ROLE OF THE MALPIGHIAN TUBULES IN ACID-BASE REGULATION IN THE DESERT LOCUST *SCHISTOCERCA GREGARIA*

by

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Abstract

Malpighian tubule fluid from *Schistocerca gregaria* adults, starved for one day, was collected by cannulation of the gut *in situ*, both before and after injecting 10 μmol of HCl or NaCl into the haemocoel. Haemolymph pH at the neck remained depressed by 0.3 units for at least 8 hours in HCl- compared with NaCl-injected locusts. Haemolymph pH remained lower compared with pre-injected haemolymph values for several hours. The pH of tubule fluid remained about 0.5 units more acid than haemolymph under all conditions. Thus, net tubular acid secretion was proportional to haemolymph acid-base status. The greater acidity of tubular fluid after acid-injection was associated with lower estimated bicarbonate concentrations and higher Pco_2, without any change in total CO_2 when compared with controls. The combined contribution of bicarbonate, phosphate, and urate to total buffer capacity of tubular fluid was estimated to be 75%, with bicarbonate responsible for 55% of the total. The maximum rate of acid removal by all Malpighian tubules of starved locusts, including H^+ trapped in ammonium ions, was calculated to be very small in relation to the acid-load injected into the haemocoel. Ligation of the locust hindgut so as to prevent posterior Malpighian tubule fluid flow significantly lowered haemolymph pH as compared with either anterior or sham ligations after HCl injection. Injection of an artificial saline into the hindgut restored haemolymph pH after HCl injection. Thus the hindgut *per se* directly contributes to haemolymph pH recovery from acidosis. Increasing the temperature to 37° C from 21° C caused a 4-fold increase in tubular secretion rates. In addition, starved locusts maintained a temperature/haemolymph pH ratio that is consistent with the alphastat hypothesis under starved conditions, but temperature does not affect haemolymph pH under fed

conditions. Feeding locusts at 21° C lowered haemolymph pH 0.28 units. At 37° C, feeding had no effect on haemolymph pH. Feeding did not initiate any changes in tubular fluid acid-base variables at either 21° C or 37° C. The buffering capacity of tubular fluid was not affected by temperature or feeding. Micropuncture of the gut indicated feeding initiated a movement of alkaline midgut contents posteriorly into the hindgut. The maximum rate of acid removal was calculated to double for starved locusts at 37° C as compared to 21° C, but feeding reduced net acid excretion to zero at both 21° C and 37° C.

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List of Abbreviations

μequiv	-microequivalent (s)
μl	- micrcoliter (s)
ANOVA	-analysis of variance
ATP	- adenosine 5′-triphosphate
ATPase	- adenosine 5′-triphosphatase
Ca^{2+}	-calcium ion
cAMP	- adenosine 3′:5′-cyclic monophosphoric acid
CC	-corpora cardiaca
C_{CO_2}	-complete carbon dioxide
Cl^-	-chloride ion
cm^2	-square centimetre (s)
Cn	-colon
CO_2	-carbon dioxide gas
DH	-diuretic hormone
DIDS	-4,4′-Diisothiocyanato-stilbene-2,2′-disulfonic acid
g	-gram (s)
h	- hour (s)
H^+	-hydrogen ion
H_2O	-water
Ha	-haemolymph
HCl	-hydrochloric acid

HCO_3^-	-bicarbonate ion
HPO_4^{2-}	-monobasic phosphate ion
Il	-ileum
J_{amm}	- rate of ammonia secretion
J_{H^+}	- rate of acidification
J_{NH_3}	-rate of molecular ammonia secretion
$J_{NH_4^+}$	-rate of ammonium ion secretion
K^+	-potassium ion
KCl	-potassium chloride
kg	- kilogram
KOH	-potassium hydroxide
l	- liter (s)
M	- moles per liter (molar)
mequiv	-milliequivalents
Mg^{2+}	-magnesium ion
ml	- milliliter (s)
mm	-millimetres
mM	- millimolar
mmol	- millimole (s)
N	- number
Na^+	-sodium ion
NaCl	-sodium chloride

NaOH	-sodium hydroxide
NH_3	-molecular ammonia
NH_4^+	-ammonium ion
nm	- nanometer
O_2	-oxygen gas
OH^-	-hydroxyl ion
Pco_2	-partial pressure carbon dioxide
pH	-negative log hydrogen ion concentration
pK	-negative log dissociation constant
Re	-rectum
ß	-non-bicarbonate buffer value
J_V	-rate of fluid secretion
S. gregaria	-*Schistocerca gregaria*
S.E.	- standard error
VG	-ventral ganglia
Δ	-change
°C	-degrees centigrade

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CHAPTER 1

General Introduction

The regulation of acid-base status is fundamental to organismal homeostasis. Many proteins of physiological importance are bathed in blood or haemolymph and are sensitive to changes in H^+ concentration of these fluids. Maintaining blood pH is critical to physiological function, but surprisingly little is known of the regulatory mechanisms in insects.

Vertebrates use the ventilatory and renal systems to maintain blood Pco_2, HCO_3^-, and pH at constant levels and they have elaborate systems of blood buffers that contribute to maintaining pH within tolerable boundaries. Temperature and metabolism also influence these regulatory processes. I will first review some of the general mechanisms of acid-base homeostasis in air and water breathing vertebrates and crustaceans, and then outline available information on acid-base regulation in insects.

General Mechanisms of pH Homeostasis

a) Ventilation

Metabolic CO_2 dissolves in water to form carbonic acid, which further dissociates into H^+ and HCO_3^-. Although the primary function of the respiratory system is gas exchange, the ratio of ventilation to CO_2 production influences the tissue Pco_2. Therefore, because CO_2 produced in actively metabolizing tissues will tend to acidify the local tissue and blood, the rate of ventilation affects the acid-base status of tissue and blood in air breathing animals (Heisler, 1986).

b) Ionic transport

In the vertebrate nephron, active proton secretion is a secondary transport process in the proximal tubule, where H^+ is exchanged for Na^+ (Sullivan, 1986). Extrusion of Na^+ from the cells into the interstitial space via the Na^+-K^+ ATPase sets up a favourable gradient for Na^+ to enter the cells from the lumen (Sullivan, 1986). Reabsorption of HCO_3^- takes place in the proximal tubule primarily as a result of Na^+-H^+ secondary transport (see below). There is also a Cl^--HCO_3^- exchanger found in other segments of the nephron (Sullivan, 1986). Renal absorption of HCO_3^- shifts the blood bicarbonate buffer system to the alkaline side.

The blood protons translocated to the nephron lumen may have several effects, dependent upon the composition of the primary urine formed in the glomerulus (Pitts, 1968). Firstly, if the primary urine HCO_3^- levels are high, the extruded protons will titrate HCO_3^- to carbonic acid and the resulting CO_2 diffuses into the cell and dissociates back into HCO_3^- and H^+. Secondly, if phosphate is present in the alkaline dibasic form, protons will form the titratible monobasic acid which can be excreted as a neutral salt. Finally, if tubular buffers such as HCO_3^- and HPO_4^{2-} are low, then ammonia derived from glutamine and other amino acids in kidney epithelia will trap protons to form NH_4^+ in the lumen. Ammonium is relatively lipid insoluble and therefore remains in the tubule lumen, thereby enhancing H^+ removal from the body (Pitts, 1973).

Fish regulate blood Pco_2 mostly by ionic movements of HCO_3^-, because the difference between inspired and expired Pco_2 is low (Heisler, 1986). Diffusional gas exchange of CO_2 takes place across the gill epithelium; however transport of CO_2 to the gill site is via H^+ and HCO_3^-. Transepithelial transfer of H^+ for Na^+, NH_4^+ for Na^+, and HCO_3^- for Cl^- also occurs across the fish gill (Heisler, 1986).

Aquatic crustaceans have the same limitations for eliminating CO_2 as fish; i.e. ventilation is largely dependent on O_2 requirements (Cameron, 1986). The gills again appear to be the major site of acid-base regulation. Gill Cl^-/HCO_3^- and Na^+/H^+ exchange are the dominant mechanisms.

c) Buffers

Changes in pH from an acid or alkaline load will be greatest when blood non-bicarbonate buffer value is lowest (Heisler, 1986). The higher the concentration of blood buffers, the greater the resistance to change in pH. Therefore, mobilization of blood buffers in response to a change in blood pH is a mechanism of pH homeostasis. In vertebrates, the CO_2/HCO_3^- buffer system predominates as the major extracellular blood buffer (Truchot, 1987). In addition, major non-bicarbonate buffers include residues on the polypeptide chain of proteins, and inorganic and various organic phosphates (Heisler, 1986).

d) Temperature

Regulation of blood acid-base status is affected by temperature, and models of how ectothermic animals maintain acid-base balance in the face of a change in temperature is currently a matter of considerable debate. One model, the alphastat hypothesis, predicts that changes in blood pH reflect an attempt to maintain fractional dissociation state of proteins when the temperature changes (Reeves, 1977). Reeves has argued that the imidazole moieties of histidine residues of proteins, with a pK near 7 and a heat of enthalpy around 7 kcal mol^{-1}, produce a $\Delta pK/\Delta T$ relationship in the range of -0.017 to -0.023, which fits many observed $\Delta pH/\Delta T$ relationships (Cameron, 1988). However, many animals show changes in pH with

temperature that are not consistent with the alphastat model (Heisler, 1984). A second model predicts that blood pH should change with temperature to maintain a constant relative alkalinity: i.e. the difference between blood pH and the pH of neutral water is maintained constant (Heisler, 1984).

e) Metabolic compensation

Metabolic reactions can influence the pH of blood (Hochachka and Mommsen, 1983; Portner, 1987). For example, Atkinson and Bourke (1987) maintain protein metabolism results in large amounts of released bicarbonate, about 1 mole/100 g protein. However, the traditional view of renal physiologists is that the acid formed from sulphur and phosphate groups associated with protein lead to net acid production.

Insect Acid-Base Regulation

Preliminary Observations

Insects have recently been shown to also maintain acid-base status, and attention is now focused on mechanisms of this regulation. There are a few early studies of haemolymph acid-base parameters (Craig and Clark, 1938; Levenbrook, 1959); however CO_2 loss during measurements in these studies may have led to questionable values for haemolymph pH. Strange (1982) conducted the first detailed study of insect haemolymph pH regulation using saltwater mosquito larvae.

More recently, acid-base regulatory mechanisms have been investigated in a terrestrial insect, the locust, by Harrison (1988, 1989) at different environmental Pco_2's and temperatures.

Locusts regulate ventilation in response to environmental hypercapnia, thus controlling haemolymph pH changes due to changes in blood Pco_2. Harrison (1989) also showed that haemolymph pH is regulated with changes in temperature. In two locusts, *M. bivittatus* and *S. nitens*, a pattern of haemolymph pH regulation consistent with the alphastat hypothesis was observed over the temperature range 25° to 35° C (Harrison, 1989). From these studies it is clear locusts regulate haemolymph pH in response to temperature and changing blood Pco_2 levels.

Haemolymph buffer systems have also been investigated in the desert locust. Harrison *et al.*, (1990) found that, as in vertebrates, the major buffers of physiological importance are bicarbonate and protein, with HCO_3^- accounting for 60% of the total haemolymph buffer value.

Haemolymph acid-base regulation has also been investigated in response to exercise and acid injection. Harrison *et al.* (1991a) found that regulation of pH back to resting levels after vigorous hopping can be largely explained by increased tracheal ventilation.

Haemolymph recovery from severe acidosis initiated by HCl injection (blood pH reduced by 0.5 units) has also been investigated in locusts by Harrison *et al.*, (submitted). Haemolymph pH values returned to pre-injection levels within 8 hours primarily by transfer of additional HCO_3^- into the haemocoel while Pco_2 remained constant. This recovery of haemolymph pH after injection of acid (10 μmol) was accompanied by significant lowering of luminal pH in the crop, midgut, and at the point of Malpighian tubule entry to the gut (see Fig. 1.1). Haemolymph buffer values are not altered according to acid-base status (Harrison *et al.*, 1990b). Harrison (1989) has calculated that the alimentary canal contains greater than 30% of total body water and a large fraction of bicarbonate. Therefore, it seems the lumen of the alimentary canal is an important potential sink for net acid equivalents during regulation. A great deal is now known about the

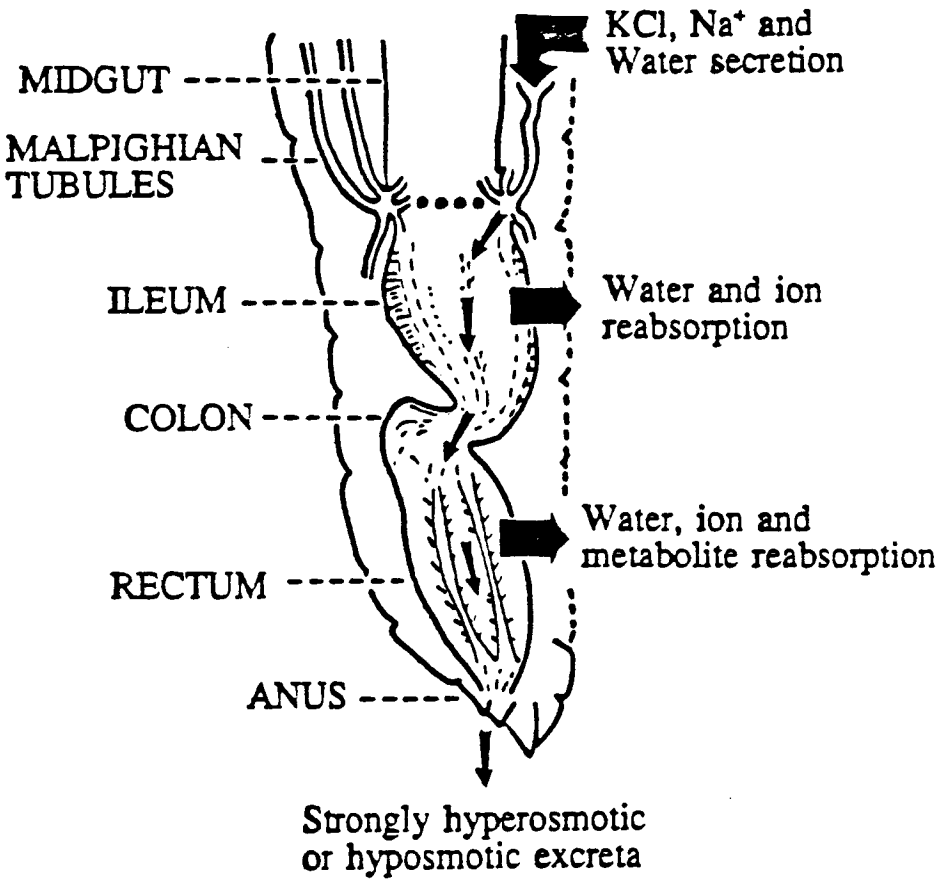

Figure 1.1. Anatomy of the locust excretory system. The flow of urine is indicated by the *thin arrows* and transfer across the epithelia is indicated by the *thick arrows*. (From Audsley, 1991).

acid-base transport mechanisms in locust hindgut epithelia but the anatomy of the locust excretory system should first be described.

Anatomy of the Locust Excretory System

The locust excretory system consists of approximately 250 Malpighian tubules that insert between the midgut and hindgut complex (see Fig. 1.1; Garrett *et al.*, 1988). The hindgut is divided into 3 segments: ileum, colon, and rectum. The ileum and rectum are the main sites of selective ionic transport and water reabsorption with the ileum reabsorbing the bulk of fluid isosmotically, while the rectum adjusts the final osmolarity of the faeces (Phillips *et al.*, 1986).

The Malpighian tubules consist of a simple epithelium, are 10-23 mm in length, and are distally blind-ended (Bradley, 1985; Garret *et al.*, 1988). Approximately 30% of the tubules are directed anteriorly and anchored to trachea and caecae. The rest of the tubules run posteriorly along the alimentary canal and rest on rectal pads with their associated tracheal connections or float freely in the haemocoel cavity. Malpighian tubules can be categorized into 3 sections on the basis of histology but there is no evidence for physiological differentiation (Garrett *et al.*, 1988).

Insect Malpighian tubules are functionally analogous to the vertebrate glomerulus in that both produce a primary isosmotic urine (Phillips, 1981). Locust Malpighian tubules drive fluid secretion by active KCl transport (Maddrell and Klunsuwan, 1973) rather than by pressure-driven filtration at the glomeruli. This K^+ rich isosmotic primary urine flows into the hindgut (and midgut; Dow, 1986) where selective reabsorption occurs in the ileum and rectum. The rate of fluid secretion is usually proportional to the concentration of K^+ in the external medium bathing *in vitro* preparations. This is true for most insects studied except in the case of *Rhodnius* (and

other blood-sucking insects), where Na^+ ingestion is high and therefore fluid secretion is both Na^+ and K^+ driven (Phillips, 1981).

