion movement out of the lumen). Recta were bathed bilaterally with phosphate- and CO$_2$/HCO$_3^-$-free salines; pH = 7.00. $^\dagger$ measured at end of experiment. Values are means ± SE; $n$=6. $^a,b$ values marked with common symbols not significantly different by paired t-test ($P > 0.60$).
Acidification were not significantly different (see Table 2.4). A possible explanation for this observation lies with the nature of the acid/base equivalent transported across the basolateral membrane. If $J_{\text{OH}^-}$ is mediated by $\text{HCO}_3^-$ efflux, $\text{HCO}_3^-$ must be converted to $\text{CO}_2$ relatively instantaneously for $J_{\text{OH}^-}$ to equal $J_{\text{H}^+}$. It is reasonable to assume that this conversion would be influenced both by the rate of stirring and the rate of $\text{CO}_2$ removal from the system (both a function of the bubbling rate).

Although extremely unlikely, it was possible that rectal acidification might be due to passive diffusion of $\text{H}^+$ equivalents into the lumen from the intracellular compartment of the epithelium. This hypothesis was tested by measuring intracellular pH ($\text{pHi}$) and apical and basolateral membrane potentials ($V_a$ and $V_b$ respectively) with ion-sensitive double-barrelled microelectrodes. Double-barrelled electrodes did not give significantly different values for membrane potentials than single-barrelled electrodes nor was there any evidence of impalement damage as ascertained by voltage divider ratios or $R_t$. Impalements were often exceptionally stable and allowed continuous measurements for 30-45 min (see Fig. 2.6 for typical trace). $V_a$ and $V_b$ showed very little variation between cells in the same preparation and mean values (Table 2.5) were remarkably similar to previously published values for the same tissue under similar conditions (Hanrahan and Phillips, 1984). Measured pHi values are distinctly unimodal and appear to be normally distributed about their mean (Fig. 2.7), consistent with the sampling of a single cell population (see Chapter One). Intracellular pH measurements made under phosphate- and $\text{CO}_2$/HCO$_3^-$-free conditions yielded a mean pH value of 7.36±0.02 (Table 2.5). This value is substantially higher than the equilibrium value predicted for pHi if $\text{H}^+$ was passively distributed across the apical membrane (ie. predicted pHi = 6.01 when luminal pH = 7.00 and $V_a = -58$ mV). Clearly the electrochemical gradient favours passive movement of protons in the opposite direction (ie. lumen to cell; Table 2.5); the observed rectal acidification must therefore be due to an active transport process located at the apical membrane.
Figure 2.6. Typical trace obtained with double-barreled ion-sensitive microelectrode under CO$_2$/HCO$_3^-$-free conditions (luminal and contraluminal pH maintained at 7.00). Voltage deflections in A. and B. are the result of passing 13 µAmp transepithelial current pulses. A. Measurements of Vt are referenced to the contraluminal bath. B. Apical (Va) and basolateral (Vb) membrane potentials were obtained by referencing the voltage barrel of the microelectrode to a luminal or contraluminal 3M KCl agar bridge respectively during the same impalement. In this example, current pulses passed during recording of Va resulted in downward voltage deflections (hyperpolarizing direction) whereas pulses passed while recording Vb resulted in upward voltage deflections (depolarizing direction). The break at mid-panel indicates a second impalement. C. This trace is the result of a single impalement with the same electrode used in B (current pulses were not passed in this example). Potential differences measured with the pH-sensitive barrel ($V_{H^+}$) are referenced to the lumen. The dashed line indicates the relative magnitude of Va for comparative purposes. The intracellular pH (pHi) scale is placed to demonstrate conversion from $V_{H^+}$ to actual pHi values.
Table 2.5  Intracellular pH and calculated $H^+$ electrochemical potentials for apical and basolateral membranes of locust recta bathed bilaterally by phosphate- and $CO_2/HCO_3^-$-free saline.

<table>
<thead>
<tr>
<th>pH$i$</th>
<th>$V_a$ (mV)</th>
<th>$V_b$ (mV)</th>
<th>$V_t$ (mV)</th>
<th>$\Delta\mu_{a/F}$ (mV)</th>
<th>$\Delta\mu_{b/F}$ (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.36 ± 0.02</td>
<td>-58.4 ± 0.6</td>
<td>-50.2 ± 0.2</td>
<td>8.1 ± 0.4</td>
<td>79.3 ± 0.8</td>
<td>-71.7 ± 0.6</td>
</tr>
</tbody>
</table>

pH$i$, intracellular pH. $V_a$ and $V_b$, apical and basolateral membrane potentials (cytoplasm relative to luminal or contraluminal bath respectively). $V_t$, transepithelial potential (luminal bath relative to contraluminal bath). $\Delta\mu_{a/F}$ and $\Delta\mu_{b/F}$, apical and basal electrochemical potentials (negative values indicate favourable gradients for net $H^+$ movement in the contraluminal to luminal direction; positive values indicate opposing gradients). Values are means ± SE; $n=27$ impalements on 6 recta; luminal and contraluminal pH = 7.00.
Figure 2.7. Distribution of intracellular pH values in rectal epithelial cells under open-circuit conditions. Recta were bathed bilaterally with a phosphate- and CO$_2$/HCO$_3$-free saline; luminal and contraluminal pH maintained at 7.00; $n=8$ recta, 33 cells.
Acid secretion versus base reabsorption

The identification of the exact mechanism responsible for mediating pH changes in acid/base transporting epithelia (ie. acid secretion or base reabsorption) has historically been one of the most difficult (and often controversial) issues to resolve in acid/base physiology (see Rector et al. 1965, Brodsky and Schilb 1974, Steinmetz 1974, and DuBose 1983 for examples). The volatile nature of the CO₂/HCO₃⁻ buffer system and the interactions of free protons and hydroxyl ions with the primary solvent make it particularly awkward to apply standard ion transport techniques to the solution of this problem. To complicate matters further, the epithelium itself could conceivably contribute significantly to the relative concentrations of pH relevant ions in the unstirred layers adjacent to the apical and basolateral borders. Bearing this in mind, it is not realistic to expect simple ion substitutions to necessarily give the full answer to this question. Nevertheless, it is the simplest approach and the logical first step.

The concentration of phosphate in rectal contents in vivo is known to be high (>40 mM; Speight, 1967) and it was originally thought that the observed acidification might be due to phosphate movements into or out of the lumen. Preliminary studies by Speight (1967) ruled out selective reabsorption of HPO₄²⁻, but the possibility remained that acidification might be due to secretion of H₂PO₄⁻. The persistence of acidification in the absence of luminal and contraluminal phosphate in the present study with the isolated rectal preparation (Table 2.1) and conclusive evidence demonstrating net phosphate (particularly H₂PO₄⁻) movement in the wrong direction (ie. reabsorption; Andrusiak et al., 1980) confirms that basal rates of J_H⁺ are not dependent on selective secretion or reabsorption of either phosphate moiety.

Much more difficult to determine accurately is the relationship between J_H⁺ and CO₂/HCO₃⁻. Thomson and Phillips (1985) have reported significant rates of net HCO₃⁻ reabsorption in everted rectal sacs incubated in 2% CO₂ and 5 mM HCO₃⁻. This observation
immediately suggests that rectal acidification might be due to HCO₃⁻ reabsorption per se rather than acid secretion. Although most of the experiments in the present study are conducted under nominally CO₂/HCO₃⁻-free conditions to guard against this possibility, the continued production of metabolic CO₂ makes it very difficult to ensure a completely CO₂/HCO₃⁻-free environment in the unstirred layers next to the epithelium. This in turn makes it difficult to state with absolute certainty that acidification is due to acid secretion rather than HCO₃⁻ reabsorption.

This problem was addressed by monitoring $J_{\text{H}^+}$ and bath total CO₂ content both in the presence and absence of transepithelial pCO₂ gradients. Recta were mounted as indicated above and bathed bilaterally in the standard CO₂/HCO₃⁻-free saline (pH 7.00). Contraluminal perfusion was maintained throughout the entire experiment and luminal perfusion was discontinued once a steady-state (as defined above) had been reached. As a first approximation of the efficacy of the attempts to blow off metabolic CO₂ (and hence maintain a nominally CO₂/HCO₃⁻-free environment), I measured the total CO₂ content of the initial saline (before exposure to the tissue), the contraluminal perfusate, and the luminal saline after 4h exposure to the tissue. All samples contained < 10 μM total CO₂ (this represents the practical lower limit of the CO₂ assay used in this study; see Materials and Methods; $n=6$ preparations). Although this suggests that the experimental protocol is effective for maintaining a CO₂⁻-free state in the bulk solution, it could be argued that a significant CO₂/HCO₃⁻ concentration might exist in the unstirred layers next to the apical membrane without necessarily being detected in the bulk solution. If this is true and rectal acidification occurs by HCO₃⁻ reabsorption rather than proton secretion, one would predict that increasing the pCO₂ (and hence the HCO₃⁻ concentration) of the unstirred layers should result in an increase in net rectal acidification.¹

¹ This assumes that if $J_{\text{H}^+}$ was mediated by HCO₃⁻ reabsorption that it would not be maximally stimulated by the sub-micromolar quantities of HCO₃⁻ present in the luminal bath when the tissue was bathed bilaterally with nominally CO₂/HCO₃⁻-free salines (see Schwartz and Steinmetz, 1971).
This hypothesis was tested by adding 1.5 mM HCO₃⁻ to the contraluminal perfusate and aerating the contraluminal chamber with 1% CO₂/99% O₂ (contraluminal pH was maintained at 7.00). The luminal bath was nominally CO₂-free, as above, and was aerated with 100% O₂. Based on the CO₂ permeabilities reported in the literature (see Schwartz et al., 1981), this maneuver would be expected to increase both the intracellular pCO₂ and the pCO₂ (and concomitant HCO₃⁻ concentrations) of the unstirred layers next to the apical membrane. Measurements of the total CO₂ content of the initial luminal saline (before exposure to the tissue) and the luminal saline after 4h exposure to the tissue (in the presence of contraluminal 1.5 mM HCO₃⁻/1% CO₂) were again always <10 μM (Table 2.6). This chamber design clearly permits vigorous enough stirring (with 100% O₂ in the absence of luminal perfusion) to maintain a nominal CO₂-free state in the bulk solution in the presence of a significant transepithelial pCO₂ gradient. Consistent with the previous observations, the addition or removal of contraluminal 1% CO₂/1.5 mM HCO₃⁻ had no effect on J_{H⁺} or Isc (Table 2.6).

The possibility remained, however remote, that CO₂ induced changes in J_{H⁺} were mitigated by diffusion of contraluminal HCO₃⁻ into the lumen (see Schwartz and Steinmetz, 1971). This hypothesis was tested by repeating the above experiment in the absence of exogenously added HCO₃⁻ at a contraluminal pH of 5.50 (luminal saline was CO₂/HCO₃⁻-free; pH 7.00). At this pH, equilibrium levels of HCO₃⁻ should be several orders of magnitude less than in the previous experiment and corresponding rates of HCO₃⁻ diffusion into the lumen should be substantially lower. Under control conditions (ie. bilateral CO₂/HCO₃⁻-free; lumen pH 7.00) a decrease in contraluminal pH from 7.50 to 5.00 had no effect on J_{H⁺}, Vt, or Isc (see Chapter 4, Fig. 4.1). The addition of contraluminal 1% CO₂ at pH 5.50 (lumen pH maintained at 7.00) also had no discernible effects on J_{H⁺} or Isc

---

2. CO₂ and HCO₃⁻ were not added directly to the luminal bath due to the limitations of the pH-stat measurement technique described above. CO₂ and HCO₃⁻ were added to the contraluminal bath instead in an effort to increase the HCO₃⁻ concentration of the unstirred layers adjacent to the apical membrane without significantly affecting the buffering capacity of the luminal bath.
Table 2.6 Changes in rates of rectal acidification ($\Delta J_{H^+}$) induced by contraluminal addition of $CO_2/HCO_3^-$.

<table>
<thead>
<tr>
<th>contraluminal bath</th>
<th>luminal bath</th>
<th>$\Delta J_{H^+}$</th>
<th>$J_{HCO_3^+}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>%$CO_2$</td>
<td>HCO$_3^-$ (mM)</td>
<td>pH</td>
<td>total CO$_2$ (µM)</td>
</tr>
<tr>
<td>1</td>
<td>1.5</td>
<td>7.15</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>1</td>
<td>0.0</td>
<td>5.50</td>
<td>&lt; 10</td>
</tr>
<tr>
<td>2</td>
<td>5.0</td>
<td>7.09</td>
<td>19.5 ± 1.6</td>
</tr>
<tr>
<td>2</td>
<td>0.0</td>
<td>5.50</td>
<td>20.2 ± 0.9</td>
</tr>
<tr>
<td>5</td>
<td>10.0</td>
<td>7.03</td>
<td>26.8 ± 1.7</td>
</tr>
<tr>
<td>5</td>
<td>0.0</td>
<td>5.50</td>
<td>24.9 ± 1.4</td>
</tr>
</tbody>
</table>

* $Isc$ was completely unaffected by contraluminal $CO_2/HCO_3^-$ changes in every preparation tested.

All experiments performed under short-circuit current conditions. Recta were initially brought to steady-state (as defined in the text) under bilateral $CO_2/HCO_3^-$-free conditions (pH 7.00 bilaterally). The mean rate of rectal acidification ($J_{H^+}$) measured under $CO_2/HCO_3^-$-free conditions (for all preparations included in this table) was 1.61 ± 0.09 µequiv cm$^{-2}$h$^{-1}$. $\Delta J_{H^+}$ denotes the change in $J_{H^+}$ observed after the contraluminal bath was switched from the control $CO_2/HCO_3^-$-free saline (pH 7.00) to the various contraluminal treatments listed in the table and was calculated as: final $J_{H^+}$ measured in the presence of contraluminal $CO_2/HCO_3^-$ minus the initial $J_{H^+}$ measured under $CO_2/HCO_3^-$-free conditions. The luminal bath was continuously aerated vigorously with 100% $O_2$ and the luminal pH maintained at 7.00 by pH-stat. $J_{HCO_3^+}$ denotes the apparent rate of bicarbonate formation in the lumen and was calculated as final HCO$_3^-$ concentration – initial HCO$_3^-$ concentration and expressed per cm$^2$ of tissue per hour. Luminal total CO$_2$ and $\Delta J_{H^+}$ measurements were made 1h after contraluminal CO$_2$ or $CO_2/HCO_3^-$ additions. The detection limit for total CO$_2$ measurement with the assay used in this study was 10 µM (see Materials and Methods); $J_{HCO_3^+}$ was not calculated when total CO$_2$ concentrations were below this threshold. Values are means ± SE; $n=6$ for each treatment. $^{a,b,c,d,e,f}$ values with common symbols not significantly different by Student’s $t$-test ($P > 0.05$). $^{g,h,i,j}$ values with common symbols not significantly different by paired $t$-test ($P > 0.05$). $^{k}$ significantly different from control values (determined under bilateral $CO_2/HCO_3^-$-free conditions; bilateral pH 7.00) by paired $t$-test ($P < 0.05$).
It would appear, therefore, that HCO$_3^-$ diffusion was not a factor in the previous experiment and that the addition of contraluminal CO$_2$/HCO$_3^-$ at these low levels has no effect on rectal acidification. In view of these results it is unlikely that the trace levels of metabolic CO$_2$ produced by the rectum under CO$_2$/HCO$_3^-$-free conditions could generate sufficient HCO$_3^-$ to account for the observed rates of rectal acidification.

It was possible to induce significant increases in $J_{H^+}$ and luminal total CO$_2$ concentration by increasing contraluminal CO$_2$ tensions to 2% or 5% (Table 2.6). Based on the increased total CO$_2$ content of the lumen 1h after contraluminal CO$_2$/HCO$_3^-$ addition, it is clear that CO$_2$ was entering the luminal bath faster than it could be removed (i.e. "blown off") by vigorous aeration with 100% O$_2$. As observed in the previous experiment with 1% CO$_2$ and 1.5 mM HCO$_3^-$, removal of contraluminal HCO$_3^-$ (while maintaining a constant CO$_2$ tension) had no effect on $J_{H^+}$, Isc or luminal total CO$_2$ content (Table 2.6). This suggests that there was not a significant diffusional pathway for HCO$_3^-$ in the contraluminal to luminal direction under these experimental conditions and that the increase in luminal total CO$_2$ content was due solely to CO$_2$ diffusion across the epithelium.

Without concomitant measurements of pH$_i$ it is difficult to determine whether the apparent increases in $J_{H^+}$ ($\Delta J_{H^+}$) were due to increased luminal proton secretion, increased reabsorption of HCO$_3^-$, or increased rates of CO$_2$ hydration in the lumen (with its attendant formation of H$^+$ and HCO$_3^-$). If $\Delta J_{H^+}$ was due to elevated levels of proton secretion, the apparent rate of bicarbonate formation in the lumen ($J_{HCO_3}$) should have been consistently less than $\Delta J_{H^+}$. This would be expected since the epithelial addition of protons to the luminal bath would reduce rates of CO$_2$ hydration and subsequent HCO$_3^-$ formation in the lumen (see Eq. 7).

$$\text{CO}_2 \leftrightarrow \text{H}_2\text{CO}_3 \leftrightarrow \text{H}^+ + \text{HCO}_3^-$$

(7)
Likewise if $\Delta J_{H^+}$ was due to bicarbonate reabsorption from the lumen, rates of apparent $\text{HCO}_3^-$ formation should again have been less than $\Delta J_{H^+}$. Since $J_{\text{HCO}_3^-}$ is calculated as a change in luminal $\text{HCO}_3^-$ concentration, $J_{\text{HCO}_3^-}$ will only reflect true rates of $\text{HCO}_3^-$ formation if $\text{HCO}_3^-$ is not removed from the system. If $\text{HCO}_3^-$ is removed from the system (eg. by epithelial transport), $J_{\text{HCO}_3^-}$ will underestimate actual rates of $\text{HCO}_3^-$ formation (by the rate at which $\text{HCO}_3^-$ is removed from the system) and will be less than the rate of proton addition to the system by CO$_2$ hydration (in this scenario estimated as $\Delta J_{H^+}$; see Eq. 7). The results clearly do not support either of these hypotheses (Table 2.6). In all cases, apparent rates of bicarbonate formation ($J_{\text{HCO}_3^-}$) were not significantly different from the apparent increases in luminal acidification rates ($\Delta J_{H^+}$). The simplest explanation for this observation is that $\Delta J_{H^+}$ was merely due to CO$_2$ hydration in the lumen (see Eq. 7) and was not the result of an epithelial acid/base transport mechanism being stimulated by contraluminal (or luminal) CO$_2$ or the presence of luminal $\text{HCO}_3^-$. Since even measurable quantities of luminal $\text{HCO}_3^-$ did not appear to directly affect the apical acid/base transporter, it is difficult to believe that luminal acidification under bilateral CO$_2$/HCO$_3^-$-free conditions could be due to a bicarbonate reabsorptive mechanism.

Having removed the two major buffer groups (ie. phosphate and CO$_2$/HCO$_3^-$) from the saline, it was also unlikely that rectal acidification was due to transcellular transport of some unidentified exogenous weak acid or base. Nevertheless, it was possible that acidification was due to secretion (or leakage) of an acidic buffer from the intracellular compartment of the epithelium. This possibility was investigated by comparing the buffering capacity of freshly prepared saline with saline taken from the luminal chamber at the end of 6h pH-stat experiments. There was no discernible difference in buffering characteristics between the two salines when titrated down to pH 3.00, suggesting that acidification was not due to luminal addition of an unidentified buffer component (Fig. 2.8). Lactate measurements made on these same saline samples also showed no detectable increase in lactic acid concentration
Figure 2.8 Effect of 6h tissue exposure on buffering characteristics of CO₂/HCO₃⁻-free saline. Control saline (open circles): titration performed after aerating with 100% O₂ for 6h; Experimental saline (open triangles): titration performed on saline samples taken from the luminal chamber of Ussing chambers after 6h pH-stat experiments. Mean values plotted (standard errors were within the symbol size for all points plotted); n=6 recta for each titration curve.
over the 6h period (lactate concentrations were all less than 40 μM, the lower detection limit for the assay used in this study; see Materials and Methods; n=6). Again, considering the available evidence, one must conclude that rectal acidification is due to active transport of protons into the lumen rather than selective reabsorption of HCO₃⁻ or some other unidentified buffer component.

DISCUSSION

This is the first study to successfully demonstrate luminal acidification in the locust rectum in vitro, making it possible at last to conclusively identify both the source and the nature of the acidification. The persistence of high rates of luminal acidification after vigorous bilateral perfusion and the concomitant occurrence of contraluminal alkalinization make it clear that it is the epithelium per se rather than bacterial metabolism which is responsible for maintaining the observed rates of \( J_{H^+} \). Rates of \( J_{H^+} \) under both open- and short-circuit current conditions are very similar to the in vivo observations originally reported by Phillips (1961) and Speight (1967) (Table 2.1). This confirms the viability of the in vitro preparation and demonstrates unequivocally that rectal acidification is the result of an active transport process rather than passive transepithelial distribution of acid/base equivalents. Measurements of intracellular pH, and apical and basolateral membrane potentials further show that luminal pH is not in equilibrium with pHi and that acidification must occur actively against electrochemical gradients of at least 79 mV at the apical membrane (Table 2.5).

This is also the first time that intracellular pH has been measured in a terrestrial insect epithelium. Strange and Phillips (1985) have previously reported pHi values of 7.67 ± 0.03 for the epithelial cells of the anterior rectal salt gland of the larvae of the salt water mosquito, *Aedes dorsalis*. Considering the extreme environments in which *A. dorsalis* are found (ie. alkaline salt lakes with pH values often in excess of 10) and the severe acid/base challenges associated with those environments, it is not surprising that rectal salt gland pHi values are
substantially different from those reported here for the locust rectum (7.38 ± 0.02). Locust rectal pH values are, however, very similar to pH values reported for other invertebrate preparations (e.g. leech neurone: 7.31, Schlue and Thomas, 1985; snail neurone: 7.4, Evans and Thomas, 1984; squid axon: 7.3, Boron, 1984; barnacle muscle: 7.3, Keifer, 1981).

The luminal alkalinization previously reported by Hanrahan (1982) is clearly an artifact of passing large amounts of current between simple silver electrodes. When Hanrahan’s alkalinization experiments were repeated with Ag-AgCl electrodes isolated from the bath with 100 mM NaCl agar bridges, luminal alkalinization following cAMP stimulation was abolished. Although the use of the Ag-AgCl:agar bridge electrode configuration had a pronounced effect on measurable rates of acid/base transport (see Fig. 2.5), there was no discernible difference in cAMP stimulated Isc between this electrode configuration and the simple silver electrodes used by Hanrahan (Table 2.3).

It is unlikely that Hanrahan’s conclusions regarding the apical electrogenic Cl− pump would have been different had he used a Ag-AgCl:agar bridge configuration rather than silver electrodes to pass short-circuit current (see Hanrahan and Phillips 1984a,b). However, had the proper electrode configuration been used, his arguments for an uncoupled electrogenic Cl− pump would have been more convincing. The concomitant occurrence of luminal alkalinization and Cl− reabsorption clearly complicated the elucidation of the Cl− reabsorptive mechanism. The findings of the present study finally remove that nagging enigma (i.e. luminal alkalinization) and fully support Hanrahan’s original conclusion that Cl− transport in the locust rectum does not occur by an exchange for either HCO3− or OH− at the apical membrane.
**Proton secretion versus bicarbonate reabsorption**

Selective secretion or reabsorption of phosphate, lactate, or other unidentified buffer components does not appear to be involved in luminal acidification in the locust rectum under the experimental conditions used in this study. To account for the observed rates of $J_{\text{H}^+}$ under CO$_2$/HCO$_3^-$-free conditions, a HCO$_3^-$ reabsorptive (e.g. Cl$^-$/HCO$_3^-$ exchange) or proton secretory (e.g. Na$^+$/H$^+$ exchange, H$^+$/ATPase, etc.) mechanism must be invoked. In theory, it should be relatively simple to distinguish between these two modes of acidification. Removal of luminal HCO$_3^-$ should abolish $J_{\text{H}^+}$ if acidification is due to HCO$_3^-$ reabsorption, or it should have no effect on $J_{\text{H}^+}$ if acidification is due to proton secretion. In practice, the distinction between these two mechanisms is rarely that simple. The difficulty lies in insuring that all CO$_2$ and HCO$_3^-$ are removed from the system. Since CO$_2$ production is a natural consequence of cellular metabolism, it is not enough to merely remove exogenous CO$_2$ and HCO$_3^-$ from the initial bathing saline. Active measures must be taken to remove the CO$_2$ from the system as rapidly as it is produced by the cell.

In the present study, experimental chambers were aerated vigorously with 100% O$_2$ to “blow off” metabolically produced CO$_2$. The success of this approach was evaluated by monitoring the total CO$_2$ content of the luminal saline both in the presence and absence of transepithelial pCO$_2$ gradients. This protocol was effective for maintaining an essentially CO$_2$/HCO$_3^-$-free environment in the luminal bath even in the presence of contraluminal 1% CO$_2$/1.5 mM HCO$_3^-$ (see Table 2.6). Assuming that locust rectal epithelial CO$_2$ permeabilities are similar to those reported for other tissues, this method should be more than adequate for removing metabolically produced CO$_2$ from the lumen.

