Chapter 11

Ion-sensitive microelectrodes

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1. Introduction

Electrophysiologists are generally interested in studying ion movements across cell membranes because such movements play critical roles in the function of the cells. Other chapters in this book elaborate on the techniques that are used to study fast electrical events related to ion transfer (e.g. single and two electrode voltage clamp). In this chapter we focus on a technique that allows the study of changes in ion activity at a cellular level. Ion-sensitive microelectrodes provide a means of directly assessing the extracellular or intracellular activity of an ion and for making prolonged measurements of these. It is the activity of an ion which is important because it, rather than the total or free concentration, determines, for example, the membrane potential, equilibrium potentials, or the thermodynamic conditions for ion transport mechanisms.

Other techniques used in intracellular ion measurements include optical indicators, radio-isotopic tracer and atomic absorption methods. The usefulness of the latter two is limited since these methods do not account solely for the activity of the ion in question, but give a measure of the total concentration of the ion including those sequestered in intracellular organelles and bound to intracellular buffers. In addition they cannot continuously monitor ionic changes.

A technique that could be compared with ion-sensitive electrodes is that using fluorescent indicators and one is often asked how the advantages and disadvantages of these two techniques compare. We have summarised the general features of these two methods below.

ION-SENSITIVE MICROELECTRODES

Advantages	Disadvantages
(1) Organelles excluded	(1) Need large cells (limit≈50×10 μm)
(2) Continuous measurement	(2) Specificity occasionally inadequate
(3) Two or more ions simultaneously	(3) Electrical interference
(4) Low cost and simple equipment	(4) Some degree of manipulative skill needed
(5) Calibration usually simple	(5) Response time of seconds

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FLUORESCENT INDICATORS

Advantages	Disadvantages
(1) Response time of milliseconds	(1) May not exclude organelles
(2) Continuous measurement	(2) Calibration can be difficult
(3) Good specificity -though not always	(3) May alter intracellular buffering
(4) Can be used on small preparations	(4) Equipment costly
(5) Little manipulative skill needed	(5) Photo-toxicity
	(6) Light interference
	(7) Photo-bleaching
	(8) Good indicators for some important ions not yet available (e.g. Cl^-)

Let us now consider these in more detail.

Response times. Many changes in intracellular ion activities occur quite slowly - on a second to minute timescale - and the response times of ion-sensitive microelectrodes are well suited to record such changes. However, some intracellular ionic events occur much faster and it is the examination of such fast events which would benefit from fluorescent indicators.

Organelles. Ion-sensitive microelectrodes measure only activities of the cytoplasm as their tip size precludes proper and unruptured impalement of organelles. As discussed in the chapter on fluorescent indicators, a major problem of fluorescent indicators is that one of the main methods for their incorporation into cells can lead to the indicator becoming trapped in intracellular organelles. This makes it difficult to know what one is actually measuring: ion activity in the cytoplasm, organelle or a mixture of the two.

Continuous measurement. It is possible to record ionic changes inside cells with ion-sensitive microelectrodes for as long as one can maintain impalement of the cell (often many hours). The drawback of fluorescent indicators is that they bleach to greater or lesser extents and emission intensity of the dye can decrease over a period of time, which can preclude continuous recording. It is difficult, though not impossible, to measure two ions simultaneously with fluorescent dyes but this is more straightforward to do with ion-sensitive microelectrodes provided the cell under study can withstand the multiple impalements.

Equipment. The peripheral equipment required to use ion-sensitive microelectrodes is simple to make and cheap. Most electrophysiology laboratories will have a spare oscilloscope and a strip chart recorder. In contrast, the equipment for using fluorescent indicators can be an order of magnitude more expensive.

Cell size. The technique requires that two microelectrode tips should be in the cell at the same time and this becomes more difficult as cell size decreases. There are techniques that are partial solutions to this problem but cells around 50×10 μm in size are about the smallest on which we would contemplate using ion-sensitive microelectrodes. For small cells there are advantages in using fluorescent indicators.

Specificity. Many ion-sensitive microelectrodes are not uniquely selective for the

ion one is trying to measure. Other ions cause interference and may combine with the true signal thus making it difficult to assess the exact change in activity of the ion one wishes to measure. In practice what this does is to set a detection limit for the electrode. Generally, fluorescent dyes are selective for the ion in question, but they can suffer from direct interference or changes in autofluorescence produced by, for example, pharmacological compounds.

Calibration. This is relatively straightforward for ion-sensitive electrodes, which can be calibrated separately, but can be difficult with fluorescent dyes.

Electrical interference. The sensitivity of ion-sensitive microelectrodes to electrical interference requires that they be well shielded. Fluorescent dyes obviously do not suffer from such problems but they must be used in the dark which can also put constraints on the design of the experimental area.

Buffering. Ion-sensitive microelectrodes themselves are inert measuring devices in that they do not add or remove anything from the cell under test. They do, however, require impalement of the cell membrane. Fluorescent dyes are not inert in the same way. Ca^{2+} indicators, for example, may signifcantly increase the Ca^{2+} buffering power of the cell.

2. Ion-sensitive electrode measurements

Ion activity and ion concentration

If we were to grind up a biological preparation and assess the calcium content of the tissue by atomic absorption spectrophotometry and then compare this value with one produced by an ion-sensitive microelectrode or fluorescent indicator we would find that the total calcium concentration would be of the order of millimoles per litre whilst the free intracellular calcium ion concentration would be 100 or so nanomoles per litre. Thus a huge percentage of the calcium is bound to moieties within the cell e.g. intracellular organelles and buffers. The differentiation between free ion concentration and total ion concentration is a simple matter. However, what is the difference between free ion concentration and activity? As another illustration consider a solution of 150 mM NaCl in water. The NaCl would fully ionise so the concentration of Na^+ ions ($[Na^+]$) in the solution would be 150 mM. However, the mutual electrostatic repulsion between the similarly charged species and attraction between anions and cations reduces their mobility and freedom especially as the concentration of the solution becomes higher. In other words, the ions exhibit non-ideal behaviour, which is not due to incomplete ionization but to the existance of inter-ionic forces. Thus the activity of the Na^+ ions in solution or their effective concentration is less than their total concentration. Only when the solute is infinitely diluted will its concentration equal its activity. It is the activity of the ion which is used in calculations related to thermodynamic processes. The constant that relates the ion's activity to concentration is the activity coefficient (γ_i) such that:

$$a_i = [i] \cdot \gamma_i \qquad (1)$$

where a_i is the activity of the ion (i) and the square brackets denote free ion concentration (i.e. ions which are not bound). The activity coefficient is a correction factor for thermodynamic calculations. For a 150 mM solution of NaCl at 20°C, γ_{Na}=0.75. The activity of Na^+ in the solution is therefore 112.5 mM.

The inter-ionic forces are influenced by the number and valency of the ions in solution and so the activity coefficient will vary with the ionic strength of the solution and the valency of the ion. This departure from non-ideal behaviour was recognised by Debye and Hückel who produced a quantitative expression for activity coefficients in dilute (<0.01 M) solutions. At 25°C:

$$\log\gamma = -0.5091 z^2 \sqrt{I} \tag{2}$$

where z is the valency of the ion and I is the ionic strength of the solution, which is calculated as follows for n number of ionic species (i):

$$I = 0.5 \sum_{k=1}^{n} [i_k] z_k^2 \tag{3}$$

In more concentrated solutions (>0.01 M but <0.1 M) expression (2) has to be modified so that:

$$\log\gamma \approx -A \left(\frac{\sqrt{I}}{1 + \sqrt{I}} \right) \tag{4}$$

where:

$$A = \frac{1.8246 \times 10^6}{(\varepsilon T)^{3/2}} \tag{5}$$

ε being the dielectric constant of the solvent (water = 80.1 at 20°C; 78.3 at 25°C) and T the absolute temperature.

One has a choice whether one expresses the voltages from the ion-sensitive microelectrodes in terms of free concentration or activity. The advantages and disadvantages of either way are reviewed by Thomas (1978) and Tsien (1983). Our own view is that stated by Tsien as follows: “When one says ... that the free calcium concentration in a cell was measured to be 1 μM, that really means that the calcium activity in the cell was the same as the calcium activity in a certain calibrating solution in which the other major constituents were considered to be similar to those of cytosol and which contained 1 μM of calcium ions not tightly bound to ligands”. In other words we normally calibrate the electrodes in solutions of known concentration which are usually chosen to mimic the cytoplasm. When these electrodes are pushed into a cell we compare the voltages then obtained with the calibration curves. Provided the activity coefficient is the same inside the cell and outside, then the value that we read from the calibration will be the intracellular free ion concentration. We do not need to know the value of the activity coefficient. Because the ionic strength of the calibration solution is chosen

to be nearly the same as that of the cytoplasm, then our value for the intracellular ion concentration does not get any better or become more exact if we multiply by γ. If, however, one is not prepared to accept that γ is similar on both sides of the cell membrane, then by knowing its value in your calibration solutions you can simply read off the intracellular ion activity from your curve.

One of the problems is in knowing with certainty the value of γ for the ion in question. Values can be found in the literature or they can be calculated using equations (3) and (4) above, but these will only yield an approximation which is good for monovalent ions but poorer for divalents. Mainly because ionic strengths are usually similar in calibrating and intracellular solutions it is acceptable to express results in free ion concentration. One advantage of expressing your results in activity is that this is precisely the quantity upon which biological processes depend. However, if you wish to compare an ion activity measurement with a biochemically derived one - say the K_m of a plasmalemmal exchange process - then you will need to convert your activity measurement back to concentration. The tendency in the literature is to use free ion concentration for divalents and activity for monovalents. This is largely because of uncertainty about γ for divalents. There is certainly great scope for confusion and it is largely a matter of preference but, whatever you decide to do, you should provide sufficient details of calibrations so that others know what you have done.

The Nernst relationship

Assume we have a membrane that is permeable to only one ion. If we separate, by this membrane, two solutions that have different activities of this ion, a net diffusion of the permeant ion will start to take place through the membrane. This flux gives rise to a net movement of electrical charge and therefore an electrical potential difference is rapidly generated across the membrane. At equilibrium, there is no net diffusion and the potential difference across the membrane is given by the well-known Nernst equation:

$$E = \frac{RT}{zF} \ln \frac{a_s}{a_f} \tag{6}$$

where R is the gas constant, T is the absolute temperature, z is the valency of the ion, F is the Faraday constant and a_s and a_f are the activities of the permeant ion on the two sides of the membrane.

Ion-sensitive electrodes are devices with a selectively permeable membrane sealing a small volume of a solution with a constant activity of the permeant ion (a_f) (see Fig. 1). When such an electrode is exposed to a sample of a solution with an unknown activity of the permeant ion (a_s) the equilibrium potential which develops between the two solutions can be measured and used to calculate the activity of the permeant ion in the sample.

For practical measurements a closed circuit is set up as shown in Fig. 2A. In this system we have an ion-selective electrode which is measuring the activity (a) of an

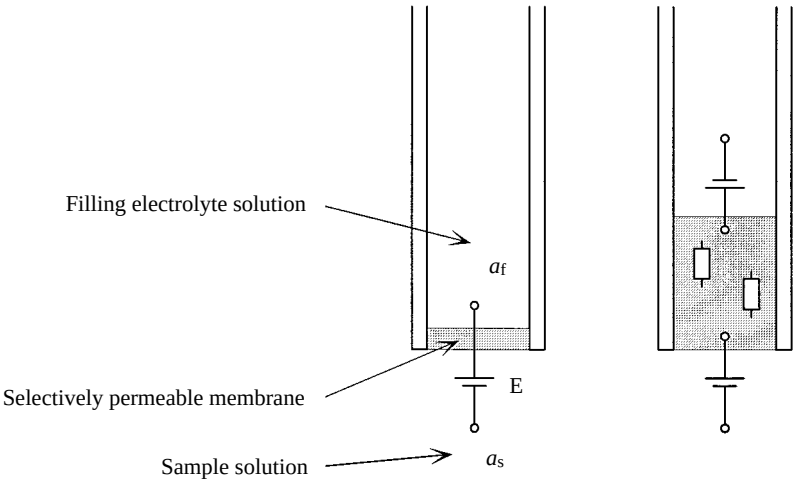


Fig. 1. Basic structure of an ion-sensitive electrode. Left: at equilibrium, the potential difference E, which exists across a selectively permeable membrane is given by the Nernst equation. a_f and a_s denote the activity of the permeant ion in the filling solution and in the sample solution respectively. Right: in practice, the selectively permeable membrane has a finite thickness and a potential difference is generated at each of the two membrane-aqueous solution interfaces. At the inner surface the potential difference can be assumed to be constant (but for its temperature sensitivity, see Section 3, *Temperature sensitivity and membrane column length*). Such a membrane column behaves in a manner analogous to a selectively permeable membrane displaying Nernstian characteristics, i.e. the two batteries can be lumped together and they generate E shown on the left.

ion (i) in solution. The potential difference (E_1) measured between the two electrodes in this solution (number 1) is:

$$E_1 = E_o + \frac{RT}{zF} \ln \frac{a_{i(1)}}{a_{i(f)}} \tag{7}$$

where E_o is the reference or offset potential (a constant consisting of several terms) and $a_{i(f)}$ is the activity of the ion in the filling solution of the electrode (which is also constant). Note that E_o includes the liquid junction potential of the reference electrode which, in the following discussion, is assumed to remain constant. If we now move the electrodes into a new solution (number 2) then E in this solution is:

$$E_2 = E_o + \frac{RT}{zF} \ln \frac{a_{i(2)}}{a_{i(f)}} \tag{8}$$

The difference in potential is dependent upon the ratio in ion activity between the two solutions because subtracting equation (7) from equation (8) yields:

$$\begin{aligned} E_{2-1} &= \frac{RT}{zF} \ln \frac{a_{i(2)}}{a_{i(1)}} \\ &= m\log \frac{a_{i(2)}}{a_{i(1)}} \end{aligned} \tag{9}$$

where *m* is the slope of the relationship and at room temperature (20°C) equals (58/z) mV. Equations (9) are statements of the Nernst equation and what this means is that for a 10-fold change in monovalent ion activity one would expect a 58 mV change in potential.

