AUTOMATED  $K^+$  SCANNING MICROELECTRODE ANALYSIS

## Automated Scanning Microelectrode Analysis of Epithelial K<sup>+</sup> Transport in Malpighian Tubules of *Drosophila melanogaster*: Evidence for Spatial and Temporal Heterogeneity

By

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### A Thesis

Submitted to the School of Graduate Studies

in partial Fulfillment of the Requirements

for the Degree

**Master of Science** 

McMaster University Hamilton, Ontario

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McMaster University Hamilton, Ontario

TITLE:Automated Scanning Microelectrode Analysis of Epithlial K<sup>+</sup><br/>Transport in Malpighian tubules of Drosophila melanogaster:<br/>Evidence for Spatial and Temporal Heterogeneity

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NUMBER OF PACIES: xi, 189

#### ABSTRACT

Malpighian (renal) tubules of the fruit fly *Drosophila melanogaster* consist of three functional domains: a non-secretory distal segment, a secretory main segment and a reabsorptive lower segment. In this study a computer-controlled micropositioning system and a self-referencing  $K^+$  microelectrode were used to measure  $K^+$  concentration gradients of extracellular unstirred layers associated with specific epithelial domains.  $K^+$  fluxes were calculated from the measured gradients. This is the first time that an accurate assessment of the concentration gradients of the unstirred layer of *Drosophila melanogaster* could be assessed due to the enhanced sensitivity of this self-referencing technique over conventional ion-selective microelectrodes. The technique permits high resolution spatial and temporal mapping of the flux patterns in response to stimulation or inhibition of ion transport. Variations in K<sup>+</sup> transport over time and at different sites suggest that transport is non-uniform within any one functional domain.

### **ACKNOWLEDGEMENTS**

I would like to thank Dr. O'Donnell, my thesis supervisor, for his continued support, patience, and cooperation during my last two years. In addition I would like to thank Dr. Nurse, the other member of my supervisory committee, for his support.

A special thanks goes to Dr. Stuart Linton, Juan Ianowski, Kim Dube, and Kim Barrett. A great bunch of people to work with and a great bunch of friends. Your friendship and support will never be forgotten

On a personal note I would like to thank my family, especially my parents for their continued support of my education goals. And last but not least I would like to thank Tania Barozzi for here undying support and whose sacrifices have enabled me to follow my dreams.

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## CHAPTER 1: Methods for Analysis of Ionic Composition of Extracellular Fluids: General Introduction

#### **Insect Malpighian Tubules**

Insects occupy almost every niche of the global environment. Environmental stresses placed on insects requires that they be able to maintain their internal environments and body fluid composition within a narrow range for proper biological function. This regulation is primarily accomplished through the excretory system or more specifically the Malpighian Tubules (MT) and hindgut of the insect. Specific systems for the active transport of ions are believed to be the driving force for fluid secretion. Movement of water is believed to be a secondary and passive osmotic consequence of active ion transport. Secreted fluid is then processed and modified by specialized downstream segments of the Malpighian tubules. Phytophagous insects such as *Drosophila melanogaster* secrete a urine rich in KCl while blood feeding insects such as *Rhodnius prolixus* secrete a urine rich in NaCl. As a consequence the Malpighian tubules present themselves as very complex regulatory machinery in a simple single layered epithelium which is easily accessible for study.

Many methods have been developed that are amenable to the analysis of Malpighian tubule ion transport. This chapter will highlight some of those methods along with their advantages and disadvantages to this field of study.

#### **Ramsay Assay**

The most common method for analysis of secreted fluid was developed by Ramsay (1952). In this technique tubules secrete a volume of fluid if the blind end of the tubule is placed in a droplet of physiological saline under paraffin oil. The open end of the tubule or ureter is pulled out in the oil and is held by wrapping it on a pin or by holding it in a block of Sylgard (Fig 1.1). Droplet diameters are measured with an eyepiece micrometer and droplet volume ( in nL) is calculated using the standard formula for the volume of a sphere. Secretion rate is then calculated by dividing droplet volume by the time over which the droplet was formed.

Ion flux rates ( pmol/min) can be calculated if the ion concentration in the droplet is known. Ion flux rates are given by multiplying the droplet concentration ( mol.  $1^{-1}$ ) by the secretion rate ( nl.min<sup>-1</sup>). O'Donnell and Maddrell (1995) calculated the flux rates of unstimulated *Drosophila* Malpighian tubules in this manner. They concluded that K<sup>+</sup> influx into the main segment was 67 ± 14 pmol.min<sup>-1</sup>, which was significantly greater than the value for fluid leaving the whole tubule, 40.5 ± 11 pmol.min<sup>-1</sup>, resulting in a recovery rate of K<sup>+</sup> by the lower tubule of about 26 pmol.min<sup>-1</sup>.

### Fig. 1.1

The Ramsay Assay (1952):

Isolated insect Malpighian tubules are positioned in bathing saline droplets under paraffin oil. The arrangement seen here is the common assay used for *Drosophila melanogaster* tubules in which one tubule is bathed in the saline while the other tubule is wrapped around a steel pin. Secreted fluid emerging from the opening of the ureter is collected at predetermined time intervals for analysis.



This is a powerful technique because it requires very little equipment and expense, as well as being relatively easy to learn. This technique also lends itself well to the study of the effects of pharmacological agents on fluid secretion, simply by adding the appropriate concentration of the agent to the physiological saline bathing the tubule. An additional advantage of this experimental preparation over flat sheet epithelium studies is that only the saline bathing the basolateral side of the epithelium needs to be maintained. In addition secreted fluid can be collected over time to determine secretion rate and ion fluxes in a very simple manner. In contrast the determination of ion movement in flat sheet epithleiums requires the extensive and labour intensive use of radioactively labelled ions in an Ussing chamber setup.

#### **Analysis of Secreted Fluid Ion Composition**

Many methods have been used over the years for the analysis of ion concentrations of the secreted fluid. Some of the more common methods will be discussed here.

#### Flame Photometry

Flame photometry allows for the quantitative analysis of alkali metals (Na<sup>+</sup>, K<sup>+</sup>) in aqueous solutions. This technique quantifies the alkali metal by measurement of a characteristic light emitted when a solution of the metal is atomized into a gas flame. The characteristic radiation is isolated by passage through filters and an electrical

impulse is produced when the filtered light strikes a photosensitive element. The magnitude of this electrical impulse is related to the quantity of the alkali metal present in the aqueous solution. Ramsay (1950) and Ramsay et al. (1952), and Maddrell and Phillips (1975) successfully used flame photometry to determine alkali metal concentrations in MT secreted fluid. This method of analysis is advantageous because it is relatively inexpensive and requires very little preparation of the collected fluid for analysis. In this case the fluid containing the alkali metal is atomized and any organic material is simply burned away in the flame.

There are several disadvantages to flame photometry. The rate of atomization of the sample into the instrument is quite fast (5 - 10 ml.min<sup>-1</sup>), and only 10% of the aspirated solution reaches the flame (Brodie, 1982), therefore large volumes of the sample are required for accurate measurements (~1-5 mL). Unfortunately Malpighian tubules secrete fluid at rates on the order of nl.min<sup>-1</sup>, requiring that an appropriate sample volume be collected from a pooled sample of tubules. Another disadvantage is that the system can only detect alkali metals whose flame spectra have available filters. There is also the possibility that foreign substances (salts, organic molecules) may alter the amount of light emitted resulting in an over or underestimation of the alkali metal in the aqueous sample.

#### **Atomic Absorption**

The development of absorption flame photometry, more commonly called Atomic Absorption (A.A.) came as a direct result of the advancement of flame photometry. In this case the flame is used as a medium for absorbing radiation rather than as a source of excitation of emission. Atomic absorption is, " based on the measurement of the absorption produced in a beam of radiation of suitable wavelength proceeding from an emitting source of constant intensity, by a medium composed of atoms of the element to be determined" (Ramirez-Munoz, 1968). The amount of light absorbed increases directly with the concentration of the absorbing medium within the sample solution. The system emits a spectrum characteristic of the element to be measured. An aerosol of the unknown is injected and a series of monochromatic filters and photodetection system measure the atomic absorbance (Ramirez-Munoz, 1968). Specific absorption spectra are within a range of 1800 - 9000 Å, and only elements with absorption spectra within this range can be detected by this method. The absorption of a given element to be measured must therefore be measured at a wavelength corresponding to the peak of its respective reabsorption line. Atomic absorption spectroscopy has been successfully used to measure Ca<sup>+2</sup>, Mg<sup>+2</sup>, Na<sup>+</sup>, and K<sup>+</sup> content in tissue, water, and biological fluid samples ( Shephard and McWilliams, 1989; Hwang et al. 1994; Wessing et al. 1993).

The advantages of atomic absorption are: the absorption process is independent of the excitation potential, making the analysis of concentration of alkali metals far more accurate than emission spectrophotometry; A.A. systems are highly sensitive and relatively easy to operate, and can detect elements in the p.p.m. or p.p.b. range, allowing the use of smaller sample volumes. Also A.A. is almost free of spectral interference that is common in other emission methods.

There are however disadvantages to the use of atomic absorbance spectrophotometry. The range of sample concentrations of a given element measurable by A.A. is smaller than that of emission spectrophotometry. Also, some of the absorption lines for certain elements are in the ultraviolet region of the spectrum, outside the detectable spectrum of the A.A.

The main difference between emission spectrophotometry and atomic absorbance is that the former uses the flame to excite atoms and measure their emitted light as the atoms return to their ground state, whereas A.A. measures the absorbance of energy of the ground state atom. Whichever method is chosen depends largely on the elements being measured, the type of sample, the sensitivity required and the quantity of the sample. For measurement of alkali cations in the secreted fluid of MTs A.A. would be the appropriate choice largely due to the quantity of the sample produced by the fluid secretion assay.

#### **Graphite Furnace**

A related method of atomization for absorption measurements commonly used is the Graphite furnace. In this system the first thing to happen is the sample undergoes a drying stage during which the solvent is removed from the sample. An ashing stage then occurs which removes organic or inorganic material. The final stage is an atomization stage in which free atoms are generated within the confines of the graphite tube. The absorption signal produced in the atomization stage corresponds to a specific elemental peak whose height can be related to the amount of the element of interest present. It is most commonly used for the detection of trace metal elements such as nickel, copper, cadmium, and lead (Brodie 1982). Shalmi *et al.* (1994) used a graphite furnace to quantify the handling of lithium, sodium, and potassium along the proximal convoluted tubule of the anesthetized rat.

Advantages of the graphite furnace include the fact that it is ~100 times more sensitive than conventional flame methods. As well the "flameless" atomization virtually eliminates sample wastage allowing for very small sample volumes to be used (~5 - 10  $\mu$ L) (Brodie 1982). However, the graphite furnace is not recommended when the sample is already in a liquid form and the concentration of the element in question permits a simple analysis by conventional flame techniques. Additionally the time to analyze samples in the graphite furnace is on the order of a ~2 minutes per sample while flame photometry is much faster ~20 seconds.

#### Radioisotopes

Radioisotopes decay with emission of alpha or beta particles or gamma rays. The maximum energy emitted by each of these rays or particles is different and is specific for any given element. Alpha and beta particles are quantified using a liquid scintillation counter. In this method an organic scintillant (e.g. toluene) in close contact with a radioactive sample can absorb the radiation energy of the sample and re-emit the absorbed energy as a photon of light. The energy absorbed from the radioactive sample by the toluene is re-emitted in the ultraviolet or visible spectrum range. Photomultiplier tubes sensitive to these specific wavelengths can then detect and quantify the photons of light emitted. The greater the energy of the emitted radiation ray, the greater the number of photons of light that are detected by the photomultiplier. The scintillation counter's electronic circuitry registers an electrical event that corresponds to the detection of each photon interacting with the photomultiplier. These electrical events are continuously averaged and displayed as counts per minute.

Analysis of Malpighian tubule secreted fluid ion concentration has been done previously (Maddrell 1978) In this case the lumen directed  $K^+$  flux was measured using radioactively labeled  ${}^{42}K^+$  in both unstimulated and 5-hydroxytrptamine stimulated tubules.

Radioisotopic labeling detects elements in their ionic form as well as complexed forms (proteins, organic acids, etc.). A major disadvantage of this method

is the hazards that come with the handling and exposure to radioactive materials. The expense of the scintillation fluid and the time (1-15 minutes) required for sample analysis are major disadvantages.

#### X-ray microanalysis

X-ray microanalysis makes use of the fact that atoms struck by electrons from an external source yield x-rays which are characteristic of that element. Consequently, the x-rays can be used to identify and quantify the elements present. Suitable detectors, placed close to the specimen, collect the x-rays and the information obtained is displayed for immediate interpretation of the sample composition.

There are two distinct methods for the collection and counting of x-rays leaving an electron irradiated sample; Wavelength dispersal spectrosocopy (WDS) and Energy dispersal spectroscopy (EDS). WDS was the original method and is currently the method of choice for the analysis of specimens in the scanning electron microscope. EDS is the conventional method of analysis in TEM (Morgan 1985). These two types of x-ray microanalysis can be distinguished from each other by the detector system used to collect and quantify the x-rays.

Wavelength dispersal analysis utilizes a crystal lattice detector and quantifies the x-rays based on their wave characteristics. In this method electron bombardment of various elements in the specimen emit x-rays with a range of wavelengths. These various x-rays leave the specimen as a group of different wavelengths which then hit the crystal lattice detector. The crystal lattice has a specific spacing that accepts only a small portion of the emitted wavelengths. These x-rays are then refracted into the detector for quantification. The fraction of the x-ray population that is refracted depends on the principle of diffraction. Only one particular wavelength ( $\lambda$ ) is strongly reflected at a given angle of the crystal lattice to the exclusion of all others. This principle is based on Bragg's Law (Chandler 1977):

#### $n\lambda = 2d \sin \theta$

where n is an integer,  $\lambda$  is the wavelength of the x-rays which are precisely diffracted, d is the lattice spacing of the particular planes in the crystal giving rise to the diffracted beam, and  $\theta$  is the angle of incidence ( and of reflection) of the x-rays arriving at the crystal. The wavelength of an x-ray is inversely proportional to its energy. Some advantages of WDS are that the crystal detectors have good energy resolution ( typically ranging from 1-10 eV), and a tolerance for very high count rates ( ~ 500 000 cps) compared to solid state detectors of EDS. However the major disadvantage of this method is that analysis is restricted to only one element in the sample at any one time. Analysis of multiple elements in a sample is difficult because repeated exposure to a high energy electron beam may damage biological samples.

Frequently an analysis of multiple elements in a sample is required. The EDS ( solid state) detector permits the simultaneous display of all the collected x-rays during a single exposure period. This detector consists of a silicon wafer located between two metal electrodes across which voltage is applied. A layer of lithium is diffused into a

silicon crystal forming a semi-conductor. The detector crystal is kept under high vacuum and at liquid nitrogen temperatures. X-rays entering the detector through a thin beryllium window create electron hole pairs within the detector. " The number of these ion pairs is proportional to the X-ray energy and is equal to the ratio of the x-ray energy and the energy required to create a pair" (Chandler 1977). The detector is connected to a pre-amplifier and pulse processor to filter out background noise. The output voltage pulse is then further amplified and passed into a multi-channel analyzer which sorts the pulses from the amplifier based on pulse amplitudes. These channels can then be expressed as a complete elemental spectrum of the electron-irradiated sample. The most obvious advantage to this type of solid state detector is that a multielemental analysis of a sample can be done with a single exposure of the sample to the electron beam. Another advantage is the ease of use of this system, because it does not require the researcher to rotate the detector through a range of angles to find the peak wavelength amplitude. The disadvantage to this method is that it does not give as accurate an estimate of elemental composition with regard to each individual element as does Wavelength dispersal analysis.

X-ray microanalysis has been used previously for analysis of secreted fluid ion composition. It is important to remember here that x-ray microanalysis does elemental analysis, and not ion analysis. Therefore, this method of study also takes into account any element which is precipitated as an organic or inorganic salt or complexed with a protein or organic substrate, etc., that may be in the secreted fluid. Van Kerhove *et al.*  (1989) working on *Formica* Malpighian tubules, set up the tubules following the Ramsay method (Ramsay 1952) previously described in this chapter. Secreted fluid was collected at predetermined time intervals in a glass pipette sealed with paraffin oil. These samples could be analyzed immediately or could be kept in a glass capillary tube between two columns of oil, shock frozen in liquid nitrogen and then stored for subsequent analysis (Van Eekelen *et al.*, 1980; Van Kerhove *et al.*, 1989). Liquid propane cryofixed samples of both tissue and haemolymph could then be sectioned ( Wessing *et al.*, 1993 ; Wessing and Zierold, 1993), or placed on a dry nickel grid and then flash evaporated (Quinton, 1979; Van Kerhove *et al.*, 1989). Flash evaporation is important because it minimizes crystal formation in the sample. Once the sample preparation was done it is mounted in a standard TEM or ESEM system with an x-ray detector for quantitative analysis of its elemental composition. Marshall *et al.* (1993) analyzed the ion content of secreted fluid of the Black field cricket using x-ray microanalysis.

#### Ion selective microelectrodes

Ion selective microelectrodes (ISMEs) are commonly used for measuring specific ion activity for analysis of intracellular and extracellular ion concentrations ( e.g. K<sup>+</sup>, Cl<sup>-</sup> Haley *et al.*, 1997; pH, Ca<sup>+2</sup>, K<sup>+</sup>, Na<sup>+</sup> O'Donnell *et al*, 1996; Maddrell *et al.*, 1993). Liquid membrane ISMEs are commonly fabricated from borosilicate glass micropipettes. These micropipettes are pulled to small tip diameters ( < 1  $\mu$ m) using a single stage vertical puller and then are broken back to larger tip diameters to decrease their resistance. After pulling, and prior to the filling stage the micropipettes are made hydrophobic by exposing the acid treated and dried pipettes to dimethlyamine trimethysilane for 10-15 minutes at ~250 °C (Deyhimi and Coles, 1982). Silanization is a very important step because it makes the glass hydrophobic, thereby retaining the organic cocktail in the tip when the electrode is place in an aqueous solution. Without silanization aqeous solution enter into the microelectrode tip. Silanization also has another advantage. The hydrophobic barrier prevents short circuiting of the potential across the liquid membrane. Without this hydrophobic barrier the hydration of the glass would provide a lower resistance pathway along the inner surface of the electrode.

The electrodes are made ion selective by filling the tip with an organic cocktail containing an ion selective ionophore. In neutral carrier ionophores the neutral carrier acts as a shuttle, transporting ions across the interface between the liquid membrane and the external solution. A neutral carrier ionophore is composed of a carrier molecule (often an antibiotic) which determines the ionophores selectivity and a plasticizer agent that enhances the stability of the ionophore column and increases its resistivity (Ammann, 1986).

