

THEORETICAL ASPECTS AND METHODOLOGY OF
PLANT ELECTROPHYSIOLOGY

Martin Gray Stanton

A Thesis Submitted for the Degree of PhD
at the
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THEORETICAL ASPECTS AND
METHODOLOGY OF
PLANT ELECTROPHYSIOLOGY.

BY

MARTIN GRAY STANTON.

A thesis presented to the
University of St. Andrews
(Department of Botany)
for the Degree of
Doctor of Philosophy.

St. Andrews
July 1981.



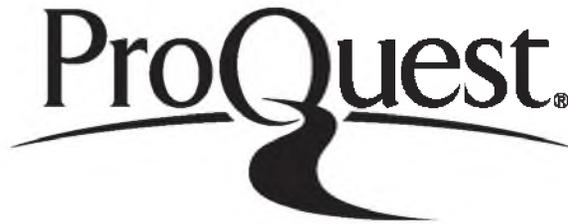
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Abstract.

Theoretical Aspects and Methodology of Plant Electrophysiology. M.G. Stanton. Ph.D. Thesis. University of St. Andrews, Scotland. July 1981.

Possible sources of electrical transmembrane potentials in living cells are examined. The equations of Nernst, Ussing and Goldman cannot predictively explain the origin of membrane potential because they all require knowledge of concentrations of ions both outside and inside the cell, and these internal concentrations are themselves generated by activity of the cell. Therefore a new electrochemical theory for the steady state has been developed, which takes into account both active transport and the Donnan effect. The theory, which should be general to all living cells, successfully predicts membrane potential in examples examined. Certain hitherto unknown effects have been predicted, the most important of which have been named (a) the "nebenion effect", whereby the presence of other ionic species of the same charge sign as the actively transported ionic species depresses membrane potential, and (b) the "Donnan enhancement effect", whereby the membrane potential when both active transport and a Donnan system co-exist is greater than the sum of the potentials produced by each acting separately. Two important consequences follow: (i) It is impossible for hydrogen or hydroxyl ion transport to generate a significant membrane potential in the face of environmental concentrations of nebenions. Thus the potential found across any membrane must be due to transport of majority ionic species and/or Donnan effects. (ii) The membrane potential in animal cells can only be explained by the "Donnan enhancement effect" in face of the "nebenion effect". Double Donnan systems and both linked and twin independent transports of two ionic species are considered. The effect of fixed charge either in the cell wall or as ζ -potential on either side of the membrane is examined.

Experimental procedures for the measurement of membrane potential are examined and a new integral microscope-manipulator system design is presented. Investigations are described of causes, and ways of avoiding, artefacts in measurements with micropipette electrodes, by studies both on model systems and directly on maize root cells.

Experimental techniques for the measurement of cell membrane resistance and capacity are reviewed, and a new method is introduced to produce AF impedance spectra of cells, from which both membrane resistance and capacity can be calculated. The electronic system used a phase-sensitive detector to simplify analysis of the a.c. bridge network, as well as to remove noise. It is believed this was in 1973 the first use of such a system in electrophysiology. The technique was tested on dummy circuits to represent the living cell. The properties of micropipette electrodes were investigated. Membrane resistance and capacity were successfully measured in maize root cells. This new technique makes these measurements possible on smaller cells than hitherto, since it uses a lone single-barrelled microelectrode. Finally the significance of such measurements in terms of cell and tissue anatomy is considered, and the theory of "vergence" resistance of small connecting bridges between cells is extended to cover the multiperforate septum.

Declaration.

I hereby declare that this thesis is based on my own readings and research, that it has been composed by myself, and that it has not been accepted in any previous application for a higher degree here or elsewhere. I also declare that I was duly admitted as a research student and candidate for the degree of Doctor of Philosophy of the University of St. Andrews under Ordinances Nos. 350 (General No. 12) and LXXIX (St. Andrews No. 16), that I have complied with the relevant Regulations, and that I have maintained my matriculation.

Candidate.

Certificate.

I certify that Martin Gray Stanton, M.A.(Cantab.) has devoted not less than nine terms to research work under my supervision, and that he has fulfilled the conditions of the Ordinances and Regulations above mentioned, and that he is qualified to submit the accompanying thesis in application for the degree of Doctor of Philosophy.

Supervisor.

Statement of Qualifications.

Designatory
letters.

1. In October 1957 I matriculated as an undergraduate in the University of Cambridge, as a member of St. Catharine's College, to read the Natural Sciences.
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4. On 11 Feb. 1974 I was admitted as a Fellow of the Society of Antiquaries of Scotland F.S.A.(Scot.)
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6. On 6 Oct. 1978 I was admitted to the class of Member of the Institute of Biology. M.I.Biol.
7. On 9 June 1979 I was admitted to the class of Member of the Institution of Electrical Engineers M.I.E.E.
and consequently was recognised as a Chartered Engineer by the Council of Engineering Institutions. C.Eng.
8. In July 1979 I was admitted to the class of Member of the Institute of Physics. M.Inst.P.

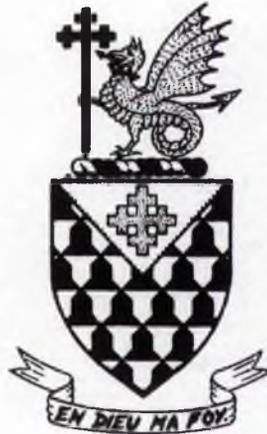
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I would also like to express my thanks to Professors J.A. MacDonald, FRSE and D.H.N. Spence, FRSE, Department of Botany, and to Professor M.S. Laverack, FRSE, Gatty Marine Laboratory, University of St. Andrews, for providing the opportunity and facilities for doing this research; also to Comm. C.J. Roemmélé, RN, M.Sc., C.Eng., MIERE, MRIN, Senior Administrator and Head of Electronics at the Gatty Marine Laboratory, for many helpful and mutually useful discussions; also to the technical staff of the Gatty Marine Laboratory for their helpful assistance. I owe particular debts of gratitude to Mr. John Brown, Head Technician for countless occasions of help, and likewise to Mr. Roland Jack, Senior Technician in charge of the Mechanical Workshops at the Gatty Marine Laboratory.

I would also like to record my thanks to Professor F.R. Whatley, FRS, School of Botany, University of Oxford, for kindly accommodating me within his Department during my period of study leave Oct. 1979 - Apr. 1980, when a large part of the theory set out in Chapter 1 of this Thesis was devised.

DEDICATION



To the Memory of my Father,
HUBERT BOLTON STANTON, C.Eng., MIBE,
who first awakened my interest in science,



and to my Mother,
NORAH BEATRICE STANTON,
who has encouraged me at every stage.

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Abbreviations in lists.

AC	alternating current
AF	audio frequency
AI	anion import
CE	cation export
CT	coupled transport
D	Donnan system
DB	double-barrelled
DC	direct current
DD	double Donnan system
N	nebenions
R	resistance
T	transport
μ-	micro-

Definitions of Principal Quantity Symbols.

(Minor symbols are explained in their contexts).

Subscripts.

o = outside
 i = inside
 w = water

Full symbols.

E = potential at any point designated by subscript
 E_m = cell transmembrane resting potential, $E_i - E_o$,
 inside referred to outside.
 (Quoted in millivolts throughout, where figures given).
 (Strictly this is a potential difference, loosely
 called "transmembrane potential".
 R = "gas constant" - enthalpy of one gram-mole
 8.314 J/°K/mole.
 T = absolute temperature, °K = 273.16 + °C.
 F = charge on one gram-ion, the Faraday, 96.49 kC/mole.
 N' = Avogadro's number, 6.022×10^{23} /mole
 k = Boltzmann's constant, 1.381×10^{-23} J/°K/molecule
 e = magnitude of charge on electron or proton,
 1.60×10^{-19} C/particle.
 z = valency of an ion, including charge sign.
 a = chemical activity of an ion = concentration x f, where:
 f = activity coefficient.
 \bar{a} = electrochemical activity
 Element symbols represent concentrations of ions.
 μ = chemical potential
 $\bar{\mu}$ = electrochemical potential
 μ_o = reference value of above (dashed for $\bar{\mu}$).
 $\Delta\bar{\mu}$ = head of electrochemical potential actually achieved
 by active transport of an ion species across the
 membrane. Positive for import, negative for export.
 Subscript refers to ion species - if no subscript,
 context indicates.

ΔH = enthalpy change.

ϕ_o, ϕ_i = outward and inward flux rates as measured by isotopic tracer.

G = frictional force on one mole of the solute ion under study diffusing in water at unit velocity.

G_w = frictional force on one mole of water diffusing in water at unit velocity.

c_o, c_i = exchange equivalence concentration of membrane-impermeant macro-ion outside and inside the cell.

S = surface area

P = permeability of the membrane to the subscript ion. (For P without subscript, see below).

P = concentration outside of actively transported (pumped) ion species (context indicates which charge sign.)

N = concentration outside of nebenion to pumped ion species, superscript referring to charge sign, and subscript to location. Where no super- or sub-script, context indicates. It follows P .

\mathcal{R} = N/P , ratio of nebenion to pumped ion, outside.

δ = mP_{Na}/P_K , where:

m = stoichiometric linkage between Na^+ and K^+ (see text)

u = free solution mobility of an ion in water.

In Henderson equation (Eqn.(7)):

c = concentration of ion species, i refers to species.

J = junction

' & '' = first and second solutions respectively in contact across liquid junction.

Mathematical operators.

$\exp(x)$ = Napierian base, 2.718..., raised to power x .

\ln = Napierian logarithm.

\log_{10} = logarithm to base 10, (common logarithm).

pX = $-\log_{10} X$

\sum = sum of

Mathematical substitutions.

$A = \exp(\Delta\bar{\mu}/RT)$

$B = \exp(FE_m/RT)$

PREFACE .

Things rarely turn out as planned. Research is often far from logical in the way it actually happens and yet, to make it intelligible, one tries to make the presentation logical, which sometimes, as here, means altering the order.

The work began with the intention of investigating the electrophysiology of maize roots (how naive I was !), and was intended to be allied to studies of their ionic relations. As I began to apply the existing state of the electrophysiological art, I became uneasy about the rigour and validity of the techniques then established, and so I decided to concentrate upon an investigation of the techniques themselves. The result was the work reported in Chapters 2 and 3. During the course of this study I felt it necessary to make a number of improvements and innovations. In particular I designed an improved system for inserting microelectrodes into cells and I designed an electronic system for the measurement of membrane potential and resistance, a system which eventually grew into the total a.c./d.c. system, described in Chapter 3, which also enables membrane capacity to be measured. The application of a phase-sensitive detector to a bridge in a novel way proved valuable for removing noise and capable of yielding resistance and capacity information with a simple resistive network.

Then I began to wonder just what it was I was measuring, a question perhaps I should have considered before I began experimenting - though I fancy if I had, I should perhaps have decided that I should be rash to try to do it in face of the problems ! Nevertheless I am glad I did. The theoretical study of membrane resistance and capacity lead me to marshall a number of lines of thought which needed bringing together, but the magnitude of the problem rather alarmed me, and it became necessary to limit the scope of the study. However I was able to extend the theory on symplastic connections and to point the way for further approaches.

Over the course of a number of years, as I taught to students the accepted dogmas on membrane potential, I became increasingly dissatisfied with the explanation, or rather lack of it, to account for the origin of membrane potential. I began to do a bit of thinking from scratch and before I realised it, found myself launched into a full-scale new theory. I soon realised that there was no reason in principle why this theory should not be universal to both plants and animals in all situations provided enough factors were included. To my delight, I found I could solve the problems by a stepwise approach, arriving at a truly predictive theory which, I believe, really does explain how cells create and maintain a membrane potential, and how large it should be. I was also able to clarify the distinction between steady state and transient effects. I present this work as Chapter 1 because much of the material in it helps understanding in Chapters 2 and 3. However since this theory was actually worked out late in this project, I present few results from my own experiments and have relied on published data, not all entirely suitable, but theoreticians often have to do that. In one sense however, the new theory is already partially tested in so far as it actually does deliver, as far as I can tell with data available, the correct values of membrane potential - and in those cases where at present I cannot tell, for lack of data, the indicators are still that the theory should be valid. Experiments specifically designed to test the theory must come later.

Therefore I make no apology on two counts. Firstly, the purpose of the experimental studies (Chapters 2 and 3) was to establish techniques and was not primarily to measure parameters on any particular living material, so "results" in that sense are few. Secondly, statistical tests of accuracy are not normally applied here because there seems no point in doing so unless the actual measurements taken are to be used for themselves. Also calculation of statistical spread does not help one to detect systematic errors, and it was these I was trying to assess.

The net result has been that the dissertation grew to a size rather larger than intended, but I felt I had a story to complete. I have been deliberately discursive so that the reader may follow hopefully without loss of comprehension (as can so easily happen in mathematical presentations!) as I proceed through lines of argument. I hope at least I hold the reader's interest.

CHAPTER I.

ORIGIN AND MAGNITUDE OF TRANSMEMBRANE RESTING POTENTIAL IN THE LIVING CELL .

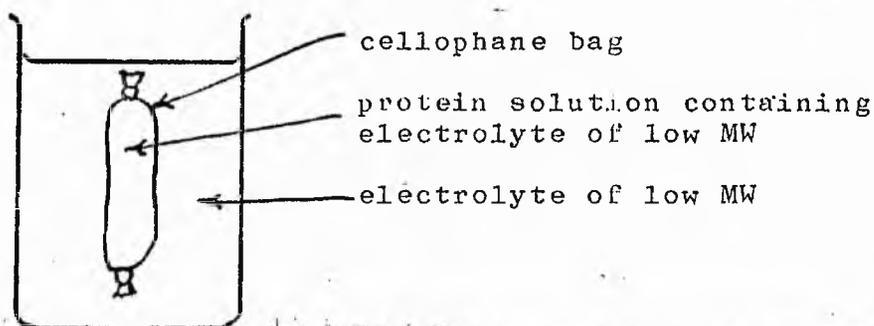
In this chapter is presented first a review of established approaches, together with a demonstration of their inadequacies as explanations of the origin of the membrane potential in living cells. Then a new theory is developed which adequately and predictively accounts for cell membrane potential. Finally, the new theory is examined for limitations and compared with current knowledge.

The Gibbs-Donnan System.

Although this system is normally attributed to Donnan, who published his analyses in 1910 and 1911, attention was first drawn to this membrane effect by Ostwald (1890). The first experimental observation of the effect was made by Donnan and Harris (1911), but unknown to them J. Willard Gibbs had already made a theoretical analysis of heterogeneous systems in which at least one phase was confined by a diffusional barrier (1906) (see also Bolam 1932 for review).

The Donnan System may be defined as that state of affairs which pertains, once equilibrium has been reached, between an electrolyte solution, normally dilute, containing a macromolecular electrolyte, such as protein not at its iso-electric pH, such solution being contained in a closed bag or membrane through which the macromolecule cannot penetrate, the whole being immersed in a dilute electrolyte solution whose ions may all pass the membrane.

What distinguishes a Donnan System then is the presence of a macromolecular electrolyte trapped in a bag, and the presence on both sides of the bag of electrolyte ions which can pass freely through. The diagram below indicates a typical case:



The importance of this system is, of course, well realised in biology, where the cell membrane behaves as the semi-permeable bag. Examples of its occurrence without the complications of active transport are however fairly rare, but one may cite the oncotic pressure in the kidneys of animals, in the problem faced by fish and plants living in freshwater, etc. Donnan effects are, of course, much reduced in sea water, as will be indicated, but they are not absent nevertheless. It is necessary to understand the Donnan system before one can approach the problem of active transport of mineral ions in plants, and so an analysis now follows. It will be indicated later that in some restricted but important respects an active transport system may be treated as a special case of a Donnan system.

The approach to an understanding of the Donnan system will be made by taking the simplest situation in which it would arise, and working up from there.

The simplest case is where one encloses a neutralised protein solution in a membrane bag and then immerses this in a limited volume of initially distilled water. By starting this way, one can see how the situation develops.

By a neutralised protein solution is meant a protein (or for that matter, any other macromolecular electrolyte) associated with mineral counterions. Thus, if the protein, (not at iso-electric pH), is represented by

Pr^{n-} , it would be associated with $n \text{Na}^+$ ions (if Na^+ were the only mineral cation species present, of course).

As initially set up, protein solution in a bag in distilled water is not at equilibrium. The protein cannot disperse because the membrane restrains it, but the associated sodium ions can do so. Some will leave the bag, each leaving behind an unbalanced negative charge and carrying a positive charge to the external solution. Thus an electrical potential difference will arise between the solution inside and that outside creating a potential gradient across the membrane tending to draw back the sodium ions. It was specified deliberately that the outside solution was to be of limited volume so that a build-up of sodium concentration outside would occur.

Thus equilibrium is reached when the diffusing tendency of the sodium ions is exactly counteracted by the electrical field which it sets up. The process is therefore naturally self-limiting. The question then is, how far does it go?

Diffusing tendency is determined by the difference in chemical potential which exists between two points for the given ionic/molecular species. This should be distinguished from the rate of diffusion, which is governed by Fick's Law, which involves the difference in chemical concentration between the two points. Chemical potential is, of course, an energy term. Thus there is a change in free energy when solute diffuses

down its concentration gradient. Similarly there is a free energy change when a charged species goes through an electrical potential gradient.

Consultation of any treatise on thermodynamics will reveal that there are recognised two sets of expressions for free energy - those when the system remains at constant pressure, Gibbs Free Energy (G), and those when the system remains at constant volume, Helmholtz Free Energy (F), (Alexander & Johnson, 1950, p.55).

The difference between these two clearly involves terms concerning work done by or on a system during volume or pressure changes or both. For almost all circumstances one is likely to encounter in biology, except under imposed or exotic conditions, the constant pressure case applies. In any case, in liquid media, the difference between G and F is small compared to other changes, because water is virtually incompressible to a first approximation, so the difference can be ignored. Necessarily the whole of a Donnan system is in the liquid phase.

Chemical potential, μ , which is of the Gibbs free energy type, is given by :

$$\mu = \mu' + RT \ln a$$

where :

μ' is a reference value

R is the "Gas constant", i.e. the enthalpy per degree K per mole.

T is the temperature in $^{\circ}\text{K}$, and

a is the chemical activity, equal to cf where :

c is the concentration, and

f is the activity coefficient.

ln is the mathematical operator, Napierian Logarithm

A reference level, μ' , has to be included because chemical potential, like electrical potential, has no absolute value, but only values reckoned as differences from somewhere else.

Thus, if we imagine one gm.-ion of an ionic species to cross the membrane from inside to outside, the free-energy change due to chemical activity gradient will be

$$(\mu' + RT \ln a_i) - (\mu' + RT \ln a_o) = RT \ln \frac{a_i}{a_o}$$

$i = \text{inside} \qquad o = \text{outside}$

Similarly for the electrical work, an expression can be derived; as follows. Since chemical potential has the units of energy, we need an expression involving electrical potential having dimensions of energy.

Such is given by the expression :

$$G + G' = z F (E + E')$$

where :

- G is Gibbs free energy with its reference value, G'
- z is valency of the ionic species
- F is the Faraday of electricity, 96500 coulombs
- E is the local electrical potential, with its reference value, E' .

Thus, when 1 gm.-ion of the ionic species crosses the membrane from inside to outside, the change in free energy due to electrical field is :

$$z F (E_i + E') - z F (E_o + E') = z F (E_i - E_o)$$

We now have expressions for changes in free energy due to changes in both concentration and electrical

potential in crossing the membrane. If the system is at equilibrium, we know that there must be no net change in free energy when an ion crosses the membrane so that these two expressions must be equal and opposite, thus :

$$RT \ln \frac{a_i}{a_o} = -zF(E_i - E_o)$$

Hence re-arranging, and inverting the logarithms to remove the minus sign:

$$\underline{\underline{E_i - E_o = \frac{RT}{zF} \ln \frac{a_o}{a_i}}}$$

This is the well-known Nernst equation, which is of fundamental importance, and applies to many circumstances where ions are involved. A more convenient form of the equation is evaluated, thus:

$$E_{io} = E_i - E_o = \left(2.3026 \frac{RT}{F} \right) \frac{1}{z} \log_{10} \frac{a_o}{a_i}$$

The value 2.3026 is the conversion factor from Napierian to Common logarithms, i.e. $\ln 10$. The value of the expression in brackets, of course, varies with temperature, but, if E_{io} is expressed in millivolts, the values are given below for a few temperatures:

$$R = 8.314 \text{ joules } ^\circ\text{K}^{-1} \text{ mol}^{-1}$$

$$F = 96487 \text{ coulombs}$$

$$0^\circ\text{C} = 273.16^\circ\text{K}$$

So at $T^\circ\text{C}$ () is

0	54.20 mV/10 fold concentration change
10	56.18 for a monovalent ion.
15	57.17
20	58.16
25	59.16
37	61.54

It should be pointed out that the Nernst equation has been derived thermodynamically, and therefore, as always with thermodynamic results, it is independent of arguments about mechanisms, in this case concerning the way an ion crosses the membrane, and therefore the properties of the membrane are totally irrelevant to the applicability of the equation. The only condition set was that the system be in equilibrium, which implies no net movement of ions across the membrane. The other consequence of the derivation of the Nernst equation is that it is true for each ion species independently of all others present, provided the ion species is in equilibrium and is truly independent. This should be contrasted with the Goldman equation and other similar relations, such as those of Hodgkin, Nikolsky and others, which deal with several ion species at once and are concerned with diffusional steady states and not equilibrium. The significance of these remarks will be seen when active transport is discussed.

When there are several ion species present in a Donnan system able to diffuse through the membrane, each one independently will fulfil the Nernst equation. Diffusion will occur until each ion species is held in a concentration ratio by the Donnan system such that all of them together "see" the same potential difference across the membrane. Clearly there can only be one potential difference across the membrane at any given moment, and the value to which it will settle will be the value which satisfies all the diffusible ions present.

The Nernst equation may be re-arranged into the form :

$$E_{io} = \frac{RT}{F} \ln \left(\frac{a_o}{a_i} \right)^{1/z}$$

from which it is clear that the expression $\left(\frac{a_o}{a_i} \right)^{1/z}$ must be equal for all the diffusible ions present.

Note that the valency, z , takes account of charge sign.

Thus if the Na^+ ion activity ratio were 10, then the Cl^- ion activity ratio would be 0.10, since $z = -1$ for Cl^- , and also the Ca^{++} ion activity ratio would be 100, and for Al^{+++} , 1000. Note however that there may be other complicating factors with calcium and aluminium ions, such as chelate or complex formation which could distort the concentration ratios very considerably, but it would still be true that the activity of the independent uncomplexed ions present would follow the expression quoted above. It is a general rule that chemical thermodynamics deals with individual ion or molecular species quite independently. Thus a complex containing Ca^{++} ions does not count in the reckoning of free Ca^{++} ions, although there will be an equilibrium between the two, of course.

Magnitude of Membrane Potential in a Donnan System.*

Although an expression has been derived relating activity ratio of an ionic species to the electrical potential difference across a membrane in a Donnan system at equilibrium, this equation does not help us to decide what value either should take in any given system.

* (This section is original to myself, but it has since been found that Bolam (1932) published a similar approach).

For the purposes of this discussion it will be convenient to modify the former simple case of a Donnan system as follows.

Initially a semi-permeable membrane bag full of protein solution, of concentration C_p gm./litre is placed in an unlimited volume (and therefore of fixed concentration) of dilute salt, NaCl, solution of concentration, C_o . This is then allowed to proceed to equilibrium. (Incidentally, C_p has been quoted in gm./l. here rather than as molarity for the protein because usually this is a more convenient quantity when dealing with macromolecules, especially in cases, perhaps most cases, where one is dealing with a polydisperse preparation - i.e. one containing polymers of different lengths mixed together.)

Of course, there are already associated with the protein cationic counterions (we assume also that the protein is in a solution of pH greater than its iso-electric pH), and it is convenient to assume these cations are sodium ions, for the present. Let the concentration of associated (but not complexed) Na^+ ions with the protein be C_i molar.

As equilibrium is approached both Na^+ and Cl^- ions will diffuse into the bag. Now, to preserve electrical neutrality within each solution, the number of Na^+ ions diffusing in will equal the number of Cl^- ions diffusing in - i.e. it will behave as if total salt

diffused in, although in fact the ions are dissociated in solution. It should be observed that the apparent paradox that electrical neutrality has to be preserved and yet an electrical p.d. develops is resolved when it is realised that within each individual liquid phase (that outside or that inside the bag) electrical neutrality must be preserved because if it were not, any net charge would speedily migrate to the boundaries of each phase, as demonstrated by the well-known "Faraday Ice Pail Experiment", in which charge always moves to the outside surface of any conductor by repulsion of the individual charge carrying elements for each other, which will necessarily be of like sign, should there be a net charge. By this same rule, the p.d. which does exist in a Donnan system does indeed reside at the boundary, which is, of course, the membrane. The transition through the potential difference does occur over a non-zero distance, sudden transitions being impossible, but the range is usually very short, and the volume of liquid contained in this thin sheet at the membrane is usually infinitesimal compared to the volumes of the phases on either side. Nevertheless it is true to say that in this boundary layer charge equality actually does not apply; in the case of cell membranes, containing a dielectric lipid layer, the membrane electrical capacity increases the imbalance between anions and cations in the peri-membrane layers.

Thus, we may now write down quantities for the total Na^+ and Cl^- inside and outside the bag.

Inside the bag:

$$Na_i = x + c_i$$

where x represents the as yet unknown quantity of Na^+ which has diffused in, up to equilibrium, and c_i that already there associated with the protein

and also $Cl_i = x$

using the condition that equal numbers of Na^+ and Cl^- ions diffuse into the bag.

Outside the bag :

$$Na_o = Cl_o = c_o$$

Now, in a Donnan system, as shown above, $\left(\frac{a_o}{a_i}\right)^{\frac{1}{z}}$ applies

for all the freely diffusible ions present. Thus the

ratio $\frac{a_o}{a_i}$ for Na^+ will equal the inverse of the same

ratio for Cl^- . To proceed further a simplifying,

although not strictly accurate, assumption will be made, namely that activity may be replaced by concentration.

Provided the concentrations are low - dilute salt - this assumption is justified to a first approximation, in order to grasp how the system behaves.

Thus we take it that :

$$\frac{Na_o}{Na_i} = \frac{Cl_i}{Cl_o}$$

We may now fill in the quantities:

$$\text{For } Na^+ \text{ and } Cl^-$$

$$\frac{c_o}{x + c_i} = \frac{x}{c_o}$$

This is a quadratic :

$$c_o^2 = x^2 + c_i x$$

or

$$x^2 + c_i x - c_o^2 = 0$$

or

$$\left(x + \frac{1}{2}c_i\right)^2 - c_o^2 - \frac{c_i^2}{4} = 0$$

Therefore

$$x + \frac{1}{2}c_i = \pm \sqrt{\left(c_o^2 + \frac{c_i^2}{4}\right)}$$

and so

$$x = \pm \sqrt{\left(c_o^2 + \frac{c_i^2}{4}\right)} - \frac{c_i}{2}$$

Only the positive root has any chemical meaning, of course.

It is now of interest to see how this system will behave in extreme circumstances, i.e. in low salt or high salt concentration.

Low Salt Concentration.

This is the situation which applies to living tissue in freshwater.

Low salt will be taken to imply that $c_o \ll c_i$

and so the root x reduces to: (δ = a small quantity)

$$\sqrt{\left(\delta + \frac{c_i^2}{4}\right)} - \frac{c_i}{2} = \frac{c_i}{2} + \delta' - \frac{c_i}{2} = 0 + \delta'$$

in other words, a very small quantity, almost zero.

Thus the ratio for Na^+ ions inside to outside, $\frac{\text{Na}_o}{\text{Na}_i} = \frac{c_o}{x + c_i}$ which reduces to $\frac{c_o}{c_i}$ which will be a small fraction, and gets smaller the lower the external salt concentration in comparison to the Na^+ associated with the protein, c_i . Thus when $\frac{c_o}{c_i}$ is put into the Nernst equation we get:

$$E_{io} = \frac{RT}{F} \ln \frac{c_o}{c_i}$$

Now the logarithm of a small fraction is a large negative

number, so we expect a high Donnan potential with the cationic macromolecule-containing solution negative when the salt concentration is low.

High Salt Concentration.

This is the situation which applies for living tissue in contact with sea water or blood, although in the latter case blood itself contains proteins in the plasma to add an extra complication. Indeed the glomerular filtrate of kidneys forms a Donnan system against the blood, because the proteins are held back by the filtration membrane. This will be referred to again when osmotic pressure is dealt with.

High salt may be taken to imply that $c_o \gg c_i$. Therefore, in the root, x , $c_o^2 \gg \frac{c_i^2}{4}$ so $x = c_o - \frac{c_i}{2}$ to a close approximation (very slightly more). Now the ratio of Cl^- ions inside to outside, $\frac{Cl_i}{Cl_o}$, will be $\frac{x}{c_o} = \frac{c_o - \frac{c_i}{2}}{c_o} = 1 - \frac{c_i}{2c_o}$ which is near unity since $c_i \ll c_o$.

(Note that $\frac{Cl_i}{Cl_o}$ was taken for simplicity of calculation here.)

Of course, $\frac{Na_o}{Na_i}$ gives the same result, i.e.

$$\frac{x}{x + c_i} = \frac{c_o}{c_o - \frac{c_i}{2} + c_i} = \frac{c_o}{c_o + \frac{c_i}{2}} = \frac{1}{1 + \frac{c_i}{2c_o}}$$

which reduces to $1 - \frac{c_i}{2c_o}$ if one remembers that $c_i \ll c_o$ and takes the usual binomial series expansion for $(1+x)^n$ where $n = -1$, in this case. In the low salt case, the alternative expression for Cl^- was indeterminate and therefore not used, but it would clearly have been a small fraction, as for Na^+ .)

Thus the system in high salt displays only a very weak Donnan effect and virtually no Donnan potential since $\ln 1 = 0$ in the Nernst equation. In other words the Donnan system is swamped by salt.

The net result of the considerations above is that a Donnan system is seen to be most pronounced both in regard to ratio of concentrations and Donnan potential when salt concentration is low, falling off as salt concentration rises.

It will be apparent from the discussions above that the criterion which determines whether the Donnan system is to be considered in high salt or low salt, etc., is the concentration of salt relative to the concentration of mineral ion associated with the macromolecule. Thus an uncharged macromolecule will be in effect always in the high salt situation, and will not therefore show any Donnan effect, as expected. Indeed the Donnan effect becomes greater the greater both the charge on the protein and its concentration.

A general formula for the Donnan potential in terms of salt concentration and concentration of ion associated with the macromolecule could obviously be constructed by substituting in the Nernst equation the root in its general form, and such an equation will be introduced later (Eqn. (24), p. III). However a note of caution must be sounded. The accuracy of such an equation may not be very good at all salt concentrations since activity coefficient, f , has not been taken into account. This

objection applies less to the low salt case, where $f \sim 1$ than at intermediate or high salt concentrations where f may be much less than 1 and not necessarily the same inside and outside the system. Nevertheless the above treatment does show the trend to be expected in a Donnan system. It will soon be apparent to anyone who tries to work out a more rigorous analysis that some difficult mathematics quickly arises, since one would have to replace f , the activity coefficient, by $\exp(A\sqrt{c})$ (from the Debye-Hückel theory), where A is a constant, characteristic of the ion, the solvent and temperature, and c is the concentration of the ion (McInnes, p.144).

It should finally be observed, before passing on, that the derivation of the Nernst equation ignored the effect of pressure on the system, which would exist because of the development of osmotic pressure. Indeed, for a system to be truly in equilibrium, osmotic pressure development is inevitable, since, were it not present, the volume of the solution in diffusional contact with one of lower osmolarity would continually expand. However, the contribution of osmotic pressure to the total free energy is small because liquid is very nearly incompressible. However a quantitative demonstration that this assertion is justified must await calculation of osmotic pressure in a Donnan system, as now follows.

Osmotic Pressure in a Donnan System.

A Donnan system can produce a remarkably high osmotic pressure, and this fact is of vital importance to all living creatures. Thus their cells and tissues either have to withstand the osmotic pressure which they may do by means of a tough cell wall, or else they have to circumvent the problem by some kind of continuous active process such as active solute transport, usually of mineral ions, or by avoiding the circumstances which cause it.

In the derivation of an expression to describe osmotic behaviour of a solute/solvent system, there are several progressive steps of simplification which can be made using reasonable assumptions (Alexander and Johnson, Chs. 4 and 7, and Glasstone, 1948, Sec. 30).

A rigorous thermodynamic derivation arrives at the expression (see Alexander & Johnson, p.75) :

$$\pi \bar{V}_1^0 \left(1 - \frac{1}{2} \kappa \pi\right) = RT \ln \frac{p_0}{p}$$

where :

- π is the osmotic pressure
- \bar{V}_1^0 is the partial molar volume of solvent at zero pressure
- κ is the compressibility of the solvent
- p_0 is the vapour pressure of pure solvent
- p is the vapour pressure of the solution.

Even the derivation of this expression makes the assumptions that:

(a) the vapour behaves as a perfect gas - a fair assumption when, as is usual, the vapour pressure is only a small

fraction of the total pressure in the system.

(b) the partial molar volume follows the compression law described by the bracketed function above. If it is assumed that for practical purposes the solvent may be regarded as virtually incompressible, this bracketed factor may be omitted yielding :

$$\Pi \bar{V}_1 = RT \ln \frac{p_0}{p}$$

This may be simplified further using Raoult's Law, which states that the vapour pressure of a solvent is depressed in solutions, as compared to the pure solvent, by the inverse ratio of partial molar volumes of solvent in each case. Partial molar volume is correctly defined as "the change in volume resulting from the addition or removal of 1 mole of solvent from a large quantity of the solution at a given temperature" (Glasstone, p.275).

Thus, by Raoult's Law, which itself applies to dilute solutions : $\frac{p}{p_0} = 1 - x_2$ where x_2 is the mole

fraction of solute in the solution. Provided the

solution is dilute, i.e. x_2 is small, a further

mathematical simplification, that $\ln(1-x_2) \approx -x_2$

may be applied, yielding the expression :

$$\Pi \bar{V}_1 = RT x_2$$

where now :

Π is the osmotic pressure

\bar{V}_1 is the molar volume of pure solvent

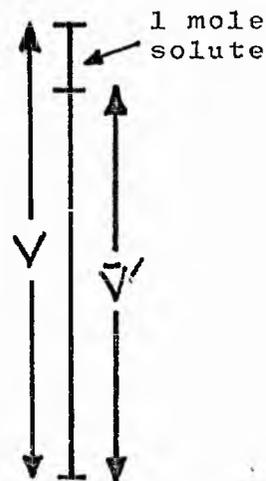
x_2 is the mole fraction of solute

Now, $\frac{\bar{V}_1}{x_2}$ may be replaced by \bar{V}' , the volume of solvent associated with one mole of solute in the solution (i.e. the reciprocal of molal concentration). This yields the equation derived empirically by Morse for osmotic

pressure : $\pi \bar{V}' = RT$

If the solution is very dilute, the quantity \bar{V}' may be replaced by V , the volume of solution containing 1 mole of solute. The difference between V and \bar{V}' lies in the volume occupied in solution by the solute.

Thus for anything but very dilute solutions V will be an over-estimate, as the diagram here inset shows, and thus osmotic pressures calculated from V will be under-estimated. Nevertheless, a useful expression is found for dilute solutions in the van 't Hoff equation so derived :



$$\pi V = RT \quad \text{or} \quad \underline{\underline{\pi = cRT}}$$

where c represents the concentration of solute in molar. Even though this equation is limited by the various simplifying assumptions, it will be adequate to obtain an insight into the osmotic behaviour of a Donnan system. In any case, when dealing, as here, with ions in solution, once again c should be replaced by α , since the Debye-Hückel depression of activity below concentration applies just as much to colligative properties as to electrochemical.

Now, in a Donnan system, there are several species of solute co-existing in the solvent. Using the model described on p.2. there are :

inside :	protein molecules (charged)
	Na ⁺ ions
	Cl ⁻ ions
outside :	Na ⁺ ions
	Cl ⁻ ions

Each ionic or molecular species makes its own contribution to the osmotic pressure and so :
 Referred to distilled water, the osmotic pressure of the solution in the bag would be :

$$\Pi_i = \left(\frac{c_p}{M} + (x + c_i) + x \right) RT$$

where $\frac{c_p}{M}$ is the molarity of the protein in the bag, M being the molecular weight of the protein.

$x + c_i$ is the total Na^+ ion in the bag at equilibrium as before (see p. 12), and x is the Cl^- ion in the bag.

Also referred to distilled water, the solution outside the bag would have an osmotic pressure of :

$$\Pi_o = 2 c_o RT$$

where Na^+ and Cl^- each have concentration, c_o .

Thus in the Donnan system when the two solutions are in equilibrium, the resultant osmotic pressure will be the difference $\Pi_i - \Pi_o$ thus :

$$\Pi_{\text{Donnan}} = RT \left(\frac{c_p}{M} + 2x + c_i - 2c_o \right)$$

Once again it is instructive to see how this system behaves for extreme cases, by substituting appropriate values for x .

Low Salt Concentration.

As before $x =$ a very low value - see p. 13.

Also, the condition set is that $c_o \ll c_i$, therefore c_o can be ignored, and we have :

$$\Pi_{\text{Donnan}} = RT \left(\frac{c_p}{M} + c_i \right)$$

Now the value of c_2 may be related to $\frac{q}{M}$ by the number of charges carried by the protein, n , if we assume that the pH is above the iso-electric pH, and that there are no negative charges on the protein juxtaposed to remanent positive charges on the molecule. In other words, we set n to represent the number of unbound monovalent counterions associated with a protein molecule. It is now clear that :

$$\Pi_{\text{Donnan}} = RT(n+1)\frac{q}{M}$$

in very dilute salt solution.

Now, it is quite likely that n could be as high as 90 for a protein of molecular weight 60 kD (kD = thousand Daltons). Thus the osmotic pressure can be quite high, and furthermore most of the osmotic pressure arises not from the protein itself, but from an ion which can itself pass through the membrane (in this case Na^+) held in the bag merely by electrical forces.

The importance of this result is that it shows the extent of the problem faced by living organisms in a freshwater environment. For instance, a 3% solution of a protein of 60 kD, and with 90 charges per molecule, when immersed, enclosed in a semi-permeable membrane, in freshwater, would give an osmotic pressure of just over 1 atmosphere at 20°C . A living organism will be quite likely to contain at least a 3% solution of protein, as well as other molecular and ionic species all contributing to the osmotic pressure. Notice that the charge state of the protein is affected by pH and

therefore the Donnan osmotic pressure would be itself sensitive to pH. Thus it may be expected that plants which live in an acid (pH \sim 4-5) environment would suffer less osmotic stress than those in neutral environments.

Different strategies are adopted by different organisms to cope with the osmotic problem, but this can only be mentioned briefly here. Thus plant cells may be protected by a cell wall which can withstand high turgor pressures. Incidentally, it should be observed that the cell wall, which may be likened to a basketwork of cellulose fibrils, affords protection against bursting to the protoplast membrane because it changes the radius of curvature of a rupture surface. At first sight, it may seem strange that a holey structure like a cell wall could protect the membrane from bursting, but it can do so because any protuberance that would lead to a rupture would have to pass through a hole in the mesh of cell wall fibrils. The minimum radius which is approached as the protuberance forms is in fact half the distance between the fibrils. An unprotected cell would expand as a whole, and so the effective radius would be that of the cell. Now the resistance to explosion is derived from the surface tensile strength of the plasma membrane, which is only a few dynes/cm., but the pressure inside a bubble, balloon or other spherical object is given by $\frac{2t}{r}$, where t is the tension in the wall across unit length, and r is the radius of curvature of the wall. Thus the presence of a cell wall, by reducing the effective rupture radius from that of the cell itself down to that of a hole in the wall basketwork, greatly increases the pressure a

cell can withstand. As a protuberance forms in a hole in the cell wall, it progresses from the relatively long radius of the cell to the short radius of the hole, thus the pressure it can withstand increases rapidly with expansion. This is therefore a negative-feedback or self-limiting situation. However with a naked protoplast, increase in size leads to a fall in the internal pressure that surface tension can provide. It is true that volume increases and therefore, by dilution, osmotic pressure may fall, but cells tend to maintain actively a constant internal medium. Therefore the naked protoplast could be in a most precarious positive-feedback situation. Of course, in the short term, before new material were accumulated or synthesised within the cell to maintain intracellular concentrations, osmotic swelling would still be self-limiting because concentration would fall as r^3 whereas pressure only as r . This explains why, for example, red blood cells with ion transport pumps blocked by ouabain, etc., do not immediately burst, and can withstand mildly hypotonic media.

This now raises the question as to what stratagems are available to naked protoplast cells, such as found in animals in most cases, to avoid osmotic catastrophe. Certainly for animals who live in freshwater there will be an osmotic problem, because their solute content is certain to be higher than that in freshwater for life processes to be carried on. Therefore water will tend to be accumulated osmotically. The rate at which it is

accumulated will depend on the permeability of surface barriers such as cell membranes or even extra cuticles. The cell membrane is partly lipid, and therefore will only allow slow permeation of water, but the water will nevertheless build up unless steps are taken to prevent it. If surface tension of the membrane were not enough to resist the osmotic pressure then, by some means or other, water would have to be re-exported through some kind of metabolic pump. A cell of 10 μm diameter, assuming a membrane tensile strength of 5 dynes/cm. (a typical order of magnitude), could withstand only a mere 0.02 atmospheres. This amounts to only 0.8 milliosmolar difference between inside and outside. Osmoregulation is therefore essential, and furthermore it would have to be fine-tuned and presumably under the control of cell size, membrane stretching, or some other such parameter. It has not been established that water itself is actually transported directly in any biological system. .

Therefore various other mechanisms have been proposed. One which is now generally regarded with favour for secretory epithelia has been examined theoretically by Diamond and Bossert (1967), their so-called "standing gradient osmotic theory", and is a scheme whereby Na^+ is pumped out of the cell into a blind cleft between neighbouring cells, to be followed by Cl^- for electrical reasons and by water in osmotic response to the consequent build up of salt in the cleft. This process leads to the outflow of a relatively concentrated salt solution from the cleft. The salt may then be recovered by the cell by inward directed transport or by passive

diffusion of Na^+ down its electrochemical gradient over the external faces of the cells. For this scheme to work there must be specialised regions of cell membrane : in the cleft with high outward rate of Na^+ pumping, low passive permeability to Na^+ , high to Cl^- and high to water, and on the outer faces, higher permeability to salt ions and very much lower to water. The net result of the total process, of course, is the export of water at the expense of metabolic energy. Such a mechanism is believed to exist on frog skin, etc., but variants of it occur frequently in animals. However, the scheme is not without its critics, some of whom seek to revise and extend it (Sackin and Boulpaep, 1975), or others who wish to replace it by alternative, though perhaps less probable, mechanisms (Hill, 1975 a,b). Kidneys in animals and the rectum in insects also perform osmoregulatory functions by a variety of mechanisms which cannot be discussed further here. Of course, maritime plants often have salt glands which serve to relieve the plants of an over-burden of salt. A most useful review of the physics and mechanisms of turgor and osmo- regulation has been presented by Zimmermann (1978), with special reference to plants.

High Salt Concentration.

Reference back to p.14 will indicate that now the value of α to insert in the formula for osmotic pressure, p.20 would be $c_o - \frac{c_i}{2}$ and therefore

$$\Pi_{\text{Donnan}} = RT \left(\frac{c_p}{M} + 2c_o - c_i + c_i - 2c_o \right) = RT \frac{c_p}{M}$$

In other words, the osmotic pressure in the presence of high salt concentration reduces to that produced by the protein alone.

To revert again to the example of a 3 % solution of protein of 60 kD, this time in highly concentrated saline, the osmotic pressure would be in the lowest limit just over 1/100 atmosphere, which is now actually within the capabilities of cell membrane surface tensile strength to contain without osmoregulation. The problem is therefore greatly reduced for sea creatures, and for cells in animals with a blood system, although the Donnan enhancement is not zero even so.

In passing, it may be mentioned that the formula quoted above for the high salt case is the basis of a technique for measuring protein molecular weight developed extensively by Adair and Adair and published in a series of papers beginning in 1925 (Adair). It should be noted that it is necessary to correct for deviation from ideal behaviour (in terms of the van't Hoff formula) by extrapolating a series of results to both zero protein concentration and infinite salt concentration, if accuracy is to be achieved.

Note that no attempt is being made here to construct a general formula for osmotic pressure in a Donnan system because the treatment given above assumes ideal behaviour which does not apply accurately in regard to either the van 't Hoff relation or the use of concentration in place of activity, and again any attempt to extend this to a rigorous treatment soon becomes unwieldy.

Nevertheless, the above analysis serves well enough

to show that there is a clear trend in a Donnan system for osmotic pressure to decline as salt concentration increases. Even in the presence of considerable quantities of salt, the Donnan osmotic pressure, called the "oncotic pressure" by animal physiologists, is by no means negligible, for example between blood and glomerular filtrate before selective re-absorption has occurred in the nephron tubules. A value of oncotic pressure of about 25 mm. Hg is quoted (Bell, et al. 1968) in this case, i.e. about three times the pressure quoted above for a 3 % solution of protein of 60 kD. However, it has to be remembered that (a) there is 9 % protein in plasma, and (b) the Donnan effect is not zero even at the level of salt in plasma.

A further point worth observing here is that as the salt concentration falls in a Donnan system (assuming that time is allowed for equilibration to be reached at all concentrations - i.e. any such experiment were done very slowly), the osmotic pressure will rise to a limiting value, namely $RT(n+1) \frac{q}{M}$, see p. 21, whereas the Donnan potential will continue to increase without limit, as inspection of the equation $E_D = RT \ln \frac{c_o}{c_i}$, derived on p. 13 will indicate.

Estimate of Error due to Ignoring Pressure Work Term.

We are now in a position to return to the point alluded to on p. 16, following discussion of the Nernst equation. (This is a consideration I have never seen mentioned in any text, in regard to the Nernst equation for a Donnan

system, but clearly it cannot be ignored unless it can be shown to be insignificant.)

In deriving the Nernst equation, the difference in electrochemical potentials between the two sides of the membrane for a given ion species :

$$(zFE_i + RT \ln a_i) - (zFE_o + RT \ln a_o)$$

was set to zero. However if there is a pressure difference due to osmosis between the two sides, then the difference in electrochemical potential will equal the pressure work term thus :

$$zF(E_i - E_o) + RT \ln \frac{a_i}{a_o} = \text{osmotic pressure work term}$$

Now this may be evaluated as follows :

$$\left. \begin{array}{l} \text{Compression energy in} \\ \text{compressed volume of liquid} \end{array} \right\} = \int_0^P P dV$$

The compression law gives :

$$V = V_o (1 - \kappa P)$$

where V_o is the volume at zero pressure
 κ is compressibility of liquid
 P is pressure

$$\text{So } dV = -V_o \kappa dP$$

and

$$\left. \begin{array}{l} \text{Energy of compressed} \\ \text{volume of liquid} \end{array} \right\} = \int_0^P -V_o \kappa P dP = -\frac{1}{2} P^2 V_o \kappa$$

(Minus sign implies sense of the energy - i.e. absorbed by the liquid on compression - but it can be ignored for this study where magnitude only is relevant).

We now need to know what volume, V_o , is to be associated with this quantity of compression energy. What is needed is the volume of solution associated with the appropriate amount of solute. The osmotic pressure observed is due to summation of osmotic pressure contributions made by each of the ionic and molecular species present, and it is the total Donnan osmotic pressure which leads to the compression energy. The Nernst equation itself is concerned with only one ion-species at a time but the difference in free energy between the two sides due to osmosis is not necessarily caused chiefly by the ion under consideration, any more than is the potential across the membrane (which also leads to an energy difference). Indeed the ion species under consideration "sees" a difference in free energy between the two sides caused by both electrical potential and osmosis, and all ion species in the system see the same total free energy difference. However in assigning the volume, V_o , it is appropriate to choose the ion species that makes the chief contribution to osmotic pressure to assess the error arising from ignoring the pressure work term. Clearly to make a rigorous and full analysis for all ions present, it would require a very tedious and complicated calculation; it will soon appear however that this is not worth doing. Therefore the volume associated with the most abundant ion species present will be taken, even though not complete.

In the example given, of a 30 % solution of 60 kD protein carrying 90 charges/molecule, the concentration

of Na^+ associated with the protein will be the most abundant ion present, and will be at $\frac{30}{60k} \times 90 \text{ M} = 0.05 \text{ M}$. Thus the volume of solution associated with 1 mole of Na^+ is 20 l. Note that there will also be extra Na^+ which will have diffused in from outside, together with its associated counterion (probably Cl^-), so that V_o will be over-estimated in the figure above, setting a maximum limit on the compression energy.

Therefore, if one substitutes for K , $4.6 \times 10^{-10} \text{ m}^2 \text{ N}^{-1}$, the compressibility of water, and for P , 1 atmosphere, being the osmotic pressure calculated for the 3% solution of protein in low salt solution, then :

Compression energy $= \frac{1}{2} P^2 V_o K$ comes to 4.73×10^{-2} joules/mole Na^+

which appears to be the correct value to insert to examine the error in the Nernst equation.

What is this compression energy equivalent to, in terms of either $\frac{a_o}{a_i}$ or E_{i_o} ?

1. If it all appeared in $\frac{a_o}{a_i}$ then :

$$\text{deviation of } \ln \frac{a_o}{a_i} = \frac{4.73 \times 10^{-2}}{RT} = 1.9 \times 10^{-5}$$

So the multiplier to correct $\frac{a_o}{a_i}$ would be $e^{1.9 \times 10^{-5}}$ which is 1.00002, an error of 2 parts in 100,000 !

2. If it all appeared in E_{i_o} then :

$$\begin{aligned} \text{deviation of } E_{i_o} &= \frac{4.73 \times 10^{-2}}{zF} \times 1000 \text{ mV} \\ &= 4.9 \times 10^{-4} \text{ mV,} \end{aligned}$$

which in comparison to commonly encountered values of E_{i_o} of the order of 100 mV, represents an error of

1 part in 200,000 - equally insignificant.

Conclusion : It is therefore perfectly justified to ignore the compression energy term in deriving the Nernst equation (which is fortunate, since its inclusion would lead to intractable calculations !) This result should be compared with the Gibbs-Duhem treatment of the effect of pressure on electrode potentials, with equally slight magnitude.

Surface Donnan Effect. Zeta Potential.

Finally, before leaving the Donnan system, mention should be made of the surface Donnan effect. This is the situation which applies near the surface of a charged membrane or large charged macromolecule. The point of nearest approach to such a body, for practical purposes, is the shear surface between the free solvent and solvent bound in an unstirred layer to the surface. At the shear surface there will be an electrical potential, governed by the local charged surface, and this is normally designated the zeta-potential, ζ . However, at all planes parallel to the surface in the solution, as one comes away from the shear surface, there will be local potentials, which may be designated ψ , falling to zero at infinite distance according to an exponential law (for a justification of this statement, see Appendix I). Now we may see that the influence of charged membrane, etc., will be expressed in deviations in the local concentrations of ions in the electrolyte solution in contact with the membrane. Of course, the local concentration of any ionic species will differ from that at infinite distance,

i.e. in the bulk of solution in contact with the membrane, to an extent given by the Nernst equation, just as in the Donnan system :

$$\psi = \frac{RT}{zF} \ln \frac{a_{\infty}}{a_{local}}$$

where a_{∞} and a_{local} refer to activities at infinite distance and close to the surface,

or
$$a_{local} = a_{\infty} \exp(-zF\psi/RT)$$
 (where $\exp(x) = e^x$).

Clearly the greatest difference of local ionic concentrations from that in the bulk phase appears at the shear surface, governed by the ζ -potential. A consequence of this surface Donnan effect is that pH is not the same at the surface of a macromolecule or membrane as in the bulk phase, indeed for the usual case of a negatively charged surface, the pH at the surface will be lower, governed by the expression :

$$pH_{local} = pH_{bulk} + \frac{\zeta}{58} \quad \text{at } 20^{\circ}\text{C.}$$

where ζ is in mV, with its sign.

How much the pH, etc., will differ from the bulk phase, and also the value of the ζ -potential at the shear surface, depend on the ionic strength, as explained and defined in Appendix I. Thus, as with the Donnan system considered earlier, one would not expect surface pH to differ from bulk pH in high salt solutions as much as in low. This effect of salt concentration on the surface Donnan effect has important consequences in several contexts, namely :

1. Interpretation of experiments to determine pH optima

of enzymes.

2. Gating behaviour of ions and drugs at the surface of active membranes.

3. The form, and therefore the range, of ζ -potential profile (i.e. the local values of ψ) as one comes away from a charged surface. This is relevant to the phenomena of electro-osmosis and streaming potential, and will be discussed in regard to artefacts in micro-electrode measurements, p.247.

The concept of ζ -potential is clearly only of limited usefulness. The trouble is that the value of the ζ -potential at the shear surface varies depending upon the ionic strength of the solution. The quantities in any given system which govern the value of the ζ -potential are the ionic strength, as mentioned, and the charge on the surface. Unfortunately, even this latter itself is not constant since the amount of charge will depend on pH via the dissociations of carboxyl, hydroxyl and quaternary ammonium groups, etc., and on conformational changes resulting in the pairing or unpairing of oppositely charged groups on the surface of large macromolecules. Clearly the subject is extremely complicated, and cannot be pursued further here.

Non-Equilibrium Processes at Membranes.

It has already been shown how an electrical potential can appear across a membrane (henceforth to be called a transmembrane potential or TMP) in a purely passive system at equilibrium. However there are several other circumstances in which transmembrane potentials can appear, all of which arise in non-equilibrium, but steady-state conditions. For the purposes of this discussion equilibrium is defined as any process in which there is no change in entropy with time, which means it will stay as it is for ever without expenditure of energy. Conversely a steady state is defined as one in which entropy is steadily increasing with time and in which certain parameters remain constant in value, but only at the expense of the continuous discharge of some form of energy from a lower to a higher entropy state. Such a system will, of course, run down to some kind of equilibrium after the energy source is exhausted. How long it takes to run down depends on the rate constants of the system, and therefore if the time is long, care has to be taken not to mistake a decaying quasi-steady-state, as one could call such a case, for an equilibrium, since no energy input is being provided in any obvious way. One should perhaps reserve the term "steady-state" for those cases where a continuous input of energy at a steady rate maintains the system, although it should be observed that this merely widens the system, and that it is still a quasi-steady-state if one includes the system which supplies the energy. There are some very common and important examples of

decaying quasi-steady-states and often, as will be indicated later, equations derived from equilibria can be used to describe to a very close approximation such systems, since in the limit at which the time to run to true equilibrium is infinite, we would be dealing with an equilibrium for all practical purposes - indeed there can be no philosophical distinction, as such a distinction becomes devoid of meaning in this limit.

Of importance in relation to the ionic relations of living organisms are the following :

Equilibrium States :

Donnan system - as already described.

Decaying Quasi-Steady States :

Liquid junction potential

Membrane diffusion potential

Streaming potential (arises in steady-state also)

Transmembrane potential in cells treated with transport pump blocking drugs

Changes in transmembrane potential occasioned by changes in the bathing medium.

These decaying quasi-steady states will now be described in turn, followed by steady states, which deserve a special section, since we are here referring to the most important example of all in biology, the resting membrane potential - which is not a decaying quasi-steady state - but more of that later.

Liquid Junction Potential.

An electrical potential difference will arise across the boundary between two differing electrolyte solutions in most cases without the presence of a membrane.

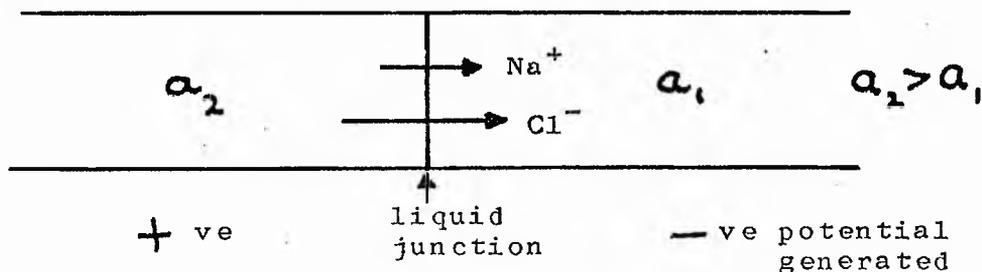
This may be understood to be so as follows :

Suppose that two solutions of NaCl at different concentrations are brought into contact across a liquid junction.

Both Na^+ and Cl^- ions will tend to diffuse down their concentration gradients. However, the mobilities, u , of the two ions are not the same. Na^+ ions move at a rate of 4.44 $\mu\text{m}/\text{sec}$ in a voltage gradient of 1 V/cm., whereas the figure for Cl^- is 6.84. (For a discussion of mobility units, see Appendix 2).

Now it should be recalled that measurement of mobility is, in effect, a measure of diffusion rates, D , for ions of the same charge,* and therefore the figures show that Cl^- ions diffuse faster than Na^+ ions and therefore we would expect the weaker solution to become charged negative with respect to the stronger, the p.d. appearing across the liquid junction. The value to which the p.d. will settle will be just enough so that the potential gradient retards Cl^- migration and accelerates Na^+ migration until the two are equal, an electrophoretic effect, so that total salt diffusion results, as required to preserve charge balance within each bulk phase in contact across the junction - see earlier, p. 11.

* Indeed the relationship is $u = \frac{zF}{RT} \cdot D$. See Appendix 4.



It is obvious here that we do not have an equilibrium situation, and it is clear that the potential difference in this system arises as a result of diffusion and furthermore is maintained in existence by the continued diffusion of the salt, resulting in actual movement. Liquid junction potential therefore arises in a decaying quasi-steady state and it would be correct to call this a diffusion potential. It is to be contrasted with the Donnan system wherein the potential difference across the membrane arises due to an initial diffusion but reaches its maximum and is maintained at equilibrium when no further net movement of salt occurs. This is correctly an equilibrium potential. This distinction between diffusion potential and equilibrium potential has been made explicit here (and all subsequent usage in this text will follow these definitions) because regrettably these terms are not always correctly used in published research literature, causing much confusion.

The potential difference across a liquid junction when a single mono-mono-valent salt is diffusing down its concentration gradient, as above, is given by a special case of the Henderson equation (which will

be given later in its general form) :

$$E_{1|2} = \frac{RT}{F} \cdot \frac{u_+ - u_-}{u_+ + u_-} \ln \frac{a_2}{a_1}$$

where : $E_{1|2}$ is potential of solution 1 with respect to solution 2 across the junction

a_1, a_2 are activities of solutions 1 and 2 respectively

u_+, u_- are the mobilities of the cation and anion respectively.

Alternatively a liquid junction could be established between solutions of two different salts at the same concentration, for example between 0.1 M KCl and 0.1 M NaCl. The Cl^- ions will not immediately move since they have no driving force for diffusion, but the K^+ ions will diffuse into the NaCl solution and the Na^+ ions into the KCl solution. However the mobility of K^+ is greater than Na^+ and therefore the NaCl solution will acquire a positive potential relative to the KCl solution. The establishment of this liquid junction potential will accelerate Na^+ and retard K^+ but also it will then cause a migration of Cl^- from the KCl solution to the NaCl solution. The value of liquid junction potential will be such that, at steady state, the rates of charge transfer by all ions in the two directions are equalised. This situation is described by another special case of the Henderson equation :

$$E_{||'} = \frac{RT}{F} \cdot \ln \frac{u_+' + u_-' }{u_+'' + u_-''}$$

where $E_{||'}$ is the potential of solution " relative to solution ' .

u_+, u_- are the mobilities of cation and anion in the superscribed solutions.

The third case, which is of special importance in electrochemical measurement techniques, is where two dissimilar salts at different concentrations are brought into contact. Let us take as an example, a concentrated solution of KCl in contact with a dilute solution of NaCl. Clearly there is a concentration gradient to drive diffusion for all ionic species present. However the rate of transport of an ionic species depends not only on its mobility but also on its concentration. This will have the result that, once the steady-state liquid junction potential has developed, its value will be determined as before by the condition that the charge transfer in the two directions must balance and hence the salt at higher concentration will dominate the liquid junction potential. In the extreme limit, if one of the salts at the junction were diminished in concentration to zero, it is clear that the liquid junction potential will then be determined entirely by the other salt diffusing down its own concentration gradient, as in the first case, (above p. 36). Therefore, if it so happens that $u_+ = u_-$ for the concentrated solution, then no liquid junction potential may be expected, or at most only a low value. This is the basis of the use of KCl in salt bridges, at near saturated concentration (see later, when discussing microelectrodes).

The general case of a liquid junction is described by the full equation of Henderson (1907, 1908) :

$$E_{"J'} = \frac{RT}{F} \cdot \frac{\sum_n (u_i/z_i) \cdot (c_i'' - c_i')}{\sum_n u_i (c_i'' - c_i')} \cdot \ln \frac{\sum_n c_i' u_i}{\sum_n c_i'' u_i}$$

where $E_{"J'}$ is the liquid junction potential of solution " relative to solution ' .

u_i is mobility
 c_i is concentration
 z_i is valency

} of the i th ionic species.

\sum_n , summed over all species present.

This equation is the result of mathematical integration of the general differential equation for liquid junction potential provided that all layers of solution through the junction region may be regarded as mixtures in various proportions of the two solutions, a condition which therefore becomes an assumption implicit in the Henderson equation. It is however a very reasonable assumption. Nevertheless it should be observed that it is not the only assumption one could take. Planck (1890 a, b) adopted the assumptions that diffusion at the junction could be considered to take place in a porous plug having of itself no influence on the solution in its pores and that mobilities of ions are constant, independent of concentration. These assumptions, the so-called "constrained diffusion boundary" lead to a very complex integration and an equation which could not be made explicit in E_J except in special cases, when the forms concur with those from the Henderson equation. Goldman (1943) made yet another assumption, namely that electric field could be assumed constant throughout a membrane (see Appendix 4), but his equations could equally be applied to a liquid junction.

Membrane Diffusion Potentials.

A potential difference will arise if a membrane displaying a certain type of selective permeability, a so-called permselective membrane, is placed between two dissimilar solutions. A potential difference will arise here even in some case when no liquid junction potential would appear, as for example when the membrane separates two concentrations of KCl. Of course, there are many ways in which a membrane may be permselective. The semipermeable membrane discussed earlier in relation to the Donnan effect was permselective in that it allowed passage of small ions and molecules but restrained macromolecules - it was in fact acting as a molecular sieve. The type which concerns us now however is where the membrane allows either free passage of cations, but is virtually impermeable to anions or vice versa, or allows some ions to pass more freely than others even if of the same sign of charge, e.g. K^+ passes more easily than Na^+ .

It may be seen that a potential difference will arise with such a membrane as follows : Suppose two concentrations of KCl are separated by a cation-permeable anion-impermeable membrane (note that KCl concentration gradients do not produce a potential across a liquid junction when the permselective membrane is absent because $u_+ = u_-$ for KCl). K^+ ions will diffuse ahead of Cl^- because they pass the membrane easier, thus charging the more dilute solution positive relative to the more concentrated. Obviously this situation has close

parallels to the liquid junction potential except that here differential permeabilities cause the effect instead of differential mobilities. Of course, there is no reason why both effects should not appear together and contribute to the transmembrane potential observed and clearly it can be very complicated. It will be referred to again (vide infra, Goldman's equation).

Perhaps it should be remarked here that the transmembrane potentials or resting potentials of living cells have much in common with these membrane diffusion potentials, making this a most important topic, but the question of the origin of potentials in living cells will be dealt with later. The concern here is with purely passive systems which must be understood before active situations can be considered. Of course, the membrane diffusion potential referred to here is again a decaying quasi-steady state in that the potential persists only so long as the concentration gradients persist and diffusion resulting in net movement is necessarily and continuously taking place, which would, in a finite system, eventually dissipate the concentration gradients, unlike in the equilibrium case (Donnan).

Various authors have derived equations for membrane diffusion potentials, and it is not always realised that these equations are essentially the same, some being merely special cases of the more general equations. The whole field of the electrochemistry of membranes has been excellently reviewed in an important paper by

Sollner (1969). It is clear from his review that the history of this field goes back to the 1880's and 1890's with authors such as Nernst, Planck and Bernstein, and somewhat later Henderson, references for whose papers are given by Sollner (1969).

One of the most widely quoted authors on membrane diffusion potentials is Goldman (1943), who derived an expression for the potential, which may be written thus :

$$E_{io} = \frac{RT}{F} \cdot \ln \frac{P_K K_o + P_{Na} Na_o + P_{Cl} Cl_i}{P_K K_i + P_{Na} Na_i + P_{Cl} Cl_o}$$

if put in the practical form usually quoted, where :

P is the permeability of the membrane for the subscript ion.

Element symbols refer strictly to activity, but concentration is often substituted as a best approximation where activity coefficient is unknown.

Subscripts o and i refer to outside and inside solutions.

There are several points to be noted concerning this equation :

1. It is calculated on the assumption that constant electric field applies throughout the membrane. This is rather unlikely to be true of a layered biological membrane, but it was a necessary simplification to enable Goldman to make the calculation. For practical purposes it may not lead to too great an error, and it does give us a starting point. See later, p.172.
2. In the form quoted above, which is derived from equations 16 and 18 of Goldman's paper (1943), P replaces

u (mobility), but in the context of his paper, it is clear that while he uses u , permeability is the relevant quantity for an ion passing through a membrane.

3. Note that all the ions quoted are mono-valent.

Goldman's paper does not deal, for reasons of avoiding complexity, with cases which include divalent, etc., ions. However this limitation is not serious in biological systems since those ions which exist at significant concentrations, that is, those ions which principally determine the membrane potential, are all mono-valent.* Note that the terms for anions are inverted relative to those for cations. If further mono-valent ionic species are to be included, then they are simply added as extra terms in the obvious appropriate places following the analogy of those indicated. Generally K^+ , Na^+ and Cl^- principally determine E_{ω} , being the most concentrated ions present.

4. It is not always realised; indeed I have not seen reference to it; that the Goldman equation, while it refers to a decaying quasi-steady state potential arising while diffusion is actually occurring, caused by the differential permeability of the membrane to different ionic species, does not include any information on the free mobilities of the ions. In other words, it is not complete in that it ignores any liquid junction potential between the two electrolytes. It should be noted that, since net diffusion is actually occurring, without which there would be no membrane potential, ions have to diffuse up to the membrane before passing through

* Except in certain special cases, such as the "calcium spike" of ciliated protozoa, etc. For extension to polyvalent ions, see Spangler (1972) and Shamoo & Goldstein (1977).

it, and then away from it on the other side. This will mean, except in special cases such as an electrolyte of a single salt which happens to have $u_+ = u_-$, e.g. KCl, that a liquid junction potential - or perhaps better called a diffusion gradient potential here - will occur between the bulk phase and the solution at a point very near the surface of the membrane but just outside it. The same will apply on the other side of the membrane. Stirring of the electrolytes might reduce these errors to some extent but (a) stirring at a rate required is hardly likely in living cells even with cytoplasmic streaming and (b) stirring will not cope with the ordered unstirred layers close to the membrane surface.

The saving factor regarding this limitation of the Goldman equation is that the permeability of membranes, certainly those of biological interest when they contain lipid materials, to ions tends to be very much lower than would be that of an equivalent thickness of free aqueous medium, with the result that the diffusional constraints in the system are almost entirely due to the membrane itself and the rates of diffusion are relatively slow so that only very small concentration gradients would build up in the aqueous phases on either side of the membrane. Therefore it would be incorrect simply to add on to the Goldman equation an extra contribution to E_{L} in the form of the Henderson equation evaluated for the two bulk electrolytes as if they were in direct liquid contact. The contribution due to diffusion in the aqueous phases would be less than that, and would

depend on the characteristics of the membrane. Thus a full treatment would lead to a very complicated equation. However this point should be borne in mind if it were contemplated to use Goldman's equation to cope with hydrophilic membranes of very low resistance, when terms involving the free ion mobilities would certainly have to be brought in.

Perhaps it is now appropriate to mention what would happen if, in an experiment to determine transmembrane potential by means of a microelectrode inserted into a living cell, a tear or rent were made in the membrane, or the membrane did not seal to the microelectrode shank. The result of such a leak would be to bring the cell sap into liquid junction with the external bathing medium. Now the potential which would be registered would not be the true transmembrane potential of the whole cell, but the weighted average of the true transmembrane potential and the liquid junction potential between cell sap and external medium, the weighting factors being the input conductance of the whole cell and the conductance of the hole or rent. Fortunately the fatty nature of the cell membrane mostly results in a seal to the glass micropipette of the electrode occurring spontaneously in most well executed impalements. One can usually judge this by the recorded voltage and its steadiness.

5. The Goldman treatment does not include any possible effect on transmembrane potential of any ordered fixed charges in the membrane. It is not immediately obvious that this is unimportant, but this will now be demonstrated.

Selective permeability of a membrane to different ionic species is often caused by the presence in the membrane, as part of its permanent structure, of fixed charge sites, perhaps lining pores or ionophore channels. The theory of ion permeation through membranes has been the subject of much work by many authors, but, as Eisenman(1962) points out, the permeability of a membrane for any ionic species is unexceptionably given by the "free energies of interaction of the cations (he is considering the permeation of cations at this point) with water on the one hand and with anionic "sites" in the glass (i.e. in this case what the membrane he is considering happens to be made of) on the other". While this statement is undoubtedly true, in practical terms it is not very useful and we must be more specific, and so he goes on to show that permeability depends on :

1. Charge, radius and hydration energy of the permeating ion
2. Charge and field, i.e. charge density, of charged sites in the membrane.

Thus permselective membranes invariably carry fixed charges in their structure to confer this permselectivity. However a further consequence may arise from this.

If charges are fixed, then, in an ordered system, the chances are they will be fixed in such a way that each charged molecule of the same type has the same orientation as its fellows, especially in a biological membrane where vectored biosynthesis and thermodynamics determine the structure. Now it is perfectly possible, indeed probable, that molecules such as protein, assembled

into a membrane on the Singer model (e.g. Singer 1971, after Lenard & Singer 1966) will constitute oriented dipoles in the membrane. This sort of structure is reminiscent of the "electret". We shall now therefore consider the electret and its possible rôle, if any, in the development of a transmembrane potential.

Electrets.

The possibility of making devices, later to be named "electrets" by Heaviside, was first shown by Eguchi in 1925. He constructed his electrets by melting a wax, such as beeswax or Carnauba wax or a mixture of the two, and subjecting the melt to a high electric field, several 1000 V/cm., and allowing it to solidify while under this field. The waxes he used were polar, and it has since been discovered that non-polar waxes such as paraffin wax, produce poor electrets or fail to show any electret properties. The device so produced has properties which may be described as the electrostatic equivalent of the magnet, hence the name. The dipolar molecules of the wax were locked in the solid, as it froze, in the oriented position so that a permanent dipole moment was displayed by the whole slab of wax.

Of course, it has long been known that crystals in certain crystal classes involving a lack of a centre of symmetry (Phillips 1951) can display both piezo-electricity (the appearance of charge displacement across opposing faces, under pressure) and pyro-electricity

(charge displacement with change of temperature), but these effects persist only so long as the imposed stress remains and/or until the potential generated is discharged electrically. The distinction between piezo-electricity in crystals and the behaviour of electrets is important to an understanding of the rôle of fixed dipoles in the living cell membrane, and so it will now be pursued as follows.

If one considers a passive material such as wax or a crystal with a built-in electric dipole moment, it is clear that any voltage difference which may appear between its external surfaces could not be expected to persist. Even in air, such a device would be in the presence of a few free charges producing a conductivity through air between the front and back faces not quite zero, and neither can the material itself be expected to be a perfect insulator. Thus, any initial voltage difference between its faces would not be able to persist indefinitely. This is what we find with piezo-electric crystals under steady pressure, but surprisingly electrets made of wax do show what appears to be a permanent voltage difference across their faces, often reversed in sign from the polarising voltage used during formation (so-called heterocharge electrets). This property of electrets was initially very puzzling since it is clear that there is no source of energy in an electret, as there is in an electrochemical cell (e.g. Leclanché cell) to regenerate the charge displacement. It should be noted

that the persistence of a permanently oriented set of polar molecules would not be detectable externally or directly once the initial surface charges have been dissipated because one would expect migration of mobile charges within and outside the material to lead to neutralising charges of opposing sign clustering round the head and tails of each dipole.

The solution to this puzzle was first presented by Adams (1927), who proposed that the charge displacement is regenerated in the electret precisely because the bulk orientation of dipoles is not permanent. It appears to be permanent because electrets can be stored for many years, especially in dry conditions between "keepers" (shorted foils) without any noticeable loss of electret properties.. It so happens however that the time constant of decay of the dipolar orientation is very long but that because the effective electrical capacity between the faces of the electret is small (a few pF/cm²) a remarkably high potential difference between faces can be developed with such a slow decay of dipolar orientation. This, of course, also depends on the fact that the wax has a very low conductivity. Adams has derived expressions for the behaviour of the electret which appear to be substantially borne out by experiment. His expression for the free charge on the surface, η , is :

$$\eta = \frac{\lambda P_0}{\beta - \lambda} (e^{-\lambda t} - e^{-\beta t})$$

where : λ is the rate constant of decay of polarisation, P , according to the reasonable assumption that it is first order, following $\frac{dP}{dt} = -\lambda P$.

- P_0 is the polarisation at time $t = 0$, when η is also taken to be 0.
- β is the "surface conductivity" = $\frac{4\pi}{\kappa\tau}$ where (as Adams calls it)
- κ is the dielectric constant (permittivity relative to vacuum)
- τ is the specific resistivity of the material of the electret.

What this equation indicates is the way in which charge builds up across an initially shorted electret, once the short is removed. Of course, the voltage between faces is related to η by the usual capacitor formulae, but is limited by the breakdown voltage of air - 30 kV/cm. - which remarkably is frequently achieved by electrets! At moderate temperatures the rate constant of decay of polarisation, λ , is very small compared to the "surface conductance", β , so that the equation reduces to :

$$\eta = \frac{\lambda P_0}{\beta} (1 - e^{-\beta t})$$

which will run to a maximum, at a long time, of :

$$\eta_{\max} = \frac{\lambda P_0}{\beta}$$

Adams further discusses the piezo-electric and pyro-electric properties of electrets, but that need not concern us here.

Of course, the distinction between electrets, where a persistent potential difference is observed, and piezo-electric crystals, where the potential difference dissipates while under steady pressure, is now clear. In the crystal the dipolar molecules are permanently locked in position by the crystal lattice whereas in the electret, relaxation is possible, and this regenerates the potential

difference continually. This property of electrets has enabled them to be used in certain technical applications such as position transducers and microphones (the latter being first introduced by the Japanese in World War II much to the puzzlement of Western technicians at the time!).

Returning now to the biological membrane, at first sight it might appear that fixed dipoles in the membrane could be responsible for some part of the observed transmembrane potential. However the above discussion of electrets now makes it clear that this is unlikely for two chief reasons. Firstly, the membrane, although a dynamic structure in the sense that it is being continually resorbed and regenerated, tends to maintain a fairly constant average structure. The exceptions to this are in growing cells - although that is probably merely a matter of more of the same - and in cells undergoing some biochemical adjustment, such as specialisation. Where the membrane structure is constant, no potential will be generated by any mechanism of the electret type, since potential generation across the electret depends on decay of polarisation. Secondly, and probably crucially, the cell membrane exists in contact with conducting aqueous electrolyte solutions and itself has a considerable conductivity due to ionophore channels. Incidentally, the high conductivity of the internal and external media would not of itself, in this case, dissipate the potential produced by any electret mechanism, because the cell is entirely enclosed in a continuous cell

membrane, thus providing no path for conduction between inside and outside of the cell (cf. air conduction path between opposing faces of a wax electret). The exception to this is of course found in the ionophore channels which raise the conductivity from that of insulating dielectric by several orders of magnitude.

Although, as it turns out, the presence of fixed dipoles in the membrane makes no significant contribution to membrane potential in living cells, this point seems to have been very little discussed in published literature, hence the reason for discussing it at length here. A short note was published by Berg (1968), including much less detail, in which he comes to similar conclusions.

Streaming Potential (and other electrokinetic phenomena).

In this text the question is being addressed as to possible sources of transmembrane potential in the living cell and of errors which can occur in the measurement of transmembrane potential. It is therefore necessary to mention streaming potential. However, although streaming potential is probably the most important in the biological context, it must be observed that streaming potential is actually only one of a class of related effects known collectively as the "electrokinetic phenomena". All these phenomena arise in the presence of electrolyte solutions and a surface carrying a charge - in other words where the surface displays a ζ -potential, the distinguishing feature being the presence of an insoluble matrix bearing charged sites. The complete set of electrokinetic phenomena is displayed in Table 1, overleaf, together with the quantitative relations which apply (see Butler 1951).

It will be seen that all these effects arise when there is relative movement between the charged surface and the electrolyte solution, either as cause or effect.

The phenomenon of streaming potential is of importance in the biological context in the following situations :

A. In the living material itself :

1. During uptake of the bathing medium by osmosis. The osmotic drive is for the uptake of water, but since the external bathing medium contains dissolved mineral ions, these will be carried

TABLE I.

ELECTROKINETIC PHENOMENA

Phenomenon	Causative Force	Resultant Effect
Streaming Potential	Pressure Difference	Electrical p.d. between high and low pressure zones
Dorn Effect	Gravity	Electrical p.d. along direction of gravitational gradient
Electro-osmosis (converse of streaming potential)	Electrical potential difference	Movement of electrolyte or pressure development
Electrophoresis (converse of Dorn effect)	Electrical potential gradient in electrolyte	Movement of charged particles through electrolyte solution

For symbols, limitations, and assumptions, see next sheet.

When it occurs	Quantitative Relations
When electrolyte solution is forced to stream through porous plug or capillary	$E = - \frac{3D}{4\pi\eta\kappa} \cdot P$
When charged particles move through electrolyte under gravity	$X = \frac{3D(\rho - \rho_s)g}{3\pi\eta\kappa}$
When a fixed porous plug or capillary separates two volumes of electrolyte	$Y = \frac{a^2 3D}{4\eta} \cdot X$ <p>for volume flow unopposed by pressure</p> $P = \frac{23DE}{\pi a^2}$ <p>for pressure to stop flow</p>
When charged particles or macromolecules are suspended in electrolyte solution.	$u = \frac{D\zeta}{6\pi\eta} \quad \text{Hückel}$ <p>for "small" spherical particles</p> $u = \frac{D\zeta}{4\pi\eta} \quad \text{Helmholtz-Smoluchowski}$ <p>for "large" particles of any shape</p>

Electrokinetic Phenomena. Notes on Table 1.

Symbols :

E	= potential difference	} between ends of porous plug or capillary
P	= pressure difference	
X	= electrical field gradient in electrolyte solution	
κ	= specific conductivity of electrolyte solution	
η	= viscosity of electrolyte solution	
D	= dielectric constant of solvent	
ζ	= ζ -potential at surface of shear on solid or particle	
V	= volume transported per unit time	
ρ	= density of particle	
ρ_s	= density of solution	
g	= gravitational acceleration.	
u	= mobility of particle.	

Limitations and Assumptions, etc.

1. In the derivation of the streaming potential relation it was assumed that the pore or capillary bore is much larger than the effective thickness of the electrical "double-layer".
2. Streaming potential is independant of capillary bore, with the proviso of 1, above.
3. The constancy of E/P in streaming potential only holds good for low pressures, i.e. pressures below 1 atmosphere.
4. In the Dorn effect, and in electrophoresis, "particles" must be large enough that the applied field (X or g) can produce a frank migration and not merely a concentration or population gradient counteracted by diffusional dispersion.
5. Electro-osmosis effects are dependant on the square of bore radius, therefore statistical information is needed with a porous plug with non-uniform channels.
6. In electrophoresis, "small" means when the particle size is of the order of the "double-layer" thickness and "large" vice versa.

along with the solvent and will tend to create a streaming potential. This effect may therefore be expected with growing cells.

2. During flow of xylem sap up a tree under transpiration tension.
3. During pinocytotic uptake of bathing medium by a cell - this situation is not quite the same but rather similar. The charged surface of the membrane, on invagination, will take up a drop of solution not quite identical in ionic composition to the bathing medium, due to its proximity to the charged surface. (This is more of a speculative suggestion!)

B. In measuring techniques applied to living cells :

Micro-electrodes are generally constructed using a micropipette, and the insertion of the tip of such a structure into the cell may lead to a streaming potential if there is a positive turgor pressure inside the cell. Is this effect important? The question will be examined in detail later (Ch. 2 p.200)

It is perhaps rather unexpected that the streaming potential difference produced between the ends of a capillary should be independent of the bore (see Table I) especially when (as is assumed in the derivation) the ζ -potential profiles of the two opposing walls are far apart. The explanation lies in the fact that pressure difference determines the rate of flow, and that increasing

the diameter greatly increases the flow rate for a given maintained pressure difference, thus also increasing the charge transfer rate in the layers of solution near the wall wherein there exists an imbalance between populations of anions and cations, while a compensatory increase occurs in the return path conductance of the electrolyte in the bore of the tube. It must be remembered that the observed streaming potential is the expression of a balance between charge displacement due to streaming at the edges, and its dissipation by return conductance down the centre of the capillary.

It is now clear that micro-electrodes may well be expected to produce an artefactual streaming potential in the tip of the micropipette, the more so for the fact that the dimensions are there so small ($\sim 0.2 \mu\text{m}$) that ζ -potential profiles may well overlap, a situation likely to produce much higher streaming potentials than predicted by the formula in Table 1, based on calculations which assume a broad bore tube in which ζ -potential profiles do not overlap.

Transient Effects on Transmembrane Potential.

In conclusion of this section on decaying quasi-steady states, mention must here be made of the effect of blocking drugs, etc., and of changes in the bathing medium on the observed transmembrane potential of living cells.

It is well known that a sharp change in transmembrane potential is produced when the bathing medium around a living cell is changed, especially when the chief change is in the external concentration of a permeant ion. Changing the concentration of a relatively impermeant ion, when in the presence of others much more permeant, may not be expected to have much effect. However it is not always realised that such a step-change in transmembrane potential is in the nature of a transient effect - at least, some part of it will be. In the next section I go on to consider the origin of transmembrane potential and its value, but suffice it here to say that a change in transmembrane potential occasioned by a change in bathing medium cannot be more than merely temporary because the balance of forces that produce it (see next section) will have been upset. Temporary is, of course, a relative term. How long it will take the cell to run to a new steady-state transmembrane potential depends on rate constants relevant to the system, and if these are long the effect observed may appear to be permanent as far as the practical duration of an experiment is concerned. This particular effect, while

certainly observed, has been the source of some misunderstanding. Of course, it is true that the value of the step-change in transmembrane potential observed in such experiments is in accord with the Goldman equation, but it would be incorrect to conclude from this that the transmembrane potential has its origin in processes to which the Goldman equation strictly applies. Thus the Goldman equation gives the value of transmembrane potential to be expected when two dissimilar solutions are set against each other with a permselective membrane between them. The misunderstanding arises if one concludes from the fact that this happens to give a reasonable agreement with the observed value that the Goldman equation in some way explains the existence of the transmembrane potential - it does not - see next section.

The effect of drugs which either block ion channels (e.g. TTX for Na^+ channels) or open up high conductance channels (e.g. actinomycin) may be expected to cause a step-change in transmembrane potential in an analogous fashion to changes in medium. The mechanism is different but the effects are similar, and are accounted for by the Goldman equation (in the short term), where permeabilities are being manipulated instead of concentrations, as in the case of the bathing medium changes mentioned above.

Further consideration of the Goldman equation must be made in regard to so-called "electrogenic pumps", see p.179.

The Steady State. Origin of Resting Transmembrane
Potential in Living Cells.

So far the cases discussed have been the equilibrium state (Donnan system) and certain decaying quasi-steady states. An equilibrium cannot account for the origin of the transmembrane potential observed in living cells. The reason is because the steady state observed in a cell had to be set up by active processes acting on indifferent surroundings. Life processes invariably produce local order of a kind which had not existed there before, but this is always at the expense of an increase in entropy in the wider environment. It may be that equilibria (as will presently appear) will be associated with the living system, but wherever they appear, they will invariably have been created by that living system, and can therefore in no way be used to explain their own origin. Also a decaying quasi-steady state is not what pertains in cells because such a state refers to a mechanism that is running down. It is however proper to describe the resting transmembrane potential of a living cell as a steady state. What is now being addressed is the question as to how the steady state transmembrane potential arose and is maintained.

Transmembrane potential is often described as being essentially a potassium diffusion potential, usually with the qualification that the actual potential is deviated from the equilibrium potential by a small amount caused by the presence of other ions. Such a

concept is, in my view, very misleading. It may be true that the potential observed is close to the potassium equilibrium potential, but it is not correct to infer from this that it is the diffusion of potassium which creates the potential. It does not.

It has already been shown that two dissimilar solutions of electrolytes in contact will lead to the appearance of a potential difference between them whether the contact be across a liquid junction (to which the Henderson equation applies, p. 36) or whether the contact be through a permselective membrane (to which the Goldman equation applies, p. 41), but the same problem arises with these relations as in the case of the Donnan system. It was pointed out (p. 9) that the Nernst equation related concentrations (strictly, activities) and the membrane potential in a Donnan system at equilibrium, but that this relation did not help us to decide what either the ratio of ionic concentrations or membrane potential would be, unless we knew the other one already. So it is with the Goldman equation as applied to the living cell. We can calculate the transmembrane potential from already known values of concentrations and permeabilities, but the equation does not help us to decide what values the internal concentrations of a cell would set themselves to, when the cell is bathed in a particular medium. The Goldman equation is therefore of limited value in the predictive sense. It cannot tell us how the steady state transmembrane potential would respond to changing the bathing medium in which the cell is

placed except in the short term, as observed in the previous section, p. 57. However the short term effect is a decaying quasi-steady state, and eventually after a change of bathing medium, the potential will drift to a new value - but what would we expect that value to be? For this the Goldman equation offers no assistance.

In order to proceed from here, it is necessary to assume that the membrane carries a vectorial transport pump for one or more ionic species, and that this pump is capable of generating and maintaining a certain maximum level of free energy difference across the membrane. The action of this pump generates the potential (whether it is directly "electrogenic" or not - see later) and creates the concentration gradients. Having created these gradients, it is true to say that to a fair approximation for many, but not necessarily all,* commonly encountered living systems, the potential relates to them by an equation such as that of Goldman, but it must be pointed out that this is only so for reasons essentially fortuitous, and may not always be so, as will appear presently. It is now of interest to examine models in which we can derive the transmembrane potential from imposed parameters. We will start with the simplest case, and build from there. The treatment which follows is original to myself. No reference to a similar approach has been found in published literature, surprisingly, despite careful search. Comparison with other approaches will follow this treatment.

* see p.179.

Let us now consider the simplest system. Consider a closed membrane, such as a cell would have, in NaCl solution (and no other ions), but for the moment without ion transport pumps. We also assume no trapped macromolecular ions to be present, so we do not have any Donnan system, and therefore no transmembrane potential on that account. Of course, so far, as set up, the concentration of NaCl inside will be identical to that outside at equilibrium, with no transmembrane potential. Now suppose we introduce into the membrane an active export pump for Na^+ ions only. We also assume that it is capable of pumping Na^+ ions very fast compared to back leakage through the membrane. The analogy can be drawn here with a centrifugal-type water pump - it is capable of fast flow at zero head and zero flow at a certain maximum head. Suppose such a water pump carried a vertical pipe on the outlet somewhat higher than this maximum head, and suppose we then introduce a small leak in the pipe at the bottom near the pump. Provided the leak rate is not large compared to the flow rate capabilities of the pump, the head developed will be little depressed from the maximum with no leak. Thus, returning to the Na^+ pump, we can say that the head of electrochemical potential difference across the membrane will be, at steady state, virtually equal to the potential energy available from a metabolically fuelled transport system, provided also that it is fast compared to the leakage rate. Thus at steady state :

$$\Delta \bar{\mu} = (zFE_i + RT \ln Na_i) - (zFE_o + RT \ln Na_o)$$

or, rearranging, and using E_m to represent E_{i0} in line with conventional physiological usage :

$$\Delta\bar{\mu} = zFE_m + RT \ln \frac{Na_i}{Na_o} \dots \dots \dots (1)$$

where $\Delta\bar{\mu}$ is the electrochemical potential difference eventually and actually attained at steady state.

Its value will be negative for ions pumped out, as defined in Eqn.(1). (Of course, in the equilibrium state of a Donnan system $\Delta\bar{\mu} = 0$, and so we get the familiar Nernst equation). In Eqn.(1), Na_o is known and $\Delta\bar{\mu}$ may be equated to a first approximation with enthalpy change in, for example, the hydrolysis of ATP to ADP. However, in this treatment, the fact that processes are occurring at finite rates, which should require treatment by the formalism of irreversible thermodynamics, is being avoided by deliberately assigning $\Delta\bar{\mu}$ to be the actually achieved level of electrochemical potential difference for the pumped ion.

This matter will be considered further later.

Neither Na_i nor E_m are known in advance, but if Cl^- ions are passively distributed, then we can say that every Na^+ ion pumped out will be accompanied by a Cl^- ion which will follow in order to maintain ion balance within each solution phase, as observed earlier (p.10). Therefore we can say that ion balance determines that $Na_i = Cl_i$ and $Na_o = Cl_o$, there being no other ionic species present in this case. However, since Cl^- ions are passively distributed, they will obey the Nernst equation :

$$E_m = \frac{RT}{F} \ln \frac{Cl_i}{Cl_o} \dots \dots \dots (2)$$

Here is a case of an equilibrium associated with the steady state, but of course, it has its origin in the situation created by active processes and is not sui generis.

Indeed it can be said that the pumped ion in effect sets up a Donnan system for all other ionic species, while its own concentration gradient, maintained as a steady state, is equivalent to the trapped macro-ion of a true Donnan system. All other non-pumped membrane permeant ions will therefore distribute themselves passively just as in a Donnan system.

It is now possible to evaluate E_m since the ion balance condition allows us to replace Cl^- by Na^+ in equation (2), with subscripts the same way about.

Thus :

$$\Delta\bar{\mu} = FE_m + FE_m$$

\uparrow from (1) \uparrow from (2) in place of
 $RT \ln \frac{Na_i}{Na_o}$

(z = 1)

Hence :

$$\underline{\underline{E_m = \frac{\Delta\bar{\mu}}{2F} \dots \dots \dots (3)}}$$

It should be remarked that equation (3) has been derived ignoring any osmotic pressure contribution. However it is fair to ignore this for reasons already stated with regard to the derivation of the Nernst equation for the Donnan system, see p. 27.

Equation (3), then, gives a value for the steady state resting transmembrane potential, and it is interesting to observe, in face of the usual statements made about transmembrane potential, that this has here

arisen in a circumstance in which potassium is absent, and so the potential cannot be a potassium diffusion potential. It is also interesting to observe that the value of the potential is here independent of the concentration of NaCl in the bathing medium, subject of course to the condition that NaCl concentration has no influence on the efficiency of working of the sodium export pump, and hence on the value of $\Delta\bar{\mu}$.

