DISTRIBUTION AND FLUXES

OF SODIUM AND HYDROGEN

IN CRUSTACEAN MUSCLE CELLS

by

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ABSTRACT

A new technique was devised for measurement of the unidirectional sodium efflux from single striated muscle cells of the giant barnacle, Balanus nubilus. It involves the continuous measurement of the activity of sodium in the myoplasm with an intracellular sodium-specific microelectrode, during the collection of radiosodium from the same cell by a standard method. Changes in the specific activity inside the cell, which are larger than had been thought previously, can be calculated directly. Thus the sodium efflux can be calculated accurately.

It is assumed in these calculations that the only pool of intracellular sodium of appreciable size which exchanges rapidly with the extracellular solution is the sodium in solution in the myoplasm. Several experiments which test this assumption, together with results from the literature, are consistent with the hypothesis that most of the sodium associated with the cell yet not detected by the intracellular sodium-specific microelectrode resides in the extracellular space in association with the glycocalyx.

Intracellular microinjection was used to load the myoplasm of single cells with radiosodium. It was necessary to take into account the longitudinal diffusion of tracer inside the cell from injected to noninjected regions.

Use of the new technique to measure the sodium efflux from intact single muscle cells revealed several new results. Saturation of the efflux into normal Ringer's solution was not apparent even in cells with very high sodium content. However, saturation of the efflux into potassium-free solution and into ouabain-containing solution occurred at relatively low levels of intracellular sodium.

The efflux into sodium-free solution was similar to that into normal Ringer's solution. The decline in the sodium efflux reported by other workers to occur in this situation was found to be due to the rapid decline of the sodium content of the myoplasm which occurs. No 'sodium-sodium

exchange' was found. Most of the sodium efflux under normal conditions appears to be due to a mechanism which is not sensitive to external ouabain or potassium.

The sodium efflux in barnacle muscle was shown to be electrogenic. A correlation between the measured values of the active sodium efflux and the electrogenic portion of the membrane potential was found. The correlation was consistent with the predictions of a phenomenological extension of the leading model for the membrane potential, the Goldman-Hodgkin-Katz equation.

The efflux of hydrogen ions from the cell can only be measured indirectly, from changes in the intracellular pH. Measurements of the intracellular pH with an intracellular pH-specific glass microelectrode revealed no 'pH transients' of the type reported by other workers in different preparations of barnacle muscle. Measurements of the intracellular pH made with the micro-electrode and with an indicator method were in close agreement. However, the distribution of the indicator DMO (5,5-dimethyl-2,4-oxazolidinedione) exhibited unusual behavior not previously reported. A refinement of the DMO method which takes this behavior into account is described.

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A	area of membrane (cm^2)	...60
$(a_{Na})_m$	myoplasmic sodium activity (mM)	...25
c(x)	concentration of cation at distance x from origin	...69
cpm	counts per minute of radioactivity	...118
dpm	disintegrations per minute of radioactivity	...219
E_m	membrane potential (millivolts)	...50
F	charge of a mole of electrons (96,520 coulomb/mole)	...50
g_K	conductivity to potassium ions (coulomb^2/joule-cm^2-sec)	...53
I_K	current of potassium ions (coulomb/cm^2-sec)	...53
j(x)	ion flux at distance x from origin; j^P=passive, j^m=active	...69
k	rate constant (units depend on context, usually min^{-1})	...15
M_{Na}	unidirectional sodium efflux (mole/cm^2-sec)	...15
M	net cation efflux in electrogenic transport (mole/cm^2-sec)	...72
m_{Na}, m_K, m_{Cl}	net passive flux across membrane (mole/cm^2-sec)	...70
mc	millicurie of radioactivity	...219
mV	millivolt	...50
Na^*	moles ^{22}Na collected in 5 min in an efflux experiment	...60
Na^*_{cell}	moles ^{22}Na inside the cell	...60
Na^*_m	moles ^{22}Na in solution in the myoplasm	...60
Na_{cell}	moles ^{23}Na inside the cell	...60
$(Na)_{cell}$	$= Na_{cell}/V$: apparent concentration of Na in cell	...60
o.d.	outside diameter (mm or μ)	...107
pcs	picomoles/cm^2-sec	...140
P_x	permeability of membrane to species x (cm/sec)	...50
ℛ	coupling ratio of ouabain-sensitive Na-K exchange	...74
R	gas constant per mole (8.3×10^7 erg/mole-°K)	...50
SA	specific activity of sodium	...60
Slope Ratio		...122
T	absolute temperature (°K)	...50
t	time	...60
u	mobility (erg-cm/mole-sec)	...53
U	numerator of ln term of Goldman-Hodgkin-Katz equation	...72
V	volume of region indicated by subscript	...60
W	denominator of ln term of Goldman-Hodgkin-Katz equation	...72
z	valence of ion	...51
β	partition coefficient between solution and membrane	...53
$\gamma_\pm$	mean ionic activity coefficient	...25
λ	microlitre	...107
$\tilde{\mu}$	electrochemical potential (erg/mole)	...51
μ	micrometre	...81
μl	microlitre	...219
$\bar{\Phi}$	electrical potential (joule/coulomb)	...51
( )	concentration (mole/litre)	...15

subscripts: i = intracellular
          o,e = extracellular
          m = myoplasmic

ACKNOWLEDGEMENTS

SECTION 1. GENERAL INTRODUCTION

A. SCOPE OF THE THESIS

A principal function of the cell membrane is the translocation of ions and molecules. It has been estimated that between one-fifth and one-third of the resting energy production of the cell is devoted to the mechanism which transports sodium and potassium ions alone (Brinley & Mullins 1968; Whittam 1975; but see Chinet, Clausen, & Girardier 1977). The transmembrane distribution of ions and molecules is far different from that which would occur if they all were in equilibrium. In particular, the extracellular medium is poor in soluble organic molecules relative to the intracellular medium. Altogether, there is always present a force which tends to move water and electrolytes into the cell. For cells which lack rigid walls, the amount of osmotically active intracellular solute must be regulated if osmotic lysis is to be prevented and the cell is to be enabled to exist. The active extrusion of sodium is felt to be the major control of the water content of the cell (eg. Tosteson 1964; MacKnight & Leaf 1977).

The transmembrane distribution of sodium is also a store of energy, suitable for utilization by energy-requiring reactions and processes at the cell membrane. Important examples are those processes which effect transport of substances across the cell membrane, and those which rapidly transmit signals to another part of the cell or to a different cell in order to trigger chemical reactions.

Of course, whatever the other functions it serves, the regulated ionic composition of the interior of the cell appears to be required for the effective functioning of the metabolic machinery of the cell.

This thesis is concerned with the experimental measurement of ion

transport, and with certain aspects of the transport of sodium and hydrogen ions in whole cells of striated muscle. Interpretation of transport data is much more straightforward with isolated membrane preparations than with whole cells. However, with current techniques, it is only from a few cells that intact membranes can be isolated for study: red blood cells, and giant axons such as that of the squid. There are many similarities between the transport properties of these two cell types, but there are also many differences. Of necessity, then, whole cells must be examined so the nature and importance of the various transport processes can be discovered.

In addition, there are positive reasons for examining membrane transport properties in whole cells. The cells of a specialized tissue will possess a repertoire of transport mechanisms suitable for the tissue's function. A particular transport mechanism, present in most cell types, might be prominent in a particular cell type and so be more easily studied there.

The chemical species which mediates a given aspect of ion transport can only be identified in isolation if its behavior is known. This behavior must be deduced from study of the behavior of the whole cell. Once the detailed properties of an identified species are known, the part that species plays in the complete transport system of the cell can be deduced. Then the behavior due to other transport species can be deduced and an attempt made to isolate them. Eventually it is hoped that all species which contribute to ion transport will be characterized, and their behavior when acting in concert understood.

Finally, abnormalities of the transport systems can be a cause of or a feature of pathology of the tissue (Bolis, Hoffman, & Leaf 1976). As with other areas of physiology, a detailed knowledge of cellular transport mechanisms can lead to the formulation of a rational treatment plan. An

example is the use in the treatment of cholera of one transport mechanism to bypass another which is deranged (Field 1977).

There are many reasons, then, why the capability to study transport in whole cells and multicellular preparations should be developed.

Several problems are addressed in this thesis. The first is the technical problem of measuring the trans-membrane flux in whole cells. The main concern is the measurement of sodium fluxes. The measurement of hydrogen ion fluxes presents different problems, of both a conceptual and a practical nature. It is of secondary concern here.

In order to resolve the technical problem of measuring the flux, an investigation of the states of water and ions inside the cell had to be carried out. This is an active area of research in its own right.

The technique developed for flux measurement, which involves simultaneous use of an ion-specific intracellular microelectrode and radioisotopes, was applied to a brief overview of the kinetics of sodium transport in a whole cell.

Then, two specific problems were investigated: the sodium efflux into sodium-free solutions, of the type previously seen in frog skeletal muscle; and the electrogenic properties of the sodium transport.

Finally, indirect measurements of the extrusion of acid by the cell and of the heterogeneity of the intracellular pH were made. In this context, the use of an indicator for the measurement of pH was evaluated. The latter is a question of great practical interest.

The cell chosen for this work is the very large striated muscle cell of the giant barnacle Balanus nubilus. This crustacean was described by Darwin in 1854, but it was only in 1963 that Hoyle and Smyth described its neuromuscular physiology and suggested that it would be a valuable preparation for further such study. Since then, work has been published on its

ultrastructure, on the states of its water and ions, on the permeability and electrical properties of its membranes, and on its ion transport mechanisms.

Its large size makes it especially suitable for impalement by micro-electrodes (which it tolerates for long periods) and for microinjection. It was desired to use these techniques to sample the cell interior selectively and to load the cell interior with radioisotope selectively, as will be explained more fully later.

B. HISTORICAL NOTES

The study of the movement of substances into and out of cells is almost as old as the cell theory itself. The cell membrane cannot be seen with the light microscope, but permeation of solutes and osmotic effects are readily demonstrated. Nageli, a student of Schleiden's, inferred the presence of a cell membrane from his studies of plant cell permeability (Nageli & Cramer 1855). Pfeffer (1877) proposed from his work with artificial semipermeable films that a film with similar properties surrounded the cell. Overton (1899) measured the permeability of cells to many substances, and proposed that a layer of lipid on the surface of the cell was the principal barrier to penetration.

It was the plant physiologists who led the way in these studies on single cells. They found that plant cells actually accumulated certain substances, and seemed to exist in a 'non-equilibrium condition' in this respect (Hoagland & Davis 1929; Brooks 1929). Osterhout (1931) rejected the Donnan effect as the cause of the accumulation of ions, and proposed

that the continuous production of acid by the cell led to the passive inflow of potassium and chloride.

Brooks (1938) appears to have been the first to employ radioisotopes in the study of ion accumulation by individual cells.[1] He employed a radioisotope of potassium to quantitate the accumulation of potassium by a cell, and expressed the intracellular concentration in terms of the total cell water (the difference between the wet and dry weights of the 'protoplasm'). He observed a rapid penetration of potassium against the gradient of potassium concentration. He attributed this to ion exchange (Brooks 1940). Steinbach (1940) noted that the potassium accumulation theories then current required low permeability to sodium, while experiments had shown that sodium penetrates the cell quite readily. He remarked that "there must be some mechanism present for pumping out the sodium which wanders into the protoplasm." He felt that the ion distribution as a whole was due to a "physico-chemical balance between the protoplasm and the medium, with the permeability characteristics of the membrane playing only a subordinate structural role."

The efflux from cells of a radioisotope of sodium was measured by Levi and Ussing (1949) and later by others. Ussing (1949) derived a relation which should be obeyed by passive fluxes. Hodgkin and Keynes (1954) found that according to Ussing's relation, sodium was actively expelled from nerve cells.

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[1] The first use of radioisotope in uptake studies was much earlier. Hevesy (1923) measured the uptake by plants of an isotope of lead obtained as a natural breakdown product of thorium. The use of radioisotopes became more common after the development of the cyclotron and radioactivation in about 1936. It was E.O. Lawrence of the University of California at Berkeley and Niels Bohr of the Institute of Theoretical Physics in Copenhagen who supplied local physiologists with radioisotopes of phosphorous and potassium.

Keynes and Lewis (1951) explicitly formulated the 'bag model' of the animal cell for flux studies, wherein the intracellular region was assumed to comprise a single homogeneous compartment within a closed selectively-permeable membrane. The results of their experiments on squid axon seemed to be consistent with this formulation, but the results for muscle cells were more difficult to interpret. The trend has been to employ more complicated models having several cellular compartments among which ions can move (for example, Keynes & Steinhardt 1968; Rogus & Zierler 1973).

An enzymatic basis for the active transport of sodium and potassium across the cell membrane was discovered by Skou (1957) in the form of a sodium- and potassium-activated, magnesium-dependent adenosine triphosphate phosphohydrolase (the (Na + K)ATPase). This enzyme has come to be called "the sodium pump" (Glynn & Karlish 1975). Other mechanisms for the transport of sodium have been postulated, as will be discussed later, and many mechanisms for the transport of other species have been postulated.

The ability of tissues to generate an electric potential difference has been recognized for well over a hundred years (Matteucci 1840; Du Bois-Reymond 1843). The early work involved rather gross injury to the tissues, and the potential differences were called 'injury potentials'. They were thought to be due to the freeing of inorganic ions through chemical reactions in the injured tissue. The equilibrium theory of Donnan (1910) provided one model for the origin of the potential difference across the cell membrane, while the theoretical description by Teorell (1935) and Meyer and Sievers (1936) of the potential difference at the boundary between two solutions of different composition or concentration provided another.

Osterhout (1931) measured electrical potential differences across the 'protoplasm' of single plant cells, and formulated a model for their origin as diffusion potentials chiefly due to potassium. He felt that phase

boundary potentials and the Donnan potential would be too small to account
for his measured values, and that oxidation-reduction potentials would not
be measured with his apparatus. He stated that the equations of Nernst
and Henderson describing diffusion potentials "enable us to predict poten-
tial differences with sufficient accuracy to justify their use quite aside
from all other considerations." He identified differences in ionic mobil-
ities as the key feature of diffusion potentials, and set out to test the
model by measuring the mobilities of the ions in the "nonaqueous layers"
(membranes) of cells.

In the theoretical description formulated by Teorell (1935) and Meyer
and Sievers (1936) for the concentration potential developed across
membranes separating two electrolyte solutions, there was a Donnan potential
at each interface and a diffusion potential in the membrane.

Steinbach (1940) noted that the maintenance of a diffusion potential
requires "continued production of electrolytes, and as such is linked to
the metabolism of the cell."

Boyle and Conway (1941) analyzed the accumulation of potassium by
muscle and concluded that it must be due to a Donnan equilibrium, while the
sodium permeability of the muscle cell membrane must be extremely low.

Goldman (1943) applied the theory of diffusion potentials to simplified
models of the cell membrane, and obtained a good qualitative description of
the rectification and membrane potential in squid axon.

Hodgkin and Katz (1949) used Goldman's equations under the assumption
that the resting squid axon membrane was more permeable to potassium than
to sodium, while the sodium permeability could increase greatly to bring
about the reversal of polarization of the membrane which occurs during an
action potential. The passage of sodium across the membrane was proposed
to occur "in combination with a lipoid-soluble carrier in the membrane

which is only free to move when the membrane is depolarized." They
regarded their expression for the value of the membrane potential as "no
more than a rough approximation." However, it was sufficiently simple and
flexible that it could be applied to almost any result under quite reasonable
assumptions. Goldman's equation and variations of it continue to be used to
describe the membrane potential (eg. Schwartz 1971).

Cell water came under scrutiny very early on, because of the failure
of cells to act as perfect osmometers (for example, Overton 1902). It was
felt that "a considerable portion of the water in the cell or body is
physically 'bound' in the colloidal structure of the protoplasm and must be
considered an integral part of the living system" (Sharp 1934). The
presence of 'bound ions' was indicated by the presence of slowly-exchanging
fractions in ion uptake and depletion studies. With the construction of
ion-specific electrodes small enough to be placed into single cells (Taylor
& Whitaker 1927; Caldwell 1954; Hinke 1959; Walker 1971) it became possible
to study the interior of the cell directly, and it was apparent that not
all of the ions measured by chemical analysis of the cell were present in
free solution inside the cell. Some workers have concluded that the
asymmetrical ion distributions and osmotic behavior of the cell are due to
the association of the ions with fixed charge groups in cytoplasmic
macromolecules and to organization of the cell water, with the membrane
playing only a passive role (Troschin 1961; Ling 1962; Ling, Miller &
Ochsenfeld 1973).

A problem addressed by many present-day investigators is the elucida-
tion of the detailed mechanism of ion transport in cells. Most attention
is addressed to the cell membrane, but for the interpretation of studies of
the transport properties of the membrane in whole cells, it is necessary to

characterize the states of the intracellular water and ions. Why this is

so is described in the next section.

SECTION 2. TRANS-MEMBRANE FLUXES OF SODIUM AND HYDROGEN IONS

A. GENERAL CONSIDERATIONS

Almost all of the observed passage of sodium ions and indeed of all lipid-insoluble inorganic ions across the cell membrane is associated with membrane proteins. The permeability of a pure phospholipid bilayer to sodium is several orders of magnitude lower than that of a cell membrane (Jain 1972; Lauger & Neumcke 1973). The translocation of the ions can thus be regarded as an enzyme-mediated reaction in which one product is the translocated ion.

The efflux of sodium from cells normally involves a considerable increase in the electrochemical potential of the translocated ions, and so requires energy. This energy must come ultimately from metabolism. (Exchange and the passive unidirectional flux are considered below.) In theory, the direct source of energy for sodium efflux could be other ions (or sodium) which pass spontaneously to a region of lower electrochemical potential, or it could be the hydrolysis of 'high-energy' phosphate compounds or other products of metabolism. A mechanism involving the cytochromes of the electron transport system directly has also been proposed (Mitchell 1969).

Experiments have indicated that most and perhaps all of the metabolism-dependent sodium efflux depends directly on adenosine triphosphate (ATP) for energy (Dunham 1957; Whittam 1958; Caldwell 1960; Hoffman 1960). Inosine triphosphate (ITP), guanosine triphosphate (GTP), uridine triphosphate (UTP), phosphoarginine (PA), cytidine triphosphate (CTP), acetyl phosphate (AcP), phospho(enol) pyruvate (PEP), D-glyceraldehyde-3-phosphate (G-3-P), adenosine diphosphate (ADP), and adenosine monophosphate (AMP) do

not support sodium efflux, while deoxyadenosine triphosphate (d-ATP)

supports about half of the normal sodium efflux (Hoffman 1960; Brinley &

Mullins 1968).

When the ATP concentration in squid axon is reduced to very low levels

by internal dialysis, the sodium efflux approaches the rate expected from

passive mechanisms (Brinley & Mullins 1968). If the axon is treated with

cyanide (2 mM. CN for 1 - 3 hours) and then dialyzed, the ATP concentration

is reduced to about 2 μM. and the sodium efflux is reduced to the value

estimated for passive movement (Brinley & Mullins 1967). CN alone has

little immediate effect on the sodium efflux (Keynes & Maisal 1954; Hodgkin

& Keynes 1956; Carey, Conway, & Kernan 1959).

An ATPase found in the cell membrane and activated by sodium and

potassium has come to be regarded as being largely if not entirely respon-

sible for the metabolism-dependent sodium efflux (Skou 1965), at least in

red blood cells.[1] It is currently being referred to as "the" sodium pump

(Glynn & Karlish 1975).

Five modes of behavior have been described for this (Na+K)ATPase:

(i) exchange of internal sodium for external potassium, requiring ATP and

accompanied by a net hydrolysis of ATP; (ii) reversal of (i); (iii) exchange

of internal sodium for external sodium, requiring ATP and ADP but accom-

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[1]Sodium has also been alleged to be involved in the efflux of calcium
from nerve and muscle, where internal calcium is exchanged for external
sodium with no net hydrolysis of ATP (Baker 1972; Requena, DiPolo, Brinley,
& Mullins 1977), and in the efflux of magnesium, where once again sodium
enters and there is no net hydrolysis of ATP (Baker & Crawford 1972;
Ashley & Ellory 1972; Mullins, Brinley, Spangler, & Abercrombie 1977).
Sodium efflux apparently is associated with the transport of sugars
(Schultz & Curran 1970; Kimmich 1972) and amino acids (Colombini & Johnstone
1974; Johnstone 1974). A sodium-hydrogen exchange has been suggested but
is not clear (Keynes 1965; Biro 1965; Bittar et al. 1973, 1976).
An association of sodium and iodide transport has also been suggested
(Skou 1965).

panied by no net hydrolysis of ATP; (a separate sodium-sodium exchange found in nerve and muscle is discussed later; it is thought to comprise as much as half of the sodium efflux in muscle cells and is insensitive to inhibitors); (iv) exchange of internal potassium for external potassium, requiring inorganic phosphate and ATP but accompanied by no net hydrolysis of ATP; this apparently is associated with operation in mode (i); and (v) efflux of sodium without coupling to influx of another ion, requiring ATP and probably accompanied by a net hydrolysis of ATP (or much less effectively, CTP, ITP, or UTP; this mode is much less fastidious than the others) (Glynn & Karlish 1974, 1975).

The contribution of a given mode of the (Na+K)ATPase or of other transport mechanisms to the net flux might be difficult to deduce from simple experiments, since alteration of the substrate concentrations (ions and ATP) should cause a redistribution among all of the modes, rather than simply an alteration of the contribution of a single mode. An example from the literature is that in the absence but not in the presence of external potassium, both the influx and efflux of sodium in frog skeletal muscle are reduced by ouabain (Keynes & Steinhardt 1968). That is, removal of external potassium disables the sodium-potassium exchange but unmasks a ouabain-sensitive sodium-sodium exchange not apparent in solutions which contain potassium. This type of occurrence must be acknowledged in the interpretation of experiments.

Nevertheless, experiments in which the intra- or extracellular sodium or potassium ion concentration is altered and the effect on the sodium efflux is observed are of value in the study of the sodium efflux. Results from such experiments and variations on them are known for many cells, and by consideration of this abundance of data it is hoped that the general features of the different routes of sodium efflux can be deduced.

Like the efflux of sodium, the efflux of hydrogen ions from cells involves an increase in the electrochemical potential of the translocated ions, and so requires energy. It is only relatively recently that convincing evidence for specific transport mechanisms has been found. The study of the efflux mechanism for hydrogen ions is more difficult than is the case for sodium because processes equivalent to the expulsion of protons appear to be operating, in addition to the expulsion of protons themselves. Also, the cell is not conservative with respect to hydrogen ions. (Further difficulties of a more technical nature will be discussed later.)

Mitchell (1969) proposed a model, originally for mitochondria, wherein the electron transport system of aerobic metabolism was embedded in the membrane, and the energy derived from the flow of electrons through this system was used directly to expel protons to the outer side of the membrane. He assumed that there was a membrane-bound ATPase which had the capability of expelling protons as it hydrolyzed ATP. It was thought to 'run backwards' as protons passed into the cell through it, and ATP was thus formed from ADP and inorganic phosphate. This is called the chemiosmotic hypothesis.

Rehm (1972) proposed that the acidification of the lumen of the stomach was due to an electrogenic hydrogen ion pump directed toward the lumen. In addition, a chloride pump was thought to be directed toward the lumen and a sodium pump toward the blood. Acidification would occur when the hydrogen ion pump became active and the sodium pump became inactive. Coupling of the pumps was not rigid. The respiratory chain was invoked directly to pump protons, as in the Mitchell model, and it was thought that ATP was not directly involved.

Stoeckenius and co-workers (Osterhelt & Stoeckenius 1973; Danon & Stoeckenius 1974; Stoeckenius 1976) found that the 'purple membrane' of the

bacterium <u>Halobacterium halobium</u> could expel protons from the bacterium when exposed to light, and that the action spectrum for ATP production in response to light was similar to the absorption spectrum of the purple membrane. The bacterium could also expel protons in the dark under aerobic conditions. Stoeckenius proposed that the bacterium contained three membrane-bound systems capable of expelling protons: (i) a purple membrane protein which could expel protons when exposed to light; (ii) a respiratory chain which could expel protons using the energy from aerobic metabolism; and (iii) an ATPase which could expel protons using the energy from the hydro-lysis of ATP, but which usually operated in the opposite sense, synthesizing ATP from ADP and inorganic phosphate using the energy stored in the 'proton gradient' created by the other two systems. This seems to be a unique case, but it provided evidence that an ATPase capable of causing proton transport <u>per se</u> exists. Nature has tended to employ extended and improved versions of primitive cellular mechanisms in the more sophisiticated cells which evolved later. It would not be too surprising if the 'proton pump' of nucleated cells is found to be built on these mechanisms.

A sodium-hydrogen exchange was suggested by many workers (Keynes 1965; Biro 1965; Bittar <u>et al</u>. 1973, 1976) but only recently has the dependence of the alkalinization of the cell on extracellular sodium been demonstrated, in mouse skeletal muscle cells (Aickin & Thomas 1977), snail neurone (Thomas 1977), and barnacle muscle cells (Boron & Ross 1978).

Finally, a chloride-bicarbonate exchange has been proposed. The exchange of internal chloride for external bicarbonate would be equivalent to the extrusion of HCl, since at a given CO_2 tension excess bicarbonate would quickly combine with a proton to yield carbonic acid and then, under catalysis by carbonic anhydrase, water and CO_2. The CO_2 would leave the cell passively. Similar systems had been proposed for the cerebrospinal

fluid (eg. review by Siesjo 1972). Good evidence for chloride-bicarbonate exchange has been found in snail neurone (Thomas 1976), squid axon (Boron & DeWeer 1976a), mouse skeletal muscle (Aickin & Thomas 1977), and barnacle muscle (Boron & Roos 1978).

A particular mechanism for sodium or hydrogen transport is characterized by its kinetic behavior. Much more work has been done on the sodium efflux kinetics than on the proton efflux kinetics. The connection between the efflux and the kinetics of each of the sodium transport systems will be discussed in part (C) of this section.

In the general case, the connection is made via a chemical reaction model in which the substrate S binds with an enzyme E to form a complex ES, which then dissociates into enzyme and products Pr, with no back reaction:

$$E + S \; \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \; ES \; \overset{k_2}{\longrightarrow} \; E + Pr \quad .$$
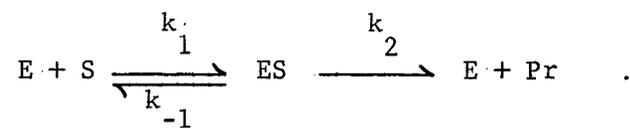
The overall rate, and hence the efflux rate if this models the dominant mechanism for sodium extrusion, is proportional to the concentration of the complex, (ES). It is assumed that the dissociation of this complex to yield products is so much slower than the reactions which form it that the reactions preceding this dissociation are essentially at equilibrium (steady state assumption).

The reaction mechanism for the (Na+K)ATPase has been characterized in some detail through the use of in vitro preparations of the enzyme, but a simplified model will be adopted here. Only one parameter, (S) = (Na), is assumed to be variable, and the rest is concealed in the rate constants.

In this, the elementary 'Michaelis-Menten' model of enzyme kinetics, (ES) can be expressed in terms of (S) and the equilibrium constant for the reaction which forms the complex, and the overall rate, eg. of sodium efflux M_{Na}, depends on (Na) as

$$M_{Na} = M_{max} / (1 + \frac{k}{(Na)})$$

For three sodium ions binding successively to E at equivalent independent sites, a realistic model of the (Na+K)ATPase (Glynne & Karlish 1975), a similar treatment yields

$$M_{Na} = M_{max} / (1 + \frac{k}{(Na)} + (\frac{k}{(Na)})^2 + (\frac{k}{(Na)})^3)$$

where k of course has a meaning different from that in the previous case. If the three sodium ions bind simultaneously, the relation is

$$M_{Na} = M_{max} / (1 + \frac{k^3}{(Na)^3})$$

(Mullins & Frumento 1963), where again k has a different significance.

More complicated versions will be described later. It is conceivable that the effective number of binding sites is different at different sodium concentrations, for example.

It should be noted at this point that Baker, Blaustein, Keynes et al. (1969) and Garay and Garrahan (1973) appeared to take a different approach, in that they assumed instead that three sodium ions had to bind to equivalent independent sites on the enzyme, and that the efflux was proportional to the fraction of the independent enzyme units which were fully occupied by three sodium ions. This fraction they took to be the cube of the probability of having an enzyme unit occupied with sodium at one site. This probability is

$$\frac{(ES)}{(E) + (ES) + (ES_2) + (ES_3)}$$

but they took it to be

$$\frac{(ES)}{(E) + (ES)} = \frac{1}{1 + k/(S)}$$

for $k = (S)(E)/(ES)$. Since

$$k = \frac{(S)\,(E)}{(ES)} = \frac{(S)\,(ES)}{(ES_2)} = \frac{(S)\,(ES_2)}{(ES_3)}$$

then

$$(E) + (ES) + (ES_2) + (ES_3) =$$
$$(E)\left[1 + \frac{(S)}{k} + \left(\frac{(S)}{k}\right)^2 + \left(\frac{(S)}{k}\right)^3 \right].$$

Writing $(ES) = (S)\,(E)\,/\,k$, one sees that the probability is actually

$$\frac{1}{\frac{k}{(S)} + 1 + \frac{(S)}{k} + \left(\frac{(S)}{k}\right)^2}$$

for having one site occupied by sodium, while the probability of having all

three sites occupied is

$$\frac{(ES_3)}{(E) + (ES) + (ES_2) + (ES_3)} = \frac{1}{1 + \frac{k}{(S)} + \left(\frac{k}{(S)}\right)^2 + \left(\frac{k}{(S)}\right)^3}$$

or just as for the Michaelis-Menten case for three equivalent sites. This

had to be so, of course, since the assumptions were equivalent. The fact

that a good fit to the experimental data was obtained with the incorrect

model illustrates the ease with which a smooth curve can be approximated by

a polynomial, and the limitations of this sort of modelling.

Continuing in this vein, the applicability of this type of model to the

(Na+K)ATPase can be considered. In reality, the steps followed by the

enzyme to transport sodium out of the cell via the (Na+K)ATPase involve the

binding of ATP, magnesium, and "n" sodium ions. The enzyme becomes

phosphorylated and the conformational changes required to make the

sodium available to the outside of the cell occur. It has generally

been assumed that these steps of conformational change will be rate

limiting, so that the steady state approximation can be applied.

Recently, Mardh and Post (1977) found evidence that with each binding of

ligand to E, the conformation shifted significantly towards the "potent"

complex which can proceed to phosphorylation of E. That is, the initial rate of phosphorylation of the enzyme was increased over that when ATP, magnesium, and sodium were all made available at once, if one or two of the ligands were added first, and then the missing ligands were added. This suggests that the steady state assumption cannot be applied with impunity in the case of the sodium efflux.

Nevertheless, it should be possible to obtain semi-quantitative agreement with the data if the basic notion of a 'dominant mode' with n sodium ions binding is correct.

Thus, the approach has been to compare the kinetic curves of the sodium transport out of cells with the curves produced by kinetic models such as the above. Even with just two adjustable parameters, k and M_{max}, it is usually easy to get a reasonable fit for data over most of the concentration range. It is at low substrate (sodium) concentration that differences between models are most apparent.

It is important, then, to measure as accurately as possible the concentration of the reactant (eg. sodium) in the transport reaction. (In fact, the quantity of interest is the chemical potential, but discussion of such refinements will not be presented here.) This is the concentration at the internal reaction sites of the transport enzymes, and it is on this point that a complication arises.

As noted above, the cell is a heterogeneous structure, and it has been found that the water and solutes inside the cell do not behave as if they were in a simple aqueous solution bounded by a protein-lipid membrane. Before the experimental work on fluxes could be done, it was felt that the current notions about the states of water and sodium ions inside the barnacle muscle cell had to be clarified. In particular, the amount and distribution of the cellular sodium which will participate in flux studies,

and the concentration of sodium in the solution which bathes the internal surface of the cell membrane must be known. This interesting general problem is reviewed next.

B. STATES OF WATER AND IONS IN CELLS

The current wiew of the state of water and ions inside living cells can be summarized briefly as follows (Tait & Franks 1971; Hinke, Caille, & Gayton 1973; Palaty & Friedman 1973; Cooke & Kunta 1974; Berendsen 1975; Lee & Armstrong 1974; Edzes & Berendsen 1975; Lev & Armstrong 1975).