Once the ionophore is placed in the tip of the electrode the rest of the electrode is backfilled with an appropriate electrolyte (0.5 mol  $l^{-1}$  KCl for K<sup>+</sup>- sensitive microelectrodes). An AgCl coated silver wire is placed in the electrolyte filling solution and connected to a high impedance electrometer (Fig 1.2). Potential

differences can then be monitored and recorded as the ion distribution across the liquid membrane interface changes (Vaughan-Jones and Aickin, 1988). The potential measured by the microelectrode is due to the charge separation created across the sample solution/ionophore interface (see  $E_A$ , Fig 1.3). The potential registered by the electrometer is actually the addition of separate potentials at two aqueous interfaces  $(E_D)$ . The first being the sample solution / ionophore interface  $(E_A)$  and the second being the ionophore / backfill solution interface ( $E_B$ ). However the potential of  $E_B$ essentially remains constant so the changes observed in the voltage are due to the potential changes across the sample solution / ionophore interface. It is important to realize that the ion being measured is not transported across the whole length of the ionophore column. The ion in question is carried by the neutral carrier across the sample solution / ionophore interface, and very few ions actually have to move to cause a distinct charge separation and measurable potential change. This is also important because it means that the ionophore does not alter the concentration of the ion in the sample solution. Potential differences are then converted into concentrations using the following equation:

$$[ion]_{USL} or [ion]_{Bath} = [ion]_{cal} *10^{(\Delta V/S)}$$
(Eq1.1)

where:  $[ion]_{USL}$  or  $[ion]_{Bath}$  is the concentration of the ion in the unstirred layer or

### Fig 1.2

Schematic diagram representing a standard ISME filled with ion-selective ionophore and backfilled with an electrolyte solution (left), and a reference electrode (right) both with a AgCl coated silver wire connected to a high impedance electrometer.



bathing saline (mmol  $\Gamma^1$ ), [ion]<sub>cal</sub> is the corresponding concentration in the calibration solution  $\Delta V$  is the change in electrical potential ( in mV) between the calibration solution and the USL or the bathing saline, and S is the slope ( in mV) mesured for a 10-fold change in the ion concentration. Although electrodes measure ion activity, data can be expressed as concentration if it is assumed that the activity coefficients of the calibration solutions, and sample solution are the same. For Malpighian tubules secreting isoosmotic fluid, the conversion of ion activity to ion concentration is accurate to within  $\pm$  5% (Ammann, 1986).

The selectivity of an ISME for a particular ion is based on the selectivity of the ionophore used. Selectivity coefficients are measured using the separate solution method. The potential of the ISME ( compared to its reference) is measured in a solution of the test ion "x" and then in a solution of the interfering ion "y". The selectivity coefficient (K) is then given by:

$$K_y = a_x / a_y \cdot 10 (E_y - E_x) / S$$
(Eq. 1.2)

where  $E_y$  is the potential measured in a solution containing an activity  $a_y$  of the interfering ion,  $E_x$  is the potential measured in a solution containing an activity  $a_x$  of the test ion, and S is the slope of the electrode, defined as the change in electrode potential in response to a 10-fold change in  $a_x$ . The smaller the selectivity coefficient for an ionophore, the more selective the ionophore is over the interfering ion (Thomas 1978). Usually selectivity coefficients are reported as logs. For example Fluka

Potassium Ionophore I - Cocktail B has a selectivity coefficient of -3.9 for potassium over sodium. This means that the ionophore is  $\sim 10~000$  times more sensitive for potassium ions than it is for sodium ions.

The advantage of ISMEs according to Thomas (1978) are: rapid response times; a direct measurement of ion activity; continuous measurements may be taken over long periods of time; simple construction and relatively low cost.

One disadvantage of conventional ISMEs is the lack of highly selective ionophores for some ions (Thomas 1978). For extracellular fluids highly selective ionophores are available for H<sup>+</sup>, K<sup>+</sup>, and Ca<sup>+2</sup>. Accurate measurements of Na<sup>+</sup> can be made providing there is a correction made for interference by K<sup>+</sup>. Another disadvantage is their sensitivity limit. These standard ISMEs are limited by their sensitivity to significant activity changes. This is due to the inherent characteristics of the ionophore resulting in a theoretical detection limit of ~100  $\mu$ V corresponding to a flux of ~16 pmol.cm<sup>-2</sup>.s<sup>-1</sup> for static K<sup>+</sup>-sensitive microelectrodes in a background of 0.3 mmol l<sup>-1</sup> (Kochian *et al.* 1992). Sensitivity of the static ion-selective microelectrodes increases as the background concentration of the ion in question decreases. Unfortunately this is not always the case or possible in biological situations. Often the background concentration of the ion trying to be measure (e.g K<sup>+</sup>) is relatively high in biological fluids. Fig.1.3

Schematic representation of the calculation of the potential difference measured across the liquid membrane interface of ionophore column in a conventional ISME.



 $E_D = E_A + E_B$ 

NB:  $E_B$  remains essentially constant while  $E_A$  changes with changing ion activities in the sample solution

membrane A (sample solution / LIX interface)

Analysis of Malpighian tubule ion transport mechanisms using static ionselective microelectrodes has been done previously by two separate methods. The first method involved the determination of ion concentrations in drops of fluid secreted by isolated tubules measured under paraffin oil (Maddrell and O'Donnell, 1992; Maddrell et al. 1993; O'Donnell and Maddrell 1995; and O'Donnell et al. 1996). Ion concentrations were calculated from the change in electrical potential when ionselective and reference microelectrodes were moved between droplets of secreted fluid and calibration solutions. The second method used by Collier and O'Donnell (1997) on Rhodnius prolixus Malpighian tubules and Kochian et al. (1992) on Maize root cell suspensions involved the measurement of unstirred layers next to the tissue using a microelectrode positioned at two different distances from the surface of the tissue. In both cases the microelectrode measured the voltage at each of these two points and compared that voltage to that of a known calibration solution. In doing so it was then possible to calculate a concentration at each of these points and use this concentration difference to calculate an ion flux into or out of the tissue. This second method is acceptable for large voltage signals in relatively low background ion concentrations, but falters when the voltage signals are small  $< \sim 200 \,\mu V$  in relatively high background ion concentrations. Another problem with static electrodes used in this manner is associated with there tendency to drift with time. Therefore any measurement made between two points at different times or with two separate electrodes will suffer from a component of voltage drift. In general it appears that static ion-selective

microelectrodes are appropriate for measurement of ion concentrations in isolated fluids but are not amenable to measurement of small voltage signals associated with dyanamic steady state situations, such as the measurement of unstirred layer gradients in high background concentrations.

#### Shielded ion-selective microelectrodes (SIM)

Often the desire to measure transient changes in ion concentration in the extracellular environments are hampered by the rapid diffusion of the ions into the virtually infinite extracellular bathing solution. The use of shielded ion selective microelectrodes is a novel way of measuring these small transient changes in ion concentration which are too small to be detected by conventional ion-selective microelectrodes. Danker *et al.* (1996) have successfully fabricated a  $K^+$ - selective SIM that increases the electrodes signal to noise ratio by a factor of 3.

The electrode consists of a K<sup>+</sup> sensitive microelectrode equipped with a shielding pipette. The electrode is produced as described previously for ion-sensitive microelectrodes but with some minor modifications. The electrode is pulled to a tip diameter of  $\sim 1 \ \mu m$  whose shank does not increase to more than 10  $\mu m$  over the first 5 mm. The electrode is then silanized and front filled with the ionophore, under a microscope, by dipping the tip of the electrode into an ionophore reservoir and applying negative pressure to adjust the column length to 150 - 200  $\mu M$ . Except for its larger size the shielding pipette is the same shape as the inner electrode. The tip
diameter is ~ 10  $\mu$ m and is beveled at a 30° angle to allow sealing of the electrode on the biological sample. The inner and outer electrodes are assembled under a microscope with the tip of the inner electrode adjusted to within 5  $\mu$ m of the opening of the outer electrode. The electrodes are then fastened together using dental wax.

The advantages of the SIM are that it is no more expensive than standard microelectrodes but is three times more sensitive. The electrodes are fabricated relatively easily and have proven to be useful for the study of mechanically stimulated ion channels (Danker *et al.* 1996).

The disadvantage of the SIM is that it creates a localized environment near the surface of the membrane which may not reflect the normal environment. Therefore the biological preparation and more specifically ion channels may respond to this concentrated gradient in a manner that does not represent their normal physiological function. Another disadvantage is that the spatial resolution of this electrode is equivalent to the diameter of the opening of the outer shielding. Also, because the shielding electrode must contact the sample the electrode can be considered invasive.

#### Limitations of previously described techniques

All of the above methods, although suitable for ion analysis, exhibit restrictions for analysis of Malpighian tubule ion secretion. All of the above methods, except SIM, have poor temporal resolution. They require that enough secreted fluid be collected for analysis. This means that it would typically take ~5-10 minutes to collect enough fluid, preventing the temporal mapping of ion transport in real time. Also the above methods all have poor spatial resolution. Ion transport across different functional domains of a Malpighian tubule may utilize different mechanisms. Analysis of secreted fluid provides infromation on the combined effects of these functional domains. It would be beneficial to determine the effect on ion transport of each of the functional domains independently. As a result, the previous mentioned methods would have a difficult time spatially mapping these ion transport processes.

### Self-referencing electrodes

Recent development of self-referencing microelectrodes facilitates non-invasive anlaysis of spatial and temporal aspects of cellular transport. The development of a self-referencing current density probe (CDP) (Jaffe and Nucitelli 1974) has enabled the study of net ionic currents due to ion transport in various tissues (Scheffey *et al.* 1983; Kropf *et al.* 1984; Foskett and Ussing, 1986; Marcus *et al.* 1994; Somieski and Nagel, 1998). The current density probe has also been used to determine the pathway of chloride conductance in the isolated Malpighian tubules of *Drosophila melanogaster* (O'Donnell *et al.* 1998). The further development of ion-selective self referencing microelectrodes (Jaffe and Levy 1987; Kuhtreiber and Jaffe 1990) have enabled the elucidation of the specific ion fluxes that contribute to the net ion current detected by the current density probe. Electrodes specific for many ions have been developed and used to characterize ion fluxes in a number of tissues (Kuhtreiber and Jaffe 1990; Kochian *et al.* 1992, Smith *et al.* 1994; Pineros *et al.* 1998). A further detailed description of the fabrication and function of these self-referencing microelectrodes is given in Chapter 2.

### Goals for this thesis

This thesis will examine the use of a non- invasive self-referencing  $K^+$ -selective microelectrode for the study of K<sup>+</sup> transport by the secretory and reabsorptive segments of Drosophila melanogaster Malpighian tubules. In theory ion transport via the tubules should increase or decrease the concentration of ions in the unstirred layer adjacent to the basolateral surface of the epithelium. Previous study on the Malpighian tubules of Rhodnius prolixus using static microelectrodes confirmed that it was possible to measure a  $K^+$  concentration gradient in the USL adjacent to tubules ( Collier and O'Donnell 1997) as long as the voltage signals were relatively large and the background concentration of the ion in question was low. In Drosophila the voltage signals to be measured are below the sensitivity of conventional static microelectrdoes and the background concentration of potassium is relatively high (20 mmol 1<sup>-1</sup>). The use of the non-invasive self-referencing system should allow for the detection of these small voltage signals. Chapter 2 will provide a description of the fabrication of the ion-selective microelectrodes, their calibration and a description of the general use and principles of the relevant software and hardware. As well, the specific application of the self-referencing  $K^+$  probe to the study of ion transport in the Malpighian tubules of *Drosophila melanogaster* is also discusseed in chapter 2. Chapter 3 will discuss the spatial and temporal analysis of ion transport by isolated *Drosophila* Malpighian tubules using the non-invasive self-referencing  $K^+$ -selective microelectrode. In doing so the role of main, lower segments and non-secretory distal segment will be discussed. The role of the distal segment is of particular interest due to the fact that the determination of its function is not amenable to previously used techniques. The role of both stimulants and inhibitors of fluid secretion will also be described in chapter 3. Chapter 4 will be a general discussion regarding the suitability of the non-invasive self-referencing system for Malpighian tubule ion transport study. As well future uses of the ASET software and improvements of the system will be discussed in chapter 4.

# Chapter 2: Self-referencing Ion-selective Microelectrodes Principles of Use and Construction

# Introduction

Living organisms and cells have the ability to regulate their internal ionic environment at compositions different from those in their external environment. They expend energy to accomplish this, and the resulting electrochemical gradients are central to membrane excitability, vectoral transport of ions and water, cellular homeostasis, and establishment of cell polarity. Cellular ion transport generates significant steady-state transmembrane currents, driven by differences in the distribution of ion channels and pumps. All of these processes involve the asymmetric distribution of ion transporters in the plasma membrane. Many of these membrane associated transport mechanisms are easily studied using well established electrophysiological techniques such as cellular and epithelial voltage clamping, patch clamping and standard intracellular microelectrode recording. However, the relatively small steady state signals generated by the activity of ATPases and non-electrogenic exchangers are not always detectable by these techniques.

The most significant problem in measuring weak spatial distributions of voltage or ion concentration generated by steady state current flow is the noise and instability found in the electrode. This problem has been solved by both the selfreferencing voltage-sensitive and ion-selective probes. Whereas the voltage-sensitive

probe measures current densities in the nanoamp range, the ion-selective probe measures signals in the microvolt range, which are equivalent to picomolar fluxes of a specific ion species. Self-referencing probes provide several advantages over conventional techniques. Firstly, the sensitivity of these probes is unparalleled in comparison to conventional microelectrodes. Secondly, the electrodes are noninvasive and their use, therefore, does not perturb cell function. Patch clamping for example involves driving changes in the natural membrane potential, and is invasive due to the required contact with the plasma membrane. Also only cells where a gigaohm seal can be established can be studied. As well, one could realistically sample only a small area of the tissue in question. Thirdly, because the tip of the electrode is within a couple of microns of the cell surface, the spatial resolution is determined by the electrode tip diameter. This can be as little as 5-10 µm for the current density probe and ~ 2-4  $\mu$ m for the ion-selective probes as discussed below. Lastly, because these probes are stable over long periods of time and can be used with automated positioning and measurement systems, they can be used to map current patterns and ion fluxes from many different regions of a single sample, and can be used over extended periods of time (hours to days).

### **General Principles of Self-referencing Microelectrodes**

The original aim of the self-referencing probe was to map the steady state voltage fields in the extracellular medium and then deduce the underlying current. Theoretically this could be accomplished by placing two conventional glass microelectrodes filled with electrolyte at a known distance apart and a known distance from a cell or tissue. In theory current can be calculated from the voltage difference between the two separate electrodes by applying a modification of Ohm's Law appropriate for a continuous medium (Smith et al 1994):

(Eq. 2.1)

$$\mathbf{E} = \mathbf{J}\mathbf{p}$$

where E, is the voltage gradient in volts.cm<sup>-1</sup>,J is the current density in amps.cm<sup>-2</sup>, and p is the medium resistivity in ohm.cm<sup>-1</sup>. In practice this approach is unfeasible for several reasons. Firstly, because of variations in tip potentials and thermal noise liquid-filled electrodes cannot be used to reliably detect differences smaller than about a millivolt (Jaffe and Nuccitelli 1974). For example, in a typical case with two electrodes 30  $\mu$ m apart in a medium with a resistivity of 300 ohm.cm<sup>-1</sup>, current densities smaller than ~ 1 mA.cm<sup>-2</sup> would not be detectable. Secondly, both electrodes would drift independently of one another precluding any assurance that the measured voltage difference was due to changes in current rather than drift of the electrodes. In

response to these problems a method for determining transcellular currents from extracellular voltage gradients and Ohm's Law was developed by Jaffe and Nucitelli (1974). The voltage sensitive vibrating probe, hereafter referred to as the Current Density Probe can measure voltage differences down to a range of nanovolts, a signal orders of magnitude below the sensitivity of conventional glass microelectrodes.

### **Current Density Probe**

The current density probe first developed by Jaffe and Nucitelli (1974) was a one dimensional probe which measured currents in a single plane (x, y or z). Elucidation of a two dimensional current density pattern required a second scan in a direction orthogonal to the first. This was difficult because it required the repositioning of the sample or the vibrational array, compromising the precision and repeatability of the scanning technique. Solving these shortcomings led to the creation of a two dimensional current density probe (Scheffey 1986, 1988), which vibrated in both the x and y axis, enabling the calculation of the current density in two dimensions as well as the angle of current flow between the tissue and current sink. The description given here will refer to the two dimensional configuration in use although the theory and general principles are applicable to both one and two dimensional probes.

The current density probe resolves steady state current densities in the nanoamp range. The probe uses a platinized metal electrode vibrated at ~200 Hz over a vibrational distance which is typically twice the probe tip diameter (  $5-10 \mu m$ ).

Extracellular current flow through the medium bathing the surface of the cell produces a voltage difference. The probe measures this voltage difference (a few nV) between both extremes of the vibration. Knowledge of the medium resistivity can then be used to convert these minute voltage drops over the vibrational amplitude into a current density ( $\mu$ A.cm<sup>-2</sup>). The current density probe circumvents drift because it is selfreferencing, The probe minimizes surface noise by increasing the vibrational frequency to reduce the electrical impedance at the metal-saline interface. In theory, the frequency required to reduce this impedance to negligible levels, in the absence of a Pt-black coating, is on the order of 10 kiloHz. This problem is overcome by plating the metal electrode with a high capacitance (> 10 nF) platinum black ball (Jaffe and Nucitelli 1974). The construction of platinum black electrodes has been previously described in detail (Scheffey 1988).

The primary advantage of the current density probe is it's great sensitivity (1-100 nV) relative to that of conventional glass microelectrodes ( $\sim 1 \text{ mV}$ ). Also the current density probe is not affected by ion gradients within the bathing medium due to the mixing of the bath by the high frequency vibration, nor are these metal electrodes affected by dissolved chemicals or toxins in the bathing medium.

There are several limitations to the current density probe technique. Firstly, although the probe easily measures steady state currents that last seconds or longer, it is not amenable to measurement of fast transient currents. Secondly, the study of nonelectrogenic ion transporters is impossible because they produce no net current flow. Thirdly, the probe measures only net electrical currents and does not directly identify which ions carry the current. Such identification requires additional experiments involving ion substitution and pharmacological blocking agents. However, the current carried by one specific ion may be only small fraction of net current and therefore difficult to detect with the current density probe. Other limitations include the disturbance of less robust preparations by the vortex produced during probe vibration and the need for a relatively large sampling area (~100  $\mu$ m<sup>2</sup>). Although a very powerful and informative technique on its own the limitations of the current density probe led to the development of the self-referencing ion-selective probe.

# **Self-referencing Ion-selective Microelectrodes**

Although the measurement of extracellular current density can be very useful, it is often preferable and biologically more significant to determine the specific ions whose fluxes underlie net extracellular current flow. In this regard a major advancement was the introduction of the self-referencing ion-selective microelectrode by Jaffe and Levy (1987) and its further refinement by Kuhtreiber and Jaffe (1990).