If the hydrolysis of ATP were the energy source then $\Delta\bar{\mu} = 31 \text{ kJ/mole}$, and for a single ion transfer per ATP, this results in a value of E_m of -160 mV , at maximum.

In the presence of KCl.

We must consider, next, what would happen if we were now to introduce KCl also, into the bathing medium. We will assume that K^+ is not pumped for the present, but that Na^+ is actively exported, as before. At steady state, the ion balance condition demands that inside $Na_i + K_i = Cl_i$ and outside $Na_o + K_o = Cl_o$. As before, equation (1) applies for the sodium pump. Both K^+ and Cl^- are now passively distributed at steady state and so :

$$E_m = \frac{RT}{F} \ln \frac{K_o}{K_i} = \frac{RT}{F} \ln \frac{Cl_i}{Cl_o} \dots \dots (4)$$

which for these ions represents equilibrium (Nernst).

Replacing E_m in equation (1) by RH side of equation (4).

we have :

$$\Delta\bar{\mu} = RT \ln \frac{Cl_i Na_i}{Cl_o Na_o}$$

or, rearranging :

$$\exp\left(\frac{\Delta\bar{\mu}}{RT}\right) = \frac{Cl_i Na_i}{Cl_o Na_o} \dots \dots \dots (5)$$

But $Na_i + K_i = Cl_i$ for ion balance, and from (4) :

$$K_i = K_o \exp(-FE_m/RT) \quad \text{and} \quad Cl_i = Cl_o \exp(FE_m/RT)$$

and so :

$$Na_i = Cl_o \exp(FE_m/RT) - K_o \exp(-FE_m/RT)$$

and so, replacing Cl_i and Na_i in equation (5) :

$$\exp\left(\frac{\Delta\bar{\mu}}{RT}\right) = \frac{Cl_o \exp\left(\frac{2FE_m}{RT}\right) - K_o}{Na_o}$$

After re-arrangement, this yields :

$$E_m = \frac{RT}{2F} \ln \left\{ \frac{K_o}{Cl_o} \left[\frac{Na_o}{K_o} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + 1 \right] \right\} \dots (6)$$

This is the required general equation for this case.

However there are three extreme cases worth examining which allow of simplification :

1. If $\frac{Na_o}{K_o} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) \ll 1$ then $E_m = \frac{RT}{2F} \ln \frac{K_o}{Cl_o} \dots (7)$

This applies if either :

- (i) $Na_o \ll K_o$, unlikely in a biological situation, or
- (ii) $\Delta\bar{\mu} < 0$ and large in magnitude compared to RT .

A negative value of $\Delta\bar{\mu}$ is exactly what we do expect from the outward pumping of Na^+ on the definition implicit in Equation (1), since $\bar{\mu}$ for Na^+ outside is clearly higher than $\bar{\mu}$ for Na^+ inside if Na^+ is pumped out. It is also likely that $|\Delta\bar{\mu}| \gg RT$ since $\Delta\bar{\mu}$ represents the hydrolysis for example of ATP, which yields in itself 31 kJ/mole, but see later, whereas

$RT = 2.44 \text{ kJ/mole at } 20^{\circ}\text{C. or } 2.58 \text{ kJ/mole at } 37^{\circ}\text{C.}$

It therefore appears that Equation (7) may be expected to apply to active Na^+ extrusion, and it gives the interesting result this time that E_m is largely independent of the value of $\Delta\bar{\mu}$ and therefore it does not much matter what hydrolysis of which energy carrier, or indeed what other energy coupling, serves to fuel the pump, provided that it achieves a head for Na^+ in which $|\Delta\bar{\mu}| \gg RT$, which indeed it is likely to be for almost any energy coupling mechanism one might propose, on the assumption that there are enough pump sites in the membrane capable of running fast enough to overcome back diffusion of Na^+ by passive leakage - which usually will be so. The value of E_m however does depend on the external concentration of K^+ and Cl^- , which should be contrasted with the previous simplest case, wherein the concentration of external medium had no effect on E_m . The situation here can be generalised a little further. We have considered only Na^+ , K^+ and Cl^- . Real biological situations are likely to contain other ionic species also. An immediate extension can be made if we assume that only one monovalent cationic species is pumped and that all other ionic species, assumed monovalent, both cations and anions, are passively distributed. It should be noted that, if the cations are A^+ , B^+ , C^+ , etc., then passive distribution means that they all follow the Nernst equation at steady state (for them equilibrium) and so :

$$\frac{A_o}{A_i} = \frac{B_o}{B_i} = \frac{C_o}{C_i} = \dots = \frac{(A+B+C+\dots)_o}{(A+B+C+\dots)_i}$$

with a similar argument for passive anions. Ion balance considerations follow a similar argument also. Thus Equation (7) may be re-written in the form :

$$E_m = \frac{RT}{2F} \cdot \ln \left\{ \frac{\text{total equivalence of all non-pumped monovalent cations outside}}{\text{total equivalence of all non-pumped monovalent anions outside}} \right\} \dots (8)$$

The difference between top and bottom under the logarithm is Na^+ , i.e. the pumped ion in our example, which is the major external cation in most cases. This fact renders the result that E_m is negative inside the cell, which it is bound to be if a cation is pumped out.

2. The second extreme case we could apply to Eqn. (6) is if $0 < \Delta\bar{\mu} \ll RT$, when the exponential would approximate unity. Then Eqn. (6) reduces to :

$$E_m = \frac{RT}{2F} \ln \frac{K_o}{Cl_o} \left(\frac{Na_o}{K_o} + 1 \right) = \frac{RT}{2F} \ln \left(\frac{Na_o}{Cl_o} + \frac{K_o}{Cl_o} \right)$$

and since $Na_o + K_o = Cl_o$ for reasons of ion balance, $E_m \doteq 0$, which is trivial, but hardly unexpected if $\Delta\bar{\mu}$ is so small - i.e. pumping is ineffectual.

3. For the sake of completeness the third extreme case we could apply to Eqn. (6) would be to take :

$$\frac{Na_o}{K_o} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) \gg 1$$

when :

$$E_m = \frac{RT}{2F} \ln \left\{ \frac{Na_o}{Cl_o} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) \right\} = \frac{\Delta\bar{\mu}}{2F} + \frac{RT}{2F} \ln \frac{Na_o}{Cl_o} \dots (9)$$

which is reminiscent of Eqn. (3) for the case of NaCl only, except that we now have an extra term representing the ratio :

$$\left\{ \frac{\text{concentration of pumped monovalent cation outside}}{\text{total equivalence of all non-pumped monovalent anions outside}} \right\}$$

in place of $\frac{Na_o}{Cl_o}$ in Eqn. (9). This would give a small correction term on Eqn. (3) of -0.8 mV on the external concentrations to be quoted below, and would slightly reduce a positive value of E_m . However, Eqn. (9) will apply only if $\Delta\bar{\mu}$ is positive and large compared to RT , a situation which in magnitude is quite likely, but in sign refers to a monovalent cation pumped into the cell, generating a membrane potential positive inside the cell, which is not ever found, as far as I am aware.

Finally it should be observed that Eqn. (6) reduces to Eqn. (3) in the limit as KCl concentration is reduced since $Cl_o = K_o + Na_o$ and the first term in the square brackets is in any case likely to be greater than 1 because $\Delta\bar{\mu}$ is invariably greater than RT , and also in any case the decreasing level of K_o increases Na_o/K_o thus rendering Eqn. (3) in the limit as K_o approaches zero, as is only to be expected since it now reduces to the first simplest case discussed earlier.

Examination of practical cases.

1. Equation (6), or rather the derived form, Eqn. (8) is highly suitable for application to the case of many animal cells, in which the chief membrane pump is a sodium export pump. It is true that most animal cells carry a membrane bound pump which exchanges

Na_i for K_o , often with a stoichiometry varying between 1 : 1 and 3 Na : 1 K (Finean et al. 1978) but to a first approximation it is fair to ignore the transport of K^+ when the membrane is very leaky to potassium as compared to sodium, as it is for nerve and muscle ($P_{\text{Na}} : P_{\text{K}} = 1 : 75$ Katz 1966). Thus the pump will be virtually stalled once the electrochemical gradient for Na^+ has built up to the maximum the pump can sustain ($\Delta\bar{\mu}$), and any further activity of the pump will be merely replacement of sodium leakage.* The potassium transport associated with such a low rate of transport will clearly not lead to a significant electrochemical gradient for potassium in the face of its permeability being so much larger than that for sodium. To a good approximation then, the fact that potassium is exchanged to some extent for sodium may be ignored, and K^+ taken to distribute itself passively, which is what was assumed in deriving Eqns. (6) and (8). It is therefore once again clear that potassium diffusion is not responsible for generating the observed trans-membrane potential, although it is hardly surprising to find that the potential is close to equilibrium potential for potassium. But of course equilibrium implies no net diffusion, as discussed earlier, p. 34.

For practical purposes we may take values of Na_o of 145 mM and external anion equivalence of 154 mM (Bell, et al. 1968, p. 738), when Eqn. (8) gives :

* This statement, it should be emphasised, refers to the ability of the pump to generate a certain level of $\Delta\bar{\mu}$ for Na^+ and is not meant to imply that the coupled metabolic processes are necessarily stalled - that is a matter of efficiency of coupling of metabolism to transport.

$$E_m = \frac{RT}{2F} \ln \frac{154 - 145}{154} = -38 \text{ mV.}$$

Clearly this result is too low compared to the accepted value of -70 mV for many animal cells, but as yet the model is far from complete in certain important respects for a real cell. We will return to this case when refinements to the theory are considered later, p.118.

2. Next we will consider a botanical example. There is much information available on salt transport in the Characean algae, and in particular Nitella species (MacRobbie, 1970, 1975, Hope and Walker, 1975). It is reported in these reviews that Nitella translucens actively pumps Na^+ out and K^+ in at the plasmalemma and also H^+ out and Cl^- in at the plasmalemma. For the moment the tonoplast transports will be ignored, on the reasonable basis that what is likely to decide the electric potential between cytoplasm and outside will be active transports at the plasmalemma. The fact that at least four important ions are pumped complicates the issues, and at this stage we cannot cope with these complications all at once, but it will be interesting to see what values of E_m (plasmalemma) will be indicated by application of Eqn.(8), derived above. To do this it will be necessary to take the transport of one ionic species at a time to see if it could be the chief cause of the transmembrane potential. Again we will ignore K^+ because it will be fairly close to its

equilibrium potential, since membrane permeability to K^+ is so high, (although in the case of Nitella translucens there would appear to be a difference of 31 mV for K^+ with E_m lower than E_K - in other Characeae the difference is negligible - Higinbotham 1973 a,b). As yet we have not dealt with anion transport, so the present examination will be confined to Na^+ and H^+ transport.

(a) Na^+ export as putative determinant of E_m .

The external medium concentrations of inorganic ions in which Nitella was grown were (MacRobbie 1970) :

Na^+ 1.0 mM, K^+ 0.1 mM, Cl^- 1.3 mM.

Na^+ + K^+ is not quite equal to Cl^- , the difference being accounted for by other ions. So for Eqn. (8) we will take "total equivalence of all non-pumped monovalent cations outside" as $Cl^- - Na^+ = 0.3$ mM, and "total equivalence of all non-pumped monovalent anions outside" as $Cl^- = 1.3$ mM. Thus Eqn. (8) gives :

$$E_m = \frac{RT}{2F} \ln \frac{0.3}{1.3} = \frac{58}{2} \log_{10} \frac{0.3}{1.3} = -18 \text{ mV.}$$

for 20°C., which is clearly an under-estimate. Even if we were to take for non-pumped cation equivalence 0.1 (i.e. K^+), the calculated value would still be only -32 mV. Clearly these values are far below the observed value of -140 mV.

(b) H^+ export as putative determinant of E_m . See also p.102.

Hope and Walker (1975) report that artificial pond water in which most Characean experiments were normally carried out, had a pH of 5.5. Let us take external pH as 5 for simplicity, and as a probable lowest value. In

Eqn. (8) now, the values above and below the line under the logarithm give the following :

$$E_m = \frac{RT}{2F} \ln \frac{\text{approx. } 1.3 - [H^+]_{\text{ext.}}}{\text{approx. } 1.3}$$

(The exactitude of the value 1.3 is somewhat in doubt, but is unimportant, as will appear below).

$[H^+]$ for pH 5 is $10^{-5}M$ or 10^{-2} mM, so

$$E_m = \frac{RT}{2F} \ln \frac{1.3 - .01}{1.3} = -0.1 \text{ mV.}$$

It is abundantly clear that higher pHs would produce even smaller values of E_m , and so one is bound to the conclusion that H^+ pumping is never likely to be responsible for establishing the transmembrane potential at the plasmalemma in the face of what I could now call the "Nebenion Effect". By this term, I mean the effect that the presence of ions of the same charge as the one pumped has on the value of E_m . In deriving Eqn. (3) (p. 64) for the case of Na^+ pumping in the presence of $NaCl$ and no other ions, and in deriving Eqn. (6) (p. 66) when KCl was also included in the bathing medium, comparison of the two indicates that the more K^+ (or any other non-pumped cation) there is, the lower is the value of steady-state E_m that will be produced. This is not to say that a high level of $\Delta\bar{\mu}$ for the pumped ion is not achieved in the presence of unpumped nebenions, because this is assumed to be the case in the derivation of Eqns. (6) and (8). Notice that the inclusion of other non-pumped anions, in the examples used to derive Eqns. (3) and (6), such as, say Br^- and NO_3^- , etc., would have no effect since they may

be lumped in with the equivalence of counterions to the pumped ion, as shown in the derivation of Eqn.(8) from Eqn.(6), whereas inclusion of a nebenion (i.e. of same charge) to the pumped ion does influence the value of E_m produced. It is true that introduction of nebenions brings along counterions as their neutralising partners but that is not the point at issue, as derivation of Eqn. (6) shows.

Actually, one may add that it is not perhaps surprising to find that E_m is not primarily or even significantly controlled by H^+ pumping because, as Raven and Smith (1974) have pointed out, the primary significance of H^+ pumping is likely to be that of controlling cytoplasmic pH for the sake of intracellular enzymes, whereas pumping of other cations, especially Na^+ , K^+ and Cl^- is probably more important with regard to turgor pressure control, and also I may add the provision of a correct ionic strength for metabolic reactions to take place. I add this because clearly Na^+ , K^+ and Cl^- pumping cannot be serving entirely to achieve high internal ionic strength solely for turgor pressure generation because animal cells, which, lacking a cell wall, cannot withstand high turgor pressure, also pump some or all of these ions. Therefore there must be another reference (at least) besides those mentioned by Raven and Smith (1974) and clearly ionic strength is important with regard to the solubilisation and hence conformation of globulins, which many enzymes are.

Further pursuit of botanical examples at this stage

would be fruitless, and so must be left until later. However, we must next consider the possibility that anion transport could generate the transmembrane potential, before returning to botanical cases.

Electrogenic and Electroneutral ?

Perhaps now is a good point at which to discuss the term "Electrogenic", over which in my view there hangs a cloud of mystery. In fact, one cannot generate electric charge as such, although by its etymology, the term "electrogenic" should most probably mean this. One can only either transfer the position of an already existing charged entity or alternatively separate charges leading to the appearance of equal and opposite charges held apart at the expense of potential energy. Therefore the only permissible meanings of the term suggest that it ought to refer to any process which generates electric current (charge transfer) or electric potential gradients (charge separation), although better terms for these perhaps might be "rheogenic" and "potentiogenic" respectively. The problem then arises as to what they are the current flow or potential gradient of, and so one could propose even more precise but unfortunately rather clumsy terms such as "electrorheogenic" and "electropotentiogenic". The clumsiness however could be avoided by use of the unit names, ampère and volt, since these are unique, giving the possible terms "amperogenic" and "voltagenic", terms which as far as I know have no other use already. I here suggest that these terms be seriously considered

in view of the confusion that can arise in the use of the unqualified term "electrogenic", as set out below.

Even if we confine the term "electrogenic" to refer to a pump transferring net charge of itself, what is not always clear from the term, or the context of its usage, is the consequence of the activity of such a pump. The term has been defined by Rapoport (1970) to refer to an active transport of an ionic species in such a way that the transport itself amounts to a direct transfer of charge, (i.e. to an "amperogenic" pump on my definition). Clearly such a metabolically-coupled charge transfer is bound to lead to some level of potential difference across the membrane in which the transport system is located. How large it will be will depend on the presence of other ions and the passive permeability of the membrane to the pumped ion and all other ions present, as already shown (Eqns. (3) & (6)). It should be noted that pumps which achieve partial exchange can also produce charge transfer. An example is the Na/K exchange pump so well-known in animal cells. The stiochiometry of the exchange seems to be different in different cells and tissues, as remarked earlier, but it can be as high as 3 Na : 1 K transported in opposite directions (antiport) (Finean et al. 1978). Clearly the transfer of 3 Na⁺ out to 1 K⁺ in results in a charge transfer of 2 +, and is therefore in this first sense "electrogenic". This is the sense defined by Rapoport (1970).

However there is a second possible meaning for the

term "electrogenic", which although strictly outside the definition given by Rapoport (1970), is nevertheless natural enough in terms of its etymology to lead, I submit, to confusion. This is when active processes lead to the appearance of an electric transmembrane potential, irrespective of how it has been caused, i.e. to a "voltagegenic" situation in the broadest sense. In this instance, one may cite an exchange pump with 1 : 1 stoichiometry, e.g. 1 Na⁺ out to 1 K⁺ in, i.e. so-called "electroneutral". Such stoichiometry apparently does occur (Finean et al. 1978). Another example particularly relevant here, discussed in the literature in regard to the debate on the energy coupling which drives Cl⁻ transport in higher plants is a possible OH⁻/Cl⁻ exchange pump (antiport) or a possible H⁺/Cl⁻ cotransport (Poole, 1978, Raven & Smith, 1974). If the stoichiometries were again 1 : 1, then clearly no charge transfer would result in such cases, directly from the action of the pump, but that does not mean that a transmembrane potential will fail to appear as a result. In the example of 1 Na : 1 K antiport in animal cells, a potential due to action of the pump will generally appear for the following reason. The membrane of living cells is in many cases much more permeable passively to K⁺ than to Na⁺, and therefore while the pump is building up an appreciable electrochemical gradient for Na⁺, the reverse gradient for K⁺ is being largely dissipated by passive back diffusion. The net result of these processes will therefore be largely as if only Na⁺ were being pumped out. Such would amount to a net transfer of charge and would therefore lead to

the appearance of a transmembrane potential, a state of affairs that does not offend the etymology of the term "electrogenic", even if not precisely in accord with the definition given by Rapoport (1970). Indeed in the consideration of the animal example, using Eqn.(8) on p.68, this was taken as the reasonable assumption. It should be noted that a stoichiometric relationship between the transports of two ionic species necessarily implies that their transports are linked, and therefore that stalling the transport of one will stall that of the other.

These considerations make it clear that the term "electrogenic" can, in fairness to its etymology, be used in either of two senses, and in reading published literature one is often left in confusion as to which is meant. By this I do not mean to imply that authors themselves are necessarily unclear in their own minds (although I fancy sometimes they may be !) as to what they mean, but the term itself carries with it, I suggest, a certain built-in ambiguity. It is hard to decide, in fact, which should be the correct meaning, but I submit that the meaning of a word must include all reasonable senses consistent with its etymology, because if it does not, confusion is bound to arise. Nevertheless one has to concede that many scientific terms have been given a more precise meaning than their etymology would sustain, and this is inevitable when science forces us to coin many new words for which there is no antecedent in ancient languages..

It would therefore appear that, to clarify matters, authors may be recommended (a) to define, on first use

in a paper precisely (if they must use the term at all - see below) what they mean by "electrogenic" - i.e. referring to a pump which of itself directly transfers charge, and (b) to add qualifications, such as "directly" or "indirectly voltagenic", if they can - i.e. if they know. In fact an "electrogenic" pump is bound to be to some extent "directly voltagenic" (see p.177), but it may also be "indirectly voltagenic"; indeed this latter may be the principal component of the total membrane potential generated as a result of the activity of the pump.

An "electroneutral" pump cannot of course be "directly voltagenic", although it may be "indirectly voltagenic". This fact makes it clear that the term "electroneutral" is also fraught with similar problems since the term unqualified could mislead one into thinking that an electroneutral pump would not lead to the appearance of a membrane potential, which, as demonstrated above, is not necessarily so.

A very high standard of clarity and precision is clearly demanded on all counts in the use of these potentially confusing terms. My own view is that in some ways it may be preferable to abandon the term "electrogenic" and use the perhaps clearer term "amperogenic" to refer to pumps which of themselves transfer net charge, and the term "non-amperogenic" to refer to pumps which do not transfer charge of themselves, in place of the confusing term "electroneutral", although this latter is perhaps less objectionable than "electrogenic".

In my view, the term "electrogenic" is in any case an unfortunate one to have been chosen, since electricity, presumably in essence, charge, cannot be created, except in nuclear reactions and/or relativistic situations, which are clearly excluded from the situation being discussed here. A flow of charge, amperogenesis, can however be created, as also can charge separation, voltogenesis. It therefore seems to me on etymological grounds more appropriate to use the terms "amperogenic" and "voltagenic" to refer to the characteristics and effects respectively of active ion transport pumps, quite apart from the confusion the term "electrogenic" can produce.

The term "electrogenic" does not seem to have been applied to the Donnan system, and perhaps that is reasonable, since, although a Donnan system is undoubtedly a source of E_m , indeed a fundamental one, as will be shown later, p.108ff, it does not arise out of an active transmembrane transport system as such, but out of biosynthesis, which is itself, of course, another form of active process of the cell.

A further discussion of the criteria for "electrogenicity" or as I would prefer to call it "amperogenicity" will be found on p.172, when the Goldman equation is also re-examined in the light of my fully developed theory.

We are now in a position to return to the case of anion transport. It was however necessary to clarify the above first, because Cl^- transport may perhaps be

linked to OH^- transport, and the question of whether it is electrogenic in the first or second sense is immediately raised.

Anion Transport - no nebenion present.

Next we consider a case which may be of considerable importance in botany. Many of the higher plants have, as one of their primary membrane ion transport pumps, a chloride import pump (Higinbotham 1973). At present there is considerable debate as to how it functions and what powers it. However, as a start, let us now examine a model situation which is admittedly a gross oversimplification of the real situation in higher plants - but one has to start somewhere! We will examine how a Cl^- import pump could generate a transmembrane potential, if it were acting alone, and on the assumption that no nebenions to chloride are present (i.e. no other anions). This situation is the exact parallel of the first case examined, that for Na^+ pumped out in the presence of NaCl only, p.62. The procedure is exactly the same as before. Thus for the Cl^- pump, we can say that :

$$\Delta\bar{\mu} = (zFE_i + RT \ln \text{Cl}_i) - (zFE_o + RT \ln \text{Cl}_o)$$

We note that $z = -1$ for Cl^- and so rearranging :

$$\Delta\bar{\mu} = -FE_{i_o} + RT \ln \frac{\text{Cl}_i}{\text{Cl}_o} \dots \dots \dots (10)$$

where E_{i_o} has been retained to remind us that it is the potential inside referred to outside, as would be measured by microelectrode. If Cl^- is pumped in, then Cl^- will be at a higher internal electrochemical potential than external. So $\Delta\bar{\mu}$ is positive for this case.

We are proceeding from here on the assumption that the bathing medium is NaCl only for simplicity, although unlike the two previous cases, it is unimportant this time if K^+ is also present so long as there is no other anion, and only Cl^- is pumped. The reason we can ignore K^+ is because both Na^+ and K^+ will distribute passively this time, and so Na^+ and K^+ can be lumped together as already explained on p. 68 (top) in the derivation of Eqn. (8) from Eqn. (7).

Thus with NaCl as bathing medium, Na^+ will distribute passively according to the Nernst equation :

$$E_{io} = \frac{RT}{F} \ln \frac{Na_o}{Na_i}$$

But again ion balance in both internal and external solutions demand that $Na_o = Cl_o$ and $Na_i = Cl_i$. So we can say that :

$$\begin{aligned} \Delta\bar{\mu} &= -FE_{io} + RT \ln \left(\exp -\frac{FE_{io}}{RT} \right) \\ &= -2FE_{io} \end{aligned}$$

or

$$E_{io} = \underline{\underline{E_m}} = -\frac{\Delta\bar{\mu}}{2F} \dots \dots \dots (11)$$

This form is hardly surprising in view of Eqn.(3) because the ion being pumped is now negative but since $\Delta\bar{\mu}$ is positive by definition in the case of import pumping, E_m will be negative as expected.

What value to assign to $\Delta\bar{\mu}$ is not clear since the mechanism and energy coupling of chloride transport remains as yet obscure, but if a value of about 31 kJ/mole were appropriate then the membrane potential would be -160 mV by Eqn. (11) and on this model.

Now we must examine the case of a plant cell importing chloride ions, bathed in a solution containing additional anions.

Anion Transport - nebenions present.

The salts present will now be taken as NaCl and NaNO₃ assuming for this discussion that NO₃⁻ is not actively transported.* Now (following a similar approach to that used for the earlier case of Na⁺ pumping in the presence of Na⁺, K⁺ and Cl⁻), we may say that for chloride (Eqn.(10) again) :

$$\Delta\bar{\mu} = -FE_{io} + RT \ln \frac{Cl_i}{Cl_o}$$

* Nitrate is a likely environmental ion in some quantity. Reisenauer (1966) (quoted in Epstein 1972) analysed many soil types and found that the concentrations of macro-nutrients in these soils varied widely but the greatest number of soils were found to contain soil solutions with concentrations, as follows, expressed as millimolar :

Element	Low end of range	Predominant value	Upper end of range
Na	Not quoted by Epstein (1972) but likely to be about	5 mM.	
K	0.2	1.5	5.0
Ca	0.5	1.75	7.5
Mg	0.5	3.0	12.5
N as NO ₃	0.9	8.6	18
P as PO ₄ ³	-	.001	.01
S as SO ₄ ⁴	0.4	1.2	6.3
Cl	Not quoted by Epstein (1972) but likely to be about	6.0 mM, depending on level of Na.	

Necessarily these values have required some interpretation of the data presented by Epstein (1972).

$\Delta\bar{\mu}$ is a positive quantity as before for Cl^- import. In this case Na^+ and NO_3^- will distribute themselves passively, in effect according to a Donnan distribution, governed by the prevailing value of E_{i0} at steady state, and so following the Nernst equations :

$$E_{i0} = \frac{RT}{F} \ln \frac{Na_o}{Na_i} = \frac{RT}{F} \ln \frac{NO_{3,i}}{NO_{3,o}} \dots (12)$$

The condition that each solution must be ionically balanced demands that :

$$Na_i = Cl_i + NO_{3,i} \quad \text{and} \quad Na_o = Cl_o + NO_{3,o} \dots (13a, b)$$

Replacing E_{i0} in Eqn.(10) by the Na^+ form of Eqn.(12) we have :

$$\Delta\bar{\mu} = RT \ln \frac{Na_i}{Na_o} \cdot \frac{Cl_i}{Cl_o}$$

or rearranging :

$$\exp\left(\frac{\Delta\bar{\mu}}{RT}\right) = \frac{Na_i}{Na_o} \cdot \frac{Cl_i}{Cl_o} \dots (14)$$

and from (12) and (13) it follows that

$$Na_i = Na_o \exp\left(\frac{-FE_{i0}}{RT}\right)$$

and so

$$Cl_i = Na_i - NO_{3,i}$$

$$= Na_o \exp\left(\frac{-FE_{i0}}{RT}\right) - NO_{3,o} \exp\left(\frac{FE_{i0}}{RT}\right)$$

and so Eqn.(14) becomes, by substitution :

$$\Delta\bar{\mu} = RT \ln \frac{[Na_o \exp\left(\frac{-FE_{i0}}{RT}\right)][Na_o \exp\left(\frac{-FE_{i0}}{RT}\right) - NO_{3,o} \exp\left(\frac{FE_{i0}}{RT}\right)]}{Na_o Cl_o}$$

Therefore :

$$\exp\left(\frac{\Delta\bar{\mu}}{RT}\right) = \frac{Na_o \exp\left(\frac{-2FE_{i_o}}{RT}\right) - NO_{3,o}}{Cl_o}$$

which may be rearranged to give :

$$E_{i_o} = E_m = -\frac{RT}{2F} \ln \left[\frac{NO_{3,o}}{Na_o} \left(\frac{Cl_o}{NO_{3,o}} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + 1 \right) \right] \quad (15)$$

This is the general equation showing the nebenion effect on anion transport, and is, not surprisingly, similar in form to Eqn.(6). The - sign at the beginning arises because Cl^- is being pumped and is its valency sign.

Let us now examine extreme cases of this, as was done for Eqn.(6) earlier.

$$1. \text{ If } \frac{Cl_o}{NO_{3,o}} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) \ll 1 \text{ then } E_m = -\frac{RT}{2F} \ln \frac{NO_{3,o}}{Na_o} \quad (16)$$

This applies if either :

(i) $Cl_o \ll NO_{3,o}$ which is unlikely since Cl^- tends to be at least equal to, if not more than NO_3^- in soil solutions and pondwaters.

(ii) $\Delta\bar{\mu} < 0$ and large in magnitude compared to RT .

Unlike the former case, p. 66, $\Delta\bar{\mu}$ for inward pumping of Cl^- would be positive, so this does not apply.

We therefore realise that in fact it is far more likely that :

$$2. \quad \frac{Cl_o}{NO_{3,o}} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) \gg 1$$

and so :

$$E_m = -\frac{RT}{2F} \ln \left[\frac{Cl_o}{Na_o} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) \right]$$

or

$$E_m = -\frac{\Delta\bar{\mu}}{2F} - \frac{RT}{2F} \ln \frac{Cl_o}{Na_o} \quad (17)$$

is the form that will apply for Cl^- pumping in the presence of nebenions. However :

(a) the value of $\Delta\bar{\mu}$ does affect the value of E_m in this case unlike the former case, discussed for Na^+ export pumping in the presence of a nebenion, e.g. K^+ (p. 68).

(b) the ratio of the concentration of Cl_o to the concentration of the counterions affects the value of E_m .

Notice that Na_o stands for the sum total of all monovalent cations present outside, by the same reasoning that derived Eqn.(8) from Eqn. (7). The principle cations present are Na^+ and K^+ in soil water and pondwater, in free ionic form. Although there are appreciable amounts of Ca and Mg also present in soil, but lower in many pondwaters, it is unlikely that these ions will have an appreciable free ionic activity, since (a) there will be, for these alkaline earth elements, appreciable chelation to soil constituents, especially soluble organic matter and (b) the activity coefficient for divalent ions is lower than that for the corresponding concentration of monovalent ions (Robinson & Stokes, 1959/1965).

Nevertheless the effect of the divalent ions will doubtless not be entirely negligible, but examination of the derivation of Eqn.(15) will show that it would be tedious to include the divalent ions, and is not very profitable to attempt at this stage since the present aim is to find the likely chief cause of the membrane potential, which purpose will be served without recourse to such a refinement.

1. Application of Eqn.(17) to Plants.

In making an assessment of the possible value of E_m as predicted by Eqn.(17) that Cl^- import pumping in higher plants might produce, we may take Cl_o and (total counterion) $_o$ as being in the ratio of between 1 and 0.5, as examination of the table in footnote on p. 83 indicates. When $Cl_o : \sum_o(\text{counterions}) = 1$, Eqn.(17) reduces to Eqn.(11) and E_m would calculate to -160 mV, and when $Cl_o : \sum_o \text{counterions} = 0.5$, E_m would calculate to -152 mV ... i.e. only 8 mV lower, if we assume in each case that $\Delta\bar{\mu} = 31$ kJ/mole (but see below). It is worth noticing that the ratio $Cl_o : \sum_o(\text{counterions})$ does not have such a marked effect as the change in the ratio itself. The depression is a "nebenion effect" however, and not a "counterion effect" despite the form of Eqn.(17), because the cause of the effect is the inclusion of nebenions. This is implicit in the ratio, since the condition must apply that $\sum_o(\text{anions}) = \sum_o(\text{cations})$ for ion balance, on the assumption that only monovalent ions are present in the system as the model stipulated. Therefore $Cl_o + \sum_o \text{nebenions} = \sum_o \text{counterions}$, and so

$$\frac{Cl_o}{\sum_o \text{counterions}} = \frac{Cl_o}{Cl_o + \sum_o \text{nebenions}}$$

which renders a form for Eqn.(17) of :

$$E_m = -\frac{\Delta\bar{\mu}}{2F} - \frac{RT}{2F} \ln \left\{ \frac{\text{concn. of pumped monovalent anion outside}}{\text{total concn. of all monovalent anions outside}} \right\} \dots \dots (18)$$

It should perhaps be remarked that it is not cheating, on account of the electronic charge, to apply Eqns. (15) or (18) to a plasmalemma transport coupled to electron transport reactions, because the electron transfer reactions

occur entirely within the cell, either at the mitochondrial or at the chloroplast membranes and do not involve a charge transfer across the plasmalemma itself.

The assumption of 31 kJ/mole is the value for ATP hydrolysis, taken for the sake of discussion, but it is much in doubt if ATP does power Cl^- transport for plants (Poole 1978 has reviewed this) in either storage tissues of roots where photosynthesis is clearly not occurring or in green parts. The coupling appears to be to electron transport and not to ATP in both cases. The value of 31 kJ/mole taken above is therefore not necessarily applicable and at this stage the present author is not able to comment competently on the value to assign to $\Delta\bar{\mu}$ for electron transport driven systems.

However an assessment of the value to assign to $\Delta\bar{\mu}$ may be approached as follows. One electronic charge taken through one volt corresponds to an energy of 96.5 kJ/mole. In the electron transfer chain in mitochondria, the largest energy difference in any single redox step would appear to be 0.29 volt corresponding to a $\Delta\bar{\mu}$ of 28 kJ/mole, and the smallest energy step to 0.03 volt corresponding to 2.9 kJ/mole (calculated from data in West & Todd 1961). Since however we do not know at this stage which electron transfer reaction is, or reactions are, coupled to Cl^- transport, nor indeed how many steps are involved, it is not possible to say what value of E_m may be expected from active chloride import on this model, but the value will certainly be more than -15 mV even for the lowest energy transfer reaction

(to which it is unlikely to be coupled) and if it were coupled to the highest single energy transfer step the value would be -145 mV. The value can be even higher if the coupling were to several electron transfer reactions in tandem. It would therefore appear that, for plants at least, the plasmalemma membrane potential could be explained in terms of active chloride import - or indeed active import of any suitable monovalent anion (but the theory sets limits on which are suitable candidates, see below).

As has already been shown (p. 72) H^+ export at the plasmalemma cannot account for the observed value of E_m because of the cationic nebenion effect in face of such low values of $[H^+]$. OH^- import would also appear to offer no explanation of E_m because of the same objection. $[OH^-]$ is too low compared to its nebenions, and the offset term (second) in Eqn. (18) would be large. For instance at $pH_o \sim 5$, $[OH^-] = 10^{-(14-5)} = 10^{-9} M = 10^{-6} mM.$; total nebenion concentration will be of the order of 10 mM in pondwater or soil water (and worse in brackish or sea water), so that the offset term becomes :

$$- \frac{RT}{2F} \ln \frac{10^{-6}}{10+10^{-6}} = + 203 \text{ mV.}$$

which is more than the first term! Actually this figure is, of course, absurd, because these values invalidate the approximations on which Eqn. (18) was derived, and we have to revert to the full Eqn. (15) which gives the value of only -0.43 mV. An external pH of 6 only improves the situation very slightly. Therefore OH^- import cannot be causative of E_m . This will be examined further on p. 102.

The only further plausible alternative to Cl^- pumping as causative of E_m would seem to be NO_3^- import but there are problems with this suggestion. Firstly, in the case of marine algae, the concentration of NO_3^- is so low in the sea (micromolar) that the "nebenion effect" would certainly nullify NO_3^- transport as a source of E_m although there is strong evidence that the NO_3^- ion as such is actively uptaken (Eppley et al. 1969 for the marine diatom, Skeletonema costatum). Uptake of NO_3^- also appears to be by an active mechanism (but with a higher half-maximal concentration for its kinetics than for the marine diatom, as is to be expected) in higher plants (van den Honert & Hooymans, 1955) and in this case soil and pondwater often do contain sufficiently high levels of soluble NO_3^- ion to allow of the generation of an appreciable E_m in the face of nebenion. However, as Epstein (1972, Chap. 10, p.257 ff) has pointed out, the interpretation of NO_3^- uptake is fraught with difficulty because of the high level of metabolic conversion and incorporation of N inside the cell. In discussion of sources of E_m by ion transport, as above, it is strictly the ionic form, NO_3^- , and not any other species, ionic or otherwise, containing the uptaken N which would be applicable to the electrochemical thermodynamics. We do not as yet know the precise ionic activity of NO_3^- ion as such inside the cytoplasm of any cell and until this information is available we cannot pursue this enquiry further. However, one may hazard a guess that active NO_3^- ion uptake is unlikely to be a major factor in determining E_m since the reference of NO_3^- uptake,

as for NH_4^+ uptake, is likely to be more related to the nitrogen requirements in protein synthesis, etc. Also these conversion and incorporation processes will very probably lead to a low value of NO_3^- ion activity inside the cytoplasm, even in face of active uptake, not a situation likely to generate either significant levels of $\Delta\bar{\mu}$ for NO_3^- uptake or any appreciable contributions to E_m . It all depends on the relative rates of (a) NO_3^- uptake and (b) NO_3^- conversion to other species.

Thus, as far as anion transport is concerned, we are driven back to the possible rôle of Cl^- import pumping as the only likely candidate to account for the development of a transmembrane potential at the plasmalemma. But there are further considerations yet. It was taken as an assumption in the derivation of all these electrochemical equations for active transport that the active process was able to achieve a high level of electrochemical potential energy difference, $\Delta\bar{\mu}$, between the two sides of the membrane. This assumption rests on the further assumption that the rate capabilities of the pump are much faster than the rate of back diffusion of the pumped ion by passive processes (facilitated exchange has not been considered, but would amount to a transport coupled to another ionic or other chemical species, and comes under the same considerations as does K^+ transport linked to Na^+ transport as discussed earlier, p. 69). Whether this assumption is justified or not is clearly subject to dispute, but it is also subject to experimental test. Thus passive diffusion permeability can be measured by radio-tracer flux studies, if the active pump sites are

blocked by a binding drug, and maximal rates of active transport can be measured by tracer flux studies in conditions of voltage clamp, when the membrane is held at a potential such that there is no electrochemical gradient for the ion under study.

The fact still remains however that a membrane is always leaky to any ion to some extent and therefore a pump, however fast it can run maximally, will not achieve precisely the value of ΔH which the biochemical reaction which fuels it could yield, expressed as $\Delta\bar{\mu}$ for the ion. The downgrading is related to the relative rates of back-diffusion and maximal pumping. Notice however that, at steady state, the fact that the effective actual value of $\Delta\bar{\mu}$ is lower than the theoretical maximum ΔH , by a larger or smaller amount does not invalidate the use of equilibrium (reversible) thermodynamics, as has been applied above in the derivation of Eqns. (6) and (15), because steady state implies that equilibrium will apply to all other chemical (e.g. ionic) species present except those through which the continuous dissipation of energy flows to maintain the steady state, such energy always ending up as heat. Thus it is fair to use Nernst equilibria for non-pumped ions, as was done above.

The argument then turns on whether the available biochemical reaction to which any given ion transport is considered to be coupled can deliver to the electrochemical potential difference, $\Delta\bar{\mu}$, a significant proportion of the available ΔH for the reaction.

Where we observe a high difference in electrochemical potential for an ion across a membrane, we may safely conclude not only that it is actively transported (the Ussing-Teorell criteria)* but also that a significant portion of ΔH is delivered. In other words we conclude that the pump can run maximally at zero head much faster than back-diffusion at the level of $\Delta\bar{\mu}$ achieved.

In the case of Na^+ export pumping, already considered, this is certainly true in animal cells, and appears to be true in the giant Characean and other algae, but how far it applies to higher plants is uncertain. In those cases Na^+ export pumping must be considered as an important, if not always the chief, cause of E_m (but see later, where two pumps running simultaneously are considered, p.143.)

In the case of Cl^- import pumping, in many animal cells the membrane is much more permeable to Cl^- than to Na^+ . Figures are $P_{\text{Na}} = 0.2 \text{ nm}\cdot\text{s}^{-1}$, $P_{\text{Cl}} = 40 \text{ nm}\cdot\text{s}^{-1}$ and $P_{\text{K}} = 20 \text{ nm}\cdot\text{s}^{-1}$ for frog skeletal muscle (Hodgkin & Horowicz, 1959). With the exception of the oxyntic cells of the stomach wall pits (which secrete HCl into the lumen by both active H^+ and active Cl^- transport, it appears) and possibly a few other specialised cells, animal cells do not apparently maintain a significant electrochemical gradient for Cl^- ions across their outer membranes. In view of the very much higher P_{Cl} than P_{Na} it is clear that a higher continuous power consumption would be needed to maintain a high $\Delta\bar{\mu}$ for chloride, than for sodium. Therefore on these two counts we can probably discount active chloride pumping as a causative

* See p. 161 ff.

for E_m in animal cells. This is interesting because it seems general that animal cells have E_m values between -15 mV (red blood cells) and -70 mV (most other cells, nerve, muscle, etc.) but not higher, whereas E_m values as high as -170 mV (Chara corallina) and even -200 mV (Neurospora crassa, Slayman 1965, 1970) are found in freshwater algae and micro-organisms respectively, although E_m in marine algae seems to be lower - more in line with animal cells (MacRobbie, 1970). These differences between animal cells and plant cells in freshwater are in line with expectations based on Eqns. (6) and (15) above. As we saw Eqn. (6) gave a lower if under-estimated value of E_m on Na^+ export for animal cells, whereas Eqn. (15) on Cl^- import could account for the much higher value of E_m for certain plant cells which are known to maintain electrochemical gradients of accumulation for Cl^- (MacRobbie, 1970). There appears to be additionally the possibility that Cl^- pumping is coupled to several steps in tandem of an electron transfer chain, capable of yielding values of E_m up to the membrane rupture potential. It is however true to say that Cl^- flux rates ($\sim 2 \text{ p-moles.cm}^{-2}.\text{s}^{-1}$) in Characean algae are of not too dissimilar order to the Na^+ flux rates ($\sim 0.5 \text{ p-moles.cm}^{-2}.\text{s}^{-1}$), presumably indicating that P_{Cl} is close to P_{Na} and low, in these cells, in contrast to animal cells. In such a situation active Cl^- transport as the source of E_m is reasonable since it would not waste undue power in re-circulating leaked ions if P_{Cl} is low. An anomaly however appears to be found in the marine Chlorophyte, Acetabularia mediterranea. Saddler (1970 a, b, c) found an E_m of -174 mV whereas

the Cl^- flux rate was several hundred p-moles. $\text{cm}^{-2}.\text{s}^{-1}$ as against Na^+ and K^+ flux rates of a few tens of p-moles. $\text{cm}^{-2}.\text{s}^{-1}$. If we need to postulate that Cl^- pumping accounts for the high value of E_m , then presumably we must expect a high level of continuous power consumption to maintain it in face of so high a Cl^- flux rate in A. mediterranea, but much more detailed analysis would be needed of this case than is so far available.

There still remains the problem of accounting for an E_m value of -70 mV in animal cells, where Na^+ export pumping would appear to be the only active ion transport contributing significantly to it. As we saw, in the face of the nebenion effect, a value larger than -38 mV cannot be expected on the model so far considered (p. 71). There remain two further important models one could postulate which could be invoked and must soon be considered. They are :

- (1) Active ion transport and a Donnan system existing together, and
- (2) Two or more independant active ion transport pumps existing together in one system - to be contrasted with Na/K exchange pumping which, whatever its stoichiometry, is a linked transport, and therefore subject to kinetic considerations consequent on P_{Na} being much smaller than P_{K} .

Internal Ion Concentrations.

Before however proceeding to these considerations it will be instructive to see how closely the models so far proposed reproduce internal ion concentrations. We will not examine further the first simplest case

(Na^+ export in the presence of NaCl only) because it does not represent any real biological situation, but was used to introduce the concepts. In the second case (Na^+ export in the presence of NaCl and KCl), Eqn.(6) was derived, and from it Eqn.(8) as applicable in practical circumstances. We saw that, assuming $\text{Na}_o = 145$ and $\sum \text{anions} = 154$ mM for human body cells, then E_m calculated to -58 mV, on the model used to derive Eqn.(8). To find Na_i , we now recall Eqn.(1) and by rearranging we find that :

$$\text{Na}_i = \text{Na}_o \exp \left\{ \frac{\Delta\bar{\mu} - zFE_m}{RT} \right\} \dots \dots \dots (19)$$

This is interesting because, although the value of $\Delta\bar{\mu}$ does not have much effect on E_m (see Eqn.(8)), it does affect Na_i , since $|\Delta\bar{\mu}| \gg |zFE_m|$. The value calculates to $1.9 \mu\text{M}$ if we take $\Delta\bar{\mu} = 31$ kJ/mole for ATP hydrolysis. This value of Na_i is clearly far too low, intracellular Na^+ being about 10 mM (Bell et al. 1968). This must mean, if we accept this model, that the pump is not achieving the full 31 kJ/mole of electrochemical activity difference. Actually, of course, intracellular Na^+ ion activity will not be as high as the concentration for two reasons (1) some of the intracellular ion will be bound into chelates, and (2) the Debye-Hückel effect depresses ion activity below concentration. These points will be dealt with later in more detail, but for now we will take 40% of the Na^+ present as ionically active (Lev and Armstrong, 1975). This figure is much lower for reason (1) than that in free aqueous solution at similar concentrations. Nevertheless the difference between 10 mM and $2 \mu\text{M}$ cannot be accounted for in this

way, and we are bound to expect a lower value of $\Delta\bar{\mu}$ achieved, due presumably to leakage of ion through the membrane and to inefficiencies of energy coupling between ATP hydrolysis and pumping. If we take $Na_i = 10$ mM, $f_i = 40$ % and $Na_o = 145$ mM, $f_o = 76$ %* with a real value for E_m of -70 mV, we find that $\Delta\bar{\mu}$ (using Eqn.(1), with f's included) is actually -14.8 kJ/mole. This is factual and assumes no model. Even using this value in Eqn.(6) yields a value for E_m hardly perceptibly different from that calculated when $\Delta\bar{\mu}$ was taken as 31 kJ/mole - i.e. the nebenion effect still predominates. It is however interesting, although probably merely accidental, that Eqn.(5) on -14.8 kJ/mole gives E_m as -76.7 mV ! Further consideration of this must be deferred until we have examined the two further models : active transport with a Donnan system present and two or more independent active transports coexisting.

To calculate K_i on the present model (Eqns. (6) & (8)) we observe that the assumption was taken that K^+ was passively distributed or nearly so. The effect of any K^+ import pumping in exchange for Na^+ will be virtually nullified by the high value of P_K compared to P_{Na} in face of the rate limit on K^+ transport set by P_{Na} which itself sets the rate of actual Na^+ transport and hence by linkage that of K^+ transport at steady state. Thus for $E_m = -38$ mV and $K_o = 5$ mM (Bell et al. 1968), K_i calculates to 23 mM. Actually $K_i = 150$ mM in real cells (Bell et al. 1968) and again, even if $f_{Ki} = 40$ %, it would not explain the

* Robinson & Stokes, 1959/65.

shortfall, but, of course, we already know that the value of E_m estimated by Eqn.(8) is too low, so this is to be expected.

To calculate Cl_i , we note that (a) Cl_i follows the Nernst equation for passive distribution which, taking Cl_o as 100 mM (Bell et al. 1968) gives $Cl_i = 22$ mM, and (b) charge neutrality must be preserved inside - hence $Cl_i = K_i + Na_i$ and this checks as about right on these calculations. However Cl_i is over-estimated, the measured value being 10 mM (Bell et al. 1968), but again this is a consequence of under-estimated E_m .

We now turn to a freshwater alga, Nitella translucens (data in MacRobbie, 1970). Earlier we saw that the nebenion effect on Cl^- import pumping at the plasmalemma does not depress the value of E_m as much from its maximum value in the absence of nebenions as was the case for Na^+ export pumping in animal cells. It was seen that the effect of nebenions in pondwater only depressed the value of E_m about 8 mV below its maximum value - which calculates to something around -150 mV on the model taken, the exact value depending on what the energy coupling was taken to be. Cl^- is the ion pumped, inwards, and so it is now the one not following the Nernst equation. The appropriate equation is Eqn.(10) which rearranges to give :

$$Cl_i = Cl_o \exp\left(\frac{\Delta\bar{\mu} + FE_m}{RT}\right) \dots \dots \dots (20)$$

Taking data from MacRobbie (1970) and Hope & Walker (1975) for Nitella translucens, $E_m = -140$ mV (plasmalemma) and

$Cl_o = 1.3 \text{ mM}$, and if we take $\Delta\bar{\mu} = 30 \text{ kJ/mole}$ (for the sake of discussion), Cl_i calculates on Eqn.(20) to 1150 mM, which is clearly far too high, the value being estimated to be about 65-87 mM or 240 mM depending on exactly which cytoplasmic fraction is taken (MacRobbie, 1970). This must mean that $\Delta\bar{\mu}$ has been over-estimated at 30 kJ/mole. Calculating back using the measured values : $E_m = -140 \text{ mV}$, $Cl_i = 75 \text{ mM}$ (say) and $Cl_o = 1.3 \text{ mM}$, we get that $\Delta\bar{\mu} = 23 \text{ kJ/mole}$, and if $Cl_i = 240 \text{ mM}$, the value of $\Delta\bar{\mu}$ would be 26 kJ/mole. These values are fair in terms of a reasonably efficient coupling to an electron transfer step, as mentioned earlier. As may be seen however, a relatively small change of $\Delta\bar{\mu}$ for Cl^- leads to a large change of Cl_i . On these values and Eqn.(17), E_m calculates to a few mV less than -119 mV if $\Delta\bar{\mu}$ were 23 kJ/mole or a few mV less than -134 mV if $\Delta\bar{\mu}$ were 26 kJ/mole (the few mV less being for the nebenion effect). These do not tally with the measured value of -140 mV, although clearly agreement is not bad. The discrepancy may however point to an inadequacy of the model on which Eqn.(17) was derived, and the next paragraph tends to confirm this.

On the model of Eqn.(15) with K^+ passively distributed, and taking $K_o = 0.1 \text{ mM}$, then -140 mV would produce $K_i = 26 \text{ mM}$. This seems to be very much an under-estimate, since the value appears to be between 119 mM and 150 mM, so evidently K^+ is being actively accumulated as MacRobbie (1970) says. Likewise passive distribution of Na^+ would produce $Na_i = 260 \text{ mM}$ if $Na_o = 1.0 \text{ mM}$ and $E_m = -140 \text{ mV}$, whereas it is apparently pumped out,

Na_i being either 14 or 55 mM depending which cytoplasmic fraction one takes to be in contact with the plasmalemma.

General Display of the Nebenion Effect.

At this point it is interesting to see, on the basis of Eqns. (6) and (15) what effect the presence of nebenions has on E_m , displayed in a general way in graphical form. We must use the forms of Eqns. (6) and (15) for this rather than the simplified forms (8) and (17) because these latter depend on assumptions concerning quantities in the full equations reasonable for real situations, but which we are now about to break in order to examine the full quantitative effects of nebenions.

It may be seen that Eqns. (6) and (15) are essentially the same. The similarity arises, of course, from the symmetry of the situations they describe (symmetrical up to a point, see below), and can be brought out more clearly if we replace the concentration of pumped ion in each case by P and the concentration of nebenion by N. We may then represent the ratio N/P by \mathcal{R} and the sign of the pumped ion by z in the usual place. Then on either Eqn. (6) or (15) :

$$E_m = \frac{RT}{2zF} \ln \left\{ \frac{N}{P+N} \left(\frac{P}{N} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + 1 \right) \right\}$$

which, on substitution of \mathcal{R} for N/P, yields the form :

$$E_m = \frac{RT}{2zF} \ln \left\{ \frac{\exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + \mathcal{R}}{1 + \mathcal{R}} \right\} \dots \dots \dots (21)$$

This equation lends itself conveniently to the construction of families of graphs showing :

(a) E_m as a function of \mathcal{R} for various values of $\Delta\bar{\mu}$ in cation export pumping.

(b) E_m as a function of $\Delta\bar{\mu}$ for various values of \mathcal{R} in cation export pumping.

(c) E_m as a function of \mathcal{R} for various values of $\Delta\bar{\mu}$ in anion import pumping

(d) E_m as a function of $\Delta\bar{\mu}$ for various values of \mathcal{R} in anion import pumping.

The tabulated values, Tables 2 & 3, were calculated by inserting a programme for Eqn.(21) into a Hewlett-Packard Model 25C Calculator. See Appendix 3 for programmes.

Notes on Tables 2, 3, and Figs. 1, 2, 3, 4.

1. It is clear that, although the situations seem symmetrical and produce equations closely similar (6 and 15), there is a sharp asymmetry in the results, between cation export and anion import. The explanation of the appearance of asymmetry here is because the conditions under which the two types of transport operate are actually not symmetrical; thus we impose known concentrations of solution to the outside of the membrane, and ask the theory to tell us what the inside concentrations will settle to, and what value of E_m will be generated, but in one case the outside solution is the one being pumped from, and in the other case it is the solution being pumped into.
2. It is clear that the nebenion effect is much more severe on cation export than it is on anion import. Also the nebenion effect sets an upper limit on the value of E_m whatever the value of $\Delta\bar{\mu}$ for cation export whereas no such restriction appears for anion import.

Table 2. CATION EXPORT WITH NEBENIONS.

Values of $-E_m$ on Eqn.(21) for various values of \mathcal{R} and $\Delta\bar{\mu}$ at 20°C.

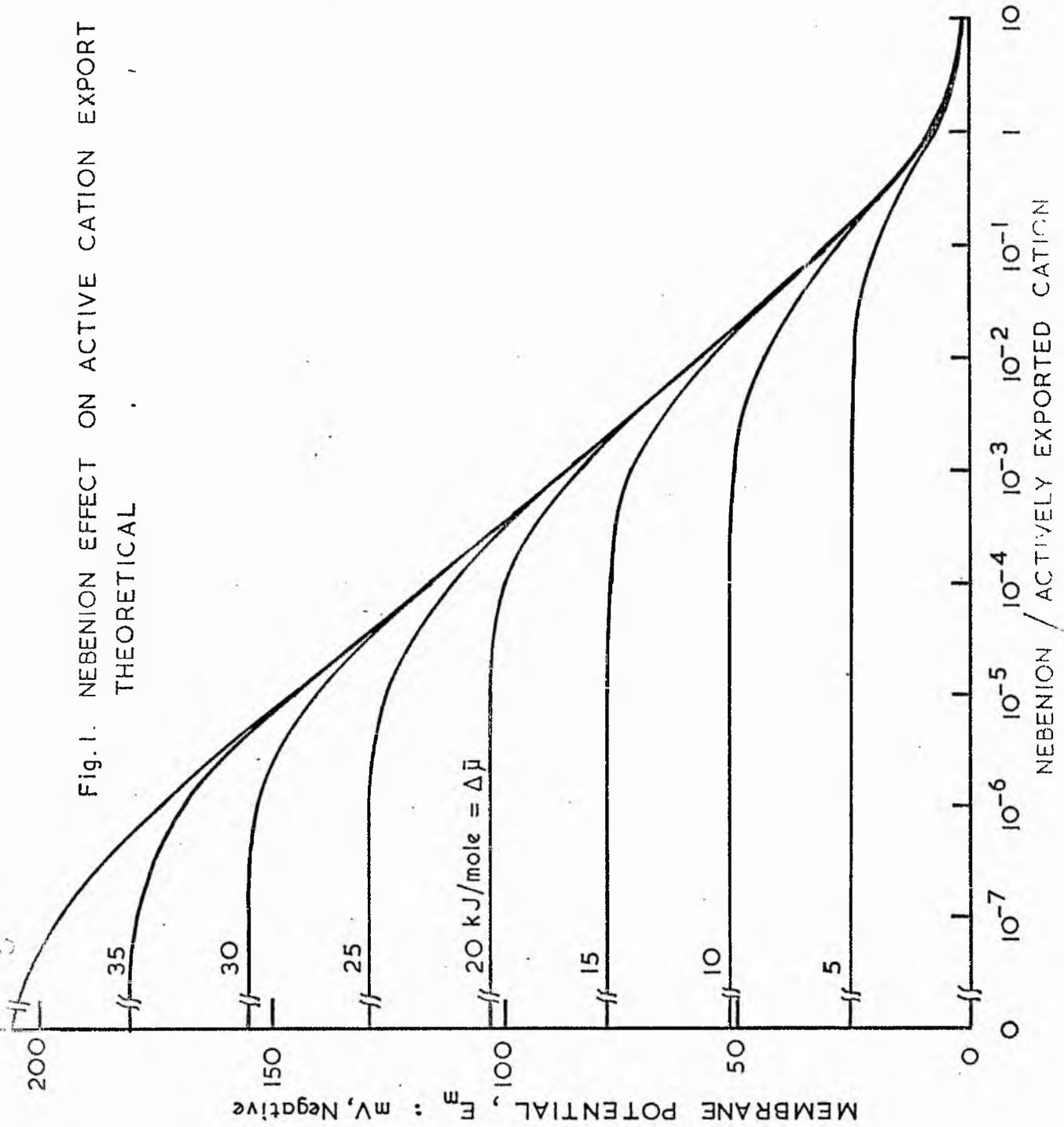
$\Delta\bar{\mu}$, kJ/mole \rightarrow	Millivolts.							
	5	10	15	20	25	30	35	40
\mathcal{R} \downarrow								
10	1.0	1.2	1.2	1.2	1.2	1.2	1.2	1.2
1	7.2	8.5	8.7	8.8	8.8	8.8	8.8	8.8
0.1	20	28	30	30	30	30	30	30
0.01	25	46	56	58	58	58	58	58
0.001	26	51	73	84	87	87	87	87
0.0001	26	52	77	100	113	116	116	116
0.00001	26	52	78	103	126	141	145	145
0.000001	26	52	78	103	129	153	169	174
0.0000001	26	52	78	103	129	155	179	197
0	26	52	78	103	129	155	181	207

Table 3. ANION IMPORT WITH NEBENIONS.

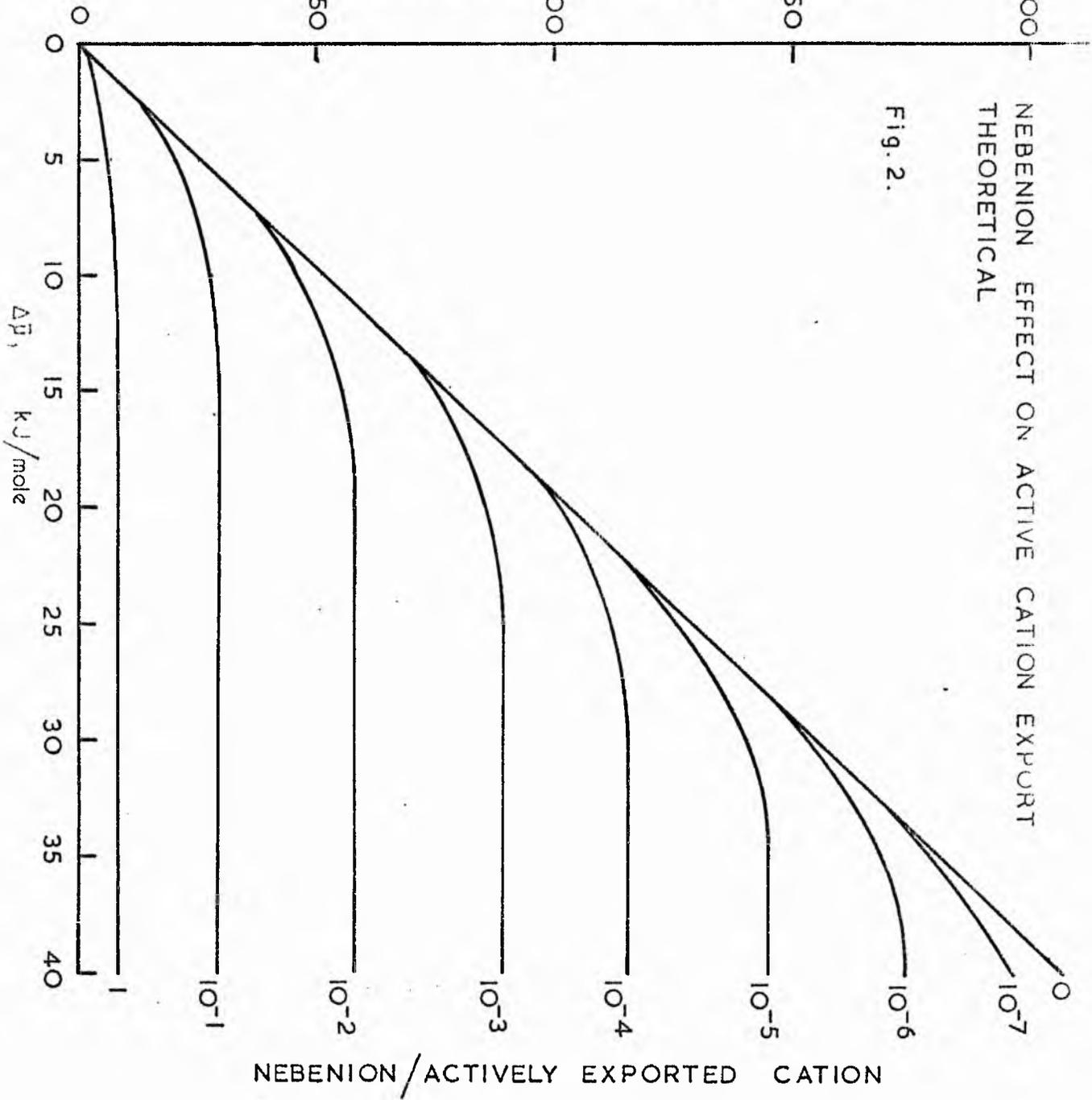
Values of $-E_m$ on Eqn.(21) for various values of \mathcal{R} and $\Delta\bar{\mu}$ at 20°C.

$\Delta\bar{\mu}$, kJ/mole \rightarrow	Millivolts							
	5	10	15	20	25	30	35	40
\mathcal{R} \downarrow								
1000	0.09	0.7	4.9	19	43	68	94	120
100	0.8	5.9	22	46	71	97	123	149
10	6.0	23	48	73	99	125	151	177
1.0	19	43	69	95	121	147	172	198
0.1	25	51	77	102	128	154	180	206
0.01	26	52	78	103	129	155	181	207
0	26	52	78	103	129	155	181	207

Fig. 1. NEBENION EFFECT ON ACTIVE CATION EXPORT
THEORETICAL



MEMBRANE POTENTIAL, E_m : mV, Negative



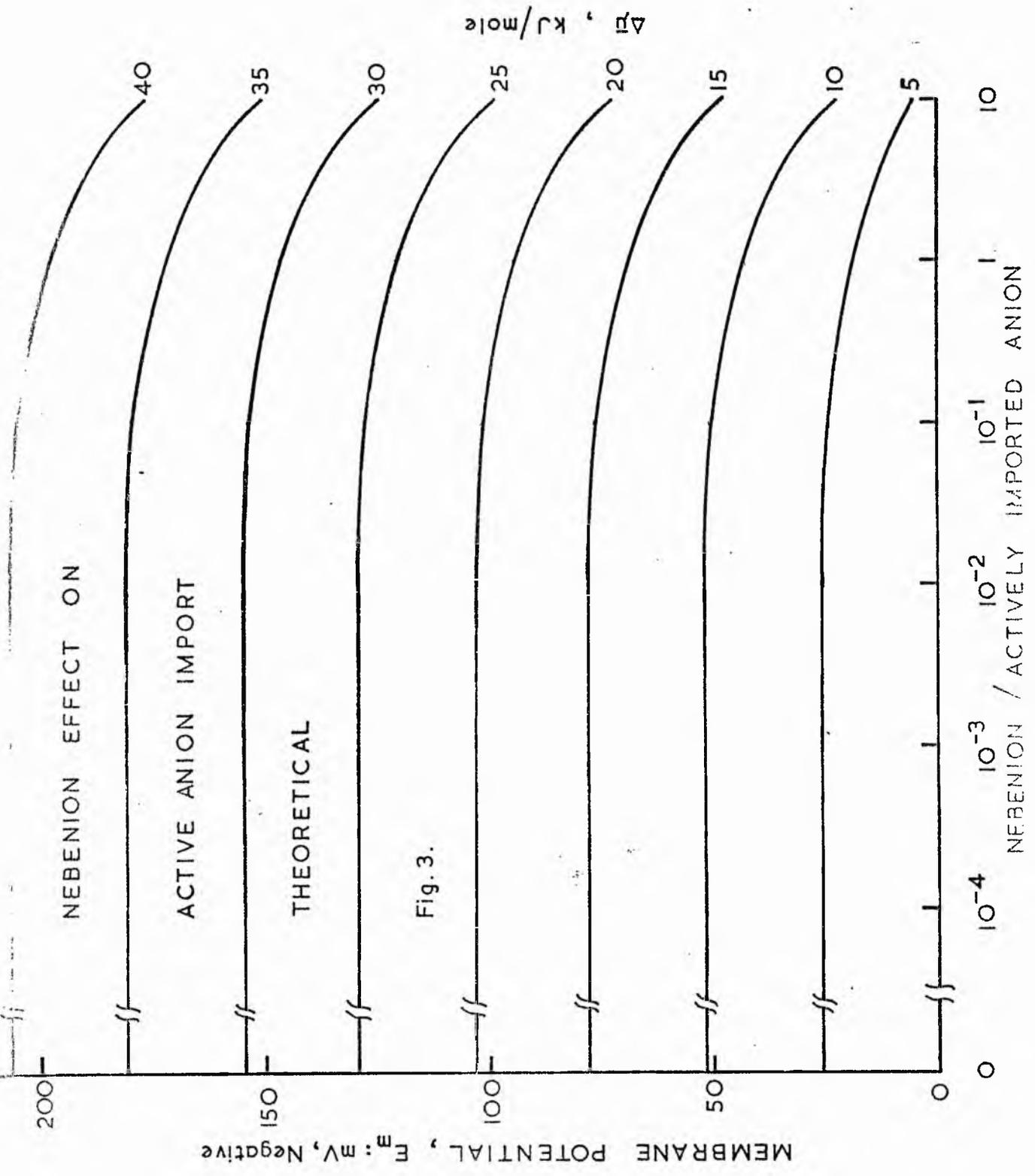


Fig. 3.

MEMBRANE POTENTIAL, E_m : mV, Negative

$\Delta\mu$, kJ/mole

NEBENION EFFECT ON

ACTIVE ANION IMPORT

THEORETICAL

NEBENION / ACTIVELY IMPORTED ANION

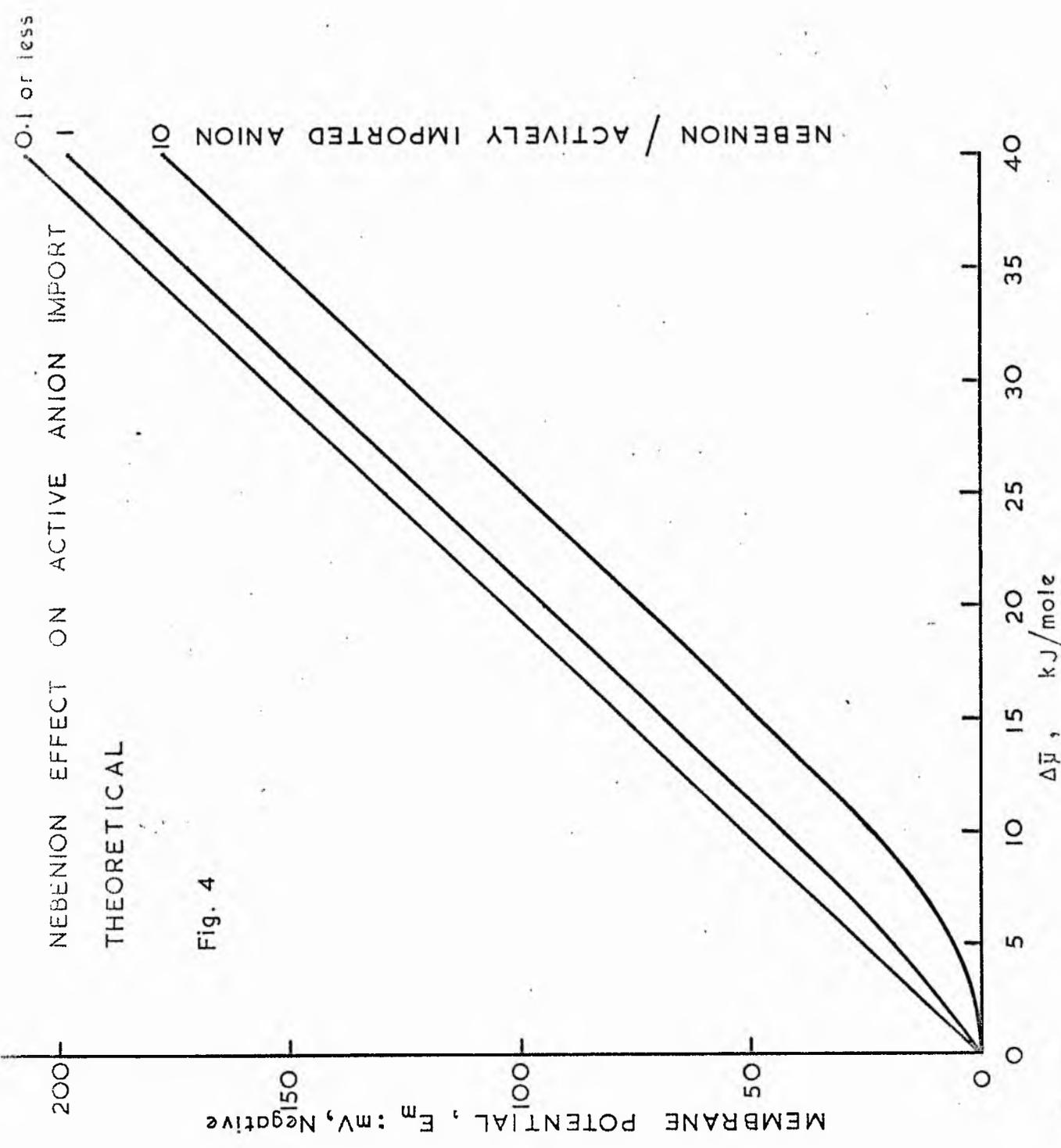


Fig. 4

Hydrogen and Hydroxyl Ion Transport as Causative of E_m ..?

It has already been shown (pp. 72 and 89) that neither H^+ export nor OH^- import can account for the observed transmembrane potential, E_m , in living cells. It was not however examined as to whether H^+ import or OH^- export could give rise to significant contributions to E_m . Both of these latter would, of course, if acting alone, cause a positive value of E_m , which is never found in living cells. However the matter is of some importance because H^+ and OH^- are actively pumped at cell membranes, and their transport could lead to significant depolarisation of a negative E_m arising from other sources. Also mitochondria and plastids establish pH gradients by active processes (where the cytoplasm now becomes equivalent to the external medium), and it is of interest to see if H^+ or OH^- transport in either direction would give rise to a potential difference across the plastid membrane.

It should be noted that Eqn.(21) certainly can apparently generate appreciable values of E_m , at least in theory, but it must be remembered that this only occurs because either H^+ or OH^- is being actively transported, which necessarily implies the establishment of a different pH inside to that outside. This fact must be taken into account because it may be that the pH inside is then either physiologically improbable or physically impossible in those cases where an appreciable E_m would be predicted due to transport of one of these ions.

The problem may be approached as follows. E_m may be calculated from Eqn.(21). (Note that there is nothing in the derivations leading to Eqn.(21) which disallows its use for H^+ import or OH^- export, even though it was designed for the reverse in each case - it is a question of the sign of $\Delta\bar{\mu}$).

pH_i after H^+ Transport.

From Eqn.(1) it follows that for H^+ pumping :

$$H_i = H_o \exp\left(\frac{\Delta\bar{\mu} - FE_m}{RT}\right)$$

So :

$$-\log_{10} H_i = -\log_{10} H_o - \log_{10} \exp\left(\frac{\Delta\bar{\mu} - FE_m}{RT}\right)$$

or, since $\log_{10} \exp(x) = 0.434 x$:

$$\underline{\underline{pH_i = pH_o - \frac{0.434}{RT} (\Delta\bar{\mu} - FE_m)}} \quad (21a)$$

pH_i after OH^- Transport.

From Eqn.(10) it follows that for OH^- pumping :

$$OH_i = OH_o \exp\left(\frac{\Delta\bar{\mu} + FE_m}{RT}\right)$$

or

$$-\log_{10} OH_i = -\log_{10} OH_o - \log_{10} \exp\left(\frac{\Delta\bar{\mu} + FE_m}{RT}\right)$$

But, $pH + pOH = 14$ since the ion product of water is 10^{-14} .

Therefore :

$$\underline{\underline{pH_i = pH_o + \frac{0.434}{RT} (\Delta\bar{\mu} + FE_m)}} \quad (21b)$$

Table 4 has been constructed using the full form of Eqn. (21) for E_m and Eqns. (21a, b) for pH_i , taking two values of nebenion concentration, N , which bridge the range from freshwater to seawater. Also two values of pH outside have been taken, namely, 5 and 8 (upper and lower figures in each box, respectively), these values of pH being probable environmental limits for living cells. Note that N is nebenion to the pumped ion and therefore, in working out $\mathcal{R} = N/P$, it is necessary at pH 5 (e.g.) to use 10^{-5} for P with H^+ pumping, but 10^{-9} for P with OH^- pumping. At such low values of P , N is in effect equivalent to external total salt concentration.

It is seen from this table that, although there are theoretically conditions in which an appreciable E_m would be predicted by Eqn. (21), in no case is this achieved at a really feasible internal pH. The only cases where an appreciable (i.e. \sim mV) E_m appears at even slightly probable pH_i values are marked with an asterisk (*). However as may be seen from those cases, at most only a few mV appear at possible pH_i , and even these are improbable for cytoplasmic chemistry to function. Even these few cases occur only when the external nebenion is very dilute even for freshwater. Only in very extreme acid or alkaline external conditions would transport of either H^+ or OH^- generate appreciable contributions to E_m at feasible internal pHs in face of the nebenion effect. One is therefore driven to the conclusion that, as a generality, H^+ and OH^- transport cannot contribute significantly to E_m under any circumstances, either in plastids or at

Table 4. HYDROGEN AND HYDROXYL ION TRANSPORT.

Values of E_m (mV) on Eqn.(21) and of pH_i (in brackets) on Eqn.(21a) (H^+ pumping) and Eqn.(21b) (OH^- pumping). $20^\circ C$.

$pH_0 = \frac{5}{8}$ in each box. Some values of pH_i are, of course, impossible, but they are theoretical values, based on this model.

 H^+ IMPORT.

Values of N, meq/l.

 E_m positive.

		1.0		500	
+5		0.82	(4.1) *	2 μ	(4.1)
		1 μ	(7.1)	2n	(7.1)
+10		5.86	(3.3) *?	0.02	(3.2)
		0.01	(6.2)	15n	(6.2)
+20		46	(2.2) *?	0.9	(1.5)
		0.46	(4.4) *	0.9 μ	(4.4)
+40		149	(0.44)	71	(-0.9)
		62	(1.9)	3.0	(0.95)

 H^+ EXPORT. E_m negative.

-5		0.11	(5.9)	0.2 μ	(5.9)
		0.11	(8.9)	0.2n	(8.9)
-10		0.12	(6.8)	0.2 μ	(6.8)
		0.12	(9.8)	0.2n	(9.8)
-20		0.13	(8.6)	0.2 μ	(8.6)
		0.13	(11.6)	0.2n	(11.6)
-40		0.13	(12.1)	0.2 μ	(12.1)
		0.13	(15.1)	0.2n	(15.1)

 OH^- IMPORT. E_m negative.

+5		86n	(5.9)	0.2n	(5.9)
		0.09	(8.9)	0.2 μ	(8.9)
+10		0.8 μ	(6.8)	1.5n	(6.8)
		0.73	(9.8) *	1.5 μ	(9.8)
+20		0.05	(8.6)	92n	(8.6)
		19	(11.2) *?	0.09	(11.6)
+40		54	(11.6)	0.34	(12.1)
		120	(15.1)	42	(14.4)

 OH^- EXPORT. E_m positive.

-5		11n	(4.1)	0.02n	(4.1)
		0.01	(7.1)	22n	(7.1)
-10		12n	(3.2)	0.02n	(3.2)
		0.01	(6.2)	24n	(6.2)
-20		13n	(1.4)	0.02n	(1.4)
		0.01	(4.4)	25n	(4.4)
-40		13n	(-2.1)	0.02n	(-2.1)
		0.01	(0.9)	25n	(0.9)

μ = μV n = nV Others mV. * See text.

the plasmalemma (with the possible exception of oxyntic cells of the stomach which secrete gastric juice at pH \sim 1, but this treatment cannot be applied to a frank secretory situation in any case). This general conclusion is quite important, and may well have far reaching consequences, for example, regarding mitochondria and chloroplasts.

However, it should not be concluded from the above that this treatment denies the possibility of existence of H^+ or OH^- transports; it does not, nor that such pumping has any useful effect. Indeed it shows rather clearly that transport of one or both of these ions can easily produce pH gradients across membranes, but that any E_m found to be present is not directly due to their pumping, but to another cause. Although H^+ or OH^- pumping evidently cannot generate a significant membrane potential, this fact does not preclude the use of H^+ or OH^- gradients once generated from providing energy to drive other processes, as in the chemiosmotic theory of Mitchell (1966, 1970). Thus it may well turn out that the active transport of another ion, e.g. Cl^- , if shown not to be obeying the Ussing-Teorell criteria, may actually be responsible for generating E_m in accord with my theory, while its own transport is fuelled by the electrochemical gradient of either H^+ or OH^- , ions not themselves capable of generating any appreciable proportion of E_m in face of the nebenion effect. In this regard it is pertinent to mention that Smith (1970) has indeed proposed that the link between Cl^- transport and the electron transfer

systems might be H^+ gradients. Recently Sanders (1980 a, b) has followed this approach, but clearly the theoretical limitation I have discovered sets the bounds that must be drawn around theories attempting to account for the generation of cell membrane potential. The same must also be said of the theories of Spanswick (1972, and subsequent papers) in which he proposes an electrogenic H^+ pump as source of E_m , a proposition which is evidently incorrent, as we now are able to see. The literature on membrane potential of mitochondria has been reviewed by Tedeschi (1980), but the theories he reviews must also be treated with caution in the light of my discoveries.

Obviously pumping of H^+ and OH^- would in general have to be considered either as a coupled transport if another ion were actively transported at the same time as one of these ions, or as a twin independant transport with one or more other ion(s) with which there is no coupling, although a fuller treatment in this latter case would merely reduce the effect that transport of one of these ions could have on E_m (see later, p.143).

Anticipating the sections to follow, it should be added for completeness here that the findings described above still hold good when H^+ or OH^- transport accompanies a Donnan system. The "Donnan enhancement effect" (to be introduced on p.108) does not lift E_m above the level of insignificance with H^+ or OH^- transport as Table 5, calculated on Eqns. (26) and (28), shows.

Table 5. H^+ and OH^- TRANSPORT WITH
DONNAN SYSTEM.

$\Delta\bar{\mu}_{H^+}$ (kJ/m)	c_i (meq/l)	E_m (mV)
<u>Hydrogen ion export.</u>		
1. Assume $pH_0 = 5$ (i.e. $P = 10^{-2}$ meq/l) and $N = 1.0$ meq/l.		
-20	100	-123
-20	0	-0.13
0	100	-122.7
2. Assume $pH_0 = 5$ and $N = 500$ meq/l.		
-20	100	-2.67
-20	0	-2.7×10^{-4}
0	100	-2.67
3. Assume $pH_0 = 8$ and $N = 1.0$ meq/l.		
-20	100	-123
-20	0	-1.3×10^{-4}
0	100	-123
4. Assume $pH_0 = 8$ and $N = 500$ meq/l.		
-20	100	-2.67
-20	0	-2.67×10^{-7}
0	100	-2.67
<u>Hydroxyl ion import.</u>		
1. Assume $pH_0 = 5$ and $N = 1.0$ meq/l.		
+20	100	-123
+20	0	-0.03
0	100	-123
2. Assume $pH_0 = 5$ and $N = 500$ meq/l.		
+20	100	-2.67
+20	0	-6×10^{-5}
0	100	-2.67
3. Assume $pH_0 = 8$ and $N = 1.0$ meq/l.		
+20	100	-123
+20	0	-16.0 *
0	100	-123
4. Assume $pH_0 = 8$ and $N = 500$ meq/l.		
+20	100	-2.72
+20	0	-0.06
0	100	-2.67

* Reference to Table 4 shows that pH_i is improbable.

Notice that line 1 in each section is virtually identical to line 3. Line 1 corresponds to active transport of H^+ or OH^- ($\Delta\bar{\mu} \neq 0$) with a Donnan system ($c_i \neq 0$), whereas line 3 corresponds to a Donnan system alone ($\Delta\bar{\mu} = 0$). Clearly transport makes little effect. Line 2 in each section corresponds to transport alone ($c_i = 0$), as in Table 4. Values of pH_o and N have been chosen to bridge the likely ranges. Notice that, in the only case where a significant value of E_m arises (* in Table 5), it does so only when pH_i is improbable (see Table 4). In this present table, H^+ import and OH^- export have not been included because these would merely act to depolarise the Donnan potential, but the effects are equally insignificant, as is obvious from the fact that $\Delta\bar{\mu}$ has virtually no effect on E_m , and its reversal of sign can hardly be expected to have a greater effect than shown in the table.

This examination of the role of H^+ and OH^- pumping in the development of E_m brings to light one cause for caution. It must always be borne in mind that the application of any of these equations in a predictive sense for E_m , also when ions such as Na^+ or Cl^- are the principal pumped ions, must always be tempered by the consideration as to whether the internal concentration of any ion will run to a physiologically improbable or physically impossible value. This warning applies particularly to the pumped ion, and at the higher levels of $\Delta\bar{\mu}$. The point however will normally be resolved in practise by actual measurement of $\Delta\bar{\mu}$ in any given organism.

Active Ion Transport and a Donnan System Co-existing.

What characterises a system of the kind we now treat is the existence of a certain concentration of charged molecules inside the cell which cannot pass through the membrane - e.g. protein - at the same time as the membrane surrounding the cell is capable of actively transporting across it at least one ionic species present. We now direct our attention to an examination of the effect of inclusion of these impermeant macro-ions on the value of the transmembrane potential, E_m , which will appear. Such a system is virtually certain to apply to almost every living cell to a greater or lesser extent. All cells contain dissolved macromolecules and usually the concentration is higher than that outside, although the meaning of "concentration" in this context must be defined with care. We are dealing with a "polydisperse" situation; one in which there will be a mixture of different proteins, and the mixture will be different inside and outside the cell. There will also be some organic acids in the cytoplasm, although in plants these tend to be dumped into the vacuole, but nevertheless they do not always pass the plasmalemma, and to that extent may contribute to the content of trapped ions in the cytoplasm. In the case of simple organisms growing in freshwater or seawater, clearly there will be little protein outside the cell, and a large Donnan contribution may be expected. Cells of the human body (or other "higher" animal) will be in contact with blood plasma and interstitial fluid, where a high level of protein exists outside the cell. One might expect the Donnan contribution here to be quite small since

captive charge inside the cell may be largely offset by macroions outside the cell in the plasma, but see p.129.

As an approach to this problem, we will again start with a simple model and build from there.

Active Cation Export Plus Donnan System - no diffusible nebenions present.

We will take a system in which the membrane actively exports Na^+ , and the cell is bathed in NaCl solution. We also assume that the cell contains a certain exchange equivalence of captive charge in the form of macroions. We do not need to know the nature of the captive macroions but merely their total ionic exchange equivalence. We will further assume that the pH is such that their charge is negative. In line with earlier usage (p.12), let the concentration of Na^+ associated with the ionic charge of the protein be c_i molar or equiv./l. (which are identical for monovalent Na^+). As before active Na^+ export provides that :

$$\Delta\bar{\mu} = FE_m + RT \ln \frac{\text{Na}_i}{\text{Na}_o} \quad (1)$$

and passive distribution of Cl^- requires that :

$$E_m = \frac{RT}{F} \ln \frac{\text{Cl}_i}{\text{Cl}_o} \quad (2)$$

However we now observe that ion balance considerations make $\text{Na}_o = \text{Cl}_o$ but $\text{Na}_i = \text{Cl}_i + c_i$. This last follows because not all of the Na^+ is balanced by Cl^- from the bathing medium, since some is already balanced by the protein negative charges. Nevertheless, since all the Na^+ present inside is assumed to be freely diffusible, all of it must be taken into account in Eqn.(1) whatever

its origin. Therefore :

$$\Delta\bar{\mu} = FE_m + RT \ln \frac{c_i + c_i}{c_l_0}$$

and using Eqn. (2) :

$$\Delta\bar{\mu} = FE_m + RT \ln \frac{c_l_0 \exp\left(\frac{FE_m}{RT}\right) + c_i}{c_l_0}$$

This equation now contains all given quantities except E_m , which we now require to make explicit, as follows.

$$\begin{aligned} \Delta\bar{\mu} &= RT \ln \left(\exp \frac{FE_m}{RT} \right) + RT \ln \frac{c_l_0 \exp\left(\frac{FE_m}{RT}\right) + c_i}{c_l_0} \\ &= RT \ln \left\{ \exp \frac{FE_m}{RT} \times \left(\frac{c_l_0 \exp\left(\frac{FE_m}{RT}\right) + c_i}{c_l_0} \right) \right\} \end{aligned}$$

and so :

$$\begin{aligned} \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) &= \exp \frac{FE_m}{RT} \times \left(\frac{c_l_0 \exp\left(\frac{FE_m}{RT}\right) + c_i}{c_l_0} \right) \\ &= \exp\left(\frac{2FE_m}{RT}\right) + \frac{c_i}{c_l_0} \exp\left(\frac{FE_m}{RT}\right) \quad (22) \end{aligned}$$

As for the earlier simple Donnan system we have a quadratic (see p. 12) :

$$\text{Let } \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) = A, \quad \exp\left(\frac{FE_m}{RT}\right) = B$$

Then Eqn. (22) becomes :

$$B^2 + \frac{c_i}{c_l_0} B = A$$

Completing the square, we have :

$$\left(B + \frac{c_i}{2c_l_0} \right)^2 = A + \left(\frac{c_i}{2c_l_0} \right)^2$$

and so :

$$B = \pm \sqrt{A + \left(\frac{c_i}{2c_l_0} \right)^2} - \frac{c_i}{2c_l_0}$$

$A = \exp(\dots)$ is always positive; c_i and Cl_o , being concentrations are always positive, and only the + root is physically meaningful, since B must also be positive, so :

$$\exp\left(\frac{FE_m}{RT}\right) = \sqrt{\exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + \left(\frac{c_i}{2Cl_o}\right)^2} - \frac{c_i}{2Cl_o}$$

or

$$\underline{\underline{E_m = \frac{RT}{F} \ln \left\{ \sqrt{\exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + \left(\frac{c_i}{2Cl_o}\right)^2} - \frac{c_i}{2Cl_o} \right\}}}} \quad (23)$$

This is the general equation for this case.

Tests.

We observe that Eqn.(23) reduces to Eqn.(3) when $c_i = 0$ as expected, i.e. the system would then be a pure Na^+ export with no Donnan effect.

When $\Delta\bar{\mu} = 0$, i.e. we have a pure passive Donnan system, Eqn.(23) reduces to :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{1 + \left(\frac{c_i}{2Cl_o}\right)^2} - \frac{c_i}{2Cl_o} \right\}$$

If we refer back to p.13, we see that for a pure Donnan system :

$$\begin{aligned} E_m &= \frac{RT}{F} \ln \frac{x}{c_o} = \frac{RT}{F} \ln \left\{ \frac{\sqrt{c_o^2 + \left(\frac{c_i}{2}\right)^2} - \frac{c_i}{2}}{c_o} \right\} \\ &= \frac{RT}{F} \ln \left\{ \sqrt{1 + \left(\frac{c_i}{2c_o}\right)^2} - \frac{c_i}{2c_o} \right\} \quad (24) \end{aligned}$$

and since $c_o = Cl_o$, we see that this checks. Note that $Na_o = Cl_o$ so Cl_o stands for $(NaCl)_o$.

These two extremes above correspond to cases of "high salt" and "low salt" for this model on the same definitions as on p.13ff for a pure Donnan system. Therefore the

situation of interest is "intermediate salt". Let us examine it numerically. The values in Table 6, following, were obtained by inserting a programme for Eqn.(23) into a Hewlett-Packard HP25C calculator. (Programmes for all equations appear in Appendix 3).

Table 6 demonstrates the very dramatic effect that the inclusion of an active pump has on a Donnan system (comparing columns) and also the dramatic effect that the presence of captive macroions (a Donnan system) has on the value of E_m generated in active transport (comparing figures down one column). Note also that the combined effect is not merely the sum of a Donnan potential with a simple transport-generated potential. There is a strong enhancement effect. This effect is perhaps unexpected, and is clearly very important. I believe it has not been reported before, and therefore I will henceforth refer to it as the "Donnan Enhancement Effect".

Now we need an assessment of the likely value of $\frac{c_i}{(\text{salt})_o}$ for a practical case, although of course we have yet to consider the effect of nebenions to the pumped ion, which will be present certainly in a practical case, before this becomes fully applicable. However, at this stage an examination is still worth pursuing. Probably no exact data exist to establish c_i because the problem has not previously been considered on this model before. However an assessment may be arrived at, as follows. Let us say, as a rough estimate, that protein in the cytoplasm could be between 2 and 10 % concentration as reasonable ends of range. The mix of proteins will

Table 6. CATION EXPORT PLUS DONNAN SYSTEM. No nebenions.

Values of $-E_m$ for various values of $\Delta\bar{\mu}$ and $\frac{c_i}{(\text{salt})_0}$ on Eqn.(23) at 37°C , (20°C). $-\Delta\bar{\mu}$, kJ/mole
Millivolts.