Ion-sensitive microelectrodes are a miniaturisation of the system shown in Fig. 2A. In order to make intracellular ion measurements one must have two electrodes in the same cell (Fig. 2B). One electrode measures the reference potential while the other is the ion-sensitive device. Ion-sensitive microelectrodes are usually glass micropipettes plugged at the tip with an organic membrane solution. This medium does not provide electrical interactions for ions as water dipoles do in the water phase and therefore ions can only enter the membrane phase if they bind to specific carrier molecules (ionophores) in the membrane solution. These form

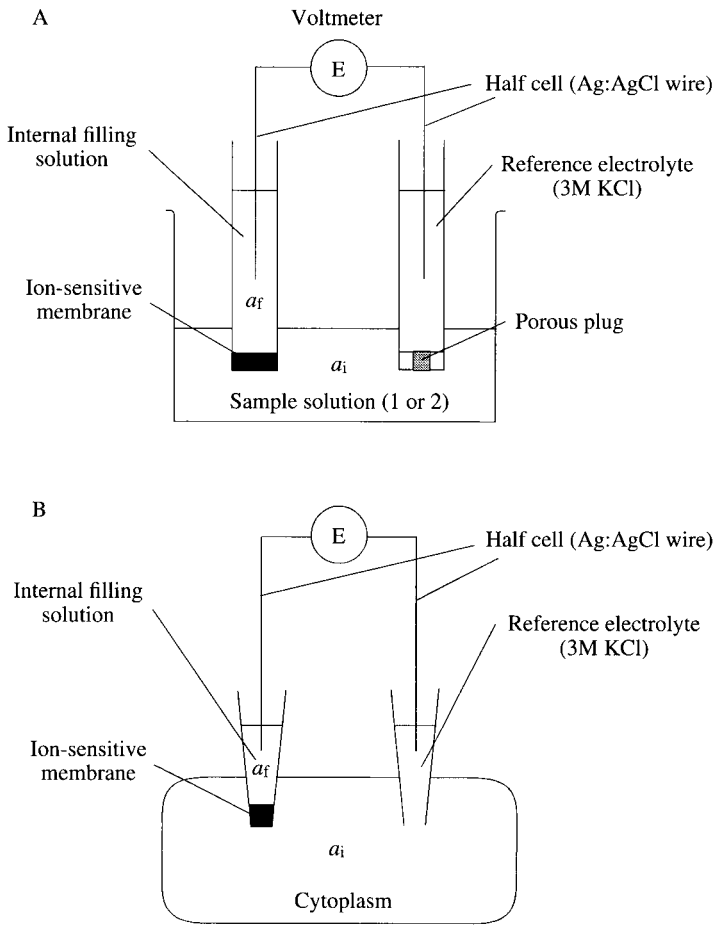


Fig. 2. Experimental set-up for ion-sensitive electrodes. The potential difference *E*, which exists across a selectively permeable membrane is measured by a voltmeter. a_f and a_i denote the activity of the permeant ion in the filling solution and in the sample solution respectively. For details, see text.

lipophilic complexes with ions and so promote the transfer of hydrophilic ions into and across a hydrophobic region. This then generates the potential across the membrane between the inner filling solution and the external solution being measured. Ion-sensitive microelectrodes will also record the membrane potential in addition to the intracellular ion changes. Thus an independent measure of membrane potential needs to be made (this is done by the separate reference electrode) and the voltage due to the membrane potential is then subtracted from the combined signal.

Detection limits and selectivity factors

The ion-sensitive electrode response tends to deviate from the Nernst relationship at low activities of the ion. The reason for this is that the total potential change is also governed by the presence of other "interfering" ions in the sample solution, which compete with the primary ion at the aqueous solution/ion-sensitive membrane interface. In mixed solutions the electrode response (E) is better described by the Nicolsky-Eisenman equation:

$$E = E_o + m\log(a_i + \Sigma K_{ij}^{pot}(a_j)^{z_i/z_j}) \quad (10)$$

where j are the interfering ion(s) and K_{ij}^{pot} are the selectivity constants of the ion-selective electrode for the interfering ions. K_{ij}^{pot} or more simply, K_{ij} are a measure of the preference by the electrode for the interfering ion (j) relative to the primary ion (i) being detected. The potential measured by an ion-sensitive electrode will thus be a combination of an offset potential and the potential due to the primary ion and the interfering ions in solution. In practice, it is the logarithm of the selectivity constant which is usually given. As an example, consider the selectivity of a Na^+-sensitive electrode (made from the ETH 227 sensor and containing sodium tetraphenylborate) for K^+. The log K_{NaK} is quoted as −2.3 so K_{NaK} is 0.005. This means that the sensor is approximately 200 times more sensitive to Na^+ than to K^+. When log K_{ij} is given, a negative value means that the sensor is more selective for the primary ion and a positive value means that the sensor is more selective for the interfering ion. Therefore, the more selective sensors will have the more negative log K_{ij} values. When the selectivity of the sensor for the primary ion is the same as for the interfering ion, log K_{ij} will equal zero.

In equation (10) when:

$$a_i = \Sigma K_{ij}(a_j)^{z_i/z_j} \quad (11)$$

then the limit of detection for the electrode has been reached. This is about the lowest activity of the primary ion at which the electrode can discriminate; thereafter a decrease in primary ion activity will produce a progressively attenuated potential change because the influence of a constant background of interfering ion will predominate. It is then apparent why, at low activities of the primary ion, the slope of the relation between potential and primary ion activity (measured in a constant background of interfering ion) deviates from one which is Nernstian.

3. Practical electrode design and construction

Ion-sensitive glass electrodes

Ion-sensitive microelectrodes can be made using special glass with ion-sensitive properties. H^+-, Na^+- and K^+-sensitive glasses have been applied to physiological measurements. The development of a potential at the glass-solution interface of a glass pH-sensitive electrode is related to the transfer of protons into the glass in exchange for sodium ions passing into solution on the other side of the membrane. The glass behaves as a semi-permeable membrane, although the potential is developed by a different mechanism on each surface (see Bates, 1954). While some glass ion-sensitive microelectrodes are more selective than their liquid-membrane counterparts, practically their use is limited to the measurement of pH or Na^+. The main advantage of this type of ion-sensitive microelectrode, which is important in certain cases, is that they are very insensitive to substances that are known to interfere with the organic membrane of liquid membrane microelectrodes. The disadvantages with electrodes of this type are associated with tip size and response time and a lot of experience is required in their construction. The main difficulty in manufacturing microelectrodes of this type is sealing the tip of an ordinary micropipette with a small piece of ion-sensitive glass. For these reasons most ion-sensitive microelectrodes are now made from the liquid membrane cocktails. An interested reader can find a detailed description of ion-sensitive glass microelectrodes and different methods used to produce them in Thomas (1978). On the following pages, we concentrate on liquid-membrane microelectrodes.

Liquid-membrane microelectrodes

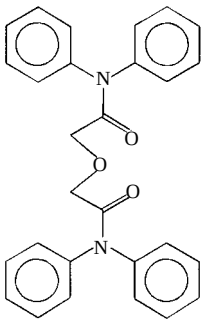
Liquid-membrane solutions generally have three components: the ion-selective compound or carrier (most often a neutral ligand), a membrane solvent or plasticizer in which the carrier is dissolved and a membrane additive (a lipophilic salt).

There are a variety of neutral carriers or ligands to bind (and hence detect) a variety of ions that are biologically important: Na^+, K^+, H^+, Ca^{2+}, Mg^{2+} and Cl^-. The carrier is dissolved in a solvent which must be non-polar (to reject hydrophilic ions and allow solubilization of lipophilic compounds) and be of moderate viscosity (to allow a microelectrode to fill easily). Additives produce significant improvements in selectivity and decrease membrane resistance and electrode response time. As an example, the components of a Ca^{2+}-sensitive membrane solution are shown in Fig. 3.

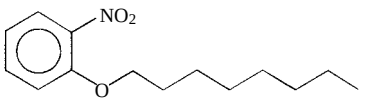
Single-barrelled microelectrodes

Single-barrelled liquid-membrane microelectrodes are the most widely used type of ion-sensitive microelectrodes. The detailed ways of making these electrodes vary between different laboratories, but basically the aims of all steps in the work are the same. In what follows, we have divided the manufacturing procedure into four main steps in the same order as they take place in practical work.

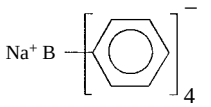
Pulling the micropipettes. Micropipettes suitable for ion-sensitive microelectrodes



ETH 129 - Ca^{2+} ionophore



o-nitrophenyl octyl ether - membrane solvent



Sodium tetraphenyl borate - membrane additive

Fig. 3. Components of a Ca^{2+}-sensitive membrane solution.

can be made by almost any puller. The shape at the extreme tip is not critical as it is bevelled away in most cases. We have, however, successfully used thin-walled tubing without any bevelling or breaking in crustacean preparations (e.g. Voipio *et al*. 1991). These electrodes have a small tip diameter combined with a reasonable electrode resistance, but their applicability is limited by easy breaking of the tips while impaling cells or tissue. We have also used thicker-walled tubing without bevelling in mammalian heart cells (MacLeod, 1989) but one has to be careful since such electrodes may suffer from poorer selectivity and sensitivity. Thick-walled borosilicate glass without filament (like GC150 from Clark Electromedical Instruments) is good for general use in short-column electrodes, but filamented glass is better if the pipettes are to be backfilled with membrane solution. The use of other glass materials (e.g. aluminosilicate) is not common despite speculation concerning non-specific cation permeability of hydrated borosilicate glass in the tip region (c.f. e.g. Tsien & Rink, 1981). For these reasons it is preferable to start with ordinary borosilicate tubing.

In our experience, the tubing rarely needs cleaning. Recommendations vary from avoidance of cleaning (Thomas, 1978) to very effective washing procedures (e.g. Tsien and Rink, 1980). If silanization fails, tubing may be simply soaked overnight in butanol, rinsed in distilled water and ethanol and finally dried at 200°C. Since pulling pipettes is not time consuming, it is best to pull pipettes on the day they will be used. Pipettes are mounted in a metal holder in a Petri dish with a lid to protect them from dust. Never use modelling wax or any sticky material e.g. plasticine or Blutack™ to hold the pipettes, since the remains of oily components on the glass may evaporate and prevent proper silanization. It is best to start with about 20 pulled micropipettes. After a few days of training, one should be able to obtain more than ten working ion-selective electrodes in a few hours.

Bevelling of the micropipette tip. Micropipettes are bevelled in order to obtain tips with the shape of a hypodermic needle. This results in a large tip opening without compromising sharpness. An increase in tip inner diameter decreases the resistance of an ion-sensitive microelectrode and improves electrode sensitivity and selectivity.

Bevelling techniques can be divided into two categories. In wet bevelling, a micropipette filled with an electrolyte solution is lowered towards a rotating surface covered with aluminium oxide particles or diamond dust (Brown & Flaming, 1975) or with loose particles as a "thick slurry" (Lederer *et al.* 1979). The surface is covered by an electrolyte solution, which makes it possible to control bevelling of the tip while monitoring electrode resistance. These methods have the disadvantage that pipettes must be filled for bevelling and therefore silanization should be undertaken first. In addition, impaling properties indicate that slurry-bevelled electrodes do not necessarily have sharp tips. We know of several laboratories where both wet and dry bevelling techniques are available and all prefer the latter at least in construction of ion-sensitive electrodes.

Our dry-bevelling technique (Kaila & Voipio, 1985) was originally developed to meet the needs of making ion-sensitive microelectrodes, but it has been successfully used also in making low-resistance microelectrodes for voltage clamping of cardiac (e.g. Kaila & Vaughan-Jones, 1987) and crustacean (e.g. Kaila & Voipio, 1987) preparations. With this method, tips with a 0.6 μm outer diameter at the base of the bevel still appeared to have a neatly bevelled shape when examined with scanning electron microscopy. The dry bevelling equipment is simple and is described in Section 4, *Microelectrode dry bevelling equipment.* A pipette to be bevelled is lowered with a micromanipulator until its tip touches a rotating bevelling surface at an angle of 30-45°. The total bevelling time is controlled by amplifying and listening to the noise originating from the shank of the pipette. The correct bevelling time is found by trial-and-error and it is seldom longer than a few seconds unless large diameter tips are desired. Each bevelled tip should be inspected with a high quality microscope.

If bevelling equipment is not available, one may try breaking the tips by pushing them against a piece of glass under a microscope. This often gives tips with a reasonable diameter and with sharp edges facilitating impalements (Thomas, 1978).

Silanization. The organic liquid membrane solution must be in tight contact with the glass wall of the electrode, otherwise the aqueous electrolyte solution will find a

pathway along the luminal glass surface thus short-circuiting the ion-sensitive signal source. This can be avoided if the glass surface is made hydrophobic by silanization. Reactive silanes replace hydroxyl groups on the glass surface and bind to it with covalent bonds resulting in a monomolecular hydrophobic coating (for references, see Ammann, 1986). Vapour treatment with N,N-dimethyltrimethylsilylamine (TMSDMA; Fluka or Sigma) is a very effective and widely used silanization method. The disadvantages are that this compound evaporates rapidly and is extremely toxic. Therefore, the correct place for handling TMSDMA (including the placement of the oven used for silanization) is a fume-cupboard.

Silanization with TMSDMA is easy. Bevelled micropipettes are mounted horizontally on a metal holder in a Petri dish which is taken with its lid open to an oven. The pipettes are baked at 200°C for 15 min after which time 20-40 μl of TMSDMA is added and the lid of the dish immediately closed. Because TMSDMA evaporates rapidly even at room temperature, it is best added to the dish in a small open glass vial. After another 15 min the lid of the dish is opened and baking is continued for a few more minutes to let the remains of TMSDMA disappear. The micropipettes are now ready for filling. They may be stored in the oven at e.g. 110°C, but we usually keep them in a closed Petri dish on a lab bench for a day. Storage of pipettes for longer periods should be avoided. Some workers store pipettes in a hot oven or in a desiccator but despite keeping them dry you may find resilanization necessary if the pipettes have been stored for several days. The silanization temperature used in different laboratories ranges from 110 to 200°C, but we have found that, within these limits, higher temperatures give better results. Silanization may fail if some other use has lead to contamination of the oven by other organic substances which evaporate and stick to the inside surfaces of the oven. Cleaning the inside surfaces with a solvent and/or prolonged baking at a temperature much higher than that needed for silanization are tricks that usually help.