Acid-Base Transport in the Locust Hindgut

The pH of the gut content differs from that of haemolymph over much of the gut length (see Fig. 1.2; Phillips *et al.*, 1986; Thomson *et al.*, 1988). Earlier *in situ* studies showed that the locust rectum was capable of actively acidifying the luminal contents even after rinsing several times with an alkaline buffer (Phillips, 1961). Thompson *et al.*, (1988a) showed conclusively that protons are actively secreted across the apical membrane of the rectum against a large electrochemical gradient. This active proton extrusion is Na^+, K^+, Cl^-, Mg^{2+}, and Ca^{2+} independent and can be inhibited by azide. This locust proton pump is similar in properties to that described in the turtle bladder (Thomson, 1990).

Both the ileum and rectum have been identified as sites of HCO_3^- reabsorption. Thompson and Phillips (1985) showed that HCO_3^- reabsorption is 100% inhibited by DIDS. Thompson (1990) has proposed that the powerful proton pump in the apical membrane acidifies the rectal lumen, thereby titrating HCO_3^- to CO_2, which diffuses across the apical membrane and combines in the cell with OH^- formed behind the H^+ pump. The resulting HCO_3^- moves to the haemocoel.

Lechleitner *et al.*, (1989) showed that HCO_3^- reabsorption occurs in everted ileal sacs and this absorption is inhibited by extracts of ventral ganglia (VG) and corpora cardiacum (CC). The locust ileum also secretes acid equivalents (Audsley, 1991), and although the mechanism has not been described, it seems probable that this is what generates the high rates of HCO_3^- reabsorption observed in this segment. Acid secretion in the ileum is inhibited by extracts of CC, VG, and by addition of cAMP (Audsley, 1991). HCO_3^- reabsorption can also be inhibited by addition of these

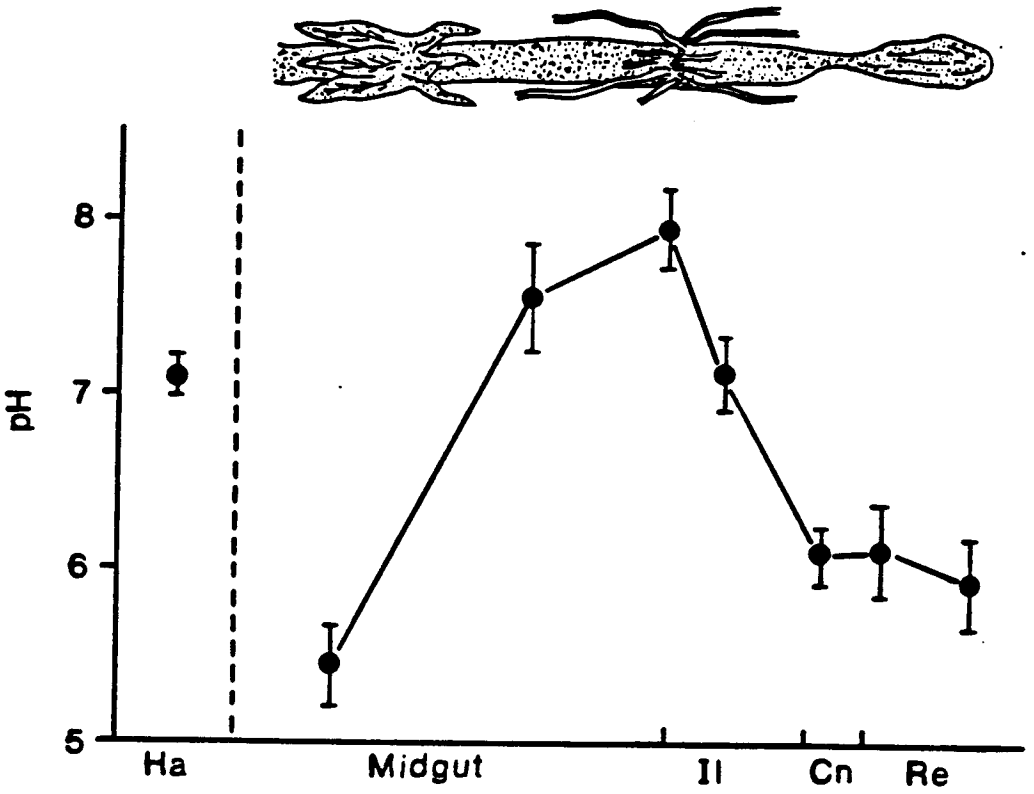

Figure 1.2. *In vivo* profile of the locust alimentary canal. Ha, haemolymph; Cn, colon; Re, rectum. *N*= 10 for each value; Mean ± S.E. (From Thomson, 1990).

factors to the serosal side of flat sheet preparations. Acid secretion rates are significantly reduced in the rectum by serosal addition of cAMP (Thomson *et al.*, 1988a). Therefore, both the rectum and ileum are sites where titratible acidity can be modified via putative hormonal mechanisms. Hence these sites could be implicated in haemolymph pH homeostasis. In support, the pH of rectal contents increases to 6.2 after feeding from below 5.0 in starved individuals (Speight, 1967; Harrison, *et al.*, submitted).

Ammonia Secretion in Locusts

The Malpighian tubules secrete large amounts of amino acids (50mM), particularly proline, into the hindgut (Chamberlin, 1981). Proline and other amino acids have been shown to act as respiratory substrates to support active Cl^- transport and hence fluid reabsorption in both the rectum and ileum (Chamberlin and Phillips, 1982; Lechleitner and Phillips, 1989; Peach and Phillips, 1991). Metabolism of these amino acids leads to the production of ammonia.[1] Thomson *et al.*, (1988b) have demonstrated that the rectum secretes significant quantities of ammonia into the rectal lumen as NH_4^+ (rather than NH_3) primarily by apical, amiloride-sensitive Na^+/NH_4^+ exchange. More recently, Peach (personal communication) has characterized ammonia secretion in the ileum and found even higher rates of ammonia secretion which is independent of luminal Na^+ levels. If the diffusion-trapping mechanism proposed for the vertebrate kidney (Good and Knepper, 1985) were present in the excretory system of the locust, one would expect the rate of ammonium secretion ($J_{NH_4^+}$) to increase as the hindgut lumen becomes more acidic. This is not the case for either the rectum (Thompson *et al.*, 1988b) or the ileum (Peach, personal

[1] Ammonia refers to total ammonia: i.e. molecular ammonia (NH_3) plus ammonium ion (NH_4^+).

communication). Therefore an alternative model to diffusion trapping must be proposed for both locust ileum and rectum.

The acidic rectal contents, coupled with the large amounts of ammonia production, again suggest the rectal epithelium is involved in pH regulation. Moreover, a low luminal pH could increase phosphate reabsorption and precipitate urate and ammonium salts (Harrison and Phillips, submitted). The discovery of high ammonia secretion rates in locust hindgut also raises the question of the relative importance of ammonia and uric acid as excretory end-products (Phillips 1986). The traditional dogma is that NH_4^+ excretion is associated with copious water loss whereas urates are the major nitrogenous end-product in dry environments where water conservation is critical to survival (Cochran, 1985). Harrison *et al.* (submitted) report, however, that ammonium urate is in fact less soluble than uric acid and excess NH_4^+ is precipitated apparently with organic anions in locust excreta, which can be quite dry. It is not known whether insect Malpighian tubules might secrete NH_3, and if so, how important this might be compared with ammonia secretion demonstrated in hindgut.

Despite the recent research into acid-base regulation in terrestrial insects, no research has been done on the involvement of Malpighian tubule secretion in haemolymph acid-base regulation. In Chapter 2, I measure major acid-base parameters in Malpighian tubule secretion collected by cannulation *in vivo*. The response of these tubules to acid injection into the haemocoel was assessed by measuring changes in pH, CO_2, calculated Pco_2 and HCO_3^-, and total buffer value in the secretion. Ammonia and urate values for tubular fluid were also measured to assess their relative contribution to nitrogenous excretion and acid-base balance. In Chapter 3, I describe the effect of feeding and temperature on acid-base physiology of Malpighian tubules

and implications for haemolymph pH regulation. Finally, in Chapter 4, I discuss the similarities and differences between the vertebrate nephron and the locust excretory system in whole body acid-base regulation.

CHAPTER 2

Acid-Base Parameters of the Malpighian Tubules and Response to Acidosis

Introduction

Regulation of acid-base balance in terrestrial insects has been a neglected area (Chapter 1). Phillips *et al.* (1986) proposed that the process is probably similar to that in vertebrates: ie. the respiratory (tracheal) system regulates haemolymph Pco_2 and the excretory system (consisting of Malpighian tubules and hindgut) regulates haemolymph bicarbonate levels. As a result, haemolymph pH is maintained within narrow limits as recently reported for locusts during rest, temperature change and activity (Harrison, 1988; 1989; Harrison *et al.*, 1991a).

Indeed, during hopping and recovery at 35° C, regulation of pH back to resting values can be largely explained by increased tracheal ventilation (Harrison *et al.*, 1991a). In contrast, recovery from acidosis caused by injection of HCl into desert locusts is almost entirely due to increase in haemolymph bicarbonate levels (Harrison *et al.*, submitted). Although the source of this additional haemolymph bicarbonate has not been firmly established, the excretory system of the locust seems likely. A significant fraction of the acid removed from the haemocoel within a few hours of acid injection appears (temporarily at least) in the midgut contents (Harrison *et al.*, submitted). This transfer may be partially mediated by the Malpighian tubules.

It is now firmly established that locust ileum and rectum secrete H^+ and NH_4^+, and absorb OH^- and HCO_3^-; moreover, these processes can be substantially modified by cAMP, and by corpora cardiaca and ventral ganglia factors (Thomson *et al.*, 1988a, 1988b, 1991; Lechleitner *et al.*, 1989; Audsley, 1991; Chapter 1). However, the potential contribution of Malpighian tubules to acid-base regulation has not been investigated in any terrestrial insect. Even simple

measurements of the primary determinants of acid-base excretion (ie the concentrations of CO_2, levels of bicarbonate, phosphate, ammonia, organic acids, and total buffer capacity) have never been measured in tubule secretion. The exception is some *in vitro* values for tubular bicarbonate in corixids living in alkaline lakes, an unusual situation for most insects (Cooper *et al.*, 1987). Previous relevant information for terrestrial insects is restricted to some isolated estimates of pH, urate or phosphate levels in fluid collected from Malpighian tubules isolated *in vitro* (reviewed by Phillips, 1981; also O'Donnell *et al.*, 1983; Andrusiak *et al.*, 1980; Hanrahan *et al.*, 1984). In no insect has the response of tubular acid-base parameters to changes in haemolymph pH been investigated. In this chapter I describe such a study, using tubules of the desert locust cannulated *in situ* and with acidosis initiated by HCl injection into the haemocoel.

Materials and Methods

Animals

Adult female *Schistocerca gregaria*, 2-3 weeks past final moult, were used in all experiments. Locusts were maintained as described previously (Thomson *et al.*, 1988a).

Collection of Malpighian Tubule Fluid in situ

Animals were restrained under dry cotton to prevent any visual stimulus. An incision was made in the third and fourth abdominal segments, and the gut was gently lifted with glass hooks. The gut was ligated just anterior to the point of tubule entry and at the posterior end of the ileum. The ileum was then partially severed near the tubule junction and the gut contents were gently teased out with a glass hook. A glass cannula (1.0 mm wide) was inserted into the anterior ileum

and secured with surgical thread. (The end of the cannula was thinly coated with wax to ensure a tight seal). The gut and cannula were then gently inserted back into the abdomen and the incision sealed with wax. The cannula and gut were then rinsed with 100mM KCl to ensure no blockage was present and the rinse solution was removed with a syringe. Rates of tubule fluid production were calculated by measuring the fluid advancement through the glass cannula at intervals and calculating volume from the inside diameter of the tubing.

Experimental Protocols

Locusts were placed in individual containers and fed lettuce *ad libitum* for 2 hours at 35° C the day before cannulation. Animals were then starved for 18-24 hours at 21° C (the experimental temperature) with access to cotton soaked with distilled water, and then cannulated as above.

The influence of haemolymph acidosis on composition of Malpighian tubule fluid was assessed as follows: When 20 µl of tubular fluid had been collected in the cannula (usually after 2-3 hours), this fluid was drawn into a length of fine PE 20 tubing by a Hamilton syringe. This constituted the pre- injection sample. Then acidification was initiated by injecting 25 µl of 0.4 M HCl into the abdominal haemocoel through the 7th or 8th intersegmental membrane. A further 20 µl of tubular secretion was collected after another 2-3 hours. This constituted the post-injection sample on the same locust. A second 20 µl post injection aliquot was often collected , but no significant difference in solute composition were observed as compared to the first post-injection aliquot. Only data for the first period is therefore included in this report. Since my method precluded reabsorption of tubule fluid in the midgut and hindgut, the resulting reduction

in haemolymph volume must be considered. In most later experiments, 20 µl was collected before and another 20 µl of tubule fluid collected after injection of acid or NaCl into the haemocoel. This represents roughly 10% of haemolymph volume. No more than 20% of haemolymph volume was estimated to be secreted in any of the long experiments lasting up to 8 hours. Preparations continued to secrete at low rates for 1-2 days.

As a control for possible diuresis resulting from fluid injection, other locusts were treated in the same way, but they were injected with 25 µl of 0.4 M NaCl. Finally a third group were not injected but otherwise treated identically to assess changes in composition of tubular secretion and haemolymph with time due to hindgut cannulation.

Acid-Base Measurements

Tubular fluid pH was measured with glass-microelectrodes on 1-2 µl samples as previously described (Harrison *et al.*, 1990b). Total CO_2 was measured with 4 µl aliquots using the technique of Boutilier *et al.* (1985). Bicarbonate and Pco_2 were calculated from total CO_2 and pH assuming carbonic acid pK values and CO_2 solubility coefficients were identical to those in locust haemolymph. (Harrison, 1988).

The method of Chamberlin and Phillips (1982) was used to verify that collection of fluid from a cannula did not result in CO_2 loss. As before, an incision was made in the third and fourth abdominal segments. Instead of placing a cannula in the gut, ligatures were made just anterior and posterior to the point of tubule entry. Fluid was allowed to collect in the distended sac, and pH and total CO_2 determinations were made on fluid collected by puncturing the sac with a Hamilton syringe. In preliminary experiments, the pH and total CO_2 values estimated in this way

were similar to values determined by cannulation.

Biochemical Measurements

Total urate and inorganic phosphate were determined on 5 µl samples of tubular fluid diluted 100-fold with distilled water. Urate concentrations were determined spectrophotometrically at 520 nm with a commercial kit (Sigma) and uric acid stock solutions as standards. Inorganic phosphate was determined spectrophotometrically at 820 nm by the technique of Chen *et al.* (1956). Total ammonia (ammonia + ammonium) concentrations were determined by the enzymatic assay of Kun and Kearny (1974). The non-bicarbonate buffer value (ß) for tubular secretion was determined using samples diluted 100-fold with 100 mM KCl. Dilute samples were acidified with 20 mM HCl until the pH was below 4.0. Samples were then stirred for 2 hours to drive off CO_2 and HCO_3^-. Buffer value was then determined from pH 6.4 to 7.0 by titrating with 20 mM NaOH using a PHM 84 research pH meter, TTT 80 titrator, and ABU 80 autoburrette (Radiometer, Copenhagen, Denmark).

Statistics

All data are presented as mean ± standard error (S.E.) with *N* indicating the number of locusts. Paired *t*-tests were used to determine significant differences in composition of secretion caused by acid injection. Where paired *t*-tests were not appropriate, significance of difference between means was determined by one way ANOVA, with $p<0.05$ accepted as significant.

Results

Time course of fluid secretion

The rate of fluid secretion (J_V) by insect Malpighian tubules isolated *in vitro* generally falls with time (reviewed by Maddrell, 1980). I therefore first followed J_V with time for the full complement of locust tubules in starved animals, as determined by hindgut cannulation *in situ* (Fig. 2.1). The initial mean J_V over the first 2 hours varied between 8 and 15 µl h^{-1} in different experiments and gradually fell by 20-40% over 9 hours. These rates are within the range of values previously reported for the full complement of *Schistocerca* tubules using *in vitro* and other *in vivo* methods (reviewed by Phillips, 1981).

The injections of 25 µl of 0.4 M NaCl or HCl into locusts between the 2nd and 3rd hour after cannulation had no measurable effects on J_V as compared to uninjected controls (Fig. 2.1). There was no statistical difference between J_V values for the three treatment groups except for HCl injected locusts at the third hour.

Time Course of Tubular Acid-Base Parameters in Uninjected Locusts

The pH of tubular fluid from starved, uninjected locusts did not change significantly over 7 hours and averaged 6.63 (Fig. 2.2). This is 0.61 pH units below the initial haemolymph value of 7.24, which fell slightly to 7.11 over 6 hours of cannulation (Fig. 2.3). Initial values in tubular fluid over the first 2 hours were: total CO_2 (8.9mM), Pco_2 (55 torr), and HCO_3^- (6.5 mM), and these did not change significantly over 8 hours (Fig. 2.4).