	0	5	10	20	50
0	0	26	52	104	155
0.005	0.07	26	52	107	176
0.01	0.13 (0.13)	26 (26)	53 (53)	110 (111)	190 (196)
0.02	0.27	27	54	116	207
0.05	0.67	28	56	131	231
0.1	1.3	29	61	147	249
0.2	2.7	33	69	165	268
0.5	6.6	42	87	189	292
1.0	13 (12)	55 (55)	104 (104)	207 (207)	311 (311)
2.0	23	71	122	226	329
5.0	44	95	147	250	354
10	62	113	165	269	372
20	80	132	184	287	391
50	105	156	208	312	415
100	123 (116)	175 (168)	227 (220)	330 (323)	453 (430)

Note that the column $\Delta\bar{\mu} = 0$ represents a pure Donnan system and row $\frac{c_i}{(\text{salt})_0} = 0$ represents Eqn.(3) for pure sodium export pumping. It is also clear that, between 20°C . and 37°C ., temperature has very little effect on the value of E_m .

be polydisperse, but let us assume that the "average" protein is ~60 kD and has 90 charges per molecule (see p. 21). At 2 % we have 20 g/l. protein and so the exchange equivalence will be $\frac{20 \times 90}{60k} = 30 \text{ meq/l.}$, and the corresponding figure for 10 % would be 150 meq/l. In theory, we can check this by deducting total free permeant anions inside from total free permeant cations inside (expressed as equivalences) and the difference would presumably be total captive anion equivalence inside. For an animal cell in contact with blood plasma, there is the additional complication that externally there are dissolved plasma proteins so that the Donnan effect may be partially nullified - to what extent requires a further study which will be presented later. Even when we come to examine plant examples in contact with freshwater or seawater, data in published literature are far from specific enough about the concentrations of mineral ions in cytoplasm, since analyses of the total plant are useless, the vacuole occupying much of the cell volume, and the layer of cytoplasm is often so thin that accurate measurements on it are hard to make. The best available data are from the Characeae and certain marine algae, and data in Hope and Walker (1975) indicate that the concentrations in cytoplasm are :

<u>Species</u>		<u>K⁺mM</u>	<u>Na⁺mM</u>	<u>Cl⁻mM</u>	<u>Na + K - Cl</u> * (see text)
Nitella flexilis	FW	125	9	20	114
Tolypella intricata	FW	~90	~15	~27	~76
Hydrodictyon africanum	FW	137	46	86	97
Valonia ventricosa	SW	454	40	138	336

FW = freshwater. SW = seawater.

Obviously these data must be regarded with the utmost caution and interpretation of the difference $(Na + K) - Cl$ as captive anion (* in table above) may not be justified because there are other permeant mineral ions in cells, but it does seem probable that the figures in the final column will largely represent at least an order of magnitude of captive anion equivalence. It is comforting that the figures quoted in the final column above tally remarkably well with the range calculated on the basis of an estimated concentration of cytoplasmic protein. Therefore we may say with some confidence that in freshwater $\frac{c_i}{(salt)_o}$ will be about $100/1.3 = 77$ and in seawater it will be about $100/534 = 0.19$. Referring to the first column in Table 6 , we see that a large Donnan potential would appear in freshwater algae even without pumping, though not as high as the observed E_m (~ -170 mV for *N.flexilis*), and, with pumping, E_m could be enormous on this model. Even in seawater, an algal cell could easily generate the observed E_m (which anyway tends to be lower than for freshwater algae, i.e. for *Valonia* -71 mV) with Donnan and active transport acting synergistically, but a pure Donnan system would then certainly fall far short of accounting for E_m . Clearly this extension of the theoretical model is promising, but next we must consider the effect on E_m of the presence of nebenions to the pumped ion.

Active Cation Export Plus Donnan System - Nebenions Present.

As before we assume $c_i =$ equivalence of captive anion, but now we bathe the cell in NaCl and KCl and regard Na^+ as the actively exported ion.

Again :

$$\Delta \bar{\mu} = FE_m + RT \ln \frac{Na_i}{Na_o} \quad (1)$$

and

$$E_m = \frac{RT}{F} \ln \frac{Cl_i}{Cl_o} = \frac{RT}{F} \ln \frac{K_o}{K_i}$$

since both Cl^- and K^+ are passively distributed, but now :

$$Na_o + K_o = Cl_o \quad \text{and} \quad Na_i + K_i = Cl_i + c_i$$

$$\text{so } Na_i = Cl_i + c_i - K_i$$

$$= c_i + Cl_o \exp\left(\frac{FE_m}{RT}\right) - K_o \exp\left(-\frac{FE_m}{RT}\right)$$

and so substituting in Eqn. (1) and setting $\exp\left(\frac{FE_m}{RT}\right) = B$ we get :

$$\Delta \bar{\mu} = FE_m + RT \ln \left\{ \frac{c_i + BCl_o - \frac{K_o}{B}}{Na_o} \right\}$$

or

$$\Delta \bar{\mu} = RT \ln \exp\left(\frac{FE_m}{RT}\right) + RT \ln \left\{ \right\}$$

and substituting A for $\exp\left(\frac{\Delta \bar{\mu}}{RT}\right)$

$$A = B \left\{ \frac{c_i + BCl_o - K_o/B}{Na_o} \right\}$$

or

$$ANa_o = Bc_i + B^2Cl_o - K_o$$

and so we have the quadratic in B :

$$B^2 + \frac{c_i}{Cl_o} B = \frac{K_o}{Cl_o} + A \frac{Na_o}{Cl_o}$$

which, by completing the square, gives :

$$\left(B + \frac{c_i}{2Cl_o} \right)^2 = \frac{K_o + ANa_o}{Cl_o} + \left(\frac{c_i}{2Cl_o} \right)^2$$

or

$$B = \pm \sqrt{\frac{K_o + ANa_o}{Cl_o} + \left(\frac{c_i}{2Cl_o}\right)^2} - \frac{c_i}{2Cl_o}$$

Again only the + root has physical meaning, so that:

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{K_o + Na_o \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)}{Cl_o} + \left(\frac{c_i}{2Cl_o}\right)^2} - \frac{c_i}{2Cl_o} \right\} \quad (25)$$

This is the general equation for this case. We may note that there are here five irreducible degrees of freedom, namely T , $\Delta\bar{\mu}$, Na_o (the pumped ion, P), K_o (the nebenion ion, N) and c_i (the Donnan captive anion inside). $Cl_o = Na_o + K_o = P + N$ of course. Nothing is gained by setting $N/P = \mathcal{R}$ this time (cf p.100), so we must be content with the more complicated form:

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{N+P \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)}{N+P} + \left(\frac{c_i}{2(N+P)}\right)^2} - \frac{c_i}{2(N+P)} \right\}$$

(26)

This form is suitable for evaluation by automatic programme fed into the HP25C calculator.

Tests.

1. When $\Delta\bar{\mu} = 0$, we have a pure Donnan system. The equation reduces to Eqn.(24) (p.111) as expected.
2. When $P = 0$, the equation again reduces to Eqn.(24) as expected.
3. When $N = 0$, we have the previous case, without nebenions. The equation reduces to Eqn.(23) (p.111) as expected.
4. When $c_i = 0$, we have the earlier situation of Na^+ export

in the presence of nebenions. Eqn.(26) then becomes :

$$E_m = \frac{RT}{2F} \ln \left\{ \frac{N + P \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)}{N + P} \right\}, \text{ or taking } \mathcal{R} = \frac{N}{P}$$

$$= \frac{RT}{2F} \ln \left\{ \frac{\mathcal{R} + \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)}{\mathcal{R} + 1} \right\}$$

which is Eqn.(21), (p.100)

In order to examine the behaviour of this equation and the model which it implies, we may for the present reduce the degrees of freedom to 4 by setting $T = 293^\circ\text{K}$ i.e. 20°C . Further simplification can be made if c_i (the captive Donnan anion) is set to 100 meq/l. on the basis of the discussion on p.112. E_m then calculates on Eqn.(26) to the values shown in Table 7. This table shows certain interesting properties of this model, in themselves worth observing :

1. Notice there is a reversal phenomenon between $N = 0.03$ and $N = 1.0$ when $\Delta\bar{\mu} = -30$ kJ/mole and between $N = 3.0$ and $N = 10$ when $\Delta\bar{\mu} = -15$ kJ/mole.
2. Nebenion effect is still evident - a decline in E_m with increased nebenion concentration.
3. Saturation still appears - vertical columns.
4. Between $N = 0.1$ and $N = 0.3$, E_m is independant of external pumped ion concentration when $\Delta\bar{\mu} = -30$ kJ/mole and at $N = 3.0$ when $\Delta\bar{\mu} = -15$, offering remarkable stability of E_m , its value also being largely independant of $\Delta\bar{\mu}$ between these limits. This fact could have considerable significance in regard to the maintenance of a constant E_m in the face of varying external and metabolic factors - it is in fact a form of inherent

Table 7. CATION EXPORT PLUS DONNAN SYSTEM WITH NEBENIONS.

Values of $-E_m$ for various values of P and N on Eqn. (26) with c_i set at 100 meq/l. captive anion

$\Delta\bar{\mu} = -15$ to -30 kJ/mole in each box

Millivolts 20°C

Values of N

10

3.0

1.0

0.3

0.1

0.03

0.01

0.003

0.001

0

0.1	330	*	286	291	261	263	232	232	204	205	174	174	147	147	116	116	88	89	58	58
0.3	302	*	278	291	258	263	231	232	204	205	174	174	147	147	116	116	88	89	58	58
1.0	272	425	262	291	250	263	228	232	203	205	174	174	147	147	116	116	88	89	58	58
3.0	244	399	240	290	234	263	220	232	200	205	173	174	146	147	116	116	88	89	58	58
10	214	369	212	289	210	263	204	232	191	205	169	174	145	147	116	116	88	89	59	59
30	186	341	185	287	185	262	183	232	176	205	162	174	142	147	115	116	88	89	59	59
100	156	311	155	281	155	260	154	232	152	204	146	174	133	147	112	117	88	89	60	61
300	128	283	128	269	128	254	127	229	127	204	124	174	119	147	105	117	86	91	62	64
1000	101	253	101	248	101	240	101	223	101	201	100	174	99	147	93	118	83	94	67	70

* Cannot be evaluated by HP25C - runs off number store and produces a calculation error.

homeostasis, if one may so describe it as distinct from a homeostasis which works via a negative feedback loop.

For the immediate purpose of attempting to account for the observed transmembrane potential (i.e. resting potential in excitable cases) in living cells, it will be more profitable to examine Eqn.(26) in the context of certain specific environments. We will therefore examine Eqn.(26) for (1) human blood plasma, (2) fresh-water and (3) seawater, as the solution external to the cell. Now the tabulated variables will be set as $\Delta\bar{\mu}$, to see what effect the pumping energy achieved has on E_m , and c_i , since it is not clear in most cases what precise value should be assigned for the captive anion equivalence inside the cell. Clearly experimental measures are needed here. In each of the three environments there is also uncertainty about the effective nebenion concentration, since this cannot be equated precisely with K^+ , there being minor quantities of other ions also (in some cases not so minor). Therefore the tables following (8 , 9 , 10) show the effect of changing the values of N (nebenion) on the diagonal in each box in each table. The following remarks are now pertinent :

In Human Blood Plasma.

It appears from Table 8 that E_m is relatively insensitive to $\Delta\bar{\mu}$, provided it is greater than -15 kJ/mole, but that the observed value in real cells of -70 mV requires about 100 meq/l. of captive anion equivalence inside the cell. Since there is a considerable amount of protein in plasma,

Table 8. CATION EXPORT PLUS DONNAN SYSTEM WITH NEBEXIONS IN HUMAN BLOOD PLASMA.

Values of $-E_m$ on Eqn. (26) for various values of $\Delta\bar{\mu}$ and c_i , taking $P = 145$ meq/l. (Na^+)₀ at 37°C.

$\Delta\bar{\mu}$, KJ/mole	N = 7 meq/l. in each box						Millivolts.												
	0	1.0	3.0	10	30	100	300	0	1.0	3.0	10	30	100	300					
0	0	0.09 0.09 0.09	0.27 0.26 0.26	0.89 0.88 0.87	2.7 2.6 2.6	8.8 8.6 8.5	24 23 23	0	0	0	0	0	0	0					
5	23	24	23	22	24	23	23	26	25	24	30	29	28	43	42	40	67	65	63
10	39	40	37	34	40	37	35	43	40	37	50	46	43	70	65	61	97	91	87
15	44	40	41	38	46	42	38	49	44	41	58	52	48	80	72	66	107	99	93
20	45	41	41	38	47	42	39	50	45	41	59	53	48	82	73	67	109	100	94
25	45	41	41	38	47	42	39	50	45	41	59	53	48	82	73	67	110	101	94
30	45	41	41	38	47	42	39	50	45	42	59	53	48	82	74	67	110	101	94

(Lowering the Temperature to 20°C reduces E_m by 0 at top left increasing to 5 mV at bottom right of table).

an offsetting effect might be expected, and therefore a further model must be constructed in which membrane-impermeant anions are considered to exist both sides of the membrane, but in general at different concentrations. This model will be presented later. The value of N is also important and requires further investigation. However this table does indicate that Eqn.(28) and the model on which it is based can successfully account for the observed value of E_m , in contrast to the earlier simpler model described in Eqn.(21), on reasonable values for all the parameters (with some doubt about c_i).

In Freshwater.

In Table 9 it has been assumed that the concentration of Na^+ (the pumped ion, P) may be taken reasonably as 1.0 mM as representative of "average" freshwater. As regards the value of N , there is some uncertainty. K_0 is quoted as 0.1 mM by Hope and Walker (1975), but there are other ions present in freshwater, and in any case a range exists in natural freshwaters. The table therefore starts at a baseline of 0.1 meq/l. for N , and goes through 0.3 to 1.0 as the highest possible value for nebenion to be encountered. However rather surprisingly this time, it turns out that Eqn.(26) (implying the export of cation as the active transport) is still capable of accounting for the observed E_m in freshwater algae (Characeae) if we take $c_i = 100$ meq/l., at which point E_m is insensitive to the value of $\Delta\bar{\mu}$ provided it is greater than -15 kJ/mole. The surprise arises from the fact that this model merely postulates

Table 9. CATION EXPORT PLUS DONNAN SYSTEM WITH NEBENIONS IN FRESHWATER.

Values of $-E_m$ on Eqn. (26) for various values of $\Delta\bar{\mu}$ and c_i , taking $P = 1.0$ (corresponding to Na^+).

$-\Delta\bar{\mu}$, KJ/mole	Millivolts 20°C.									
	0	1.0	3.0	10	30	100	300			
	c_i , meq/l.									
	0	1.0	3.0	10	30	100	300			
0	0	11 9.5 6.3	28 25 18	56 52 42	84 79 68	114 110 99	142 137 126			
5	20	14 72	42 30 15	66 51 29	96 80 56	123 107 83	154 138 113	181 165 141		
10	28	18 8.5	57 36 17	82 58 32	112 87 59	140 115 86	171 145 116	198 173 144		
15	30	18 8.7	60 37 18	86 59 32	116 88 59	143 116 86	174 147 116	202 174 144		
20	30	19 8.8	61 37 18	86 59 32	116 89 59	144 116 86	174 147 116	202 174 144		
25	"	"	"	"	"	"	"	"		
30	Then	no	further	change	in	these	columns			
35	30	19 8.8	61 37 18	86 59 32	116 89 59	144 116 86	174 147 116	202 174 144		

Na^+ export only, and the effect of Cl^- import, considered to be so important in plants, has so far not been discussed in connection with a Donnan system. This however will be done later.

In Seawater.

The values of E_m on Eqn.(26) assuming 460 meq/l. for Na^+ are given in Table 10. The first point to notice is the extremely low level of Donnan potential in seawater - this is shown in the top row of the table, when $\Delta\bar{\mu} = 0$. This is entirely to be expected because of the high level of salt (see p. 14). Nevertheless a strong "Donnan enhancement effect" is still seen - the value in a box is much greater than the sum of the corresponding values for $\Delta\bar{\mu} = 0$ and $c_i = 0$, respectively alongside and above the value concerned. In seawater we observe that E_m is lower than in freshwater, or indeed in blood plasma, and there is a rather strong nebenion effect. The lower value of E_m in seawater as compared to freshwater as calculated on this model is generally in agreement with findings on marine algae, but again this must be regarded as provisional until Cl^- import has been considered with a Donnan system. Nevertheless, as for freshwater algae, it is probably true to say that most marine organisms do transport cations actively at the cell membrane. It is now appropriate to consider the case of the converse situation as below :

Active Anion Import Plus Donnan System - no nebenions present.

It is assumed that there are no diffusible nebenions in the present system, although of course the captive anions

Table 10. CATION EXPORT PLUS DONNAN SYSTEM WITH NEBENIONS IN SEAWATER.

Values of $-E_m$ on Eqn.(26) for various values of $\Delta\bar{\mu}$ and c_i , taking $P = 460$ meq/l. $(Na^+)_0$

$N = 10$
 50 meq/l. in each box
 100

Millivolts $20^\circ C.$

c_i , meq/l.

		0	1.0	3.0	10	30	100	300	
$-\Delta\bar{\mu}$, kJ/mole	0	0	increasing to 0.03 0.02	0.03 0.08 0.07	0.08 0.26 0.23	0.27 0.77 0.68	0.81 2.6 2.25	2.7 7.6 6.7	
	5	24 21 16	no change to 24 22 16	24 22 16	25 22 16	26 23 17	31 28 20	43 38 28	
	10	41 32 21	"	42 33 21	42 33 21	43 33 10	46 35 22	55 42 26	74 57 35
	15	47 35 22	"	48 35 22	48 35 22	49 36 22	53 38 23	64 45 27	85 61 37
	20	48 35 22	"	49 35 22	49 36 22	50 36 22	54 38 23	65 45 27	87 61 37
	25	49 "	"	"	"	"	"	"	"
	30	Then	no	further	change	in	these	columns	
	35	49 35 22	"	49 35 22	49 36 22	50 36 22	54 38 23	66 45 27	87 61 37

of the Donnan system do constitute nebenions in charge sign to the pumped ions this time. The model we consider now is where there is captive anion of equivalence c_i together with active anion import at the membrane, which we will assume to be Cl^- transport. As before active import of Cl^- provides that :

$$\Delta\bar{\mu} = -FE_m + RT \ln \frac{Cl_i}{Cl_o} \quad (10)$$

$\Delta\bar{\mu}$ will be positive for import pumping.

We assume that the only other ions present outside are Na^+ , which, in the assumed absence of Na^+ pumping now, distribute passively. Thus by Nernst equation :

$$E_m = \frac{RT}{F} \ln \frac{Na_o}{Na_i} \quad \text{or} \quad \frac{Na_o}{Na_i} = \exp\left(\frac{FE_m}{RT}\right)$$

Ion balance requires that :

$$Na_o = Cl_o ; \quad Na_i = Cl_i + c_i \quad \text{or} \quad Cl_i = Na_i - c_i$$

Therefore, using both of the above, Eqn. (10) becomes :

$$\Delta\bar{\mu} = -FE_m + RT \ln \frac{Na_i - c_i}{Na_o}$$

But we can substitute for Na_i using the Nernst relation.

Thus, putting $\exp\left(\frac{FE_m}{RT}\right) = B$ we obtain :

$$\begin{aligned} \Delta\bar{\mu} &= -FE_m + RT \ln \left\{ \frac{\frac{Na_o}{B} - c_i}{Na_o} \right\} \\ &= RT \ln \left\{ \exp\left(-\frac{FE_m}{RT}\right) \times \frac{Na_o - c_i}{B Na_o} \right\} \end{aligned}$$

and putting $\exp\left(\frac{\Delta\bar{\mu}}{RT}\right) = A$:

$$A = \frac{\frac{Na_o}{B} - c_i}{B Na_o} = \frac{Na_o - c_i}{B^2 Na_o}$$

which re-arranges to the quadratic :

$$B^2 + \frac{c_i}{ANa_o} B = \frac{1}{A}$$

and, completing the square :

$$\left(B + \frac{A^{-1}c_i}{2Na_o} \right)^2 = A^{-1} + \left(\frac{A^{-1}c_i}{2Na_o} \right)^2$$

Therefore :

$$B = \pm \sqrt{A^{-1} + \left(\frac{A^{-1}c_i}{2Na_o} \right)^2} - \frac{A^{-1}c_i}{2Na_o}$$

which again only has physical significance for the + root.

Therefore, resubstituting :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\exp\left(-\frac{\Delta\bar{\mu}}{RT}\right) + \left(\frac{c_i \exp\left(-\frac{\Delta\bar{\mu}}{RT}\right)}{2Na_o}\right)^2} - \frac{c_i \exp\left(-\frac{\Delta\bar{\mu}}{RT}\right)}{2Na_o} \right\} \quad (27)$$

This is the general equation for this case. Note that Na_o represents the external concentration of NaCl, rather than either Na_o or Cl_o specifically, since $Na_o = Cl_o$ when only NaCl is present.

Tests.

When $\Delta\bar{\mu} = 0$ (i.e. a pure Donnan system), Eqn.(27) becomes :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{1 + \left(\frac{c_i}{2Na_o}\right)^2} - \frac{c_i}{2Na_o} \right\}$$

which is Eqn.(24), if we remember that $Na_o = c_o$ formerly, p.12.

When $c_i = 0$ (i.e. a pure active anion import), Eqn.(27) becomes :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\exp\left(-\frac{\Delta\bar{\mu}}{RT}\right)} \right\} = -\frac{\Delta\bar{\mu}}{2F}$$

which is Eqn.(11).

It will be observed that the symmetry between Eqn.(23) and Eqn.(27) is not nearly as close as between Eqn.(6)

and Eqn.(15), but now an element of asymmetry has been introduced which did not exist in the former models, namely the captive anion on one side of the membrane only.

Eqn.(27) is rather unwieldy, but we may now examine it by means of an automatic programme for it fed into the HP25C calculator. However, despite its rather unwieldy nature, it, like Eqns.(6), (15) and (23), has only 3 degrees of freedom, namely T , $\Delta\bar{\mu}$ and $\frac{c_i}{(\text{salt})_o}$. We will therefore tabulate values of E_m for various values of $\Delta\bar{\mu}$ and $\frac{c_i}{(\text{salt})_o}$ at 20°C. - Table II.

It is now apparent that the behaviour of this system contrasts sharply with the former cases of cation export with a Donnan system. The "Donnan enhancement effect" is now virtually non-existent. It is now seen that, although the presence of captive anions of the Donnan system do invariably raise the value of E_m above its value due to active anion import alone (apparent in vertical columns of figures), the level of enhancement is now not even equal to the sum of Donnan potential and transport-generated potential. Indeed at high levels of captive anion compared to external salt, active anion import has no appreciable effect on E_m (which is thus almost pure Donnan potential) until $\Delta\bar{\mu}$ for the transported ion becomes greater than about 10 kJ/mole. Eventually at $\Delta\bar{\mu}$ of 30 to 55 kJ/mole the potential is almost entirely dominated by active transport, with the Donnan system making virtually no contribution.

Table 11. ANION IMPORT PLUS DONNAN SYSTEM. No nebenions.

Values of $-E_m$ for various values of $\Delta\bar{\mu}$ and $\frac{c_i}{(\text{salt})_0}$ on Eqn. (27) at 20°C.

Millivolts.

	$\Delta\bar{\mu}$, kJ/mole					
	0	5	10	20	30	35
0	0	26	52	104	155	181
0.1	1.3	26	52	104	155	181
1.0	12	30	53	104	155	181
2.0	22	35	55	104	156	181
5.0	42	46	56	105	156	181
10	58	60	67	106	156	181
20	76	76	79	108	156	182
50	99	99	99	114	157	182
100	116	116	116	123	158	182

$\frac{c_i}{(\text{salt})_0}$

Active Anion Import Plus Donnan System. Nebenions Present.

We will now consider the case in which we assume there is present in the external solution an equivalence of diffusible permeant nebenions to the pumped ion, and these we will designate N_o (corresponding to NO_3^- of Eqn.(15), but avoiding the implication of assumptions as to its chemical identity - see earlier discussion). Again we will assume the pumped ion to be Cl^- , for which again Eqn.(10) applies :

$$\Delta\bar{\mu} = -FE_m + RT \ln \frac{Cl_i}{Cl_o}$$

with $\Delta\bar{\mu}$ positive for import pumping.

Ion balance now requires that :

$$N_o + Cl_o = \sum_o^+ \quad \text{and} \quad N_i + Cl_i + c_i = \sum_i^+$$

where we use the term \sum^+ to represent the sum of all diffusible cations, rather than identifying them by chemical symbols - we know there will be several species present in any real situation, but they may be lumped since we are not here assuming any of them are pumped actively.

The nebenions will distribute passively according to the Nernst Equation :

$$E_m = \frac{RT}{F} \ln \frac{N_i}{N_o} \quad (\text{N are negatively charged})$$

We also have the further Nernst relation that :

$$E_m = \frac{RT}{F} \ln \frac{\sum_o^+}{\sum_i^+} .$$

We see that $\sum_i^+ - N_i - c_i = Cl_i$ and so Eqn.(10)

becomes :

$$\Delta\bar{\mu} = -FE_m + RT \ln \frac{\sum_i^+ - N_i - c_i}{Cl_0}$$

and using the substitutions $A = \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)$ and $B = \exp\left(\frac{FE_m}{RT}\right)$ and replacing \sum_i^+ and N_i by their Nernst equivalents :

$$A = \frac{1}{B} \left(\frac{\sum_0^+ / B - N_0 B - c_i}{Cl_0} \right)$$

which, on re-arrangement, yields the quadratic :

$$B^2 + \frac{c_i}{ACl_0 + N_0} B = \frac{\sum_0^+}{ACl_0 + N_0}$$

Therefore, completing the square :

$$\left(B + \frac{c_i}{2(ACl_0 + N_0)} \right)^2 = \frac{\sum_0^+}{ACl_0 + N_0} + \left(\frac{c_i}{2(ACl_0 + N_0)} \right)^2$$

Hence the roots are :

$$B = \pm \sqrt{\frac{\sum_0^+}{ACl_0 + N_0} + \left(\frac{c_i}{2(ACl_0 + N_0)} \right)^2} - \frac{c_i}{2(ACl_0 + N_0)}$$

of which again only the + root is relevant. Therefore :

$$E_m = \frac{RT}{F} \ln B \text{ by definition of } B$$

which, on replacing N_0 by N , Cl_0 by P and \sum_0^+ by $N + P$

and re-substituting for A and B , yields :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{N+P}{P \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + N} + \left(\frac{c_i}{2(P \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + N)} \right)^2} - \frac{c_i}{2(P \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + N)} \right\}$$

This is the general equation for this case. Again, like (28)

Eqn.(26), it has 5 degrees of freedom, namely T , $\Delta\bar{\mu}$,

N , P and c_i .

Tests.

When $\Delta\bar{\mu} = 0$, the equation reduces to Eqn.(24) for a pure

Donnan system. The same also occurs when $P = 0$.

When $N = 0$ the equation reduces to Eqn.(27).

When $c_i = 0$:

$$E_m = \frac{RT}{2F} \ln \left\{ \frac{N+P}{P \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + N} \right\}$$

$$= - \frac{RT}{2F} \ln \left\{ \frac{P \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + N}{N+P} \right\}$$

which is Eqn.(21), if we recall that $\frac{N}{P} = \mathcal{R}$.

We will now explore Eqn.(28) numerically, by means of a programme inserted into the HP25C calculator.

Calculated values are tabulated in Tables 12, 13, 14 & 15.

In Table 12 it is seen that the reversal effect (down columns) again occurs. $N = 0$ corresponds to the former model, Eqn.(27). Nebenions invariably depress E_m , but the effect is less marked the higher the value of P . At low values of P and N , E_m is not much affected by $\Delta\bar{\mu}$, but at high values of P and N it is steeply sensitive to $\Delta\bar{\mu}$. This is worth bearing in mind with regard to the stability of membrane potential.

In Human Blood Plasma.

Table 13 shows values of E_m on Eqn.(28) on the assumption that P may be taken as 100 meq/l. for Cl^- . The assignment of a value for N is not straightforward, but data of Bell et al. (1968) suggest an upper limit for N of 30 meq/l. on the assumption that HCO_3^- (27), PO_4^{---} (1) and SO_4^{--} (1) are all in fully ionised form. It should be noted however that Table 13 probably has little relevance (and is only included for the sake of completeness) because Cl^- is not normally a pumped ion in human cells (with certain notable exceptions), so this will not be pursued.

Table 12. ANION IMPORT PLUS DONNAN SYSTEM WITH NEBENIONS.

Values of $-E_m$ on Eqn.(28) with c_i set at 100 meq/l. captive anion and T at 20°C. for various values of N and P.

$\Delta\bar{\mu} = \begin{matrix} 15 \\ 30 \end{matrix}$ kJ/mole in each box.

Millivolts. 20°C.

		Values of N									
		0	0.001	0.01	0.03	0.1	0.3	1.0	3.0	10	30
Values of P	0.1	174 179	174 179	172 177	168 173	157 164	139 117	114 133	88 117	59 100	35 85
	0.3	147 164	147 164	146 164	144 163	140 159	129 153	110 141	87 128	61 128	38 98
	1.0	117 158	117 158	117 158	117 158	115 157	111 154	101 149	85 139	64 126	45 113
	3.0	96 156	96 156	96 156	95 156	95 156	94 155	90 153	82 147	68 137	53 125
	10	83 156	83 156	83 156	83 156	83 156	83 155	82 155	79 152	73 147	63 138
	30	80 156	80 156	80 156	80 156	80 156	80 155	79 155	78 154	76 152	70 147
	100	78 155	78 155	78 155	78 155	78 155	78 155	78 155	78 155	77 154	75 152
	300	78 155	78 155	78 155	78 155	78 155	78 155	78 155	78 155	78 155	77 154
1000	78 155	78 155	78 155	78 155	78 155	78 155	78 155	78 155	78 155	77 155	

Table 13. ANION IMPORT PLUS DONNAN SYSTEM WITH NEBENIONS IN HUMAN BLOOD PLASMA.

Values of $-E_m$ on Eqn. (28) for various values of $\Delta\bar{\mu}$ and c_i , taking $P = 100$ meq/l. (Cl)₀.
 Millivolts 37°C. * See notes in text.

$\Delta\bar{\mu}$, kJ/mole.	$N = 10$ meq/l. in each box					
	0	10	30	100	300	
0	0	1.3 1.2 1.0	3.9 3.6 3.1	13 12 10	31 30 26	
5	26 25 23	26 25 23	27 26 24	31 30 27	40 39 36	
10	51 51 48	52 51 49	52 51 49	53 52 50	57 56 53	
15	77 76 74	77 77 74	78 77 74	78 77 75	79 79 76	
20	103 102 100	103 102 100	103 102 100	104 103 100	104 103 101	
25	129 128 126	129 128 126	129 128 126	129 128 126	129 128 126	
30	155 154 152	155 154 152	155 154 152	155 154 152	155 154 152	
35	181 180 178	181 180 178	181 180 178	181 180 178	181 180 178	

In Freshwater.

Table 4 shows values of E_m on Eqn. (28) for cells bathed in freshwater. This set of results is highly relevant to the case of freshwater plants, and should be compared and contrasted with the results on the former models : namely, for anion import in the absence of captive Donnan anions following Eqn. (21) (see p. 100 and Table 3), and for cation export in the presence of both nebenions and captive Donnan anions following Eqn. (26) (see p. 116 and Table 9). It is seen, on the present model, Eqn. (28), that the presence of captive Donnan anions only slightly increases the value of E_m (comparing first column, $c_i = 0$ with those to its right) as compared to the former model Eqn. (21), but on p. 89 it was already established that anion import in the presence of nebenions was capable of delivering the high values of E_m observed in freshwater algal cells, provided $\Delta\bar{\mu}$ achieved for Cl^- was high enough (but still of quite feasible value). Although the nebenion effect does depress the value of E_m , it is not severe at fairly high values of $\Delta\bar{\mu}$ and at reasonable values of c_i , thus this model can adequately account for E_m in freshwater algal cells. Stability of E_m is also good under probable physiological conditions.

What is perhaps surprising is that the present model (Eqn. (28)) yields rather lower values of E_m than cation export with nebenions and captive Donnan anions (Eqn. (26), see p. 116 and Table 9), but the nebenion effect is less steep (noting that nebenions for the two models are opposite in sign, of course, because of the

Table 14. ANION IMPORT PLUS DONNAN SYSTEM WITH NEBENIONS IN FRESHWATER.

Values of $-E_m$ on Eqn. (28) for various values of $\Delta\bar{u}$ and c_i , taking $P = 1.3 \text{ meq/l. (Cl}^-)_0$, 20°C .

$N = \begin{matrix} 0.1 \\ 0.5 \\ 1.0 \end{matrix}$ meq/l. in each box. Millivolts.

c_i , meq/l.

$\Delta\bar{u}$, kJ/mole	c_i , meq/l.									
	0	1	3	10	30	100	300			
0	8.8	7.8	24	50	77	108	136			
		5.5	21	47	74	104	132			
			15	48	65	95	123			
5	25	28	35	53	78	108	136			
	24	27	33	50	74	104	132			
	20	22	27	42	66	95	123			
10	51	52	54	63	80	108	136			
	49	50	53	60	77	105	132			
	45	46	48	54	69	96	123			
15	77	77	78	81	89	110	136			
	75	76	76	79	87	107	132			
	71	71	72	74	80	98	123			
20	103	103	103	104	107.	117	137			
	101	101	101	102	105	115	134			
	96	97	97	98	100	108	126			
25	129	129	129	129	130	134	144			
	127	127	127	127	128	132	142			
	122	122	122	123	124	127	135			
30	155	154	155	155	155	156	160			
	153	153	153	153	153	155	158			
	148	148	148	148	149	150	153			
35	180	180	180	181	181	181	183			
	179	179	179	179	179	179	181			
	174	174	174	174	174	175	176			

signs of their respective pumped ions). Even more surprising is the fact that the mechanism yielding the higher values of E_m (cation export + $N + c_i$) is likely to prevail in organisms displaying generally lower values of E_m in practise, namely freshwater protozoa and metazoa, as against the mechanism yielding not so high (but adequate) values of E_m (anion import + $N + c_i$) likely to prevail in freshwater algae where E_m is actually very high. Of course, this fact does not invalidate the models, but may merely reflect the actual values of $\Delta\bar{\mu}$ for the pumped ion achieved in each case by the relevant organisms. Of course, Table 14 does not apply to higher animals in either fresh or sea water, such as fish, since their cells are largely bathed in a regulated "milieu interne", i.e. the blood, for which Table 13 would be a closer analogy.

In Seawater.

Table 15 shows values of E_m on Eqn.(28) for cells bathed in seawater. Relevance here is mainly to marine algae. These results should be compared and contrasted with those on p.89 and Table 3 and p.120 and Table 11. On the present model, Eqn.(28), at the sort of values of $\Delta\bar{\mu}$ and c_i likely to prevail, the presence of captive Donnan anion (c_i) has practically no effect, the value of E_m being determined almost totally by $\Delta\bar{\mu}$ and little affected by nebenions. It is noteworthy however that E_m is much higher for anion import (Eqn.(28)) than for cation export (Eqn.(26) in seawater. This is in general agreement with findings on plant and animal cells in seawater, but as previously observed, marine algae generally show lower E_m

Table 15. ANION IMPORT PLUS DONNAN SYSTEM WITH NEBENIONS IN SEAWATER.

Values of $-E_m$ on Eqn. (28) for various $\Delta\bar{\mu}$ and c_i , taking $P = 534$ meq/l. 20°C .

Millivolts.

$N = 3.0$ meq/l. in each box

$\Delta\bar{\mu}$, kJ/mole	c_i , meq/l.			
	0	30	100	300
0	0	0.71 0.70 0.67	2.4 2.35 2.2	7.0 7.0 7.0
5	26 26 26	unchanging to	26 26 26	28 28 28
10	52 52 52	"	52 52 52	52 52 52
15	78 78 78	"	78 78 78	78 78 78
20	103 103 103	"	103 103 103	103 103 103
25	129 129 129	"	129 129 129	129 129 129
30	155 155 155	"	155 155 155	155 155 155
35	181 181 181	"	181 181 181	181 181 181

values than do freshwater algae, and this is indicated if Table 15 is compared with Table 14.

We now turn attention to the problem of the extended model needed to cope with active transport in the presence of membrane-impermeant macro-anions on both sides of the membrane, as applies in the case of cells in contact with blood.

Cation Export Plus Double Donnan System with Nebenions.

We assume in this model that there exists an equivalence, c_i , of exchangeable charge associated with captive macro-anions inside the cell and exchange equivalence c_o confined to the outside, as is the case with cells bathed in whole blood plasma, which contains dissolved protein. Again we assume there are present monovalent nebenions, N , to the pumped ion present in the system.

For Na^+ export, we have Eqn.(1) again, namely :

$$\Delta \bar{\mu} = FE_m + RT \ln \frac{Na_i}{Na_o}$$

Monovalent nebenions are passively distributed according to :

$$E_m = \frac{RT}{F} \ln \frac{N_o}{N_i} \quad (N \text{ are positive})$$

Counterions (e.g. Cl^-) are passively distributed according to :

$$E_m = \frac{RT}{F} \ln \frac{Cl_i}{Cl_o} \quad (\text{negative ions})$$

Ion balance in each phase demands that :

$$Na_o + N_o = Cl_o + c_o \quad \text{and} \quad Na_i + N_i = Cl_i + c_i$$

and so :

$$\frac{Na_i}{Na_o} = \frac{Cl_i + c_i - N_i}{Cl_o + c_o - N_o}$$

but $Cl_i = Cl_o \exp\left(\frac{FE_m}{RT}\right)$ and $N_i = N_o / \exp\left(\frac{FE_m}{RT}\right)$

Eqn. (1) may be written, as before :

$$\Delta\bar{\mu} = RT \ln \exp\left(\frac{FE_m}{RT}\right) + RT \ln \frac{Na_i}{Na_o}$$

yielding the now familiar form :

$$\exp\left(\frac{\Delta\bar{\mu}}{RT}\right) = \frac{Na_i}{Na_o} \exp\left(\frac{FE_m}{RT}\right)$$

which on replacing $\frac{Na_i}{Na_o}$ as above, yields, with the usual substitutions of A and B for the exponentials :

$$A = \frac{Cl_o B + c_i - N_o/B}{Cl_o + c_o - N_o} \cdot B$$

which again is a quadratic in B :

$$A(Cl_o + c_o - N_o) = Cl_o B^2 + c_i B - N_o$$

or

$$B^2 + \frac{c_i}{Cl_o} B = \frac{N_o + A(Cl_o + c_o - N_o)}{Cl_o}$$

which, on completion of the square, yields :

$$\left(B + \frac{c_i}{2Cl_o}\right)^2 = \frac{N_o + A(Cl_o + c_o - N_o)}{Cl_o} + \left(\frac{c_i}{2Cl_o}\right)^2$$

which has roots :

$$B = \pm \sqrt{\frac{N_o + A(Cl_o + c_o - N_o)}{Cl_o} + \left(\frac{c_i}{2Cl_o}\right)^2} - \frac{c_i}{2Cl_o}$$

only the + root of which is meaningful since B must be positive as an exponential. Hence on resubstitution

we have :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{N_o + (Cl_o + c_o - N_o) \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)}{Cl_o} + \left(\frac{c_i}{2Cl_o}\right)^2} - \frac{c_i}{2Cl_o} \right\} \quad (29)$$

We may now make the following generalisations and replacements $N_o = N$, $Na_o = P$ and therefore $Cl_o = P + N - c_o$, where we assume that Cl_o represents the sum of all passively distributing monovalent counterions to the pumped ion.

Eqn.(29) now becomes :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{N + P \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)}{N + P - c_o} + \left(\frac{c_i}{2(N + P - c_o)}\right)^2} - \frac{c_i}{2(N + P - c_o)} \right\}$$

This is the general equation for this case. (30)

Tests.

When $c_o = 0$, the equation reduces to Eqn.(26) as expected.

There is also a special case of interest; when the exchange equivalence of membrane-impermeant macro-anions is the same on both sides of the membrane, i.e. when

$c_i = c_o = c$. Eqn.(30) then becomes :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{N + P \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)}{N + P - c} + \left(\frac{c}{2(N + P - c)}\right)^2} - \frac{c}{2(N + P - c)} \right\}$$

(31)

This equation makes it clear that the presence of impermeant macro-anions is not irrelevant even when they are at the same exchange equivalence on the two sides of the membrane, a situation which would produce no Donnan potential in the absence of pumping. This gives a further test to the equation - by putting $\Delta\bar{\mu} = 0$, when Eqn.(31) becomes :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{N + P}{N + P - c} + \left(\frac{c}{2(N + P - c)}\right)^2} - \frac{c}{2(N + P - c)} \right\}$$

Now replace $N + P$ by S for salt :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{S}{S - c} + \left(\frac{c}{2(S - c)}\right)^2} - \frac{c}{2(S - c)} \right\} = 0$$

The above expression may be seen to be zero if one observes

that $\frac{s}{s-c}$ may be replaced by $\frac{s-c+c}{s-c}$ or $1 + \frac{c}{s-c}$ whereupon it becomes clear that we have a perfect square under the $\sqrt{\quad}$, namely $\left(1 + \frac{c}{2(s-c)}\right)^2$.

Examination of Eqns. (30) and (31).

It should be observed that there is a physical constraint on the values which may be put into Eqn. (30) to have real meaning. The equation, as a mathematical function, behaves very oddly when $N + P$ is almost identical in value to c_0 . If c_0 is somewhat less than $N + P$ (i.e. $N+P-c_0 > 0$) then E_m falls in magnitude (running in a positive direction) as the value of c_0 approaches $N + P$, whereas if c_0 is somewhat greater than $N + P$ (i.e. $N+P-c_0 < 0$) then E_m rises in magnitude as the value of c_0 approaches $N + P$. At the identity $N+P = c_0$, the equation becomes inevaluable. However the second of these situations has no physical meaning because it is impossible for an electrolyte solution to be constructed in which the sum of cationic charge ($N + P$) is less than the total anionic charge (c_0). The reverse situation ($N+P-c_0 > 0$) is however perfectly meaningful in terms of the model, the shortfall of c_0 as against $N + P$ being accounted for by counterions to P , for example Cl^- . This was assumed in the derivation of Eqn. (31). These considerations therefore set the limit that c_0 must never be greater than $N + P$.

Next it should be observed that Eqn. (30) has 6 independent degrees of freedom, namely: T , $\Delta\bar{\mu}$, N , P , c_0 and c_i . This makes a full numerical or graphical display of Eqn. (31) rather difficult. However for

practical purposes, we may reduce the number of degrees of freedom to a manageable number as follows. First we fix T . Since this equation has been designed primarily to cope with human cells in contact with blood, T may be set to 37°C . (i.e. $273+37^{\circ}\text{K}$). With this reduction in the degrees of freedom the equation may first be examined in the form of Eqn.(31) with c_o set equal to c_i . This is a state of affairs not likely to be too far away from that pertaining in the human body, although until actual measurements have been made of c_o and c_i , it remains a somewhat open question, (but see below as to how important this is). We now have the following four degrees of freedom, namely : $\Delta\bar{\mu}$, N , P , and c . Table 16 shows values obtained with the value of P (i.e. Na^+ in plasma) set to 145 meq/l.

It is clear from Table 16 that, with increasing values of c , E_m rises from the baseline level (when $c = 0$, corresponding to Eqn.(21)), to a maximum set by the limit when $c = N+P$. It is also evident that, as $\Delta\bar{\mu}$ is increased, E_m reaches a maximum plateau for values of $\Delta\bar{\mu}$ greater than -20 kJ/mole. Since -20 kJ/mole is quite a modest level of electrochemical potential difference for Na^+ , it is now reasonable to set $\Delta\bar{\mu}$ above this level and examine the effect on E_m of c_o and c_i not being identical - reverting to the general equation Eqn.(30). Such a display of the equation is given in Table 17.

Table 17 shows the perhaps surprising result that the amount of external membrane-impermeant macro-ion

Table 16. CATION EXPORT PLUS DOUBLE-DONNAN SYSTEM WITH NEBENIONS IN HUMAN BLOOD PLASMA.*

Values of $-E_m$ (mV) on Eqn. (31) for various values of $\Delta\bar{\mu}$ and c ($c_i = c_o$), taking $P = 145$ meq/l. for Na^+ in plasma. Millivolts 37°C .

$N = \frac{5}{9}$ meq/l. in each box.

c , meq/l.

almost
absolute
limit
|

		0	10	20	50	100	120	140
$-\Delta\bar{\mu}$, kJ/mole.	0	0	0	0	0	0	0	0
	5	23	25	26	31	39	42	45
		23 22	24 23	25 25	29 28	37 36	41 39	43 42
	10	39	42	45	55	68	73	77
		36 34	39 36	42 42	50 46	63 58	67 62	71 66
15	44	48	52	64	79	83	87	
	40 37	43 40	47 47	57 52	70 64	75 69	79 72	
≥ 20	45	49	54	65	80	85	89	
	41 38	44 41	48 44	58 52	72 65	76 70	80 73	

* Uncertainty exists as to values to assign for N and c , and this table displays how N and c affect E_m . For the purposes of this table c_o (external macro-anion equivalence) is assumed equal to c_i (internal macro-anion equivalence), and given as c .

Table 17. CATION EXPORT PLUS DOUBLE-DONNAN SYSTEM WITH NEBENIONS IN HUMAN BLOOD PLASMA.*

Values of $-E_m$ (mV) on Eqn.(30) for various values of c_o and c_i , taking $P = 145$ meq/l. for Na^+ in plasma. $\Delta\bar{\mu} > 20$ kJ/mole. Millivolts $37^\circ C.$

$N = \frac{5}{9}$ meq/l. in each box.

		c_i , meq/l.						
		0	10	20	50	100	120	140
c_o , meq/l.	0	45 41 38	50 45 42	55 49 45	67 60 55	82 73 67	86 78 71	90 81 75
	10	45 40 37	49 44 41	54 49 44	67 60 54	82 73 67	86 78 71	90 81 75
	20	44 39 36	49 44 40	54 48 44	67 59 54	82 73 67	86 77 71	90 81 75
	50	40 36 33	46 41 37	52 46 41	65 58 52	81 73 67	86 77 71	90 81 75
	100	31 27 24	39 34 30	47 40 36	64 56 50	80 72 65	85 77 70	89 80 74
	120	24 20 18	35 29 25	44 37 32	63 55 48	80 72 65	85 76 70	89 80 74
	140	9 7 6	27 21 17	40 32 27	62 53 47	80 71 65	85 76 69	89 80 73

* Uncertainty exists as to the values to assign to N , c_o and c_i . Note that c_o is limited to a maximum of $N+P$ whereas c_i is not, but in practise it is hardly likely to be above 140.

equivalence, c_o , has little effect on the value of E_m , over quite a wide range of values. It is therefore relatively unimportant what value should be assigned to c_o . However, the value of c_i , the captive Donnan macro-ion equivalence inside the cell, does markedly affect the value of E_m . The "Donnan enhancement effect" is therefore essentially due to the macro-ion captive inside the cell, and we therefore cannot really refer to the effect seen in Table 17 (based on Eqn.(31)) as a "Double-Donnan enhancement effect", but that table does underline the fact that there is still a Donnan enhancement effect even in a circumstance where no E_m (Donnan) would result in the absence of active transport, an undoubtedly unexpected result.

It is highly satisfying to observe, on this now very much more complete model, intended to represent certain human cells in contact with blood plasma, that the generally accepted figure for the resting cell membrane potential of -70 mV (or thereabouts) is found in Table 17 at probable values for all the parameters.

Effect of Coupled Transport of K^+

There yet remains the need to trim the above model further by introducing the import of K^+ coupled to the export of Na^+ , the expected effect of which would be to depolarise E_m by a few millivolts. However, this will involve consideration of rate processes, hitherto unnecessary, involving the permeabilities of the membrane

to sodium, P_{Na} and to potassium, P_K . This will be approached as follows. It will be assumed, for the purposes of discussion, that a stoichiometry of $m K^+$ to $1 Na^+$ applies to the Na/K exchange pump. (The value of m varies between 1 and $1/3$ - see p.70). It will further be assumed that the Na/K pump runs only* to the extent required to make good the leakage of Na^+ back into the cell through the indifferent passive permeability, P_{Na} . Since it has been found with many cells that P_{Na} is very much less than P_K , then K^+ will leak out so fast that its import, at the rate of Na^+ leakage times m , will result in a rather small $\Delta\bar{\mu}$ being achieved for K^+ . Note however that the total work done by the pump on ionic gradients is equal to $\Delta\bar{\mu}_{Na} + \Delta\bar{\mu}_K$, and this total is likely to be the effective limit in the general case (to which the treatment below still applies) when P_K and P_{Na} are more nearly equal. However, in the example here, it is fair, to a close approximation, to assume that the limiting energy the pump can transmit to ionic gradients is $\Delta\bar{\mu}_{Na}$, since $\Delta\bar{\mu}_{Na}$ will be much greater than $\Delta\bar{\mu}_K$.

Permeability follows a form of law, similar to Fick's Law, where the driving force for the rate is concentration (strictly activity) difference. Thus the rate of passive transit of a neutral species across a membrane is given by :

* This assumption may not be precisely true in real cells, of course, but it provides a reasonable starting point.

$$\Phi = - P \Delta C$$

where : Φ = flux rate per unit area of membrane
 P = permeability of membrane for solute
 C = concentration of solute.

However, for an ion, since it is charged, the situation is much more complicated. There are two forces acting on an ion, namely those resulting from concentration gradients (as for a neutral species) and also electrostatic forces due to the presence of an electric field. The rate of flux of an ionic species is given therefore by the equation :

$$\Phi = - \frac{uRT}{zF} \frac{\partial C}{\partial x} - uC \frac{\partial E}{\partial x}$$

where : u = electrophoretic mobility of the ion*
 and where the first term refers to diffusional movement in the concentration gradient, and where the second term refers to electrophoretic drift due to the electric field. (In this treatment it will be assumed that the activity coefficient does not vary throughout the system - admittedly an approximation, but one which does not usually lead to very serious error, see p.159).
 Now, the differential equation for Φ , above, cannot be integrated directly for the full thickness of the membrane without knowledge of the profiles of C and E across the membrane. However a simplifying assumption was made by Goldman (1943) that the electric field could be taken as constant, enabling him to derive

* For a full treatment of units and defining formulae, see Appendix 4, where also the Goldman flux equation is reproven.

an equation for flux rate, which in the symbols of this text, is :

$$\Phi = - P_z \frac{FE_m}{RT} \cdot \frac{C_o - C_i \exp\left(\frac{zFE_m}{RT}\right)}{1 - \exp\left(\frac{zFE_m}{RT}\right)} \quad \begin{array}{l} \text{flux rate} \\ \text{per unit area} \end{array} \quad (32) *$$

Now the stoichiometric linkage of the pump determines that the rate of leakage of K^+ be equal to m times the rate of leakage of Na^+ . Thus :

$$\Phi_K = - m \Phi_{Na} \quad \text{where the - sign indicates opposition of direction.}$$

or, using Eqn. (32) and replacing $\exp\left(\frac{zFE_m}{RT}\right)$ by B :

$$P_K \frac{FE_m}{RT} \cdot \frac{K_o - K_i B}{1 - B} = - m P_{Na} \frac{FE_m}{RT} \cdot \frac{Na_o - Na_i B}{1 - B}$$

Now let $m \frac{P_{Na}}{P_K} = \gamma$ and recall that active Na^+ transport is described by Eqn. (1) which in the substituted form is :

$$A \frac{Na_i}{Na_o} = B$$

Now Na_i can be eliminated, thus :

$$K_o - K_i B = - \gamma Na_o (1 - A)$$

which after re-arrangement gives :

$$K_i = \frac{Na_o}{B} \gamma (1 - A) + \frac{K_o}{B} \quad (33)$$

This should be contrasted with the assumption in previous models that K^+ distributes according to the Nernst equation, when, in the present notation, K_o/B was taken to apply.

* Note that there is an alternative flux equation to Eqn. (32), derived by Henderson (1907, 1908) on the assumption of constant concentration gradient, but its adoption instead would make little difference since E_m is not much affected by K^+ import anyway when $P_K \gg P_{Na}$. In other cases however it may repay examination.

In the present model, the system contains :

Outside.

$Na_o, K_o, N_o, Cl_o, c_o$

Inside.

$Na_i, K_i, N_i, Cl_i, c_i$

N_o and N_i now stand for nebenion not including K^+ .

Their values are therefore likely to be lower than N of the former model (Eqn.(30)). We are still assuming that no counterions (i.e. anions) are pumped.

Ion balance demands that :

$Na_o + K_o + N_o = Cl_o + c_o$ and $Na_i + K_i + N_i = Cl_i + c_i$

Passive distribution of nebenions and counterions give the Nernst expressions :

$$N_i = \frac{N_o}{B} \quad \text{and} \quad Cl_i = Cl_o B$$

and active export of Na^+ gives the now familiar expression :

$$A = \frac{Na_i}{Na_o} B \quad (\text{a form of Eqn.(1)})$$

$Na_i = Cl_i + c_i - K_i - N_i$ by ion balance, so, using Nernst substitutions and Eqn.(33) and (1) as above :

$$ANa_o = \left(Cl_o B + c_i - \gamma \frac{Na_o}{B} (1-A) - \frac{K_o}{B} - \frac{N_o}{B} \right) B$$

which is once again a quadratic in B :

$$ANa_o = Cl_o B^2 + c_i B - \gamma Na_o (1-A) - (K_o + N_o)$$

or

$$B^2 + \frac{c_i}{Cl_o} B = \frac{ANa_o + \gamma Na_o (1-A) + (K_o + N_o)}{Cl_o}$$

which has roots :

$$B = \pm \sqrt{\frac{ANa_o + \gamma Na_o (1-A) + (K_o + N_o)}{Cl_o} + \left(\frac{c_i}{2Cl_o}\right)^2} - \frac{c_i}{2Cl_o}$$

only the + root having meaning, as before.

We may now recall that, by ion balance outside :

$$\begin{aligned} Cl_o &= (Na_o + K_o + N_o) - c_o \\ &= \sum_o^+ - c_o \end{aligned}$$

Therefore, resubstituting for A and B and re-arranging :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{(K_o + N_o) + Na_o \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) + Na_o \gamma \left(1 - \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)\right)}{\sum_o^+ - c_o}} + \left(\frac{c_i}{2(\sum_o^+ - c_o)}\right)^2 - \frac{c_i}{2(\sum_o^+ - c_o)} \right\}$$

where $\sum_o^+ = (Na_o + K_o + N_o)$, $\gamma = mP_{Na}/P_K$ and $\Delta\bar{\mu}$ refers to Na^+ export. The equation is clearly similar to

Eqn.(30), but with the addition of the extra term :

$$\gamma Na_o \left(1 - \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)\right) \text{ under the square root.}$$

It is seen that this equation reduces to Eqn.(30) when

$m = 0$ (i.e. $\gamma = 0$) implying that there would then be no

coupling between Na^+ transport and K^+ . $K_o + N_o$ is

equivalent to N of the former treatments, and it is

interesting to observe that, although the model assumes

K^+ alone to be coupled to Na^+ transport, with other positive

nebenions not transported, no distinction appears between

them in this equation for E_m . However, this fact must

not be taken to imply that unpumped nebenions distribute

in the same concentration ratio as K^+ , since clearly the

model assumes that they do not. \sum_o^+ is now again seen to

be $N + P$. Terms in $\exp\left(\frac{\Delta\bar{\mu}}{RT}\right)$ may be collected, yielding

the form :

$$E_m = \frac{RT}{F} \ln \left\{ \sqrt{\frac{N + P \left(\gamma + (1 - \gamma) \exp\left(\frac{\Delta\bar{\mu}}{RT}\right)\right)}{N + P - c_o}} + \left(\frac{c_i}{2(N + P - c_o)}\right)^2 - \frac{c_i}{2(N + P - c_o)} \right\}$$

Eqn.(34) has seven degrees of freedom, namely : T, $\Delta\bar{\mu}$, (34)

N, P, γ , c_i , c_o . Note that although both N and γ comprise

more than one variable each, they do not increase the degrees of freedom of the equation as a mathematical expression on that account. Again c_o must not be greater than $N + P$.

It is now of interest to see in quantitative terms what effect is found upon E_m by the inclusion of K^+ import coupled to Na^+ export. P may be set to 145 meq/l. for Na_o as before. N may be taken as 7 meq/l. to represent K_o + unpumped nebenions. c_i may be assumed equal to c_o , since it has already been shown (p.134) that c_o has little effect on E_m for practical values of c_o and c_i . The value for c may be taken provisionally as 100 meq/l. P_{Na}/P_K may be set to 1 : 75 = 0.013 (see Katz 1966) and if m is taken as 1 Na : 1 K then $\gamma = 0.013$ or if $m = 2/3$ then $\gamma = 0.0089$ or if $m = 1/3$ then $\gamma = 0.0044$. When γ is set to zero it implies no transport coupling as observed above. T may be set to $37^\circ C$. With these values and using an automatic programme in the HP25C calculator, values of $-E_m$ (mV) are :

Table 18. Effect of Coupling of Transports.

$\Delta\bar{\mu}$, kJ/mole	$\gamma = 0.013$ (1Na:1K)	$\gamma = 0.0089$ (3Na:2K)	$\gamma = 0.0044$ (3Na:1K)	$\gamma = 0$ (K unpumped)
-15	65	66	68	70
-20	66	67	69	72
-25	66	68	70	72
-30	66	68	70	72

It is now evident that the fact that K^+ import is coupled to Na^+ export does indeed produce a depolarisation of a few millivolts. It so happens that the value of -68 mV for the resting membrane potential, with 3Na:2K pumped, is precisely the value frequently quoted for nerve, but

it must be borne in mind that this value is dependant on the captive macro-anion content of the cell having an exchange equivalence of 100 meq/l. This figure, although reasonable as indicated earlier (p. 112 to 114) is clearly open to question, and experimental measurements are needed. It is however noteworthy that cells which may be expected to have a rather high value of c_i due to high concentration of intracellular protein, for example muscle cells, do tend to show larger E_m values than cells with lower c_i , for example nerve cells. For instance, Katz (1966) quotes -90 mV for E_m in frog muscle as against -60 mV for squid axon, although of course other factors besides c_i differ, such as Na_o , external nebenion, and species. In any case the value of E_m will be dependant on $\Delta\bar{\mu}$ for the primary pumped ion (Na^+) and although E_m saturates when $\Delta\bar{\mu}_{Na}$ exceeds -20 kJ/mole, it must not be assumed that $\Delta\bar{\mu}$ will necessarily always be so high. Thus the low value of -9 mV for E_m in red blood cells (Hladky and Rink 1976) could be due to a number of causes of which low $\Delta\bar{\mu}$ for Na^+ could be one, but detailed consideration of this unusual case cannot be entered into here, where the purpose is to establish the theoretical framework on which such studies can be carried out.

However, one further point worthy of consideration here, is the difference between E_m and the potassium equilibrium potential, E_K . This difference is, of course, of considerable interest to neurophysiologists since the "after-potential hyperpolarisation" following an action potential sequence is caused by the momentary expression

of E_K due to the temporarily very high value of P_K , the permeability of the membrane to potassium. As already observed above in the derivation of Eqn.(34), K^+ is not quite in passive equilibrium across the membrane when its transport is coupled to Na^+ transport. The action of the Na/K pump, with the aid of Donnan effects, generates E_m and also sets up the level of K_i (and other ions). Now the value of E_K is that value to which the potential across the membrane would set when bathed on its two sides by these values of K_i and K_o , assuming that K^+ and no other ion could permeate freely. This is in effect the case when P_K rises to such a high value during an action potential sequence. To evaluate $E_K - E_m$ then, we need to calculate K_i . K_o is already known, of course, as an imposed condition. Eqn.(33) states that :

$$K_i = \frac{\gamma(1-A)Na_o + K_o}{B} = \frac{\gamma(1-A)Na_o + K_o}{\exp\left(\frac{FE_m}{RT}\right)}$$

Now E_K by definition follows the Nernst equation on the values of K_i and K_o as established by active transport plus Donnan effects, namely :

$$E_K = \frac{RT}{F} \ln \frac{K_o}{K_i}$$

which, using Eqn.(33), is seen to be :

$$\begin{aligned} E_K &= \frac{RT}{F} \ln \frac{K_o}{\gamma(1-A)Na_o + K_o} \exp\left(\frac{FE_m}{RT}\right) \\ &= E_m - \frac{RT}{F} \ln \left(\gamma(1-A) \frac{Na_o}{K_o} + 1 \right) \end{aligned}$$

so that, replacing A :

$$\underline{\underline{E_K - E_m = - \frac{RT}{F} \ln \left(\gamma \left\{ 1 - \exp\left(\frac{\Delta\bar{\mu}}{RT}\right) \right\} \frac{Na_o}{K_o} + 1 \right)}}} \quad (35)$$

Taking $\chi = 0.0089$ (assuming 3Na:2K stoichiometry), $\text{Na}_o = 145 \text{ meq/l}$, $\text{K}_o = 5 \text{ meq/l}$. (Bell et al. 1968) and $\Delta\bar{\mu} \gg -20 \text{ kJ/mole}$, $E_K - E_m$ on Eqn.(35) calculates to -6 mV , or in other words, E_K should be 6 mV more negative than the resting potential, E_m . This figure appears to be somewhat of an under-estimate compared to the generally accepted value of about -10 mV . Nevertheless it is clearly of the right order, and the error may lie in the values assigned to the parameters. It may be more appropriate to take $\chi = 0.013$ (assuming 1K:1Na stoichiometry) and K_o as 4 meq/l , when $E_K - E_m = -10.3 \text{ mV}$ on Eqn.(35). However this may be, Eqn.(35) indicates how $E_K - E_m$ may be expected to depend on imposed parameters in the long term, as distinct from short term effects where the Goldman equation is appropriate.

It should be noted in Eqn.(35) that a distinction now appears between K_o and external nebenions, in contrast to Eqn.(34). Also the point should be made that $E_K - E_m$ is not the same as the depression in the value of E_m generated occasioned by the linkage of K^+ import to Na^+ export.

Simultaneous Independent Cation Export and Anion Import with Donnan System and Nebenions.

There now remains one final model worth considering because of its probable relevance to plants. It is known in Characeae (e.g. MacRobbie 1971) that there are active transport pumps at the plasmalemma for both

Na/K exchange and Cl^- import. The same situation appears to apply also in higher plants (Higinbotham, 1973 a,b). However the Cl^- and Na/K pumps are not directly coupled to each other, it is believed, since the evidence is that Cl^- pumping derives its energy from electron transfer reactions whereas Na/K pumping is driven by ATP.

For this present treatment, it will be assumed that K^+ is not pumped as in all former models up to that embodied in Eqn.(34). However, as Eqn.(34) has illustrated, K^+ import linked to Na^+ export produces a depolarisation of only a few millivolts, provided $P_{\text{K}} \gg P_{\text{Na}}$. Thus now, with two independent transport systems operating together, the effect of linked K^+ transport will be even less, and so may reasonably be ignored.

In this present model, Na^+ export gives again the familiar Eqn.(1):

$$\exp\left(\frac{\Delta\bar{\mu}_{\text{Na}}}{RT}\right) = \frac{\text{Na}_i}{\text{Na}_o} \exp\left(\frac{FE_m}{RT}\right) \quad (1)$$

and for Cl^- import, Eqn.(10) applies :

$$\begin{aligned} \Delta\bar{\mu}_{\text{Cl}} &= -FE_m + RT \ln \frac{\text{Cl}_i}{\text{Cl}_o} \\ &= RT \ln \exp\left(-\frac{FE_m}{RT}\right) + RT \ln \frac{\text{Cl}_i}{\text{Cl}_o} \\ &= RT \ln \left(\frac{\text{Cl}_i}{\text{Cl}_o} \cdot \exp\left(-\frac{FE_m}{RT}\right) \right) \end{aligned}$$

or

$$\exp\left(\frac{\Delta\bar{\mu}_{\text{Cl}}}{RT}\right) = \frac{\text{Cl}_i}{\text{Cl}_o} \exp\left(-\frac{FE_m}{RT}\right)$$

As before we can use the substitution B for $\exp\left(\frac{FE_m}{RT}\right)$

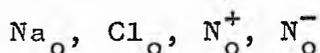
and A for $\exp\left(\frac{\Delta\bar{\mu}}{RT}\right)$, now adding appropriate subscripts, thus :

$$A_{Na} = \frac{Na_i}{Na_o} B$$

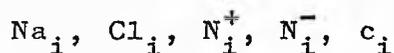
$$A_{Cl} = \frac{Cl_i}{Cl_o} \cdot \frac{1}{B}$$

Of course, the B s in the two equations are identical since only one E_m can exist in a system at any one time. We assume that the system contains nebenions, and now it is necessary to consider nebenions to the pumped cation, N^+ , and nebenions to the pumped anion, N^- . The cell cytoplasm will be assumed to contain captive Donnan macro-anion equivalence, c_i , and the bathing medium will be assumed free of macroions, a fair assumption for the purposes of this model, namely for plants (mainly) in freshwater or seawater. Situations where there would be a significant level of macroion solute outside the cell are hard to envisage*. Thus the constituents of the system are :

Outside.



Inside.



Therefore ion balance in each phase requires that :

$$Na_o + N_o^+ = Cl_o + N_o^- \quad \text{and} \quad Na_i + N_i^+ = Cl_i + N_i^- + c_i$$

The nebenions follow passive distribution according to the Nernst equations :

$$N_i^+ = \frac{N_o^+}{B}$$

$$N_i^- = N_o^- B$$

Substitutions can now be made into the ion balance equation for inside :

$$Na_o \cdot \frac{A_{Na}}{B} + \frac{N_o^+}{B} = Cl_o A_{Cl} B + N_o^- B + c_i$$

* The effect, if any, of "Donnan Free Space" in the wall will be considered on p.150.

$$A_{Na} \cdot Na_o + N_o^+ = (A_{Cl} \cdot Cl_o + N_o^-) B^2 + c_i B$$

which is once more a quadratic in B :

$$B^2 + \left(\frac{c_i}{A_{Cl} \cdot Cl_o + N_o^-} \right) B = \frac{A_{Na} \cdot Na_o + N_o^+}{A_{Cl} \cdot Cl_o + N_o^-}$$

which has roots (of which only the positive root has physical meaning) :

$$B = \sqrt{\frac{A_{Na} \cdot Na_o + N_o^+}{A_{Cl} \cdot Cl_o + N_o^-} + \left(\frac{c_i}{2(A_{Cl} \cdot Cl_o + N_o^-)} \right)^2} - \frac{c_i}{2(A_{Cl} \cdot Cl_o + N_o^-)}$$

giving the form :

$$E_m = \frac{RT}{F} \ln \left\{ \frac{N_o^+ + Na_o \exp\left(\frac{\Delta\bar{\mu}_{Na}}{RT}\right)}{N_o^- + Cl_o \exp\left(\frac{\Delta\bar{\mu}_{Cl}}{RT}\right)} + \left(\frac{c_i}{2(N_o^- + Cl_o \exp\left(\frac{\Delta\bar{\mu}_{Cl}}{RT}\right))} \right)^2 - \frac{c_i}{2(N_o^- + Cl_o \exp\left(\frac{\Delta\bar{\mu}_{Cl}}{RT}\right))} \right\}$$

(36)

This rather lengthy expression has seven degrees of freedom, namely : T , $\Delta\bar{\mu}_{Na}$, $\Delta\bar{\mu}_{Cl}$, Na_o , Cl_o , N_o^+ , N_o^- , c_i , minus one. The minus one follows because $Na_o + N_o^+ = Cl_o + N_o^-$.

Tests.

When $\Delta\bar{\mu}_{Cl} = 0$, the equation reduces to Eqn.(26), as may be seen if one recalls the equivalences of symbols.

When $\Delta\bar{\mu}_{Na} = 0$, the equation reduces to Eqn.(28), as appropriate.

The equation may now be examined numerically using an automatic programme in the HP25C calculator.

Examination of Eqn.(36).

Tables 19 and 20 show the values of E_m calculated for organisms living in, respectively, freshwater and seawater.

(No table has been constructed for blood plasma because

Table 19. SIMULTANEOUS INDEPENDANT CATION EXPORT AND ANION IMPORT, WITH DONNAN SYSTEM AND NEBENIONS. IN FRESHWATER.

Values of $-E_m$ (mV) on Eqn. (36) for various values of $\Delta\bar{\mu}_{Na}$ and $\Delta\bar{\mu}_{Cl}$, taking $c_i = 100$ meq/l. and $Na_0 = 1.0$ meq/l. and $Cl_0 = 1.3$ meq/l. Millivolts $20^\circ C$.

* $N_0^+ = 0.4$ meq/l. in each box. $\Delta\bar{\mu}_{Cl}$, kJ/mole.
 1.3
 10.3

	0	5	10	15	20	25	30
0	108 95	108 95	108 96	110 98	117 108	134 127	157 150
5	132 107	132 107	132 108	133 109	137 117	150 134	170 156
10	138 109	138 109	138 110	139 111	142 119	154 135	173 157
Saturation $\gg 15$	139 110	139 110	139 110	140 111	143 119	154 135	174 157

* Ion balance outside requires that N_0^- be respectively : 0.1, 1.0 and 10 meq/l.

Table 20. SIMULTANEOUS INDEPENDANT CATION EXPORT AND ANION IMPORT, WITH DONNAN SYSTEM AND NEBENIONS. IN SEAWATER.

Values of $-E_m$ (mV) on Eqn. (36) for various values of $\Delta\bar{\mu}_{Na}$ and $\Delta\bar{\mu}_{Cl}$, taking $c_i = 100$ meq/l. and $Na_0 = 460$ meq/l. and $Cl_0 = 534$ meq/l. Millivolts $20^\circ C$.

* $N_0^+ = 77$ meq/l. in each box.
 104

$\Delta\bar{\mu}_{Cl}$, kJ/mole.

	0	5	10	15	20	25	30
0	2.4 2.2	27 26	52 51	78 77	104 103	130 129	155 155
5	22 20	45 42	70 67	95 93	121 119	147 145	173 170
10	29 25	51 48	76 72	101 98	127 124	153 149	179 175
Saturation ≥ 15	30 26	52 48	77 73	102 99	128 124	154 150	180 176

* Ion balance requires that N_0^- be respectively : 3.0 and 30 meq/l.

Eqn.(36) really has little application to human body cells since only a few tissues (e.g. oxyntic cells of stomach and cells of the thick portion of the ascending limb of the loop of Henle in the kidney) pump both a cation and an anion, both at high enough levels to be dominant, although an extension of Eqn.(36) should be used for such cases including both internal and external macro-anion, except that the effect of external macro-anion may well be no more significant than before (but this has not been tested)).

What is particularly interesting, revealed in Tables 19 and 20, is that there appears a marked difference in response between freshwater and seawater to twin independent ion pumps. It might perhaps be expected that the value of E_m achieved by two pumps acting simultaneously but independently would be the sum of those values of E_m which each pump would produce if acting alone. This is clearly not true in either case, but inspection of the tables will reveal that the shortfall in freshwater is quite large, whereas in seawater the sum is almost achieved. This would suggest that the higher the level of external salt, the more does E_m reflect the sum of the two ion pumps, but a complicating factor in freshwater is that the higher the nebenion content, the lower is the shortfall - a fact which still argues in basically the same direction.

When $\Delta\bar{\mu}_{Na}$ and $\Delta\bar{\mu}_{Cl}$ are both zero, the system reduces to a pure Donnan system, of course, and as expected, there is a large Donnan potential indicated for freshwater

but a very small one for seawater. The columns below $\Delta\bar{\mu}_{Cl} = 0$ in the tables correspond of course to the single transport model of Eqn.(26) (cation) and the rows beside $\Delta\bar{\mu}_{Na} = 0$ correspond to the complementary single transport model of Eqn.(28) (anion).

The choices of nebenion levels to insert into Eqn.(36) have necessarily been rather arbitrary, but the intention has been to bridge the range likely to be encountered. The effect is evidently more marked in freshwater than in seawater as expected, although the upper figure chosen in freshwater may well not be encountered except in eutrophic lakes where there is a heavy inflow of agricultural fertiliser from neighbouring fields. As far as this model is concerned KNO_3 and K_2SO_4 would supply nebenions although obviously NO_3^- and $SO_4^{=}$ may well be actively uptaken by many plants. However this possibility may not in itself invalidate the present model since in both cases uptaken ion is largely internally converted into other compounds, leaving little free ion in the cytoplasm.

In this present model, the fact that K^+ import is often, perhaps always, coupled to Na^+ export has been ignored. This is because P_K is generally much greater than P_{Na} (except in erythrocytes and certain strains of mammalian cells in prolonged tissue culture for which this model was not constructed), when (see p.140) the depression of E_m by K^+ import then amounts to only a few millivolts. Here there is likely to be even less depression than with the Na/K pump alone, because exchange pumping is essentially a single transport pump whereas this present model has

two unlinked pumps. Also the effect of K^+ import is much more important in the animal case in regard to excitable tissues, where K^+ import is responsible for generating the "hyperpolarisation after-potential", $E_K - E_m$ (see Eqn.(35)).

In those cases in plants where something akin to an action potential may be elicited (e.g. *Nitella* or the Ca^{++} "spikes" of the Venus fly trap) perhaps a model for linked transport and twin transport together may be required, but it would have to be constructed to fit the particular case under study.

Further examination here of Eqn.(36) would be fruitless beyond this present purpose of demonstrating its capability to account adequately for the observed values of transmembrane potential in a general way. An equation with so many parameters can really only be examined in the context of specific organisms and environments, but clearly it is an equation which may be subjected to experimental tests, since all the parameters are experimentally accessible quantities. It is now satisfying to observe that the present model is quite capable of generating transmembrane potentials at levels actually observed in freshwater and marine organisms. This now completes the series of models which will be discussed. Eqn.(34) (with Eqn.(35)) and Eqn.(36) may be regarded as the most important of the series since they imply all the others, and are the most complete in terms of those factors likely to govern E_m in living cells.

There yet remains the following important topic to discuss.

Cell Walls and Membrane Surface Charge.

So far no attempt has been made to consider what effect, if any, the presence of either a cell wall around the protoplast or surface charge on the protoplast membrane itself would have on the membrane potential. When a microelectrode is inserted into a cell, assuming its tip is in cytoplasm (a controversial assumption, see Chapter 2), what we measure is the potential difference between cytoplasm and outside, where the reference electrode is situated. However around a plant cell, there is usually a cell wall made of celluloses and pectins which can carry a very large equivalence of ion exchange sites. The matrix sites are largely anionic and concentrations in the range 300 - 800 meq/l. were calculated by Briggs (1957) using data of himself and others. Dainty and Hope (1959) estimated the exchange equivalence concentration of Chara australis to be 400 - 600 meq/l. However, as Briggs et al. (1961) pointed out, some of this ion exchange capacity will be occupied by divalent ions such as Ca^{++} . My own experimental investigations of a model Donnan system (alginate solution contained in a cellophane bag) show that the behaviour of such a system to divalent ions is much more complicated than Donnan theory would predict, and that some, perhaps most, of the divalent ion associated with the macro-anion is held by chelation as well as by

electrostatic interaction. Therefore it is probably fair to say that the matrix sites in the cell wall neutralised by Ca^{++} and Mg^{++} can be regarded as not available for ion exchange. This means that the level of exchange equivalence will certainly be less than 800 meq/l., and for the purposes of this discussion the figure of 500 meq/l. will be assumed for matrix sites in equilibrium with the mobile monovalent ions, Na^+ , K^+ , Cl^- , etc. The cell wall round the protoplast constitutes the so-called "Donnan Free Space" (Briggs et al. 1961), and this region will be in Donnan equilibrium with the external bathing medium. The concentrations of mobile ions will be much different from those in the external medium, and there will be a Donnan potential in the cell wall, E_w , negative with respect to the external medium.

The concentrations immediately external to the active protoplast membrane (plasmalemma) will be those prevailing in the wall, and therefore the membrane potential, E_m , will be developed, according to the theory already presented, against the wall as "outside" medium. The total potential difference observed between cytoplasm and bathing medium will thus be $E_m + E_w$, which we may call E_{mw} . The question of importance now is whether E_{mw} differs from E_m calculated on the assumed absence of a wall.

The problem may be approached as follows :

1. The wall Donnan potential, E_w , can be calculated

using the given data of bathing medium concentrations of mobile ions and the wall concentration of exchange sites, c_w . This may be done using Eqn.(24).

2. Now the wall concentrations of mobile ions may be calculated using E_w and the external concentrations in the Nernst equation.

3. These wall concentrations may now be used in Eqn.(36) to calculate the membrane potential, E_m , if we assume values for $\Delta\bar{\mu}_{Na}$, $\Delta\bar{\mu}_{Cl}$ and c_i , the internal macro-anion content (but taking all quantities in "o" in Eqn.(36) as if they were in "w").

4. E_m and E_w may now be added to give E_{mw} , and this may be compared with the case in which the wall was not considered.

It will suffice to take a case already calculated. We may take the freshwater case as most likely to reveal any discrepancy, since the wall Donnan potential will be high in freshwater. Let us therefore take the case where, (Table 19) :

$$Na_o = 1.0 \text{ meq/l.} \quad Cl_o = 1.3 \text{ meq/l.}$$

$$N_o^+ = 1.3 \text{ meq/l.} \quad N_o^- = 1.0 \text{ meq/l.}$$

$$c_i = 100 \text{ meq/l.}$$

$$\Delta\bar{\mu}_{Na} = -15 \text{ kJ/m}$$

$$\Delta\bar{\mu}_{Cl} = 15 \text{ kJ/m}$$

Reference to Table 19 shows that, without a cell wall, the membrane potential, E_m , would be :

$$\left. \begin{array}{l} -110 \text{ mV with } Na^+ \text{ pump only} \\ -98 \text{ mV with } Cl^- \text{ pump only} \\ -111 \text{ mV with both pumps} \end{array} \right\} \text{ operating.}$$

Let us now interpose a cell wall, with ion exchange equivalence concentration 500 meq/l. between external bathing medium and protoplast membrane.

Following the procedure outlined above, the quantities quoted below are calculated :

1. Wall interior Donnan potential, $E_w = -136$ mV.

2. Wall interior concentrations of mobile ions :

$$Na_w = 217 \text{ meq/l.} \quad Cl_w = 0.00598 \text{ meq/l.}$$

$$N_w^+ = 283 \text{ meq/l.} \quad N_w^- = 0.00460 \text{ meq/l.}$$

These give ion balance with the 500 meq/l. of c_w .

3. Calculation of the protoplast membrane potential,

E_m , now proceeds using Eqn.(36). (However the

programme for Eqn.(36) in Appendix 3 may not be

used because N_o^- was therein derived from Na_o , Cl_o and N_o^+

but with no external macro-anion present. In this

case the value of N_w^- given above must be used, which

then automatically takes account of the wall charge, c_w .

Therefore a new programme was written, which is

designated 36.1 in Appendix 3. Also calculations

were done at the full accuracy of which the HP25C

calculator is capable, although the results are

presented here rounded to 3 significant figures).

The values of E_m and E_{mw} are given below.

(a) Na^+ only pumped : $E_m = +26.3$ mV, so $E_{mw} = -110$ mV

(b) Cl^- only pumped : $E_m = +37.7$ mV, so $E_{mw} = -98$ mV

(c) Both pumped : $E_m = +24.5$ mV, so $E_{mw} = -111$ mV.

These values are exactly the same as were obtained

when the wall was absent. However such an illustration

in a particular case does not prove that this conclusion is generally true. Therefore I present below the general proof.

We may proceed along the lines set out above.

Thus the Donnan potential in the wall is given by :

$$E_w = \frac{RT}{F} \ln \left\{ \sqrt{1 + \left(\frac{c_w}{2(Na_o + N_o^+)} \right)^2} - \frac{c_w}{2(Na_o + N_o^+)} \right\}$$

which when converted to the substituted form becomes :

$$B_w = \left\{ \text{above} \right\} \quad \text{where} \quad B_w = \exp \frac{FE_w}{RT}$$

Now B_w is in fact the Donnan ratio for ion distribution such that :

$$\begin{aligned} Na_w &= Na_o / B_w & Cl_w &= Cl_o \cdot B_w \\ N_w^+ &= N_o^+ / B_w & N_w^- &= N_o^- \cdot B_w \end{aligned}$$

Now, Eqn. (36) may be written also in the substituted form :

$$B_m = \left\{ \sqrt{\frac{N_w^+ + Na_w A_{Na}}{N_w^- + Cl_w A_{Cl}}} + \left(\frac{c_i}{2(N_w^- + Cl_w A_{Cl})} \right)^2} - \frac{c_i}{2(N_w^- + Cl_w A_{Cl})} \right\}$$

whence we see that :

$$B_m = \left\{ \sqrt{\frac{N_o^+ + Na_o A_{Na}}{B_w^2 (N_o^- + Cl_o A_{Cl})}} + \left(\frac{c_i}{2B_w (N_o^- + Cl_o A_{Cl})} \right)^2} - \frac{c_i}{2B_w (N_o^- + Cl_o A_{Cl})} \right\}$$

from which we observe that :

$$B_m \cdot B_w = \text{same form as in Eqn. (36).}$$

$$\text{Now :} \quad \exp \left(\frac{FE_m}{RT} \right) \cdot \exp \left(\frac{FE_w}{RT} \right) = \exp \left(\frac{FE_{mw}}{RT} \right)$$

which shows that the intervention of the wall is irrelevant to the value of potential difference we may expect between cytoplasm and bathing medium in the general case.

However, relative to the wall, the potential difference across the membrane itself is profoundly altered, as the numerical example showed. Nevertheless, the electrochemical gradient for each pumped ionic species, $\Delta\bar{\mu}$, is exactly the same between cell interior and bathing medium as it is between cell interior and wall. This follows since in a Donnan equilibrium (which will apply between outside and wall when the whole system is in steady state), $\Delta\bar{\mu} = 0$ for all mobile ions. The electrochemical gradient for the pumped ion is that which actually prevails, and that which therefore can be inferred by analysis of cell sap, external medium and E_{mw} as measured. It does of course remain an open question as to whether the concentrations of ions in the wall, being different from those in the bathing medium, may alter the value of $\Delta\bar{\mu}$ which a pump could achieve.

However the level of $\Delta\bar{\mu}$ which a pump can achieve is related to three principal factors, namely the free energy available from the driving reaction and the rate of back leakage compared to the rate maximum of the pump. The free energy available will not be altered by the situation external to the plasmalemma. The rate maximum may perhaps be influenced by ion concentrations outside, if binding controls the rate. It also seems possible that leakage permeability may be influenced by the concentrations of ions in contact with the plasmalemma sites, but again little effect

may be expected. The force which determines whether an ion binds on a site depends not merely on concentration but on the local value of $\bar{\mu}$, which takes into account both chemical concentration and electrical potential, both of which are relevant at all times when considering ions. Now, $\bar{\mu}$, as we have seen, is invariant in a Donnan system. Therefore there is no obvious reason to suppose either the rate maximum of the pump or the permeability of the membrane would be altered by the presence of a wall.

The Donnan potential in the wall, E_w , as calculated in the numerical example above, is high and one may perhaps expect to be able to measure it when the tip of a microelectrode touches the cell wall. However, it is not usually observed, and in fact it is unlikely that it would be on account of the mechanism by which a salt bridge half cell (a micropipette filled with 3 M KCl) acquires the potential of any solution around its tip. A Donnan system, being passive, cannot yield work directly to charge up an electrometer, but we can nevertheless measure a Donnan potential in a "macro" system because ions from the tip of the micropipette diffuse to neutralise the liquid junction potential (see p.36) and this net actual diffusion provides the work necessary to charge up the electrometer. However when a micropipette of end diameter, say, 0.2 μm is brought into contact with a cell wall, itself of high conductivity, with thickness of similar magnitude,

backed by a plasmalemma of very low relative conductivity, the ion concentrations in the patch of wall under the micropipette tip will be vastly distorted by ions diffusing from the tip, and the patch of wall will be shorted to bathing medium. Thus we do not measure the undisturbed Donnan potential, but a much reduced (if not negligible) value due to the swamping (see p. 14) of the local patch with KCl. This consideration indicates that we can only expect a micropipette electrode to give a "genuine" readout of local potential when the diameter to a boundary of the volume of solution in which its tip is placed is very much greater than the diameter of the micropipette tip. This will be so for cell interiors generally because we are then dealing with diameters of at least several μm , but the point needs attention in measurements on very small cells or structures of cells, such as root hairs, flagella, etc. (if one can impale them!).

The surface of a membrane such as the plasmalemma carries net charge. Evidence for this comes from various sources, but one of the most straightforward is that from microelectrophoresis. It has long been known that cells in suspension will move under the influence of an electric field (see Ambrose 1966). This can only be so if net electric charge resides on their surfaces, and indeed it is possible, using the equation of Smoluchowski to calculate the ζ -potential at the surface of shear. The potential, however,

varies with distance away from the surface of the membrane (see Appendix I) and falls to zero at infinite distance. However each infinitesimally thin layer of external solution may be regarded as a miniature Donnan system at equilibrium with the bathing medium. Since it has been established above that the interposition of a Donnan system between the active membrane of the cell and bathing medium does not alter the potential difference between cell interior and bathing medium, then neither will a surface charge on the membrane itself. This argument applies as much to the internal face of the membrane as to the external, and actually brings us back to the discussion on "electrets", (p.48).

General Points and Limitations of the Theory.

Concentration and Activity.

In all thermodynamic equations the appropriate quantity to insert would strictly be activity not concentration. However in ion charge balance equations it would be concentrations not activities that would apply. This latter is because charge balance counts the number of ionic charges of each charge sign, whereas in the former, thermodynamic evaluation takes account of the ion as seen from a distance through its cloud of counterions, in accordance with Debye-Hückel theory.

To amend the models presented, it would be necessary to observe the above guidelines. However this would lead to intractable equations because (a) activity varies with concentration according to a logarithmic function ($-\log f = A\sqrt{c}$, (McInnes 1939) where f = activity coefficient, c = concentration and A = a constant) in free electrolyte solutions, and (b) the variation of activity as against concentration in biological electrolytes containing protein and other ion chelators (see further on p.169) is likely to follow a much more complicated law than that predicted by the Debye-Hückel theory (as in (a)).

However the evident remarkable success of the present set of models and equations to predict correctly the values of E_m found in practise would appear to render such a difficult exercise hardly profitable.

Nevertheless, of course, these theoretical equations do require further test by experiments on living organisms - the greatest doubt being about the value of captive anion equivalence, c_i - and should these models prove to be inadequate, this point should be borne in mind.

Internal Ion Concentrations.

The main thrust of the present theoretical study has been to account satisfactorily for the plasmalemma transmembrane potential, E_m . However, as was made clear at the outset, of as much interest is the level to which ion constituents present in the bathing medium settle inside the cytoplasm. These internal concentrations are clearly derivative from the metabolic activity.

In the course of constructing these models, equations have been set up for each of the ion constituents in accordance with the assumptions of the model. Once E_m has been calculated from the final equation, each of these constituent equations can then be used to calculate the internal concentrations of each ion constituent in turn. However, it should be noted that this can only be done for the actively pumped ion(s) if the actual value of $\Delta\bar{\mu}$ achieved in pumping is known, even although the level of $\Delta\bar{\mu}$ may be already above saturation for E_m when therefore its value is not needed to calculate E_m .

Regulatory Responses of the Cell.

These models have been treated in the only way possible for a first approach, on the assumption that the cell is

acting as a machine with fixed parameters. $\Delta\bar{\mu}$ is assumed to be fixed by the mechanism of energy delivery from metabolism; leakage, etc. Captive anion, c_i , is assumed to be fixed. The permeability properties of the membrane are assumed to be static. In practice none of these fictions is likely to apply to a living cell which is capable of making adjustments to any of these parameters - in other words there is likely to be regulatory feedback mediated by genetic material in the nucleus (if there is one - vide red blood cells!). How these regulatory feedback mechanisms operate is likely to be a very complex question, but although their existence may point to inadequacies of the models presented here, nevertheless these models do present a means of detecting the nature and extent of such regulatory responses. However, whatever regulatory responses may exist, the fact remains that the values of E_m actually observed do seem to be predicted very precisely by the set of equations derived, suggesting that regulatory responses play a minor role in determining E_m .

Growing Cells. Effect of Solute/Solvent Drag.

Before examining this in relation to the models presented it is necessary to set the scene. The criterion which is frequently applied to establish if an ion species is actively transported at a membrane or is merely passively distributed is to discover if the ion species is distributed across the membrane in accordance with the Nernst equation. If it is not in obedience to the Nernst equation, active transport may be inferred.

However this strictly only applies to cells in a steady state and not growing. A hidden assumption in the Nernst equation is that there is no net flux of the ion species under consideration across the membrane.

In fact if the cell is growing - or indeed possibly for other reasons - then, in order to maintain constant concentrations of internal ions, the cell must take up water and salts. Therefore there will be a net flux of ions, and the Nernst equation then does not provide a correct criterion for the transport of an ion species. Ussing (1949) and Teorell (1949) independantly published their analyses, providing what is now known as the Ussing-Teorell equation, which copes with situations of this kind.

Although the concept is clear enough that individual ions will cross the membrane in both directions at all times, as a result of thermal motion, whether there is equilibrium or net flux, such a concept is of little value in practise unless it can be applied to some experimental measurement. The advent of isotopic tracers (radioactive or stable, the latter being sortable by mass spectrometry) provided the means of measuring these "partial" fluxes, the method depending on the fact that the various isotopes of an element behave chemically and physically (except in radio-analysis or mass spectrometry) in almost identical ways. Thus the initial rate of uptake of an isotopic tracer into an unloaded cell (i.e. containing no isotopic tracer, although of course containing the usual complement of non-tracer normal isotope of the ion) gives the partial

influx, ϕ_i , and the initial rate of loss of tracer from a pre-loaded cell gives the partial efflux, ϕ_o . The net rate of influx is then $\phi_i - \phi_o$.

Criteria that can include these measurements are provided by the Ussing-Teorell flux ratio equation, which may be derived as follows. If an ion is not subjected to active transport but is nevertheless being accumulated or lost by a cell due to size change, etc., then $\phi_i \neq \phi_o$, but ϕ_i is proportional to \bar{a}_o , the electrochemical activity outside, since this is driving the inflow of isotopic tracer into zero \bar{a} inside for the tracer, initially, and likewise for ϕ_o as defined by tracer measurements. Now \bar{a} may be derived as follows :

$$(1) \quad \mu = \mu' + RT \ln a \quad - \text{chemical potential}$$

$$(2) \quad \begin{aligned} \bar{\mu} &= \mu'' + RT \ln a + zFE \quad - \text{electrochemical potential} \\ &= \mu'' + RT \ln a + RT \ln \exp(zFE/RT) \\ &= \mu'' + RT \ln a \exp(zFE/RT) \quad - \text{same form as (1)} \end{aligned}$$

Therefore by analogy we see that $\bar{a} = a \cdot \exp(zFE/RT)$.