Water in cells behaves largely as it does in bulk solutions. 75 to 90% of the water has normal liquid properties as far as diffusion of ions and molecules, osmotic effects, and solvation are concerned, and responds like bulk water in NMR, infrared spectroscopy, and x-ray and neutron diffraction studies. About 1% is tightly bound to macromolecules as 'structural water'. The remaining 8 - 24% is influenced by the macromolecules and the structural water, apparently through the formation of short-lived extended clusters of water molecules, by means of hydrogen bonding, in the so-called 'hydrophobic interaction'. This fraction is of special interest in that it is conceivable that its behavior is different from that of bulk water. There is disagreement about its exact size. Techniqes which reflect the freedom of motion of individual water molecules, such as NMR yield the lower estimates, while measurements of solvent properties yield the higher estimates.

Water passes the cellular membranes very readily, and quickly flows to or from any region of the cell where its chemical potential deviates from that of the rest of the cell, or indeed of the vicinity of the cell. Because the cell membranes can transport substances and are selectively-permeable, however, this is not the case for the major inorganic ions. They can be

confined within or barred from the cell or membrane delimited organelles inside the cell. Further, in a given compartment inside the cell or in the extracellular space they can be in free solution, or associated with large or small organic molecules via specific or nonspecific binding. Finally, even though an ion can pass from a free state to one of the non-free states listed, in response to a non-uniformity in its chemical potential, the characteristic time of the exchange might be very slow relative to that of other cell processes, notably diffusion in bulk solution and transmembrane transport.

The activity of some ions inside the cell can be measured with ion-specific microelectrodes. These actually reflect the chemical potential of the ion inside the cell, but the activity and concentration of the free ion can be estimated under reasonable assumptions about the solvent proper- ties of the water in which the free ion is thought to reside. If the volume of distribution of the free ion can be estimated, then the amount of free ion in the cell can be calculated.

This is the conceptual heart of the question. The ions in solution are no more free than those participating in ion pairing, during steady conditions, in the sense that the chemical potential is the same for the two groups. The distinction is made because both are measured by chemical analysis, while only the solvated ion is assumed to be measured in microelec- trode studies. The assumption involved is that all of the free ion is in a homogeneous compartment as far as concentration is concerned, although it is clear that near charge inhomogeneities on membranes or macromolecules, considered as a group, the concentration will be different from that in the bulk even though the chemical potential is the same.

The total ion content of the cell, including that in extracellular locations, can be determined quite accurately from chemical analysis of the

ashed tissue. The ion content of the extracellular space is often difficult
to determine, because fixed negatively-charged sites abound in
the polysaccharide-rich glycocalyx. If this ion fraction can be estimated,
then by subtraction one can calculate the amount of ion which is truly
intracellular but not in free solution. This is especially difficult for
sodium, which has a high extracellular but low intracellular concentration.

The individual ions can be considered in turn. They all can pass the
resting cell membrane, but at very different rates.

Sodium is actively expelled from the cell (that is, during steady
conditions the chemical potential for sodium ions is lower inside the cell
than outside the cell). No local intracellular accumulations of sodium
have been found in analyses of subcellular fractions. (This will be reviewed
below.) However, a large amount is thought to be associated with fixed
negative sites on intracellular macromolecules, in competition with other
cations.

Hydrogen is actively expelled from the cell. The intracellular pH is
buffered by the intracellular proteins, and by phosphate and bicarbonate.
Hydrogen ions are also produced and consumed in many reactions in the cell.

Potassium is actively accumulated by the cell, but because the permea-
bility of the cell membrane to potassium is relatively high the chemical
potential for potassium ions is about the same inside and outside many cells.
A quantity of potassium which is small relative to the amount of potassium
in solution probably associates with fixed negative sites inside the cell.

Calcium is actively expelled from the cell. It is also actively
sequestered in mitochondria and in the sarcoplasmic reticulum of muscle.
An additional buffering mechanism of very large capacity appears to exist
(Brinley, Tiffert, Scarpa, & Mullins 1977).

Magnesium is actively expelled from the cell. About half of the

intracellular magnesium is bound to ATP in barnacle muscle (Brinley, Scarpa, & Tiffert 1977).

The situation with chloride is not clear. Microelectrode measurements indicate that there is a slight accumulation of chloride inside the cell (see also Bolton & Vaughan Jones 1977; Dulhunty 1978). Diffusion studies show little chloride associated with fixed intracellular sites, but large bound fractions have been reported. Chloride, like sodium, is abundant in the extracellular space, and this makes accurate allocation of chloride to compartments difficult.

The situation in a given cell type often differs in detail from these generalizations. This thesis is primarily concerned with sodium and hydrogen in barnacle muscle cells.

The hydrogen ion exemplifies the problems of interpretation of measurements of the chemical potential discussed above. The hydrogen ion differs in several fundamental respects from the sodium ion in living systems. First, the cell is not conservative with respect to hydrogen: hydrogen ions participate as reactant and product in many chemical reactions in the cell. Changes in these reactions might occur with any manipulation of the cell or its environment. Second, hydrogen is buffered by the bicarbonate-CO_2 system and, more importantly inside the cell, by the phosphate system and the protein system. Only 0.001% of the available hydrogen ion is free in solution at pH 7.0 (Waddell & Bates 1969). To calculate changes in the amount of hydrogen ions with any manipulation, it is not sufficient to measure only pH changes. The buffering capacity at each stage of the manipulation must be known. Third, hydrogen is a labile part of the solvent (water) in which the entire cellular system is embedded. The effective translocation of hydrogen ions can occur by the forming and breaking of hydrogen bonds and hydrogen-oxygen bonds in the water. This is

much more rapid than the self-diffusion of hydrogen, and means that no radioisotope can be used to measure fluxes. Finally, the concentration of hydrogen ions in solution in the cell is usually about 10^5 times smaller than that of sodium.

The question of the relationship between measurements of pH (which is defined in terms of the potential difference developed in a standard electrochemical cell) and 'hydrogen ion concentration' troubles even the physical chemists, and they maintain that the quantity of practical interest in almost all contexts is the chemical potential (Waddell & Bates 1969). Nevertheless, processes equivalent to the movement of hydrogen ions do occur across the cell membrane, and it is reasonable to calculate the effective quantity of hydrogen ions involved. The reasonable approximation that pH is the negative of the logarithm of the hydrogen ion activity will be adopted in these qualitative discussions.

The question of the inhomogeneity of the pH inside the cell arose early because measurement of the distribution of an indicator (weak acid or weak base) was the usual technique for measuring pH (Fenn & Maurer 1935). When these measurements indicated that the pH was too high inside cells, the existence of alkaline organelles was suggested as the cause. Otherwise the active extrusion of protons from the cell would have had to be postulated.

Recently, Garthwaite (1977) examined the difference in the pH measured by acidic (DMO - see section 9) and basic (nicotine) indicators in various tissues, with reference to the number of mitochondria present. A weak acid will yield a pH value closer to the higher pH in the inhomogeneous tissue, and a weak base will yield a pH value closer to the lower pH (Waddell & Bates 1969). The difference ($pH_{acid} - pH_{base}$) in the pH results of the two indicators was 1.0 in brown fat, which has many mitochondria, about 0.8 in

most cells, and about 0.08 in mature red blood cells, which have no organelles. Mitochondria are thought to have a high pH. Further, in most cells the difference in the measured pH values was reduced by exposure to DNP (dinitrophenol). This would uncouple oxidative phosphorylation in mitochondria and presumably prevent them from maintaining a high internal pH.

For barnacle muscle cells, the pH measured with the acidic indicator DMO has been reported to be higher than that measured with the basic indicator methylamine by about 0.1 (Boron & Roos 1976). These values were lower than the value measured with an intracellular electrode, which is consistent with the existence of an acidic intracellular compartment.

This phenomenon presents a practical problem, in that the intracellular pH for most tissues can only be measured with indicators, and the meaning of what they measure is not certain. For this reason, experiments were done, as part of the work presented in this thesis, in which the pH was measured with DMO, as a measure of the pH of the whole cell in the sense discussed above, and with a pH microelectrode, as a measure of the pH of the major aqueous intracellular compartment, over a wide range of intracellular pH, in identically-prepared barnacle muscle cells. The result, discussed in section 9, was that pH(DMO) was consistently higher than pH(electrode). This in itself is consistent with the existence of an alkaline intracellular compartment, but the technical difficulties of the indicator method are such that this cannot be stated with any degree of certainty.

Attention will now be turned to the states of water and sodium in barnacle muscle.

Hinke (1970) adopted a working hypothesis for single barnacle muscle cells wherein the intracellular water was divided into two fractions: one

("ideal water") was completely like bulk water; the other was not behaving as bulk water in that it did not act as solvent for sodium, potassium or chloride, and was not osmotically active. His experiments indicated that the bulk water comprised about 68% of the water in a blotted cell, which is about 73% of the intracellular water since about 7% of the cell water lies in the extracellular space. The measured size of the extracellular space depends on the technique of blotting. In the same study, it was found that the mean ionic activity coefficient $\gamma\pm$ in the myoplasm was 0.65, the value in a bulk solution at the ionic strength of normal barnacle Ringer's solution. A similar value can be deduced from the data of Hagiwara, Chichibu, and Naka (1964).

The volume of distribution of the free ion was assumed to be the volume of "ideal water", so free ion contents could be determined from microelectrode measurements. It was found that only a part of the intracellular sodium, potassium, and chloride measured by chemical analysis of whole cells is in free solution in the "ideal water" (McLaughlin & Hinke 1966; Hinke, Caille, & Gayton 1973). The "missing fractions" were typically 13% of the potassium, 73% of the sodium, and 31% of the chloride (Hinke et al. 1973). One estimate was that fully 83% of the intracellular sodium could be inaccessible to the sodium microelectrode (Hinke 1969b).

The results of microelectrode studies by other workers in other cells have been similar (reviewed by Lev & Armstrong 1975). However, explicit allowance for 'bound water' is seldom made. Lee & Armstrong (1972;1974) made no allowance for 'bound water' in their calculations of free ion concentrations in frog skeletal muscle, although they acknowledge the concept and the physical meaning of the calculated concentrations. They based their conclusions about the existence of sequestered sodium and potassium on the observed changes in the apparent activity coefficient $(a_{Na})/(Na)_i$ when the

sodium content of the cell was altered, where (a_{Na}) is the activity of sodium deduced from the microelectrode measurement and $(Na)_i$ is the quotient of the total analyzed cellular sodium and the total water content of the cell, excluding the extracellular space.

The location of the 'missing sodium' in barnacle muscle cells is not certain, but some conclusions can be drawn from a critical review of morphological and physiological studies. The ultrastructure of the barnacle muscle cell was examined by Hoyle *et al.(1973).*The structure is qualitatively similar to that of vertebrate striated muscle, but there are several unusual features. The cell membrane is deeply furrowed by an extensive, unordered system of clefts. These were classified as "major clefts", deep furrows opening directly into the bathing solution all along their length, and "minor clefts", branches opening into the major clefts or the bathing solution only at their ends. The clefts contained "mucopolysaccharide-like" material, and comprised about 8% of the total cell volume as measured from micrographs. A system of flattened tubules oriented both longitudinally and radially, and devoid of the 'mucopolysaccharide', comprised less than 1% of the total volume. The latter system was identified as the analogue of the transverse tubular system (TTS) of vertebrate striated muscle. The tubules open into clefts or to the exposed surface of the cell. The sarcoplasmic reticulum is small, comprising about 0.5% of the total cell volume. By comparison, it is about 13% of the total cell volume for frog sartorius muscle (Peachey 1965). There are longitudinal and cisternal elements, and diadic (rarely: triadic) contacts are made with TTS.

Mitochondria and nuclei are located just under the exposed sarcolemma and the membrane of the major clefts. Together the latter organelles probably comprise less than 1% of the cell volume.

The remaining almost 90% of the cell volume is occupied by the

contractile proteins and the myoplasmic solution.

It is among all of these structures that the compartments of a flux model should find counterparts.

If the sodium not detected in the myoplasm by the microelectrode is isolated in the other intracellular compartments, very high concentrations must be attained. Few studies on the ionic content of organelles are available. Size alone was considered to rule out the nuclei and mitochondria of barnacle muscle cells as significant repositories of sodium.[2] The cell membrane itself is probably slightly more important, since sodium, potassium, magnesium, and calcium interact competitively with the membrane polar groups.

The cleft system is directly open to the bathing solution, and so the solution filling it will have the sodium-rich composition of the bathing solution. The solid material in the clefts is the negatively-charged polysaccharide of the glycocalyx and will have sodium associated with it, perhaps in large quantities (Harris & Steinbach 1956; Brading & Widdicombe 1977). The amount of sodium associated or adsorbed will depend on the concentration of sodium in the bathing solution. The amount of glyco-protein in barnacle cells has not been determined. An indication of its binding capacity can be found in experiments on smooth muscle, where at least half of the extracellular space cation content was found not to be in solution in the sucrose space (Brading & Widdicombe 1977).

Correction of the total analyzed cell sodium by assuming that all extracellular sodium is in a volume of fluid equal to the inulin (or other

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[2]Some sodium is sequestered in the nuclei and mitochondria of frog skeletal muscle (Sorokina & Kholodova 1970) and in the nuclei of rat hepatocytes (Hooper & Dick 1976). However, electron microprobe analysis has shown that nuclear and cytoplasmic sodium concentrations are the same in toad oocytes (Dick 1978), and some accumulation of sodium in nuclei and mitochondria of thymus and liver cells has been reported in different species (Itch & Schwarta 1957; Thiers, Reynolds, & Valee 1960).

marker) space, at the concentration of the bathing solution, thus probably is inadequate.

The TTS of crayfish muscle has been shown to swell when chloride is caused to enter it from the myoplasm (Girardier, Reuben, Brandt, & Grundfest 1963). This occurs when the extracellular chloride or potassium concentration is reduced so that potassium chloride is caused to leave the cell, but not during an osmotic stress when the product $(K)_{bath} \times (Cl)_{bath}$ of concentrations is kept constant so that potassium chloride does not leave the cell. The TTS therefore might comprise more than 1% of the cell volume under some conditions. It appears to open directly into the extracellular solution, and indeed in frog skeletal muscle is accessible to extracellular sucrose (Birks & Davey 1969). Flux studies by Harris (1963) had indicated that 15 - 30% of the cell volume was a "special region" freely accessible to sodium, chloride, and sucrose. The work of Birks and Davey plus that of many others (Vinogradova 1967, 1968; Vinogradova, Nikolsky, & Troshin 1967; Sperelakis, Shigenobu, & Rubio 1978; Rogus & Zierler 1973) indicated that this was the sarcoplasmic reticulum. However, Neville (1979) has shown from the kinetic behavior of this "special region" that it cannot be sarcoplasmic reticulum, and Somlyo, Shuman, & Somlyo (1977a) found no accumulation of sodium in the sarcoplasmic reticulum on electron microprobe analysis of toadfish striated muscle. It is possible that the special compartments of flux studies are artifacts of analysis.

In any case, the TTS of the barnacle cells used in the experiments described in this thesis were subjected to treatment which would cause swelling in only a few special cases. Altogether, it is very unlikely that the TTS in barnacle contains much of the 'missing sodium'.

The contractile proteins form a large compartment, and have not yet been considered here. It might reasonably be expected that most of the

intracellular sodium not detected by the electrode is associated as counter-ion with the fixed anionic sites on the proteins in this compartment (Hinke et al. 1973). Myosin is known to associate with cations (Szyent-Gyorgi 1947; Fenn 1957; Lewis & Saroff 1957), and is unique among the major proteins of the cell in showing a modest preference for sodium over potassium. Studies of the diffusion of ions and molecules inside barnacle muscle cells have indicated that the muscle protein has sites which can sequester cations but admit to very rapid exchange with the cations which are free in solution inside the cell (Caille & Hinke 1972, 1973, 1974). The total capacity of these sites for sodium and potassium was estimated to be about 68 millimoles per kilogram of dry weight. Again, a simple model, with only very rapidly exchanging sites, was assumed, so the capacity might be larger if some sites have longer residence times.

Experiments with a different time resolution, in which the cell membrane was destroyed and the protein allowed to equilibrate via a jacket of porous glass, with bathing solutions of different sodium and potassium content, yielded capacities about twice as large (Fenn 1957; McLaughlin 1968; Hinke et al. 1973). About half of the dry weight of a barnacle muscle cell apparently is due to soluble organic molecules (M.E. Clark, personal communication), accounting for the larger apparent capacity, but the experiments with membrane-damaged cells might reflect compartmentalization with less-rapid exchange.

From these morphological and physiological studies, then, it seems reasonable to conclude that, in barnacle muscle cells, the cell membrane and membrane-delimited organelles which sequester sodium and are not directly open to the extracellular space contain only a small portion of the intracellular sodium not detected by the microelectrode (in the model of the cell outlined above). Most of this small pool of sodium should engage in

rapid exchange with sodium in free solution in the cell and so influence

flux experiments, but it appears that accurate measurement of the extra-

cellular sodium might be a more significant problem.

The plan of experimental investigation of this problem for this thesis

was to follow changes in the sodium content of the compartment measured by

the microelectrode, as the total sodium content of the cell was manipulated.

It was found that indeed a great deal of the 'missing sodium' appears to

reside in the extracellular space, but that there appears to be some

sequestered inside the cell as well. These experiments are described in

section 3.

It was concluded from these experiments that the intracellular sodium

activity measured by a sodium-specific microelectrode was the most suitable

parameter against which the sodium efflux should be compared in kinetic

studies. An additional advantage of the use of continuous measurements

with the microelectrode was that rapid changes in the intracellular sodium

activity could be followed. It was anticipated that these might occur in

sodium-free solutions under certain conditions, as they had in frog skeletal

muscle (White & Hinke 1976).

It was also concluded that an attempt should be made to load the inside

of the cell with radiosodium selectively, so that efflux from the extra-

cellular space would not conceal any part of the transmembrane flux (White

& Hinke 1976), although this is not the only way to accomplish this end.

This meant that a study of the effects of microinjection had to be done,

and this is described in section 4. Microinjection studies can also yield

information about the states of sodium inside the cell, as will be described

later.

C. THE SODIUM EFFLUX

As noted above, the general experimental approach to the sodium efflux has been to compare the data with the predictions of kinetic models in hope of determining the general kinetic properties of the sodium transport systems. The behavior of the transport systems when the cells are in physiological saline in vitro should be close to that in vivo. However, it is likely that there is more than one transport mode, so the results in the physiological saline (normal Ringer's solution) probably reflect the contributions of several modes.

It has been of interest to compare the kinetics in normal Ringer's solution with the kinetics in solutions where one possible mode has been altered. For example, a sodium-potassium exchange mode should be markedly reduced if potassium is omitted from the saline, and the contribution of other modes to the sodium efflux seen in this situation will be greater.

It bears repeating that this maneuver possibly does not just reduce the sodium-potassium exchange mode, but rather causes in addition a change in the contribution of the other modes to the efflux. Neither the size of the sodium-potassium exchange mode nor the size of the contribution of the other modes in normal Ringer's solution can be measured. Nevertheless, it is possible to obtain information from experiments such as these. An example from the literature is the finding that in invertebrate giant axons, the reduction in the sodium efflux which follows removal of external potassium can be appreciably greater than the size of the potassium influx. This was strong support for the hypothesis that potassium ions act as activators of the sodium efflux as well as engaging in exchange for sodium (Hodgkin & Keynes 1955a; Sjodin & Beauge 1967; Mullins & Brinley 1967, 1969; Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt 1969).

Examination of the kinetics of the total sodium efflux traditionally has been done for efflux into sodium-<u>free</u> solutions, because some experiments indicated that a great deal of sodium-for-sodium exchange occurred across the cell membrane (Keynes & Swan 1959, but see Mullins & Frumento 1963). This was considered to be entirely separate from the 'active efflux', which was the efflux of most interest (Keynes 1966, but see Keynes & Steinhardt 1968). Such experiments defined the problem of the dependence of the efflux on the internal sodium concentration, so they will be reviewed briefly here with reference to this question.

Keynes and Swan (1959) found that a plot, for selected experiments, of the efflux of radiosodium from frog skeletal muscle versus the total amount of radiosodium remaining in the muscle, as radiosodium and ^{23}Na were washed out into sodium-free lithium-substituted Ringer's solution, implied a power law relationship between efflux and sodium content. They suggested that the carrier in the membrane can only operate when "n" sodium ions were bound to it, where n appeared to be three. This was assumed to be independent of the sodium-sodium exchange seen in normal Ringer's solution. This model was attractive because it required only one intracellular compartment, hence no complicated exchanges of sodium between intracellular compartments, and yet explained the data fairly well. Of course, a power law takes no account of saturation of the transport system, and should fit best at very low values of the cellular sodium content. Keynes and Swan found deviations at low rather than at high levels of sodium. Mullins and Frumento (1963) extended these experiments to higher values of sodium content. They found a "cube law" fit best at low sodium concentration, but that the 'power' decreased as the sodium content increased, and that saturation occurred. (At very high sodium content, a very rapid efflux was seen. This will be discussed with the sodium-free effect below.) They used a relation very

similar to the Michaelis-Menten case of three sodium ions binding simultaneously, discussed above, but did not use the Michaelis-Menten model.

Later, Keynes and Steinhardt (1968) recanted on the power law model because the large sarcoplasmic reticulum of the frog muscle cell had been described (Peachey 1965), providing a morphological basis for two-compartment models. In addition, the large fraction of the intracellular sodium not detected by microelectrodes had been described for frog muscle (Lev 1964). A "series-parallel" model with first-order kinetics was proposed, and explained some of the observations.

However, the properties of the (Na+K)ATPase had been further elucidated in the meantime, and it appeared that about three sodium ions were transported per ATP molecule hydrolyzed (Glynn 1962; Bonting & Caravaggio 1963; Sen & Post 1961, 1964). The mechanism suggested by Keynes and Swan thus could not be ignored. The kinetics of the various modes of the ATPase were verified (eg. Glynn & Karlish 1975), but attempts to clarify the kinetics of the sodium efflux from intact cells other than erythrocytes tended instead to reveal added complications (eg. White & Hinke 1976).

(i) Sodium efflux into normal Ringer's solution.

The efflux of sodium M_{Na} into normal Ringer's solution, that is, where the extracellular sodium concentration has not been reduced, has been measured in several cell types, and the question of how the efflux varies with the internal sodium concentration $(Na)_i$ has been considered.

In the squid axon, M_{Na} is a strict linear function of the intracellular sodium concentration over the range of 1 to 220 mM. (Hodgkin & Keynes 1956; Sjodin & Beauge 1967; Brinley & Mullins 1968). No saturation was seen. It is known that nerve cells have high concentrations of the (Na+K)ATPase (eg. Bonting, Simon, & Hawkins 1961), but it is not clear why such a large

pumping capacity is needed by squid axon.

In snail neurones, the rate of fall of the intracellular sodium activity (a_{Na}) following iontophoretic injection of sodium ions is an affine function of (a_{Na}), that is, linear above a threshold value of (a_{Na}) (Thomas 1972b).

In frog ventricular muscle, no saturation was seen over the range of sodium content studied, but M_{Na} rose as $(Na)_i^n$ for n between 1.0 and 1.6 in different experiments (Van der Kloot & Dane 1964).

In red blood cells (Garay & Garrahan 1973) and in frog skeletal muscle (Harris 1965) the relationship between M_{Na} and $(Na)_i$ is sigmoidal.

In barnacle muscle cells, Brinley (1968) found saturation of M_{Na} at high $(Na)_i$, with the shoulder at ca. 20mM.

The experiments to be described in section 5 of this thesis revealed that the true behavior of the sodium efflux from barnacle muscle into normal Ringer's solution is similar to that from the squid axon and snail neurone. Saturation does not occur at the low level found by Brinley (1968).

(ii) <u>Sodium efflux into potassium-free solution.</u>

Steinbach (1940) showed that when frog skeletal muscle is soaked in potassium-free Ringer's solution, it loses potassium and gains sodium. Return of potassium to the solution enables the cells to extrude some of the accumulated sodium. The effect was ascribed to a reduction in the sodium efflux in potassium-free media. A similar effect was found in red blood cells by Harris and Maizels (1951), and in giant axons by Hodgkin and Keynes (1954). It was not due to permeability changes or to changes in the membrane potential. Harris and Maizels (1952) proposed that the potassium influx and the sodium efflux in red cells were linked. As described above, linked sodium and potassium transport was found to be the principal mode of the 'sodium pump' under normal conditions. The stoichiometry of the coupling

appears to be fixed in red cells (Garrahan & Glynn 1967c) but to be variable in frog skeletal muscle (Cross, Keynes, Rybova 1965) and in squid axon (Mullins & Brinley 1969). However, the decrease in the sodium efflux from squid axon upon removal of the external potassium can far exceed the magnitude of the potassium influx, as discussed by Sjodin (1971). It is implied that potassium ions act as activators for sodium transport.

In barnacle muscles, too, removal of the extracellular potassium results in a decrease in the sodium efflux (Brinley 1968; Bittar et al. 1972). Elevation of the potassium concentration of the bathing solution, to a value between 8 (the normal value) and 40 mM, had no effect on the sodium efflux in barnacle, even though the cells contract at concentrations greater than 20mM. (Bittar et al. 1972). However, further increases above 40 mM caused a marked stimulation of the sodium efflux.

A similar effect was found in frog skeletal muscle by Horowicz and Gerber (1965a,b), and they proposed that the increase in sodium efflux was mediated by the coincident alterations of the membrane potential due to the change in the potassium concentration. Beauge and Sjodin (1976) have shown that for frog skeletal muscle cells in which E_m is made unresponsive to changes in the external potassium concentration by prolonged incubation in potassium-rich solutions, changes in the external potassium concentration between 0 and 10 mM activate the sodium efflux along an activation curve almost identical to that obtained for untreated control cells. The potassium-activated sodium efflux also was shown to differ from the azide-stimulated sodium efflux, which also was independent of membrane potential changes. Beauge and Sjodin suggest that external potassium activates the sodium pump in frog muscle by altering the transport enzyme directly, at least for external potassium concentrations between 0 and 10 mM. The site at which activation by potassium occurs is distinct from the catalytic site

for potassium transport. The activation at very high concentrations of external potassium remains to be explained.

Only the effect of reduction of the external potassium concentration was examined experimentally in the present work, as descirbed in section 5. It was found that the efflux into potassium-free solutions behaved much like the efflux into ouabain-containing Ringer's solution.

(iii) Sodium efflux into sodium-free solution.

The removal of sodium from the extracellular medium reverses the gradient of electrochemical potential which is the driving force for 'passive' sodium ion movement across the cell membrane. This should make the efflux of sodium from the cell less costly in terms of energy, and so result in an increase both in the passive and in the active efflux. However, for enzyme-mediated transport of intracellular sodium to occur, sodium might be required at an external nontransport site. It has also been suggested that an exchange of internal for external sodium ions might occur, with no net flux of ions and no net consumption of energy.

There are two classes of 'sodium-free effects' on the basis of the time course. The difference between the steady sodium efflux into normal Ringer's solution and the steady sodium efflux into sodium-free solution has been the one more studied. There is also a transient rapid loss of sodium from the myoplasm of frog skeletal muscle and crab striated muscle and from the snail neurone upon removal of sodium from the bathing solution. The better-known effect will be reviewed first.

Ussing (1947, 1949) first suggested the possibility that a one-for-one exchange of intracellular sodium for extracellular sodium could occur, with no net expenditure of metabolic energy. This would complicate the interpretation of radiosodium fluxes. Subsequently it was found (Keynes & Swan

1959) that the radiosodium efflux from frog skeletal muscle was reversibly reduced to about half when the external sodium was replaced by lithium or choline, with external potassium unchanged. The fractional reduction was less when the sodium content of the muscle was elevated by incubation in an appropriate solution, but could be restored by lowering the sodium content again by a further incubation. External potassium did not affect the sodium-free response.

In squid axon, however, choline- or lithium-substituted sodium-free solutions caused an <u>increase</u> in the efflux of radiosodium (Hodgkin & Keynes 1955; Mullins <u>et al</u>. 1962). Mullins (appendix to Mullins & Frumento 1963) suggested that accumulation of incoming sodium near the internal site of an active transport enzyme, due to restricted diffusion from this location to the bulk cytoplasm, could account for both of these observations, where the intracellular sodium concentration $(Na)_i$ was the determining factor.

Subsequently it was shown that removal of external sodium does indeed cause a decrease in the radiosodium efflux from squid axons of low sodium content (lowered by stimulation of the axon in lithium solution) (Frumento & Mullins 1967; Mullins & Brinley 1967; Sjodin & Beauge 1968a), and an increase in the radiosodium efflux from frog skeletal muscle of high sodium content (loaded by soaking for 20 hours at 2 deg.C in potassium-free solution) (Keynes 1965; Beauge & Sjodin 1968). (The glycoside sensitivity of the effect differs in the two tissues (Sjodin & Beauge 1968b; Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt 1969)). In toad oocyte, however, there was little effect of removal of external sodium, whatever the sodium content (Dick & Lea 1964).

A cardiac glycoside-sensitive sodium-sodium exchange of the type seen in red blood cells (Garrahan & Glynn 1967) (there is no glycoside <u>in</u>-sensitive sodium-sodium exchange in red blood cells) was thought to be

responsible for only part of the external sodium-dependent efflux seen in muscle since the effects of glycosides and the removal of external sodium appeared to be independent and additive (Horowicz 1965; Keynes 1966; Sjodin & Beauge 1968a). Glycoside decreased both sodium efflux and influx by about 20% in frog muscle in potassium-free solution but the influx was unaffected by glycoside in potassium-containing solution (Keynes & Steinhardt 1968; Keynes 1966), as for red blood cells (Garrahan & Glynn 1967).

Lithium appeared to stimulate the active sodium efflux from frog skeletal muscle in the same way that potassium, rubidium, and cesium do (Beauge & Sjodin 1968). The stimulation was abolished by cardiac glycosides. A "dual effect" of lithium-substituted sodium-free solutions on sodium efflux was proposed: (a) removal of extracellular sodium prevents the sodium-dependent efflux, and (b) lithium stimulates the sodium efflux by acting like potassium externally. Thus as the internal sodium content rises, the pump rate will rise, and so the stimulatory effect of lithium will increase. Eventually this stimulated efflux will surpass the inhibitory effect of the lack of external sodium.

A complication of these studies in whole muscle preparations is that in potassium-free solutions, potassium which leaves the cells passively can accumulate in the extracellular space in sufficient amounts to stimulate sodium efflux appreciably (Sjodin & Beauge 1973). Beauge (1975) estimated that almost half of the stimulation of the sodium efflux seen in sodium-free, potassium-free, lithium-substituted solution in frog muscle was due to this reaccumulation of potassium.

Sachs (1977) has shown that the effects of external sodium on sodium extrusion by red blood cells are consistent with sodium acting as a "dead-end competitive inhibitor and as a heterotropic allosteric effector" on the (Na+K)ATPase.

It was found that a small glycoside-sensitive increase in the sodium efflux from frog skeletal muscle was obtained if calcium or magnesium were used to replace external sodium, as well as when lithium was so used (Horowicz, Taylor, & Waggoner 1970). It was concluded that most of the glycoside-insensitive sodium efflux required external sodium, but of the glycoside-sensitive sodium efflux, part can be inhibited by external sodium (?sodium-sodium exchange). The former part of the glycoside-sensitive efflux appears when $(Na)_i$ is high, and the latter when $(Na)_i$ is low. Similar results were obtained by another worker (Sjodin 1971). That the glycoside-sensitive effects are due to the (Na+K)ATPase is supported by results on the isolated enzyme (Robinson 1975). Further, Kennedy and DeWeer (1976) have demonstrated a strophanthidin-sensitive increase in sodium efflux requiring external sodium but not potassium, in frog skeletal muscle in which the ATP/ADP ratio had been lowered by poisoning. A strophanthidin-sensitive sodium influx of similar size also occurs under these conditions.

In squid axons, Baker, Blaustein, Keynes et al. (1969) saw sodium-sodium exchange under conditions of partial poisoning, contrary to the results of Frumento and Mullins (1964). The exchange was glycoside-sensitive. Poisoning with CN or DNP, or injection of apyrase (which hydrolyzes ATP) all give rise to an external-potassium-independent, external-sodium-dependent sodium efflux (DeWeer 1970, 1974). Apparently the presence of ADP in high concentrations is the critical factor. This is similar to the results in red blood cells (Garrahan & Glynn 1967b; Glynn & Hoffman 1971).