Filling. The method chosen for filling micropipettes with the liquid membrane and filling electrolyte solutions determines the length of the membrane column within electrodes. Since this is a critical factor concerning the temperature sensitivity of the finished ion-sensitive microelectrode, this matter will be discussed before going into the details of pipette filling.

(1) Temperature sensitivity and membrane column length. The electrical output of ion-sensitive electrodes is the sum of potentials at the two liquid membrane - aqueous solution interfaces (Fig. 1). These are the two energy barriers where the ion to be measured experiences a step change in electrochemical potential which is, along with other factors as outlined in section 2, a function of temperature. If the liquid membrane column within a microelectrode is so long that part of it remains above the surface level of a warmed experimental bath, changes in bath temperature or surface level can give rise to changes in the temperature gradient along the membrane column and therefore, cause changes in electrode output, thereby causing serious noise and errors in quantitative ion measurements (Vaughan-Jones & Kaila, 1986). For this reason, the column length should be shorter than the part of the electrode that is immersed in the experimental bath during measurements.

Obviously, the temperature sensitivity of long-column microelectrodes is much less of a problem if the experiment is performed at room temperature. However, they still suffer from two other minor drawbacks: backfilling consumes a lot of (often expensive) liquid membrane solution and capacitance compensation is more difficult since there is no low resistance connection for some distance up the shank. In spite of these problems, long-column electrodes are widely used because they are very easy to fill and they usually have a longer lifetime than their short-column counterparts.

(2) Filling long-column microelectrodes. Micropipettes are back-filled through the stem with a small sample of liquid membrane solution. If a filamented pipette is well silanized, it fills spontaneously up to its tip resulting in a column several millimetres long. Back pressure is required when using non-filamented pipettes. The rest of the pipette is then filled with an electrolyte solution and the electrode is ready for calibration.

(3) Filling short-column microelectrodes. The filling of a short-column microelectrode involves two steps: the whole pipette is first filled with an electrolyte solution and then a short column of a liquid membrane solution is taken up into its tip. If the pipettes are properly silanized, they should not show any self-filling properties with an aqueous solution. A pipette is first back-filled up to its shoulder by injection of an electrolyte solution (see below) and it is then coupled to a pressure source. When pressure is applied it is possible to see (provided light comes from a suitable angle) the air-solution interface moving along the shank while the air in the tip is first compressed and then is pushed out through the tip opening. If very little pressure is needed, one should suspect either poor silanization or a broken tip. Pressurised air or nitrogen are suitable gases, or more simply, a 10-20 ml syringe can be used if a very high pressure is not needed. To couple the pipette to the pressure source either use tightly fitting silicone tubing or a special adapter like a patch-clamp holder. Dental wax has been used to seal a copper capillary into the pipette stem. Whatever the connection is, never point the pressurised pipette at anyone - if it gets loose, it flies like a bullet!

Pipettes may be checked after filling by resistance measurement, which will reveal broken or blocked tips. At this stage (when the electrode contains its electrolyte filling solution only) the higher resistance values, compared with conventional microelectrodes of the same size, result from the lower conductivity of the internal filling solution. A DC current of 1 nA induces a voltage change of 1 mV/MΩ. In AC measurements, a low-frequency sine-wave signal is preferable. Problems with filling can occur if the tip tapers at a steep angle, i.e. its diameter changes rapidly. With this type of pipette tip the release of pressure after filling may be followed by air being taken up into the tip. Such a phenomenon is not seen if tips have a more tubular shape.

The filling electrolyte solution must contain the ion to be measured in addition to chloride, which is required for stable operation of the Ag:AgCl electrode. NaCl- and KCl-containing solutions are used in Na^+ and K^+ electrodes, respectively, as well as in Cl^- electrodes. Buffer solutions are used in pH and Ca^{2+} electrodes. Some examples of filling solutions are given in Table 1.

Sometimes bubbles appear in the filling solution within the shank of the

Table 1. *Typical examples of ion-sensitive microelectrodes as constructed in the authors' laboratories*

Measured ion	Liquid membrane	Filling solution (mmol.l^{-1})	Comments
H^+	Fluka 95291/95293	NaCl 100, Hepes 20, NaOH 10, (pH 7.5)	for pH 5.5-12
H^+	Fluka 95297	NaCl 100, Hepes 20, NaOH 10, (pH 7.5)	for pH 2-9
K^+	Fluka 60031/60398	NaCl 100, KCl 5	Valinomycin
Na^+	Fluka 71176	NaCl 100	
Na^+	Fluka 71178	NaCl 100	(see note 1)
NH_4^+	Fluka 09879	NH_4Cl 10 (see note 2)	
$TeMA^+$	Corning 477317	KCl 100 or TeMACl 150 (see note 3)	volume meas.
Ca^{2+}	Fluka 21048/21191	pCa 6 solution of Tsien & Rink (1981) or Hove-Madsen & Bers (1992)	ETH 1001±PVC
Ca^{2+}	Fluka 21196	pCa 6 solution of Tsien & Rink (1981) or Hove-Madsen & Bers (1992)	ETH 129
Mg^{2+}	Fluka 63085	KCl 100, $MgCl_2$ 5	
Cl^-	Fluka 24902	NaCl 100	
Cl^-	Corning 477913	NaCl 100	

$TeMA^+$=tetramethylammonium ion. Note 1: suitable for extracellular measurements due to higher selectivity against Ca^{2+}. Note 2: see Fresser, Moser & Mair (1991). Note 3: see p. 232 in Nicholson & Phillips.

microelectrode. Filamented tubing does not help in this case, since after silanization the self-filling properties with aqueous solutions are lost. If the bubble is initially small and it is attached to the inner glass wall, a fall in pressure during suction used to take up membrane solution will increase the bubble diameter until it breaks the continuity of the filling solution. Returning to normal pressure does not necessarily result in the reverse process since the hydrophobic glass surface provides no route for the filling solution to cross the gas phase. The resulting disk-shaped bubble appears as a transverse stripe on the shank and causes a break in electrical conductivity which is seen as an infinite electrode resistance. Such a bubble can usually be broken down by a gentle tap on the electrode or by pushing a thin fibre (e.g. the classical cats whisker or a hair) through it.

Small bubbles often consist of air that was originally dissolved in the filling solution. The reduction in pressure during suction or a slight increase in temperature during handling will favour bubble formation, since the solution becomes over-saturated with air. If bubbles occur frequently, it is a good idea to de-gas all filling electrolyte solutions now and then. This can be done either by shaking the solution under low pressure or at a slightly elevated temperature (about 40°C).

When a pipette has been filled with electrolyte solution, it is advisable first to dip its tip in distilled water before quickly immersing it in the liquid membrane solution. Sometimes, a sufficient amount of membrane solution is taken up by capillarity, but suction (by mouth or from a syringe or a suction pump) lasting from a few seconds to minutes is often required to obtain a proper column length. Keep the tip in membrane solution during the suction so that air is not taken into the pipette. The membrane

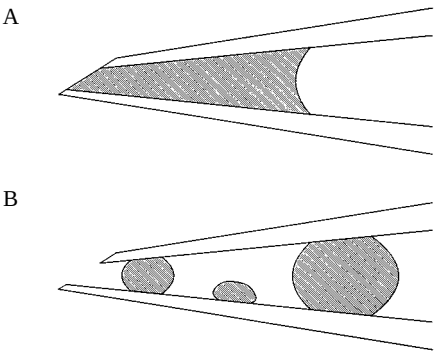


Fig. 4. (A) In a properly silanized micropipette, the liquid membrane solution forms a uniform column with a concave interface against the aqueous filling solution. (B) In a poorly silanized micropipette, the organic membrane solution is easily replaced by water at the hydrophilic glass surface which results in convex interfaces and, often, breaking of the membrane column.

column should appear as a 50-200 μm long continuous region ending in a concave surface against the filling electrolyte solution, which indicates proper silanization of the glass. A convex membrane-water interface, or breaking of the membrane phase into multiple sections, or its withdrawal into the shank are typical signs of bad silanization (see Fig. 4).

(4) Choice of membrane solution. Ready-to-use liquid membrane solutions - 'cocktails' - are commercially available for H^+, K^+, Li^+, Na^+, NH_4^+, Ca^{2+}, Mg^{2+} and Cl^-. Fluka Chemie AG publishes a separate catalogue entitled "Selectophore®, Ionophores for Ion Selective Electrodes and Optodes" which contains detailed information (including cited literature) on numerous products for ion-sensitive electrodes. Other sources for liquid membrane solutions are given in Table 2. For some ions there are several liquid membrane solutions available. Fluka supplies H^+ cocktails based on two different hydrogen ionophores. They differ in measuring range (pH 5.5-12 and 2-9) and electrode response time. Fluka also has different cocktails for Ca^{2+} microelectrodes. Those based on Calcium Ionophore I (ETH 1001) yield microelectrodes with a detection limit at or slightly below typical resting intracellular Ca^{2+} levels, but microelectrodes made using the cocktail containing Calcium Ionophore II (ETH 129) are able to record much lower Ca^{2+} levels and should be preferred for intracellular experiments. They also have two different valinomycin-based cocktails for K^+, which make highly selective low- and high-resistance microelectrodes, and two cocktails for Na^+, one of which is widely used but the other has better selectivity against Ca^{2+} and therefore it has been applied to extracellular measurements (see Table 1 and Coles & Orkand, 1985). A potassium ion-exchanger solution (Corning cat.no. 477317; or 5 mg potassium tetrakis p-chlorophenylborate in 0.1 ml 3-nitro-O-xylene, Alvarez-Leefmans *et al.* 1992; see also Ammann, 1986) is very selective to tetra-alkylammonium ions and is used in extra- and intracellular volume measurements. This technique, as well as a recently

developed CO_2 microelectrode, is briefly discussed below. In general, Cl^- liquid membrane electrodes are not ideal with respect to anion interference (bicarbonate, acetate, lactate and other intracellular anions). Fluka supplies a Cl^- cocktail (cat.no. 24902) with a reported selectivity coefficient of 0.03 against bicarbonate. In our experience, the widely used Cl^- exchanger (Corning cat.no. 477913) yields microelectrodes with a similar selectivity coefficient against bicarbonate of 0.03-0.04 (Kaila *et al.* 1989) which is much better than that reported previously (Baumgarten, 1981). The improvement is likely to be due to dry bevelling of the micropipettes used in the electrode construction. A membrane solution, originally published as a HCO_3^- sensor (Wise, 1973) but later shown to be sensitive to CO_3^{2-} (Herman & Rechnitz, 1974), has been used also in intracellular microelectrodes (Wietasch & Kraig, 1991). A nonspecific ion exchanger solution for reference electrodes (Thomas & Cohen, 1981) avoids problems related to electrolyte leakage into the cytoplasm, but such electrodes are now rarely used.

Electrode testing. Physiological experiments should never be made with electrodes that have not been properly tested. Different calibration methods are discussed in detail below (Section 5). An essential step in testing an ion-sensitive microelectrode is measurement of its resistance. This is done by passing a DC current of 1.0 or 0.1 pA through the electrode, which induces a voltage drop of 1.0 mV/GΩ or 0.1 mV/GΩ respectively, across the membrane column. Typical resistance values depend on tip

Table 2. *Sources for components used in the construction of ion-sensitive electrodes*

Components	Sources
Borosilicate, aluminosilicate and pH-sensitive glass, fused and theta-style capillaries, teflon-coated silver wire	Clark Electromedical Instruments, P.O. Box 8, Pangbourne, Reading, RG8 7HU, England. 0734 843888
Glass including thick-septum theta tubing, membrane solutions	World Precision Instruments, Inc., 175 Sarasota Central Blvd., Sarasota, Florida 34240, USA or World Precision Instuments Ltd., Astonbury Farm Business Centre, Aston, Stevenage, Hertfordshire, SG2 7EG, England
Liquid membrane solutions and components for practically all ions, silanizing agents	Fluka Chemie AG, CH-9470 Buchs, Switzerland or Fluka Chemicals Ltd., The Old Brickyard, New Road, Gillingham, Dorset, SP8 4JL, England. 0800 262300 or 0747 823097
A widely used Cl^- exchanger (cat. no. 477913) and a K^+ exchanger used in volume measurements (no. 477317)	Ciba Corning U.K., Colchester Road, Halstead, Essex, CO9 2DX, England. 0787 472461
High quality 100 GΩ resistors (type RX-1M). Useful when testing equipment	Victoreen Inc., 6000 Cochran Road, Solon, Ohio 44139, USA or LG Products Ltd., c/o ECl International, 17 Trident Industrial Estate, Blackthorne Road, Colnbrook, Slough, SL3 0AX, England. 0753 686667
Details of the construction of the dry beveller and of electrometer amplifiers	Information available from the author J.V. Fax +358 0 191 7301

diameter as well as on the liquid membrane solution being used and they range from a few GΩ to 100-200 GΩ.

Electrode holders and silver wires. Ion-sensitive microelectrodes should not be mounted on holders that might generate any pressure difference (positive or negative) between electrode interior and ambient air, since the membrane column within the tip is easily dislodged. Simple clamps attaching to the stem of a microelectrode give good results. Such clamps may be made of metal and be grounded without a risk of electrical leakage, since silanized glass has an exceedingly low surface conductivity under typical experimental conditions.

The AgCl coating on silver wires should be in perfect condition. We have obtained very good stability with wires chlorided by a DC current of +0.1-1.0 mA in 250 mM NaCl. Teflon coated wires are very useful, but only a few millimetres of teflon should be removed from each end, so that the chlorided area will be completely immersed in the electrode filling solution.