The technique relies upon the increases or decreases of ion concentration in the unstirred layer (USL) next to a cell or tissue. Although ion movement through biological membranes can be either an active or passive process, diffusion through the external medium is passive and follows Fick's Law (Kuhtreiber and Jaffe 1990):

$$\mathbf{J} = -\mathbf{D} \left( \Delta \mathbf{C} / \Delta \mathbf{x} \right)$$

where J is an ion flux in the x direction,  $\Delta C/\Delta x$  is its concentration gradient, and D is the diffusion constant. Patterns of ion flux across a cell or tissue surface can be resolved by measuring and mapping gradients in the concentration of the ion near the surface of the cell or tissue. In some instances this can be done using a single conventional static ion-selective microelectrode positioned at two different points in the bath. Kropf et al. (1984) successfully determined the pattern of proton currents through fungal hyphae using a non-vibrating pH-sensitive microelectrode. pH differences as small as 0.01 pH units could be reliably detected, corresponding to a change in voltage of 500  $\mu$ V. Kropf's simple method of slowly moving a single nonvibrating pH microelectrode around a cell could successfully resolve moderate proton fluxes ( $1.0 \times 10^{-4}$  picomoles.cm<sup>-2</sup>.s<sup>-1</sup>) because of the low background concentration of protons ( $0.3 \mu M$ ) in the bathing solution. This is orders of magnitude lower than the concentration of most other important ions (Na<sup>+</sup>, Ca<sup>+2</sup>, K<sup>+</sup>, Cl<sup>-</sup>) in physiological media. Since the sensitivity of any measurement of a concentration difference depends upon the fractional or relative difference of  $\Delta C/C$ , not  $\Delta C$ , their simple method of slowly moving a static microelectrode around a cell would not work for most studies. In another study, Collier et al. (1997) successfully determined the pattern of  $K^{+}$ currents across the lower segment of the Malpighian tubules of Rhodnius prolixus using a non-vibrating K<sup>+</sup>-sensitive microelectrode positioned at two different points in

the bath. The flux rate determined was 2.03 nanomol.cm<sup>-2</sup>.s<sup>-1</sup> corresponding to about a 200  $\mu$ V voltage difference and a 12% relative change in concentration (background of 3.6 mmol l<sup>-1</sup> K<sup>+</sup>) over the distance measured (0.0042 cm). It was possible to measure a voltage signal near the limit previously mentioned for static microelectrodes for two reasons. Firstly, because the potassium fluxes under study were relatively large, and secondly, the background concentration of K<sup>+</sup> was relatively low in this study (3.6 mmol l<sup>-1</sup>). Ryan *et al.* (1990) have suggested that the theoretical limit for non-vibrating electrodes was ~ 13  $\mu$ V. In practice, however non-vibrating electrodes have an actual working resolution of ~200-500  $\mu$ V.

Assuming that the self-referencing electrode has a sensitivity of ~ 5-10  $\mu$ V ( Jaffe and Nucitelli, 1990), the self referencing electrode would be able to measure a relative concentration difference in potassium of 0.03% when the background concentration of K<sup>+</sup> is 20 mmol  $\Gamma^1$ . However, conventional ion selective microelectrodes, assuming a realistic minimum limit of sensitivity of 200-500  $\mu$ V, could detect a minimum relative concentration difference of ~1% or greater in a corresponding background potassium concentration. In most cases, therefore where the K<sup>+</sup> background concentration is relatively high and the fluxes are relatively small (subpicomoles to picomoles per cm<sup>2</sup> per second) the use of a self-referencing ionselective probe will be advantageous.

The most significant problems with slowly moving one electrode around a system or with using two microelectrodes positioned a known distance apart is voltage

drift and electrode noise. A stationary electrode drifts too quickly to allow for the measurement of small voltages and two paired stationary electrodes drift independently of each other. Vibrating the electrode essentially allows for a self-referencing system in which the drift of the electrode can virtually be eliminated. The frequency of vibration of the electrode is very important; it must be sufficiently fast enough to avoid the inherent drift associated with liquid ion exchanger (LIX) filled electrodes, but not so fast that the probe movement does not allow for re-establishment of the concentration gradients which one is trying to measure.

The advantages of the self-referencing ion-selective microelectrode are that the system is a non-invasive measure of specific ion fluxes, and is highly sensitive. It is capable of measuring a voltage signal of ~5  $\mu$ V, which is equivalent to fluxes less than 1 picomole.cm<sup>-2</sup>.s<sup>-1</sup>. Another advantage is the spatial resolution of the probe, which is determined by the tip diameter of the glass microelectrodes (usually ~2-4  $\mu$ m), and the vibrational distance. However it must be noted that there is a trade-off between vibrational amplitude and signal resolution. Resolution of small signals will require larger vibrational amplitudes resulting in a decreased spatial resolution.

However, although the self-referencing ion-selective microelectrode provides many advantages over the current density probe and it has some limitations. Firstly, it is limited by the selectivity and availability of commercial ionophores. Secondly, the temporal resolution of the technique is on the order of seconds, limiting the speed of events that can be monitored. Thirdly, the probes are somewhat fragile and delicate so great care must be taken when using them. Fourthly, the electrodes may be easily poisoned by some experimental compounds. Finally, the probes must be positioned close to the membrane but must not touch it because contact may cause loss of the ionophore.

## **Fabrication of Ion-selective Microelectrodes**

Successful use of the self-referencing ion-selective microelectrode technique is highly dependent on efficient fabrication of both the ion-selective electrode and the reference electrode. Construction details differ slightly for each ion species and the procedures detailed below are those which have been found to be most effective for the analysis of  $K^+$  transport by insect Malpighian tubules. Since these procedures have not been documented previously they are provided here in part so that this chapter may serve as a reference manual for subsequent users.

#### **Microelectrode Shape**

Glass micropipettes were pulled from 1.5 mm diameter non-filamented glass capillary tubes (TW150-4; World Precision Instruments Inc., Sarasota, Florida). The capillary tubes were cleaned by washing in nitric acid for 5 minutes, then rinsed with deionized water and dried on a hot plate at 200 °C for 1 hour. The micropipettes were

pulled on a programmable horizontal puller (P-97 Flaming-Brown, Sutter Instrument Co., Novato, CA) using a 3 stage pulling procedure (Table 2.1). The resulting microelectrodes had a bee stinger like appearance (see Fig 2.1) with a shank of ~4mm and a tip diameter of ~2-4  $\mu$ m. The shank of the potassium electrode used in my studies is longer than those previously reported for studies using Ca<sup>+2</sup> and H<sup>+</sup> ionophores (Kuhtreiber *et al.* 1990, Kochian *et al.* 1992). Also a longer column length of ionophore is used for the potassium ionophore (~180-200  $\mu$ m) instead of the conventional 10-40  $\mu$ m column length used for Ca<sup>+2</sup> and H<sup>+</sup> ionophores.

Table 2.1

Three stage program for pulling ion-selective microelectrodes on P97 Flaming Brown programmable horizontal puller.

	HEAT	PULL	VELOCITY	TIME	
LINE 1	480	0	5	250	_
		•	C C		
		_	_		
LINE 2	500	0	5	250	
I INTE 2	460	10	15	25	
	-+00	10	10	43	

Figure 2.1

Ion-selective microelectrodes are produced by a 3 stage pulling procedure generating an electrode with a bee stinger like appearance. Scale bars are (A) 1mm and (B) 10  $\mu$ m.



### Silanization

Glass surfaces are quite hydrophilic and as a result tend to repel organic based liquids such as the ionophores used in these microelectrodes. Moreover, an ionophore cocktail placed in the tip of a micropipette will tend to be displaced by aqueous (i.e. hydrophilic) solutions. To overcome this problem the procedure of silanization was introduced (Walker, 1971) to render the glass hydrophobic. The silanization procedure is important because it chemically alters the surface of the glass so that hydroxyl groups on the glass surface condense with the unstable silanol generated from the silane (Ammann, 1986). In this study the electrodes were silanized by vapour phase treatment with N.N-dimethyltrimethylsilylamine (Fluka Chemical Co. Ronkonkoma, NY) in a simple setup illustrated in Fig 2.2. A batch of micropipettes that had been previously pulled and predried on a hot plate for 30 min at 200 °C are placed under a large glass petri dish inverted on a hot plate. A small amount (~50µL) of silanization reagent was injected onto the inside of the petri dish. The reagent vapourized immediately and was allowed to react with the glass for 15 min at 200 °C. The petri dish is then removed to allow the excess vapour to escape and then replaced. The micropipettes were then baked on the hot plate for a further 20 min at 200 °C. The micropipettes were then be removed and stored in an air tight chamber over a desiccant for up to 3 months before use.

Figure. 2.2

Simple arrangement for the vapour-phase silanization of micropipettes (not drawn to scale)



An additional advantage of silanization is that this procedure helps to stabilize the short column length and prevent electrical shunting along the interface of the ionophore and the glass surface by increasing the resistance of the glass surface ( Dagostino and Lee 1982, Smith *et al.* 1994).

### **Electrode Filling**

The ease with which a micropipette can be filled with an organic solution is dependent on many factors including the viscosity of the solution, the quality of the silanization procedure, the tip diameter as well as the method for filling the electrode. For this study the ionophore was introduced into the electrode using the front-filling technique. Prior to front-filling with the ionophore the electrolyte used as a backfill must be injected into the electrode. A solution of 100 mmol  $l^{-1}$  KCl, filtered through a 0.2 µm Acrodisc filter to remove particulate matter was used as the backfill. It has been reported previously that an agar solution confers significantly better stability on the ionophore column during vibration than a simple aqueous electrolyte (Smith et al. 1994). This study used a 100 mmol  $l^{-1}$  KCl without agar because no noticeable disruption was noted in the signal during vibration. The electrolyte is injected into the shank of the microelectrode to a 15 mm column length, using a 1 mL polystyrene syringe pulled to a fine tip over an open flame. This column length was the minimum sufficient to ensure contact with the AgCl wire (see below). When back filling, the electrolyte does not flow to the tip of the silanized micropipette. The air at the tip was

forced out by application of pressure, using a syringe connected to the microelectrode holder by flexible tubing, to the back of the micropipette prior to loading the electrode with the ionophore cocktail ( see below).

There are several commercially available potassium ionophores. The ionophore used in these studies was purchased from Fluka and was chosen based on its preferable selectivity coefficients and short response time (Table 2.2). Selectivity coefficients are usually presented in logarithmic format; a value of -3, for example, indicates that the ionophore is 1000X more selective for  $K^+$  than for the interfering ion. Potassium Ionophore I - Cocktail B (Fluka Chemical Co., Ronkonkoma, NY) contains a potassium selective neutral carrier in a mix of 93% 1,2,-dimethyl-3-nitrobenzene and 2% potassium tetrakis (4-chlorophenyl) borate.

Front-filling the micropipette was done under an upright microscope with a 40X objective (Fig 2.3). A silanized pipette with the final taper broken back to an aperture of 50-100  $\mu$ m was used as an ionophore reservoir. Positive pressure was applied to this pipette using a micrometer connected to a 1 mL syringe until the ionophore bulged out the tip and the microscope was focused on the leading edge of the bulge. The microelectrode to be filled was fitted into a WPI Microelectrode holder with a sidearm luer through which pressure could be applied using a hand held 10 mL syringe connected via polyethylene tubing. Prior to front filling positive pressure was applied to expel a small amount of electrolyte and remove any air

Table 2.2

Table of selectivity coefficients for Potassium Ionophore I - Cocktail B

(Reprinted from Fluka Selectophore Catalog 1991; Fluka Chemical Co. Ronkonkoma,

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NY)

Selectivity Coefficients	$\log K^{Pot}_{KM}$ as obtained by the separate solutions method	
	$(0.1 \text{ mol } l^{-1} \text{ solutions of the chlorides})$	

$\log K^{Pot}_{KLi}$	-4.2
$\log K^{Pot}_{KNa}$	-3.9
log K <sup>Pot</sup> KMg	-4.6
log K <sup>Pot</sup> KCa	-4.9
$\log K^{Pot}_{KAcetylcholine}$	-3.5

Electrode Function: slope of linear regression;  $57.8 \pm 1.2 \text{ mV} (20^{\circ}\text{C}, 10^{-1} \text{ to } 10^{-4} \text{ mol } 1^{-1} \text{ KCl.}$ 

Electrical Resistance: tip diameter  $\sim 1 \ \mu m$ ; 1.2 \* 10<sup>10</sup> ohms

Response time: time constant of < 1 s.

Figure 2.3

Photograph of setup used for front-filling micropipettes.



spaces. The tip of the micropipette was then brought into the same focal plane as the tip of the ionophore reservoir using a hydraulic micromanipulator (Narishige, Tokyo, Japan). The micropipette was advanced until the tip was in the ionophore at which time negative pressure was applied to pull the ionophore into the microelectrode to the desired column length. The ionophore column length could be accurately adjusted at that time by appropriate adjustments of pressure. It was important at this point make sure that no air bubbles were in the electrode as this would result in increased noise. Also one can check the efficiency of silanization qualitatively by examining the meniscus of the ionophore column. A flat meniscus indicates poor silanization and a highly convex meniscus facing the shaft indicates over silanization.

After filling the micropipette is inserted into a standard microelectrode holder ( WPI EHB1; WPI, Sarasota, Florida). The holder comes fitted with a silver wire, which must be plated electrolytically with silver chloride. For best results the wire was cleaned with fine sandpaper prior to electroplating to remove any corrosion. The electrode was then plated by immersing the wire in a 0.5 N solution of hydrochloric acid while passing current supplied by a 9V battery through the electrode. The other pole of the battery was connected to a silver wire immersed in the acid solution and current polarity was reversed using a switch every 2 seconds for a total of 10 seconds. Experience has shown that properly plated electrode wires are considerably less noisy than wires which are poorly plated or corroded.

### **Reference Electrode**

The reference electrode used in this system consisted of a 10 cm long, 1.5 mm diameter glass capillary tube (TW150-4; WPI Inc, Sarasota, Florida). The tube was held over an open flame and bent to a 60° angle 2 cm from one end. The tube was then filled with a mixture of 3 mol  $\Gamma^1$  KCl and 1% agar. The agar served to stabilize the electrolyte and prevent rapid dilution of the reference electrode by bulk inflow of water from the bathing solution, while at the same time minimizing K<sup>+</sup> leakage into the bath. After reference electrodes were filled in this manner they could be stored in a sealed jar containing a mixture of 3 mol  $\Gamma^1$  KCl and 0.5% agar for repeated use over a number of months. Electrical contact was made by inserting the reference electrode into 3mol  $\Gamma^1$  KCl filled a standard microelectrode holder half cell (WPI MEH3S; WPI Inc., Sarasota, Florida) (Fig 2.4). Air bubbles were removed since their presence in the reference electrode or the microelectrode holder resulted in no signal or at best an unreliable signal.

Figure 2.4

Photograph of the 3 mmol l<sup>-1</sup> KCl filled reference electrode in its standard microelectrode holder half cell in the experimental apparatus.



### **Probe Positioning and Vibration**

The original design published by Kuhtreiber and Jaffe (1990) positioned the probe using Newport linear motors and probe vibration was produced by three piezoelectric microstages (PZS-100; Burleigh Instruments Inc., Fishers, NY) stacked in an orthogonal array and holding the preamplifier. This system had many drawbacks. The size of the array close to the condenser of the inverted microscope limited the range of probe movement. As well, the mass of the array on the manipulator affected positioning accuracy. The system was further limited by the use of linear motors whose accuracy was  $>> 1 \ \mu m$ . One of the major deficiencies was poor precision especially during repeated scans of particular sites due to the absence of a computer controlled positioning system.

In the present configuration used in this study many of these problems have been resolved. The positioning system relies on an orthogonal array of stepper motors (Fig 2.5) that control both vibration angle and length in three dimensions. The stepper motors are fitted to a set of Newport 360 translator stages and can control movement over a wide range from 3-5 cm down to submicron accuracy (0.003  $\mu$ m), with a bidirectional repeatability of ~ 1.0  $\mu$ m. Both the probe and the preamplifier are vibrated together in order to prevent capacitative changes in the linking cable and mechanically generated electrical noise. The motors are controlled by Pentium PC running a Windows 95 compatible version of the Automated Scanning Electrode Technique (ASET) software Figure 2.5

Photograph of microstepper motors (A) mounted on Newport 360 translator stages

(B) used for probe positioning and vibration.



package (Science Wares; East Falmouth, Massachussetts) through an intelligent motion system ( Applicable Electronics; East Falmouth, Massachussetts). A major advantage of this programmable computer controlled motion system is the ability to perform repeated complex three dimensional scans with submicron precision. The probe is vibrated only at right angles to the long axis of the electrode, so as to minimize the pressure dependent movement of the ionophore column and the resultant instabilities in the electrode signal (Smith et al. 1994). Vibration frequency is a critical parameter. It must be sufficiently high to avoid the inherent drift associated with LIX filled electrodes but not high as to preclude re-establishment of the ion concentration gradient under study. The signal measured within a gradient is dependent on the background concentration and the amplitude of the vibration. Background concentration is dependent on the bathing medium ( e.g. sea water versus physiological saline or fresh water). According to Kuhtreiber and Jaffe (1990) the optimal distance for vibration of a  $Ca^{+2}$  electrode is ~30  $\mu$ m. They have shown that the measured voltage difference increases linearly with vibration distance up to an amplitude of  $\sim$ 30  $\mu$ m. The optimal vibrational distance depends on the ionophore used, and the biological preparation under study.
#### **Stationary Calibration of Electrodes**

As with conventional ion selective microelectrodes the self-referencing ionselective microelectrode must be calibrated. Ion selective microelectrodes measure ion *activity* but it is often more convenient to express the data in terms of concentration. This requires the assumption that the activity coefficient of the ion in question is the same in the sample bathing medium as it is in the calibration solutions. In solutions whose ionic strength is comparable to that of physiological saline, this assumption is justifed. For example, the activity coefficient of NaCl:KCl mixtures with a total concentration of 150 mmol  $\Gamma^1$  is 0.750, whereas the activity coefficients for 100 mmol  $\Gamma^1$  and 200 mmol  $\Gamma^1$  NaCl are 0.778 and 0.735 respectively (Robinson and Stokes, 1965). The sensitivity of ion-selective electrodes can be described by the Nikolsky-Eisenman equation (Ammann, 1986):

(Eq. 2.3)  

$$V = E_o + s \log \left[ a_i + \sum_i K^{Pot}_{ii}(a_i)^{2i/2j} \right]$$

where V is the potential (mV) measured,  $E_o$  is the reference potential (mV), s is the Nernstian slope (See Eq 2.4 below),  $a_i$  and  $a_j$  are the activities of the ion of interest and the interfering ion respectively,  $z_i$  and  $z_j$  are the charge of both the ion of interest and interfering ion respectively, and  $K^{Pot}_{ij}$  is the activity coefficient or selectivity factor for the ion of interest over the interfering ion (See Eq 2.5 below). The Nernstian slope (s) can be calculated as (Ammann, 1986):

(Eq. 2.4)

$$s = 2.303 \text{ RT}/z_i F = 59.16 \text{ (mV } @ 25^{\circ}\text{C})$$

where R = gas constant (8.314 JK <sup>-1</sup> mol<sup>-1</sup>), T = absolute temperature (K), and F = Faraday constant (9.6487 x  $10^4$  C mol<sup>-1</sup>).