Thus the ratio of fluxes :

$$\frac{\phi_o}{\phi_i} = \frac{\bar{a}_i}{\bar{a}_o} = \frac{a_i \exp(zFE_i/RT)}{a_o \exp(zFE_o/RT)}$$

or, observing that $E_i - E_o = E_m$, and re-arranging :

$$E_m = \frac{RT}{F} \ln \frac{a_o}{a_i} \cdot \frac{\phi_o}{\phi_i} \quad \begin{array}{l} \text{a form of the} \\ \text{Ussing-Teorell} \\ \text{flux ratio equation} \end{array} \quad (37)$$

This is the equation which Ussing (1952) quotes when water does not form a continuous liquid phase penetrating the membrane, but passes the membrane in essentially the vapour phase. It is now seen that the Nernst equation is a special case of this equation when $\phi_i = \phi_o$, at flux equilibrium. This equation now

provides a better test for active transport, obedience to it implying passive distribution, disobedience implying active transport.

Even this flux ratio equation however is incomplete since growing cells take up water as well as solutes, and the inward drift of water tends to entrain solute ions and molecules - the so-called "solvent drag" effect. To cope with this, the Ussing-Teorell equation has been extended by Ussing (1952) as follows (expressed in the symbols of this text and re-arranged to make E_m explicit) :

$$E_m = \frac{RT}{F} \ln \left[\frac{a_o}{a_i} \cdot \frac{\phi_o}{\phi_i} \cdot \left(\frac{a_{wi} \phi_{wi}}{a_{wo} \phi_{wo}} \right)^{G/G_w} \right] \quad \begin{array}{l} \text{a form of Ussing's} \\ \text{solvent drag, flux} \\ \text{ratio equation.} \\ (38) \end{array}$$

(For derivation see Ussing 1952)

where : a_{wi}, a_{wo} = activity of water inside and outside the cell respectively

ϕ_{wi}, ϕ_{wo} = flux of water into and out of the cell respectively

G = frictional force on one mole of the solute ion under study diffusing in water at unit velocity

G_w = frictional force on one mole of water diffusing in water at unit velocity

However, as Ussing (1952) points out, the value of the extra drag term (in brackets to the power) is not likely in most cases to be far from unity because :

(a) there can not be much difference between the activity of water inside and outside, solutes never being more than a few percent of the total,

(b) in many instances ϕ_{wi} and ϕ_{wo} will not differ by more than 1 % even when quite rapid water uptake is occurring because the partial fluxes of water across the membrane are quite large anyway (see for frog, Hevesy,

et al. 1935, although for dog intestine, ϕ_i was 1.5 ϕ_o according to Visscher et al. (1944), except that it is probably fair to say that this is not a very typical case for cells in general as it is an active secreting/absorbing tissue), and (c) G/G_w is unlikely to exceed 2 for mineral ions in water (Ussing 1952).

Therefore the absolute maximum value of the final term on these values would be 2.2, giving a shift in the potential, E_m , of 20 mV positive.

Clearly it is not very satisfactory to rely on references as old as 1935 and 1944 for flux rates. It seems from published literature that there has been rather little use of Ussing's solvent drag flux ratio equation, as compared to his simple flux ratio equation. The reasons are doubtless twofold. Firstly he himself (1952) indicated that the effect of solvent drag was likely to be small, and secondly his first simpler flux ratio equation is easier to apply and probably provides a good enough criterion in most cases for active transport. Even so it is hard enough to measure reliably and simultaneously on one cell or tissue all the parameters for the simpler equation in any case!

However, as an attempt at a more modern assessment of water fluxes, Sorensen (1971) was consulted. It is clear from his paper that measurement of water fluxes is beset with difficulties, especially of interpretation of measurements. He distinguishes two types of flux, giving two permeability coefficients,

namely, P_{wf} , the "non-diffusional" permeability coefficient as measured by osmotic uptake of water, the water being considered to travel into the cell mainly through aqueous channels, and P_{wd} , the "diffusional" permeability coefficient, as measured using tracer fluxes (in this case deuterium in DHO). Sorensen (1971) lists measured values of both P_{wf} and P_{wd} of himself and earlier workers (for references see his paper), and in most cases (with the notable exception of red blood cells) P_{wf} is of the order of 100 times P_{wd} , which appears to be itself about 10^{-4} cm/sec. The difficulty is which permeability coefficient to apply to Ussing's solvent drag equation. P_{wf} applies by definition for osmotic uptake, but it has to be remembered that external solution travelling into a cell by osmotic uptake is going in through aqueous pores (the size of which it was the purpose of Sorensen (1971) to measure, but he had problems). Now aqueous pores in membranes are invariably lined with fixed charge sites, either positive or negative, conferring selective permeability to mineral ions. Uptaken external solution will therefore be modified in passage through such pores, a state of affairs not catered for in the derivation of Ussing's solvent drag equation. It seems therefore that the best one can do at present is to assume that Ussing's solvent drag equation does nevertheless apply and examine what the magnitude of the drag term would be. Sorensen (1971) shows that osmotic uptake, after an external medium change, equilibrates in about 30 sec. in a crab muscle fibre, quoted as measuring 1.16 mm. wide by 0.64 mm. in thickness. If one treats the

cell surface as smooth (in fact it is thrown into clefts) then the surface area is about $0.36 \times L \text{ cm}^2$ and the volume $0.0074 \times L \text{ cm}^3$. (L = length of fibre).

If one assumes that the cell doubles its volume in 30 sec. after an osmotic change, then the inward flux is $\frac{0.0074 \times L}{30 \times 0.36 \times L} = 6.8 \times 10^{-4} \text{ g/cm}^2/\text{sec}$. Now the appropriate permeability for tracer exchange would be P_{wd} ($= 10^{-4} \text{ cm/sec}$). The "partial" flux due to thermal motion in either direction is given by :

$$\frac{dn}{dt} = -PA\Delta c \quad \text{where} \quad \frac{dn}{dt} = \text{amount (in g.)}$$

crossing the membrane per second. Now therefore the flux per unit area $\phi = -P\Delta c$ with units $\text{cm/sec} \times \text{g/cm}^3$, i.e. $\text{g/cm}^2/\text{sec}$. as before for the osmotic flux.

Therefore the appropriate value for Δc is 1 since the "partial" flux in one direction is reckoned as if water were at 100 % concentration on one side and zero on the other. Therefore we now have that

$\phi_{wi} - \phi_{wo} = 6.8 \times 10^{-4}$ for osmotic uptake. Now both ϕ_{wi} and ϕ_{wo} contain a component of 1×10^{-4} for "partial" flux due to thermal motion exchange. Therefore we may estimate that $\phi_{wi} / \phi_{wo} = 7.8$. This would produce a drag correction term in the Ussing solvent drag equation of $(7.8)^2$ (at maximum taking $G/G_w = 2$), i.e. 61, which would be equivalent to 103 mV positive displacement of membrane potential. If the fact that the cell surface is thrown into clefts is taken into account, this figure would be reduced. However, it must be remembered that this high figure is calculated from an imposed osmotic shock, equilibrating in 30 sec., not from growth of the cell. It is therefore not strictly

applicable since in any case it is in the nature of a transient effect for which neither the Ussing equations nor my own derivations presented earlier are applicable. A more realistic assessment would be to take a cell of say 50 μm diameter and assume that the rate of growth amounted to a doubling in size in say 1 hour (this is obviously very much of a guess, and would depend anyway on the cell type and its maturity.)

Area of a sphere = $4\pi r^2$, and volume = $\frac{4}{3}\pi r^3$.

Size-increase flux is therefore $\frac{4}{3}\pi r^3 / 4\pi r^2 \times t \text{ g/cm}^2/\text{sec}$.

where t (sec) is the time to double volume. That is $r/3t \text{ g/cm}^2/\text{sec}$. Now the thermal exchange flux will be $P_{wd} \times 1 \text{ g/cm}^2/\text{sec}$, as observed earlier. Therefore

$$\phi_{wi} / \phi_{wo} \text{ will be } \frac{P_{wd} + r/3t}{P_{wd}} = 1 + \frac{r}{3 t \cdot P_{wd}}$$

This shows that the drag term depends on cell size and obviously also on the rate of growth (measured by $1/t$). However growth rate itself will doubtless depend on cell size because uptake is likely to be limited by surface area. Therefore t may well be proportional to r , making the ratio independent of cell size. For a cell 50 μm diameter then: ϕ_{wi} / ϕ_{wo} will be about $1 + 2 \times 10^{-3}$, which would give a membrane potential shift of a mere 0.12 mV positive. Doubling in size in 1 minute (rather rapid for growth?) would produce a shift of 6.6 mV.

The potential shift due to solvent drag is of course given by:

$$58 \log_{10} \left(\frac{\phi_{wi}}{\phi_{wo}} \right)^2 = 116 \log_{10} \frac{\phi_{wi}}{\phi_{wo}} \quad (\text{mV})$$

since we take a_{wi} / a_{wo} as 1 and G/G_w as 2 (Ussing 1952) for the sake of argument. It therefore appears that, for practical growth rates, the effect of solvent drag

produces a negligible shift on transmembrane potential.

Therefore to return to present considerations, the point at issue is whether solvent drag, which has not been taken into account in the development of the series of equations presented in this text, would invalidate these equations, by neglect of a serious contribution to membrane potential in expanding cells. The answer would appear to be that any such error is likely to be small, of the order of a few millivolts at the very most, in rapidly expanding cells. Of course there would be no error in cells not expanding, which is probably the case, to all but a small extent, in most cells of specialised tissues, where the attention of the physiologist is likely to be most closely directed. The most noteworthy exceptions are in plants, in such cells as stomatal guard cells, and cells of the stems or roots responsible for controlling orientation, where turgor or size change are of the essence of their specialised function. Shape change in animals tends to be controlled by contractile elements within cells not by changes in cell volume.

Incidentally, in passing but of some general importance, the point should be made here that, if active transport of an ion species has been inferred from experimental data that disobey one of Ussing's equations (or the Nernst equation, with its limitations taken into account), then it follows that the transport of the ion is not achieved by direct use of the electrostatic charge of the ion, for this would go

contrary to the derivations of the equations. Thus the ion cannot be transported merely as an ion-pair, associated with some carrier of opposite charge; the carrier must attach to the ion by some means other than its electrostatic charge - e.g. by some form of chelation. Now, it had not been realised until Marullo and Lloyd (1966) published their findings on the formation of a "co-ordination structure" between sodium and racemic p,p',diamino-2-3-diphenylbutane, a neutral molecule, attachment being via N to Na, that alkali metals other than lithium would readily form chelates, if at all. Of course, there is another line of indirect evidence indicating that such chelates are to be expected in biological systems, which is that the ionic activity of both Na^+ and K^+ , as measured by ion sensitive micro-electrodes and NMR (Na only) is found to be very much lower than concentration in cytoplasm. Depression due to the Debye-Hückel effect is to be expected, of the order of 0.7, but in fact the depression in cell cytoplasm may be as low as 0.3 (see micro-electrode work of Hinke 1959, 1961, Ohara and Newton 1968, MacLaughlin 1968 and the NMR work of Jardetzky and Wertz 1960, Rotunno et al. 1967, Martinez et al. 1969, and of Ling and Cope 1969 with their unorthodox conclusions ! But see the cautionary note by Magnuson and Magnuson (1973) on interpretation of NMR data.) (For a detailed review of the development of ion-sensitive glass electrodes see Eisenman 1962). The implications of these findings, however, and the consequences of disobedience of the Ussing equations do not seem to be generally appreciated as much as perhaps they should be.

by those who are seeking the carrier molecules.

Perhaps it should be pointed out that, while Ussing's equations are useful for deciding if a given ionic species is actively transported* his equations cannot predict the value of E_m that a cell would achieve in any given environment, by active processes, from starting conditions, any more than can the Nernst or Goldman equations, so that the treatment set out earlier in this dissertation is still entirely necessary.

Divalent Ions.

The models presented earlier have only been concerned with monovalent ions, but of course, there are several divalent ion species which exist partly in the free ionic form in and around living cells, notably Ca^{++} , Mg^{++} , SO_4^- , and the various forms of phosphate. While some or all of these ions are certainly actively transported in many biological situations, their concentrations, at least in external medium, tend to be much lower than Na^+ and Cl^- , and in cytoplasm some are certainly extremely low in free ionic form, even though appreciable quantities may be present as complexes or compounds. It therefore seems rather unlikely that these ions could have a major role in determining membrane potentials, except in special circumstances, such as Ca^{++} in ciliate protozoa and Ca^{++} in the sarcoplasmic reticulum, and these cases do merit special study. Nevertheless, the agreement between the theory presented earlier and measured values

* However Ussing's equation has been found to be not precisely valid, requiring some correction - see Coster and George (1968) and others they quote.

of E_m in those cases where adequate data are available would suggest that no serious error has been introduced by ignoring divalent ions or by treating them simply as part of the total nebenion equivalence.

Goldman Equation and the Steady State.

Assertions were made when the steady state was introduced, p. 59 to 61, to the effect that the membrane potential maintained by living cells in steady state should not properly be described as a Goldman diffusion potential. Now, it can be argued, once steady state has been reached, that the processes of membrane active transport then merely replenish the leakages of ions which necessarily occur when such transport has generated electrochemical potential gradients for transported ions to which the membrane is not entirely impermeable, the resulting electrical potential difference being described by the Goldman equation. This argument is not quite right, however, as will be demonstrated below. In any case, it does not explain how the ion gradients were generated, nor predict their extent, both of which involve more than ion transport, as has been shown earlier.

The situation may be clarified by considering what would happen subsequent to the blockage of the Na/K ATPase by, say, ouabain, and here we will assume only Na^+ and K^+ are pumped, as the available evidence suggests in nerve and muscle. Until the blockage, the resting membrane potential is generated by the two primary processes acting synergistically, namely by Donnan processes and by

active transport, as already shown. At this stage, there is no net diffusion of any ionic species. The actual transmembrane movements of ions are pumping one way and leakage the other way of those ion species actively transported, with additionally some passive diffusional movements of all permeant ions present, of both pumped and non-pumped species. For a cell with a Na/K transporting ATPase only and no Cl^- transport, it has already been shown (see p.140) that the linkage of K^+ import to Na^+ export has rather little effect on E_m (a mere 4 mV depolarisation, to be distinguished from "after-potential hyperpolarisation" in an action sequence, (see p.143). Therefore it is fair to say that E_m in the steady state is not caused by diffusion of K^+ , nor is it caused by diffusion of Cl^- , since this ion is in equilibrium in this case. It is hardly surprising that these ions follow or nearly follow the Nernst equation, of course, if they are at or near equilibrium, but distribution of membrane-permeant ions according to the Nernst equation is a consequence of a membrane potential existing for other reasons, not a cause of it. This point may be underlined by drawing attention to the use of cationic dyes such as diS-C₃-(5) to determine membrane potential in cells too small to be penetrated by microelectrodes (Sims et al. 1974, Hladky and Rink 1976, Salama et al. 1980). The basis of the technique is that the dye is uptaken by the cells and hence partially removed from the external solution, because the cationic dye molecules are able to pass through the cell membrane to the more negative cell interior. It is assumed, on good evidence, that the dye comes to a distribution following the Nernst equation, but in this

instance it is perfectly clear that no-one is suggesting that diffusion of the dye causes the membrane potential. It merely follows a pre-existing potential, but nevertheless one could include terms in the Goldman equation for the dye, except that such terms would be relatively insignificant compared to terms for other ionic species present, owing to the very low concentrations of dye used. (Incidentally this argument is just as much applicable to H^+ ions, whose contribution to the Goldman equation also may well be small because H^+ concentration is invariably very low at physiological pHs, even bearing in mind the higher permeability of membranes for H^+ ions. This makes the hypothesis of Kitasato (1968) and Spanswick (1972), that H^+ ions are directly implicated in generation of E_m , just as much untenable from this point of view as from the nebenion effect, as outlined on p.102). See also p.187.

As soon as the Na/K ATPase is blocked, the active part of the twin synergistic mechanisms generating E_m disappears, though of course the Donnan system still persists. Immediately after blockage, however, the cell contains concentrations of permeant ions not in accord with Donnan processes alone, and so the ion gradients do now cause net diffusion which will eventually take the system to a new equilibrium, (Donnan), and the potential meanwhile follows the Goldman equation on the instantaneous values of ion concentrations. (As a matter of fact, the Goldman equation will also describe a Donnan equilibrium, but the pairs of terms for each ion species will all have the same ratios at that time, thus telling us no more than Nernst equations.)

Examination of the Goldman equation shows that the terms in K^+ dominate the equation if P_K is much greater than either P_{Na} or P_{Cl} . In fact, since Cl^- is not pumped in nerve and muscle, and is therefore in equilibrium, terms for Cl^- are often omitted to give the equation of Hodgkin (1958), an equation not however applicable to cells transporting Cl^- , as do many plant cells. However, the domination of the K^+ terms in the Goldman equation again tends to obscure the mechanism underlying the origin of the membrane potential even in a decaying quasi-steady state. It is not exactly the diffusion of K^+ which is generating the potential, but the fact that the dissipation of the electrochemical gradients of Na^+ , and also perhaps of Cl^- , are relatively slow. Thus the more rapidly permeating K^+ introduces a tendency to charge separation which reflects itself in the appearance of a membrane potential, which then opposes further diffusion, K^+ then being held in quasi-equilibrium. In effect we have a leaky Donnan system. The Na^+ ions (or Cl^- ions, whichever have the lowest membrane permeability) are behaving in a similar way to Donnan macro-ions in providing a relatively immobile pool of captive charges, whose captivity is the root cause of the membrane potential.

One should not here be misled back to the concept that K^+ diffusion is generating E_m in a steady state by the appearance of an after-potential hyperpolarisation in the action sequence of a nerve, when P_K has become temporarily very high. The fact is that K^+ is not quite in equilibrium across the membrane due to its coupling to Na^+ transport, and

therefore naturally elevated P_K leads to hyperpolarisation (i.e. a shift to E_K) since it was the restricted P_K that made it possible for the pump to generate a $\Delta\bar{\mu}$ for K^+ in the first place, albeit small (see derivation of Eqn. (34)). However, that hyperpolarisation, generated initially by the non-equilibrium of K^+ , would persist only as long as other ions do not diffuse with it, on the basis that charge neutrality always has to be preserved in any volume of electrolyte. Thus initially enough K^+ ions diffuse to charge up the electrical capacity of the boundary, but continued diffusion can only occur if counterions accompany K^+ or nebenions counterdiffuse. As it happens, the action potential sequence is so short (a few milliseconds) that K^+ gradients do not decay very far before P_K returns to its resting value. Of course, when P_{Na} rises rapidly at the beginning of an action sequence, Na^+ no longer behaves as a quasi-Donnan captive ion and E_m swings towards its equilibrium potential for the Na^+ concentrations prevailing, but here again the ion reserve of the cell is sufficiently large that the gradient of Na^+ is not significantly changed before the Na^+ gate closes again.

It is now clear that the rate of transit of a cell from a steady state to either another steady state or to an equilibrium (whichever is appropriate) is controlled by the rate of leakage of the ion to which the membrane is least permeable, and that this ion, not the most permeant ion, in effect controls E_m during transitional states.

Set out below is an examination which has been made of the potentials predicted by my theory presented earlier, and those predicted by the Goldman equation. Values were calculated as follows, and are shown in Table 21: $\Delta\bar{\mu}_{Na}$ was the imposed condition, taken with the plasma concentrations stated. By means of Eqn.(34), E_m was calculated. Then using E_m , Na_i was calculated from Na_o using Eqn.(1); K_i was calculated from K_o using Eqn.(33) and Cl_i was calculated from Cl_o using the Nernst equation in appropriate form. Then these derived data were used in the Goldman equation (see p.43) to calculate a "Goldman potential", E_G , which should persist immediately after blockage. It was assumed that (a) P_K/P_{Na} was 75 as before, and (b) P_{Cl}/P_{Na} was as given in Table 21 to show any effect of varying the permeability to Cl^- , which, if the equation of Hodgkin (1958) were exactly true would be unimportant. Two values of m are also shown in Table 21. When $m = 1$, it corresponds to a pump of itself not transferring charge ("non-amperogenic" or electroneutral), whereas when $m = 2/3$, it corresponds to a net transfer by the pump itself of positive charge outwards (electrogenic, or in my proposed terminology, "amperogenic").

Without entering into arguments about how representative all these values are for any particular tissue, since they are theoretical values consequent upon the assumed values for the purposes of discussion, the following conclusions can be drawn :

(1) The Goldman potentials agree very well with the

Table 21. STEADY STATE AND GOLDMAN POTENTIALS.

Assumed values :

$N_{a_0} = 145 \text{ meq/l.}$, $K_0 = 7.0 \text{ meq/l.}$, $c_0 = c_i = 100 \text{ meq/l.}$, so by ion balance, $Cl_0 = 52 \text{ meq/l.}$

$P_K/P_{Na} = 75$ $\Delta\bar{u}_{Na} = -15 \text{ kJ/m.}$ $T = 37^\circ\text{C.}$

Calculated values :

Nature of pump	m	$-E_m, \text{ (mV)}$	Na_i	K_i	Cl_i	$-E_G, \text{ (mV)}$, when $P_{Cl}/P_{Na} =$
						(meq/l.)
(*1) Non-amperogenic	1K:1Na	64.5	4.81	99.8	4.65	64.5 no change
(*2) Amperogenic	2K:3Na	66.3	5.15	99.2	4.35	64.3 64.4 65.1

(*1) generally hitherto called "electroneutral"

(*2) " " "electrogenic"

predicted E_m on the present theory. This is interesting because the Goldman equation was derived on the admitted assumption, not likely to be true in a real living membrane, that constant field prevailed throughout the membrane. While it is true that the effect of linkage of K^+ transport to Na^+ transport in Eqn. (34) was derived using Goldman's flux equation which depends on the same assumption, it must be observed that most of E_m arose from Na^+ transport and Donnan effects, as we saw when $\gamma = 0$. Now when $\gamma = 0$, the Goldman assumption is not involved. However this agreement here cannot be used as confirmation of Goldman's equation for E_G because when P_{Na} is very much less than P_K and P_{Cl} , K^+ and Cl^- terms will dominate E_G , and also when $\gamma = 0$, K^+ and Cl^- will be in equilibrium. So we expect the steady state E_m to be close to E_G by the form of the Goldman equation.

(2) In the amperogenic example, when $m = 2/3$, it appears that the steady state potential can be some 2 mV negative (in this example) to the potential which the ion concentrations derived from the active processes would yield when placed in contact across a passive membrane of the same permeability properties as the cell membrane, according to the Goldman equation. However the accuracy of this calculated shift compared to reality depends on how well the Goldman equation describes the diffusion potential for a real living membrane. The approach described on p. 180 fares no better in this respect, of course, but we do have here an approach which goes some way towards relating the shift on blockage of a pump to parameters of the system.

Comparison with Earlier Work of Others and the
"Electrogenic Pump" Hypothesis.

One of the earliest attempts to analyse quantitatively the distribution of electrolytes in living cells was presented by Conway and Boyle (1939). This was extended by Dean (1941) who merely assumed that there was maintained by active processes a constant ratio by which the concentration ratio of sodium was made to deviate from that which would prevail in a Donnan system. It is improbable that such a ratio would be constant, and it was therefore not a very reasonable assumption to make.

There has been a series of papers presenting treatments following the formalism of irreversible thermodynamics (e.g. Kedem and Katchalsky 1958, Jardetzky and Snell 1960, Essig and Caplan 1968, Rapoport 1970, Higinbotham and Anderson 1974, Katchalsky and Curran 1975, Caplan and Essig 1977, etc.). However, unexceptionably true as these approaches may be, it would not seem that such treatments are very useful in practise, since they involve so many phenomenological cross-coefficients, many of which are not accessible to measurement, or even if they are, certainly are subject to considerable interpretative controversy. Even in those cases where specific equations have been presented for the cell membrane potential (e.g. Essig and Caplan 1968 and Rapoport 1970) it is not readily possible to evaluate them. Rapoport's equation 24b for instance contains within it the equilibrium potentials of K^+ and Na^+ , which are precisely the kind of parameters we do not know when trying to

calculate E_m from starting conditions. Essig and Caplan's equation 26, for zero current through the membrane, also involves quantities hard to determine such as phenomenological resistance coefficients and degree of coupling.

An approach which has received prominence in recent literature has been the idea that the membrane potential can be regarded as a combination of a Goldman potential and a potential produced by the current flow generated by a so-called "electrogenic pump" (or as I would prefer to call it an "amperogenic pump") through the finite electrical conductance of the membrane (Kitasato 1968, Spanswick 1973, Higinbotham and Anderson 1974, Anderson 1976, Keifer and Spanswick 1978, etc.) according to an expression of the form :

$$E_m = E_q + \sum_j \frac{\Phi_j z_j F}{g_j} \quad (39)$$

where : Φ_j = flux rate of pumped ionic species j
 z_j = valency of ionic species j
 g_j = chord conductance of the membrane to ionic species j .

and where the ionic species j are all actively transported species, excluding the passively distributing species.

It is clear however from published literature that there has been difficulty in reconciling experimental data with this theory, despite strenuous attempts to do so.

Nevertheless it seems that these and other authors take the basic theory itself to be unquestionably correct.

Indeed some authors justify the expression on the basis of irreversible thermodynamics (e.g. Higinbotham and

Anderson 1974), but my feeling is that this approach has the danger of loss of intuitive comprehension.

My theory presented earlier did not take account of the continuing maintenance current of the transported ionic species through the pump sites and back through the leakage sites. Nevertheless there can be no doubt that active transport of an ionic species would yield a definite value of $\Delta\bar{\mu}$ across the membrane for that ionic species at steady state. Also the charge balance condition must apply in all solution compartments and the Nernst equation must apply to those ionic species distributing passively. These conditions can only be mutually fulfilled in the way I have already demonstrated and it is not possible to build into the electrochemical relationships the current in the pump/leak loop, nor is there any need to do so. Therefore there is no way of avoiding the conclusions I have drawn earlier.

The treatment I presented in the previous section, p. 177, showed that the Goldman potential, for the internal ion concentrations prevailing, expresses itself immediately after blockage of the pump, and that there is a shift in potential when an amperogenic pump is blocked. These conclusions could certainly give an expression for the steady state of the form $E_m = E_G + E_P$, where E_P is a component created by an amperogenic pump, equal in magnitude but opposite in sign to the shift on blockage of the pump, but how useful is such an expression?

The problem is that this sort of expression does not explain the origin of membrane potential although presumably it is aimed at doing so. The generation of the component E_G , the Goldman potential*, depends on the previous existence of the internal concentrations of the ionic species present for this diffusion potential to express itself, so the approach misses the point that those very concentrations were produced by active ion transport and other metabolic activity in the first place, without explaining how.

However, there also remains the question as to whether the term $\sum_j \frac{\Phi_j z_j F}{g_j}$ in Eqn.(39) is valid for the amperogenic component. It is based on the idea that the membrane current for the transported ionic species induce a potential difference across the membrane resistance summed for the ionic species in question.

It is tempting to think of an active transport pump for an ion as either a source of fixed current or of fixed electromotive force (e.m.f.), leading to equivalent generator or battery models. An active transport pump for an ionic species is driven by some metabolic process in the cell, necessarily a biochemical reaction. What is available from a chemical reaction is a certain amount of free energy per mole reacted. This free energy may then be used to drive the active transport

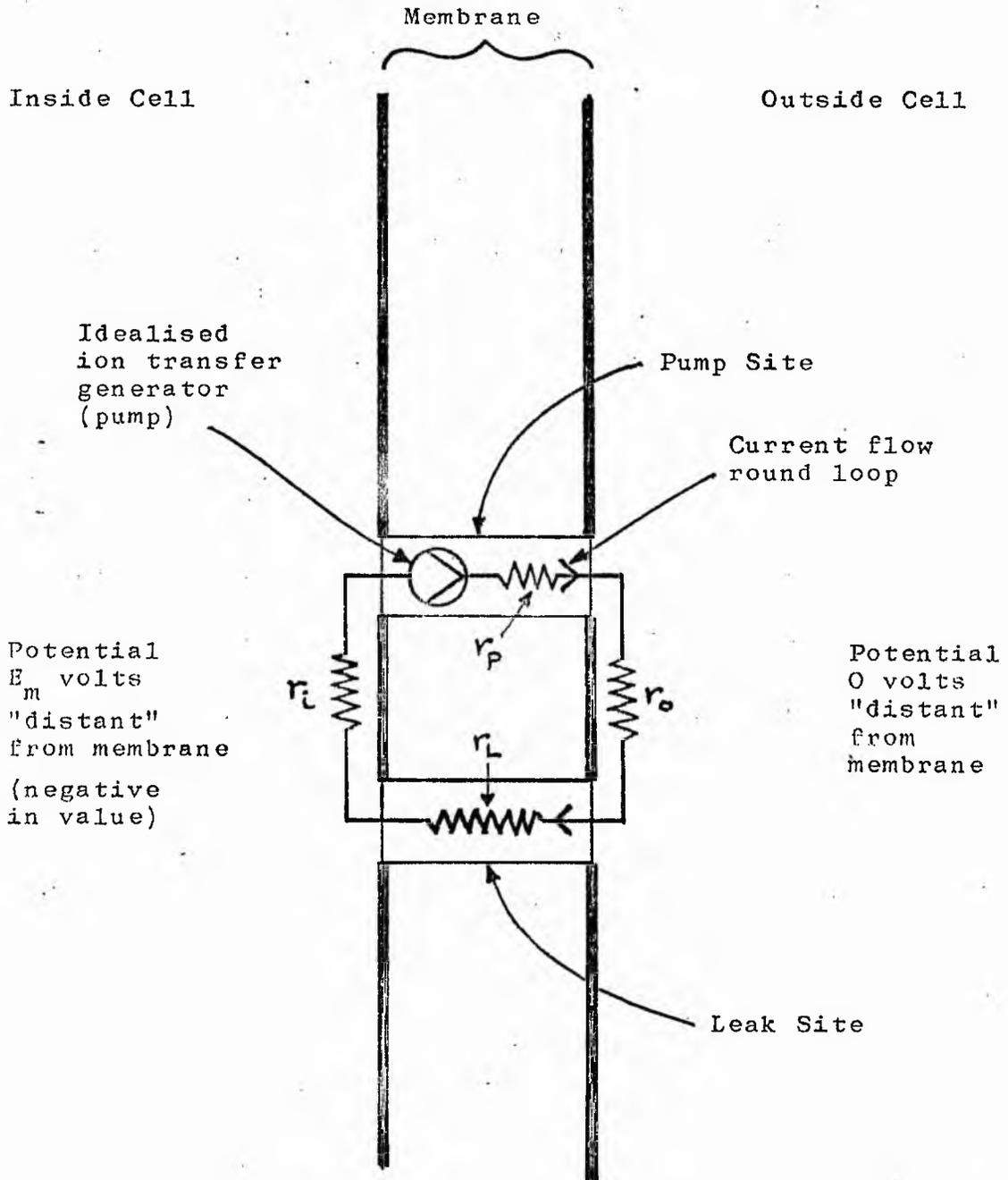
* E_G is sometimes symbolised as $E_m(0)$ for the potential difference across the membrane when $\Phi = 0$, in a notional situation which apologists of this hypothesis identify as E_G (Spanswick 1973).

mechanism. There will of course be inefficiency since the reactions and the coupling to transport must proceed at finite rates for practical effect. Sources of loss will be such factors as (a) rate limit on production of the immediate fuel substance driving the pump (e.g. ATP), (b) diffusional drag, on the supply across distance of the fuel substance from the site of production (e.g. mitochondria) to the site of utilisation (e.g. the plasmalemma), (c) inefficiency in coupling to the pump, (d) diffusional drag on the products of reaction (e.g. ADP and Pi) as they diffuse away from the site of reaction, (e) frictional losses in the pump itself and (f) frictional losses in the return leakage path in the steady state. All these losses, which generate heat, must be fuelled by the free energy from chemical reactions. It is therefore inevitable that something less than the whole of the free energy of the fuelling reaction available per mole in reversible (i.e. infinitesimally slow) reactions can find its way into the electrochemical potential difference, $\Delta\bar{\mu}$, for the pumped ion. Nevertheless it would seem reasonable to assume that the quantity which is essentially generated at steady state by any given active transport system would be the actual level of $\Delta\bar{\mu}$ achieved, taking all these sources of loss into account. Provided that the pump is not running too close to its rate limit, once steady state has been reached, it would seem reasonable to suppose that $\Delta\bar{\mu}$ would be at least a sizeable fraction of the free energy of the fuelling reaction.

However it would not be correct to assume that the generation of a certain level of $\Delta\bar{\mu}$ can of itself guarantee a constant e.m.f. for the transport pump, except in constant circumstances. What the pump, we may assume, can do is to act as a device for transferring on demand, up to a limiting rate, leaked ions back across the membrane to maintain $\Delta\bar{\mu}$. It cannot therefore be said that the pump is inherently a constant current generator either, even though in a steady state it will transfer a steady current. Therefore the prime motion of an active transport pump must be the generation of $\Delta\bar{\mu}$ rather than either e.m.f. or current. These latter are consequences not causes.

The current of ions through the membrane may be considered in the steady state as travelling in a closed loop, Fig. 5, out through the pump sites and in through leakage sites (or vice versa depending on what ionic species is pumped). Let us for the moment consider a pump site in the membrane to contain an idealised ion transfer generator in series with a pump resistance, r_p . A leak site may be considered to contain a leak resistance, r_L . Between the mouth of a pump site and the mouth of a leak site, the ions must travel through medium in contact with the membrane. These paths external to the membrane itself will have resistance, which we may designate r_i and r_o . Current flow in these external paths will be shallow, extending only to a depth away from the membrane of the order of the spacing of pump and leak sites. At distance, the potential on

Fig. 5. THE "ELECTROGENIC" PUMP HYPOTHESIS.



one side of the membrane will be uniform and equal to that prevailing half way along the external path resistance. In practise, r_i and r_o are probably negligible compared to r_p and r_L . Across the whole membrane there will be the transmembrane potential, E_m .

The danger of perpetrating a fallacy enters here, when one sets out to analyse this loop quantitatively. It is tempting to think of this system by analogy with electrical circuits. If one were to do so, then the current, carried by ions, through the leak path for instance would be given by $-E_m / (r_L + \frac{r_i + r_o}{2})$. However this is incorrect, since ions are influenced by concentration gradients as well as electrical gradients. The movement of an ionic species, indeed, may be taken to follow the flux equation of Goldman (1943) (see Appendix 4) when the current density :

$$j = -P \frac{z^2 F^2}{RT} \cdot \frac{C_o - C_i \exp\left(\frac{zFE_m}{RT}\right)}{1 - \exp\left(\frac{zFE_m}{RT}\right)} \cdot E_m \quad \text{amps/cm}^2.$$

This current density may be considered to flow through r_L on the leak path, so that at steady state the amperogenic term would be jr_L . Note that r_L is here the chord resistance and must be distinguished from the incremental or slope resistance (see Chapter 3). Any given system will contain in general several ionic species, but terms of the kind, jr_L , will apply only to those ionic species which are actively transported since others will be at equilibrium requiring therefore no actively circulated

maintenance current. An amperogenic pump would result if say Na^+ alone were exported, Cl^- alone were imported or if Na^+ and K^+ were antiported in a stoichiometry not 1 : 1. In this last case, there will be two circulating maintenance currents, one for Na^+ and one for K^+ , both of which will contribute to the component of E_m generated amperogenically. Although this approach is doubtless correct, it does not overcome the fundamental objection raised earlier that it is not predictive, requiring as it does a prior knowledge of internal ion concentrations in order to calculate E_G . In any case this approach would seem to be unnecessary to explain the origin and magnitude of E_m , and also of doubtful usefulness to account for the shift in E_m upon blockage of the pump, since I have shown earlier how this may be calculated on another basis (p.177).

Immediately this will raise the objection that much larger shifts of E_m have been observed than I calculated earlier, when so-called "electrogenic" pumps are blocked by inhibitors (Davis & Higinbotham 1969, etc.) or when other disturbances are imposed such as changes in illumination upon photosynthetic tissues (Spanswick 1973). The shift in E_m as an experimental fact cannot be disputed, but care must be taken in proposing an explanation. In my derivation of the expected shift in E_m upon blockage of the pump, p.177, it was taken for granted that the only effect of the blocking agent was to block the pump, but of course it would be rash to suppose that the effect of

any given metabolic inhibitor would be so narrow and specific. If the inhibitor were to bind to the pump sites, the chances are that it would distort the steric arrangement of the membrane and could well alter permeabilities in general, which in the short term would cause shifts in E_m according to the Goldman equation on the new permeabilities. Indeed great care must be exercised to maintain the distinction between short term and long term effects. Certainly measurements taken within minutes of the imposition of a disturbance, whether it be switching a light on or off with photosynthetic tissues, or the introduction of a blocking agent, or sudden changes in pH_o or other external ion concentration changes, must be considered as measurements in the short term, when the steady state no longer exists.

Now it has been shown earlier, p.146, that appreciable levels of E_m may be achieved by Na^+ or Cl^- pumping, enough to account, with the Donnan effect, for the whole of E_m . However, it has been suggested (Spanswick 1973) that the extrusion of H^+ would maintain cytoplasmic pH close to neutrality "and at the same time polarise the membrane to give the observed membrane potential". Earlier (p.102) I have demonstrated that the active transport of either H^+ or OH^- cannot of themselves polarise the membrane (except at improbably extreme environmental pHs) in face of the nebenion effect. Nevertheless, although Spanswick's interpretation of the data must be incorrect, this does not of itself

invalidate Eqn.(39). In fact H^+ transport may well establish an electrochemical gradient for H^+ , and of course, the membrane potential, E_m , will contribute to $\Delta\bar{\mu}_{H^+}$ according to the H^+ form of Eqn.(1), but the generation of E_m will not be due to H^+ pumping itself, but to the transport of one or more other ionic species, and it will be to those other ionic species that Eqn.(39) will refer. However, once an electrochemical gradient for H^+ has been created, of course, it can then be harnessed as an energy source to drive another ion transport. Therefore I propose that it is the transport of Cl^- , driven by $\Delta\bar{\mu}_{H^+}$ (see Smith 1970, Sanders 1980a,b) which may be responsible for generating E_m , along with Donnan effects, but this is very different from saying that H^+ transport itself directly polarises the membrane. If my proposed mechanism is correct, then blockage of H^+ transport might well lead to a sudden fairly large depolarisation (quite apart from possible permeability effects), which might possibly mislead one into thinking that the H^+ pump had itself generated some part of the total E_m . However, immediately after blockage of the H^+ pump, the electrochemical gradient for H^+ would persist, but would then very quickly dissipate, removing the driving force for the Cl^- pump. (However pH gradients may well persist longer, but that can be so when $\Delta\bar{\mu}_{H^+}$ has gone to zero and H^+ is in equilibrium). Indirectly then the inhibitor of H^+ transport would be blocking the Cl^- pump. Now, as shown earlier, (Table 3) a Cl^- pump is quite capable of generating observed values of E_m in face of the nebenion effect, but my proposed mechanism must remain speculative for the present.

However, there are certain worrying aspects of this proposal which require detailed quantitative study. Firstly, to pump the considerable quantity of Cl^- required to generate E_m , a very rapid turnover of H^+ would be required in the face of the low concentrations of H^+ ions that prevail at physiological pHs. This actually tends to support the idea that Cl^- pumping would stop immediately H^+ transport is blocked. Secondly, if the membrane is highly permeable passively to H^+ , a matter upon which there seems to be some debate, then the H^+ pump would waste much of its effort, ending as heat, merely to maintain the electrochemical gradient for H^+ . This would seem to be contrary to expectation, but may still be so. It needs careful study.

It may seem strange that the circulation current necessary in the steady state to maintain $\Delta\bar{\mu}$ for the pumped ion in the face of leakage should produce heat and require the continuous expenditure of metabolic energy when, in a purely passive symmetrical situation in which a membrane separates identical solutions, individual ions will still cross the membrane due to thermal motion although no energy is put into this latter situation. This passive two-way movement of ions could also be described as a circulation current, so what is the difference? The explanation lies in the nature of molecular kinetic events in the two cases.

In an equilibrium, where no energy input is necessary to maintain the system, ions and molecules are in thermal

motion, but rebounds between them may be thought of as perfectly elastic, so that there is conservation of total kinetic energy in the system.

Likewise in the pump/leak loop, individual ion-molecular collisions are conservative in kinetic energy, but where there exists an electrochemical gradient for an ionic species, ions will tend to drift through the membrane along the leakage paths. Drift amounts to a particle velocity slightly higher on average than that of surrounding particles. That is, drifting particles are effectively at slightly higher temperature than their surroundings, and the effect of encounters with neighbouring particles will be to share kinetic energy, thus raising the general temperature and tending to slow down, or apply drag to, the drifting particles. This drag loss has to be made good by energy delivered to the transport pump in its effort to maintain $\Delta\bar{\mu}$ for the pumped ion.

Finally, I would like to remind the reader that the value of E_m in the steady state, as I have shown earlier, is determined by several factors, namely $\Delta\bar{\mu}$ for the pumped ion, external concentrations of all permeant and non-permeant ionic species present, and content of captive Donnan macro-ions. It is a serious lack in all previous theories designed to account for membrane potential that no attempt has been made to take account of captive Donnan macro-ion in conjunction with active transport. This alone is enough to

indicate that earlier approaches cannot be complete, and indeed, as shown on p.112, Donnan macro-ions play a very crucial role, for without them in our own body cells, the value of E_m observed could not be achieved.

SUMMARY OF FINDINGS.

In the search for the origin of cell transmembrane potential, first the Gibbs-Donnan system was examined (p. 2) whereby a "cell" is considered to contain captive macro-anion of multiple charge per molecule - e.g. protein. It was shown that although such a Donnan system was certainly capable of producing high potentials across the diffusional barrier (the membrane here considered to be purely passive) for organisms in freshwater, it was not capable of producing anything near to the value of transmembrane potential, E_m , actually observed in marine organisms and cells in contact with blood in higher animals, where the high level of salt swamped the Donnan system almost completely. Besides that, such a system would be incapable of presenting an energy gradient across the membrane which could perform work as we know it does in excitable tissues like nerve and muscle. The Donnan potential arises in an equilibrium

situation and although ions are in concentration imbalance across the membrane, they are not in electrochemical imbalance, and will not cross the membrane in response to an increased permeability (as indeed we know they do at a synapse and in subsequent propagation of an action potential sequence). This may be illustrated by the fact that a Donnan potential may be observed between the inside of a lump of ion-exchange gel and external bathing medium, where clearly there is no membrane to restrict the movement of ions - such a system acts as a Donnan system not because of a semi-permeable barrier, but because the charge sites are captive as part of the gel matrix. It is, of course, a fundamental of equilibrium processes that rate-limiting parameters such as permeability are irrelevant because the net rate is zero at equilibrium.

Attention was therefore directed to non-equilibrium processes at membranes (p. 34) and firstly decaying quasi-steady states were examined. Clearly none of these could account for (a) how a cell arrived in the first place at the state in which we find it, with ion concentrations inside clearly very different from those outside, with an accompanying cell transmembrane potential, nor (b) how such a state was maintained. It was pointed out that the well-known Goldman equation is really only applicable to a decaying quasi-steady state or a transient state (which states are actually formally equivalent) and that furthermore the Goldman equation is an equation describing a particular kind

of junction potential between two dissimilar electrolyte solutions - that in which the solutions are separated by a membrane of selective permeability - and should therefore be classed with the Henderson equation for liquid junction potential (when the two solutions come into direct contact without a permselective barrier). This however does not reduce the usefulness of the Goldman equation to predict transient effects caused by drugs or by concentration changes in the medium bathing a cell, but merely draws limits to its power, showing that it cannot of itself account for the origin of cell transmembrane potential and the concomitant ion gradients. These facts made it necessary to take issue with the statement so frequently made, that cell transmembrane potential is essentially a diffusion potential. It is not, and it is very misleading to imply that its origin lies in diffusion.

Next a somewhat unusual case of a quasi-steady state was considered, namely the electret (p.48). It was examined as to whether oriented charged dipoles in the membrane could give rise to a persistent transmembrane potential. In electrets it was shown that the appearance of a potential difference across its two faces resulted from the slow decay of an orientation of dipolar molecules initially introduced, and that crystals with a permanent dipolar orientation locked into the crystal lattice could never display electret properties, although they could display temporary piezo- or pyro-electricity. Electrets therefore clearly fell into the quasi-steady state class, and

although they can re-generate a discharged potential difference, the rate at which they can do so in terms of charge transfer is very slow, and so can never be expected to account for the cell transmembrane potential, because the cell membrane has far too high a conductivity. Electret potentials are always associated with highly insulating situations. Besides that, electrets, being quasi-steady and decaying cannot account for how a cell first arrived at the state we find.

Streaming potential likewise cannot account for E_m . It will only appear in cells that are expanding continuously, and although there will doubtless be a contribution to E_m from streaming potential in growing cells (but see discussion on p.161), it cannot account for the existence and persistence of E_m in non-growing cells in the steady state.

It has therefore been established that the second kind of non-equilibrium state, namely the true steady state must account for the origin of cell transmembrane potential. Although it has long been a widely accepted belief that active transport was responsible for the appearance of a transmembrane potential, it does not appear from published literature that any thorough attempt (except by the formalism of irreversible thermodynamics, which is not very helpful in practise - see p.179) has previously been made to apply quantitative electrochemical analysis to this situation, nor to assess the relationship between active transport and a Donnan system. This gap has now been filled in this

text. A series of models, of increasing complexity approaching what we suppose to be the real situation in living cells, has been developed and examined using such electrochemical quantitative analysis.

This study has revealed a number of important effects hitherto unknown. These effects are as follows :-

(1) The "Nebenion Effect" See Figs. 1 to 4.

When there are present in the bathing medium surrounding a cell nebenions (i.e. ions of the same charge) to the pumped ion, a marked depression of the value of transmembrane potential, E_m , occurs even though the ion is pumped up to an actual head of $\Delta\bar{\mu}$, which may be high. It was seen that the nebenion effect was much more severe on active cation export than on active anion import - the assymetry arising from the direction of the transport not from the sign of the pumped ion. The assymetry arises because the inside of a cell has limited volume where ion concentrations can be modified by active transport whereas the bathing medium is considered to be of inexhaustibly large volume where active processes of the cell can have no effect on the ion concentrations.

It appears that E_m is generally limited, in cases where cation export is the primary cause of E_m , more by nebenion concentration than by the ability of the pump to achieve adequate levels of $\Delta\bar{\mu}$. For active anion import, no such limitation by nebenions applies at practical values of P and N, thus allowing organisms

which rely mainly on anion import for the generation of E_m to achieve a very much higher transmembrane potential than those relying on cation export. This asymmetry is clearly very important, and will have influenced evolutionary development.

It was further established (p.102) that the nebenion effect rendered it impossible for active H^+ or OH^- transport in any circumstance of physiological pH to make any significant contribution to transmembrane potential, and this conclusion must equally apply to internal organelle membranes as to the plasmalemma. However, H^+ and OH^- transport can nevertheless have relevance in regard to the generation of pH gradients at mitochondrial and chloroplast membranes, etc. and at the plasmalemma itself, for example in oxyntic cells of the stomach and cells of the thick ascending limb of the loop of Henle in the kidney (although both of these are frankly secretory) and in plants such as the Characean algae. (In the Characeae pH banding is observed suggesting that transport of these ions is not generalised over the whole cell surface.) Electrochemical gradients for H^+ may also drive the transport of other ionic species, such as Cl^- , which latter may be responsible for generating E_m (p.188) but such a mechanism would require a very rapid turnover of H^+ in comparison to the concentration of H^+ at physiological pHs, but may nevertheless still occur.

(2) The "Donnan Enhancement Effect".

When a cell contains captive macro-anions (a Donnan system) and the membrane is also capable of active transport, it is found that the value of E_m generated is dramatically higher than the sum of the Donnan effect and active transport acting alone in the case of active cation export. This is shown in Fig. 6, included merely for illustration, (in which the ratio of $c_i/(\text{salt})_o$ to $\Delta\bar{\mu}$ has been artificially fixed (abscissae) for the sake of representation in two dimensions on a graph. Note also that the potential of a pure Donnan system appears to remain zero until $c_i/(\text{salt})_o$ has reached 1. This is merely a reflection of the logarithmic nature of the expression for E_m while the display of E_m is linear, but the value is actually increasing.)

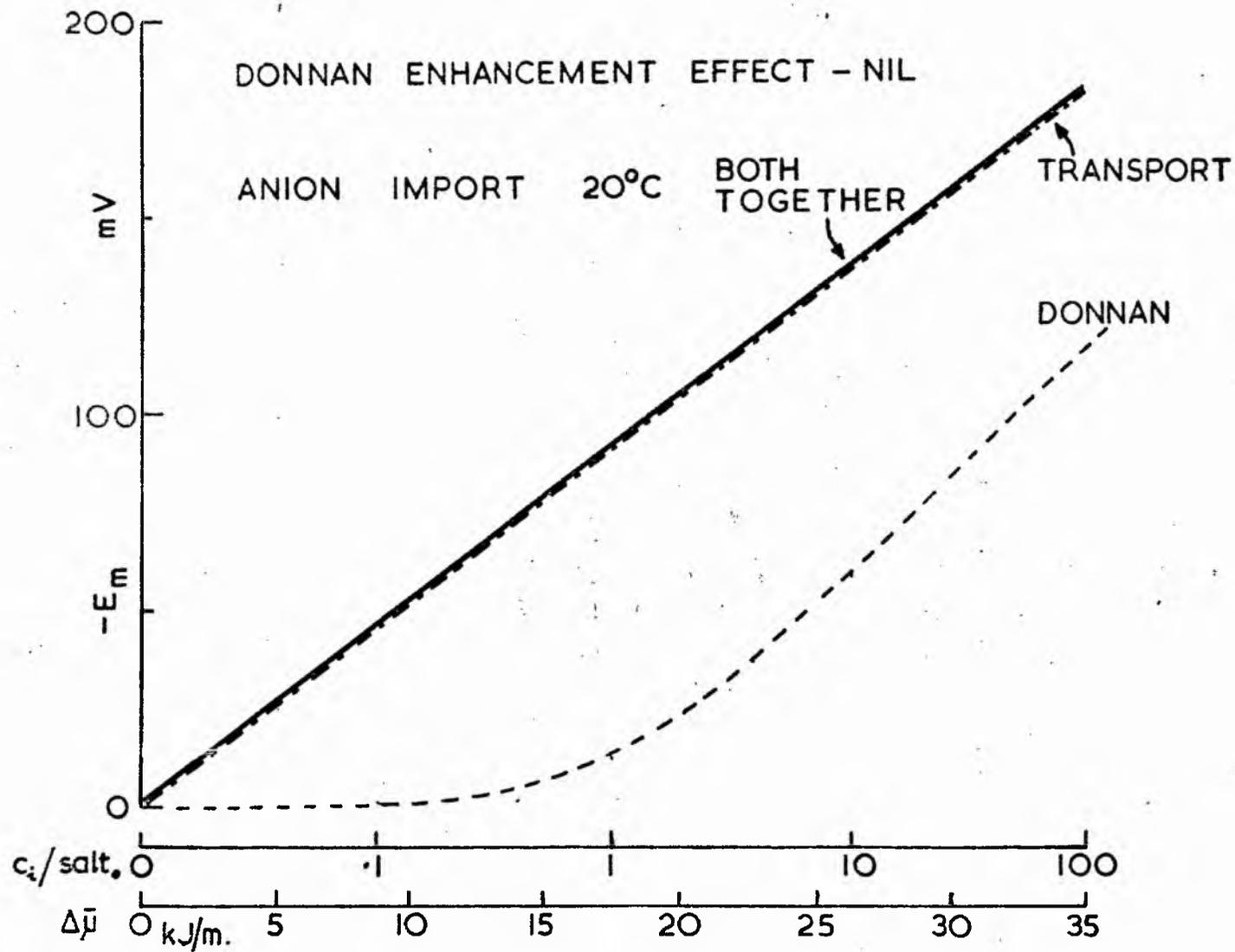
However, with active anion import, the Donnan enhancement effect is virtually non-existent and active transport dominates the system, as illustrated in Fig. 7, (with the same provisos as for Fig. 6).

The inclusion of nebenions again causes depression of E_m for both cation export and anion import with a Donnan system, but the presence of nebenions does not abolish the Donnan enhancement effect.

(5) Weak "Double Donnan Effect". See Fig. 8.

When a cell, itself containing captive Donnan macro-anions, is bathed in a medium also containing macro-anions in

Fig. 7.



addition to the usual mineral ions, it is found that active cation export once again produces a "Donnan enhancement effect" in regard to the internally captive macro-anions, but that the presence outside of macro-anions has little depressive effect on the enhancement. This should be observed particularly in the case where the exchange equivalence of internal and external macro-anion are equal (i.e. $c_o = c_i$, the cross-over point on Fig. 8), a situation in which no Donnan potential would arise in the absence of active transport at the membrane. It is seen that a marked Donnan enhancement effect appears even in this case, when the ion transported is cation outwards. (Active anion import was not considered in the context of a double Donnan system because such a situation only rarely exists in practise, and such cases tend to be secretory in the higher animal body. It may well not exist elsewhere). Fig. 9 shows the nebenion effect in the presence of a double Donnan system.

(4) Effect of Coupled Transports. See Table 18.

The effect was examined of having the transport of a second ionic species coupled to the transport of the primary pumped ionic species. The case examined was that wherein K^+ import was coupled by a defined stoichiometry to Na^+ export, a situation common in animal cells and indeed in many plant cells also (though possibly never unaccompanied by active anion import in plants).

Fig. 8.

DOUBLE DONNAN + NEBENIONS
CATION EXPORT
BLOOD PLASMA

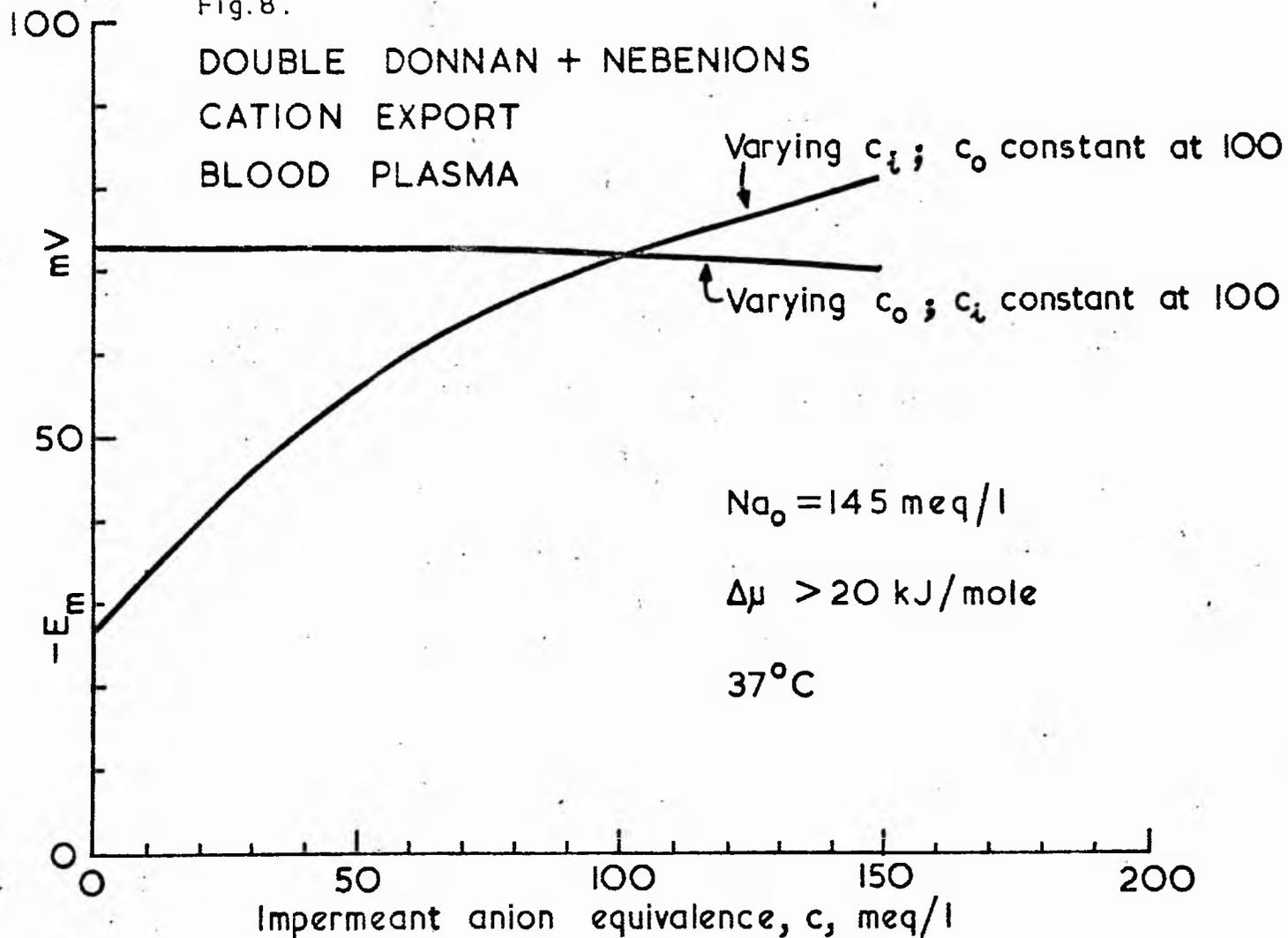
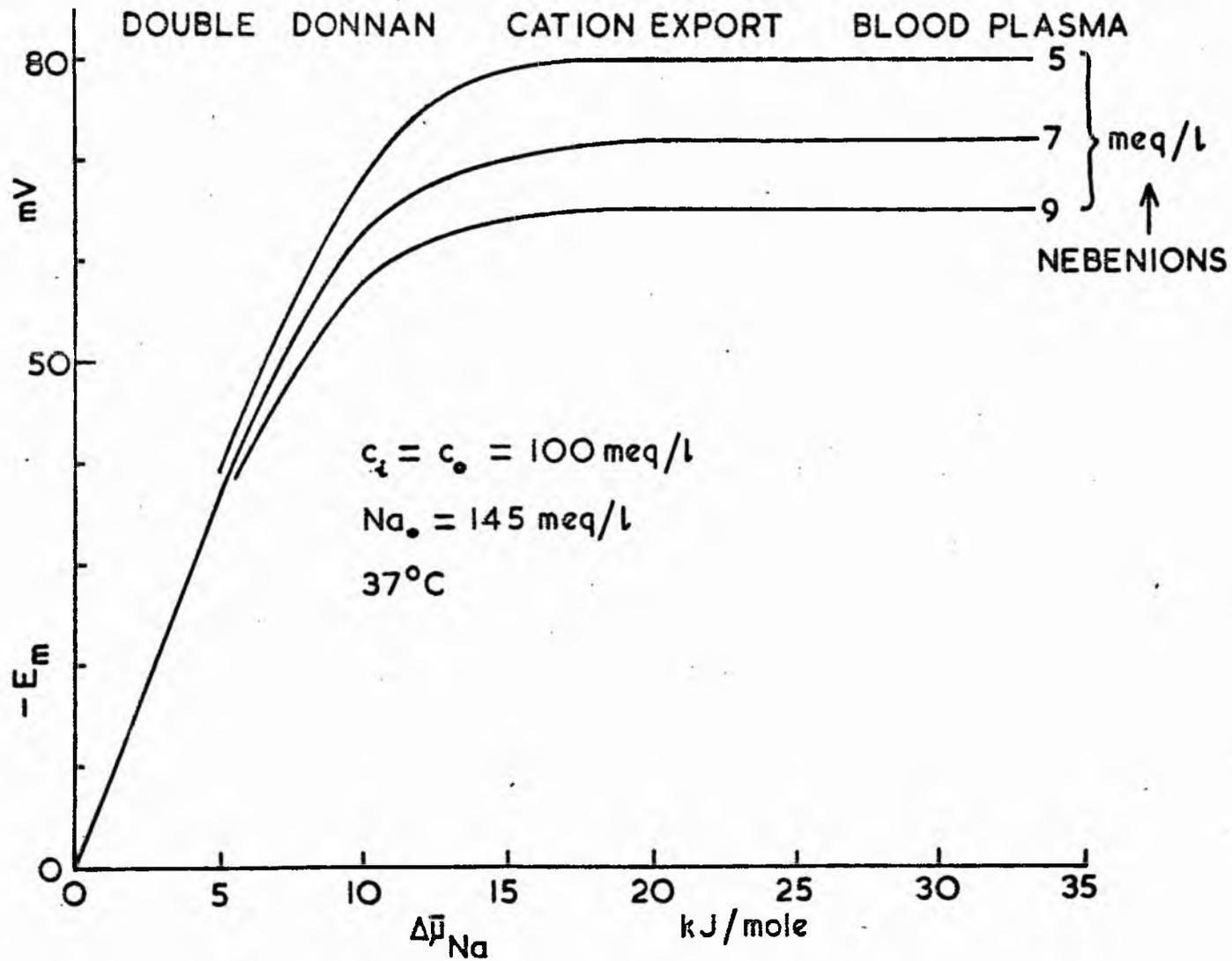


Fig. 9.



It was seen that K^+ import depresses the value of E_m by a small amount when $P_K \gg P_{Na}$. The value of $E_K - E_m$, of interest to neurophysiologists, was also derived, and it was pointed out that this is not equal to the depression of E_m consequent upon introducing the coupled import of K^+ , being in the reverse sense and not of the same magnitude.

(5) Effect of Twin Independent Transport.

The case was examined in which there existed simultaneously in the system active cation export and active anion import, but with the two systems not coupled (as would appear to be the case in many plants). It appears that the level of E_m achieved is never quite equal to the sum of values of E_m which each pump acting alone would achieve, but that the shortfall is larger the lower the external salt concentration for any given level of captive internal Donnan macro-anion. Indeed in freshwater the shortfall is very large, whereas in seawater the sum is almost achieved.

Conclusion.

These models have amply demonstrated their capability of accounting for the origin and magnitude of cell transmembrane potential, E_m , in all situations envisaged, and what is now needed is experimental test in detail of the predictions of this theory. The form of the equations derived does offer the possibility of practical application. This work has been communicated to the Physiological Society (Stanton 1980), and the full paper has been accepted by the Royal Society (London).

CHAPTER 2.

EXPERIMENTAL DETERMINATION OF CELL TRANSMEMBRANE POTENTIAL

This chapter presents first a discussion of the technique for inserting microelectrodes into living plant cells, and improvements in equipment are described. Next follows a description of the method of preparation and filling of microelectrodes, with critical appraisal. The electronic system for measurement of E_m and d.c. resistance is described. Finally, artefacts in the measurement of membrane potential are examined in detail, and ways of overcoming them are presented. Some typical results on maize roots are given at the end of the chapter.

Microelectrodes.

In order to measure the transmembrane potential of a living cell, it is necessary to make electrical connection with the internal solution - the cytoplasm hopefully (see later, p.266) of the cell. This is now regularly achieved by the use of a "microelectrode", which may be extremely small at the tip. The microelectrode is inserted into the cell by some means of micromanipulation, under microscopic examination. What is generally used, and loosely called a "micro-electrode" is in fact a glass micropipette filled with an electrolyte, into the shank end of which is inserted a metal electrode wire for connection to the electronic measuring system. The introduction of such a technique is generally credited to Ling and Gerard (1949).

It would not be satisfactory to insert a bare metal wire into a cell, of course, because the potential between a metal wire and the solution in which it is placed depends on the ionic composition of that solution. Thus, since in general we do not know the composition of cell sap - or, even if we do, it introduces a further measurement variable - it is desirable to have an "electrode" the potential of which is insensitive to solution composition. In any case, a bare wire would need to be insulated along its entire length except for its tip, at which point it must be small enough to penetrate a cell wall (if there is one) and a cell membrane without doing damage. Although such insulation can be achieved on a needle-shaped electrode, it is not easy to prevent some

lateral electrical leakage along the electrode shank, which can be disastrous when one bears in mind that the cell membrane itself has an electrical resistance of the order of megohms. Thus any shunt resistance to the outside medium must be at least one and preferably two decade orders of magnitude higher if measurement is to be reasonably accurate.

These considerations have dictated that the system of choice be a glass micropipette drawn out to a very fine tip (less than 1 μm diameter), open at the tip, and that it be filled with an electrolyte of composition such that there is no change in liquid junction potential (q.v., p.36) when the micropipette is transferred from bathing medium, through the membrane to cell sap. As already indicated on p.39 the filling electrolyte chosen is one which gives as low a liquid junction potential as possible. Such is usually achieved by using 3 M KCl. Potassium chloride (KCl) is chosen because the mobilities of its constituent ions are equal (i.e. $u_{\text{K}} = u_{\text{Cl}}$) to a close approximation. (Other possible salts are KNO_3 , NH_4NO_3 , NH_4Cl .) KCl is usually used at 3 M because at that concentration it is about as high as is convenient to use. Above this, there is the danger that crystals will form in the tip of the micropipette, which would cause electrical blockage.

Although 3 M KCl would give only a very small liquid junction potential against most solutions of biological importance, there is a further problem.

Glass, of which the micropipette is made, is to some extent capable of ion exchange on its surface, which means that it has a non-zero ζ -potential at its surface. The tip of a micropipette may well have a hole as small as $0.1 \mu\text{m}$ (see later), and at such small dimensions the ζ -potential profiles of opposing sides of the micropipette lumen may well overlap. This would produce a region close to the tip where ions of the same charge as the wall charge are excluded to some extent and ions of opposite charge are enhanced in concentration. Such is in effect an ion-selective pore, and as already seen (p. 41) the diffusion of KCl down such a pore would produce a diffusion potential, even although diffusion gradients of KCl in free aqueous media do not. Such a permselective diffusion potential does occur with micropipettes and sometimes it can be embarrassingly large (especially if the tip becomes blocked with a plug of protein or other ion-exchange material from the cell into which it is impaled) compared to the cell membrane potential, introducing serious error. This error will henceforth be referred to as "tip potential".

Electrical connection to the 3 M KCl salt bridge in the micropipette is made by insertion of a metal wire into the rear end of the shank. It is usual practise to use a silver wire coated with silver chloride. The electrode should be reversible, as little toxic as possible, and it should display a steady potential in 3 M KCl. A reversible electrode is one which follows reversible thermodynamics, and should display no change

in potential whether an infinitesimal current is passed from wire to solution or from solution to wire. This can only be achieved if there are present in the medium surrounding the electrode, ions of the electrode metal. (A bare wire in the absence of ions of its own metal will be subject to overpotentials arising from electrode reactions which evolve gas, for one direction of current flow - therefore the potential displayed on the wire relative to the solution would vary with direction of current flow, and would be inappropriate for electrometry). The desired result of metal in a solution of its own ions could be achieved by deliberately dissolving a salt of the metal in the salt bridge solution, but this is not very desirable since metal ions would then diffuse from the tip into the living cell, with possible toxic consequences. Simpler is to choose a metal which has a barely soluble salt which may be coated on to the wire as a melt. Silver, coated in AgCl is found to be admirable. In fact the present author has found that coating with AgCl is hardly necessary in electrometric experiments, since the tarnish on a silver wire seems to be quite capable of supplying the charge required to charge up a modern high impedance, low capacity electrometer. However, silver wires normally have been chloridised in this study.

The potential on a silver wire follows, of course, the Nernst equation :

$$E = E_0 + \frac{RT}{F} \ln a_{Ag}$$

where E_0 is a reference level, and a_{Ag} is the activity of Ag^+ ions in the solution

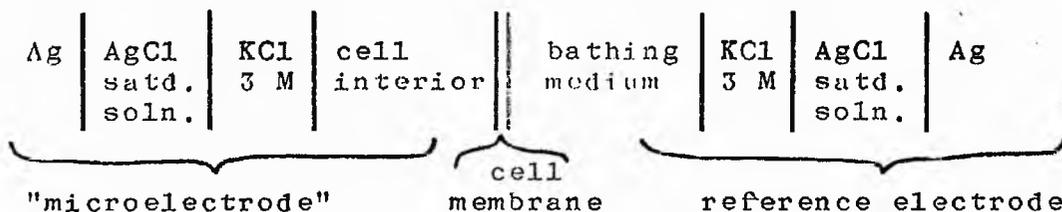
surrounding the electrode. Provided a_{Ag} is held constant, the electrode will display a steady potential relative to the salt bridge solution. When the surface of the electrode is coated with AgCl, it immediately produces on insertion into the micropipette a saturated solution of AgCl in the salt bridge solution in the shank of the micropipette. Eventually Ag^+ ions will diffuse down to the tip, but this is unimportant with freshly prepared micropipettes. AgCl is a barely soluble salt following the usual solubility law :

$$SP = a_{Ag} \times a_{Cl}$$

where: SP is the solubility product of AgCl (about 10^{-10} at $20^{\circ}C$). a_{Cl} is controlled by the Cl^- in the shank of the micropipette, from 3 M KCl, and is therefore very high. a_{Ag} will therefore be very low, but it will be steady, which means that the electrode potential will be steady. The presence of solid AgCl in intimate contact with the metal wire ensures an adequate ion reserve to cope with current flow, notwithstanding the very low level of dissolved Ag^+ . In fact current flow is extremely small in electrometry. However the layer of AgCl should not be too thick, otherwise there is a danger of introducing unwanted electrical resistance.

The electrode wire with its micropipette filled with 3 M KCl thus forms an electrochemical half cell. Another half cell, the reference electrode, but of larger size, is placed in the external medium surrounding the cell, usually in the outflow from the tissue bath,

so that KCl diffusing from its tip does not contaminate the medium near to the cell under study. The electrochemical arrangement is therefore :



As a matter of fact, in the studies reported here, a Pye dipping mercury-calomel electrode with sintered glass end channel was usually used as reference electrode rather than an Ag/AgCl/KCl reference electrode.

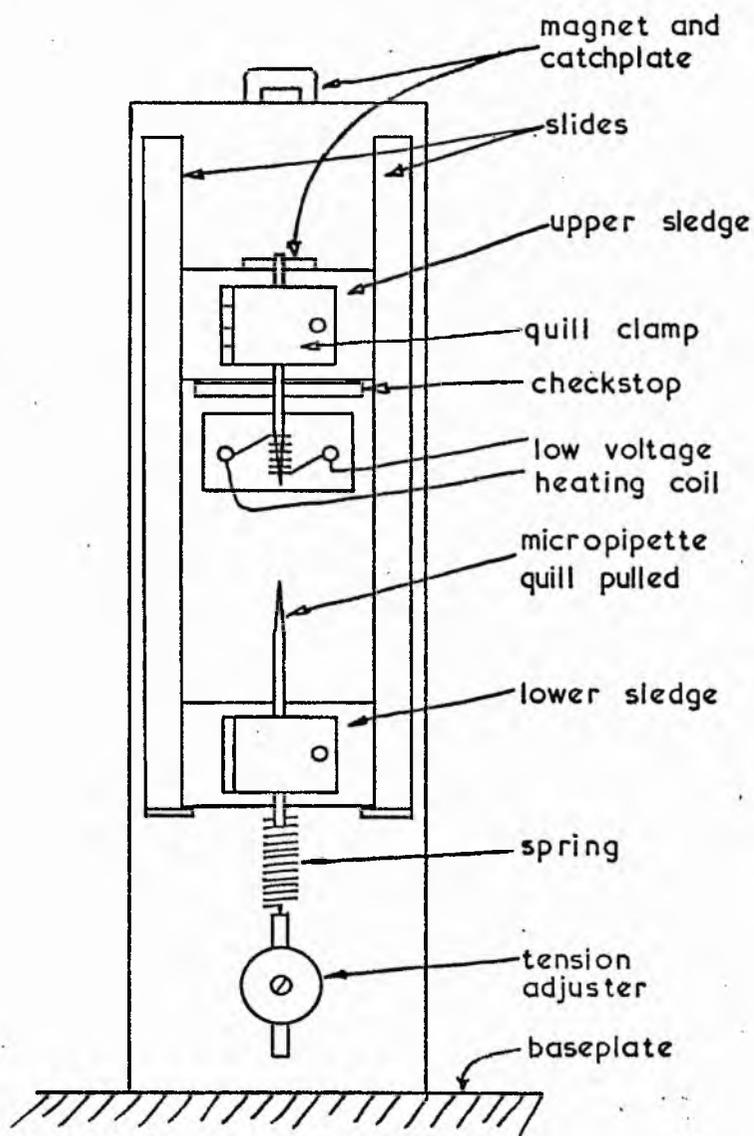
This was merely a matter of convenience, since the Pye electrode required virtually no maintenance. To set a silver electrode against a mercury electrode does however introduce a standing potential difference, but this is unimportant since the electronic system must in any case include a variable voltage source to back off any standing voltage when both microelectrode and reference electrode are in the external bathing medium. A backing off device would be needed here, even if both electrodes were silver because the tip potential of the microelectrode (strictly micropipette) is rarely zero, so it is convenient to use the same device also to back off the potential difference between the silver wire in the microelectrode and the mercury in the reference electrode.

Preparation of Micropipettes.

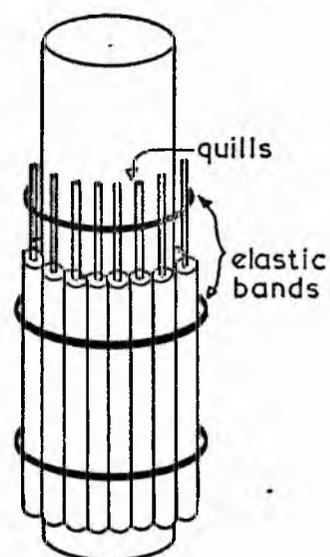
The micropipettes were made on a special electrode puller (Fig. 10). A glass quill 10 cm. long is clamped in the upper clamp while the upper sledge is in its uppermost position, caught on the magnet. It is

Fig. 10

ELECTRODE PULLER



QUIVER



then lowered, so that the quill passes through the heating coil, and the lower end of the quill is then clamped in the lower clamp with the lower sledge in its uppermost position. The upper sledge does not move during pulling, but is raised afterwards to retrieve the drawn pipette. The lower sledge falls under gravity with spring assistance when the quill melts. Between the two clamps the glass quill is heated by a small hollow heating coil of nichrome ribbon or wire about 3 mm. diameter by 5 mm. long, through which an adjustable current is passed. When the glass melts, the tube is drawn out to a long fine constriction which finally snaps off to leave two open-ended micropipettes. It will be seen that the arrangement is asymmetrical, the break occurring below the heating coil, with only one clamp moving. This is essential. Symmetrical arrangements have been constructed, but they generally produce sealed micropipettes due to radiation from the heating coil (which cannot be avoided even with timed switch off of the current, because of the thermal delay in cooling).

The machine used for preparing micropipettes for this work was made in the workshops of our laboratory to a design evolved over several years in the laboratory, by Mr. Roland Jack.

Glass quills were of Corning Pyrex capillary tubing, 1.75 mm. outer and 1.15 mm. inner diameter. After cutting to lengths of 10 cm., they were quickly flame smoothed at the ends, so that, when later a

chloridised silver wire was inserted, the chloride was not scraped off.

After pulling, micropipettes were stored in a quiver to protect the tips and to allow easy handling while they were being filled. The quiver consisted of a length of wide bore glass tube (15 cm. long x 2 cm. diameter) around which were clustered a ring of shorter tubes, with bore just large enough comfortably to clear the micropipette quills. These tubes were held to the larger tube by elastic bands. A newly formed micropipette was taken straight from the puller and inserted, back end first, into a vacant quiver tube, and allowed to slide gently down until its end emerged against the ball of the thumb, the latter acting as a soft cushion. The micropipette was then clamped in place under an elastic band. When required for use, micropipettes were removed from the quiver, tip first, by a reversal of the above process, using a piece of quill to push them out of the then wet tubes. Fig. 10 shows diagrammatically the electrode puller and the quiver for micropipettes.

Before use a quiver was prepared carefully to remove dust. The tubes were washed in clean distilled water and finally in clean ethanol (95%), and then drained and dried with a current of warm air.

Filling Micropipettes.

Various methods have been used by others for filling

micropipettes. One popular method has been to use three solutions in succession. The quiver of pipettes is first immersed in clean methanol, and the air is then removed by boiling under reduced pressure at room temperature. Next the quiver of pipettes is transferred to distilled water. A day or two must be allowed for diffusional exchange before the quiver is finally transferred to 3 M KCl solution. Again several days must be allowed for diffusion. The process is slow and unless a long waiting period is allowed after the last step, the electrodes have rather a high resistance due to incomplete diffusion of potassium chloride into the narrow lumen near the tip.

Prepared micropipettes may not be stored for more than a few days after filling because the tips tend to erode away and also the glass becomes brittle near the tip. For this reason the three-stage process for filling them was considered to be too slow besides being inconvenient. The usual argument against direct filling with 3 M KCl is that the pipettes do not fill properly, a small bubble often blocking the lumen. A way was sought to avoid this.

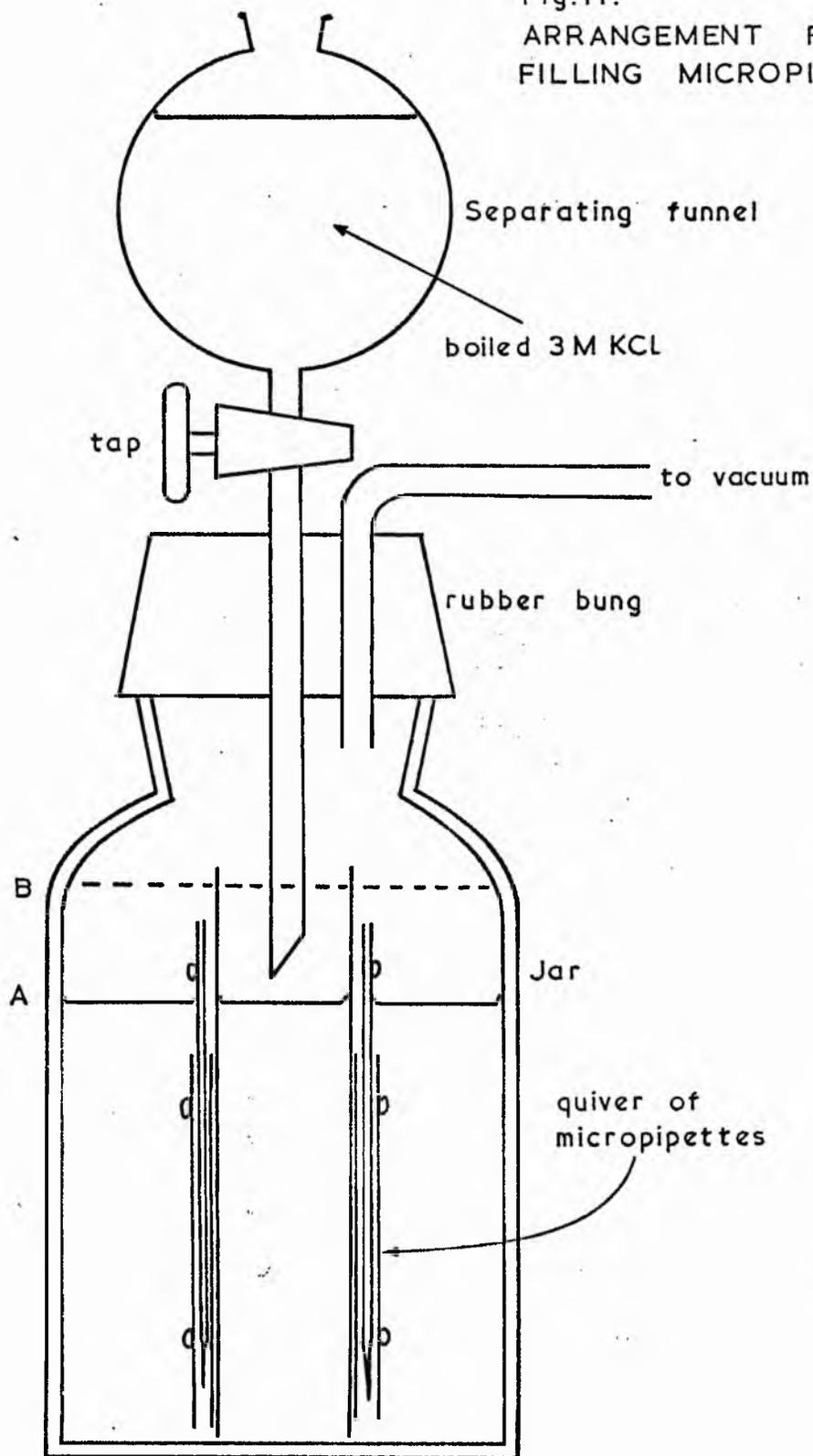
When 3 M KCl comes into contact with glass it does not wet it well, and no doubt this is largely responsible for the failure rate of direct filled micropipettes. Addition of a few drops of Teepol detergent to a litre of 3 M KCl was found to improve the problem only marginally, and more Teepol was not risked on account

of its possible bad effects on living cells when microelectrodes were inserted. Of course, very little would be introduced into cells from the lumen, if the outside of the micropipette was washed in distilled water. This was usual practise anyway, to remove adhering KCl, but nevertheless the advantage of using Teepol was not enough to warrant its use and it was abandoned.

Finally, filling micropipettes was made easy by use of the arrangement shown in Fig. 11. A stout jar capable of withstanding a vacuum was filled to level A with 3 M KCl, then the quiver of micropipettes was carefully lowered in. Over the jar was held the assembly of the separating funnel, filled with pre-boiled 3 M KCl, with the spout extending through a large rubber bung. The bung was larger than the jar mouth to save the vacuum sucking it in; seal was effected by the vacuum itself quite satisfactorily pulling the bung against the smooth jar rim.

First the micropipettes were evacuated in this vertical position, with their back ends well above the surface of the solution. (Care has to be taken that splashes during boiling do not block back ends of pipettes at this stage). Since this end of the micropipette was now dry, the pressure inside the quill was equal to that above the solution, which is its vapour pressure. If however the back end were also immersed, the pressure in the quill lumen would be held above that over the free

Fig.11.
ARRANGEMENT FOR
FILLING MICROPIPETTES



surface of the solution due to its depth of immersion, plus the more serious pressure required to form a bubble against surface tension. For a tube of 1 mm. bore, this will be of the order of 6 cm. head of water. Thus complete removal of air would be impossible from a fully immersed micropipette. It is nevertheless true that the vapour pressure of water itself is about 12 cm. head of water at room temperature, but the point is that an open-ended unobstructed micropipette may be flushed out, to remove all air, by alternating cycles of pulling down to vacuum sufficient to boil the solution and slight release of vacuum, but not enough to re-introduce a gross amount of air. It was necessary to use a rotary two stage vacuum pump (Edwards), fitted with gas ballast device and with a spray trap; a Geissler filter pump on the water main is inadequate. Once vacuum was established, the solution was allowed to boil for a few minutes in order to clear it of dissolved air. Tapping the jar down on to the bench aided this, bringing up a cloud of fine bubbles, but care was needed not to overdo this, and cause splashing. After this, the vacuum was held while further degassed 3 M KCl was run in from the separating funnel until the micropipettes were fully immersed to level B (Fig. 11). The vacuum was then gently released. Often a small bubble would still remain in a micropipette near the tip, but, if a day or so were allowed, the bubble usually dissolved in the air-free potassium chloride solution. A few percent of the micropipettes never filled properly this way, but this was discovered by running a magnifying glass along

each fresh micropipette before use. Naturally, a persistent bubble lead to an electrode of infinite resistance. Despite this failure rate, which is higher than by the three-step process, the method was considered still to be advantageous. Successful micropipettes could be prepared from start much quicker; an hour or two always produced a group of workable electrodes, which was sometimes very useful, but the cost of speed was a somewhat higher failure rate. Nevertheless, the bubble often could be seen in the quiver, and if left, would usually disappear later. The particular advantage of this method, especially important in cell membrane resistance and capacity measurements (see later, p.268) was that successful specimens were always filled throughout their length with 3 M KCl, and there was no uncertainty that perhaps anomalously high resistance might perhaps be due to incomplete diffusion of KCl into the lumen of the micropipette. Therefore resistance measurement was a fairly reliable index of the size of the micropipette tip, when measured with the tip in external bathing medium. No evidence was seen to suggest that damage had occurred to the tip by this method of filling, as judged by microscopic examination. One suspects that the fear of other workers that this method damages the tip arises from the lower resistance which they interpret as due to a larger tip diameter. However the higher resistance from the three-step process is almost certainly due to incomplete diffusion of KCl into the lumen near the tip, where it is so narrow.

The actual typical resistance will be discussed later.

Chloridisation of Electrodes.

The actual electrode proper, which was used with these micropipettes, was a length of 28 s.w.g. silver wire. This was coated with silver chloride (AgCl) to make it reversible, as already discussed. Two methods were used to chloridise wires. In one, the wire was passed through molten AgCl. The conventional way of doing this, in a crucible of molten AgCl, is messy, fiddly and wasteful of expensive chemical. It is tricky to pass a wire through a pool of molten AgCl in the bottom of a crucible and one is liable to crack off the coating when the wire is straightened afterwards. The crucible is liable to shatter as it cools, thus wasting AgCl. It was much simpler to use a 15 cm. length of 3 mm. bore Pyrex tubing. One end of the tube was heated to near red heat in a flame, and then dipped into AgCl crystals. Some stuck and melted to form a blob across the end. The silver wire was passed into and drawn out of the molten blob, to produce an even coat on a straight wire. The glass tube with its AgCl was then kept till next time.

The second method of chloridising silver wires was by electrolysis. Two silver wires were connected as terminations of a 6 v. a.c. supply. They were lowered in and slowly withdrawn from 3 M KCl solution. If this was repeated several times, eventually no bubbles

were evolved and each electrode had become coated with AgCl, but it did not adhere as well as melted AgCl. This however is a useful method if AgCl is not available.

Sometimes it has been claimed in this laboratory that silver wires must be covered with a pore-free layer of AgCl if potentials are to be steady. I do not believe this. Indeed to cover a wire with too thick a layer of AgCl will lead to slow equilibration of the electrode and high resistance.

Insertion of Microelectrodes into Plant Cells.

The cells of maize roots can be up to 30 μm across when adult. To insert a microelectrode (strictly the tip of the micropipette) into such a cell requires a delicate movement, and must be done under microscopic examination.

It has now become a standard technique to insert microelectrodes into living cells using the following arrangement. The living tissue is mounted on the stage of a microscope, preferably fitted with mechanical lateral stage adjustments. Beside the microscope is placed a micromanipulator of some kind, carrying the microelectrode. Both microscope and micromanipulator are usually placed on a thick slab of concrete or iron separated from the bench by soft padding intended to prevent vibrations from the building from disturbing the delicate microelectrode, once in a cell. The microelectrode is inserted into the cell by mechanical adjustment of

the micromanipulator.

This arrangement was followed in this laboratory, but it was notorious for being a difficult technique with certain plant tissues, especially those with tough cell walls, such as Enteromorpha intestinalis. Despite all care and elaborate arrangements to eliminate vibration, the success to failure rate on attempting to insert microelectrodes was poor, not to mention the tedium and exasperation it caused! The usual result was that the micropipette broke off before it had penetrated into the cell. And once the pipette was successfully implanted, vibration was then liable to cause a rent in the cell, with consequent loss of reading from the microelectrode. Elaborate cushioning of the whole setup failed to produce much improvement.

Evidently the conventional arrangement left much to be desired, so an investigation was made of the causes of the trouble. The following criticisms of the conventional arrangement were brought to light :

1. The arrangement was mechanically sloppy. There should be no relative movement between tissue and microelectrode tip except that produced by deliberate adjustment. But an arrangement with a micromanipulator standing beside a microscope is open to considerable movement by distortion of frame members. Thus, if one traverses the loop, through solid members, from the microelectrode tip, via the micromanipulator, across the baseplate, up the microscope stand and to the tissue on the stage,

a distance of 50 cm., and probably more will come between micropipette tip and the impaled cell. Thus vibration was amplified to give a severe wobble of the microelectrode relative to the cell. This can only be overcome by making the mechanical linkage between tissue and microelectrode very rigid, and as short as possible. In this kind of engineering, it is easy to be misled into thinking that delicate movement needs delicate parts. But delicate parts are flimsy. Chunky solid parts are called for, and fine movement should be achieved by suitable design in which care is taken to see that rigidity is not lost at the points of movement.

2. The direct adjustment of the micromanipulator by hand upon its controls introduced wobble from the hand. Remote control of the final movement of insertion is therefore desirable.

3. When the micropipette tip touched the tissue, it began to bend like a bow, until often it broke before it had entered the cell. Obviously this fault arose because the shank of the pipette was able to move sideways. This meant that the clamp and micromanipulator holding the micropipette was not rigid enough against lateral movement. A device may appear reasonably rigid to the naked eye, but be far from rigid when examined under the microscope. This particular problem is more troublesome to those wishing to insert microelectrodes into some plant tissues, than into animal cells which of course lack a cell wall.

4. As the microelectrode was advanced, there was a tendency for it to proceed in jumps rather than steadily with the conventional screw micromanipulator. (There was also some backlash in the screws of the micromanipulator which was more of a nuisance than disastrous for advance in one direction, as during the actual insertion). The jumping was doubtless due to the interplay of dynamic and static friction in the sliding elements of the micromanipulator. Static friction is invariably greater than dynamic friction between two surfaces. Thus, when an adjustment is made, first elastic distortion of the mechanical parts occurs, until sufficient force is built up, to be relieved in a sudden jump. From then on, smooth movement may occur or the static friction may re-establish itself to await another jump. Oil or grease between the sliding members will not solve this problem entirely. Oil is a Newtonian non-thixotropic liquid so that pressure between the slides will squeeze it out until the metal surfaces touch, only to establish static friction once again. Movement will draw oil in, and temporarily smooth movement will be possible, but as soon as movement ceases, the device will drift as the oil flows out again. The drift may be lateral rather than along the micromanipulated axis, but it is nevertheless unacceptable. Grease is thixotropic, and requires a certain minimum shear stress to break the gel. The gel reforms again however, as soon as movement ceases. Thus juddering will occur to some extent even with grease, just as with dry or oiled slides. Sliding members are thus fundamentally inappropriate for a micromanipulator.

Design of a Suitable Micromanipulator.