It can always be argued that HCO$_3^-$ might be formed in the unstirred layers without necessarily being detected in the bulk solution and that this low level of HCO$_3^-$ might be sufficient to bring about the observed rates of rectal acidification. Although this implies a transport mechanism with an extremely high affinity for HCO$_3^-$, it is not inconceivable
considering the affinities which must be postulated for a proton translocating mechanism
given the very much lower concentrations of protons under typical physiological conditions.
However, for HCO₃⁻ reabsorption to be the source of \( J_{H^+} \) under exogenous CO₂/HCO₃⁻-free
conditions, metabolic CO₂ must be produced by the epithelium at rates at least commensurate
with the observed rates of acidification.

Under similar experimental conditions, Chamberlin (1981) has shown that isolated
recta consume oxygen at approximately 16.07 nmoles min⁻¹ mg⁻¹ dry weight. An average dry
rectal weight of 1.5 mg and a mean surface area of 0.64 cm² rectum⁻¹ allows a conversion of
Chamberlin’s oxygen consumption value to 2.3 μmoles cm⁻² h⁻¹. If one assumes an RQ ratio
of 1 (for maximum conversion to CO₂), an equivalent rate of CO₂ diffusion across the apical
and basolateral membranes (see Schwartz et al., 1974), a luminal pH of 7, an instantaneous
hydration of CO₂ to HCO₃⁻, and no significant loss of CO₂ from the system (ie. no stirring
with 100% O₂), there would only be enough HCO₃⁻ formed in the lumen to account for
approximately 75% of \( J_{H^+} \) (ie. 1.15 μmoles CO₂ cm⁻² h⁻¹ as compared to \( J_{H^+} \) values of 1.54
μequiv cm⁻² h⁻¹). Obviously these are optimistic assumptions. Taking into account a more
realistic RQ value (8-9), a much lower pH in the unstirred layer, and a loss of at least 30% of
the CO₂ from the system (Schwartz et al., 1974, were reporting CO₂ losses of at least 50%
with a similar flat sheet preparation), rates of luminal HCO₃⁻ formation would be
substantially lower. Under these conditions, the likelihood that sufficient HCO₃⁻ could be
generated in the unstirred layers to account for the observed rates of rectal acidification is
extremely remote.

Notwithstanding the availability of metabolic CO₂, if acidification was mediated by
HCO₃⁻ reabsorption and sufficient HCO₃⁻ was being generated in the unstirred layer, any
manipulation which affected the rate of HCO₃⁻ formation should consequently affect \( J_{H^+} \).
This clearly did not happen in this study. When 1% CO₂/1.5 mM HCO₃⁻ was added to the
contraluminal bath neither \( J_{H^+} \) nor Isc was affected (see Table 2.6). Unless the epithelium
has an extremely low CO₂ permeability, this procedure should have increased CO₂ diffusion into the lumen and concomitant rates of HCO₃⁻ formation in the unstirred layers. That this had no effect on J₇⁺ strongly supports the original conclusion that acidification is not mediated by HCO₃⁻ reabsorption.

When contraluminal CO₂ tensions were increased to 2% or 5%, there were significant increases in luminal total CO₂ concentrations and J₇⁺ (Table 2.6). The observation that apparent rates of bicarbonate formation (J₇⁻⁻) equaled the increases in J₇⁺ (ΔJ₇⁺) under all conditions studied (Table 2.6) suggests that ΔJ₇⁺ was due to hydration of CO₂ in the lumen rather than stimulation of an apical acid/base transport mechanism. In support of this conclusion is the observation that there were no changes in Isc with the contraluminal additions of either CO₂ or HCO₃⁻. Data presented in Chapter 4 clearly demonstrate that J₇⁺ is due to an apical electrogenic transport mechanism under CO₂/HCO₃⁻-free conditions. Therefore, if contraluminal CO₂/HCO₃⁻ was directly affecting the apical transporter responsible for luminal acidification, one would expect to see an increase in Isc. The fact that this was not observed strongly supports the conclusion that rectal acidification under CO₂/HCO₃⁻-free conditions is mediated by proton secretion rather than bicarbonate reabsorption.

Summary

The data thus far clearly supports a model in which rectal acidification is mediated by an active proton secretory mechanism located at the apical membrane (Fig. 2.9). Although the nature of the contraluminal alkalinization has not yet been determined, the favourable electrochemical gradient for protons at the basolateral border (in the haemocoel to lumen direction; Fig. 2.9c) implies that an active step is not required at the basolateral membrane to drive the observed rates of contraluminal alkalinization. Moreover, the insensitivity of J₇⁺ and Vt to contraluminal pH (see Chapter Four, Fig. 4.1) and the effect of O₂ bubbling rates
on $J_{\text{OH}^-}$ suggests that like other urinary epithelia (see Schwartz and Steinmetz, 1971 and Giebisch and Aronson, 1987 for examples), contraluminal alkalinization in the locust rectum is likely due to HCO$_3^-$ efflux (Fig. 2.9b) rather than passive movements of H$^+$ or OH$^-$ (Fig. 2.9a).
Figure 2.9. Proposed models for rectal acidification under bilateral CO$_2$/HCO$_3^-$-free conditions. A. luminal acidification mediated by active proton secretory mechanism located at the apical membrane; contraluminal alkalinization mediated by movement of H$^+$ out of or OH$^-$ into the contraluminal bath; metabolic CO$_2$ diffuses freely out of the cell in all directions without affecting either transport process. B. luminal acidification mediated by active proton secretory mechanism located at the apical membrane; contraluminal alkalinization mediated by HCO$_3^-$ efflux from the cell into the contraluminal bath; fraction of metabolically produced CO$_2$ (equivalent to quantity of protons translocated across the apical membrane) buffers the OH$^-$ generated behind the apical proton pump (to form HCO$_3^-$) whereas the remainder diffuses freely out of cell across both membranes. C. Electrochemical profile for protons ($\Delta\mu_H/F$) across apical and basolateral membranes; electrochemical potentials calculated from measurements of pH$_i$ and intracellular membrane potentials made under CO$_2$/HCO$_3^-$-free conditions (bilateral pH=7.00); negative values indicate favourable gradients for net H$^+$ movement in the haemocoel to lumen direction whereas positive values indicate opposing gradients. The polarities of $V_a$ and $V_b$ are referenced to the cell interior.
Lumen | Cell | Haemocoel

A.

\[ H^+ \quad \rightarrow \quad H^+ \]

\[ \text{CO}_2 \]

B.

\[ H^+ \quad \rightarrow \quad OH^- \quad \rightarrow \quad HCO_3^- \]

\[ \text{CO}_2 \]

C.

\[ V_a \]

58 mV

\[ \text{pHi 7.38} \]

\[ V_b \]

50 mV

\[ \Delta \mu_a/F \]

79.3 mV

\[ \Delta \mu_{bl}/F \]

-71.7 mV
CHAPTER THREE: Characterization of rectal ammonia secretion

INTRODUCTION

The rectum of the desert locust secretes acid against pH gradients of up to two units in vivo and is believed to play a significant role in whole-animal pH regulation. An ongoing study of the mechanisms of acid-base transport in this epithelium has revealed that substantial quantities of ammonia\(^1\) accumulate in the rectum concurrent with luminal acidification. This observation suggests that ammonia secretion (\(J_{\text{Amm}}\)) may play a role in acid-base transport similar to that in other acid-secreting epithelia (ie., enhancement of luminal proton secretion via diffusion trapping). Moreover, if ammonia is secreted as \(\text{NH}_3\), and diffusion trapping does occur, ammonia secretion rates should be added to titratable acidity to obtain accurate values of hydrogen ion secretion in the rectum. It is the aim of the present chapter to determine the nature of the relationship between ammonia production and acid secretion in the locust rectum and particularly to determine the actual species of ammonia which crosses the apical membrane.

The locust hindgut performs a variety of homeostatic functions analogous to those found in vertebrate renal and gastrointestinal systems, and as seen in Chapter Two, the rectum itself is amenable to study using a wide variety of electrophysiological techniques. A primary isosmotic urine containing most blood solutes (including high levels of free amino acids) is formed in the Malpighian tubules, and selective reabsorption of desirable solutes and water occurs in the rectum (Phillips et al., 1986). Luminal amino acids secreted by the Malpighian tubules (particularly proline) are the principal metabolic substrates for the locust rectum, and Chamberlin and Phillips (1982) have shown that their oxidation produces significant quantities of endogenous ammonia.

---

\(1.\) The terms, ammonia, total ammonia, and \(J_{\text{Amm}}\) refer to the sum of \(\text{NH}_3\) and \(\text{NH}_4^+\). The terms \(\text{NH}_3\) and \(\text{NH}_4^+\) refer to the nonionic and ionic forms of ammonia, respectively.
Ammonia secretion may occur by a number of different mechanisms, depending on the form of ammonia transported. Transmembrane movements of NH$_3$, such as that found in mammalian proximal tubules (Pitts, 1973), are presumed to occur largely by passive diffusion through the hydrophobic phase of the membrane (Good and Knepper, 1985). Passive diffusion of NH$_4^+$, on the other hand, is much more likely to occur through hydrophylic pathways (eg. channels) rather than the lipid bilayer itself. Secondary active transport of NH$_4^+$ via coupling to energetically favourable movements of other ions (eg. Na$^+$/NH$_4^+$ exchange) is well documented in several cell types (see for example Evans and Cameron, 1986, and Kinsella and Aronson, 1981). Primary active transport directly dependent on metabolic energy has been postulated (eg. Na$^+$/K$^+$ (NH$_4^+$) ATPase; Aichin and Thomas, 1977; Kurtz and Balaban, 1986), but has not been clearly demonstrated to be significant under physiological concentrations of extracellular K$^+$.

Mechanisms of ammonia excretion have received relatively little attention in terrestrial insects. Mullins and Cochran (1972) demonstrated that ammonia was the predominant nitrogenous waste excreted by cockroaches under a variety of conditions and suggested that ammonia may be a major end product of terrestrial insects. The site of ammonia clearance in the insect excretory system is virtually unknown, with one exception; Prusch (1972) showed that ammonia was secreted in the unsegmented hindgut of Sarcophaga bullata larvae living in decaying meat and that the ammonia was actively secreted as NH$_4^+$ by a cation-independent mechanism.

The present study clearly demonstrates significant rates of luminal ammonia secretion in the locust rectum. Under the conditions studied, the secreted ammonia originated almost exclusively as the oxidative end product of amino acids reabsorbed from the lumen. There is very little correlation between hydrogen ion secretion ($J_{H^+}$) and $J_{Amn}$, and the experimental evidence strongly suggests that the bulk of transported ammonia is primarily in the ionic
form rather than \( \text{NH}_3 \). \( \text{NH}_4^+ \) secretion apparently occurs by an amiloride inhibitable \( \text{Na}^+/\text{NH}_4^+ \) exchange mechanism in the apical membrane.

**MATERIALS AND METHODS**

*Ammonia secretion.* Recta were mounted as flat-sheets in the modified Ussing chambers described in Chapter Two. Recta were brought to steady-state conditions (as defined by stable Isc or \( V_t \); ~2 h) under bilateral perfusion, and then flow was stopped unilaterally for the experimental period (1 h). \( J_{\text{Amn}} \) was determined as (total final ammonia) – (total initial ammonia) and expressed as a flux rate per square centimeter per hour. \( \text{NH}_4^+ \) concentrations were determined by the enzymatic assay of Kun and Kearny (1974), which utilizes the reductive amination of 2-oxoglutarate by glutamate dehydrogenase (GLDH) to bring about a change in extinction (at 365 nm) proportional to the ammonia content of the sample:

\[
\text{GLDH} \quad \text{NADH} + \text{NH}_4^+ + 2\text{-oxoglutarate} \xrightarrow{\text{GLDH}} \text{L-glutamate} + \text{NAD}^+ + \text{H}_2\text{O} \quad (1)
\]

Extinction was measured at 340 nm for the amiloride experiments due to amiloride interference at 365 nm.

*Solutions.* The composition of experimental salines was based on the content of locust haemolymph and Malpighian tubule fluid as outlined in Chapter Two and unless otherwise stated contained (in mM): 100 NaCl, 5 K\(_2\)SO\(_4\), 10 MgSO\(_4\), 10 Na\(^+\)-isethionate, 10 glucose, 100 sucrose, 5 CaCl\(_2\), 10 3-(N-morpholino)propanesulfonic acid (MOPS; pKa 7.2 at 25°C), 1.0 arginine, 1.5 serine, 13.1 proline, 1.3 asparagine, 11.4 glycine, 2.9 alanine, 1.8 valine, 5.0 glutamine, 1.0 tyrosine, 1.4 lysine, and 1.4 histidine. Salines titrated to pH < 6.50 also contained 10 mM 2(N-morpholino)ethanesulfonic acid (MES; pKa 6.1 at 25°C) to ensure adequate buffering capacity at all saline pH values used in this study. All salines were
CO$_2$/HCO$_3^-$-free to facilitate comparisons with the work on acid secretion in the rectum and to obviate the effects of volatile buffer components other than NH$_3$. All salines were also initially ammonia free (always < 20 μM) to confine the scope of the investigation to the effects of endogenously produced ammonia. The pH and gas tension of all solutions were adjusted as described in Chapter Two. Sodium and chloride were replaced with choline and gluconate salts, respectively, to prepare Na$^+$- and Cl$^-$-free salines. The free base form of L-Lysine was used rather than the monohydrochloride salt in Cl$^-$-free salines. K$^+$- and amino acid-free salines were prepared by replacement with isosmotic concentrations of sucrose.

Amiloride was made up in sulphate-free salines immediately before use and added to the luminal compartment at least 30 min prior to the experimental period to allow complete exposure of the membrane to the inhibitor. Amiloride was a generous gift from Merck Frosst Canada, Pointe-Claire, Quebec. Amino acids and adenosine 3',5'-cyclic monophosphate (cAMP; Na$^+$ salt) were obtained from Sigma Chemical (St. Louis, MO).

**Calculations.** $J_{\text{Amn}}$ was calculated as an apparent flux rate per square centimeter of tissue per hour. This designation was not intended necessarily to imply transepithelial transport, but rather a net accumulation in either the luminal or contraluminal bath regardless of the origin of the transported species (e.g. cellular or contraluminal).

**RESULTS**

**Characterization of ammonia secretion.** Recta exposed bilaterally to the standard saline (pH 7.00) secreted ammonia at a substantial rate almost exclusively into the lumen under both open (0.58 ± 0.12 μmoles·cm$^{-2}$·h$^{-1}$) and Isc conditions (0.62 ± μmoles·cm$^{-2}$·h$^{-1}$; Fig. 3.1). The difference between these values was not statistically significant ($P > 0.05$), and unless otherwise specified, the remaining experiments were performed under Isc conditions.
Figure 3.1 Luminal and contraluminal ammonia secretion rates ($J_{\text{amm}}$) measured under open- and short-circuit current conditions. Bilateral pH=7.00. Values are means ± SE; $n=6$ recta for each value.
In long-term flux studies where the contraluminal chamber was continuously perfused and the luminal chamber regularly rinsed with fresh saline at 60-min intervals (standard \(\text{CO}_2/\text{HCO}_3^-\)-free saline; pH 7.00), recta maintained constant \(J_{\text{Amm}}\) for at least 8h (0.65 ± 0.08 \(\mu\text{moles}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}; n = 6\)). Likewise, preparations with luminal perfusion stopped for 3 h yielded constant \(J_{\text{Amm}}\) (as determined by sequential sampling every 15 min) over the entire experimental period (0.60 ± 0.10 \(\mu\text{moles}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}; n = 6\)). This is clearly an extremely viable preparation capable of maintaining stable rates of ammonia secretion over long periods of time. Over the course of the 3h period, ammonia concentrations typically rose from < 20 to 200-250 \(\mu\text{M}\), indicating that \(J_{\text{Amm}}\) is not affected by changes in luminal ammonia concentrations of at least 250 \(\mu\text{M}\). Since all remaining experiments utilized only 60 min sampling periods and luminal ammonia concentrations never rose above 100 \(\mu\text{M}\) in that time, this method of flux measurement should enable accurate detection of even small changes in \(J_{\text{Amm}}\).

The large luminal secretion rate in the absence of exogenous ammonia suggests that the ammonia is generated intracellularly rather than transported transepithelially. With consideration to the role of amino acids in ammoniagenesis in both vertebrate kidney (Good and Knepper, 1985) and \textit{S. bullata} hindgut (Prusch, 1972), amino acid metabolism was considered a likely source of the secreted ammonia in the locust rectum. Reduction of \(J_{\text{Amm}}\) to near zero with bilateral removal of exogenous amino acids (Table 3.1) supports this hypothesis and rules out a significant contribution from intracellular ammonia precursors.

Salines containing the full complement of amino acids typically had background ammonia concentrations 10-15 \(\mu\text{M}\) higher than amino acid-free salines. When the amino acid-free experiments were repeated in the presence of 20 \(\mu\text{M}\) \((\text{NH}_4)_2\text{SO}_4\), \(J_{\text{Amm}}\) did not change significantly (Table 3.1). This indicates that the increase in \(J_{\text{Amm}}\) accompanying amino acid addition is due to the amino acids per se and not the concomitant increase in background ammonia levels.
Table 3.1 *Amino acid precursors of luminal ammonia secretion.*

<table>
<thead>
<tr>
<th>Ammonia Substrate</th>
<th>Bilateral Concentration (mM)</th>
<th>Ammonia Secretion Rate (μmoles/cm² h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Full saline complement</td>
<td>*</td>
<td>0.62 ± 0.08 †</td>
</tr>
<tr>
<td>Glutamine</td>
<td>5.0</td>
<td>0.27 ± 0.04</td>
</tr>
<tr>
<td>Proline</td>
<td>13.1</td>
<td>0.21 ± 0.05</td>
</tr>
<tr>
<td>Alanine</td>
<td>2.9</td>
<td>0.18 ± 0.06</td>
</tr>
<tr>
<td>Serine</td>
<td>1.5</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Glycine</td>
<td>11.4</td>
<td>0.04 ± 0.01 ‡</td>
</tr>
<tr>
<td>Glu, Pro, Ala, Ser</td>
<td>a</td>
<td>0.58 ± 0.10 †</td>
</tr>
<tr>
<td>Amino acid free</td>
<td>0</td>
<td>0.03 ± 0.01 ‡</td>
</tr>
<tr>
<td>Amino acid free + 20 μM ammonium sulphate</td>
<td>0</td>
<td>0.05 ± 0.01 ‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n=6 recta for each value. Amino acids replaced with isosmotic concentrations of sucrose. Bilateral pH=7.00. * see text for composition and concentrations. †,‡ values marked with common symbols are not significantly different by Student’s t-test (P>0.50 and P>0.20 respectively). a Glu, glutamine (5.0 mM); Pro, proline (13.1 mM); Ala, alanine (2.9 mM); Ser, serine (1.5 mM).
A series of substrate replacement experiments was conducted to determine which amino acids were capable of supporting $J_{\text{Amm}}$. Recta were brought to steady state with the full complement of saline amino acids listed above and then exposed bilaterally to salines containing only a single amino acid (Table 3.1). The five amino acids tested in this study were selected on the basis of their high concentrations in haemolymph and hindgut fluid (Phillips et al., 1986). Glutamine, proline, alanine, and serine were each capable of supporting significant fractions of control $J_{\text{Amm}}$. However, consistent with earlier studies by Balshin and Phillips (1971), glycine was incapable of supporting significant rates of $J_{\text{Amm}}$. A saline containing only glutamine, proline, serine, and alanine sustained secretion rates not significantly different from control values of $J_{\text{Amm}}$ ($P > 0.50$; Table 3.1), confirming that these amino acids were the principal precursors of the secreted ammonia.

There is considerable evidence for active luminal uptake of neutral amino acids in the locust rectum, and it has been shown that luminal proline is the principal metabolic substrate for electrogenic Cl$^{-}$ transport in this tissue (Balshin and Phillips, 1971; Chamberlin and Phillips, 1982). It is not surprising therefore that luminal amino acids alone (full amino acid complement) were capable of supporting control values of $J_{\text{Amm}}$ (Fig. 3.2). The low ammonia secretion rate supported by contraluminal amino acids alone (full amino acid complement) suggests that there might be a small component of basolateral substrate uptake but that it is of much less importance than the apical route.

**Effect of pH and $J_{\text{H}^+}$ on ammonia secretion.** As defined by Fick's law, bulk diffusion of the uncharged NH$_3$ molecule can only occur if there is a transmembrane concentration gradient to provide the driving force. Such a gradient may be established by unilateral addition or removal of exogenous ammonia or unilateral pH changes (diffusion trapping hypothesis; Pitts, 1973). The diffusion trapping hypothesis predicts that, once diffusion equilibrium is attained (ie. no transmembrane concentration gradient for NH$_3$), NH$_4^+$ will be passively distributed across the membrane in proportion to the pH gradient.
Figure 3.2 Effect of amino acid source on luminal ammonia secretion rates ($J_{\text{Amm}}$). Bilateral pH=7.00. Values are means ± SE; $n=6$ recta for each value.
This implies that the magnitude and direction of $J_{\text{Amm}}$ should vary directly with changes in transmembrane pH gradients and $J_{H^+}$ if ammonia crosses the apical membrane as NH$_3$ rather than NH$_4^+$. Moreover, if ammonia is transported as NH$_3$ rather than NH$_4^+$, $J_{\text{Amm}}$ will not be directly affected by membrane potentials, ion substitutions, or specific transport inhibitors.

When recta were exposed to transepithelial pH gradients, $J_{H^+}$ decreased dramatically, but $J_{\text{Amm}}$ remained relatively unaffected (Fig. 3.3). To ensure that the imposed transepithelial pH gradient was reflected by a similar pH gradient across the apical membrane, pH$_i$ was measured over the range tested above (Fig. 3.4). pH$_i$ did not decrease from control values (7.38 ± 0.03) until luminal pH was less than 6.00; therefore, changes in luminal pH were a direct indication of the relative magnitude of the apical transmembrane pH gradient above this pH value. The fact that $J_{\text{Amm}}$ did not vary with either $J_{H^+}$ or the transepithelial pH gradient strongly suggests that the bulk of ammonia crosses the apical membrane as NH$_4^+$ rather than NH$_3$.

It could be argued that a pH gradient sufficient to drive NH$_3$ diffusion might exist in the unstirred layers next to the apical membrane without necessarily being detected in the bulk solution. This possibility was addressed by altering the buffering capacity of the saline bathing the tissue; in this case, by increasing the concentration of MOPS from 2 to 10 mM (Table 3.2). A similar maneuver was effectively used in the isolated turtle bladder to reduce the extent of a pH disequilibrium driving transepithelial NH$_3$ diffusion (Schwartz and Tripolone, 1983). The fact that this manipulation had no effect on $J_{\text{Amm}}$ in the locust rectum is again consistent with NH$_4^+$ transport rather than NH$_3$ diffusion.

If the relative permeabilities of the apical and basolateral membranes to NH$_3$ are the same (or at least similar), the direction of NH$_3$ diffusion should be determined by the direction of the transmembrane pH gradient. Likewise, if the pH gradient is equal across both membranes, the rate of luminal $J_{\text{Amm}}$ should equal the rate of contraluminal $J_{\text{Amm}}$. Neither of these predictions was borne out by the locust rectum. When apical and basolateral
Figure 3.3 Effect of transepithelial pH gradients on luminal ammonia secretion rates ($J_{\text{Amn}}$; closed circles) and rates of luminal acidification ($J_{\text{H}^+}$; open circles). Contraluminal pH maintained at 7.00 by continuous perfusion; luminal pH maintained at respective pH values by pH-stat. Transepithelial potential clamped at 0 mV. Values are means ± SE; n=6 for each value.
Figure 3.4 Effect of transepithelial pH gradients on intracellular membrane potentials (top), transepithelial potential difference (Vt; middle; open circles), and intracellular pH (pHi; bottom; closed circles). Va and Vb, apical (open triangles) and basolateral (closed triangles) membrane potentials (relative to luminal and contraluminal baths respectively). Vt, lumen relative to contraluminal bath. Contraluminal pH maintained at 7.00 by continuous perfusion; luminal pH maintained at respective pH values by continuous perfusion. All measurements made under open circuit conditions. Values are means ± SE; n=6 recta and 18 cells for each value.
Table 3.2 *Effect of saline buffer concentration on luminal ammonia secretion rates.*

<table>
<thead>
<tr>
<th>Concentration of MOPS Buffer (mM)</th>
<th>Ammonia Secretion Rate (µmoles·cm⁻²·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>0.60 ± 0.06 †</td>
</tr>
<tr>
<td>6</td>
<td>0.62 ± 0.08 †</td>
</tr>
<tr>
<td>10</td>
<td>0.64 ± 0.12 †</td>
</tr>
</tbody>
</table>

MOPS, 3-(N-morpholino)propanesulfonic acid. Values are means ± SE; *n* = 6 recta for each value. Bilateral pH = 7.00. † values marked with common symbols not significantly different by Student’s *t*-test (*P* > 0.50).
pH gradients were equal there was a large asymmetry between luminal and contraluminal $J_{\text{Amm}}$ (Table 3.3). Reversal of the transepithelial pH gradient only brought about a slight change in the magnitude of luminal $J_{\text{Amm}}$ (Table 3.3; not significant; $P > 0.50$). Once again the data suggests that the bulk of net ammonia movement occurs as NH$_4^+$ transport rather than NH$_3$ diffusion.