In lengthy experiments, water may evaporate from electrodes to such an extent that the activity of the electrolytes does not remain constant. This is, of course, seen as electrode drift, which can be avoided simply by sealing the upper end of each electrode. Evaporation of water is seldom a problem with ion-sensitive microelectrodes, but occurs sometimes in KCl-filled reference electrodes (affecting the electrode potential of their Ag:AgCl electrodes), where the filling solution has a tendency to creep up along the hydrophilic glass surface. To ensure stability of the potential in the bath, it should be grounded by a saturated KCl bridge with a conical tip (Strickholm, 1968).

Ion-sensitive surface electrodes

Net transmembrane ionic fluxes cause changes in intracellular ion activities, but they can also give rise to measurable ionic gradients within the unstirred water layer surrounding individual cells. Ion-sensitive surface microelectrodes, made using blunt

Table 3. *Steps in the construction of short-column single-barrelled liquid-membrane ion-sensitive microelectrodes*

0.*	Clean the tubing and de-gas the filling electrolyte solution
1.	Pull micropipettes with sub-micron tips
2.	Bevel the tips to the desired diameter
3.	Silanize the bevelled pipettes
3.1	Bake at 200°C for 15 min
3.2	Add 20-40 µl of TMSDMA in a glass vial, immediately close the Petri dish containing the pipettes and continue baking for 15 min
3.3	Open the dish and continue baking for 5 min
4.	Back-fill a pipette with the filling electrolyte solution
5.*	Measure the resistance of the electrode
6.	Take a column of membrane solution into the tip
7.	Look at the membrane column with a microscope
8.	Calibrate the electrode and measure its resistance

Less essential steps are indicated by an asterisk.

micropipettes in the shape of a patch pipette, can be gently pressed against a cell to measure changes in ion activity at the external surface (see also Vanheel *et al.* 1986). An example of the application of a surface electrode is given in Fig. 5. In this experiment, surface pH and intracellular pH were measured simultaneously in a crayfish muscle fibre. A prolonged application of γ-aminobutyric acid (GABA) gives rise to an extracellular surface alkalosis coupled to a fall in intracellular pH, both of which are due to a channel mediated efflux of bicarbonate ions (Kaila & Voipio, 1987). Surface electrodes have also been used in a double-barrelled configuration to measure changes in extracellular K^+ (Kline & Kupersmith, 1982) and depletion and accumulation of Ca^{2+} at the surface of cardiac muscle cells (Bers & MacLeod, 1986; MacLeod & Bers, 1987).

Double-barrelled electrodes

As explained in Section 2, *The Nernst relationship*, the ion-dependent signal is measured with respect to a reference electrode which will sense the electrical potential prevailing around its tip. For intracellular measurements it is important that both microelectrodes measure the same membrane potential and the same changes in membrane potential. This necessitates both microelectrodes being in the same cell but because of cell size, fragility or visual difficulties it is often not possible to make multiple impalements. When making extracellular electrode measurements significant voltage gradients can appear, especially within excitable tissues and if the

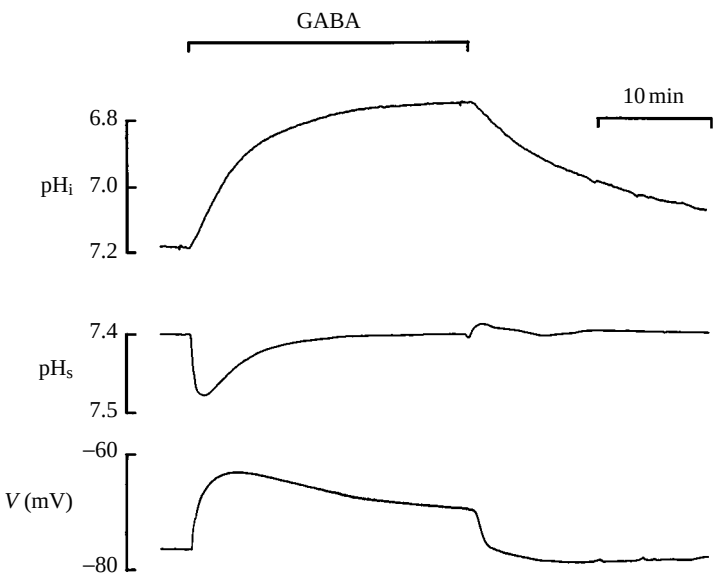


Fig. 5. Simultaneous measurement of intracellular pH (pH_i), extracellular surface pH (pH_s) and membrane potential (V) in a crayfish muscle fibre exposed to γ-aminobutyric acid (GABA, 100 μM). The experimental solutions were equilibrated with 5% CO_2 (30 mM HCO_3^-). The GABA-induced effects are due to a channel-mediated efflux of HCO_3^-. (Reproduced with permission from Kaila & Voipio, 1987).

reference electrode is not situated close to the ion-sensitive electrode, spurious changes in ion activity can be recorded. These difficulties can be overcome by using double-barrelled microelectrodes which contain the reference and the ion electrode in the same tip.

Several methods have been used to produce double-barrelled micropipettes. In the twist-and-pull method, two glass capillaries are first glued together and then twisted around each other whilst being pulled into micropipettes (see Zeuthen, 1980). An easier approach may be to use filamented and non-filamented capillaries which have been already fused together (e.g. 2GC150FS from Clark Electromedical Instruments). An eccentric positioning of two capillaries has also been used (Thomas, 1987). Theta-tubing is easy to pull and gives a spherical cross-section up to the tip, but other steps in making ion-electrodes are sometimes more tricky with them. Thick-septum theta tubing has a lower electrical coupling (for DC and AC signals) between the two barrels and it has been used also in making ion-electrodes. (Note: Bending theta-tubing easily causes cracks to form in the septum, which causes an inter-barrel electrical shunt). Making double-barrelled ion-sensitive microelectrodes differs from making single-barrelled electrodes in only one respect: only one of the two barrels should be silanized. If the tip of the reference barrel becomes hydrophobic, it is easily blocked by membrane solution. Therefore, the main objective is to obtain a specific silanization of one barrel only. Barrel-specific silanization has been done using wet silanizing methods and vapour treatments have been used in combination with sophisticated barrel-specific pressure/suction systems. However, good results are obtained by simply letting TMSDMA vapour evaporate into one barrel at room temperature and then moving the pipette to an oven. A simple holder, like the one shown in Fig. 6, makes it possible to silanize many pipettes simultaneously. TMSDMA is added to a Petri dish which is covered by the holder. Silane vapour is then allowed to flow into the non-filamented barrels for 5-15 min, thereafter the holder with the pipettes is rapidly moved into an oven at 200°C for 15 min.

If bevelling equipment is available, it is advisable to silanize the pipettes before bevelling. This will reduce the possibility of inter-barrel silane contamination at the

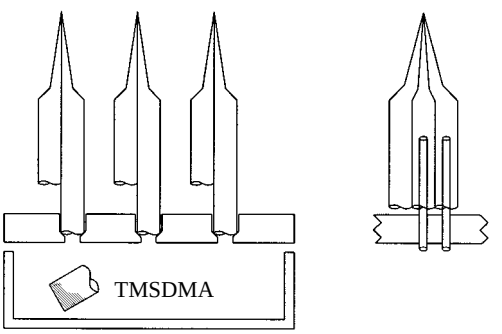


Fig. 6. Holder that allows selective silanization of individual barrels of double or triple-barrelled micropipettes. For details, see text.

tip. In addition, the opening of the reference barrel, which may still get silanized by vapour leaking from the other barrel, is then bevelled away after silanization.

If silanization has been successful, filling the pipettes is easy. Both barrels are first filled with their electrolyte solutions and the liquid membrane solution is then taken up into the ion-sensitive barrel by suction. The reference barrel will not become blocked with the membrane solution unless it has been contaminated by silane vapour. To check this, its resistance should be measured as the first step of electrode testing.

One should be cautious about the filling solution used in the reference barrel. Leakage from the tip makes a point source of ions at the very site of ion measurement. Just imagine the effect of 3 M KCl leaking out of the reference barrel of a double barrelled Cl^- microelectrode!

Mini-electrodes

Mini ion-sensitive electrodes (tip size $\geq$1-2 mm) are easy and cheap to make and can be very useful. An example is titration of the free Ca^{2+} concentration in calibration solutions (see Appendix, Section 7) and in physiological test solutions containing Cl^- substitutes which bind Ca^{2+}, such as glucuronate or gluconate. The relative amounts of constituents in liquid membrane solutions used in micro- and mini-electrodes are usually different. In addition, the solution has to be bound to a mechanically durable membrane matrix. PVC membranes are widely used, but silicone rubber membranes can also be made. Membrane compositions and instructions for making solvent polymeric membranes can be found in Ammann (1986) and in Fluka's Selectophore® catalogue. In brief, the liquid membrane components (usually a neutral ligand, a lipophilic salt and a plasticizer) are mixed in a small glass vial and dissolved in tetrahydrofuran (THF) together with high molecular weight polyvinylchloride (PVC). When all constituents are fully dissolved (this can often take 1-2 hrs) the solution is poured onto a glass-plate or glass Petri dish and the THF left to evaporate overnight. The membrane that forms upon evaporation of the THF can be stored for months. A mini-electrode is made by cutting out a piece of membrane, placing this over the end of a piece of PVC tubing and sealing the tubing and membrane together using a few drops of THF to dissolve the PVC. The tube is then filled with an appropriate electrolyte solution. In this state the finished electrode can be kept for anything from a week or so to a month depending on the membrane. Thereafter, the membrane may have deteriorated resulting in a decrease in the steepness of the calibration slope and a decrease in the detection limit.

Special applications of ion-sensitive microelectrodes

Volume measurements and related techniques

Extracellular (see e.g. Dietzel *et al.* 1980; Hablitz & Heinemann, 1989) as well as intracellular (Serve *et al.* 1988; Alvarez-Leefmans *et al.* 1992) volume changes can be detected and measured with ion-sensitive microelectrodes. If the extracellular fluid or cytoplasm contains an impermeable ion species, its activity will undergo transient

changes upon transmembrane water movements. This basic idea has been applied to volume measurements using K^+ ion-exchanger microelectrodes (see above), which are far more sensitive to the membrane-impermeable tetramethylammonium ion than to the potassium ion. In addition to this, probe ions of different size have also been used to estimate the maximum pore size of unidentified permeability pathways, e.g. those activated by spreading depression in brain tissue (Nicholson & Kraig, 1981). The recovery of a transient change in the extracellular activity of an impermeable ion reflects volume changes and also its diffusion in the extracellular space. This has been used to examine the diffusional properties of the interstitial space in brain tissue (Nicholson & Phillips, 1981).

CO_2-sensitive microelectrodes

In general, changes in the partial pressure of CO_2 (P_{CO_2}) can result from cellular metabolism and from the operation of the CO_2/HCO_3^- buffer system. It appears that a frequent implicit assumption in physiological work done under *in vitro* conditions is a constancy of P_{CO_2}. However, this view may need re-evaluation. Direct evidence obtained with a recently developed CO_2-sensitive microelectrode clearly indicates that considerable changes in P_{CO_2} take place upon neuronal activity in rat hippocampal slices (≤400 μm) (Voipio & Kaila, 1993) that are kept in an interface-type chamber, which provides a route for gas exchange on both sides of the preparation. As shown in Fig. 7, surprisingly large changes in P_{CO_2} are induced by

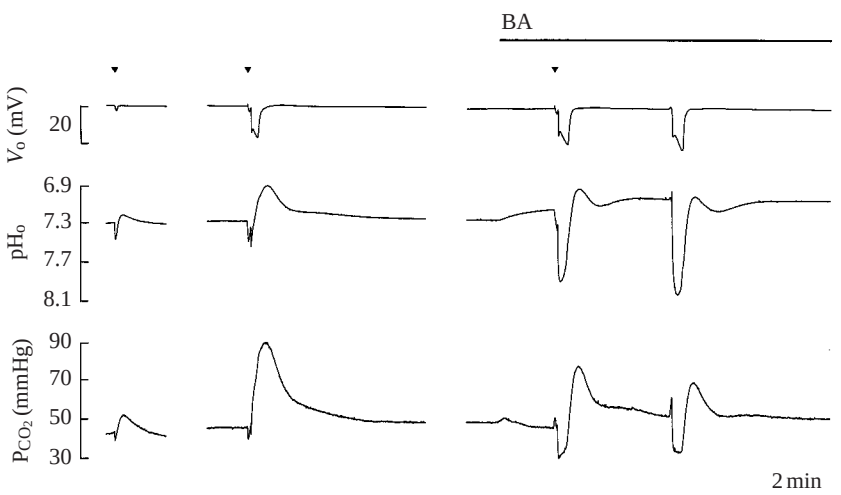


Fig. 7. Simultaneous recordings of changes in the interstitial partial pressure of CO_2 (P_{CO_2}), interstitial pH (pH_o) and extracellular potential (V_o) induced by neuronal activity (left) and by spreading depression (SD) in a rat hippocampal slice (stratum pyramidale of area CA1). Triangles (▼) indicate trains of stimuli applied to the Schaffer collaterals used to initiate SD (SDs are seen as pronounced negative deflections in V_o; one of the SDs occurred spontaneously). In the latter part of the experiment benzolamide (BA, 10 μM) was used to inhibit extracellular carbonic anhydrase activity. (Voipio, Paalasmaa, Taira & Kaila, unpublished observations.)

spreading depression in the same preparation (Voipio, Paalasmaa, Taira, Kaila, unpublished observations).