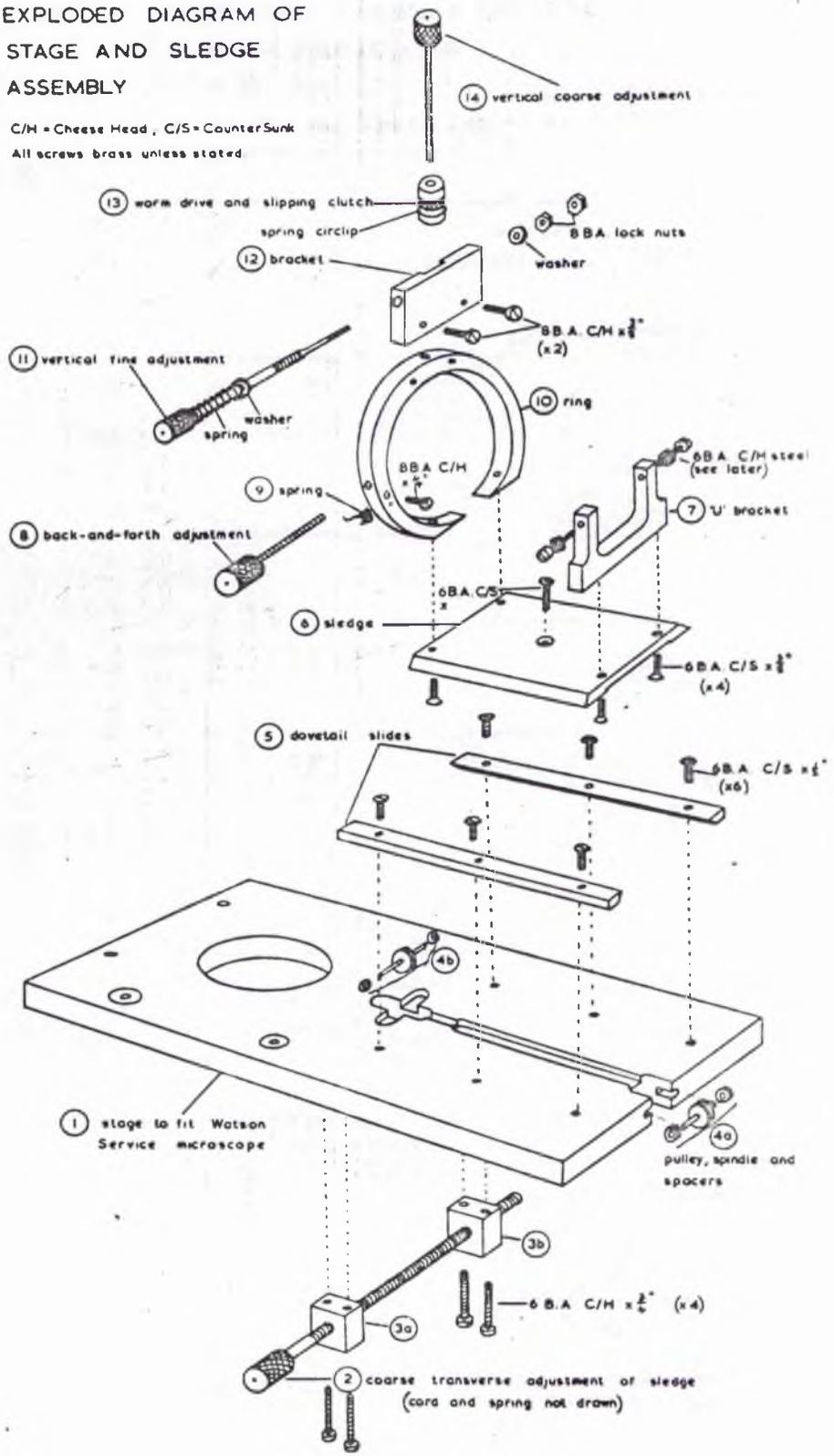
There seemed little possibility of modifying existing arrangements to overcome these objections because the faults were fundamentally inherent in the design. Part of the trouble arose because separate units had been used together, as explained in relation to flexibility of frame members. These two considerations indicated that an integral design for a microscope/micromanipulator assembly would be better than the conventional approach.

In the first model of the new design, the necessary rigidity between the tissue and the micromanipulator was achieved by mounting both the tissue bath and the micromanipulator head on the same thick metal stage, which was mounted on a conventional microscope in place of the standard stage. Thus the flexibility of the frames of the microscope and micromanipulator were eliminated. The aim was to provide a unit which would not permit relative movements within itself so that vibration from outside should have a minimal effect.

The standard stage of a Watson "Service" microscope was replaced by a specially designed long stage, 30 cm. by 13 cm. by 12 mm. ($\frac{1}{2}$ ") thick of aluminium alloy (see Fig. 12). This was machined about one third from one end with a hole for the microscope condenser, and near the other end for the slides on which the micromanipulator head was carried. It was fixed so that the optics of the microscope were unaffected. A Watson mechanical stage device was fixed to the stage, and to it was

Fig. 12.
EXPLODED DIAGRAM OF
STAGE AND SLEDGE
ASSEMBLY

C/H = Cheese Head, C/S = CounterSunk
All screws brass unless stated



Exploded Diagram of Stage and Sledge Assembly. Fig. 12.

Legend.

1. Stage to replace conventional microscope stage - Al alloy
2. Coarse transverse adjustment screw of sledge - brass
- 3, a,b. Carriers for 2 - brass
- 4, a,b. Pulley wheels for coarse adjustment cord - brass
& steel.
5. Dovetail slides
6. Sledge baseplate - brass
7. U-bracket for rear gimbals suspension - brass
8. Lateral adjustment screw - steel
9. Spring bearing on screw 8 to prevent backlash in thread
10. Front suspension ring - brass
11. Vertical fine adjustment - steel
12. Bracket to carry vertical adjustment system
13. Worm drive and slipping clutch on vertical adjustment
14. Vertical coarse adjustment - steel.

attached a special Perspex tissue bath (see Fig. 20).

A hydraulic device was designed as the micromanipulator. Remote control was thus easily provided by separating the micromanipulator head on the stage from the hand-operated drive unit (Fig. 13). Hydraulic devices have been designed before for micromanipulation, but some of them have used syringes. A syringe is a sliding device itself and is thus open to the same objections as slides. Indeed, one arrangement available in the laboratory was of this type, and it suffered badly from judder.

However, it is not necessary to use syringes in a hydraulic system. Metal bellows were chosen for this new design. These involve no friction since motion arises through elastic distortion of the metal. One difficulty with a metal bellows however is that, if it is long enough to be easily extensible over a useful range within its elastic limits, it is also flexible and therefore will flop when a weight is carried on its end, as shown in the diagram, Fig. 14(a). Thus some means must be provided to maintain the free end normal to the axis, while still allowing movement. This was done with a second bellows, open to the atmosphere, separated along the axis by several centimeters, Fig. 14(b). The back ends of both bellows were fixed to the frame, and the front ends were both soldered to a 1 cm. diameter brass tube into a rigid structure. The only remaining point was to decide how long each bellows element should be. Even in an arrangement with axial maintenance, there remained the possibility of poor lateral rigidity

Fig. 13.

PRINCIPLE OF THE MICROMANIPULATOR.

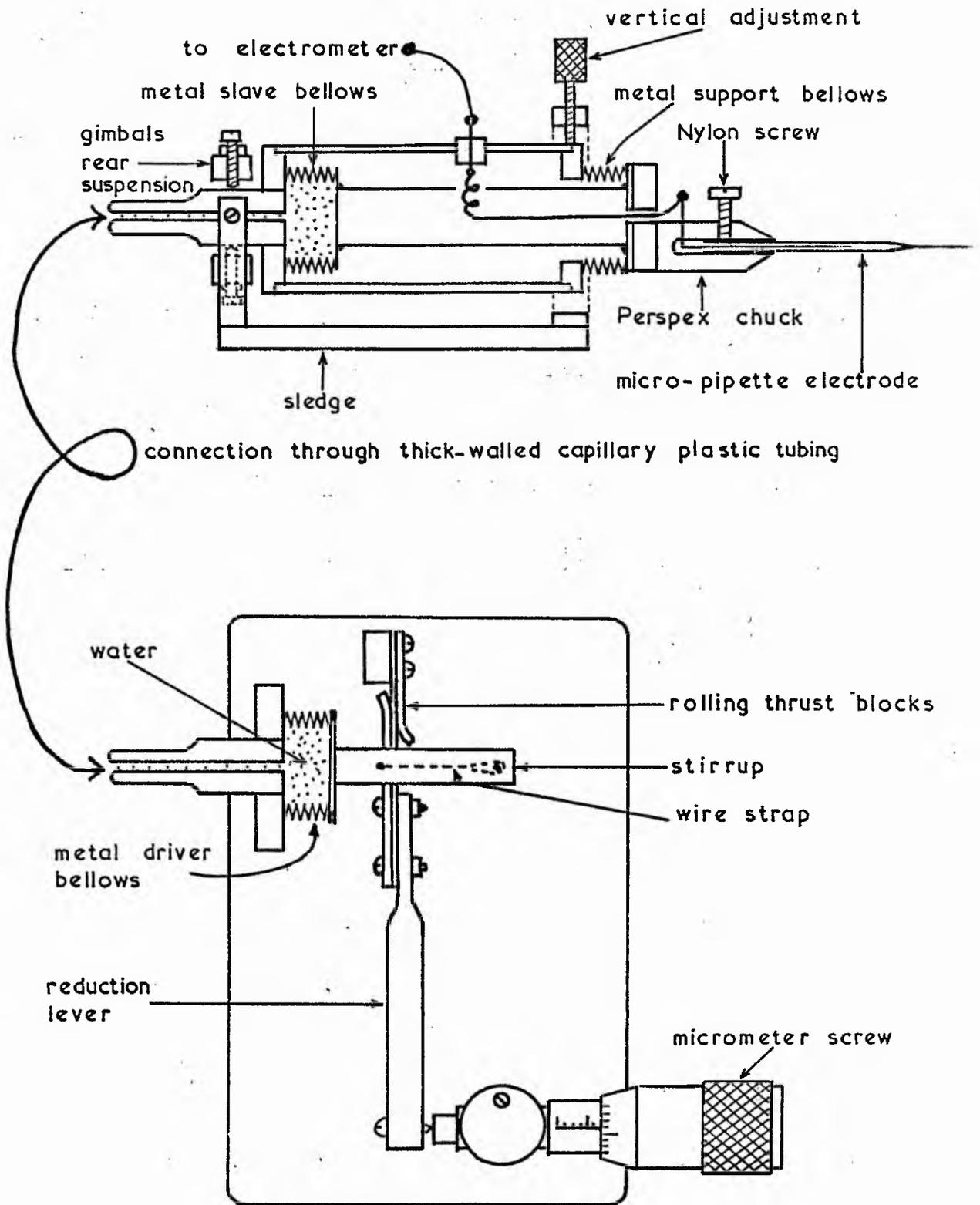
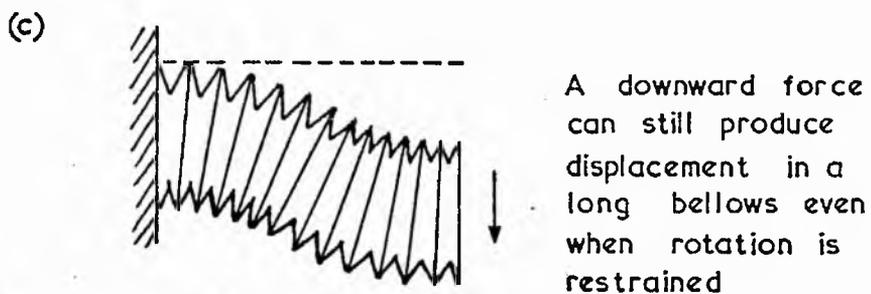
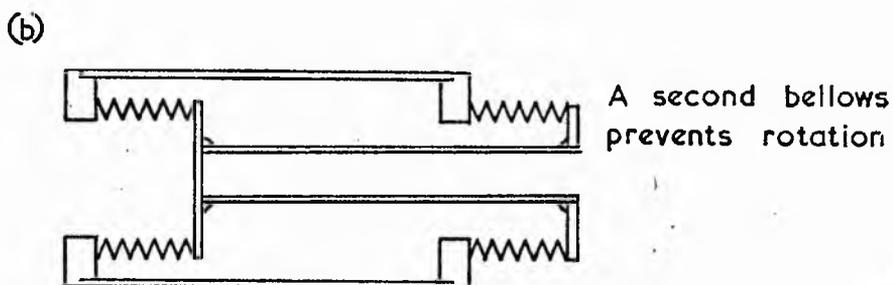
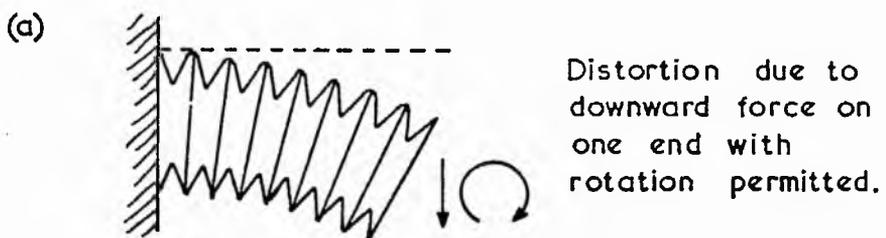


Fig.14.

METAL BELLOWS



due to distortion of the bellows in a mode illustrated in Fig. 14(c). It was found that about 6 - 8 convolutions of the bellows gave the best compromise between lateral rigidity and axial extensibility. It is important that the bellows should never be stretched beyond their yield point in the movement required, and the length of the bellows chosen allowed up to 5 mm. of longitudinal movement which is generously adequate for microelectrode impalement, as the final fine adjustment. Bellows were cut from lengths of Boa Gilding Metal Bellows, Cat. No 115, of the Power Flexible Tubing Co., Ltd., Derby Works, Vale Road,, London, N4 (nominal external diameter 1.14 in., single ply, 11.5 corrugations per inch). Diagrams of the complete head are shown in Fig. 15 and 16. The micromanipulator head was mounted on the stage in conventional greased slides to provide a coarse longitudinal movement, used only in setting up. These slides were maintained tight by two springs in pockets milled in one side of the slide. Movement was achieved using a nylon line attached to the moving member and running over pulley wheels, and round a 2 B.A. threaded control shaft. The line formed a continuous loop closed by a spring. In later versions, conventional rack and pinion was used. The objection to slides raised earlier (p.217) does not arise here because the slide was used only for setting up and was greased, not oiled, so that drifting did not occur.

Lateral movements were also provided. The micromanipulator head was suspended at its back end on

Fig.15.

SECTIONAL DIAGRAM OF MICROMANIPULATOR HEAD ASSEMBLY. ENLARGED.

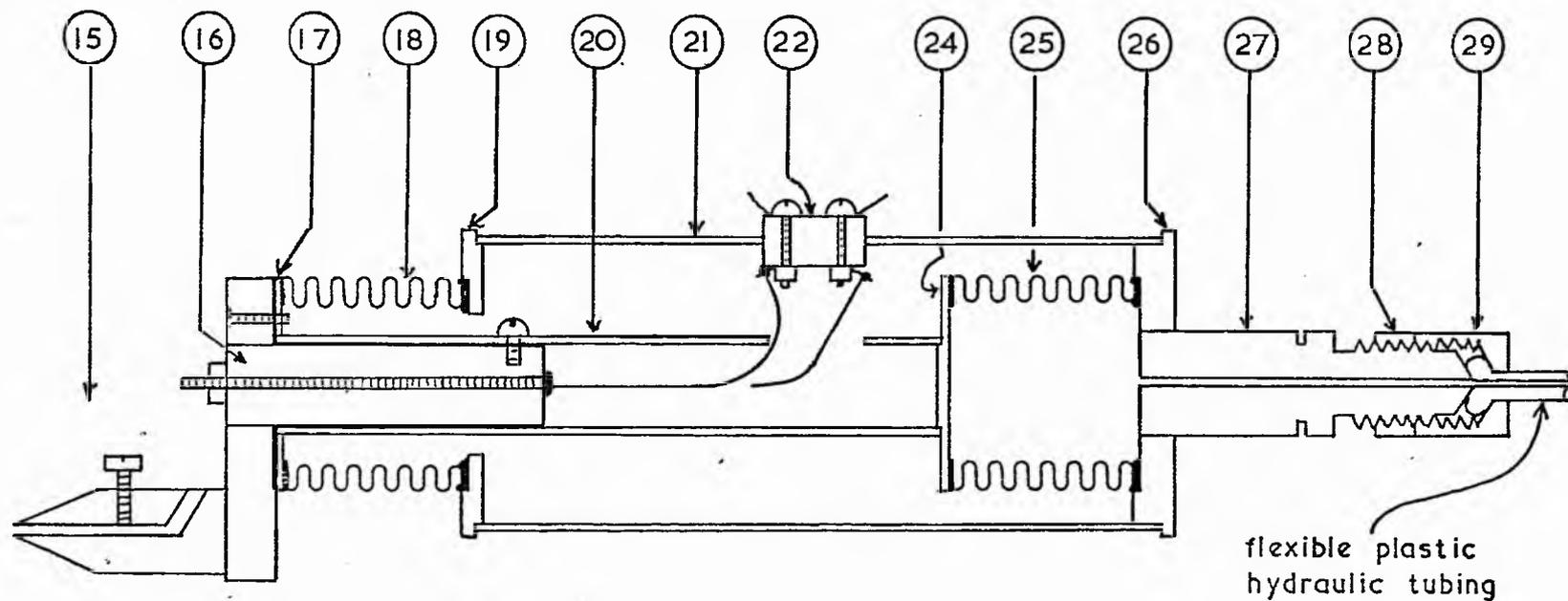
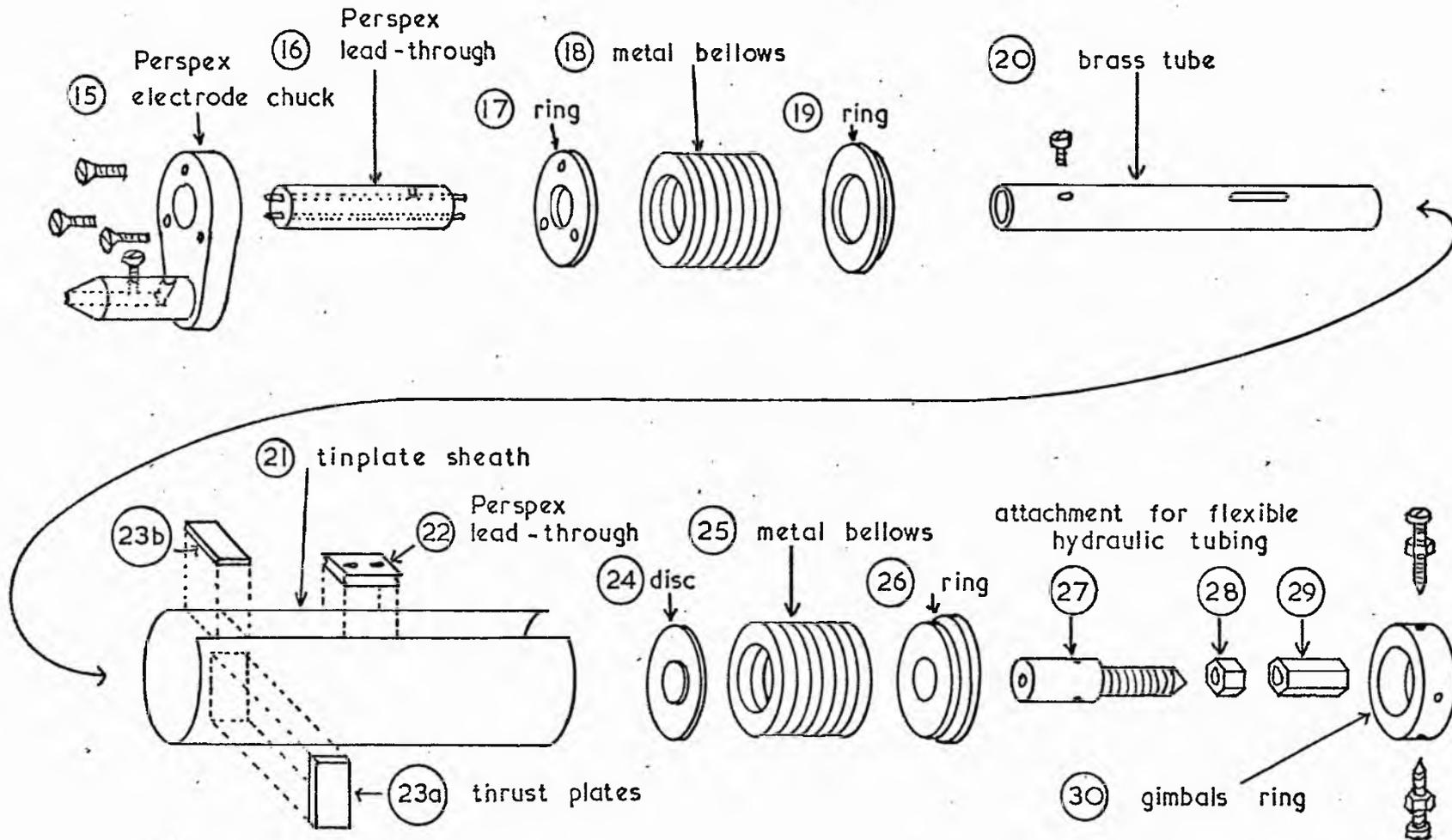


Fig. 16.

EXPLODED DIAGRAM OF MICROMANIPULATOR HEAD ASSEMBLY.



pivot gimbals (30) and at its front end by a light long coil spring wrapped around the sheath (21) and attached at its ends to hooks on the ring (10) against adjusting screws penetrating radially through the ring (10) mounted on the sledge. The vertical movement was also provided with a fine worm adjustment, since this adjustment was particularly critical when setting up a microelectrode. These parts are displayed in the exploded view, Fig. 12.

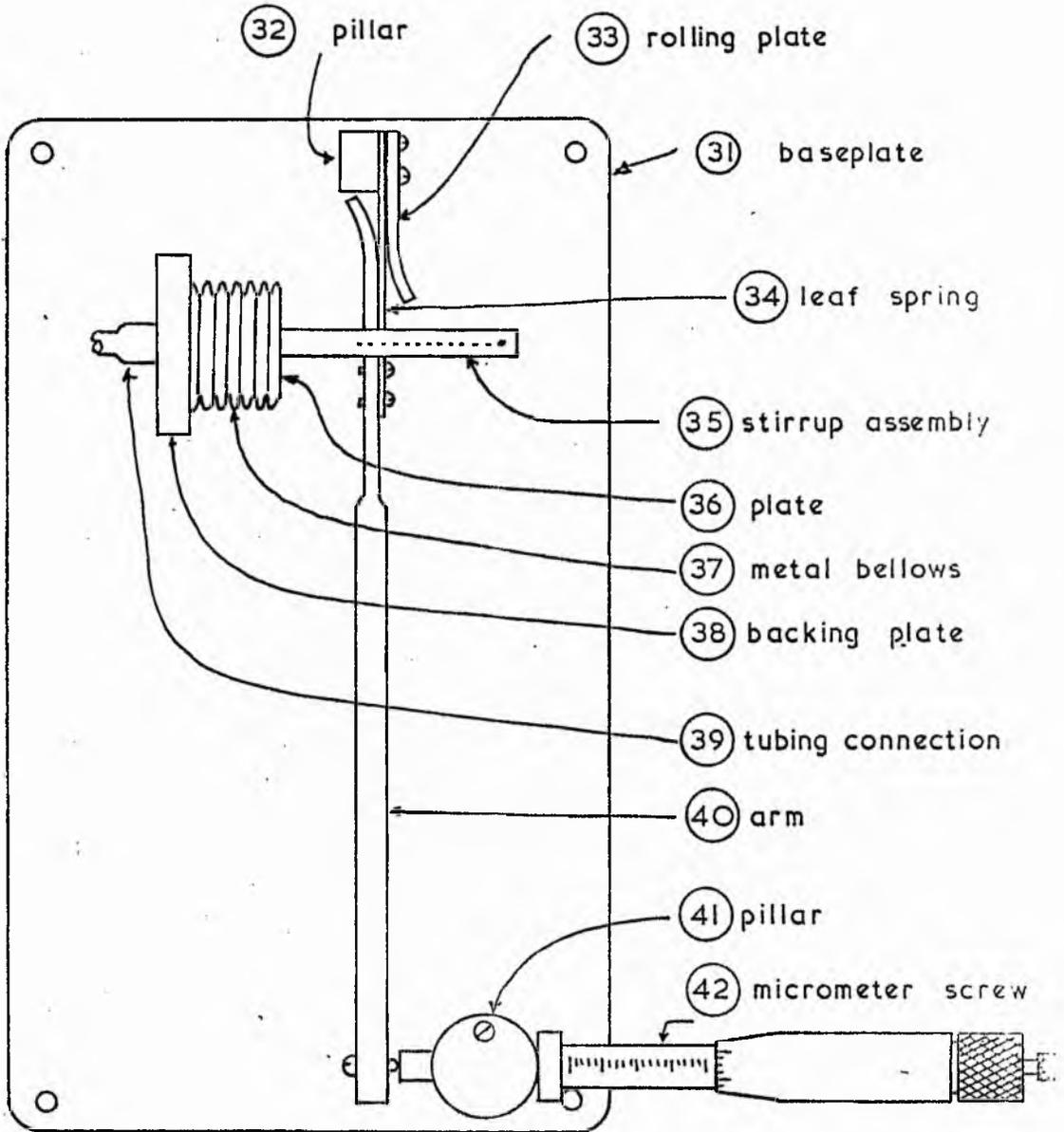
The hydraulic control unit involved no sliding parts (Fig. 17). There was one length of metal bellows only, mounted with its axis horizontal on a baseplate. The free end was compressed by a lever system. The fulcrum of the lever was so constructed that pivots were avoided by use of a leaf spring between rolling blocks as shown in Fig. 17, and pressure was applied to the bellows through a stirrup, about 1 cm. away from the fulcrum. The distal end of the lever was pushed laterally by the end of a micrometer screw calibrated in thousandths of an inch. The lever produced a mechanical reduction of about 17, and the fine thread on the micrometer made delicate adjustment very easy.

Initially some difficulty arose concerning the flexible tubing used to connect the hydraulic control unit to the micromanipulator head. The first tubing tried was thick-walled 3 mm. bore hard plastic tubing. When an adjustment was made, the micromanipulator would first advance, and then slowly drift back

Fig.17.

DIAGRAMMATIC PLAN OF DRIVER UNIT.

2/3 SCALE



again slightly, which is clearly unacceptable. It was realised that the increased pressure on advance was causing plastic deformation of the tubing with consequent change in internal volume. Thick-walled rubber tubing was tried, as was also canvas-reinforced rubber tubing, but these were both similarly unsuccessful. In the case of rubber slow plastic deformation seems unlikely to occur, but perhaps a change in tension in the rubber may have lead to a momentary adiabatic temperature change which slowly relaxed diathermally. At last, thick-walled plastic capillary tubing commended itself for the following reasons. First the thick wall would restrain stretching, secondly the stretching force both longitudinally and radially of a small bore is small, and thirdly its internal volume is in any case small so that any stretching will have minimal effect on the volume of the hydraulic system. A suitable tubing was obtained from standard $\frac{1}{4}$ inch coaxial single stranded core solid polythene insulated radio aerial cable. The sheath and screen were removed and the centre conductor was withdrawn (a tricky operation!) from the solid polythene insulation, which latter was then used as thick-walled capillary tubing. (This was used because no suitable tubing seemed to be readily available !)

The hydraulic system was filled with clean air-free (boiled) distilled water as working liquid, and great care was taken to see that no air bubbles remained in

any part of the system. Water was chosen as the working liquid because, of readily available liquids, it so happens that water has the lowest thermal expansion coefficient. However since water can be the source of corrosion with parts of mixed metals in contact - e.g. solder with bronze or brass - it is essential that the system be thoroughly cleaned first and that boiled de-ionised water be used. No trouble has been experienced on this count over several years of use.

When finally assembled, the micromanipulator was tested carefully. It was found to be completely free of drift as far as one could see under high power microscopy (x400). The movement was completely "dead-beat" and there was no backlash or judder, thus the finest settings were possible. One thousandth of an inch on the micrometer screw was equivalent to $1.44 \mu\text{m}$ at the head. One interesting effect of using capillary tubing to join together the hydraulic parts was that there was a very slight delay between adjustment of the micrometer screw and response of the head for sudden movements. In practise, this was not disadvantageous because microelectrodes were always introduced slowly into tissue. However a bonus advantage of this effect was that vibration at the control unit due to hand contact was not transmitted to the electrode. Indeed even gently tapping the lever showed no visible effect.

Lateral stability at the head was excellent and vibration from the bench produced remarkably little vibration at the microelectrode tip, so little, in fact, that only minimal precautions were needed to avoid vibration. The complete device, on its microscope, was merely placed on a 30 cm. square of plywood on top of a foam polythene mat 25 cm. thick. The arrangement was so stable that reagent bottles could be moved on the bench without upsetting a microelectrode actually implanted in a plant cell! Insertion of microelectrodes into plant cells was very easy, and accidental breakage of electrodes was very rare. It was possible to insert the same electrode into one cell after another virtually indefinitely and even to propel the electrode right through a root 1 mm. or so in diameter without fear of breaking the fine tip. (Of course, the repeated use of one microelectrode may be limited by changes in its electrical properties due to breakage at the extreme tip, unseen under the microscope, or to blockage with foreign materials, such as cell cytoplasm.)

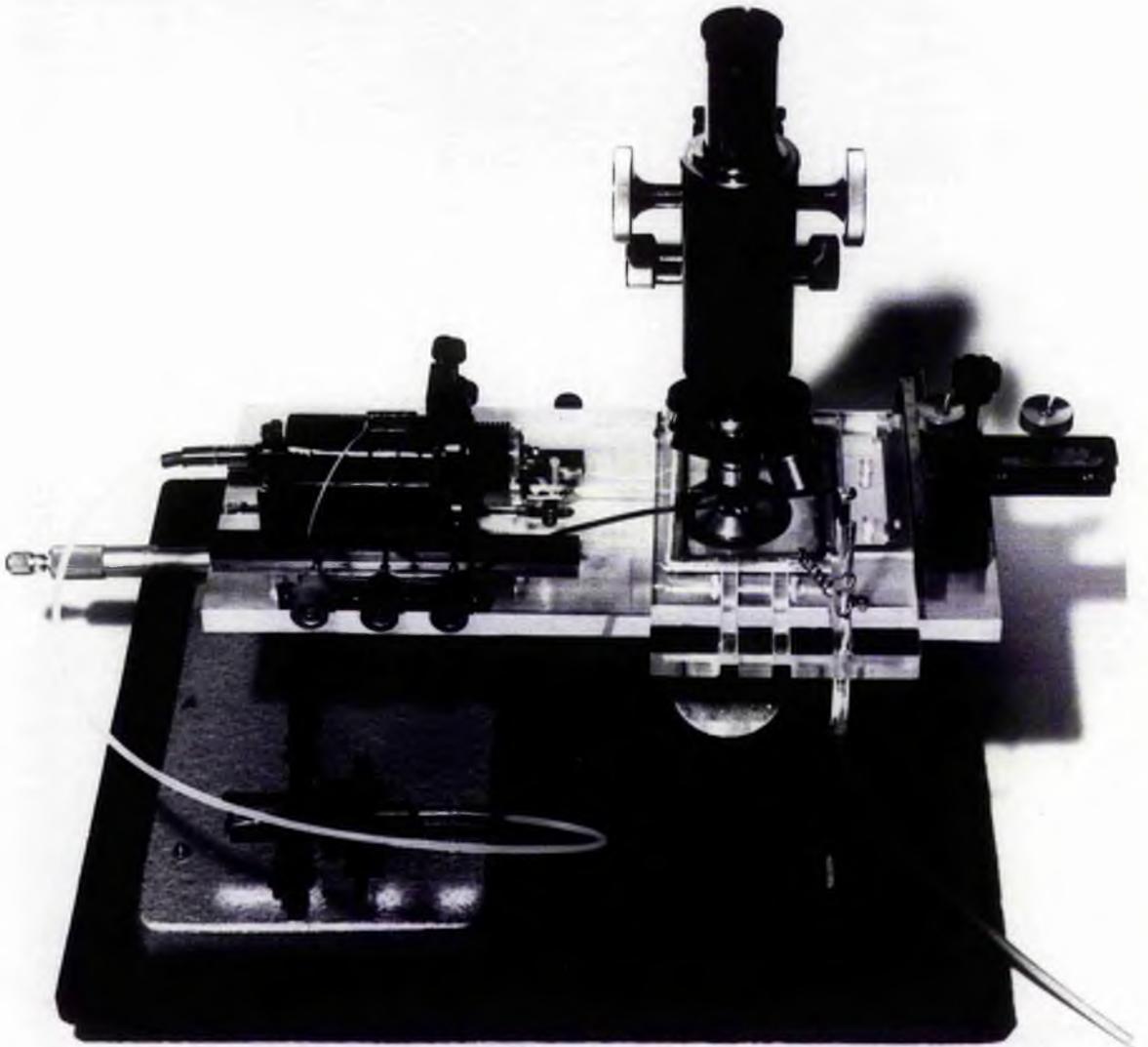
This micromanipulator system, using metal bellows as the extensible element was invented and devised by myself as an independent and original design, and applications have been made to obtain patents on the design (see below). However the idea of using a single metal bellows in micromanipulators had been anticipated by May in 1952 with a design for 1- and

3-dimensional micromanipulators (May 1956). The chief improvement in this design over those of May was the inclusion of a second bellows to act as a steady to preserve axial alignment. Subsequent tests of devices constructed to the designs of May indicated that his devices are inconvenient to use, especially the 3-dimensional micromanipulator in which x, y and z axial adjustments are not independent of each other. Also lateral stability of May's design was poor.

There were hydraulic designs earlier than that of May (1956), in particular the design of de Fonbrune (1932-4), which used syringes as the driver units actuated by cams, and aneroid barometer elements as slave units in a 3-dimensional arrangement. Clearly his design also partly anticipates mine, but it was a pity he used syringes as driver units since they involve slides (see p.217). Also, as he designed it, lateral stability was not as good as in my design. A Provisional Specification of my improved micromanipulator was filed at the Patent Office on 1 April 1975 and the Complete Specification was published on 15 Feb. 1978 (Stanton 1978a) under Patent no. 1,501,253, which is still in force. The design therefore may not be copied or manufactured without my express permission.

The complete prototype microscope/manipulator unit is illustrated in the photograph, Fig. 18.

Fig. 18 MICROMANIPULATOR



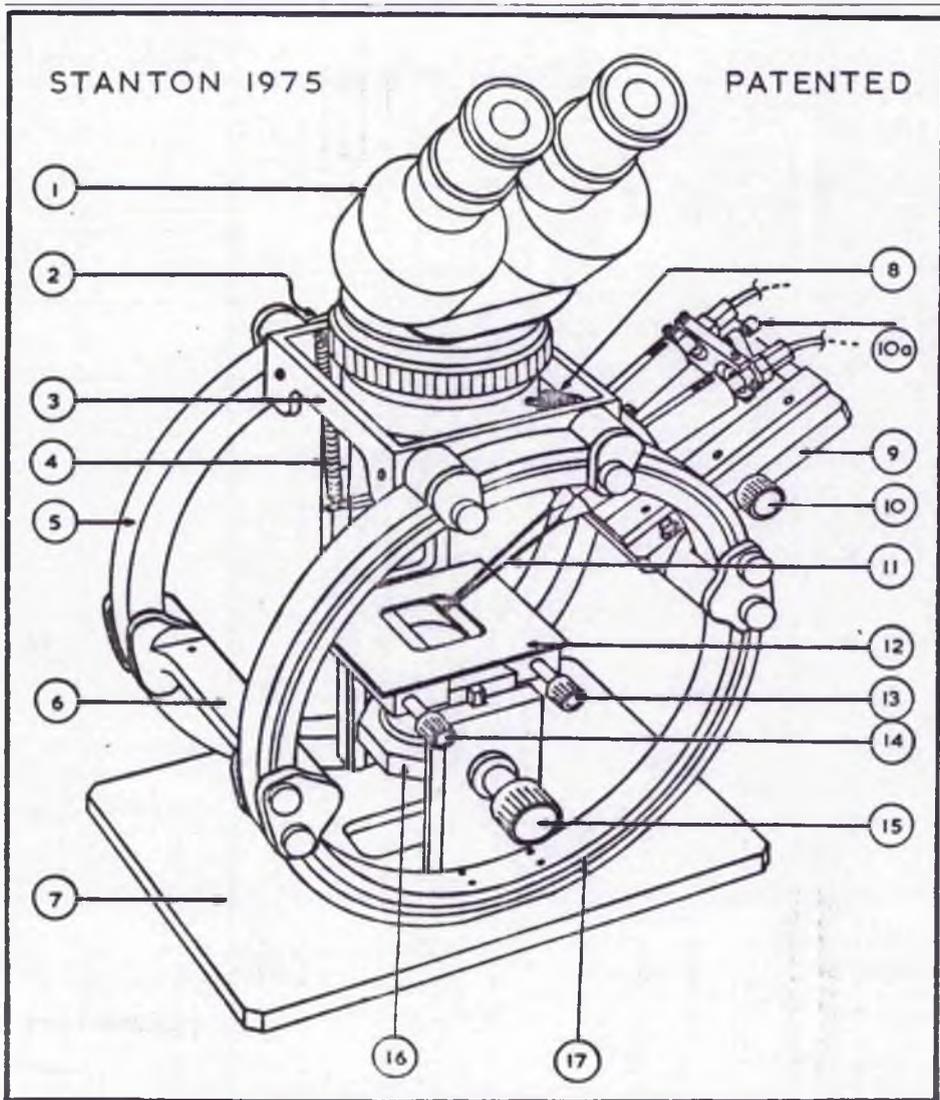
An Improved Microscope/Manipulator Unit.

The design for the new micromanipulator and the integral assembly on the stage of a microscope proved so successful in microelectrode impalement experiments that several units were made for use by others in this laboratory (Gatty Marine Laboratory) and elsewhere. However it soon became clear that a unit of more general application was needed for electrophysiologists who wished to do other things with it than myself. The chief shortcomings of the prototype design were that (a) entry of the microelectrode to the tissue could only be achieved at 90° to the optical axis, there being no provision to tilt the micromanipulator, (b) only one microelectrode could be presented to the tissue at one time and (c) only compound microscopy was available in the original design. These limitations were overcome in a second design, illustrated in Fig. 19, shown with a binocular zoom stereo microscope head fitted. The new unit features the following :

1. Both microscope, on its subframe, and micromanipulators may be tilted relative to the stage which remains level at all times. (It is not usual to be able to tilt a microscope independantly of the stage). The microscope is mounted on a subframe which extends below the stage to carry substage optics. Thus when the microscope is tilted the substage optics go with it and remain in alignment. However only a small degree of tilt is useful with a compound microscope and substage illumination since refraction of light

Fig. 19. MICROTACTOSCOPE

INTEGRAL MICROSCOPE / MANIPULATOR UNIT



Integral Micromanipulator - Microscope Unit. . Fig. 19.Legend.

1. Stereo zoom microscope head. (interchangeable).
2. Focus knob for microscope (operates rack and pinion to raise or lower head)
Not seen in this view as it is behind.
3. Microscope subframe.
4. Springs to carry weight of head.
5. Frame rings.
6. A carrier for a micromanipulator unit.
7. Baseplate.
8. Microscope head clamping screw.
9. Micromanipulator baseplate.
10. Coarse micromanipulator advance.
11. Micropipettes in place.
12. Top surface of stage - manipulable and magnetic.
- 13, 14. Stage lateral movement manipulators.
15. Stage rise and fall control.
16. Substage optics carrier, with adjusters.
17. Groove in ring to locate carriers.

Not visible, behind microscope subframe,
rise and fall control for substage optics.

Also not shown - illumination system.
This may be either by means of a mirror
beneath the substage optics or from
beneath the whole unit via a hole provided
in the baseplate (7). Alternatively a
compact illuminator may be used. This
was not built into the unit because so many
different arrangements are used.

in the tissue bath soon causes misalignment of the sub- and super-stage optic axes. Some adjustment however is available to cope with this. A tilting stage, with a bath carrying liquid on it, would of course be useless.

2. The components of the system are all mounted between two stout metal rings fixed to the baseplate. Tilt is obtained by moving components round these rings so that the salient points of microscope and micromanipulators always point to the centre of the stage. When the clamps are tightened the whole is locked into a rigid box structure.

3. The unit is based on a modular construction concept. Thus the microscope may be either zoom stereo (illustrated) or compound, and any degree of optical complexity may be built in, such as Nomarski, phase contrast or polarising. Also the microscope head may be rotated so that viewing may be from any position round the unit.

4. Up to four micromanipulator carriers may be mounted, and since each carrier carried a double headed unit, this allowed up to eight microelectrodes or other microtools to be directed at the preparation!

5. A double micromanipulator head was introduced, with provision for longitudinal relative adjustment to accommodate micropipettes of slightly different lengths. Of course, each micropipette is also independantly adjustable along its axis in the same way as in the single case. The double head is particularly useful when two microelectrodes are to be inserted close to each other in one cell or in

neighbouring cells of a tissue.

6. The unit is provided with both rise and fall stage and rise and fall microscope adjustments. Most microscopes do not have both, but in micromanipulation experiments this facility is often very useful.

7. The stage is fitted with mechanical adjustment in the two horizontal dimensions (i.e. normal to the usual position of the optic axis). The whole of the top surface of the stage is moved, rather than merely having slide clips move as on conventional stage devices. The top surface of the stage is also made of magnetic material (a suitable magnetic stainless steel is ideal) so that a tissue bath can be clamped to it very simply by means of ferrite-filled plastic strips (such as used in magnetic gaskets and to hold notices on noticeboards) fixed with rubber cement to the underside of the bath. The stage adjustments are also finer than is usual on mechanical stage devices. Fine control is an advantage when accurate positioning of a point on a piece of tissue is necessary, as with microelectrode impalement experiments.

The frame system of the unit has been the subject of a Provisional Specification filed at the Patent Office on 18 Dec. 1975 and published as a Complete Specification on 28 June 1978 (Stanton 1978b) under Patent no. 1,516,219. The unit has also been patented in the United States of America (Stanton 1978c) under US Patent no. 4,128,944. These patents remain in force, and the design may not be copied or manufactured without my express permission.

The whole of the machining to make the parts for the micromanipulators and the frame system and their assembly into the complete unit were carried out by myself in the workshops of the Gatty Marine Laboratory, but all the parts were purchased at my expense.

The complete microscope/manipulator unit has been given a unit name, for which I am indebted to Prof. Kenneth Dover (now Sir Kenneth Dover, President of Corpus Christi College, Oxford)* for suggesting, namely the "Microtactoscope". The reason for seeking a new name was that the combined name "Microscope/manipulator unit" was rather long. Also this new unit embodies a new concept, wherein the chief concern is with the accurate positioning of a microtool (e.g. a microelectrode) relative to something on the stage, while retaining the ability to view under microscope with top quality optics.

The complete Microtactoscope was exhibited at the 100th Anniversary Meeting of the Physiological Society in Cambridge in July 1976 (Stanton 1976). It is expected that the unit will be offered for sale through a reputable distributor in due course. Meanwhile development work on certain details continues.

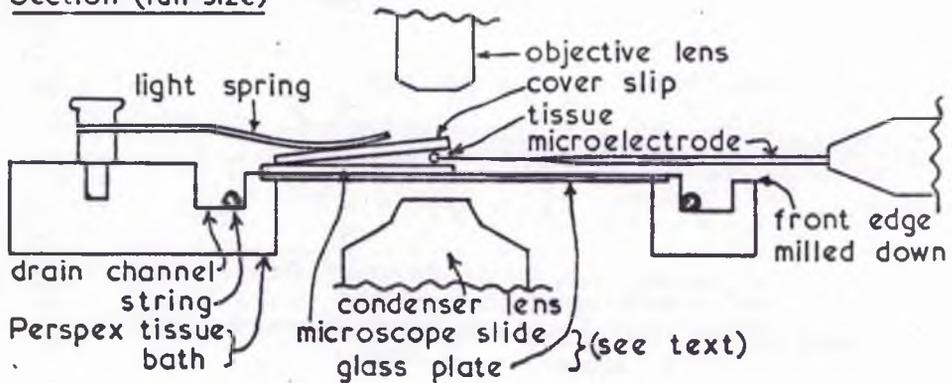
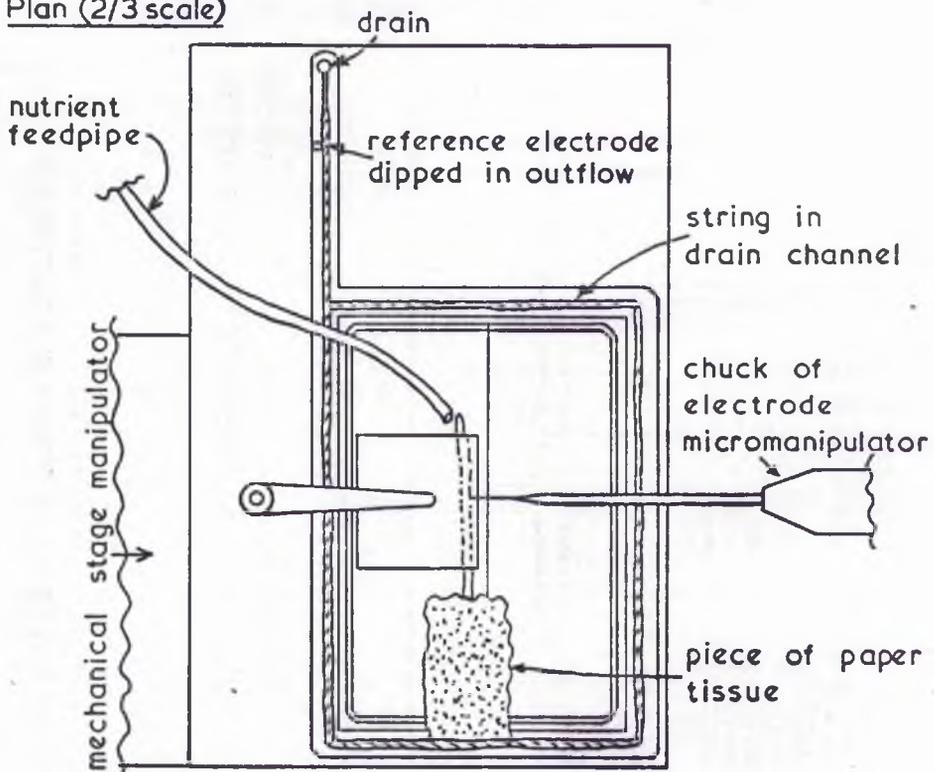
* - and since this was typed, elected Chancellor of this Univeristy !

Technique for Setting up Tissue and Inserting a
Microelectrode.

The first prototype model of micromanipulator was used for most of the work reported in this dissertation. The tissue bath, attached to the moving member of a conventional mechanical stage device (Fig. 20) was provided with a raised platform of glass in the middle surrounded by a drain channel. The microscope condenser could be brought up underneath this glass platform. On top of it was placed a standard microscope slide, made optically continuous with the platform glass using a drop of Zeiss lens immersion oil. The slide covered only half of the platform so that there was a step halfway across the platform (see Fig. 20a). This was to allow the tip of the microelectrode to be brought down to the lower levels of the tissue without its shank fouling with the platform. For the same reason the wall of the drain channel was milled lower where the microelectrode shank passed over it. A piece of string was wrapped round the drain channel to facilitate drainage by overcoming surface tension. In the plan view of Fig. 20b, it will be seen that a piece of absorbent paper was also laid between the end of the plant tissue and the drain channel to aid drainage and to maintain electrical continuity between the tissue and the reference electrode. The reference electrode was placed in the drain channel, supported by a wire loop (to which it was not connected) attached

Fig. 20.

INSERTION OF MICROELECTRODES INTO PLANT TISSUE.

(a) Section (full size)(b) Plan (2/3 scale)

to the bath, so that ions diffusing from its tip did not upset the ion concentrations in the nutrient bathing medium near the tissue. Nutrient medium was supplied from a large stock bottle through a capillary polythene tube to the opposite end of the tissue from the absorbent paper so that a steady flow past the tissue was maintained. Nutrient in the stock bottle was sometimes continuously aerated, although it was found that this had little advantage during short experiments.

A piece of plant tissue (maize* root) was clamped on the top of the slide near its edge under a square of microscope slide glass. (Conventional cover slip glass was too fragile). Gentle pressure was maintained with a light spring. Application of pressure was necessary to hold the tissue steady while the micro-electrode was pushed into it, but it was necessary to take care that this pressure was not so great that it damaged the tissue or altered its physiology. The tissue was immediately bathed in nutrient medium.

A freshly chloridised silver wire was fixed to the electrical terminal on the micromanipulator head followed by a fresh micropipette filled with 3 M KCl, which was clamped firmly under the nylon screw of the chuck, all with the micromanipulator head wound right back by the coarse adjustment so that

* Zea mays. Details of the culture technique used will be found in Appendix 5.

the tip of the microelectrode was well clear of the tissue bath and raised above it. The tissue was then adjusted into the optic axis of the microscope and the microscope focussed using the 10 x objective with 10 x eyepiece. Once focussed on the tissue the microscope controls were not touched again. The tissue bath was now withdrawn laterally until the lower step of the platform was in the optic axis. Next the micromanipulator head was advanced, using the coarse adjustment, until the microelectrode tip was well under the objective lens, care being taken, with observation from the side, to see that the tip did not crash into anything. The vertical control of the micromanipulator head was next adjusted to bring the microelectrode tip into focus. Of course, the cover slide on the tissue upset the apparent light path length between the tissue and the objective lens. To correct this, another piece of identical slide glass was momentarily held immediately under the objective lens while the microelectrode was adjusted vertically. This manoeuvre brought the microelectrode tip into the same plane as the tissue. It was then safe to wind back the tissue cautiously into the field of the microscope. Final minor adjustment of the height of the microelectrode tip was often necessary, with the fine control. The greatest danger was that the tip might crash into the edge of the underslide or coverslide. Once in the bathing medium, the microelectrode was

routinely tested for resistance. A good electrode in "freshwater" bathing medium measured certainly much less than 25 M Ω . Electrodes blocked with an air bubble read infinity and those with a broken tip read much below 1 M Ω . Either of the latter were rejected. However care was needed in interpreting electrode resistance data, as will be explained later.

All was now ready for the final insertion of a microelectrode into the tissue. Starting from about 100 μm away from the tissue surface the tip was slowly advanced using the hydraulic micromanipulator control only until the electronics indicated a recorded cell membrane potential. During this adjustment visual observation of progress was necessary at the same time as one needed to know the electrical state of the electrode tip. It was soon realised that an audible aid to tell its state would be very useful, because one cannot look down the microscope, and at the electrometer or oscilloscope simultaneously. Accordingly I designed a simple electronic device to give a change of pitch with change of input voltage (a voltage controlled oscillator, VCO). This unit was coupled to the recorder output point of the electrometer. Its circuit is shown in Fig. 21.

The circuit was designed in 1966 (Stanton unpublished) and was an early example of the use of what is now a very important technique, namely opto-electronics.

Valves were still widely in use in 1966, and although obviously it would be easy now to make a VCO using transistors or integrated circuits, the device has been retained because it continues to be functional! It functions as follows. The first valve acts as a high-impedance input amplifier driving a miniature neon lamp. The neon lamp is set between two CdS photoresistors in a dark box. The CdS cells form part of the frequency controlling network in a multivibrator oscillator, the output from which is amplified and fed into a loudspeaker. Voltage stabilisation of the HT line was necessary to keep the pitch of the note insensitive to power line fluctuations. There is a large electric immersion heater in the building which causes bad sudden voltage changes on switching, which in this case could mislead one into thinking that a cell had been penetrated!

Electronics for Transmembrane Potential Measurement.

To measure correctly a potential difference which includes in its circuit a high resistance requires an electrometer with input resistance at least 100 times greater, if error is to be less than one percent. The microelectrode alone carried up to 25 M Ω in "freshwater" bathing medium and the cell itself contributed a further few megohms, so that the electrometer must have an input d.c. resistance of at least 3000 M Ω . For these measurements of steady transmembrane potential, it was however unnecessary for the response rate of the electrometer

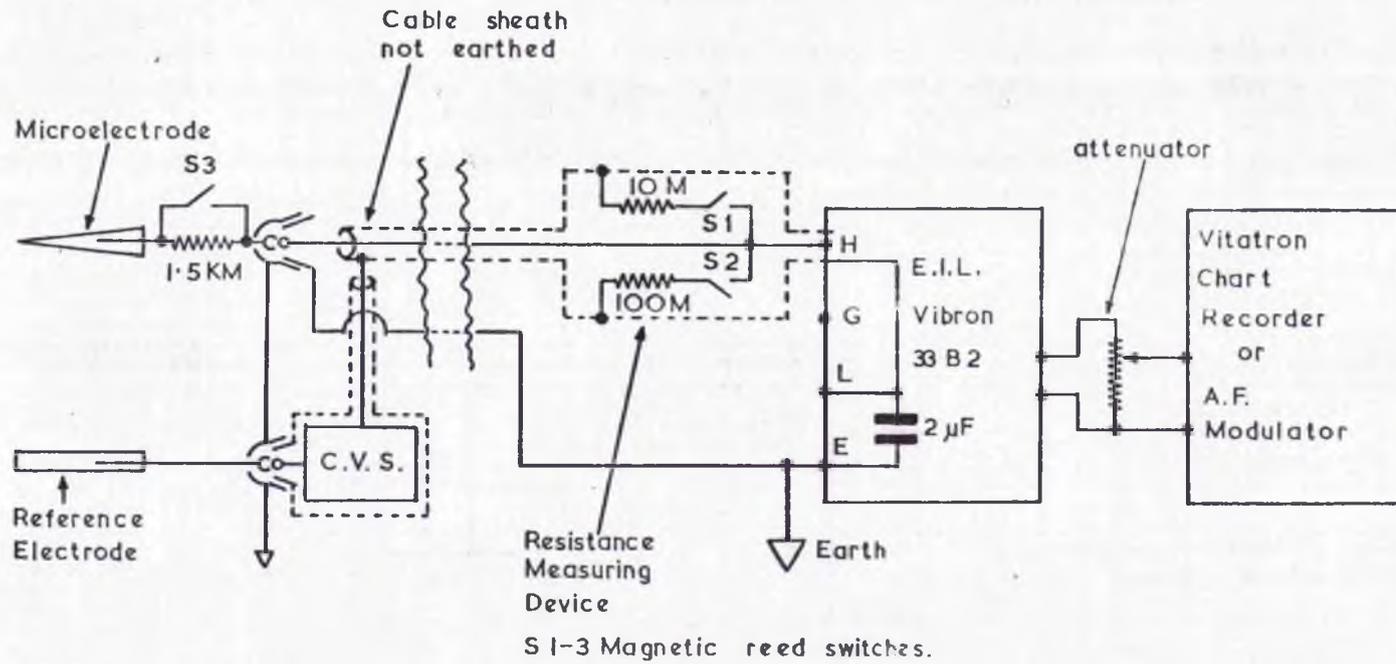
to be fast, nor was it necessary for the input stage of the electrometer to have a very high a.c. impedance, i.e. very low capacitance, although the capacitance must not be too high otherwise it imposes too much of a charge burden on the impaled cell (see Chapter 3).

Initially in this study a Pye Dynacap pH meter was used, but later a Vibron 33B2 of Electronic Instruments Ltd. of Richmond, Surrey was used. Both of these electrometers convert the d.c. input signal into an alternating signal of proportional magnitude by means of a vibrating reed capacitor. The alternating signal is then amplified at precisely defined gain, rectified and presented on a panel meter. The advantages of such instruments are exceedingly high d.c. input resistance and very stable gain with inherently no zero drift. Zero drift with direct coupled d.c. amplifiers is a persistent hazard from which these instruments are free. One disadvantage is that they are somewhat slow to respond, but, as above, this was unimportant. The Pye Dynacap is slower than the E.I.L. Vibron by a factor of about 5. Vibrating reed electrometers would of course be unsuitable for electrophysiology of nerves, etc., when rapid voltage excursions are to be observed, but their extremely high input resistance (about $10^{15}\Omega$) stable gain and absence of zero drift make them ideal for accurate membrane potential measurements where the absolute value is important, as in ion studies.

Fig.22 shows the first circuit which was devised for potential measurements and simple d.c. resistance measurements (see below). The screening sheath of the coaxial cable to the microelectrode was not connected to earth but to the LOW impedance input terminal of the Vibron. This was done so that the circuit was floating and yet buffered to earth against transients by the internal capacitor of $2 \mu\text{F}$ between the EARTH and LOW terminals of the Vibron. The microelectrode was connected to the HIGH impedance terminal of the Vibron through a resistance of $1.5 \text{ G}\Omega$, which was included because without it a recording artefact occurred in the form of a momentary overshoot on the chart record. This was caused by a peculiarity of the Vitatron recorder itself. However the inclusion of this very high value resistor proved necessary for another reason (see Chapter 3) when a.c. detection was also attached to this system, since it isolated the a.c. signal from the input capacitance of the electrometer. The value of this resistor was discovered by trial and error. Across the $1.5 \text{ G}\Omega$ resistor was connected a magnetic reed switch so that it could be removed from circuit during resistance measurements. Magnetic reed switches were chosen rather than conventional switches because, in the 'off' position, their impedances between contacts and to earth are extremely high, so that their inclusion has virtually no effect on the high impedance circuit.

Fig. 22.

CIRCUIT FOR MEASURING RESTING POTENTIAL AND D.C. RESISTANCE.



C.V.S. Calibrated Voltage Source

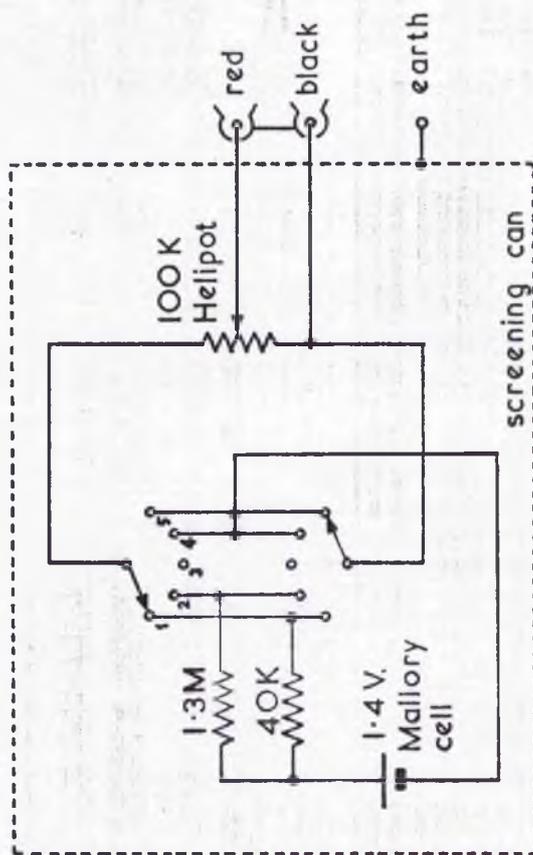
A calibrated voltage source (CVS) for backing off unwanted standing potentials (Fig. 23) was included in the low impedance lead connected to the reference electrode. It will now be apparent why the sheath to the electrometer input needed to be floating. If bathing medium inadvertently shorted the bath to earth (microscope frame), then the tip of the salt bridge of the reference electrode (but not its wire) would become earthed. This happens rather easily, by flooding the bath! Thus the inclusion of a backing-off source had to be between the reference electrode wire and the LOW terminal. It could not, of course, be included in the HIGH impedance circuit of the microelectrode. The calibrated voltage source was able to provide up to 1 volt in either direction, and was used (a) in potential measurements, to back off any potential difference between the two electrodes with both in the external medium, and (b) during d.c. resistance measurements.

D.C. Resistance Measurements.

D.C. resistance was measured by connecting a load resistor of either nominally 10 M Ω or 100 M Ω across the electrometer input. The actual resistances of these resistors were measured accurately by current and voltage measurements. Their actual values were 14.4 M Ω and 125 M Ω . Both load resistors were housed in a Perspex tube with coaxial socket one end and coaxial plug the other end, so that it could

Fig. 23.

CALIBRATED VOLTAGE SOURCE (CVS)

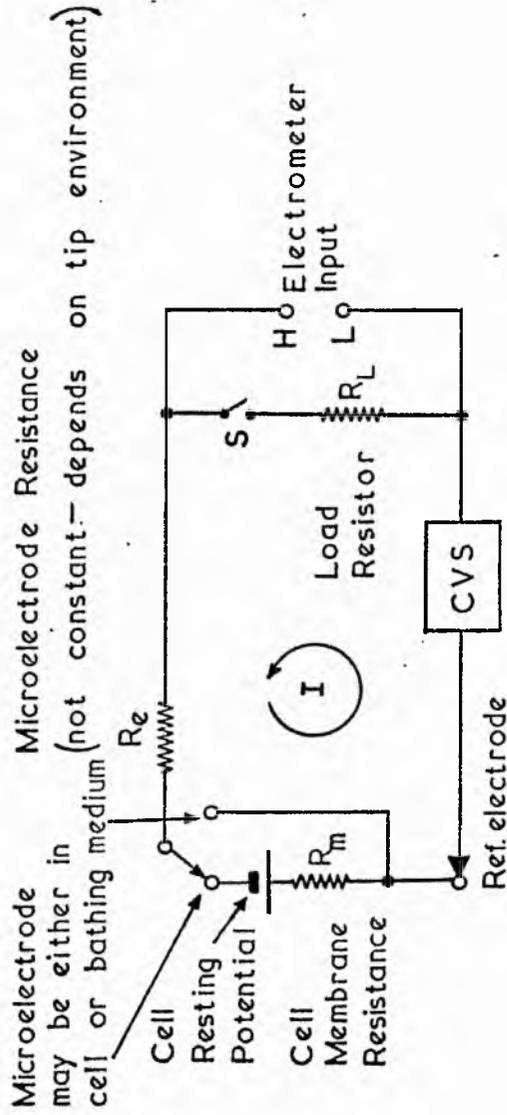


Switch positions:
 1,2 forward ; 3 off ; 4,5 reverse
 2,4 0-0.1V. ; 1,5 0-1 V.

be simply interposed between the electrometer and the input lead to its HIGH terminal. Also enclosed inside the Perspex tube were two magnetic reed switches which could be actuated individually by suitable placement of an external small bar magnet.

Reference to Fig.24 will show that resistance was measured by observing the drop in reading of the electrometer when the load resistor was connected across its terminals. It will be clear that the position of the potential source is unimportant provided none of it is backed off within the electrometer itself. Thus although the Vibron 33B2 itself contained a backing-off source, it could not be used here. (In fact it was not very useful because its sense was always in opposition to the input voltage, and this was not always required.) The microelectrode is regarded as a straight resistance, but the cell must be regarded as a potential source in series with a resistance, most of which will reside in the cell membranes. However, the voltage in the loop driving current through the various resistances is the sum of the backing-off potential and any other potential deriving from asymmetry of the electrodes, unless the microelectrode tip is outside the cell when voltage is derived from the electrodes and backing-off circuit only. Although this latter had an internal resistance of about $100\text{ k}\Omega$, this was negligible compared to the resistance being measured. The Pye Calomel Reference electrode presented only

Fig. 24.
EQUIVALENT CIRCUIT FOR D.C. RESISTANCE MEASUREMENT.



$$R_e + R_m = R_L \left(\frac{V_0}{V_L} - 1 \right)$$

6 k Ω to the circuit.

The procedure for d.c. resistance measurement was as follows. With S open, CVS was adjusted to give a conveniently high reading on the electrometer, say 100 on the scale, V_o ; S was then closed and the electrometer read again, V_L . By application of Ohm's Law to the loop in Fig. 24, it may be seen that when the micropipette tip is in the cell :

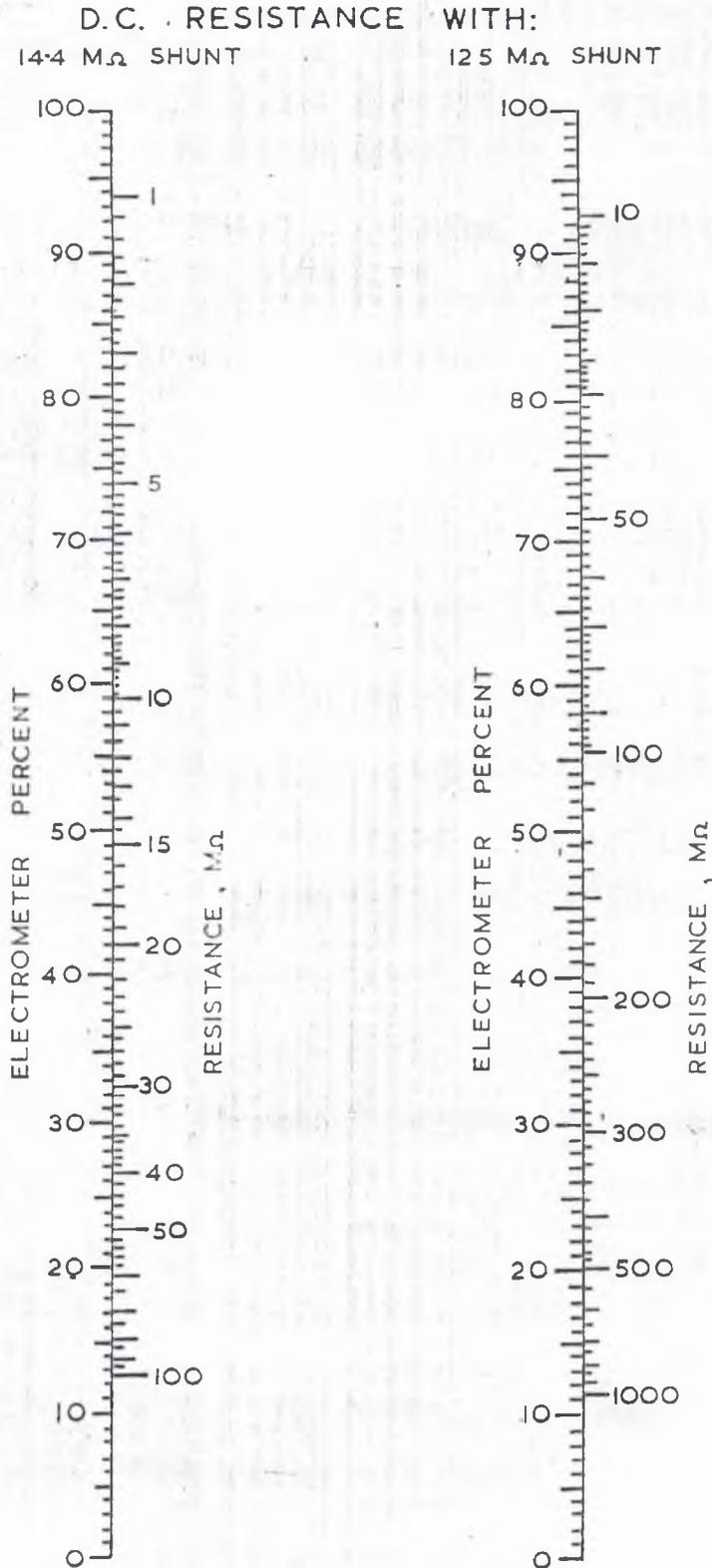
$$R_e + R_m = R_L \left(\frac{V_o}{V_L} - 1 \right)$$

In practise, the value of $R_e + R_m$ was obtained quickly by reference to the nomograms (Fig. 25) of $V_L/V_o \times 100$ (second reading expressed as percentage of first), against the factor $(V_o/V_L - 1)$ multiplied by the load resistor in use. This was easy to read if R_L was such as to bring $V_L/V_o \times 100$ down to 50 %, but see p.319.

In this procedure, there was no way of separating electrode resistance and cell membrane resistance, when the microelectrode was in the cell. The membrane resistance cannot be calculated by taking the difference between the resistances with the microelectrode tip in and out of the cell for reasons which will be discussed later (Chapter 3). For the present discussion the value of this circuit was in the measurement of microelectrode resistance with the tip in bathing medium, to check that the micropipette was "good" - i.e. well filled and not broken.

Fig. 25.

NOMOGRAMS FOR RESISTANCE COMPUTATION



Electrical measurements on plant cells were mostly recorded on chart paper. The recorder was the Model UR 100 by Vitatron of Dieren, Holland. Most records were taken at a chart speed of 20 secs./cm. The span was adjusted by means of an external attenuator, to give convenient calibrations in millivolts on the chart, and the zero was often deliberately offset by 20 mV from the zero of the paper, using the CVS, because occasionally the potential reversed slightly when the microelectrode was withdrawn from a cell. Results, when this happened, were not regarded as reliable (but see below, p.242), but it was valuable to retain the information on the chart. The voltage controlled AF oscillator (see p.233) was also included, usually driven in parallel with the chart recorder.

Sources of Measurement Artefacts.

The accuracy of measurement of transmembrane potential unfortunately must be better than that for concentrations or activities of ions if they are to be compared by the Nernst or Ussing equations or any of those equations which I derived (Chapter 1), because the potential is raised to the power of the exponential, whereas concentrations are not. Hence artefacts in E_m measurement are serious. An error of 17 mV corresponds to a twofold change in ionic concentration ratio or flux ratio. The following sections therefore discuss in some detail the investigations carried out to ensure that a procedure was adopted likely to measure

the real transmembrane potential.

1. Liquid Junction Potential.

In general, when two solutions are brought into contact, a potential difference soon develops across the boundary between them, as described on p. 36. This potential is known as the liquid junction potential.

When electrodes are used to measure potential differences between points spaced apart in an electrolytic cell (of which a plant cell plus electrode saltbridges is an example), it is evidently important that there should be no liquid junction potential where the solution surrounding the metal electrode comes into contact with the electrolyte of the system under study, or else this liquid junction potential must be known, or must not vary with location of the electrode. In practise, the only useful situation is to avoid liquid junction potentials, if possible, because the probing electrodes are usually placed in an unknown environment.

Thus an electrode used for local potential measurements in cells must be provided with a salt bridge, as was stated on p. 39, and the reason for the choice of 3 M KCl was there explained. Sometimes 3 M KCl is unsuitable for chemical reasons, and then alternatives would be KNO_3 or NH_4Cl .

2. Tip Potential.

While the treatment above would be satisfactory for wide bore salt bridges, there are yet further complications with micropipette salt bridges. Firstly, a tip potential arises due to the very small size of the tip. The existence of this phenomenon is well known (e.g. Nastuk 1963, Vol.VI, p.64, and Adrian 1956) but it does seem likely, contrary to their assumptions, that it could be due to the properties of the glass as much as to contamination of the tip. Glass is capable of ion exchange at its surface, and so its surface will behave, in electrolyte solutions, as a surface Donnan system (p. 31). As the surface is approached from the bulk aqueous phase, the electrical potential will go progressively negative (for an anionic glass). The potential at the surface of shear is normally called the ζ -potential. The effective thickness of this layer will depend on the concentration of salt in the solution in contact with the surface (Appendix 1). In this region, cations will be held at higher, and anions at lower, concentration than in the bulk phase. When the lumen of the tube is large compared to the dimensions of this layer, the effect will lead to little tip potential, but as the lumen becomes narrower, eventually the electric field profiles of the opposing surfaces will overlap. Then the region in the lumen will be filled with solution depleted in anions and enhanced in cations. The system is now seen to be similar to a permselective membrane separating two

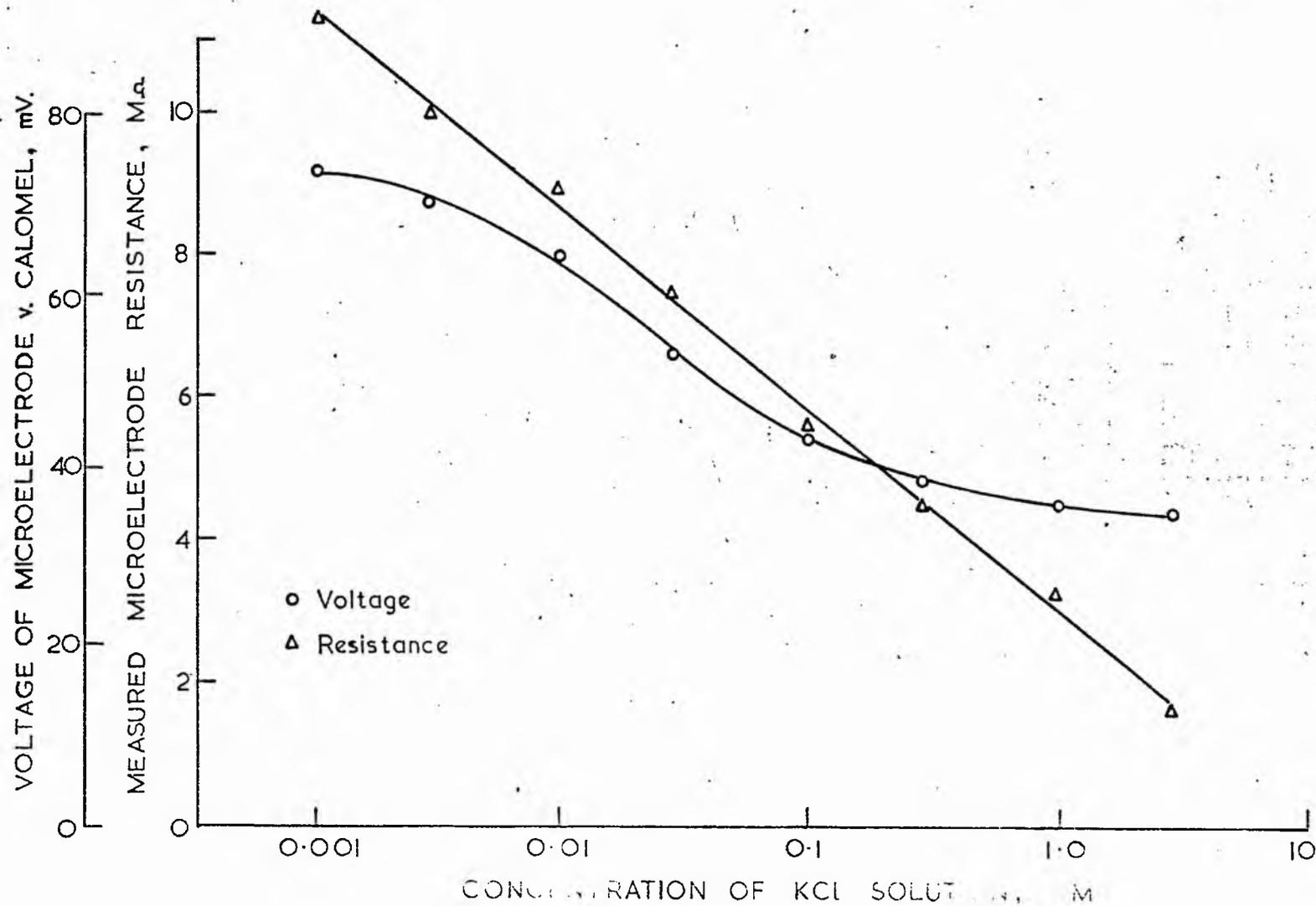
differing solutions (see p. 41). The effect of enhanced cation concentration on the apparent mobility of the cations will be to raise it above the free diffusion mobility. This is so because the increased concentration leads to an increased permeability of the ion through the pore in a way parallel to the increase in permeability with solubility of substances in a membrane material (see Alexander and Johnson, 1949, pp.808-820).

In effect the negative Donnan potential increases the "solubility" of cations in the pore. The opposite occurs for the anions. Thus the presence of a Donnan potential or ζ -potential profile right across the lumen at the tip of a micropipette filled with KCl solution produces a situation in which K^+ diffuses more rapidly than Cl^- despite the near identity of their free diffusion mobilities. As was seen on p. 41, this leads to a diffusion potential.

Immersion of the micropipette in a solution identical to its filling solution should be expected to eliminate tip potential, as would also breaking off the tip, to a point where the bore was "wide". Tip potential was investigated experimentally as described below.

The extent of variation of tip potential with concentration of external KCl is shown in Fig. 26 (circles). At the small dimensions of the tip Adrian (1956) has also shown a variation of tip potential according to whether the external solution contains Na^+ or K^+ . This may be explained due to a difference in permeability

Fig. 26. BEHAVIOUR OF MICROELECTRODES IN POTASSIUM CHLORIDE SOLUTIONS.



of the pore for Na^+ and K^+ ions. Adrian adopted the practise of selecting only those micropipettes which showed small tip potentials when fresh. However this does not help to avoid this artefact because, once a micropipette has been inserted into a cell, it will have become contaminated with cell contents. This is especially likely to arise with plants where the inside of the cells are at turgor pressure. The ingress of cell contents into the micropipette may occasionally be seen under the microscope. Application of pressure to the micropipette would not appear to solve the problem because there is then the danger of filling the cell with concentrated KCl solution. However, see the experiments described on p.245ff. In fact, the ingress of cell contents into the micropipette may be a help. It will be clear, from the argument above, that tip potential will be eliminated if the external solution flows into the micropipette far enough to pass the "tip" region (here understood as the region of the lumen wherein the ζ -potential profiles overlap). This ingress of cell contents will push back the 3 M KCl in the tip and the liquid junction between cell contents and pipette filling solution will occur at a wider part of the pipette where the ζ -potential profiles do not overlap. Thus inside a cell initially under turgor pressure, a microelectrode may well be reading correctly. However, when the electrode is withdrawn the "zero" will be shifted by the cell contents now in the tip. In any case neither the "zero" before or after insertion

and withdrawal will be true because in both cases there will be a tip potential in the dilute "freshwater" nutrient medium bathing the root. Perhaps the truest measurement would be obtained between a fresh microelectrode "zeroed" in 3 M KCl and then inserted into a turgid cell, but this would certainly be inconvenient to set up on the microscope stage. The unreliability of the tip potential may be noted from the fact that if a new microelectrode was first "zeroed" in the bathing medium, and then inserted into a cell and withdrawn, the reading was no longer zero by as much as 20 mV, due presumably to alteration of the contents of the micropipette tip. Usually the zero became more nearly reproducible after a fresh micropipette had been inserted and withdrawn from the root a few times.

Instead of using an external solution of 3 M KCl to establish the zero of the fresh micropipette, it might be possible to obviate tip potential by first measuring the potential inside a cell and then, after withdrawal, deliberately to break off the tip of the micropipette and record the potential outside the cell. Minus tip, the micropipette should give the true "zero", so that the difference between the reading in a cell with tip unbroken and the reading in bathing medium with tip broken should be the true transmembrane potential. A comparison of these treatments is shown in Table 22.

It is clear from these results that micropipettes vary considerably in the tip potential which they

Table 22, continued.

Micropipette tip in :	3 M KCl	Nutrient medium	Cell
<hr/>			
<u>Micropipette 5.</u>	(new)	-2 (5.6 MΩ)	
Insertions into region of root hairs.		-6/18 (144 MΩ) unsteady	-88
		? unsteady	-86
	break tip :	0	
<hr/>			
<u>Micropipette 6.</u>			
	(new)	-7 (6.2 MΩ)	
Insertions into region of root hairs.		-9 (37 MΩ)	-97
		-9 (42 MΩ) unsteady	-109
		-6 (20 MΩ)	-94
Insertions into root tip (not cap).		-8 (18.5 MΩ)	-98
		-11 (56 MΩ)	-96
		-36 (9.2 MΩ)	-106
		-8 (9.6 MΩ)	-96
	break tip :	0	
		Average :	-99 ± 4.8

produce even when new. However voltages recorded in the cell are much more nearly constant when referred to a broken-tip zero than when referred to the zero for the unbroken micropipette in bathing medium. For micropipette 4, for instance, the standard deviation of readings is 4.0 when the consecutive readings with unbroken tip in and out of the cell are subtracted from each other. Also the mean is shifted to -95 mV. Likewise with micropipette 6 in the root tip, the standard deviation of readings is 9.1 and the mean is shifted to -83.3 mV, if the unbroken-tip zero is taken. It is also interesting to note, from the two breakage treatments on micropipette 2, that the tip potential is confined to a region very close to the tip.

These experiments cannot provide a definitive decision as to which "zero", with or without tip, should be taken, but theory suggests that the zero with broken tip is more likely to be correct, and the improved standard deviation of readings by this method tends to confirm this. It appears that generally an underestimate of cell transmembrane potential would be made if intracellular measurements were referred to an unbroken tip zero outside. It therefore seems reasonable on the basis of this investigation to regard the difference between voltage recorded with a micropipette with unbroken tip in a cell and with the tip broken outside the cell as probably the most reliable measure of transmembrane potential one can make.

3. Zero Shift.

It was seen in several recordings in which the micropipette was propelled right through a root that the "zero" of the microelectrode was shifted when the micropipette tip was protruding from the far side of a root, but that it returned to the set zero when it was withdrawn. (This set zero was with a used electrode). Usually the shift was towards a more negative value, although this was not perfectly consistent. This effect could perhaps be attributed to a slight sensitivity of the walls of the micropipette to pH or cations in the root in much the same way as glass electrodes used for pH measurement respond to pH - by permeation of ions through the glass. The shift was rarely more than 10 mV. It is of course not relevant to measurements on cells at the surface of a root on the near side.

4. Streaming Potential.

When an electrolyte solution is forced under pressure through a capillary with charged walls, a potential develops between the ends of the tube. This is known as the streaming potential (see p. 54).

It is possible that streaming potentials could produce artefacts in measurements of transmembrane potentials in two ways. Firstly, when a micropipette is inserted into a turgid plant cell, the turgor pressure will force a flow of intracellular solution

up the micropipette, or even by leakage past the tip, due to a rent in the wall or membrane made during insertion of the micropipette. Consequently the turgor pressure may well be released, and osmotic inward flow of external medium through the wall and membranes may occur. Since both the wall and membranes will have some capacity for ion exchange (i.e. they carry fixed matrix charge) there may be a streaming potential which will be artefactual as far as measurements of transmembrane potential in the intact cell are concerned.

Secondly, either the slow relief of turgor pressure or the continuous uptake of external medium by the cell would cause a continuous, if small, flow into the micropipette. This may also induce a streaming potential in the micropipette tip. Unfortunately, if both cell boundary and micropipette tip are anionic, as is most likely, these two effects will be additive.

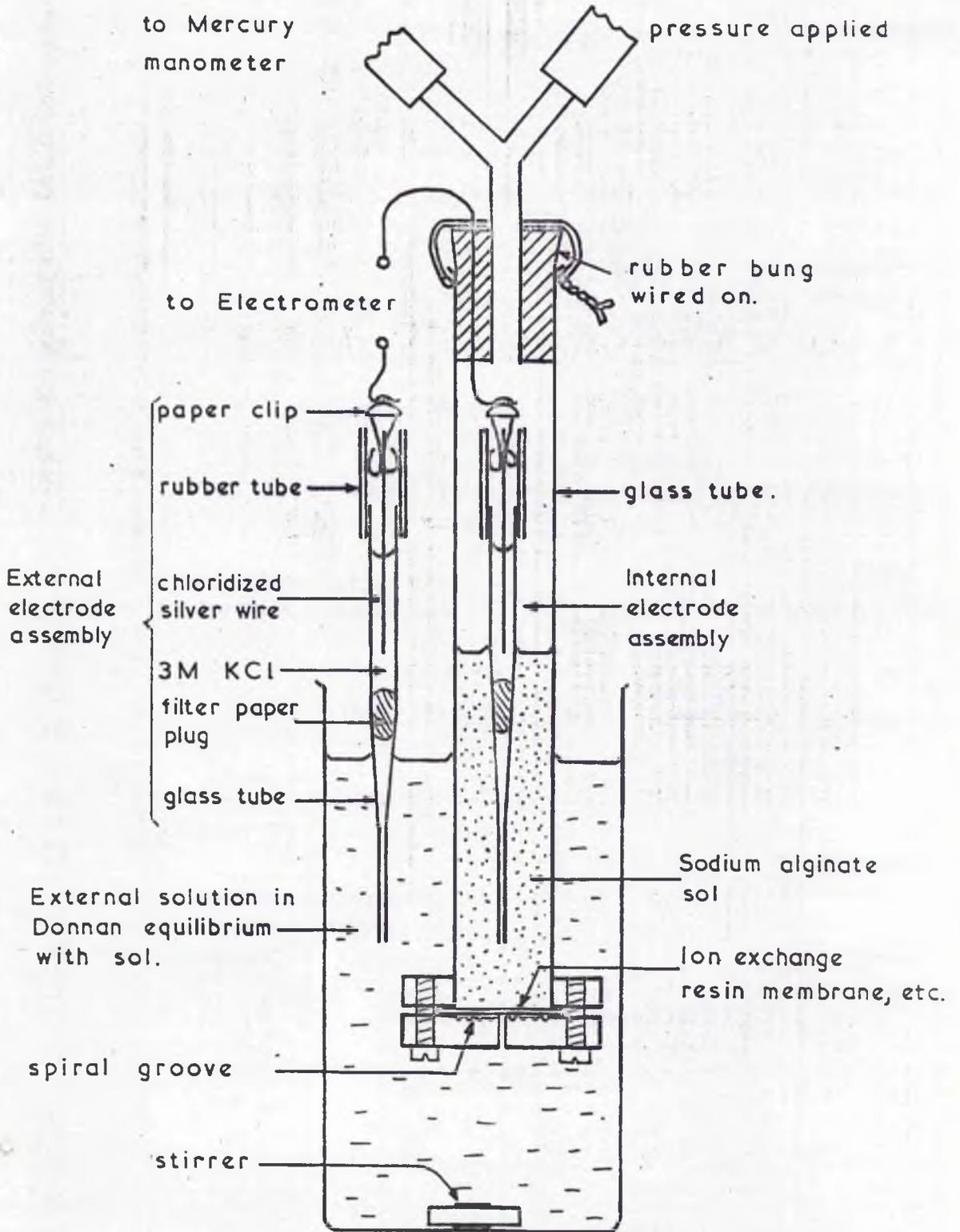
It was thus of interest to discover the magnitude of these effects in comparison to membrane potential.

(a) Streaming Potential in Membranes.

To investigate the streaming potential behaviour of the cell wall/membrane system directly is difficult, so a model system was set up as follows. Fig. 27 represents the arrangement used. Two reversible electrodes with salt bridges were connected to an electrometer, to measure the potential difference across a membrane. On one side of the membrane

Fig.27.

APPARATUS USED TO STUDY THE EFFECT OF 'TURGOR' PRESSURE ON THE DONNAN POTENTIAL.



was placed a solution of sodium alginate, about 1 % to represent cell sap, and on the other the very dilute solution obtained by dialysis of 20 ml of this alginate solution against a single 500 ml volume of distilled water for 12 hours, to represent bathing medium. (It is not important for the purposes of this experiment that the concentrations should be exactly the same as in a cell.) During the dialysis, of course, the cellophane bag (Visking tubing) containing the alginate solution became turgid, the osmotic pressure being almost entirely due to Na^+ ions held by the alginate in the bag. The system constituted a Donnan system. Also the external solution, initially distilled water, became weakly alkaline due to diffusion of some of the Na^+ ions from the bag, and counterdiffusion of H^+ ions from the water.

Pressure was applied to the system by means of an adjustable rubber pipette pump. It will be seen from Fig. 27 that pressure caused no flow of electrolyte into the internal reference electrode because it was all inside the system. The change in potential of the reference electrode itself with pressure is given by the Gibbs-Duhem equation (MacInnes 1939) :

$$\left(\frac{\partial E}{\partial P}\right)_T = -\frac{\Delta V}{zF}$$

where : $\left(\frac{\partial E}{\partial P}\right)_T$ = the change of reference voltage with pressure at constant temp.

ΔV = the volume change between products and reactants of the electrode process

z = valency, and F = Faraday.

Where both reactants and products are solids (Ag and AgCl) this turns out to be of the order of microvolts per atmosphere pressure and so may be neglected here.

Three membranes were tested. First cellophane was used, and the voltage change on application of up to 2 atmospheres of pressure was negligible (i.e. was less than 1 mV for a Donnan potential of 70 mV). However the ion exchange capacity of cellophane is small. Therefore both cation and anion exchange resin membranes on glass-cloth base were also tested. The resin membranes were Permutit Permaplex C-20 and A-20 respectively. Even with these, no significant steady voltage change with pressure was observed. A small transient voltage change was observed when pressure was released. This was probably caused by a change in electrical capacity of the system with displacement of the membrane relative to its support grid, remembering that this capacity was charged by the Donnan potential. There may be another unknown explanation however, since the same effect was observed with micropipettes.

It seems unlikely therefore on this evidence that streaming potential through the cell wall and membranes will alter the measured transmembrane potential significantly. This conclusion will be qualified on p.255, but it does seem to be in line with expectations based on the Ussing flux ratio solvent drag equation discussed on p.162 ff.

Of course, the objection can be raised that this model system is not a very close model to the living cell, and this must be recognised, but at present I do not see how to improve the model with a measurable system.

(b) Streaming Potential in Micropipettes.

The micropipette of the electrode presents a rather complicated situation. Near and in the tip, the lumen diameter is so small that the ζ -potential profiles of the opposing walls overlap. Here also is the region where most of the resistance to liquid flow will be located. A simple equation for streaming potential is not possible in a tube of varying bore, but an equation could perhaps be derived by integrating infinitesimally thin discs normal to the axis of the tube, if the form of the tube and the form of the ζ -potential profile were known. However a complete analytical solution of the problem would be tedious and of little practical value.

According to the treatment of Lens (1933) the streaming potential should increase as the tube becomes narrower, for a constant ζ -potential, although McInnes (1939, p.440) presents data of White, Urban and van Atta (1932) which suggests the opposite. Clearly we have here an embarrassing discrepancy! Unfortunately the standard analysis of streaming potential assumes (p.58) that the ζ -potential profiles of opposite walls of the tube do not overlap. When they do, the formula of Helmholtz and Smoluchowski quoted in Table I cannot be expected to apply. With Lens, I would expect a

higher streaming potential when ζ -potential profiles overlap because there will be charge transfer with flow right across the bore instead of only at the edges, although even here there will be more transfer at the edges than in the centre due to the close proximity of the wall charge at the edges. Therefore a circulation current will still flow down the centre of the bore. It is clearly a very complicated situation, and would require a full study in itself to arrive at satisfactory theoretical understanding.

Experimental tests on glass micropipettes was carried out in the arrangement shown in Fig. 28. It was found that the streaming potential produced by forcing various dilute solutions up the micropipette at a pressure of 1 atmosphere (76 cm. Hg) could be sometimes as high as 30 mV. There was considerable difficulty in obtaining consistent results. Eventually it was thought that the difficulty was due to blockage of the tip perhaps by specks of dust, unseen to the naked eye, in the solution. Of course, it is impossible to observe, let alone measure the rate of flow of solution up the micropipette by any simple method. It was remarked on p.244 that it was possible sometimes to see the inflow of cell sap, but this was because of very fine visible inclusions from the cell in the sap. No such aid was present with pure aqueous solutions, and it would be hazardous to introduce particles deliberately in case they cause blockage. Table 23 shows the kind of results obtained with aqueous

Fig.28

ARRANGEMENT TO INVESTIGATE STREAMING POTENTIALS IN MICROELECTRODES.