**Mechanism of ammonia transport.** The previous results clearly demonstrate that net $J_{\text{Amm}}$ must occur by some mechanism other than passive NH$_3$ diffusion. With this in mind, a series of bilateral ion substitution experiments was conducted to determine the ionic dependency of $J_{\text{Amm}}$ (Fig. 3.5). Recta were mounted in standard saline (full ion and amino acid complement) and then perfused bilaterally with the specific ion-free saline for 3 h. By use of this protocol, it was found (unpublished observations) that background saline concentrations of omitted ions (always < 50 μM) did not rise significantly over the 60 min sampling period following cessation of mucosal perfusion. Na$^+$ removal reduced $J_{\text{Amm}}$ by 63%, Cl$^-$ removal increased $J_{\text{Amm}}$ by 30%, and removal of K$^+$ had no significant effect ($P > 0.05$).

The inhibition of $J_{\text{Amm}}$ by Na$^+$ removal strongly suggests the presence of an apical Na$^+$/NH$_4^+$ exchange mechanism. However, the effect of Na$^+$ removal might not be on NH$_4^+$ exit per se, but rather on amino acid uptake from the lumen and subsequent de novo synthesis of ammonia. This possibility was investigated by adding 1 mM amiloride, a putative inhibitor of Na$^+$/NH$_4^+$ exchange in other cell types (Evans and Cameron, 1986), to the lumen in the presence of either 110 or 20 mM Na$^+$ (Table 3.4). Under these conditions amino acids should still be able to enter the cell to fuel ammonia synthesis. Black et al. (1987) have shown that amiloride is capable of reaching Na$^+$ transport sites on the apical membrane of the locust rectum without first cutting the cuticular intima covering the apical surface of the epithelium. In agreement with that study, I found that cutting the cuticle had no significant effect on the efficacy of amiloride action on luminal ammonia secretion ($n = 9$; data not
Table 3.3  *Effect of reversing transepithelial pH gradients on luminal and contraluminal ammonia secretion rates.*

<table>
<thead>
<tr>
<th>Luminal pH</th>
<th>Contraluminal pH</th>
<th>Luminal $J_{\text{Am}}$ (μmoles/cm²·h⁻¹)</th>
<th>Contraluminal $J_{\text{Am}}$ (μmoles/cm²·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.00</td>
<td>7.00</td>
<td>0.62 ± 0.06</td>
<td>0.02 ± 0.01</td>
</tr>
<tr>
<td>7.00</td>
<td>6.50</td>
<td>0.70 ± 0.06 †</td>
<td>0.02 ± 0.01 ‡</td>
</tr>
<tr>
<td>6.50</td>
<td>7.00</td>
<td>0.67 ± 0.05 †</td>
<td>0.03 ± 0.01 ‡</td>
</tr>
</tbody>
</table>

Luminal $J_{\text{Am}}$, rate of ammonia accumulation in the luminal bath. Contraluminal $J_{\text{Am}}$, rate of ammonia accumulation in the contraluminal bath. Values are means ± SE; $n=6$ recta for each value. †, ‡ not significantly different from respective controls by Student’s $t$-test ($P>0.50$ and $P>0.20$ respectively).
Figure 3.5 Effect of bilateral ion substitution on luminal ammonia secretion rates ($J_{\text{AMM}}$). Bilateral pH=7.00. Values are means ± SE; $n=6$ recta for each value. a,c significantly different from control by Student’s $t$-test ($P<0.005$ and $P<0.05$ respectively). b not significantly different from control by Student’s $t$-test ($P>0.20$).
Table 3.4 Effect of amiloride on luminal ammonia secretion rates under short-circuit current conditions.

<table>
<thead>
<tr>
<th></th>
<th>$J_{\text{Amm}}$ (μmoles cm$^{-2}$ h$^{-1}$)</th>
<th>Vt (mV)</th>
<th>Isc (μequiv cm$^{-2}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>110 mM Na$^+$ saline †</td>
<td>0.54 ± 0.05 $^a$</td>
<td>6.5 ± 0.8</td>
<td>0.98 ± 0.16</td>
</tr>
<tr>
<td>+ 1 mM luminal Amiloride</td>
<td>0.23 ± 0.03 $^a$</td>
<td>7.2 ± 1.2</td>
<td>1.01 ± 0.12</td>
</tr>
<tr>
<td>20 mM Na$^+$ saline ‡</td>
<td>0.59 ± 0.06 $^b$</td>
<td>4.7 ± 1.0</td>
<td>1.05 ± 0.11</td>
</tr>
<tr>
<td>+ 1 mM luminal Amiloride</td>
<td>0.21 ± 0.05 $^b$</td>
<td>5.1 ± 1.6</td>
<td>1.11 ± 0.09</td>
</tr>
</tbody>
</table>

$J_{\text{Amm}}$, rate of luminal ammonia secretion. Vt, transepithelial potential (lumen relative to haemocoel). Isc, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). † tissues were brought to steady-state in sulphate- and CO$_2$/HCO$_3^-$-free saline with an otherwise full ion complement (including 110 mM Na$^+$; see Materials and Methods). ‡ tissues were brought to steady-state in the same saline used in the previous trial except that bilateral Na$^+$ concentrations were 20 mM rather than 110 mM (the Cl$^-$ deficit was made up with choline chloride). Bilateral pH=7.00. Vt was measured at the end of each experimental period. Values are mean ± SE; n=6 for each treatment. $^a,b$ values with common symbols are significantly different by paired t-test ($P<0.001$).
shown). The data presented in Table 3.4 are taken from experiments performed without the cuticle previously cut. At both Na\(^+\) concentrations studied, amiloride inhibited \(J_{\text{Amm}}\) by 55-65%, supporting the hypothesis that a significant fraction of the transported ammonia crosses the apical membrane by Na\(^+\)/NH\(_4^+\) exchange.

The component of ammonia efflux remaining after amiloride addition or Na\(^+\) removal suggests that there might be other pathways involved in luminal ammonia secretion. Movement of \(J_{\text{Amm}}\) through K\(^+\) channels is reported in other cell types (eg. frog skin; Zeiske and Van Driessche, 1983), and it has been shown that there is an apical K\(^+\) conductance in this tissue that can be increased approximately five-fold with contraluminal applications of cAMP (Hanrahan and Phillips, 1984a). If NH\(_4^+\) crosses the apical membrane of the locust rectum by this route, contraluminal cAMP should increase \(J_{\text{Amm}}\) significantly. However, this treatment had no significant effect on \(J_{\text{Amm}}\) under any of the conditions studied (Table 3.5). Furthermore, if NH\(_4^+\) exit was via an undisclosed channel of some sort, \(J_{\text{Amm}}\) should be directly affected by changes in apical membrane potential. Again, this was not the case. The changes in \(V_a\) accompanying both cAMP stimulation (-11 mV; unpublished observations and Hanrahan and Phillips, 1984b) and the imposition of a transepithelial pH gradient (+17 mV; Fig. 3.4) had no effect on \(J_{\text{Amm}}\). These observations suggest that NH\(_4^+\) exit across the apical membrane is neither electrogenic nor via apical K\(^+\) channels.

**DISCUSSION**

**Characterization of ammonia production and secretion.** In the absence of exogenous ammonia, rectal epithelial cells secrete metabolically derived ammonia at high rates preferentially into the lumen. Moreover, specific amino acids must be present in the bathing solutions (primarily the luminal) to support the intracellular synthesis of the secreted ammonia.
Table 3.5 *Effect of contraluminal cAMP on luminal ammonia secretion rates under short-circuit current conditions.*

<table>
<thead>
<tr>
<th></th>
<th>$J_{Amn}$ (μmoles/cm$^2$-h$^{-1}$)</th>
<th>$V_t$ (mV)</th>
<th>$I_{sc}$ (μequiv/cm$^2$-h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control saline</td>
<td>0.61 ± 0.08 a</td>
<td>8.2 ± 0.9 †</td>
<td>1.10 ± 0.15 ‡</td>
</tr>
<tr>
<td>+ 1 mM luminal cAMP</td>
<td>0.57 ± 0.09 a</td>
<td>43.7 ± 2.1 †</td>
<td>9.34 ± 0.61 ‡</td>
</tr>
</tbody>
</table>

$J_{Amn}$, rate of luminal ammonia secretion. $V_t$, transepithelial potential (lumen relative to haemocoel). $I_{sc}$, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). Bilateral pH=7.00. $V_t$ was measured at the end of each experimental period. Values are mean ± SE; $n=6$ for each treatment. a not significantly different by paired $t$-test ($P>0.50$). †, ‡ values marked with common symbols are significantly different by paired $t$-test ($P<0.001$).
The observed $J_{\text{Amm}}$ (0.58 ± 0.12 μmoles-cm$^{-2}$-h$^{-1}$; Fig. 3.1) is significantly higher than values reported by Chamberlin (1981; 0.05-0.08 μmoles-cm$^{-2}$-h$^{-1}$) but not that dissimilar to whole-animal values calculated from Mullins for *Periplaneta americana* (Mullins, 1974; 0.22 μmoles-cm$^{-2}$-h$^{-1}$). The low values reported by Chamberlin might be due to amino acid interference with the salicylate-based ammonia assay used in that study. I found that moderate concentrations of exogenous amino acids significantly reduced the reliability of similar salicylate-based ammonia assays (data not shown) and for that reason chose the enzymatic assay used in this study.

In mammalian systems the bulk of urinary ammonia is derived from the metabolism of specific amino acids in renal tubule epithelial cells (Pitts, 1973). The principal pathway for ammonia synthesis in this system involves hydrolytic deamidation of glutamine (via glutaminase 1) to L-glutamate and ammonia and subsequent oxidative deamination of glutamate (via L-glutamate dehydrogenase) to α-ketoglutarate and ammonia. Although glutamate is the major source of synthesized renal ammonia, other amino acids can provide significant quantities of ammonia if they are present in high enough concentrations (Shalhoub *et al.*, 1963). These amino acids are utilized by transaminating α-ketoglutarate to form L-glutamate. The glutamate is then oxidized by glutamate dehydrogenase as above to form α-ketoglutarate and ammonia.

Insects are capable of producing ammonia by essentially the same pathways. The enzymes required for transamination to glutamate and glutamate dehydrogenase have been identified in a wide variety of insect tissues for several amino acids (Cochran, 1975). The predominant metabolic route for proline oxidation in the locust rectum has been shown to involve the formation of glutamate and subsequent oxidative deamination (via L-glutamate dehydrogenase) to ammonia and α-ketoglutarate (Chamberlin and Phillips, 1983). If this

---

2. By use of a conversion factor of 1.5 μmoles-cm$^{-2}$-h$^{-1}$ = 1 μmoles-rectum$^{-1}$-h$^{-1}$ (Phillips *et al.*, 1986), whole-rectum values of ammonia secretion for the locust would be 0.39 μmol·h$^{-1}$. 

pathway exists for proline, other amino acids may be metabolized in the locust rectum in a similar fashion.

Luminal amino acids were required to maintained high rates of $J_{A_{\text{mm}}}$ in the locust rectum under the present experimental conditions (Table 3.1); therefore it is unlikely that other nitrogenous compounds (such as tissue urea or intracellular proteins) could be acting as ammonia precursors. The residual component of $J_{A_{\text{mm}}}$ remaining after amino acid removal (~5% of the control value) could be the result of transepithelial transport of trace levels of background ammonia present in the saline (< 10 μM). Preliminary investigations have indicated that the rectum may be capable of significant rates of transepithelial ammonia transport in the absence of amino acids if exogenous ammonia levels are raised > 500 μM (data not shown). Alternatively the residual component of $J_{A_{\text{mm}}}$ could be the result of metabolism of endogenous amino acid stores. This is quite likely, since it takes > 6 h to completely deplete recta of endogenous metabolic substrates (Chamberlin, 1981).

Unlike mammalian proximal tubules, a single amino acid does not support the bulk of $J_{A_{\text{mm}}}$ in the locust rectum. Glutamine, proline, alanine, and serine are each capable of sustaining significant rates of $J_{A_{\text{mm}}}$ (Table 3.1). Given that proline constitutes 80% of the total amino acids entering the rectum in vivo (Phillips et al., 1986), it is probable that it is the major natural source of secreted ammonia. It is interesting to note that, in experiments with only one amino acid removed at a time (data not shown), $J_{A_{\text{mm}}}$ did not change significantly, suggesting that rectal cells are capable of metabolizing other amino acids to a greater degree when one of the primary substrates is removed.

**Nature of secreted ammonia.** The rate of ammonia transport in the locust rectum is independent of transmembrane pH gradients and luminal proton secretion and is directly affected by Na+ substitution and amiloride addition. Therefore, by the criteria outlined above, luminal $J_{A_{\text{mm}}}$ in the locust rectum must be occurring primarily as NH$_4^+$ transport rather than NH$_3$ diffusion.
In rat and rabbit cortical-collecting ducts net NH$_3$ diffusion was demonstrated in the absence of both pH and ammonia gradients (Knepper et al., 1984, 1985). In both epithelia an acid pH disequilibrium in the unstirred layer provided the driving force for NH$_3$ movement. This is unlikely to be the case in the locust rectum for several reasons. Increased saline buffering capacity (Table 3.2) and reduced rates of $J_{\text{H}^+}$ (Fig. 3.3) had no significant effects on luminal ammonia secretion; both maneuvers should have reduced the extent of a pH disequilibrium and decreased the driving force for NH$_3$ diffusion. Moreover, the significance of a pH disequilibrium in the unstirred layer is questionable when one considers that large changes in the imposed pH gradient had no effect on $J_{\text{Amm}}$ (Fig. 3.3). Finally, $J_{\text{Amm}}$ was strongly inhibited by Na$^+$ removal and addition of amiloride, whereas $J_{\text{H}^+}$ (and hence the pH of the unstirred layer) was not greatly affected by these treatments (see Chapter Four).

A further possibility is that apparent $J_{\text{Amm}}$ occurs as a result of deamination of luminal amino acids by membrane-bound enzymes (eg. $\gamma$-glutamyltransferase has been found in the apical membrane of this tissue; Lechleitner, personal communication). This would explain the preferential addition of ammonia to the lumen, the sidedness of amino acid support of ammonia synthesis, and possibly the lack of a pH effect on $J_{\text{Amm}}$. However, this hypothesis cannot explain the inhibitory effect of Na$^+$ removal (Fig. 3.5) or amiloride addition (Table 3.4) on $J_{\text{Amm}}$. Nor is there any other evidence to support significant rates of amino acid deamination in the lumen per se. Uptake studies with [$^{14}$C]proline (Meredith, personal communication) have shown that the isotope is largely accumulated without alteration.

Ammonia secretion via NH$_4^+$ transport was somewhat unexpected considering that most acid secreting urinary epithelia studied to date rely primarily on diffusion trapping of NH$_3$ (eg. Knepper et al., 1984,1985; Pitts, 1973; Schwartz and Tripolone, 1983). There are, however, several epithelial cell types that do support significant rates of $J_{\text{Amm}}$ as NH$_4^+$ (see above). Most pertinent of these is the isolated hindgut of larval S. bullata; this tissue is
capable of secreting NH$_4^+$ against large concentration gradients and against what would be considered an unfavourable pH gradient for NH$_3$ diffusion (ie. lumen alkaline; Prusch, 1972).

**Mechanism of NH$_4^+$ transport.** The effect of bilateral Na$^+$ substitution (Fig. 3.5) on $J_{\text{Amm}}$ suggests that NH$_4^+$ enters the lumen via an apical Na$^+$/NH$_4^+$ exchange mechanism. On this basis alone it is difficult to distinguish between a possible effect of Na$^+$ removal on amino acid uptake or NH$_4^+$ efflux. Na$^+$-coupled amino acid uptake mechanisms are known to occur in a number of different cell types (Christensen, 1984), and a significant fraction of glycine uptake in the locust rectum is Na$^+$ dependent (Balshin and Phillips, 1971). However, Chamberlin and Phillips (Chamberlin and Phillips, 1982) and Meredith and Phillips (1988) have found very little evidence to support Na$^+$-dependent proline uptake in the locust rectum. It is not known how other amino acids cross the apical membrane in this tissue, but it is possible that they are also reabsorbed by Na$^+$-independent mechanisms. If this is the case, then Na$^+$ removal should have very little effect on amino acid uptake.

The strongest evidence for a direct link between luminal Na$^+$ and apical ammonia efflux is the comparable degree of inhibition of $J_{\text{Amm}}$ obtained with luminal amiloride (60%; Table 3.4) or bilateral Na$^+$ removal (63%; Fig. 3.5). A similar amiloride-inhibitable mechanism has been postulated to occur in renal microvillus membrane vesicles (Aronson and Igarashi, 1986). It is possible that the observed effect is a result of the high concentrations of amiloride used in this study. Millimolar concentrations of amiloride have been shown to inhibit renal Na$^+$/K$^+$-ATPase activity by up to 50% in some tissues and have been implicated in a number of other unspecific effects (Soltoff *et al.*, 1986; Soltoff and Mandel, 1983). There is no evidence to suggest that this concentration of amiloride elicits similar unspecific responses in the locust rectum. I have not observed any effects (neither long nor short term) on cAMP-stimulated Isc (ie. electrogenic chloride transport) or unstimulated Isc or Vt and only marginal effects on luminal proton secretion (10-20%; see Chapter Four). All these rectal transport processes are extremely sensitive to changes in
aerobic respiration (Phillips et al., 1986); it is unlikely, therefore, that this level of amiloride had a pronounced effect on general rectal metabolism. As well, addition of amiloride at this concentration had virtually the same effect on $J_{Amm}$ as bilateral Na$^+$ removal (Fig. 3.5 and Table 3.4). Nagami and Kurokawa (1985) found that 1 mM amiloride at this concentration had no unspecific effects on either amino acid uptake or subsequent metabolism. Similarly, millimolar concentrations of amiloride have been shown to have no significant effects on normal renal oxidative metabolism (Soltoff et al., 1986). In light of these observations, it is reasonable to conclude that the amiloride effect is most likely due to specific interaction with an apical Na$^+$/NH$_4^+$ exchange mechanism rather than the result of interactions at unspecific transport sites or interference with amino acid uptake or subsequent ammoniagenesis.

It is interesting to note that a significant unidentified fraction of $J_{Amm}$ remained after both Na$^+$ removal and amiloride addition. NH$_4^+$ substitution for K$^+$ on the Na$^+$/K$^+$-ATPase has been demonstrated in a number of different cell types (e.g. Aickin and Thomas, 1977; Claiborne et al., 1982; Kurtz and Balaban, 1986), but this possibility was not pursued in this investigation. The orientation and apical location necessary to explain NH$_4^+$ secretion make Na$^+$/K$^+$-ATPase involvement highly improbable. As well, the lack of effect of cAMP (Table 3.5) and insensitivity to changes in Va rule out a significant component of electrogenic NH$_4^+$ efflux. It is entirely possible that the remaining component is due solely to passive NH$_3$ diffusion and is a result of inhibition of the normal ammonia efflux pathway. With the Na$^+$/NH$_4^+$ exchange mechanism blocked, intracellular ammonia levels might rise (due to continuing amino acid metabolism) to levels high enough to set up a significant NH$_3$ diffusion gradient.

I have no clear explanation for the stimulatory effect of Cl$^-$ removal on $J_{Amm}$ in this tissue (Fig. 3.5). One possibility is that bilateral Cl$^-$ removal blocks basolateral HCO$_3^-$ exit (see Chapter 4) and causes pH$i$ to rise. This might increase the NH$_3$-to-NH$_4^+$ ratio in the cell high enough to increase passive NH$_3$ efflux (via diffusion trapping) and accelerate $J_{Amm}$. 
Alternatively, Cl⁻ removal might somehow increase amino acid uptake or metabolism and subsequent ammonia production within the cell.

Ammonia secretion rates are routinely added to titratable acidity to obtain corrected values of hydrogen ion secretion in many acid-base transporting epithelia. This correction is frequently made without prior knowledge of the transported form of ammonia and is based on the observation that NH₃ addition reduces apparent titratable acidity to the extent that it no longer accurately estimates proton secretion. The applicability of this correction factor depends on the magnitude of $J_{\text{Amm}}$ and the proportion of total ammonia secreted as NH₃.³

Clearly, addition of net $J_{\text{Amm}}$ to $J_{\text{H}^+}$ in the locust rectum would result in very significant estimation errors of luminal proton secretion.

**Summary**

The rectum of the desert locust secretes NH₄⁺ at a substantial rate preferentially into the lumen in the absence of exogenous ammonia. Luminal amino acids are the primary source of the secreted ammonia under these conditions, and $J_{\text{Amm}}$ occurs primarily as NH₄⁺ transport via an apical Na⁺/NH₄⁺ exchange mechanism.

This study suggests that ammonia may be a significant end product of nitrogen metabolism in the locust and that the rectum is the major site of $J_{\text{Amm}}$. Given the similar findings in the cockroach (11), it is perhaps time for reevaluation of the traditional view of strict uricotelism in terrestrial insects. It is difficult to say whether rectal $J_{\text{Amm}}$ plays a role in whole-animal acid/base balance in the locust. Certainly it cannot act as a luminal proton trap as it does in the vertebrate kidney, since it is already largely in the protonated form when it crosses the apical membrane. Since Na⁺ is often in short supply in the typical herbivore diet, rectal reabsorption of Na⁺ in exchange for metabolically produce NH₄⁺ may play an important role in maintaining the overall cation balance in the locust.

³ This correction may be appropriate for NH₄⁺ movement if a measure of net acid secretion rather than direct proton secretion *per se* is desired.
CHAPTER FOUR: Characterization of Rectal Acid Secretion

INTRODUCTION

The data presented in Chapter Two confirm that the rectal epithelial cells *per se* are capable of actively maintaining the low luminal pH values observed *in vivo*. Measurements of intracellular pH and apical and basolateral membrane potentials clearly show that pH_i is not in equilibrium with either luminal or contraluminal pH and that the active mechanism responsible for luminal acidification must reside on the apical membrane. The persistence of luminal acidification in the nominal absence of exogenous CO_2 and HCO_3^- , and the lack of correlation between J_H^+ and trace levels of luminal HCO_3^- , indicate that rectal acidification must be due to active proton secretion rather than bicarbonate reabsorption. The present chapter further characterizes the nature of the luminal acidification observed under CO_2/HCO_3^- -free conditions and identifies the cellular mechanisms responsible for active proton translocation across the apical membrane.

As outlined in Chapter One (General Introduction), active proton translocation may be accomplished either by coupling net proton movements to energetically favourable movements of other ions (eg. Na^+/H^+ exchange) or by directly utilizing energy derived from cellular metabolic events (eg. H^+/K^+ ATPase). Measurements of apical electrochemical gradients for Na^+, K^+, and Cl^- in the locust rectum (Hanrahan and Phillips, 1984b) reveal that only the Na^+ gradient has enough potential energy to drive active proton secretion under the conditions used in the present study (ie. 110 mM NaCl, 10 mM K^+, and bilateral pH=7.00). In the intact animal, levels of Na^+ entering the rectum are often less than 20 mM (Phillips, 1981) and luminal pH values range from 4.5 to 6.5 (Speight, 1967). It is doubtful if the apical electrochemical gradient for Na^+ present under these conditions could maintain the rates of rectal acidification reported by Phillips (1961) and Speight (1967). Since net acid secretion was maintained in the *in vitro* preparation (see Chapter Three; Fig. 3.3) against
transepithelial pH gradients similar to those observed *in vivo*, it is reasonable to propose that rectal acidification occurs by the same mechanism under both conditions. If this is true, the bulk of net proton secretion in the locust rectum clearly must occur by a primary active transport mechanism rather than a secondary coupling to spontaneous fluxes of other ions.

Despite the fact that active proton secretion is probably not driven by electrochemical gradients for Na\(^+\), K\(^+\) or Cl\(^-\), the possibility remains that the apical proton pump may have a direct requirement for other ions in a manner similar to that proposed for acid secretion in the gastric mucosa (reviewed by Forte and Machen, 1987) or electrogenic Cl\(^-\) uptake in the locust rectum (reviewed by Phillips *et al.*, 1986).

The data presented in this chapter confirms that the bulk of net acid secretion in the locust rectum occurs by a primary electrogenic proton translocating mechanism located on the apical membrane. Under control conditions (ie. bilateral pH=7.00), 10%-20% of net acid secretion could be attributed to amiloride-inhibitable Na\(^+\)/H\(^+\) exchange. Contrary to what might be expected for an acid-secretory epithelia, the apical membrane also appears to possess a significant proton diffusional pathway in parallel with the active proton transport mechanisms.

**MATERIALS AND METHODS**

Experimental chambers, tissue dissection, microelectrode methods, and measurement of \(J_{H^+}\), \(V_t\), \(I_{sc}\), and \(R_t\) were all as described in Chapter Two.