So far, all CO_2-sensitive electrodes have suffered from a slow response time of minutes rather than seconds. One of the electrode designs makes use of a membrane which is permeable to both H^+ and CO_2 (Niedrach, 1975; Funck *et al.* 1982). The pH affecting the inner surface of such a membrane depends on the external P_{CO_2} if the electrode is filled with an unbuffered solution. Therefore, the electrode output is a function of both pH and P_{CO_2}. In our novel CO_2-microelectrode (Voipio & Kaila, 1993), a Hepes-buffered solution and an unbuffered solution are used in a theta micropipette to obtain a pH-sensitive signal from one barrel and a P_{CO_2}-sensitive signal as the difference of the outputs of the two barrels. The membrane in both barrels consists of the same proton carrier solution. A response time of only a few seconds is obtained upon addition of carbonic anhydrase (CA) to the unbuffered filling solution which speeds up the equilibration of the CO_2 hydration reaction. By using PVC in the membrane solution, it is possible to produce very short (4-7 μm) yet mechanically stable membrane columns (a prerequisite for fast diffusion of CO_2 through the column). A useful membrane is obtained simply by using 14% (w/w) PVC in a H^+ cocktail (Fluka 95291) and by dissolving them in THF (Voipio & Kaila, 1993). The fast response time, stability, insensitivity to lactate and a P_{CO_2} measuring range from the level prevailing in nominally CO_2-free solutions to over 350 mmHg (unpublished observations) make the above microelectrodes suitable for a wide variety of applications.

Quantification of the pH signal that is measured simultaneously with P_{CO_2} requires a reference microelectrode if the potential around the tip is unknown. Clark Electromedical Instruments has started supplying a special triple-barrelled tubing (3GC150SF) which consists of one filamented and two non-filamented capillaries fused together. This material is advantageous in the construction of CO_2-microelectrodes, since the side-by-side positioning of the three pieces of tubing makes it possible, with the use of a microscope, to see the two membrane columns at the tip. The filling solution containing CA should be made daily from its three components (4.0 mg CA (C-7500, Sigma) + 1.0 ml 150 mM NaCl + 23.6 μl 0.1 N NaOH) and must not be shaken. The triple-barrelled micropipettes should be silanized before bevelling. Plastic tubing can be inserted into the barrels and sealed e.g. by modelling wax to obtain barrel-specific pressure/suction for filling.

Limitations imposed by electrical properties

The high resistance (10^{10}-10^{11} Ω) of ion-sensitive microelectrodes combined with a total capacitance of several picofarads means that their electrical time constant (τ) is generally between 0.1-1 sec (τ=RC). This results in a significant low-pass filtering of both electrical and ion signals if no attempt is made to compensate for the filtering. However, electrical compensation can greatly reduce the effect of long time constants. This matter is dealt with in detail in Section 4, *Differential amplifier and filtering.*

4. Equipment design and construction

Microelectrode dry bevelling equipment

As discussed above, a simple method for dry bevelling of micropipettes (Kaila & Voipio, 1985) has proven advantageous in the construction of ion-sensitive microelectrodes. The equipment consists of a rotating unit with a commercially available bevelling film, a detector coupled to the shank of a micropipette and a low-noise audio amplifier.

A rotating plate, which is free of vertical movements, can be made using two optically flat glass plates (Brown & Flaming, 1975). A piece of commercially available lapping film with 0.3 μm particle size (ILF-film 3M261X A/O 0.3 Mic; contact local subsidiary of 3M, or 3M, St. Paul, MN 55144-1000, USA) is used on top of the rotating unit, which is mounted on a rigid plate together with a micromanipulator. We cut the lapping film to a diameter of 8 cm and use a speed of 50-80 rotations per minute. A simple DC motor is mounted elastically to the system, in order to avoid mechanical coupling of its vibrations.

A cheap piezocrystal record-player pick-up can be used as the detector (moving magnet or moving coil pick-ups give a much weaker signal), but better sensitivity is obtained with a 70 mm long piezocrystal bimorph element (Philips order code 4322 0200823 is ideal, but the minimum order is 600 pieces!). A low-noise audio amplifier is easily built from standard components. Fig. 8 shows the circuit diagram of a simple amplifier, which can be used with headphones.

Electrometer amplifier design

Ion-sensitive microelectrodes have an extremely high resistance so they must be coupled to electrometer amplifiers that are designed to cause as small a load as

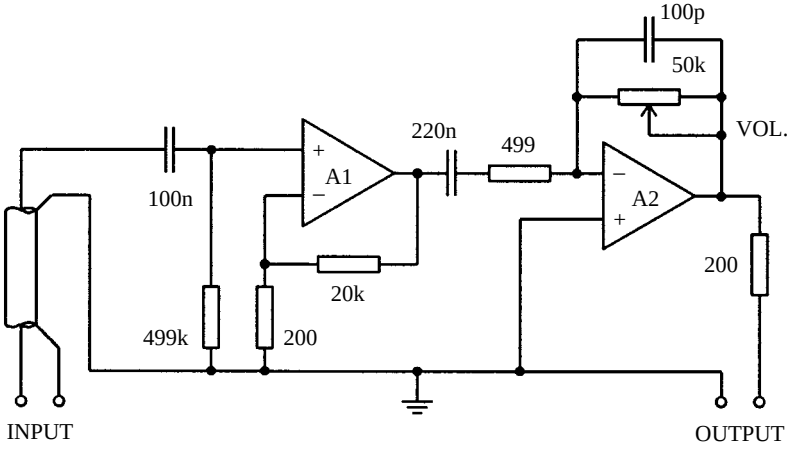


Fig. 8. Circuit diagram of a simple audio amplifier for headphones and a piezocrystal detector used in dry-bevelling of micropipettes. A1 is a low-noise op. amp. (e.g. LM 607), A2 is a general purpose op. amp. (e.g. LF 356). The circuit may be powered by two 9 V batteries or from a conventional ±15 VDC source.

possible to the signal source. The input impedance (Z_{in}) of an amplifier is usually expressed as the input resistance (R_{in}) from input to ground paralleled by the input capacitance (C_{in}). The effect of R_{in} is to short circuit the liquid membrane column of an ion-sensitive microelectrode. The input resistance of an amplifier is defined as the ratio of a given change in input voltage to the corresponding change in input current at steady state. Due to this relationship being often non-linear in electrometer circuits and the input current seldom vanishing with zero input voltage, the concept of input resistance is not very meaningful in this context. Instead, the performance of an electrometer amplifier is best described if an upper limit is given for the input bias current (I_b) throughout the whole input voltage range.

A common practice in electrophysiological laboratories is to build electrometer amplifiers instead of buying them. An operational amplifier with an I_b as low as possible is used as the input stage. Since the Ag:AgCl wire is frequently touched with fingers, the input must also tolerate some static discharge.

The best operational amplifier for use with ion-sensitive microelectrodes is no longer available. It is the ultra-low bias current, varactor-bridge operational amplifier AD311J from Analog Devices. In our amplifiers, I_b has stayed at about 6 fA (6×10^{-15}A) for years. However, other components that are both cheaper and smaller and have a reasonable I_b are available. The rather old AD515L (AD515AL) is available from both Analog Devices and Burr Brown and has a maximum input bias current of 75 fA. Its input is reliably protected by a series resistor (1 MΩ is probably sufficient but we have used resistors up to 100 MΩ). The AD549L (Analog Devices) is its improved version and has a typical I_b of 40 fA (60 fA max.). An OPA128LM (40 fA typ., 75 fA max., Burr Brown) may also be used. Other components exist, but do not try to combine an unprotected operational amplifier with an input protection circuit including ultra low-leakage diodes (e.g. FDH300 or FDH333) since this will result in an increase in I_b.

A small capacitor is used to couple an adjustable positive feedback signal to the amplifier input. This compensates for the effect of stray capacitances, which would otherwise reduce the input impedance to all AC signals (capacitance compensation, ‘negative capacitance’). If a linear ramp is added to the feedback signal, a constant current flows to the amplifier input ($I = C \cdot \delta V/\delta t$) making it possible to measure the electrode resistance.

A poor lay-out can totally ruin the performance of an ambitiously designed electrometer circuit. Optimal performance cannot be achieved by using the often recommended guard loops around input lines on a printed circuit board. Instead, all connections of the input signal to the circuit board should be avoided and, if necessary, teflon stand-offs should be used for input wiring. Most BNC connectors use pure teflon as the insulator and are suitable as the input connector. Ordinary capacitors are too leaky to be used in coupling the capacitance compensation signal to amplifier input. Since the required capacitance value is only a few pF, it is preferable to construct a teflon or air insulated capacitor for this purpose.

The easiest way of measuring the I_b of an amplifier is to connect its input to ground

via a low leakage capacitor. After instantaneously grounding the input, I_b starts to charge the capacitor causing a drift on the recorded signal, and I_b is obtained as $I_b = C \cdot \delta V/\delta t$. Fig. 9 shows a circuit diagram of an electrometer amplifier that allows capacitance compensation and measurement of the electrode resistance. For simplicity the circuit is based on standard operational amplifiers and a separate bias current compensation circuit is not included. Indeed, bias current with the AD 549L is low enough not to require compensation.

Differential amplifier and filtering

The gains of an electrometer amplifier, a reference electrode amplifier and a differential amplifier are easily adjusted to show no change at the differential output upon a common mode input signal (a test signal connected simultaneously to both inputs). However, the different time constants of ion-sensitive and conventional microelectrodes requires that the reference signal is slowed down

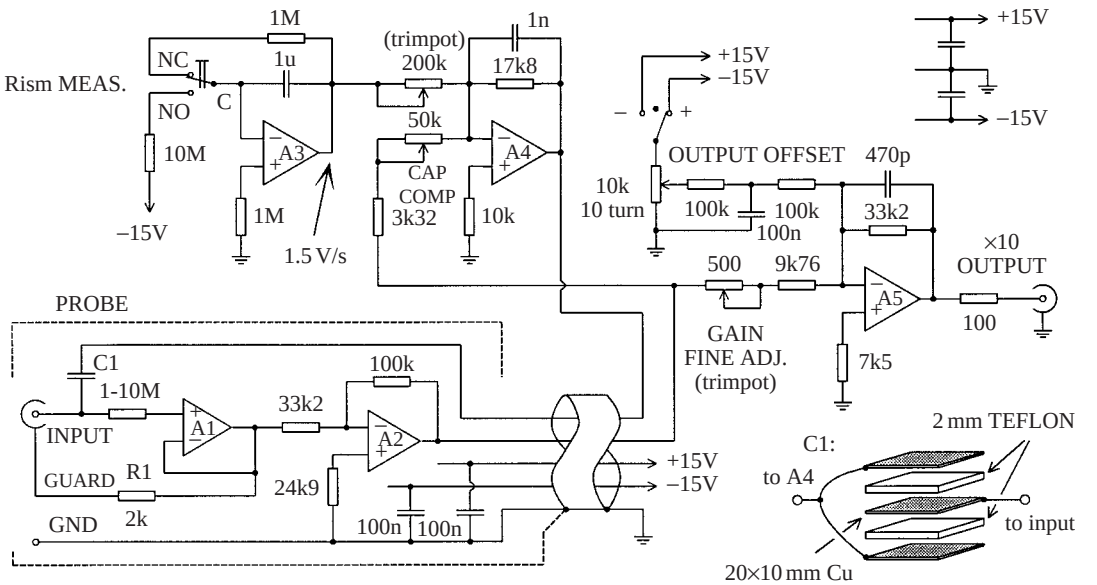


Fig. 9. Circuit diagram of an electrometer amplifier designed for use with ion-sensitive microelectrodes. The amplifier provides capacitance compensation, electrode resisitance (Rism) measurement and an output with offset and a gain of 10. The active guard signal can be obtained via R1 as shown, but it is advisable to replace this resistor by a voltage follower. The feedback capacitor C1 should be constructed from thin copper and 2 mm teflon plates sandwiched as shown in the inset. The 200 kΩ trimpot is adjusted to obtain a constant current of 1 pA through C1 for a few seconds when pressing the Rism MEAS. pushbutton. For the adjustment, note that a current of 1 pA induces a voltage change of 1 mV/1 GΩ (10 mV at output) when flowing through an electrode or a test resistor, or a linear ramp of 1 $mV.s^{-1}$ (10 $mV.s^{-1}$ at output) if the input is connected to ground with a 1 nF capacitor and briefly short-circuited just before the measurement. A1 is preferably an AD 549L (Analog Devices), but an AD 515L, AD 515AL or an OPA 128LM (Burr Brown) may also be used. A2-A5 e.g. LF 356 (A2 must tolerate the capacitive load of the probe cable), resistors up to 1 MΩ with 1% tolerance.

before subtraction to mimic the frequency response of the ion electrode. A simple adjustable RC filter gives a reasonable approximation of the distributed resistance and stray capacitances of an ion-sensitive microelectrode. This kind of adjustment is important when recording from excitable cells and in voltage clamp experiments, where step changes in membrane potential are otherwise seen as large transient deflections on the differential ion signal. Suggested circuitry is shown in Fig. 10A.

The correct procedure to minimise transient deflections on the differential signal due to rapid membrane potential changes includes the following steps made in this order: (1) capacitance compensation adjustment of the ion-sensitive electrode, (2) slowing down of the reference signal and (3) low pass filtering of the differential signal. Fig. 10B shows the effect of these steps performed on an equivalent circuit mimicking an intracellular recording with step changes in membrane potential at a constant ion concentration.

Noise reduction

In practical work with ion-sensitive microelectrodes, capacitive coupling is the main source of noise. Stray capacitances from surrounding structures to the electrode and the Ag:AgCl wire mediate small currents, which flow through the high resistance of the membrane column, thereby giving rise to a voltage signal seen as noise in the recording. Capacitive current is usually given by $I = C \cdot \delta V/\delta t$ but it includes the assumption of a constant C. The general equation for capacitive current is obtained from $Q = CV$ and it is:

$$I = \left(C \cdot \frac{\delta V}{\delta t} \right) + \left(V \cdot \frac{\delta C}{\delta t} \right) \tag{12}$$

Therefore, capacitive coupling to ion-sensitive microelectrodes is minimised if: (1) cables etc. with AC (mains) signals are removed (to minimise $\delta V/\delta t$), (2) the total surface area of the wiring from the electrode to the amplifier input is as small as possible (to minimise C), (3) solutions have a steady surface level (pay particular attention to experimental bath design if the superfusate is to be pre-bubbled and if necessary, shield the waste line from the bath by a grounded metal tube and fix all wires and superfusion tubing so that they cannot move (to minimise $\delta C/\delta t$)), and (4) the lab has a proper antistatic floor coating (to minimise V associated with static charges on your clothes and skin). (Static charges can be a real problem if the relative humidity of air is very low. A portable humidifier helps in such cases).