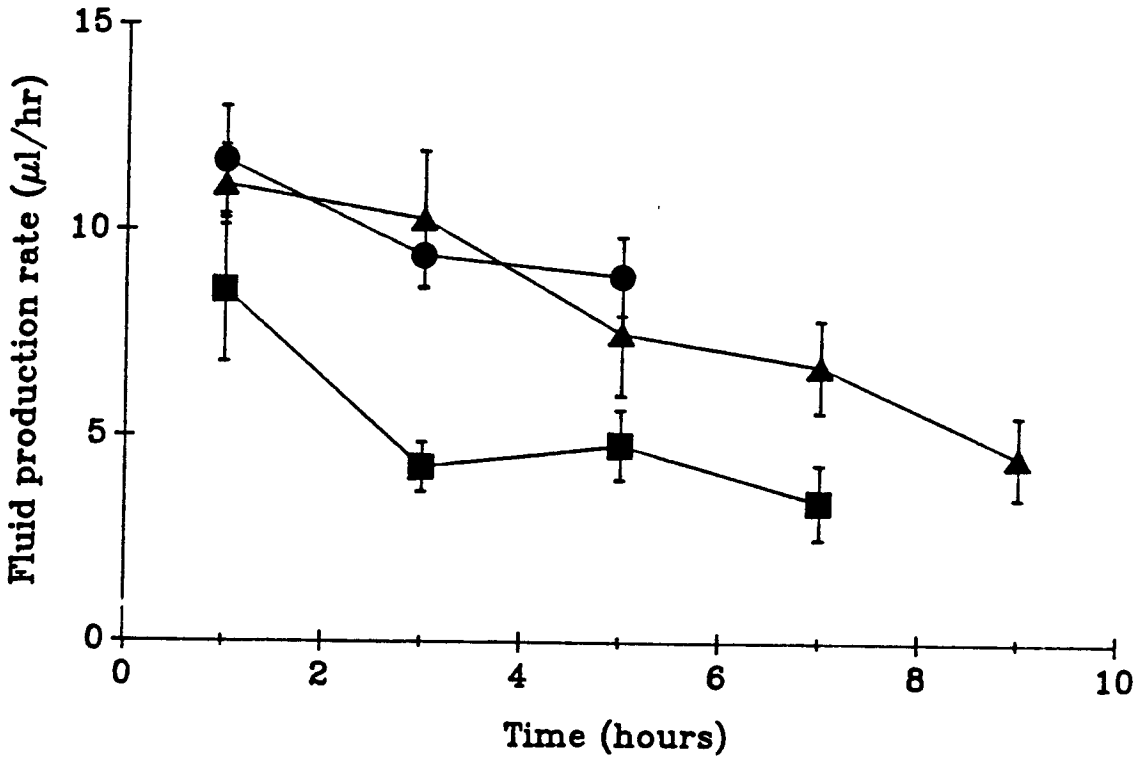

Figure 2.1. Fluid production rates (J_V) for the full complement of Malpighian tubules of starved locusts cannulated *in situ* at 21° C. Mean ± S.E. (N=10-20) for J_V over an equal period before and after the point of injection (ie: 1 or 2 hour total period). Values for the three treatment groups [uninjected (●), HCl-injected (■), and NaCl-injected (Δ)] are not significantly different except for the HCl group at 3 hours after cannulation.

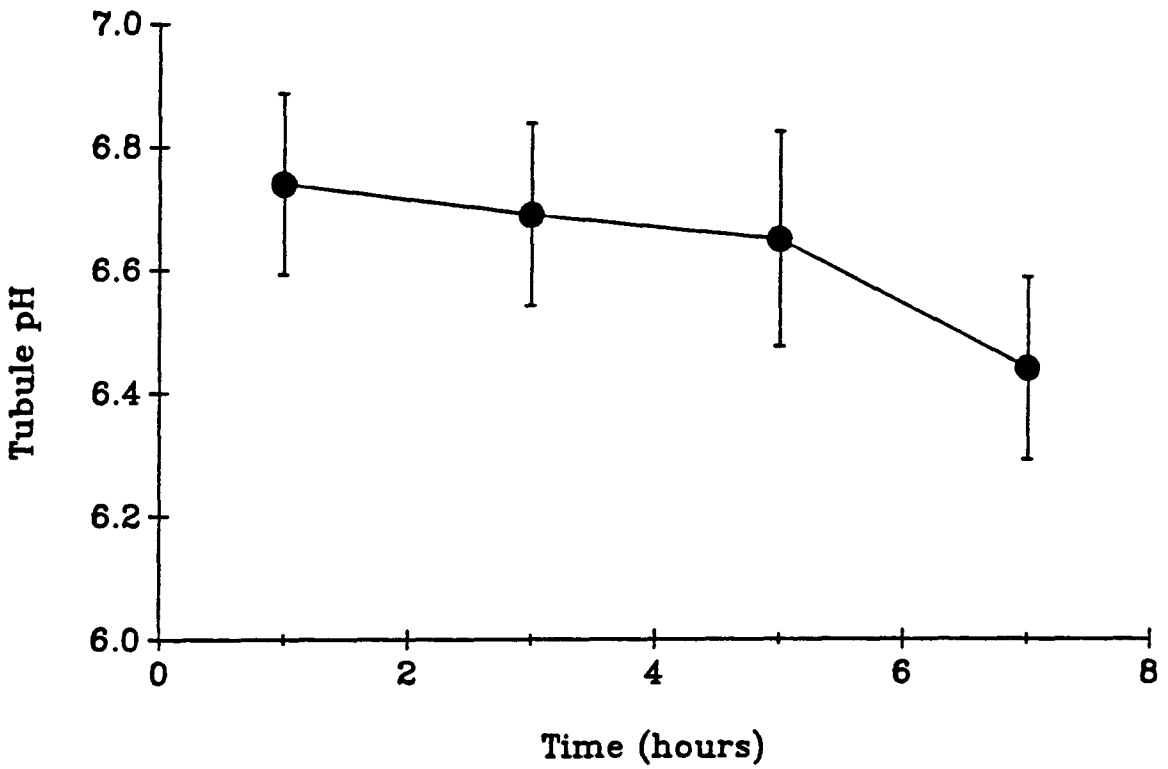

Figure 2.2. Tubule pH in uninjected locusts cannulated *in situ* at 21° C (Mean ± S.E., N=5-9). The apparent decrease in pH over 8 hours is not significant (p>0.5).

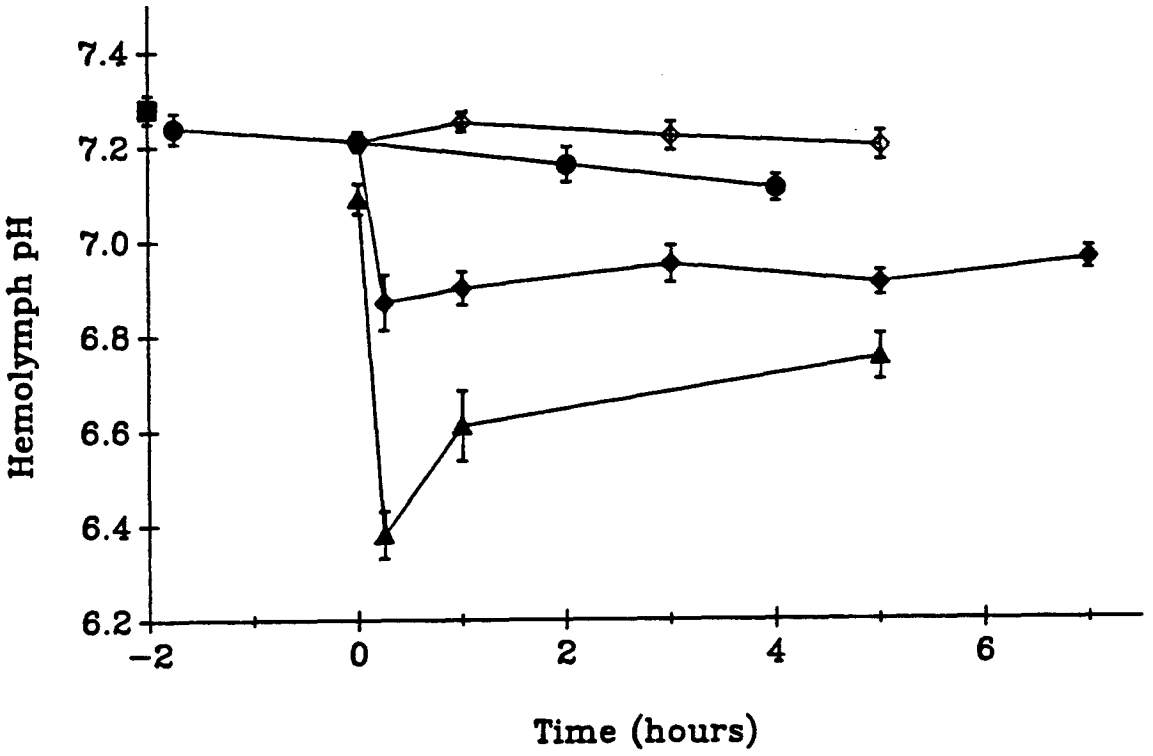

Figure 2.3. Haemolymph pH relative to the time of injection (0 h) for different experimental groups of locusts starved one day at 21° C. Mean ±S.E. (*N*=4-9). Values are for haemolymph collected at the neck (all symbols except ▲), or near the abdominal injection site (▲), of locusts injected with 25 µl of 0.4 M NaCl (◊) or 0.4 M HCl (♦) or uninjected (●). The pH of haemolymph sampled from the neck prior to cannulation is also given (■). HCl injection caused a significant decrease (p=0.000) in haemolymph pH within 15 min (first sample) of injection. Haemolymph pH remained depressed after HCl injection for 7 hours.

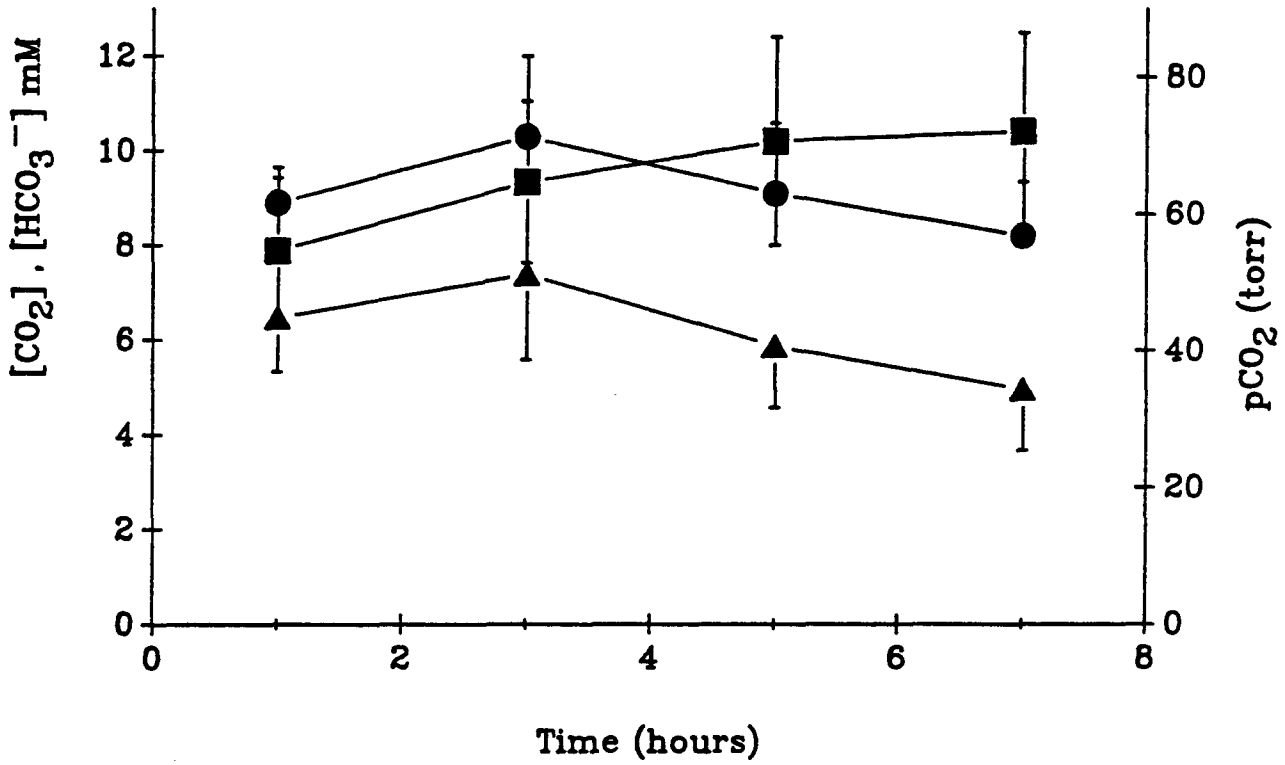

Figure 2.4. Total CO_2 (●), Pco_2(■), and HCO_3^- (▲) concentrations in haemolymph collected at the neck from uninjected, starved animals at 21° C as a function of time after cannulation. (Mean ± S.E. *N*=4-5). There was no significant change in any of these variables due to injection, or with time after injection.

Effect of HCl or NaCl injection on Haemolymph Acid-Base Parameters

Before cannulation, locust haemolymph pH averaged 7.28. Injection of 25 µl of 0.4 M NaCl into the abdomen of locusts (control) did not significantly change haemolymph pH, which remained above 7.2 for at least 5 h after injection (Fig. 2.3). Likewise haemolymph total CO_2, Pco_2 and HCO_3^- were not significantly changed by NaCl injection and remained close to 8.0 mM, 18 torr, and 7.5 mM respectively (Fig. 2.5). Within 15 min of injecting 25 µl of 0.4 M HCl into the abdomen, haemolymph pH fell by 0.34 units to 6.87, as measured at the neck (30-50 µl sample) and to 6.4 as measured by abdominal sample near the site of injection. Apparently, complete mixing in the haemolymph compartment is not attained over several hours.

Abdominal injection of HCl had a marked effect on acid-base parameters of haemolymph collected at the neck (Fig. 2.6). Initial values before injection were similar to those for the NaCl injected group (Fig. 2.5). Over the first 15 min after HCl injection, total CO_2 fell significantly by over 30% to 5.2 mM, Pco_2 increased temporarily but not significantly, and calculated HCO_3^- fell by 40% to 4.2 mM. Total CO_2 and HCO_3^- remained significantly depressed 7 hours after injection (Fig. 2.6).

In summary, changes in haemolymph acid-base parameters (as measured at the neck) caused by acid injection were sustained for at least 7 hours. Subsequent studies on composition of tubule fluid were normally made within 4 hours of acid injection.

Effect of Haemolymph Acidosis on Composition of Malpighian Tubule Fluid

Acid injection into the haemocoel initially caused a significant decrease in the pH of tubular fluid by 0.46 pH units as compared to NaCl injected controls (Fig. 2.7). However tubular

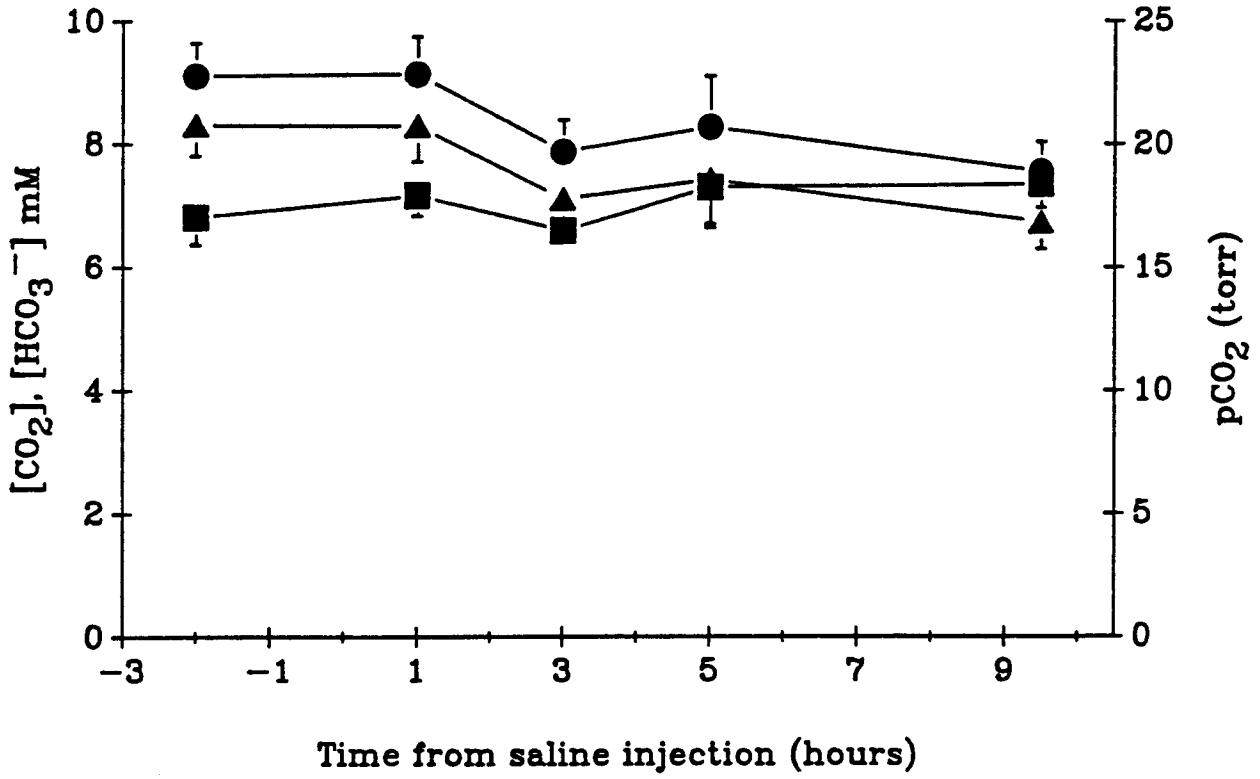

Figure 2.5. Total CO_2 (●), Pco_2 (■), and HCO_3^- (▲) concentrations in haemolymph collected at the neck from NaCl-injected, starved locusts at 21° C as a function of time (injection at 0 hour). Mean ± S.E. (*N*=5-9). There was no significant change in any of these parameters due to injection, or with time after injection.