Since the potassium-free and sodium-free effects are highly correlated (Sjodin & Beauge 1969; DeWeer 1970), it was concluded that the 98% of the sodium efflux from squid axon which is not passive is due to: (a) potassium-stimulated sodium efflux, inhibited by external sodium (ascribed to the sodium-potassium exchange mode of the pump), and (b) external-sodium-

stimulated sodium efflux, inhibited by external potassium (ascribed to the sodium-sodium exchange mode plus a different, glycoside-insensitive mechanism).

Sodium-sodium exchange conceivably could occur by a mechanism other than a closely-linked one-for-one exchange. For example, if from the measured sodium influx into frog muscle one subtracts the passive influx calculated in the constant field approximation, there remains a component of the sodium influx which is about 38% of the total (in normal Ringer's solution) (Venosa 1974). If the cell is maintaining a constant sodium content, this must be balanced by sodium extrusion, (as is the passive influx). If this influx is via an enzyme, the net result is enzyme-mediated sodium-sodium exchange comprising 38% of the total sodium efflux, but it need not be one-for-one exchange by one enzyme. The notion of one-for-one exchange of sodium ions eliminates the need to specify a counterion motion, but clearly creates other difficulties. It might be that one enzyme mediates sodium influx and a nearby enzyme mediates sodium efflux. The key question is how far apart can such a pair of different enzymes be yet operate in a 'thermodynamically permissible' manner.

Perhaps another indication of the existence of a separate sodium transport enzyme is the effect of ethacrinic acid on muscle. Ethacrinic acid appears to inhibit the external-sodium-dependent sodium influx without reducing the glycoside-sensitive, external-potassium-sensitive sodium efflux (Erlij & Leblanc 1971), but only in glycoside-treated muscles: ethacrinic acid alone stimulates the sodium efflux. Ethacrinic acid has no effect on the sodium efflux from barnacle muscle normally, but prevents the increase in sodium efflux which usually follows exposure to CO_2 (Danielson et al. 1972).

In summary, it appears that in nerve (under some conditions) and in muscle (normally), there probably is a component of the sodium efflux which requires external sodium. It is usually regarded as strict one-for-one exchange of internal for external sodium. On the basis of the effect of poisons, it would appear that much of this exchange proceeds by a mechanism other than the (Na+K)ATPase, although sodium-sodium exchange of the (Na+K)ATP-ase type can be demonstrated under some conditions. Since removal of external sodium causes a decrease in the sodium efflux when $(Na)_i$ is low, but an increase when $(Na)_i$ is high, there appears to be a mechanism by which external sodium can inhibit the sodium efflux. This could be the sodium-potassium mode of the (Na+K)ATPase. The sodium-free effect is the combined result of (at least) these two effects, and the net effect observed in experiments in vitro depends on the experimental conditions (Sjodin 1971).

In frog skeletal muscle, 20 to 50% of the sodium efflux under normal conditions is of the sodium-sodium exchange type. In squid axon, none of the sodium efflux under normal conditions is of the sodium-sodium exchange type. The situation in barnacle muscle was not clear. Brinley (1968) found that replacement of external sodium by lithium reduced the radiosodium efflux by 67%, while replacement by sucrose reduced it by 47%. (Potassium-free solutions reduce the radiosodium efflux by 51%.) In cells with higher sodium content, an increase in efflux was seen, but the absence of external sodium always caused a contracture, so the effects could not be measured.

Experiments on this type of sodium-free effect are described in section 5. Both an inhibitory and a stimulatory effect of the removal of external sodium were seen in barnacle muscle cells, but on the whole the kinetic characteristics of the efflux into sodium-free solution were the same as those of the efflux into normal Ringer's solution. That is, much of the 'sodium-free effects' in barnacle muscle is due to changes in the sodium

content of the cells which occur when they are placed in sodium-free

solution.

A second 'sodium-free effect' has been reported. Exposure of frog

skeletal muscle to sodium-free solutions (substituted with lithium or tris)

causes a large rapid fall of the intracellular sodium activity measured with

an intracellular microelectrode (White & Hinke 1976). A similar rapid effect

has recently been reported in crab muscle (Vaughan-Jones 1977), and a

similar but slower effect entirely consistent with the continuation of the

normal sodium efflux in the absence of influx, was found in snail neurone

(Thomas 1972b).

For frog muscle (White & Hinke 1976), the time course of the fall could

be fitted by a sum of two exponential terms. The two rate constants were

unchanged by ouabain treatment, but the 'capacity' of the kinetic compartment

defined by the more rapid rate was reduced. The slower rate was comparable

to that for sodium influx, and was identified as "passive" leakage. The

more rapid rate was identified as active sodium extrusion, and the rate

constant was the same as that found for the washout of labelled sodium from

the extracellular space.

With a muscle which had been loaded with radiosodium by passive uptake,

the washout of radiosodium from the extracellular space would mask such a

rapid efflux unless the extracellular space was cleared of radiosodium

before the muscle was exposed to sodium-free solution (White & Henke 1976).

Chemical analysis of the muscles indicated that the fall in myoplasmic

sodium activity is due to movement of sodium ions out of the cell (White &

Hinke 1976), but this has not been confirmed. Some earlier workers attached

some significance to the initial rapid exchange of radiosodium in whole

frog muscle (Carey & Conway 1954). Others discarded the first twenty

minutes of the isotope efflux data from single muscle cells, which they

quite reasonably assumed to represent washout of the extracellular space
almost exclusively (Hodgkin & Horowicz 1959).

The situation in crab muscle is quite different (Vaughan-Jones 1977).
The rapid fall of the myoplasmic sodium activity on exposure of the cell to
sodium-free lithium-substituted or tris-substituted solution was unaffected
by ouabain, the removal of extracellular potassium, calcium, or magnesium,
or by changes in the extracellular pH. It was blocked by manganese, cobalt,
and lanthanum, which are known to block the movement of divalent cations
across membranes, but not by D600 or Verapamil, which block calcium fluxes
in nerve. However, lanthanum and, to a lesser extent, manganese themselves
often caused a rapid fall of the myoplasmic sodium activity in the presence
of external sodium.

If the rapid efflux of sodium is not accompanied by potassium influx,
nor by chloride efflux (the myoplasmic chloride activity is not changed in
low external sodium solutions), one would expect a considerable electrogenic
contribution to the membrane potential to occur. Only a slight depolariza-
tion was found. Perhaps there is some counterion or co-ion transport, or
the permeability to lithium might be greater than that to sodium and a
concurrent depolarization thus result in the resting membrane potential,
approximately countering the electrogenic effect.

Calcium influx is suggested by the results with manganese, cobalt,
and lanthanum, and by the similar time course of the rise of $(Ca)_i$ in crab
muscle and squid axon under similar conditions. Removal of external calcium
prevents the rise of the sodium efflux normally seen in squid axon when
exposed to lithium solutions (Baker, Blaustein, Hodgkin et al. 1969).
However, with crab muscle removal of extracellular calcium and/or magnesium
had no effect. Further, a sudden rapid influx of calcium would trigger a
contraction, and this did not occur. It might be that the extracellular

space of the whole-muscle preparation used was not completely cleared of calcium or potassium, but this is unlikely because long washout times were used. The similarity of the response to lanthanum and removal of external sodium was noted above. Lanthanum can displace quite large quantities of membrane-bound calcium, and intracellular calcium stimulates sodium efflux from barnacle muscle (Bittar et al. 1972, 1973). Altogether, though, the processes which lead to a decline in the myoplasmic sodium activity remain unknown.

Measurements with the intracellular microelectrode can only detect loss of sodium from the major intracellular compartment. They cannot reveal the fate of the lost sodium. Therefore an experiment in which the micro-electrode measurements were combined with radiosodium efflux measurements was devised, as described in section 6. It was found that a rapid fall in the myoplasmic sodium activity similar to that in frog and crab muscle occurs in barnacle muscle under certain conditions, and that it is accom-panied by a rapid loss of sodium from the cell. As with snail neurone, this was found to be due to the continuing normal operation of the sodium efflux in the absence of sodium influx.

(iv) The effect of ouabain on the efflux of sodium.

Cardiac glycosides, principally ouabain (g-strophanthin) and its aglycone strophanthidin, have long been known to inhibit the transport of sodium and potassium in red blood cells and frog skeletal muscle (Schatzmann 1953; Matchett & Johnson 1954). Some such action had been suspected because toxic doses of the drugs were known to cause a loss of potassium from heart muscle (Schatzmann & Witt 1954). It was found later that cardiac glycosides also inhibit the activity of the (Na+K)ATPase (Skou 1965).

The current theory of the action of cardiac glycosides (Schwartz et al.

1975; Glynn & Karlish 1975) is that they bind specifically to a single site on the (Na+K)ATPase which is separate from the catalytic sites. The binding site is exposed only at the external surface of the cell membrane, and there is a particular conformation of the enzyme which favours binding of the glycoside. The binding is very strong. The half time for dissociation of the isolated enzyme-glycoside complex at $37^{o}C$ is about 2.5 hours although the physiological effect of glycosides can be reversed much more rapidly. The binding does not render the enzyme completely inactive. Although the sodium-potassium exchange appears to be prevented, some partial or side reactions can still occur (Glynn et al. 1974). It is conceivable that the effects of glycoside are different for the isolated and the in situ enzyme, however.

In whole cells, cardiac glycosides can promote some modes of ion flux. In squid axons with low ATP content, strophanthidin increased the rate of sodium efflux (Brinley & Mullins 1968). Strophanthidin also increases the potassium efflux from frog skeletal muscle (Harris 1957; Sjodin & Beauge 1968a) and squid axons (Mullins & Brinley 1969).

The inhibition of the sodium efflux by strophanthidin increases with increasing sodium content in 'aged' frog sartorius muscle (Sjodin & Beauge 1968a) but decreases with increasing sodium content in freshly-dissected frog sartorius muscle (Horowicz et al. 1970).

Dependence of fluxes on ATP does not always correlate with the sensitivity to glycosides (Mullins & Brinley 1969). In ATP-depleted squid axon, strophanthidin causes a marked increase in the sodium efflux, against the gradient of electrochemical potential, while leaving the sodium influx unchanged (Mullins 1972).

Recent experiments on red blood cell ghosts (Bodeman & Hoffman 1976) revealed that in the presence of external potassium, the rate at which

ouabain bound decreased when either the internal sodium or the internal

potassium was raised. When external potassium was not present, such

variations in ion content had no effect on ouabain binding. In other

experiments, the final amount of ouabain bound was not affected by such

manipulations (Schwartz et al. 1975), and it is not known if an increase in

the internal ion concentrations can promote the disassociation from the

enzyme of ouabain which is already bound to the enzyme.

The effects of strophanthidin on the sodium efflux from barnacle muscle

cells were studied by Brinley (1968). He found that there was little or no

inhibition at 10^{-8}M. strophanthidin and that as the concentration of stroph-

anthidin was increased to 10^{-5}M, the percent inhibition increased. The

maximum inhibition was about 90% and occurred for concentrations of stroph-

anthidin greater than or equal to 10^{-5}M. In one cell of very high sodium

content, there was a delayed increase in the sodium efflux after an initial

fall at 10^{-4}M strophanthidin. Ouabain appeared to be slightly less

effective. The inhibition was less in cells which had a larger sodium

content. Strophanthidin produced a greater reduction of sodium efflux

than did removal of external sodium and/or potassium, and removal of external

sodium and potassium did not increase the inhibition in strophanthidin-

treated cells.

The effects of ouabain on the sodium efflux from barnacle muscle cells

were studied by Bittar et al. (1973). They obtained dose-response curves

by exposing isolated cells to increasing concentrations of ouabain, as

Brinley had done, and obtained a similar curve but with a maximum inhibition

of about 70%. They used the reduction in the fraction of radiosodium lost

per unit time as their measure of inhibition, while Brinley had calculated

the size of the sodium efflux using an estimated value for $(Na)_i$. At 10^{-3}M,

after about twenty minutes of exposure to ouabain, the efflux of radiosodium

began to rise again. The inhibition was greater in cells which had "slope ratios" close to 1, that is, in cells having a lower sodium content (see section 4). Injection of ouabain into the cells caused no change in the sodium efflux. The increase in the sodium efflux caused by depolarization of the cells, by raising the external potassium concentration or by injecting $CaCl_2$, was not inhibited by ouabain. Nor was the increase in the sodium efflux caused by CO_2 treatment. Bittar et al. concluded that there are at least two separate sodium extrusion systems, located in different parts of the membrane.

The effects of several cardiac aglycones on the sodium efflux in barnacle muscle cells have also been studied (Bittar & Brown 1977). They all appear to bind to the cardiac glycoside site and to have the same effects as cardiac glycosides. Only the potency differs.

Three aspects of the effect of ouabain on the sodium efflux in barnacle muscle were examined experimentally as part of the work reported in this thesis. First, the effect on the dose-response curve of the use of the sodium electrode to measure the rising intracellular sodium activity after ouabain begins to act was investigated. Impariment of the extrusion mechanism by ouabain should result in an immediate increase in the sodium content of the cell as the passive influx continues unchanged, and the increased sodium content should be reflected in the measured sodium efflux. Second, the kinetic characteristics of the sodium efflux in cells which are maximally inhibited by ouabain were to be measured. It turned out that this flux is an increasing function of the intracellular sodium activity at low levels of internal sodium, but reaches a plateau at higher levels. These two aspects are described in section 5. Third, an electrogenic contribution of the sodium flux to the membrane potential was sought. Experiments which demonstrate the existence of an electrogenic potential in barnacle muscle will be described in section 7.

Although the membrane potential has only now been mentioned in connection

with the ion fluxes, it is of course intimately involved with them. In much

of the early work on ionic currents, the models of electrical circuits were

adopted operationally. The result has been a mixture of molecular physics

and circuit theory, with emphasis on the latter. It was felt to be worthwhile

to review the origins of the most commonly used models of the membrane poten-

tial and ionic currents, and to indicate how they could be improved. This

review is presented in the next part of this section, and completes the intro-

duction. It is followed by a summary of the problems to be addressed in this

thesis, and a description of three models used later in the interpretation

of experiments but gathered together for the convenience of the reader.

D. THE TRANSMEMBRANE DIFFERENCE IN ELECTRICAL POTENTIAL

In all cells there is an electrical potential difference measurable

across the cell membrane. Almost always, the electrical potential measured

in the interior of the cell is negative with respect to that measured in

the bulk solution bathing the cell.

In a number of cells it has been found that if the rate of the sodium

pump is suddenly altered, there is an immediate change in the resting mem-

brane potential. A decrease in the pump rate, caused for example by applica-

tion of ouabain, is accompanied by a depolarization of the cell. That is,

the inside of the cell becomes less negative with respect to the outside.

An increase in the pump rate, caused for example by injection of sodium into

the cell interior, is accompanied by a hyperpolarization (see reviews by

Kernan 1970; Thomas 1972; also DeWeer & Geduldig 1973; DeWeer 1974).

Different modes of the pump appear to have different degrees of

electrogenicity (DeWeer 1974). The stoichiometry of the net sodium-potassium exchange by the transport enzymes appears to be fixed for red blood cells at 3Na : 2K, but to vary from 1:1 to 3:1 in squid axon (Glynn & Karlish 1975). A separation of charge such as is implied by this could only be effected with a very large investment of energy. The question of how the charge separation necessary to produce the observed change in membrane potential comes about is essentially the same as the question of how active sodium extrusion comes about.

The development of ideas on the electrogenicity of the sodium pump has been reviewed by Thomas (1972). Theories of the origin of the contribution of the electrogenic sodium pump to the resting membrane potential are phenomenological extensions of the approach of Goldman (1943) and Hodgkin and Katz (1949). The notions of ionic permeability and conductance, and the ionic mechanisms involved in this approach, often are employed in other contexts.

It is generally felt that the membrane potential E_m can be regarded as basically a diffusion potential, which arises in nerve and striated muscle because of the selective permeability of the cell membrane to potassium and the elevated intracellular potassium concentration created by active transport of ions. (Other likely contributions to the measured value are considered below.) KCl tends to leak out of the cell across the membrane, but since the mobility in the membrane of the potassium ions is greater than that of the counterion chloride (the mobilities are almost equal in bulk solution), a small local charge separation occurs. This charge separation results in an electrical force which retards the potassium ion movement and promotes the chloride ion movement. A steady state is attained where the unidirectional efflux of K and Cl are equal. This is just like the familiar liquid junction or diffusion potential (eg. Lakshminarayanaiah 1969), but there is no net loss of K because of the

sodium-potassium exchange pump, there is no <u>net</u> loss of Cl at the steady

value of the resting potential, and the diffusion front is fixed in space

by the membrane, like the "constrained liquid junction" of Planck

(Lakshminarayanaiah 1969).

From a model of the cell membrane as a homogeneous lamella, charac-

terized by mobilities (empirical ratios of average diffusion velocity to

driving force) for each major ion which are much lower than the mobilities

in bulk solution and in which the electric field is constant, Goldman (1943)

derived an expression for the transmembrane flux of an ion by integrating,

over the thickness of the membrane, a relation between the flux and the

driving force at each point in the membrane (the Nernst-Planck equation).

The integration can be carried out for sodium, potassium, and chloride ions,

and when the total current is zero,

$$E_m = \frac{RT}{F} \cdot \ln\left\{\frac{P_K \cdot (K)_o + P_{Na} \cdot (Na)_o + P_{Cl} \cdot (Cl)_i}{P_K \cdot (K)_i + P_{Na} \cdot (Na)_i + P_{Cl} \cdot (Cl)_o}\right\}$$

(Hodgkin & Katz 1949), where subscript i refers to intracellular and o to

extracellular, and P is the permeability of the membrane to the ion (essen-

tially the mobility). (This will be referred to as the 'GHK equation'.)

Clearly this model of diffusion through a homogeneous slab does not

coincide with the situation in a real membrane. In reality, the ions pass

the membrane only at certain locations, ie. in association with proteins.

At the molecular level the movement of the ions bears no simple relation

to that seen in diffusion in a bulk solution. Yet the equation describes

the membrane potential very well. Therefore the essential physical basis

of the equation must <u>describe</u> what is occurring at the molecular level.

The basis of the equation is the Nernst-Planck equation, which simply

states that the flux of an ion is proportional to the driving force. The

driving force for a system at constant and uniform pressure and temperature can be deduced from an expression for the energy change accomplished by the resulting flow, the flow being easy to define. The energy change is -dG (where G is the Gibbs free energy) and the force conjugate to a flow of ions is $-\vec{\nabla}\tilde{\mu}$, where $\tilde{\mu} = \mu_0 + RT \cdot \ln(a) + zF\phi$ is the electrochemical potential for the ion. (a) = activity of the ion, ϕ = electrical potential. (The driving force can also be deduced in the framework of irreversible thermodynamics, from the entropy production - eg. see Katchalsky & Curran 1967.) This "phenomenological force" is an approximation, useful in a macroscopic representation of a system in which 'all gradients are sufficiently gradual.' The thermodynamic functions cannot actually be defined at each point in space, since they represent the interactions of a large number of particles, and unless the system can be regarded as an aggregate of macroscopically-small volume elements, each containing a large number of particles, the representation stated above cannot be applied with any expectation of success. Goldman (1943) stated that "the current carriers pass through more or less randomly distributed interstices in the structure (membrane), which is assumed uniform normal to the direction of flow." The integration was along a direction parallel to the direction of flow, passing through a pore. In the pore the ions were regarded as diffusing as they do in bulk solution, but in one dimension and with much lower mobility. The result, not surprisingly, was similar to that of Planck for a 'constrained liquid junction'. Hodgkin and Katz wrote the solution for the major ions and solved for the transmembrane potential difference at zero net current, as stated above.

A mechanistic model at the molecular level for the origin of the membrane potential can be envisaged. The physical origin of the potential difference is indeed similar to that in the case of a liquid junction. In

the cell membrane, the protein channels through which cations pass are thought to be lined with electronegative moieties, such as carbonyl or carboxyl groups, so that the cationic charge can be partially or completely balanced when it is in the channel. (These models are called the "neutral polar pore" (Eisenman 1968; Mueller & Rudin 1967) and the "fixed charge pore" (Eisenman 1968)). There is a finite concentration of cation in the channel. When a cation enters the channel, it leaves its counterion behind. This can occur only occasionally on the molecular scale of space and time, since a local concentration of negative charge would retard further egress of cation (via the channel or otherwise). Such an inhomogeneity of charge could be balanced by a movement of cations from the region to which the cations in the channel are heading, in a one-for-one exchange on average, but it is more likely that the anions will be drawn after the cations if a path is available. The anion clearly cannot readily follow through the cation channel, so there is assumed to be an anion channel nearby. The anions normally would pass through the channel in a manner similar to that of the cations, but the cations can do so more readily ("higher mobility in the membrane"). A separation of charge occurs, and an electrical potential difference results.

The GHK equation has proved to be a good qualitative and quantitative or semiquantitative description of the membrane potential in many situations. However, there are several ways in which a quantitative relation between the potential difference across the membrane and the driving force for the flow of ions could be formulated. The net effect alone can be considered, so that the membrane channels are regarded as 'black boxes' characterized by a resistance or mobility. Then the current equals the quotient of the voltage and a resistance, or the flux velocity equals the product of the net difference in chemical potential and a permeability (or mobility,

denoted u). Such a relation underlies the usual definition of membrane conductance (Hodgkin & Horowicz 1959b) and the usual conception of the 'electrogenic component' of the resting membrane potential (Hodgkin & Keynes 1955a).

If one regards the potassium flux in this manner, for example, then

$$M_K \text{ (moles/cm}^2\text{sec)} = -u_K \cdot (K)_i \cdot \Delta\tilde{\mu}$$
$$= -u_K \cdot (K)_i \cdot (R \cdot T \cdot \ln \frac{(K)_i}{(K)_o} + F E_m)$$

while $I_K \text{ (coulomb/cm}^2\text{sec)} = g_K \cdot (E_m - E_K)$
$$= g_K \cdot (E_m - \frac{R \cdot T}{F} \cdot \ln \frac{(K)_o}{(K)_i})$$

and since $I_K = M_K \cdot F$, the conductance g_K would be $F^2 u_K (K)_i$. In fact, Hodgkin and Horowicz (1959b) related g_K to P_K by substituting from the constant field solution for I_K in $I_K = g_K (E_m - E_K)$. They mixed their models, in a sense, and the resulting relationship between g_K and P_K is a complicated function of E_m and the concentrations. Thus the mobility in the pure "net effect" model is also a complicated function of E_m and the concentrations, as might have been expected when the complications of the process are forced into the mobility as a proportionality factor.

The Goldman-Hodgkin-Katz (GHK) treatment, as already stated, regards the ions as diffusing through a regime of reduced but constant mobility, after entering the channel by an unspecified process. The entry into the channel is included in the permeability P as a partition coefficient (β): the concentration of ion in the membrane is β times the concentration in the bulk solution. A more detailed model would treat entry into and exit from the channel as a mass-action situation, again with diffusion assumed to occur in the channel. For example, Teorell (1935) regarded this as a Donnan equilibrium.

This sort of treatment was used by Eisenman et al. (1968) to describe ion transport via neutral mobile carriers in the membrane, but the result can be taken over to the channel model almost intact. The result is the GHK equation plus an extra term, where the partition coefficient has been characterized explicitly. Perhaps a better example is the model proposed for the potential developed in a glass microelectrode (articles by Doremus, by Eisenman, and by Nicolsky in Eisenman 1967). The interactions at the surfaces, which provide the selectivity, and the diffusion through the matrix of the glass (described by a constant mobility) are treated separately, to yield a relation like the GHK equation, although the counter-ion transport occurs via an external circuit.

A still better representation would take into account the "single-file" effects which must occur in a pore (Hodgkin & Keynes 1955; Lakshminarayanaiah 1969). The mobility would be further characterized, but a liquid-junction-type equation would still be obtained. The essential feature of all of the models is the different mobility of the cation and anion.

The usual approach taken to include electrogenic pumping in the expression for E_m has been to include the flux of ions which occurs through the sodium pump as a phenomenological term in a flux balance wherein the passive fluxes are described by the Nernst-Planck equation (eg. Mullins & Noda 1963; Moreton 1969; Schwartz 1971). The electrogenic potential then arises in the same way that the rest of the membrane potential arises. In a sense, the 'mobility' of sodium ions in the membrane (for outward movement) is enhanced by the pump, so that when there is an apparent net expulsion of cations, a potential develops in the manner described above as counterion movement occurs.

If this hypothesis for the origin of the electrogenic contribution to the resting membrane potential is essentially correct, a measurement of the

electrogenic potential is a measurement of the net ion current through the

sodium pump. Similarily, in a voltage-clamped cell, the current required

to keep E_m steady is a measurement of the net ion current through the sodium

pump. With the simultaneous use of radioisotopes and intracellular micro-

electrodes, it is possible to measure the sodium flux and the membrane

potential simultaneously, and to detect the simultaneous changes in the two

when the sodium pump is selectively impaired by exposure of the cell to

ouabain. Such experiments are described in section 7, and it is shown that

the two measurements can be related in a theoretical model like those

described above, to yield measurements of permeabilities or of the coupling

ratio of the sodium pump. Voltage clamp studies by other workers are also

described in section 7.

Before concluding this discussion of the membrane potential, further

mention must be made of the contribution of other potentials to the

measured value of the membrane potential. The liquid junction and tip

potentials of micropipette electrodes are well known, and are technical

problems. Several workers have found that an electrical potential difference

qualitatively and quantitatively like a Donnan potential can be measured in

muscle cells which have been 'chemically skinned' by extraction with

glycerol or with detergent (Collins & Edwards 1971; Pemrick & Edwards 1974;

J.A. Hinke - personal communication). The Donnan potential due to fixed

charges on the lattice of contractile proteins must be confined to a region

of at most a few hundred Angstroms diameter around the charges, yet it

appears to influence the intracellular microelectrode.

The membrane potential measured with intracellular microelectrodes thus

might be different from the electrical potential difference between the bulk

intracellular solution and the external solution.

Tasake and Singer (1968) review several problems involved in electrical

measurements of biological systems. They remark that "no right-minded electrochemist would even attempt to perform meaningful measurements under the complex conditions which are required to maintain living biological systems," but conclude that meaningful measurements can be made if the "proper precautions are observed."

E. SUMMARY OF THE PROBLEMS TO BE ADDRESSED

The principal object of this thesis is the measurement and interpretation of the sodium and hydrogen ion efflux from whole cells. Hydrogen ion efflux can only be measured indirectly; most of the work to be described concerns the sodium efflux.

It is currently believed that it is mainly the cell membrane which controls the ion content of the cell, by its passive permeability properties and its ability to translocate ions against the forces which effect passive flow.

The membrane transport reactions can be viewed as enzyme reactions and characterization of the transport can be carried out in the context of enzyme kinetics.

One enzyme system, the (Na+K)ATPase, has been isolated in an active form. Discussion of sodium transport tends to be dominated by discussion of the (Na+K)ATPase, but there appear to be other membrane mechanisms by which sodium can be transported in whole cells. The characterization of the activity of the enzyme in the whole cell is required before a component of the measured flux can be ascribed to a particular enzyme which has been extracted and studied in isolation.

The actual measurement of the sodium efflux from whole cells involves several problems. The state of sodium inside the cell has not been well characterized, a prominent problem being the estimation of the amount of sodium which resides in extracellular regions, that is, in regions outside the cell membrane per se. The sodium concentration in the solution which bathes the internal surface of the cell membrane should be measured, because it is the correct parameter to use for the interpretation of experiments in the context of the enzyme kinetics model. An intracellular sodium-specific microelectrode will measure this parameter, even when it changes rapidly.

In the last part of this section is described a revised equation by which the sodium efflux from whole barnacle muscle cells can be calculated from simultaneous microelectrode and radioisotope measurements. Two other models, related to the steady state cation distribution and to the relation between the sodium efflux and the membrane potential, are also described there.

In section 3 are described measurements made with an intracellular sodium-specific microelectrode on cells whose sodium content had been altered. The results and those of other workers are consistent with a simple model of the states of sodium in the cell which included both intracellular and extracellular pools of sequestered sodium.

In section 4 is described an investigation of the use of intracellular microinjection. This was carried out because other workers had interpreted their results on microinjected barnacle muscle cells in terms of a model for the states of sodium in the cell which contradicts the model described in section 3. An alternate interpretation of their results is presented. Microinjection was also studied because it was desired to use it to load the interior of cells with radioisotope rapidly and selectively, as a convenience in carrying out efflux experiments. Microinjection is shown to

be relatively, although not entirely benign as far as the barnacle muscle cell is concerned. It is shown to be equivalent to a passive method for loading the cell with radioisotope, aside from its failure to load the extracellular space, once certain corrections are applied.

In section 5 a survey of the sodium efflux from barnacle muscle cells is presented. It is shown that the dependence of the sodium efflux on the intracellular sodium concentration is similar to that in squid axon and snail neurone. Saturation of the efflux into normal Ringer's solution does not occur over the wide range of sodium content studied. The efflux into sodium-free solution is shown to be very similar to that into normal Ringer's solution. The behavior of the sodium efflux into potassium-free and into ouabain-containing solutions is shown to be almost identical. The results for barnacle differ in several respects from those of previous workers because of difficulties with the microinjection and radioisotope techniques which have not been recognized before.

In section 6 the measurements with the sodium-specific microelectrode and with radiosodium are compared for efflux into sodium-free solutions. Consistency of the model and techniques developed in the preceding sections is demonstrated, and the nature of the sodium-free effect is considerably clarified.

In section 7 an electrogenic contribution to the membrane potential of barnacle muscle is demonstrated. The correlation between the electrogenic membrane potential and the active sodium efflux is measured, and is interpreted in terms of an extension of the GHK model for the membrane potential.

In section 8 the results of measurement of the intracellular pH using pH-specific glass microelectrodes are presented. It is shown that in the barnacle muscle cell preparation used in the present work, there are no "pH transients" of the type reported by other workers. Also, a relationship

between the steady distribution of hydrogen ions and the resting membrane

potential is described, and an estimate of the size of the active hydrogen

ion efflux is made.

In section 9 is presented a direct comparison of measurements of the

intracellular pH made with the microelectrode and with an indicator method.

Such a comparison can reveal the existence of subcellular compartments

having a relatively low or high pH, but the most important results of this

study turned out to be those concerning the applicability of the indicator

method.

In section 10 is presented a brief discussion of the significance of

the results and some suggestions for further work.

F. SUMMARY OF MODELS

Part of the development of the models presented here relies on results

which have not yet been described. The reader might wish to proceed

directly to section 3 and to refer back to this section when references to

these models are encountered.

(i) <u>Efflux of sodium from a whole cell</u>.

It was desired to formulate an expression by which the sodium efflux

from a whole cell could be calculated from experimental measurements.

In the model of the cell used by early workers (Keynes & Lewis 1951;

Keynes 1951), it was assumed that the intracellular medium is a simple

solution of salts and organic molecules bathing the internal surface of the

cell membrane, and that only a negligibly small fraction of the transmembrane

passage of ions occurs by simple diffusion. In such a situation, if one could replace some of the intracellular sodium with labelled sodium ions, the unidirectional efflux of sodium ions from the cell (which is different from the net efflux) could be deduced from the efflux of the labelled ions into a large bathing solution containing no labelled sodium ions. It is assumed that the labelled ion behaves just as the abundant ion does as far as ion movements are concerned, an assumption satisfied by ^{22}Na and ^{23}Na.

Thus: the specific activity of sodium in the cell can be approximated

$$SA_{cell} = \frac{\text{moles } ^{22}Na \text{ in the cell}}{\text{moles } ^{23}Na \text{ in the cell } + \text{ moles } ^{22}Na \text{ in the cell}}$$

$$\doteq \frac{\text{moles } ^{22}Na \text{ in cell}}{\text{moles } ^{23}Na \text{ in cell}}$$

$$= \frac{Na^*_{cell}}{Na_{cell}}$$

where Na^*_{cell} (moles of ^{22}Na in the cell) is much smaller than Na_{cell} (moles of ^{23}Na in the cell) in practice.