Selectivity Factors are often determined using the separate solution method (SSM). This method measures the potential obtained for the ion of interest and the interfering ion, in 100 mmol  $\Gamma^1$  pure single electrolyte solutions. The values are compared using a transformation of the Nikolsky-Eisenmann equation (Ammann, 1986):

$$K_{ii}^{Pot} = 10^{[(E_j - E_i)Z_i F/2.303 RT]} a_i / (a_i)^{Z_i/Z_j}$$

where  $E_i$  and  $E_j$  are the potentials (mV) measured in the pure solutions of the ion of interest and the interfering ion respectively.

For the highly selective valinomycin based  $K^+$  ionophore used in this study ( Table 2.2) at 10-fold change in concentration of the major interfering ions (Na, Ca, Mg) produces a signal 8000-80,000 times less than that produced by a 10-fold change in  $K^+$  concentration. Under these circumstances the contributions of the interfering ions can be ignored and the Nicolsky-Eisenmann equation effectively reduces to the Nernst equation.

(Eq. 2.6)

$$V = E_0 + s \log a_i$$

When a slope is determined by a voltage change between two solutions of different activities for the same ion, equation 2.6 can be reduced to:

$$\Delta V = 59.16 (\log [ion]_1 / [ion]_2)$$

where  $\Delta V$  is the change in voltage (mV), and [ion]<sub>1</sub> and [ion]<sub>2</sub> are the concentrations ( mmol l<sup>-1</sup>) of the calibrating solutions. The electrodes are calibrated by measuring the mV signal in response to a 10-fold concentration difference of the ion of interest.

The ASET software automatically calculates the slope of the electrode using its calibration window (See Fig 2.6). A 2 point or 3 calibration can be made. Preliminary studies have indicated that a 2 point calibration is sufficient. The effects of changes in the compositions of the backfilling solutions on the Nernstian behaviour of the electrode in 1mmol  $1^{-1}$  to 300 mmol  $1^{-1}$  KCl were examined (Fig 2.7). Although there were changes in offset, the slope of all electrodes with the different backfilling solutions did not vary significantly from the global mean value of  $53.3 \pm 0.3$  mV/decade over the range 15 to 150 mmol l<sup>-1</sup> KCl.

# Efficiency

After the determination of electrode slope the self-referencing ion-selective electrode must be dynamically calibrated to account for its sampling properties and responses during vibration. A dynamic calibration is required because the microelectrode has a definitive time constant for measuring a voltage signal on the order of seconds. During vibration of the probe, the signal measured may only be a percentage of the true signal. The efficiency of the electrode is that portion of the signal that the probe measures using its current sampling parameters. The dynamic calibration is done by filling a blunt micropipette ( tip diameter  $\sim 10 \ \mu m$  ) with 100 mmol 1<sup>-1</sup> KCl plus 0.5% agar and placing this in a 35 mm diameter petri dish containing a 1:1 mixture of Drosophila saline and Schneider's Insect Medium, containing the normal background  $K^{\dagger}$  concentration (C<sub>B</sub>). Thirty minutes is allowed to elapse to allow a steady state to be established between  $K^{+}$  ions diffusing between the source ( i.e. the micropipette) and the sink (i.e. the saline in the dish). Inclusion of the agar in the source pipette minimizes the effects of bulk water flow into the pipette. Convective disturbances are minimized by placing the tips of the

Screenshot of calibration window used in ASET software package (Version 1.05).

# K+ y-plane Probe Calibration

Nernst Slope 58.08 mV/decade Intercept: -49.16	mV	Probe
		-0.00 mV
Auto Bath Ulfset Update Zero Bath Ulfset 0.00	mV	Watch Off
Solution 1: 15. mM Solution 1 19.15	mV	Output Gain
Solution 2: 150. mM Solution 2 77.23	mV	100.
Solution 3: 10. mM Solution 3	mV	A/D Chan: 4

X

Effects of various micrelectrode backfills on microelectrode slope. Electrode calibration plots of the voltage versus the log of the concentration of KCl calibration solutions. The calibration solutions in each experiment were 1, 3, 6, 10, 30, 60, and 100 mmol  $I^{-1}$  KCl. A 180  $\mu$ m ionophore (Potassium ionophore I-Cocktail B) was used in all cases. The electrolyte was backfilled to a column length of 15 mm in all cases.

- A) Backfill consisted of 100 mmol  $l^{-1}$  KCl. Slope = 52.7 mV with y-intercept of -20.9 mV ( $R^2 = 0.9923$ )
- B) Backfill consisted of 80 mmol  $l^{-1}$  KCl / 20 mmol  $l^{-1}$  NaCl. Slope = 53.7 mV with y-intercept of -4.4 mV ( $R^2 = 0.9785$ )
- C) Backfill consisted of 60 mmol  $l^{-1}$  KCl / 40 mmol  $l^{-1}$  NaCl. Slope = 53.6 mV with y-intercept of +20.3 mV ( $R^2 = 0.9802$ )







Α

в



68

source pipette as well as the measuring electrode relatively close to the bottom of the dish.

In the steady state, the concentration at the mouth of the pipette ( $C_0$ ) is much smaller than the concentration deep within the source pipette ( $C_s$ ). This is because the solid angle inside the pipette's mouth, and thus its diffusional conductance, is much smaller than the solid angle available for diffusion outside the pipettes mouth. In actual experiments,  $C_0$  is found to be 0.5 to 3% of  $C_s$  (Kuhtreiber and Jaffe 1990).

Theoretical values for the  $K^+$  gradient generated at the tip of the source pipette were calculated according to the following equation (Pineros *et al.* 1998):

(Eq. 2.8)

$$\Delta V = S \left[ \left( -U\Delta r \right) / \left( C_B r^2 + Ur \right) \right] / 2.3$$

where  $\Delta V$  is the change in millivolts over the vibration excursion of the electrode, S is the slope of the electrode calibration, r is the distance from the source,  $\Delta r$  is the amplitude of vibration,  $C_B$  is the background concentration of  $K^+$  (20 mmol  $\Gamma^1$ ), and U is an empirical constant. Empirical measurements were made by vibrating the electrode over and amplitude of 100 µm using the Move, Wait and Sample parameters of the ASET software. This protocol allows the probe to move to the designated position without data collection. A designated wait period in which no data collection occurs then takes place in order to allow the ion gradients to re-establish themselves. The probe then samples the probe signal over a designated time period and averages the signal reporting the average voltage gradient. In this study wait and average times of 6 seconds and 1 second respectively were used.

The constant U was calculated by first generating a calibration curve to characterize the response of the electrode ( as described in the preceding section), and then taking a series of static millivolt readings at known distances from the source pipette. The millivolt readings were then converted to  $K^+$  concentration values using the calibration curve. A plot of these concentration values ( C ) versus the inverse of the distance from the  $K^+$  source ( 1/r) yields whose slope as determined by linear regression is U :(Fig 2.8)

$$C = C_{\rm B} + U/r$$

The empirical constant can then be substituted into the previous equation and used to calculate the theoretical voltage change over a vibrational distance at a known distance from the source. Theoretical voltages at known distances from the source over a known vibrational amplitude are then calculated and plotted against the distance from the source. Actual experimental measurements of the voltage are then done using the self-referencing  $K^+$  electrode at the same distances from the source and plotted similarly. The ratio of the experimental to theoretical slopes of both lines yields the

efficiency of the electrodes (Fig 2.9). The experimentally measured K<sup>+</sup> gradient had a slope of -229.4 mV/cm between 0.035 and 0.01 cm from the source (b = 8.9 mV and  $r^2 = 0.975$ ), in contrast to the theoretical value of -245.8 mV/cm (b = 8.7 mV and  $r^2 = 0.817$ ) over the same range of distances. The ratio between the experimental and theoretical slopes yielded an efficiency of 85 % for the self-referencing K<sup>+</sup>-selective microelectrode system. Thus, for our measurements of epithelial K<sup>+</sup> fluxes, flux values had to be corrected to account for self-referencing K<sup>+</sup> microelectrode; the correction required dividing measured fluxes by 0.85. It should be noted here that efficiencies should be calculated for all types of electrodes used and also when sampling rules are altered (e.g. when vibrational amplitude is changed).

# **Measurement of Ion Fluxes**

Ion fluxes were calculated using Fick's first law of diffusion:

(Eq. 2.10)

$$J_{K^+} = D_{K^+} (C_1 - C_2) / \Delta x$$

where  $J_{K^+}$  is the net flux of potassium ions in picomol.cm<sup>-2</sup>.sec<sup>-1</sup>,  $D_{K^+}$  is the diffusion constant for K<sup>+</sup> (  $1.9*10^{-5}$  cm<sup>2</sup>.sec<sup>-1</sup> [Robinson and Stokes 1959]), C<sub>1</sub> and

Calculation of the empirical constant, U. Static measurements were made at a series of distances from the  $K^+$  source and then millivolt outputs were converted to concentration values. A plot of these concentration (C) values versus the inverse of the distance from the  $K^+$  source (1/r) yields a line with a slope of U, according to the equation:

$$C = C_b + U/r$$

where  $C_b$  is the background concentration of  $K^+$  (20 mmol l<sup>-1</sup>), and U in micromoles per square centimeter defines the diffusion characteristics of the gradient source ( $R^2 = 0.9692$ ).



Theoretical (•) and experimental (o) measurements of  $K^+$  gradient as a function of distance from the artificial  $K^+$  gradient source. Theoretical values were calculated according to the equation:

$$\Delta \mathbf{V} = \mathbf{S} \left( [-\mathbf{U}\Delta \mathbf{r}] / [\mathbf{C}_{\mathbf{b}}\mathbf{r}^2 + \mathbf{U}\mathbf{r}] \right) / 2.3$$

where  $\Delta V$  is the change in millivolts over the vibration excursion, S is the slope of the electrode, r is the distance from the source,  $\Delta r$  is the amplitude of vibration, C<sub>b</sub> is the background concentration of K<sup>+</sup>, and U is an empirical constant. Experimental measurements were made by vibrating the electrode through a 100 µm amplitude at different distances from the K<sup>+</sup> source. The ratio of the experimental slope to the theoretical slope yielded an efficiency of 85% for these K<sup>+</sup> electrodes.



 $C_2$  are the K<sup>+</sup> concentrations at the two extremes of the vibration ( in µmol.cm<sup>-3</sup>), and  $\Delta x$  is the amplitude of vibration (cm). It should be noted here that the units for concentration of mmol.l<sup>-1</sup> are equivalent to µmol.cm<sup>-3</sup> for the purposes of this calculation.

## ASET SOFTWARE OPERATION

#### Introduction

The Automated Scanning Electrode Technique (ASET) software was developed and supplied by Science Wares (East Falmouth, Massachussetts) and is used for data collection by self-referencing voltage, ion-selective and polarographic microelectrodes. This windows based software provides a simplified graphical interface for control of all scanning options and calculations.

The remainder of this chapter will be a brief description of the software and its components for use in the study of ion fluxes using a self-referencing ion-selective microelectrode. These procedures can be used for any ion-selective microelectrode but the description given refers to  $K^+$ -selective electrodes.

# **Equipment Setup**

The experimental setup was located in a space where traffic was minimal and away from any sources of electrical or mechanical interference. For measurement of minute voltage signals it was important to isolate the system on a properly grounded power supply in order to have the "cleanest" power possible. Personal experience has shown that an Uninterrupted Power Supply (UPS) was very beneficial for this purpose. Electrical noise is a constant problem when trying to measure very small voltage signals, therefore proper grounding ( single point grounding) of all equipment is important. To further isolate that system and minimize noise the experimental setup is enclosed within an aluminum mesh Faraday cage. Faraday shielding is necessary because the ion-selective probes have a very high resistance ( 200 to 500 megaohms) or more ( Eric Karplus, personal communication), mostly due to the properties of the LIX.

The following section describes the general setup of the ASET system and its components. ASET involves three subsystems for: 1) control of probe movement; 2) recording and amplification of probe signal, and; 3) collection and storage of a video signal (Fig. 2.10).

#### 1) Probe movement

The microelectrode is vibrated together with the preamplifier. The microstepper motors responsible for the vibration are connected to an Intelligent Motion Systems control box which then interfaces with the I/O board of the PC for computer controlled movement

#### 2) Recording and amplification of probe signal.

ASET relies upon a two stage amplification system in which a low gain (10X) amplification occurs prior to subtraction of the bath offset and then a further high gain amplification (100X) occurs before the voltage is reported by the software. The bath offset serves to zero the electronics around a mV signal that is typical of the bath. This allows small voltage signals to be measured with minimal error. The system is designed to have a maximal signal resolution of  $\sim \pm 5$  mV around the bath offset signal. Voltage changes larger than this maximal offset cause the amplifier to saturate ("rail") at its supply voltage (5 V=1000 x 5 mV). It should be noted that conventional ion-selective microelectrodes would be sufficient to measure voltage differences > 1 mV.

The probe signal is amplified 10-fold by the preamplifier, then sent to the main amplifier where the meter switch determines the channels to be used in signal processing. The signal then passes to the A/D board of the computer where the bath offset is subtracted (A/D channel 5). The signal is then returned to the amplifier via D/A channel 0 where a further 100-fold amplification occurs, yielding a final amplification of 1000 times the original signal. This signal is then returned to the computer via A/D channel 4 for interpretation and manipulation by the ASET software.

Schematic diagram showing the general setup of the ASET system and its components. Three subsystems for control of probe movement, recording and amplification of probe signal, and collection and storage of a video signal are represented. Interactions between these subsytems are represented by arrows. As well the signal amplification process is represented.



#### 3) Collection and storage of a video signal.

The digital image system is comprised of a Nikon upright microscope attached to a Sony SSC-M350 black and white video camera. The camera is connected to a Sony Trinitron external video screen and a Computer Eyes/ 1024 frame grabber I/O board in the PC for digital image capture and processing.

#### Software Setup

Once the software is installed it must be configured to the hardware. The A/D board converts an analog voltage into a digital value and vice versa. The A/D board used in the current setup is a CIO-DAS1602/16 I/O board at a base address of 310 hex, A/D range of +/- 10 volts, D/A range of +/- 5 volts and a sampling pace of 200 samples per second. The Frame grabber I/O board captures digital images form a video signal. The current setup uses a Computer Eyes /1024 board at a base address of 230 hex, NTSC standard, and 8-bit grey scale. The motion control system used for probe positioning and vibration has a base address of 710 hex.

#### Sampling Rules

The sampling rules refer to the general movement parameters of the tip of the electrode at each point in it's specified sampling path. Each type of probe will

Screenshot of sampling rules window used in ASET software package (Version 1.05).

y-plane Probe Sampling Rules	
Universe	Previews
Rotation: 0. degrees from 3	o'clock Show Local Ref
Tilt: 0. degrees from ho	orizontal Show Universe Obs
Update Bath Wait 9.00 sec, average for 1.	D/A chan 0 from A/D chan 5 C At Beginning of Path .00 sec, set C D/A chan 1 from A/D chan 7 At End of Path
Path Name X Y Z W origin: 0.0 0.0 0.0 0.0	wait average a/d d/a Coordinate system:  Absolute /  Relative
Path Name X Y Z ₩ origin: 0.0 0.0 0.0 0. Y: 0.0 -100.0 0.0 0.0 0.0	wait       average       a/d       Coordinate system: C Absolute / C Relative         0.0       6.00       1.00       4+0 (0:712>0)       Add       Move Parameters         6.00       1.00       4+0 (0:712>0)       Add       Move Parameters         Delete       Move Type:       C Linear Interp         1       repetitions
Path Name X Y Z W origin: 0.0 0.0 0.0 0. Y: 0.0 -100.0 0.0 0.0 Gradient Calculations	wait       average       a/d       Coordinate system: <sup>C</sup> Absolute / <sup>C</sup> Relative         0.0       6.00       1.00       4+0       [0:712>0]         6.00       1.00       4+0       [0:712>0]         Add       Move Parameters         Delete       Move Type: <sup>C</sup> Linear Interp <sup>C</sup> Tracking         1
Path Name X Y Z W origin: 0.0 0.0 0.0 0. Y: 0.0 -100.0 0.0 0.0 Gradient Calculations Initial # Final # origin-Y	wait       average       a/d       Coordinate system: <sup>C</sup> Absolute / <sup>C</sup> Relative         0.0       6.00       1.00       4+0 (0:712>0)       Add       Move Parameters         6.00       1.00       4+0 (0:712>0)       Add       Move Parameters         Delete       Move Type: <sup>C</sup> Linear Interp <sup>C</sup> Tracking          1       repetitions
Path Name X Y Z W origin: 0.0 0.0 0.0 0. Y: 0.0 -100.0 0.0 0.0 Gradient Calculations Initial # Final # origin-Y	wait       average       a/d       Coordinate system: <sup>C</sup> Absolute / <sup>C</sup> Relative         0.0       6.00       1.00       4+0 [0.712>)       Add       Move Parameters         0.0       1.00       4+0 [0.712>0]       Add       Move Parameters         0       Delete       Move Type: <sup>C</sup> Linear Interp. <sup>C</sup> Tracking          1       repetitions

have its own sampling rules as defined in the sampling rules dialog box. For  $K^{\dagger}$  probe moving in the y-axis (Fig 2.11), the probe vibrates over a 100 micron distance at each point with a wait and average time of 6.00 and 1.00 seconds respectively. The probe samples using A/D channel 4 and D/A channel 0. The probe may move by linear interpolation or tracking. Linear tracking requires that the probe move in a straight line between two points using computer determined acceleration and deceleration parameters. Tracking movement causes the probe to move between points at a constant speed along each axis. The bath offset measurement which zeros the electronics for sampling has a defined wait and average time of 9.00 and 1.00 seconds respectively and samples using D/A channel 0 from A/D channel 5. Periodically updating the bath while sampling is important because it ensures that the electrode does not encounter large voltage signals due to drift that would obscure the minute voltage signals that the operator is trying to measure. Subtract reference gradient must be checked if the gradient calculation is to take the reference measurement into account. The apply calibration box is used for direct calculation of concentrations. This option should not be used as the current software does not support this option. Rotation refers to counterclockwise rotation about the z-axis, and tilt refers to the specified rotation up from the horizontal axis applied to sampling rules during data acquisition. The current system is sampling only in one plane but it does have the capability to calculate gradients in multiple planes.