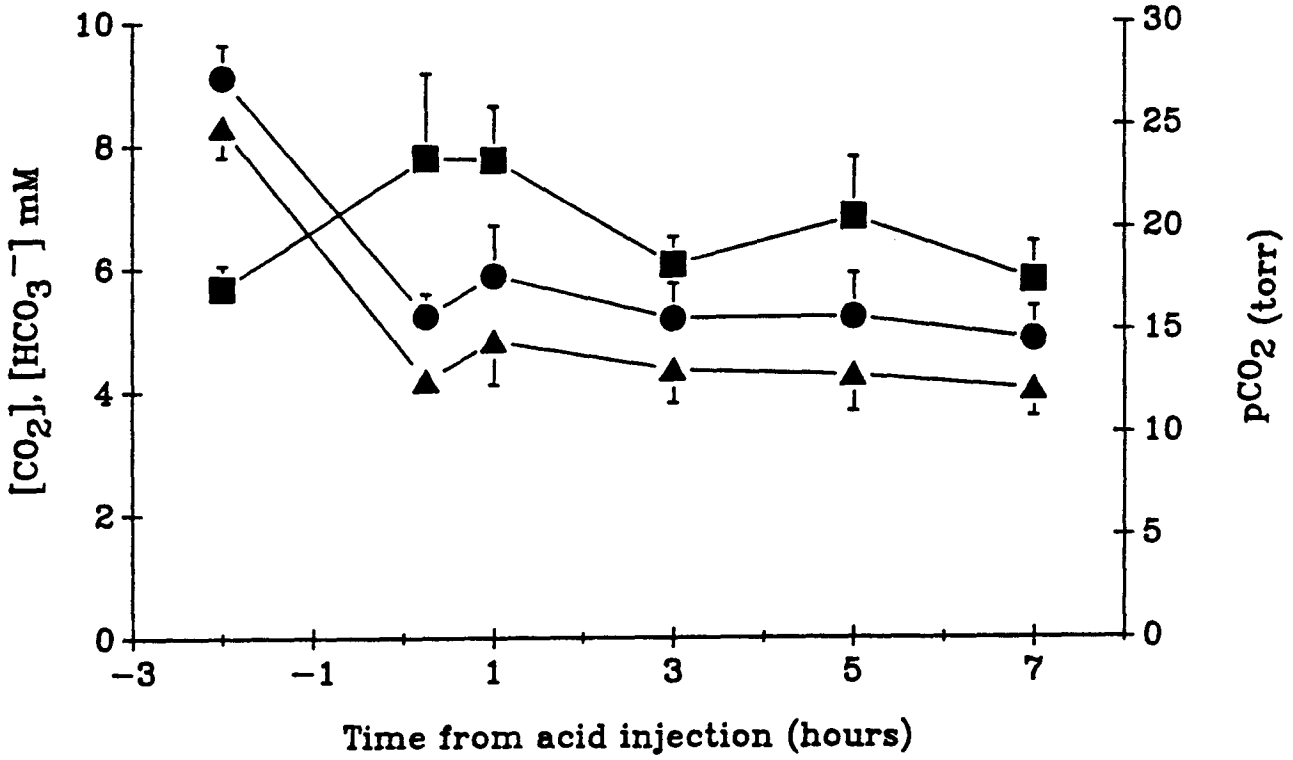

Figure 2.6. Total CO_2 (●), Pco_2 (■), and HCO_3^- (▲) concentrations in haemolymph collected at the neck from HCl-injected starved animals at 21° C, as a function of time (injection at 0 hour). Mean ± S.E. (N=5-8). Pco_2 did not change significantly after acid-injection (p=0.13); but both total CO_2 and HCO_3^- levels significantly decreased (p=0.028, 0.007 respectively) after injection and remained depressed for the next 7 hours.

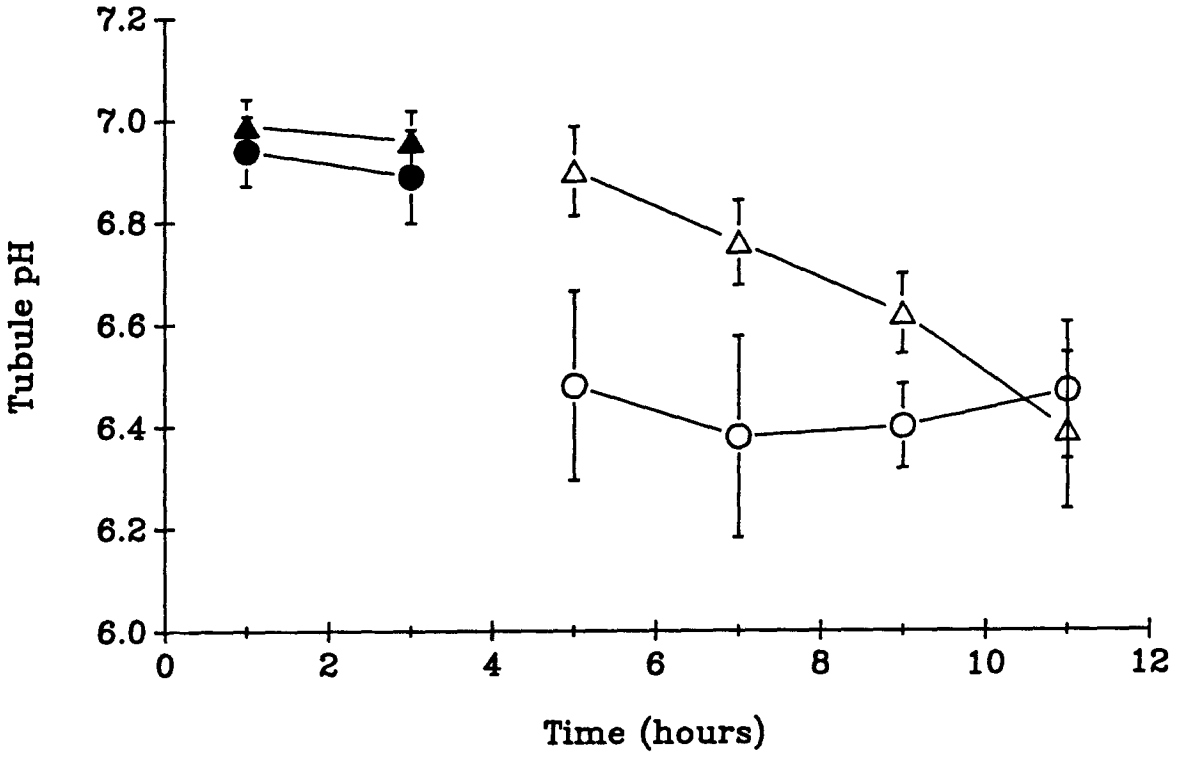

Figure 2.7. The pH of Malpighian tubule fluid prior to injection (filled symbols) and after injection (open symbols) with either NaCl (Δ) or HCl (O). Mean ± S.E. (N=10-14). The pH was decreased significantly (p=0.001) immediately after HCl injection relative to NaCl injected controls.

fluid pH in the NaCl injected controls also gradually fell with time so that after 6-8 hours there was no difference between the acid and salt injected groups. Thus the cannulation/injection procedure itself caused a slow increase in tubule acidity. Therefore, in the remaining series of experiments, I compared the composition of Malpighian tubule fluid pooled over approximately 2.0-2.5 hours before injection with tubular fluid collected within 3 hours after injection from the same locust.

The effect of injecting either 10 μmol of NaCl or HCl on tubular acid-base parameters is shown in Table 2.1. Before injection, the tubule fluid pH of 6.9 was similar in acid and NaCl injected groups. Acid but not NaCl injection caused a significant decrease in tubule fluid pH by 0.46 units to pH 6.48. Total CO_2 did not change significantly in the acid injected group, so that the effect of increased tubular acidification was to titrate HCO_3^- (which fell significantly from 5.5 to 3.2 mM), to CO_2. As a result, tubule fluid Pco_2 doubled after acid injection. There is no indication from these results that Malpighian tubules contributed additional HCO_3^- to the haemolymph after acid injection.

By comparison, the NaCl injected controls showed no change in Pco_2 after injection. However total CO_2 and HCO_3^- in tubule fluid were initially higher in this group and both declined slightly but significantly after injection.

There is no indication that acid injection stimulated long- term active acid secretion by Malpighian tubules, because the final pH difference between haemolymph and tubular fluid was not significantly different in uninjected, NaCl injected and HCl injected groups (Table 2.2).

After 4 hours, tubule fluid was on average 0.56 to 0.75 units acid to haemolymph regardless of treatment. The lower tubule fluid pH initially observed (2 hours) after acid injection

Table 2.1. Effect of HCl and NaCl injection into the haemocoel on acid-base status of Malpighian tubule fluid in *Schistocerca gregaria* at 21° C. Pre samples were secreted over a 2-3 hour period before injection, post samples were secreted over a 2-3 hour period after injection.

	HCl injected		NaCl injected	
	pre-inj	post-inj	pre-inj	post-inj
pH	6.89 ± 0.07	6.42* ± 0.1	6.91 ± 0.08	6.81 ± 0.07
Total CO_2 (mM)	6.2 ± 0.8	5.5 ± 0.7	11.8 ± 1.3	9.1* ± 0.9
Pco_2 (torr)	24 ± 2.8	51* ± 11.3	45 ± 5.0	42 ± 3.8
HCO_3^- (mM)	5.1 ± 0.7	3.2* ± 0.4	9.8 ± 1.3	7.3* ± 0.9
Non bicarbonate buffer value (ß) (mmol l^{-1} pH $unit^{-1}$)	9.5 ± 1.0	10.4 ± 1.2	10.4 ± 1.1	15.5* ± 2.6

Mean ± S.E. (*N*=9-14)

* Significantly different from pre-injection value, paired t-test, $p<0.05$.
Non-bicarbonate buffer value is for the pH range 6.4-7.0.

Table 2.2. Final pH determinations of haemolymph (neck sample) and tubule fluid at the end of experiments** on three groups of locusts at 21° C.

	Uninjected	HCl injected	NaCl injected
1) Hemolymph pH	7.15 ± 0.04	6.95 ± 0.01	7.12 ± 0.03
2) Tubule pH	6.39 ± 0.21	6.40 ± 0.11	6.51 ± 0.11
3) Difference (1-2)	0.75 ± 0.18	0.56 ± 0.11	0.60 ± 0.13

Mean ± S.E. (N=9-14). The pH difference across the tubule wall (3) was not significantly different between treatments.

**Values were made on fluids collected between 3 and 6 hours after injection time and 6 to 9 hours after cannulation.

(reported in Table 2.1 and Fig. 2.7) may simply reflect greater haemolymph acidity of the former group rather than enhanced acid secretory activity. In particular, different pH values of haemolymph collected from the abdomen and neck (Fig. 2.3) make it difficult to decide the effective pH experienced by tubules after acid injection (see Table 2.3).

Tubular Fluid Buffer Values

The rate of acid secretion is determined not only by tubular pH, but by the buffer capacity (ß) which might change following acid injection. I measured the non-bicarbonate buffer value (ß) for Malpighian tubule fluid over the pH range 6.4-7.0 observed in earlier experiments (Table 2.1). Titration indicated a constant value for ß between pH 6.4 and 7.0 (data not shown). The value of ß was 9.5 mequiv l^{-1} pH $unit^{-1}$ before injection, and this was unchanged after HCl injection. However, NaCl injection caused a significant increase in ß to 15.5 mequiv l^{-1} pH $unit^{-1}$. The nature of this increased buffer capacity was investigated by measuring the levels of major potential buffers previously reported in tubule fluid, namely phosphates and urates. Moreover, ammonia will trap secreted H^+ to form NH_4^+. Indeed enhanced ammonia production by vertebrate proximal tubules is a major mechanism of removing excess acid (Pitts, 1973). I therefore also measured total ammonia concentrations in locust tubular fluid.

Phosphate, Urate, and Total Ammonia Levels in Tubule Fluid

As shown in Table 2.3, phosphate levels in tubule fluid increased with time in uninjected locusts, and this was significantly enhanced (4-fold increase) by NaCl injection but not HCl injection. This difference in phosphate secretion may account for the greater ß value of tubular

Table 2.3. Composition (mM) of *Schistocerca gregaria* Malpighian tubule fluid collected *in situ* at 21° C.

	Uninjected		HCl injected		NaCl injected	
	pre	post	pre	post	pre	post
Urate	1.6 ± 0.4	1.6 ± 0.5	5.3 ± 0.8	2.2* ± 0.2	3.6 ± 0.4	3.8 ± 1.3
Phosphate	2.7 ± 0.7	4.8* ± 1.2	4.5 ± 1.0	6.7* ± 1.0	4.5 ± 1.2	16* ± 3.9
Ammonia	5.2 ± 1.0	8.1* ± 1.8	4.6 ± 0.7	6.7* ± 0.9	5.3 ± 0.9	6.2 ± 1.3

Mean ± S.E. (*N*=9-21 locusts) for fluid collected by cannulation over 2-3 hours before and 2-3 hours after HCl or NaCl injection, and during same time periods for uninjected locusts.

* Indicates were significantly different from pre- injection values on the same animals.

fluid from NaCl- as opposed to HCl-injected locusts (Table 2.1). However the contribution of phosphate to acid removal depends on the form of this anion that is actively secreted by tubules, and this is unknown (reviewed by Phillips, 1981). Average urate concentrations varied from 1.6 to 5.3 mmol l^{-1} in the three treatment groups prior to injection. Urate levels decreased significantly from 5.3 to 2.2 mmol $^{-1}$ following HCl injection (Table 2.3).

Total ammonia was 4 to 5 mM in tubular fluid prior to injection in all three groups. While there was a small but significant increase in total ammonia to 6.7 mM following acid injection, this could reflect a change with time because a similar significant change with time was observed in uninjected locusts (Table 2.3).

Discussion

This study provides the first comprehensive measurements of the major acid-base parameters for Malpighian tubule secretion in a terrestrial insect *in situ*. In control locusts starved for one day, both total CO_2 and HCO_3^- levels are similar in haemolymph and tubular fluids at 8-9 and 7-8 mM respectively. In contrast, calculated Pco_2 levels (55 torr) are nearly 3 times higher in tubular fluid than haemolymph. Tubule fluid, at pH 6.63 is 0.5 pH units more acid than haemolymph in the control group, suggesting that locusts already may experience an acidotic state within one day of starvation. In support, Harrison *et al.* (submitted) found that the pH of faecal pellets from similarly starved locusts averaged 4.62, compared to 6.2 in feeding animals. Thus control locusts used in my study may have already been responding to a natural acid load prior to acid injection, and this may have diminished the response to my experimental acidotic challenge. Regardless, my results permit an estimate of the maximum capacity of the full

complement of locust tubules to eliminate excess acid equivalents.

Using measured J_V (initial average of 10 µl hour^{-1}; Fig. 2.1), a median value for the measured pH difference between haemolymph and tubular fluid under the three conditions studied (0.6 pH units), and the measured highest buffer values for tubular fluid (15 mmol l^{-1} pH unit^{-1}) for NaCl injected locusts (Table 2.1), the estimated maximum rate of excess acid removal by locust Malpighian tubules (J_{H^+}) is 0.09 µmoles h^{-1}. This value could be considerably less if more conservative values are used in these calculations. While tubule fluid was initially much more acid after HCl injection (Table 2.1), so was the haemolymph. It is therefore not possible to conclude that active tubular acid secretion rate was stimulated by HCl injection, especially given the heterogeneity in haemolymph pH observed between the neck and abdomen (Fig. 2.3). Clearly mechanisms and possible control of tubular acid secretion will require *in vitro* studies, where the composition of the fluid bathing isolated tubules can be precisely controlled. Nevertheless my *in vivo* studies do provide the necessary groundwork for such future studies.