A short interval of time "t" is considered. (The extension from the discrete collection intervals used in practice to a function applicable at any instant is assumed implicitly in this development.) If, in time t, Na^* moles of radiosodium leave the cell with no backflux of radiosodium, then the total number of moles of sodium leaving the cell is Na^* / SA_{cell} and the efflux density is

$$M_{Na}(\text{moles/cm}^2\text{-sec}) = \frac{Na^*}{SA_{cell}} \cdot \frac{1}{A} \cdot \frac{1}{t} \qquad \ldots(1)$$

where A is the area of the membrane across which the sodium ions pass. If V is the volume of the intracellular fluid, then

$$M_{Na} = \frac{V}{A \cdot t} \cdot \frac{Na^*}{Na^*_{cell}} \cdot (Na)_{cell} \qquad \ldots(2)$$

where $(Na)_{cell} = Na_{cell}/V$.

In an actual experiment, M_{Na} as a function of time is determined by collection of Na^* for each of a succession of time periods. The value of Na^*_{cell} at the beginning of each collection period is found by "back-addition" of the Na^* values to the radioactivity left in the cell at the end of the experiment. It is required that $(Na)_{cell}$ be known, and that it not change much during a collection period.

The results of experiments on squid axons seemed to be consistent with this formulation. The rate at which radiosodium came out of the cell fell off as a simple exponential with time, and was approximately equal to the influx rate, which can be measured directly from separate isotope uptake experiments (Keynes & Lewis 1951; Keynes 1951). The results for muscle cells are more difficult to interpret. The trend has been to employ more complicated models having several cellular compartments which can exchange sodium ions, such as that illustrated in Fig. 1(b) (eg. Keynes & Steinhardt 1968). These interpretations have not been completely successful, but recognize in part the current view of the heterogeneous structure of the cell and the state of ions and water inside the cell which would seem to make the above model untenable for most cells.

The features which probably give rise to error are as follows. First, the relevant concentrations are those of the 'myoplasm', the large aqueous compartment inside the cell which is much like a bulk aqueous solution and which is assumed to bathe the internal surface of the cell membrane. (Any structural layer deep to the 'bimolecular leaflet' will be assumed to be part of the 'functional membrane'.) $(Na)_{cell}$ must be replaced by the concentration of sodium in free solution in the myoplasm $(Na)_m$. The latter can be measured reliably as $(Na)_m = (a_{Na})_m / \gamma_{\pm}$. The value for $\gamma_{\pm}$ is taken to be 0.65. This is justified by the concept of the myoplasmic compartment as a solution like a bulk solution, and by an experimental determination
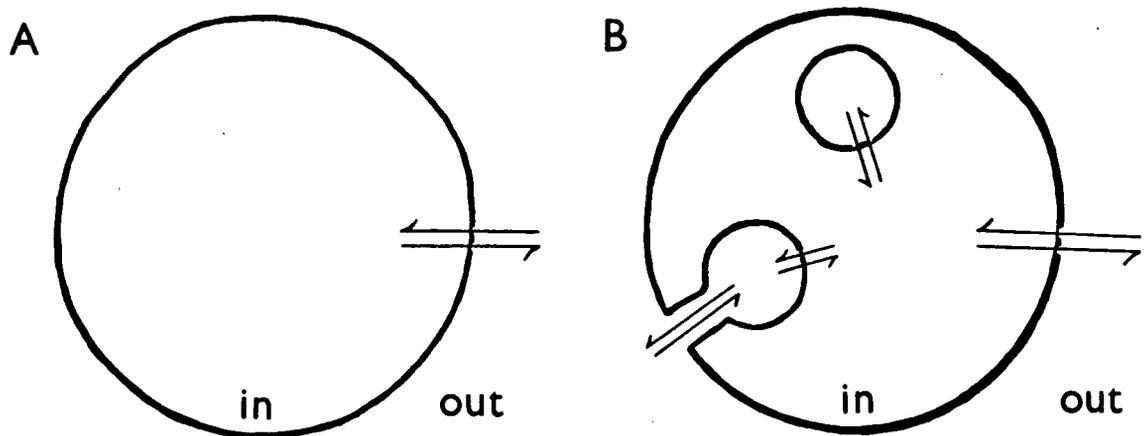
Figure 1. Models of the cell used in calculation of ion fluxes.

(A) Model on which equation (2) is based. All of the intracellular sodium is in solution in all of the intracellular water, and can only exchange with the extracellular compartment (arrows).

(B) Current model. Intracellular sodium can reside in a nonmyoplasmic compartment exchanging only with the myoplasmic compartment, or in a nonmyoplasmic compartment which also exchanges with the extracellular compartment. 'Nonparticipatory' sodium and water are not shown, nor are all possible exchanges for a multicompartment system.

(Hinke 1970).

It is suggested by previous work (and confirmed by the present experiments) that M_{Na} is a nondecreasing function of $(a_{Na})_m$. Continuous monitoring of $(a_{Na})_m$ during efflux experiments, especially those in which $(a_{Na})_m$ changes, should yield a more reliable measurement of M_{Na} than is possible from chemical analysis of total cellular sodium and back-addition of changes due to transmembrane transport.

Na^* is readily and unequivocally measured as the amount of radioisotope collected in the solution bathing the cell during a given time interval. However, Na^*_{cell} is a problem. As far as can be measured with the present techniques, injected radiosodium is deposited only in the myoplasm and in a small compartment which exchanges sodium with the myoplasm very quickly. This was concluded from the close similarity of the sodium efflux from cells loaded with radiosodium by microinjection and from cells loaded by immersion in normal Ringer's solution which contained radiosodium, as described in section 4.

Morphological and physiological studies rule out the membrane-delimited organelles as important sites of sequestration of sodium. They do not have a large enough capacity, nor rapid enough exchange with the myoplasm in barnacle muscle cells.

The contractile proteins are known to bind sodium and to exchange rapidly with the myoplasm. Their total capacity, ca. 68 millimoles per kg dry weight for rapidly exchanging sites,[3] would largely be taken up by potassium and hydrogen since they show only a modest preference for sodium over potassium and the intracellular concentration of potassium is much

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[3] $$\frac{\text{moles Na}}{\text{kg dry weight}} = \frac{\text{moles Na}}{\text{kg cell water}} \cdot \frac{\text{\%water}}{1 - \text{\%water}}$$

greater than that of sodium. They certainly should bind some sodium, though.

From experiments in which the total sodium content of barnacle muscle cells was altered (section 3), it was concluded that of the total amount of sodium associated with the cell after account had been taken of extracellular sodium by the usual techniques, a large amount of extracellular sodium was still included. If this is taken into account, then about 30% of the intracellular sodium is not accessible to the microelectrode. About 20% exchanges so slowly as to be nonparticipatory in the flux experiments discussed here. The most generous estimate was that less than 15% (and probably less than 10%) of the intracellular sodium is in rapid exchange with the free sodium, where the free sodium represents about 70% of the sodium which is truly intracellular.

More important is the existence of an effective intracellular sink of injected radiosodium due to longitudinal diffusion of radiosodium in injected cells, as discussed in section 4. For the cells in which the sodium content was not raised by microinjection of NaCl solutions, this plus radiosodium associated with the contractile proteins, in a realistic estimate, appears to be equivalent to about 15% of injected radiosodium in the experiments reported here. The effect is greater when NaCl is injected.

However, the amount lost to the sink is different in different cells, and increases from the initial value of zero as the experiment progresses. Thus a value Na_m^* for the 'myoplasmic radiosodium' should be used in place of Na_{cell}^*.

A correction can be applied to the data to account for this. In the definition of the efflux density (equation (1)) it is assumed that the amount of sodium which comes out of the cell in unit time is related to the amount of radiosodium which comes out in unit time, Na^*, via the specific activity of radiosodium in a homogeneous intracellular compartment contain-

ing only exchangeable sodium. An equivalent statement of this fundamental assumption of the tracer method is that the rate at which radiosodium appears in the bath, Na^*/t, is directly proportional to the amount of exchangeable radiosodium inside the cell, Na_m^*. Thus

$$\left(\frac{dNa_m^*}{dt}\right)_{via\ membrane} = \frac{Na^*}{t} = k \times Na_m^*$$

under steady conditions, where the interior of the cell is well-mixed and the size of the efflux doesn't change too much over a collection period t = 5 minutes. Note that it is assumed in addition that the continuing loss of radiosodium from the myoplasmic compartment to the intracellular sink is entirely independent of the loss of radiosodium across the cell membrane. The 'constant' k is then the instantaneous slope of the plot of $lnNa^*$ versus time. It bears repeating that the approximation will be closest to reality where the semilog plot ($ln\ Na^*$ versus time) is linear and not too steep, for then it is most likely that the myoplasmic compartment is well-mixed, and that the use of a time resolution of 5 minutes will not introduce too much error.

The effect of the contractile proteins could be corrected for via a model for the competition of sodium and potassium for the sites on the protein, if such a relatively small correction were felt to be necessary.

Another problem arises with V/A. The membrane in the clefts presumably is similar to that at the surface (the two develop from the same source as the syncytial muscle fibre is formed, and functionally it is reasonable that they should have similar properties, although the membrane of the TTS probably is different - Girardier et al. 1963). If the function of the cleft system is to ensure that all parts of the interior of the cell are within a certain distance from some part of the cell membrane, the surface-

to-volume ratio might well be independent of the diameter of the cell. A further complication is that the volume represented by V should be that of the myoplasmic compartment, as discussed above and in the introduction.

It was concluded that reasonable approximations would have to be made in the formulation of a practical equation. Thus: conductance measurements indicate that the values for the membrane capacitance and resistance for the barnacle muscle cell can largely be reconciled with those for the membranes of other cells if the effective area of homogeneous cell membrane is about ten times the apparent surface area of the cylindrical cell (Hagiwara $\underline{et}$ $\underline{al}$. 1964; Brinley 1968). For a cylinder of radius r, V/A = r/2 so here V/A will be taken to be equal to r/20.

Thus:

$$M \text{ (moles/cm}^2\text{sec)} = \frac{r}{20} \frac{1}{300\text{sec}} \frac{Na^*}{Na_m^*} \frac{(a_{Na})_m}{0.65}$$

$$= 2.56 \times 10^{-10} \frac{\text{litre}}{\text{cm}^3} r \frac{Na^*}{Na_m^*} (a_{Na})_m \qquad \ldots(4)$$

where r (cm) is taken to be the average of the cell radii measured in perpendicular directions, and $(a_{Na})_m$ is in millimoles/litre, to yield M_{Na} in moles/cm^2-sec. Na^*/Na_m^* is the ratio of counts per minute in a 300 second perfusate sample to calculated counts per minute in the cell at the start of the collection period for those cells which have a "slope ratio" close to unity, ie. in essence those cells not microinjected and some of those injected with solutions of very low sodium concentration. For other cells, Na^*/Na_m^* should be calculated from the slope k of a plot of ln Na^* versus time, ideally only where such a plot is linear and not too steep, as

$$\frac{Na^*}{Na_m^*} = k \cdot 5 \text{ minutes.}$$

This limits the use of microinjected cells to measurements of steady effluxes. Such will occur into normal Ringer's solution, and in most cases into sodium-free solution, but in potassium-free or ouabain-containing solutions the cell will gain sodium continuously. The efflux increases as $(a_{Na})_m$ increases, and the slope of the plot of $\ln Na^*$ versus time can become zero or even positive. A reasonable estimation of Na^*/Na_m^* usually can be made in these cases, but the resulting calculated value of M_{Na} is more uncertain than it is for steady effluxes.

Nevertheless, microinjection does have some virtues. The selectivity, convenience, and economy of the microinjection technique offset to some degree the uncertainty due to longitudinal diffusion. All 'participatory' intracellular compartments appear to be loaded, just as with passive loading, but the extracellular space is not loaded. Only a small amount of radio-isotope is required for each experiment. Finally, cells can be used shortly after dissection, when they should be in a state most like that _in vivo_.

The determination of Na_m^* would be more accurate if only the central part of a long segment of injected cell were perfused in microinjection experiments. The strategy adopted in the present experiments was to perfuse only a long injected segment of the cell rather than of perfusing a long cell only part of which had been injected. This was only partially success-ful in eliminating the problem of longitudinal diffusion. The problem is eliminated by the use of passive loading, but this method requires a great deal of isotope and long periods of incubation of the cell after dissection, and loads the extracellular space with isotope.

The 'time constant for exchange' Na^*/Na_{cell}^* has been taken to reflect the operation of the transport systems of the membrane most directly (eg. Dick & Lea 1967; Brinley 1968). It can be asked why the _size_ of the efflux should be calculated. Quite aside from the problems of using Na^*/Na_{cell}^*

for microinjected cells, it can be seen from equation (4) that the sodium content of the myoplasm must be taken into account if the activity of the transport mechanisms is to be deduced from isotope measurements. Identical values of Na^*/Na_m^* in cells of different sodium content reflect different 'pumping rates'. As can be seen from the manner in which equation (2) is derived from the definition of flux density (equation (1)), the appearance of the 'time constant' in the efflux equation is in intimate association with the specific activity of radiosodium inside the cell.

Similarily, the appearance of $(a_{Na})_m$ explicitly in equation (4) is misleading. It might appear that this imposes a spurious dependence of M_{Na} on $(a_{Na})_m$. Again, examination of equations (1) and (2) reveals that this is not so. M_{Na} and $(a_{Na})_m$ are mutually dependent in several ways in the context of a living cell during an efflux experiment _in vitro_. Equation (4) simply separates four measurable quantities from which M_{Na} can be calculated.

It must be asked at this point what advance all of this is over the method of Keynes and Lewis (1951). Conceptually, it is certainly a more realistic model of the cell and the ion movements. The main advance is the introduction of the sodium-specific microelectrode. This permits measurement of the true specific activity inside the cell. The microelectrode also enables one to measure the efflux in experiments where $(a_{Na})_m$ changes rapidly.

Several fundamental problems remain, however. The question of surface-to-volume ratio cannot be taken further without the performance of exacting morphological measurements, which themselves are plagued with uncertainties in the form of changes in cell volume during fixation. The much more significant and difficult question of the homogeneity of the cell membrane with respect to transport properties has not been pursued at all. In

practice, the best strategy is to use cells of about the same size, as was done here, so any error due to these factors will be about the same for each cell. Finally, the question of film-controlled diffusion ("unstirred layers") has not been addressed, aside from the explicit statement of the assumption that boundary layers are considered to be part of the 'functional membrane'. If a really good estimate of the magnitude of the sodium efflux is to be obtained, these difficult problems must be solved.

In the remainder of this thesis, equation (4) is used to calculate the sodium efflux from barnacle muscle cells, whether loaded with radio-sodium by microinjection or by immersion in a solution containing radio-sodium. The appropriate correction should be applied in the former case.

(ii) <u>Steady state distribution of cations.</u>

In the experiments described in section 8 concerning the distribution of hydrogen ions measured during steady conditions (the use of the term 'steady state' in this context has been criticized), a relationship between the membrane potential and the transmembrane difference in pH was found. Such a relationship had been sought by other workers, but had not been found. In the discussion of the results, the relationship derived here from elementary theory will be employed. These considerations apply to sodium ions as well as hydrogen ions.

Assume the cell membrane is a lamella uniform in the y and z directions of a Cartesian coordinate system having the x axis directed perpendicular to the membrane surface. At a point x, in the membrane, the net flux density $j(x)$ (moles/cm^{2}sec) is assumed to be the sum of a flux density $j^p(x)$ due to diffusion of hydrogen ions as described by the Nernst-Planck equation, and an additional flux density $j^m(x)$ which is not further specified. Thus

$$j(x) = j^m(x) - u(x) \cdot c(x) \left[R \cdot T \frac{\partial}{\partial x} \ln c(x) + F \frac{\partial}{\partial x} \phi(x) \right] \qquad \dots (5)$$

where $u(x)$ and $c(x)$ are the mobility and concentration (or activity) of

hydrogen ions free to diffuse at x, $\phi(x)$ is the electrical potential at x,

R is the gas content, T is the absolute temperature, and F is the Faraday

constant. Note that an efflux is positive in sign. To obtain a relation

between the net transmembrane flux density and the transmembrane difference

of c and ϕ, equation (5) must be integrated across the membrane. Only

steady conditions are considered here, so the total flux density j is

independent of x in the membrane, although j^m need not be. Multiplying

both sides by $\left[\exp(F\phi(x)/RT) \right]/u(x)$ and making use of the identity

$$\frac{\partial}{\partial x} \left[c(x) \exp(F\phi(x)/RT \right] = \exp(F\phi(x)/RT) \left[\frac{\partial}{\partial x} c(x) + \frac{c(x)F}{R \cdot T} \frac{\partial}{\partial x} \phi(x) \right]$$

one finds $\quad j = \frac{1}{Q} \left[M - RT \left[c(a) - c(0) \exp(FE_m/RT) \right] \right]$

where $\quad Q = \int_{x=0}^{a} \frac{\exp\left[\frac{F}{RT} \cdot (\phi(x) - \phi(a)) \right]}{u(x)} \cdot dx$

$$M = \int_{x=0}^{a} \frac{j^m(x) \exp\left[\frac{F}{RT} \cdot (\phi(x) - \phi(a)) \right]}{u(x)} \cdot dx$$

and $E_m = \phi(0) - \phi(a)$ is the membrane potential, and is generally negative.

Also, $Q > 0$ and if $j^m(x)$ represents an efflux, $M > 0$. (This relation has

been derived by Schwartz (1971)).

If at least one compartment is finite, the net total flux density must

vanish in the steady state, so $M = RT \left[c(a) - c(0) \exp F\phi/RT \right]$. (Note

that this argument could have been applied at equation (6).) If the internal

pH is $pH_i = -\log_{10} c(0)$ and the external pH is $pH_o = -\log_{10} c(a)$, re-

arrangement yields

$$pH_e - pH_i = \log_{10}\left[1 - \frac{M}{R \cdot T \cdot c(a)}\right] - \frac{F}{2.3\ RT}\ E_m \qquad \ldots (6)$$

Note that the constraint on M is now $0 < M < RT \cdot c(a)$. Also, $x = 0$ can be taken to be in the myoplasm, away from the surface of the membrane, and $x = a$ similarily to be in the external bathing solution, so surface effects need not be considered with respect to ϕ or c except in the evaluation of M, and the values of $(pH_o - pH_i)$ and E_m in equation (6) are those measured with microelectrodes.

The corresponding relationship for sodium ions is:

$$\log_{10}(Na)_i - \log_{10}(Na)_o = \log_{10}(1 - \frac{M}{R\ T\ (Na)_o}) - \frac{F}{2.3\ R\ T}\ E_m \qquad \ldots (7)$$

(iii) <u>Electrogenic contribution to the resting membrane potential</u>.

A number of physiochemical models of the contribution of electrogenic transport to the resting membrane potential have been made (see review by Thomas 1972a). The usual strategy is to carry out a derivation of the membrane potential as a diffusion potential, with the inclusion in the flux balance of an extra flux of ions due to active transport. The derivation of Moreton (1969) will be adapted for use in the analysis of the results of the experiments described in section 7.

Moreton (1969) adopted the Goldman-Hodgkin-Katz model, and calculated the passive transmembrane fluxes for sodium (m_{Na}), potassium (m_K), and chloride (m_{Cl}) from the Nernst-Planck equation in the constant field approximation:

$$m_{Na} = -\frac{R\ T}{F} \cdot u_{Na} \cdot \frac{d}{dx}(Na) - (Na) \cdot u_{Na} \cdot \frac{d}{dx}\phi$$

m_K, m_{Cl} similarily.

If there are no fluxes but these, then it must happen that

$$m_{Na} + m_k - m_{Cl} = 0$$

since the cell cannot accumulate a net charge. The result is the Goldman-Hodgkin-Katz equation, as discussed above.

If there is an additional component M of ion flux due, for example, to an active exchange of sodium for potassium which is not one-for-one, then the sum of this and the passive fluxes is zero:

$$m_{Na} + m_K - m_{Cl} + M = 0$$

and the following expression is obtained (Moreton 1969):

$$E_m = \frac{R T}{F} \cdot \ln \left[\frac{P_K (K)_o + P_{Na} (Na)_o + P_{Cl} (Cl)_i + \frac{R T}{F E_m} M}{P_K (K)_i + P_{Na} (Na)_i + P_{Cl} (Cl)_o + \frac{R T}{F E_m} M} \right] \quad \text{or}$$

$$E_m \cong \frac{R T}{F} \cdot \ln \left[\frac{U + \frac{R T}{F E_m} M}{W + \frac{R T}{F E_m} M} \right] \quad \text{for convenience.}$$

This is the familiar GHK equation, with an additional term representing the net pumped cation efflux. When the net efflux increases, the magnitude of the membrane potential increases because the numerical value of the numerator is less than that of the denominator.

This is not presented as an exact description of what occurs in reality, but rather is developed in the spirit of the GHK formulation. Some further approximations can be made to facilitate comparison of this relationship with experimental results. Most of the resting membrane potential is not due to the pump, ie. $U \ll W$, so it is a good approximation to neglect the pump term in the denominator to yield

$$E_m \doteq \frac{R \cdot T}{F} \cdot \ln \left[\frac{U + \frac{R\,T}{F\,E_m}\,M}{W} \right] \quad .$$

When the cell is exposed to ouabain, the pump term changes from M to a new value M', and the membrane potential changes from E_m to E_m'. Thus:

$$\Delta E_m \equiv E_m' - E_m$$

$$= \frac{R\,T}{F} \cdot \ln \left[\frac{U + \frac{R\,T}{F\,E_m'}\,M'}{U + \frac{R\,T}{F\,E_m}\,M} \right] > 0 \quad ,$$

where it is assumed that U and W are unchanged in the time it takes to complete the measurement of E_m'. Upon rewriting this as

$$\Delta E_m = \frac{R\,T}{F} \ln \left[1 + \frac{R\,T}{U\,F\,E_m'}\,M' \right] - \frac{R\,T}{F} \ln \left[1 + \frac{R\,T}{U\,F\,E_m}\,M \right]$$

it is seen that the second term in the argument of each of the logarithmic functions is much less than unity. It is thus a reasonable approximation to expand each of the logarithmic functions in a Taylor series and retain only terms to first order in the small quantities $\frac{R\,T}{U\,F\,E_m'} \cdot M'$ and $\frac{R\,T}{U\,F\,E_m} \cdot M$. This yields:

$$\Delta E_m \doteq \left(\frac{R\,T}{F} \right)^2 \cdot \frac{1}{U\,E_m} \cdot \Delta M$$

where $\Delta M \equiv M' - M < 0$

and $\left(\frac{R\,T}{F} \right)^2 \cdot \frac{1}{U \cdot E_m'}$ has been assumed to be equal to $\left(\frac{R\,T}{F} \right)^2 \cdot \frac{1}{U \cdot E_m}$ as a matter of convenience.

With one further step, the measured change in the sodium efflux on exposure of the cell to ouabain can be introduced. If the coupling ratio of sodium to potassium transported by the pump is introduced:

$$\mathcal{R} \equiv - \frac{M_{Na}}{M_K} > 0$$

then the <u>net</u> pumped cation flux is

$$M = M_{Na} + M_K = (1 - \frac{1}{\mathcal{R}}) M_{Na}$$

and

$$\Delta E_m \doteq \left(\frac{R\,T}{F}\right)^2 \cdot \frac{1}{U\,E_m} \cdot (1 - \frac{1}{\mathcal{R}}) \cdot \Delta M_{Na} \qquad \ldots(8)$$

This approximate expression relates the change in membrane potential ΔE_m to the change in sodium efflux ΔM_{Na} in a relatively simple manner, in the context of the usual formulation for the origin of the membrane potential. Either the coupling ratio $\mathcal{R}$ or the permeabilities in U can be evaluated from experimental data if one or the other is already known from independent measurements.

The proportionality factor between the unbalanced cation efflux and the change in E_m is decreased if the cell is hyperpolarized or if the concentration gradients are reduced. This feature reflects the imposition of the pumped fluxes on the Nernst-Planck equation. The forces which tend to oppose the charge separation which arises passively likewise oppose the unbalanced flux added phenomenologically, because of how the latter has been imposed in the current balance. Although mechanistically the unbalanced pumped cation efflux is like an increased mobility for sodium going out of the cell, the existence of this efflux is first apparent only as a sort of boundary condition of the steady state passive fluxes. A better and potentially very useful model would specify some driving force for the active efflux, such as the affinity of a chemical reaction, and would acknowledge the interaction between the active and passive fluxes. It bears repeating that such a model would reflect the mechanism which brings about active cation transport.

SECTION 3.   THE STATES OF SODIUM IN CELLS

The cell is known to be composed of several distinct morphological compartments.   These were described in the Introduction.   The sodium ions in the cell are distributed among these compartments.   If in an experiment some labelled sodium ions are introduced into one compartment, the exchange of sodium ions via diffusional and non-diffusional processes which occurs continuously between communicating compartments will tend to bring about a steady distribution in which the specific activity of sodium is the same in every compartment.

The rate at which sodium exchange occurs is not the same for every communicating pair of compartments.   One can conceive of a very rapid exchange, as between sodium ions in the myoplasmic compartment and the sodium ions which are acting as counterions to the fixed negative charges on a macromolecule in a protein matrix immersed in the myoplasmic compartment.   On the other hand, one can conceive of a very slow exchange, as between myoplasmic sodium ions and sodium ions which are counterions isolated by the hydrophobic barrier of a coiled and folded macromolecule. In the latter case, the exchange rate is probably so slow that, as far as in vitro isotope flux experiments are concerned, such sodium ions are non-participatory.

Between these extremes are known to lie most of the ion transport and exchange processes of the cell membrane.   In order to interpret flux experiments, one would like to know the exchange rates between communicating intracellular compartments, as well as the fraction of the cell sodium contained in each compartment.

Ion-specific glass microelectrodes of the type used to make intra-cellular measurements are assumed to sample the myoplasmic compartment.

This is the intracellular compartment which behaves very much like a bulk solution and is not enclosed by subcellular membranes, (see, for example, Hinke, Caille, & Gayton 1973 and the discussion following it, and Edzes & Berendsen 1975). Microelectrode measurements have indicated that only a part of the intracellular sodium, potassium, and chloride measured by chemical analysis of whole cells is in free solution in the bulk water of the myoplasm (McLaughlin & Hinke 1966; Dick & McLaughlin 1969; Lee & Armstrong 1972; Hinke et al. 1973; Lev & Armstrong 1975). The size of the fraction not accessible to the microelectrode in each case is not certain, because of uncertainty about the volume of the myoplasmic compartment and, especially for sodium, because of great uncertainty about the portion of the ions which is extracellular, in solution or sequestered (Lev & Armstrong 1975). One extreme estimate is that fully 83% of the intracellular sodium can be inaccessible to the sodium microelectrode (Hinke 1969b).

The sodium content of the barnacle muscle cell can be increased by immersion of the cell in a potassium-free solution (Beauge & Sjodin 1967), and can be decreased by immersion in a sodium-free solution (Allen & Hinke 1971). It was expected that the distribution of sodium inside the cell would also change during such manipulations. Thus, changes in the amount of myoplasmic and 'nonmyoplasmic' intracellular sodium were measured as the total sodium content of the cell was changed.

METHODS

Specimens.

The specimens used in all of the Na experiments were obtained from Georgia Strait. Those used in the pH experiments were obtained from Puget Sound. The morphology of the different species of giant barnacles has been described by Pilsbry (1916). He states that the overall form is highly variable, and that the shape of the opercular valves, the details of the structure of the plates of the wall, and the structure of the feet are the important characteristics. The largest specimens, which are specifically chosen for microinjection work, are Balanus nubilis, and occasionally B. aquila. (These are the largest North American barnacles found in shallow water.) As noted by Pilsbry, these larger barnacles are old, so their shell is worn and often riddled by boring animals. This does not appear to affect the health of the barnacle, but makes the identification of a species difficult. B. nubilis is unique in that as it grows it increases its internal volume by excavation of the basis, and this excavation is easily seen during dissection. Apparently there is no physiological difference between the muscle fibres of these two species.

Specimens were obtained by divers, and kept in a holding tank at the Vancouver Public Aquarium. Seawater drawn from Burrard Inlet was run through the tank continuously. The osmolarity of this seawater varied somewhat over the year, (950±50 mOsm on a Fiske osmometer). The residence time in the tank was not closely monitored, but never exceeded three months. Barnacles were moved to a controlled artificial seawater aquarium (Instant Ocean) at the laboratory 1-3 weeks prior to use. The artificial seawater was made up to 960 mOsm (the value for normal Ringer's solution) and maintained at 10-12° C. The barnacles actively extended their cirri in both

aquaria.

Dissection.

Great care was taken to minimize the trauma experienced by the muscle fibres. The barnacle was quickly killed by cutting through the opercular adductor and removing the cirri, and the digestive and reproductive organ mass. This left the six large depressor muscles attached to the opercular plates and to the basis. In the early experiments the shell was cracked with bone shears to isolate each muscle bundle intact (Hoyle 1963), with its fibres attached at one end directly (without visible tendon) to a fragment of the basis, and at the other end via tendons to a fragment of the opercular plate. For most of the experiments, however, a lapidarist's saw was used. This was far superior, and enabled one to isolate a muscle bundle very quickly, with a minimum of manipulation, on a compact fragment of the basis. The isolated bundle was immediately suspended by the fragment of operculum, in a beaker of normal barnacle Ringer's at 5 - 10° C. The muscles used were the Depressor Scutorum Lateralis and Rostralis. The tergal depressor was not used because its fibres were heavily invested with connective tissue and were more difficult to isolate.

Single muscle cells were typically 1.0 - 1.5 mm in diameter and 4 - 5 cm in length. To facilitate the separation of these single fibres from one another, a bundle was attached by the opercular and basal fragments to a frame which held the bundle horizontally at about the resting length, in a dish of cold Ringer's solution. Fat and connective tissue were carefully removed with jeweller's forceps and iridectomy scissors, under a dissection microscope. To isolate the fibres, the tendon of an accessible fibre was grasped with the forceps and cut from the operculum. The connections with the other fibres (connective tissue and a few small nerve fibres) were then

cut, proceeding from tendon to basis. (Damage to the fibre membrane would become apparent immediately as a local contraction.) This dissection was carried out to as close to the basis as was possible, and the fibre was left attached to the basis, since removal from the basis without causing damage to the cell membrane is impossible. This procedure was repeated for each of the fibres in the bundle, and then the bundle was left in normal Ringer's at $5 - 10^{\circ}$ C for 1 - 3 hours before being used.

This procedure assured that any damaged fibres would be identified, even if the damage was slight. Only fibres which were uniform in contour (ie. without contractures) and uniform in translucency were chosen for experiments. Fibres were only removed from the basis at the end of the experiment, when they were taken for weighing and chemical analysis. This use of intact fibres is different from the practice of other workers (Hagiwara, Chichibu, & Naka 1964; Brinley 1968; Bittar, Chen, Danielson, & Tong 1972), who cut the cells off at the basis and then cannulated the cut end of the cell.

Solutions.

The artificial seawater was prepared from Instant Ocean ingredients, to 960 mOsm. The Ringer's solutions were as in Table I. A ouabain (Schwartz-Mann) stock solution (100 mM) was prepared and used to prepare all solutions for ouabain experiments, by addition of the appropriate amount of ouabain stock solution in making up one of the solutions shown in Table I.

TABLE I

COMPOSITION OF SOLUTIONS

All values are mM. All solutions are 960±5 mOsm.

	Normal	Zero-K	Li	Li-½Cl	Choline	Tris**	Sucrose	Rinse
Na	450	458	--	--	--	--	--	--
K	8	--	8	16	8	8	8	--
Ca	20	20	20	20	20	20	20	20
Mg	10	10	10	10	10	10	10	10
Cl*	543	543	522	269	543	543	93	85
Tris*	25	25	25	25	25	475	25	25
Li	--	--	429	421	--	--	--	--
Choline	--	--	--	--	450	--	--	--
Sucrose	--	--	--	--	117	117	675	665
CH_3SO_4	--	--	--	268	--	--	--	--

** Tris is tris-hydroxymethyl aminomethane

* TrisCl is not completely dissociated: at pH 7.6 there are 19 mM Cl⁻ for 25 mM TrisCl (Gayton & Hinke 1968).

Microelectrodes.