**Solutions.** The composition of the standard CO\(_2\)/HCO\(_3\)-free saline used throughout this chapter (ie. the "control saline") is identical to that outlined in Chapter Two. Na\(^+\), Cl\(^-\), and K\(^+\)-free salines were prepared as described in Chapter Three. Background levels of Na\(^+\) and K\(^+\) in the respective ion-substituted salines were measured by atomic absorption spectrophotometry (Techtron AA 120; Varian Ltd., Melbourne, Australia). Background Cl\(^-\) levels were measured by the titrimetric method described by Ramsay *et al.*, 1955.
In all ion-substitution experiments, the composition of agar bridges reflected the salt composition of the respective bathing salines to maintain the nominally Na\(^{+}\), K\(^{+}\), or Cl\(^{-}\)-free state. During Na\(^{+}\)-substitution experiments, the pH-stat titrant was changed from 0.01N NaOH to 0.01N KOH. Luminal K\(^{+}\) levels rose by less than 0.04mM during the 15 min pH-stat measurement period and appeared to have no significant effect on rectal transport processes as judged by stable Vt and Isc values.

Amiloride containing solutions were prepared as described in Chapter Three. Omeprazole, 4-acetamido-4'-isothiocyanatostilbene-2,2'-disulfonic acid (SITS), and 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS) were presolubilized in dimethyl sulphoxide (DMSO) immediately before use. The volume of DMSO (+ inhibitor) added to experimental salines was always <1% of the final saline volume. Similar volumes of DMSO were added to control salines to ensure that DMSO alone was not affecting \(J_{H^{+}}\), Vt, or Isc. Salines containing SITS and DIDS were amino acid-free (replaced with sucrose) as suggested by Strange and Phillips (1984). Acetazolamide and vanadate were dissolved directly in the standard CO\(_2\)/HCO\(_3\)\(^{-}\)-free saline used throughout the study. Omeprazole was a generous gift from Hässle, Mölndal, Sweden. SITS, DIDS, vanadate and acetazolamide were obtained from Sigma Chemical (St. Louis, MO).

RESULTS

Effect of transepithelial electrochemical gradients on rectal acid secretion

Measurements of \(J_{H^{+}}\) have thus far been largely confined to conditions where both luminal and contraluminal pH have been set to 7.00. Although this arrangement is essential for obtaining accurate measurements of rectal acid secretion under true short-circuit current conditions (ie. no transepithelial electrochemical gradients), it does not necessarily reflect the conditions found in the intact animal. Phillips (1964) and Speight (1967) consistently
measured significant transepithelial pH gradients in vivo with rectal pH values often less than 5.0. To study the effect of similar pH gradients on rectal acid secretion in vitro (under CO₂/HCO₃⁻-free conditions), isolated recta mounted in Ussing chambers (see Materials and Methods, Chapter Two) were exposed to a series of luminal and contraluminal pH changes. To obviate the effects of associated transepithelial electrical gradients, experiments were conducted with Vt clamped at 0 mV, except for brief intervals to allow measurement of open-circuit transepithelial membrane potentials.

In the first series of experiments, luminal pH was maintained at 7.00 by pH-stat and contraluminal pH was varied from 5.00 to 7.50 by rapid perfusion (15 ml/min). This manipulation had no effect on J₅⁺, Vt, or I_app (the current required to clamp Vt=0 mV) over the entire range of contraluminal pH values tested (Fig. 4.1). This observation suggests that under these conditions, there is not a significant paracellular leak pathway for protons (or hydroxyl ions) in this epithelium. Moreover, it implies that either the basolateral border¹ is extremely impermeable to H⁺/OH⁻ or that pHi is regulated very closely by some unidentified mechanism located at the basolateral membrane.

In the second set of experiments, contraluminal pH was maintained at 7.00 by continuous perfusion and luminal pH was varied by a combination of rapid perfusion (15 ml/min) and pH-stat. J₅⁺ decreased linearly to zero as luminal pH was lowered from 7.00 to 5.25 (Fig. 4.2; the actual point at which J₅⁺ equaled zero is estimated at 5.27 by regression analysis; r²=0.9904). Further luminal pH reductions beyond 5.25 forced net acid movements in the opposite direction (ie. net acid absorption). Based on the observations in the previous set of experiments, it appears that acid influx must have been occurring across the apical membrane rather than through an unspecified paracellular route. It is not clear at this point, however, whether the acid influx was occurring through the apical proton transport

¹ The term "basolateral border" includes both the basolateral membrane and the adjacent secondary cell layer. Refer to Chapter One for details of the morphology of the epithelium.
Figure 4.1 Effect of contraluminal pH on $J_{H^+}$, $V_t$, and $I_{app}$. $J_{H^+}$, rate of rectal acidification. $V_t$, transepithelial potential (lumen relative to haemocoel). $I_{app}$, current required to clamp $V_t=0$ mV (a positive value indicates net cation movement into or anion movement out of the lumen). All tissues brought to steady-state in the standard CO$_2$/HCO$_3^-$-free saline with the full ion complement (see Materials and Methods). Luminal pH maintained at 7.00 by pH-stat; Contraluminal pH maintained at appropriate pH values by continuous perfusion. All experiments performed with $V_t$ clamped at 0 mV. Values are means ± SE; $n=6$ recta.
Figure 4.2 Effect of luminal pH on $J_{H^+}$, $V_t$, and $I_{app}$. $J_{H^+}$, rate of rectal acidification. $V_t$, transepithelial potential (lumen relative to haemocoel). $I_{app}$, current required to clamp $V_t=0$ mV (a positive value indicates net cation movement into or anion movement out of the lumen). All tissues brought to steady-state in the standard $\text{CO}_2/\text{HCO}_3^-$-free saline with the full ion complement (see Materials and Methods). Contraluminal pH maintained at 7.00 by continuous perfusion; luminal pH maintained at appropriate pH values by a combination of rapid perfusion and pH-stat. All experiments performed with $V_t$ clamped at 0 mV. Values are means ± SE; $n=6$ recta. Dashed line fit by regression analysis to facilitate determination of apparent PMF (least squares method; $r^2$, coefficient of determination).
mechanism *per se* (i.e. operating in reverse) or an unidentified parallel pathway across the apical membrane.

Under certain conditions it is possible to estimate the true force of an active ion pump from the transmembrane electrochemical gradient at which net flux equals zero. An accurate estimate requires a low parallel conductance for the ion in question and a reliable measurement of the transmembrane electrochemical gradient. Since there are no measurements of pH**i**, V_a, or parallel proton conductance for the conditions used in this portion of the study, it is difficult to assess how accurately an apparent proton motive force (PMF) calculated from transepithelial rather than transmembrane parameters reflects the true PMF of the proton pump on the apical membrane. Nevertheless, one could argue that a PMF based on net transport rates rather than actual pump rates *per se* is the more biologically relevant statistic of the two and is a better indicator of the proton transport capabilities of the epithelium as a whole. An apparent transepithelial PMF of 101 mV was calculated from a regression analysis of the data in Fig. 4.2 (see Chapter Two for details on calculation of electrochemical potentials).

When luminal pH was reduced from 7.00 to 5.00 in the previous set of experiments, V_t and I_app decreased concomitantly with J_{H^+} (Fig. 4.2), indicating that this maneuver was affecting net charge movement across the apical membrane. If the changes in V_t and I_app were at least partially due to proton conductive movements (in either direction), it should be possible to abolish net proton flux by increasing the opposing electrical gradient across the epithelium. This hypothesis was tested by voltage clamping recta at progressively greater transepithelial potentials (lumen positive) in the absence of a transepithelial pH gradient and measuring resultant rates of luminal acid secretion. Under these conditions, J_{H^+} decreased linearly to zero as the luminal bath was made progressively more positive (Fig. 4.3). A regression analysis of the data estimates the transepithelial potential at which J_{H^+}=0 (the apparent transepithelial PMF) to be 107 mV (r^2=-0.9973). As observed with the pH gradient
Figure 4.3 Effect of transepithelial membrane potential on net acid secretion. $J_{H^+}$, rate of rectal acidification. $V_{\text{CLAMP}}$, transepithelial potential set by voltage clamp (lumen relative to haemocoel). All tissues brought to steady-state in the standard CO$_2$/HCO$_3^-$-free saline with the full ion complement (see Materials and Methods). Contraluminal pH maintained at 7.00 by continuous perfusion; luminal pH maintained at 7.00 by pH-stat. Values are means ± SE; $n=6$ recta. Dashed line fit by regression analysis (least squares method; $r^2$, coefficient of determination).
$J_{H^+}$ (µequiv.cm$^{-2}$.h$^{-1}$) vs $V_{CLAMP}$ (mV)

$r^2 = -0.9973$
study above, when the electrical gradient was increased beyond the apparent PMF, net acid movement was forced to proceed in the opposite direction (ie. absorption). The modulating effect of the applied electrical gradients and the very good agreement between the apparent PMF values measured under the two different conditions (ie. electrical or chemical transepithelial gradients) demonstrates very clearly that there is a proton conductive pathway across the apical membrane. On the basis of these results, it is very tempting to speculate that the H⁺ conductive pathway and the active proton secretory mechanism are one and the same (ie. active electrogenic proton secretion). However, these results could be explained equally well by an electroneutral proton secretory mechanism in parallel with a proton conductive pathway in the opposite direction (eg. proton/organic cotransport or nonspecific leak pathways). Clearly, the ionic dependency of the apical proton secretory mechanism must be established before any conclusions can be drawn regarding the electrogenicity of this transporter.

Ionic requirements of rectal acid secretion

Sodium. Na⁺ is actively reabsorbed from the rectal lumen at rates of 1.5 to 2.5 μequiv cm⁻² h⁻¹ (Black et al., 1987). Biochemical studies with isolated membrane fractions (Lechleitner and Phillips, 1988) and electrophysiological measurements of intracellular Na⁺ activities and apical and basolateral membrane potentials (Hanrahan and Phillips, 1984b) clearly show that the active step for Na⁺ uptake is located at the basolateral border and that net Na⁺ reabsorption is driven by basolateral Na⁺/K⁺-ATPase activity. The mechanisms by which Na⁺ crosses the apical membrane are still largely unknown. Hanrahan and Phillips (1984b) have shown that under similar experimental conditions to those used in the present study (ie. bilateral 110 mM Na⁺) there is an electrochemical potential of 123 mV favouring passive Na⁺ uptake at the apical membrane. The magnitude of this electrochemical gradient conceivably represents a very significant energy source for driving active movements of
other ions and solutes across the apical membrane (via Na\(^+\) coupling). Na\(^+\)/NH\(_4\)^+ exchange (see Chapter Three) and Na\(^+\)/Glycine cotransport (Balshin and Phillips, 1971) are the only Na\(^+\) coupled mechanisms identified to date, and together account for less than 40% of net Na\(^+\) uptake. Electrophysiological measurements made in the absence of luminal organic substrates suggest that close to 50% of the Na\(^+\) crossing the apical membrane does so by an organic-independent electrogenic pathway (ie. not amino acid or glucose coupled; Black et al., 1987). Although an apical Na\(^+\) channel has been postulated, cotransport or exchange with other ions (apart from Cl\(^-\); Black et al., 1987) has by no means been ruled out.

To test the possibility that proton secretion might be coupled to transepithelial Na\(^+\) flux, recta were mounted in Ussing chambers and subjected to long-term bilateral Na\(^+\) removal. All experiments were performed under short-circuit current conditions with CO\(_2\)/HCO\(_3\)^-free salines (bilateral pH = 7.00). Long-term (4h) bilateral Na\(^+\) replacement under these conditions caused a significant decrease (40%) in net acid secretion (Table 4.1). The fact that such a large component of \(J_{H^+}\) remained after bilateral Na\(^+\) removal suggests several possibilities: 1) the apical proton pump has a very high affinity for Na\(^+\) and luminal Na\(^+\) was not reduced to low enough levels to completely abolish \(J_{H^+}\), 2) the apical proton pump does not require luminal Na\(^+\), but long-term bilateral Na\(^+\) removal is affecting other epithelial parameters which subsequently indirectly affect \(J_{H^+}\), or 3) rectal acid secretion has several components, only one of which is Na\(^+\)-dependent.

Background Na\(^+\) levels were continuously monitored in all Na\(^+\)-free salines used in this portion of the study. Perfusate samples ranged from 12 to 56 μM Na\(^+\). To insure that bath Na\(^+\) levels did not rise significantly, tissues were continuously perfused (bilaterally) at 10-14 ml/min, except for two brief periods when luminal flow was stopped for 15 min to allow a pH-stat measurement (see Table 4.1). Luminal saline samples taken immediately after each 15 min pH-stat sampling period contained from 13 to 69 μM Na\(^+\) (\(n=6\) preparations). Hanrahan and Phillips (1984b) have reported that passive Na\(^+\) entry across the
Table 4.1 *Effect of bilateral and luminal Na\(^+\) removal on rectal acid secretion (J_{H^+}) under short-circuit current conditions.*

<table>
<thead>
<tr>
<th>Condition</th>
<th>(J_{H^+}) ((\mu)equiv cm(^{-2}) h(^{-1}))</th>
<th>(V_t) (mV)</th>
<th>Isc ((\mu)equiv cm(^{-2}) h(^{-1}))</th>
<th>(R_t) ((\Omega) cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.62 ± 0.11</td>
<td>6.9 ± 1.4</td>
<td>1.22 ± 0.24</td>
<td>211.1 ± 10.0</td>
</tr>
<tr>
<td>30 min bilateral Na(^+) replacement</td>
<td>1.17 ± 0.08 (^a)</td>
<td>14.3 ± 1.8 (^a)</td>
<td>2.02 ± 0.27 (^a)</td>
<td>264.2 ± 10.7 (^a)</td>
</tr>
<tr>
<td>4 h bilateral Na(^+) replacement</td>
<td>0.98 ± 0.09 (^a)</td>
<td>5.9 ± 0.7</td>
<td>1.26 ± 0.20</td>
<td>175.9 ± 8.5     (^a)</td>
</tr>
<tr>
<td>Control</td>
<td>1.54 ± 0.14</td>
<td>8.7 ± 1.3</td>
<td>1.35 ± 0.19</td>
<td>239.4 ± 9.5</td>
</tr>
<tr>
<td>30 min luminal Na(^+) replacement</td>
<td>1.34 ± 0.12 (^a)</td>
<td>19.0 ± 1.1 (^a)</td>
<td>2.30 ± 0.25 (^a)</td>
<td>308.0 ± 11.4    (^a)</td>
</tr>
<tr>
<td>4 h luminal Na(^+) replacement</td>
<td>1.30 ± 0.11 (^a)</td>
<td>17.8 ± 0.8 (^a)</td>
<td>2.27 ± 0.24 (^a)</td>
<td>292.5 ± 10.9    (^a)</td>
</tr>
</tbody>
</table>

\(J_{H^+}\), rate of rectal acidification. \(V_t\), transepithelial potential (lumen relative to haemocoel; measured at end of each experimental period). Isc, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). \(R_t\), transepithelial resistance (calculated from \(V_t\) and Isc by Ohm's Law). All tissues were brought to steady-state in the standard CO\(_2\)/HCO\(_3\)-free saline with the full ion complement (see Materials and Methods). Contraluminal pH was maintained at 7.00 by continuous perfusion; luminal pH was maintained at 7.00 by continuous perfusion except at 30 min and 4 h after Na\(^+\) removal, when it was maintained at 7.00 by pH-stat for 15 min sampling periods. Control values of \(J_{H^+}\), \(V_t\), Isc, and \(R_t\) were determined on each tissue immediately before bilateral or luminal Na\(^+\) replacement. Values are mean ± SE; \(n=6\) for each treatment. †, ‡ control and experimental values for 30 min and 4 h time periods collected from same preparations. † contraluminal bath contained 110 mM Na\(^+\) throughout the entire experimental period. * current required to clamp \(V_t=0\) in the presence of the transepithelial Na\(^+\) gradient (ie. not true Isc). \(^a\) significantly different from control values by paired \(t\)-test (\(P < 0.05\)).
apical membrane can not continue once luminal Na\(^+\) levels drop below 1 mM because the electrochemical gradient for Na\(^+\) at the apical membrane decreases to the point where it can no longer drive Na\(^+\) uptake. At the micromolar levels of luminal Na\(^+\) maintained in the present study, Na\(^+\)-coupling could not energize active proton secretion into the lumen. If rectal acid secretion was entirely dependent on the energy potential supplied by the apical Na\(^+\) electrochemical gradient, \(J_{H^+}\) should have been abolished by luminal Na\(^+\) removal. That this did not happen, indicates that the bulk of active luminal proton secretion is not coupled to apical Na\(^+\) flux.

Hanrahan and Phillips (1984a) reported that long-term bilateral Na\(^+\) removal reduced cAMP-stimulated Cl\(^-\) flux in the locust rectum by 42%. They could find no relationship between Cl\(^-\) movements and trace levels of Na\(^+\) in the luminal bath or electrochemical gradients for Na\(^+\) across the apical membrane. They concluded that the inhibitory effect of long-term Na\(^+\) removal was probably due to a generalized nonspecific effect (eg. reduced metabolite uptake, volume regulatory defect, or pH\(_i\) perturbation) rather than a direct requirement of the Cl\(^-\) transport mechanism for Na\(^+\).

This possibility was addressed in the present study by comparing transepithelial electrical parameters and rates of proton secretion measured at 30 min after bilateral Na\(^+\) removal with the same parameters measured 4h after Na\(^+\) removal (Table 4.1). Although \(J_{H^+}\) did not change dramatically over the 3.5h period, \(V_t\), \(I_{sc}\), and \(R_t\) were all much reduced after prolonged exposure to Na\(^+\)-free salines. These observations are consistent with those reported by Hanrahan and Phillips (1984a) and support their conclusion that long-term Na\(^+\) removal has a generalized adverse effect on the epithelium as a whole.

To circumvent this problem, the above experiments (ie. luminal Na\(^+\) removal; \(V_t\) clamped at 0 mV) were repeated with 110 mM Na\(^+\) present in the contraluminal bath (Table 4.1). Background levels of Na\(^+\) were monitored in the freshly prepared salines before each experiment and in the luminal bath immediately after each 15 min pH-stat sampling period
(see above). Salines contained from 7 to 48 µM Na⁺ before exposure to the tissues (n=6 preparations) and from 32 to 73 µM Na⁺ after the pH-stat sampling period. Although the trace levels of Na⁺ increased significantly during the 15 min period when luminal perfusion was stopped, luminal Na⁺ concentrations never rose to the point where the electrochemical gradient for Na⁺ at the apical membrane favoured passive Na⁺ uptake (see above). J_{H⁺}, V_t, and R_t were all virtually unchanged over the 3.5h period over which measurements were taken, indicating that this protocol was effective in preventing the decrease in viability observed with long-term bilateral Na⁺ removal. Under these conditions, net proton secretion was only reduced by 13-16% as compared to the 40% reduction in J_{H⁺} observed with bilateral Na⁺ removal. The bulk of net luminal proton secretion (>80%) is clearly not coupled to transepithelial Na⁺ fluxes and is not energized by the large electrochemical gradient for Na⁺ at the apical membrane.

The results from the previous set of experiments indicate that a significant fraction of rectal acid secretion (=20%) requires luminal Na⁺. The presence of a Na⁺/NH₄⁺ exchange mechanism on the apical membrane (see Chapter Three) and the occurrence of Na⁺/H⁺ exchangers in many of the acid/base transporting epithelia studied to date (reviewed by Seifter and Aronson, 1986), suggests that the ubiquitous Na⁺/H⁺ exchanger might also be present in the locust rectum. To explore this possibility further, recta were exposed to 1 mM amiloride under short-circuit current conditions in the presence of either 110 or 20 mM bilateral Na⁺ (Table 4.2). Consistent with the results of the previous experiment, amiloride inhibited J_{H⁺} by 11-19% (20 mM and 110 mM Na⁺ salines respectively; both inhibitions significant by paired t-test; P<0.05). V_t and I_sc were completely unaffected by addition of amiloride in either of the Na⁺ salines tested. Taken together, the results from the amiloride and Na⁺ substitution studies support a model in which 15-20% of net active proton secretion

---

2. Due to the competitive nature of amiloride inhibition of Na⁺/H⁺ exchange, it is sometimes possible to improve the efficacy of inhibition by decreasing the concentration of the competing ion (in this case Na⁺). It is important not to decrease the Na⁺ concentration to a level where Na⁺/H⁺ exchange is noticeably affected before amiloride addition.
Table 4.2 *Effect of amiloride on rectal acid secretion (J_{H^+}) under short-circuit current conditions.*

<table>
<thead>
<tr>
<th>Condition</th>
<th>J_{H^+} (μequiv cm^{-2} h^{-1})</th>
<th>V_{t} (mV)</th>
<th>Isc (μequiv cm^{-2} h^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>110 mM Na^+ saline †</td>
<td>1.49 ± 0.11 ^a,c</td>
<td>6.5 ± 0.8</td>
<td>0.98 ± 0.16</td>
</tr>
<tr>
<td>+ 1 mM luminal Amiloride</td>
<td>1.21 ± 0.07 ^a</td>
<td>7.2 ± 1.2</td>
<td>1.01 ± 0.12</td>
</tr>
<tr>
<td>20 mM Na^+ saline ‡</td>
<td>1.41 ± 0.09 ^b,c</td>
<td>4.7 ± 1.0</td>
<td>1.05 ± 0.11</td>
</tr>
<tr>
<td>+ 1 mM luminal Amiloride</td>
<td>1.25 ± 0.10 ^b</td>
<td>5.1 ± 1.6</td>
<td>1.11 ± 0.09</td>
</tr>
</tbody>
</table>

J_{H^+}, rate of rectal acidification. V_{t}, transepithelial potential (lumen relative to haemocoel). Isc, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). † tissues were brought to steady-state in sulphate- and CO_{3}/HCO_{3}^-free saline with an otherwise full ion complement (including 110 mM Na^+; see Materials and Methods). ‡ tissues were brought to steady-state in the same saline used in the previous trial except that bilateral Na^+ concentrations were 20 mM rather than 110 mM (the Cl^- deficit was made up with choline chloride). Bilateral pH was maintained at 7.00 by contraluminal perfusion and luminal pH-stat. V_{t} was measured at the end of each experimental period. Control values of J_{H^+}, V_{t}, and Isc were determined on each tissue immediately before luminal addition of 1 mM amiloride. Values are mean ± SE; n=6 for each treatment. ^a,b values marked with these common symbols significantly different by paired t-test (P < 0.05). ^c values marked with this symbol not significantly different by Student’s t-test (P > 0.50).
occurs by electroneutral Na⁺/H⁺ exchange on the apical membrane and the remainder occurs by an as yet unidentified proton secretory mechanism.

**Potassium.** The bulk of net K⁺ uptake in the rectum of the desert locust is passive and electrically coupled to active, electrogenic Cl⁻ reabsorption (see Phillips *et al.*, 1986 for review). Under open-circuit conditions, net K⁺ flux is equivalent to net Cl⁻ flux both before and during cAMP exposure (0.9 and 4.5 μequiv·cm⁻²·h⁻¹ respectively; Hanrahan, 1982). Under short-circuit current conditions, active K⁺ reabsorption in unstimulated recta has been estimated at 0 to 0.2 μequiv·cm⁻²·h⁻¹ (Hanrahan and Phillips, 1983 and Williams *et al.*, 1978 respectively). The similarity of \( J_{\text{H}^+} \) under both open- and short-circuit current conditions (see Chapter Two), despite the changes in net K⁺ flux under these same conditions, implies that active proton secretion is not directly coupled to net K⁺ flux at the apical membrane.

Under both open- and short-circuit current conditions, the electrochemical gradient for K⁺ across the apical membrane is near zero (Hanrahan and Phillips, 1984b). This indicates that K⁺-coupled influx could not provide the driving force for active proton secretion across the apical membrane (remember that the electrochemical gradient opposing active proton secretion at the apical membrane is 79 mV; see Chapter Two). However, the possibility remains that luminal potassium might be required for net acid secretion in a manner similar to that proposed for active Cl⁻ reabsorption in the locust rectum (eg. as an enzyme activator; reviewed by Phillips *et al.*, 1986) or HCl secretion in the vertebrate gastric muscosa (reviewed by Forte and Machen, 1987).