Given that 10 µmoles of acid was injected into locusts, the Malpighian tubules clearly do not have the capacity (at a maximum J_{H^+} of 0.09 µmoles h^{-1}) to return haemolymph pH to normal values within an 8 hour period, as observed by Harrison *et al.* (submitted) in a parallel study on uncannulated animals. The potential regulatory capacity of the hindgut is many times greater. Both locust ileum and rectum actively secrete acid *in vivo* at 1.5 µmoles h^{-1} cm^2 in the absence of a pH difference (Thomson *et al.*, 1988a, 1988b, 1991). Correcting for surface area of these hindgut segments, the locust rectum (0.62 cm^2) and ilea (0.4 cm^2) can still secrete H^+ at 0.6 and 0.3 µmoles hour^{-1} respectively against a gradient (0.6 pH units) comparable to the maximum developed by the tubule epithelium. Clearly all three segments of the locust excretory system

(tubules, ileum, and rectum) contribute to acid excretion, with H^+ secretory capacity increasing and luminal pH decreasing posteriorly. Fall in luminal pH during passage through the hindgut has been observed by Thomson *et al.*, (1988a) and Harrison *et al.* (submitted).

Haemolymph pH measured either from the neck or abdomen did not recover substantially to control values even after 8 hours (Fig. 2.3). This lack of recovery in the cannulated animals is evidence for a role of the locust hindgut in haemolymph pH regulation, suggesting interruption of tubule fluid reabsorption in the midgut and hindgut prevents bicarbonate reabsorption necessary for haemolymph pH recovery. In a parallel study using uncannulated desert locusts, haemolymph pH measured at the neck recovered to control values within 8 hours of a similar acid injection, accounted for by a rise in haemolymph HCO_3^- (Harrison *et al.*, submitted).

Calculations of J_{H^+} presented above using buffer values and pH differences do not evaluate the additional acid removal which may be associated with total ammonia excretion. I did not observe increased tubular ammonium secretion attributable specifically to HCl injection; however, using the typical total ammonium concentration in tubular fluid of 5.5 mM (Table 2.3), the maximum rate of ammonium secretion (J_{amm}), assuming J_V of 10 µl h^{-1}, is 0.06 µmoles h^{-1}. Thus J_{amm} could potentially increase net acid secretion by locust tubules to a maximum of 0.15 µmoles h^{-1}, assuming all secreted ammonium trapped H^+ which came from the haemolymph. Again, the locust hindgut is a much more important site for potential acid removal in the form of ammonia. Oxidation of amino acids by the locust rectum supports a J_{amm} to the lumen of 0.4 µmoles $hour^{-1}$ (Thomson *et al.*, 1988b: corrected for surface area). However the locust ileum is by far the major source of excreted ammonia, with an unstimulated J_{amm} of 0.6 µmoles h^{-1}, and this can be stimulated to 1.4 µmoles h^{-1} by adding 5 mM cAMP. (Lechleitner, 1988; Audsley, 1991).

A surprising observation is that ammonia concentrations equal or exceed those of urate in locust tubular fluid (Table 2.3). As a result of the additional and much larger J_{amm} in locust hindgut, the final excreta in locusts would be expected to contain ammonia rather than urate as the predominant nitrogenous end-product, contrary to previous dogma that urate is the major end product in locusts (reviewed by Cochran, 1975). (There is no evidence for urate production or secretion by locust hindgut). In a companion study (Harrison *et al.*,1990a; Harrison and Phillips, submitted), the high ammonia content prediction is confirmed. The faecal concentrations of ammonia and urate are 270 and 68 (mM Kg^{-1} H_2O) respectively in locusts starved for a day, when care is taken to prevent ammonia loss on exposure of excreta to air. A comparison of these faecal concentrations with those of Malpighian tubule fluid (Table 2.3) provide interesting new information. Assuming no urate secretion in hindgut, the 15-fold increase in urate levels during passage through the hindgut suggests that about 95% of tubular fluid is reabsorbed in the hindgut, in agreement with previous estimates (reviewed by Phillips, 1981). The increase in ammonia-to-urate ratio from near 1:1 at the tubules (Table 2.3) to about 4:1 in the faeces, confirms that hindgut contributes over 70% of total ammonia excreted.

Finally, I have assessed the contribution of individual potential buffers to the total buffer capacity of locust Malpighian tubule fluid (Table 2.4). Using average measured values of pH and solute concentrations in tubule fluid, and pK values from the literature (Robinson and Stokes, 1959), phosphate (3.2 mequiv l^{-1} pH $unit^{-1}$) is the major non-bicarbonate buffer in Malpighian tubule fluid, while urate (0.84 mequiv l^{-1} pH $unit^{-1}$) plays a minor role. The contribution of phosphate to non-bicarbonate buffer value after NaCl injection is 8.4 mequiv l^{-1} pH $unit^{-1}$, which accounts for the increase in non-bicarbonate buffer value shown in Table 2.1. However,

Table 2.4. Estimated buffer contribution (ß) of measured solutes in Malpighian tubule fluid of HCl-injected *Shistocerca gregaria* at 21° C, pH 6.71, and constant Pco_2.

		ß (mequiv/l/pH unit)
a)	Bicarbonate	9.90
b)	Phosphate	3.16
c)	Urate	0.84
d)	Total value of a+b+c	13.9

Component ß values calculated according to Heisler (1986), using pK values from Robinson and Stokes (1959) and measured solute concentrations in tubule fluid post injection (Table 2.3).

bicarbonate ion is overall the major buffer (9.9 mequiv l^{-1} pH $unit^{-1}$) in tubule fluid, accounting for greater than 50% of total buffer capacity (Table 2.4). Only about 40% of non-bicarbonate buffer value is represented by total inorganic phosphate and urate. Despite the variability in the tubule fluid concentrations, there must be some additional unmeasured ionic species that account for the remaining 20-30% of total buffer capacity of tubule fluid.

CHAPTER 3

Effect of Temperature, Feeding, and Malpighian Tubule Fluid Flow on Haemolymph Acid-Base Regulation

Introduction

In Chapter 2 I observed that prevention (by cannulation) of Malpighian tubule fluid flow into the hindgut reduces the ability of locusts to regulate haemolymph pH. After 8 hours, haemolymph pH of cannulated locusts still had not recovered to pre-HCl injection values. In a companion study, Harrison *et. al.* (submitted) report haemolymph pH recovery from HCl induced acidosis in uncannulated locusts within 8 hours. Harrison suggests haemolymph recovery is due to a transfer of net acid equivalents to the alimentary canal, associated with movement of HCO_3^- into the haemocoel. I suggested (Chapter 2) that the lack of haemolymph recovery from acidosis in cannulated animals may be due to reduced HCO_3^- reabsorption in the hindgut. Previous studies have shown high rates of HCO_3^- transport in locust hindgut (Thomson and Phillips, 1985; Lechleitner *et al.*, 1989) and cannulation would deprive the hindgut of respiratory substrates secreted by Malpighian tubules into the gut lumen.

Temperature affects the acid-base status of blood and haemolymph (Chapter 1). Harrison (1988) has shown haemolymph pH of locusts decreases with a slope of -0.017 pHU/ $^{\circ}$C, consistent with temperature/blood pH relationships observed for other taxa. Since locusts normally experience high temperatures (35-40° C) during the day, acid secretion by tubules may be much more important than suggested by my previous study at 21° C (Chapter 2).

The state of feeding can also affect the acid-base status. Possibly hemolymph pH can be regulated faster when food is passing rapidly through the gut after feeding. Harrison *et al.*

(submitted) have suggested that the alimentary canal might be utilized as a temporary storage site for acid equivalents. Speight (1967) showed that feeding state of locusts affects the pH of rectal luminal contents, which has since been implicated circumstantially in haemolymph pH regulation.

In this chapter, I examine the importance of respiratory substrates and HCO_3^- content in the hindgut lumen in haemolymph pH regulation. In addition, since temperature and feeding are likely to affect acid-base status, I examine the effects of feeding at two different temperatures on acid-base parameters in Malpighian tubule fluid and on haemolymph acid-base status.

Materials and Methods

Animals

Adult female *S. gregaria*, 2-4 weeks past their final molt, were used in all experiments. Locusts were maintained and their Malpighian tubules cannulated *in situ* as previously described (Chapter 2; Thomson *et al.*, 1988a).

Feeding Protocols

Groups of locusts were isolated in a container for 2 days with access to cotton wool soaked with distilled water. The day before the experiment, animals were placed in individual containers at the experimental temperatures (21° C or 37° C), again with access to distilled water. Half of these animals were fed lettuce and bran *ad libitum* in individual containers for at least 2 hours prior to experiments. Net food uptake was determined by weighing individual locusts before and after feeding.

Hindgut Ligation and Malpighian Tubule Fluid Collection

LIGATION: Animals were restrained under dry cotton wool to prevent any visual stimulus. An incision was made in the third and fourth abdominal segments, and the gut gently lifted with glass hooks. A curved glass rod was carefully dragged from the midgut posteriorly along the alimentary canal, allowing clear access anteriorly to the point of tubule entry, without any Malpighian tubules lying close to the gut. This procedure does not damage the tubules. A surgical ligature was tied immediately anterior to the point of tubule entry. A similar procedure was performed posterior to the point of tubule entry. Loops of surgical thread, remaining loosely tied around the gut just anterior or posterior to the tubule entry point, constituted a sham operation. The ligated gut was then gently inserted back into the haemocoel cavity, and the incision sealed with sealing wax. Animals remained restrained throughout the experiment.

TUBULE FLUID COLLECTION: Locusts were cannulated as previously described (Chapter 2). Fluid production rates were measured by the advancement of fluid through the cannula. When 20-30 µl of tubular fluid had collected in the cannula, the sample was mixed by withdrawal into a length of PE tubing attached to a Hamilton syringe. Total CO_2 and pH were measured immediately on the pooled sample and biochemical measurements were made as outlined below.

Experimental Pertubations

Haemolymph acidosis was initiated by injection of 25 µl of 0.4 M HCl (10 µmol) through the 7th or 8th intersegmental membrane. Haemolymph acid-base parameters were measured on samples collected from the neck membrane as described in Chapter 2.

In some experiments, a saline mimicking Malpighian tubule fluid was injected into the posterior ligated hindgut complex by injecting the saline through PE50 tubing wedged into the anus. The volume of saline injected into the hindgut equalled the average initial rate of Malpighian tubule fluid production of 10 µl/hr (Chapter 2) over 6 hours (=60 µl). The saline had the following composition (mM): $NaH_2PO_4 \cdot H_2O$ (6), NaCl (35), K_2SO_4 (126), $CaCl_2$ (7), $Mg(C_2H_3O_2)_2 \cdot 4H_2O$ (4), $MgSO_4 \cdot 7H_2O$ (16), KCl (39), glucose (4.6), alanine (1.0), aspartate (0.5), glutamate (0.8), glutamine (0.5), glycine (4.0), proline (38.0), serine (1.0), $NaHCO_3$ (6), pH 6.63.

Acid-Base Measurements

CANNULATION AND LIGATION EXPERIMENTS: Haemolymph pH was measured on 2 µl samples collected from the neck as previously described (Harrison *et al.*, 1990b). Total CO_2 was measured on 8 µl aliquots using the technique of Boutilier *et al.*, (1985). Tubular fluid values were determined on samples collected as described above. Bicarbonate and Pco_2 were calculated from total CO_2 and pH assuming that carbonic acid pK values and CO_2 solubility coefficients were identical to those for locust haemolymph (Harrison, 1988).

MICROPUNCTURE EXPERIMENTS: Tubule fluid pH was also determined *in situ*. The abdominal cavity of animals was quickly dissected open and a small incision was made in the gut at the

point of tubule entry into the gut. The glass pH microelectrode was inserted directly into the gut lumen, along with a KCl/agar reference electrode. Similar measurements were made on the posterior midgut contents. These measurements were completed within 2 minutes of capture.

Biochemical Measurements

Total ammonia (ammonia + ammonium) concentration was determined on 10 µl of the pooled fluid sample that was transferred directly into 40 µl of 5% tricarboxylic acid (TCA). The enzymatic assay used was that of Kun and Kearney (1974). Total urate and inorganic phosphate were determined on 5 µl samples of tubular fluid diluted 100-fold with distilled water. Urate concentrations were determined spectrophotmetrically at 520 nm with a commercial kit (Sigma) using uric acid stock solutions as standards. Inorganic phosphate was determined spectrophotometrically at 820 nm by the technique of Chen *et al.* (1956).

The non-bicarbonate buffer value (ß) for tubular secretion was determined using samples diluted 100-fold with 100 mM KCl. Dilute samples were acidified with 20 mM HCl until the pH was below 4.0. Samples were then stirred for 2 hours to drive off CO_2 and HCO_3^-. Buffer value was then determined from pH 6.4 to 7.0 by titrating with 2 mM KOH using a PHM 84 research pH meter, TTT 80 titrator and ABU 80 autoburrette (Radiometer, Copenhagen, Denmark).

Statistics

All data are presented as mean ± standard error (S.E.) with *N* indicating the number of locusts. Paired *t*-tests were used to determine significant differences in composition of secretion

caused by acid injection. Where paired *t*-tests were not appropriate, significance of difference between means was determined by one-way ANOVA, with $p< 0.05$ accepted as significant.

Results

Effect of Hindgut Ligation on Recovery of Haemolymph pH After Acid Injection

Haemolymph pH is closely regulated under starved conditions in locusts and returns to normal within 8 hours of acid injection (Harrison *et al.*, submitted). In my initial study (Chapter 2) it was observed that cannulation of the gut to collect Malpighian tubule secretions reduced the ability of locusts to recover haemolymph pH after injection of HCl. I first repeated an earlier experiment by Harrison *et al.* (submitted) to confirm their observation on pH recovery after acid injection. Unmanipulated animals injected with 10 μmol HCl did indeed recover from the acidosis to pre-injection haemolymph pH values within 8 hours (Fig. 3.1).

The reduced regulatory ability after cannulation was attributed to reduced bicarbonate reabsorption in the hindgut complex (Chapter 2). Alternatively, the operation to cannulate Malpighian tubules might itself interfere with haemolymph pH regulation by unknown feedback mechanisms. I therefore first studied haemolymph pH recovery in sham operated locusts (Fig. 3.2). Before HCl injection, haemolymph pH was 7.31, which is similar to pre-cannulated values in Chapter 2. After 6 hours, haemolymph pH from uninjected sham operated animals was slightly lower at 7.22. Within 20 minutes of HCl injection haemolymph pH had fallen 0.36 pH units to 6.95. Total CO_2 was reduced by over 30% to 5.8 mM and Pco_2 temporarily increased at that time. These results are very similar to those observed previously after HCl injection into unoperated animals (Chapter 2; Harrison *et al.*, submitted).

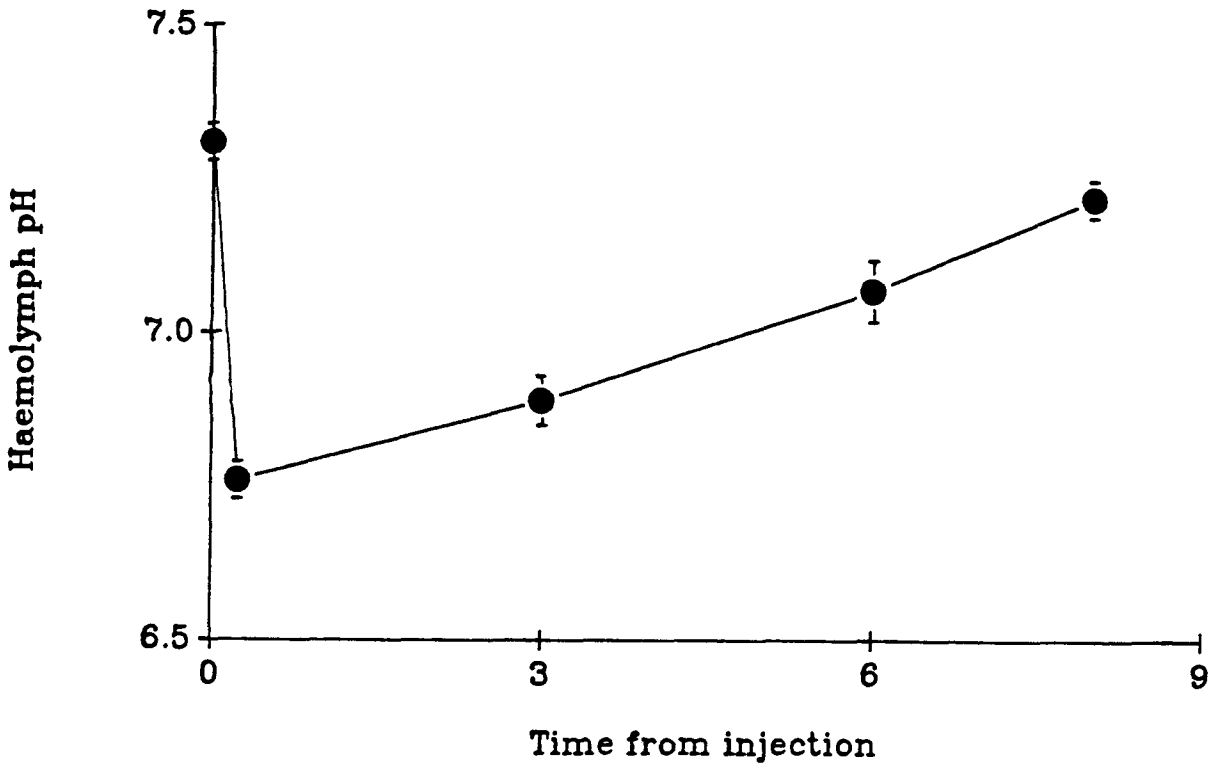

Figure 3.1 Haemolymph pH as a function of time after injection (0 h) of 25 µl 0.4 M HCl. Mean ± S.E. (N = 5-9). Locusts were starved for 1 day at 21° C.