The experiments should be performed within a Faraday cage which intercepts stray capacitances to external noise sources. The headstages of all microelectrode amplifiers should be positioned in the vicinity of the electrodes and ion-sensitive microelectrodes should be connected to their amplifiers with thin teflon-coated or non-insulated wires. (Some insulating materials generate a slowly declining noise current upon bending the cable.) Shielded cables even with the shield driven by an

active guard signal should not be used to connect ion-sensitive microelectrodes, because the insulation resistance between the signal wire and the shield is not necessarily high enough. Direct capacitive coupling from a current-passing microelectrode can be reduced by wrapping a small piece of aluminium foil round it and grounding the foil.

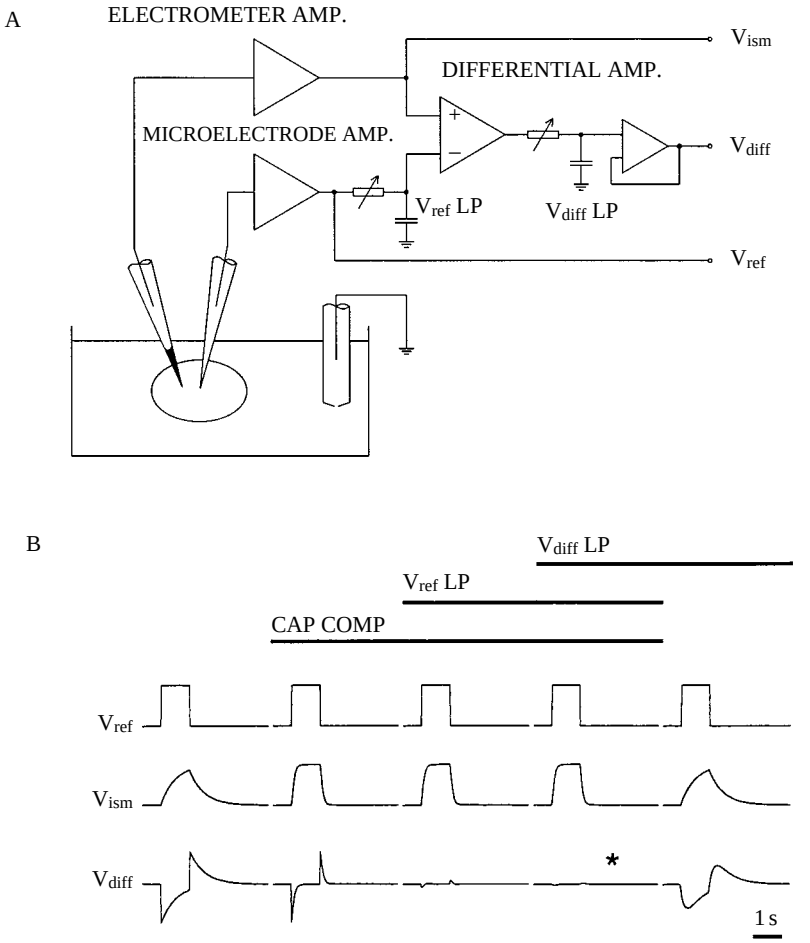


Fig. 10. (A) Circuitry for intracellular ion measurements. V_{diff}LP and V_{ref}LP indicate adjustable low-pass filters for the subtracted true ion signal (V_{diff}) and for the reference electrode signal (V_{ref}) respectively. The ion-sensitive microelectrode signal is V_{ism}. (B) Reduction of transient artifacts on the ion signal V_{diff}, caused by rapid changes in membrane potential in an intracellular recording, can be obtained by sequential adjustment of the capacitance compensation (CAP COMP) and of the two low-pass filters shown in A as described in the text (optimal response indicated by *). The measurements were made using an equivalent circuit with a 100 GΩ resistor as an ion-sensitive microelectrode and a 10 MΩ resistor as the reference electrode. Square pulses were connected as a common mode signal to both resistors to mimic step changes in membrane potential taking place at a constant intracellular ion activity. Note that low-pass filtering of the differential signal alone gives a poor result, as is evident from the trace on the right.

5. Practical electrode calibration

Empirical methods

There are various methods for calibrating ion-sensitive microelectrodes and the easiest and most practical from a biologists point of view are empirical techniques where no effort is made to ascertain the selectivity coefficients and there is no absolute requirement for knowledge of the activity coefficient (see Section 2, *Ion activity*). In addition to the extracellular solution which is used as a reference point one simply makes up solutions that are representative of the intracellular environment containing a fixed amount of the major interfering ion(s) likely to be encountered intracellularly and varying amounts of the primary ion. It is important to maintain constant ionic strength (so that γ is the same, for the reasons outlined in Section 2, *Ion activity*) and one way of achieving this is to make so-called "reciprocal dilutions" with a substituting ion of the same valency which does not interfere with the sensor. Calibration of the electrode should also be undertaken at the same temperature at which the experiment is performed because, as equation 9 shows, the slope obtained is dependent upon temperature. The reference point for calibration is obtained in the physiological solution which is used as a perfusate during impalements. The intracellular measurements obtained after such a calibration procedure are a measure of the intracellular free ion concentration (assuming γ is the same in the cytoplasm as in the calibration solutions). This method should be used with care for its approach assumes that the intracellular activity of the interfering ion does not change during the experiment. If it does, one has to find out by how much the interfering ion changes and calibrate using best and worst case solutions. The empirical approach to electrode calibration is outlined in Fig. 11.

Another way to calibrate electrodes is by a more formal approach involving calculation of the selectivity coefficient, K_{ij}. While this technique is certainly more rigorous it is probably of less benefit to the biologist than the physical chemist. There are two main reasons for this which stem from problems in the determination of K_{ij}. Firstly, the value of K_{ij} differs depending upon which method is used (see below); secondly, K_{ij} varies with the size of the ion-sensitive microelectrode tip (see e.g. Bers & Ellis, 1982) so it cannot be assumed to be constant from one electrode to the next.

Separate solution method

The potentials (E) are recorded from an electrode in single electrolyte solutions of varying activity of the primary ion (a_i) and are compared with potentials recorded in single electrolyte solutions of varying activity of the interfering ion (a_j). The selectivity coefficient is then calculated from the following equation:

$$K_{ij} = \frac{a_i}{a_j^{z_i/z_j}} \times 10^{(E_j - E_i)/m} \qquad (13)$$

where m is the slope of the electrode (negative for anions).

Fixed interference method

With this method, potentials are recorded from an electrode in solutions of varying activity of the primary ion (a_i) and which also contain a fixed activity of the interfering ion (a_j). The selectivity coefficient is then obtained at the intercept of the asymptotes of the two linear portions of the calibration curve (i.e. at the detection limit):

$$K_{ij} = \frac{a_i}{a_j^{z_i/z_j}} \tag{14}$$

The empirical approach to electrode calibration is to be recommended but as we remarked earlier: however you calibrate, state how you did it!

6. Analysis of results

Ion fluxes

The net flux of an ion across the cell membrane can often be deduced by measuring the change in the intracellular activity of this ion over time, i.e. $\delta a_i/\delta t$. The net flux (J) is then defined as:

$$J = \frac{V{:}A}{\gamma_i} \cdot \left(-\frac{\delta a_i}{\delta t} \right) \tag{15}$$

where γ_i is the intracellular activity coefficient for the ion, V:A is the volume/surface area ratio of the cell. Flux is then a change in amount per area and time, commonly expressed as $mmol.min^{-1}.cm^{-2}$, and an outward flux has a positive value. Usually $\delta a_i/\delta t$ is taken as the instantaneous rate of change of a_i, which in a recording with an ion-selective electrode corresponds to the slope of the recorded trace at a given instant of time. As discussed above (Section 2, *Ion activity*) γ_i is usually assigned the value of the extracellular activity coefficient as a value for γ_i can normally not be obtained. The volume and/or surface area of a cell is often not known and so flux is expressed as a change in concentration per time ($mmol.l^{-1}.min^{-1}$), i.e.:

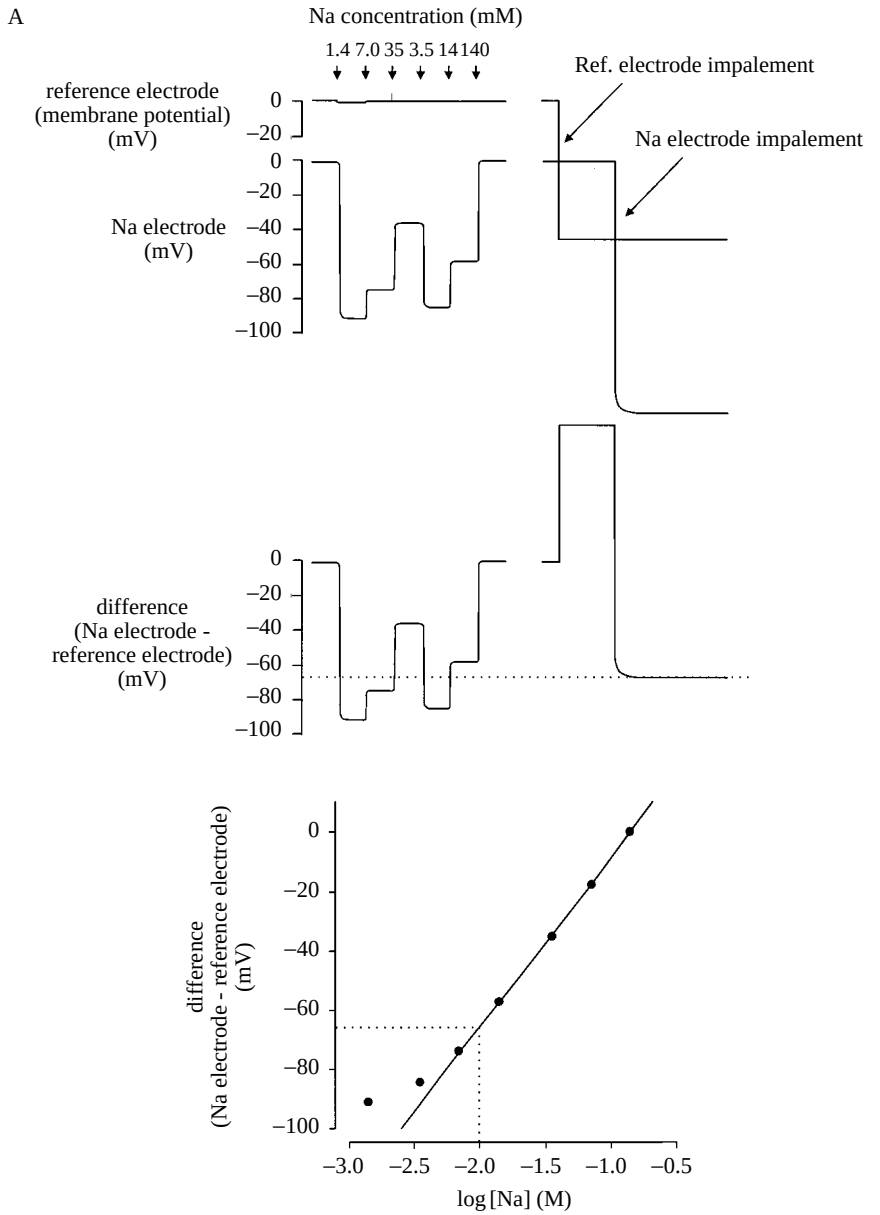
$$J = \frac{1}{\gamma_i} \cdot \left(-\frac{\delta a_i}{\delta t} \right) \tag{16}$$

A measured change in the intracellular ion activity can be converted to net flux *only* if it is known to be solely the result of transmembrane movement of the relevant ion and if the amount of intracellular buffering for this ion is known. It should be stressed that a change in the a_i can also result from, for example, a change in cell volume, or from movements of the ion into or out of subcellular compartments, like mitochondria or the nuclei.

In some cases it is possible to detect a transmembrane movement of an ion by means of an ion-sensitive electrode measuring a change in ion activity in an extracellular compartment (e.g. in brain slices) or at the outer surface of a cell

membrane (see Fig. 5, Section 3). This type of measurement can be used to verify transmembrane movements of H^+, Ca^{2+} and K^+ as the transmembrane gradients and the physiological extracellular activities of these ions favour the detection of such movements with an ion-selective electrode. Convincing evidence for transmembrane fluxes of a given ion using ion-sensitive electrodes is often provided by the coupling of an intracellular and a surface ion measurement. An example of a simultaneous measurement of intracellular and extracellular pH is seen in Fig. 5.