To test the possibility that proton secretion might be dependent on the presence of luminal or contraluminal K⁺, recta were mounted in Ussing chambers and subjected to long-term (4h) bilateral K⁺ removal under short-circuit current conditions. Hanrahan and Phillips (1984b) have shown that under these conditions, \( V_a \) and \( V_b \) depolarize by 25-30 mV and intracellular K⁺ activites fall from 61 to <5 mM. In the present study, this treatment reduced net acid secretion by approximately 65% (Table 4.3). Consistent with observations reported
Table 4.3 *Effect of bilateral and luminal K\(^+\) removal on rectal acid secretion (J_{H^+}) under short-circuit current conditions.*

<table>
<thead>
<tr>
<th></th>
<th>(J_{H^+}) (μequiv cm(^{-2}) h(^{-1}))</th>
<th>(V_t) (mV)</th>
<th>(I_{sc}) (μequiv cm(^{-2}) h(^{-1}))</th>
<th>(R_t) (Ω cm(^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control †</td>
<td>1.52 ± 0.17</td>
<td>5.7 ± 1.2</td>
<td>0.94 ± 0.20</td>
<td>228.3 ± 9.6</td>
</tr>
<tr>
<td>Bilateral K(^+) replacement †</td>
<td>0.53 ± 0.03 (^a)</td>
<td>4.1 ± 0.9</td>
<td>0.57 ± 0.22</td>
<td>271.0 ± 11.2</td>
</tr>
<tr>
<td>Control ‡</td>
<td>1.59 ± 0.15</td>
<td>6.6 ± 1.2</td>
<td>1.33 ± 0.17</td>
<td>186.2 ± 8.4</td>
</tr>
<tr>
<td>Luminal K(^+) replacement ‡</td>
<td>1.12 ± 0.14 (^a)</td>
<td>27.0 ± 2.1</td>
<td>4.06 ± 0.34 (^*)</td>
<td>248.3 ± 12.9</td>
</tr>
</tbody>
</table>

\(J_{H^+}\), rate of rectal acidification. Vt, transepithelial potential (lumen relative to haemocoel; measured at end of each experimental period). Isc, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). Rt, transepithelial resistance (calculated from Ohm's Law using Vt and Isc). All tissues were brought to steady-state in the standard CO\(_2\)/HCO\(_3\)-free saline with the full ion complement (see Materials and Methods). Contraluminal pH was maintained at 7.00 by continuous perfusion; luminal pH was maintained at 7.00 by continuous perfusion until 4 h after K\(^+\) removal, after which time it was maintained at 7.00 by pH-stat. Control values of \(J_{H^+}\), Vt, Isc, and Rt were determined on each tissue immediately before bilateral or luminal K\(^+\) replacement; all other measurements were made 4 h after commencement of the experimental treatment. Values are mean ± SE; \(n\)=6 for each treatment. †, ‡ control and experimental values from same preparations. ‡ contraluminal bath contained 10 mM K\(^+\) throughout the entire experimental period. \(^*\) current required to clamp Vt=0 in the presence of the transepithelial K\(^+\) gradient (ie. not true Isc). \(^a\) significantly different from control values by paired \(t\)-test (\(P<0.01\)).
by Hanrahan and Phillips (1984a), Vt and Isc were similar to control values after the 4h K⁺-free period; Rt, on the other hand, was significantly increased (19% in this study as compared to 25% reported by Hanrahan and Phillips, 1984a). On the basis of these results and the findings of Hanrahan and Phillips (1984b), it does not appear that long-term K⁺ removal reduces \( J_{H^+} \) simply by altering the electrochemical gradient opposing net acid secretion. If this was the case, one would expect the depolarization of the apical membrane associated with bilateral K⁺ removal to increase rather than decrease \( J_{H^+} \) (assuming pHᵢ was not indirectly affected by K⁺ removal).

The possibility existed that the decrease in \( J_{H^+} \) was the result of nonspecific effects associated with long-term K⁺ removal rather than a direct effect on the H⁺ translocating mechanism per se (similar to that postulated for long-term Na⁺ removal). To test this hypothesis, \( J_{H^+} \) was measured in recta where K⁺ was removed from the luminal bath and contraluminal K⁺ concentrations were maintained at 10 mM. Hanrahan and Phillips (1984b) reported that intracellular levels of K⁺ did not decrease significantly under these conditions. In the present study, \( J_{H^+} \) decreased by only 30% when luminal K⁺ was removed and contraluminal K⁺ was left at 10 mM (significant by paired t-test; \( P<0.01 \); Table 4.3). Vt and Rt were significantly greater than corresponding control values. The substantial increase in the current required to clamp Vt=0 was due to the K⁺ diffusion potential generated by the enlarged concentration gradient for K⁺ across the apical membrane when luminal K⁺ was removed (see Hanrahan, 1982). Again, there does not appear to be any relationship between the electrochemical driving force for K⁺ across the apical membrane and net proton movements.

Background levels of K⁺ were monitored carefully to insure that K⁺ contamination of nominally K⁺-free salines did not occur. Luminal and contraluminal bath samples taken immediately before the pH-stat measurement period (see previous section) ranged from 15 to 45 µM K⁺ (\( n=6 \)). Saline samples taken from the luminal bath immediately after the 15 min
pH-stat measurement period ranged from 51 to 87 μM K⁺ (n=6). This increase was significant by paired t-test (P<0.01), but the electrochemical gradient for K⁺ at the apical membrane still did not favour K⁺ uptake and could not drive active proton secretion into the lumen. Although the bulk of active proton secretion is clearly not dependent on luminal K⁺, a significant fraction of $J_{H^+}$ (30%) was affected when luminal K⁺ was removed. At this point, it is not apparent whether the K⁺-dependent fraction can be attributed to generalized nonspecific effects of luminal K⁺ removal, a stimulatory role for luminal K⁺ similar to that observed for Cl⁻ uptake, or simply a small K⁺-coupled component of net acid secretion.

Apical, electroneutral H⁺/K⁺ exchange has been described in a number of vertebrate gastric mucosae (see Forte and Machen, 1987 for review). In many of these preparations, the substituted benzimidazole, omeprazole, has been shown to significantly inhibit both rates of acid secretion and net K⁺ reabsorption. In an effort to identify the nature of the remaining K⁺-dependent proton flux, 1 mM omeprazole was added bilaterally to recta mounted in Ussing chambers and bathed bilaterally in the standard CO₂/HCO₃⁻-free saline used throughout this study. As evident from Table 4.4, this treatment had absolutely no effect on $V_t$, $I_{sc}$, or $J_{H^+}$. Although the lack of an effect does not necessarily rule out the existence of such a mechanism, taken with the incomplete inhibition of $J_{H^+}$ observed with bilateral K⁺ removal, it at least supports the conclusion that the bulk of net acid secretion in the locust rectum occurs by a K⁺-independent mechanism.

**Chloride.** Cl⁻ is actively reabsorbed from the lumen of the locust rectum by a cAMP-stimulated electrogenic transport mechanism located on the apical membrane (reviewed by Phillips et al., 1986). Under unstimulated short-circuit current conditions, net Cl⁻ uptake has been estimated at 0.85 μequiv cm⁻² h⁻¹; contraluminal addition of 1 mM cAMP causes net Cl⁻ flux to increase to 9.46 μequiv cm⁻² h⁻¹ (Hanrahan and Phillips, 1984a). Although Hanrahan (1982) has shown that the cAMP-stimulated component of transepithelial net Cl⁻ flux is not linked to transepithelial proton gradients, he has not ruled out the possibility that acid/base
Table 4.4  Effect of omeprazole on rectal acid secretion ($J_{H^+}$) under short-circuit current conditions.

<table>
<thead>
<tr>
<th></th>
<th>$J_{H^+}$ (μequiv·cm⁻²·h⁻¹)</th>
<th>$V_t$ (mV)</th>
<th>$I_{sc}$ (μequiv·cm⁻²·h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>1.62 ± 0.12</td>
<td>8.4 ± 1.1</td>
<td>1.44 ± 0.17</td>
</tr>
<tr>
<td>+ 1 mM bilateral Omeprazole †</td>
<td>1.66 ± 0.12 *</td>
<td>7.8 ± 1.0  *</td>
<td>1.35 ± 0.17 *</td>
</tr>
</tbody>
</table>

$J_{H^+}$, rate of rectal acidification. $V_t$, transepithelial potential (lumen relative to haemocoel). $I_{sc}$, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). All tissues were brought to steady-state in the standard CO₂/HCO₃⁻-free saline (see Materials and Methods). Bilateral pH was maintained at 7.00 by contraluminal perfusion and luminal pH-stat. $V_t$ was measured at the end of each experimental period. Control values of $J_{H^+}$, $V_t$, and $I_{sc}$ were determined on each tissue immediately before bilateral omeprazole addition. † measurements made 1h after bilateral omeprazole addition. Values are mean ± SE; $n=6$ for each treatment. * values not significantly different from control by paired t-test ($P>0.2$).
transport in the rectum might somehow be dependent on local Cl⁻ movements across either the apical or basolateral membrane. The widespread involvement of Cl⁻ in acid/base transport in a number of different cell types (e.g., mosquito salt gland: Strange and Phillips, 1985; turtle urinary bladder: Fischer et al., 1981; erythrocytes: Knauf, 1987) suggests that this possibility be carefully considered in the locust rectum.

The first step in assessing the role of chloride in active proton secretion was the bilateral removal of Cl⁻ under both open and short-circuit current conditions (salines were CO₂/HCO₃⁻-free; bilateral pH=7.00). In light of the extremely high Cl⁻ affinity observed for the Cl⁻/HCO₃⁻ exchanger found on the basolateral membrane of the turtle bladder (Kₘ=200 μM; Fischer et al., 1981), it was imperative that background Cl⁻ levels in the nominally “Cl⁻-free” salines used in the present study remain low throughout the entire experimental period. Luminal and contraluminal bath samples taken immediately after measurements of J_H⁺ ranged from 24 to 61 μM Cl⁻ (n=6 preparations). Four hour bilateral removal of chloride had no significant effect on net proton secretion in unstimulated recta under both open- and short-circuit current conditions (Table 4.5). Although Vₜ and Iₛₖ were very similar to control values for both treatments, Rₜ increased significantly (P< 0.01; paired t-test) after bilateral Cl⁻ removal under both open- and short-circuit current conditions. Unless Cl⁻ affinities are sub-micromolar, it would appear that active proton secretion (under CO₂/HCO₃⁻-free conditions) has no requirement whatsoever for extracellular Cl⁻. Hanrahan and Phillips (1983) have reported that intracellular Cl⁻ activities in the locust rectum fell to <5 mM after exposure to Cl⁻-free salines for 3h. Considering that recta in the present study were exposed to Cl⁻-free salines for 4h before measurement of J_H⁺, I would expect intracellular Cl⁻ activities to fall to at least the same levels, if not lower. Based on this assumption and the lack of effect of Cl⁻ removal on J_H⁺, it would appear that active proton secretion also has no requirement for intracellular chloride.
Table 4.5  Effect of bilateral Cl⁻ removal on rectal acid secretion ($J_{H^+}$) under open- and short-circuit current conditions.

<table>
<thead>
<tr>
<th></th>
<th>$J_{H^+}$ (µequiv/cm²·h⁻¹)</th>
<th>Vt (mV)</th>
<th>Isc (µequiv/cm²·h⁻¹)</th>
<th>Rt (Ω·cm²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control † Isc conditions</td>
<td>1.69 ± 0.11</td>
<td>8.0 ± 1.3</td>
<td>1.19 ± 0.17</td>
<td>251.4 ± 8.8 b</td>
</tr>
<tr>
<td>Bilateral Cl⁻ replacement † Isc conditions</td>
<td>1.76 ± 0.11 a</td>
<td>9.2 ± 1.1 d</td>
<td>1.02 ± 0.18</td>
<td>337.2 ± 9.5 b</td>
</tr>
<tr>
<td>Control ‡ Open-circuit conditions</td>
<td>1.58 ± 0.13</td>
<td>5.6 ± 1.5</td>
<td>0.96 ± 0.21 e</td>
<td>218.2 ± 9.7 c</td>
</tr>
<tr>
<td>Bilateral Cl⁻ replacement ‡ Open-circuit conditions</td>
<td>1.53 ± 0.09 a</td>
<td>6.6 ± 1.7</td>
<td>0.83 ± 0.20 e</td>
<td>297.6 ± 10.1 c</td>
</tr>
</tbody>
</table>

$J_{H^+}$, rate of rectal acidification. Vt, transepithelial potential (lumen relative to haemocoel). Isc, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). Rt, transepithelial resistance. All tissues were brought to steady-state in the standard CO₂/HCO₃⁻-free saline with the full ion complement (see Materials and Methods). Contraluminal pH was maintained at 7.00 by continuous perfusion; luminal pH was maintained at 7.00 by continuous perfusion until 4 h after Cl⁻ removal, after which time it was maintained at 7.00 by pH-stat. Control values of $J_{H^+}$, Vt, Isc, and Rt were determined on each tissue immediately before bilateral Cl⁻ replacement; all other measurements were made 4 h after commencement of the experimental treatment. Values are mean ± SE; n=6 for each treatment. †, ‡ control and experimental values from same tissues. b calculated from Vt and Isc using Ohm’s Law. c determined by passing transepithelial constant current pulsed (13.6 µA). d measured at end of each treatment period. e calculated from Vt and Rt using Ohm’s Law. a not significantly different from control values by paired t-test (P>0.3).
**Divalent cations.** Mechanisms of Ca$^{2+}$ and Mg$^{2+}$ uptake in the rectum of the desert locust are completely unknown. Rates of net absorption of both Ca$^{2+}$ and Mg$^{2+}$ were too low to be measured reliably in everted rectal sacs by atomic absorption (reviewed by Phillips *et al.*, 1986). Nevertheless, considering the widespread involvement of calcium in cellular control processes and magnesium in enzyme function and protein synthesis, one would expect that closely regulated membrane transport mechanisms for these ions exist in most cell types. If the large fraction of active proton secretion in the locust rectum unaccounted for by Na$^+$, K$^+$, or Cl$^-$ dependence occurs by an electroneutral mechanism, proton movements across the apical membrane must be linked to movements of either Ca$^{2+}$ or Mg$^{2+}$. To test this possibility, recta were mounted in Ussing chambers and exposed to Ca$^{2+}$- or Mg$^{2+}$-free salines for 1h under short-circuit current conditions (Table 4.6). Bilateral removal of Ca$^{2+}$ (with or without 5 mM EGTA) had no significant effects on rectal acid secretion. However, a small, but consistently significant increase in Vt and Isc was observed immediately after Ca$^{2+}$ removal; control values of Vt and Isc were likewise restored immediately after return to control salines with the full ion complement. Although the increases in Vt and Isc are clearly not related to net acid secretion, they are consistent with an increase in net cation movement into the lumen (loss of intracellular Ca$^{2+}$?) or an increase in net anion uptake from the lumen (increase of Cl$^-$ reabsorption under unstimulated conditions?). Bilateral removal of magnesium had no significant effects on $J_{H^+}$, Vt, Isc, or Rt under these experimental conditions.

Unless a very subtle form of local ion recycling is involved (with extremely low ion affinities), it would appear that the bulk of net active proton secretion in the locust rectum is not directly dependent on the presence of Na$^+$, K$^+$, Cl$^-$, Ca$^{2+}$, or Mg$^{2+}$ under short-circuit current conditions. The results from the ion substitution studies as a whole consistently support the conclusion that the bulk of active proton secretion across the apical membrane
Table 4.6  Effect of bilateral removal of Ca\textsuperscript{2+} or Mg\textsuperscript{2+} on rectal acid secretion (\(J_{H^+}\)) under short-circuit current conditions.

<table>
<thead>
<tr>
<th></th>
<th>(J_{H^+}) ((\mu\text{equiv}\text{-cm}^{-2}\text{-h}^{-1}))</th>
<th>(V_t) (mV)</th>
<th>(I_{sc}) ((\mu\text{equiv}\text{-cm}^{-2}\text{-h}^{-1}))</th>
<th>(R_t) ((\Omega\text{ cm}^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>control (\dagger)</td>
<td>1.38 ± 0.16</td>
<td>8.1 ± 0.9</td>
<td>1.05 ± 0.19</td>
<td>289.1 ± 6.6</td>
</tr>
<tr>
<td>Bilateral Ca\textsuperscript{2+} replacement (\dagger)</td>
<td>1.25 ± 0.14 (b)</td>
<td>10.9 ± 1.4 (a)</td>
<td>1.43 ± 0.21 (a)</td>
<td>284.6 ± 6.1</td>
</tr>
<tr>
<td>control (\ddagger)</td>
<td>1.24 ± 0.11</td>
<td>6.6 ± 1.1</td>
<td>0.82 ± 0.13</td>
<td>301.8 ± 9.7</td>
</tr>
<tr>
<td>Bilateral Ca\textsuperscript{2+} replacement (\ddagger) + 5 mM EGTA</td>
<td>1.20 ± 0.11 (b)</td>
<td>7.8 ± 1.2 (a)</td>
<td>0.95 ± 0.15 (a)</td>
<td>307.9 ± 9.4</td>
</tr>
<tr>
<td>control (\ast)</td>
<td>1.48 ± 0.20</td>
<td>11.1 ± 2.2</td>
<td>1.29 ± 0.18</td>
<td>319.2 ± 8.8</td>
</tr>
<tr>
<td>Bilateral Mg\textsuperscript{2+} replacement (\ast)</td>
<td>1.52 ± 0.18 (b)</td>
<td>10.2 ± 1.7</td>
<td>1.18 ± 0.16</td>
<td>315.8 ± 8.5</td>
</tr>
</tbody>
</table>

\(J_{H^+}\), rate of rectal acidification. \(V_t\), transepithelial potential (lumen relative to haemocoel; measured at end of each treatment); \(I_{sc}\), short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). \(R_t\), transepithelial resistance (calculated from \(I_{sc}\) and \(V_t\) using Ohm’s Law). All tissues were brought to steady-state in the standard CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{−}-free saline with the full ion complement (see Materials and Methods). Contraluminal pH was maintained at 7.00 by continuous perfusion; luminal pH was maintained at 7.00 by continuous perfusion until 1 h after either Ca\textsuperscript{2+} or Mg\textsuperscript{2+} removal, after which time it was maintained at 7.00 by pH-stat. Control values of \(J_{H^+}\), \(V_t\), \(I_{sc}\), and \(R_t\) were determined on each tissue immediately before bilateral ion replacement; all other measurements were made 1 h after commencement of the experimental treatment. \(\dagger\), \(\ddagger\), \(\ast\) control and experimental values from same tissues. Values are mean ± SE; \(n=6\) recta for each treatment. \(a\) significantly different from control values by paired \(t\)-test (\(P<0.01\)). \(b\) not significantly different from control values by paired \(t\)-test (\(P>0.1\)).
(apart from the small component of Na\textsuperscript{+}/H\textsuperscript{+} exchange) occurs by an electrogenic transport mechanism.

**Characterization of the electrogenic component of rectal acid secretion.**

The decrease in net acid secretion induced by application of a transepithelial pH gradient is paralleled by changes in transepithelial membrane potential and the current required to clamp Vt at 0 mV (see Fig. 4.2). This and the ionic independence of \( J_{\text{H}^+} \) under short-circuit current conditions intimates that the changes in Vt and \( I_{\text{app}} \) induced by transepithelial pH gradients are due to changes in net proton conductive movements across the apical membrane. This proposal was investigated by monitoring apical and basolateral membrane potentials and transepithelial resistance (with intracellular microelectrodes) both before and after application of transepithelial pH gradients. All experiments were performed under open-circuit conditions with CO\textsubscript{2}/HCO\textsubscript{3}\textsuperscript{-}-free salines. Although contraluminal pH was maintained at 7.00 at all times, luminal pH was rapidly changed from 7.00 to the pH value required to reduce net proton secretion to zero (i.e. the apparent PMF). Luminal bath changes were complete in less than 5 sec (as judged by dye removal; perfusion flow rate 55-60 ml/min; chamber volume 1.5 ml) and luminal pH was maintained at the value required to abolish net acid secretion for less than 20 sec at a time to guard against large shifts in pHi. Under these conditions, reductions in luminal pH induced significant depolarizations of Vt, Va, and Vb which could be completely reversed by restoring luminal pH to 7.00 (see Fig. 4.4 for typical trace).

To determine if a component of the Vt and \( I_{\text{app}} \) changes could be attributed to pH effects on other ion conductances, these experiments were also performed in the absence of luminal Na\textsuperscript{+}, K\textsuperscript{+}, or Cl\textsuperscript{-} (full ion complement maintained in the contraluminal bath at all times). Since luminal ion substitutions under open-circuit conditions substantially altered transepithelial membrane potentials, it was necessary to do a preliminary series of \( J_{\text{H}^+} \)
Figure 4.4 Typical recording of $V_a$, $V_b$, and $V_t$ under open-circuit conditions when luminal pH was decreased stepwise from 7.00 to the value required to abolish net acid secretion. $V_t$, transepithelial potential (lumen relative to haemocoel). $V_a$ and $V_b$, apical and basolateral membrane potentials (cytoplasm relative to luminal or contraluminal bath respectively). All salines were CO$_2$/HCO$_3^-$-free, but otherwise contained the full ion complement (see Materials and Methods). Contraluminal pH maintained at 7.00 by continuous perfusion; luminal bath changes made by rapid perfusion (see text for details). Voltage deflections are the result of transepithelial constant current pulses used to calculate voltage divider ratios and transepithelial resistance (13.2 $\mu$A; pulse frequency: 0.7 hz; pulse duration: 0.5 s).
<table>
<thead>
<tr>
<th>Contraluminal pH</th>
<th>7.00</th>
</tr>
</thead>
<tbody>
<tr>
<td>Luminal pH</td>
<td>7.00</td>
</tr>
</tbody>
</table>

![Graph showing voltage changes](image)

**Contrast data:**
- **V_a, V_b (mV)**: 
  - V_a: Voltage changes indicating pH variation.
  - V_b: Voltage changes indicating pH variation.

**Graph details:**
- **V_t (mV)**: Voltage changes over time, labeled with **20 sec**.
measurements under varying transepithelial pH gradients with each ion substitution to
determine the respective luminal pH value at which net acid flux equaled zero (see Table
4.7). Calculated apparent PMF values under these conditions (ie. transepithelial pH gradient;
open circuit) agree well with those observed in earlier experiments when Vt was clamped at 0
mV (see Table 4.7 and Fig. 4.2 for comparison).

Vt, Va, Vb, Rt, and I_{app} were all significantly reduced when luminal pH was decreased
from 7.00 to the value required to abolish net acid secretion (with every treatment tested;
Table 4.8). The similarity of the reductions in each of these parameters between the control
and ion-substituted salines suggests that the changes are largely due to H^{+}/OH^{-} conductive
movements as predicted, rather than altered apical conductive movements of Na^{+}, K^{+}, or Cl^{-}.
Although recta were exposed to salines in which luminal Na^{+}, K^{+}, or Cl^{-} was removed, it
must be remembered that intracellular activities of Na^{+} and K^{+} (and quite likely Cl^{-}) are not
dramatically reduced by this treatment (Hanrahan and Phillips, 1984b). Therefore, luminal
ion substitution can not rule out the possibility that decreased luminal pH might somehow
alter basolateral ion conductances as has been shown in other systems (eg. rabbit proximal
tubules; Kuwahara et al., 1989). However, if a change in basolateral ion conductance was
responsible for the electrophysiological changes observed, one would expect to see a
significant change in the ratio of apical to basolateral membrane resistances (estimated by the
voltage divider ratio; see Materials and Methods, Chapter Two). Under control conditions
(ie. full ion complement bathing both sides of the epithelium), the voltage divider ratio was
virtually unaffected by a reduction in luminal pH from 7.00 to 5.18 (1.25 ± 0.04 and 1.26 ±
0.02 respectively; not significantly different by paired t-test; P>0.80; n=43 cells in 6 recta),
indicating that a specific basolateral ion conductance was not affected by this treatment and
that the electrical changes observed in Table 4.8 are primarily due to H^{+}/OH^{-} conductive
movements.
Table 4.7  Transepithelial pH gradients required to abolish net acid secretion after luminal ion substitution under open-circuit conditions.

<table>
<thead>
<tr>
<th>luminal bath</th>
<th>$J_{H^+}$ (µequiv cm⁻² h⁻¹)</th>
<th>$V_t$ (mV)</th>
<th>PMF (mV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-0.02 ± 0.01</td>
<td>0.4 ± 0.3</td>
<td>107</td>
</tr>
<tr>
<td>Na⁺-free</td>
<td>-0.01 ± 0.01</td>
<td>8.3 ± 1.2</td>
<td>102</td>
</tr>
<tr>
<td>K⁺-free</td>
<td>0.01 ± 0.01</td>
<td>19.7 ± 1.6</td>
<td>111</td>
</tr>
<tr>
<td>Cl⁻-free</td>
<td>0.03 ± 0.02</td>
<td>-0.1 ± 0.3</td>
<td>95</td>
</tr>
</tbody>
</table>

$J_{H^+}$, net rate of luminal proton secretion (negative values indicate net proton movements in the opposite direction; ie. absorption). $V_t$, transepithelial potential (luminal bath relative to contraluminal bath). PMF, transepithelial proton motive force (calculated from mean $V_t$ values and transepithelial pH gradients). All experiments were performed under open-circuit conditions. All tissues were brought to steady-state (as defined in the text) in the standard $CO_2/HCO_3^-$-free saline with the full ion complement (see Materials and Methods) before being exposed to the respective treatments listed in the table. The contraluminal surface of each epithelium was continuously perfused with the standard $CO_2/HCO_3^-$-free saline (full ion complement; pH 7.00) in all treatments. Luminal bath changes made by rapid perfusion (see text for details). Recta were exposed to each transepithelial pH gradient for 10-15 min to allow accurate measurements of $J_{H^+}$. Values are mean ± SE; $n=6$ for each treatment.
Table 4.8 Effect of transepithelial pH gradients and luminal ion substitutions on membrane potentials, \(R_t\), and \(I_{app}\).