#### Data File Structure

Ion-selective probe measurements are recorded in either a .VPO (Fig 2.12) or SCN document. The .VPO document screen is the main screen of interest over which all other windows open. The information recorded is the average millivolt reading from the signal amplifier and most recent reference value for each point in the sampling rules. Both D/A channel (D/A channel 5 and 0) voltages are also recorded. The tip millivolt values are then transformed into microvolt gradients as specified by the sampling rules. The reference microvolt gradients can be subtracted if specified in the sampling rules. Subtracting reference gradients eliminates that portion of the signal that can be attributable to background activity of the bath. The left side of this screen allows for manipulation of the motion control system parameters. The Enable Kbd/Mouse button should be activated in order to have control of the probe using the keyboard. Directly below this button there is a window which allows the operator to choose the optical system in use. The sensitivity window allows the operator to specify the precision of movement of each keystroke from 0.7 µm - 20,000 µm, and the number of microns per 50 pixels on the video screen. The probe window allows the operator to specify control of the probe by keyboard or mouse and whether movement is by linear interpolation or tracking. This box also allows the operator to define the probe position on the video monitor by using the locate button. The present position of the probe is also reported with regard to each individual axis.

Screenshot of .VPO document used in ASET software package (Version 1.05). This is the main window in which data is displayed and is the main document from which all other windows can be opened.

ASET for Windows v1	.05 - D:\MARKASET\98090901.VP0	_ 8 ×
<u>File Edit Video</u> <u>I</u> echniqu	ue (K+y-plane) Mode Analysis Yiew Window Help	
Motion System	D:\MARKASET\98090901.VP0	
Disable Kbd/Mouse		
Nikon Plan 20 Ph2	Events from 09/09/98 10:03:29 to 08/04/99 14:01:04	1285
Sensitivite		
20 um/keshit	10:03:29.350 K+ y-plane ISP/PVP Calibration: Concentrations: c1=15.0 c2=150.0	c3=10.0 Volta
	10:37:26.000 K+ y-plane Gradient Sampling Rules: reps=6, points=2, calcs=1, ro	t=0.0, tilt=0.0
10.0 um/50pix	10:39:36 220 Log Entry' Begin Vector Scan Parameters (5 points at 180.00 secon	ads intervals for
Probe	10:39:36.000 K+ v-plane Gradient Sampling Rules 1: reps=6, points=2, calcs=1,	rot=0.0, tilt=0.0
Kbd A	10:39:36.000 K+ y-plane Gradient Sampling Rules 2: reps=6, points=2, calcs=1,	rot=0.0, tilt=0.0
Mouse 140.3	10:39:36.000 K+ y-plane Gradient Sampling Rules 3: reps=6, points=2, calcs=1,	rot=0.0, tilt=0.0
C Track 80.9	10:39:36.000 K+ y-plane Gradient Sampling Rules 4: reps=6, points=2, calcs=1,	rot=0.0, tilt=0.0
Z O	10:39:36.000 K+ y-plane Gradient Sampling Rules 5: reps=6, points=2, calcs=1,	rot=0.0, tilt=0.0
Locate 0.0	10:39:36.490 Log Entry: End Vector Scan Parameters	
MoveTo W 0	10:39:48 580 K+ v-plane Gradient Bef1' D/A=0.0.0.4 [6x2]; 40.981 40.978	40.977 40.976
	10:40:26.470 K+ y-plane Gradient2: D/A=0.0.0.4 [6x2]: 41.455 41.456 41.462 41.	460 41.461 41.4
1 Zmin: -100.00	10:41:03.220 K+ y-plane Gradient3: D/A=0.0,0.4 [6x2]: 41.666 41.666 41.664 41.	664 41.666 41.E
Sample	10:41:39.960 K+ y-plane Gradient4: D/A=0.0,0.4 [6x2]: 41.775 41.776 41.776 41.	779 41.773 41.7
☐Kbd A	10:42:16.710 K+ y-plane Gradient5: D/A=0.0,0.4 [6x2]: 41.845 41.841 41.841 41.	842 41.843 41.8
Mouse U.U	10:42:43.240 Log Entry: End Vector Scan 1 of 12: 5 points	
C Track 0.0	10:42:43.400 Log Entry: Begin Vector Scan 2 of 12: 5 points	20 756 41 606
ZO	10:42:34.770 K+ y-plane Gradient?: D/A=0.0.0.4 [0x2]; 41.001 40.324	293 A5 283 A5 5
Locate 0.0	10:44:09.470 K+ v-nlane Gradient3: D/A=0.0.0.4 (6x2): 43.519 43.512 43.511 43	505 43.505 43.4
MoveTo W 0	10:44:46.160 K+ y-plane Gradient4: D/A=0.0,0.4 [6x2]: 43.298 43.195 43.189 43	.183 43.183 43.1
0.0	10:45:22.850 K+ y-plane Gradient5: D/A=0.0,0.4 [6x2]: 43.208 43.203 43.207 43	.202 43.203 43.2
1 Zmin: -100.00	10:45:49.380 Log Entry: End Vector Scan 2 of 12: 5 points	
15.52M / 0.92K	10:45:49.490 Log Entry: Begin Vector Scan 3 of 12: 5 points	<u>ت</u>
S:84 / G:91 / U:84		
For Help, press F1		INUM
Start Microsoft Of	fice Shortcut Bar MASET for Windows v	3:25 PM

# Watch Modes

## Raw A/D

This mode allows for the display of channel voltages (volts) as a function of time and allows recording in real time. (Fig 2.13) The Raw A/D mode is useful for quickly assessing the nature of a sample or for looking for "hot spots" of activity. In the current setup the voltage recorded is from A/D channel 5 as set out in the sampling rules. It is important to note that the signal observed in the Raw A/D mode has undergone only a low gain (X10) amplification before it is displayed. The watch interval specified must be 10 % longer than the sampling period. As well, personal experience has shown that it is best to leave the autoscale option off and to manually set the voltage scale (In Fig. 2.3 the scale is from -0.273 V to 0.102 V). Data can be recorded at single instances using the Record One button or continuously using the Record button. The span window specifies the length of the time plot of the graphical display window. The actual voltages sampled are reported as they are sampled at the bottom of the dialog box. Once an obvious signal has been established it is best to examine it using the ion-selective probe watch mode or the appropriate scan mode.

#### **Ion-selective probe**

This mode displays microvolt gradients as a function of time and allows for recording in real time (Fig 2.14). The ion-selective probe watch mode makes gradient observations at regular intervals specified by the sampling rules and displays the results both graphically and numerically. Much like the Raw A/D watch mode the ionselective probe watch mode can be used for quick scans to search for active areas of a sample. This mode can also be used to do long-term time dependent measurements at a single point. This watch dialog box displays signals which have undergone both the low gain (10X) and high gain (100X) amplification. The bath offset, sample, and reference values are expressed in millivolts in the Tip voltage dialog box. The Log Entry button allows for text messages to be inserted in the .VPO document. Both a graphical and numerical display of the microvolts gradients are displayed in the gradient calculations dialog box, if the Show  $\mu V$  and Show bargraph boxes are checked. The watch control window allows the operator to access the sampling rules through a single button and also displays the time interval required for execution of the sampling rules. The Resume button activates or terminates the sampling procedure and graphical display as required. The time plot dialog box graphically displays the microvolt gradient versus time. Experience has shown that periodic bath offset updates should be done manually through this dialog box during long sampling periods.

Screenshot of Raw A/D Watch mode window used in ASET software package (

Version 1.05).

Watch Raw A/D Values		×
	Record	Close
Interval 1.89 seconds Resume Samp Rules	Record One	Log Entry
Month Day Year Hour Min Sec Day Hour Min	n Sec ⊠On	
From: 8 4 1999 15 44 43 Span: 0 0 0	30 Auto	oscale Split
0.102		
+ -		
Zoom Out		
Volts		
Zoom In		
+ -		
-0.273		
Actual Voltages: Ch5: -0.144 Volts		

Screenshot of Ion-selective probe Watch mode window used in ASET software

package (Version 1.05).

itch K+ y-plane Probe	
Tip Voltage Bath Offset -14.6, 0 V Show Sample (mV): -14.6,-14.6 / Reference -0.00366,-0.00366 /	Close Log Entry
Gradient Calculations IF Show uV: 1:0 / IF Show bargraph: IF Autoscale +/-20. microVolts IF Use Calibration IF Subtract Reference IF Show as p[] units	Bath Offse Reference Record
Watch Control Interval 17.91 seconds Rotation 0. Tilt	Samp Rules
38.6 + - Zoom Out /\ microVolts V Zoom In + -	
-8.87 0 seconds	120

# **Scan Procedures**

#### **Grid Scans**

The Grid scan moves the probe through a rectangular grid or along a straight line collecting data at evenly spaced intervals. Data for each repetition of the scan is stored in a file with a .SCN extension. This file is referenced to the parent observation document (.VPO) by the grid scan. The grid scan is best used when it is necessary to scan a large uniform surface of a sample such as a flat sheet of an epithelium. When setting up a grid scan the first thing specified is the type of probe being used in the Acquisition rules dialog box (Fig 2.15). When defining the scan area it is best to first enter the start and end points of the scan. This can be done in one of three ways. The first way is by using the Show button, which allows the operator to indicate a point by moving a cursor on the video screen with the mouse. Second, one can use the Move To button and then use the keyboard to move the probe on the video monitor to indicate position. Thirdly, the coordinates can be entered directly in the start and end windows. The number of points in the grid are then entered for each plane of the scan. It is necessary to define the grid points as 'n' points +1. For example, if the grid is 100 microns by 100 microns and 10 points 10 microns apart are required, it is necessary to specify 11 points in the grid to have 10 equal intervals. It is also best to use a zig-zag grid type not only because it is the fastest but also because it minimizes the stirring of the bath. The reference location is specified in a previous manner as previously described for the start and end points of the scan. The home position refers to a point,

usually a point far away from the sample in the bath, to which the probe returns to after each repetition of a scan is completed. This is necessary during prolonged scanning periods. Pre-programmed log entries can also be included in the scan definitions using the Comments window.

#### Vector Scan

The Vector scan is used to move the probe through an arbitrary set of programmed points. Vector scans store their data directly in the parent document and are set up through the vector scan definition dialog box (Fig 2.16). Vector scans are excellent for scanning irregular objects or for repeated scanning of points separated by large distances. As with grid scans the probe type is first specified. Operations can then be added individually for any indicated probe position. A reference observation should be the first operation added followed by the rest of the points to be sampled. Points can be added in any order but should be added so as to minimize the scan time required. Experience indicates that when selecting sampling points it is best to move the probe using keyboard control to that point to ensure that the probe does not contact the sample. Contact with the sample may damage the probe or cause the loss of the ionophore . In cases where the sample is irregular or the probe must move around an obstruction an

Screenshot of Grid scan setup window utilized by the ASET software package (

Version 1.05).
Target Directory: SG080412			I SI	now Status	Autosave every 10		] samples	OK Cal Cancel
d:\mai Start ever Estimated	kaset y 30. I time per s	• seconds • minutes • hours can: 12.1 se	for 2 continuou	repetitions sly up to 999 << Calc	CDP obse	rvations observations obs: Del	Define F	Locate Prob Cal Bath
Scan Area	a (in micror	ns relative to o	origin)				Grid Type	
Start: End: Origin: C Log Gri C Grid:	91.15 93.91 0. d X	88.56   -86.75   0.   Y 5	199.83 0. 0. Z 1	0. 0. 0. W 1	Show Show Show C Line / 5 C Log Inc /	Move To Move To Move To steps C Log Dec	C Zig Za C Flybac Mor Scan Too C Probe C Sample	e C X axis first W axis first W axis first We along axes only Move Type C Linear Interp C Tracking
Reference	e (in micro X 186.91	ns relative to Y 144.58	origin) Z	W	Show	Move To	CDP: 🔽 Gradient: Г Bath: Г	before 「after scar before 「after scar before 「after scar
Home Po	sition (in m	icrons relative	to origin)					Store Video Image
Probe:	131.66	•69.58	0.	0.	Show	Move To	∏ 0n	Before each sca
Sample:	0.	0.	0.	0.	Show	Move To	[] On	After each scan
Comment Pre-Acq Post-Acq	s uisition: [ uisition: [							

intermediate point can be programmed to clear the obstruction. No data collection occurs at these specified intermediate points. The preview button allows the operations to be executed with no data collection occurring. Scans can be scheduled by the operator as long as the scan interval is 10% longer than the estimated time per scan. Experience has shown that the autosave option slows down scanning. It is best to leave this option off, but a manual save must be done prior to exiting the ASET software or all data will be lost.

Figure 2.16

Screenshot of Vector Scan setup window utilized by ASET software package (

Version 1.05).

C second Start every 180. C mine C hou Autosave every 10 sar	onds utes for 12 repetitions rs nples Calc >> Estimated tim	✓ Update status Set CDP Resisti e per scan: 164.6 se	vity Cancel
add an operation X Locate probe at 146.31 With Locate sample at 0.00	Y Z W 80.90 0.00 0.00 0.00 0.00 0.00	Show Move To Show Move To	Set Selected Op Get Selected Op
Prompt when adding: C None C Show \ probe C Move / sample C Move / sample Move with mouse	Current Density Probe Obs Current Density Probe Reference ISP/PVP Obs ISP/PVP Reference Raw A/D Obs	e V Potassium Static Potass K+ y-plane	mediate Point ideo Image sium <u>Bat</u> Grid Scan
Iperations   # I Probe [ X Y Z W   1: P [108.69,-608.77,0.00,0.0]   2: P [108.69,31.21,0.00,0.0]   3: P [28.91,26.20,0.00,0.00]   4: P [-30.82,21.23,0.00,0.00]   5: P [-86.63,3.23,0.00,0.00]	] Sample [X Y Z W] 00] K+ y-plane Gradient Reference 0] K+ y-plane Gradient Observation K+ y-plane Gradient Observation ] K+ y-plane Gradient Observation K+ y-plane Gradient Observation	Operation e Observation: reps= n: reps=6, rot 0.0, tilt : reps=6, rot 0.0, tilt 0 n: reps=6, rot 0.0, tilt 0 reps=6, rot 0.0, tilt 0	Clear All 6, rot 0.0, tilt 0.0; bath upd 0.0; bath upd 0.0; bath upd 0.0; bath upda 0; bath upda Unse Copy

# CHAPTER 3: Analysis of Ion secretion and Reabsorption by Malpighian tubules of *Drosophila Melanogaster*

# Introduction

Chapter 2 described the functioning of the non-invasive self-referencing system and the ASET control software. This system is effective at measuring small ion concentration gradients created by ion fluxes in a variety of specimens. The Malpighian tubules of *Drosophila* are an excellent tissue for study of ion transport and fluid secretion because they are easily dissected and are small enough to allow physiological testing to be done on an intact and complete tissue sample. Non-invasive analysis of ion concentrations in the unstirred layers adjacent to the Malpighian tubules of *Drosophila melanogaster* may lead to further knowledge of the transport process.

# Structure and Function of the excretory system:

#### Organization of the excretory system.

The excretory system in insects consists of the Malpighian tubules and the hindgut. The Malpighian tubules are the sites of fluid and ion secretion between the alimentary system and the haemolymph of the insect. In *Drosophila melanogaster* there are two pairs of blind ended tubules which open to the alimentary system at the junction of the midgut and the hindgut (Fig 3.1). Each pair of tubules arises from

opposite sides of the pyloric ventriculus on a single ureter. The ureter of the anterior tubules projects forward while the stalk of the posterior tubules projects posteriorily into the abdominal cavity. Both the anterior and posterior tubules are bathed in the haemolymph which serves as the circulatory fluid of the insect (Demerec, 1950). Each Malpighian tubule of *Drosophila* is approximately 1.5 mm in length and 35  $\mu$ m in diameter (Demerec, 1950). The anterior tubule is divided into three distinct morphological segments; the distal (white) segment is non-secretory, the main segment is secretory and the lower segment is reabsorptive. Sozen *et al.* (1997) have further subdivided these domains according to their genetic characteristics. The posterior tubule has the same function except that it has no obvious distal segment (Dow et al. 1994b).

#### Anatomy and Ultrastructure of the Malpighian tubule.

The Malpighian tubules of *Drosophila melanogaster* are composed of a single layer of squamous epithelial cells. Cells of the main segment are of two types, the primary (principal) cells and the secondary (stellate) cells (Satmary and Bradley, 1984). The principal cells are large fusiform cells which can fold upon themselves to create a lumen while the stellate cells are found between the principal cells (Pannabecker et al. 1993). Typically there are 146 principal cells and 33 stellate Figure 3.1

Drawing of the gut and excretory system of *Drosophila Melanogaster* showing the point of attachment of the Malpighian tubules at the junction between the midgut and the hindgut of the alimentary canal. Note that their are an anterior and posterior pair of tubules which can be functionally divided into segments. Tubules are composed of a singular epithelial layer comprised of two distinct cell types; principal cells (darker fusiform cells) and stellate cells (lighter irregular cells).

(Reprinted from Wessing and Eichelberg 1978)



cells (Sozen *et al.* 1997). The tubule is made up of two to five cells in cross section with each principal cell resembling the shape of a half cylinder up to 35  $\mu$ m long and are joined to adjacent cells by septate junctions to form a tubule.

Each Drosophila tubule can secrete fluid at rates of 0.6-1.0 nl/min unstimulated and up to 6 nl/min when stimulated (Dow et al., 1994). Given that the outside diameter of tubules is 35um, the luminal diameter is 17um and the active length of a tubule is 2mm, it has been calculated that the main segment of the tubule must secrete its own volume of fluid in less than 15s. This is comparable to rates achieved by tubules of the blood feeding insect, Rhodnius prolixus (Maddrell 1991). Ultrastructural studies have revealed the sorts of membrane modifications that contribute to these high rates of transport. Electron micrographs of tubules show that the apical and basal membranes are highly folded. The total surface area of an insect Malpighian tubule is about 20 times greater than that of the vertebrate glomeruli per unit body weight (Phillips 1981). In Rhodnius, it has been calculated that the apical side of the cell is increased 150 fold due to the presence of microvilli and that the surface area of the basal side of the cell is increased by a factor of 40 due to the extensive infolding of the membrane. The increases in surface area as a result of this folding are presumed to be necessary for the incorporation of membrane proteins that are utilized for transport of ions, excretion of solutes such as uric acid, and osmotic water flow in Malpighian tubules. Based on cell dimensions and the width of the intercellular spaces the ratio of the area of the basal surface of the tubule to that of the intercellular cleft is 300:1. When the 40-fold amplification of the basal surface is taken into account, the ratio of area of the membrane to the intercellular cleft becomes 120,000:1. Given the high value of this ratio it has been suggested that the dominant route for fluid and ion transport in *Rhodnius* tubules is transcellular ( i.e through the cells) rather than paracellular (i.e around the cells) (O'Donnell and Maddrell 1983).

#### Fluid and ion secretion by Malpighian tubules.

Secreted fluid is slightly hyper-osmotic to the bathing saline or the haemolymph of the insect. Water transport is apparently a passive osmotic process that is directly coupled to the active transport of  $Na^+$ ,  $K^+$  and  $Cl^-$  (O'Donnell and Maddrell 1983). Based on the osmotic permeability it has been determined that very small osmotic gradients are sufficient to allow for passive water flow. Gradients that are as small as 0.7 mOsm across the basal membrane, and 2.6 mOsm across the apical membrane have been found to be sufficient to explain the observed secretion rates of *Rhodnius* Malpighian tubules.