Flux measurements made with ion-selective microelectrodes can be used for



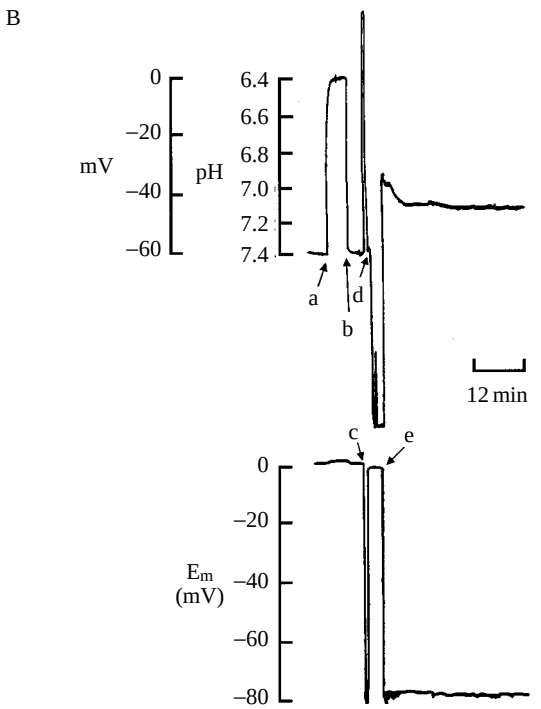


Fig. 11. (A) Representation of a typical calibration procedure for a Na^+-sensitive microelectrode. At the left side of the figure the reference electrode and the Na^+-sensitive microelectrode were exposed to a physiological saline including 140 mM NaCl and 4mM KCl and five other solutions containing various amounts of NaCl and KCl at a total concentration of 144 mM in addition to 1 mM $MgCl_2$, 10 mM glucose, 5 mM Pipes, 1 mM EGTA + 0.3 mM $CaCl_2$ to give a free [Ca^{2+}] of 0.1 μM, pH, 7.20. In this way, calibration for intracellular levels of Na^+ was carried out under conditions of constant ionic strength, with a high background of K^+ (the major interfering ion expected in the cytoplasm) and with values of pH and free [Ca^{2+}] also near those likely encountered in the cytoplasm. The calibration curve on the right was constructed from the values obtained from the difference signal. The solid line drawn through the points has a slope of 57 mV. Notice that at low concentrations of Na^+, the response of the electrode deviates from linearity as interference from K^+ begins to predominate. After the calibration was completed, the preparation was taken to the bath and superfused with a physiological saline. The reference electrode was pushed into the cell giving a value of –81mV for membrane potential. This produced an equal but opposite movement on the difference signal. Some minutes later, the Na^+-sensitive electrode was pushed into the cell. This produced a large deviation on the trace due to the electrode measuring (1) a much lower [Na^+] than outside the cell and (2) the membrane potential. The membrane potential is subtracted from the combined signal, producing, on the difference trace, a value of –64 mV which corresponds to an intracellular Na^+ concentration of 10.7 mM (dotted line). If $\gamma_{Na} = 0.75$ then intracellular Na^+ activity is about 8 mM. (B) Electrode calibration and subsequent impalement of a sheep cardiac Purkinje fibre with a pH-sensitive microelectrode. At point a, the solution bathing the tips of the reference (E_m) and pH-sensitive microelectrode was changed from a pH of 7.4 to a pH of 6.4. This 10-fold change in H^+ resulted in a 60 mV change in voltage from the pH electrode. At point b the solution was changed back to a pH of 7.4. Next, an impalement of the cell by the reference electrode was made (point c) and because the pH trace already has the reference potential subtracted (i.e. it is the difference) it moved an equal amount in the opposite direction. This impalement was soon lost while pushing in the pH electrode (point d). Finally, the reference electrode was put back into the cell (point e) which allows the intracellular pH (difference) to be read directly from the calibration. This gives an intracellular pH of about 7.15 and a membrane potential (E_m) of about –80 mV.

dissecting the ionic components of a current carried by more than one ion. An example of this is shown in Fig. 12. A crayfish muscle fibre is penetrated with conventional microelectrodes for measuring membrane voltage and injecting current, as well as with a Cl^--sensitive and a H^+-sensitive microelectrode. In a solution buffered with CO_2 and HCO_3^- the cell is clamped at different voltages and short pulses of GABA (γ-aminobutyric acid) are applied. The recording shows that the GABA-induced changes in intracellular chloride are dependent on the membrane voltage. Their direction reverses, by definition, at the chloride reversal potential, which is approximately at the resting membrane potential (RP). The current evoked by GABA is, however, not solely carried by chloride, since it reverses at a potential more positive to the chloride reversal potential. The other component of the GABA-induced current is now known to be HCO_3^-, to which GABA channels are also permeable. In the absence of HCO_3^- the GABA-induced current reverses at the chloride reversal potential.

Buffering power

The capability of a solution to "resist" changes in the activity of a given ion upon a

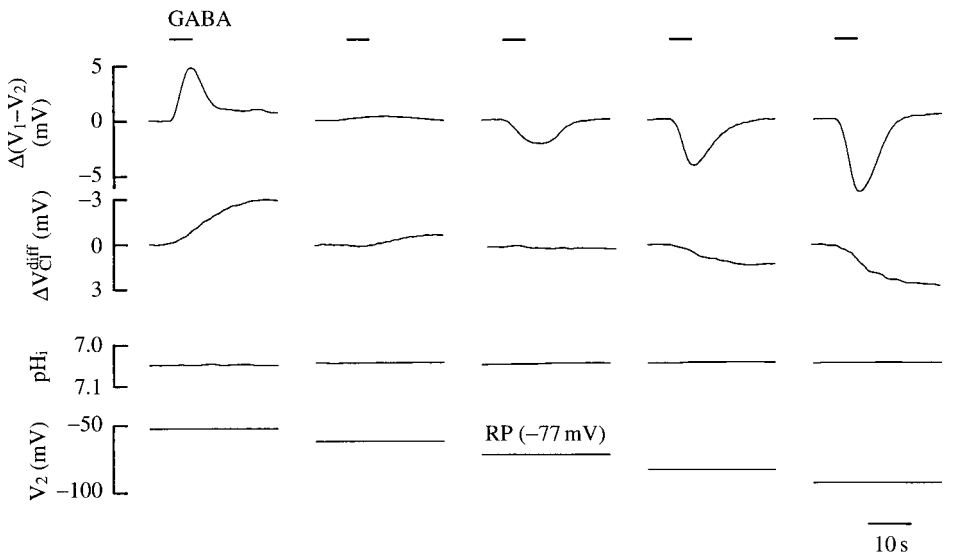


Fig. 12. Simultaneous measurement of the GABA-current and chloride reversal potentials in a crayfish muscle fibre. The muscle fibre is penetrated with five electrodes: three conventional ones for voltage-clamping in a three electrode voltage-clamp configuration, and two ion-sensitive electrodes for measuring changes in the intracellular chloride level (ΔV_{Cl}^{diff}) and the intracellular pH. $\Delta(V_1\text{-}V_2)$ is the difference in voltage seen by the electrode placed in the middle of the fibre (V_1) and that placed halfway between the middle and end (V_2). $\Delta(V_1\text{-}V_2)$ is proportional to the membrane current at the site of V_2. pH_i is measured to show that no changes in intracellular pH occur during the short GABA applications, as changes in pH_i would change the driving force for HCO_3^-. The chloride reversal potential is approximately at the resting membrane potential (RP; middle traces) where the intracellular Cl^- does not change upon the GABA application. The GABA-induced current (carried by both Cl^- and HCO_3^-) reverses at a more positive holding potential, where GABA induces a Cl^- influx. (Reproduced with permission from Kaila *et al.*1989).

change in its concentration is called the buffering power of this solution for the ion. As stated above, a measured change in the ion activity cannot be converted to a flux unless the extent of buffering of this ion is known. Usually cells have appreciable buffering of H^+ and Ca^{2+} but other ions can be considered to be unbuffered. For this reason changes in K^+, Na^+ and Cl^- are usually directly converted to transmembrane fluxes, but in doing so one should bear in mind the possibility of cellular volume changes (see below).

Buffering of H^+

The H^+ buffering power (β) is defined as:

$$\beta = \frac{\Delta A}{-\Delta \mathrm{pH}} \left(= \frac{\Delta B}{\Delta \mathrm{pH}} \right) \tag{17}$$

where ΔA and ΔB are the changes in acid or base concentration respectively, which cause a change in pH (ΔpH). Intracellular H^+ buffering is usually divided into two categories: the intrinsic buffering power (β_i) which is attributable to intracellular proteins and the "CO_2 buffering power" (β_{CO_2}) which is due to the presence of CO_2 and bicarbonate (HCO_3^-). β_i is commonly between 10 and 50 mmol.l^{-1} and is, in many cells, fairly constant within the physiological intracellular pH range. β_{CO_2} is the result of the familiar equilibrium between CO_2 and HCO_3^-:

$$CO_2 + H_2O \rightleftharpoons H_2CO_3 \rightleftharpoons H^+ + HCO_3^- \tag{18}$$

In an open buffer system, i.e. when CO_2 is held constant and the above reactions are fast (see below), it can be shown that $\beta_{CO_2} = 2.3[HCO_3^-]$ and hence β_{CO_2} is steeply dependent on pH. All buffering is additive, i.e. an increase in β_{CO_2}, for example, will add linearly to the total buffering power of the cell.

Equation 15 can now be rewritten for proton fluxes to give:

$$J^{H^+} = (\beta_i + \beta_{CO_2}) \cdot (V{:}A) \cdot \frac{\Delta \mathrm{pH}}{\Delta t} \tag{19}$$

As pH is a logarithmic function, the above equation can also be rewritten replacing ΔpH with the voltage change recorded with a H^+-sensitive electrode (ΔV_{pH_i}):

$$J^{H^+} = (\beta_i + \beta_{CO_2}) \cdot (V{:}A) \cdot \left(-\frac{\Delta V_{pH_i}}{m \Delta t} \right) \tag{20}$$

where m is the slope of the electrode. Again the $V{:}A$ ratio is often omitted and the flux given as mmol.l^{-1}.min^{-1}.

One should note however, that rapid CO_2 buffering requires catalysis of the reversible hydration reaction of CO_2 to H_2CO_3, since this reaction is fairly slow in the absence of a catalyst (the half time is about 10-30 s at 21-37°C). In biological systems the catalyst for the reaction is the enzyme carbonic anhydrase (CA). Most cells

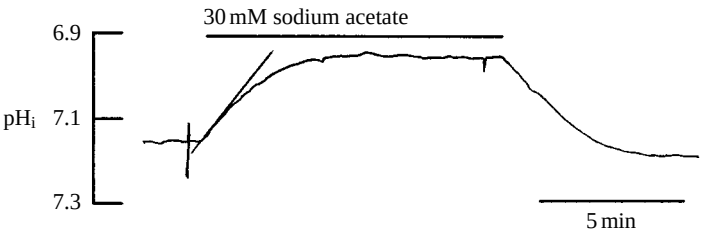


Fig. 13. Measurement of the intrinsic buffering power (β_i) of a crayfish muscle fibre. The intracellular pH (pH_i) is recorded with a H^+-selective microelectrode while the fibre is subjected to a solution containing 30 mM sodium acetate. The measurement gives a value for β_i of 53.2 mmol.l^{-1}. A line is drawn to indicate the initial slope of the change in pH_i ($\Delta pH_i/\Delta t$) used for calculating the initial acid flux into the cell (see text). (From an experiment made by the authors and a group of students in 1989 at the Plymouth workshop).

possess intracellular CA activity and some cells even have membrane-bound, extracellularly active CA (e.g. Kaila et al., 1990, 1992). In the absence of rapid catalysis, the CO_2 buffering becomes time-dependent and pH measurements may be more difficult to interpret quantitatively.

β_i can be estimated by introducing an acid or base load of known magnitude to the cell and measuring the evoked changes in intracellular pH in the absence of pH regulation. An example of the estimation of β_i of a crayfish muscle fibre using a membrane permeant weak acid is given in Fig. 13.

A crayfish muscle fibre is penetrated with two microelectrodes, a conventional electrode for measuring the membrane voltage and a H^+-sensitive microelectrode. In the crayfish muscle fibre the regulation of intracellular pH is almost totally dependent on HCO_3^-. When the cell is subjected to 30 mM of the Na^+-salt of acetic acid, sodium acetate (CH_3COONa), in a Hepes-buffered solution (pH 7.40) in the absence of CO_2 and HCO_3^-, the intracellular pH decreases with an exponential time course until it reaches a new, more acidic steady-state value. This is because the electrically neutral acetic acid is capable of penetrating the cell membrane causing an intracellular acidification. The acidification proceeds with an exponential time course until acetic acid has equilibrated across the membrane. The dissociation constant for an acid is defined as:

$$K_a = \frac{[H^+]\,[A^-]}{HA} \tag{21}$$

where HA is the non-dissociated acid and A^- is the acid anion. Assuming that the extracellular (K_a^o) and intracellular (K_a^i) dissociation constants are equal, one can write:

$$K_a^o = K_a^i = \frac{[H^+]_o\,[A^-]_o}{[HA]_o} = \frac{[H^+]_i\,[A^-]_i}{[HA]_i} \tag{22}$$

When *HA* is in equilibrium across the membrane, i.e. $[HA]_o = [HA]_i$ the equation can be solved for $[A^-]_i$:

$$\begin{aligned} [H^+]_o\,[A^-]_o &= [H^+]_i\,[A^-]_i \Leftrightarrow \\ [A^-]_i &= \frac{[H^+]_o}{[H^+]_i} \cdot [A^-]_o \Leftrightarrow \\ \log[A^-]_i &= \log[H^+]_o - \log[H^+]_i + \log[A^-]_o \Leftrightarrow \\ [A^-]_i &= 10^{pH_i - pH_o} \cdot [A^-]_o \end{aligned} \tag{23}$$

Since acetic acid is almost completely dissociated at physiological pH, $[A^-]_o$ can be considered equal to the total amount of acetate added, i.e. 30 mM. In this cell the intracellular pH change (ΔpH) is –0.2 pH units (from pH 7.15 to pH 6.95) and at this pH_i calculation of $[A^-]_i$ from equation 23 gives a value of 10.64 mM, which is equal to the intracellular acid load ($[H^+]_i$, or ΔA in equation 17). Fitting the values for ΔpH and $[H^+]_i$ to equation 17 gives β_i a value of 53.2 mmol.l^{-1}. The initial acid flux into the cell, J^{H^+}, can now be calculated using the measured value of β_i and the initial rate of change in pH_i ($\Delta pH_i/\Delta t = -0.08.min^{-1}$). Fitting these values into equation 19 gives a value of –4.26 mmol.l^{-1}.min^{-1}. The remarkable H^+ buffering power is seen in that the above measured changes in H^+ activity are in the submicromolar scale, whereas the acid load is expressed in mmol.l^{-1}!

In many cell types pH regulation involves several mechanisms and measurements of β_i with manipulations like the above may give incorrect results because of an attenuation of the evoked acidosis by the pH regulatory mechanism(s). It may be possible in some cells to use a weak base instead of a weak acid to circumvent the problem of pH regulation. However the cells may be equipped with a mechanism for pH regulation capable of intake of acid equivalents. An alternative possibility is to use a pharmacological inhibitor of the pH regulating mechanism(s).