<table>
<thead>
<tr>
<th>Saline</th>
<th>pH</th>
<th>(V_t) (mV)</th>
<th>(V_a) (mV)</th>
<th>(V_b) (mV)</th>
<th>(R_t) ((\Omega)cm(^2))</th>
<th>(I_{app}) ((\mu)equivcm(^{-2})h(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.00</td>
<td>8.5 ± 0.5</td>
<td>59.7 ± 1.1</td>
<td>51.2 ± 1.2</td>
<td>188.0 ± 4.9</td>
<td>1.72 ± 0.08</td>
</tr>
<tr>
<td>Control</td>
<td>5.18</td>
<td>0.7 ± 0.6</td>
<td>44.6 ± 0.8</td>
<td>44.0 ± 1.2</td>
<td>183.0 ± 6.7</td>
<td>0.17 ± 0.11</td>
</tr>
<tr>
<td>(\Delta) Control (\dagger)</td>
<td></td>
<td>-7.8 ± 0.4</td>
<td>-15.1 ± 0.8</td>
<td>-7.2 ± 0.6</td>
<td>-5.0 ± 0.5</td>
<td>-1.55 ± 0.07</td>
</tr>
<tr>
<td>Na(^+)-free</td>
<td>7.00</td>
<td>19.3 ± 0.8</td>
<td>62.6 ± 1.1</td>
<td>43.2 ± 0.6</td>
<td>312.6 ± 5.7</td>
<td>2.33 ± 0.11</td>
</tr>
<tr>
<td>Na(^+)-free</td>
<td>5.40</td>
<td>6.7 ± 0.7</td>
<td>43.6 ± 1.2</td>
<td>37.2 ± 0.7</td>
<td>306.9 ± 5.6</td>
<td>0.81 ± 0.09</td>
</tr>
<tr>
<td>(\Delta) Na(^+)-free (\dagger)</td>
<td></td>
<td>-12.6 ± 0.7</td>
<td>-19.0 ± 0.9</td>
<td>-6.0 ± 0.5</td>
<td>-5.7 ± 0.6</td>
<td>-1.52 ± 0.08 (\text{a})</td>
</tr>
<tr>
<td>K(^+)-free</td>
<td>7.00</td>
<td>30.1 ± 0.9</td>
<td>89.6 ± 1.0</td>
<td>59.5 ± 0.9</td>
<td>249.5 ± 7.6</td>
<td>4.49 ± 0.13</td>
</tr>
<tr>
<td>K(^+)-free</td>
<td>5.44</td>
<td>22.5 ± 0.7</td>
<td>75.0 ± 2.1</td>
<td>52.7 ± 1.9</td>
<td>242.6 ± 8.4</td>
<td>3.46 ± 0.15</td>
</tr>
<tr>
<td>(\Delta) K(^+)-free (\dagger)</td>
<td></td>
<td>-7.6 ± 0.6</td>
<td>-14.6 ± 0.9</td>
<td>-6.8 ± 0.7</td>
<td>-6.9 ± 0.8</td>
<td>-1.03 ± 0.10 (\text{b})</td>
</tr>
<tr>
<td>Cl(^-)-free</td>
<td>7.00</td>
<td>10.2 ± 0.4</td>
<td>62.2 ± 1.9</td>
<td>51.9 ± 1.2</td>
<td>252.0 ± 4.3</td>
<td>1.51 ± 0.05</td>
</tr>
<tr>
<td>Cl(^-)-free</td>
<td>5.37</td>
<td>0.5 ± 0.3</td>
<td>43.8 ± 1.0</td>
<td>43.2 ± 0.9</td>
<td>244.4 ± 3.8</td>
<td>0.08 ± 0.04</td>
</tr>
<tr>
<td>(\Delta) Cl(^-)-free (\dagger)</td>
<td></td>
<td>-9.7 ± 0.3</td>
<td>-18.4 ± 0.8</td>
<td>-8.7 ± 0.6</td>
<td>-7.6 ± 0.8</td>
<td>-1.43 ± 0.03 (\text{c})</td>
</tr>
</tbody>
</table>

\(V_t\), transepithelial potential (luminal bath relative to contraluminal bath). \(V_a\) and \(V_b\), apical and basolateral membrane potentials (cytoplasm relative to luminal or contraluminal bath respectively). \(R_t\), transepithelial resistance (calculated from deflections in \(V_t\) produced by transepithelial constant current pulses; see Materials and Methods). \(I_{app}\), applied current which would be required to clamp \(V_t=0\) mV (calculated from \(V_t\) and \(R_t\)). All experiments were performed under open-circuit conditions. All tissues were brought to steady-state (as defined in the text) in the standard CO\(_2\)/HCO\(_3\)-free saline with the full ion complement (see Materials and Methods) before being exposed to the respective treatments listed in the table. The contraluminal surface of each epithelium was continuously bathed with the standard CO\(_2\)/HCO\(_3\)-free saline (full ion complement; pH 7.00) in all treatments. \(\dagger\) values represent the mean difference ± SE of the respective parameters at pH 7.00 and pH 5.20. All other values are means ± SE; \(n = 40-85\) cells per treatment (6 recta per treatment). All parameters measured at the luminal pH values required to abolish \(J_{H^+}\) were significantly different than their respective values measured at pH 7.00 in every treatment tested (paired \(t\)-test; \(P < 0.001\)). \(\text{a}\), \(\text{c}\) not significantly different from control by Student's \(t\)-test (\(P > 0.10\)). \(\text{b}\) significantly different from control by Student's \(t\)-test (\(P < 0.001\)).
Estimates of the currents which would be required to clamp $V_t=0$ ($I_{app}$) under each condition were calculated using Ohm's Law and measurements of $V_t$ and $R_t$ (Table 4.8). Since these are not true short-circuit current conditions (i.e. transepithelial electrochemical gradients are present with most of the treatments tested), the value of $I_{app}$ reflects both the active transport processes in the epithelium and any diffusional currents established by luminal ion substitution or decrease of luminal pH. The decrease in luminal pH does not appear to affect conductive movements of $Na^+$, $K^+$, or $Cl^{-}$; consequently, the component of $I_{app}$ due to the respective diffusional currents of these ions should be the same at both luminal pH values tested. The difference in $I_{app}$ observed between luminal pH 7.00 and the pH required to abolish net acid secretion ($\Delta I_{app}$) should therefore be a measure of the electrogenic component of $J_{H^+}$. Under control conditions (i.e. full ion complement bilaterally), this value was $1.55 \pm 0.07 \mu$equiv·cm$^{-2}$·h$^{-1}$. Although paired values of $J_{H^+}$ are not available, the magnitude of $\Delta I_{app}$ is consistent with the conclusion that the bulk of net active proton secretion occurs by an electrogenic mechanism. The similarity of control values of $\Delta I_{app}$ with those observed under $Na^+$- and $Cl^{-}$-free conditions confirms that net acid secretion is largely independent of these ions and that their removal has no effect on the electrogenic portion of $J_{H^+}$ (remember that a small component of $J_{H^+}$ ($\approx 0.2 \mu$equiv·cm$^{-2}$·h$^{-1}$) is due to electroneutral $Na^+/H^+$ exchange). The significantly reduced value of $\Delta I_{app}$ observed when luminal $K^+$ was removed (compared to control values of $\Delta I_{app}$) is consistent with the net reduction of $J_{H^+}$ observed previously when luminal $K^+$ was removed under voltage clamped conditions (see Table 4.3). This observation suggests that $K^+$ removal only affects the electrogenic component of $J_{H^+}$ and that there is likely not a $K^+$-coupled electroneutral component as previously proposed (in agreement with the lack of an omeprazole effect on $J_{H^+}$; see Table 4.4).
The application of a transepithelial pH gradient could abolish net electrogenic proton secretion by directly inhibiting the proton pump *per se* or by increasing the conductive movement of protons in the opposite direction (ie. increased backflux). If the abolition of $J_{H^+}$ in the locust rectum was due solely to a direct inhibition of the electrogenic proton pump, one would expect to see a decrease in $V_t$ and an increase in $R_t$. Under the present experimental conditions, $R_t$ did not increase concomitantly with the decrease in $V_t$ as predicted. Rather, $R_t$ decreased by 5-7 $\Omega \text{cm}^2$ in every preparation examined (significant by paired $t$-test; $P<0.001$; see Table 4.8). The similarity of the change in $R_t$, regardless of luminal ion substitutions, suggests that the decrease in $J_{H^+}$ was at least partially due to an increased back flux of protons from the lumen. The lack of effect of contraluminal pH on $J_{H^+}$ (Fig. 4.1) suggests that this back flux must be occurring across the apical membrane rather than through a nonspecific paracellular pathway.

*Effect of putative acid/base transport inhibitors on luminal acid secretion in the locust rectum*

The data thus far supports a model in which net luminal acid secretion is the result of two separate proton translocating mechanisms located at the apical membrane: a primary electrogenic proton pump accounting for $>80\%$ of net acid secretion and a secondary electroneutral $\text{Na}^+/H^+$ exchange mechanism accounting for the remainder. The exact nature of the electrogenic pump (eg. proton ATPase or redox pump), the role of metabolic $\text{CO}_2$ in net acid secretion, and the mechanisms of contraluminal alkalinization (ie. base exit) are all outstanding issues which have yet to be resolved. Although a detailed investigation into each of these questions is beyond the scope of the present study, a brief survey of putative inhibitors of acid/base transport in other epithelial systems might provide some preliminary answers and establish a direction for future research.
Acetazolamide (ACTZ) has been shown to significantly inhibit net acid/base transport in a wide variety of epithelial cell types (e.g. mosquito salt gland, Strange and Phillips, 1984; turtle bladder, Steinmetz, 1969; mammalian gastric mucosa, reviewed by Forte and Machen, 1987). ACTZ is a potent inhibitor of carbonic anhydrase activity and has in the past been proposed to inhibit net acid secretion by forcing the accumulation of hydroxyl equivalents behind the ACTZ-blocked enzyme (the resulting increase in pHi was proposed to be the factor responsible for inhibition of net acid secretion). The effect of this inhibitor on net proton secretion in the locust rectum was investigated by exposing isolated recta (mounted in Ussing chambers; CO₂/HCO₃⁻-free saline; bilateral pH 7.00) to 1 mM bilateral ACTZ. Although this treatment reduced $J_{H^+}$ by approximately 39%, both $V_t$ and $I_{sc}$ increased slightly (all changes significant by paired t-test; $P<0.05$; Table 4.9). If the ACTZ effect was restricted to inhibition of net proton secretion, one would expect to see a decrease in $V_t$ and $I_{sc}$ concomitant with the decline in the activity of the electrogenic proton pump (unless the inhibitory effect was restricted to the Na⁺/H⁺ exchanger). That both $V_t$ and $I_{sc}$ increased with application of this compound suggests that conductive pathways for other ions are also affected. It has recently been shown that ACTZ exhibits a broad range of effects completely unrelated to carbonic anhydrase suppression (see Graber et al., 1989). Moreover, Graber et al. (1989) have proposed that ACTZ inhibition of net acid secretion in the turtle bladder occurs independent of its inhibition of carbonic anhydrase activity or its effect on intracellular pH. It is clear that ACTZ significantly reduces net proton secretion in the locust rectum. However, the unexpected effects on $V_t$ and $I_{sc}$ and the observations reported by other researchers make it very difficult to define a role for carbonic anhydrase and metabolic CO₂ in net acid secretion on the basis of this inhibition.

The mechanisms of contraluminal alkalinization (see Table 2.5) in the locust rectum are as yet unknown. Measurements of electrochemical gradients for protons across the basolateral membrane indicate that an active step at that membrane is not required to drive
Table 4.9  *Effect of acetazolamide on rectal acid secretion* ($J_{H^+}$) *under short-circuit current conditions.*

<table>
<thead>
<tr>
<th></th>
<th>$J_{H^+}$ ($\mu$equiv cm$^{-2}$ h$^{-1}$)</th>
<th>$V_t$ (mV)</th>
<th>$I_{sc}$ ($\mu$equiv cm$^{-2}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>2.08 ± 0.18</td>
<td>5.8 ± 1.1</td>
<td>0.99 ± 0.13</td>
</tr>
<tr>
<td>+ 1 mM bilateral Acetazolamide †</td>
<td>1.28 ± 0.11 *</td>
<td>7.5 ± 1.4 *</td>
<td>1.21 ± 0.15 *</td>
</tr>
</tbody>
</table>

$J_{H^+}$, rate of rectal acidification. $V_t$, transepithelial potential (lumen relative to haemocoel). $I_{sc}$, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). All tissues were brought to steady-state in the standard CO$_2$/HCO$_3^-$-free saline (see Materials and Methods). Bilateral pH was maintained at 7.00 by contraluminal perfusion and luminal pH-stat. $V_t$ was measured at the end of each experimental period. Control values of $J_{H^+}$, $V_t$, and $I_{sc}$ were determined on each tissue immediately before bilateral acetazolamide addition. † measurements made 1h after bilateral acetazolamide addition. Values are mean ± SE; $n=6$ for each treatment. * values significantly different from controls by paired t-test ($P<0.05$).
the observed rates of contraluminal alkalinization. Preliminary observations (see Chapter Two) suggest that the contraluminal alkalinization might be due to passive movements of HCO$_3^-$ rather than protons or hydroxyl ions. Although the fact that bilateral Cl$^-$ removal had no effect on $J_{H^+}^-$ suggests that base exit does not occur by a Cl$^-$ dependent exchange mechanism (see Table 4.5), preliminary studies with rectal sacs (incubated in CO$_2$/HCO$_3^-$ containing salines) have shown that contraluminal application of 1 mM DIDS completely inhibits CO$_2$/HCO$_3^-$ uptake from the rectal lumen (Thomson and Phillips, 1985). DIDS is known to inhibit Cl$^-$/HCO$_3^-$ exchange in a wide range of epithelial cell types (eg. turtle bladder, Steinmetz, 1974; rabbit medullary collecting ducts, Zeidel et al., 1986; Necturus gall bladder, Reuss and Constantin, 1984; mosquito salt gland, Strange and Phillips, 1984). To test whether base efflux across the basolateral membrane occurs by the same mechanism under both conditions (ie. with or without exogenous CO$_2$/HCO$_3^-$), recta were mounted in Ussing chambers and exposed to 1 mM contraluminal SITS or DIDS (under CO$_2$/HCO$_3^-$-free conditions). Consistent with the lack of effect of bilateral Cl$^-$ removal, neither SITS nor DIDS had a significant effect on $J_{H^+}$, Vt, or Isc (Table 4.10). The lack of a DIDS effect under CO$_2$/HCO$_3^-$-free conditions (as opposed to when CO$_2$ and HCO$_3^-$ were present in the sac preparation), suggests that either the mechanisms for electrogenic proton secretion and CO$_2$/HCO$_3^-$ reabsorption are completely unrelated, or that under CO$_2$/HCO$_3^-$-free conditions, base equivalents cross the basolateral membrane in a different manner than when CO$_2$ is present (eg. OH$^-$ efflux rather than Cl$^-$/HCO$_3^-$ exchange).

Active electrogenic proton transport in the locust rectum occurs against electrochemical gradients of up to 107 mV at the apical membrane. Ion substitution studies have clearly shown that the bulk of this transport is not linked to passive fluxes of other ions across the apical membrane (ie. active electrogenic H$^+$ transport is not energized by the electrochemical potentials of other ions); therefore, one must postulate a direct metabolically dependent mechanism to account for this transfer (eg. proton ATPase or redox pump).
Table 4.10  *Effect of inhibitors on rectal acid secretion (J_{H^+}) under short-circuit current conditions.*

<table>
<thead>
<tr>
<th></th>
<th>$J_{H^+}$ ($\mu$equiv cm$^{-2}$ h$^{-1}$)</th>
<th>Vt (mV)</th>
<th>Isc ($\mu$equiv cm$^{-2}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.43 ± 0.13</td>
<td>4.6 ± 1.3</td>
<td>0.82 ± 0.23</td>
</tr>
<tr>
<td>+ 1 mM contraluminal SITS</td>
<td>1.39 ± 0.13</td>
<td>4.2 ± 1.3</td>
<td>0.76 ± 0.22</td>
</tr>
<tr>
<td>Control</td>
<td>1.56 ± 0.10</td>
<td>8.3 ± 1.7</td>
<td>1.29 ± 0.18</td>
</tr>
<tr>
<td>+ 1 mM contraluminal DIDS</td>
<td>1.58 ± 0.09</td>
<td>8.6 ± 1.6</td>
<td>1.31 ± 0.18</td>
</tr>
<tr>
<td>Control</td>
<td>1.73 ± 0.13</td>
<td>9.7 ± 1.5</td>
<td>1.95 ± 0.26</td>
</tr>
<tr>
<td>+ 1 mM bilateral Vanadate</td>
<td>1.77 ± 0.12</td>
<td>9.1 ± 1.5</td>
<td>1.83 ± 0.25</td>
</tr>
</tbody>
</table>

SITS, 4-acetamido-4’-isothiocyanatostilbene-2,2’-disulfonic acid. DIDS, 4,4’-diisothiocyanatostilbene-2,2’-disulfonic acid. $J_{H^+}$, rate of rectal acidification. Vt, transepithelial potential (lumen relative to haemocoel; measured at end of each treatment period). Isc, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). All tissues were brought to steady-state in the standard CO$_2$/HCO$_3^-$-free saline (see Materials and Methods). Bilateral pH was maintained at 7.00 by contraluminal perfusion and luminal pH-stat. Contraluminal amino acids were replaced with osmotically equivalent amounts of sucrose for both the control and experimental periods in the experiments with SITS and DIDS. Control values of $J_{H^+}$, Vt, and Isc were determined on each tissue immediately before inhibitor addition. All other measurements were made 1h after inhibitor addition. Values are mean ± SE; n=6 for each treatment. Neither SITS, DIDS nor Vanadate had a significant effect on $J_{H^+}$, VT, or Isc by paired t-test ($P>0.50$).
Plasma membrane proton ATPases have been proposed in a number of acid secreting epithelia (eg. toad bladder, Beauwens et al., 1981; turtle bladder, Gluck et al., 1982; rat proximal tubule, Kinne-Saffran and Kinne, 1986). Vanadate, a putative inhibitor of the so-called “P” class of ATPases (those ATPases which form a covalent phosphorylated intermediate as part of their reaction cycle; formerly referred to as “E1-E2” type ATPases; see Pedersen and Carafoli, 1987), has been shown to inhibit active proton secretion (presumably by inhibiting an apical proton ATPase) in both the toad bladder (Beauwens et al., 1981) and the turtle bladder (Arruda et al., 1981; Steinmetz et al., 1981). The possibility that active proton secretion in the locust rectum might be mediated by a similar vanadate-sensitive mechanism was tested by exposing recta mounted in Ussing chambers (CO$_2$/HCO$_3^-$-free; bilateral pH 7.00; short-circuit current conditions) to 1 mM bilateral vanadate. Under these conditions, vanadate had no significant effects on $J_{\text{H}^+}$, $V_t$, or $I_{sc}$ (Table 4.10), suggesting that active proton secretion in the locust rectum was not mediated by the typical “P”-class plasma membrane proton ATPase. As with any inhibitor study, however, the lack of an effect does not necessarily mean that the transport mechanism in question is absent from the system. It is possible that the inhibitor was simply not reaching the appropriate target site on the membrane or that it was somehow being inactivated before it could reach the target binding site. For example, although Lechleitner and Phillips (1988) were able to show substantial vanadate inhibition of Na$^+$/K$^+$-ATPase activity in crude homogenates of locust recta (98% inhibition with 100 µM vanadate), Lechleitner (1988) was unable to show any inhibition at all in intact recta mounted in Ussing chambers with concentrations of up to 1 mM vanadate. Whether or not this indicates that vanadate was unable to reach the appropriate binding site on the Na$^+$/K$^+$-ATPase in the intact tissue is unclear. What it does indicate is that a certain amount of caution is required when interpreting inhibitor results obtained with intact cell preparations.
Effect of anoxia on rectal acid secretion

The results of the previous sections intimate that the apical membrane of the locust rectum possesses a proton conductive pathway (leak?) in parallel with the electrogenic proton pump. At this stage, it is not known whether this conductance is only expressed at low luminal pH values or if it is always present in the apical membrane. The simplest way to approach this question would have been to selectively inhibit both active proton secretory mechanisms in the apical membrane (ie. the electrogenic proton pump and Na⁺/H⁺ exchanger) and monitor the resultant changes in luminal pH (which would presumably reflect passive proton movements through the parallel pathway). As pointed out in Chapter Two, when recta are bathed bilaterally with a pH 7.00 CO₂/HCO₃⁻-free saline, there is a large driving force favouring passive proton diffusion from the lumen into the cell (see Table 2.5). Therefore, one would expect to see an increase in luminal pH consistent with the magnitude of the passive conductance once luminal proton secretion had been abolished. The drawback with this approach is that it requires a fairly complete and reasonably rapid inhibition of the active proton secretory mechanisms. Thus far, the only compound tested which approaches that degree of inhibition is potassium cyanide (see Fig. 2.3); unfortunately, 95% inhibition of \( J_{H^+} \) took 15-35 min to achieve with KCN \((n=6 \text{ recta})\). Given that length of time and the nonspecific nature of KCN inhibition, driving forces for protons across the apical membrane could change dramatically. Moreover, even after 1 hour, KCN did not completely inhibit net acid secretion into the lumen.

Anoxia is known to rapidly and reversibly inhibit net acid secretion in both the turtle bladder (Steinmetz, 1967) and the gastric mucosa (see Sachs et al., 1978). To test the effect of anoxia on net acid secretion in the locust rectum, isolated recta were mounted in Ussing chambers and exposed to 100% N₂ (rather than 100% O₂) under open-circuit conditions. As observed in the turtle bladder and gastric mucosa, anoxia abolished net acid secretion in the locust rectum virtually instantaneously (Fig. 4.5). Also, as predicted for a passive proton
Figure 4.5 Effect of anoxia on $J_{H^+}$, $V_t$, and $R_t$ under open-circuit conditions. $J_{H^+}$, rate of rectal acidification (negative values indicate net acid uptake rather than acid secretion). $V_t$, transepithelial potential (lumen relative to haemocoel). $R_t$, transepithelial resistance (determined by passing transepithelial constant current pulses; see Materials and Methods). Recta were bathed bilaterally with the standard CO$_2$/HCO$_3^-$-free saline with the full ion complement (see Materials and Methods). Contraluminal pH maintained at 7.00 by continuous perfusion; luminal pH maintained at 7.00 by pH-stat. Values are means ± SE; $n=6$ recta.
conductance in the apical membrane, luminal pH began to increase (as indicated by a negative \( J_{\text{H}^+} \) value; Fig. 4.5) as soon as net acid secretion was abolished. Within 30-40 min of the onset of anoxia, rates of luminal alkalinization had reached a steady-state value of 1.03 ± 0.11 μequiv cm\(^{-2}\) h\(^{-1}\).

It is not clear if the gradual increase in luminal alkalinization after oxygen removal is an indication of the length of time it took to completely abolish active proton secretion, of changes in driving forces for protons across the apical membrane, or of a general deterioration of the integrity of the epithelium. The fact that Rt increased immediately after \( \text{O}_2 \) removal suggests that epithelial integrity (ie. "leakiness") was not compromised significantly within the first 5-10 min of anoxia. Even after 1 h of anoxia, values of Rt were not dramatically different than control values measured when \( \text{O}_2 \) was present. It is unlikely, therefore, that the observed rates of luminal alkalinization are merely the result of a decrease in paracellular resistance or a deterioration of the apical membrane. Under short-circuit current conditions, rates of luminal alkalinization rose to 0.83 ± 0.13 μequiv cm\(^{-2}\) h\(^{-1}\) within 15 min of oxygen removal (not significantly different from open circuit values by Student’s \( t \)-test; \( P>0.2; n=6 \) recta). Since there is no driving force for paracellular proton movement under these conditions (Isc; bilateral pH 7.00), luminal alkalinization must be the result of proton movements across the apical membrane.

The problem with using anoxia to unmask the passive proton conductance is the fact that many cellular processes other than active proton secretion will be affected by oxygen removal. It is unrealistic, therefore, to expect rates of luminal alkalinization measured 30 min after oxygen removal to necessarily provide accurate estimates of passive proton influx under control conditions (ie. oxygen present; bilateral pH 7.00). Nevertheless, the observations that Rt increased rather than decreased over the first 5-10 min after \( \text{O}_2 \) removal, that significant rates of luminal alkalinization were measured within that same period, and that rates of luminal alkalinization were similar under both open- and short-circuit current...
conditions all strongly suggest that the inward movement of protons observed under anoxic conditions (ie. lumen to cell) is also present under control conditions when O$_2$ is present and the active proton secretory mechanisms are functioning.

*Effect of cAMP on rectal acid secretion*

Contraluminal cAMP has been shown to increase both electrogenic chloride uptake and Isc by up to 10 µequiv·cm$^{-2}$·h$^{-1}$ in the locust rectum (see Phillips *et al.*, 1986 for review). Concomitant with the stimulatory effect on the apical Cl$^-$ pump, is an increase in apical K$^+$ conductance and an increase in basolateral Cl$^-$ conductance, collectively manifested as a decrease in transepithelial resistance of up to 100 Ω·cm$^2$ (Hanrahan and Phillips, 1984b). Under open-circuit conditions, the increased Cl$^-$ flux is accompanied by an equivalent increase in passive K$^+$ uptake from the lumen (4-5 µequiv·cm$^{-2}$·h$^{-1}$; Hanrahan and Phillips, 1983). Since the increase in Isc observed after cAMP addition can be entirely accounted for by increased Cl$^-$ flux, it would appear that cAMP does not enhance electrogenic proton secretion. However, it is possible that cAMP addition might specifically enhance the electroneutral component or inhibit net proton secretion altogether.