The current model of ion transport for the Malpighian tubule is presented in (Fig 3.2). It is now generally accepted that the central role in active ion transport is accomplished by an apical vacuolar-type  $H^+$  -ATPase which is insensitive to ouabain but is inhibited by bafilomycin A<sub>1</sub> (Bertram et al. 1991; Bowman et al. 1988). This V-type ATPase maintains a proton gradient by pumping protons from the cell to the

lumen, providing a driving force for the secondary active transport of alkali cations from the cell to lumen through amiloride sensitive apical Na<sup>+</sup>/ H<sup>+</sup> or K<sup>+</sup>/H<sup>+</sup> antiporters. Potassium movement across the basolateral membrane is accomplished primarily by a K<sup>+</sup>:Cl<sup>-</sup> cotransporter with a minor contribution of a ouabain sensitive Na<sup>+</sup>/K<sup>+</sup>-ATPase ( Linton and O'Donnell, 1999). Also as a result of the active transport of cations from the cell to the lumen an electrical gradient is created which favours transport of Cl<sup>-</sup> from haemolymph to lumen (Williams and Beyenbach 1984).

It now appears that cation and anion transport in *Drosophila* Malpighian tubules are controlled separately. The endogenous peptide  $CAP_{2b}$  (cardio-acceleratory peptide), stimulates cation (Na<sup>+</sup>, K<sup>+</sup>) transport through cGMP, and also raises the fluid secretion rates. It appears likely that  $CAP_{2b}$  first elevates intracellular levels of nitric oxide which in turn stimulates the production of cGMP. Fluid secretion rates and cGMP levels are elevated by nitric oxide donors, and nitric oxide synthase is present in the tubules (Davies *et al* 1995). Cation transport can also be stimulated by cAMP. These effects are not additive to the effects of cGMP, and the first messenger (i.e hormone) leading to cAMP production in tubule cells is unknown (Davies *et al* 1995; O'Donnell et al. 1996).

The peptide leucokinin acts through intracellular calcium levels independently of cAMP or cGMP to raise the chloride permeability of the epithelium by a factor of five-fold and the rate of fluid secretion two-fold (Hayes *et al* 1989; O'Donnell *et al* 1996, 1998; and Pannabecker *et al*, 1993). Leucokinin, an endogeneous neuropeptide found in a number of insects, is believed to use intracellular calcium as a second messenger because it has been observed that, thapsigargin, a  $Ca^{+2}$ -mobilising agent ( Thastrup et al. 1990), has an additive effect on fluid secretion rates, which is indistinguishable from the effects of leucokinin, but not additive to the effects of leucokinin (Davies et al 1995). In contrast the effects of cGMP and cAMP are additive to those of leucokinin or thapsigargin, suggesting that cGMP or cAMP are not the secondary messengers utilized by leucokinin. These results also indicate separate control of cation and anion transport in the epithelium.

Electrophysiological data provide further support for the proposal of separate control of cation and anion transport in *Drosophila* Malpighian tubules. Treatment with cyclic nucleotides (1mmol  $1^{-1}$  cAMP and 1mmol  $1^{-1}$  cGMP) makes the lumen of the tubule more electropositive, by 40% and 28% respectively. These messengers act by stimulating the electrogenic cation transporting apical V-ATPase and have little effect on anion conductance or intracellular calcium (O'Donnell and Maddrell 1984; Maddrell and O'Donnell 1992; O'Donnell *et al.*, 1996). Again it was shown that the effects of cAMP and cGMP on the TEP were not additive (Dow et al. 1994), and therefore act in parallel on the same transporter(s).

Fig. 3.2

Schematic Drawing summarizing the current proposals for the cellular mechanisms of ion transport in the main segment of the Malpighian tubule of *Drosophila melanogaster*. The overall process of secretion of Na<sup>+</sup>, K<sup>+</sup>, and Cl<sup>-</sup> into the lumen is driven by the electrochemical gradients established by the V-type H<sup>+</sup>-ATPase. cGMP and cAMP stimulate the V-ATPase, which pumps H<sup>+</sup> into the lumen, which are then exchanged for K<sup>+</sup> via a K<sup>+</sup>/H<sup>+</sup> antiporter in the principal cells. K<sup>+</sup> enters basolaterally via a DIOA sensitive K<sup>+</sup>:Cl<sup>-</sup> cotransporter and by the Na<sup>+</sup>/K<sup>+</sup>-ATPase. Cl<sup>-</sup> is transported via a transcellular pathway in the stellate cells using leukokinin as a primary signal and Ca<sup>+2</sup> as a second messenger.

(Reprinted from Linton and O'Donnell 1999)



The chloride conductance of the epithelium has also been examined using cellular electrophysiological techniques and it has been found that the addition of leucokinin or thapsigargin have a depolarizing effect on the TEP towards 0 mV in a dose dependent manner. Subsequently, it was also shown that TEP becomes more lumen positive when the Cl<sup>-</sup> concentration of the bathing saline of LK-stimulated tubules was reduced 10-fold (Hayes et al 1989; Pannabecker et al 1993, and O'Donnell et al 1996). This observation suggests that leucokinin increases the Cl permeability of the epithelium thereby short-circuiting the voltage across the epithelium. Pre-treatment of tubules with the cell permeant calcium chelator BAPTA-AM significantly reduced the effect of leucokinin on both fluid secretion rates and on TEP. Evidence suggests that chloride conductance could be through transcellular pathways in either the principal cells e.g. Rhodnius (O'Donnell and Maddrell 1984) or stellate cells of Drosophila (O'Donnell et al. 1998) or through the paracellular pathways between stellate cells in Aedes (Panabecker et al. 1993). The participation by the principal cells of Drosophila has been refuted through observations which show the absence of appreciable effects of unilateral and bilateral Cl<sup>-</sup> concentration changes on the apical and basolateral membrane voltages of the principal cells, indicating that the CI<sup>-</sup> conductance of both these membranes is very low (Pannabecker et al. 1993).

In summary, CAP2b is believed to stimulate the V-ATPase through cGMP, and thereby increase  $Na^+$  and  $K^+$  transport. In contrast leukokinin or thapsigargin increases Cl<sup>-</sup> permeability of the epithelium through elevation of intracellular calcium. Cation and anion transport are thus controlled separately.

#### Fluid and ion reabsorption by Malpighian tubules

The functional properties of Malpighian tubules of *D. melanogaster* change as the fluid moves downstream of the main segment. O'Donnell and Maddrell (1995) have shown that the lower tubules reabsorb significant amounts of KCl and water from the fluid secreted by the main segment. In addition, they also demonstrated that the lower segment helps acidify the urine and at the same time secretes  $Ca^{+2}$  into the lumenal fluid. The lower segment has also been shown to be electrophysiologically different from the main segment. Whereas the main segment has a lumen positive potential, the lower segment becomes lumen-negative close to its junction with the common ureter. All of these observations suggest that the primary role of the lower segment is to reduce the amount of K<sup>+</sup> and water that enters the hindgut.

This chapter examines if transport of  $K^+$  fluxes can be mapped to specific segments or cells of the *Drosophila melanogaster* MT using a non-invasive selfreferencing microelctrode to measure  $K^+$  fluxes in the unstirred layer (USL). Temporal and Spatial patterns of flux in all functional domains of the tubule are assessed. Comparison of the direct flux measurements collected using ASET will be compared to those flux measurements calculated from fluid secretion experiments. Of particular interest is the previously described non-secretory distal segment. The presence or absence of any discernable  $K^+$  flux would provide clues as to the function of this segment. The effects of typical fluid secretion inhibitors and stimulants on  $K^+$  flux will also be examined on each functional segment of *Drosophila melanogaster* Malpighian tubules. The effects of stimulants on the patterns of  $K^+$  flux adjacent to both principal and stellate cells will also be assessed to determine if stellate cells have any effect on  $K^+$  transport. The hope is that this technique will enhance the current knowledge of function when the tubule is both unstimulated and stimulated.

## **Materials and Methods**

### Insects

Oregon R strain of Drosophila melanogaster were maintained in tubes of standard fly medium at 21-25 °C and ambient humidity (Ashburner, 1989). Adult flies were fed on a yeast diet which lightly covered approximately two thirds of the surface of the standard fly medium. The second generation of adult flies emerged approximately 10 days later, and were transferred to new tubes with fresh yeast. The remaining larvae and pupae remained in the initial tube, and the newly emerged adults were transferred to new tubules on a daily basis. Age of adult flies could thus be determined within a 24 hour range. Anterior Malpighian tubules of females, 4-7 days post-emergence were used for all experiments in this study for three reasons. Firstly, only the anterior tubules of *D. melanogaster* have a morphologically distinct distal segment, which of was interest in this study. Secondly, females were used because they are large and easier to dissect than their male counterparts. Thirdly, only tubules from females were used in order to preclude any undetectable differences that could arise between the sexes (Dow et al. 1994).

#### Dissection procedures and collection of secreted fluid

After insects were sacrificed by crushing their head with forceps, the Malpighian tubules were dissected under standard *Drosophila* saline which consisted of ( in mmol  $l^{-1}$ ): NaCl (117.5), KC; (20), CaCl<sub>2</sub> (2), MgCl<sub>2</sub>.6H<sub>2</sub>O (8.5), glucose (20),

NaHCO<sub>3</sub> (10.2), NaH<sub>2</sub>PO<sub>4</sub> (4.3), HEPES (8.6). Saline was titrated with NaOH to pH 7.0. Dissection involved gripping the dorso-ventral margin of the abdomen with two pairs of fine forceps to tear open the body wall and uncoil the alimentary canal. The anterior tubules unravel and part from their tracheal connections relatively easily. The posterior pair of tubules were dissected free using fine glass probes. Once the tubules were free the anterior tubules were cut free at the junction of the common ureter and the alimentary canal.

Fluid secretion rates were determined using methods previously described by Dow *et al.* (1994). Isolated tubules were transferred on fine glass probes from the dissecting saline to droplets of standard bathing medium, under paraffin oil. The standard bathing medium (SBM) was a 1:1 mixture of standard saline and Schneider's *Drosophila* medium (Sigma), used because it maintains higher secretion rates for longer periods of time than saline alone (Dow *et al.* 1994). One tubule of each pair was pulled out of the drop and wrapped around a fine steel pin, to which it adhered by surface tension. The use of one tubule as an anchor for the other allowed virtually the entire length of one tubule to be immersed in the bathing saline medium. Secreted fluid emerged from the aperature at the cut end of the common ureter. Due to the fact that the ureter must be in the paraffin oil to allow the secreted fluid to form a droplet, a small portion of the lower segment of the tubule was outside the bathing droplet. Great care was taken to minimize the portion of the tubule which remained outside the bathing droplet. Droplets of secreted fluid were collected at regular intervals using fine glass probes (See fig 1.1). Droplet diameters were measured under a stereoscopic microscope using an ocular micrometer, and droplet volume was calculated using the standard formula for volume of a sphere. Secretion rates (nl/min) were calculated by dividing the droplet volume by the time (min) over which the droplets were formed.

#### Addition of stimulants or inhibitors to isolated tubules

All inhibitors or stimulants used were obtained from Sigma-Aldrich Chemical Co. Stock solutions of NaCN, cAMP, cGMP, and Leucokinin-I (LK-I) were all solubilized in standard bathing saline. All agents were diluted to their final concentration by micropipetting the appropriate volume into the droplet bathing the surface of the tubule epithelium. Fine glass probes were used to mix the bathing droplet to ensure proper dilution of agents in the bathing medium.

In the experiments in which only one agent was added the tubules were allowed to secrete fluid over a 60 minute period. Droplets of fluid were collected at 10 minute intervals. Agents were added at 30 minutes and secreted fluid droplets were collected at 10 minute intervals for a further 30 minutes. For control tubules the solvent (i.e. standard bathing saline) was added at 30 minutes. It is important to note however that because fluid secretion rates are stable over time, each tubule may serve as its own control prior to the addition of the inhibitors or stimulants. In the experiments where a combination of two agents were added (i.e. cAMP & NaCN or cGMP & LK-I) the tubules were allowed to secrete fluid over a 90 minute period with secreted fluid being collected at 10 minute intervals. In these experiments the tubules were allowed to secrete fluid for 30 minutes in standard bathing medium at which time the first agent was added ( cAMP or cGMP). The tubules were then allowed to secrete for a further 30 minutes at which time the second agent (i.e. NaCN or LK-I) was added. The tubules were then allowed to secrete fluid for a further 30 minutes at which time the second agent (i.e. NaCN or LK-I) was added. The tubules were then allowed to secrete fluid for a further 30 minutes at which time the experiment was stopped. It is important to note here that isolated tubules have been shown to secrete fluid at constant rates for up to 15 hours (Dow *et al.* 1994b).

# Self-referencing K<sup>+</sup>-selective microelectrode analysis of potassium gradients

The procedures for fabrication, calibration, and data acquisition of self-referencing  $K^+$ -selective microelectrodes has been described in chapter 2.

# Measurement of Artificial K<sup>+</sup> Sources Fluxes

A K<sup>+</sup> source, which generated a standing K<sup>+</sup> gradient, was used to test the stability of the probe signal and the repeatability of measurements using separate probes. The K<sup>+</sup> source was constructed by backfilling a blunt tipped micropipette (10  $\mu$ m) with a solution of 100 mmol l<sup>-1</sup> KCl. The source micropipette was held by a manual micromanipulator (Narishige, Tokyo, Japan) and advanced into a Petri dish containing a 1:1 mixture of standard bathing medium and Schneider's insect medium (

20 mmol  $l^{-1} K^+$ ), where it was allowed to equilibrate for 30 minutes. This equilibration time allows for a steady state to be established between the potassium concentration deep in the pipette and the large sink in the dish. Convective disturbances in the gradient were minimized by placing the source close to the bottom of the dish (~ 150  $\mu$ m from the bottom).

#### Raw A/D measurements

The Raw A/D sampling rules used in these experiment had a wait time of 1 second and a sample average time of 1 second. The experiments began with the K<sup>+</sup>-selective microelectrode positioned within 5  $\mu$ m of the opening of the source pipette. The voltage signal was continuously recorded and the probe was then moved to 100  $\mu$ m in the -Y direction (i.e. downward) and then back to the source, then to 100  $\mu$ m in the +Y direction ( i.e. upward) and back to the source, and then 100  $\mu$ m in the +X direction (i.e. right) and back to the source ( see Fig 3.3). At each point the probe signal was allowed to stabilize before returning to the source.

#### Gradient scans

The  $K^+$ -Y sampling rules used for these experiments had a wait time of 6 seconds and a sample average time of 1 second. Each scan was set up using the

# Figure 3.3

Schematic representation of the orientation of K<sup>+</sup>-selective microelectrode sampling near a 100 mmol l<sup>-1</sup> KCl source electrode. The density of the stippling indicates the approximate KCl concentration within the source. Tip diameter of the source electrode is ~10  $\mu$ m, while the tip diameter of the K<sup>+</sup>-selective microelectrode is ~2  $\mu$ m. Points A,B,and C represent the position of the K<sup>+</sup>-selective microelectrode relative to the source during RawA/D scans.



vector scan mode with the first point within 5  $\mu$ m of the tip of the source pipette. The probe was then moved at regular steps 10  $\mu$ m from the previous point in the -Y direction. The probe was vibrated over a distance of 100  $\mu$ m in the -Y direction at each point. Sixty-four points were analysed in each scan for a total distance moved away from the source pipette of ~650  $\mu$ m.

# Measurement of Malpighian tubule epithelial K<sup>+</sup> fluxes

#### **Isolation of Malpighian tubules**

Drosophila melanogaster Malpighian tubules were dissected as described previously in this chapter. Tubules were then transferred to a 35mm x 10 mm Falcon dish and bathed in a 1 ml of standard bathing medium. A 1.25 cm diameter hole was cut out of the bottom of the dish and a 22 x 22 mm glass cover slip (VWR Scientific Inc., thickness #1) was sealed in place with paraffin wax. Optical properties of the cover slip are better than those of the plastic bottom of the dish. The glass slide was covered with a 50  $\mu$ l droplet of poly-L-lysine (125  $\mu$ g/ml) to facilitate tubule adhesion and allowed to air dry overnight. Anterior tubules were positioned on the glass slide such that the pair of tubules spanned a straight line, with the ureter bisecting the line. Once the tubules were secured to the bottom of the dish 1 ml of Schneider's medium was added to the standard bathing medium bathing the tubule to create a 1:1 mixture of standard bathing medium and Schneider's insect medium. Schneider's insect medium was added after the securing of the tubule because it was found that addition of the Schneider's medium prior to securing of the tubule interferred with the adhesion of the epithelium to the poly-L-Lysine coated dish.

#### Sampling rules

In all subsequent experiments the probe sampled using a wait of 6.00 seconds and a sample averaging time of 1 second. In all cases the excursion distance of the probe was 100  $\mu$ m with the first point being within 5  $\mu$ m of the tubule surface, and the second point 100  $\mu$ m away from the first in the bath. Reference measurements used the same sampling protocol and were taken at 1000  $\mu$ m from the tubule surface in the surrounding bathing medium. All differences in ion gradients are reported as voltage differences over the excursion distance of the probe.

#### Conversion of voltage differences to Flux values

Voltage differences are first converted to a concentration difference using:

(Eq. 3.1)

$$\Delta C = \frac{2.3 (\Delta V \times C_B)}{S \times 0.85}$$

where:

 $\Delta C = \text{concentration difference (mmol.cm}^3)$ 

 $\Delta V$  = voltage difference (mV)

 $C_B$  = background concentration of ion (µmol.cm<sup>-3</sup>)

S = slope of the electrode (mV/decade DC)

and 0.85 is the predetermined efficiency of the electrode.

Concentration differences can then be converted to fluxes using Fick's Law:

(Eq. 3.2)

$$\mathbf{J}_{\mathbf{K}}^{+} = \frac{-\mathbf{D}_{\mathbf{K}}^{+} \times \mathbf{D}\mathbf{C}}{\Delta \mathbf{r}}$$

where:

 $J_{K}^{+} = K^{+} \text{ flux (pmol.cm}^{-2}.s^{-1})$   $D_{K}^{+} = \text{Diffusion coefficient for } K^{+} (1.96 \times 10^{-5} \text{ cm}^{-2}.s^{-1})$  $\Delta r = \text{vibration distance (cm)}$ 

#### Temporal Scans of single point K<sup>+</sup> Fluxes

In these experiments  $K^+$  fluxes were measured over a 15 minute period at single points in the lower, main and distal domains of the Malpighian tubules. The excursion distance for probe vibration was set at 100 µm throught the experiment which was run using the Watch mode. The probe began sampling at a point 1000 µm away from the tubule surface. After 5 minutes the probe was then moved to within 5 µm of the tubule surface and sampled for a further 5 minutes. The probe was then returned to the previous point in the bath and sampling continued for a further 5 minutes.