Buffering of Ca^{2+}

Intracellular calcium ions are buffered in several ways: (1) Like H^+, Ca^{2+} can bind to intracellular proteins, (2) Ca^{2+} can be sequestered into endoplasmic or sarcoplasmic reticulum and cellular organelles, such as mitochondria, and (3) Ca^{2+} can bind to sites on the intracellular plasmalemma surface. Some of the buffering sites for Ca^{2+} may be shared with those for H^+ and so changes in intracellular pH may affect the levels of free Ca^{2+} concentration (often given as the logarithmic term pCa, akin to pH) and *vice versa*. The release and uptake of Ca^{2+} from and into internal stores, as well as the competing action of H^+ for Ca^{2+} buffering sites makes it difficult to assess the cellular Ca^{2+} buffering power.

The changes in intracellular pCa are often rapid and cannot be detected accurately with ion-sensitive electrodes. The response times of a Ca^{2+}-sensitive microelectrodes are, even with proper capacitance compensation (see Section 4, *Electrometer amplifier design* and *Differential amplifier and filtering*), in the order of a second. Ca^{2+}-sensitive electrodes can be used for the detection of *slow* changes in pCa (i.e.

those occurring over time courses from seconds to minutes). However, in the interpretation of Ca^{2+}-electrode measurements one should bear in mind that undetectable fast transients in pCa may be involved in the development of the slower changes, e.g. a rapid undetected increase in pCa may trigger an enhancement in intracellular Ca^{2+} storage and thus result in a decrease in pCa. In such a case the experimental manipulation would cause a biphasic change in pCa but only the slow decrease would be apparent to the experimenter.

Changes in cellular volume

A change in cellular volume will obviously result in a change in the activities of all intracellular ions, if the volume change is not parallelled with ion movements. On the other hand, fluxes that are not balanced with osmotically equivalent counterfluxes will change the cellular osmolarity and result in changes in cell volume. This may introduce errors in the quantitative analysis of ion-selective electrode recordings, unless the volume change is taken into account during the analysis.

The overall changes in intracellular ion activities produced by cotransport mechanisms, e.g. the K^+-Cl^--cotransport or the Na^+-K^+-Cl^- cotransport, may be complex, since water movements are perhaps more pronounced than those related to countertransport.

Fig. 14 depicts an example of the consequences of the activation of a Na^+-K^+-Cl^- cotransport. The stochiometry of this transport is assumed here to be 1 Na^+:1 K^+:2 Cl^-. In this example 5 mM of Na^+ and K^+ together with 10 mM of Cl^- are transported into the cell. Assuming no other ion movements, the end result of this transport is an increase of about 6.5% in the cell volume. The final ion activities are, due to this swelling, decreased by about 6% from those expected in the absence of water movements, resulting in the activities shown in the figure. As can be seen, intracellular Na^+ activity increases by 88% and intracellular Cl^- by 90% of the values expected with no swelling. What is perhaps more difficult to comprehend at first glance, is that K^+ activity *decreases* from its initial value. This is because the final activities of all solutes are decreased in the same proportion (6%) resulting in a larger absolute decrease in K^+ activity than in Na^+ or Cl^- activity.

In the flux equations given above the cellular volume to surface area ratio (V:A) is assumed to be constant. Obviously a change in this ratio will cause some error in the estimate of the flux, unless the flux is taken as the instantaneous change in ion activity and the V:A of the cell at this point in time is known.

At first glance a countertransporter mediating equal fluxes of ions in and out of the cell is not expected to induce volume changes. However, the activation of an equimolar countertransport may change cell volume if one of the transported ions is buffered. The activation of Na^+/H^+ exchange or Cl^-/HCO_3^- exchange can lead to changes in cell volume, since H^+ is buffered and changes in pH therefore do not contribute equally to the cellular osmolarity. HCO_3^- on the other hand may, together with H^+ (see equation 18), exit the cell as CO_2 and thus cause a decrease in intracellular osmolarity.

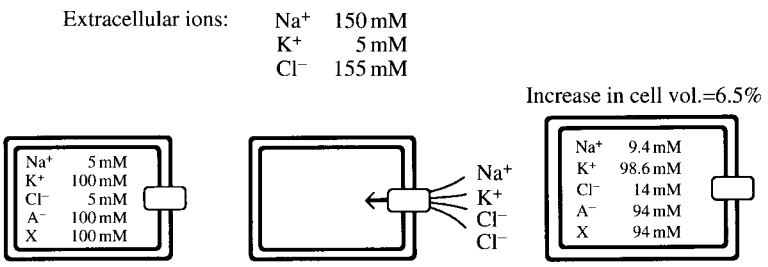


Fig. 14. Effect of Na^+:K^+:Cl^- cotransport on cell volume and ion activities. Activation of a Na^+:K^+:Cl^- cotransport results in an increase of the cell volume, which attenuates the increase in intracellular ions. For further explanation, see text. (X, intracellular impermeant solutes; A^-, intracellular impermeant anions).

7. Appendix: Ca^{2+}-sensitive electrodes and their use in measuring the affinity and purity of Ca buffers

One important use of Ca^{2+}-sensitive microelectrodes which is often overlooked is in the preparation of solutions of known Ca^{2+} concentration. These solutions can be used subsequently to, for example, calibrate Ca^{2+}-sensitive electrodes, fluorescent indicators or bathe skinned muscle preparations. Recall that the association constant (K_a) describes how well a calcium buffer (e.g. EGTA, NTA) binds Ca^{2+}:

$$K_a = \frac{[Ca]_b}{[Ca]_f \cdot [Ca\ buffer]_f} \tag{24}$$

where the subscripts b and f denote bound and free concentrations respectively. One of the difficulties in making up solutions of varying Ca^{2+} concentration is that association constants for calcium buffers are sensitive to ionic strength, temperature and can be particularly sensitive to pH (Bers & MacLeod, 1988; Harrison & Bers, 1987, 1989). Thus if any of these variables change then the free [Ca^{2+}] in solution may be quite different from the expected value. The effect of pH on K_a should not be underestimated. It can be easy to make an error of 0.1 pH units particularly if the electrode is poorly calibrated and it is very worrying that many electrodes incorporating a ceramic porous plug can generate large liquid junction potentials when the ionic strength of the solution is altered which, in turn, can cause the pH measurement to be in error by an average of 0.2 pH units (Illingworth, 1981). Our advice when making pH measurements is to use a separate reference electrode with a flowing junction, calibrate carefully, check this with a known standard (i.e. a solution of 25 mM KH_2PO_4/25 mM Na_2HPO_4 which should give a pH of 6.865 at 25°C and when diluted 10 times with distilled water the pH should be 7.065) and calibrate often (every hour if you are using the electrode continuously). Not only is it important to be careful with consistency of the above variables, but also it is important to consider the purity of your sample of calcium buffer (Bers, 1982; Miller & Smith, 1984).

Variations in the purity can cause significant errors in the final free [Ca^{2+}]. It is important to realise to what extent an error in each of these will alter the final free [Ca^{2+}]. Table 4 shows for EGTA how a 5% change in purity, or a 0.2 unit error in pH or changing the temperature of the solution from 25 to 35°C alters the expected value of free [Ca^{2+}]. Making up solutions with accurate and precise Ca^{2+} concentrations is not difficult provided care is taken with weighing any calcium buffer, adding Ca^{2+} usually as $CaCl_2$ and making sure the solution pH is correct to within ±0.005 units (especially if using EGTA as the Ca^{2+} buffer). The pH of the solutions need not be as accurate if one uses BAPTA or dibromoBAPTA (Tsien, 1980) as the calcium buffer. These compounds exhibit good selectivity for Ca^{2+} over Mg^{2+} (like EGTA) but their affinities are much less sensitive to pH changes around physiological pH values (Tsien, 1980; Bers & MacLeod, 1986; Harrison & Bers, 1987).

Steps for making up Ca^{2+} calibration solutions:

(1) Calculate the binding constant for your buffer in the solution composition you will use

(2) Determine the purity of your batch of buffer

(3) Knowing the above, calculate the total [Ca^{2+}] required to be added to your solutions using, for simple solutions equation (25) or, for more complex, multi-ligand solutions, a computer programme (Fabiato & Fabiato, 1979)

(4) Make up your solutions to about 90% of the required volumes weighing accurately and precisely the calcium buffer

(5) pH your solutions to pH 7.000 and add $CaCl_2$

(6) Re-pH to desired pH ± 0.005 unit

(7) Make up to near target volume and check pH - adjust if necessary and then make up to final volume.

Table 4. *The effects of altering purity, pH or temperature on free [Ca2+] in buffered solutions*

expected free [Ca^{2+}]		observed free [Ca^{2+}] (µM)		
pCa	µM	Ca buffer 95% pure	pH error +0.2 unit	Δ temp. to 35°C
8.000	0.010	0.011	0.004	0.010
7.699	0.020	0.021	0.008	0.019
7.301	0.050	0.053	0.019	0.048
7.000	0.100	0.108	0.039	0.096
6.699	0.200	0.221	0.079	0.191
6.301	0.500	0.600	0.199	0.480
6.000	1.000	1.379	0.399	0.956
5.699	2.000	3.920	0.802	1.919
5.301	5.000	22.21	2.098	4.778
5.000	10.000	43.70	4.769	9.805

Ca buffer is EGTA (1 mM). K_a (expressed as $\log_{10} M^{-1}$) of 6.45 is derived from constants in Martell & Smith (1974); 0.1 M ionic strength, pH 7.00, 25°C. pH sensitivity of K_a = 0.2/0.1 pH unit (see Bers & MacLeod, 1988). Temperature correction made using ΔH=16.6 kJ mol^{-1} (Harrison & Bers, 1987).

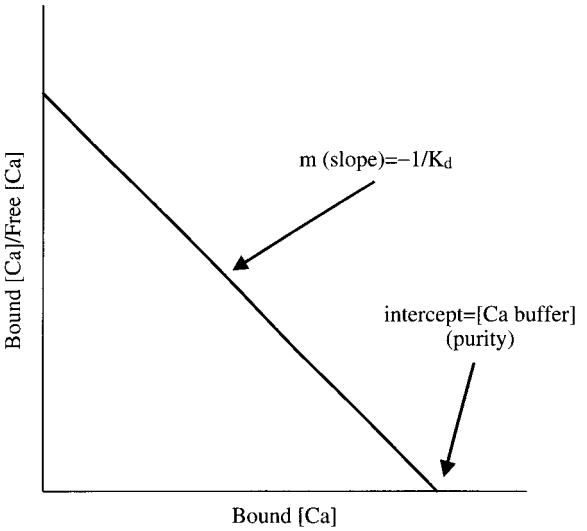


Fig. 15. Scatchard plot showing how K_a ($1/K_d$) and the purity of the Ca buffer are obtained from a plot of Bound [Ca]/ Free [Ca] against Bound [Ca].

Assuming that there is only one calcium buffer in your solutions then the total $[Ca^{2+}]$ can be calculated from the following equation:

$$[C_a]_t = \frac{[Ca\ buffer]_t + [Ca]_f + \frac{1}{K_a}}{1 + \frac{1}{K_a[Ca]_f}} \tag{25}$$

where the square brackets denote concentration of the species and the subscripts f and t denote free and total respectively. It is often useful to be able to calculate the free $[Ca^{2+}]$ knowing the total $[Ca^{2+}]$ added and this can be done by solving the quadratic:

$$[C_a]_f = \frac{\left([Ca]_t - [Ca\ buffer]_t - \frac{1}{K_a}\right) + \sqrt{\left([Ca\ buffer]_t - [Ca]_t + \frac{1}{K_a}\right)^2 + \frac{4[Ca]_t}{K_a}}}{2} \tag{26}$$

Ca^{2+}-sensitive electrodes can be used to calculate the binding constant of the buffer and its purity (Bers, 1982) although there are other methods (Miller & Smith, 1984; Smith & Miller, 1985). The procedure involves making an educated guess at K_a, assuming buffer purity is 100% and then making (say) 5-10 solutions aiming to obtain target pCa values of (say) 8.0, 7.5, 7.0, 6.5, 6.0, 5.5, 5.0, 4.5 and 4.0. You then measure the free $[Ca^{2+}]$ with a Ca^{2+}-sensitive electrode and calculate the

corresponding bound [Ca^{2+}] in each solution. Once you have done this you will be able to produce a Scatchard-type plot of bound Ca/free Ca against bound Ca. The slope of this relationship gives you the affinity (K_a) and the intercept the purity of the buffer. Equilibrium binding is analysed by a Scatchard plot which is a modification of the Eadie-Scatchard rearrangement of Michaelis-Menten enzyme kinetics. Applying it to our needs yields the equation:

$$\frac{[Ca]_b}{[Ca]_f} = -\frac{1}{K_d}[Ca]_b + \frac{[Ca\ buffer]_{total}}{K_d} \quad (27)$$

where K_d is the dissociation constant for the Ca buffer, the square brackets denote concentration of the species and the subscripts b, f and t denote bound, free and total respectively. Thus a plot of the bound:free [Ca] against bound [Ca] is linear with a slope of $-1/K_d$ or simply $-K_a$ (see Fig. 15). The intercept with the x-axis gives the total concentration of Ca buffer in the solution. Assuming you weighed out the Ca buffer with a good degree of accuracy then this figure allows you to calculate the purity of the batch of EGTA or BAPTA etc. you are using. The advantage of the method is that you can establish the K_a for the calcium buffer in the solutions you wish to use and at the pH you desire.

Ca^{2+}-sensitive electrodes can be obtained commercially (Orion) or they can be made more cheaply yourself. The advantage of making them yourself is that you can make small minielectrodes (1 mm≤tip size≤5 mm) which are useful for measuring small amounts of solution. Good methods for making Ca^{2+} minielectrodes can be found in Bers (1982) or Hove-Madsen & Bers (1992), see also Section 3, *Mini-electrodes*.

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