These possibilities were addressed by adding 1 mM cAMP to the contraluminal bath of isolated recta mounted in Ussing chambers and bathed bilaterally with the standard CO$_2$/HCO$_3^-$-free saline used throughout this study (bilateral pH 7.00). Under both open- and short-circuit current conditions, cAMP significantly reduced $J_{H^+}$ by 66% and 42% respectively (Table 4.11). Consistent with the findings reported by Hanrahan and Phillips (1984a, 1984b), both Vt and Isc increased dramatically with contraluminal addition of cAMP.

The fact that $J_{H^+}$ did not increase concomitantly with net Cl$^-$ flux and net K$^+$ flux after cAMP addition (as estimated by the increase in Isc; Table 4.11) provides additional evidence that net proton secretion is not directly linked to movements of either of these ions across the
Table 4.11  Effect of contraluminal cAMP on rectal acid secretion ($J_{H^+}$) under open- and short-circuit current conditions.

<table>
<thead>
<tr>
<th></th>
<th>$J_{H^+}$ ($\mu$equiv cm$^{-2}$ h$^{-1}$)</th>
<th>$V_t$ (mV)</th>
<th>$I_{sc}$ ($\mu$equiv cm$^{-2}$ h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (Open-circuit)</td>
<td>1.83 ± 0.15</td>
<td>12.2 ± 1.8</td>
<td>-</td>
</tr>
<tr>
<td>+ 1 mM contraluminal cAMP †</td>
<td>0.63 ± 0.10 *</td>
<td>43.7 ± 2.6 *</td>
<td>-</td>
</tr>
<tr>
<td>Control (Isc)</td>
<td>1.40 ± 0.14</td>
<td>8.9 ± 1.3 ‡</td>
<td>1.38 ± 0.24</td>
</tr>
<tr>
<td>+ 1 mM contraluminal cAMP †</td>
<td>0.81 ± 0.11 *</td>
<td>46.7 ± 2.9 ‡, †</td>
<td>17.95 ± 1.31 *</td>
</tr>
</tbody>
</table>

$J_{H^+}$, rate of rectal acidification. $V_t$, transepithelial potential (lumen relative to haemocoel). $I_{sc}$, short-circuit current (a positive value indicates cation movement into or anion movement out of the lumen). All tissues were brought to steady-state in the standard CO$_2$/HCO$_3^-$-free saline (see Materials and Methods). Bilateral pH was maintained at 7.00 by contraluminal perfusion and luminal pH-stat. Control values of $J_{H^+}$, $V_t$, and $I_{sc}$ were determined on each tissue immediately before contraluminal cAMP addition. † measurements made 1h after cAMP addition (the approximate time required to attain steady-state, as defined by a stable $I_{sc}$ or $V_t$). ‡ measured at end of each treatment period. Values are mean ± SE; $n$=6 for each treatment. * values significantly different from respective controls by paired t-test ($P<0.001$).
apical membrane. Although cAMP has been shown to inhibit Na⁺/H⁺ exchange in *Necturus* gallbladder (Reuss and Petersen, 1985), chick intestine (Semrad and Chang, 1987), and rabbit proximal tubule cells (Weinman *et al.*, 1988), it is unlikely that a similar inhibition of apical Na⁺/H⁺ exchange could account for the degree of inhibition of net acid secretion observed in the locust rectum (remember that Na⁺/H⁺ exchange accounts for <20% of $J_{H^+}$; see Table 4.2). On the basis of these experiments alone, it is difficult to know whether the cAMP induced inhibition of $J_{H^+}$ is due to a combined effect on both proton secretory mechanisms, on the electrogenic mechanism alone, or on some other mechanism which promotes H⁺/OH⁻ movement in the opposite direction (eg. the diffusional proton pathway postulated above). Nevertheless, it is clear, that by whatever mechanism, cAMP induces a significant reduction in net acid secretion in the locust rectum.

**DISCUSSION**

*Nature of active proton secretion in the locust rectum*

Phillips (1961) has reported that locust recta actively generate and maintain transepithelial pH gradients of 1.5-2.5 pH units *in vivo*. The ability of isolated recta to secrete acid against similar transepithelial pH gradients *in vitro* under CO₂/HCO₃⁻-free conditions (see Fig. 4.2), intimates that the same mechanisms are responsible for net acid secretion in both the isolated and intact preparations regardless of the presence or absence of exogenous CO₂ or HCO₃⁻ (remember that CO₂ and HCO₃⁻ would be present in the *in vivo* preparation). This observation is consistent with the model for rectal acidification proposed in Chapter Two where the decrease in luminal pH observed under CO₂/HCO₃⁻-free conditions was shown to be due to a proton secretory rather than bicarbonate reabsorptive mechanism. Thomson and Phillips (1985) have shown that isolated rectal sacs incubated in 2% CO₂/5 mM HCO₃⁻ actively effect net HCO₃⁻ movement from the lumen to the
haemocoel. The similarity of the maximum pH gradients which can be developed in both the intact and isolated preparations, despite the occurrence of \( \text{HCO}_3^- \) reabsorption in the in vivo preparation, raises the possibility that \( \text{HCO}_3^- \) reabsorption in the locust rectum may be the result of luminal titration (facilitated by active proton secretion) and subsequent \( \text{CO}_2 \) diffusion into the cell, as proposed for the vertebrate kidney (reviewed by Aronson and Giebisch, 1987), rather than a direct ionic \( \text{HCO}_3^- \) reabsorptive mechanism per se.

Net acid secretion in the isolated locust rectum is extremely sensitive to both electrical and chemical driving forces for protons across the apical membrane. \( J_{\text{H}^+} \) could be abolished by reducing luminal pH to 5.27 (\( V_t \) clamped at 0 mV; Fig. 4.2) or clamping \( V_t \) at 107 mV lumen positive (bilateral pH=7.00; Fig. 4.3). The striking similarity between the rates of inhibition of \( J_{\text{H}^+} \) and apparent transepithelial PMF values obtained by applying either an electrical or chemical transepithelial gradient (Fig. 4.6) implies that both treatments are modulating the same transport process. The ability to eliminate net acid secretion by application of electrical or chemical transepithelial gradients has also been observed in the turtle bladder (Al-Awqati et al., 1977) and is considered indicative of a proton conductive pathway in the epithelium. The fact that contraluminal pH did not affect \( J_{\text{H}^+} \), \( V_t \), or \( I_{\text{app}} \) (Fig. 4.1) suggests that this pathway does not have a paracellular component (as would be expected for a tight epithelium).

The transepithelial electrochemical potential for protons (\( \Delta \overline{\mu}_{\text{H}^+}/F \)) at which net acid flux equals zero is a measure of the apparent transepithelial proton motive force of the acid secretory mechanism. The degree to which the apparent PMF reflects the true PMF of the apical pump depends on how well the transepithelial \( \Delta \overline{\mu}_{\text{H}^+}/F \) approximates the apical \( \Delta \overline{\mu}_{\text{H}^+}/F \) and how well \( J_{\text{H}^+} \) reflects unidirectional \( \text{H}^+ \) transport rates through the pump (remember that \( J_{\text{H}^+} \) is a measure of net acid secretion; ie. pump rate minus leak rate). Although there are no direct measurements of \( V_a \) or \( pHi \) for the exact conditions used in this set of experiments, it is
Figure 4.6 Effect of transepithelial electrochemical proton gradients on rates of net acid secretion. $J_{H^+}$, rate of rectal acidification (negative values indicate acid uptake rather than acid secretion). Data points obtained by converting abscissa values from Fig. 4.2 ($J_{H^+}$ vs $\Delta p$H) and Fig. 4.3 ($J_{H^+}$ vs $\Delta Vt$) to transepithelial $H^+$ electrochemical gradients ($\Delta \bar{u}_{H/F}$). Open and solid circles represent values measured when the transepithelial $\Delta \bar{u}_{H/F}$ was created by imposing transepithelial voltage or pH gradients respectively. Dashed and dotted lines fit by regression analysis (least squares method) and correspond to solid and open circles respectively.
possible to calculate approximate electrochemical proton gradients for the apical membrane from measurements made under slightly different experimental conditions.

The fact that apical and basolateral membrane resistances are roughly equal (α=1.26 ± 0.02; Hanrahan and Phillips, 1984b report voltage divider ratios of 0.8-1.44) indicates that an applied transepithelial voltage drop will be expressed more or less equally across both membranes. Under short-circuit current conditions when Vt=0 mV, both apical and basolateral membrane potentials are approximately -55 mV (relative to luminal or contraluminal bath respectively; Hanrahan, 1982; Thomson, unpublished observation). Based on a voltage divider ratio of 1.26, voltage clamping the epithelium at 107 mV (the transepithelial potential required to abolish \( J_H^+ \) when bilateral pH=7.00) should hyperpolarize Va by 60 mV (Va=-115 mV) and depolarize Vb by 47 mV (Vb=-8 mV). If pHi was between 6.56 (the interpolated pHi value predicted to occur when luminal pH is decreased to 5.18 under open-circuit conditions; see Fig. 3.4 and Table 4.7) and 7.38 (the pHi value observed under open-circuit conditions when bilateral pH=7.00; Table 2.5), the apical \( \Delta \bar{\mu}_H/F \) would be between 89 and 137 mV respectively (pHi would have to equal 6.86 for the apical \( \Delta \bar{\mu}_H/F \) to equal 107 mV).

Under open-circuit conditions, when luminal pH is decreased to 5.18, pHi is predicted to decrease to approximately 6.56 (Fig. 3.4). In Table 4.18, Va, Vb, and Vt approximate a short-circuited state (ie. Vt=0 mV and Va=Vb) when luminal pH was decreased to 5.18. An intracellular pH of 6.56 and an apical membrane potential of -45 mV corresponds to an apical \( \Delta \bar{\mu}_H/F \) of 125 mV.

Remembering that these calculations are based largely on predicted values and measurements made on different tissues under differing experimental conditions (eg. open-circuit vs. short-circuit), the agreement between measured transepithelial \( \Delta \bar{\mu}_H/F \) values and calculated apical \( \Delta \bar{\mu}_H/F \) values is actually quite good. The excellent agreement in apparent PMF values predicted by applying either electrical or chemical gradients to the epithelium
considerably strengthens the conclusion that the transepithelial $\Delta \bar{\mu}_{H}/F$ is a realistic estimate of the apical $\Delta \bar{\mu}_{H}/F$ under the conditions required to abolish net acid secretion (see Fig. 4.6 and Table 4.7) and hence that the measured transepithelial PMF is a reasonable estimate of the proton motive force which can be generated across the apical membrane.

In order for the apical PMF to equal the PMF of the proton secretory mechanism *per se*, the apical membrane must have a very low parallel proton conductance and by implication $J_{H^{+}}$ must equal the active rate of proton secretion through the pump (ie. $H^{+}$ backflux must be negligible). There are several lines of evidence which suggest that this condition is not met in the locust rectum. When luminal pH was reduced to the value required to abolish $J_{H^{+}}$ under open-circuit conditions, $R_{t}$ did not increase as would be expected if the active proton pump represented the only proton conductive pathway in the apical membrane; rather, $R_{t}$ decreased by approximately 5 $\Omega \text{cm}^{2}$ in every preparation tested (Table 4.8). The similar magnitude of the decrease in $R_{t}$ regardless of the ionic composition of the luminal saline (eg. Na$^{+}$-free, Cl$^{-}$-free, or K$^{+}$-free; Table 4.8) indicates that the effect is not due to a pH-mediated increase in the conductance of Na$^{+}$, Cl$^{-}$, or K$^{+}$. It appears, therefore, that the decrease in $R_{t}$ can be attributed to conductive movements of protons across the apical membrane in parallel with and opposite to the $H^{+}$ conductive movements through the active secretory mechanism.

To test whether the parallel pathway was present in the absence of the transepithelial pH gradient and to get an estimate of the magnitude of the passive $H^{+}$ flux, net acid secretion was abolished by making the tissue anoxic (Fig. 4.5). Consistent with the presence of a passive, apical proton conductive pathway, this treatment caused an immediate reversal from net acid secretion to net acid uptake (remember that the apical $\Delta \bar{\mu}_{H}/F$ favours passive proton movements from the lumen to the cell; see Table 2.5). When the same treatment was applied to the turtle bladder (an epithelium known to have a low passive $H^{+}$ permeability; reviewed by Al-Awqati and Dixon, 1982), net acid secretion was abolished, but there was no evidence
of passive proton movements from the lumen to the cell (Steinmetz, 1967). The increase in Rt and the observation of similar rates of luminal alkalinization under both open- and short-circuit current conditions suggests that the passive movement of protons observed under anoxic conditions is not merely the result of a decrease in paracellular resistance or a deterioration of the apical membrane. Rather, I am proposing that the passive flow of protons from the lumen to the cell is always present and that the inhibition of the active proton secretory mechanism merely unmask the passive backflux.

The passive movement of protons from the lumen to the cell does not necessarily have to occur through a nonspecific leak pathway in the apical membrane. Meredith and Phillips (1988) have proposed that up to 43% of net proline uptake (ie. \( =1.4 \mu \text{equivcm}^{-2}\cdot\text{h}^{-1} \)) from the rectum of the desert locust may occur by a proton/proline cotransport mechanism on the apical membrane. The presence of such a mechanism would be entirely consistent with the changes in \( J_{\text{H}^+} \) and Rt observed when \( \Delta \mu_{\text{H}^+}/F \) is increased to the PMF of the apical membrane or when the active component of net acid secretion is abolished by anoxia.

The existence of a parallel \( \text{H}^+ \) conductive pathway in the apical membrane implies that \( J_{\text{H}^+} \) (the measure of net acid secretion) underestimates the true rate of active proton secretion by the magnitude of the \( \text{H}^+ \) backflux. Based on the anoxia study, the rate of \( \text{H}^+ \) backflux could be between 0.5 and 1.03 \( \mu \text{equivcm}^{-2}\cdot\text{h}^{-1} \) when luminal pH=7.00 (apical \( \Delta \mu_{\text{H}^+}/F = 79 \text{ mV} \)). Considering the differences in experimental protocols and salines, this range of values is certainly very similar to the magnitude of proton/proline cotransport reported by Meredith and Phillips (see above; 1988). When \( \Delta \mu_{\text{H}^+}/F \) is increased to 107 mV (ie. lumen pH=5.18 or \( V_t \) clamped at 107 mV), the driving force for passive proton movements is obviously increased and one would expect the magnitude of passive proton flux to increase concomitantly. Clearly, the conclusion to be drawn from these observations is that the apparent transepithelial (and hence apical) PMF is not a valid estimate of the true PMF of the active proton secretory mechanism per se. Although the apparent PMF is of little value for
describing pump energetics, it is useful for predicting the maximum pH gradients which could be sustained under a variety of conditions in the in vitro preparation (eg. Table 4.7; despite the major differences in saline composition, the apparent PMF values were remarkably similar) and may prove useful in explaining luminal pH changes in the whole animal.

Mechanisms of active proton secretion

Inhibition of net acid secretion by application of either chemical or electrical transepithelial gradients (ie. ΔpH or ΔVt) suggests that net acid secretion might occur by an electrogenic transport mechanism on the apical membrane. From this observation alone, however, it is difficult to distinguish between the effects on the active proton secretory mechanism and passive proton movements in the opposite direction. The observed changes in $J_{H^+}$, $V_t$, and $I_{app}$ could be easily explained by an increase in passive proton conductive movements from the lumen to the cell without having to propose an electrogenic proton secretory mechanism on the apical membrane.

The electrogenic nature of active proton secretion was subsequently confirmed by demonstrating that $J_{H^+}$ was largely independent of Na$^+$, K$^+$, Cl$^-$, Ca$^{2+}$, or Mg$^{2+}$. The only ions which had any effect at all on rates of net acid secretion when they were removed from the bathing salines were Na$^+$ and K$^+$. The 10-20% inhibition of $J_{H^+}$ observed after luminal Na$^+$ removal could be completely accounted for by an electroneutral, amiloride sensitive Na$^+$/H$^+$ exchange mechanism on the apical membrane (see Table 4.1 and Table 4.2). This conclusion is entirely consistent with the proposed apical Na$^+$/NH$_4^+$ exchange mechanism reported in Chapter Three. Kinsella and Aronson (1981) have shown that this class of transporter is fully able to exchange either protons or ammonium ions for external sodium in isolated renal microvillus membranes. It remains to be seen whether there are distinct populations of Na$^+$/H$^+$ and Na$^+$/NH$_4^+$ exchangers in the locust rectum or whether the same
exchangers pump either Na\(^+\) or H\(^+\) depending on which ion interacts with the appropriate binding site.

Hanrahan and Phillips (1984b) reported that K\(^+\) removal reduced active, electrogenic Cl\(^-\) flux by up to 70% in the locust rectum under short-circuit current conditions. On the basis of changes in electrochemical gradients for Cl\(^-\) and K\(^+\) across the apical membrane, they concluded that the K\(^+\) effect was due to a direct interaction of K\(^+\) with the apical Cl\(^-\) pump rather than an indirect effect of K\(^+\) on intracellular membrane potentials or a strict requirement for a K\(^+\)/Cl\(^-\) cotransport mechanism. The reduction in \(J_{H^+}\) observed after long-term bilateral K\(^+\) removal (65% reduction; Isc conditions; Table 4.3) could not be attributed to a K\(^+\) induced modulation of intracellular membrane potentials. Under these conditions, intracellular K\(^+\) activities have been shown to decrease to <5 mM and Va and Vb depolarize by 25-30 mV (Hanrahan and Phillips, 1984b). Since \(J_{H^+}\) did not increase as would be predicted by the decrease in Va, long-term K\(^+\) removal must have either affected the H\(^+\) transport mechanism directly in a manner similar to that proposed for the electrogenic Cl\(^-\) pump or indirectly by unspecified cellular effects similar to those proposed for long-term Na\(^+\) removal (see above and Hanrahan and Phillips, 1984b). When the above experiments were repeated with K\(^+\) present in the contraluminal bath (luminal bath K\(^+\)-free; Vt clamped at 0 mV), \(J_{H^+}\) was only reduced by 30%. Since intracellular K\(^+\) activities were maintained at control levels by maintaining 10 mM K\(^+\) in the contraluminal bath (60-65 mM; Hanrahan and Phillips, 1984b), it is likely that most cell functions were not adversely affected by this treatment. When luminal K\(^+\) was removed under open-circuit conditions, Va and Vb hyperpolarized to 90 and 60 mV respectively (see Table 4.8) and the voltage divider ratio rose to 2.38 ± 0.05 (n=38 cells in 6 recta). On the basis of this voltage divider ratio, when Vt was clamped at 0 mV (Table 4.3), Va and Vb should have equaled ~69 mV and the electrochemical gradient opposing active proton secretion at the apical membrane should have increased by ~15% (assuming that pHi did not change significantly). Under these
conditions (K\(^+\) removed from the luminal bath only) it is reasonable to propose that the inhibition of \(J_{H^+}\) could be attributed almost entirely to K\(^+\) modulation of the apical membrane potential and hence the apical \(\Delta \mu_{H^+}/F\) rather than a direct requirement of the proton pump for K\(^+\) (as proposed for the Cl\(^-\) pump) or nonspecific cellular effects as proposed above for long-term bilateral K\(^+\) removal.

Having ruled out other ion gradients as the energy source for active proton secretion, one is forced to consider the possibility of either a redox pump or a proton ATPase. Redox pumps similar to those described in bacteria, chloroplasts, and mitochondria (see Harold and Altendorf, 1974, Dilley and Giaquinta, 1975, and Wikstrom, 1982, for respective reviews) have in the past been proposed for renal brush border membranes (Gimenez-Gallego et al., 1980) and gastric mucosa (Rehm, 1972). Although the proton pump in the gastric mucosa has since been shown to be an ATPase (reviewed by Forte and Machen, 1987), it has been difficult to completely rule out the existence of the redox pump in the renal brush border (see Aronson, 1983). Nevertheless, the current view is that primary active transport of protons in eukaryote plasma membranes is facilitated by proton ATPases rather than redox pumps. Plasma membrane proton ATPases have been proposed for a variety of acid/base transporting epithelia including gastric mucosa (reviewed by Forte and Machen, 1987), turtle bladder (reviewed by Steinmetz and Andersen, 1982), rabbit renal medullary collecting ducts (Zeidel et al., 1986) and rabbit proximal tubules (Kuwahara et al., 1989). The fact that vanadate had no effect on net acid secretion in the locust rectum by no means rules out the possibility that a proton ATPase is present on the apical membrane. Vanadate-insensitive plasma membrane proton ATPases have previously been reported in other tissues (eg. Tobacco Hornworm midgut, Schweikl et al., 1989; rat renal brush border, Turrini et al., 1989) and the possibility exists that vanadate might not even have reached the target site or that it was somehow being inactivated in the epithelium (see above). Other putative proton ATPase inhibitors such as \(N\)-ethylmaleimide or \(N,N'\)-dicyclohexylcarbodiimide were not tested on this preparation in
consideration of the vast array of nonspecific effects they exert on intact cell systems. An isolated membrane preparation must be developed before these inhibitors can be used with confidence and quite likely before the exact nature of the apical proton pump can be conclusively determined.

Acetazolamide (ACTZ) and DIDS have been reported to inhibit HCO$_3^-$ uptake in everted rectal sacs incubated in exogenous CO$_2$ and HCO$_3^-$ by 37% and 98% respectively (Thomson and Phillips, 1985). The similar degree of inhibition of net acid secretion in the flat-sheet preparation under CO$_2$/HCO$_3^-$-free conditions with ACTZ (39%; Table 4.9) implies that both transport processes share a common pathway or that HCO$_3^-$ reabsorption may depend on active proton secretion as proposed for the vertebrate kidney (see Giebisch and Aronson, 1987). The lack of effect of contraluminal DIDS on net acid secretion under CO$_2$/HCO$_3^-$-free conditions, on the other hand, suggests fundamental differences depending on whether CO$_2$ and HCO$_3^-$ are present or not. DIDS has been shown to reduce net HCO$_3^-$ uptake in vertebrate proximal tubule cells by inhibiting the basolateral exit step whereas ACTZ is thought to act by modulating the intracellular concentrations of HCO$_3^-$ and hydroxyl ions (reviewed by Preisig and Alpern, 1989). The disparate responses to contraluminal DIDS in the locust rectum suggest a difference in the basolateral exit step depending on the availability of intracellular HCO$_3^-$.

In the vertebrate proximal tubule, the principal means of base efflux across the basolateral membrane when exogenous CO$_2$ and HCO$_3^-$ are present is a SITS and DIDS inhibitable Na$^+$/3HCO$_3^-$ cotransport mechanism (reviewed by Preisig and Alpern, 1989). Under CO$_2$/HCO$_3^-$-free conditions, base efflux is maintained, but sensitivity to disulfonic stilbenes is lost. Burckhardt and Fromter (1987) have proposed a parallel stilbene-insensitive H$^+$/OH$^-$ conductive pathway on the basolateral membrane (which is normally silent when exogenous CO$_2$/HCO$_3^-$ is present) to account for this observation. It is possible that a similar phenomenon is also occurring in the locust
rectum. Specific experiments aimed directly at this question must be performed before any conclusions can be drawn.

KCl uptake in the rectum of the desert locust is increased 10 fold by the addition of glandular extracts from the retrocerebral complex of the locust brain (reviewed by Phillips et al., 1986). The factor (CTSH; Chloride Transport Stimulating Hormone) which stimulates transport is proposed to operate through a cAMP- rather than Ca\textsuperscript{2+}-mediated second messenger network. Consistent with this proposal, contraluminal applications of cAMP stimulate KCl uptake in a dose-dependent manner similar to applications of crude brain homogenates (Hanrahan and Phillips, 1984c). Addition of 1mM contraluminal cAMP to the locust rectum reduced net acid secretion under both open- and short-circuit current conditions by 66% and 42% respectively (Table 4.11), implying that rectal acid secretion might also be under direct hormonal control. The mechanism by which cAMP inhibits net acid secretion is unknown. Without concomitant measurements of pH\textsubscript{i} it is difficult to attribute the effect solely to changes in $\Delta\mu_{\text{H}^+}$ across either the apical or basolateral membranes. As reviewed above, cAMP has been shown to inhibit Na\textsuperscript{+}/H\textsuperscript{+} exchange in a variety of acid/base transporting epithelia; it is possible, therefore, that at least a component of the reduction in $J_{\text{H}^+}$ may be due to a similar effect.