## Spatial K<sup>+</sup> fluxes of unstimulated Malpighian tubules

These experiments used the vector scan dialog box of the ASET software to program the scan. Scans were done in the lower, main, or distal segment of unstimulated tubules at a number of sites along the tubule surface 100  $\mu$ m apart. Each scan was repeated six times for each set of points and the resultant voltage changes were averaged. Between each repetition a reference was taken 1000  $\mu$ m away in the bath and subtracted from the reported voltage difference.

# K<sup>+</sup> flux during stimulation or inhibition of MT ion transport

In these experiments 4-8 sites on lower, main and distal segments of tubules were scanned according to procedures used for unstimulated tubules. However, points chosen were randomly selected and were not evenly spaced. Sites in each domain were scanned unstimulated for six repetitions, then treated with 1mmol  $1^{-1}$  NaCN, cAMP, cGMP or 100 µmol  $1^{-1}$  LK-I. After 5 minutes the same points were scanned again for 6 repetitions. All inhibitors or stimulants were added by micropipetting a stock solution into the bathing medium to the appropriate final concentration.

#### K<sup>+</sup> fluxes for unstimulated and stimulated principal cells and stellate cells

In these experiments 5 points on the main segment of a Malpighian tubule were scanned. One point was adjacent a stellate cell, two points were adjacent principal cells on each side of that stellate cell, and the last two points were adjacent principal cells with no stellate cell as an immediate neighbour. In each case the 5 points were scanned six times unstimulated and then 1mmol l<sup>-1</sup> cAMP was added. After 5 minutes the same points were scanned again for 6 repetitions.

#### Data Analysis

Data were analyzed using Excel (Microsoft). All experiments were conducted at room temperature (21-25°C). Values are reported as mean  $\pm$  S.E.M. (n), where n equals the number of tubules for fluid secretion studies and n equals # of sites on 'x' number of tubules for K<sup>+</sup> flux measurements. Where error bars are not visible in the figures they are smaller than the symbol used. Where appropriate, the significance between control and experimental groups or different treatments was assessed using Student's t-tests (two tailed) using P = 0.05 as the critical level.

#### RESULTS

ASET requires use of the same probe at different times or in some cases, the use of more that none probe because of limited probe lifetime (typically 2-3 days). For these reasons, it was important to determine that the responses measured by ASET were independent of the particular probe used or its age. These determinations were made using an artificial  $K^+$  source, as described above.

# Analysis of Artificial K<sup>+</sup> Sources

A 100 mmol  $l^{-1}$  KCl source was used to provide a predictable and repeatable potassium gradient, where the concentration of potassium was higher near the source than farther away. An increase or reduction in the voltage signal of the K<sup>+</sup> selective microelectrode corresponds to an increase or decrease in K<sup>+</sup> activity respectively.

When the probe was moved from the source to 100  $\mu$ m away in either the y plane (above or below the source) or in the x-plane, the drop in voltage at each point was similar (Fig. 3.4a). This result was expected because diffusion of ions away from a point source in a uniform bath should be equal in any plane of diffusion. Fig 3.4a also shows that the 90% response time of the electrode was typically < 2 seconds.

Repeated scans of the same point source with 12 different  $K^+$ -selective probes showed a high degree of repeatability and low variability

# Figure 3.4

A) Sample tracing of static K<sup>+</sup>-selective microelectrode moved from 100 mmol  $\Gamma^1$ source to (A) 100  $\mu$ m in the -Y direction, returned to the source, then moved to (B) 100  $\mu$ m in the +Y direction, returned to the source, then moved to (C) 100  $\mu$ m in the X direction ( see inset in Fig 3.4b). All measurements at the source were made by positioning the microelectrode 5  $\mu$ m from the tip of the source micropipette.

B) Graph showing the mean voltages measured with 12 static K<sup>+</sup>-selective microelectrodes at the source and points A, B, and C (see inset). Voltages at points A,B, and C were significantly different (P<0.05) from the voltages measured at the point source but not from each other. The same 100 mmol l<sup>-1</sup> KCl source was used for all experiments.



between microelectrodes when data were pooled (Fig 3.4b). The variability (S.E.M.) in the pooled data was 5.5%, 7%, and 6.7% from points A,B, and C respectively. In all cases the voltages are significantly different from the voltage measured at the source but not from each other. In Fig 3.4 the measurements were static, i.e. the probe was not vibrating at the point 100 µm distant from the source. When the probe is vibrated, a gradient is measured between the two extremes of vibration. According to the principles of diffusion of ions from a point source into an infinite bathing medium, it is expected that at some distance from the point source the ion concentration will approach the bath concentration asymptotically and the concentration gradient will therefore be indistinguishable from zero. Repeated scans in which the K<sup>+</sup>- selective microelectrode was stepped away from the source at regular distance intervals showed that the voltage gradient due to ion activity dissipated and had consistently disappeared when the microelectrodes were  $\sim 100 \,\mu\text{m}$  away from the source (Fig 3.5) Slight changes in the slopes of these scans between 0 and 60 µm were presumably a combination of factors, including temperature, convective disturbances in the bath (e.g. due to air currents), or to differences in the Nernstian slope of the  $K^+$  electrode.

#### Analysis of secreted fluid

The validity of  $K^+$  flux measurements using ASET was assessed by comparison with independent estimates of  $K^+$  flux calculated from the product of

# Figure 3.5

Graph of concentration gradient scans of 100 mmol  $\Gamma^{1}$  KCl source, where the K<sup>+</sup>selective microelectrode was moved away from the source 10  $\mu$ m at each step for a total of 640  $\mu$ m. The K<sup>+</sup>-selective microelectrode was vibrated over a 100  $\mu$ m excursion distance at each point sampled. n=4 K<sup>+</sup>-selective microelectrodes. The same 100 mmol  $\Gamma^{1}$  source was used in all experiments.



the secretion rate of isolated tubules and the  $K^+$  concentration of secreted fluid droplets. In figures 3.6 - 3.9 the effects of stimulants and inhibitors of fluid secretion are presented. Flux calculations and comparison with flux measurements determined by ASET are presented in the discussion.

#### Effects of NaCN on unstimulated and stimulated tubules

Fluid secretion by both unstimulated and cAMP (1mmol  $l^{-1}$ ) stimulated tubules was blocked completely within 10 min by 1 mmol  $l^{-1}$  NaCn (Fig 3.6a & b). In the case of stimulated tubules (Fig 3.6 b) application of 1 mmol  $l^{-1}$  cAMP at 30 minutes increased fluid secretion rates by 38.3% and maximal stimulation was achieved within 10 - 20 min.

#### Effects of cAMP, cGMP, and LK-I on secretion rates

Fluid secretion rates were significantly increased by the addition of various stimulants to the standard bathing medium (Figs. 3.7, 3.8 & 3.9) The addition of 1 mmol  $\Gamma^1$  cAMP, cGMP, or 100 µmol  $\Gamma^1$  LK-I increased the fluid secretion rates by 70%, 29%, and 194% respectively. The addition of 1 mmol  $\Gamma^1$  cGMP followed by subsequent stimulation with 100 µmol  $\Gamma^1$  LK-I increased the fluid secretion rate by 43% and then a further 115% for a total increase of 158%. Not all stimulants were equally potent; the hierarchy of effectiveness was LK-I >> cAMP>cGMP. Previous studies (Dow *et al.* 1994 a,b) have shown similar effects of these stimulants on
Time course of effects of 1 mmol  $l^{-1}$  NaCN on (A) unstimulated and (B) 1mmol  $l^{-1}$  cAMP stimulated fluid secretion rates of *Drosophila melanogaster* Malpighian tubules. Arrows on the abcissa indicate the time of addition of (A) NaCN and (B) cAMP then NaCN. Control and experimental tubules are indicated by circles and squares respectively. n=10 tubules in (A) and n=15 tubules in (B). All points aremean  $\pm$  SEM at each time. NaCN decreases secretion rate significantly in both A and B (P<0.05).



% increase in fluid secretion rate in response to 1 mmol  $l^{-1}$  cAMP,cGMP, 100 µmol  $l^{-1}$  LK-I and cGMP + LK-I. The mean unstimulated secretion rate was ~0.33 nl/min. All of the increases in fluid secretion rate are significantly different from zero (P<0.05; n=12-18 tubules).



Time course of effects of (A)1 mmol  $\Gamma^1$  cAMP and (B) 1mmol  $\Gamma^1$  cGMP on secretion rates of *Drosophila melanogaster* Malpighian tubules. Arrows on the abcissa indicate the time of addition of both cAMP and cGMP. Control and experimental tubules are indicated by circles and squares respectively. n=12 tubules in (A) and n=15 tubules in (B). All points are mean ± SEM at each time. cAMP and cGMP increase fluid secretion rates significantly compared to their controls using P<0.05.



Α

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Time course of effects of (A) 100 $\mu$ mol l<sup>-1</sup> LK-I and (B) 1mmol l<sup>-1</sup> cGMP then 100 $\mu$ mol l<sup>-1</sup> LK-I on secretion rates of *Drosophila melanogaster* Malpighian tubules. Arrows on the abcissa indicate the time of addition of (A) cGMP and (B) cGMP then LK-I. Control and experimental tubules are indicated by circles and squares respectively. n=13 tubules in (A) and n=18 tubules in (B). All points are mean ± SEM at each time. LK-I significantly icreased fluid secretion rates of previously unstimulated and previously stimulated tubules (P<0.05). The effects of LK-I are additive to those of cGMP.



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В

fluid secretion rates of *Drosophila* Malpighian tubules. Mean secretion rates of stimulated and control tubules are presented in Figures 3.8 and 3.9. The time course of stimulation was similar for cAMP, cGMP, and LK-I; maximal stimulation was achieved within 10-20 minutes.

#### ASET analysis of K<sup>+</sup> fluxes in unstimulated Malpighian tubules

In these experiments a positive voltage difference denotes potassium efflux consistent with an increase in  $K^+$  concentration of the unstirred layer near the basolateral surface of the tubule. This increase would occur in response to  $K^+$  reabsorption from tubule lumen to bath, as in the lower tubule of *Drosophila melanogaster* (O'Donnell and Maddrell, 1995). A negative voltage difference denotes potassium influx consistent with a decrease in  $K^+$  concentration of the unstirred layer near the basolateral surface of the tubule. This decrease would result from  $K^+$  secretion from bath to lumen, by the main segment of the tubule.

Temporal scans of single points in the lower (reabsorptive), main (secretory), and distal (non-secretory) segments of a Malpighian tubule are shown in Figure 3.10. Scans of the lower segment consistently showed a positive voltage difference when the  $K^+$ -selective microelectrode was moved from the background concentration (20 mmol  $I^{-1}$ ) of the bath to the tubule surface (Fig. 3.10a), indicating an increase in the concentration of potassium ions in the USL. Scans of the main segment showed a negative voltage difference when the  $K^+$ -selective

Sample of temporal K<sup>+</sup> flux analysis of single points in the (A) Lower (B) Main and (C) Distal segments of the Malpighian tubules of *Drosophila melanogaster*. Measurement of K<sup>+</sup> fluxes was conducted in a background concentration of 20 mmol l<sup>-</sup> <sup>1</sup> K<sup>+</sup>. The electrode was vibrated over an excursion distance of 100  $\mu$ m at a distance of ~5  $\mu$ m from the tubule surface (TS). BKG denotes positioning of the vibrating electrode in the background solution (1000  $\mu$ m from the tubule). When the vibrating microelectrode was brought to the tubules surface: (A) a positive voltage difference indicating a K<sup>+</sup> efflux was observed in the lower segment. (B) a negative voltage difference, indicating no K<sup>+</sup> influx or efflux was observed in the distal segment.



В

С

microelectrode was moved from the bath to the tubule surface (Fig 3.10b), indicating a depletion of  $K^+$  from the USL adjacent to the basolateral membrane of the tubule. This is consistent with an influx of  $K^+$  into the tubule and therefore  $K^+$  secretion by the main segment. Repeated scans of the distal segment of tubules showed no significant voltage difference when the electrode was moved from the bath to the tubule surface ( Fig 3.10c). Accordingly, no significant influx or efflux and therefore secretion or reabsorption appeared to be occurring in the distal segment of anterior Malpighian tubules. These results are the first direct evidence consistent with the proposal ( Dow *et al.* 1994b) that the distal segment of the anterior tubules do not secrete  $K^+$ , and moreover the data also showed that it did not reabsorb  $K^+$ .

It should be noted that in all these experiments the electrode response was rapid, and stabilized in less than 10 seconds when the probe was moved from the bath to the tubule surface and vic versa (Figs. 3.10a,b,c). The oscillations in voltage observed in the lower tubule (Fig. 3.10a) are much slower than the response time of the electrode, and must therefore reflect actual oscillations in potassium flux. Similar oscillations to those of Fig. 3.10a were seen in 8 of 10 lower tubules analysed.

#### Spatial analysis of K<sup>+</sup> flux in unstimulated tubule segments

In these experiments the spatial distribution of potassium flux along the lower, main, and distal segments of unstimulated tubules was studied. Each point was seperated from the previous point by  $\sim 100 \ \mu m$ .

The scans of all points in the lower segment yielded a surprisingly variable range of voltage differences ranging from  $31.5 \pm 21.1 \,\mu\text{V}$  at site 7 of MT1 (Fig. 3.11a) to  $343.3 \pm 49.1 \,\mu\text{V}$  at site 2 of MT2 (Fig. 3.11b). This variation indicates that in spite of its morphological homogeneity, the lower segment exhibits a very heterogeneous pattern of K<sup>+</sup> reabsorption. Also in contrast to studies of the reabsorptive segments of *Rhodnius* Malpighian tubules (Collier and O'Donnell 1997) in which the peak K<sup>+</sup> reabsorption consistently occurred ~25% along the length of the lower tubule, no consistent pattern of spatial K<sup>+</sup> reabsorption was evident in the lower Malpighian tubules of *Drosophila*.

Scans of the main secretory segment yielded voltages ranging from  $-36.1 \pm 10.4 \,\mu\text{V}$  at site 2 of MT5 (Fig. 3.12b) to  $-313.2 \pm 6.4 \,\mu\text{V}$  at site 8 of MT4 (Fig. 3.12a) indicating that not all cells contribute equally to K<sup>+</sup> secretion. However the spatial variability seen in the main segments that were scanned was not as dramatic as that seen in the lower tubule (Fig. 3.12a,b,c).

In contrast to the lower and main segments the voltage differences measured adjacent to the basolateral membrane of the distal segment were

Representative spatial scans of  $K^+$  efflux along the lower segment of *Drosophila* melanogaster Malpighian tubules. All sites in A,B, and C are 100  $\mu$ m from the previous site. n=19 sites on 6 tubules (3 tubules shown)





Representative spatial scans of  $K^+$  influx along the main segment of *Drosophila* melanogaster Malpighian tubules. All sites in A,B, and C are 100  $\mu$ m from the previous site. n=24 sites on 7 tubules (3 tubules shown)



В

С

Representative spatial scans of  $K^+$ flux along the distal segment of *Drosophila* melanogaster Malpighian tubules. All sites in A,B, and C are 100  $\mu$ m from the previous site. n=46 sites on 11 tubules (3 tubules shown)



Table 3.1

Mean voltage differences ( $\mu$ V) measured using a self-referencing K<sup>+</sup>-selective microelectrode and averaged for all sites within lower, main, and distal segments. The K<sup>+</sup>-selective microelectrode was vibrated over a 100  $\mu$ m excursion distance. Voltage differences are converted to relative concentration differences (mmol l<sup>-1</sup>). All measurements were done in a 20 mmol l<sup>-1</sup> K<sup>+</sup> background.

Tubule Domain	N	Voltage Difference ( μV)	Concentration Difference (mmol.l <sup>-1</sup> )
Lower	19 sites (6 tubules)	137 ± 10	0.13 ± 0.009
Main	24 sites (7 tubules)	-218 ± 7.4	-0.21 ± 0.007
Distal	46 sites ( 11 tubules)	<b>20.2</b> ± <b>7.1</b>	0.019 ± 0.007

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Graph of mean  $K^+$  flux values converted from the voltage differences given in Table 3.1 for the lower, main, and distal segments. The flux values for both the lower and main segments are significantly different from zero. The flux of the distal segment is not significatly different from zero. n=19-46 sites on 6-11 tubules with P<0.05.



found not to be significantly different from zero (Figs. 3.13a,b,c), where zero was the voltage difference measured in the bathing medium surrounding the tubule.

Integration of all points across each tubule segment yields a mean voltage differences of  $137 \pm 10 \ \mu\text{V}$  for K<sup>+</sup> reabsorption in the lower segment,  $-218 \pm 7.4 \ \mu\text{V}$  for K<sup>+</sup> secretion in the main segment and  $20.2 \pm 7.1 \ \mu\text{V}$  in the non-secretory distal segment. These voltage differences correspond to K<sup>+</sup> concentrations changes of 0.6% for the lower, 1% for the main and 0.009% for the distal segments. Actual concentration differences expressed in mmol l<sup>-1</sup> are given in Table 3.1. The mean flux for each functional tubule domain was calculated according to the manner outlined previously, and can be seen in Figure. 3.14.

#### Effects of stimulation or inhibition of K<sup>+</sup> flux

A representative example of the effects of 1 mmol  $l^{-1}$  cAMP on voltage differences and K<sup>+</sup> flux at 6 different principal cells on the main segment of a single tubule is shown in Figure 3.15a & b. cAMP significantly increased K<sup>+</sup> flux at sites 1-5 (Fig 3.15b) but had no significant effect at site 6. The basis for the latter result is discussed below.

The results of inhibition with 1 mmol  $\Gamma^1$  NaCN, or stimulation with 1 mmol  $\Gamma^1$  cAMP, cGMP, or 100  $\mu$ mol  $\Gamma^1$  LK-I on fluxes averaged across all sites within each segment of isolated Malpighian tubules are shown in Figure 3.16. The active nature of K<sup>+</sup> reabsorption in the lower segment was indicated by the 95% reduction

Representative graphs of (A) mean voltage differences and (B) corresponding mean  $K^+$  fluxes for the effects of 1mmol 1<sup>-1</sup> cAMP on the principal cells of the main segment of *Drosophila melanogaster* Malpighian tubules.  $K^+$  flux is a linear transformation of the  $K^+$  concentration data, which are in turn a logarithmic transformation of voltage differences. The relative magnitude of open and closed bars in A and B are almost identical because of small size of the voltage gradient (i.e. the logarithmic value of a near zero is ~1). Effects of cAMP on voltage differences due to  $K^+$  concentration gradients and  $K^+$  flux rates are significantly different for sites 1-5 (open vs closed bars) but not for site 6 (open vs closed bars). n=27 sites on 5 tubules (1 tubule shown) with P<0.05





A) Mean  $K^+$  efflux along the lower segment of *Drosophila melanogaster* Malpighian tubules before (closed bars) and after (open bars) the addition of 1 mmol l<sup>-1</sup> NaCN, cAMP, or cGMP. Only the effect of NaCN is significant using P<0.05. n=18-24 sites on 5-6 tubules

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B) Mean K<sup>+</sup> influx along the main segment of *Drosophila melanogaster* Malpighian tubules before (closed bars) and after, (open bars) the addition of 1 mmol l<sup>-1</sup> NaCN, cAMP, or cGMP or 100  $\mu$ mol l<sup>-1</sup> LK-I. The effects of NaCN, cAMP, and cGMP are significant using P<0.05. n=16-27 sites on 4-8 tubules

C) Mean  $K^+$  flux along the distal segment of *Drosophila melanogaster* Malpighian tubules before(closed bars) and after (open bars) the addition of 1 mmol l<sup>-1</sup> NaCN, cAMP, or cGMP on . None of the stimulants or inhibitors had a significant effect using P<0.05. n=10-15 sites on 3-5 tubules



in  $K^+$  flux in the presence of 1 mmol  $l^{-1}$  NaCN. Stimulation with cAMP or cGMP did not significantly change  $K^+$  reabsorption in the lower segment of the tubule.