Sodium-specific glass microelectrodes were constructed and calibrated by the method of Hinke (1967; 1969a), except that the sensitive glass was drawn by hand, and the final glass-to-glass seal at the tip was performed with the electrode held horizontally in the microforge and the heater wire brought in horizontally to touch and melt the tip of the sensitive glass.

The heat was sufficient to make the glass-to-glass seal, and when the heater wire was then withdrawn horizontally the sensitive glass was drawn into a fine tip and sealed. The outside diameter of the insulating glass at the seal was typically $20-25\mu$, and the length of the sensitive tip was typically 75μ. These electrodes were quite durable, and usually broke before they lost their Na selectivity. With use, the response time lengthened, but an electrode could be 're-activated' by a 10 sec. immersion of the tip in 0.1 M hydrofluoric acid. This treatment made the tips more fragile.

Conventional micropipette electrodes were pulled on a mechanical puller, either from the same lead glass used in the construction of the Na electrode, or from borosilicate glass (Hinke 1969). The potential change between 0.2 M NaCl and 0.2 M KCl was less than 2 mV for electrodes accepted for use (Adrian 1956). New electrodes were constructed the day before or the day of each experiment.

Potassium-specific ion exchanger microelectrodes were constructed by the method of Walker (1971), and tested by the method of Hinke (1969a).

For most of the experiments, the sodium or potassium electrode was referred to an extracellular calomel electrode. The potential difference was measured by a Cary 401 electrometer, and recorded on a chart recorder. The micropipette electrode was also referred to the calomel electrode, and the potential difference was monitored by a Vibron 33B electrometer or by a Kiethley 616 electrometer. For a few experiments the sodium electrode was referred directly to the intracellular micropipette electrode. Electrical interference was more of a problem with the latter method, but the two methods yielded similar results.

The experimental apparatus was housed in a small metal-walled room, and all electrometers, recorders, light sources, and power syringes were outside the room, except for the pre-amp of the Cary electrometer. The

separation of the above equipment from the experimental chamber was less than one metre. Leads, tubing, and fibre optic conduit were passed through small ports cut in the wall of the shielded room. With this arrangement and appropriate grounding, the electrometer readings were very stable throughout.

The axial insertion of the ion-specific electrode into the cannulated cell and the radial insertion of the micropipette electrode are depicted in Fig. 2. The calculation of the intracellular sodium and potassium activities from the potential differences in the microelectrode circuits was by the method of Hinke (1969a).

Chemical Analysis.

In a particular experiment, a cell was cut near its connection to the basis, rinsed for 30 sec in sucrose rinse solution, and blotted on filter paper. A short segment of the cell was cut from the tendon end and from the other end, and the remaining central part was placed in a pre-weighed stoppered vial. The wet weight was measured, and the dry weight was measured after drying of the cell fragment in an oven overnight. The cell was then wet ashed for analysis for sodium and potassium by atomic absorption spectrophotometry.

Extracellular Space.

The volume of the extracellular space was measured for barnacles from the same lot as those used in the experiments as the volume of distribution of (^3H) inulin or (^{14}C) sorbitol (New England Nuclear), by standard methods. The composition of the fluid in this volume was assumed to be that of the bathing solution, and the total sodium and potassium contents of the cell as determined by chemical analysis were corrected for the contribution of
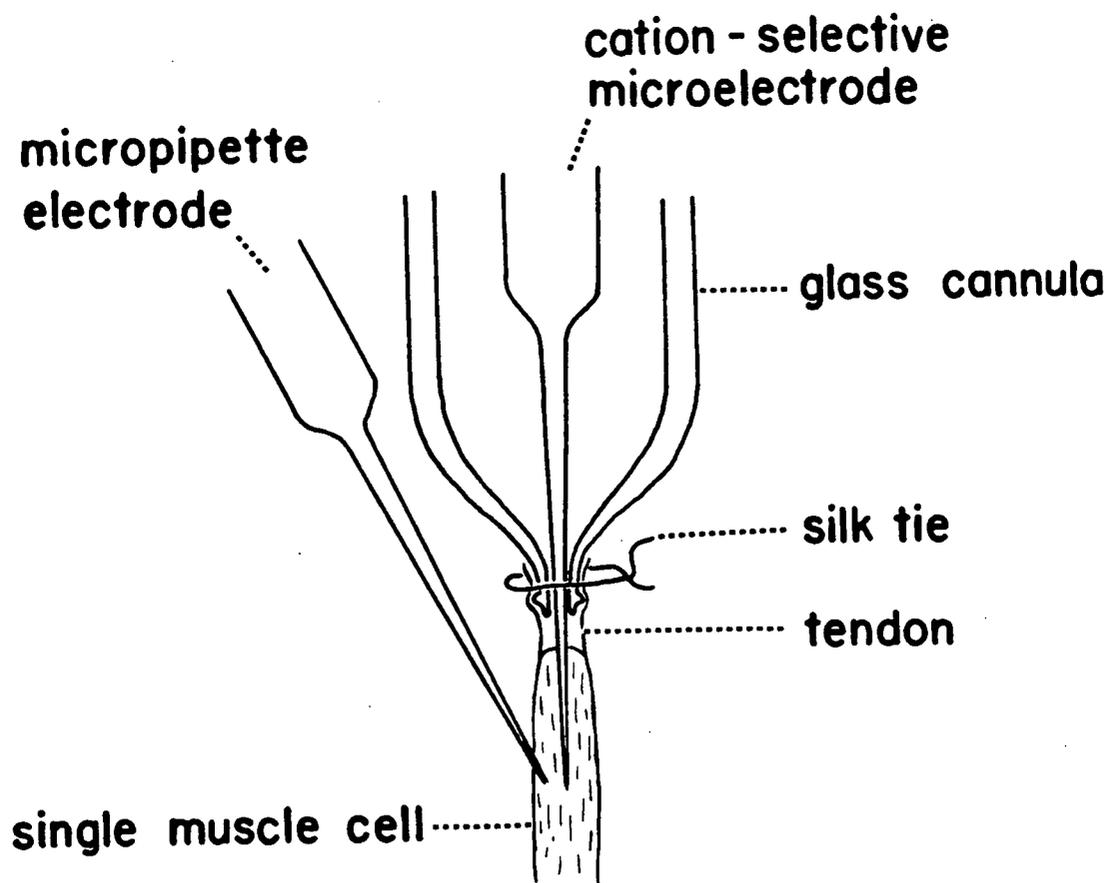
Figure 2. Configuration of the microelectrodes and cannulated cell during an experiment. Not to scale.

this extracellular fluid in the usual manner.

Myoplasmic and Nonmyoplasmic Intracellular Sodium.

For a given cell, the myoplasmic sodium activity $(a_{Na})_m$ in millimoles/litre and the intracellular sodium concentration $(Na)_i$ in (millimoles intracellular sodium)/(kilogram intracellular water) were measured. The total amount of intracellular sodium is thus $(Na)_i \times V_i$ where V_i is the weight of intracellular water. This amount is uncertain insofar as the fraction of the chemically analyzable sodium residing in the extracellular space is uncertain. The myoplasmic sodium content is $(\gamma_\pm)^{-1} \times (a_{Na})_m \times V_m$, where $\gamma_\pm$ is taken as 0.65 and V_m, the volume of the myoplasmic solvent water, is taken as the solvent water fraction measured for the barnacle (Hinke 1970), $(0.73 \times V_i)$, rather than the higher figure quoted in the Introduction as a general figure for cells. The calculated myoplasmic sodium content thus might be an underestimate. A similar calculation can be done for potassium.

The form in which the sodium content of each compartment will be presented, for the purpose of direct comparison of the _amount_, rather than the concentration, in each compartment, is arrived at as follows. The weight of water in the cell is $V_t=$ (wet weight) - (dry weight). The extracellular space is assumed to be 6% of the total water. The sodium concentration in normal Ringer's solution is 450 mM. The sodium content in solution in the extracellular space is thus $0.06 \times V_t(kg) \times 0.450(mole/litre)$. The tabulated total intracellular sodium concentration $(Na)_i =$ (mmoles intracellular Na)/(kg intracellular water) $= [$ (total analyzed Na) - (extracellular Na)$]/(0.94 \times V_t)$. 'Analyzed' refers to flame photometry.

The values in Table II part b and in Fig. 6 as 'sodium content' have been normalized by division by V_t. Thus the sodium content of the myoplasmic compartment is (moles Na in myoplasm)/$V_t = (a_{Na})_m 0.68/0.65$ and the sodium content

of the nonmyoplasmic compartment is (moles Na inside cell but not in myoplasm)/V_t = 0.94(Na)$_i$ - (sodium content of the myoplasmic compartment).

Two separate experiments will be described in turn. For clarity, the Methods, Results, and Discussion for each experiment will be presented separately.

A. INCREASE OF CELL SODIUM

Methods.

Cells were loaded with sodium by immersion overnight in potassium-free Ringer's solution. Four muscle bundles from the same barnacle were dissected as described above. Two were assigned to be experimental and two to be controls. $(a_{Na})_m$, (Na)$_i$, $(a_K)_m$, and (K)$_i$ were measured on six cells from each group. The remaining experimental cells were then placed in potassium-free Ringer's solution at 2° C for 20 hours. The remaining control cells were kept in normal Ringer's solution at 2° C.

After 20 hours, the experimental cells were transferred to potassium-free Ringer's solution at room temperature and $(a_{Na})_m$, (Na)$_i$, $(a_K)_m$, and (K)$_i$ were measured for six more cells. Similar measurements were also done for six control cells.

Half of the remaining experimental cells were then set in normal Ringer's solution at 10° C for 18 hours, and half were set in normal Ringer's solution to which had been added ouabain to 10^{-4} M, and left at 10° C for 18 hours. The remaining control cells were kept in normal Ringer's solution at 10° C.

At the end of 18 hours, measurements of $(a_{Na})_m$, $(Na)_i$, $(a_K)_m$, and $(K)_i$ were performed on cells from each group.

The sodium and potassium content of the two intracellular compartments were calculated for each cell as described in Methods.

This procedure was modelled after a method for measurement of a sodium extrusion dependent on external potassium $(K)_o$ and inhibited by ouabain (Steinbach 1940; Beauge & Sjodin 1967). The features relevant to the present problem are the changes in ion content of the myoplasmic and non-myoplasmic compartments.

Results.

The results of this experiment in which cells were 'passively loaded' with sodium by immersion in potassium-free solution in the cold and then were allowed to recover in normal Ringer's solution, are detailed in Table II.

The control cells, which were maintained in normal Ringer's solution throughout the ca. 40 hours of the experiment, underwent a continuous rise in sodium content, amounting to almost 40% overall. The potassium content was unchanged over the first 22 hours, but showed an increase of about 4% over the final 20 hours. It had been anticipated that a decline in the potassium content of the cells would accompany the rise in the sodium content. The membrane potential was very close to the potassium equilibrium potential for barnacle muscle cells (Hinke & Gayton 1971), and it showed no change during this long experiment. The water content of all cells increased slightly over the final 20 hours of the experiment.

The changes in the total amount of sodium and potassium in the experimental cells (where correction was made for extracellular sodium and potassium on the basis of an extracellular space containing 6% of the cell

TABLE IIa

SUMMARY OF MEASUREMENTS ON PASSIVELY-LOADED CELLS

Condition	$(a_{Na})_m$	$(Na)_i$	$(a_K)_m$	$(K)_i$	E_m	% water
Initial:						
Control	7.08	14.57	158.07	195.75	72.3	74.5
n = 6	(1.00)	(1.82)	(16.99)	(4.47)	(1.2)	(0.3)
Experiment	10.12	13.05	139.47	193.88	71.4	74.9
n = 6	(2.45)	(1.78)	(21.58)	(2.25)	(2.7)	(0.3)
Loaded:						
Control	7.82	16.98	149.68	194.47	72.5	74.4
n = 6	(2.30)	(2.56)	(24.28)	(6.21)	(4.4)	(0.4)
Experiment	15.47	25.82	113.68	178.17	88.6	74.8
n = 6	(4.18)	(4.44)	(37.41)	(1.91)	(9.4)	(0.2)
Recovered:						
Control	9.58	20.35	127.85	202.45	60.6	75.1
n = 4	(4.09)	(3.45)	(34.74)	(5.88)	(15.0)	(0.3)
Experiment	5.83	12.60	136.30	196.05	69.1	75.5
n = 4	(0.79)	(1.21)	(10.28)	(5.11)	(2.1)	(0.1)
Ouabain	25.48	50.48	106.83	159.98	55.4	75.5
n = 4	(7.20)	(9.93)	(22.89)	(12.97)	(12.0)	(0.4)

$(a_{Na})_m$ and $(a_K)_m$ are millimoles/litre myoplasmic water.

$(Na)_i$ and $(K)_i$ are millimoles/kg cell water, corrected for extracellular space ions as detailed in Methods.

E_m is membrane potential, in -millivolts. Note that the measurement on the Loaded-Experiment cells was carried out in potassium-free solution.

The numbers in parenthises are the standard deviation of the measured values, and n is the number of cells examined.

TABLE IIb

ION CONTENT OF THE MYOPLASMIC AND NONMYOPLASMIC COMPARTMENTS

Condition	Myoplasmic		Nonmyoplasmic		Total	
	Na	K	Na	K	Na	K
Initial:						
Control	7.4	165	6.3	19	13.7	184
Experiment	10.6	146	1.7	36	12.3	182
Loaded:						
Control	8.2	157	7.8	26	16.0	183
Experiment	16.2	119	8.1	49	24.3	168
Change	4.8	-19	4.9	6	9.7	-14
Recovered:						
Control	10.0	134	9.1	56	19.1	190
Experiment	6.1	143	5.7	41	11.8	184
Change	-11.9	47	-3.7	-38	-15.6	9
Ouabain	26.6	112	20.8	38	47.4	150
Change	8.6	16	11.4	-41	20.0	-25

All values are millimoles/kg total cell water, as detailed in Methods.
The change in the ion content of the experimental cells was corrected by
subtraction of the corresponding change in the control cells.

water, as determined by inulin and sorbitol uptake on barnacles from the same lot using the same blotting technique) were similar to those found by Beauge and Sjodin (1967) in a similar experiment on barnacle muscle where only chemical analysis was done. When the changes in ion content of the control cells were subtracted from the changes in the experimental cells over the corresponding time period, it was found that the total sodium gain and potassium loss was about one-for-one on incubation of the cells in potassium-free solution. When cells which had been incubated in potassium-free solution were allowed to recover in normal Ringer's solution, sodium was lost and potassium gained, again very roughly on a one-for-one basis. When companion cells which also had been immersed in potassium-free solution were set to 'recover' in normal Ringer's solution to which ouabain had been added (to 10^{-4}M), there was no recovery. Rather, a further gain of sodium and loss of potassium occurred, again on about a one-for-one basis.

The changes in the sodium and potassium content of the myoplasmic compartment, calculated from the measurements made with ion-specific intracellular electrodes as described in Methods, were for the most part just like those for the entire cell. On immersion of the cells in a potassium-free solution, sodium was gained and potassium lost by the myoplasmic compartment on about a 4Na:1K basis. When cells which had been immersed in a potassium-free solution were allowed to recover in normal Ringer's solution, sodium was lost and potassium gained by the myoplasmic compartment, but in this case on about a 1Na:4K basis. For those cells set to 'recover' in the solution which contained ouabain, sodium was not lost, but rather a further gain occurred. The potassium content of the myoplasmic compartment also showed an apparent increase, but it should be noted that there was a rather large correction applied for the behavior of the control cells. This is the only instance in which the correction for changes in the ion

content of the control cells caused a change in the <u>qualitative</u> result for the experimental cells. The uncorrected data show a roughly one-for-one sodium gain and potassium loss by the myoplasmic compartment for cells which were set to recover in a normal Ringer's solution which contained ouabain.

The changes in the sodium and potassium content of the <u>non</u>myoplasmic compartment, calculated as the difference between the change in ion content of the whole cell and that of the myoplasmic compartment, were qualitatively different. On immersion in potassium-free solution, there was an equal gain of sodium and of potassium by the nonmyoplasmic compartment. On recovery in normal Ringer's solution, there was a loss both of sodium and potassium by the nonmyoplasmic compartment, on about a 1Na:10K basis. For those cells set to 'recover' in the solution which contained ouabain, sodium was gained and potassium lost by the nonmyoplasmic compartment, on roughly a 1Na:4K basis.

The initial sodium distribution in the experimental group was quite different from that in the control group. Large reciprocal differences in the sodium and potassium content of barnacle muscle cells have been noted before (McLaughlin & Hinke 1966). This and the differences in the total ion content of barnacles from different populations (Brinley 1968; Gayton, Allen, & Hinke 1969) apparently are normal. It is certainly correct to try to isolate changes in the experimental cells which are due solely to the experimental manipulation, but the unexpected gain of potassium by the control cells over the final 20 hours of the experiment suggests the possibility of a similar caprice by the experimental cells, quite unrelated to the experimental manipulation. For this reason, it was felt that credibility should be assumed only for the qualitative changes described here.

Thus: the myoplasmic compartment behaved like the cells as a whole. The behavior is consistent with the model of the myoplasmic compartment as

a compartment whose ion content is governed by a sodium-potassium exchange mechanism which is stimulated by extracellular potassium and inhibited by ouabain. The nonmyoplasmic compartment, on the other hand, gains sodium and potassium together in the absence of external potassium, so there must in effect be a shift of potassium from the myoplasmic to the nonmyoplasmic compartment. The nonmyoplasmic compartment loses sodium and potassium together when external potassium is restored. Finally, the nonmyoplasmic compartment gains sodium but loses potassium on exposure to Ringer's solution which contains ouabain but is otherwise normal.

Discussion.

The main problem of concern here is the location of the sodium not detected by the sodium-specific intracellular electrode. The model employed in the above calculations, in terms of solvent water and partition of ions, is due to Hinke (McLaughlin & Hinke 1966; Hinke et al. 1973). He proposed that the nonmyoplasmic ions were associated with proteins inside the barnacle cell.

Experiments on intact cells and on membrane-damaged cells indicate that this compartment has a maximum capacity for ions of about 68 milli-equivalents/kilogram dry weight of intact cell (Hinke et al. 1973),[1] and a binding preference for sodium ions over potassium ions. In earlier experiments, it was assumed that all of the potassium was free in the myoplasm (Hinke 1970), but further experiments indicated that some potassium was

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[1]The membrane-damaged preparation has lost soluble organic molecules, which account for about half of the dry weight of the barnacle cell. The binding capacity found in experiments on membrane-damaged cells is roughly twice that found for intact cells, when no account is taken of the difference in the dry weight in the two situations.

'bound' as well (Hinke et al. 1973). The actual amount of potassium associated with fixed intracellular anionic sites should be greater than the amount of sodium so bound, though, because the myoplasmic potassium activity is much greater than the myoplasmic sodium activity. However, the total amount of nonmyoplasmic cation was about twice the capacity of the proteins in whole cells. Thus there probably is an additional component of the nonmyoplasmic compartment, which contains more sodium than potassium.

Experiments in which the water content of the barnacle cell was changed by exposure of the cell to hypertonic or hypotonic solutions indicated that most of the nonmyoplasmic sodium in barnacle muscle cells remained immobile despite large changes in cell water (Hinke 1969b). The myoplasmic potassium was unchanged when the cell water was increased, but decreased when the cell water was decreased. Competitive binding of sodium and potassium to intracellular proteins should follow a mass action rule, so should not be changed by a change in the amount of water in the myoplasmic compartment. It is interesting, however, that the potassium behaved slightly differently from the sodium in these experiments, as it did in the present experiments.

The effect of potassium-free solution on the myoplasmic sodium and potassium content of frog skeletal muscle has been reported (Armstrong & Lee 1971; Lee & Armstrong 1974). The results differed from those found here in barnacle muscle, in that the nonmyoplasmic compartment of frog muscle lost potassium on incubation in potassium-free solution. Thus the behavior of the myoplasmic and nonmyoplasmic compartments in frog muscle was qualitatively the same. However, it was found that if the potassium-free solution used for incubation contained much less calcium than frog Ringer's solution does, then all of the sodium gained by the cell entered the myoplasmic compartment, although potassium was still lost from both the myoplasmic and nonmyoplasmic compartments. Further, exposure to the calcium-poor

solution resulted in a decrease in the capacity of the nonmyoplasmic compartment. Finally, when the loading period was extended to 48 hours, the myoplasmic compartment accounted for most of the accumulation of sodium over the final 24 hours, as if the nonmyoplasmic compartment had become saturated.

Aside from the potassium loss by the nonmyoplasmic compartment, then, the behavior of frog muscle was the same as that of barnacle muscle on exposure to potassium-free solution. The effects of calcium are particularly relevant to the second likely site of residence of nonmyoplasmic sodium, the polysaccharides in the extracellular space.

The possibility that the sodium not detected by the microelectrode might be extracellular was mentioned by Caldwell (1968). Harris and Steinbach (1956) had measured cation binding by sugars. Brading and Widdicombe (1977) recently published a careful study of the capacity of the extracellular cation-exchanging sites in mammalian smooth muscle. They used the trivalent ion lanthanum to displace sodium, potassium, magnesium, and calcium from the tissue, and calculated the contribution of the intra-cellular and extracellular space to the effect. These cations all compete for anionic binding sites outside the cell. The amount of extracellular cation displaced from specific sites by lanthanum should be less than the total amount of cation bound to extracellular sites. It amounted to about 4 mmole potassium per kg dry weight, and about 60 mmole sodium per kg dry weight (assuming 80% water). It was also found that lanthanum reduced the passive sodium and (to a lesser extent) potassium movement across the cell membrane, but did not appear to affect the active ion movements. It was suggested that the binding of cations to extracellular sites is a stage of passive transmembrane passage of the cations, and that potassium behaves quite differently from sodium in its passive passage of the cell membrane.

If the extracellular polysaccharide is similar in barnacle muscle, as seems likely, there is then a credible second component to the nonmyoplasmic compartment, containing mostly sodium and having an ion-binding capacity similar to that of the intracellular proteins. Together, these two components appear to have a capacity adequate to contain all of the nonmyoplasmic sodium (mostly extracellular) and potassium (mostly intracellular).

It is not clear how the behavior of the nonmyoplasmic potassium in barnacle muscle can be accounted for with binding to these compartments according to the mass action rule. It might be that the extracellular compartment is indeed involved directly in the transmembrane transport of potassium by a method different from that for sodium, but this certainly could not be concluded from the present experiments alone. It seems more likely that potassium shifts to the TTS from the myoplasm when external potassium is removed (Birks & Davey 1969), while binding of potassium to fixed charges is less important.

The behavior of the nonmyoplasmic sodium is accounted for by a model of the nonmyoplasmic compartment as two regions which can bind sodium, one intracellular and containing relatively little sodium in comparison with potassium, and one extracellular, containing relatively little potassium in comparison with sodium.

At least some of this extracellular sodium should engage in rapid exchange with the sodium in the bathing solution, although in smooth muscle these cations were mobilized only when lanthanum was introduced. Such exchange might be revealed in an experiment in which the efflux of sodium into sodium-free solutions is measured. The results of such an experiment are described next.

B. DECREASE OF CELL SODIUM

Methods.

In a separate series of experiments, single barnacle muscle cells were

depleted of sodium by immersion in isotonic sodium-free lithium-substituted

Ringer's solution (Table. I).

A muscle bundle was dissected as described above, and measurements of

$(a_{Na})_m$ and $(Na)_i$ were performed on several cells in normal Ringer's solution

at room temperature. The bundle was then immersed in a sodium-free lithium-

substituted Ringer's solution for 30 seconds, then transferred to a large

volume of this solution. Measurements of $(a_{Na})_m$ and $(Na)_i$ were performed

on each of a succession of cells over the next three hours.

This procedure was carried out on three different muscle bundles, from

barnacles from the same lot. In one case, some measurements were made

after 16 hours of immersion in sodium-free solution, where the cells were

kept at 10° C between the third and sixteenth hours.

The sodium content of the myoplasmic and nonmyoplasmic compartments

was calculated, as described above.

Results.

The results are presented in Fig. 3 as the change in the sodium content

of the myoplasmic (closed symbols) and nonmyoplasmic (open symbols) com-

partments with time, while the cells were immersed in sodium-free lithium-

substituted Ringer's solution. The zero of time corresponds to the moment

of immersion. The lines were drawn by eye as a visual aid.

Under certain conditions, a large rapid fall of the myoplasmic sodium

activity can occur in frog skeletal muscle (White & Hinke 1976) and in

crab striated muscle (Vaughan-Jones 1977). Such an effect had been sought
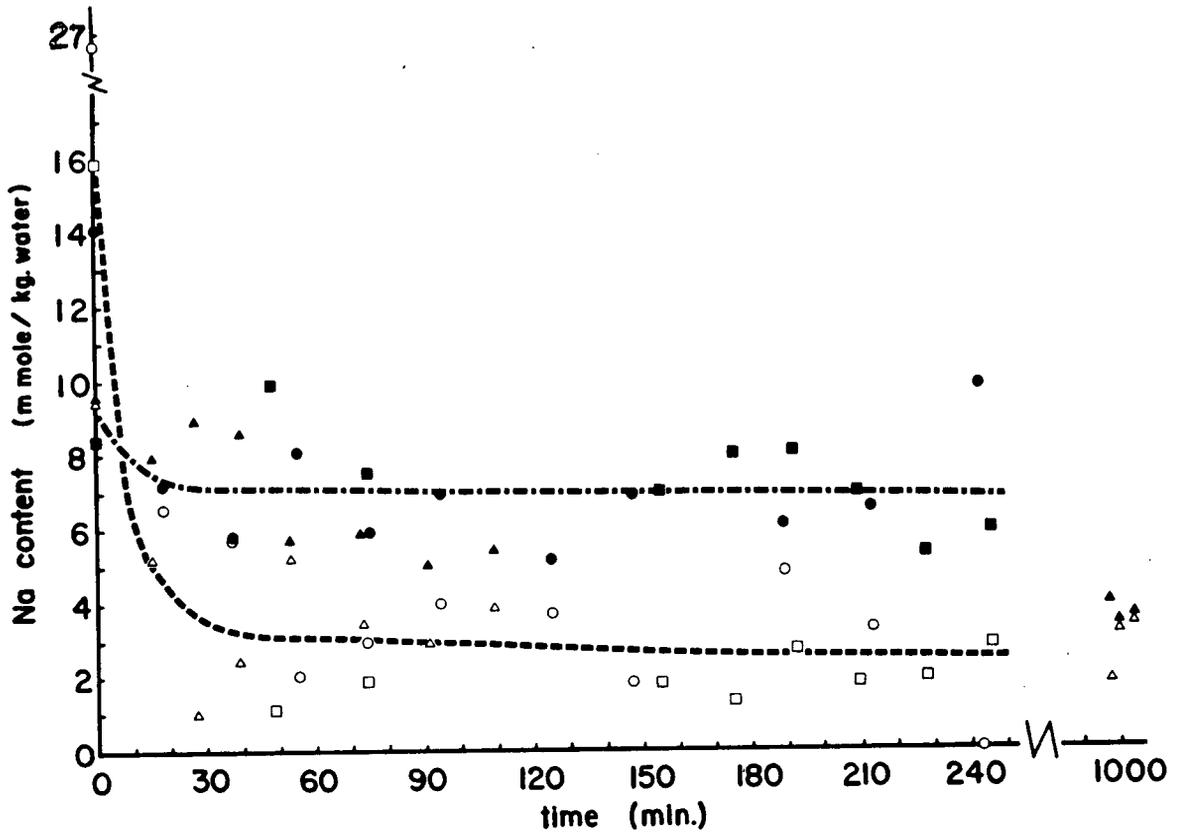
Figure 3. Changes in the sodium content of cells during incubation in sodium-free lithium-substituted solution. Correction for extracellular sodium, by standard methods (see text), was made for the cells at zero time (normal Ringer's solution). Each point represents one cell, where the closed symbol at a given time represents the myoplasmic sodium, and the open symbol at that time represents the nonmyoplasmic intracellular sodium. The three different symbol shapes represent three different experiments. The irregularly broken line represents the myoplasmic intracellular sodium, and the regularly broken line represents the nonmyoplasmic sodium. The lines were drawn by eye to summarize the three experimental runs.

and found in barnacle striated muscle, and is described in section 6. However, it is only seen in barnacle muscle cells which have an elevated sodium content. Two of the muscle bundles used in this experiment had relatively low sodium content (triangular and square symbols in Fig. 3), and it was not expected that the effect would be seen in them. The small initial fall in the calculated sodium content of the myoplasmic compartment, shown in Fig. 3, might reflect a rapid efflux from the third muscle bundle (round symbols), whose initial sodium content was higher.

Aside from this variation, the decline of the sodium content of the myoplasmic compartment with time was rather slow. Even after 16 hours of immersion in the sodium-free solution (much of which time was spent at 10° C as noted above), the myoplasmic compartment had retained half of its initial sodium.

The decline of the sodium content of the nonmyoplasmic compartment was markedly different. There was a large fall over the first 30 to 40 minutes of immersion, but then almost no loss over the next 15 hours.

The variation of the myoplasmic sodium activity $(a_{Na})_m$ in barnacle muscle cells on brief (less than 60 min) immersion in sodium-free solution has been measured by other workers (McLaughlin & Hinke 1968; Allen & Hinke 1971). They found that $(a_{Na})_m$ increased initially, then decreased. However, the lithium solution they used was prepared by substituting LiCl for NaCl on a one-for-one basis (McLaughlin & Hinke 1968, Table I). Such a solution is hypertonic, so the initial behavior of $(a_{Na})_m$ reflects the movement of water out of the myoplasm. This by itself has been found not to affect the sodium content of the myoplasmic and nonmyoplasmic compartments (Hinke 1969b). A similar experiment of 25 minutes' duration using isotonic sodium-free sucrose-substituted Ringer's solution showed behavior similar to that in Fig. 3 (Hinke 1969b).

A rough approximation of the size of the various fractions of cellular sodium can be made from Fig. 3. If the rapidly-lost fraction of the non-myoplasmic sodium is assigned to the extracellular space, there remains about 30% of the intracellular sodium not accessible to the microelectrode (in the model described earlier). This is about 10 millimole/kg dry weight. The data for very long time of immersion suggest that in barnacle muscle perhaps 20% of the cellular sodium is both not accessible to the micro-electrode and so slow to exchange as to be nonparticipatory in the type of in vitro experiments described in this thesis.

It has often been found that a fraction of the cell sodium exchanges only very slowly with radioactively-labelled sodium in the bathing solution (Conway & Cary 1955; Troschin 1961; Dunham & Gainer 1968; Allen & Hinke 1970). In particular, Allen and Hinke (1970) found good quantitative agreement in barnacle muscle for the amount of cellular sodium which ex-changes slowly as calculated from isotope flux studies with the amount of 'bound' (nonmyoplasmic) sodium calculated from microelectrode studies.

Discussion.

In the first experiment, where the sodium content of the cell was increased by immersion of the cell in potassium-free solution, it was found that the behavior of the nonmyoplasmic sodium could be accounted for by a model of the nonmyoplasmic compartment as two regions which can bind sodium: one intracellular, containing a relatively small amount of sodium; and one extracellular, containing a relatively large amount of sodium. The second experiment showed that indeed much of the nonmyoplasmic sodium can be washed out very rapidly in sodium-free solution, while some cannot be washed out even with long immersion in sodium-free solution. It seems reasonable to assign the former to the extracellular component of the non-

myoplasmic compartment, and the latter to the intracellular component of the nonmyoplasmic compartment, although some of the extracellular sodium probably is tightly-bound, as in smooth muscle.

To summarize the rough quantitative estimates, about 30% of the sodium which is truly intracellular is not accessible to the microelectrode. Most of this (about 20%) cannot be washed out during long immersion of the cell in sodium-free solution. If this 20% is identified with the fraction of the cell sodium which exchanges very slowly with radioisotopic sodium, then the amount of nonmyoplasmic intracellular sodium which exchanges rapidly with the myoplasmic sodium is probably less than 10% of the intra-cellular sodium. This conclusion is important because it allows the model for the measurement of the sodium efflux from barnacle muscle cells to be relatively simple, as discussed in section 2.D.

The possibility that the extracellular nonmyoplasmic sodium and potassium is within the overall mechanism by which these ions pass the cell membrane was raised in the discussion of the first experiment. It is possible that some of the extracellular nonmyoplasmic sodium can exchange directly with the myoplasmic sodium. However, it seems prudent to adopt the simpler hypothesis first, and test it in practice. The results of the next two sections support this choice.