Source of short-circuit current in unstimulated locust recta

Williams et al. (1978) reported that net fluxes of Na\textsuperscript{+}, K\textsuperscript{+}, and Cl\textsuperscript{-} could not account for the short-circuit current values observed in unstimulated locust recta. On the basis of the magnitude of the unidentified component of the short-circuit current ($\approx3.1$ $\mu$equiv$\cdot$cm$^{-2}$$\cdot$h$^{-1}$), the direction of the current (indicative of net cation movement into or net anion movement out of the lumen), and the rates of luminal acidification reported by Speight (1967; 2.15 $\mu$equiv$\cdot$cm$^{-2}$$\cdot$h$^{-1}$), they proposed that the unidentified component of Isc might be due to electrogenic movements of protons or hydroxyl ions across the rectal epithelium.
In the present study I have shown that the bulk of net acid secretion is due to an active, electrogenic proton secretory mechanism located at the apical membrane. Under short-circuit current conditions, rates of net acid secretion were 1.53 ± 0.03 μequiv cm⁻² h⁻¹ (n=90 recta). Although this value is approximately half of that required to account for the unidentified component of Isc observed by Williams et al., the Isc and net flux values for Na⁺, K⁺, and Cl⁻ reported by them are substantially larger than corresponding values reported by other researchers (e.g. Hanrahan and Phillips, 1984a; Black et al., 1987). Different experimental salines, measurement of short-circuit current without compensation for saline resistance, and data collection prior to steady-state conditions have all been suggested as possible reasons for this discrepancy (Hanrahan, 1982). To date, no one person has measured Na⁺, K⁺, Cl⁻, and H⁺ fluxes under exactly the same conditions with the same population of experimental animals. This and the significant variability associated with measurement of very small ion fluxes makes it very difficult to collectively reconcile reported flux values with observed short-circuit currents. Nevertheless, if one considers only those flux values obtained from more or less similar preparations, it would appear that active movements of Na⁺, Cl⁻, H⁺, and NH₄⁺ can account for most (if not all) of the observed short-circuit current in unstimulated locust recta (Table 4.12).

Summary

Isolated locust recta are able to maintain net acid secretion against transepithelial H⁺ electrochemical gradients of up to 105 mV under CO₂/HCO₃⁻-free conditions. The presence of a proton diffusional pathway on the apical membrane suggests that the apparent PMF values calculated from net acid secretion rates significantly underestimate the true proton motive force which can be generated by the active proton secretory mechanism. As proposed for vertebrate proximal tubule cells (Kuwahara et al., 1989), net acid secretion in the locust rectum is a composite of electroneutral Na⁺/H⁺ exchange and electrogenic proton secretion.
Table 4.12 *The source of short-circuit current in unstimulated locust recta.*

<table>
<thead>
<tr>
<th>Ion</th>
<th>Net flux (μequiv·cm⁻²·h⁻¹)</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cl⁻</td>
<td>+0.85</td>
<td>Hanrahan and Phillips (1984a)</td>
</tr>
<tr>
<td>Na⁺</td>
<td>−2.0</td>
<td>Black <em>et al.</em> (1987)</td>
</tr>
<tr>
<td>K⁺</td>
<td>0</td>
<td>Hanrahan and Phillips (1983)</td>
</tr>
<tr>
<td>NH₄⁺</td>
<td>+0.6</td>
<td>Chapter Three</td>
</tr>
<tr>
<td>H⁺</td>
<td>+1.5</td>
<td>Chapter Four</td>
</tr>
<tr>
<td>Expected Isc</td>
<td>+0.95</td>
<td>calculated from net flux values</td>
</tr>
<tr>
<td>Observed Isc</td>
<td>+0.82 to +1.95</td>
<td>range observed in Chapters Two to Four</td>
</tr>
</tbody>
</table>

Isc, short-circuit current. Positive flux or Isc value indicates movement of positive charges into or negative charges out of the lumen.
(ATPase?); the major difference between the two systems being that Na\(^+\)/H\(^+\) exchange accounts for the bulk of net acid secretion in the vertebrate kidney whereas electrogenic proton secretion accounts for the major fraction of acid secretion in the locust rectum. Concomitant reports of HCO\(_3\)\(^-\) reabsorption and active proton secretion in the locust rectum (Thomson and Phillips, 1985) suggest, that like most vertebrate urinary epithelia, locust recta reabsorb HCO\(_3\)\(^-\) by titration of luminal HCO\(_3\)\(^-\) to CO\(_2\), subsequent CO\(_2\) diffusion into the cell, carbonic anhydrase catalyzed hydration of intracellular CO\(_2\) to HCO\(_3\)\(^-\), and HCO\(_3\)\(^-\) exit from the cell across the basolateral membrane through a stilbene-sensitive pathway. The presence of a significant proton diffusional pathway in the apical membrane and reports of proton driven proline uptake in the locust rectum (Meredith and Phillips, 1988) suggest that rectal epithelial cells utilize the enlarged electrochemical gradient for protons across the apical membrane to energize solute uptake (specifically proline in this instance) from the lumen to the cell in a manner similar to that proposed for prokaryotes (reviewed by Eddy, 1978) and more recently, rabbit renal proximal tubules (Roigaard-Petersen et al., 1987).
CHAPTER FIVE: General Discussion

Mechanisms of acid/base transport in the locust rectum. The data presented in this thesis confirm that the rectal epithelial cells per se are capable of generating and maintaining the low pH values observed in the locust rectum in vivo. The results of preliminary in vitro studies which suggested that the isolated rectum alkalized rather than acidified luminal contents under short-circuit current conditions (Hanrahan, 1982) were shown in Chapter Two to be an artifact of improper short-circuit current technique rather than the result of an active transport process in the rectal epithelium. Ion-substitution and microelectrode studies (Chapter Two) indicate that the active mechanism responsible for the rectal acidification is located on the apical membrane and that acidification under CO₂/HCO₃⁻-free conditions is mediated directly by proton secretion rather than movements of bicarbonate, phosphate, or unidentified buffer components. Under short-circuit current conditions, the bulk of net acid secretion is not dependent on Na⁺, K⁺, Cl⁻, Mg²⁺, or Ca²⁺ and is due to a primary electrogenic proton translocating mechanism (proton ATPase?) located on the apical membrane (Chapter Four). A small component (10-16%) of the net acid secretion measured under these conditions could be attributed to an apical amiloride-inhibitable Na⁺/H⁺ exchange mechanism. The similarity of the maximum sustainable transepithelial pH gradients reported for the in vitro (see Chapter Four) and in vivo (Phillips, 1961; Speight, 1967) preparations strongly suggests that the same mechanism is responsible for the bulk of rectal acidification under both conditions regardless of the presence or absence of exogenous CO₂ or HCO₃⁻. Given this assumption, it is possible to integrate the observations made in this study with those reported for the intact animal to formulate a working model for acid/base transport in the locust rectum (Fig. 5.1).

Thomson and Phillips (1985) have reported significant rates of HCO₃⁻ reabsorption in everted rectal sacs incubated in exogenous CO₂ and HCO₃⁻. Bicarbonate uptake was
Figure 5.1 Proposed model for acid/base transport in the *in vivo* locust rectum. ACTZ, acetazolamide. DIDS, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid. ca., carbonic anhydrase. Dotted lines indicate proposed sites of inhibitor activity. Dashed lines indicate passive transport steps. Solid lines with circles indicate primary or secondary active transport processes. See text for complete details.
partially inhibitable with bilateral acetazolamide (37%) and completely inhibitable with contraluminal DIDS. Since net fluxes of Na\(^+\), K\(^+\), and Cl\(^-\) in isolated locust recta are not affected by bilateral CO\(_2\)/HCO\(_3\)\(^-\) removal (reviewed by Phillips et al., 1986), one would expect that HCO\(_3\)\(^-\) uptake from the lumen likewise does not occur by a Na\(^+\), K\(^+\), or Cl\(^-\)-coupled mechanism at the apical membrane. In support of this hypothesis, DIDS had absolutely no effect on cAMP stimulated- or unstimulated short-circuit current or active Cl\(^-\) uptake from the lumen (Hanrahan, 1982, and Chapter Four). Considering the low luminal pH values maintained in the rectum in vivo (presumably by the apical proton pump described in Chapter Four) and the ionic independence proposed above for HCO\(_3\)\(^-\) uptake, it is likely that the bulk of luminal HCO\(_3\)\(^-\) crosses the apical membrane as CO\(_2\) rather than bicarbonate ions per se in a manner similar to that proposed for the vertebrate proximal tubule (see Chapter One and Giebisch and Aronson, 1987 for review).

In the scheme illustrated in Fig. 5.1, luminal HCO\(_3\)\(^-\) is titrated to CO\(_2\) by protons actively transported into the lumen by the apical proton translocating mechanism. CO\(_2\) then diffuses passively across the apical membrane into the cytoplasm where it is hydroxylated by OH\(^-\) equivalents generated behind the apical proton pump. Carbonic anhydrase is implicated in the intracellular hydroxylation of CO\(_2\) on the basis of limited ACTZ inhibition of both net acid secretion (Chapter Four) and HCO\(_3\)\(^-\) uptake (Thomson and Phillips, 1985)\(^1\) and histochemical evidence confirming that the enzyme is indeed present in the epithelium (Hanrahan, 1982). Measurements of electrochemical gradients for protons across the basolateral membrane (Chapter Two) suggest that an active exit step for HCO\(_3\)\(^-\) need not be postulated. Since changes in contraluminal HCO\(_3\)\(^-\) concentration had no effect on Isc (Chapter Two) or Vt (unpublished observation) it is unlikely that HCO\(_3\)\(^-\) exit is electrogenic. Although it is not known for certain that HCO\(_3\)\(^-\) exit is coupled to movements of other ions

\(^1\) As indicated in Chapter Four and Fig. 5.1, it is unclear whether the effects of ACTZ are limited to inhibition of intracellular carbonic anhydrase activity. However, until conclusive evidence can be presented to the contrary, I will assume for simplicity's sake that the principal means by which ACTZ reduces net proton secretion and HCO\(_3\)\(^-\) uptake is inhibition of carbonic anhydrase.
(eg. Cl⁻/HCO₃⁻ exchange or Na⁺/HCO₃⁻ cotransport), it appears that the pathway is stilbene sensitive (Thomson and Phillips, 1985), as reported for other bicarbonate reabsorptive epithelia (eg. turtle bladder, reviewed by Steinmetz and Andersen, 1982; vertebrate proximal tubule, reviewed by Giebisch and Aronson, 1987). Further work is clearly needed in this area.

Despite the significant component of net acid secretion which could be attributed to Na⁺/H⁺ exchange in the *in vitro* preparation under control conditions (ie. bilateral pH=7.00; Chapter Four), it is questionable whether this mechanism contributes significantly to rectal acid secretion in the intact animal. Remembering that levels of Na⁺ entering the rectum *in vivo* are usually <20 mM (reviewed by Phillips *et al.*, 1986) and that intracellular Na⁺ activities are normally around 8 mM (Hanrahan and Phillips, 1984b), it is unlikely that the apical electrochemical gradient for Na⁺ has enough potential energy to provide the necessary driving force for proton secretion when luminal pH drops much below 7.0 ² (which by all estimates is >90% of the time; Phillips, 1961; Speight, 1967; Harrison, unpublished observation). These considerations suggest that Na⁺/H⁺ exchange is induced by the conditions used in the *in vitro* preparation (ie. Na⁺ concentration of 110 mM and luminal pH=7.00) and that in the intact animal, the bulk of amiloride sensitive Na⁺ uptake probably occurs via exchange for NH₄⁺ rather than H⁺.

In many acid/base transporting epithelia (eg. vertebrate proximal tubule; reviewed by Good and Knepper, 1985), ammonia secretion is thought to enhance luminal acid secretion by the so-called "diffusion trapping" hypothesis (see Pitts, 1977). This hypothesis predicts that luminal NH₃ acts as a proton sink by combining with free protons in the lumen to form NH₄⁺. Ammonia secretion in the locust rectum clearly does not perform this function. In Chapter Three it was shown that significant rates of ammonia were secreted preferentially

---

² An apical membrane potential of 58 mV (see Fig. 3.4), a luminal Na⁺ concentration of 20 mM, and an intracellular Na⁺ concentration of 8 mM (Hanrahan and Phillips, 1984b) yields an apical electrochemical gradient for Na⁺ of about 81 mV favouring passive movement of Na⁺ from the lumen to the cell. The corresponding electrochemical gradient for protons at luminal pH=7.00 is 79 mV opposing passive movements of protons from the cell to the lumen (Table 2.5).
into the lumen, but that the ammonia was largely protonated before it crossed the apical membrane. Ion-substitution and inhibitor studies indicate that the ammonia is actively secreted into the lumen by an apical, amiloride-inhibitable Na\(^+\)/NH\(_4\)\(^+\) exchange mechanism (see Fig. 5.1).

In the *in vitro* preparation used in the present study, the secreted ammonia originated almost exclusively from the oxidative metabolism of amino acids reabsorbed from the lumen (specifically proline, glutamine, alanine and serine; see Table 3.1). Since luminal amino acids (and particularly proline; Chamberlin and Phillips, 1982) appear to be the principal respiratory substrate for the rectum, it is quite possible that amino acid metabolism and subsequent NH\(_4\)\(^+\) excretion are completely unrelated to pH regulation in the desert locust. This is in marked contrast to the vertebrate proximal tubule, where rates of glutamine metabolism, bicarbonate formation (from \(\alpha\)-ketoglutarate), and ammonia excretion are all directly related to the acid/base status of the animal (see Warnock and Rector, 1981, and Brosnan *et al.*, 1986, for reviews). Given the importance of renal ammoniagenesis to vertebrate pH regulation (especially during chronic acidosis; Brosnan *et al.*, 1986), it is clear that this line of investigation should not stop with this thesis. It is entirely possible that, as in the vertebrate proximal tubule, rates of amino acid metabolism (and subsequent ammonia production) in the locust rectum are strongly influenced by chronic acid/base perturbations in the intact animal.

The recent proposal of proton driven proline uptake in the locust rectum (Meredith and Phillips, 1988; see Fig. 5.1) suggests that luminal acidification might be important for more than just HCO\(_3\)\(^-\) reabsorption. The large proton electrochemical gradient developed across the apical membrane by the active proton pump described in Chapter Four clearly represents a large potential energy source for driving active solute uptake from the lumen to the cell. Considering the magnitude of this driving force, the widespread occurrence of proton coupled solute transport in prokaryotes (Eddy, 1978), and the recent report of proton coupled
proline uptake in the vertebrate kidney (Roigaard-Petersen et al., 1987), it would not be surprising if a significant fraction of net solute uptake in the locust rectum occurred by proton coupled mechanisms.

**Regulation of acid/base transport in the rectum.** Rates of luminal acidification in the *in vitro* preparation used in the present study were unaffected by changes in contraluminal pH (Chapter Four), pCO₂, or HCO₃⁻ (Chapter Two), implying that the respective haemolymph parameters do not directly modulate rectal acid secretion in the locust *in vivo*. Contraluminal pH was also shown to have no effect on J_H⁺ in the turtle bladder, but changes in contraluminal pCO₂ (while maintaining a constant contraluminal pH) strongly influenced rates of luminal acid secretion (Schwartz and Steinmetz, 1971). The lack of an effect of contraluminal CO₂ and HCO₃⁻ on rates of luminal acidification in the locust rectum suggests that either this treatment was not significantly affecting the intracellular pH of the rectal epithelial cells, or that pHi does not modulate J_H⁺. Measurements of pHi under these conditions are clearly required to address this question.

In the isolated locust rectum, rates of luminal acidification were significantly reduced by contraluminal addition of 1 mM cAMP under both open- and short-circuit current conditions (Chapter Four). From the results presented in this thesis, it is not clear whether cAMP was acting directly on the apical proton pump *per se*, the electrochemical gradient opposing active proton secretion, or the passive proton conductance in the apical membrane. If the bulk of passive proton movements from the lumen to the cell could be accounted for by proline/proton cotransport (as suggested in Chapter Four), then it is unlikely that J_H⁺ inhibition was due to increased rates of proton backflux, since Meredith and Phillips (1988) have shown that cAMP stimulation reduces rather than enhances net rates of proline uptake from the lumen. Measurements of pHi under cAMP-stimulated conditions are required to distinguish between the other two possibilities. Although it has yet to be tested, it is expected that crude homogenates of corpora cardiaca (CC) and ventral ganglia (VG) (and therefore
CTSH)\(^3\) will reduce rates of net acid secretion in the \textit{in vitro} preparation in a manner similar to that reported for cAMP (see Chapter Four).

In a preliminary study of HCO\(_3^-\) reabsorption in the locust ileum, Lechleitner (1988) showed that both the pH and the HCO\(_3^-\) concentration of the ileal absorbate decreased substantially after contraluminal application of crude homogenates of either CC or VG. Based on calculated rates of HCO\(_3^-\) reabsorption before and after stimulation, he concluded that the decrease in absorbate HCO\(_3^-\) concentration was largely due to an increase in fluid uptake rather than a direct effect on rates of HCO\(_3^-\) reabsorption \textit{per se}.\(^4\) In other words, he is proposing that the decreased HCO\(_3^-\) concentrations in the absorbate are primarily due to a dilution effect. If rates of HCO\(_3^-\) uptake reflect rates of luminal acidification, then one must conclude that the acid/base transport mechanism postulated to occur on the apical membrane (see Irvine \textit{et al.}, 1988) was also unaffected by addition of homogenates of CC or VG.

Considering the similar stimulatory effect of CC and VG homogenates on fluid reabsorption in the rectum, it is quite likely that the HCO\(_3^-\) concentration of the rectal absorbate will be significantly reduced by increased rates of fluid reabsorption as proposed for the ileum. However, in contrast to the ileum, it would be expected that rates of HCO\(_3^-\) uptake \textit{per se} would also be reduced by addition of gland homogenates (assuming that cAMP accurately mimics the effects of the glands and that rates of HCO\(_3^-\) reabsorption reflect rates of proton secretion). The net effect would be a greater reduction in the concentration of HCO\(_3^-\) in the absorbate than could be achieved by dilution alone.

**The role of the rectum in haemolymph pH regulation.** As reviewed in Chapter One, the mechanisms by which terrestrial insects regulate levels of non-volatile acids and bases to maintain long-term pH homeostasis is largely unknown. Given that the HCO\(_3^-\) buffer system plays a major role in haemolymph pH regulation in the locust (Wong \textit{et al.}, 1989) and that

---

\(^3\) These neuroendocrine organs are believed to contain high levels of CTSH (see Phillips \textit{et al.}, 1986)

\(^4\) In the intact animal, increased levels of CTSH in the haemolymph are believed to significantly increase rates of fluid reabsorption in both the rectum and the ileum.
both segments of the hindgut (ie. ileum and rectum) have the ability to reabsorb substantial quantities of \( \text{HCO}_3^- \), it is reasonable to propose that both the ileum and rectum play significant roles in haemolymph pH regulation.

An ongoing study of the mechanisms of haemolymph pH regulation in the desert locust by Harrison (personal communication) has suggested that animals made acidotic by injection with HCl recover from the pH insult by modulating haemolymph bicarbonate levels rather than haemolymph \( \text{pCO}_2 \) or non-bicarbonate buffering capacity. Measurements made in starved animals, both before and after acid injection, revealed significant decreases in the pH of Malpighian tubule fluid (7.2 to 6.6), and no significant differences in ileal pH (6.2), rectal pH (4.7), rectal \( \text{HCO}_3^- \) content (= 1 mM), or rectal titratable acidity.\(^5\)

What this means for the rectum in terms of a specific pH regulatory role is uncertain. If the pH gradient across the apical membrane was already the maximum which could be established by the apical proton pump under control conditions, one would not necessarily expect to see a further decrease in luminal pH with the onset of acidosis. Modulation of luminal acid secretion in the turtle bladder occurs by a change in the density of apical proton pumps rather than an alteration in the transport characteristics of the individual pumps \textit{per se} (ie. the maximum PMF which can be generated by the pump is unchanged; reviewed by Steinmetz, 1986). If this is also the case in the locust rectum, then the maximum possible rate of \( \text{HCO}_3^- \) reabsorption (as judged by luminal pH and \( \text{HCO}_3^- \) concentration)\(^6\) might already have been achieved before the animals were made acidotic. The lack of change in ileal pH with acidosis suggests that this may be happening in the anterior portions of the hindgut as well.

The significant decrease in Malpighian tubule fluid pH observed during acidosis intimates that the haemolymph pH recovery might be mediated by an active regulatory

---

5. \( \text{HCO}_3^- \) content and titratable acidity values for the midgut, Malpighian tubules, and ileum have not yet been measured

6. Assuming that \( \text{HCO}_3^- \) is reabsorbed by luminal titration to \( \text{CO}_2 \) as proposed earlier and that the rate limiting step for \( \text{HCO}_3^- \) uptake is luminal conversion to \( \text{CO}_2 \), rates of \( \text{HCO}_3^- \) uptake could be increased either by decreasing luminal pH or increasing the amount of \( \text{HCO}_3^- \) entering the rectum
processes present in this organ. However, based on $V_t$ (8-14 mV, lumen positive; Dow, 1986) and levels of pH and $\mathrm{HCO}_3^-$ in the haemolymph ($\mathrm{pH}=7.1$ and $[\mathrm{HCO}_3^-]=10-15\,\text{mM}$) and Malpighian tubule fluid ($\mathrm{pH}=7.2$ and $[\mathrm{HCO}_3^-]=10\,\text{mM}$; Harrison, personal communication), it appears that protons and bicarbonate ions are passively distributed across the epithelium under control conditions (ie. animals starved, but not acid loaded). If this is the case, one could explain the observed decrease in tubular pH (6.65) merely on the basis of the concomitant decrease in haemolymph pH observed after acid injection (ie. pH=6.6), without having to invoke an active pH regulatory process in the Malpighian tubules. Using the same argument, levels of $\mathrm{HCO}_3^-$ in the tubule fluid would be expected to fall to $<2\,\text{mM}$ simply because haemolymph $\mathrm{HCO}_3^-$ levels fell to approximately that level after acid injection.

Measurements of transepithelial potentials and tubular levels of $\mathrm{HCO}_3^-$ will be required to confirm this hypothesis.

The fact that the titratable acidity of the rectal contents was unchanged with acidosis implies that there was not a compensatory increase in net proton secretion or buffer reabsorption along the entire length of the excretory system (including the Malpighian tubules). Since Harrison has shown that the pH recovery requires the net addition of $\mathrm{HCO}_3^-$ to the haemolymph, one must postulate an increase in the production of $\mathrm{HCO}_3^-$ from metabolic substrates and a concomitant increase in non-titratable acid excretion to account for restoration of normal haemolymph pH values. Both of these objectives could be achieved by increased levels of ammoniagenesis in the rectum (or ileum)\(^7\) as proposed in the previous section. Clearly, levels of luminal $\mathrm{NH}_4^+$ must be measured along the length of the entire excretory system before any conclusions can be drawn regarding the respective roles of the Malpighian tubules, ileum and rectum in haemolymph pH regulation.

In the same study, Harrison observed that rectal pH and $\mathrm{HCO}_3^-$ content changed significantly with the feeding status of the animal; luminal $\mathrm{HCO}_3^-$ concentrations decreased

\(^7\) Lechleitner (1988) demonstrated that the ileum secreted ammonia into the lumen at two to four times the rate observed in the rectum
from >10 mM to ~1 mM and luminal pH decreased from >6 to <5 as the animals progressed from a fed to a starved state. It is not certain at this point whether the elevated luminal pH values observed in the fed animals are the result of “down-regulation” of the apical proton pump or merely the inability of the rectal epithelium to acidify the gut contents as rapidly as they enter the rectal lumen. The increased levels of circulating CTSH associated with feeding (see Phillips et al., 1986) and the inhibitory effects of cAMP (and therefore presumably CTSH) on $J_{H^+}$ suggest the former. It is not difficult to envisage a scenario whereby rates of $\text{HCO}_3^-$ reabsorption would be significantly reduced (predicted by the elevated luminal pH) at a time when $\text{HCO}_3^-$ would be at a surplus (ie. during feeding) and the animal could be facing an alkalotic rather than acidotic challenge.

Although Harrison’s study of haemolymph pH regulation is still in the early stages, it clearly supports the conclusions drawn in this thesis. Luminal pH values predicted by the apparent PMF derived in Chapter Four and the role of the apical proton pump in $\text{HCO}_3^-$ reabsorption are all in very good agreement with actual trends and values observed by Harrison in the intact animal. Reports of proton secretion (Phillips et al., 1986), $\text{HCO}_3^-$ reabsorption, and ammonia secretion (Lechleitner, 1988) in the locust ileum suggest that this segment of the excretory system also plays a significant role in haemolymph pH regulation. With regards to ion and water uptake, the ileum and rectum have proven to be roughly analogous with the vertebrate proximal tubule and the collecting duct respectively; the ileum by-in-large accommodates bulk uptake and the rectum adjusts the final osmotic and ionic content of the excreta. It will be interesting to see if the analogy extends to the handling of acid/base equivalents.
REFERENCES


Craig, R., and J.R. Clark (1938). The hydrogen ion concentration and buffer value of the blood of larvae of Pieris rapoae (L.) and Heliothis obsoleta (F.). J. Econ. Ent. 31: 51-54.


Hastings, E., and J.N. Pepper (1943), Studies on body fluid of seven orthopterans, their pH, buffering capacity, and effect on solubility of fractionated insecticides. J. Econ. Ent. 36: 857-864.