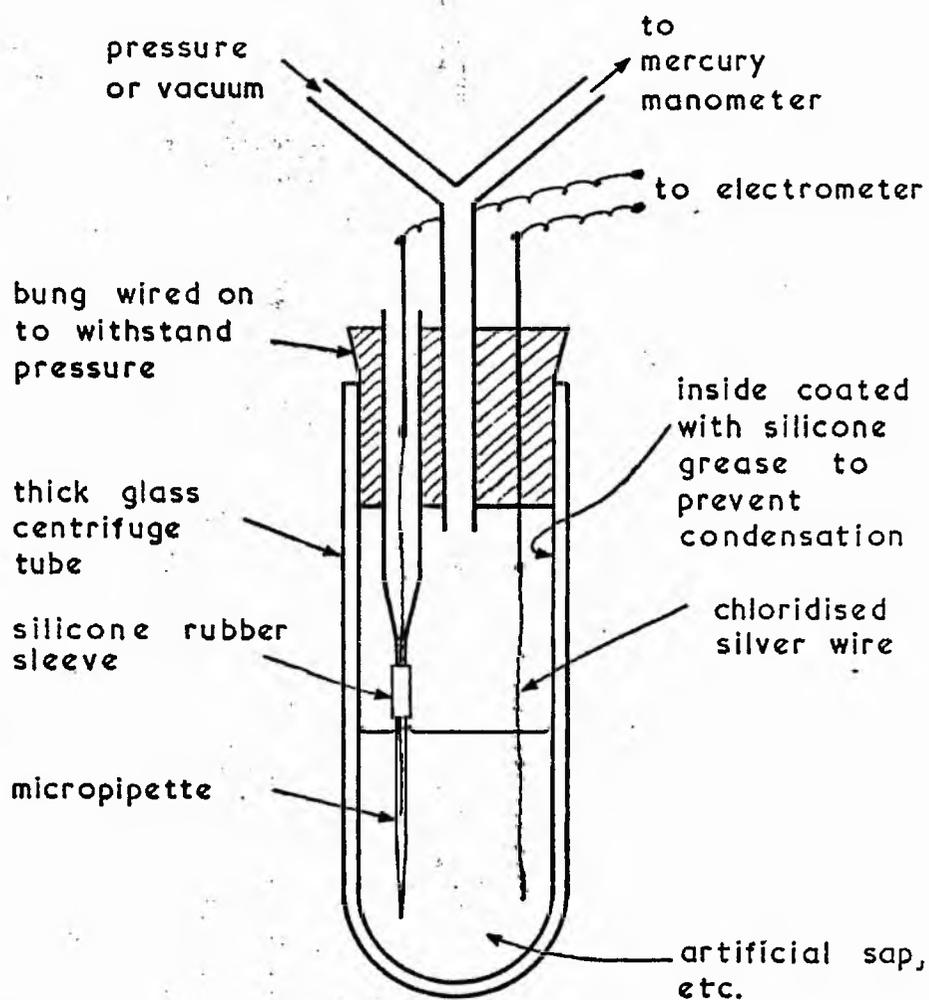


Table 23. STREAMING POTENTIALS IN MICROPIPETTES.

Micropipette number	External solution	Pressure (+) Suction (-) applied to external solution	Voltage shift mV
1.	"Artificial cell sap" without colloid 30 mM NaCl, 10 mM KCl	+	-2
2. (new)	"	-	+8
		+	-4
3. (3 weeks old)	"	+	-4
		-	+3
4. (new)	"	+	-9
		-	+8
		-	+16
5. (new)	"	-	+10
		+	-3
"	3 mM NaCl, 1 mM KCl	-	+27
		+	-5
"	100 mM KCl	+	-6
		-	+20
"	10 mM KCl	+	-1
		-	+3 ?
"	return to 100 mM KCl	+	-5
		-	+20
"	10 mM KCl	+	-20
		-	+15
"	1 mM KCl	+	-6
		-	+8 ?
"	1 M KCl	+	0
		-	0

? = unreliable.

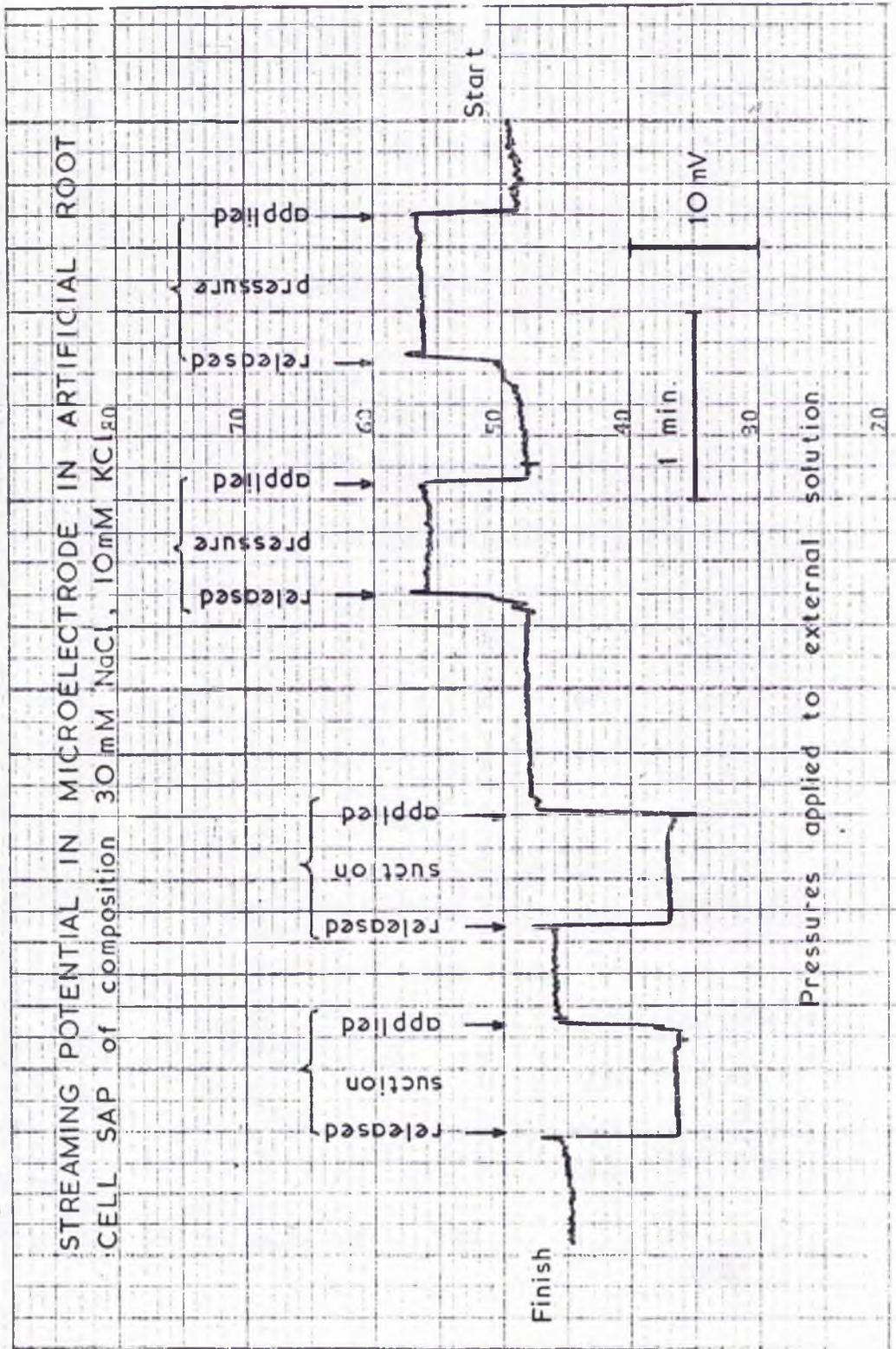
Pressure applied was 1 atmosphere (76 cm. Hg)
Suction applied was somewhat less than 1 atmosphere,
(about 66 cm. Hg).

electrolyte solutions, an "artificial cell sap", without or with added colloid. "Artificial cell sap" was based on analyses of root tissue corrected for free space to give intracellular values. Pressure of 1 atmosphere (76 cm. Hg, measured on a manometer), directed into or out of the tip, was applied either with a plastic 50 ml syringe, or by suction from a Geissler filter pump. Care was necessary to avoid boiling under reduced pressure. The d.c. resistance of the micropipette was measured before and after application of pressure, but was usually unaltered. Typical values lay between 7 and 11 M Ω . From this, one may reasonably conclude that the pressure regime did not damage the micropipettes significantly.

It would not be expected that pressure or suction would produce the same potential shift for a given pressure, because in one case external solution is forced into the micropipette and in the other the 3 M KCl solution filling the micropipette is sucked out. On this basis it might be expected that the voltage shift would be independent of external solution when the micropipette filling is sucked out. However as Table shows for micropipette 5, there is considerable influence of external solution on these readings. A typical chart record is illustrated in Fig. 29.

The sense of the voltage shift is remarkable. One would expect a shift to the positive recorded

Fig. 29.



on the microelectrode when an electrolyte solution is forced into the tip of the micropipette, if the walls were negatively charged. However the reverse occurred. (Negative moved the pen upwards on the chart as convenient for intracellular recordings). It is hard to understand how glass could be positively charged on its surface. This and the observations in the previous paragraph indicate that the behaviour of micropipettes is much more complex than expected by simple theory. However, it was not necessary to investigate these effects further for the present purpose, as will become clear shortly.

Although, during the impalement of a living cell, the micropipette tip presumably often comes to rest in the vacuole, which would not be expected to contain large molecular weight colloids, but may contain organic acids, the micropipette must pass through the cytoplasm to reach the vacuole, during which the turgor pressure may be expected to drive a plug of cytoplasm into the micropipette tip. Therefore a test was performed, again with the arrangement shown in Fig. 28, with a solution containing 0.3 % sodium alginate in 30 mM NaCl plus 10 mM KCl solution. The alginate contributed a further 6 mM Na⁺ ions. This solution was intended to imitate a little more closely the flow properties of cell cytoplasm. It was found that there was no shift in potential with pressure or suction when alginate was in the external solution. However, d.c. resistance

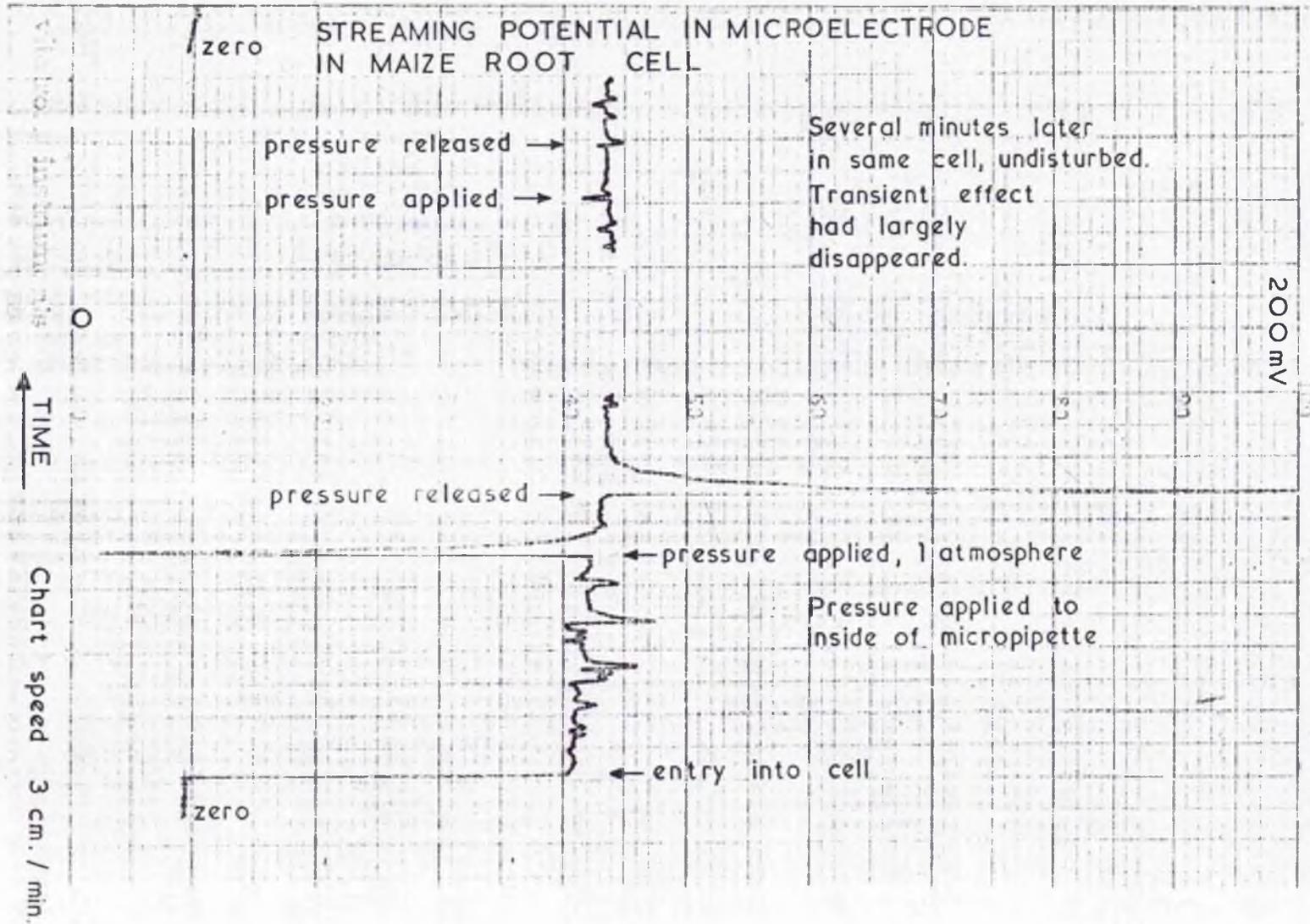
measurements of the micropipette before and after application of pressure were the same and typically between 7 and 11 M Ω as with aqueous media containing no alginate. It was also found that the resistance of a micropipette which had previously shown streaming potential in colloid-free solution was unchanged in most cases even after it had failed to give streaming potentials. Evidently, if blockage with a dust speck was the cause of such failure, the speck did not prevent ionic movements, even if it prevented bulk flow of solution. This finding throws doubt on the validity of the findings on artificial resin membranes described on p.248, as models of the living cell membranes.

Since the presence of alginate suppressed the streaming potential in this model system, it seemed possible that the fear that large streaming potentials may interfere with transmembrane potential measurements on turgid cells might not be relevant after all. This was thought possible because the cytoplasm would probably block the tip anyway. To test this, an arrangement was set up so that pressure up to 1 atmosphere could be applied to the micropipette during potential measurements on living cells. A glass T-piece was connected to the back end of the micropipette, after the latter had been mounted in the micromanipulator chuck (which was specially bored straight through). Pressure was applied to the side arm of the T-piece, and the third arm carried the silver wire electrode (a long piece) sealed in with Araldite epoxy resin.

It was found that pressure up to 1 atmosphere produced no shift in potential in any measurement made on maize root tissue. Occasionally a large shift occurred on application or release of pressure, but this always decayed back to the normal value if pressure was held steady. Fig.30 illustrates such a recording. (Noise on the recording is due to the way pressure was applied and is irrelevant). The pressure of 1 atmosphere should be approximately equal to the turgor pressure in root cells, because the osmotic pressure of cell sap, on the basis of salt analyses should be about 1.8 atmospheres, and that of the external nutrient medium 0.7 atmosphere, under the conditions of culture prevailing. Even if this pressure was not equal to the turgor pressure, its application should produce a shift of potential if a streaming potential was occurring before its application.

One is therefore reassured that streaming potential in micropipettes will not, in most cases, apparently be an artefact in transmembrane potential measurements. However, it would probably be unwise to assume that this would always be so when one begins to work with a new plant tissue. It is not however entirely practical to test routinely for streaming potential because it was found that application of pressure often lead to the onset of very high resistance of the microelectrode some seconds after its withdrawal from the tissue. This is a most peculiar effect, and never appeared while the micropipette was still

Fig. 30



implanted in a cell. However a micropipette would often recover to normal behaviour on implantation in another cell. Perhaps one must therefore revise the conclusion on p.253, that pressure does not damage micropipettes. The conclusion is probably only valid if the resistance remains stable at all stages.

5. Leak between Microelectrode and Cell Membrane.

Let us now return to consider the effect of a rent in the wall or membrane(s) of a cell on the transmembrane potential measured. It is usually assumed that the fatty cell membrane seals against the outside of the micropipette when it is inserted into a cell, so that the only communication of the cell with the outside is through the lumen of the micropipette. Walker (1955) had shown photographs of cytoplasm growing round and eventually over the end of micropipettes inserted into Nitella sp. cells, so that presumably the plasmalemma was here in contact with the micropipette shank. However it is not easy to see in the case of smaller cells and finer micropipette tips. A sharp increase in resistance would be expected if the cytoplasm grew up around the micropipette tip and plasmalemma formed across the end and blocked it. However a sharp resistance change after prolonged impalement of a cell is not observed in all plants, casting doubt as to whether sealing of the plasmalemma to the micropipette is universal. It is therefore of interest to speculate what effect might be expected

on observed potential of a cell whose contents communicate with bathing medium via a rent as well as with the micropipette contents.

It was shown in the previous discussion of streaming potential, that the mere removal of the turgor pressure will not, of itself, significantly alter the Donnan potential of a Donnan system. What determine the potential are the ionic contents of the system. For instance, in the case of the Donnan system formed between captive sodium alginate and dilute salt solution, it is the captive alginate ions which are responsible for the potential. If however the restraint on their diffusion is removed, for example by rupturing the dialysis bag, the potential will not immediately disappear, but it will eventually decay as the alginate ions become dispersed by both diffusion and osmotic dilution. In this case the time constant will be fairly long.

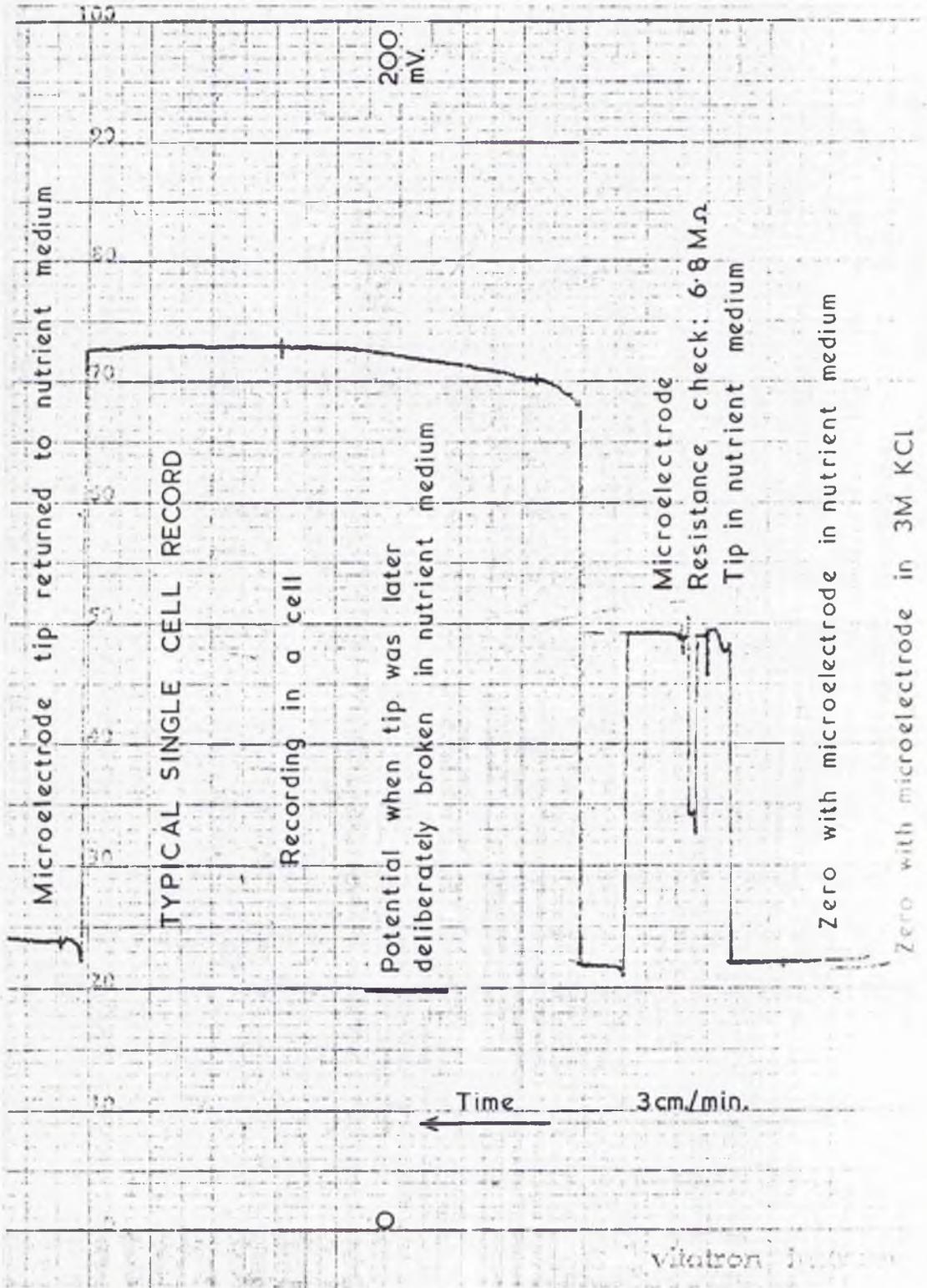
Obviously a living cell is not as simple as a Donnan system, but direct contact of the cytoplasm, through a rent in the membrane, if extensive enough (see below), would shift the potential recorded by an intracellular electrode from the true E_m to a value in the short term corresponding to a combination of a Donnan potential caused by protein, etc., in the cytoplasm and a liquid junction potential between the mineral ions in the cytoplasm and in the bathing medium. This potential would be probably close to the Nernst

potential for the most abundant ion in the system, but clearly the situation is complex. How far the potential recorded would be shifted from E_m towards this cytoplasm/bathing medium junction potential would depend on the relative conductances between cytoplasm and bathing medium of the rent and the total cell membrane, as a weighted mean.

Fortunately, it would appear from chart records that the cell membrane does, in most cases, seal against the micropipette, eventually if not immediately. When the micropipette is inserted into a cell and left to settle, after the initial sharp rise of potential on entry, the potential rises slowly in some cases to a value a few millivolts above the initial value and then it remains steady for long periods. This may be seen in Fig. 31, which is typical of such records. The slowly rising phase may perhaps be due to the opposition of streaming potential in the micropipette to the transmembrane potential, as the turgor pressure forces cell sap up the micropipette until sap viscosity prevents further flow, but one would expect any such blockage to be immediate. The slowly rising phase may last as long as 1 - 2 minutes. However it is much more likely that this slow rise is due to progressive sealing after insertion of the micropipette tip and/or recovery of the cell after the withdrawal of charge to charge up the electrometer.

As an attempt at quantifying this possible effect of charge demand on a cell we may proceed as follows.

Fig. 31



The electrometer plus connecting lead (coaxial with capacity to screening sheath of about 30 pF/foot) present suddenly a capacity of the order of 100-200 pF to the cell. (Special potential-follower guard screen techniques can be employed to offset much of this capacity but it was not applied to these d.c. potential measurements. The Vibron electrometer does in fact provide a guard screen, but there is a snag with it. It is driven by a point in the circuit of the instrument at which there is an appreciable delay between screen response and input. Thus, when the input potential changes rapidly, as on penetration of the cell membrane, the charge is drawn before it is given back. It is hard to predict the effect of such a sequence on the cell. We therefore proceed assuming the guard screen is not in use, and the full input and lead capacity is to be charged.) The cell membrane itself, for a maize root cell, say, 100 μm long by 15 μm diameter, at 1 $\mu\text{F}/\text{cm}^2$ (see Chapter 3), will carry about 50 pF. Therefore clearly the immediate effect of connection to the electrometer would be partial discharge of the membrane to between $\frac{1}{3}$ and $\frac{1}{5}$ of E_m , before any ions move. However the electrostatic polarisation of membrane lipids is in any case only a secondary result of ion movements. To charge up the electrometer to the extent of say 100 mV would require of the order of 10^{-11} coulomb, which is equivalent to 10^{-16} mole of monovalent ion. In our example cell of volume 1.8×10^{-8} cm^3 , this corresponds to a concentration of 5 μM . Now actual concentrations of the dominant permeant monovalent mineral ions are at least 1000 x greater

than this, so we may safely conclude that the ion reserve in the cell will charge up the electrometer very quickly to the Goldman potential of the ion concentrations prevailing at the time of impalement. However, as explained in Chapter 1 (p. 177), the steady state potential is not identical to the Goldman potential when there is an amperogenic pump in the membrane.

Would it therefore be fair to speculate that the slow rise phase often seen after the impalement of a maize root cell is evidence of an amperogenic pump, or must one fall back on the less informative explanation that it is due to sealing of the micropipette to the plasmalemma? If it always happened one might feel more confidence in ascribing it to an amperogenic pump, but in fact there is no way of deciding which is the true explanation of the effect, except perhaps by monitoring membrane resistance during this phase. However this has not been attempted and would be difficult to do technically in the time available (2 minutes).

6. Contamination of Cell Contents.

Yet another possible source of artefact in E_m measurement is the contamination of cell contents with potassium chloride from the micropipette.

In fact this danger is almost certainly not relevant because the turgor pressure of the cell forces back the filling solution.

7. Mains hum may cause artefacts and damage membranes, see p.29

SUMMARY OF FINDINGS
AND TYPICAL RESULTS.

First, the existing techniques for the insertion of microelectrodes into plant cells were applied. These were found to be inconvenient and unsatisfactory in certain respects, and so were subjected to a detailed examination. It was found that existing micro-manipulators were subject to lack of adequate lateral rigidity, and also backlash and judder in the advance. Also the frame system left much to be desired, when a conventional microscope was placed beside a conventional micromanipulator. Accordingly a hydraulic micro-manipulator of novel design using metal bellows as the extensible elements was constructed. The concept was introduced of integral construction of microscope and manipulator on one frame, and a prototype and later an improved universal modular design were constructed.

Next, the preparation and filling of micropipettes was examined. A direct filling method was devised, which considerably improved the reproducibility and speed of preparation of usable micropipettes. Direct filling with 3 M KCl was preferred to an exchange technique because one could be sure, with a micropipette not blocked by a bubble, that it would be filled completely with 3 M KCl, and therefore would have a more consistent resistance (see also Chapter 3).

Finally, possible sources of artefact in the measurement of cell membrane potential were examined.

The following conclusions were reached :

(1) Liquid junction potential between micropipette filling solution and intracellular fluid would generally be avoided if the micropipette were filled with 3 M KCl, and it were inserted into a turgid cell.

(2) Tip potential was evidently a hazardous source of artefact, and could only be avoided if the 3 M KCl micropipette filling were driven back up the micropipette to a point where the micropipette internal diameter exceeded the effective depth of the ζ -potential profiles of the glass. This requires the cell contents to be under positive turgor pressure, a situation which does apply in the case of maize root cells. Occasionally, blockage of the micropipette tip may be expected by particles in the cell sap before enough flow of sap has occurred into the micropipette to push back the filling solution until liquid junction forms out of the "tip" region, but it is expected that such occasions may be spotted in a series of impalements, and rejected. The sense of the tip potential is normally such as to be additive with cell membrane potential, so that E_m would be over-estimated. Unfortunately this makes it more difficult than if tip potential had been in opposition to E_m since imperfect sealing on impalement would downgrade the observed potential. There will thus be a scatter of poor results above and below the true value for different reasons.

Since tip potential is large between a micropipette filled with very concentrated KCl and the dilute

"freshwater" nutrient medium, it was a matter of concern as to what should be taken as "zero" outside the cell. An examination was therefore conducted to find the best way of determining the true zero.

A micropipette filled with 3 M KCl in 1 M KCl showed virtually no tip potential, as expected, but it would be inconvenient to refer the tip to 1 M KCl before, between, and after cell impalements. An alternative technique, which was compared with use of 1 M KCl outside, was found adequate, namely to break the last mm. or so off the end of the micropipette after a series of cell impalements was completed, and to refer the potential of the broken micropipette to bathing medium as "zero". This technique greatly reduced the statistical scatter of readings taken from cells.

(3) A shift of zero was noted when a micropipette was driven right through a root and out the other side. This was tentatively explained as a pH response of the glass by H^+ ion permeation.

(4) Streaming potential could be an artefact in two ways; either (a) through osmotic uptake by the cell through the cell membrane itself after loss of turgor, an effect which was examined as far as possible by a model system and found to be probably negligible, or (b) through flow of cell sap into the micropipette. This was investigated directly on micropipettes (i) in a model system with "artificial cell saps" and (ii) in living cells by application of back pressure

in the micropipette itself. Although "artificial cell sap" could produce appreciable streaming potentials in the model system when colloid was absent, no streaming potential was detected either in the model system in the presence of colloid or in maize root cells. It was therefore concluded that provided cells are under turgor pressure, streaming potential may well not be a problem.

5. Rents in the membrane, or imperfect sealing of the micropipette to the cell membrane were considered. A rationale for evaluating these were presented. Chart records provided evidence that sealing did usually occur and that d.c. resistance measurements were useful in evaluating this. It was also concluded that the ion reserve of a cell was generally so large that instrumental charge demand disturbed the cell very little.

6. Cell contamination by contents of the micropipette was considered not to be a probable hazard when cells were under positive turgor pressure.

Typical Results.

Although it is not the primary purpose of this dissertation to present measurement results, but rather to show how such measurements may be made, as far as possible without artefacts, it is of interest to present the following results, which will not however be discussed. A further study would be

necessary for this, and it therefore lies outside the scope of this dissertation.

Cell membrane potentials measured after application of the techniques described above for the avoidance of artefacts, on maize roots cultured as described in Appendix 5, were between -90 and -100 mV on average for cells in the region of root hairs. It is not useful to present results more accurately than this unless they are discussed in the context of specific measurements of internal and external ion concentrations, but in general it was found that, for any given root the highest membrane potentials were recorded in the region immediately behind the cap to about 2 or 3 mm. up the root. There was then a decline of about 15 or 21 mV as one proceeded into the region of root hairs 9 mm. up the root. Cells, probably dead, at the extreme tip registered about -55 mV, but micropipettes were frequently blocked here by viscid material.

It is pertinent here to speculate about the cause of this decline in E_m between the young cells near the tip and the mature cells of the region of root hairs. One cause could be the decreasing vigour of the cells, but it is also possible that what is happening is that the micropipette tip invariably comes to rest in the vacuole where this is large, as in the region of root hairs, whereas in the tip region, it may well be that the micropipette tip sometimes comes to rest in the cytoplasm and at others in the very small vacuoles of

the young cells. This would point to a tonoplast potential positive inside with respect to cytoplasm of about 15 or 20 mV. One would expect the scatter of results to be worse, and perhaps to divide into two populations, in the transition region than in either the tip or mature root hairs regions, but this has not been critically examined. It is hard to obtain unequivocal evidence as to where the micropipette tip comes to rest, and much further work is needed on this. The work of Spanswick (1972), in which he made deductions based on resistance measurements in cells and cell-cell coupling may well point the way to a resolution of this problem, see p.351.

CHAPTER 3.

EXPERIMENTAL DETERMINATION OF CELL MEMBRANE RESISTANCE AND CAPACITY.

This chapter presents a review of established techniques followed by the presentation of a new technique, which was tested experimentally and justified theoretically. Interpretation of results obtained with this system was examined in terms of fundamental processes, cable theory and the symplast.

Introduction.

In this chapter, the following symbols will be used :

R_m = cell membrane resistance of unit area.

C_m = cell membrane capacity per unit area.

The subjects of this chapter cover an enormous field, and could easily in themselves fill a further complete dissertation and more. The topics which are involved in this field are the following :

- (1) Techniques for the measurement of cell membrane resistance.
- (2) Techniques for the measurement of cell membrane capacity. Often (1) and (2) may be measured on the same setup, but not always.
- (3) Measurement of relevant cell parameters, especially surface area to convert resistance and capacity as measured to area-specific values. Problems of cell geometry and surface uniformity enter here, especially with cells in tissues.
- (4) "Kelvin cable" properties of elongated cells and processes extending from cells (cilia, flagella, root hairs, axons, etc.). Interfering effects of internal structures in cells.
- (5) Cell-cell coupling. The symplast or syncytium. Plasmodesmata and nexal regions of so-called "gap junctions".
- (6) Incremental resistance, chord resistance and rectifying properties of cell membranes.
- (7) Theories to account for the measurable quantities in terms of fundamental electrochemical processes.
- (8) Extent to which the Nernst-Einstein relation between fluxes and conductances holds good. Explanations proffered for its breakdown.

Clearly in the remaining space in this dissertation I can do no more than concentrate on a restricted part of this range. The choice falls upon methods of measurement of R_m and C_m because I have made a careful study of existing techniques, and have also introduced a new one, for reasons which will be explained. Preliminary results on maize root cells will be presented and briefly discussed. Exactly what is measured by the technique, and what this means in terms of fundamental processes will be examined. I shall also discuss the theory of cell-cell coupling and present some experimental data on a model system which has enabled me to extend the theoretical framework currently available. In the course of this I shall inevitably touch upon all of the topics listed above, but I make no apology for not making an exhaustive examination in all cases.

Measurement of Membrane Resistance. Tip Resistance.

Already in Chapter 2 (p.237), mention has been made of the technique for d.c. resistance measurement, the purpose there being primarily as a means of testing micropipettes for usability.

However, when this technique is applied to a micropipette inserted into a cell, only the sum of cell membrane and microelectrode resistances together can be measured. It is not possible to calculate the membrane resistance simply by subtracting from

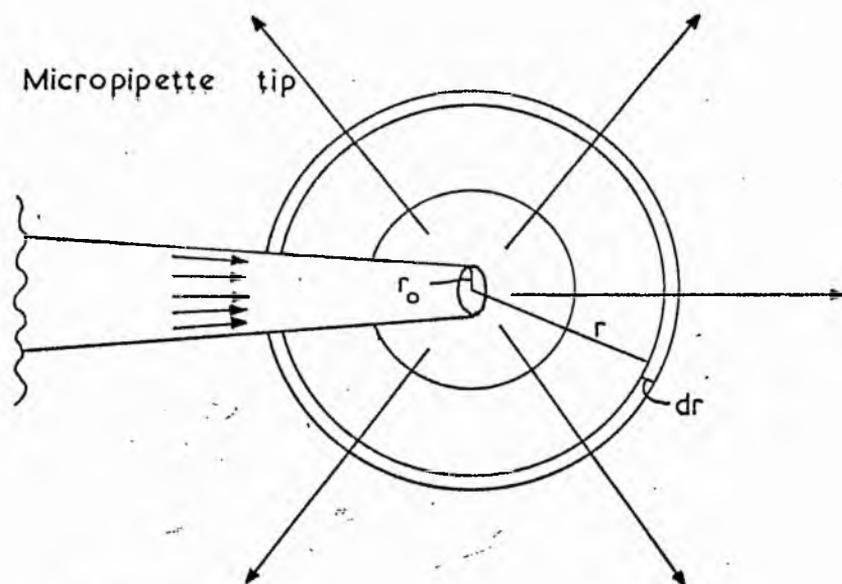
the resistance measured inside a cell, the resistance of the microelectrode measured with its tip in bathing medium. The total measured resistance when the microelectrode is implanted in the cell is usually lower than when it is in the "freshwater" bathing medium, in the case of maize. This is because the resistance of the microelectrode itself varies according to the environment of its tip. The resistance of the solution in the lumen of the micropipette will also change when cell contents enter it under turgor pressure.

However, let us concentrate for a while on the variation of tip resistance of the micropipette with tip environment. Let us consider current to flow along the lumen of the micropipette and then to emerge into the bulk of solution surrounding the tip. Unless the reference electrode is very close indeed, it is a fair approximation to regard the lines of current as spreading out radially from the tip (Fig. 32). The solution outside the tip will have a resistivity different from, usually higher than, that of the filling solution. Some contamination of the external solution may occur due to diffusion of filling solution into it, but this does not affect the argument below, since it is simply equivalent to an enlargement of tip radius.

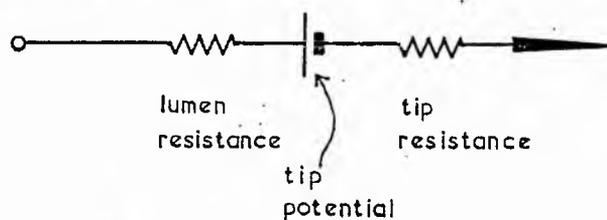
Since the total current flow through each shell of thickness dr is equal to that through every other,

Fig. 32

MICROPIPETTE TIP RESISTANCE



Equivalent circuit



The tip resistance,

$$R_t = \int_{r_0}^{\infty} \frac{\rho dr}{4\pi r^2} = \frac{\rho}{4\pi r_0}$$

where: ρ = resistivity of external solution, ohm.cm. R = total tip resistance excluding lumen. r_0 = effective radius of tip.

the resistance contributions of each shell may be integrated between r_0 , the radius of the micropipette tip and infinity, as shown on Fig. 32. (There will be a small error because the tip has been assumed to be acting as a spherical conducting knob of radius r_0 attached to a negligibly thin insulated conductor. The solid angle obscured by the pipette shank however will only be a small fraction of 4π .) This approach is essentially similar to that presented and examined experimentally by Krischer (1969a,b, & with Deichmann 1970). It is thus apparent that the tip resistance depends both on the external solution resistivity and on the tip diameter. This effect is shown experimentally in Fig. 26 (triangles) presented in Chapter 2. In 3 M KCl, a typical micropipette measures about 1 or 2 M Ω . This resistance cannot be less than the lumen resistance, and therefore the resistance of about 12 M Ω in 0.001 M KCl for this specimen, must be mostly due to tip resistance. If we take 10 M Ω as the tip resistance in 0.001 M KCl, then the expected tip diameter, using the resistivity of 0.001 M KCl, which is 6830 Ω .cm. (derived from data in Kaye and Laby 1956), is 1.0 μ m.

Tips were examined by electron microscope. The whole, unfilled micropipette was introduced directly into the AEI EM6B electron microscope at the Gatty Marine Laboratory, and was carried in a special specimen holder which would work in the vacuum lock system. The carrier was machined by myself.

This examination showed that the electrode puller (p.206) produced remarkably consistent results. The diameters of the tips were all in the range 0.15 to 0.2 μm . This is smaller than expected on the basis of resistance measurements, but diffusion of KCl from the tip could perhaps explain the discrepancy, since this would effectively make the zone of highly conducting electrolyte around the tip larger than the tip itself.

The d.c. resistance data obtained on cell insertions and withdrawals may now be used to derive a very approximate value for cell membrane resistance, as follows. Let us suppose for the purposes of discussion that the microelectrode resistance fell to half the value it had in the bathing medium (b.m.) when it was inserted into a cell. Resistance values obtained on one cell and micropipette were :

In b.m. 23 $\text{M}\Omega$, in cell 20 $\text{M}\Omega$ and again in b.m. 24.5 $\text{M}\Omega$.
Another cell and micropipette gave :

In b.m. 15 $\text{M}\Omega$, in cell 10 $\text{M}\Omega$ and again in b.m. 13.5 $\text{M}\Omega$.

These measurements suggest that the resistance for cells of maize roots in standard nutrient bathing medium was between 3 and 5 $\text{M}\Omega$. If a maize root cell is taken as a cylinder 200 μm long by 20 μm diameter, it will have a surface area of approximately $1.3 \times 10^{-4} \text{cm}^2$. This suggests a surface resistivity of the cell between 377 and 628 $\Omega \cdot \text{cm}^2$. This value is at least an order of magnitude lower than the value calculated by Walker (1960) for Nitella and Chara cells, but of

course we do have a different species here (the Characeae do seem to have high membrane resistances), and the cells would have to be 3 to 10 times larger in linear dimensions to account for the discrepancy. Even if the microelectrode resistance fell to zero inside the cell (impossible), it would still not account for so large a difference. It must therefore be assumed that at least an order of magnitude has been correctly derived here. Nevertheless, a method which requires the assumption of so many unknowns is clearly inadequate. It is very difficult to decide what the microelectrode resistance would be inside the cell because of the uncertainties about the cell contents, and changes in the micropipette itself on insertion, such as tip blockage. Let us now see what established techniques are available.

Review of Established Techniques.

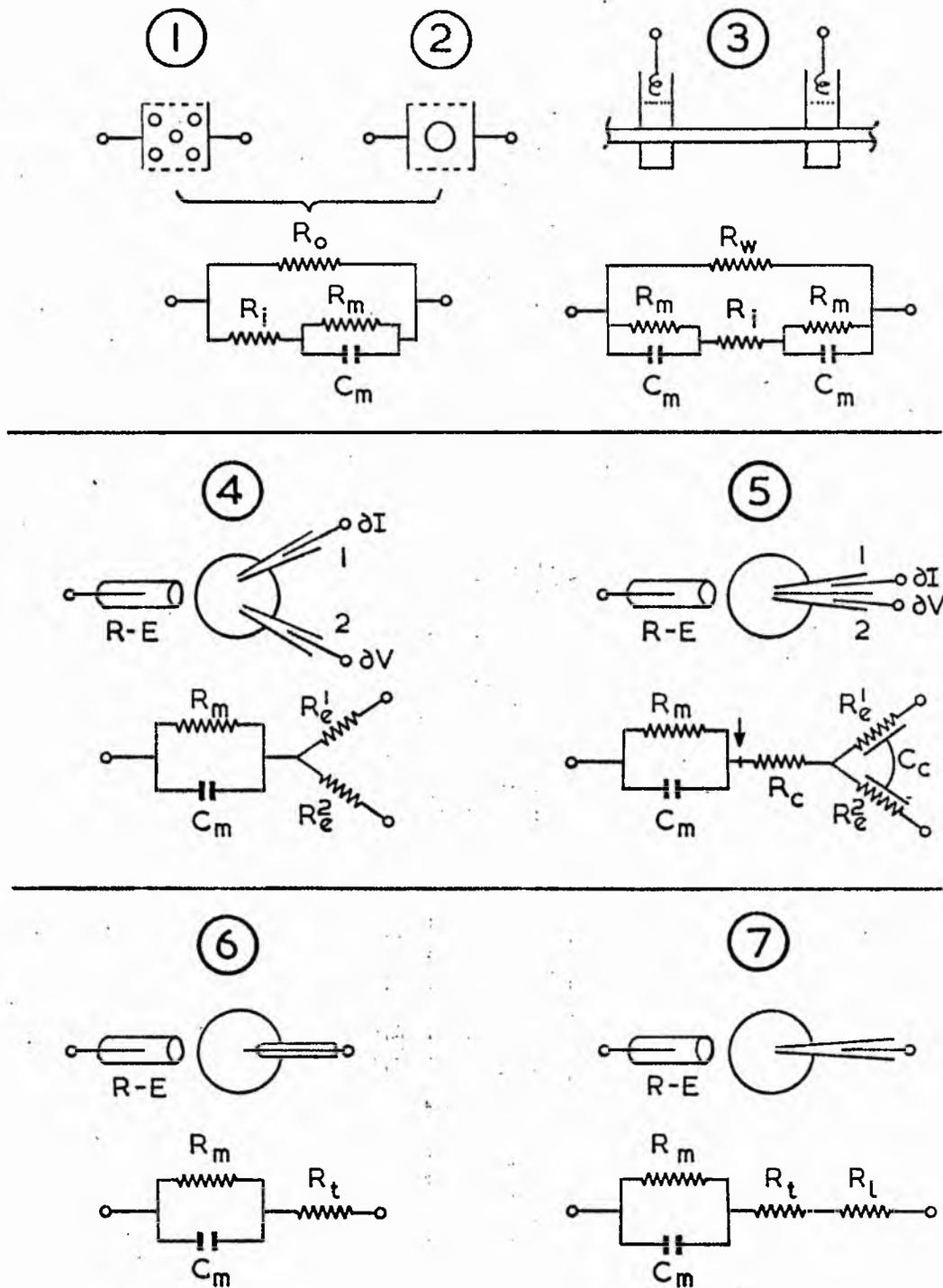
Measurement of the electrical impedance of living material attracted attention as early as 1897-9 with the measurements on blood by Stewart (1897), Bugarsky and Tangl (1898) and Stewart (1899). It was early realised that there was a reactive component in the impedance measured, and that the reactance was of the capacitive type. With the advent of modern concepts of cell boundaries, it was natural to equate the capacitive component with lipoid material in the cell membrane. This concept was well established by the 1920's when there was a considerable revival of interest in impedance measurements in biology.

Sample references only will be quoted below and Cole (1972) reviews some of them.

A number of methods have been used to determine the impedance of cells and tissues, and these are summarised in Fig. 33. The earliest measurements were performed in a device similar to the traditional Kohlrausch electrolytic conductivity cell (Philipsson 1921 and McClendon 1929). Either a suspension of cells (Fricke 1925 a,b, McClendon 1929, Cole and Cole 1936, Hope 1956) or single cells (Curtis and Cole 1937, Lord Rothschild 1946) were measured between platinum or gold electrodes as depicted in (1) and (2) of Fig. 33. The theory of impedance of cell suspensions was worked out very fully by Fricke 1924 a,b, 1925 c) following Maxwell (1873) from which it should have been possible to obtain the specific resistance and capacity of the cell membrane and the resistivity of the cell sap. While the values measured for membrane capacity and cell sap resistivity accorded well with estimates by other methods, membrane resistances were often wildly incorrect. It was originally suggested that this may have been due to leakage of electric current between cells, as represented by R_0 in Fig. 33 (1) and (2), although damage to the membrane in preparation had also been suspected. A further complication, which has been discussed by Lord Rothschild (1946), Schwan (1963) and others, was that the capacitive element was found to behave as what was called a "polarisation

Fig.33.

METHODS USED IN THE MEASUREMENT OF CELL MEMBRANE RESISTANCE AND CAPACITY.



capacity". In such a case the capacity and the resistance across it vary with frequency in such a way that the phase angle, ϕ , between current and voltage remains constant. This phenomenon seems to be associated both with the electrodes and with the living membranes themselves (McClendon 1929, Rothschild 1946, Hope 1956 and Cole 1972). This therefore throws some doubt upon the confidence to be placed on measurements of membrane capacity unless this has been examined. Phase angle however has been used, at 2 kHz, as the basis of an ingenious instrument designed for the assessment of fish freshness in markets and results correlated well with subjective scores assigned by trained panels (Jason and Richards 1975). Evidently in dead tissue, although R_m and C_m vary in an unpredictable way, individually, the power factor $R.C.\omega$ (where R and C are values measured outside the cells by electrodes placed on the surface of the fish, and $\omega = 2\pi f$) and therefore the phase angle do vary in a predictable way for any particular species as the dead tissue breaks down. This method must essentially be regarded as a low frequency technique (see below) in which ionic membrane conductance is more relevant than dielectric dispersion loss "conductance" in the membrane lipids.

Certainly some of the polarisation capacity observed in early experiments was due to the electrodes, and could have been separated or avoided by techniques described by Schwan (1963). Polarisation impedance

arises at polarisable electrodes, such as noble-metal electrodes, because of the existence of an electrical Helmholtz double-layer and diffusion rate-limiting processes close to the metal surface.

It is also significant that most of the workers who used the cell suspension technique used frequencies of 1,000 Hz or above (McClendon 1929, Hope 1956). Perhaps it is not surprising in view of our present knowledge of cell membrane resistance and capacity, that they failed to derive sensible values for membrane resistance since membrane reactance equals membrane resistance at quite low frequencies for most cells. Cole (1972) discusses in his book at considerable length this question of phase angle, and shows how he and his colleagues generally presented their data as an impedance locus in the plane of reactance and resistance, whereby a constant phase angle of the capacitive element less than 90° manifests itself by the depression of the semi-circular impedance locus such that its centre is below the resistance axis. The effect appears to be due to a lossy element in the membrane capacity which Cole and his colleagues eventually attributed to something like liquid properties of the membrane lipids. This conclusion has, of course, since been effectively confirmed by experiments using spin labels in NMR studies on lipids in membranes (Kornberg & McConnell 1971, Brûlet & McConnell 1975). The high frequencies used in studies of Cole and others on cell suspensions will reveal this behaviour of

membrane lipids, but it appears that, as Cole admits, the suspension technique does not work well at lower frequencies, where the membrane capacitive reactance rises to a value comparable with the reciprocal of ionic transmembrane conductance. Nevertheless, by application of a more complete theory, using "pedal curves" for the membrane impedance locus, including almost incredibly an inductive* component, Cole and Marmont (1942) were able to extend their measurements down to lower frequencies when, for squid axon, I calculate from their data a zero frequency membrane resistance, R_m , of $380 \Omega \cdot \text{cm}^2$ and capacity, C_m , of $1 \mu\text{F}/\text{cm}^2$.

* By a strange coincidence I have direct personal experience of the probable explanation, as given by Cole (1972), of the apparently absurd membrane inductance from a quite unconnected problem. Once I had occasion to design a circuit for a controller to work with a car alternator driven by a small petrol engine to provide power for small portable electric tools where mains was not available. When the tool was switched on, it presented a near dead-short (because the motor in the tool was stationary) which caused the field in the alternator to quench, and it would not build up again. The trouble was traced to the inability of the build up of field current to keep pace with the sudden demand. (This problem does not arise on a car, when for instance headlamps are switched on, because the field has supplementary supply from the battery). I therefore thought of using a small value inductance in the load line to limit the rate of increase of load current to a value which build-up in the alternator could follow. The inductor however had to be specially designed with an air core to maintain inductance at very high current which would saturate an iron core with consequent loss of inductance. This component, although completely effective, was a nuisance on account of its bulk, so I experimented with the idea of replacing it with a transistor/capacitor/resistor network which would also limit the rate of load current increase. The device obviously has parallels with an inductance in its behaviour, although not an electromagnetic device. It is interesting that this device manipulates conductance, and therefore parallels the behaviour of excitable cell membranes, in line with Cole's proposal as an explanation of the apparently absurd "inductance" in cell membranes.

Next came the method depicted in Fig. 33(3), whereby a long narrow cell, for example the coenocytic freshwater alga, Nitella flexilis was suspended between two saline/agar collars which acted as external electrical connections to the cell. The region between the collars was left in air saturated with water vapour (Blinks 1930). Large reversible silver/silver chloride electrodes were used, thus avoiding electrode polarisation, and direct current was used. The electrical circuit through the cell was through two membranes in series, along the central column of vacuolar sap, and out again through two membranes. Actually four membranes were involved because the main current flow was along the vacuole, thus current passed through both plasmalemma and tonoplast. Current flow would be negligible through the thin layer of cytoplasm, and the alternative pathway along the wall, represented by R_w was found to be insignificant. The value of $250 \text{ k}\Omega \cdot \text{cm}^2$ for membrane resistance measured by Blinks however seems remarkably high when compared to the value of $47.5 \text{ k}\Omega \cdot \text{cm}^2$ for the plasmalemma (tonoplast resistance was negligible) of Nitella translucens obtained by Walker and Hope (1969) by another method, Fig. 33(4). Hope and Walker (1961) suggested that the discrepancy was mostly due to the conditions in which the measurements were made and the results of Bennett and Rideal (1954) would seem to confirm this. Recently Skierczyńska et al. (1972) have combined methods (3) and (4) on Nitella and Chara cells and this promises to be a useful approach. However it is

rather difficult to understand the interpretation of their results in terms of the membranes known to exist in Characean cells.

Method (4) of Fig. 33, with two separate intracellular micropipette electrodes was used by Tyler et al. (1956) to determine the membrane potential and resistance of starfish eggs. This has become a very popular technique and has been applied to a wide variety of tissues, but it has the disadvantage that two microelectrodes must be inserted into a single cell. This is easy only where the cell is large, as in Nitella, nerve or muscle cells. The advantage electrically is that there is very little interference between one electrode and the other. The equivalent circuit in Fig. 33 (4) shows the electrode resistances as totals only because there is no complication from tip resistance, although still present, of course. It is possible to fix the current drawn down the current electrode by using a high voltage in series with a high resistance, e.g. 1,000 M Ω , constituting effectively a constant current source. Then, if the input impedance of the amplifier on the voltage sensing electrode is sufficiently high (at least 1,000 M Ω), then the microelectrode resistances are irrelevant, and a true record of voltage change within the cell is obtained for a known current. The electrodes will be far enough apart for valid measurements to be made from time-courses after the application or removal of a square pulse. Most workers appear to have used square pulses or direct current for twin microelectrode

measurements. However square pulses are somewhat inconvenient for impedance measurements, especially if the system under study contains more than one time-constant, because a square waveform may be Fourier-analysed into an infinite harmonic series of sine waves of increasing frequency and decreasing amplitude. It is thus difficult to use the fundamental frequency of the square waveform in a system involving bridge techniques. Even the analysis of rise and fall profiles is fraught with problems where multiple time-constants are involved, as is indeed sometimes the case with living cells. Nevertheless many valid measurements of membrane capacity have been made using square pulses.

Tasaki and Hagiwara (1957) and Falk and Fatt (1964) have used sinusoidal alternating current. One interesting use of sinusoidal a.c. was that devised by Norman (1972) in which he used two separate microelectrodes as in Fig. 33(4), but he measured phase angle. Phase angle measurement can be tedious by the traditional Lissajou figure method wherein X and Y deflections of the cathode ray oscilloscope (CRO) are driven by excitation and response signals respectively, both sinusoidal. Instead Norman drove the X deflection with a triangular wave which was synchronous and in phase with the sinusoidal excitation to the preparation down the current electrode. The response from the voltage electrode was fed to the Y deflection of the CRO. He showed that phase angle could be read directly as

the separation at any level in mm. of the two sides of the sigmoid loop traced on the CRO screen, provided the total width of the loop was adjusted to 90 mm. This approach has much merit when a phase sensitive detector is not available, although it does require a special signal generator providing both sine and triangular wave outputs, and it can only be applied to a twin electrode method.

It is easier to insert double-barrelled micropipette electrodes into many of the smaller cells, such as those in tissues of higher plants or animals, and such a technique was devised by Coombs et al. (1955). It is represented in Fig. 33 (5). The difficulty with this technique, as Coombs et al. admit, is that there is a relatively large capacity, of the order of 20 pF (at least, in my experience), between the barrels of the micropipette due to the glass partition dividing them, so that current changes in one barrel will lead to voltage transients in the other, and the interference will be unacceptably large. Also the resistance of the fluid immediately surrounding the tips leads to an incorrect reading on the voltage-sensing electrode. Thus, current drawn by the current-inserting electrode flows through the electrolyte solution in this region close to both tips, producing a voltage drop which will be sensed by the voltage electrode. Resistance at the tip of a micropipette was discussed above, p.271, and in double-barrelled micropipettes these tip resistances will show themselves as a mutual or coupling resistance, whereby changes in

current in one electrode cause changes in voltage in the other. However, not the whole of the tip resistance of each barrel will necessarily contribute to this coupling resistance, since this will also depend on the proximity of the two openings. Low coupling resistance will occur if one barrel broke off a micron or so behind the other during pulling, and therefore coupling resistance is very variable between individual double-barrelled micropipettes. Coupling resistance thus becomes an embarrassment which cannot entirely be allowed for. The best that can be done is to select micropipettes with low coupling resistance when immersed initially in the external bathing medium, and hope that insertion into the cell does not alter it.

The high capacity between the barrels of double-barrelled electrodes rules out their use for the study of cell membrane capacity.

Single metal electrodes have been used intracellularly, as in Fig. 33 (6). Bennett and Rideal (1954) inserted a glass micropipette into the vacuole of Nitella cells and then pushed a chloridised silver wire down this into the cell. Their method suffered with the disadvantage that the micropipette tip was 17 μm diameter and the wire 10 μm diameter, thus it can only be applied to large cells like Nitella, and even then they reported temporary trauma on insertion. The electrode however had a very low resistance in itself and allowed them to measure cell membrane impedance between 20 and 20k Hz, using a

bridge circuit. They recorded a membrane resistance of $78 \text{ k}\Omega \cdot \text{cm}^2$ for cells under "normal" conditions and they described wide variations in cell membrane resistance with external nutrient medium ion concentrations. The value of membrane capacity they reported lay between 0.003 and $0.015 \text{ }\mu\text{F}/\text{cm}^2$, with a "normal" value towards the low end of the range. These values are not in accordance with those recorded by other workers and need explanation. Cole and Curtis (1937) found $0.94 \text{ }\mu\text{F}/\text{cm}^2$ for Nitella, and Hope (1956) found $1.0 \text{ }\mu\text{F}/\text{cm}^2$ for Chlorella cells, etc. Indeed both Cole (1972) and Keynes (1972) remark on the constancy of membrane capacity as a sort of biological constant (even though the latter was expecting changes during the action potential sequence of a nerve - changes which proved to be small fractional). Even in those cases where this constancy appears to be broken, as Cole (1972) points out, it can generally be explained by multiple folding of the cell surface which had lead to under-estimates of cell surface area when judged by light microscopic examination. Electron microscopy would be needed to resolve such puckering. It therefore seems highly improbable that the low membrane capacity found by Bennett and Rideal (1954) could be correct, and Williams et al. (1964) have pointed out that Bennett and Rideal neglected to consider the "cable" properties of the long cell, leading them to an erroneous interpretation of their results.

Smaller intracellular metal electrodes have been used by others, including Grundfest et al. (1950), Bishop and

Collins (1951), who used fine steel electrodes, Wohlbarshtet et al. (1960), who used platinum electrolytically pointed and coated with glass except for the tip, Gray and Svaetichin (1951), who pulled down to tips less than 1 μm glass pipettes filled with silver solder, and Dowben and Rose (1953), later modified by Gesteland et al. (1959) and others, who filled their pipettes with an indium alloy before pulling. Gesteland et al. (1959) also discuss the relative merits of fluid-filled and metal microelectrodes in regard to noise and impedance with frequency response, and their paper is useful.

Although metal microelectrodes have the advantage of low impedance inside cells, they suffer seriously with a number of disadvantages. If the electrode tip is very small, as required to penetrate into small cells, then tip resistance is just as serious as with fluid-filled micropipette electrodes, as explained earlier, because this derives from the medium surrounding the tip, and not from the micropipette contents; it is also generally not precisely known. Because electrode reactions must occur at the surface of a metal when current flows, a large polarisation impedance will appear at the tip of noble metal electrodes, and its extent will be even more embarrassing than in methods (1) and (2) (Fig. 33) because of the small size of the electrode tip, and therefore higher current density. It is no solution to use non-noble metal electrodes coated with an insoluble salt of the metal, as with the reversible chloridised silver wire, because cell constituents react strongly

with metal ions, leading to poisoning of the cell, even though such an electrode (hardly micro!) was used with qualified success by Bennett and Rideal (1954).

We are now left with the lone single-barrelled micropipette electrode method, Fig. 33 (7). Brennecke and Lindemann (1971) had presented (in a very obscure journal!) a technique (later simplified for botanical use by Anderson et al. (1974)), using a lone single-barrelled micropipette before I first announced my own technique (Stanton 1973), but I was unaware of it at the time, and in any case they used square wave excitation, which severely complicates the analysis (p.281), and according to Etherton et al. (1977) is not entirely satisfactory. Nevertheless Brennecke and Lindemann (1974 a,b) presented a detailed theoretical analysis of their technique and a system design. Racusen (1976) and Drake et al. (1978) have used a lone single-barrelled method, in which they took advantage of the faster response of a microelectrode as compared to the cell membrane to discover the voltage shift at the membrane elicited by a sharp square pulse of current injected by the lone electrode.

My interest in devising a lone single-barrelled technique arose for the following reasons :

- (1) Double-barrelled micropipettes do not readily penetrate into some plant cells because of the tough and fibrous cell wall.
- (2) It is difficult to be sure that two separate

micropipette electrodes are inserted into one cell in complex tissues like maize roots.

(3) Micropipette electrodes need not suffer with polarisation capacity, and evidence verifying this will be presented later.

(4) Methods (1), (2) and (3) of Fig. 33 cannot be applied to single cells in complex tissues except where a high degree of uniformity exists as in potato tuber or beet root tissue (Remington 1928), and even here it is of doubtful validity, because of the possibility that a symplast exists, a problem which also applies however to all other methods. Maize roots contain a stele within a cortex separated by the endodermis layer of cells, with a suberised band in the walls, the Casparian strip, which is supposed to provide a barrier to the movement of ions and water, and hence electrical insulation in the walls of these cells, but whose properties are still not entirely certain.

(5) It is not, of course, valid to try to measure cell membrane resistance by first measuring d.c. resistance inside the cell and then repeating with the micropipette tip outside the cell, as explained above, p.270.

(6) Single micropipette electrodes are simple to make and relatively easy to insert into plant cells, especially using the special micromanipulator system described in Chapter 2. It therefore seemed worth while to see if such electrodes could be used for intracellular impedance measurements. I believe the evidence presented below establishes that they can be used successfully and that the method may well be useful for a wide variety of plant

and animal tissues, and with much smaller cells than hitherto. It is amusing to reflect that the experimental difficulties with electronics reported here are very similar to those experienced by McClendon in 1929, except that the impedances now being handled are several orders of magnitude higher than in those days!

Alternating Current Impedance Measurements.

An alternating current (a.c.) method offers a way round the difficulties described above without the need to resort to techniques involving double-barrelled electrodes, with their attendant difficulties of interpretation. The cell membrane has both resistance and capacity, as observed above. Now, if we take the value of $1 \mu\text{F}/\text{cm}^2$ as typical for membrane capacity, then at $\sim 1,000$ Hz membrane reactance would fall to a negligible value compared to its d.c. resistance, if we may assume this is of the order of $1,000 \Omega \cdot \text{cm}^2$. Since the membrane capacity is in parallel with its resistance, this means that the resistive component measurable between a microelectrode in the cell and an external electrode* will be the microelectrode resistance only, assuming that R_m and C_m behave "normally", and that the microelectrode has no reactive properties of its own. In fact, the micropipette tip also has capacity between its lumen and the outside solution and may behave as a distributed RC network or "Kelvin cable" - such behaviour will be considered later. However, apart from this complication,

* The external reference electrode usually has very low resistance - of the order of kilohms.

it should be possible to deduce the cell membrane resistance by taking the difference between the d.c. resistance and the a.c. "resistance" at some suitably high frequency, both measurements being performed with the microelectrode in the same place inside the cell. The advantage of this method is that there is no problem of the microelectrode resistance being altered between measurements, not at least as a result of being moved. As a sideline, the value of the microelectrode resistance inside the cell may also give interesting information on the cell contents.

It should also be possible to discover the cell membrane capacity by measuring a.c. impedance at intermediate frequencies. A complication here is that there will be a significant phase shift as frequency is altered, but see below, when the theory is discussed. However, the inclusion of a variable capacitor in parallel with the balancing resistor in a form of bridge network should make it possible also to balance the bridge for membrane capacity. In practise, it was found that adequate information could be obtained from a plot of resistance against frequency without the use of a balancing capacitor, for a special reason to be introduced below, p.304.

Alternating current impedance measurements can be made using a variation of the Wheatstone bridge. It is not uncommon for some form of bridge to be included in electrophysiological setups, usually to enable so-called

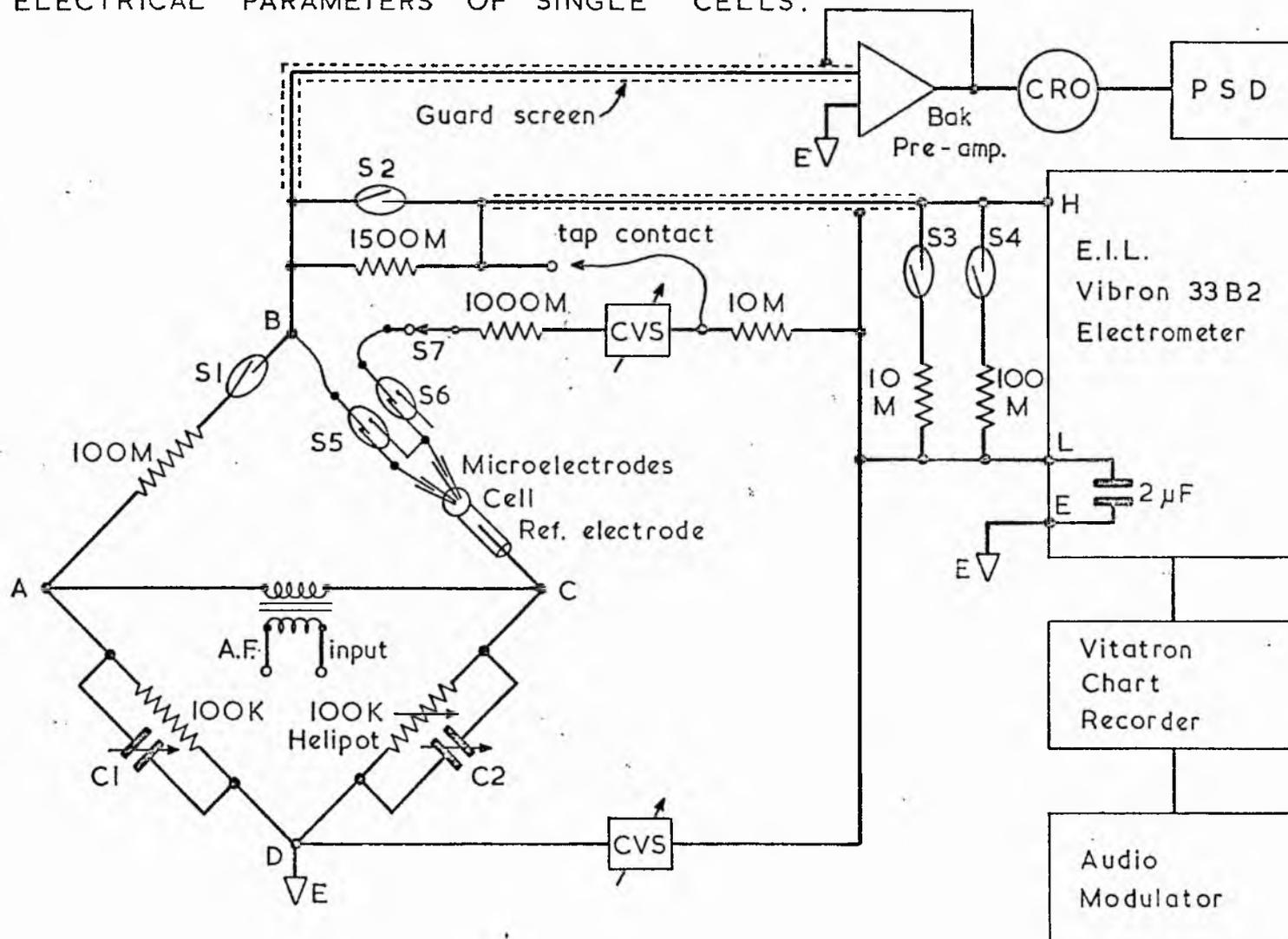
"stimulus artefacts" to be eliminated, and for checking electrode resistances. Regrettably most of these are not suitable for any measurement of accuracy. Often the power input for the bridge is derived from a square-wave pulse source, such as the CRO calibrator, or a stimulator used for excitable tissues, but, as explained earlier square pulses are not very suitable for impedance measurements, p.281.

The circuit of the arrangement which was designed to make these measurements is shown in Fig. 34.* When measuring impedances as high as one encounters with cells and microelectrodes, special precautions must be taken in the physical layout of the components. The circuit diagram does not represent this for the sake of clarity, but care was taken to avoid inter-component capacitive coupling. Point B on the bridge network was connected to the silver wire in the micropipette. This point must therefore be isolated from earth to the maximum impedance possible. Capacity to earth was kept to a minimum by mounting the piece of Veroboard (printed circuit strip board) which carried the bridge components as close to the micromanipulator head as possible and keeping the lead to the micro-electrode as short as possible. This lead was also both unscreened and thin, one strand of enamelled 32 s.w.g. wire, because the capacity between a naked

* The circuit diagram shows two electrodes in the cell, but for the present please ignore the outer one connected via S6 to S7, etc. The technique operates without this one, which was included merely for comparison of techniques, see below.

Fig. 34.

A.C. BRIDGE AND D.C. CIRCUITS FOR MEASURING ELECTRICAL PARAMETERS OF SINGLE CELLS.



All magnetic reed switches are shown in the unmagnetised position.

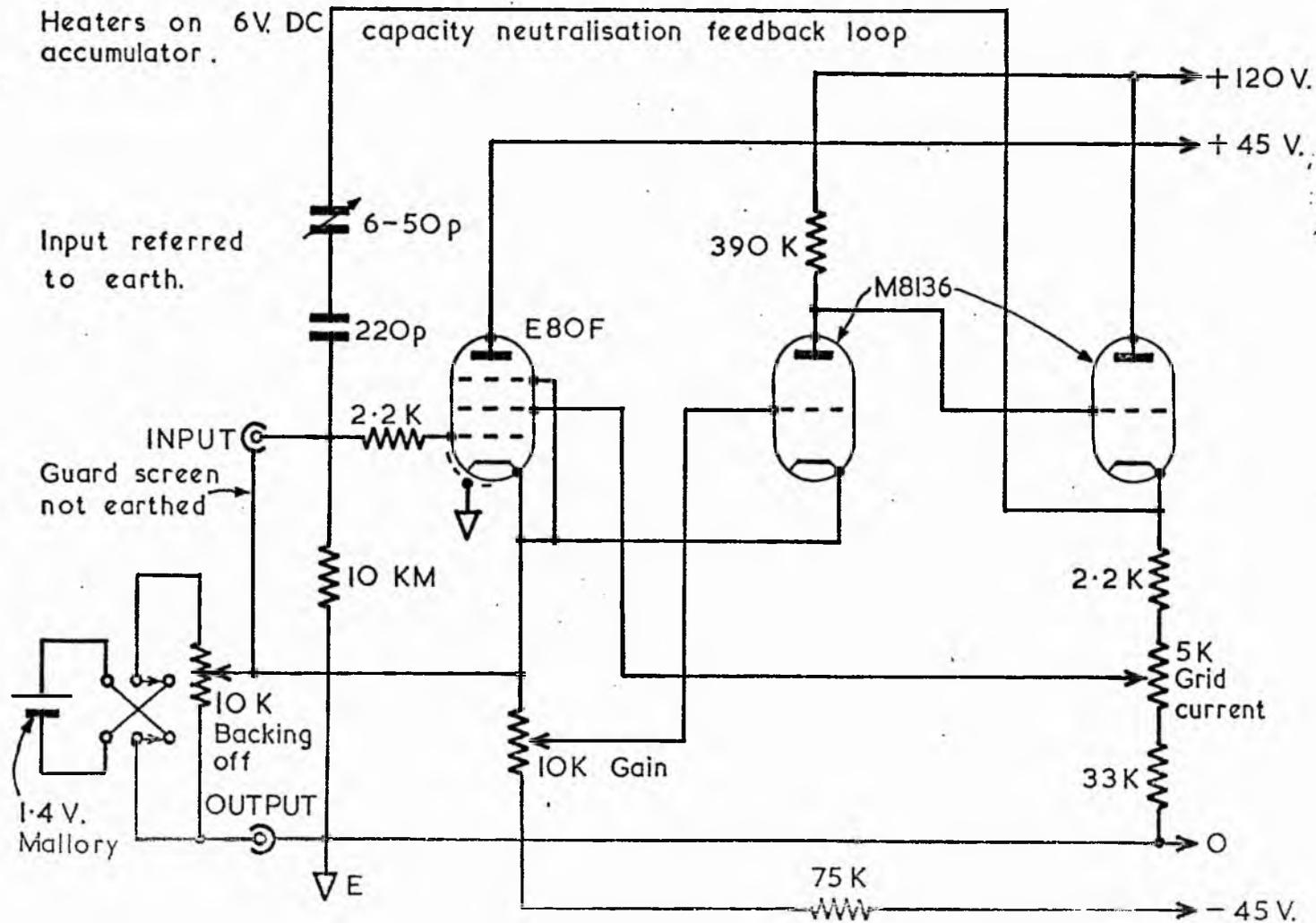
wire and earth at infinite distance depends on the diameter of the wire. Nevertheless a few picofarads is unavoidable. The seriousness of lead capacity is seen when it is realised that a cell of 30 μm cube will have a membrane capacity of only about 60 pF itself. The lead capacity in the bridge may be balanced by a trim capacitor, C1, 1,000 times larger, but it is a general rule in electronics that one should avoid rather than balance out stray capacity.

Special problems also arise in detecting the out-of-balance signal from a bridge involving such high impedances. The detector used was a special type of unity-gain amplifier designed by Bak (1958) as modified by C.J. Roemm  l   (1969, personal communication). Its circuit is shown in Fig. 35. The first stage is a pentode cathode follower. The output from this is amplified and the amplified signal is then buffered through a further cathode follower stage before being fed back to the input via an adjustable small capacitor, which is increased, with all leads connected as in an experiment, until the amplifier oscillates. It is then backed off until oscillation just ceases, at which point the system has maximum stable frequency response, and input lead capacity to earth, etc., is neutralised. Later a better circuit using integrated circuit operational amplifiers, designed by Roemm  l   was used, also incorporating capacity neutralisation. The input lead from the microelectrode, between point B on the bridge and the pre-amplifier, was also screened with a

Fig.35

BAK PRE-AMPLIFIER
as modified by C.J. Roëmmélé

A.F. BAK A Unity Gain Cathode Follower
E.E.G. Clin. Neurophysiol. 10 745 (1958)



"guard screen" connected to the output of the pre-amplifier, thus further reducing the effect of lead capacity.

The output of this pre-amplifier was coupled to a cathode ray oscilloscope (Telequipment D53) with its time-base synchronised either externally directly to the audio-frequency (AF) oscillator (Belco ARF 100, or internal oscillator in PSD, see below), or, more often, internally to the 50 Hz supply, using the CRO calibrator output. In practise it has proven easier to use the bridge with the CRO synchronised to the 50 Hz supply because it is very difficult to eliminate all 50 Hz hum from the electronics at these high input impedances. If the AF oscillator was set to beat rapidly with 50 Hz hum, and the bridge adjusted until the beating was eliminated, balance of the bridge must have been achieved at the oscillator frequency.

However a technique (my idea, modified from an old radio trick!) which has proven very useful, rather than waste time searching for sources of hum (which may be as diverse as mains wiring in the laboratory to magnetic field radiated by transformers in equipment), is to apply "hum bucking". A simple single stage Class A amplifier using a junction field effect transistor was designed (by myself, unpublished) with both gain and phase adjustments on the output. The circuit was accommodated with a small 9 v. battery in an earthed can with lid of domestic origin. The high impedance input of the transistor was driven by a disc of

aluminium foil 50 mm. diameter on a length of lead, to act as a hum pick-up aerial, which was placed in a suitable position outside the Faraday cage containing the microelectrode and bridge system. The output was connected to a similar piece of foil enclosed in a small plastic bag and bent into a half cylinder and placed about 2 cm. under the micropipette shank and electrode wire. By trial and error, searching with the input disc and adjusting phase and amplitude controls, it was possible quite easily to reduce hum to a matter of microvolts at the electrode. It was found however that the repeated strike transients of fluorescent room lighting could not be coped with, and so all work was done under tungsten lighting. This device, though simple, provides an excellent solution to a nuisance. In effect it creates a "zero hum field" around the microelectrode. Apart from the obvious instrumental advantage, this is desirable with all cells and essential with excitable cells. For a while, I collaborated with Dr. N. Sperlakis of the University of Virginia, Charlottesville, on measurements on frog heart cells. Hum fed into the tissue via the microelectrode was then disastrous, as it caused the muscle to pull off the micropipette! However, even with non-excitable tissues like maize roots, hum, which can easily be of the order of magnitude of the membrane potential, fed into a cell is highly undesirable, and may well cause membrane damage through electrostriction, (see p.299) and may also disturb the measured value of E_m if the membrane is rectifying (which it probably will be). This point,

which is clearly important, is often not realised in setups designed only to measure E_m , and a slow response electrometer (e.g. Vibron) gives no hint that hum is present. Therefore, in my view, any E_m setup is incomplete if it does not contain means of detecting and countering if necessary any hum - this means inclusion of a high impedance pre-amplifier and CRO, unless a battery operated system is used in a place remote from mains powered buildings. In buildings the Faraday cage helps, but in my experience, it rarely eliminates all hum. The hum-bucking device makes it possible to reduce this to a safe level easily.

Even so, it was very difficult to balance the bridge at low (15 - 250 Hz) frequencies. A great improvement in ease of use was achieved by introducing a phase-sensitive detector (PSD). A Lock-In Amplifier, Model JB-4 of Princeton Applied Research Corp. was used. Such a device registers only signal which is coherent with the reference signal applied to the instrument. Also noise, being random, is not registered. This is useful, because high impedance circuits are prone to noise, according to the Nyquist formula : $V_n = \sqrt{4kTBR}$ where V_n = noise voltage, k = Boltzmann's constant, T = Temp. °K, B = frequency bandwidth, and R = resistance across which the noise is developed by random thermal motion of electrons. (There are also other causes of noise in electronic circuits, and the reader is referred to Connor 1973.) Clearly, also unless the reference frequency happens to be the same or an odd multiple of

supply frequency, hum will not be registered by the PSD. It is simple to choose frequencies at which to operate the bridge avoiding these odd multiples. A point near an odd multiple causes a swinging motion of the PSD meter, as the hum and oscillator run into and out of phase with each other. Therefore any points near odd multiples were taken far enough away that this beating was too rapid to be displayed by the meter time-constant chosen.

The Princeton JB-4 Lock-In Amplifier contains its own sine-wave oscillator, which was used to drive the bridge. The instrument also incorporates a control for adjusting the phase angle between the oscillator output and the reference signal, which is internally connected in this model. It was necessary to readjust phase angle for every new frequency because there was a phase shift across the bridge input transformer, details of which will be given on p.296. This shift varied with frequency, as it was certain to do with the type of transformer chosen, since it was not feeding the bridge as a constant impedance source.

On the PSD, its meter time-constant was usually set to 1 sec. and rejection to 6 dB octave. The helipot in the bridge was set close to its balance point, established by a rough preliminary measurement, so that the transformer was loaded appropriately, the load being mostly in the arms AE and ED. S1 was opened (with S2 also open). This deliberately unbalanced the bridge, thus applying

the output from the transformer directly to the detection system. It will be noted that reactance in the cell-microelectrode assembly was then irrelevant since the point B was feeding into virtually infinite impedance (the detection pre-amplifier). Next, the phase control on the PSD was adjusted to give maximum deflection on the PSD meter. This established the condition that in-phase signals only were detected and quadrature signals due to reactance rejected. (In-phase here refers to the bridge excitation points A and D.) S1 was then closed and the helipot adjusted for null reading on the PSD meter. The helipot reading was then taken. This does not produce a "full" balance of the bridge, but see below.

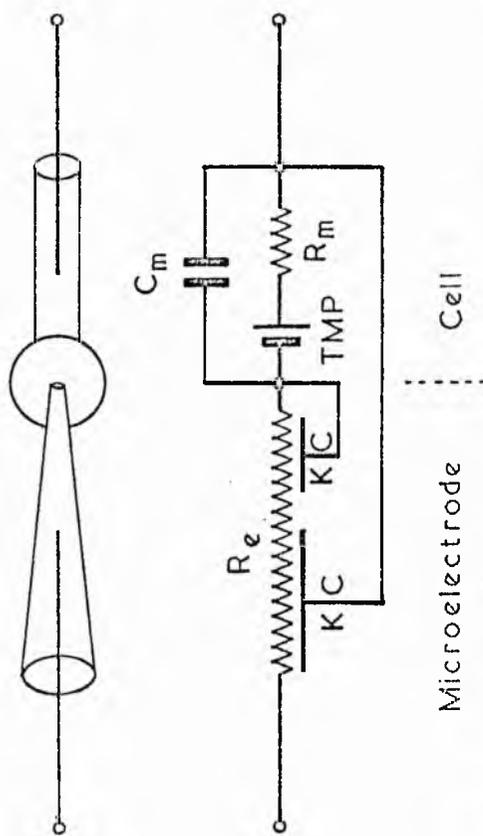
Magnetic dry reed switches were used throughout (S1 - 6) because they have exceptionally high impedance both to earth and between their contacts when open.

The AF input is introduced to the bridge through a small 1:1 transformer. This was required because both points A and C must be isolated from earth to quite a high impedance, and must also float to d.c. standing potentials. The transformer was therefore specially constructed, since standard laminated-iron transformers have rather high capacity between coils. A 5 cm. length was cut from a 1 cm. diameter ferrite aerial rod (RS Components, Blue grade). Two separate formers were turned from Perspex, each to carry a winding of about 2,000 turns of 40 s.w.g. enamelled copper wire.

The coils were spaced about 5 mm. apart and held in place with a length of rubber cut from a rubber band rammed between the formers and the rod. Capacity between these windings was down to a few picofarads. No screening can was placed over these coils since this would have increased stray capacities between the coils, and to earth, and would also have reduced the already poor efficiency of the transformer. Of course, a rod core with only an air return magnetic path, radiates rather badly (and in retrospect now I would have used a ferrite pot-core, as in the new system, p.317) and so the transformer was not mounted on the Veroboard, but was inserted in the AF feed line about 30 cm. from the Veroboard.

The arm BD of the bridge represents the microelectrode plus living cell plus reference electrode. A double-barrelled electrode has been drawn in Fig.35 but for the present we will consider just one barrel, that connected to S5. In single-barrelled experiments, the equivalent circuit of the arm BD would be as shown in Fig.36. The shank and tip of the micropipette have resistance in the lumen and capacity across the wall of the tube. Such a distributed resistance and capacity is known as a Kelvin cable. It will be considered later, but, in Fig.36, the shank outside the cell has been referred to the reference electrode and the tip to the inside of the cell. Only the former is an embarrassment. Evidence will be presented below on the impedance behaviour of micropipette electrodes. However it

Fig. 36.
 SINGLE - BARRELLED MICROELECTRODE IN A CELL
 AND EQUIVALENT CIRCUIT.



KC - Kelvin cable. TMP = transmembrane potential.

would possibly not be valid to equate the "tip" of this discussion with the "tip" which generates the "tip potential" or is responsible for "tip resistance", as described on p.242 and p.271 respectively.

Accordingly the lumen and tip resistances have not been drawn separately in Fig. 36.

Point D on the bridge is at low impedance with respect to earth so that no special care need be taken here. The helipot in arm ED balances R_e , plus R_m at low frequencies, and C2 may be used to balance C_m at intermediate frequencies, although it was not normally used. The ratio arms R1 and R2 determine the ratio of impedances in the other two arms.

Provision was also made in this circuit for measuring d.c. resistance and transmembrane potentials. During d.c. resistance measurement, S1 was opened, S2 closed and S3 or S4 used to apply a load as described on p.237. To measure transmembrane potential, reed switches S1 - 4 were opened. A resistance of 1.5 G Ω originally included to avoid the overshoot artefact of the pen recorder (p.236) also served to isolate the AF signal from the electrometer, the input capacity of which therefore did not attenuate the signal before it entered the pre-amplifier. To perform a.c. impedance measurement S1 only was closed. The fact that the transmembrane potential was then within the bridge was unimportant, because it had no effect on a.c. signals.

Further circuitry was included so that two separate electrodes or double-barrelled electrodes could be used in single cells, for the purposes of comparison of methods (4) and (5) with (7) (Fig. 33). Change-over reed switches S5 and S6 were included. These were placed alongside each other so that one magnet actuated both simultaneously. As drawn (Fig. 34) S5 connects the voltage-sensing barrel to the detection system and S6 connects the current-inserting electrode to the current source, which consisted of a calibrated voltage source, up to 1 v., in series with 1 G Ω . A further 10 M Ω was connected behind this to act as current calibrator. The 10 M Ω itself was a close-tolerance resistor, checked for value. (It would be unwise to rely on the value of a 1 G Ω resistor as being precise). The voltage across the 10 M Ω resistor could be read by tapping a contact directly on to the electrometer input. Since it was tapped on the electrometer side of the 1.5 G Ω resistor (with S2 open), the potential at point B was over-ruled without itself being disturbed.

When S5 and S6 were actuated together, the current barrel was connected to point B and the voltage barrel open-circuited. This was used after a cell insertion had been made to check that both barrels were in the cell, and in good condition, as witnessed by potential and d.c. resistance measurements.

In early experiments on living cells, it was found that high amplitude a.c. applied to the cell

through the microelectrode caused an early decline of the transmembrane potential. Presumably the cell had suffered damage, and one can speculate that the cause was mechanical vibration of the membrane. Such vibration would be set up by the periodic electrostriction of the membrane. A force of attraction exists between the parallel plates of a condenser when it is charged, and the force increases with voltage. Thus mechanical vibration will result if an alternating voltage is applied to the membrane. Perhaps mechanical vibration causes the membrane to break up into micelles, with large pores between, thus making the cell leaky.

Another possible cause of failure could have been that the electrical currents at the tip may have caused iontophoresis of micropipette filling solution into the cell. Although the current may well have been alternating symmetrically about the zero level, net efflux of KCl would still result. Large increases in internal KCl concentration might be expected to upset the cell.

It was found, however, that cells of maize root survived for long periods if the amplitude of a.c. applied to the microelectrode wire was less than 10 mV, peak-to-peak. This, of course, is not the amplitude applied to the membrane, which will be much less, owing to microelectrode resistance. To ensure that this condition was observed, the AF input voltage to the

bridge was monitored on channel Y2 of the CRO, the signal being derived from the primary side of the AF input transformer, and the display was available throughout. This signal is of course greater than the voltage applied to the bridge, due to transformer inefficiency, and therefore much greater than that applied to the cell. A further direct check was available on Y1 of the CRO, if the helipot was turned to zero, since then the AF signal is applied between point E, and, through the 100 M Ω resistor, point B. The displayed signal is still an over-estimate, so both methods allow safe signal levels to be maintained.

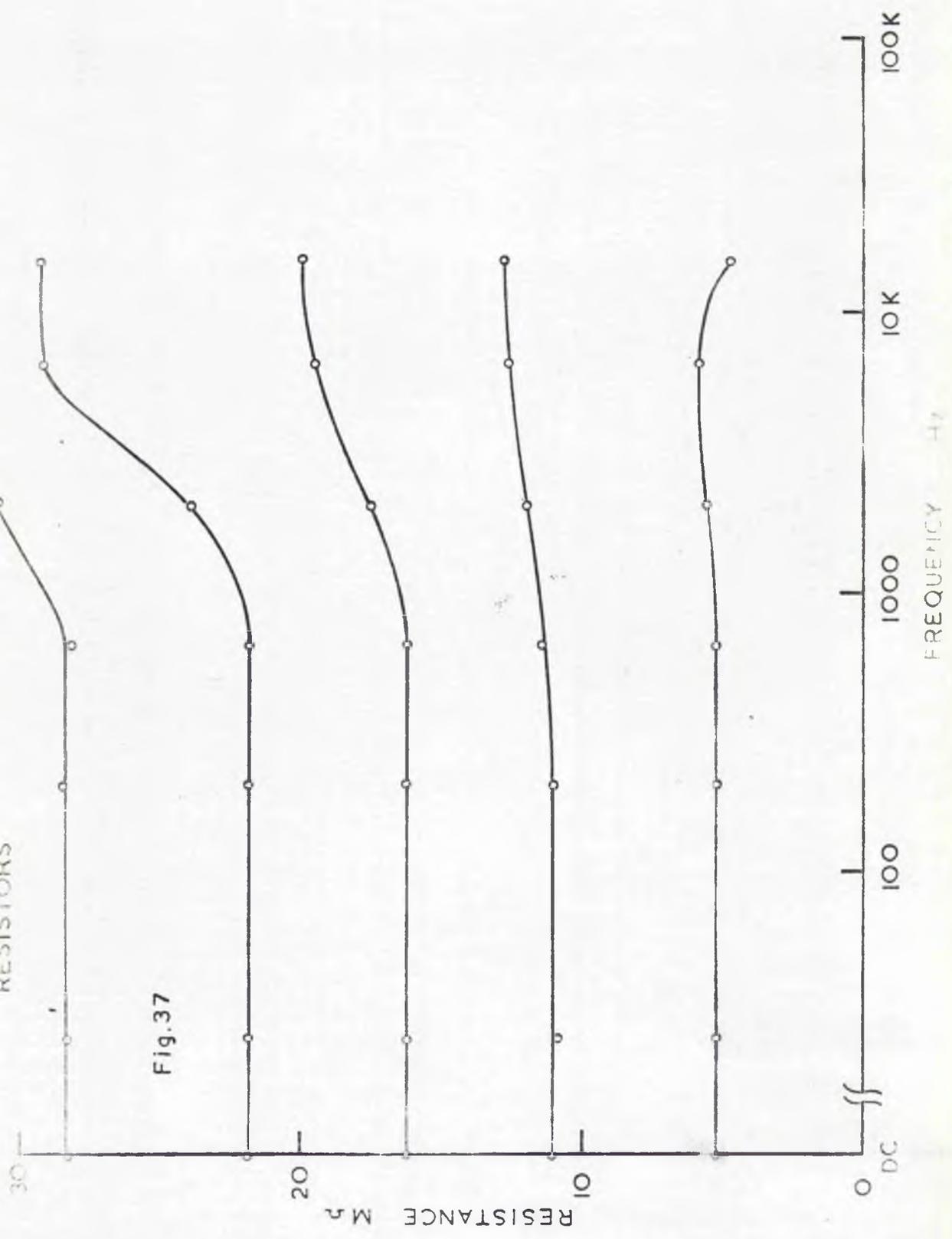
Tests on the A.C. Bridge and Calculation of Results.

First a family of impedance spectra was obtained using various standard electronic resistors in place of the cell in arm BD, to check that the bridge performed correctly.

There are various types of resistor available. For these tests it is desirable to use a type with the smallest possible reactance in the component. Obviously it is impossible to construct a resistor which has zero capacity between its ends, and in these studies a few picofarads is serious. It was found that only certain types of linear carbon resistor were suitable. A set of gold band ($\pm 5\%$) 5.0 M Ω resistors was used, and they were banked in series to obtain the family of spectra shown in Fig. 37. It is better to use a bank in series rather than single components of increasing

IMPEDANCE SPECTRA OF ELECTRONIC RESISTORS

Fig.37



value because the reactance of the bank bears a constant relationship to its resistance. This is not the case with single components of similar construction but different resistance.

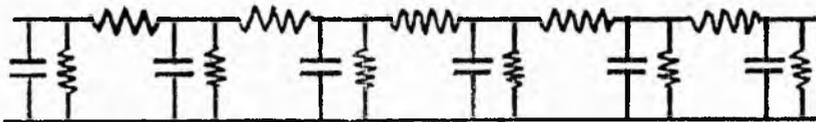
As will be seen from Fig. 37, the "pure" resistors gave constant readings between d.c. and about 6 kHz, except for the higher values of both resistance and frequency, but above 6 kHz it was difficult to be sure of balance, and was getting beyond the best range of the PSD used. Therefore results above 6 kHz were regarded with increasing suspicion. However 0 - 6 kHz usually proved adequate for studies on maize root cells.