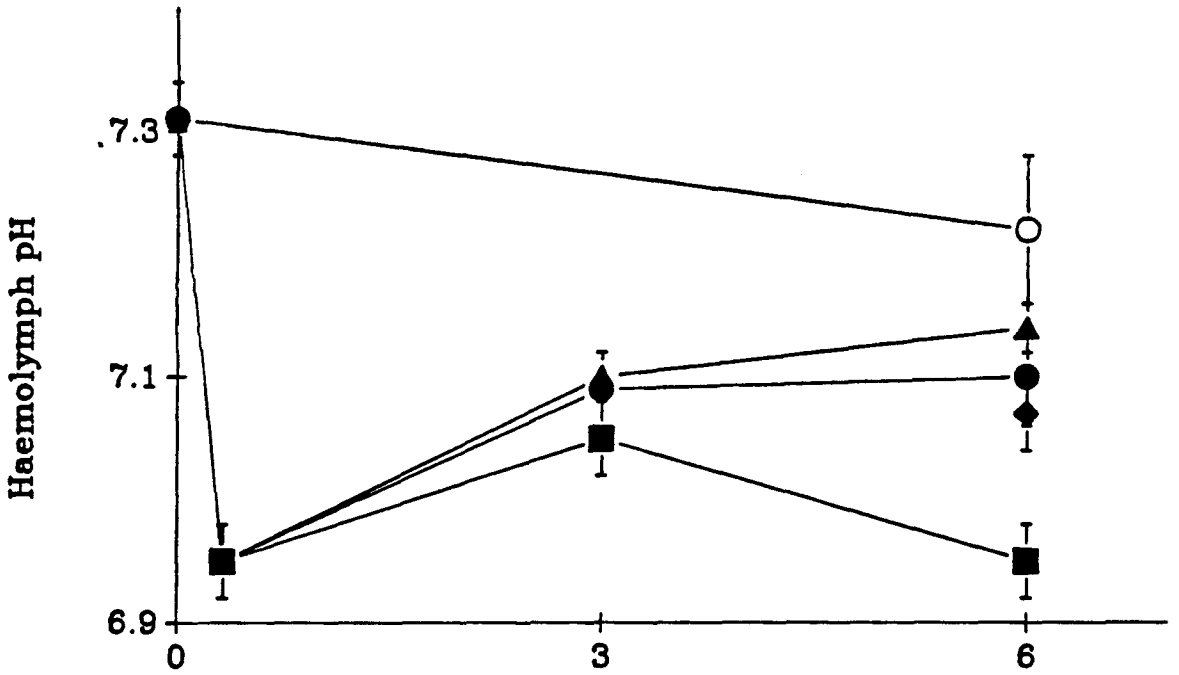

Figure 3.2. Haemolymph pH with time after injection (0 h) for different experimental groups of locusts starved for 1 day at 21° C. Mean ± S.E. (N = 6-12). Locusts were sham operated (●), anterior ligated (▲), posterior ligated (■) and then injected with 25 µl of 0.4 M HCl. A fourth group were sham operated and uninjected (O). The pH of haemolymph from posterior ligated locusts that were first injected with HCl and then injected with a saline mimicking Malpighian tubule fluid through the anus is also shown (♦).

Haemolymph from the sham operated group had recovered to a pH of 7.10 within 6 hours after injection of HCl, which is significantly higher than 20 minutes post-injection, but haemolymph pH did not recover to pre-injection values (Fig. 3.2), as shown by Harrison *et al.* (1991) and this study (Fig. 3.1). No further significant recovery of haemolymph pH was observed between 6 and 24 hours after acid injection into sham operated locusts (data not shown). Therefore, in the remaining experiments, I compared haemolymph values 6 hours post injection, using the partial (61%) recovery of haemolymph pH on sham operated locusts as the control.

A posterior ligation, which prevents fluid flow posteriorly into the hindgut complex, results in a haemolymph pH of 6.95 at six hours after injection of 10 μmol HCl, which is significantly lower than the similar means for locusts with either anterior or sham ligations. There were no significant differences in total CO_2, or calculated Pco_2 and HCO_3^- of haemolymph between any experimental group 6 hours after HCl injection (Fig. 3.3).

Effect of Saline Addition Into the Hindgut Lumen

The prevention, by ligation, of Malpighian tubule fluid flow into the hindgut resulted in reduced recovery of haemolymph pH (Fig. 3.2) compared with sham controls. Since the hindgut obtains respiratory substrates from the lumen side (Chamberlin, 1981) to sustain ion transport activities, hindgut ligation may inhibit metabolism and hence HCO_3^- reabsorption to restore haemolymph pH. To test this possibility, I injected 60 μl of an artificial saline resembling Malpighian tubule fluid into the posteriorly ligated hindgut complex 3 hours after HCl injection into the abdomen. This caused a significant recovery of haemolymph at 6 hours to values equal to the sham operated locusts (Fig. 3.2). There was no change in total CO_2 or HCO_3^- of

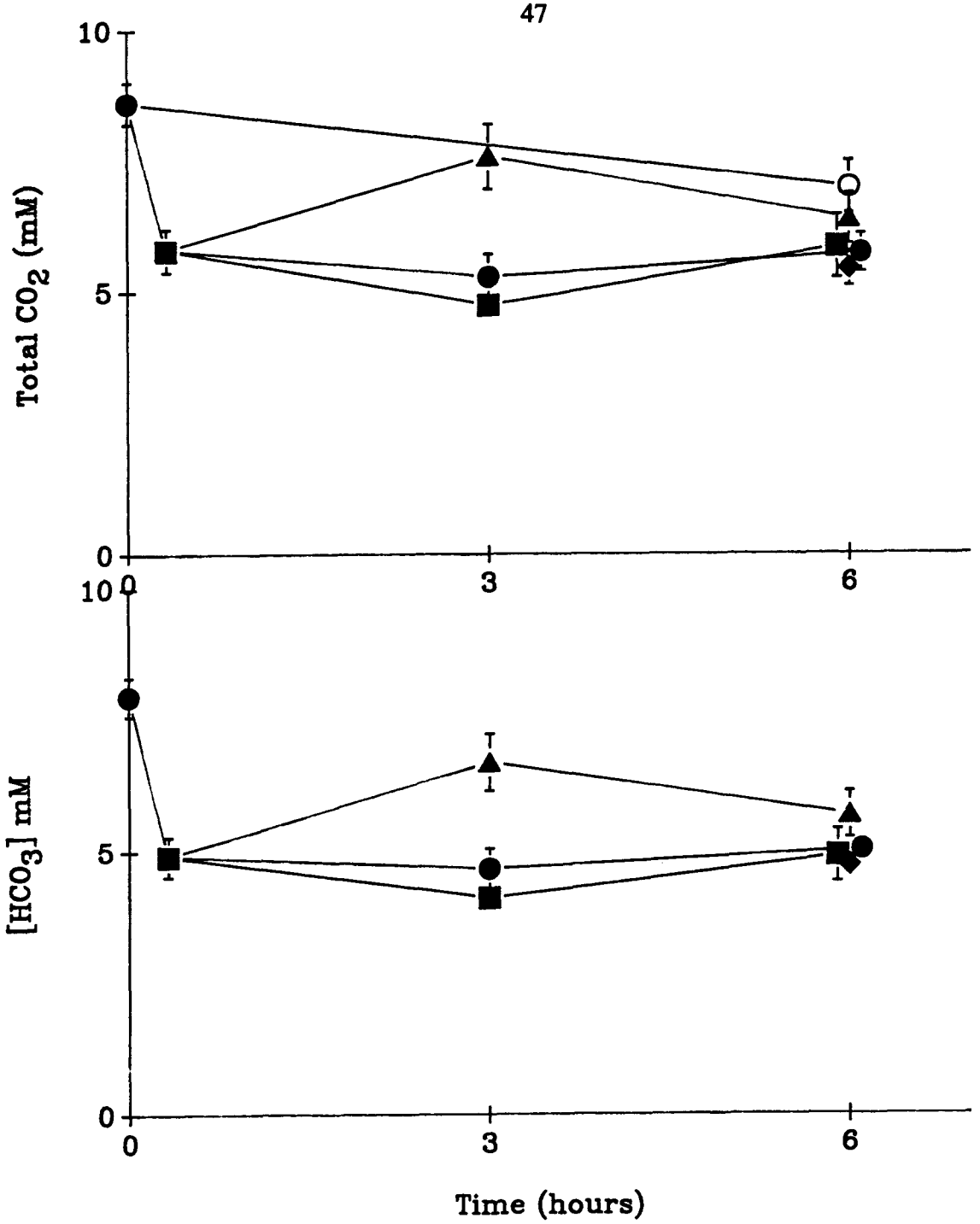

Figure 3.3. Haemolymph total CO_2 and HCO_3^- with time after injection (0 h) for different experimental groups of locusts starved for 1 day at 21° C. Mean ± S.E. (*N*=6-12). Locusts were sham operated (●), anterior ligated (Δ), posterior ligated (■) and then injected with 25 µl of 0.4 M HCl. A fourth group were sham operated and uninjected (O). Values for haemolymph from posterior ligated locusts that were first injected with HCl and then injected with a saline mimicking Malpighian tubule fluid through the anus is also given (♦).

haemolymph (Fig. 3.3). This provides the first direct evidence that hindgut transport activities contribute to haemolymph pH recovery from acidosis in locusts, or indeed any terrestrial insect. Anterior ligation did not prevent recovery of haemolymph pH as compared to sham operated controls, suggesting that midgut reabsorption was not essential to regulation.

Time Course of Fluid Secretion in Fed Locusts: Temperature Effect

All previous studies have been on starved locusts. Feeding initiates the release of diuretic hormones (DH) which stimulated fluid secretion in isolated insect tubules by several fold (Maddrell, 1980; Phillips, 1981). The effect of DH on acid-base transport by insect Malpighian tubules has not been studied. Conceivably DH stimulation could initiate a much greater capacity of tubules to eliminate acid. I therefore cannulated locusts that had been starved for 3 days and then recently fed lettuce and bran for 2 hours to ascertain the effect of feeding on tubular fluid parameters. Secretion rates were measured between 15 and 120 minutes after feeding.

Since all previous studies were done at 21° C (i.e. typical night-time temperature), possibly acid-base elimination by Malpighian tubules is much faster at typical daytime temperatures (e.g. 37° C). I therefore followed J_v for the full complement of tubules in starved and fed animals at both 21° and 37° C. Temperature affects the amount of food taken in (Table 3.1) and hence the rate of passage through the gut which could also influence pH regulation if the hindgut is involved. At 21° C, locusts ingest 0.29 g of lettuce and bran and at 37° C they ingest 0.45 g in 2 hours, representing 12% and 20% of total body weight respectively (Table 3.1). Initial fluid production rates for fed locusts at 21° C varied between 9 and 10 $\mu l\ h^{-1}$, and gradually decreased by up to 50% in 3 hours (Fig. 3.4). The J_v for fed animals gradually

Table 3.1. Food uptake in 2 hours by *S. gregaria* starved for 3 days at 21° C and 37° C.

	Locust Weight (g)	Food Uptake (g)	% Body Weight
21° C	2.42 ± 0.1	0.29 ± 0.03	12.3%
37° C	2.26 ± 0.07	0.45 ± 0.03	20.3%

Mean ± S.E. (*N*=15-16)

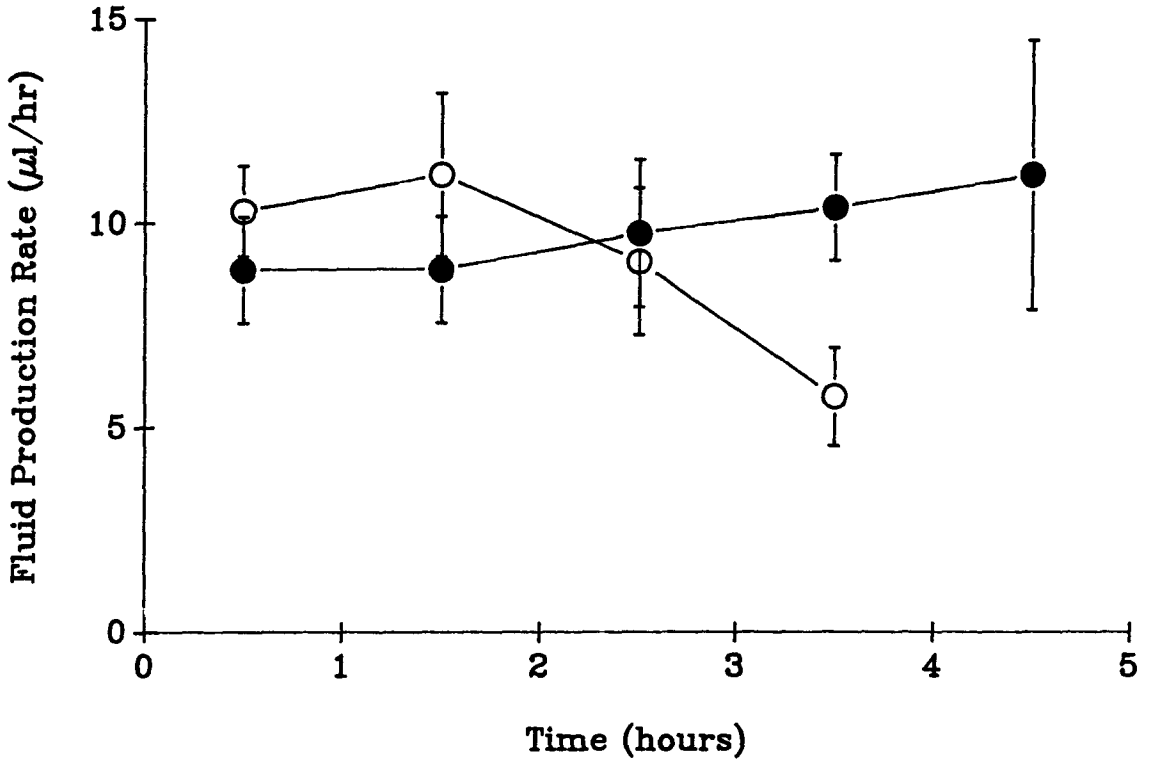

Figure 3.4. Fluid production rates (J_v) for the full complement of Malpighian tubules of starved (O) and fed (●) locusts cannulated *in situ* at 21° C. Mean ± S.E. (N =7-8).

increased but remained statistically unchanged from initial values. These values were insignificantly different from those for starved locusts in Chapter 2. At 37° C, initial fluid production rates are almost 4x higher than those at 21° C (Fig. 3.5). J_v for unfed locusts fell by up to 50% over 3 hours but fed animals exhibited no change in initial fluid production over the first hour as compared to starved animals. Again feeding did not significantly increase Malpighian tubule secretion rate, at least over the first 1.25 hours following a meal equal to 20% of body weight.

Haemolymph pH Variables

Before considering the effect of temperature and feeding on acid-base parameters in tubular secretion, it is first necessary to consider changes in the haemolymph bathing the tubules. Haemolymph pH in locusts decreases with increasing temperature (Harrison, 1988), but the effects of feeding state on blood pH is not known. I therefore compared blood pH in fed and starved locusts at two different temperatures. The different amounts of food uptake were as shown in Table 3.1.