SECTION 4. MICROINJECTION OF RADIOSODIUM INTO SINGLE MUSCLE CELLS

In the preparation of a cell for an experiment in which the efflux of radiosodium is to be measured, it is usually necessary to immerse the cell for some time in a solution which contains radiosodium. Both the intracellular and the extracellular sodium become labelled, and in the subsequent experiment both the intracellular and the extracellular sodium contribute to the measured radiosodium efflux.

The extracellular radiosodium is lost very rapidly, and after a short period of time only the intracellular radiosodium contributes appreciably to the observed efflux. However, the behavior of the efflux from the interior of the cell immediately after the experiment has begun is masked. It is not a simple matter to subtract the estimated contribution of the extracellular sodium to the total radiosodium efflux, because the efflux from extracellular sites is not always simple (eg. Rogus & Zierler 1973).

In large cells, the interior can be loaded with radiosodium selectively by microinjection. As first described by Hodgkin and Keynes (1956; see also Caldwell & Walster 1963), a fine cylindrical glass needle was inserted axially into a squid axon, and then removed while fluid was ejected from the tip. The injected fluid filled the space vacated by the withdrawing needle.

The technique is of interest here in two respects. First, the distribution of the injected radiosodium among intracellular pools of sodium can provide a test of the model for the cellular sodium described in section 3. The interpretation by Bittar and coworkers of sodium microinjection experiments in barnacle muscle seems to be at variance with the model (Bittar, Chen, Danielson, Hartmann, & Tong 1972), as will be described fully below. Second, the technique is convenient in that the cell can be loaded with

radiosodium quickly, the extracellular sodium pool can be bypassed, and the sodium content of the cell can be raised by injection of sodium solutions. All of this can be done with passive techniques as well, but at the expense of long immersion times, often in nonphysiological solutions.

Of course, the effects of microinjection on the cell and in particular on the sodium transport out of the cell must be known before the technique can be adopted as a convenience in efflux experiments.

In the remainder of this introductory passage, the results of other workers on the questions of the effect of microinjection on barnacle muscle cells, and the distribution of injected radiosodium among intracellular sodium pools are discussed. The experimental portion of this section consists of the comparison of the efflux of radiosodium from cells loaded by microinjection, with that from cells loaded passively.

Microinjection was first used in barnacle muscle by Hagiwara, Chichibu, and Naka (1964). A large needle was used (200 - 500 μ o.d.), fluid was injected as the needle was advanced down the axis of the cell, and enough fluid was injected to double the diameter of the cell. Even so, the excitability of the cell membrane was unimpaired, and the resting membrane potential showed a dependence on the transmembrane difference in the potassium concentration similar to that of intact, noninjected barnacle cells (Hagiwara et al. 1964; Hinke 1970).

Brinley (1968) used the technique of Hodgkin and Keynes, but with a microinjector needle which served as an open-tipped intracellular electrode for measurement of the membrane potential as the needle was being advanced down the axis of the cell (Brinley & Mullins 1965). He found that there was a transient membrane potential depolarization of 2 or 3 millivolts (mV) with each advance of the injector needle, with recovery occurring

within a few seconds after the advance was halted. He interpreted this as being due to the tearing and rapid resealing of the clefts and transverse tubules, which are open to the bathing solution and penetrate deeply into the cell (Hoyle 1973). Brinley found it difficult to obtain stable membrane potential readings in his preparation, even when using conventional micro-pipette electrodes. The mean value he found was -68 mV, and it did not vary with temperature (16 to 22° C). By comparison, Hagiwara et al. (1964) reported -73.5 mV; Hoyle and Smith (1963) reported -74 to -96 mV on selected intact cells not dissected from the muscle bundle; and McLaughlin and Hinke (1966) reported -71 mV on intact cells.

Bittar, Chen, Danielson, Hartmann, and Tong (1972) used the original technique (Caldwell & Walster 1963). Like Hagiwara et al. and Brinley, they cut single barnacle muscle cells from the basis at the point of attachment to the basis, and cannulated the cut end. They found that the membrane potential was usually unaffected by the process of microinjection, although their mean value for the resting potential was only -56 mV (range -42 to -72 mV).

Bittar et al. found that the efflux of injected radiosodium from the cell into normal Ringer's solution declined exponentially with time. The fraction of the total intracellular radiosodium lostpper unit time declined slowly over the first 60 minutes in the majority of the cells they studied, but was more stable thereafter. They found that the slope of the semilog plot versus time of the amount of radiosodium lost from the cell per unit time $(\frac{d}{dt} \ln(\frac{d}{dt} Na^*_{cell}))$ was greater than the slope of the semilog plot versus time of the amount of radiosodium left in the cell $(\frac{d}{dt} \ln Na^*_{cell})$, as did Hodgkin and Keynes (1956) in injected squid axon. That is, the amount of radiosodium in the cell did not decline with time at a rate commensurate to the decline with time of the rate at which radiosodium appeared in the

bath. This was not expected under the conditions of the experiment. If the rate of unidirectional sodium efflux is constant, and rapid mixing occurs inside the cell, then whatever the kinetic relation describing the dependence of this efflux rate on the intracellular concentration of sodium, the fall of the total radiosodium content of the cell is a simple exponential function of time. That is, the total intracellular sodium content and distribution should be constant while the cell is in normal Ringer's solution, but the pool of radiosodium present at the initial time is depleted as ^{22}Na exits with ^{23}Na by a random process which is slow relative to diffusion in bulk solutions. Dilution of the intracellular ^{22}Na by ^{23}Na occurs (where mixing inside the cell is assumed to be rapid compared to the efflux rate), and the rate at which the total radioactivity Na^*_{cell} due to radiosodium in the cell declines at each instant is proportional to the amount present at that instant:

$$\frac{dNa^*_{cell}}{dt} = -k \cdot Na^*_{cell} .$$

The rate constant k depends on the rate of unidirectional sodium efflux, which in turn depends, in particular, on the activity of sodium in the solution bathing the intracellular sites of the transport mechanisms. Thus:

$$Na^*_{cell} = Na^*_{cell}(t=0) \cdot \exp(-kt)$$

and

$$\frac{d}{dt} \ln Na^*_{cell} = -k = \frac{d}{dt} \ln \frac{d}{dt} Na^*_{cell}$$

since $\frac{dk}{dt}$ is assumed to be zero. Bittar et al. refer to the "slope ratio" $(\frac{d}{dt} \ln Na^*_{cell})/(\frac{d}{dt} \ln \frac{d}{dt} Na^*_{cell})$, which they found to be less than unity.

Hodgkin and Keynes (1956) considered and rejected as a possible explanation that the sodium efflux was not very sensitive to changes in

the intracellular sodium concentration $(Na)_i$, since injection of sodium

to raise $(Na)_i$ caused an appreciable rise in the sodium efflux. They

considered it possible that their preparation was slowly deteriorating, so

that the unidirectional sodium efflux M_{Na} was directly proportional to $(Na)_i$

at each instant but the proportionality constant slowly declined with time.

They demonstrated that at any given time the sodium efflux increased in

strict proportion to the amount of sodium injected into the axon.

Bittar et al. (1972) mentioned the possibility that the "sodium pump"

was running down in their preparation, but argued that the small slope

ratio was actually due to damage done to the "internal membrane system"

by the passage of the microinjector. An examination of injected fibres

with the electron microscope had revealed local disruption of the sarco-

plasmic reticulum and clefts along the injection track. They hypothesized

that sodium and calcium were compartmentalized in microsome-like vesicles

created by the injection. This requires that some of the injected radio-

sodium be sequestered at the time of injection and exchange only very slowly

with the free intracellular sodium. As Dick and Lea (1967) have pointed

out, this would cause the ratio of $\frac{d}{dt} \ln Na^*_{cell}$ to $\frac{d}{dt} \ln \frac{d}{dt} Na^*_{cell}$ to equal

the fraction of the intracellular radiosodium which is free in the myoplasm

(assuming that $\frac{d}{dt} Na^*_{cell}$ is linearly proportional to the amount of free

label, as discussed above). From such a calculation, Bittar et al. conclude

that on average about 30% of the injected radiosodium is sequestered, and

in some experiments an average of about 75% is sequestered. In addition,

they postulate that the cells in which the fraction of injected radiosodium

lost per unit time does not fall with time, and those in which it does fall

with time, form two distinct populations of normal barnacle muscle cells.

Some pharmacological experiments were also done by Bittar and co-

workers. They were interpreted in terms of bound sodium, and so will be

discussed here. Bittar and Tallitsch (1975, 1976) showed that exposure to aldosterone of muscles from a barnacle which had been exposed to aldosterone over the previous night results in a halt of the decline of the fraction of the injected radiosodium lost per unit time. This effect was reversible, the fraction resuming its decline when aldosterone was removed from the bathing solution after an exposure of less than 30 minutes. They proposed that, after the pretreatment with aldosterone, acute exposure of the cell to aldosterone caused a reversible release of radiosodium from intracellular binding sites.

The act of injecting solutions of NaCl after radiosodium had been injected (into aldosterone-pretreated cells) also caused a cessation of the decline of the fraction of the injected radiosodium lost per unit time, even with solutions of NaCl so dilute that the intracellular sodium concentration was raised by only 1 mM. Subsequent acute exposure to aldosterone had no effect unless the injected NaCl had considerably increased the intracellular sodium concentration. In the latter case, the rate of loss of radiosodium rose slowly to a new steady level.

If in the overnight pretreatment with aldosterone actinomycin D was included, it was found that no cell showed a decline with time of the fraction of injected radiosodium lost per unit time. If a cell pretreated only with aldosterone was exposed acutely to spironolactone, subsequent acute exposure to aldosterone was without effect.

It is interesting that in a later publication, Bittar, Chambers, and Shultz (1976) found that the fraction of injected radiosodium lost per unit time was constant in almost all cases (judging from the data presented in the figures). Their specimens were obtained from Puget Sound, while for the previous work barnacles both from Puget Sound and from California were used. Differences in the ion content of these two populations have been

reported (Brinley 1968; Gayton, Allen, & Hinke 1969).

The acute exposure to aldosterone of aldosterone-pretreated cells also caused a delayed transient stimulation of the radiosodium efflux. This effect was abolished by acute treatment with actinomycin D, ouabain, DPH (diphenylhydantoin), or injected ethacrynic acid, but was stimulated by maneuvers which would increase the intracellular supply of ATP.

To account for these findings, Bittar and coworkers suggested that aldosterone induces synthesis of new protein receptors in the barnacle muscle cell, some of which cause the reversible release of "bound" intra-cellular sodium (alleged in the earlier paper to be sequestered in vesicles created by the microinjector) and some of which cause a delayed stimulation of the ATP-dependent sodium efflux, upon subsequent exposure to aldosterone.

A different explanation for the observed "slope ratio", in terms of an effective intracellular sink for injected radiosodium, will be described in the discussion of the experimental portion of this section. It cannot account for the effects of aldosterone reported by Bittar and coworkers, but it seems likely that the main effect of aldosterone is on the transport systems in the membrane rather than on the state of the intracellular sodium. The suggestion that over half of the exchangeable intracellular sodium can be reversibly sequestered does not seem reasonable, given our knowledge of the morphology and ion-sequestering properties of the cell.

It is possible that in some cells the supply of metabolic energy in a suitable form for utilization by the sodium transport systems is not optimal, so that the system is indeed 'running down'. Aldosterone specifically promotes the transport of sodium in some cells by a mechanism which involves the synthesis of new protein by the cell. This could cause activation of the transport enzymes, provide additional energy for the transport enzymes, provide additional transport enzymes, or yield a combination of these

effects (Feldman, Funder, & Edelman 1972).

METHODS

Dissection of barnacle muscle bundles has been described in section 3. Previous work on the sodium efflux from barnacle muscle cells was done with cells which had been cut off at the basis, as mentioned above. Since this cannot be done without damaging the cell membrane, special measures were required to prevent the rapid occurrance of deterioration of the cell. Brinley (1968) immersed the terminal 5-10 mm of the cut end of the cell in oil for at least 30 minutes before cannulation and injection. Bittar et al. apparently adopted a similar procedure (Bittar 1966). In the present work the cells were kept intact throughout, with the tendon alone being cannulated. The cell membrane was breached only by the injection needle or the sodium electrode through the tendon end, and by the micropipette electrode radially about 20 mm from the tendon end (Fig. 2). No insulating oil was used, yet only rarely did these manipulations cause visible damage to the cell (and thereby cause the cell to be discarded).

$^{22}NaCl$ was obtained from New England Nuclear, carrier-free, in distilled water. Before use for injection, the water was evaporated off and the $^{22}NaCl$ redissolved in distilled water or in $^{23}NaCl$ solution.

Injection Apparatus.

A Hamilton microsyringe was used throughout. This had a nominal volume of 1.0 microlitre (λ), a Chaney adaptor, and a metal collar protecting the proximal part of the needle (model NCH 7001). A fine needle was drawn from 4 mm o.d. lead glass, on a mechanical micropipette puller with a long

throw (Hinke 1969a). Sufficiently durable needles had cylindrical shaft with o.d. 110-120 μ and length from the top of the shoulder to the tip of 38 mm. The tip was broken off to this length in such a manner that the tip was beveled slightly. New glass needles were made each day.

The glass needle was attached to the syringe needle with sticky wax as follows (Fig. 4). With the plunger withdrawn from the tip slightly, a small collar of hot sticky wax was put on the metal syringe needle near the tip, and allowed to harden. Distilled water was then drawn up into the syringe to 0.9 λ, the tip was dried by blotting, and the plunger withdrawn to 0.95 λ so no water was at the tip. The metal needle was then inserted into the glass needle, up to the shoulder. The shoulder (where the wax collar contacted the inside of the glass) was briefly passed through a gentle flame so that the sticky wax melted, and the glass needle was then gently pushed further onto the metal needle, until the metal needle was stopped by the tapering shoulder of the glass. The sticky wax flowed to seal the needles together without bubbles. The stem of the glass needle, which extends inside the metal protective collar, was then fixed to the metal collar with dental impression compound. The distilled water was then expelled into the glass needle, and usually filled it without bubbles. When all air had been expelled from the glass needle, the assembled microsyringe was mounted vertically on the injector (see below) with the tip of the glass needle submerged in distilled water.

The injector (Fig. 4) consisted of a brace which firmly held the microsyringe by the barrel and by the plunger, and a Prior micromanipulator rebuilt so that the entire microsyringe could be moved in the vertical direction ('positioning'), and so that the barrel of the microsyringe could be moved relative to the plunger ('injecting'). The former movement was used to position the glass needle in the cell, and the latter to withdraw
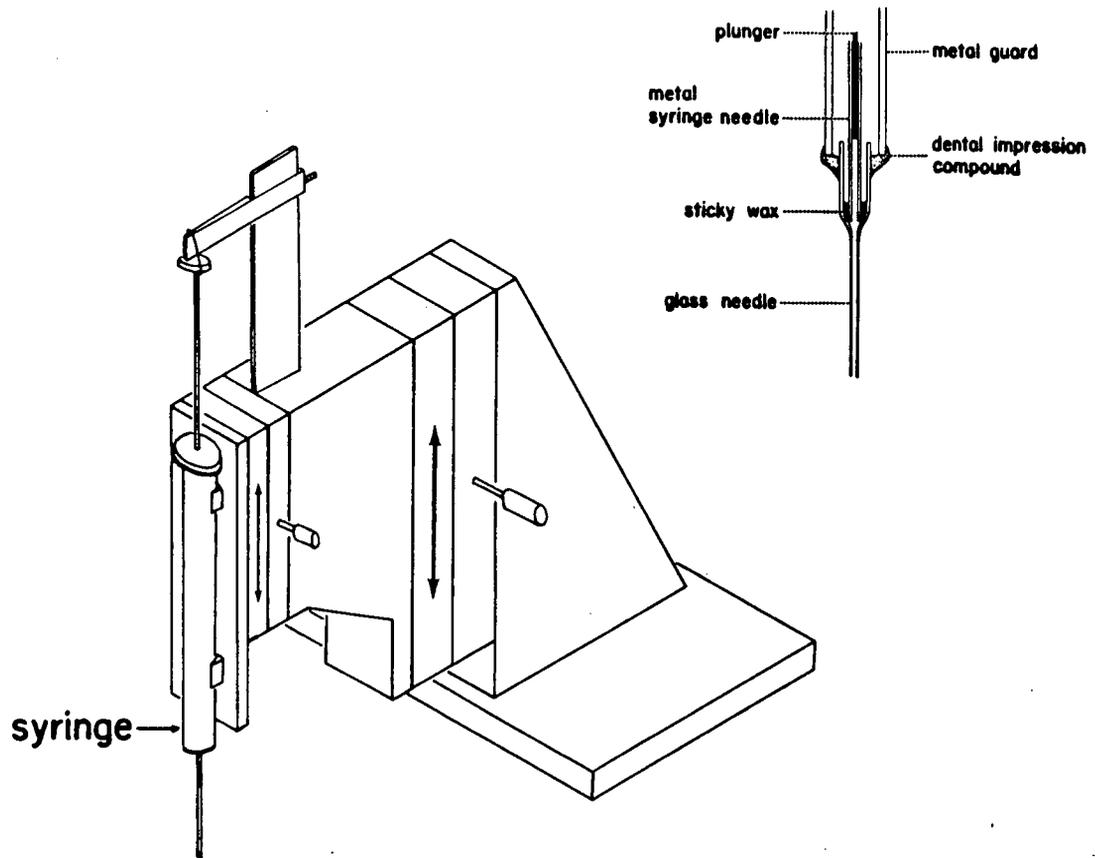
Figure 4. Microinjector. The basic features are shown, not to scale. Inset: detail of the connection of the glass injection needle to the Hamilton syringe needle. Not to scale.

the needle while expelling a column of injection fluid into the cell. The clamp holding the syringe was adjustable in two horizontal directions, so that the glass needle could be aligned with the vertical defined by the motion of the micromanipulator.

Calibration of the Microinjector.

To test the uniformity of the delivery of fluid from the syringe, a solution of ^{22}NaCl in water was drawn up into the syringe, to 0.75 λ, and ejected into gamma counter glass counting tubes containing 5 ml of distilled water, in aliquots nominally of 0.1 λ. Five successive 0.1 λ aliquots could be ejected before the counts in the tube dropped below the total for each of the preceding tubes. The dropoff presumably was due to mixing of the working fluid (distilled water) with the calibrated fluid with which it was in contact. In several such tests, and trial ejections of amounts varying from (nominally) 0.05 λ to 0.5 λ, the ejected volume calculated from the amount of radiosodium ejected agreed with the nominal volume to well within the uncertainty due to counting of the isotope (less than 4%). Injections into cells during experiments were carried out within these limits of uniform delivery.

The absolute value of the injected volume was tested by ejections of a solution of known radioactivity, and was found to agree with the nominal value to within the uncertainty of counting.

Collection of Isotope.

The chamber used during injection of intact fibres and collection of perfusion fluid was similar to that described by Allen and Hinke (1970) but considerably modified (Fig. 5). The cannulated fibre (see below) was held vertically at its rest length, with the fragment of the basis resting
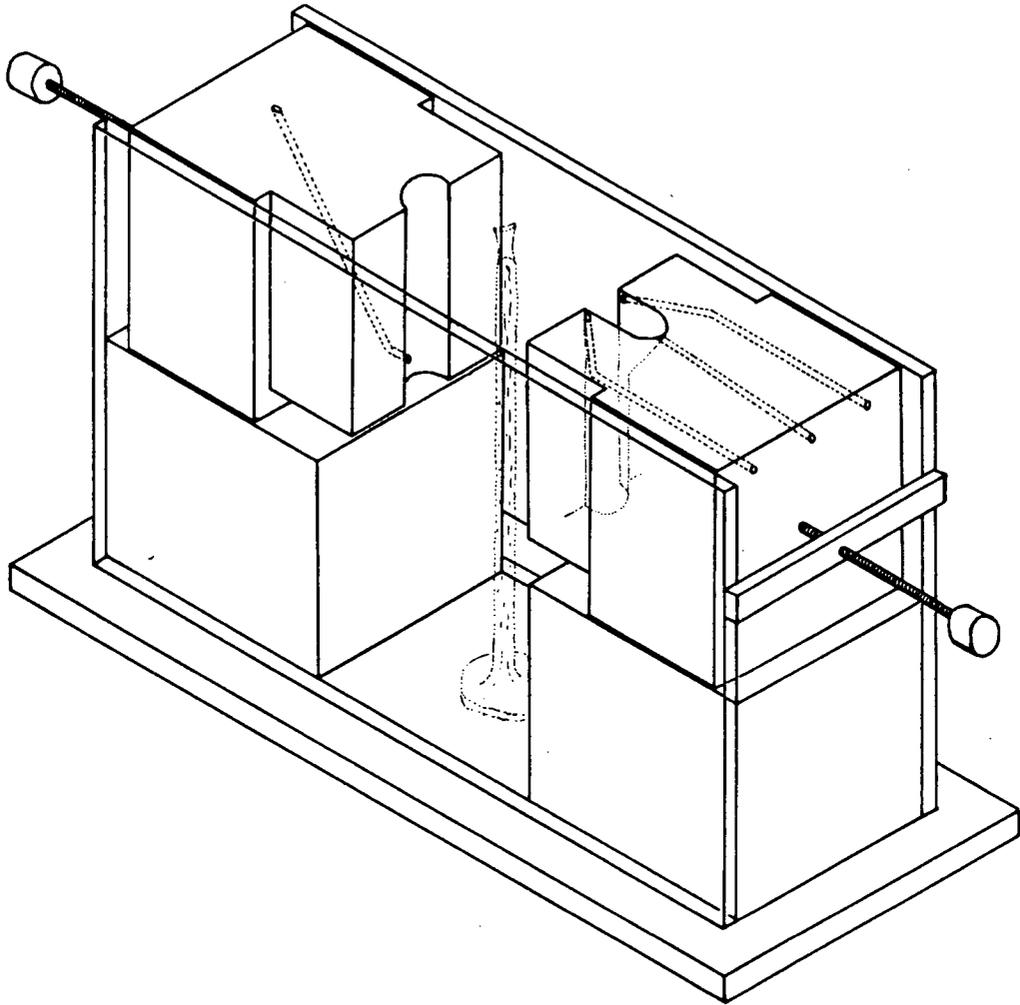
Figure 5. Apparatus for isolation of a segment of a cell for perfusion.
The moveable blocks, one containing the inflow channel and the other containing
the outflow channel, are shown in the opened position. A cell attached to a
fragment of basis is outlined.

on the false floor of the lower chamber. The height of the false floor was adjusted so that the cannulated tendon was at the desired height above the top of the chamber while undue tension was not exerted on the fibre. The movable Plexiglas blocks were retracted and the chamber filled with Ringer's solution for injection of isotope and placement of microelectrodes. The movable blocks were then brought together by turning the thumb screws, while the displaced fluid was withdrawn through the suction tubes. The grooves milled in the movable blocks formed a cylindrical chamber around the fibre when the blocks were brought together. This was separated from the lower chamber, containing the fragment of basis, by a seal of petroleum jelly (Vaseline). Vaseline was also used to seal the movable blocks to the stationary parts of the chamber. The grease seal which separated the upper and lower chambers was routinely tested by raising the fluid level in the lower chamber and observing the failure of fluid to enter the upper chamber, or by filling the upper chamber and observing no leak into the lower chamber.

Perfusion fluid was delivered from a Braun syringe pump, fitted with a 50 ml syringe (or two 50 ml syringes in parallel), at a constant rate of 1 ml/min. Two such pumps were used alternately, so that solution changes were accomplished by disconnecting the delivery tube from one syringe and connecting it to the other. This switch takes less than two seconds, so the interruption of the perfusion was negligible.

Perfusion fluid was drawn off through three exhaust ports located near the top of the upper chamber, and collected directly in a glass gamma counting tube (Fig. 6). The collection period was 5 min. The collection tube was changed manually. This interrupted the collection for 2 to 3 seconds, but caused no loss of fluid.

The volume of the upper chamber, which housed the injected portion of
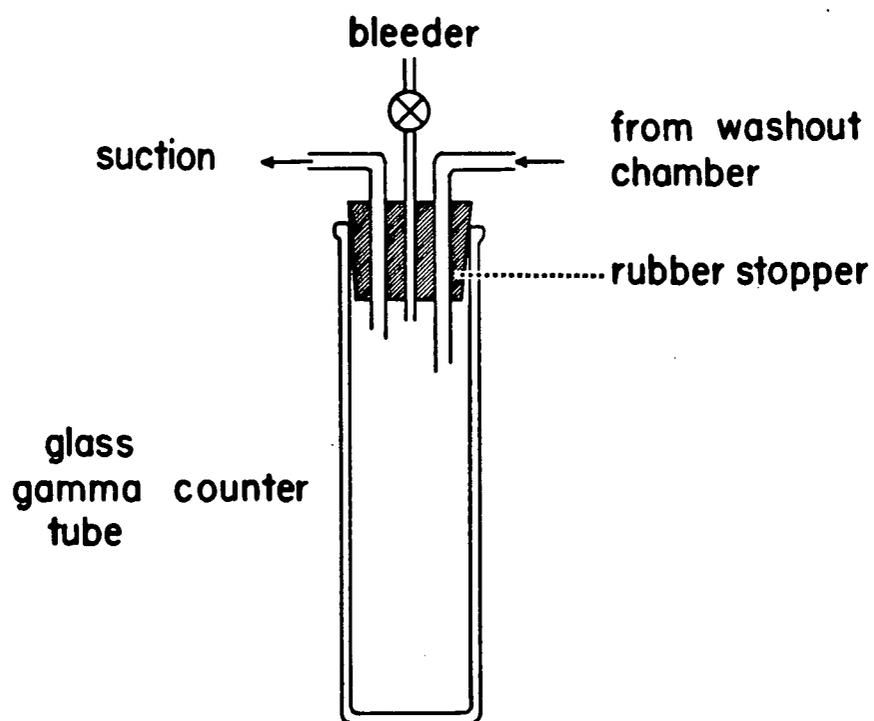
Figure 6. Vacuum system. The perfusate from the washout chamber (Fig. 5) is collected in the glass gamma-counter tube.

the fibre, was 1.0 ml. The washout time of the chamber was tested, with a glass rod placed where the fibre would normally be, and was found to be 87% complete within 2 minutes, corresponding to a time constant for exponential loss of label of about 1 min.$^{-1}$.

The effect of the finite washout time of the chamber can be estimated from a simple model. For the myoplasm (Na_1^*), the washout chamber (Na_2^*), and the collection tube (Na_3^*) considered as three compartments in series with no backflux, and

$$\frac{dNa_1^*}{dt} = -k_1 Na_1^*$$

$$\frac{dNa_2^*}{dt} = k_1 Na_1^* - k_2 Na_2^*$$

$$\frac{dNa_3^*}{dt} = k_2 Na_2^*$$

where at $t = 0$, $Na_1^* \equiv (Na_1^*)_0$ and $Na_2^* \equiv Na_3^* \equiv 0$. The general solution for this simple linear system is

$$Na_1^*(t) = (Na_1^*)_0 \exp(-k_1 t)$$

$$Na_2^*(t) = \frac{k_1(Na_1^*)_0}{k_2 - k_1} \left[\exp(-k_1 t) - \exp(-k_2 t)\right]$$

$$Na_3^*(t) = \frac{(Na_1^*)_0}{k_2 - k_1} \left[k_2(1 - \exp(-k_1 t)) - k_1(1 - \exp(-k_2 t))\right] .$$

That is,

$$-\frac{dNa_1^*}{dt} = k_1(Na_1^*)_0 \exp(-k_1 t)$$

while $\dfrac{dNa_3^*}{dt} = \dfrac{k_1 k_2 (Na_1^*)_0}{k_2 - k_1} \left[\exp(-k_1 t) - \exp(-k_2 t)\right]$,

so if dNa_3^*/dt (which is measured) is to approximate $-dNa_1^*/dt$ closely, k_1 must be much less than k_2. Since k_1 is typically 0.01 min^{-1} and k_2 is about 1 min^{-1}, this condition is well-satisfied, and except for the interval

just after t = 0 the fall in the amount of label collected with time closely
approximates the fall in the amount of label leaving the muscle cell with
time.

The 5 ml samples of perfusion fluid were counted on a well-type gamma
counter (Nuclear Chicago). Initial counting for 1 min. per sample was done
during the experiment to monitor the progress of the efflux. Later, the
counting was repeated at 20 min. per sample.

Sample tubes were re-used after washing with detergent and then chromic
sulfuric acid. Backgrounds for each tube were determined by a 10 min.
count and subtracted individually from the corresponding sample count.
This economy was possible because glassware cleaning and unlimited counter
time were available. Sample counts were almost always more than 10 times
background (the exceptions being samples of low activity at the end of a
long efflux experiment), and the background of the chemically cleaned tubes
was equal to that of new tubes.

Microinjection.

Cannulation of the tendon of the muscle fibre is readily accomplished
without affecting the cell membrane (Fig. 2). The cannulated fibre was
positioned vertically in the Plexiglas chamber as described above, so that
the silk tie was about 3 mm above the level of the fluid filling the
chambers. The cannula was held vertically, by a Prior micromanipulator.
The Plexiglas chamber was positioned on the horizontal platform of a Palmer
screw stand, and the microinjector was positioned on the horizontal plat-
form of a second Palmer screw stand. The glass needle was then advanced
down through the cannula and into the muscle fibre, as close as possible
to the long axis of the fibre. The advance of the needle was viewed through
a binocular microscope, from the front and, via a small mirror positioned

at a 45 degree angle near the muscle fibre, from the side. The outside diameters of the fibre in these two views were measured with an eyepiece micrometer. Illumination was provided by a 500 watt lamp (Volpi), directed obliquely at the muscle fibre by a fibre optic light conduit. The needle was quite visible in the opaque muscle fibre, and was always kept close to the central axis of the muscle fibre.

The injecting movement of the microinjector was then used to withdraw the needle while expelling the injection fluid into the cell. The injection track was 22 mm long, corresponding to 0.4λ, in most cases, but was shorter when it proved impossible to advance the needle far enough down the muscle cell without having the tip deviate from the axial line. Injection was terminated 5 mm from the point of impalement, to ensure that all of the fluid was deposited inside the cell.

With the more concentrated injection solutions, there would often be a slight contracture over the injected region, and if this had not disappeared by the time the microelectrodes were put into place, the experiment was not continued. In most cases, and with the more dilute injection fluids almost always, the injection was well tolerated by the muscle fibre, as far as could be detected by observation through the microscope.

The microinjector was removed and the sodium-specific microelectrode was placed in the cell axially, through the cannula, via manipulations similar to those used for positioning the injector needle. The sensitive tip of the electrode was placed in about the centre of the injected region of the muscle fibre. The micropipette electrode tip was passed obliquely across the cell membrane so it rested at the level of the sodium electrode. The movable blocks were then brought together to form the efflux chamber, the grease seal was tested, and the perfusion was started. With all of these maneuvers, the first perfusion fluid was collected 10 to 12 minutes

after the actual injection.

Passive Loading Experiments.

From a single dissected muscle bundle, three small groups of muscle cells were isolated, each group on a fragment of the basis. These were placed in a vessel with 10 ml of normal Ringer's solution to which ^{22}NaCl had been added, to 20,930 cpm per microlitre, and kept at 5° C overnight. The next day, two of the small groups were used for efflux experiments, as described above for microinjected cells, but they were not injected. The third group was loaded for 48 hours before being used for efflux measurement.

RESULTS

Effects of microinjection on the cell.

The efflux of microinjected radiosodium from a single barnacle muscle cell is shown in Fig. 7. A similar plot for a cell loaded passively, by immersion in a solution which contained radiosodium, is presented as Fig. 9 (page 128). The fall of the radiosodium content of the injected cell with time and the fall of the efflux of radiosodium with time can each be closely matched by a simple exponential function. After the initial rapid efflux from the passively-loaded cell, ascribed to the extracellular space as discussed below, the plots can each be matched by a simple exponential function during the efflux into normal Ringer's solution (Fig. 9). The rate constants are similar for the two cases. The 'slope ratio' effect is discussed below.