The bridge was calibrated for resistance, as follows. The ratio arms AB, AE and ED (with helipot set to zero) were measured carefully in separate experiments and a calibration factor was calculated. This factor was also checked by inserting calibrated resistors in arm BD and balancing. The factor was 1.17 to be multiplied by the helipot reading (0-100) to give the answer in $M\Omega$. Calibration of resistors below $10 M\Omega$ was carried out on a Marconi Universal Bridge TF 2700 (internal oscillator 1 kHz), which the manufacturers state has an accuracy of $\pm 2\%$. Secondly resistors were checked using series current measurements and a constant voltage power supply on an Electronic Avometer, Type EA113, which the manufacturers state has an accuracy of $\pm 1.25\%$. These measurements corresponded as accurately as the instruments could be read. Very high value resistors

($> 10 \text{ M}\Omega$) were measured using the electrometer in the same way as cell d.c. resistance was measured.

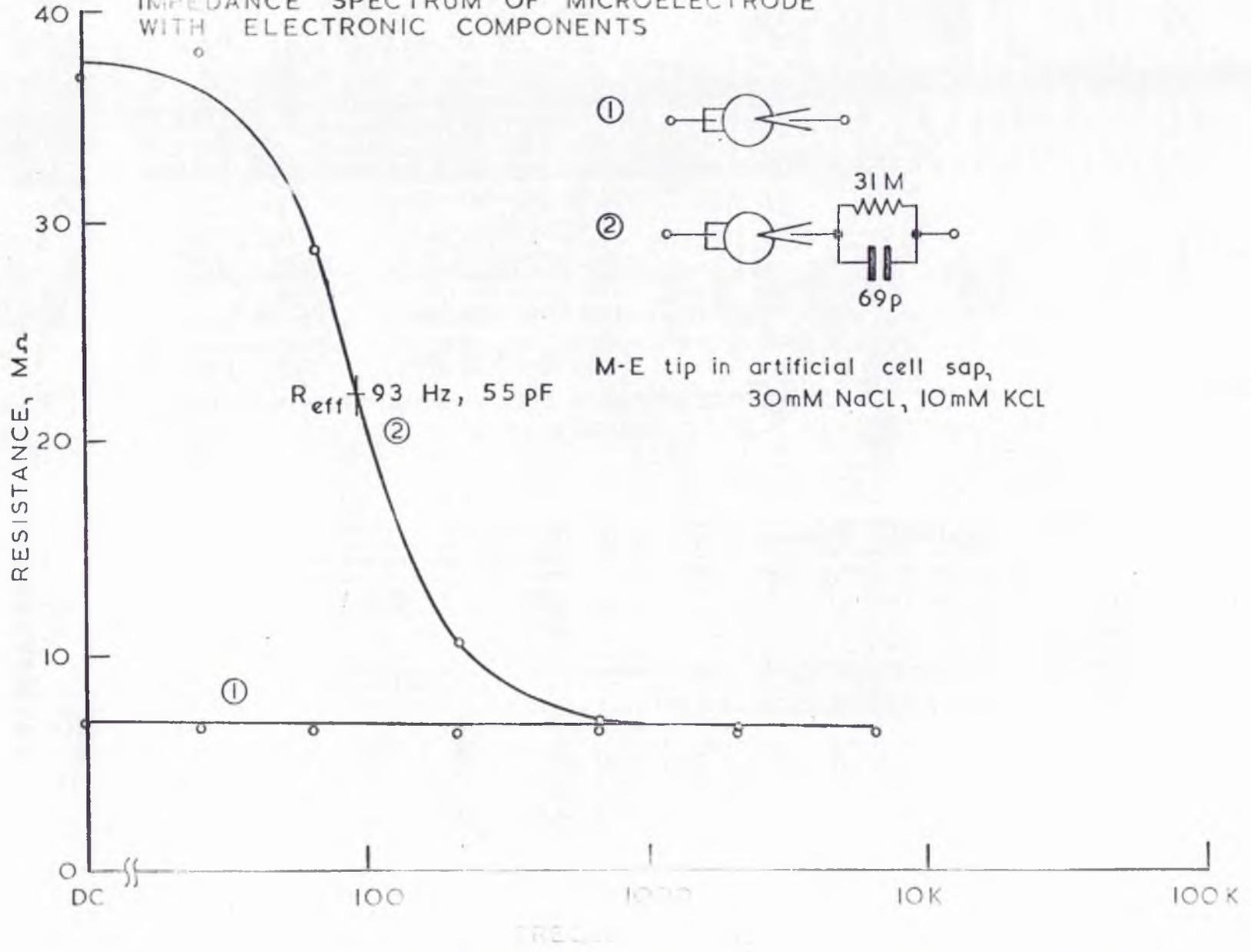
Microelectrodes were next investigated in the bridge. Fig.38 shows the impedance spectrum for a microelectrode with its tip in artificial cell sap (ACS) of 30 mM NaCl and 10 mM KCl. Up to well above 1 kHz, most of the micropipettes used behaved, with their electrode wire, as pure resistors, and it was re-assuring that no complication of the Kelvin cable type was found.

Kelvin cable properties will be discussed further later (p.326), but, what is known as a Kelvin cable arises whenever a transmission line is electrically leaky throughout its length, between the line and earth (or another parallel line), and the conductors themselves have appreciable resistance along their length. The problem originally arose in connection with long land lines for telegraph and telephone communications. Such a lossy cable, which also, of course, has capacity between the conductors, is equivalent to a distributed RC network as shown below.



The effect of such an arrangement is to attenuate higher frequencies more than lower, although the behaviour is very complex and may lead to a "notch" spectrum as described by Short (1968).

Fig 38
 IMPEDANCE SPECTRUM OF MICROELECTRODE
 WITH ELECTRONIC COMPONENTS

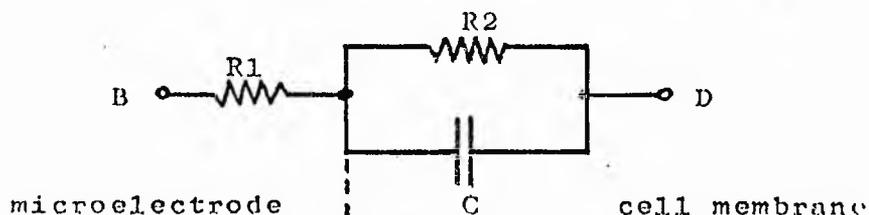


Electronic components were then included in series with the silver wire of the microelectrode to imitate the putative behaviour of a cell membrane. Preliminary measurements on maize root cells had already established the order of magnitude these values should take.

Fig.38 shows a typical impedance spectrum for such an arrangement. The true values of the components are included on the inset diagram of the circuit. However before spectra of this kind can be discussed further, we must consider the theory.

Theory of Impedances.

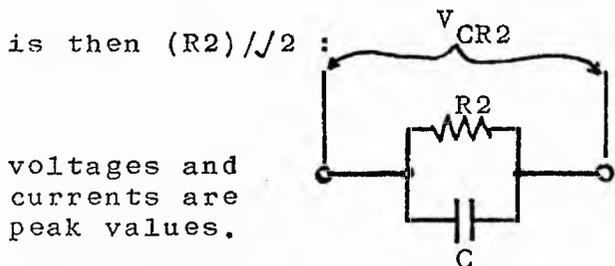
The arrangement in arm BD is equivalent to :



However it may well be an over-simplification to regard the cell membrane in just this way. C may vary with f . There may be a series resistive component in C also, due to a layer of membrane material on either side of the lipid layers. Also C itself in real membranes has been shown to be lossy especially at high f (Cole 1972). But we will assume this to be an adequate model for the present.

Under d.c. and very low frequencies this network is equivalent to $R1 + R2$, because the reactance of C, X_C , is then virtually infinite. At very high frequencies the impedance reduces to R1, since R2 is shorted by C, whose reactance has now fallen to a negligible value.

To evaluate C, it is convenient to consider the condition when the magnitude of $X_c = R_2$, as will apply at some intermediate frequency. By vector analysis, it is easy to see that the impedance of the pair CR2

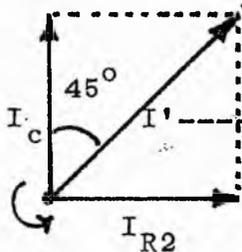


voltages and currents are peak values.

voltages across both R2 and C must be in phase. Thus I's are 90° out of phase, I_c leading I_{R2} .

Current Vectors :

$I_c = I_{R2}$ in magnitude because X_c is taken as equal to R2

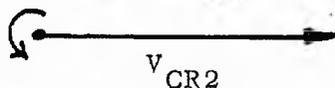


resultant of CR2 = $\sqrt{2} \times I_{R2}$

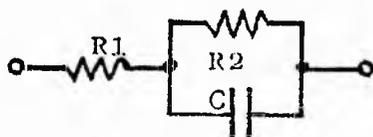
$$I_{R2} = \frac{V_{CR2}}{R_2}$$

$$I' = \frac{\sqrt{2} \cdot V_{CR2}}{R_2}$$

Voltage Vector :

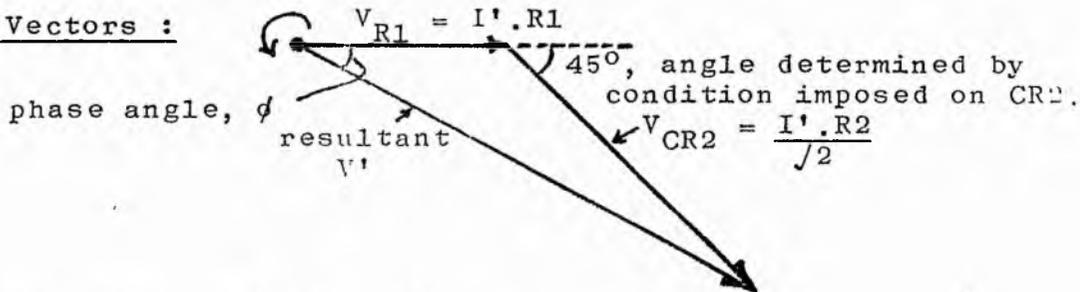


Compounding this with R1 we get :

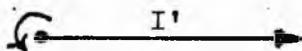


In this case, current is in phase through R1 and the pair CR2 taken together, so that we have :

Voltage Vectors :

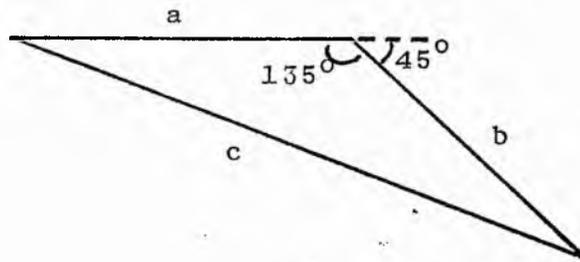


Current Vector :



Let us assume that such a voltage is applied that it produces I' through the network. That voltage, V' , will be compounded of V_{CR2} , which is $I' \cdot R_2 / \sqrt{2}$, and

V_{R1} , which is $I' \cdot R1$. In a triangle with one internal angle of 135° :



it is easy to prove from geometry that :

$$c^2 = a^2 + b^2 + \sqrt{2} \cdot ab.$$

Hence :

$$\begin{aligned} V'^2 &= (I' \cdot R1)^2 + \frac{(I' \cdot R2)^2}{2} + \frac{\sqrt{2} \cdot I' \cdot R1 \cdot I' \cdot R2}{\sqrt{2}} \\ &= I'^2 \left(R1^2 + \frac{R2^2}{2} + R1R2 \right) \end{aligned}$$

Thus impedance of the whole assembly :

$$Z = V'/I' = \sqrt{R1^2 + \frac{R2^2}{2} + R1R2}$$

in the circumstance that the magnitude of $X_c = R2$.

The value of Z calculated here refers to V' and I' ,

which, it is clear from the triangle of vectors, are

not in phase with each other, there being some angle

less than 45° between them. But the component of voltage

which is in phase with the current through the network

will be :

$$I' \cdot R1 + \frac{I' \cdot R2}{2}$$

Thus the effective resistance (which I now define as

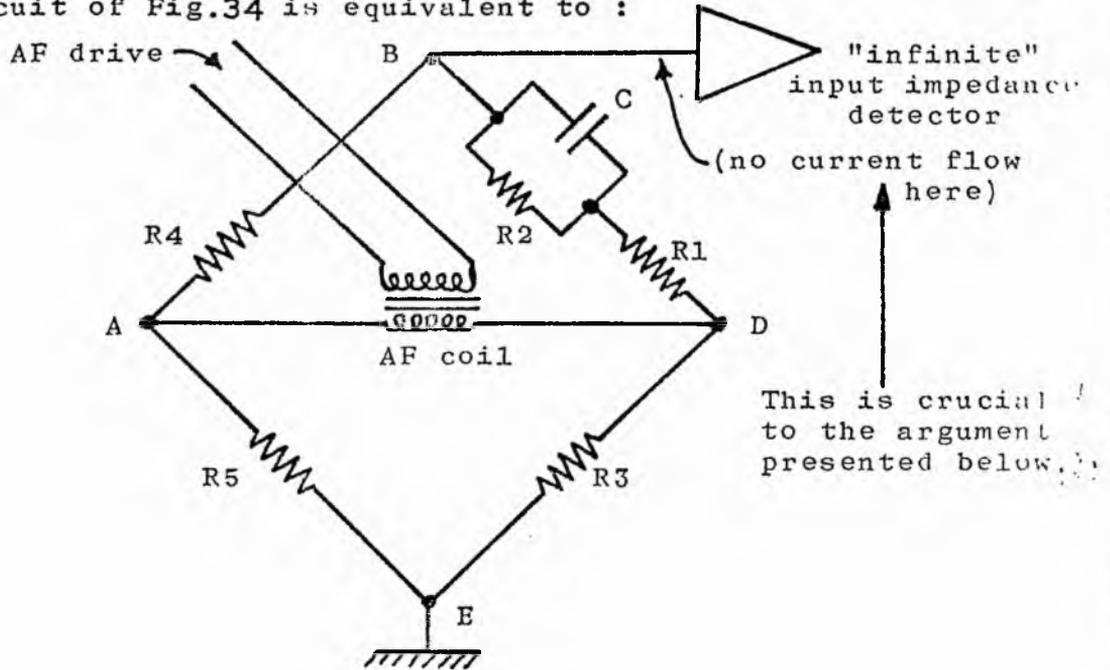
the component of impedance for voltage in phase with

current) will be simply :

$$R_{\text{eff}} = R1 + \frac{R2}{2}$$

We may now see that this is what is measured by the system employed (Fig.34), when out-of-balance signal of the bridge is detected by a PSD set to detect in-phase signals only, as follows :

The circuit of Fig.34 is equivalent to :



By earthing point E, we are effectively looking at the difference voltage between B and E by the detection system. Thus we may regard point E as on a voltage divider AED, where we refer all voltages to A and regard D as drive. Arm ABD may be regarded similarly for point B. Now in a purely resistive bridge, clearly points B and E are always in phase with point D, and balance is achieved when (as here at low f or d.c.) :

$$\frac{R1 + R2}{R4} = \frac{R5}{R3}$$

However with C, at higher frequencies we know that current and voltage in arm BD are not in phase. Let the phase angle be ϕ . It therefore follows that the in-phase component of voltage (since clearly current must be in phase through arm BD, the AF coil and R4) across BD will be $V' \cos \phi$, which for the imposed condition that $X_C = R2$, gives $I' (R1 + \frac{R2}{2})$. Since the in-phase component of voltage across R4 will be $I' \cdot R4$, it follows that if the bridge is "balanced" to give a null reading on the PSD set to detect in-phase signals only then :

$$\frac{R3}{R5} = \frac{R1 + \frac{R2}{2}}{R4}$$

at the frequency when $X_c = R2$.

The bridge however is not fully balanced by this procedure, but this is not required when a PSD is used, and the use of in-phase only balancing makes possible a very simple analysis of the network $R1$, $R2$, C , without the need for a capacitor in parallel with another arm, and the attendant problems of analysis of readings in terms of $R1$, $R2$, and C whose values are required.

This was believed to be the first use of a phase-sensitive detector in electrophysiological problems of this kind to be announced (Stanton 1973). A PSD was also used (independent thinking!) by Valdiosera, Clausen and Eisenberg (1974) to study frog skeletal muscle fibre impedances by sinusoidal a.c. Indeed I recall a fascinating conversation with Dr. Robert Eisenberg, when we were on the train between Copenhagen and Roskilde - we were both (independently again!) taking the day off from the International Congress of Biophysics in Copenhagen in the hot summer of 1975, and met on the train only to discover our mutual interests!

Thus to evaluate membrane capacity, C , by my method, R_{eff} must be calculated from $R1$ and $R2$, and the frequency found that corresponds to this value of resistance, by reading from the impedance spectrum. Then we know from the initial condition that $R2 = X_c = 1/2\pi fC$, hence C . In practise the values of R_{eff} are plotted against f , when the curve will start at value $R1 + R2$ at low f and

will fall to R_1 at high f . Thus the dip height represents R_2 and its mid-height frequency is the value required in the formula, $1/2\pi fC = R_2$.

Experimental Measurements Again.

Now we return to consider the impedance spectra of microelectrodes in series with electronic components. In order to make a fair comparison with the behaviour in cells, the micropipette tips were bathed in an artificial cell sap. It was found that there was a discrepancy between values of C calculated from R_{eff} and the correct values. However the reason for this is not understood.

Stray capacity in the leads cannot explain this discrepancy since it existed to the same extent in the tests on pure resistors and with microelectrodes alone, which all gave flat spectra. Stray capacity would also be expected to produce a fall in impedance with increasing frequency, which was not observed. Such an anomaly reduces to some extent confidence in the results obtained, but a correction procedure was worked out, and the error was at least consistent. Values measured on living cells were corrected using the nomogram, Fig. 41 constructed from data taken on known electronic components. An investigation of measurement error showed that it depended mostly on the resistance, R_2 . Figs. 39 and 40 show some of the data on which this conclusion was based.

Fig. 39
 IMPEDANCE SPECTRA FOR A RANGE OF CAPACITIES

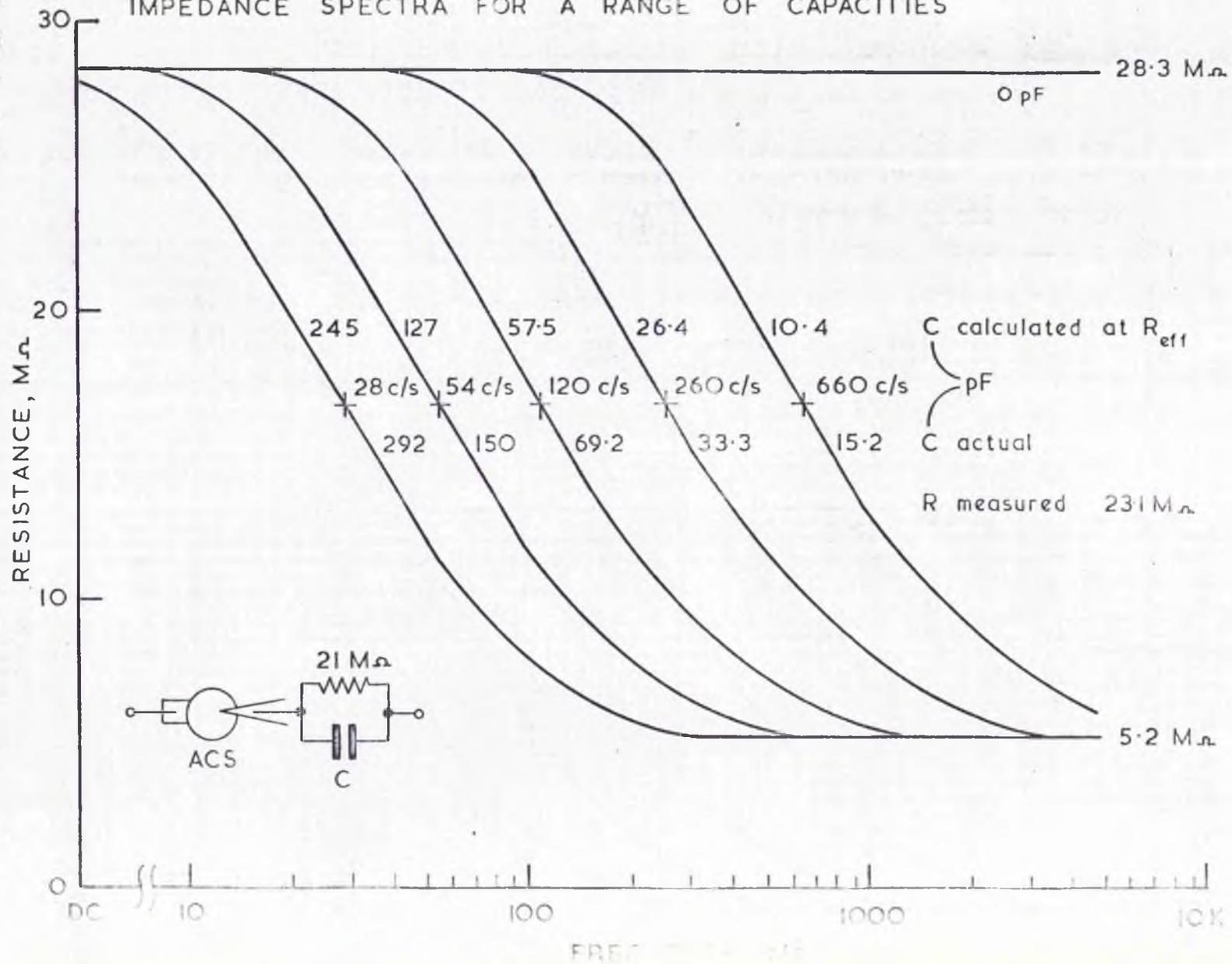
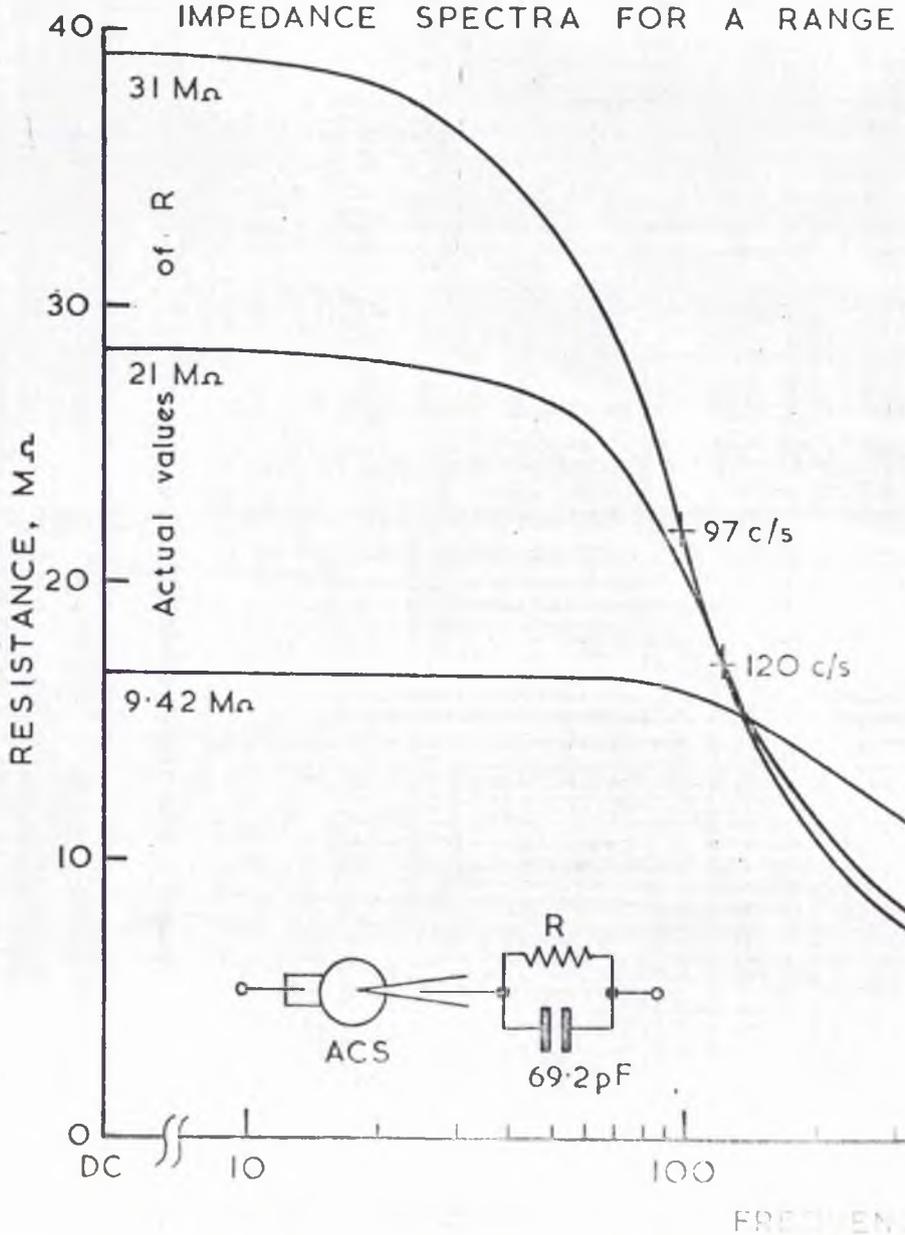


Fig. 40

IMPEDANCE SPECTRA FOR A RANGE



OF RESISTANCES

39 M Ω

33.2 M Ω
53 p

values

28.4 M Ω

22.6 M Ω
57 p

Measured

16.6 M Ω

10.7 M Ω
47 p

* 360 c/s

5.8 M Ω

1000

10 K

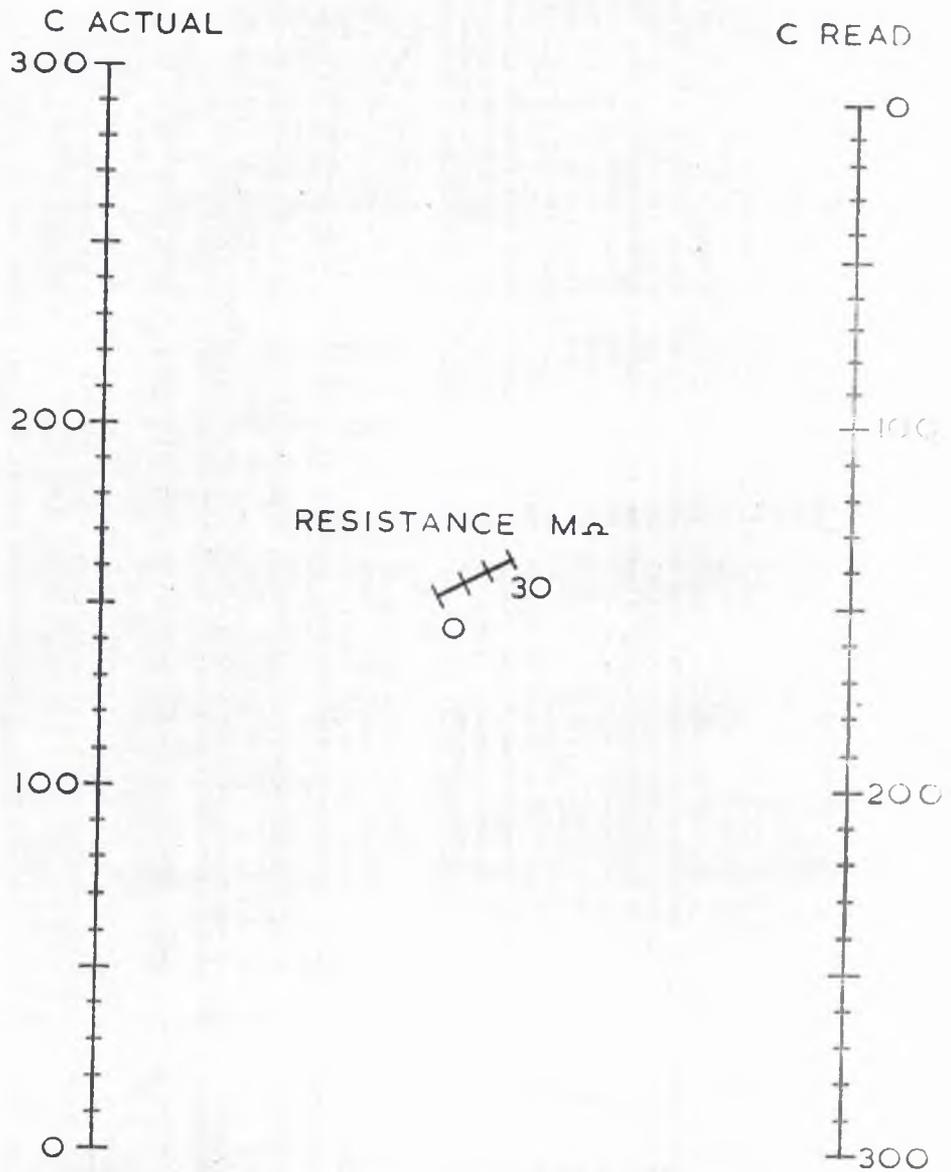
v c/s

π
Δ
○

Fig. 41

NOMOGRAM FOR CAPACITY MEASUREMENT CORRECTION

ρF



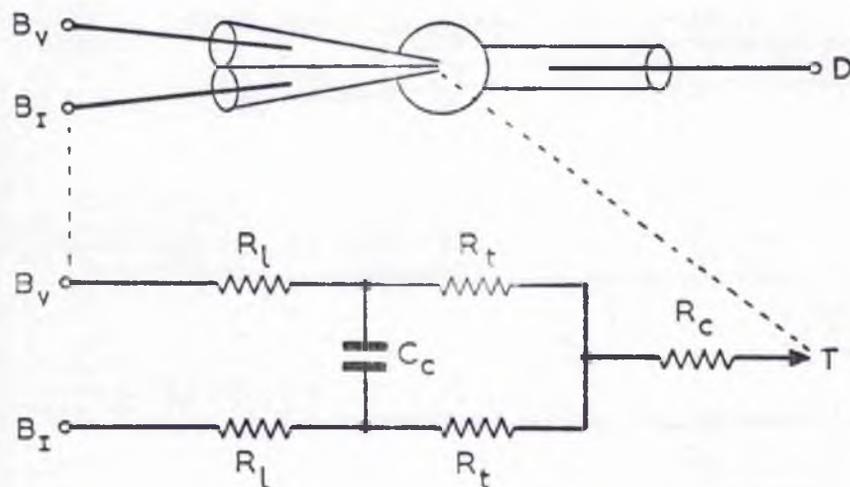
The nomogram, Fig.41, constructed from these data, shows "Resistance", referring to cell membrane resistance, and "C Read", referring to the value calculated from frequency at half the dip height.

Double-barrelled Micropipettes.

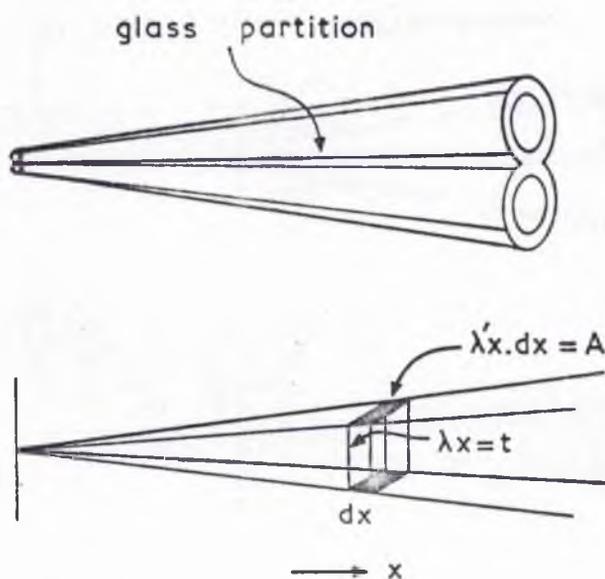
A further investigation was undertaken to discover the behaviour of double-barrelled electrodes, both in their intended double-barrelled d.c. function, and also using only one barrel in the a.c. bridge leaving the other open-circuited. This was of interest because it was highly desirable to compare the a.c. method with the d.c. method ((4) of Fig. 33) of measuring cell membrane resistance. This comparison should be carried out on the same cell, hence the use of one insertion of a double-barrelled micropipette for both measurements. The other approach of using a double-barrelled electrode for d.c. measurement and a single-barrelled electrode for a.c. measurement was also tried.

A double-barrelled electrode, plus a possible representation of its equivalent circuit is shown in Fig. 42. This has been modified somewhat from the representation in Fig. 33 (5) to include the coupling capacity, C_c , between the barrels and the separated lumen resistances, R_l , and tip resistances, R_t . As explained earlier, part of the tip resistance of either barrel constitutes also a coupling resistance, R_c , to the other barrel, such that change of current down one barrel causes a change of voltage on the other.

Fig. 42
DOUBLE-BARRELLED MICROELECTRODE AND
A POSSIBLE REPRESENTATION OF ITS
EQUIVALENT CIRCUIT (EXCLUDING CELL.)



CAPACITY BETWEEN BARRELS OF
DOUBLE-BARRELLED MICROELECTRODE.



$$dC = \frac{K \cdot \lambda' x \cdot dx}{4 \pi \cdot \lambda x} = \frac{K \cdot dx}{4 \pi} \cdot \frac{\lambda'}{\lambda}$$

$$\therefore C = \frac{K x}{4 \pi} \cdot \frac{\lambda'}{\lambda}$$

The coupling capacity was measured, as described below, and also estimated theoretically as follows. During the pulling of a double-barrelled micropipette, the bore diameter and wall thickness appear to preserve something close to a constant relationship, so that the separation between barrels through the glass partition at point x may be represented, as in Fig.42, by λx , where λ is a "thickness proportionality factor" and x is the axial distance from the vanishing point of the tip. Similarly the tangential area of an element of length dx will be $\lambda' x \cdot dx$, where λ' is a "width proportionality factor" in the plane perpendicular to the first, both planes including the pipette axis. Then from the general formula for the capacity of a parallel plate capacitor, element dx contributes :

$$dC = \frac{K \cdot \lambda' x \cdot dx}{4\pi \cdot \lambda x} = \frac{K \cdot dx}{4\pi} \cdot \frac{\lambda'}{\lambda}$$

and integrating from the tip to x :

$$C = \frac{Kx}{4\pi} \cdot \frac{\lambda'}{\lambda}$$

It thus appears that the capacity between barrels is independent of the actual dimensions, except its length, but is dependent on its shape. If we assume, for the sake of argument, that the ratio of thickness to width of the partition is unity, then, for a tip of length 1 cm. to the shanks, where the barrels are no longer fused together, the capacity will be :

$$C = 0.09 K \cdot x \frac{\lambda'}{\lambda} \text{ pF}$$

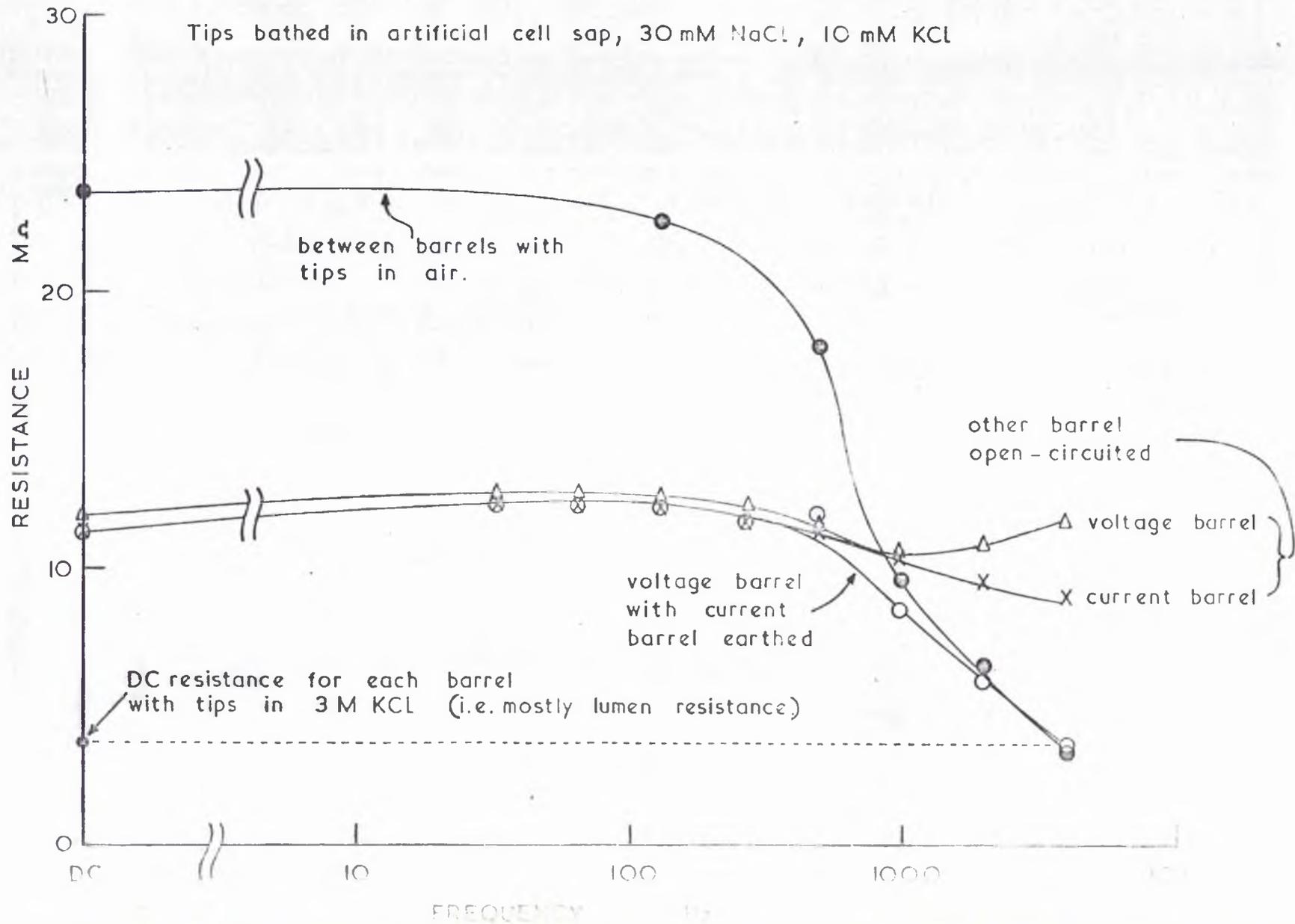
when the permittivity of free space is expressed in the units of pF/cm.

Thus, taking the relative permittivity (dielectric constant) of glass as 5, this gives a value of 0.45 pF. This value is certainly very much lower than is observed (see below). Even if we assume that the partition is 10 times wider than its thickness, this would only raise the value to 4.5 pF, which is still at least an order of magnitude too small. The value measured therefore requires explanation. It is perhaps worth mentioning that anomalously high dielectric constants are known and used in electronics for the construction of very small high value capacitors - the so-called ceramic capacitors. Again a heat treatment is used to make the dielectric, and perhaps strain introduced into the material is relevant. However this phenomenon appears only for double-barrelled micropipettes and not with single-barrelled. Thus, although an interesting question in itself, this problem is fortunately not unavoidably relevant for measurement of R_m and C_m . It will therefore not be pursued, but it does emphasise the conclusion that double-barrelled micropipettes are useless with fast changing signals.

The experimental behaviour of double-barrelled microelectrodes to a.c. was investigated using the bridge, and spectra are shown in Fig. 43. If only one barrel was connected, and the other left unconnected, it behaved much like a single-barrelled microelectrode. However if the wire in one barrel was earthed to the reference electrode at point D, a dip in impedance was observed at high frequencies. In this case one must

Fig. 43

IMPEDANCE SPECTRA OF A DOUBLE-BARRELLED MICROELECTRODE.



imagine one barrel, say B_I in Fig. 4 2, as connected to point T. Since R_L is usually low compared to R_t with the tip in physiological concentrations of ions, the equivalent circuit reduces approximately to R_t and C_c in parallel. Hence the frequency at half the dip height on the spectrum represents the reactance of C_c equal to R_t , hence C_c . On this basis values for a typical double-barrelled micropipette were measured as :

	Tip in ACS
R_L	3.2 M Ω
R_t	8.0 M Ω
C_c	146 pF
R_c	1.54 M Ω

The value of C_c measured for my double-barrelled micropipettes compares fairly favourably with the value of 20 pF reported by Coombs et al. (1955), if one remembers that C_c will depend very much on the type of glass used and on the precise shape pulled. As expected tip resistances and coupling resistance varied with external salt concentration, but lumen resistance did not.

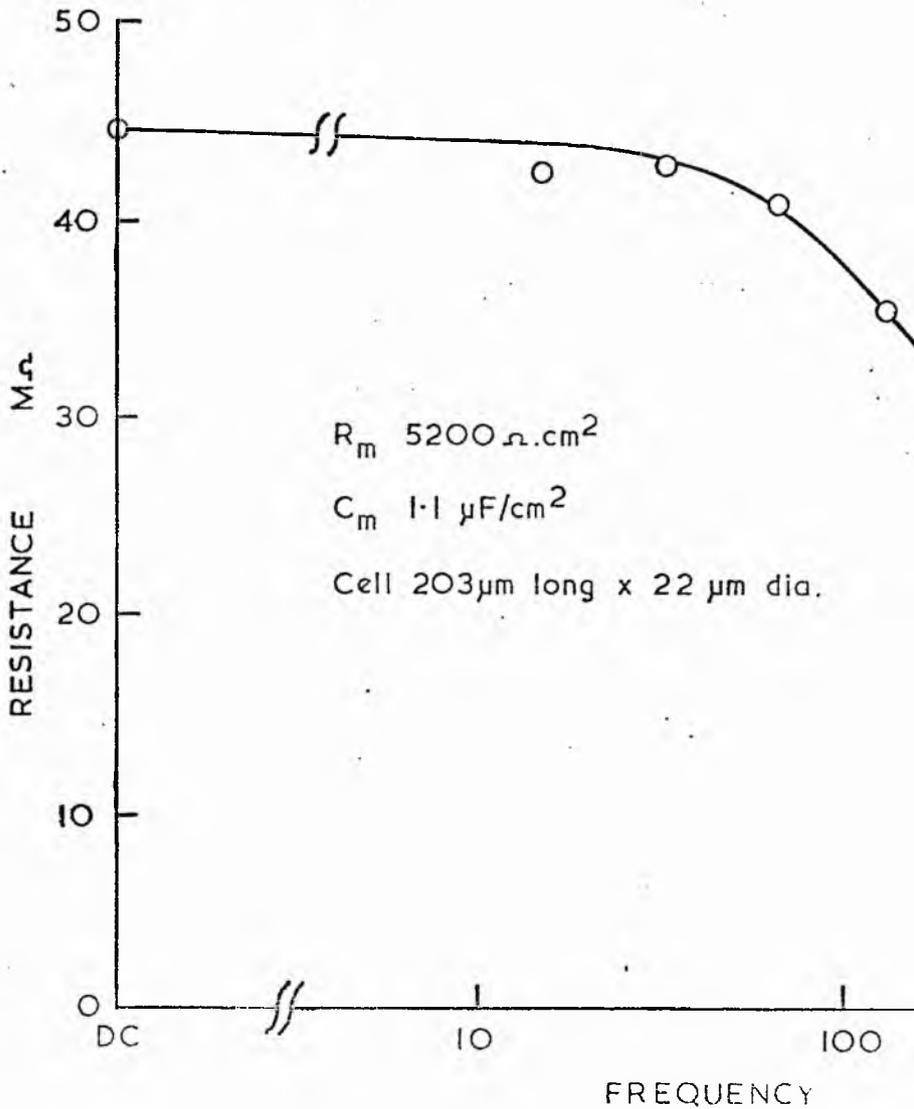
A further experiment was performed with the double tip in air, and measurement made between the two electrode wires rather than between one wire and a reference electrode. A much larger but unstable d.c. resistance was found between the barrels, but again the dip in impedance occurred with increasing frequency. This experiment is difficult to interpret however, owing to the unstable external connection between the tips through drying electrolyte.

Typical Results on Maize Root Cells.

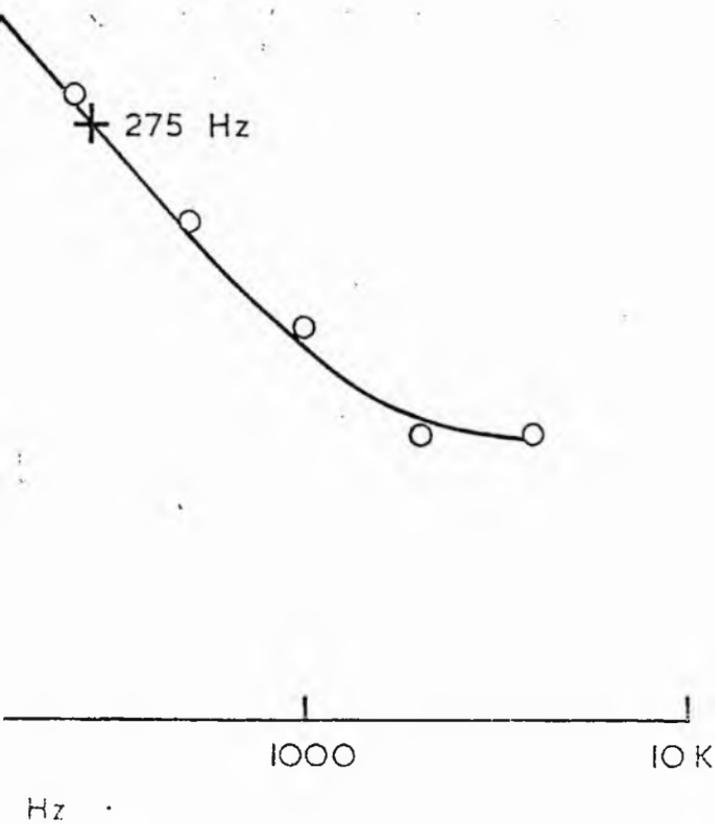
Cells in maize roots in the region of root hairs were studied using this equipment and Fig. 44 shows a typical AF impedance spectrum (average of two runs). This one was taken with a double-barrelled micropipette. The d.c. membrane resistance by the double-barrel method ((4) of Fig. 33) was also taken, and gave a value of R_m of $3360 \Omega \cdot \text{cm}^2$ before the AF spectrum run and $1510 \Omega \cdot \text{cm}^2$ afterwards. It is disconcerting that the value should have changed, but this was largely a result of the requirement to hold good impalements for long periods (up to $\frac{1}{2}$ hour) while the AF spectrum was taken (see below).

The double-barrel method gave a lower R_m than the single-barrel bridge method ($5200 \Omega \cdot \text{cm}^2$). This is also disturbing, but is in line with the findings of Etherton et al. (1977), who compared the double-barrel method and a square pulse method (after Brennecke and Lindemann 1971). Their conclusion was to place more confidence in the double-barrel method than in the single-barrel pulse method, but I do not see that they were justified in doing so. Double-barrelled micropipettes are so complex that I would be just as worried about results from them, but clearly there is a problem as to which method gives the more reliable result. At least they both give results within the same order of magnitude!

Fig. 44
IMPEDANCE SPECTRUM OF MAIZE ROOT



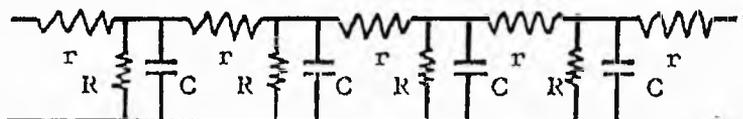
CELL.



It is however particularly gratifying but on second thoughts a little puzzling that the single-barrel bridge method consistently yielded a value of $1 \mu\text{F}/\text{cm}^2$ for C_m . It is gratifying because this is the accepted value for cell membranes (Cole 1972), but puzzling because the expectation is that the micropipette tip would generally come to rest in the vacuole, so that we would be seeing capacities of tonoplast and plasmalemma in series. Probably C_m would be similar for these, but R_m may well be much lower for the tonoplast than for the plasmalemma, and this would produce a second dip in the AF spectrum at higher frequencies, thus leaving the observed dip and C_m calculated therefrom to refer to the plasmalemma after all! In fact it was true to say that results fell into two categories which at the time of taking was not understood, but which could be explained by this possibility. There were runs like Fig.44 where a definite bottom plateau was found, and others where a rather unclear bottom plateau was found. Could it be that in the former the micropipette tip was in the cytoplasm whereas in the latter it was in the vacuole? However, it is equally possible that the resistance of the tonoplast may be higher than that of the plasmalemma. Both situations are known, thus for the freshwater alga, Nitella translucens, $R_p = 112 \text{ k}\Omega.\text{cm}^2$ and $R_T = 6 \text{ k}\Omega.\text{cm}^2$ (Spanswick 1970), whereas in the marine alga, Griffithsia monile, $R_p = 200 \text{ k}\Omega.\text{cm}^2$ and $R_T = 5 \text{ k}\Omega.\text{cm}^2$ (Findlay et al. 1970). Now there is a marked effect of external salt concentration on R_p , see later, and maize may be nearer to Nitella than to Griffithsia on that

count. On Eloidea leaves Spanswick (1972 b) found $R_p = 3.1 \text{ k}\Omega \cdot \text{cm}^2$ and $R_T = 1.0 \text{ k}\Omega \cdot \text{cm}^2$, which tends to confirm this view, since Eloidea grows submerged in freshwater.

There is however also another possibility. The AF bridge (or any other method using microelectrodes) measures a resistance and a capacity across it. To convert these measures to area-specific values, the total surface area of cell membrane electrically accessible to the microelectrode is required. Now, in the assessments given above, I have used the dimensions of the cell as seen under the microscope to calculate a surface area. However (a) the cell membrane may not be smooth, and (b) plasmodesmatal coupling to other cells in the tissue, constituting a symplast, will mean that the microelectrode is actually looking at a much larger membrane surface area than what is merely seen for one cell. A complicating factor in this is the question as to whether the a.c. signal over the whole of the accessible membrane area is oscillating in phase. Williams et al. (1964) draw attention to this. It is well known in electronics that a chain of resistors and capacitors as below



r refers to plasmodesmatal coupling, and RC to each-cell.

will produce a phase shift. This can be evaluated (see p.326) for single isolated cells fairly easily, but it becomes very complex when cells outside the impaled cell must be included due to plasmodesmatal coupling.

At this stage, the best that can be done is to assume that if R_p is very different from R_T (which itself requires establishment), then at the first time constant measured, membrane resistance of the less conducting membrane can be calculated by finding that area which renders $1 \mu\text{F}/\text{cm}^2$ from the observed capacity, on the basis that $1 \mu\text{F}/\text{cm}^2$ may be taken as a biological constant. Clearly this is far from satisfactory and much further work is needed to resolve it.

Development of the Equipment.

There were two major problems with the prototype single-barrel bridge equipment. Firstly, runs, with a barely sufficient number of frequency points, took rather long to perform and were very tedious to do because, at each f point, amplitude of applied AF, sensitivity of PSD input and phase of PSD all required resetting, followed by balancing of the bridge for the preparation. This took several minutes to perform on each point, so that a complete run took about $\frac{1}{2}$ hour. This is a long time (in my experience) to hold a good impalement in a cell.

Secondly, the Princeton JB4 PSD was stretched to its limit at 6 kHz, but it was desirable to go up to at least 10 kHz. Actually not all the problem is in the PSD, because, at the high impedances involved, it is very difficult to construct a perfect bridge due to stray capacity between components.

A new system is therefore in course of construction. A Princeton 120 Lock-In Amplifier has been chosen, with an upper frequency limit of 150 kHz! The problem of frequency changing has been approached by construction of a multiple switching system. The phase control of the "120" has been brought out to an external preset resistor. Frequency control already requires external components in this model, and AF amplitude and input sensitivity merely require external resistive attenuators. Therefore all the components for any given f point can be assembled on to a printed circuit board (PCB) etched to a suitable design. Each PCB has a row of edge contacts soldered on to the copper strips and these make contact with phosphor-bronze contacts on a rotatable drum with snap position lock. A frame has been constructed to carry the set of PCBs arranged radially, with contacts facing the drum. Each PCB slips in and can be locked in place. This arrangement allows all the system variables to be preset, and then in a run on a cell, each f point can be instantly selected and then the bridge balanced. This system is not however yet completed, but promises to speed up AF impedance spectrum runs very considerably.

Cell Membrane Resistance - Theoretical Considerations.

Three questions will be addressed, namely :

- (1) What is the nature of the membrane resistance measured by the single-barrel bridge method ?
- (2) How valid are such measurements in terms of the cable properties of a long cell ?

(3) How does communication of the measured cell to others in a tissue through plasmodesmata affect the reading ?

These questions take us into some very stormy and sometimes uncharted waters, and only a brief discussion can be presented here.

(1) The single-barrel bridge method introduced earlier functions by application of alternating voltage to the cell membrane, small in amplitude relative to the cell membrane potential. Now, in general, a membrane may be expected to display non-linear resistance or rectifying properties - i.e. the current-voltage characteristic is not a straight line. There is plenty of evidence for this kind of behaviour (Cole 1972). Therefore the single-barrel bridge method measures a slope or incremental resistance, which must be distinguished from the chord resistance (which refers to total voltage/total current). This consideration also raises a warning in regard to d.c. resistance measured, as above, by use of a load resistor. It is important that the membrane potential be not pulled down more than a few percent if a strictly incremental d.c. resistance is to be measured.

D.C. Resistance - Precautions.

If we refer again to Fig.24, we see that not all of the source of potential is in the cell. There is also CVS, and the difference between the two electrode potentials. In E_m measurement CVS is used to back off

the electrode asymmetry, but in R measurement, CVS is adjusted to produce a convenient reading on the electrometer - say, 100 on some scale - so that percent depression on applying load can be read easily.

Now Ohm's Law gives that the current flow through the load is $V_o / (R_e + R_m + R_L)$, where V_o is the sum of all voltages sources in the loop, and is measured without load. Voltage, V_L , across R_L is of course

$$V_o R_L / (R_e + R_m + R_L), \text{ hence we had that } R_e + R_m = R_L \left(\frac{V_o}{V_L} - 1 \right).$$

But this current also flows through R_m , in such a sense as to offset E_m , if CVS is used merely to adjust a forward reading of the electrometer, not to reverse it.

Thus the membrane potential will be depressed by

$$V_o R_m / (R_e + R_m + R_L)$$

Thus :

$$V_m = E_m - V_o R_m / (R_e + R_m + R_L)$$

where V_m is the loaded membrane potential. Now since :

$$V_L = V_o R_L / (R_e + R_m + R_L)$$

it follows that :

$$V_m = E_m - V_L \frac{R_m}{R_L}$$

Now, what is required to measure incremental d.c. resistance is for $V_L (R_m / R_L)$ to be small compared to E_m . In prospect this is difficult to achieve until R_m has been found by the bridge, since d.c. loading cannot give R_m separated from R_e . However, in retrospect new runs should be performed, keeping $V_L (R_m / R_L)$ much smaller than E_m .

If we take some typical figures, for example

suppose $R_L = 100 \text{ M}\Omega$, $R_m = 30 \text{ M}\Omega$ and $R_e = 15 \text{ M}\Omega$ with $E_m = -70 \text{ mV}$ and $V_o = -100 \text{ mV}$, then on these figures

$V_L = -69$ mV, a depression which is easily read. However $V_L (R_m/R_L) = -21$ mV, which is hardly small compared to an E_m of -70 mV. In practise, the measurement of d.c. resistance by loading requires a fairly large depression of reading for it to be accurately read, since it is not perfectly steady. It is always difficult to measure small changes in large quantities. Therefore it would seem that merely raising R_L would not help very much. The measurement of $R_e + R_m$ however at very low frequency (0 - 30 Hz for maize root cells; the top plateau, Fig.44) by the bridge promises therefore to be a more reliable measure of d.c. resistance, since the PSD can detect, over noise and fluctuations which render d.c. measurement difficult, the out-of-balance signal from the bridge. In most cases, the d.c. resistance measured on maize root cells was not far different (though usually a bit lower) than low-f resistance, so presumably maize root cells display only slight membrane rectifying properties.

Significance in Terms of Fundamental Processes of Membrane Resistance.

The current through the membrane is carried by ions, and the ionic current increment for a given ionic species caused by a membrane voltage increment is obtained (if we accept the constant field assumption) by differentiating the Goldman flux equation (Appendix 4) as follows :

$$j = -P \frac{z^2 F^2 E_m}{RT} \cdot \frac{C_o - C_i \exp\left(\frac{zFE_m}{RT}\right)}{1 - \exp\left(\frac{zFE_m}{RT}\right)} \quad \text{amps/cm}^2$$

Therefore :

$$\frac{\partial j}{\partial E_m} = -P \frac{z^2 F^2}{RT} \cdot \frac{\partial}{\partial E_m} \left(\frac{E_m C_o - E_m C_i \exp \frac{zFE_m}{RT}}{1 - \exp \frac{zFE_m}{RT}} \right)$$

as the contribution for one ionic species only. This leads to some rather complicated mathematics, but it has been shown that total slope conductance (Hope 1971, etc.) :

$$g_s = \frac{F^2}{RT} \cdot \frac{E_g}{\left(\frac{1}{C_o^+ - C_i^-} - \frac{1}{C_i^+ - C_o^-} \right)} \quad (40)$$

where g_s = total membrane area-specific slope conductance due to all monovalent ions present. (Divalent ions usually contribute little).

E_g = Goldman potential for ionic activities and permeabilities prevailing.

$C = \sum_j P_j a_j$, taken separately for each side of the membrane for permeant cations and anions.

Therefore g_s is equal to $1/R_m$ as measured by the single-barrel bridge method.

Obviously this is a very complex field that is being entered now, and cannot be fully discussed here.

However it is pertinent to observe that membrane slope resistance is dependent upon the voltage across the membrane and on the concentrations of ions on both sides, as well as on membrane permeabilities, which may indeed themselves be voltage and concentration dependent.

As a simplification, if it were true to say that most of the current were carried by, say, K^+ ions, on the assumption that P_K were very large compared to the permeabilities of the membrane to other ionic species and if $E_G \sim E_K$ (i.e. K^+ is either not pumped or pumping of it has little effect, see p.134), and if neither

K_o nor K_i were zero, then we may write :

$$g_{sk} = \frac{F^2}{RT} \cdot \frac{RT}{F} \cdot \ln \frac{K_o}{K_i} \sqrt{\frac{1}{P_k K_o} - \frac{1}{P_k K_i}}$$

Therefore :

$$R_{mk} = \frac{1/K_o - 1/K_i}{P_k F \ln K_o/K_i}$$

or

$$R_{mk} = \frac{1}{FP_k} \cdot \frac{K_o - K_i}{K_o K_i} \cdot \ln \frac{K_i}{K_o} \quad (41)$$

It should therefore be possible, in theory, to calculate P_k if R_{mk} can be measured by the single-barrel bridge method, and if K_o and K_i are known. Similar expressions could be written for other ionic species, providing the same conditions apply for them as we assumed above for K^+ . As a means of isolating the ionic conductivity one wishes to measure, one may reduce all other mobile ionic concentrations to zero in the bathing medium, although this procedure is not without its hazards. It will alter the membrane potential (Goldman equation), and since P is probably potential dependent in all cells in a way somewhat parallel to the known dependence in nerve and muscle cells, it would be necessary to inject current through the measuring microelectrode, as provided in Fig. 34, to repolarise the membrane back to its resting potential in normal bathing medium. This may affect the properties of the microelectrode, and so it becomes clear that resistance measurements are not easy to interpret in terms of fundamental processes.

Although, in principle, measured values of R_m can be used to calculate a permeability (which I designate P_R), there seems to be an anomaly. P_R measured in this way is usually higher for cations in biological membranes than permeability (which I designate P_ϕ) measured by radio-isotope tracer fluxes. This was observed for squid giant axons by Hodgkin and Keynes (1955) whose results for K^+ gave $P_R/P_\phi = 2.5$, and in Nitella translucens, Williams et al. (1964) found the ratio to be 10.

The breakdown of the Nernst-Einstein relation* has also been found in certain non-living membranes, but curiously the discrepancy is the other way round. Thus for activated collodion membranes, of both anion and cation permeable types, Gottlieb and Sollner (1968) found that P_ϕ was greater than P_R . An extreme case was found by Holz (1977) on artificial planar lipid bilayer membranes activated with the ionophore X537A. P_ϕ , measured by fluxes was larger than P_R measured electrically for dopamine (an ion) by as much as 10^5 times ! He suggested that the discrepancy was due to a tightly coupled electrically "silent" exchange diffusion mechanism for dopamine - but, of course, this cannot be taken as comparable with the behaviour of mineral ions. Nevertheless, a similar property has been found for Cl^- transport in barnacle muscle fibres (Russell and Brodwick 1979), so this direction of the discrepancy is not confined to non-living membranes.

* The Nernst-Einstein relation is quoted in Appendix 4, and gives that $\Phi R_m = RT/F^2$.

Clearly there are several mechanisms operating to cause discrepancy between P_ϕ and P_R . Hodgkin and Keynes (1955) proposed a mechanism by which P_R could be raised above P_ϕ . The electrochemical formulation which leads to the Nernst-Einstein relation treats ions as moving independently of each other. If however ions were confined to cross the membrane only in file down ionophore channels, then as they showed, in a brilliant piece of argument, the ratio of P_ϕ/P_R would be equal to the average number of ions in a file, which for the squid axon was therefore 2 - 3. They presented a model involving shaken balls in two chambers in communication via a channel of varying length, which showed similar properties. One may invoke an approximate analogy of a row of red snooker balls. The white ball cued up behind the row would send off a red ball from the other end, and the cue ball would not penetrate the row. It would require the exercise to be repeated with as many further balls cued up as the number in the row before the original white ball would penetrate the row. Thus isotope exchange is limited by the number in the row, but of course each transfer of an ion, whether labelled or not, contributes to conductance. Thus we expect $P_R > P_\phi$.

A mechanism operating in the reverse direction is ion-ion exchange mediated by a carrier, possibly rotating or oscillating in the membrane. Although this process will allow isotope tracer exchange, it is electrically "silent" since no net charge is transferred

at the site. This may well explain the enormously high ratio of P_{ϕ}/P_R seen by Holz (1977)

Williams et al. (1964) propose yet another mechanism involving electro-osmosis. The theory of this process has been worked out by Fensom and Dainty (1963), and Dainty et al. (1963), and the idea is that an electrical potential deviation from resting membrane potential causes water to flow across the membrane, entraining ions in the flow, thus effectively raising the mobility of the ions through the membrane. This mechanism will also lead to $P_R > P_{\phi}$.

It therefore seems that whether a membrane disobeys the Nernst-Einstein relation, and if so which way, will depend on the relative extent to which these various mechanisms prevail against or with each other. This complex situation is far from clarified, and there may be yet further mechanisms.

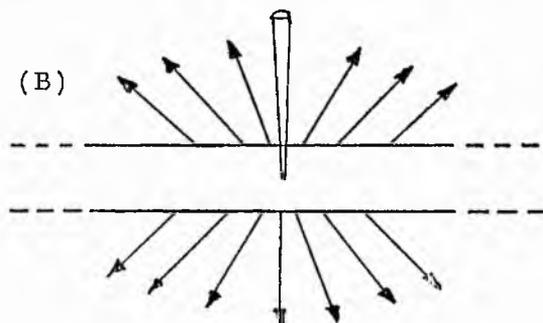
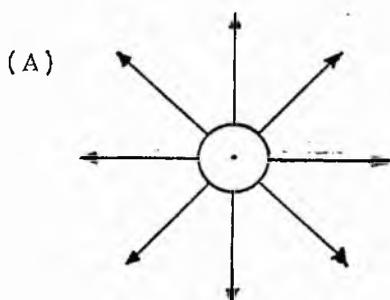
Kelvin Cable Properties of Long Cells.

(The concept of the Kelvin cable was introduced on p.303 (q.v.) in connection with micropipettes.) It has already been mentioned that a long cell may behave as a distributed RC network, and that it is unsafe to ignore such behaviour (p.283). It has also been pointed out that not only may a cell individually behave as a long cable, but also that a cell may be electrically connected to its neighbours through plasmodesmata, thus extending

the "cable" still further (p.316). It is therefore a relevant question as to whether cable properties disturb the measurement of R_m by the single-barrel bridge on long cells, and what criteria must be applied.

It is intuitively obvious, for steady current inserted by a microelectrode at some central point along the length into a long narrow cylindrical cell, that most of the current will leave the cell near the electrode and that little will flow through the ends, if the resistance of the cell sap is appreciable. It is also clear that the rate of fall-off of current density (and hence of voltage deviation across the membrane) will depend on three factors :

(i) the resistance of cell sap along unit length of the cell, r_i ($\Omega/\text{cm.}$), equal to the resistivity of cell sap, ρ_i , multiplied by the cross-sectional area of the cell, πa^2 , (ii) the resistance of the membrane over unit length along the cell, r_m ($\Omega.\text{cm.}$), equal to $R_m/2\pi a$, where a is the cell radius, and (iii) the resistance of external bathing medium to outward flow of current over unit length of the cell, r_o , a quantity which is not clearly defined in published literature. If we consider that current is flowing away from the cell radially in a plane perpendicular to the cell axis (A, below), and if we may ignore the



fact that it is also divergent in a plane containing the cell axis (B), on the basis that this rate of divergence is very much less rapid than the radial, then we may integrate shells without needing to know the profile of divergence B, but assume the flow lines are parallel. A shell at radius r and thickness dr , over unit length of cell, will present resistance $\rho_0 dr/2\pi r$; therefore integrating between limits $r = a$ and $r = b$, we have that $r_0 = \frac{\rho_0}{2\pi} \ln \frac{b}{a}$, which is indeterminate unless we know what is the sink for the current, and its geometry (which determine b). This would appear to explain why in published literature what precisely is meant by r_0 is usually rather glibly glossed over. It is usually stated that r_0 may be considered negligible. This is probably fair on two counts : (a) usually the resistivity of cell membrane material is enormously larger than external medium, whatever it is between seawater and freshwater and (b) we are concerned about the fall-off of current density at the membrane as we proceed along the cell, and fall-off outside is not of interest, provided the outside medium is sufficiently conducting not to influence the pattern of current flow. Nevertheless this question of the significance of r_0 should be better defined than it has been in any literature I have seen, but this is probably explained by the limitations inherent in the assumption made by Kelvin (see below).

By the treatment of Kelvin (1855), who assumed that inside and outside the membrane potential was

independent of radius, the theory for long cells under steady current yields a so-called "space constant", λ which is given by $\sqrt{(r_m/(r_i + r_o))}$, or since r_o may be ignored, it reduces to $\sqrt{(r_m/r_i)}$, which, if we recall the units of r_m and r_i , defines a length. Then the voltage deviation across the membrane induced by steady current injection at point $x = 0$ is given at any distance x along the cell by $V_x = V_o \exp(-x/\lambda)$ for an infinite cable or :

$$V_x = V_o \frac{\cosh \frac{L-x}{\lambda}}{\cosh \frac{L}{\lambda}} \quad (42)$$

for a terminated cable (i.e. one of finite length ending insulated), where V = voltage deviation at membrane induced by current flow, and L = cell length. The terminated cable form is appropriate for an isolated long cell.

However, the bridge technique introduced earlier applies alternating current to the cell, when of course membrane capacity plays a very important role. Whereas, for steady current (d.c.) we are only concerned about the fall-off of current density with distance along the cell, here we are also concerned about phase shift with distance. A partial theory for cables under a.c. has been presented by Williams et al. (1964), who extended the treatment of Hodgkin and Rushton (1946), itself based on Kelvin's assumption, and showed that the relevant space constant was the complex quantity

$$\lambda_c = \lambda / (1 + j\omega\tau)^{1/2} \quad \text{where } j = \sqrt{-1}, \omega = 2\pi f$$

and $\tau = R_m C_m$, the membrane time constant.

They did not however discuss phase shift, which is a severe weakness, because if the oscillation remote from the intracellular electrode is in antiphase with the electrode itself, the impedance measured will be far from simply related to membrane parameters.

What is relevant to the present study is whether the space constant is long or short compared to cell length. The following values may be calculated from Eqn. (42) :

If $L = \lambda$	$V_x/V_o = 0.731$
" = 10λ	" = 0.0067
" = $\lambda/10$	" = 0.996
" = 2λ	" = 0.410
" = $\lambda/2$	" = 0.915

The space constant :

$$\lambda = \sqrt{(r_m/r_i)} = \sqrt{\left(\frac{R_m}{2\pi a} / \frac{\rho_s}{\pi a^2}\right)} = \sqrt{\left(\frac{R_m}{\rho_s} \frac{a}{2}\right)}$$

where ρ_s = sap resistivity. It is somewhat unclear as to what should be taken here for a maize root cell, resistivity of vacuolar sap or of cytoplasmic sap.

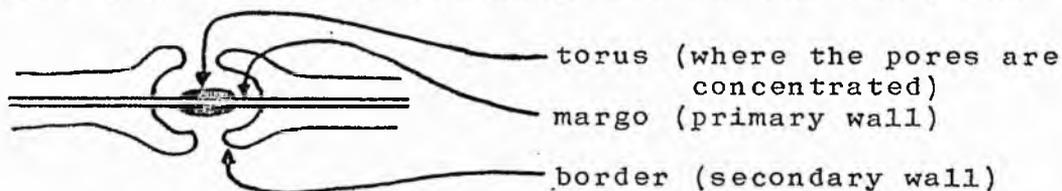
Presumably the former is more relevant, but I do not know its precise concentrations of ions. Let us therefore assume that it is equivalent to between 30 and 100 mM NaCl or KCl, which will have resistivities of the order of 250 and 70 Ω .cm. respectively (derived from data in Kaye and Laby 1956). Now the cell membrane has R_m of the order of 5 $k\Omega$.cm² (according to bridge measurement, Fig. 44). Therefore $\lambda = \sqrt{\left(\frac{5000 \cdot 10 \times 10^{-4}}{250 \cdot 2}\right)}$ for a 20 μ m diameter cell containing 30 mM electrolyte, which works out to 0.10 cm. or 1 mm. If the sap were 100 mM electrolyte, λ would be 2 mm. These dimensions are of the order of

10 x larger than cell length (200 μm), so it would be fair to assume that cable properties may be ignored at d.c. and low frequencies for maize root cells individually, if they were isolated from their neighbours.

However, the complex or quadrature component of λ_c which equals $\lambda/(\omega\tau)^{1/2}$ where τ may be taken as $5 \text{ k}\Omega\cdot\text{cm}^2 \times 1 \text{ }\mu\text{f}/\text{cm}^2$ or 5 msec., for a.c. fed to maize root cells becomes comparable with cell length at frequencies between 800 Hz and 3 kHz. It therefore appears that there is a high probability that the ends of the cell may not be closely in phase with the centre, and this may offer an alternative explanation rather than a second time-constant (due to more than one membrane in the current path, see p.315) for the poor lower plateau seen on some spectra. The position of the electrode in the cell would also influence this; thus if it were near one end, the phase difference over the cell would be more than for a centrally placed electrode. Loss of phase synchrony may well restrict the use of a.c. techniques (and it would be worse for square pulse techniques than for sinusoidal) to near spherical cells. In retrospect then, I may have been rash to try to measure R_m and C_m on so long a cell as a maize root cell, and the problems do not end here (see below). One comfort is found in the correspondence, at least in order of magnitude, between R_m derived by the bridge and R_m measured by d.c. with double-barrelled micropipette electrode on the same cell (see p.314).

Cell-cell Coupling. The Symplast.

In a tissue, cells may not be considered as isolated. There are usually cytoplasmic bridges passing through pores in the dividing wall between neighbouring cells (in plants), called plasmodesmata, thus connecting the cells to form a "symplast". In animal cells, a somewhat similar phenomenon occurs at nexal junctions, such that so-called "gap junctions", where the cell membranes of neighbouring cells run together but do not quite touch, allow electrical coupling between cells, presumably due to modified membrane permeabilities to ions at nexal regions. Animal physiologists recognise this in heart and smooth muscle, when they call it an "electrotonic syncytium". However, if I may confine my attention to plants, it is known (Hall 1976) that plasmodesmata may extend through dividing walls (usually at greater density on end walls than on side walls), generally in closed associated groups called "pits", which may or may not* be "bordered" - i.e. have an overhanging flange of secondary wall on each side, thus :



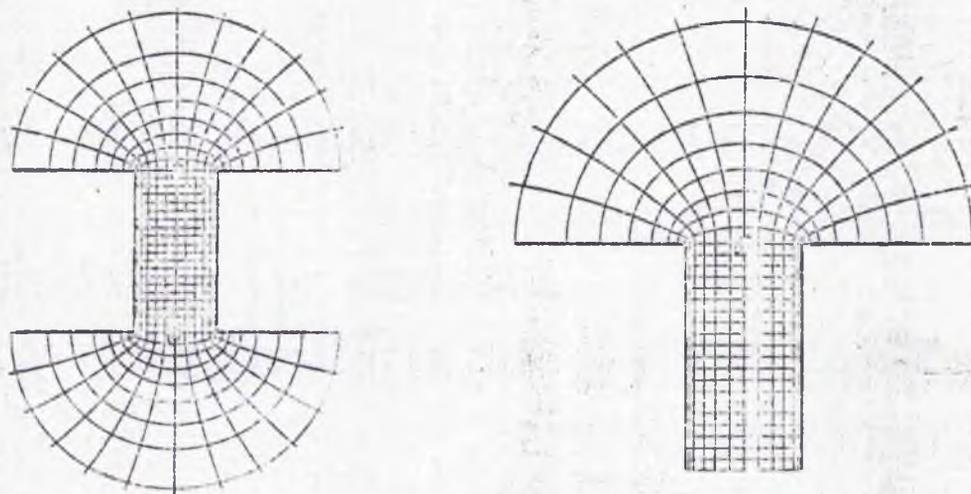
Another form of cell-cell coupling is found in sieve plates, where larger pores connect the cytoplasm of neighbouring cells. In xylem, the protoplasts may die, leaving long vessels of empty cells in line, connected through multiperforated end walls which act as fluid conducting elements, but we are here concerned

* Unbordered groups of plasmodesmata should strictly be called primary pit fields (Esau 1953).

Evidently the morphology of the situation can be very complex, but the problem of cell-cell coupling can be treated theoretically by adopting simplifying models. Tyree (1970) has presented a theoretical analysis of symplast transport, but although his paper contains much information, his theory embodies an assumption at the outset (his Eqn.(1)), which may not be true, where he states that the transport of material through pores under a driving force is proportional to the fractional area of a wall occupied by plasmodesmatal pores. This is not quite true (although, as it turns out, approximately correct for the dimensions in the case of plasmodesmata, see p.344), because it fails to take into account the fact that passage of material into or out of a small pore, follows a law very different from an area or radius-squared law, and is much closer to a law of the first power in radius. This is caused by the convergence of material to the pore on one side and its divergence again on the other. We have already met this problem for diffusion away from a spherical source in regard to resistance at the tip of a micropipette electrode (p.271), where integration of shells at equal potential (isopotential shells) yielded a first power law in radius. In the case of a plasmodesma in a cell wall, which we may regard as a plain hole, filled with cytoplasm (not necessarily identical to bulk cytoplasm), the flow lines at the surface will fan out (Fig.45, from Brown & Escombe 1900) but will not be radial, and so isopotential shells will not be spherical. To avoid having to decide which way the flow is travelling,

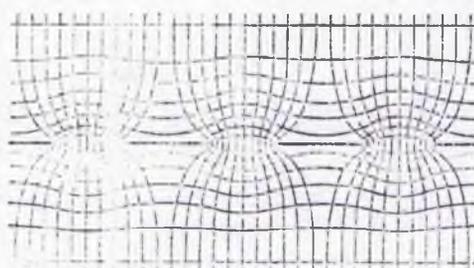
Fig. 45

FLOW PATHS AND ISOPOTENTIALS THROUGH
A SINGLE APERTURE.



from Brown & Escombe (1900)

FLOW PATHS AND ISOPOTENTIALS THROUGH
A MULTIPERFORATE SEPTUM.



from Brown & Escombe (1900)

to (con-) or away from (di-) a pore, it is simpler to drop the prefixes and simply call it a "vergence resistance".

Vergence resistance at areas of local contact or restricted access communicating with a near-limitless bulk is a common phenomenon. Thus the end effect on an organ pipe (Helmholtz 1882, Rayleigh 1870, 1877), the fringe effect on a circular plate on its electrical capacity in air, the return magnetic path on a finite solenoid in air (my novel approach to inductance calculation, using a modified vergence theory, unpublished) electrical contact resistance (Holm 1958), thermal contact resistance (pan on a hot plate, transistor on a heat sink), diffusion of water vapour and gases in general at orifices (Stefan 1873) and at stomata on leaves (Brown and Escombe 1900) and the present case of vergence resistance of plasmodesmata, are all similar phenomena in terms of their spacial relations. (There is a slight difference, which should be recognised (but wasn't always by theorists in this field) between inertial flow, such as air flow at the end of an organ pipe and flows where inertia hardly, if at all, enters into it, such as electrical resistance in electrolytes and metals).

The end correction on an organ pipe ending in an infinite flange was calculated by Helmholtz (1882) (ignoring inertial effects) to be $\frac{\pi}{8}d$, where d is the diameter of the top of the pipe. This is equivalent to extra length of diameter d , so assuming the same law holds good for electrical conduction down a channel of diameter d ,

opening at the surface into infinite bulk, we may calculate the resistance equivalent to the vergence resistance as follows :

$$R_v = \frac{\rho l}{a} = \frac{\rho \frac{\pi}{8} d}{\frac{\pi}{4} d^2} = \frac{\rho}{2d}$$

where ρ is the resistivity of electrolyte outside the pore in bulk. For a hole in a septum, there are vergence regions at each side, so the total vergence resistance will be $\frac{\rho}{d}$. This agrees with Holm (1958) for electrical contacts (and he gives a proof of the relation after Kottler (1927) and Smythe (1939)), and with Hall (1975) for pores in biological membranes. The pore channel itself presents further resistance. Once below the surface, flow lines are parallel in a pore channel of uniform diameter. Therefore the extra resistance due to the pore channel is $\frac{4\rho' l}{\pi d^2}$ where ρ' is the resistivity of electrolyte in the channel (which may not be identical in plasmodesmata to ρ outside). Total resistance of one channel with its vergences is thus $\left(\frac{\rho}{d} + \frac{4\rho' l}{\pi d^2}\right)$

However, individual pores do not exist in isolation in the middle of extensive areas of septum. They tend to be gathered into groups at pits. It is therefore necessary to extend the theory to include multiperforate septa, a problem which appears not to have been solved adequately before. Brown and Escombe (1900) mention the problem, but do not present quantitative relations for it, and as far as I am aware, noone else has done so since, surprisingly. The problem is a formidable one to tackle by mathematical analysis, so I have adopted

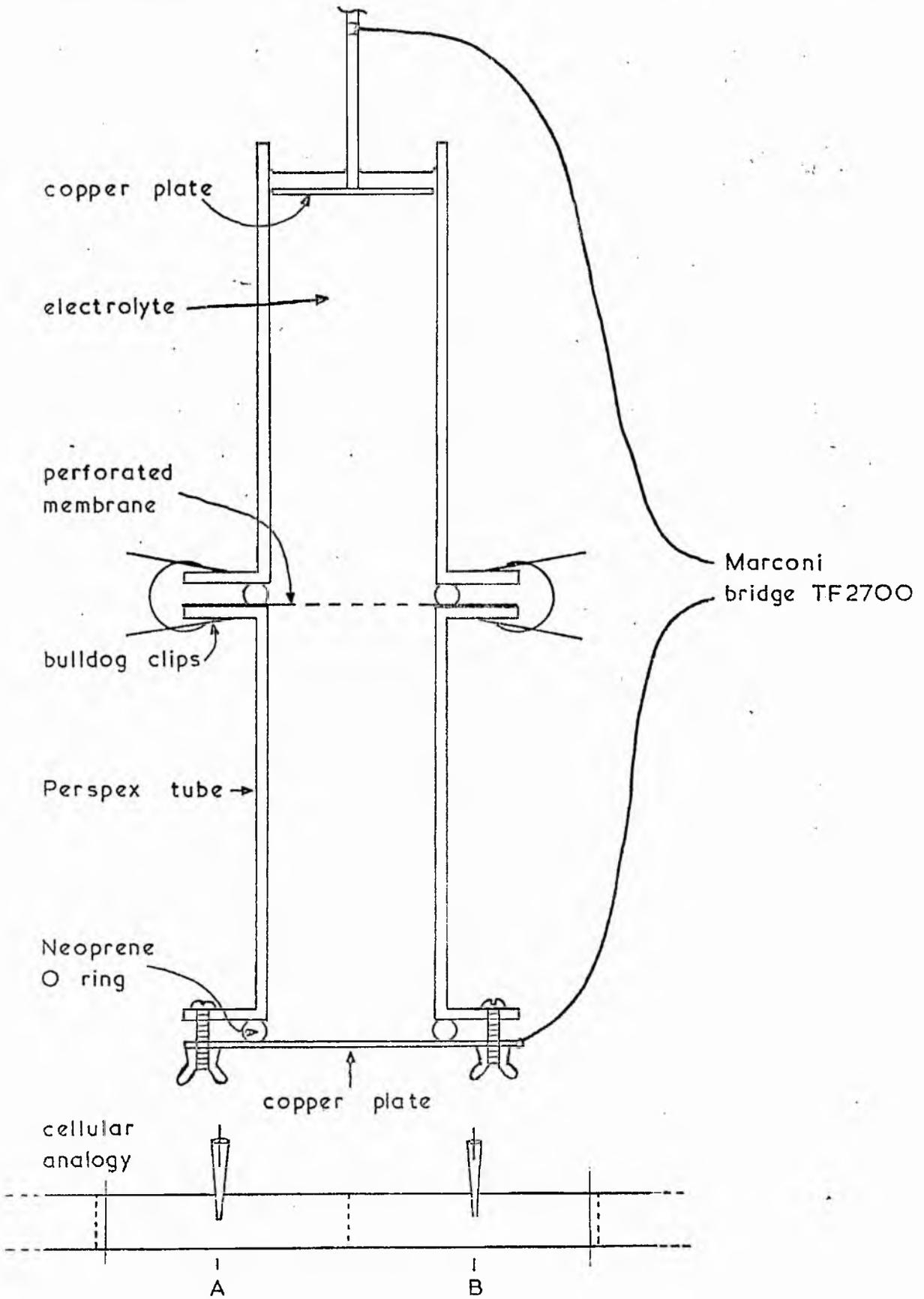
an empirical approach using a model, which should be accurate enough for practical purposes.

Model for Multiperforate Septum.

When pores in a septum are spaced far apart (i.e. their centre-to-centre spacing is many times their diameters), the flow lines interfere very little, and they may simply be summed by dividing the resistance of one pore by the total number. However when they are closely spaced, flow lines do interfere (Fig. 45). It is not convenient to tackle the multiperforate septum immediately, but instead it is easier to consider the tube of fluid standing above a pore, which may be regarded as being defined in diameter by the territory which a pore commands vis-a-vis its neighbours on the septum. Geometrical models for this will be considered below, but in order to solve the problem of a pore with its tube of fluid, a model system was constructed.

A Perspex tube (9.7 cm. diameter, 40 cm. long) was made (Fig. 46) in two parts; the upper portion ended in a flange at the bottom, and the lower portion had a flange at each end. The bottom of the lower portion was closed with a copper plate sealed with an O ring. Between the upper and lower portions was placed a piece of polythene membrane perforated with a hole, and the assembly was sealed with another O ring and held together with three "bulldog" spring clips. The assembly was filled with dilute acidified copper sulphate solution (concentration unimportant since

MODEL SYSTEM FOR STUDYING INTERCELL CONNECTION RESISTANCE.



measurements were relative and the same solution was used throughout). A second close-fitting copper disc soldered on to a brass rod was dipped into the electrolyte at the top, and resistance was measured with a Marconi TF 2700 a.c. Bridge (1 kHz sinusoidal). Because the electrodes were copper and the electrolyte contained Cu^{++} ions, there was no problem with polarisation at the electrodes and exhaustive tests without a polythene membrane (open tube channel) showed that the system behaved as a good Kohlrausch cell. In particular resistance was proportional to separation of the plates with no "end correction" for electrode effects. The polythene membrane was also tested for capacity, because this would be in parallel with the resistance of a hole. The polythene was about 5/1000 inch thick and gave a capacity of 130 pF, which presents a reactance of 1.2 M Ω at 1 kHz, and so may be ignored compared to electrolyte resistances which were of the order of hundreds of ohms.

Several membranes with centrally placed circular holes of a variety of diameters were individually introduced into the tube channel, and the resistances read. From these values, the resistance of the open channel was subtracted to give the extra resistance introduced by the constriction. The problem then was to find a mathematical law to relate hole diameter to this extra resistance.

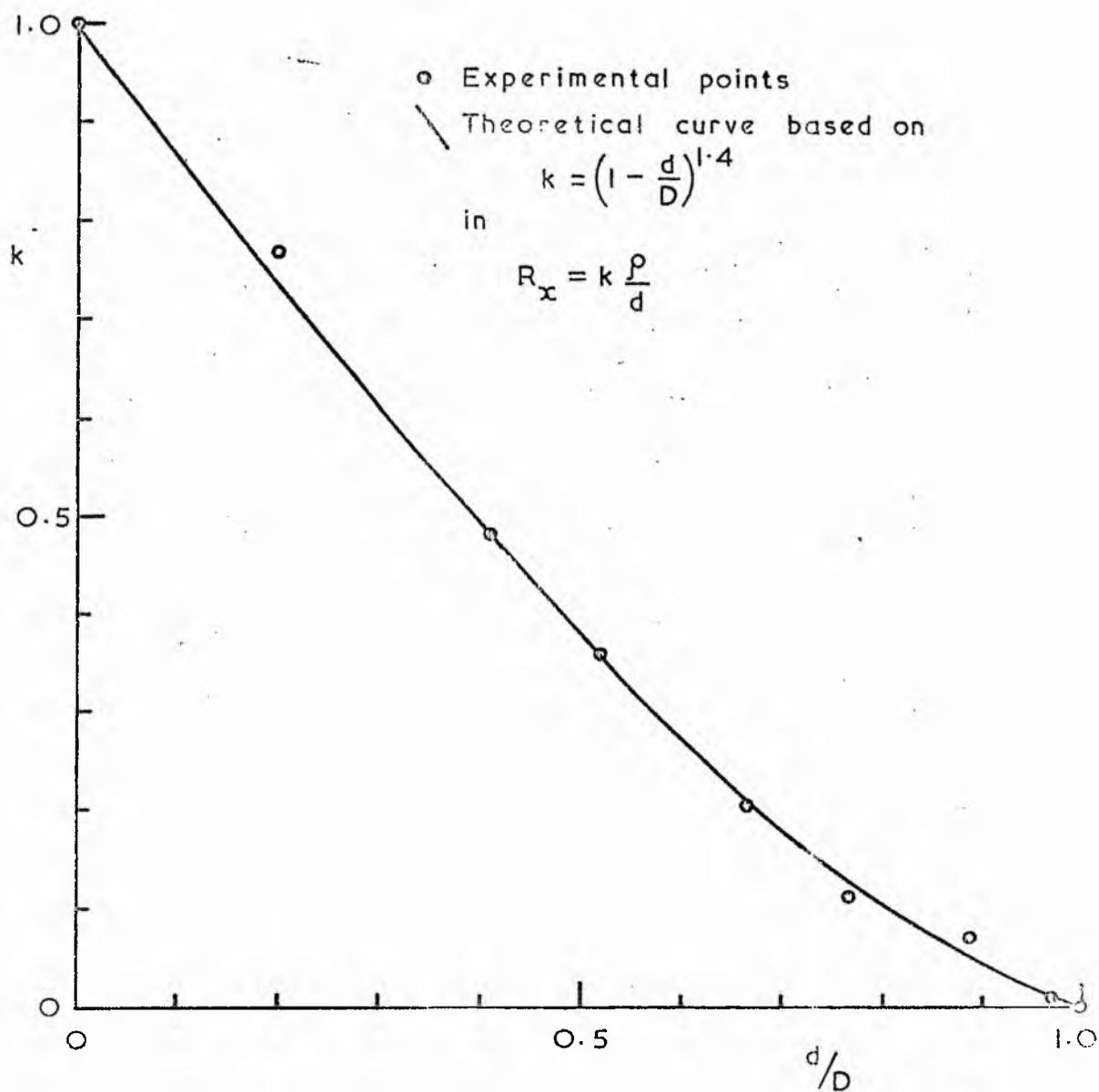
We already know that for a thin membrane (i.e. negligible pore channel resistance), vergence resistance of a pore very small compared to the tube channel diameter, D , would be ρ/d where d is the pore diameter. When the hole is equal in diameter to the tube channel, the membrane is contributing nothing to resistance between the upper and lower halves of the tube. Since what is wanted is the extra resistance introduced into the system by the presence of a perforated membrane, we now see that the desired function will run from 0 when $d = D$ to ρ/d when $d \ll D$. Therefore let the extra resistance due to the perforated membrane be R_x given by $R_x = k \cdot \rho/d$, where k embodies the desired relationship, and runs from 0 to 1 as d runs from D to 0. What is important, to make the relation general, is the ratio of d to D , not their actual values, since d is already incorporated in the equation for R_x . Thus k is some function of d/D .

Values of k were derived from the experimental data by dividing extra resistance introduced into the channel, R_x , by ρ/d , and ρ was obtained from a measure of the open-channel resistance. It was therefore possible to plot k against d/D from experimental data, and this gave a curve, Fig. 47. Various empirical relationships were tried, to see if they would fit the set of points, and eventually it was found that the form best fitting the data was $k = (1 - \frac{d}{D})^y$, where y was some unknown power. Now if $\log k$ is plotted against $\log(1 - \frac{d}{D})$ the gradient of the straight line so produced is the

Fig. 47

EFFECT OF CHANNEL SIZE ON INTERCELL
COUPLING RESISTANCE.

Measurements on a model system.



required power. It turned out to be 1.4. Thus the required form for a hole in a thin septum dividing a channel was :

$$R_x = \frac{P}{d} \cdot \left(1 - \frac{d}{D}\right)^{1.4}$$

Whether 1.4 is really $\sqrt{2}$ or not it is impossible to say from an empirical experiment, and this approach does not claim to produce a formula which would resemble the relation which a full theoretical analysis would produce, but, as Fig.47 shows, it does produce good agreement with experiment, and is certainly as accurate as is required in a biological context, where errors are likely to be much more serious in measures of the quantities involved.