Locusts starved for 3 days had a haemolymph pH of 7.23 at 21° C (Table 3.2), which is similar to the pH of haemolymph from locusts starved for one day at 21° C (Chapter 2). After feeding at 21° C, haemolymph pH decreased significantly to 6.95 (Table 3.2). Total CO_2 and HCO_3^- of haemolymph also decreased after feeding from 10.9 mM to 6.8 mM and from 9.9 mM to 5.6 mM respectively. Haemolymph Pco_2 remained statistically unchanged (Table 3.2). At 37° C, haemolymph pH in starved locusts is 6.87, which is 0.36 units lower than haemolymph pH at 21° C (Table 3.2). These values give a $\Delta pH/\Delta T$ ratio of -0.023, which is similar to the value

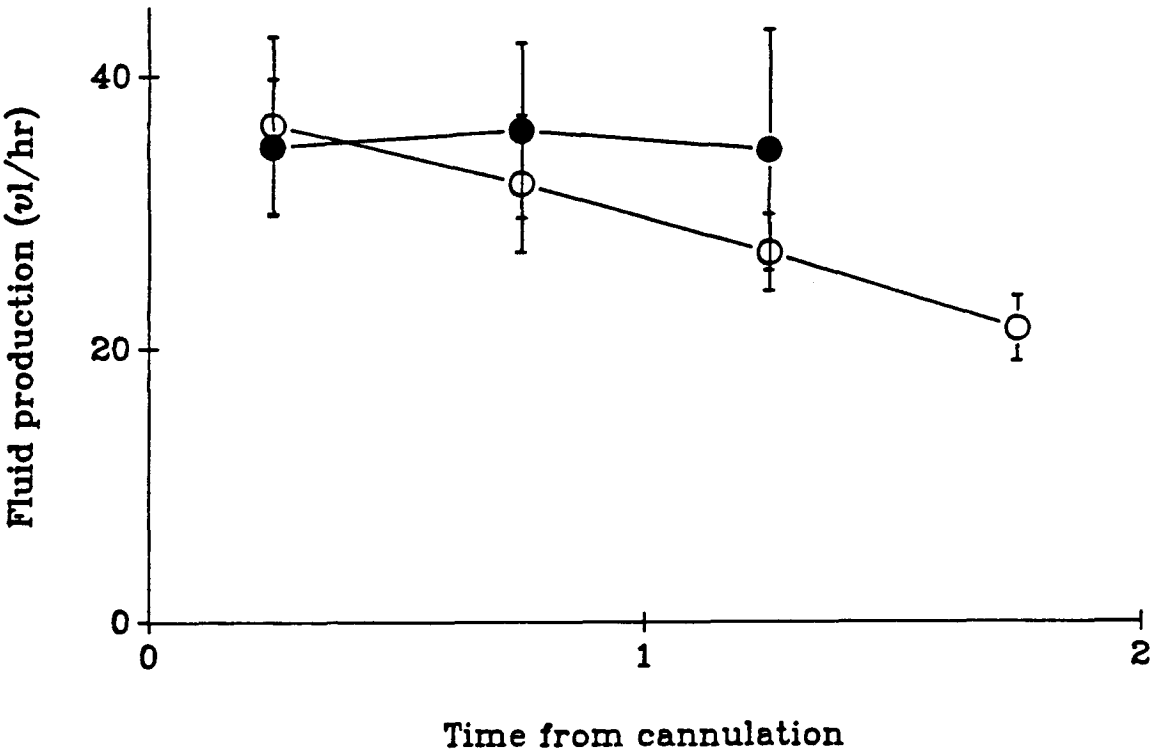

Figure 3.5. Fluid production rates (J_v) for the full complement of Malpighian tubules of starved (○) and fed (●) locusts cannulated *in situ* at 37° C. Mean ± S.E. (N =7-8).

Table 3.2. Effect of feeding and temperature on acid-base parameters of *Schistocerca gregaria* haemolymph.

	21° C		37° C	
	starved	fed	starved	fed
pH	7.23 ± 0.01	6.95* ± 0.11	6.87§ ± 0.08	6.88 ± 0.04
Total CO_2 (mM)	10.9 ± 0.3	6.8* ± 0.13	6.6§ ± 0.5	5.4 ± 0.4
Pco_2 (torr)	23.0 ±1.4	27.4 ± 6.0	32.8 ± 5.8	24.4 ± 2.4
HCO_3^- (mM)	9.9 ± 0.3	5.6* ± 0.2	5.5§ ± 0.4	4.6 ± 0.4

Mean ± S.E. (N=4-6)

* Indicates significant difference of fed from starved group at same temperature.
§ Indicates significant difference of starved group at different temperatures.

reported in a previous study of temperature/pH relationship (Harrison 1988). After feeding at 37° C, haemolymph pH was statistically unchanged at 6.88. Total CO_2 was depressed compared with starved conditions at 21° C, and feeding caused a slight decrease to 5.4 mM. Haemolymph Pco_2 (32.8 torr) and HCO_3^- (5.5 mM) both decreased to 24.4 (torr) and 4.6 mM respectively (Table 3.2), but these changes were not significant. Therefore, feeding caused changes in the acid-base status of locust haemolymph at 21° C, but not at 37° C.

Effect of Feeding and Temperature on Tubular Acid-Base Variables

The effect of feeding locusts at 21° C was to significantly lower haemolymph pH, but this did not occur at 37° C. In this series of experiments, I compared the composition of Malpighian tubule fluid pooled over 2-3 hours from starved and fed locusts at 21° and 37° C.

At 21° C, tubule fluid pH from starved locusts was 7.01 (Table 3.3), which is similar to values from locusts starved for one day (Chapter 2). After feeding, mean tubular pH was statistically unchanged at 6.86. Initial total CO_2 was 10.4 mM which was slightly, but not significantly depressed to 7.6 mM after feeding (Table 3.3).

At 37° C, haemolymph from starved animals was 6.87. Tubular pH was 6.71 before feeding, but increased to 6.89 after feeding, while blood pH remained unchanged at a pH of 6.88. Haemolymph total CO_2 decreased after feeding but tubular total CO_2 increased from 6.5 mM to 8.8 mM (Table 3.3).

Changes in acid secretion may be masked by changes in the buffer capacity (ß) of tubular fluid. I measured the non-bicarbonate buffer value for Malpighian tubule fluid for starved and fed animals at 21° C and 37 ° C. The non-bicarbonate buffer value for animals starved for 3 days

Table 3.3. Effect of feeding and temperature on acid-base parameters of Malpighian tubule fluid in *Schistocerca gregaria.*

	21° C		37° C	
	starved	fed	starved	fed
pH	7.01 ± 0.01	6.86 ± 0.13	6.71[§] ± 0.07	6.89 ± 0.14
Total CO_2 (mM)	10.4 ± 1.5	7.6 ± 1.2	6.5 ± 1.1	8.8 ± 1.6
Pco_2 (torr)	34.5 ± 5.9	33.0 ± 6.3	38.5 ± 5.1	37.4 ± 6.2
HCO_3^- (mM)	8.9 ± 1.4	6.2 ± 1.1	5.3 ± 1.0	7.6 ± 1.7
Non bicarbonate buffer value (ß) (mmol l^{-1} pH $unit^{-1}$)	19.4 ± 0.9	17.3 ± 1.1	15.8 ± 0.8	17.4 ± 3.1

Mean ± S.E. (*N*=9-14)

Non-bicarbonate buffer value is for the pH range 6.4-7.0.
[§] Indicates significant difference of starved group at different temperatures.

is 19.4 μequiv l^{-1} at 21° C, which is almost double the value for locusts starved for 1 day. After feeding at 21° C, buffer value remained unchanged at 17.3 μequiv l^{-1}. At 37° C, tubular ß value was 15.8 mequiv l^{-1} before feeding, and remained unchanged at 17.4 mequiv l^{-1} after feeding (Table 3.3).

PHOSPHATE, URATE AND TOTAL AMMONIA LEVELS IN TUBULE FLUID: The levels of these three inorganic solutes were not statistically different before and after feeding except for ammonia at 37° C (Table 3.4). Urate was elevated significantly at 37° C as compared to 21° in each feeding state. Total ammonia, phosphate and urate levels were all elevated after 3 days starvation compared with animals starved for one day at 21° C. This is probably due to desiccation. Chamberlin (1981) has shown that locusts can loose up to 50% of total body water under starved conditions.

Effect of feeding on Tubular pH as Determined by Micropuncture

It appears that feeding at 21° or 37° C has only small affects on the acid-base status of tubular fluid collected by cannulation *in situ*. However, the pH of gut contents at the tubule entry point was earlier reported to increase substantially after feeding as determined by micropuncture of the gut (Speight, 1967). This is contrary to my observation by cannulation *in situ*. I therefore measured the pH of gut contents at the point of Malpighian tubule entry without prior surgical intervention to check Speight's observation (Fig. 3.6). Animals starved for 3 days have a luminal pH of 6.9 as determined by micropuncture. After feeding for 1 hour, gut pH at the point of Malpighian tubule entry increases by over 0.5 pH units, suggesting a dramatic shift in pH of tubular fluid after feeding. This elevated luminal pH is maintained above pH 7.4 as long as

Table 3.4. Composition of *Schistocerca gregaria* Malpighian tubule fluid collected *in situ* before and after feeding at 21° and 37° C.

	21° C		37° C	
	starved	fed	starved	fed
Ammonia (mM)	7.1 ± 2.2	13.7 ± 2.8	10.1 ± 1.8	5.0[*§] ± 0.9
Phosphate (mM)	7.8 ± 1.7	8.9 ± 1.2	8.2 ± 2.0	5.5 ± 0.9
Urate (mM)	3.1 ± 0.5	2.9 ± 0.34	7.8[§] ± 0.8	6.6[§] ± 1.4

Mean ± S.E. (*N*=7-9 locusts)

[*] Indicates significant difference of fed from starved group at same temperature.
[§] Indicates significant difference of fed or starved group at different temperatures.

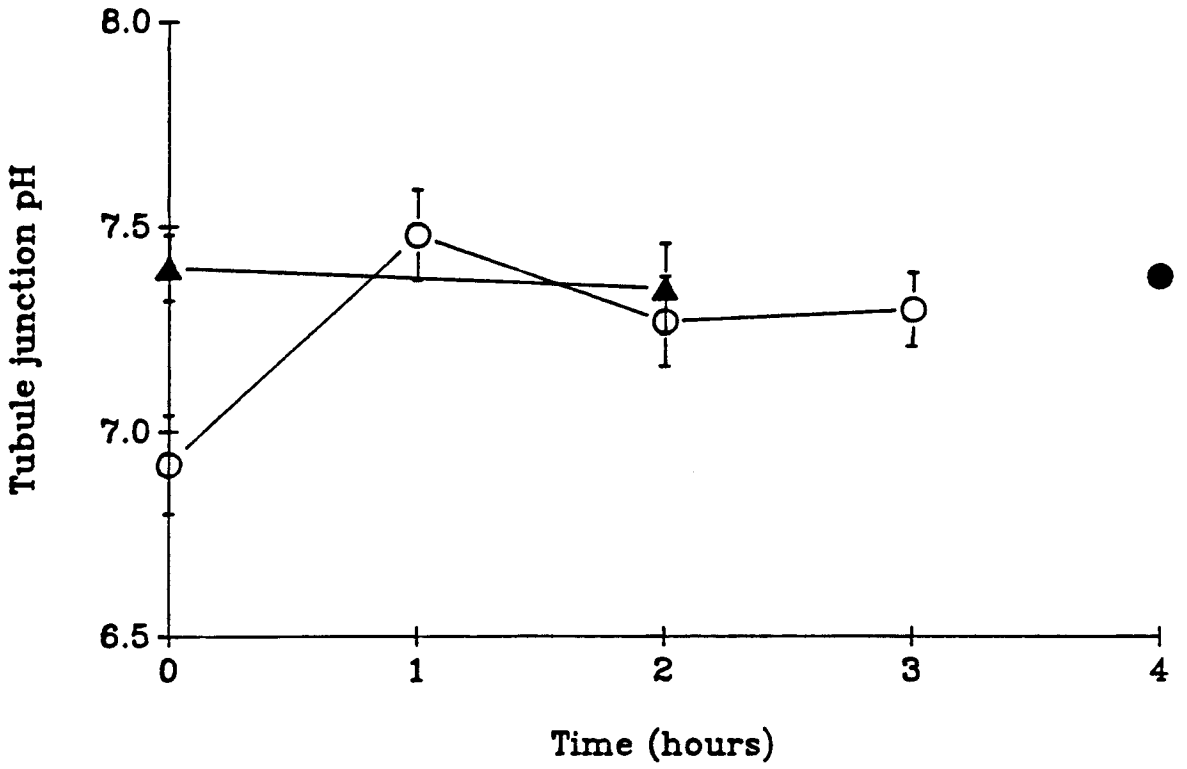

Figure 3.6. The pH of the gut contents as measured by the micropuncture technique. Mean ± S.E. (*N*=8-9). Values are for starved (0 h) and fed locusts with time from the start of feeding, for the midgut contents (▲), gut contents at the point of tubule entry (O), and from continuously fed animals at the point of tubule entry (●).

locusts are continuously fed. A clue to the cause of this alkaline lumen content at the Malpighian tubule entry point after feeding came from studying the midgut. The pH of midgut contents does not change after feeding. Contrary to previous studies, the midgut pH was found to be alkaline anterior to the point of tubule entry. Thus feeding induced substantial movement of alkaline midgut contents posteriourly to dilute the influence of more acidic Malpighian tubule fluid entering the gut.

Discussion

This study provides evidence that fluid flow from the Malpighian tubules into the hindgut is necessary for haemolymph acid-base regulation. While the Malpighian tubules *per se* have been shown to contribute very little to net acid removed from the haemolymph in starved animals at 21° C (Chapter 2) it seems that regulation of non-respiratory acid-base disturbances by the hindgut indirectly requires tubule secretions because bicarbonate reabsorption and H^+ secretion in the hindgut depends on Malpighian tubule fluid for metabolic substrates. The rectal tissue utilizes amino acids and glucose as metabolic fuels (Chamberlin, 1981), which are supplied luminally from the Malpighian tubule secretions. Thomson (1990) has shown high rates of acid and ammonia secretion by locust recta *in vitro*, and these transport processes require high energy input. I have shown that readdition of a saline that mimics Malpighian tubule fluid into the ligated hindgut complex restores haemolymph pH to values not significantly different from either an anterior or sham ligation. This is good evidence for the involvement of the hindgut in haemolymph pH regulation in locusts.

Malpighian tubule fluid secretion rates for locusts starved for 3 days at 21° C are similar to those reported previously at 21° C *in vivo* after one day starvation and are comparable to previous *in vitro* rates (see Chapter 2). There was not a dramatic increase in fluid production after feeding as has been reported for many insects taking large meals (reviewed by Maddrell, 1980). Apparently the smaller meals (20%) taken by locusts in this study do not release much DH, or the fluid secretion rate has already fallen 1 hour after feeding. Alternatively, handling animals may have stimulated DH release in all locusts used in this study. Under starved conditions, the secretion rate drops off rapidly after cannulation, possibly a result of decreasing haemolymph volume and the stressful effects of the cannulation operation. These results are consistent with observations in Chapter 2. After feeding the fluid production does not decrease, an indication feeding is causing some affect on fluid secretion. Fluid secretion is 4x higher at 37° C under starved conditions as compared to starved animals at 21° C. Also, food intake after 3 days starvation is more than 50% greater at 37° C. Even animals starved for one day ingest more food at these temperatures (unpublished observations).

Comparison of unfed haemolymph pH values at two different temperatures gives the same pattern of pH adjustment as reported for *S. nitens* over a greater temperature range (Harrison, 1989). In *S. gregaria*, I found pH to decrease by 0.017 pH units/ °C between 21° C and 37° C under starved conditions. This change in haemolymph pH is consistent with the alphastat hypothesis, but the temperature affects below 21° C have not been studied in *S. gregaria*. Under fed conditions, the $\Delta pH/\Delta T$ relationship is near zero. Clearly, these results show the alphastat model, as proposed by Reeves (1977), does not account for changes in pH with temperature in locusts under all physiological conditions.

Before feeding, the H^+ gradient across the Malpighian tubule epithelium after 3 days starvation was similar to values previously reported in cannulated locusts starved for one day (Chapter 2), the tubule fluid being 0.17-0.5 pH units more acid as compared to the haemolymph. At both 21° C and 37° C, feeding abolishes the transepithelial pH difference. Using the initial (one hour) J_v, the calculated pH difference between hemolymph and tubular fluid, and the measured buffer values for tubule fluid, I have estimated the maximum rate of excess acid removal (J_{H^+}) from the Malpighian tubules under each of these conditions. At 21° C, starved locusts have a maximal J_{H^+} of 0.05 μmol h^{-1}, which is almost half the rate of acid secretion for HCl-loaded locusts under similar conditions, but starved for one day (Chapter 2). After feeding at 21° C, the tubules no longer eliminate acid equivalents as the pH gradient is almost completely abolished. At 37° C, starved locusts eliminate excess H^+ at 0.09 μmol h^{-1}, which is almost twice the rate at 21° C, and equivalent to acid-loaded locusts at 21° C (Chapter 2). At 37° C, as at 21° C, tubules no longer eliminate acid equivalents after feeding.