In Fig. 10 (page 140) is presented a summary of the raw data and reduced
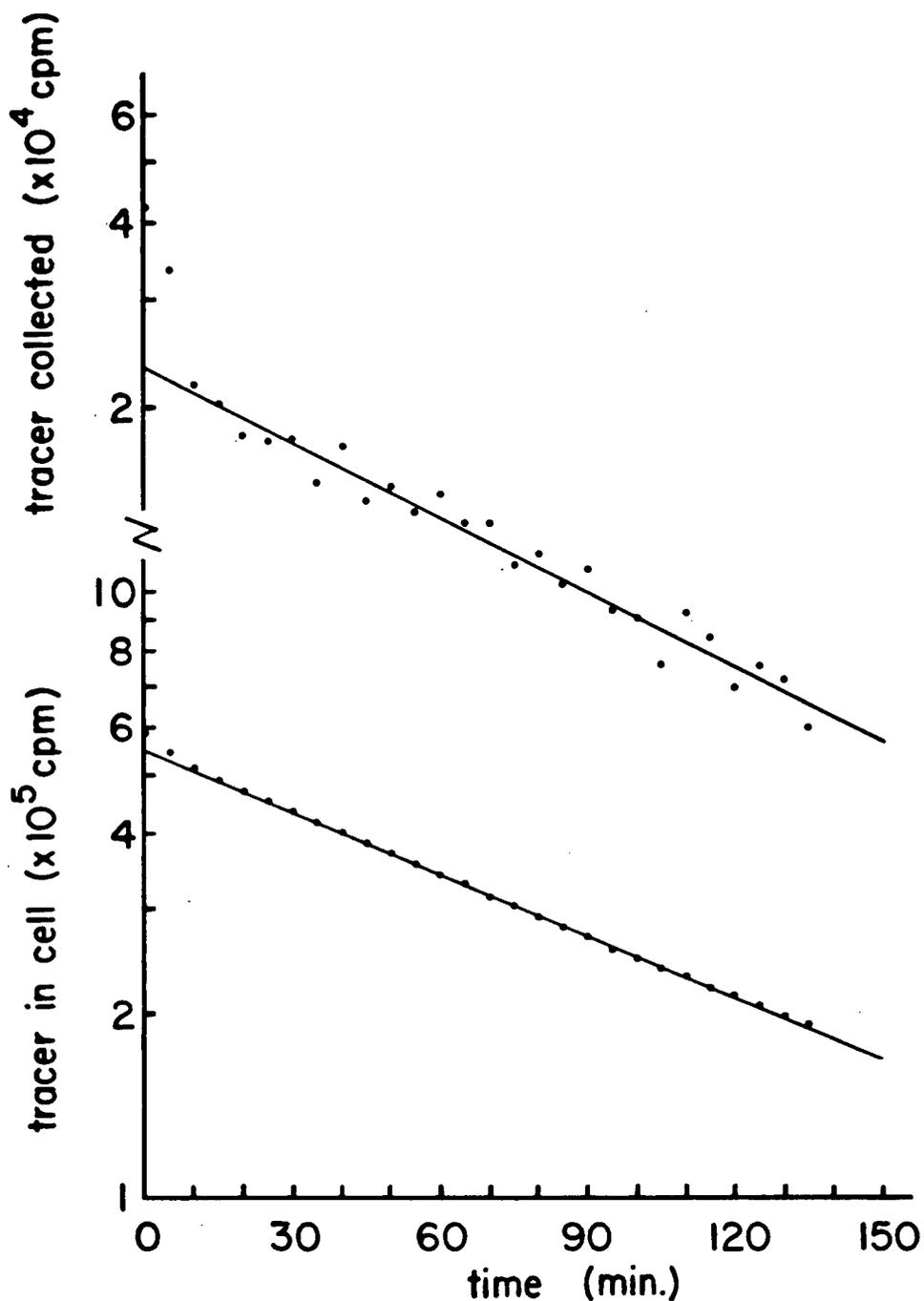
Figure 7. Semilogarithmic plot of the amount of radiosodium collected in the perfusate from a cell loaded with isotope by microinjection (upper trace), and the amount of radiosodium remaining in the cell at the start of each collection period, calculated by back-addition (lower trace), versus time. The cell was perfused with normal Ringer's solution. Lines represent the linear regression $\ln y = \ln a + bx$. Upper: $a = 23{,}112$ cpm, $b = -0.00928$ min^{-1}, $r^2 = 0.97$. Lower: $a = 548{,}947$ cpm, $b = -0.00786$ min^{-1}, $r^2 = 1.00$.

results for a typical experiment in the present series.

Measurements with the sodium-specific microelectrode show that the myoplasmic sodium activity $(a_{Na})_m$ rises after the injection in most cells, sometimes taking 30 minutes to reach a steady value. (The value of $(a_{Na})_m$ in noninjected cells is typically about 10 mM - see Table II). This rise was less marked in cells of a larger diameter.

Further, it has been reported that immersion of a barnacle muscle cell in sodium-free sucrose-substituted Ringer's solution for 5 to 10 minutes washes out most of the extracellular sodium while leaving the total intra-cellular sodium content almost unchanged (McLaughlin & Hinke 1966; Brinley 1968; Brigden, Spira, & Hinke 1971; the 'rapid' sodium-free effect in sodium-loaded cells has been mentioned in section 2 and is discussed fully in section 6). When a cell was kept in sodium-free sucrose-substituted Ringer's solution for 5 to 10 minutes prior to injection, and in normal Ringer's solution immediately after injection, the rise of $(a_{Na})_m$ after injection was much less marked. This maneuver was used in a number of the experiments to obtain data at low sodium levels. It should be mentioned that in the present series of experiments, no data was used for calculation of fluxes until $(a_{Na})_m$ had become steady, except where noted explicitly.

The observation by Brinley (1968) of a slight transient depolarization of the membrane potential with each advance of the injector needle was noted in the Introduction. Bittar et al. (1972) reported that the total sodium content of the cell was raised by injection but not by cannulation. They suggested that this was due to damage to the membrane of the clefts along the injection track, causing extracellular sodium to be released into the myoplasm.

In Fig. 7 it can be seen that the first two points in the plot of the measured amount of radiosodium leaving the cell per unit time (upper plot)

seem high. In many experiments, the first few points were lower. This conceivably could be due to a small loss of injection fluid into damaged clefts during the actual injection. It also could simply be due to the slight delay between the injection itself and the initiation of perfusion. Bittar et al. (1972) noted that a second injection did not alter the course of the efflux of previously injected radiosodium, although Brinley (1968) reported that the cell would not tolerate a second injection.

The extracellular space marker (^{3}H)inulin does not pass the intact cell membrane. When a solution containing (^{3}H)inulin was injected into a cell, no label was detected subsequently in the bathing solution. On the other hand, following injection of (^{14}C)DMO, (5,5-dimethyl-2,4-oxazolidinedione), which is known to cross the cell membrane quite readily, the label appeared promptly in the bathing solution. It was concluded that any loss of radiosodium through damaged membrane during microinjection must be quite small.

Altogether, it seems almost certain that the increase in intracellular sodium (and calcium - Bittar et al. 1972) seen after injection is due to a transient influx of extracellular fluid during injection.

The membrane potential E_m is the most sensitive indicator of the integrity of the cell membrane. As a rule, E_m was depolarized by 10 to 15 millivolts after injection, then recovered slowly (20 to 30 minutes) to assume a steady value close to but generally slightly more positive than the pre-injection value. Injected cells then showed stable membrane potentials for several hours at room temperature in normal Ringer's solution.

Altogether, it was concluded that while microinjection is not entirely benign, the injected cells recover quickly and appear to behave as non-injected cells do for several hours after injection.

The 'slope ratio'.

For the experiment presented in Fig. 7, the linear regressions of

$\ln Na^*$ and $\ln Na^*_{cell}$ on time indicate that almost all of the scatter can be

accounted for by random error in the sampling and counting ($r^2 = 0.97$ and

1.00 respectively), and yielded time constants $0.00928 \ min^{-1}$ and 0.00786

min^{-1} respectively. The slope ratio $\frac{d}{dt} \ln Na^*_{cell} \ / \ \frac{d}{dt} \ln \frac{d}{dt} Na^*_{cell}$

calculated as the ratio of the 'slopes' yielded by the linear regressions,

is 0.85.

The range of 'slope ratios' for a number of experiments is indicated

in Fig. 8. They are plotted versus the myoplasmic sodium activity for the

cell. Usually the duration of exposure to normal Ringer's solution was

shorter than in Fig. 7 (50 minutes) and the value for r^2 was slightly lower

(0.90 to 0.95 in almost all cases). Overall, these 'slope ratios' were

closer to unity than those reported by Bittar et al. (1972).

With respect to the 'slope ratios' as interpreted by Bittar et al., the

principal question is how the injected radiosodium distributes inside the

cell. For convenience, the assumptions adopted in the model of the cell

employed in this thesis will be restated.

A fundamental assumption of this model is that the intracellular

concentrations which are of direct importance for transmembrane efflux are

those of the myoplasm (given constant extracellular conditions). By

"membrane" is meant the barrier to diffusion, and any boundary layer of

glycocalyx or of water which is strongly influenced by the protein-lipid

lamina can be included as part of the membrane. Ion concentrations can

differ in the myoplasm and the boundary layer, but electrochemical poten-

tial cannot, it being assumed that transmembrane transport is almost always

slow relative to mixing inside the cell. It can be demanded formally that

ion (sodium) activities do not differ in the myoplasm and the boundary
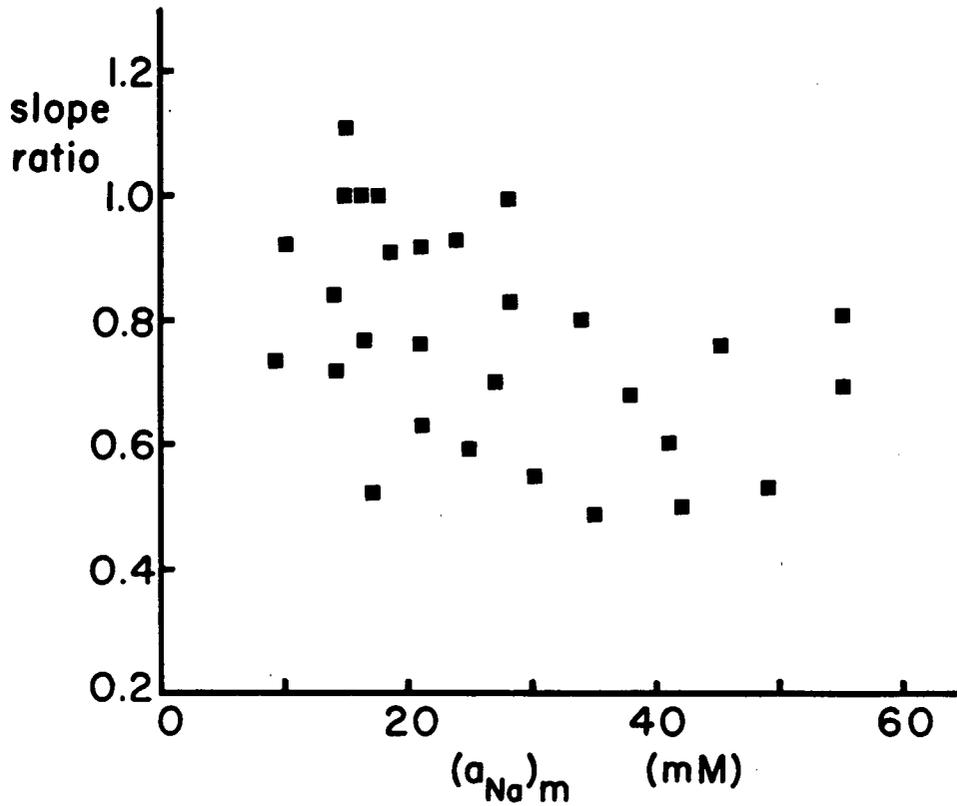
Figure 8. 'Slope Ratio' is the ratio ($\frac{d}{dt}$ ln Na^*_{cell})/($\frac{d}{dt}$ ln $\frac{d}{dt}$ Na^*_{cell}), which is the ratio of the slope of the lower line to that of the upper line in Fig. 7. Each point represents one efflux experiment in normal Ringer's solution. Not all of the experiments done in the present series are represented. Points omitted for clarity fell in the lower range of $(a_{Na})_m$.

layer, as long as this conception of the "membrane" is kept in mind.

The myoplasm bathes the internal surface of this membrane, and it is across this membrane, via various mechanisms, that the bulk of the sodium efflux from the cell occurs. That is, for the moment it is assumed that sodium efflux into the TTS and the SR is unimportant under most conditions.

Recall that it was stated above that

$$\frac{d}{dt} \ln Na^*_{cell} = \frac{d}{dt} \ln \frac{d}{dt} Na^*_{cell}$$

if and only if

$$\frac{d}{dt} Na^*_{cell} = -k Na^*_{cell} \text{ ,}$$

where $\frac{d}{dt} k \equiv 0$. The slope ratio statements must be made to refer to myoplasmic parameters: the amount of radiosodium in the myoplasm Na^*_m is substituted for Na^*_{cell}, and the myoplasmic sodium activity $(a_{Na})_m$ is substituted for the intracellular sodium concentration as the determinant of the rate of unidirectional sodium efflux. Given previous experimental results, it was postulated that the rate of unidirectional sodium efflux is an increasing function of the myoplasmic sodium activity over a wide range. Thus as long as the rate of unidirectional sodium efflux is found to be constant, the net flux of sodium must be zero, given the 'membrane-flux' model described in the preceding paragraph.

The rate constant k is, in particular, a function of the rate of the unidirectional sodium efflux. This relationship is usually expressed as a polynomial in the intracellular sodium concentration.

It has been stated that if the radiosodium is well-mixed inside the cell at all times, and the rate of unidirectional sodium efflux is constant, the slope ratio will be equal to unity. The situations in which these conditions do not obtain can be listed.

If there is a net increase in the sodium content of the myoplasm with

time, the specific activity of the myoplasm SA_m will fall more quickly than would be expected according to the observed efflux of radiosodium. A fall of SA_m will decrease $\frac{d}{dt} Na_m^*$, since a smaller fraction of the unidirectional sodium efflux will consist of radiosodium. On the other hand, as $(a_{Na})_m$ rises the rate of unidirectional sodium efflux will rise, and there will be an increase in $\frac{d}{dt} Na_m^*$. The net effect on the slope ratio cannot be specified in the absence of a specific model for the sodium efflux.

In any event, the sodium content of injected cells has been monitored with a sodium-specific microelectrode, as noted above. The intracellular sodium content increases upon injection, due to a transient influx of extracellular fluid, but the sodium content takes at most 30 minutes to stabilize, and usually much less. This cannot account for the observed slope ratios.

It is conceivable that the rate of the unidirectional sodium efflux might change independently. For example, it might fall as the supply of energy from metabolism in the isolated cell is depleted. The condition of the 'pump' can be inferred from observation of the efflux over a long period in normal Ringer's solution.

It was found that there was no obvious deviation from linearity in the semilog plots depicting the loss of radiosodium from injected cells in experiments of long duration (for example, Fig. 7). This indicates that the 'pump' is not running down.

The final possibility is that the injected radiosodium becomes compartmentalized inside the cell. Bittar et al. (1972) adopted the model of Dick and Lea (1967), wherein some of the radiosodium inside the cell was sequestered and exchanged with the free intracellular radiosodium only at a negligibly slow rate. As noted above, the slope ratio is equal to the fraction of the radiosodium left in the cell which is free. This is not a

constant, but rather declines with time as the free radiosodium is washed out of the cell.

Fig. 7 indicates that the slope $\frac{d}{dt} \ln Na^*_{cell}$ does not decline noticeably with time, contrary to the Dick and Lea model if the size of the sequestered fraction is not to be negligible. Indeed, Bittar et al. calculated this fraction as up to 70% of the injected radiosodium.

The alternative is a compartmental model in which the internal compartments can exchange sodium. Many mathematical treatments of such models have been published, but qualitative considerations can narrow the range of possibilities.

The injection loads the myoplasmic compartment, selectively and very rapidly. If there were an intracellular compartment of finite size which exchanged sodium with the myoplasm with a rate constant for exchange comparable to the rate constant for the transmembrane flux, then the efflux curve for injected cells would not be so close to a simple exponential for all times from near zero. The compartment would load from, then empty into the myoplasm in the course of the experiment.

Consideration of both the size of the hypothetical compartment and the rate at which it exchanges sodium with the myoplasm is important in drawing this conclusion.

For the range of efflux rate constants found in the present series of experiments, any such compartment of appreciable but not too large a size (as explained in the next paragraph) whose rate constant was within an order of magnitude of the rate constant for the transmembrane flux would be detectable as a deviation from linearity in a semilog plot such as Fig. 7.

A small compartment which exchanges sodium rapidly with the myoplasm would not be seen as a deviation from linearity, and is of no concern in the present context. The considerations of section 3 suggest that the

sodium associated with intracellular fixed anionic sites is such a compart-
ment. A larger compartment which exchanges very slowly with the myoplasm
would not be loaded with injected radiosodium in experiments whose duration
was a few hours, although it might be loaded in an experiment whose duration
was tens of hours. The sodium which exchanges only very slowly, such as
that reported for barnacle muscle by Allen and Hinke (1971), and the sodium
termed "inexchangeable" in section 3, which probably is the same pool,
should be such a compartment. It also is of no concern in the present
context.

A very large compartment would load with injected radiosodium from the
myoplasmic compartment throughout the duration of the experiment, and might
not be seen as a deviation from linearity in the semilog plot if its
exchange with the myoplasm can be described by a simple exponential function
of time, even if the exchange were relatively rapid. It would constitute
an intracellular sink for radiosodium, and the total amount of radiosodium
in the cell would not decline with time at a rate commensurate to the
decline with time of the rate at which radiosodium appeared in the bath.
That is, the two slopes would each be constant, but the slope ratio would be
less than unity.

In this connection, comparison of the efflux from injected cells with
that from cells loaded with radiosodium by immersion in a solution which
contains radiosodium is relevant. Three efflux experiments were done on
cells from a single barnacle which were loaded with radiosodium by immersion
at less than 5° C in normal Ringer's solution which contained some radio-
sodium, as described in Methods. Two experiments were done after a 24 hour
loading period, and one after a 48 hour loading period. The efflux determi-
nation was carried out as for injected cells, but no injection was done.

The semilog plots for the efflux from passively loaded cells differed

from those for injected cells. The plots for the second experiment are shown in Fig. 9. They were linear for time greater than 50 minutes when perfusion was with normal Ringer's solution. A linear regression was performed on the part of the curve from 50 to 135 minutes. The regression line was extrapolated back to zero time, and the difference between the data and this line over the interval from 0 to 50 minutes was re-plotted, as shown in Fig. 9. This operation yielded a simple exponential. It is reasonable to ascribe this rapid efflux to the washout of radiosodium from the extracellular space, as will be discussed below. An efflux of comparable rate from the interior of the cell has been implied by measurements in frog and crab muscle, as noted in section 2, but only in sodium-free solution. No such rapid efflux into normal Ringer's solution has ever been reported.

The slope ratios obtained in the three experiments were 1.30 and 0.98 for a 24 hour loading period, and 0.99 for a 48 hour loading period. It was noted at the conclusion of the first experiment that the cell was slightly irregular in appearance. The corresponding slope ratio for frog skeletal muscle loaded passively is also unity (Keynes & Swan 1959).

There is one difference between the two loading methods which might account for the results. Passive loading loads the entire cell, while injection deposits the radiosodium along a track which does not extend right to the tendon end of the cell and which is accessible to the uninjected portion of the cell which extends beyond the grease seal. Diffusion of sodium ions in barnacle muscle cells is about as rapid as that in a bulk solution (Caille & Hinke 1972), but the diffusion front still takes several hours to travel one centimetre. There is thus a slow continuous dilution of radiosodium throughout even the longest efflux experiment on injected cells. The result should be a slightly more rapid falloff of the radiosodium efflux as the intracellular diffusion progresses. Since the total
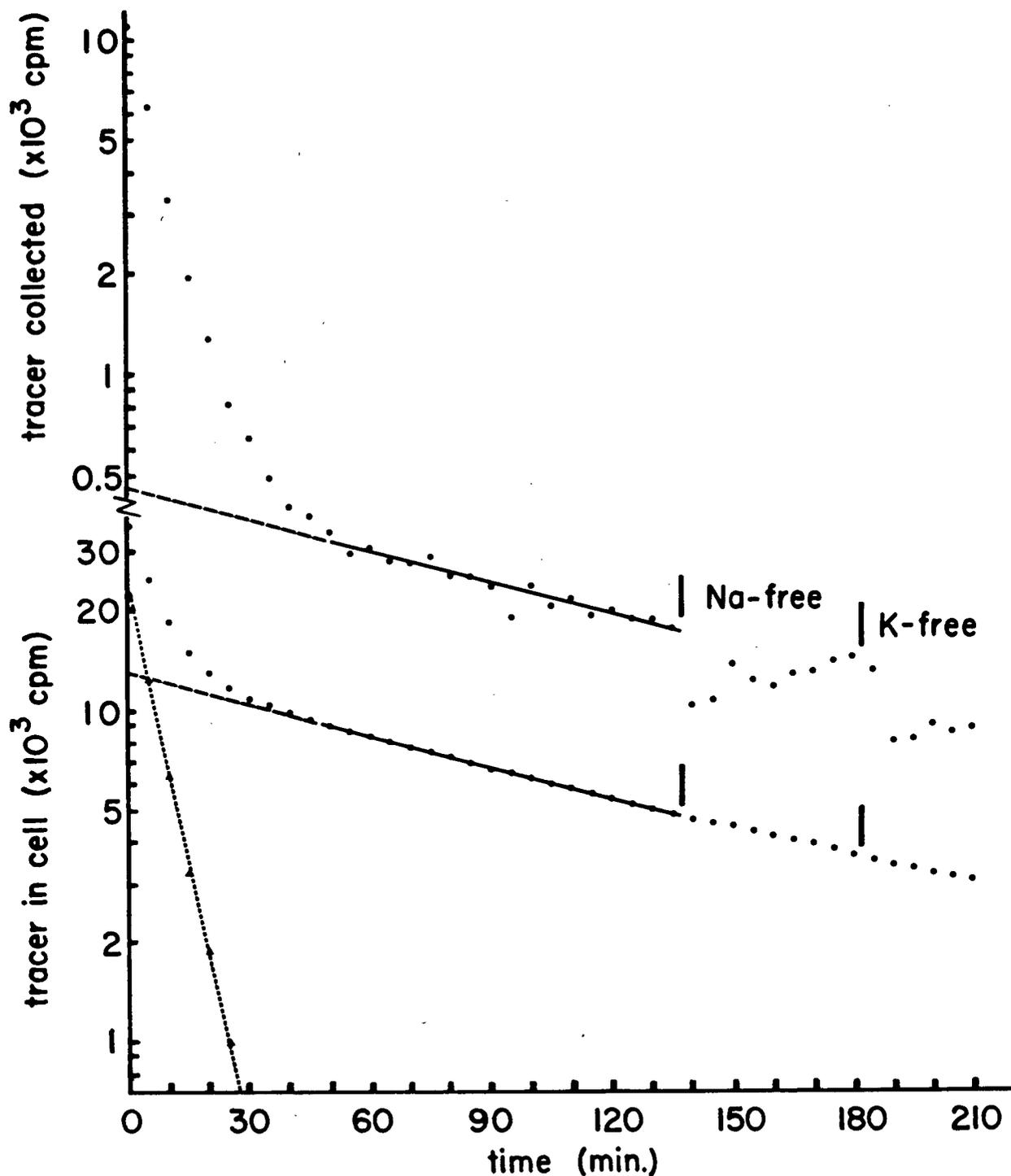
Figure 9. Semilogarithmic plot of the amount of radiosodium collected in the perfusate from a cell loaded with isotope by incubation overnight ('passive loading'), and the amount of radiosodium remaining in the cell, versus time. The cell was perfused with normal Ringer's solution initially. At ca. 140 minutes perfusion was begun with sodium-free lithium-substituted solution. At ca. 180 minutes this was replaced with potassium-free solution (Table I). Resolution of the lower curve into the sum of two exponentials is indicated: $y = A \exp(-ax) + B \exp(-bx)$; $A = 22,837$ cpm, $a = 0.125$ min^{-1}, $B = 12,830$ cpm, $b = 0.0072$ min^{-1}. Linear regression of upper curve, 55 - 135 minutes, as $y = B \exp(-bx)$ is shown: $B = 460$ cpm, $b = 0.0073$ min^{-1}.

intracellular radiosodium at each instant is calculated from back-addition of the radiosodium collected in the perfusate, the dilution effect is not taken into account. The measured radiosodium content of the myoplasm will fall too slowly.

In the present experiments, the slope ratio was often somewhat less than unity, especially when hypertonic solutions of sodium chloride were injected to raise $(a_{Na})_m$ (see caption to Fig. 8), but overall was closer to unity than in the experiments reported by Bittar et al. (1972). As explained in Methods, the injection track in the present experiments was made to extend through the entire perfused length of the cell whenever possible. Intracellular diffusion would thus occur into the part of the cell past the grease seal and into the few millimetres at the tendon end which were not injected and which were kept above the level of the perfusate. Bittar et al. (1972) injected only a 1 cm column of fluid, into cells 3 to 5 cm in length, and collected the radiosodium by immersion of the entire cell successively into a series of vials of perfusate. This difference in methods and difference in results lend support to the proposal that intracellular diffusion is responsible for the difference between the results obtained with injected cells and those obtained with passively loaded cells.

Comparability of sodium efflux in passively loaded and injected cells.

The radiosodium injected into the cell is deposited in the myoplasm, as noted above. The efflux of this radiosodium represents efflux from the interior of the cell. It exhibits a simple exponential dependence on time for efflux into normal Ringer's solution (Fig. 7).

The semilog plot for the efflux of sodium from passively loaded cells into normal Ringer's solution is more complicated (time 0 to 135 minutes in Fig. 9), as noted above. The initial rapid efflux was ascribed to the wash-

out of radiosodium from the extracellular space, and the slower efflux to the efflux of radiosodium from the interior of the cell. The process of separating the two components, known as 'curve peeling', is commonly used, and quite reasonable as long as one can be assured that the components of the net efflux which is measured can each be described by a simple exponential, and that the rate constants for the two components differ by at least an order of magnitude.

Indeed, application of the procedure to any smooth curve of approximately the shape of a washout curve (no inflection points, and steady decrease in the magnitude of the slope) will yield a sum of exponential terms whose rate constants differ by about an order of magnitude. The process is in essence the determination by a process of successive approximations of the power series expansion of the plotted function. Its success in a particular case does not in itself constitute proof of the nature of the experimental system, but as a test of a model, especially a simple model, it can be very useful.

The model in the context of which the efflux curve in Fig. 9 is to be tested is that the radiosodium is washed out from two independent pools: the interior of the cell, and the extracellular space. Actually, there must be some exchange between the interior of the cell and the extracellular space, especially deep in the cleft system. The well-known 'Huxley correction' takes account of this (A.F. Huxley 1960) but is an unnecessary refinement here. The questions to be addressed are whether the rate of the rapid component is consistent with efflux from an extracellular site but not with efflux from an intracellular site, and whether the size of the compartment which gives rise to the rapid efflux is similar to the size of the extracellular space as measured by other means.

Very rapid efflux from the interior of the cell has been seen in frog

skeletal muscle (White & Hinke 1976) and crab striated muscle (Vaughan-Jones 1977), but only during efflux into sodium-poor solution. Actually, what was observed in these two cases was rapid disappearance of sodium from the myoplasm and the rate constants for this process have a different significance -- see section 6. The demonstration that, at least in barnacle muscle, such a rapid disappearance of myoplasmic sodium is indeed accompanied by a rapid efflux of sodium from the cell is described in section 6. In normal Ringer's solution, only effluxes with rate constant of order 0.01 min^{-1} are seen. Yet this figure is the product of precisely the model it is desired to test.

It can be argued, however, that the assignment of a component of the rapid efflux to the intracellular pool, for efflux into normal Ringer's solution, while the total sodium content of the cell is steady, requires that the influx and efflux rates change together when the loading bath (normal Ringer's solution containing ^{22}Na) is replaced by a bath identical in all respects except for the absence of radiosodium. Influx experiments exhibit a similar initial rapid component, so the same argument can be applied there, and the cell supposed to sense and respond to the inclusion in or omission from the bathing solution of ^{22}Na. This does not seem reasonable.

The size of the compartment which yields the rapid efflux of radiosodium into normal Ringer's solution in Fig. 9 is approximately 5.5% of the volume of the portion of the cell which was being perfused. This is an underestimate, since a portion of the tendon end of the cell was not well perfused, and because the loss of radiosodium from the extracellular space is diffusive, but agrees well with the values of 6 to 7% found by various techniques for the size of the extracellular space in barnacle muscle cells, as noted in section 3.

Altogether, it seems reasonable to ascribe the rapid component of the radiosodium efflux entirely to the extracellular space, and the slow component to the intracellular compartments. The rate constants for the slow component in passively loaded cells and the single component in injected cells can thus be compared. In Fig. 7 it is the upper trace which reflects the efflux across the membrane, since the lower trace is declining too slowly because of the intracellular sink of radiosodium noted above. The rate constant $(0.00928 \text{ min}^{-1})$ is comparable to but larger than the rate constant for the slow efflux into normal Ringer's solution in Fig. 9 $(0.00729 \text{ min}^{-1})$. However, the sodium content of the injected cell was higher than that of the passively loaded cell (ca. 20 mM versus 12 mM), and this alone could account for the difference.

The best comparison is of the sodium efflux M_{Na} calculated from the radiosodium efflux data. In Fig. 12 (page 143) are presented the caclulated values of M_{Na} for all injected cells, in normal Ringer's solution, as a function of the myoplasmic sodium activity $(a_{Na})_m$. The results from the three passively loaded cells are included as open diamond symbols. They can be seen to lie in the region defined by the results for injected cells of comparable sodium content.

Brinley (1968) mentioned that the magnitude of the sodium efflux from passively loaded cells was within the range observed with injected cells, but did not report the results on passively loaded cells. Allen and Hinke (1970) reported an average rate constant for the slower component of the sodium efflux from passively loaded barnacle muscle cells of 0.0085 min^{-1} at 15° C. Bittar et al. (1972) reported an average rate constant for this component of about 0.010 to 0.015 min^{-1} at 23° C for injected barnacle muscle cells.

DISCUSSION

Bittar and coworkers (1972) found that the amount of radiosodium in barnacle muscle cells loaded by microinjection did not decline with time at a rate commensurate to the decline with time of the rate at which radiosodium appeared in the bath. They interpreted this in terms of a model in which a large portion of the injected radiosodium was sequestered in vesicles formed from fragments of the cell membrane created by the insertion of the micro-injector. This sodium did not exchange at all with the free sodium inside the cell over the course of the experiment, but could be released by exposure of the cell to aldosterone.

That so much of the injected sodium could be sequestered at the moment of injection, that a concomitant amount of ^{23}Na could also be sequestered (so the apparent fraction of 'bound' radiosodium reflected the fraction of 'bound' sodium), and that the cell membrane fragments which form these vesicles could become so impermeable to sodium that sodium exchange across them is negligibly slow compared to sodium exchange across the rest of the cell membrane, were not considered to be reasonable hypotheses.

On the other hand, it seems very reasonable that longitudinal diffusion of injected radiosodium is responsible for the observed slope ratios. The slope ratios were far less than unity in cells in which the length of the injection track was much less than the length of the cell which was perfused (Bittar et al. 1972). The slope ratios were closer to but still less than unity when an attempt was made to perfuse only the region of the cell which contained the injection track (present study). The slope ratios were equal to unity in cells loaded with radiosodium by immersion, in which no net longitudinal diffusion of radiosodium is expected to occur (present study and results of other workers for frog skeletal muscle). Finally, the slope

of the semilog plots of sodium efflux versus time are constant over the entire duration of long experiments in injected cells, contrary to the expectation that the magnitude of the slope should decline if a fixed amount of the injected radiosodium is sequestered.

The effects of aldosterone remain to be explained, but probably reflect an action on the transport systems in the cell membrane.

The behavior of the sodium efflux into normal Ringer's solution from microinjected cells is indistinguishable from that from passively loaded cells (Fig. 12, page 143). A detailed study of the response of the slow component in passively loaded cells to ouabain and to changes in the composition of the bathing solution was not carried out, but in the experiments which were done the behavior of the passively loaded cells was qualitatively and quantitatively the same as for injected cells.