It is now a simple matter to include the resistance of the channel in a long pore, if the membrane were of thickness l , Fig. 48. The term to be added is $\frac{4P'l}{\pi d^2}$ and to be rigorous one should subtract $\frac{4Pl}{\pi D^2}$ for the length of cell occupied by the membrane. Thus a single pore in its territory, with circular tubes of electrolyte above and below, the diameter of the territory, introduces extra resistance :

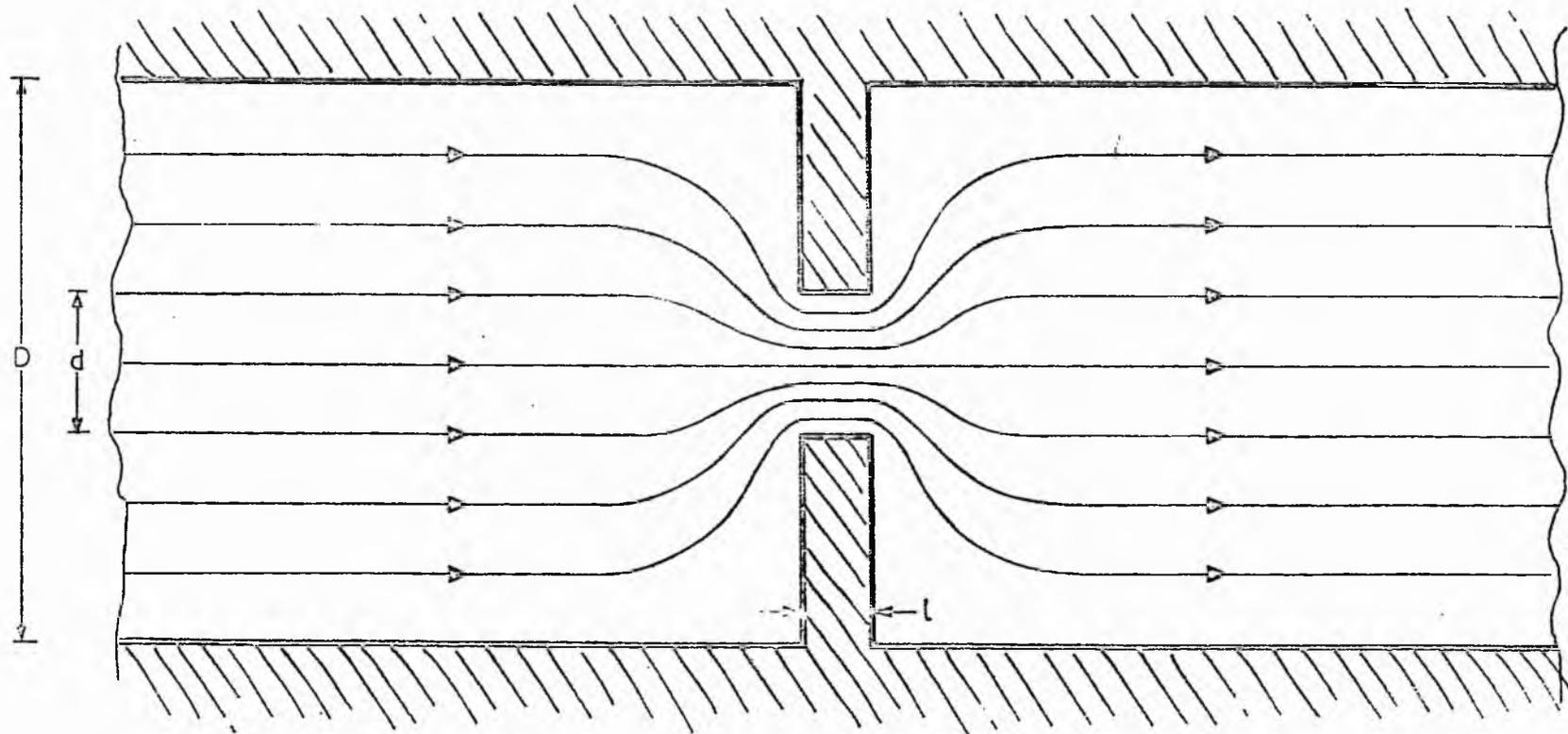
$$R_x = \frac{P}{d} \left(1 - \frac{d}{D}\right)^{1.4} + \frac{4l}{\pi} \left(\frac{P'}{d^2} - \frac{P}{D^2}\right) \quad (43)$$

It is now necessary to extend this to the multiperforate septum. This now raises the question as to how the pores are arranged over the septum. In a biological membrane they may well be randomly distributed, or nearly so, over the area of the wall in a pit. It is therefore of interest to examine two arrangements to see what effect arrangement pattern has on the summed resistance of a

Fig. 48

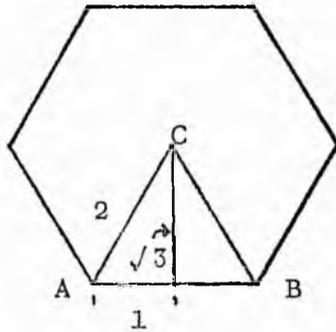
CURRENT FLOW PATTERN THROUGH A CONSTRICTION IN A CHANNEL

Constriction "large" relative to channel diameter.



Resistance introduced by constriction, $R_x = \left(1 - \frac{d}{D}\right)^{1.4} \cdot \frac{\rho}{d} + \frac{4\rho l}{\pi} \left(\frac{1}{d^2} - \frac{1}{D^2}\right)$

multiperforate septum. The two arrangement patterns which will be considered are hexagonal and square. The hexagonal arrangement is represented in Fig. 49, from which we see that the territory of any given pore is not circular. However to simplify calculation, I shall assume that Eqn.(43) may be applied to a tube of diameter D, whose area is equal to the area of the hexagonal territory of a pore. Let the spacing of two pores be S. Then let us consider the hexagon below, with side 2 units of length.



Triangle area $ABC = \sqrt{3}$ so hexagon area = $6\sqrt{3}$.

Hexagon "diameter" between flats = $2\sqrt{3}$, which is equivalent to S. Circle of equivalent area is given by :

$\frac{\pi D^2}{4} = 6\sqrt{3}$, so $D = \sqrt{\left(\frac{4}{\pi} 6\sqrt{3}\right)}$, which is equivalent to a

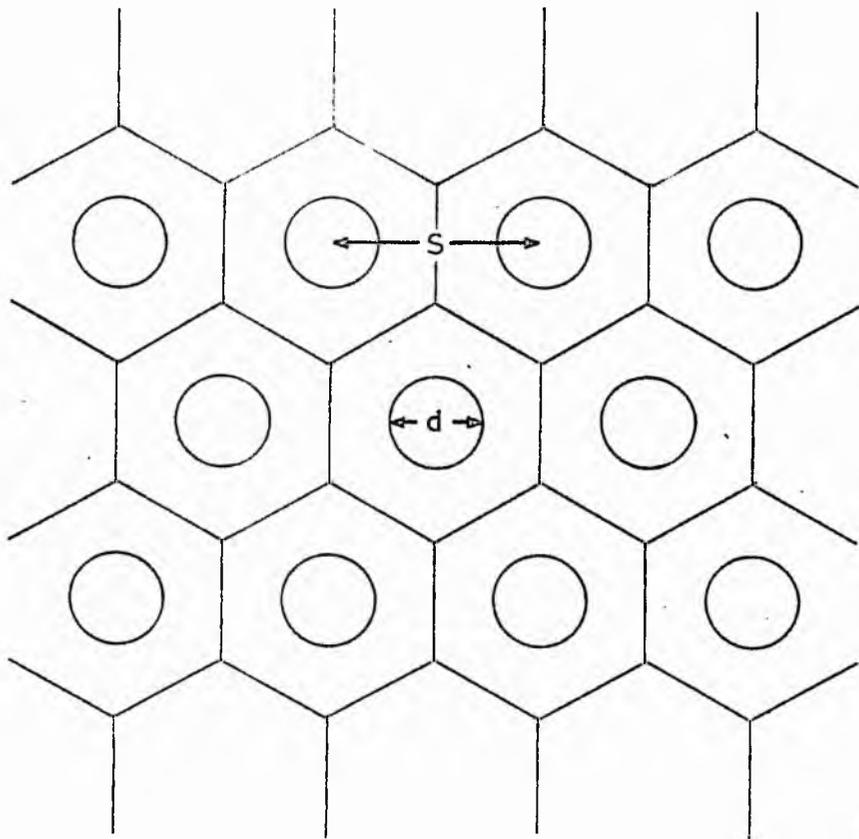
hexagon of "diameter" $2\sqrt{3}$. Therefore if hexagon "diameter" is brought to S, D becomes :

$$D = S \sqrt{\left(\frac{4}{\pi} 6\sqrt{3}\right)} / 2\sqrt{3} = S \sqrt{\left(\frac{2}{\pi} \sqrt{3}\right)} = 1.0501 S.$$

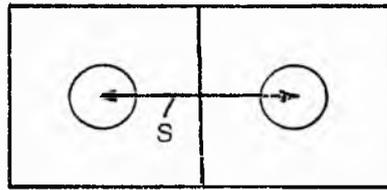
$$\text{Hexagon area} = S^2 \cdot 6\sqrt{3} / (2\sqrt{3})^2 = S^2 \cdot \frac{\sqrt{3}}{2}$$

Thus if there are n pores per unit area of septum, the value of S will be given by $\frac{1}{n} = \frac{\sqrt{3}}{2} S^2$ or $S = \sqrt{(2/\sqrt{3}n)}$
 $= 1.074 (1/\sqrt{n})$, or for Eqn.(43), $D = 1.05 \times 1.074 (1/\sqrt{n})$.

Fig. 49
MULTIPERFORATE SEPTUM. RESOLUTION INTO
SET OF EQUIVALENT CYLINDRICAL CHANNELS.



Let us now consider the square packing arrangement. If the spacing of pores is S then S will also be the side



length of a square territory. Its area is S^2 . A circle of equal area is given by $\frac{\pi D^2}{4}$, which is equal to S^2 , giving $D = 1.128 S$. If there are n pores per unit area of septum, then $1/n = S^2$, so $S = 1/\sqrt{n}$ and $D = 1.128 (1/\sqrt{n})$.

In fact the two arrangements give identical results since :

$$\sqrt{\frac{2}{\sqrt{3}} \times \frac{2}{\pi\sqrt{3}}} = \sqrt{\frac{4}{\pi}}$$

hexagonal square.

It therefore seems reasonable to conclude that the geometrical arrangement of the pores on the septum is unimportant, since D is invariant between these two dissimilar arrangements, and we may take $D = \sqrt{(4/\pi n)}$ to be general.

What we are interested to discover however is the total resistance presented by a multiperforate septum. If the septum has area A and there are n pores per unit area, the total resistance introduced by the septum is

$$R_X = \frac{R_x}{An} \text{ since the pores are in parallel.}$$

Thus :

$$R_X = \frac{1}{An} \left[\frac{\rho}{d} \left(1 - \frac{d}{\sqrt{(4/\pi n)}} \right)^{1.4} + \frac{4l}{\pi} \left(\frac{\rho}{d^2} - \frac{\rho'}{4/\pi n} \right) \right] \quad (44)$$

Eqn. (44) allows calculation of cell-cell resistive coupling if the pores are evenly spaced over the whole of the wall between two cells. However, mostly plasmodesmata are

gathered into pits or bordered pits. In both cases, each pit would have to be treated as a pore in its own right, thus the total resistance of a pitted wall would be the sum of R_x , by Eqn.(44) calculated for each pit torus, in which d is the pore diameter, n is the density of pores over the conducting torus of a pit and A is the area of the conducting torus, plus a further term for the vergence resistance of the pit areas themselves, treated as if they were pores. Let us designate the total cell wall area as A_w , the area of each pit torus as A_p , the pit density over the cell wall as n_w and the pore density in a pit as n_p . The diameter of a pit torus (assumed round) is therefore $\sqrt{(4A_p/\pi)}$, and so :

$$R_w = \frac{1}{A_w n_w} \left[R_{xp} + \frac{P}{\sqrt{(4A_p/\pi)}} \left(1 - \frac{\sqrt{(4A_p/\pi)}}{\sqrt{(4/\pi n_w)}} \right)^{1.4} \right]$$

no. of pits in parallel. resistance of pores in one pit. vergence resistance of pit itself.

where R_{xp} represents Eqn.(44) with A and n taken as A_p and n_p and d = diameter of conducting column in each individual plasmodesmatal pore, and R_w is the total coupling resistance of the cell through its walls to neighbouring cells. The equation may be simplified to :

$$R_w = \frac{1}{A_w n_w} \left[R_{xp} + \frac{P}{\sqrt{(4A_p/\pi)}} \left(1 - \sqrt{(A_p n_w)} \right)^{1.4} \right] \quad (45)$$

$A_w n_w$ is of course the total number of pits on the wall over a complete cell. For a bordered pit, the relevant area for the pit for calculation of its vergence resistance is its border orifice, but the torus area is still relevant to calculation of R_{xp} .

Evaluation of Eqn. (45) depends on the detailed morphology of cell walls in cells under study but certain generalisations can be given. Tyree (1970) states that the conducting area of a pore (excluding the plasmalemma which lines the pore) is usually about 4 - 6 nm in diameter. Hall (1976, quoting Mühlethaler) illustrates a primary pit field in a meristem cell of maize, displaying about 250 pores, and the pit field was elliptical with major axis 2.7 μm and minor axis 1.2 μm . Now further experiments I have carried out on the model system (Fig. 46), in which I varied the shape of a hole, showed that a hole followed a circumference law so long as the major to minor axial ratios were not more than 2, when thereafter the law gradually broke down. In this example, the axial ratio is greater than 2, but since only slightly, we will assume the circumference law holds. For a round hole, vergence resistance, R_x , was ρ/d . Now circumference, c , was πd . Therefore $R_x = \rho \frac{\pi}{c}$, and this will be used below.

However, in evaluating Eqn. (45), let us for the moment ignore the vergence resistance of the pit field as a whole (but see below). The pit field was 2.4 (μm)² approximately, therefore the density of pores in the pit-field was $250/2.4 = 104/(\mu\text{m})^2$ or about 10^{10}cm^{-2} . On these data, the pore vergence resistance of a single pit field amounts to about 1 M Ω , if we assume $\rho = \rho' = 130 \Omega \cdot \text{cm}$. as a mid-range value. The pore channel resistance adds per pit field another 275 M Ω , if we further assume for the sake of argument that the two juxtaposed walls total

1 μm in thickness. There is considerable variation in primary wall thickness at pit fields; thus Clowes and Juniper (1968) illustrate electron micrographs showing primary wall at pit fields with thicknesses ranging from about 0.5 μm to 0.1 μm . Even if the twin wall thickness at the pit field were to be as thin as 10 nm, the pore channel resistance totalled over the pit field would still be 2.75 M Ω , and 10 nm is of the order of the thickness of a single fibril of cellulose, so this is impossibly thin for any cell wall. This conclusion is to be expected of course, because, as stated on p.334, the vergence regions amount to an extension of length of $\frac{\pi}{4}d$, and if the pore is much longer than its diameter, then vergence effects will be small. It thus appears, on these figures only, that pore channel resistance would be the major source of resistance in plasmodesmata thus making Tyree's (1970) assumption of the area basis actually justified for this example, but he never mentioned the problem of vergence resistance, and so one cannot know whether he was aware of it or not. His assumption would not be true in all cell-cell couplings, such as macro-plasmodesmata and sieve plate pores, whose diameters are of an order of magnitude with their lengths. (Spanswick(1972) suggests that the pore core resistivity is 50-60 times higher than that of bulk cytoplasm, which if true would also help Tyree's assumption).

The vergence resistance due to the limited extent of the pit field itself, on these figures, is about

0.6 M Ω , which is surprisingly of similar magnitude to the summed vergence resistances of the pores in the pit field..

This is rather an unsatisfactory point to stop this quantitative analysis, but the discussion below will show why I cannot usefully proceed further without much more detailed information than is available to me on the tissue I used.

If we may assume that the theory above fully describes the electrical behaviour of a plasmodesmatal pit field, then, to proceed further, we need to know :

(a) what is the geometrical relationship of a cell to its neighbours, and

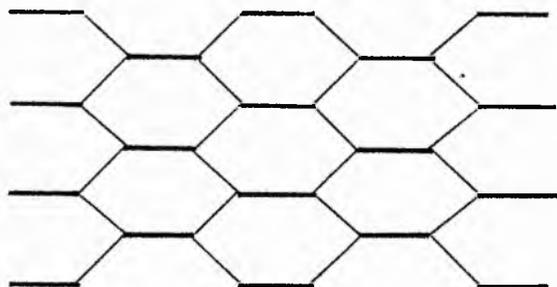
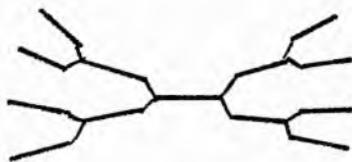
(b) how many pit fields communicate the cell under study to each of its neighbours individually.

These neighbours will then be connected via pit fields to further neighbours. Indeed to obtain a full picture of the electrical environment of a cell, an enormous amount of detailed information is needed, and the task appears to be formidable to the point of virtual impossibility.

There are three approaches published which indicate ways of proceeding from here, assuming one can reach this point satisfactorily. Spira (1971), considering dog heart muscle, has proposed a way of extending the cable theory of a single cell to include cell-cell coupling. He was concerned, of course, with intercalated discs (when gap-junctions provide the

coupling, see earlier) but the principle is the same as for pits. Like Tyree(1970) he ignores vergence resistance, and presumably he is also justified, as it happens, in doing so because intercalated disc membrane, though possibly more conducting than general cell membrane, will doubtless be much less conducting than the bulk cytoplasm, resulting in membrane resistance being much larger at the nexus than vergence resistance. Spira (1971) presented a modified cable "space-constant" which treated the intercalated discs as contributing a further drain on current as if to the outside in parallel with the plasmalemma, presumably on the basis that extensive cell-cell coupling amounts to that. This may well be a fair approach, and may circumvent the need to know in detail the tissue morphology.

The second approach, presented by George (1961), in an undoubtedly important paper, goes further and indicates how one may treat a syncytium. He pointed out that there are two (at least) possible spacial arrays for cells in a tissue which represent extremes, first the open array, two way branching with no re-entry, and secondly the closed array, two way branching with the maximum level of re-entry. This is illustrated diagrammatically below where the thick lines represent cells and thin lines represent cell-cell connections.



He did not consider the case of branching to more than two cells from each cell, nor is the treatment fully three-dimensional. He assumed that the syncytium was large compared to the space-constant, and that the resistance of extracellular fluid was negligible (as usual!). He established the following :

For the open syncytium :

(1) when $l \gg \lambda$ (l = "length of termination", effectively cell length.)

$$R_o(0) = \frac{2}{3} z \quad \text{at the end of the cell,}$$

where $z = \sqrt{(r_m r_i)}$, r_m and r_i as on p.327.

$$R_o\left(\frac{1}{2}\right) = \frac{1}{2} z \quad \text{at midpoint of cell}$$

where R_o is the observed resistance at the point in brackets. Thus when the attenuation length (λ) is short compared to the cell length, the solution reduces to the well known case of a single isolated leaky cell, as before, p.329.

2. when $l \ll \lambda$

$$R_o(0) = \frac{1}{3} z \frac{l}{\lambda} = \frac{1}{3} r_i l$$

and

$$R_o\left(\frac{1}{2}\right) = \frac{1}{2} r_i l$$

In this case, observed cell input resistance is independent of shunt resistance, r_m , of the membrane, and is only a function of longitudinal resistance in the cell sap! He presented a graph to enable the general case to be solved.

For the closed syncytium :

He used symmetry considerations to simplify the problem and produced Bessel functions to be solved. He solved these, adding the further assumption that discrete resistors may be used to replace the continuum of distributed resistance beyond a certain point away from the sampling point. By trying successive cases wherein the continuum was considered to start at the second, third and fourth mesh points out, he found that the function quickly converged, and he calculated practical examples for certain values of cell diameter and length, and these were shown on a graph of R_o , the observed cell input resistance, against r_m .

The summary of his conclusions indicates that with the kind of dimensions prevailing in maize root cells, for the open array, with $R_m = 3 \text{ k}\Omega \cdot \text{cm}^2$ or above, R_o is constant independent of R_m , but as R_m is decreased below $3 \text{ k}\Omega \cdot \text{cm}^2$, R_o does begin to fall eventually following a law $R_o \propto R_m^{0.5}$, with intermediate values of the power in the transition region ($2 \times 10^2 - 2 \times 10^3 \text{ }\Omega \cdot \text{cm}^2$). The closed syncytium follows the same law at R_m below this same transition region but the law reverts to $R_o \propto R_m^{0.25}$ above the transition. Unfortunately George (1961) dealt only with d.c. or steady current effects on a syncytium, and therefore he does not cover the a.c. case, which applies to my bridge technique.

There are however some conclusions one can draw. Although I used a.c., it is at low frequencies virtually equivalent to using d.c. At very high frequencies, it is virtually certain one would derive the microelectrode resistance on the lower plateau of the AF spectrum, because cell capacity would short circuit any cell resistance, whatever symplast complications there may be. Therefore the dip height will be equal to the input resistance of the cell. However, if George (1961) is correct, then I can state from his conclusions that, if the cells in maize root do form a symplast, open or closed, (and we know they do - of one kind), then the observed resistance cannot be equated with R_m (after multiplying by cell surface area) because either the value I observed, R_o , is independent of R_m (open array) or depends on $R_m^{0.25}$, which cannot be evaluated unless we know which kind of symplast array prevails, but see below, p.351.

Also I notice from George's conclusions that in both closed or open arrays, the value of R_o measured varies according to where in a cell the electrode is placed, with a variation of between $\frac{2}{3}z$ and $\frac{1}{2}z$ for $l \gg \lambda$ and $\frac{1}{3}r_i l$ and $\frac{1}{2}r_i l$ for $l \ll \lambda$ on the open array model and unstated by George (1961) for the closed case. Thus on the open array, electrode position will lead to a variation of $\begin{matrix} +15 \\ -0 \end{matrix}$ % above $\frac{1}{2}z$ when $l \gg \lambda$ and $\begin{matrix} -22 \\ +0 \end{matrix}$ % below $\frac{1}{3}r_i l$ when $l \ll \lambda$. The averages of large numbers of readings taken at random positions will therefore be 7.5 % above and 11 % below the midpoint values for

the two conditions.

George (1961) solved the problem for a very limited (though difficult enough) model - branch coupling was taken to be at the ends only (which may well serve for heart muscle, since intercalated discs tend to be so positioned) but in plants, although there is a greater area-density of pits at the ends than the sides, nevertheless there will be coupling to neighbouring cells along the sides as well as at the ends.

Also in all the previous discussion, it has been assumed that a cell, where not coupled through a pit to its neighbours, is coupled through its membrane to the external medium directly. In fact, even for a root surface cell, much of its surface will be coupled to external medium only through wall material. This is likely to be fairly highly conducting, but it may be incorrect to assume that this confinement of current flow has no effect on the situation. Cells in the region of root hairs also carry root hairs, of course, and these will considerably extend the surface area of a cell, and may well behave both as Kelvin cables in themselves and also as if almost equivalent to a hole in the cell membrane the diameter of the "hair", presenting only the vergence resistance of the hole to the cell. At this stage we do not know enough about the local membrane properties to comment on this further.

The third approach, namely, direct measurement of cell to cell transfer of current, with microelectrodes in at least two neighbouring cells will undoubtedly help to resolve these problems.

Spanswick (1972) has measured the electrical coupling between cells in higher plant tissues, using two electrodes (one for current injection, the other for voltage shift recording) in one cell and one electrode (to record voltage shift) in an immediately neighbouring cell and then in a next but one neighbour cell. He examined three species and found :

<u>Coupling</u>	Immediate neighbours	next but one neighbours *
Elodea canadensis	20 %	72 %
Oat coleoptile	13 %	-
Maize root cortex	24 %	-

* This refers to the coupling of the next but one neighbour to the immediate neighbour not to the current-injected cell. It leads to (a) below.

From an analysis of the results for Elodea canadensis in terms of network models to represent the symplast he was able to conclude that :

- (a) the plasmodesmata connect cytoplasms but not vacuoles
- (b) the input resistance measured on a cell is about 3 times lower than it would be due to R_m alone if there were no plasmodesmata. (This perhaps gives some hope that, after all, my measurements on maize could be used to derive a value for R_m - see p.349).
- (c) the material lining the pore of a plasmodesma has specific resistivity 50 - 60 times that of bulk cytoplasm (though I find that barely credible!).

Drake et al. (1978) and Drake (1979) have measured on oat coleoptiles the percentage of voltage displacement elicited in a cell into which current is injected which appears in neighbouring cells as about 10 %. Drake (1979) also showed how one may approach the interpretation of this in terms of the coupling resistance and membrane resistance, and drew attention to the theoretical treatment of Socolar (1977).

As yet I have myself made no such measurements (but designed the double-headed micromanipulator specifically with this in mind) on maize root cells, but Ginsburg and Laties (1973) and Ginsburg and Ginzburg (1974) have studied electrical potential profiles across and along the roots of maize, and Ginsburg and Laties (1973) attempt to interpret their data in terms of electrical conduction through stele, cortex and cell wall, drawing the conclusion that the cortical symplast excludes the vacuoles, as might be expected.

Despite the progress so far, it seems that a great deal of uncertainty will prevail for the foreseeable future about the electrical properties of membranes in syncytia and symplasts.

The final conclusion therefore is that although the a.c. bridge technique works correctly from an electronic point of view, the interpretation of results on complex tissues like maize roots is fraught with

vast and at present insuperable problems because of cell-cell coupling and cell length. It is therefore clear that much further work needs to be done on higher plants, but that probably at this stage of our knowledge, further work for the present should be concentrated on isolated single cells, such as phytoplankton, of near spherical shape.

SUMMARY OF FINDINGS AND CONCLUSIONS.

It was shown that membrane resistance could not be measured satisfactorily by means of direct or steady current because of the generally unknown and variable resistance of the tip of the micropipette electrode.

A review was then presented of established techniques from the earliest experiments on living material by examination of whole tissue or cell suspensions in Kohlrausch cells, through measurements by means of external electrodes on Characean cells to the use of intracellular electrodes in smaller cells. The merits of using electrodes of metal or provided with micropipette salt bridges were compared, and techniques using twin electrodes and double-barrelled electrodes were critically appraised for the measurements which could be obtained by them. The difficulty with twin electrodes was to impale a single cell satisfactorily with a second electrode while holding a first impalement. Double-barrelled electrodes offered a partial solution to this, but suffered with coupling resistance between the barrels leading to uncertainties in d.c.

measurements of cell membrane resistance, and capacitive coupling between the barrels rendered them unsuitable for membrane capacity measurement.

It was also pointed out that the popular use of square wave excitation leads to problems of interpretation since cells do not necessarily present one time-constant and square pulses contain all frequencies according to Fourier analysis. Sine wave excitation was therefore preferable.

Reasons were stated for seeking a lone single-barrelled micropipette electrode technique, and such methods both previous and subsequent to my new technique were discussed.

The design of an a.c. bridge was described and discussed in detail. The important novel feature was the use of a phase-sensitive detector (PSD) both to eliminate noise and to simplify interpretation of measurements in terms of an assumed model for the cell impaled on a microelectrode. Exhaustive tests with both electronic components in place of the cell and micropipette electrode, and also in series with a micropipette electrode in artificial cell sap were carried out. In particular the AF impedance spectrum of micropipettes was examined, and fortunately the specimens produced by the puller used with the glass quills used proved to be resistive up to about 6 - 10 kHz. AF impedance spectra were also taken on maize root cells.

It was pointed out that electric supply mains hum picked up by the microelectrode could easily be at a dangerously high level when fed into a cell and could well invalidate results even if it was not disastrous either due to membrane damage or, with excitable tissues like muscle, because it lead to the tissue pulling itself off the microelectrode. Such hum may not be detected in electrometer setups designed for membrane potential measurement only, and workers are advised to incorporate a high-impedance pre-amplifier and CRO. A "hum-bucking" device was introduced as a means of overcoming this nuisance (which still persists even inside a Faraday cage) without wasting time searching for causes of hum. The device produced a zero hum field around the electrode.

The theory of impedance measurement, using a PSD set to detect only the in-phase component of the out-of-balance signal from the bridge was presented, using vector analysis. It was shown that this method provides a very simple means of resolving the microelectrode plus cell into electrode and membrane resistances and membrane capacity with the use only of resistors as the balancing components in the bridge. AF impedance spectra of electronic components and living cells were then examined.

An investigation of double-barrelled micropipettes was then presented and the results obtained with them were compared with the single-barrel bridge method.

Double-barrelled micropipettes generally behaved like single-barrelled micropipettes if one barrel was left unconnected in the bridge system, but an anomalously high interbarrel capacity was found in tests using both barrels, thus reinforcing the conclusion that they could not be used with a.c. in a double-barrelled mode.

Problems with the prototype bridge system, particularly slowness of operation, were discussed and developments to improve the system were briefly described.

In the final major section, a discussion of the theoretical interpretation of resistance measurements in terms of fundamental mechanisms, membrane properties and practical difficulties was presented. A warning was sounded in regard to d.c. resistance measurement by resistive loading. Kelvin cable properties were considered both of the cells themselves and of symplasts. The effect of the symplast was examined and a theoretical analysis was given incorporating the results from a model system investigated experimentally and intended to imitate the properties of plasmodesmatal pores. An attempt was made to assess quantitatively cell-cell coupling. Published theories for the symplast and direct experimental measurements were summarised.

The general conclusions are as follows :

(1) The lone single-barrel bridge technique introduced here should enable smaller cells than hitherto to be investigated.

(2) The bridge itself worked well up to 6 kHz but it was desirable to extend the useful frequency higher. A new system should hopefully make this possible.

(3) The measurements of resistance and capacity on tissues of higher plants are extremely difficult to interpret due to cell-cell coupling in the symplast.

(4) Future work should be directed at improving our understanding of the symplast both at the theoretical and practical levels, but it may be wiser to gain much more experience on hopefully simpler material such as unicellular phytoplankton before venturing back to multicellular plants.

The new technique should prove very valuable in unicellular studies on cells much smaller than the perhaps rather untypical Characeae. The importance of allying such studies with micro-anatomy must be stressed.

APPENDICES.

APPENDIX I.

Justification that local potential, ψ , falls off exponentially with distance from a charged surface in electrolyte solutions.

The situation with which we are here concerned should be contrasted with the case, familiar in electrostatics, where the electric field intensity is constant ($4\pi\sigma$), independent of distance from an extensive conducting surface bearing a uniform charge density (σ) on each side. This is known as Coulomb's Theorem (see Starling and Woodall, 1956) and has the consequence that potential falls off linearly with distance. This situation would seem to imply that such a surface were at infinite potential relative to a plane at infinite distance, where potential is always defined as zero, by convention. This is clearly an absurd state of affairs, and is mitigated by the fact that the field lines cannot remain parallel to an indefinite distance unless the charged surface were itself truly of infinite extent, which is in practise impossible. Thus a limited plane of charge displays this linear potential gradient only so long as the distance from the plane is small compared to the dimensions of the plane - which now gives us a definition of the expression that a plane is extensive. Beyond that, the field lines begin to diverge and the linear law gives way increasingly to a law which in the limit becomes the inverse square law, which automatically limits the potential on the charged plane relative to zero at infinite distance.

The statement above that potential falls off according to an exponential law is justified in the case of a charged sheet in an electrolyte solution, because charges in the electrolyte are free to migrate, and hence alter the field distribution as one comes away from the surface. Indeed counterions tend to migrate towards the charged surface and nebulions away from it, counteracted only by thermal diffusion, leading to some screening of the surface charge as seen at a distance. The behaviour follows the Boltzmann distribution, which is merely another way of writing the Nernst equation (but for single ions instead of the gram-ion, remembering that $Nk = R$ where $N =$ Avogadro's number and $k =$ Boltzmann constant.) Of course the Boltzmann distribution merely relates the population density of any given ion to the local potential and the population density at zero potential (or any other reference potential) but it does not tell us the law which governs the fall off of either potential or concentration (i.e. population density) with distance from the charged surface.

The law relating the fall off of potential with distance has been the subject of much discussion. Helmholtz proposed the linear law as in electrostatics, not realising that Coulomb's Theorem only applies when the space in contact with the charged surface contains no movable charges. Also Gouy (1910) pointed out that such a law was improbable since there will be no layer at which the potential will have fallen to zero presenting a hard boundary to the "double-layer", as Helmholtz's theory

would require. Chapman (1913) took the matter further by writing the Boltzmann distribution law into the Poisson equation :

$$\nabla^2 \psi = -\frac{4\pi\rho}{D}$$

where D is the dielectric constant of the medium
 ρ is charge density at any point in the solution at which the potential is ψ .

The charge density, ρ , is given by $N \sum_i c_i e_i$ where

c_i is the concentration of the i th ion species and e_i its charge. c_i is then given by the Boltzmann distribution.

Stern (1924) criticised the Gouy-Chapman approach as it gave too high a value of electrical capacity for dropping mercury electrodes (which carry a double-layer), and suggested a combination of the approaches of Helmholtz and Chapman. Stern's approach gave fair agreement with the measurements of Philpots (1932) on a dropping mercury electrode, but his constants differed for every ion species. However, the Gouy-Chapman approach was a good forerunner of the well-known theory of Debye and Hückel (1923) in which they simplified by considering one ion species at a time together with the "ion atmosphere" around it.

In its application to the charge on membranes, the treatment for plane surfaces is relevant (see Butler 1951), since the surface may be considered to be "extensive", according to the definition given earlier. This would not be true for small spherical ions, which would follow another law. The treatment leads to the equation :

$$\psi = \frac{4\pi\sigma}{DK} e^{K(a-x)}$$

where σ is the charge density on the surface
 a is the shortest distance of approach of an ion to the surface (at which ζ -potential applies)
 x is the distance from the surface, and

$$K = \sqrt{\left(\frac{8\pi\epsilon^2}{DkT} \cdot \frac{N}{1000}\right) \times \mu}$$

in which : ϵ is the electronic charge
 k is the Boltzmann constant
 T is temperature in $^{\circ}\text{K}$.
 μ is the ionic strength of electrolyte, defined
 by $\frac{1}{2} \sum c_i z_i^2$

When $x=a$, $\psi = \zeta$ -potential, so :

$$\zeta = \frac{4\pi\sigma}{DK}$$

This shows that the ζ -potential on a surface is dependant not merely on the surface charge density, σ , but also on the ionic strength, μ , falling as μ rises. This has important consequences for the behaviour of charged membranes. It is in fact parallel to the point established earlier (p. 14 ff) that high salt swamps a Donnan system.

[A more accurate treatment by Müller (1928) and Abramson and Müller (1932), avoiding Debye and Hückel's approximations yields, for a plane surface the expression :

$$\sigma = 2\alpha\sqrt{c} \sinh\left(\frac{z\zeta}{2\beta}\right)$$

in which, for aqueous solutions :

$$\beta = 0.025 \text{ volt} = \frac{kT}{\epsilon}$$

$$\alpha = 17,650 \text{ at } 18^{\circ}\text{C.} = \sqrt{\left(\frac{DRT}{2\pi \times 1000}\right)}$$

z = valency.

However this expression is less convenient since it cannot readily be made explicit in ζ .]

The fall-off of local potential, ψ , as one comes away from the surface, is indeed now seen to be exponential in electrolyte solutions, and the rate of fall-off depends on the magnitude of K as compared to a . This again depends on the ionic strength, μ , such that the effect is short-range for high ionic strength, as expected. In any case, the size of a means that the range of the effect is not going to be more than a few nanometres, i.e. similar to the thickness of the layers of unstirred solvent bound to the surface.

APPENDIX 3.

Programmes for Hewlett-Packard HP25C Calculator.

Eqn. (21)
Cation or anion
transport
acting alone,
with nebenions.

STO 1 RT, kJ/m.
STO 2 F, kC
STO 3 z (1, -1)
STO 4 pH (if used)
STO 5 $\Delta\bar{\mu}$, kJ/m.
STO 6 $\mathcal{R} = N/P$

2 1 R/S

RCL 5

RCL 1

÷

e^x

RCL 6

+

RCL 6

1

+

÷

√

ln

RCL 1

x

RCL 2

÷

RCL 3

x

BEX 3

x

R/S or GTO 03

See above *

* If pH is
required on
Eqns. (21a, b)
then extend
programme
as follows :

Use R/S as
last step in
Eqn. (21)
programme,
then add :

BEX 3

÷

RCL 2

x

RCL 5

RCL 3

x

-

.434

x

RCL 1

÷

RCL 4

+

GTO 03

RT = 2.436 kJ/m.
at 20°C or
2.577 at 37°C.

F = 96.487 kC/m.

Eqn. (23)
Cation export
plus Donnan
without nebenions.

STO 1 RT

STO 2 F

STO 5 $\Delta\bar{\mu}$

STO 6 c_i/salt_o

2 3 R/S

RCL 5

RCL 1

÷

e^x

RCL 6

2

÷

STO 7

x^2

+

√

RCL 7

-

ln

RCL 1

x

RCL 2

÷

BEX 3

x

GTO 03

In all these programmes, to run, first press R/S after GTO 00 or switch-off gives Eqn.no., second press E_m and for Eqn. (21) with extension programme, third press pH_1 . Next press again gives E_m .

Eqn. (26)
Cation export
plus Donnan
with nebenions.

STO 1 RT kJ/m.
STO 2 F kC
STO 3 N meq/l.
STO 4 P meq/l.
STO 5 $\Delta\bar{\mu}$ kJ/m.
STO 6 c_i meq/l.

2 6 R/S RCL 2
RCL 5 \div
RCL 1 EEX 3
 \div x
 e^x GTO 03
RCL 4
x
RCL 3
+
RCL 3
RCL 4
+
 \div
RCL 6
2
 \div
RCL 3
RCL 4
+
 \div
STO 7
 x^2
+
 $\sqrt{\quad}$
RCL 7
-
ln
RCL 1
x

Eqn. (27)
Anion import
plus Donnan
without nebenions

STO 1 RT
STO 2 F
STO 3 blank
STO 4 blank
STO 5 $\Delta\bar{\mu}$
STO 6 c_i/NaCl_o

2 7 R/S
RCL 5
RCL 1
 \div
CHS
 e^x
STO 7
RCL 6
x
2
 \div
 x^2
RCL 7
+
 $\sqrt{\quad}$
RCL 7
RCL 6
x
2
 \div
-
ln
RCL 1
x
RCL 2
 \div
EEX 3
x
GTO 03

Eqn. (28)
Anion import
plus Donnan
with nebenions

STO 1 RT
STO 2 F
STO 3 N
STO 4 P
STO 5 $\Delta\bar{\mu}$
STO 6 c_i

2 8 R/S RCL 1
RCL 6 x
2 RCL 2
 \div
RCL 5 EEX 3
RCL 1 x
GTO 03
 \div
 e^x
RCL 4
x
RCL 3
+
STO 7
 \div
 x^2
RCL 3
RCL 4
+
RCL 7
 \div
+
 $\sqrt{\quad}$
RCL 6
2
 \div
RCL 7
 \div
-
ln

Eqn. (30)
Cation export
plus Double
Donnan with
nebenions

STO 1 RT
STO 2 F
STO 3 N
STO 4 P
STO 5 $\Delta\bar{\mu}$
STO 6 c_i
STO 7 c_o

3 0 R/S	RCL 6
RCL 5	2
RCL 1	÷
÷	RCL 3
e^x	RCL 4
RCL 4	+
x	RCL 7
RCL 3	-
+	÷
RCL 3	-
RCL 4	ln
+	RCL 1
RCL 7	x
-	RCL 2
÷	÷
RCL 6	EEX 3
2	x
÷	GTO 03
RCL 3	
RCL 4	
+	
RCL 7	
-	
÷	
x^2	
+	
√	

Eqn. (34)
Coupled cation/
cation exchange
transport plus
Donnan ($c_i = c_o = c$)
with nebenions.

STO 1	RT	+		
STO 2	F	RCL 6		
STO 3	N	-		
STO 4	P	÷		
STO 5	$\Delta\bar{\mu}$	STO 6	*	x^2
STO 6	c	x^2		+
STO 7	δ	+		√
		√		RCL 6
		RCL 6		2
3 4 R/S		-		÷
RCL 5		ln		RCL 3
RCL 1		RCL 1		RCL 4
÷		x		+
e^x		RCL 2		RCL 6
RCL 4		÷		-
x		EEX 3		÷
RCL 7		x		-
÷		GTO 03		ln
RCL 7		remember		RCL 1
-		now to		x
x		refill		RCL 2
RCL 3		STO 6		÷
+		with c.		EEX 3
RCL 3		To avoid		x
RCL 4		above, use		GTO 03
+		calculator		
RCL 6		with more		
-		than 50		
÷		steps and		
RCL 6		follow		
2		routine		
÷		to right		
RCL 3		as from		
RCL 4		this *		
		level.		

Eqn. (36) Twin Independent Cation Export plus Anion Import plus Donnan with Nebenions.

There are too many numbers for the number stores available on the HP25C, which has 7. 8 are required. Therefore $\frac{RT}{F} \times 1000$ will be multiplied in at the end of each programme execution. To convert to mV the appropriate multiplier is $\frac{2.436}{96.487} \times 1000$ for $20^\circ\text{C} = 25.25$. To reduce the number of programme steps to below 50, the exponentials in $\Delta\bar{\mu}$ will be evaluated by a second programme after the main programme, and the number will then be inserted in the appropriate number store manually.

STO 1 Internal
 STO 2 $\exp(\Delta\bar{\mu}/RT)_{\text{Na}}$
 STO 3 $\exp(\Delta\bar{\mu}/RT)_{\text{Cl}}$
 STO 4 Na_o
 STO 5 Cl_o
 STO 6 c_i
 STO 7 N_o^+

Note that :

$$\text{N}_o^- = \text{Na}_o + \text{N}_o^+ - \text{Cl}_o$$

when there is no charge captive outside the membrane.

3 6 R/S

RCL 2

RCL 4

x

RCL 7

+

RCL 4

RCL 7

+

RCL 5

-

RCL 5

RCL 3
 x
 +
 STO 1
 ÷
 RCL 6
 RCL 1
 ÷
 2
 ÷
 STO 1
 x²
 +
 $\sqrt{\quad}$
 RCL 1
 -
 ln
 25.25
 x
 R/S *
 Enter $\Delta\bar{\mu}$
 2.436
 ÷
 e^x
 GTO 03
 **

* At this point the programme will stop. Read E_m and then insert new value of one of the $\Delta\bar{\mu}$'s, press R/S and insert displayed figure in appropriate $\Delta\bar{\mu}$ STO.

** After this, pressing R/S gives a new E_m . If however both $\Delta\bar{\mu}$ values are to be changed, press GTO 39 which is the step after *.

Programme (36.1) for calculation of Eqn.(36) when there is fixed charge outside the membrane.

STO 1	N_w^- and internal	36.1 R/S
STO 2	$\exp(\Delta\bar{\mu}/RT)_{Na}$	RCL 2
STO 3	$\exp(\Delta\bar{\mu}/RT)_{Cl}$	RCL 4
STO 4	Na_w	x
STO 5	Cl_w	RCL 7
STO 6	c_i	+
STO 7	N_o^+	RCL 5

Note that N_w^- must be re-inserted after every calculation.

	x
	RCL 1
	+
	STO 1
	÷
	RCL 6
	RCL 1
	÷
	2
	÷
	STO 1
	x^2
	+
	√
	RCL 1
	-
	ln
	25.25
	x
	R/S (step 36). -Insert
	Enter $\Delta\bar{\mu}$ GTO 06
	2.436 if below
	not required.
	÷
	e^x
	GTO 04

APPENDIX 4.Diffusion, Electrophoresis, and the Goldman Flux Equation.

Although Goldman (1943) presented the original proof of the flux equation now known by his name, his proof as presented was far from clear, and indeed there was an error as printed in one of his equations (no.1.) which made comprehension even more difficult. Fortunately, it did not prevent him from correctly deriving the well known Goldman diffusion equation quoted on p.43 in this text, and the credit must go fully to him for showing one way by which the problem of ionic diffusion could be solved. Various later authors (such as Janáček 1970, Hope 1971 and Cole 1972) following and acknowledging his lead have presented their own versions of the proof of the Goldman flux equation (Eqn.(32) of this text). However the equations as presented do not seem to tally at first sight. The problem, on investigation, proved to be lack of clarity in the definitions of quantities. It therefore seemed necessary to clarify the position from first principles through to the Goldman flux equation, including a consistent set of units throughout. A mixture of CGS and practical units was chosen for convenience of intuitive understanding, although obviously other systems such as MKS or SI could be applied.

The definition formula for diffusion is:

Flux rate per unit area $\Phi_D = -D \frac{\partial c}{\partial x}$ gm.-mols./cm²/sec.

for linear diffusion in one dimension.

Therefore the units of D are as follows :

$$D = -\frac{\Phi}{\partial C/\partial x} \quad \frac{\text{gm.mols.}}{\text{cm}^2 \cdot \text{sec.}} \cdot \frac{\text{cm}^3 \cdot \text{cm.}}{\text{gm.mols.}} \text{ which is } \text{cm}^2 \text{sec}^{-1}$$

The definition formula for electrophoretic drift of an ion in an electric field is :

$$\text{velocity of drift, } v = -u \frac{\partial E}{\partial x} \quad \text{cm/sec.}$$

Therefore

$$u = -\frac{v}{\partial E/\partial x} \quad \frac{\text{cm.}}{\text{sec.}} \cdot \frac{\text{cm.}}{\text{volt}} \text{ which is } \text{cm}^2 \text{sec}^{-1} \text{volt}^{-1}$$

To convert drift velocity to flux rate across a plane perpendicular to the drift, it is necessary to multiply by C , since a length of solution $1/C$ of unit cross section area will contain 1 gm. mol., and a length v goes by per second. Flux per second per unit area is then v/length containing 1 gm.mol. Therefore :

$$\Phi_E = -uC \frac{\partial E}{\partial x} \quad \text{gm.mols./cm}^2/\text{sec.}^*$$

Flux rates for diffusion and electrophoresis are now in the same units and may be combined to give the total flux for an ion in both concentration and electrical gradients :

$$\Phi = -D \frac{\partial C}{\partial x} - uC \frac{\partial E}{\partial x} \quad \text{gm.mols./cm}^2/\text{sec.} \quad (\text{A})$$

There is however an alternative approach to this point. Electrochemical potential has the dimensions of energy.

* Note that gm.mols. are used rather than gm.ions to make it clear that we refer to the total molecular weight of the ion irrespective of the number of charges it carries.

Now an energy gradient in the spacial dimension is equivalent to a force. Electrochemical potential is given by :

$$\bar{\mu} = \mu' + RT \ln a + zFE$$

But $a=Cf$ where f is the activity coefficient, so :

$$\bar{\mu} = \mu' + RT \ln C + RT \ln f + zFE$$

This may be differentiated in one spacial dimension to give :

$$\frac{\partial \bar{\mu}}{\partial x} = \frac{RT}{C} \cdot \frac{\partial C}{\partial x} + \frac{RT}{f} \cdot \frac{\partial f}{\partial x} + zF \frac{\partial E}{\partial x} \quad \text{Joules/gm.mol./cm.}$$

Now we may ignore $\frac{\partial f}{\partial x}$ on the assumption that f varies little throughout the system. The force $\frac{\partial \bar{\mu}}{\partial x}$ acts upon ions of mobility U to give a drift velocity :

$$v = - U \frac{\partial \bar{\mu}}{\partial x}$$

and we so define U that it produces a velocity in cm/sec. Then the units of U , as so defined are :

$$U = - \frac{v}{\frac{\partial \bar{\mu}}{\partial x}} \quad \frac{\text{cm.}}{\text{sec.}} \cdot \frac{\text{cm. gm.mols.}}{\text{joule}}$$

This U is evidently not the same as u and herein has lain the confusion in published literature. However we may proceed by multiplying v by C as before to obtain a flux rate :

$$\Phi = UC \frac{\partial \bar{\mu}}{\partial x} = -URT \frac{\partial C}{\partial x} - UCzF \frac{\partial E}{\partial x} \quad \text{gm.mols./cm}^2/\text{sec.} \quad (\text{B})$$

Clearly the two equations for Φ , (A) and (B) must be identical so, comparing, we see that :

$$D = URT \quad \text{and} \quad uC = UCzF$$

or

$$D = u \frac{RT}{zF} \quad \text{and} \quad u = U z F$$

This now establishes the Einstein* relation between D and u and also the relation between the two measures of mobility, U and u . Thus we may now write for the current density carried by this ionic flux rate :

$$j = zF\bar{\Phi} = -uRT \frac{\partial C}{\partial x} - uCzF \frac{\partial E}{\partial x} \quad \text{amps./cm}^2.$$

which is the nearest form to equation 1 of Goldman (1943) except that his published version shows, incorrectly, an extra z multiplier throughout. It still does not tally with his version if U is used instead!

Let us now proceed to prove the flux equation. Equation (B) as it stands cannot directly be integrated since it contains two spacial gradients whose profiles through the membrane are unknown. There are principally two ways of overcoming this by means of simplifying assumptions, to obtain explicit expressions in $\bar{\Phi}$. Henderson (1907, 1908) adopted the assumption that the concentration gradient throughout the boundary region (here the membrane) between two solutions could be taken as constant. This approach yielded equations for both flux rates and for the diffusion potential at a liquid junction (quoted on p. 40). The Henderson flux equation could have been used in the treatments presented in this thesis, but physiologists have found the alternative approach of Goldman (1943) particularly convenient. A problem with the Henderson flux equation is that it lead to an ordinary ohmic law for membrane conductance, which is not a good representation of biological membranes.

* Also known as the Nernst-Einstein relation.

Goldman assumed that the voltage gradient throughout the membrane could be taken as uniform - the so-called "constant field" assumption. Therefore we set :

$$\frac{\partial E}{\partial x} = \frac{E_i - E_o}{\delta} = \frac{E_m}{\delta}$$

where δ is membrane thickness.

The differential flux equation now becomes :

$$\Phi = - URT \frac{\partial C}{\partial x} - UCzF \frac{E_m}{\delta}$$

By re-arrangement, this becomes :

$$\Phi + UCzF \frac{E_m}{\delta} = - URT \frac{\partial C}{\partial x}$$

whence :

$$\partial x = - \frac{URT \partial C}{\Phi + UCzF \frac{E_m}{\delta}}$$

This has the standard form :

$$\int \frac{dx}{a+bx} = \frac{1}{b} \ln(a+bx)$$

and so may be integrated directly, between the limits 0 and δ when C_o and C_i apply respectively :

$$\begin{aligned} \int_0^\delta \partial x = \delta &= - URT \int_{C_o}^{C_i} \frac{\partial C}{\Phi + UCzF \frac{E_m}{\delta}} \\ &= - \frac{URTS}{UzFE_m} \ln \frac{\Phi + UzF \frac{E_m}{\delta} C_i}{\Phi + UzF \frac{E_m}{\delta} C_o} \end{aligned}$$

which gives :

$$\exp\left(-\frac{zFE_m}{RT}\right) = \frac{\Phi + UzF \frac{E_m}{\delta} C_i}{\Phi + UzF \frac{E_m}{\delta} C_o}$$

which, after re-arrangement gives :

$$\bar{\Phi} = U z F \frac{E_m}{\delta} \cdot \frac{C_i - C_o \exp\left(-\frac{z F E_m}{RT}\right)}{\exp\left(-\frac{z F E_m}{RT}\right) - 1}$$

or, after multiplying top and bottom by $\exp\left(\frac{z F E_m}{RT}\right)$ yields :

$$\bar{\Phi} = -\frac{U}{\delta} z F E_m \frac{C_o - C_i \exp\left(\frac{z F E_m}{RT}\right)}{1 - \exp\left(\frac{z F E_m}{RT}\right)}$$

U/δ is a property of the membrane and may be referred to permeability as follows :

The definition formula for P is :

$$\bar{\Phi} = -P(C_i - C_o) \quad \text{for an uncharged species,}$$

so that units appropriate to P are $\frac{\text{gm.mols.}}{\text{cm}^2 \cdot \text{sec.}} \cdot \frac{\text{cm}^3}{\text{gm.mols.}}$
or $\text{cm} \cdot \text{sec}^{-1}$. Now the diffusion term for flux is :

$$\bar{\Phi} = -U R T \frac{C_i - C_o}{\delta} \quad \text{if we can assume } \frac{\partial C}{\partial x} = \frac{C_i - C_o}{\delta}$$

Therefore $\frac{U R T}{\delta} = P$ and so :

$$\bar{\Phi} = -P \frac{z F E_m}{RT} \cdot \frac{C_o - C_i \exp\left(\frac{z F E_m}{RT}\right)}{1 - \exp\left(\frac{z F E_m}{RT}\right)}$$

Goldman flux
equation.
 $\text{gm.mols./cm}^2/\text{sec.}$
(32)

and membrane current is $zF \times \bar{\Phi}$ amps/cm².

APPENDIX 5.

Culture of maize, *Zea mays* L., var. "White Horse Tooth".

Seeds of maize were obtained by courtesy of the University Botanic Gardens, St. Andrews, and were grown in hydroponic culture in a nutrient medium modified from one recommended by ARC Long Ashton Research Station, with composition as follows :

Stock Nutrient Concentrates :

A :	$\text{Ca}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$	106 g/l.	to give	450 mM
	$\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$	11 g/l.	"	50 mM
B :	$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	36.8 g/l.	"	150 mM
	HNO_3	17.8 ml. conc. acid/l.	"	200 mM
	H_3PO_4	25.2 ml. conc. acid/l.	"	400 mM
	$\text{Fe}^{+++}\text{EDTA} \cdot 5\text{H}_2\text{O}$	3.2 g/l.	"	9.5 mM
Na :	NaOH	24 g/l.	"	600 mM
K :	KOH	33.6 g/l.	"	600 mM

Solutions A and B were stored in a refrigerator and solutions Na and K were kept tightly stoppered. Nutrient medium was made up by mixing 5 ml. of A, 5 ml. of B, 2.5 ml. of Na and 2.5 ml. of K in 500 ml. of distilled water. (The ratio of Na to K could be varied however with this system of 4 stock solutions). The resultant nutrient medium was considerably less concentrated than the Long Ashton medium, especially in the divalent ions, to avoid precipitation, and the main constituents were separated between A and B to avoid precipitation in the concentrates. The concentrations of ions in the nutrient medium were :

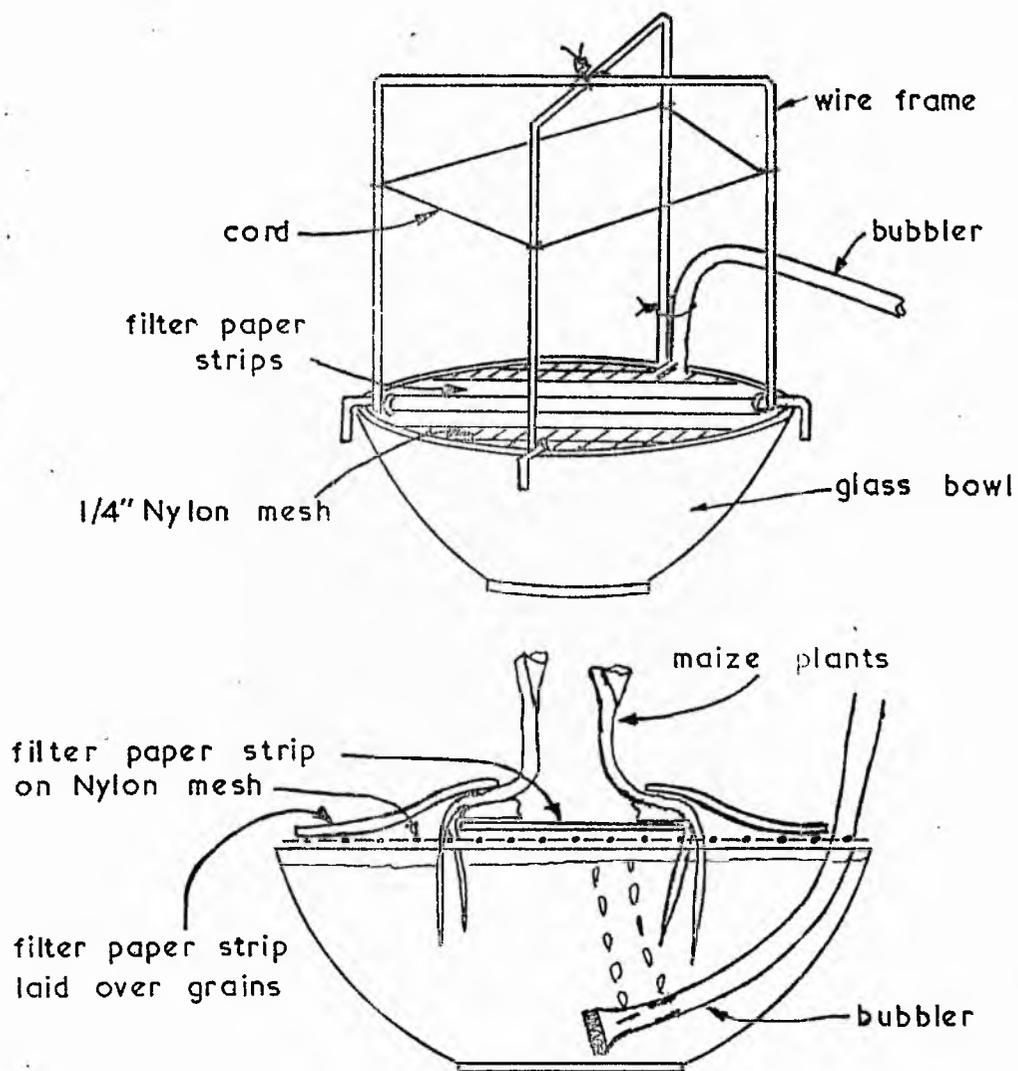
Ca ⁺⁺	5 mM	SO ₄ ⁼	1.5 mM
Mg ⁺⁺	1.5 mM	NO ₃ ⁻	11 mM
K ⁺	3 mM	H ₂ PO ₄ ⁻	4 mM
Na ⁺	3 mM	Cl ⁻	1 mM

It may be seen that the total ionic equivalence of cations and anions are equal. The pH was close to 7, slightly acid, and was checked before use.

Maize seeds were pre-soaked in distilled water until they began to germinate, and were then cultured in the arrangement shown in Fig. 50. The purpose of the arrangement was to reduce as far as possible the growth of mould. (Seeds were either pre-treated with a mercurial antifungal agent or were dusted with Vitaspor PB powder (active ingredient Zn ethylene-bis-dithiocarbamate)). For each culture bowl, six maize seeds were placed in correct orientation between strips of thick filter paper (intended for chromatography) 50 mm. wide, with the ends only dipping in nutrient medium. The seeds were thus in a damp atmosphere in contact with wet paper above and below. It was found that the chief cause of fungal attack was the availability of soluble organic nutrients mobilised from the germinating seeds. If this was allowed to dissolve in the bath by direct contact, heavy fungal infestation resulted. However, by the method adopted, soluble organic material was carried away from the seeds towards the centre of each paper strip, as evaporation caused an upward flow of nutrient. Seeds were therefore not planted in the central 8 cm. of the strips and

Fig. 50

CULTURE OF MAIZE PLANTS.



fungal infestation was much reduced. The nutrient medium was continuously aerated. The wire frame served to prevent plants, lacking root support, from falling over. Nutrient medium was changed every 4 days at the start, increasing to once every 2 days towards the end of the growing period. Lighting was provided by four 80w. 5 ft. fluorescent tubes (Ekco Warm White) and two rows of three 100w. tungsten lamps, all about 1 m. above the culture bowls. (Healthy plants did not result if the tungsten lamps were omitted). The lamps were set on a cycle of 15 hours on, 9 hours off, controlled by a time switch. Temperature in the partially enclosed growth chamber was about 21°C. Healthy plants were grown up to three weeks before use in experiments.

REFERENCES.

- ABRAMSON & MÜLLER, H. (1932) Bull. Amer. Physiol. Soc. 7 11.
- ADAIR, G.S. (1925) A critical study of the direct method of measuring the osmotic pressure of haemoglobin. Proc. Roy. Soc. A 108 627-637.
- ADAMS, E.P. (1927) On electrets. J. Franklin Institute 204 469-486.
- ADRIAN, R.H. (1956) The effect of internal and external potassium concentration on the membrane potential of frog muscle. J. Physiol. (Lond.) 133 631-658.
- ALEXANDER, A.E. & JOHNSON, P. (1950) "Colloid Science" Oxford Univ. Press.
- AMBROSE, E.J. (1966) Electrophoretic behaviour of cells. Progr. Biophys. & Mol. Biol. 16 243-265.
- ANDERSON, W.P. (1976) The electrophysiology of higher plant roots. In "Transport and Transfer Processes in Plants", ed. I.F. Wardlaw and J.B. Passioura. pp. 125-135. Academic Press.
- ANDERSON, W.P., HENDRIX, D.L. & HIGINBOTHAM, N. (1974) Higher plant cell membrane resistance by a single intracellular electrode method. Plant Physiol. 53 122-124.
- BAK, A.F. (1958) A unity gain cathode follower. EEG Clin. Neurophysiol. 10 745.
- BELL, G.H., DAVIDSON, J.N. & SCARBOROUGH, H. (1968) "Textbook of Physiology & Biochemistry" 7th ed. E. & S. Livingstone Ltd., Edinburgh.
- BENNETT, M.C. & RIDEAL, Sir ERIC (1954) Membrane behaviour in Nitella. Proc. Roy. Soc. B 142 483-496.
- BERG, H.C. (1968) Membrane dipole potentials. Biophys. J. 8 1051-1053.
- BISHOP, P.O. & COLLINS, R. (1951) Steel microelectrodes. J. Physiol. (Lond.) 112 8P.
- BLINKS, L.R. (1930) The d.c. resistance of Nitella. J. Gen. Physiol. 13 495-508.
- BOLAM, T.R. (1932) "The Donnan Equilibria". G. Bell & Sons Ltd., London.
- BRENNECKE, R. & LINDEMANN, B. (1971) A chopped-current clamp for current injection and recording of membrane polarisation with single electrodes of changing resistance. TIT J. Life Sci. 1 53-58.
- BRENNECKE, R. & LINDEMANN, B. (1974a) Theory of a membrane-voltage clamp with discontinuous feedback through a pulsed current clamp. Rev. Sci. Instrum. 45 184-188.

- BRENNECKE, R. & LINDEMANN, B. (1974b) Design of a fast voltage clamp for biological membranes, using discontinuous feedback. *Rev. Sci. Instrum.* 45 656-661.
- BRIGGS, G.E. (1957) Some aspects of free space in plants. *New Phytol.* 56 305-324.
- BRIGGS, G.E., HOPE, A.B. & ROBERTSON, R.N. (1961) "Electrolytes and Plant Cells." Blackwell, Oxford.
- BROWN, H.T. & ESCOMBE, F. (1900) Static diffusion of gases and liquids in relation to the assimilation of carbon and translocation in plants. *Phil. Trans. Roy. Soc. B* 193 223-292.
- BRULET, P. & McCONNELL, H.M. (1975) Magnetic resonance spectra of membranes. *Proc. Nat. Acad. Sci.* 72 1451-1455.
- BUGARSKY, St. & TANGL, F. (1898) Physikalisch-chemische Untersuchungen über die molecularen Concentrationsverhältnisse des Blutserums. *Arch. Ges. Physiol.* 72 531-
- BUTLER, J.A.V. (1951) "Electrical Phenomena at Interfaces" Methuen & Co.Ltd. London Chapter II.
- CAPLAN, S.R. & ESSIG, A. (1977) A thermodynamic treatment of active sodium transport. *Current Topics in Membranes and Transport* 9 145-175
- CHAPMAN, D.L. (1913) *Phil. Mag.* 25 475.
- CLOWES, F.A.L. & JUNIPER, B.E. (1968) "Plant Cells" Botanical monograph. Blackwell, Oxford.
- COLE, K.S. (1972) "Membranes, Ions and Impulses - A Chapter of Classical Biophysics" Univ. California Press, Berkeley, LA.
- COLE, K.S. & COLE, R.H. (1936) Electric impedance of *Asterias* eggs. *J. Gen. Physiol.* 19 609-623.
- COLE, K.S. & MARMONT, G. (1942) The effect of ionic environment upon the longitudinal impedance of the squid giant axon. *Fed. Proc.* 1 15-16.
- CONNOR, F.R. (1973) "Noise" Edward Arnold, London.
- CONWAY, E.J. & BOYLE, P.J. (1939) A mechanism for the concentrating of potassium by cells, with experimental verification for muscle. *Nature (London)* 144 709-710.
- COOMBS, J.S., ECCLES, J.C. & FATT, P. (1955) The electrical properties of the motoneurone membrane. *J. Physiol. (Lond)* 130 291-325.

- COSTER, H.G.L. & GEORGE, E.P. (1968) A thermodynamic analysis of fluxes and flux-ratios in biological membranes. *Biophys. J.* 8 457-469.
- C.R.C. HANDBOOK OF CHEMISTRY AND PHYSICS 55th ed. (1974) ed. R.C.Weast. CRC Press, Cleveland, Ohio.
- CURTIS, H.J. & COLE, K.S. (1937) Transverse electric impedance of *Nitella*. *J. Gen. Physiol.* 21 189-201.
- DAINTY, J., CROGHAN, P.C. & FENSOM, D.S. (1963) Electro-osmosis, some applications in plant physiology. *Can. J. Bot.* 41 953-966.
- DAINTY, J. & HOPE, A.B. (1959) Ionic relations of cells of *Chara australis*. I. Ion exchange in the cell wall. *Austral. J. Biol. Sci.* 12 395-411.
- DAVIS, R.F. & HIGINBOTHAM, N. (1969) Effects of external cations and respiratory inhibitors on electrical potential of the xylem exudate of excised corn roots. *Plant Physiol.* 44 1383-1392.
- DEAN, R.B. (1941) Theories of electrolyte equilibrium in muscle. *Biol. Symp.* 3 331-348.
- DEBYE, P. & HÜCKEL, E. (1923) *Phys. Z.* 24 185 & 305.
- DIAMOND, J.M. & BOSSERT, W.H. (1967) Standing-gradient osmotic flow. A mechanism for coupling of water and solute transport in epithelia. *J. Gen. Physiol.* 50 2061-2083.
- DONNAN, F.G. (1910) *Physiol. Soc. London, Dec.*
- DONNAN, F.G. (1911) *Z. Electrochem.* 17 572.
- DONNAN, F.G. & HARRIS, A.B. (1911) The osmotic pressure and conductivity of aqueous solutions of congo-red, and reversible membrane equilibrium. *J. Chem. Soc.* 99 1554.
- DOWBEN, R.M. & ROSE, J.E. (1953) A metal filled microelectrode. *Sci.* 118 22.
- DRAKE, G. (1979) Electrical coupling, potentials and resistances in oat coleoptiles : Effect of azide and cyanide. *J. Exp. Bot.* 30 719-725.
- DRAKE, G.A., CARR, D.J. & ANDERSON, W.P. (1978) Plasmolysis, plasmodesmata and the electrical coupling of oat coleoptile cells. *J. Exp. Bot.* 29 1205-1214.
- EGUCHI, M. (1925) On the permanent electret. *Phil. Mag. 6th Series* 49 178-192.

- EISENMAN, G. (1962) Cation selective glass electrodes and their mode of operation. *Biophys. J.* 2 259-323.
- EPPLEY, R.W., ROGERS, J.N. & McCARTHY, J.J. (1969) Half-saturation constants for uptake of nitrate and ammonium by marine phytoplankton. *Limnol. and Oceanog.* 14 912-920.
- EPSTEIN, E. (1972) "Mineral Nutrition of Plants: Principles and Perspectives." John Wiley & Sons, Inc. New York.
- ESAU, K. (1953) "Plant Anatomy" Wiley, New York.
- ESSIG, A. & CAPLAN, S.R. (1968) Energetics of active transport processes. *Biophys. J.* 8 1434-1457.
- ETHERTON B., KEIFER, D.W. & SPANSWICK, R.M. (1977) Comparison of three methods for measuring electrical resistance of plant cell membranes. *Plant Physiol.* 60 684-688.
- FALK, G. & FATT, P. (1964) Linear electrical properties of striated muscle fibres observed with intracellular electrodes. *Proc. Roy. Soc. B* 160 69-123.
- FENSOM, D.S. & DAINTY, J. (1963) Electro-osmosis in *Nitella*. *Can. J. Bot.* 41 685-691.
- FINDLAY, G.P., HOPE, A.B. & WILLIAMS, E.J. (1970) Ionic relations of marine algae. II *Griffithsia*: ionic fluxes. *Austral. J. Biol. Sci.* 23 323-328.
- FINEAN, J.B., COLEMAN, R. & MICHELL, R.H. (1978) "Membranes and their Cellular Functions" 2nd ed. Blackwell, Oxford.
- de FONBRUNE, P.H.D. (1932-4) Improvements in devices for transmitting movements from one point to another. U.K. Patent no. 418,747.
- FRICKE, H. (1924 a) A mathematical treatment of the electrical conductivity of colloids and cell suspensions. *J. Gen. Physiol.* 6 375-384.
- FRICKE, H. (1924 b) A mathematical treatment of the electrical conductivity and capacity of disperse systems. I The electric conductivity of a suspension of homogeneous spheroids. *Phys. Rev.* 24 575-587.
- FRICKE, H. (1925 a) The electric capacity of suspensions with special reference to blood. *J. Gen. Physiol.* 9 137-152.
- FRICKE, H. (1925 b) The electric capacity of suspensions of red corpuscles of a dog. *Phys. Rev.* 26 682-687.

FRICKE, H. (1925 c) A mathematical treatment of the electric conductivity and capacity of disperse systems. II. The capacity of a suspension of conducting spheroids surrounded by a non-conducting membrane for a current of low frequency. Phys. Rev. 26 678-681.

GEORGE, E.P. (1961) Resistance values in a syncytium. Austral. J. Exp. Biol. 39 267-274.

GESTELAND, R.C., HOWLAND, B., LETTVIN, J.Y. & PITTS, W.H. (1959) Comments on microelectrodes. Proc. Inst. Radio Eng. 47 1856-

GIBBS, J.W. (1906) "The Scientific Papers of JWG"
(1928) "The Collected Works of JWG"
publisher ? (New York & London)

GINSBURG, H. & GINZBURG, B.Z. (1974) Radial water and solute flows in roots of *Zea mays*. IV. Electrical potential profiles across the root. J. Exp. Bot. 25 28-35.

GINSBURG, H. & LATIES, G.G. (1973) Longitudinal electrical resistance of maize roots. J. Exp. Bot. 24 1035-1040.

GLASSTONE, S. (1946/48) "The Elements of Physical Chemistry" 5th Printing. van Nostrand, New York.

GOLDMAN, D.E. (1943) Potential, impedance and rectification in membranes. J. Gen. Physiol. 27 37-60.

GOTTLIEB, M.H. & SOLLNER, K. (1968) Failure of the Nernst-Einstein equation to correlate electrical resistances and rates of ionic self-exchange across certain fixed charge membranes. Biophys. J. 8 515-535.

GOÛY, G.L. (1910) J. de Phys. (4) 9 357-

GRAY, J.A.B. & SVAETICHIN, G. (1951) Electrical properties of platinum tipped microelectrodes in Ringer's solution. Acta Physiol. Scand. 24 278-

GRUNDFEST, H., SENGSTAKEN, R.W., OETTINGER, W.H. & GURRY, R.W. (1950) Stainless steel micro-needle electrodes made by electrolytic pointing. Rev. Sci. Instrum. 21 360-

HALL, J.E. (1975) Access resistance of a small circular pore. J. Gen. Physiol. 66 531-532.

HALL, M.A. (1976) "Plant Structure, Function and Adaptation." Macmillan, London.

HELMHOLTZ, H.V. (1882) Wiss. Abhand. (Leipzig) 1 303.

HENDERSON, P. (1907) Z. physik. Chem. 59 118.
(1908) " 63 325.

- HEVESY, G., HOFER, E. & KROGH, A. (1935) Skand. Arch. Physiol. 72 199-
- HIGINBOTHAM, N. (1973a) The mineral absorption process in plants. Bot. Rev. 39 15-69.
- HIGINBOTHAM, N. (1973b) Electropotentials of plant cells. Ann. Rev. Plant Physiol. 24 25-46.
- HIGINBOTHAM, N. & ANDERSON, W.P. (1974) Electrogenic pumps in higher plant cells. Can. J. Bot. 52 1011-1021.
- HILL, A.E. (1975a) Solute-solvent coupling in epithelia : a critical examination of the standing-gradient osmotic flow theory. Proc. Roy. Soc. B 190 99-114.
- HILL, A.E. (1975b) Solute-solvent coupling in epithelia : an electro-osmotic theory of fluid transfer. Proc. Roy. Soc. B 190 115-134.
- HINKE, J.A.M. (1961) The measurement of sodium and potassium activities in the squid axon by means of cation-selective glass micro-electrodes. J. Physiol. (Lond.) 156 314-335.
- HLADKY, S.B. & RINK, T.J. (1976) Potential difference and distribution of ions across the human red blood cell membrane : A study of the mechanism by which the fluorescent cation diS-C3(5) reports membrane potential. J. Physiol. (Lond.) 263 287-319.
- HODGKIN, A.L. (1958) Ionic movements and electrical activity in giant nerve fibres. Proc. Roy. Soc. B 148 1-37.
- HODGKIN, A.L. & HOROWICZ, P. (1959) The influence of potassium and chloride ions on the membrane potential of single muscle fibres. J. Physiol. (Lond.) 148 127-160.
- HODGKIN, A.L. & KEYNES, R.D. (1955) The potassium permeability of a giant nerve fibre. J. Physiol. (Lond.) 128 61-88.
- HODGKIN, A.L. & RUSHTON, W.A.H. (1946) The electrical constants of a crustacean nerve fibre. Proc. Roy. Soc. B 133 444-479.
- HOLM, R. (aided by HOLM, E.) (1958) "Electric Contacts Handbook". Springer-Verlag. Berlin.
- HOLZ, R.W. (1977) Exchange diffusion of dopamine induced in planar lipid membranes by the ionophore X537A. J. Gen. Physiol. 69 633-653.
- van den HONERT, T.H. & HOOYMANS, J.J.M. (1955) On the absorption of nitrate by maize in water culture. Acta Bot. Neerl. 4 376-384.

- HOPE, A.B. (1956) The electric properties of plant cell membranes : I The electric capacitance of suspensions of mitochondria, chloroplasts and *Chlorella* sp. Austral. J. Biol. Sci. 9 53-66.
- HOPE, A.B. (1971) "Ion Transport and Membranes - a Biophysical Outline." Butterworths, London.
- HOPE, A.B. & WALKER, N.A. (1961) Ionic relations of cells of *Chara australis* R. Br. IV. Membrane potentials and resistances. Austral. J. Biol. Sci. 14 26-
- HOPE, A.B. & WALKER, N.A. (1975) "The Physiology of Giant Algal Cells". Cambridge Univ. Press.
- JANÁČEK, K. (1970) In "Cell Membrane Transport - Principles and Techniques" 2nd ed. Ed. by A. Kotyk and K. Janáček. Plenum, New York 1970 and 1975.
- JASON, A.C. & RICHARDS, J.C.S. (1975) The development of an electronic fish freshness meter. J. Physics E, Sci. Instrum. 8 826-830.
- JARDETZKY, O. & SNELL, F.M. (1960) Proc. Nat. Acad. Sci. 46 616-622.
- JARDETZKY, O & WERTZ, J.E. (1960) Weak complexes of the sodium ion in aqueous solution studied by nuclear spin resonance. J. Amer. Chem. Soc. 82 318-323.
- KATCHALSKY, A. & CURRAN, P.F. (1975) "Nonequilibrium Thermodynamics in Biophysics". Harvard Univ. Press...
- KATZ, Sir BERNARD (1966) "Nerve, Muscle and Synapse" McGraw-Hill, New York.
- KAYE, G.W.C. & LABY, T.H. (1956) "Tables of Physical and Chemical Constants" 11th ed. Longmans, Green & Co., London.
- KEDEM, O. & KATCHALSKY, A. (1958) Biochim. Biophys. Acta 27 229-246.
- KEIFER, D.W. & SPANSWICK, R.M. (1978) Activity of the electrogenic pump in *Chara corallina* as inferred from measurements of the membrane potential, conductance and potassium permeability. Plant Physiol. 62 653-661.
- KELVIN, Lord (William Thompson) (1855) On the theory of the electric telegraph. Proc. Roy. Soc. 7 382-399.
- KEYNES, R.D. (1972) On the constancy of membrane capacity. In "Perspectives in Membrane Biophysics" (a tribute volume to K.S. Cole.) Ed. D.O.P. Agin. Gordon & Breach, New York.
- KITASATO, H. (1968) The influence of H^+ on the membrane potential and ion fluxes of *Nitella*. J. Gen. Physiol. 52 60-87.

- KORNBERG, R.D. & McCONNELL, H.M. (1971) Lateral diffusion of phospholipids in a vesicle membrane. Proc. Nat. Acad. Sci. 68 2564-2568.
- KOTTLER, F. (1927) Elektrostatik der Leiter. Handbuch der Physik von Geiger und Scheel, Bd.12, Springer-Verlag, Berlin.
- KRISCHER, C.C. (1969a) Theoretical treatment of ohmic and rectifying properties of electrolyte filled micropipettes. Z. für Naturforschung 24(2) 151-155.
- KRISCHER, C.C. (1969b) Current/voltage measurements of electrolyte filled microelectrodes with ohmic and rectifying properties. Z. für Naturforschung 24 (2) 156-161.
- KRISCHER, C.C. & DEICHMANN, M.M. (1970) Potential transducing properties of microelectrodes filled with electrolyte or metal. Z. für Naturforschung 25 (1) 72-79.
- LENARD, J. & SINGER, S.J. (1966) Protein conformation in cell membrane preparations as studied by optical rotatory dispersion and circular dichroism. Proc. Nat. Acad. Sci. 56 1828-1835.
- LENS, J. (1933) On the diffuse double layer. Proc. Roy. Soc. A 139 596-603.
- LEV, A.A. & ARMSTRONG, W. McD. (1975) Ion activities in cells. Current Topics in Membr. & Transport 6 59-123.
- LING, G.N. & COPE, F.W. (1969) Potassium ion : Is the bulk of intracellular K^+ adsorbed ? Sci. 163 1335-1336.
- LING, G. & GERARD, R.W. (1949) The normal membrane potential of frog sartorius muscle fibers. J. Cell. Comp. Physiol. 34 383-396.
- McCLENDON, J.F. (1929) Polarisation capacity and resistance of salt solutions, agar, erythrocytes, resting & stimulated muscle, and liver measured with a new Wheatstone bridge designed for electric currents of high and low frequency. Protopl. 7 561-582.
- McINNES, D.A. (1939) "The Principles of Electrochemistry" Reinhold, New York.
- McLAUGHLIN, S.G.A. (1968) The activity and activity coefficient of sodium in toad oocytes. Proc. Physiol. Soc. (Lond.) 13-14 Dec. 1968.
- MacROBBIE, E.A.C. (1970) The active transport of ions in plant cells. Q. Rev. Biophys. 3 251-294.
- MacROBBIE, E.A.C. (1971) Ion transport in plant cells. Phil. Trans. Roy. Soc. B 262 333-342.

- MacROBBIE, E.A.C. (1975) Ion transport in plant cells. *Curent Topics in Membr. & Transport* 7 1-48.
- MAGNUSON, N.S. & MAGNUSON, J.A. (1973) $^{23}\text{Na}^+$ interaction with bacterial surfaces : a comment on NMR invisible signals. *Biophys. J.* 13 1117-1119.
- MARTINEZ, D., SILVIDI, A.A. & STOKES, R.M. (1969) NMR studies of sodium ions in isolated frog muscle and liver. *Biophys. J.* 9 1256-1260.
- MARULLO, N.P. & LLOYD, R.A. (1966) Precipitation of sodium chloride from aqueous solution by compound formation with an organic amine. *J. Amer. Chem. Soc.* 88:5 1076-1077.
- MAXWELL, J.C. (1873) "Treatise on Electricity and Magnetism". Oxford, Clarendon Press.
- MAY, K.R. (1956) Improvements in or relating to micromanipulating devices. UK Patent no. 749,437.
- MITCHELL, P. (1966) Chemiosmotic coupling in oxidative and photosynthetic phosphorylation. *Biol. Rev.* 41 445-502.
- MITCHELL, P. (1970) Reversible coupling between transport and chemical reactions. In "Membranes and Ion Transport", Vol. I p. 192. Ed. E.E.Bittar, Wiley-Interscience, New York & London.
- MÜLLER, H. (1928) *Kolloidchem. Beihefte* 26 257.
- NASTUK, W.L. (ed.) (1964) "Physical Techniques in Biological Research. Vol. V Electrophysiological methods. Academic Press, London.
- NORMAN, R.S. (1972) Membrane time constant and impedance phase measurements. *Brain Res.* 48 401-405.
- OHARA, K. & NEWTON, J.L. (1968) Ultramicro glass electrode for sodium analysis in biological fluids. *Jap. J. Physiol.* 18 632-641.
- OSTWALD, W. (1890) *Z. physikal. Chem.* 6 71.
- PHILLIPS, F.C. (1946, 2nd new impression 1951) "An Introduction to Crystallography." Longmans, Green & Co., London, p. 155.
- PHILIPPSON, M. (1921) Les lois de la resistance electrique des tissus vivants. *Bull. Acad. Roy. Belg. Cl. Sci.* 7 387-403.
- PHILPOT, L. St.J. (1932) *Phil. Mag.* 13 775.
- PLANCK, M. (1890) *Ann. Physik.* (3) 39 161
(3) 40 561.

- POOLE, R.J. (1978) Energy coupling for membrane transport. *Ann. Rev. Plant Physiol.* 29 437-460.
- RACUSEN, R.H. (1976) Phytochrome control of electrical potentials and intercellular coupling in oat-coleoptile tissue. *Planta* 132 25-29.
- RAPOPORT, S.I. (1970) The sodium-potassium exchange pump. Relation of metabolism to electrical properties of the cell. I Theory. *Biophys. J.* 10 246-259.
- RAVEN, J.A. & SMITH, F.A. (1974) Significance of hydrogen ion transport in plant cells. *Can. J. Bot.* 52 1035-1048.
- RAYLEIGH, Lord (1870) *Phil. Trans. Roy. Soc.* 161 77-
(1877) *Phil. Mag.* 3 456-
- REISENAUER, H.M. (1966) Mineral nutrients in soil solution. In "Environmental Biology" Eds. P.L. Altman and D.S. Dittmer. *Fed. Amer. Soc. Exp. Biol.*, Bethesda.
- ROBINSON, R.A. & STOKES, R.H. (1959/65) "Electrolyte Solutions". 2nd ed., revised. Butterworths, London.
- ROTHSCHILD, Lord (1946) The theory of alternating current measurements in biology and its application to the investigation of the biophysical properties of the trout egg. *J. Exp. Biol.* 23 77-99.
- ROTUNNO, C.A., KOWALEWSKI, V. & CBREJIDO, M. (1967) NSR evidence for complexing of sodium in frog skin. *Biochim. Biophys. Acta* 135 170-173.
- RUSSELL, J.M. & BRODWICK, M.S. (1979) Properties of chloride transport in barnacle muscle fibers. *J. Gen. Physiol.* 73 343-368.
- SACKIN, H. & BOULPAEP, E.L. (1975) Models for coupling of salt and water transport. Proximal tubular reabsorption in *Necturus* kidney. *J. Gen. Physiol.* 66 671-733.
- SADDLER, H.D.W. (1970a) The ionic relations of *Acetabularia mediterranea*. *J. Exp. Bot.* 21 345-359.
- SADDLER, H.D.W. (1970b) Fluxes of sodium and potassium in *Acetabularia mediterranea*. *J. Exp. Bot.* 21 605-616.
- SADDLER, H.D.W. (1970c) The membrane potential of *Acetabularia mediterranea*. *J. Gen. Physiol.* 55 802-821.
- SALAMA, G., JOHNSON, R.G. & SCARPA, A. (1980) *J. Gen. Physiol.* 263 287-319.
- SANDERS, D. (1980a) Control of chloride influx in *Chara* by cytoplasmic Cl^- concentration. *J. Membr. Biol.* 52 51-60.
- SANDERS, D. (1980b) The mechanism of chloride transport at the plasma membrane of *Chara corallina*. I. Co-transport with chloride. *J. Membr. Biol.* 53 129-141.

- SCHWAN, H.P. (1963) Determination of biological impedances. In "Physical Techniques in Biological Research", ed. W.L.Nastuk. Vol. VI. Part B. Academic Press, London.
- SHAMOO, A.E. & GOLDSTEIN, D.A. (1977) Isolation of ionophores from ion transport systems and their role in energy transduction. *Biochim. Biophys. Acta* 472 13-53.
- SHORT, G.W. (1968) Kelvin cables. *Wireless World*, March.
- SIMS, P.J., WAGGONER, A.S., WANG, C.H. & HOFFMANN, J.F. (1974) *Biochemistry* 13 3315-3330.
- SINGER, S.J. (1971) The molecular organisation of biological membranes. In "Structure and Function of Biological Membranes" ed. L.I. Rothfield. Academic Press, London.
- SKIERCZYŃSKA, J., ZOLNIERCZUK, R., SPIEWLA, E., BULANDA, W. & PRZYGODSKA, A. (1972) Measurements of membrane resistance of Characeae with external electrodes and microelectrodes. *J. Exp. Bot.* 23 591-
- SLAYMAN, C.L. (1965) Electrical properties of *Neurospora crassa*. Respiration and the intracellular potential. *J.Gen.Physiol.* 49 93-116.
- SLAYMAN, C.L. (1970) Movement of ions and electrogenesis in microorganisms. *Amer. Zool.* 10 377-392.
- SMITH, F.A. (1970) The mechanism of chloride transport in Characean cells. *New Phytol.* 69 903-917.
- SMYTHE, W.R. (1939) "Static and Dynamic Electricity" McGraw-Hill, New York.
- SOCOLAR, S.J. (1977) The coupling coefficient as an index of junctional conductance. *J. Membr. Biol.* 34 29-37.
- SOLLNER, K. (1969) The electrochemistry of porous membranes with particular reference to ion exchange membranes and their use in model studies of biophysical interest. *J. Macromol. Sci. Chem. A* 3(1) 1-86.
- SORENSEN, A.L. (1971) Water permeability of isolated muscle fibers of a marine crab. *J. Gen. Physiol.* 58 287-303.
- SPANGLER, S.G. (1972) *Ala. J. Med. Sci.* 9 218-223.
- SPANSWICK, R.M. (1970) Electrophysiological techniques and the magnitudes of the membrane potentials and resistance of *Nitella translucens*. *J. Exp. Bot.* 21 617-627.

- SPANSWICK, R.M. (1972a) Evidence for an electrogenic pump in *Nitella translucens*. I. The effects of pH, K^+ , Na^+ , light and temperature on the membrane potential and resistance. *Biochim. Biophys. Acta* 288 73-89.
- SPANSWICK, R.M. (1972b) Electrical coupling between cells of higher plants : A direct demonstration of intercellular communication. *Planta* 102 215-227.
- SPANSWICK, R.M. (1973) Electrogenesis in photosynthetic tissues. In "Ion Transport in Plants, ed. W.P. Anderson, pp.113-128. Academic Press, London.
- SPIRA, A.W. (1971) The nexus in the intercalated disc of the canine heart. Quantitative data for an estimation of its resistance. *J. Ultrastruct. Res.* 34 409-425.
- STANTON, M.G. (1973) Cell membrane resistance and capacity measured using lone single-barrelled micro-electrodes. *J. Physiol. (London)* 234 86-87P.
- STANTON, M.G. (1976) Integral microscope/manipulator unit for electrophysiologists. *J. Physiol. (London)* 263 117P.
- STANTON, M.G. (1978a) Improved micromanipulator. UK Patent no. 1,501,253.
- STANTON, M.G. (1978b) Improved microscope. UK Patent no. 1,516,219.
- STANTON, M.G. (1978c) Microscope assembly. US Patent no. 4,128,944.
- STANTON, M.G. (1980) Origin and magnitude of cell membrane resting potential. (Paper presented at meeting of Physiological Society in St. Andrews 12 Sep. 1980). *J. Physiol. (London)* (1981) 310 17P.
- STARLING, S.G. & WOODALL, A.J. (1956) "Electricity and Magnetism" 8th ed. Longmans, Green & Co. London, p.125.
- STEFAN, J. (1873) *Wien. Akad. Ber.* 68 385.
- STERN, (1924) *Z. Elektrochem.* 30 508.
- STEWART, G.N. (1897) Elektrische Leitfähigkeit tierischer Flüssigkeiten. *Zentr. Physiol.* 11 332.
- STEWART, G.M. (1899) The behaviour of hemoglobin and electrolytes of the colored corpuscles when blood is laked. *J. Physiol. (London)* 24 211.
- TASAKI, I. & HAGIWARA, S. (1957) Capacity of muscle fibre membrane. *Amer. J. Physiol.* 188 423-
- TEDESCHI, H. (1980) The mitochondrial membrane potential. *Biol. Rev.* 55 171-206.

- TEORELL, T. (1949) Membrane electrophoresis in relation to bio-electrical polarisation effects. Arch. Sci. Physiol. 3 205-
- TYLER, A., MONROY, A., KAO, C.Y. & GRUNDFEST, H. (1956) Membrane potential and resistance of the starfish egg before and after fertilisation. Biol. Bull. 111 153-
- TYREE, M.T. (1970) The Symplast Concept. A general theory of symplastic transport according to the thermodynamics of irreversible processes. J. Theor. Biol. 26 181-214.
- USSING, H.H. (1949) The distinction by means of tracers between active transport and diffusion. Acta Physiol. Scand. 19 43-56.
- USSING, H.H. (1952) Some aspects of the application of tracers in permeability studies. Adv. in Enzymol. 13 21-65.
- VALDIOSERA, R., CLAUSEN, C. & EISENBERG, R.S. (1974) The measurement of the impedance of frog skeletal muscle fibers. Biophys. J. 14 295-315.
- VISSCHER, M.B., FETCHER, E.S., Jr., CARR, C.W., GREGOR, H.P., BUSHEY, M.S. & BARKER, D.E. (1944) Isotopic tracer studies on the movement of water and ions between intestinal lumen and blood. Amer. J. Physiol. 142 550-575.
- WALKER, N.A. (1955) Microelectrode experiments on Nitella. Austral. J. Biol. Sci. 8 476-489.
- WALKER, N.A. (1960) The electrical resistance of the cell membranes in a Chara and a Nitella species. Austral. J. Biol. Sci. 13 468-478.
- WALKER, N.A. & HOPE, A.B. (1969) Membrane fluxes and electric conductance in Characean cells. Austral. J. Biol. Sci. 22 1179-
- WEST, E.S. & TODD, W.R. (1961) "Textbook of Biochemistry" 3rd ed. Macmillan, New York.
- WHITE, H.L., URBAN, F. & van ATTA, E.A. (1932) J. Phys. Chem. 36 3152-
- WILLIAMS, E.J., JOHNSTON, R.J. & DAINTY, J. (1964) The electrical resistance and capacitance of the membranes of Nitella translucens. J. Exp. Bot. 15 1-14.
- WOHLBARSHT, M.L., MacNICHOL, E.F., Jr. & WAGNER, H.G. (1960) Glass insulated platinum microelectrodes. Sci. 132 1309-
- ZIMMERMANN, U. (1978) Physics of turgor- and osmo-regulation. Ann. Rev. Plant Physiol. 29 121-148.