Thus tubules do not contribute substantially to acid removal from locusts after feeding, unlike starved locusts where they could make a small contribution (Chapter 2). Changes in H^+ secretion activities are not masked by changes in tubule fluid buffer value (ß) (Table 3.3) before and after feeding at both 21° C and 37° C. Furthermore, the major inorganic solutes that could contribute to tubular buffering (phosphate and urate) remain statistically unchanged after feeding (Table 3.4). Total ammonia, which includes protons trapped as ammonium ions, changes significantly only after feeding at 37° C. This is consistent with a transepithelial ammonium ratio for tubules dependent on the proton gradient across the Malpighian tubule epithelium. If the epithelium moves ammonia via diffusion trapping (Good and Knepper, 1985), then alkalinization

of the tubule fluid and acidification of haemolymph would indeed result in a lower ammonium concentration in the tubular fluid.

There is no doubt that the pH of the gut lumen at the point of Malpighian tubule entry increases after feeding, as first reported by Speight (1967). From Figure 3.5, it is clear that the luminal pH increases after feeding, but the alkaline midgut would suggest this area becomes contaminated with midgut fluid after feeding and therefore doesn't reflect a change in tubular acid-base variables. Collection of Malpighian tubule fluid by cannulation indicates that tubule fluid is still acid.

CHAPTER 4

General Discussion

The overall objectives of this thesis were to 1) characterize the major acid-base parameters of Malpighian tubule fluid collected by cannulation, 2) determine the importance of Malpighian tubule secretions in the regulation of haemolymph acid-base status, and 3) study the effect of feeding state and temperature on the acid-base parameters of these fluids. Measurement of pH, total CO_2, and pertinent solute concentrations showed that the tubules do not play as major a role in haemolymph pH regulation as previously anticipated. While the regulatory response to acid loading exhibited by Malpighian tubules was not great, the tubules were clearly shown to play a secondary role in haemolymph pH regulation because of tubule fluid flow into the hindgut is essential for acid-base transport in that segment, leading to haemolymph pH homeostasis.

Effects of Cannulation

Cannulation of the gut so as to collect Malpighian tubule secretions provides a way of collecting uncontaminated urine. The fluid secretion rates observed were similar to those estimated using *in vitro* preparations or amaranth injection. The latter dye was found to be quickly concentrated in the tubule lumen without any leakage back into the haemocoel cavity. However, the effects of cannulation lead to a major observation in this thesis: i.e. the prevention of tubule fluid flow into the gut prevented normal recovery of haemolymph acid-base status after an acid challenge. It is conceivable that the operation and subsequent cannulation has unknown physiological effects, mediated through feedback mechanisms and stress, that prevent normal

recovery. However, cannulation does not prevent tubule fluid flow even one day after the operation, and locusts are always able to hop and walk when released from the restraining cage.

Malpighian Tubule Secretions

There is evidence that alkali metal pumps that are present in many insect epithelia, including Malpighian tubules, may be the active mechanism for K^+ transport. Wieczorek *et al.* (1986) partially purified an ATPase from a purified membrane preparation of tobacco hornworm midgut that has typical ion transport properties and inhibition of a vacuolar type ATPase. This ATPase activity was stimulated 2-fold in the presence of K^+. More recently, Wieczorek *et al.* (1989) proposed the ATPase activity in the midgut drives proton extrusion to the lumen, creating a 3 unit pH gradient. The resulting H^+ ion concentration gradient is then utilized to actively pump K^+ into the lumen through a H^+-K^+ antiport. This model is consistent with fluid transport being K^+ dependent. The pH of Malpighian tubule fluid is equal to or more acidic than haemolymph under all conditions tested. It is conceivable that Malpighian tubule secretions, which are K^+ dependent (Maddrell and Klunsuwan, 1973), are necessarily acid to haemolymph in order to drive K^+ extrusion for fluid transport.

Feeding initiates many metabolic and physiological changes, including well documented increases in Malpighian tubule secretion rate (Maddrell, 1980). Despite the lack of a dramatic increase in fluid production after feeding in my study, the cannulated locusts do show changes in fluid production and acid secretion rates. After feeding, the pH gradient that was observed in starved animals in Chapter 2 and Chapter 3 is abolished. If feeding is changing the rate of K^+ transport and hence fluid secretion in the tubules, this again fits well with the proposal of

Wieczorek *et al.*, (1989) that active K^+ extrusion is driven by a K^+-H^+ antiport. Therefore, under conditions that stimulate fluid secretion, one would expect the pH gradient to approach zero, as observed after feeding at both 21° C and 37° C.

In terms of acid equivalents transferred out of the haemocoel, Malpighian tubules clearly do not have the regulatory capacity of the hindgut in regulating haemolymph pH. The maximal rate of acid extrusion by Malpighian tubules is 0.09 μmol h^{-1} for starved, acid-loaded locusts and starved locusts at 37° C (Chapter 2; Chapter 3). As discussed in Chapter 2, this is not enough to account for the pH recovery observed within 8 hours in Chapter 3 and by Harrison *et al.*, (submitted). However, the effect of temperature is interesting. The maximal rate of acid clearance is temperature dependent in starved locusts and doubles between 21° C and 37° C. This is probably the result of a higher metabolic rate, leading to greater production of H^+ in the cell, and greater H^+-ATPase activity.

Comparison of Locust Excretory System with the Vertebrate Nephron

The Malpighian tubules of the locust form a primary urine by active ionic secretion causing osmotic movement of water (summarized in Figure 4.1). This contrasts with most vertebrates which have a pressure-driven filtration system (Phillips, 1981). This secretory mechanism in insect tubules might prohibit much H^+ elimination by the tubules because proton gradients must be used to drive K^+ secretion. The tubular secretions of locusts do transfer a small fraction of acid equivalents out of the haemocoel to the lumen under starved conditions, but this is reduced to insignificant values when K^+ secretion must be enhanced (by DH) after feeding.

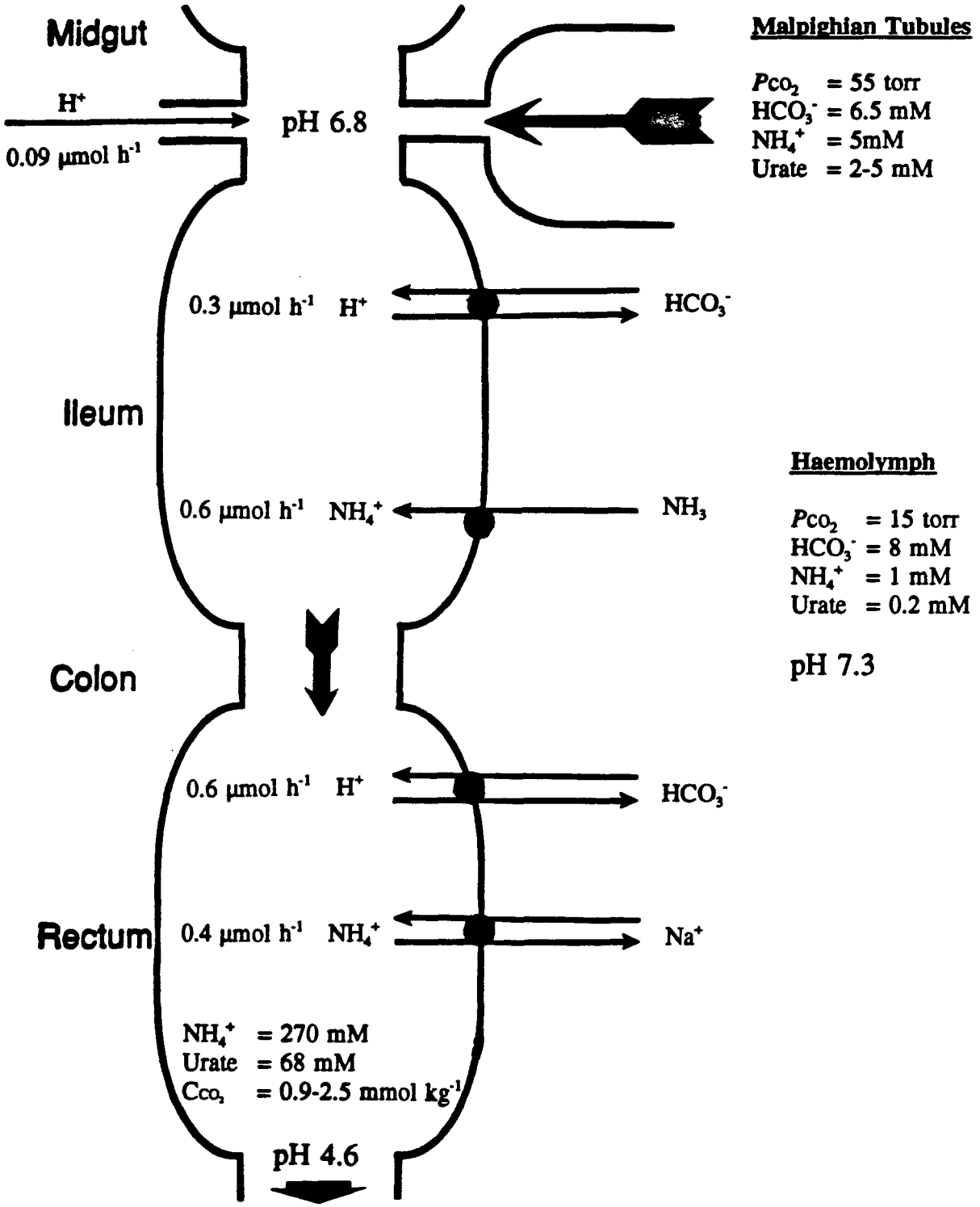

Figure 4.1. Schematic diagram of the locust excretory system. Fluid flow indicated with *thick* arrows and ionic and molecular transport indicated with *thin* arrows.

Haemolymph buffer composition in locusts is similar to that reported in many vertebrate systems (Harrison *et al.*, 1990b), and likewise, the solute concentration of the Malpighian tubule fluid I measured resembles the primary filtrate of the glomerulus (Pitts, 1968). The primary urine from Malpighian tubules flows posteriorly into the ileum where large quantities of water are reabsorbed concomitantly with Na^+, K^+, Cl^-, and HCO_3^-. Almost 100% of filtered HCO_3^- is reabsorbed in the nephron from most normal or acidotic vertebrates. Similarly, HCO_3^- reabsorption rates are high in the hindgut, reduced from an average 6.5 mM in the Malpighian tubules to near zero in the rectum. Each segment of the vertebrate nephron is capable of maintaining luminal pH below that of the filtered blood (Pitts, 1968). Accordingly, urine Pco_2 is elevated above plasma levels in the kidney tubules, thus enhancing HCO_3^- reabsorption. Similarly, Malpighian tubule fluid Pco_2 is 55 torr, some 4-fold greater than haemolymph (Summarized in Fig. 4.1).

As discussed in Chapter 1, the hindgut has been identified as a site of active proton secretion. Acid equivalents are thought to be transported in the rectum by an apical H^+-ATPase[1] and by an apical Na^+/NH_4^+ exchanger. The ileum is also capable of transporting acid equivalents. The locust hindgut exchanges NH_4^+ for luminal Na^+ and secretes H^+ in exchange for Na^+, and therefore acts much in the same manner as the vertebrate proximal tubule in eliminating acid-base equivalents.

In summary, the overall locust excretory system functions as a renal system. As outlined in Figure 4.1, the Malpighian tubules form a fluid that flows into the hindgut, where selective solute and water uptake occur. The ileum and rectum actively maintain haemolymph acid-base

[1] A small component (16%) of H^+ secretion is Na^+ dependent (Thomson, 1990).

status by utilizing Malpighian tubule fluid for metabolic substrates and a source of titratible acid. Depending upon the environmental conditions and physiological state, a very hyperosmotic or hyposmotic urine may be excreted, along with varying amounts of acid equivalents.

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Appendix

The microelectrode to measure extracellular fluid pH was modified from that described in Harrison and Walker (1977).

Glass tubing sensitive to H^+ ion concentration (pH 100-15; Clark Electromedical Instruments, Pangbourne, Reading, England) was pulled on a Kopf 700C vertical pipette puller (David Kopf Instruments, Tujunga, California, U.S.A.). Thermally matched, pH insensitive, alumina-silicate glass tubing (SM 100F-15; Clark Electromedical Instruments, as above) was pulled such that the shank angle of the pipette tip matched that of the pH sensitive glass (Fig. A.1). The tip of the pH-glass was cut near the wide end of the shank, and placed over the pH-insensitive glass. A glass-glass seal was made using a platinum microforge. The thin end of the pH-glass was then trimmed and melted closed with the microforge. A length of tygon tubing attached to a 50 ml syringe was used to blow a small bubble in the pH glass while gently heated near the tip of the microforge. Epoxy glue was applied to the edge of the glass-glass seal for strength.

The completed electrodes were backfilled and the gently boiled for 10 minutes in a pH electrode backfill solution containing (mM): KH_2PO_4 (40), NaOH (23), and NaCl (15), pH 7.0. Electrodes were stored indefinitely in the backfill solution.

Samples were measured in a small chamber made from pulled out PE 50 tubing, connected to a 3% agar bridge, which in turn was connected to a calomel electrode (Fig. A.1). Potential difference was measured with either a Keithly 602 digital electrometer or a Dagan 8800 total patch clamp. Typical electrode resistance is 10^{14}-$10^{15}\Omega$. Electrode slope criteria are given in Chapter 2.

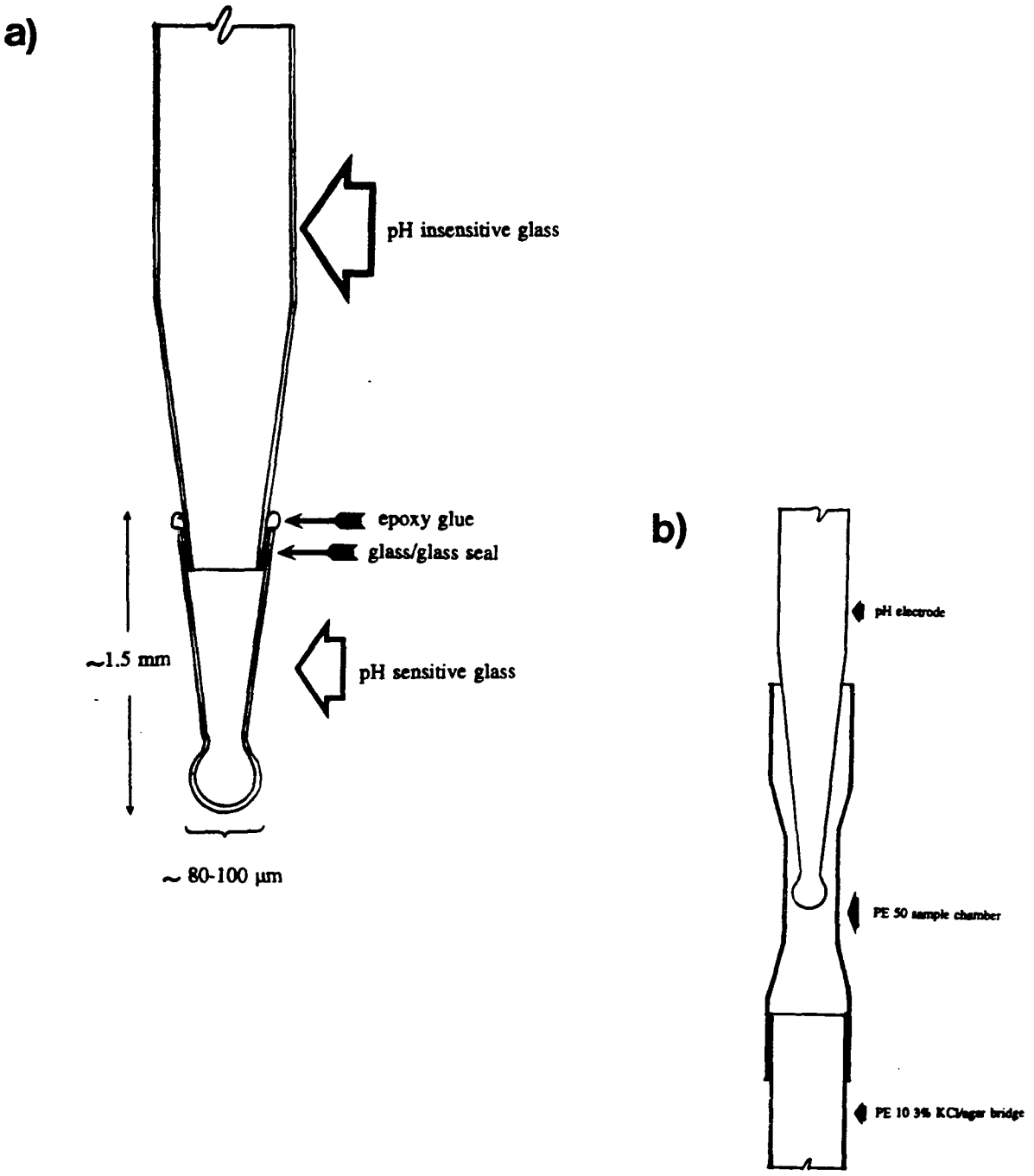

Figure A.1. a) Diagram of pH sensitive microelectrode. b) Sample chamber.