In the main segment NaCN completely blocked  $K^+$  influx. Stimulation of the tubule with cAMP and cGMP significantly increased  $K^+$  influx by 36% and 24% respectively. Suprisingly although fluid secretion was increased 194% by LK-I, there was no significant change in  $K^+$  influx. This latter result will be discussed below.

In the lower segment NaCN had no significant effect on  $K^+$  flux as was expected because prior to NaCN's addition there was no significant  $K^+$  flux in the lower segment. Additionally stimulation with cAMP or cGMP caused no increase or decrease in  $K^+$  flux of the distal segment.

#### K<sup>+</sup> fluxes for unstimulated and stimulated principal cells and stellate cells.

Previous studies (O'Donnell et al. 1996) have indicated that it is unlikely that stellate cells transport  $K^+$ . Evidence suggests (O'Donnell et al. 1998) that stellate cells are in fact the route for active transcellular Cl<sup>-</sup> secretion.

Prior to stimulation there was no significant difference between  $K^+$  flux at sites adjacent to pricipal cells versus sites adjacent to stellate cells (Fig. 3.17). This "apparent  $K^+$  influx" adjacent stellate cells is unexpected because of the bulk of evidence favouring stellate cells as sites for transcellular Cl<sup>-</sup> flux. The

The effects of 1mmol  $l^{-1}$  cAMP on (A) mean voltage differences due to K<sup>+</sup> concentration gradients and (B) K<sup>+</sup> fluxes adjacent principal and stellate cells in the main segment of *Drosophila melanogster* Malpighian tubules. The effects before and after treatment with cAMP are shown ( closed vs open bars). There is a significant effect of cAMP only on principal cells. n=19 principal cells and 5 stellate cells on 5 tubules using P<0.05. Α







explanation, discussed below, most likely resides in the dimension of the USL (and the  $K^+$  concentration gradient within it) relative to the dimensions of the cells. After stimulation with 1 mmol  $I^{-1}$  cAMP  $K^+$  flux adjacent to principal cells increased significantly by 20% (Fig 3.17b).  $K^+$  flux adjacent to stellate cells did not increase significantly. Possible explanations for those effects are discussed below.

#### DISCUSSION

This study demonstrates that both secretion and reabsorption of  $K^+$  by *Drosophila melanogaster* Malpighian tubules can be assessed both qualitatively and quantitatively by self-referencing  $K^+$ -selective microelectrode measurement of the  $K^+$  gradients in the USL adjacent to the basolateral surface of the tubular epithelium. This technique, although used only for the measurement of  $K^+$  in this study, could be used to assess transport of any cation or anion, provided that there is an appropriately selective ionophore available. Also, basolateral measurements of ion flux can be determined before and after putative stimulants or inhibitors are added to the bathing saline. Very small differences in electrical potential (~20  $\mu$ V) could be reliably resolved with a temporal resolution of ~10 seconds. The spatial resolution of this technique, using the vibration distance of 100  $\mu$ m, is comparable to the dimensions of a few cells. Spatial resolution could be increased by decreasing vibration distance, but at some point the ion concentration gradients would no longer be resolvable.

This technique revealed several new features of  $K^+$  transport in a well characterized transporting epithelium, the Malpighian tubules of *Drosophila melanogaster*. It also confirmed some previous observations about rates of  $K^+$  secretion and reabsorption.

Rates of fluid secretion by the Malpighian tubules of *D. melanogaster* were similar in this study to those observed previously (Dow *et al.* 1994; O'Donnell and Maddrell, 1995). Accuracy of the technique was assessed through comparison of  $K^+$ 

fluxes calculated from fluid secretion data and those directly measured using the ASET system. Fluid secretion rates are converted to fluxes by multiplying the measured secretion rate (nl/min) by the  $K^+$  concentrations (mmol  $l^{-1}$ ) measured in secreted fluid droplets. This calculated value is then divided by the estimated surface area  $(cm^2)$  of the tubule to yield a  $K^{+}$  flux. Tubule surface area is estimated using the formula for the surface area of a cylinder ( $\pi$ dL). Previous studies (O'Donnell and Maddrell, 1995) in which secretion rates were similar to those observed in this study indicated that the  $K^{+}$ flux into the main segment calculated in this manner is  $254 \pm 53$  pmol.cm<sup>-2</sup>.sec<sup>-1</sup> and that the K<sup>+</sup> flux out of the lower segment is  $152 \pm 41$  pmol.cm<sup>-2</sup>.sec<sup>-1</sup>. The lower segment thus reabsorbs 40% of the  $K^+$  secreted by the main segment. In this study direct measurements of  $K^{+}$  fluxes with a  $K^{+}$ -selective microelectrode yielded a  $K^{+}$  flux into the main segment of 406  $\pm$  13.8 pmol.cm<sup>-2</sup>.sec<sup>-1</sup> and a K<sup>+</sup> flux out of the lower segment of 255  $\pm$  18.6 pmol.cm<sup>-2</sup>, sec<sup>-1</sup>. This corresponds to a 63% recovery of K<sup>+</sup>. It should be noted that in the case of secretion of  $K^{+}$  by the main segment the flux measured by the ASET system is 1.6 times that calculated from fluid secretion experiments. In the case of  $K^{+}$  reabsorption by the lower segment the flux measured by the ASET system is 1.7 times that calculated from fluid secretion data. This discrepancy in the percent reabsorption by the two techniques is explainable by higher transport rates in the tubules set up for the ASET measurements. During collection of secreted fluid from isolated Malpighian tubules a short portion of the lower (reabsorptive) segment remains in the oil and is not bathed in physiological saline. As a

result this portion of the tubule does not contribute to the reabsorption of  $K^+$ . However, in the ASET technique the entire tubule is bathed in physiological saline allowing the complete lower segment to participate in  $K^=$  reabsorption. The ASET data show  $K^+$  reabsorption does in fact occur across the region of the lower tubule that is adjacent the ureter and would be positioned out of the saline droplet for fluid secretion studies.

Previous studies (Dow et al. 1994b) have shown that the distal segment of *Drosophila* Malpighian tubules is non-secretory. ASET data provided the first direct evidence that the distal segment is also non-reabsorptive. This latter result could not be shown in a secretion assay.

Although the data seem to indicate that the distal segment has a very small K<sup>+</sup> efflux (37.6  $\pm$  13.2 pmol.cm<sup>-2</sup>.sec<sup>-1</sup>) the value is not significantly different from zero. The distal segment was also unaffected by putative stimulators and inhibitors of fluid secretion. These findings do not rule out the possibility that the distal segment may perform some other vital physiological role such as transport of other ions (e.g. Ca<sup>+2</sup>) or organic solutes (Wessing *et al.*, 1988).

Spatial scans of  $K^+$  flux in both unstimulated and stimulated tubules indicated pronounced heterogeneity of  $K^+$  transport across segments which are morphologically and ultrastructurally homogeneous. Unstimulated tubules showed a highly variable pattern of  $K^+$  flux adjacent the lower segment. Variations in  $K^+$  flux adjacent the main segment were evident, but not as dramatic. These findings were unexpected for a

number of reasons. Firstly, one would expect that a tubule region that is morphologically homogeneous would be physiologically homogeneous. However, there is a precedent for physiological discontinuities in an epithleium of apparently uniform ultrastructure. Reabsorption of KCl by the lower segment of *Rhodnius* is restricted to the lower 1/3 of the segment's length (Maddrell, 1978). Osmotic permeability also varies along the length of the lower segment (O'Donnell et al. 1982). What is noteworthy, however, is that previous studies (Collier and O'Donnell, 1997; Maddrell, 1978) showed a predictable pattern of  $K^+$  reabsorption along the length of the lower segment of Rhodnius Malpighian tubules. In contrast, the present study revealed that both the lower and main segments of Drosophila Malpighian tubules exhibit variable K<sup>+</sup> flux patterns along their lengths. One explanation for these results may be found in work previously done by Sozen et al. (1997) in which the morphologically homogeneous principal cells of Drosophila Malpighian tubules show a genetic heterogeneity. This finding is important because it may suggest that the variability in physiology observed in this study reflects the genetic heterogeneity of these morphologically similar cells. In other words not all principal cells have the same K<sup>+</sup> transport capacity, consistent with the heterogeneity indicated by the molecular genetic studies of Sozen et al. (1997). Further support of this physiological heterogeneity can be seen from the effects of cAMP stimulation on spatial patterns of  $K^{+}$  flux in the main segment.  $K^{+}$  flux measurements indicate that not all pricipal cells respond equally to cAMP stimulation.
Inhibition of  $K^{+}$  flux in the main segment by addition of the metabolic inhibitor NaCN mirrored the effect of NaCN on fluid secretion. The ability of NaCN to completely block active fluid secretion and  $K^{+}$  flux can be traced back to principal cells of the main segment. The current model, now generally accepted, is that the central role in active transport of cations is accomplished by an apical vacuolar type H<sup>+</sup>-ATPase (Bertram *et al.*, 1991; Bowman *et al.*, 1988). confined to the principal cells (Sozen *et al.*, 1997). The proton gradient maintained by this H<sup>+</sup>-ATPase provides the energy source for the secondary active transport of K<sup>+</sup> from cell to lumen through a K<sup>+</sup>/H<sup>+</sup> antiporter. It is apparent that if cell metabolism is inhibited by NaCN, both fluid secretion and K<sup>+</sup> flux should stop, as observed. The blocking of K<sup>+</sup> reabsorption in the lower segment by NaCN is consistent with previous studies which have shown that reabsorption is a process of active transport (O'Donnell and Maddrell, 1995).

Comparison of cAMP stimulation of  $K^+$  flux, determined by ASET, and of fluid secretion rates reveal an unexpectedly low correlation. cAMP increased fluid secretion rates by 70% but increased  $K^+$  influx in the main segment by only 36%. The difference in these values may be attributed to the fact that transport of other positively charged ions, such as Na<sup>+</sup> may also contribute to fluid secretion rates but does not affect  $K^+$  flux measurements. In contrast, cGMP raised secretion rate by 29%, closely mirroring the 24% increase in  $K^+$  influx into the main segment. This result combined with the effect of cAMP above supports the currently held opinion that cGMP is the natural second messenger that responds to the endogenous peptide  $CAP_{2b}$  (Davies *et al.*, 1995). The first messenger (i.e hormone) leading to cAMP production in principal cells is unknown. The above results raise the intriguing possibility that cAMP may contribute to the control of more than just the V-ATPase activity inside *Drosophila melanogaster* Malpighian tubule principal cells. It is possible that Na<sup>+</sup> transport is preferentially stimulated.

It would be expected that an increase in transepithelial Cl<sup>-</sup> conductance would be accompanied by a corresponding increase in cation flux. However, although LK-I increased fluid secretion rates by 194% there was no corresponding increase in K<sup>+</sup> influx into the main segment. It is worth noting that much smaller increases in fluid secretion in response to cGMP resulted in corresponding increases in K<sup>+</sup> influx. LK-I has previously been shown to specifically stimulate a transcellular Cl<sup>-</sup> conductance in stellate cells of the main segment of *D. melanogaster* Malpighian tubules. A possible explanation for the absence of an increase in K<sup>+</sup> flux is that K<sup>+</sup> may not be the accompanying cation during LK-dependent increases in Cl<sup>-</sup> influx. Further studies are needed to address the effects of LK on ion transport (e.g Na<sup>+</sup>).

Although stellate cells have been shown to be the transcellular routes of Cl<sup>-</sup> conductance (O'Donnel *et al.*, 1998), ASET detected an "apparent" K<sup>+</sup> influx adjacent to stellate cells. The explanation may reside in the relative dimensions of the cells and the USL K<sup>+</sup> gradient produced by active ion transport. Preliminary scans of tubules (not shown) indicated that gradients in K<sup>+</sup> concentration exted outwards at least 6 tubule diameters (~200  $\mu$ m) from the basolateral surface of the tubule. As a result,

there will be extensive overlap of concentration gradients produced by  $K^+$  transport of adjacent principal cells. In short, the  $K^+$  influx associated with a stellate cell is presumably due to  $K^+$  transport of the surrounding principal cells. It is possible that when  $K^+$  transport is stimulated by cAMP, the  $K^+$  concentration gradient may be steeper and more sharply defined spatially, so that a 20% increase in  $K^+$  influx over pricipal cells is detectable. In contrast, this smaller increase may not be detectable over stellate cells because of a blunting of the  $K^+$  concentration gradient due to the presence of a cell not involved in  $K^+$  transport. A full discussion of the dynamics of  $K^+$  diffusion in 3 dimensions are beyond the scope for the present study.

# CHAPTER 4: Self-referencing ion-selective Microelectrodes for Measuring Extracellular Ion Concentration Gradients: An Evaluation, with Suggestions for Further Research

### **General Discussion**

The use of non-invasive self-referencing ion-selective microelectrodes to characterize unstirred layer ion concentrations shows great promise for further studies of epithelial ion transport. New information regarding both the temporal and spatial patterns of  $K^+$  transport in *Drosophila melanogaster* was revealed in the studies described in Chapter 3. From this work it is apparent that this technique could also be useful for the study of situations in which tissues transport ions at rates sufficient to produce differences in concentration gradients of 0.06%. For rapidly transporting tissues, where USL gradients may exceed 1% above or below background, static ion-selective microelectrodes are sufficient (e.g. Collier and O'Donnell 1997).

ASET provides numerous advantages over techniques such as flame photometry, atomic absorption spectroscopy, x-ray microanalysi, radioisotopes and static ion-selective microelectrodes. Previous methods for analysis of secreted fluid all share the same limitations; poor temporal resolution, lack of sensitivity, and long experimental periods required to gather an appropriate volume of secreted fluid for analysis. The temporal resolution of this method is dependent on the response time of the microelectrode which is in turn dependent on factors such as tip diameter and the ionophore used. In addition, temporal resolution is limited by the time required for the microelectrode to move, wait and sample over the excursion distance chosen. Most ion-selective microelectrodes with tip diameters of ~2  $\mu$ m have a response time of ~ 2-10 seconds. This allows for a rapid measurement of ion flux in both control conditions and when assessing the effects of putative stimulants and inhibitors on tubule function.

In the past, spatial resolution was limited by the length of the tubule which could be isolated in a bathing droplet of saline. Use of non-invasive self-referencing ion-selective microelectrodes allows different segments of a single tubule to be examined, minimizing variability because each tubule can act as its own control. Use of a microstepper motion control system also reduces variability in repeated measurements because it ensures that the microelectrode returns to precisely the same spot for each measurement. This study has shown that the influence of even a single cell on ion concentrations in the USL adjacent the basolateral surface of the tubule can be resolved using self-referencing ion-selective microelectrodes. Spatial resolution with this technique is determined by both the tip diameter of the probe and by the distance over which it vibrates.

Another advantage of ASET is that it allows ion transport to be tracked in real time. Previous studies required that enough secreted fluid be collected for analysis, typically taking 10 minutes. This study has shown that changes in ion concentration in the USL can typically be detected over a much shorter time period. This information is beneficial when trying to elucidate the cascade of messengers responsible for control of tubule ion transport.

Lastly, the use of ASET software enables automation of many of the functions and scanning procedures associated with this technique, thereby minimizing the need for constant attendance of the measurement apparatus.

# **Further Directions for Research**

#### Use of self-referencing CI selective microelectrodes.

Previous studies (O'Donnell *et al.*, 1998) using a current density probe in conjunction with ion substitution and pharmalogical reagents showed that transcellular Cl<sup>-</sup> conductance was confined to stellate cells. Measurement using a sel-referencing Cl<sup>-</sup> - selective microelectrode would provide direct evidence for Cl<sup>-</sup> flux associated with stellate cells.

## Use of Shielded K<sup>+</sup>-selective microelectrodes

This study observed an "apparent"  $K^+$  flux adjacent stellate cells. Use of a shielded  $K^+$ -selective microelectrode (Danker et al., 1996) would be useful to determine if this flux was due to the overlapping of the concentration gradients created by the neighbouring principal cells or was directly associated with the stellate cell.

# Characterization of H<sup>+</sup>, Ca<sup>+2</sup>, and Na<sup>+</sup> transport

There are a number of ionophores available that are selective for  $H^+$ ,  $Ca^{+2}$ , and  $Na^+$ . Use of self-referencing ion-selective microelectrodes for these ions would enable one to determine if these ions had spatial and temporal flux patterns associated with those of potassium made in this study. The lower tubule, for example, transports  $Ca^{+2}$  from bath to lumen and acidifies the urine (O'Donnell and Maddrell, 1995).

#### Effects of changes of diffusion coefficients on USL concentration gradients

Altering the diffusion coefficients for ions by addition of macromolecules (such as agar) to the bathing medium may be of interest. The resultant reduction of diffusion coefficients would tend to steepen the concentration gradient between the USL and the bulk solution. It may also reduce the overlapping effect seen in this study of principal cells on stellate cells, providing a means for increasing spatial resolution for measurements of differences in the USL ion concentration.

Further refinement of microelectrode fabrication procedures, sampling protocols, and software will enhance the suitability of this technique for the study of ion gradients in the USL adjacent the basolateral surface of Malpighian tubules.

#### Other Epithelia (e.g. Flat sheet epithelia)

Tubular epithelia can be studied using perfused tubules or Ramsay secretion assays for analysis of regional variations in ion transport. Tubular epithelia are also amenable to studies using ASET. However, the true potential of the technique may reside in its application to the study of regional variation in ion transport of flat sheet epithelia. Just as the nature of CI<sup>-</sup> transport by specific mitochondrial rich (MRC) cells in the opercular epithlia of salt water acclimated tilapia was demonstrated by the current density probe technique (Scheffey *et al.*, 1983), many other flat sheet epithelia may be amenable to the ASET technique. This will be particularly useful for instances of electroneutral ion transport, which cannot be resolved by the current density probe.

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