The use of microinjection leads to a complication in the measurement of the sodium efflux, however, due to the longitudinal diffusion. The quantity which appears in the efflux equation, equation (4) of section 2.F, is Na^*/Na^*_m, the ratio of the amount of radiosodium which leaves the cell during a collection interval to the amount of radiosodium in the myoplasmic compartment at the start of that interval. The myoplasm is continuously losing radiosodium to longitudinal diffusion as well as to the bathing solution.

The ratio Na^*/Na^*_m would be equal to the slope of the plot of $\ln Na^*$ versus time if the loss of radiosodium by longitudinal diffusion from the myoplasm being perfused were entirely independent of the loss across the cell membrane, and the rate of the loss across the cell membrane was proportional to the amount of radiosodium in the myoplasm at each instant. Then the slope of a line drawn through the data points of the semilog plot could be assumed to be equal to Na^*/Na^*_m, and used in equation (4) to

calculate M_{Na}, but only over time intervals during which the slope changed very little (ie. $\frac{dk}{dt} \triangleq 0$).

This has been done for the efflux into normal Ringer's solution for the experiment depicted in Fig. 10 (page 140). The drawing of a smooth curve averages out small variations in the raw data. The net effect is a value for M_{Na} which is about 10% higher than the average of the values calculated directly from equation (4). The slope ratio for this experiment was 0.95. The effect of ignoring the intracellular sink due to longitudinal diffusion will be greater in cells loaded with sodium, for which the slope ratio tends to be lower (Fig. 8).

This correction to the calculated size of the sodium efflux from injected cells is systematic, and involves a severe averaging of fluctuations in the raw data before M_{Na} can be calculated. It would be preferable to employ equation (4) as written, and then consider the effects of the uncertainty in Na_m^* and in V_m/A during discussions to which the absolute size of the sodium efflux is important, rather than to apply corrections to the raw data. However, comparison of the uncorrected and the corrected results of most experiments revealed a profound effect of the above effects on the data, as will be discussed in detail later.

Finally, the apparent size of the extracellular pool of sodium yielded by the efflux curves for the passively loaded cells must be mentioned. The value obtained (about 5.5%) was similar to the inulin space. However, the pool of rapidly exchanging extracellular sodium might be expected to appear much larger than this. The size of the rapidly exchanging extracellular sodium fraction proposed in section 3 is about 12 millimoles Na/kg cell water (Fig. 3). The amount of sodium in solution in the extracellular space, if the latter is taken as 6% of the cell volume, is approximately 450 mM x 0.06 or 27 millimoles Na/kg cell water. Thus one might expect the size of

the extracellular space deduced from the radiosodium washout to be somewhat larger than the inulin space. There are two main reasons why this does not occur. First, the loss of radiosodium from the extracellular space is by free diffusion, the same process which mixes the extracellular sodium which is free in solution. This causes the y-intercept in Fig. 9 to be low, and the size of the extracellular space to be underestimated. Second, the extracellular nonmyoplasmic cations are not all highly mobile. In the experiments on smooth muscle, lanthanum was used to free them. In addition, the zero of time is not precisely definable. In all, the value obtained for the extracellular space is not unreasonable.

SECTION 5.   SURVEY OF THE SODIUM EFFLUX FROM SINGLE MUSCLE CELLS

In this section, the dependence of the sodium efflux from single whole
barnacle muscle cells on the myoplasmic sodium activity is surveyed. This
serves first of all as a test of the techniques described in section 2.F for
measuring the sodium efflux, since the results of similar experiments on
barnacle muscle cells loaded with radiosodium by microinjection are avail-
able for comparison (Brinley 1968; Bittar et al. 1972).

The results of section 3 suggest that the application of the new
technique will not yield results markedly different from those found with
the usual techniques, because most of the nonmyoplasmic sodium does not
exchange rapidly with the myoplasmic sodium. This means that the use of
the sodium-specific intracellular electrode will only improve the estimate
of the size of the internal sodium concentration on which the efflux depends.
The nature of the dependence should be the same with either method.

However, the use of microinjection has been shown to give rise to a
significant uncertainty, in that most but not all of the radiosodium
injected into a cell is available for exchange with extracellular sodium
when the usual techniques are employed. There will be an underestimate of
the size of the sodium efflux, as explained in sections 2.F and 4. Further,
this underestimate will be greater in microinjected cells with an elevated
sodium content, as indicated by Fig. 8.

Therefore, the nature of the dependence of the sodium efflux on the
sodium content of the cell found by other workers using microinjected cells
might be incorrect.

The dependence of the sodium efflux M_{Na} on the myoplasmic sodium
activity $(a_{Na})_m$ reflects the contribution of more than one transport system
in muscle cells. The prominent systems are thought to be the (Na+K)ATPase,

and a system which mediates sodium-sodium exchange. The behavior of the efflux can reasonably be expected to be different during conditions which favour one or another transport mode.

The dependence of M_{Na} on $(a_{Na})_m$ was measured in normal Ringer's solution, which should correspond closely to the normal conditions of the cell in vivo; in potassium-free solution, where the major mode of the (Na+K)ATPase should be disabled; in sodium-free solutions, where the sodium-sodium exchange mode reported in muscle should be disabled; and in the presence of ouabain, where almost all of the reactions of the (Na+K)ATPase should be disabled.

It was found that application of the correction to Na^*_{cell} for cells loaded with radiosodium by microinjection changed the results appreciably.

METHODS

The method of preparing cells and the use of the sodium-specific microelectrode to measure $(a_{Na})_m$ were described in section 3. The method of injecting, collecting, and counting the radiosodium was described in section 4.

The calculation of the sodium efflux M_{Na} was carried out via equation (4) of section 2.F, both with and without the correction to Na^*_{cell} described in sections 2.F and 4. Steady conditions are of interest here for two reasons. First, the response time of the transport mechanisms to changes in the myoplasmic sodium activity is not known, so the most reliable data should be obtained during steady conditions. Second, the correction to Na^*_{cell} can only be made with confidence during steady conditions, as ex-

plained in sections 2.F and 4. Linearity of the semilog plot and steadiness of $(a_{Na})_m$ over at least four collection periods was the criterion for the selection of data.

Experiments in which the sodium efflux is impaired, as by removal of extracellular potassium or by exposure to ouabain, result in a steady rise in the myoplasmic sodium activity as the sodium influx is no longer adequately countered by sodium extrusion (for example, Fig. 10 of this section). This usually caused no problem but in a few cases the efflux fell to a minimum, then rose slowly as $(a_{Na})_m$ rose. A subjective judgment then had to be made about the value of M_{Na} to extract for analysis. The value judged to reflect the maximum effect of the experimental manipulation was extracted, along with the value of $(a_{Na})_m$ at that time. The uncertainty due to this was quite small, but the effect is of interest, as will be discussed below in connection with the dose-response curve for ouabain.

Use of Day-old Cells.

For some experiments, such as loading with radiosodium by incubation in radiosodium-containing solutions, measurements on the dissected cell cannot be performed until 24 to 48 hours after the dissection. Dissected cells kept in normal Ringer's solution at less than 5° C are found to maintain their ion gradients and membrane potential for several days (for example, Table II). In pilot experiments, the behavior of the efflux of injected radiosodium from barnacle muscle cells was not noticeably different in cells which were injected 24 hours after dissection from that in cells which were injected a few hours after dissection. Some difference (enhanced M_{Na}) was noted in a few cells tested at 48 hours after dissection. The use of cells from a barnacle dissected the preceding day makes much more efficient use of the available specimens, and saves a considerable amount of time.
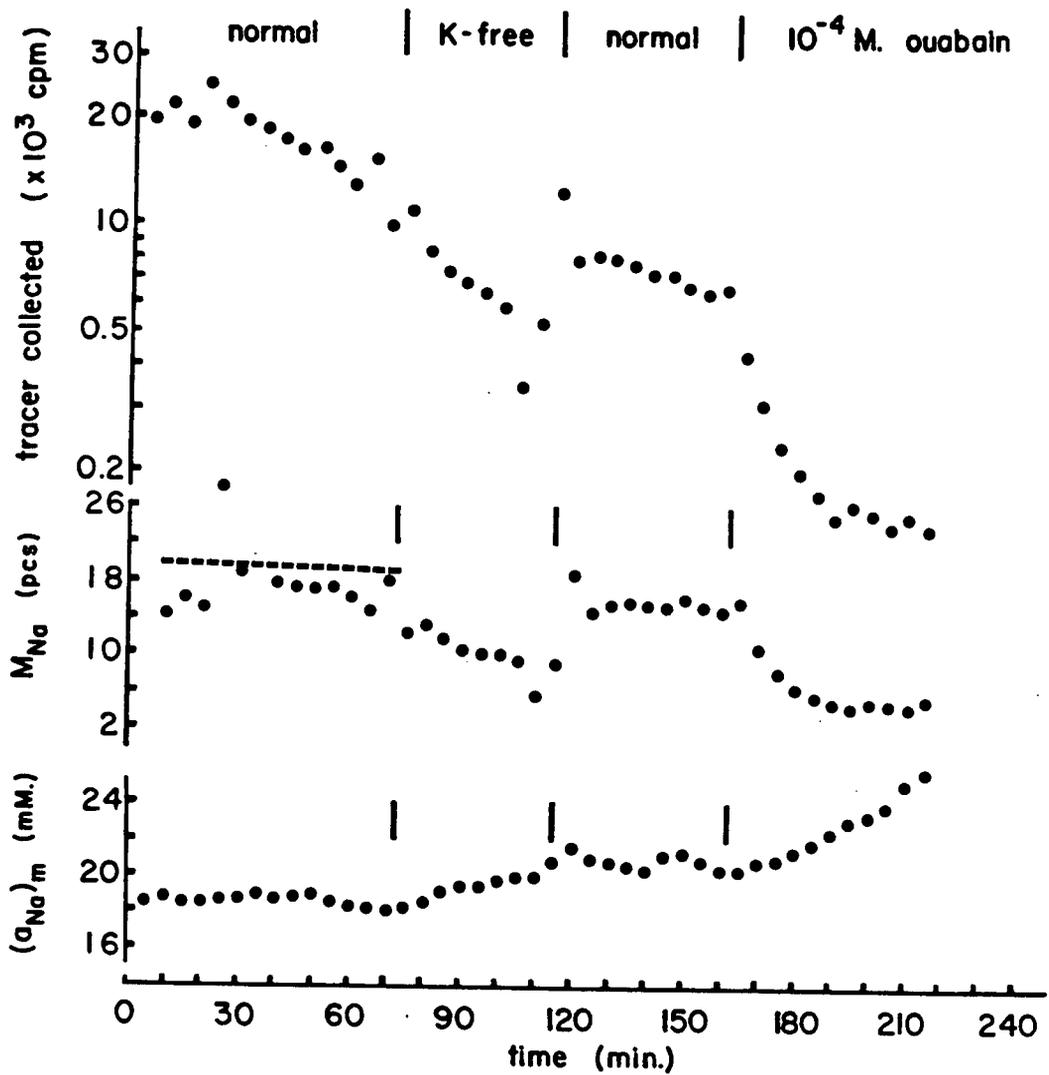
Figure 10.   Summary of the raw data and reduced results for a typical
experiment.   Upper trace: logarithm of the amount of radiosodium collected
in each 5 minute collection period, in counts per minute; lower trace:
myoplasmic sodium activity as measured by a sodium-specific glass micro-
electrode, in mM; middle trace: sodium efflux deduced from the data via
equation (4), plotted in picomoles/cm^2sec (pcs).   For the interval in normal
Ringer's solution, the corrected value of M_{Na} is indicated as a dashed line.

About half of the experiments reported on here were done on such 'day-old' cells. The data obtained from such cells is indicated in the figures. The similar behavior of fresh and day-old cells (eg. Fig. 11) is discussed below.

RESULTS

In Fig. 10 a summary of the raw data and reduced result for a typical experiment is presented. Reference will be made to this figure later.

(a) Sodium efflux into normal Ringer's solution.

The results from 58 experiments are presented in Fig. 11 and Fig. 12 as plots of M_{Na} versus $(a_{Na})_m$. In Fig. 11, M_{Na} was calculated according to equation (4) using Na^*_{cell}. In Fig. 12, the same data was employed but the corrected value of Na^*/Na^*_m was used in equation (4).

In Fig. 11, the relationship between M_{Na} and $(a_{Na})_m$ appears to be slightly sigmoidal: saturation appears to occur at higher values of $(a_{Na})_m$. Brinley (1968) used $(Na)_i$ rather than $(Na)_m$ in calculating M_{Na}, and conducted his experiments at 0° C. It had been anticipated that the present results would be similar to his (after correction for surface area by a factor of 10), but shifted to lower values on the abscissa since $(a_{Na})_m$ was used instead of $(Na)_i$, and with a larger efflux at a given sodium content due to the higher temperature. This was found. Brinley's empirical relationship is shown as a broken line in Fig. 11.

In Fig. 12, the relationship between M_{Na} and $(a_{Na})_m$ is quite different. It appears to be affine, as was found in snail neurone by Thomas (1972).
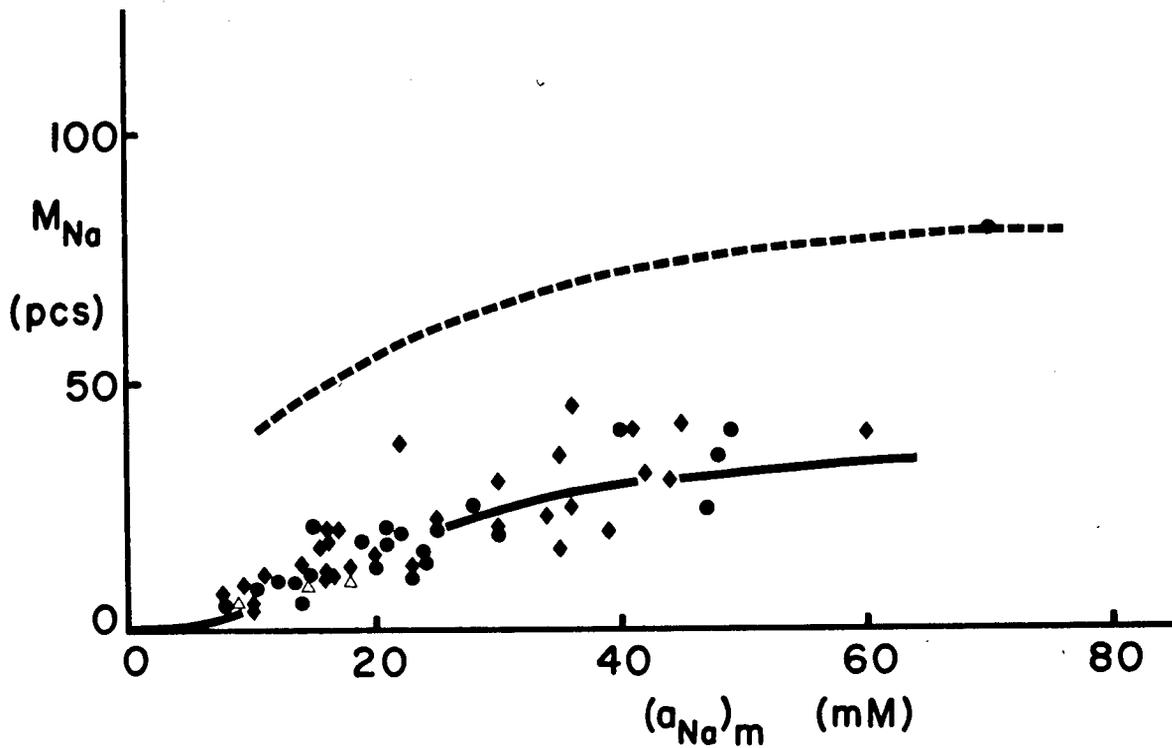
Figure 11. Sodium efflux into normal Ringer's solution, calculated from equation (4), <u>without</u> correction for Na^*_{cell}. Solid circles: cells dissected on the day of the experiment. Solid diamonds: cells dissected on the day before that of the experiment. Open triangles: cells loaded with radiosodium by immersion overnight in labelled solution.

Solid curve: model calculation for three sodium ions binding successively to equivalent independent sites per cycle of the transport enzyme (k = 15.75 mM, M_{max} = 45 pcs). Dashed curve: experimental data of Brinley (1968) as M_{Na} versus $(Na)_i$, where $(Na)_i$ is on the same numerical scale as $(a_{Na})_m$.
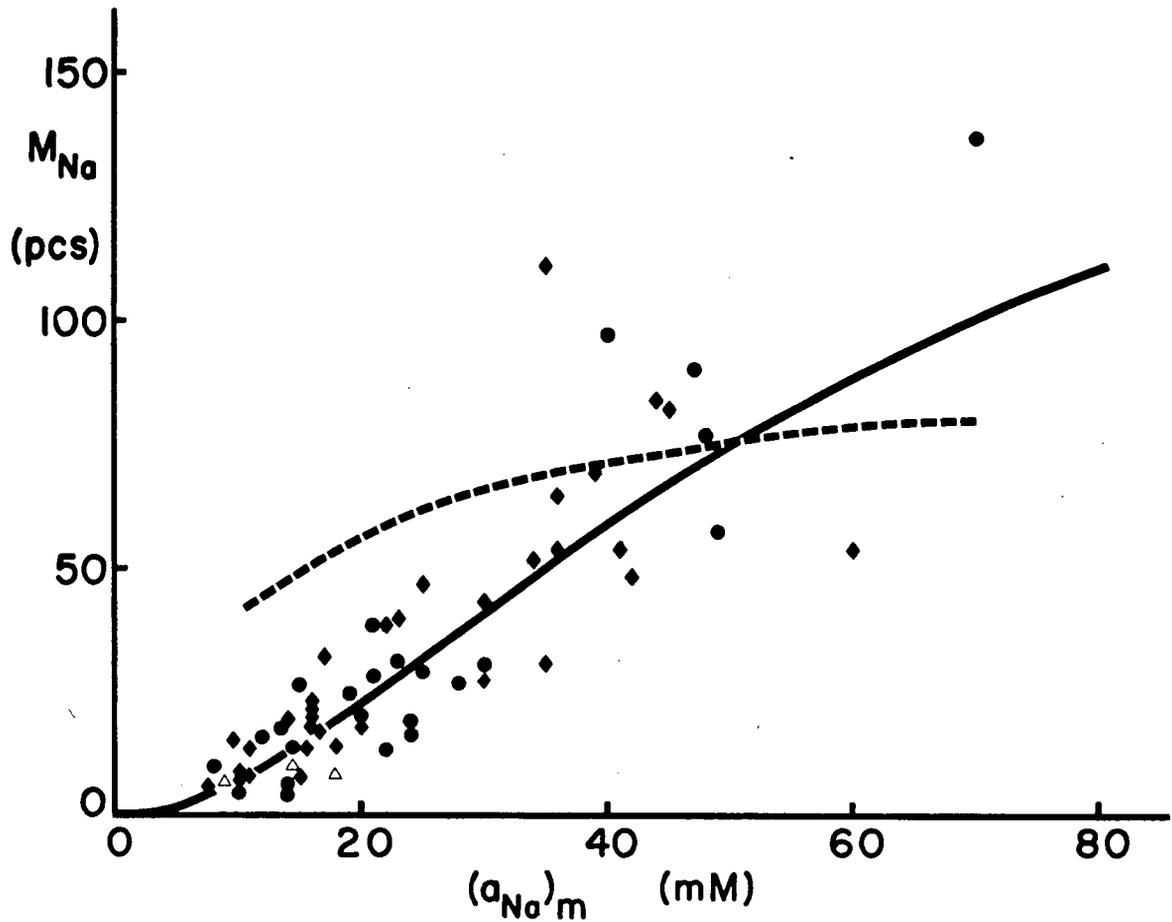
Figure 12. Sodium efflux into normal Ringer's solution, calculated from equation (4), <u>with</u> correction for Na^*_{cell}. Symbols as in Fig. 11. Solid line: curve to which kinetic models were fitted by trial and error. Dashed line: experimental data of Brinley (1968), as in Fig. 11. Elevation of $(a_{Na})_m$ above the normal range (ca. 10 mM) was accomplished by injection of NaCl into the myoplasm.

No saturation is evident. Acceptable data at higher sodium content was difficult to obtain. The value of $(a_{Na})_m$ usually did not become steady when large amounts of 5 M NaCl were injected, so it was concluded that the permeability of the cell membrane had been compromised. Very rapid effluxes were seen in such cases. High values of $(Na)_i$ (about 70 mM) have been alleged to unmask pre-formed transport enzymes in frog skeletal muscle (Erlij & Grinstein 1976a,b), although it is hard to imagine that such a challenge would ever occur in a living animal. In one acceptable experiment at $(a_{Na})_m$ = 70 mM, a relatively high efflux was found (Fig. 11). The use of prolonged immersion of the cells in potassium-free solution as a means of passively raising the sodium content was not investigated, but should be a better method for loading the cell with sodium. In this connection, note, however, that while Fig. 10 suggests that $(a_{Na})_m$ can be raised easily in potassium-free solution, Table II shows that the increase in $(a_{Na})_m$ over 20 hours in potassium-free solution is not very great.

No difference is seen between the results for fresh (solid circles) and day-old (solid diamonds) cells in Fig. 12. It has been reported that the dependence of the response of frog skeletal muscle to ouabain on the sodium content of the cell differs in fresh and 'aged' cells (Horowicz, Taylor, & Waggoner 1970), as does the response to removal of external sodium (Keynes & Swan 1959; Keynes & Steinhardt 1968).

Fig. 12 does not reveal any definitive information about the kinetics of the extrusion of sodium from the cell, because of the scatter of the data but more importantly because of the unavailability of data for low values of $(a_{Na})_m$ with the present techniques. It is clear, however, that the behavior revealed with the new method differs from that obtained with the usual method.

(b) <u>Sodium efflux into potassium-free solution.</u>

The results of 20 experiments in which the sodium efflux into potassium-free solution was measured are presented in Figs. 13 and 14 as plots of M_{Na} versus $(a_{Na})_m$. In Fig. 13, M_{Na} was calculated from equation (4) without the correction to Na^*_{cell}, while in Fig. 14, M_{Na} was calculated with this correction.

The behavior of M_{Na} is similar in the two plots, aside from the correction in Fig. 14 of the underestimate of the size of M_{Na} in Fig. 13. The definite plateau is markedly different from the behavior found in normal Ringer's solution, Fig. 12. The sodium extrusion mechanism which does not require external potassium appears to have a limited capacity, although over the 'physiological range' of $(a_{Na})_m$ it responds to an increase in $(a_{Na})_m$ by increasing its rate.

Further, it appears that the sodium extrusion mechanism which does require external potassium does not saturate at myoplasmic sodium activities up to 70 mM. By comparison, Keynes and Swan (1959) found in frog striated muscle that the reduction in the sodium efflux caused by removal of external potassium was greater as $(Na)_i$ was raised.

Two other observations on the effect of removal of external potassium can be made. As illustrated by Fig. 10, the effect of removal of external potassium is reversed by restoration of external potassium. It should be noted that Bittar <u>et al</u>. (1972) describe a rise of the sodium efflux to a level above that obtained before external potassium was removed if external potassium is subsequently restored. This occurred only for certain cells, those for which they calculated a large "sequestered fraction" of sodium by the slope ratio method. Such cells are found to have high myoplasmic sodium activity, as discussed in section 4. Bittar <u>et al</u>. reported <u>no</u> appreciable change in the ion content of cells incubated in potassium-free
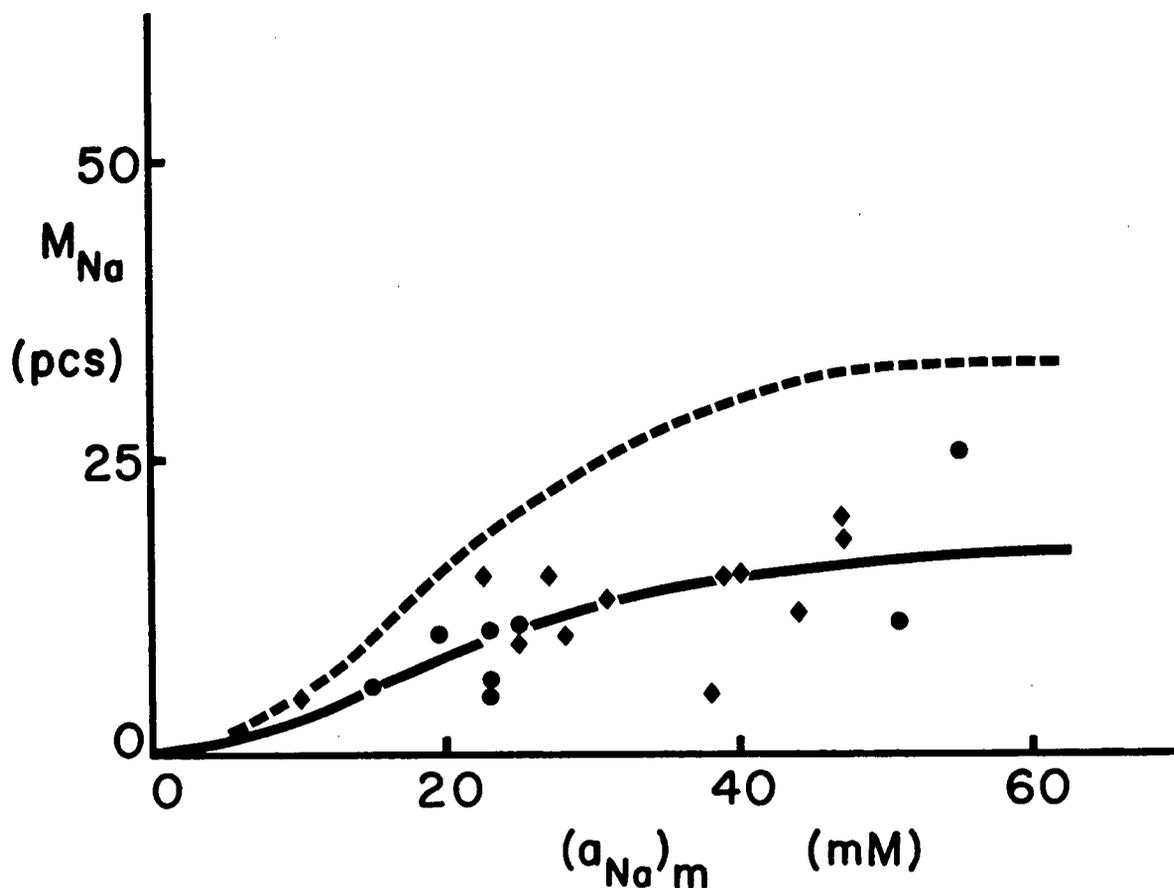
Figure 13. Sodium efflux from the cell into a potassium-free bathing solution, calculated from equation (4) <u>without</u> correction for Na^*_{cell}, versus myoplasmic sodium activity at the time of the change from potassium-containing to potassium-free solution. Circles: cells dissected the day of the experiment. Diamonds: cells dissected the day before that of the experiment. Solid line drawn by eye. Dashed line represents the sodium efflux into normal Ringer's solution calculated in a similar manner (Fig. 11). Note: the ordinate is different from that in Fig. 11 and Fig. 12.
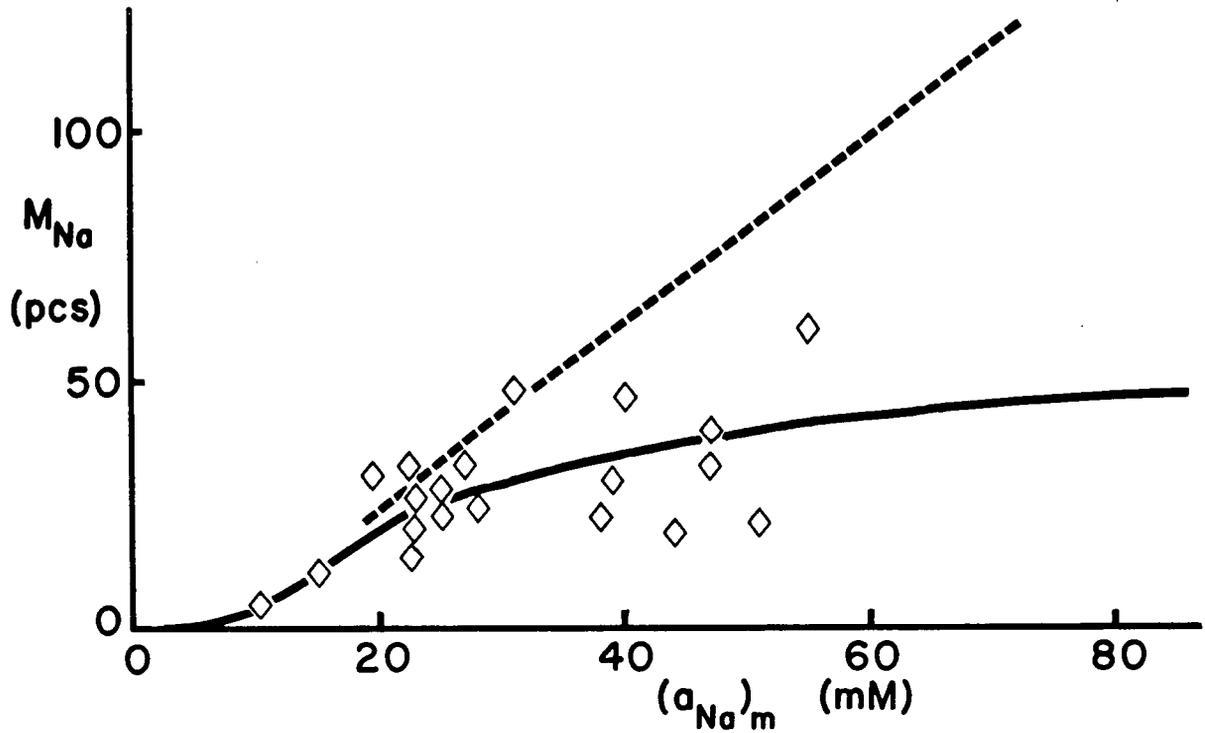
Figure 14. Sodium efflux from the cell into a potassium-free bathing solution calculated from equation (4) <u>with</u> correction for Na^{*}_{cell}, versus myoplasmic sodium activity at the time of the change from potassium-containing to potassium-free solution. Symbols as in Fig. 13. Solid line: kinetic model for three sodium ions binding successively to equivalent independent sites per cycle of the transport enzyme (k = 15 mM, M_{max} = 60 pcs). Dashed line: efflux into normal Ringer's solution, from Fig. 12.

solution for 50 to 70 minutes, although it is clear from Table II that $(Na)_i$ must rise, and from Fig. 10 that $(a_{Na})_m$ will be increased by such treatment. Bittar et al. assert that in these cells the behavior of the sodium efflux is altered, but it seems clear that the "extra efflux" can reasonably be attributed to the raised myoplasmic sodium activity. They have not demonstrated that changes in the sodium efflux caused by removal of external potassium are not reversible.

As illustrated by Fig. 24 (page 190), the membrane potential does change when external potassium is removed. The effect when E_m becomes steady is a depolarization in this particular cell, although immediately after the solution change there is a transient hyperpolarization. In most of the cells tested, the net effect was found to be a hyperpolarization. For cells in which $(a_{Na})_m$ was less than 40 mM prior to the solution change, the ratio of the membrane potential in potassium-free solution to that in normal Ringer's solution prior to the change was 1.05 (n = 11, SD = 0.02) while for $(a_{Na})_m$ greater than or equal to 40 mM the ratio was 1.10 (n = 11, SD = 0.06). (Note: there are fewer than 22 points in Fig. 13 because E_m sometimes was stable when M_{Na} was not.)

(c) Sodium efflux into sodium-free solution.

Sodium-free solutions substituted with lithium, tris, choline, or sucrose were employed (Table I). The effects on the sodium efflux of replacement of the normal Ringer's solution bathing a cell by one of the above solutions are shown in Fig. 15. M_{Na} was calculated from equation (4) without correction for Na^*_{cell}, since the correction cannot be applied with confidence when the efflux is not steady. To make comparison easier, the value plotted is the ratio of M_{Na} at each time to the steady value of M_{Na} found before the change from normal Ringer's solution. Both inhibitory and
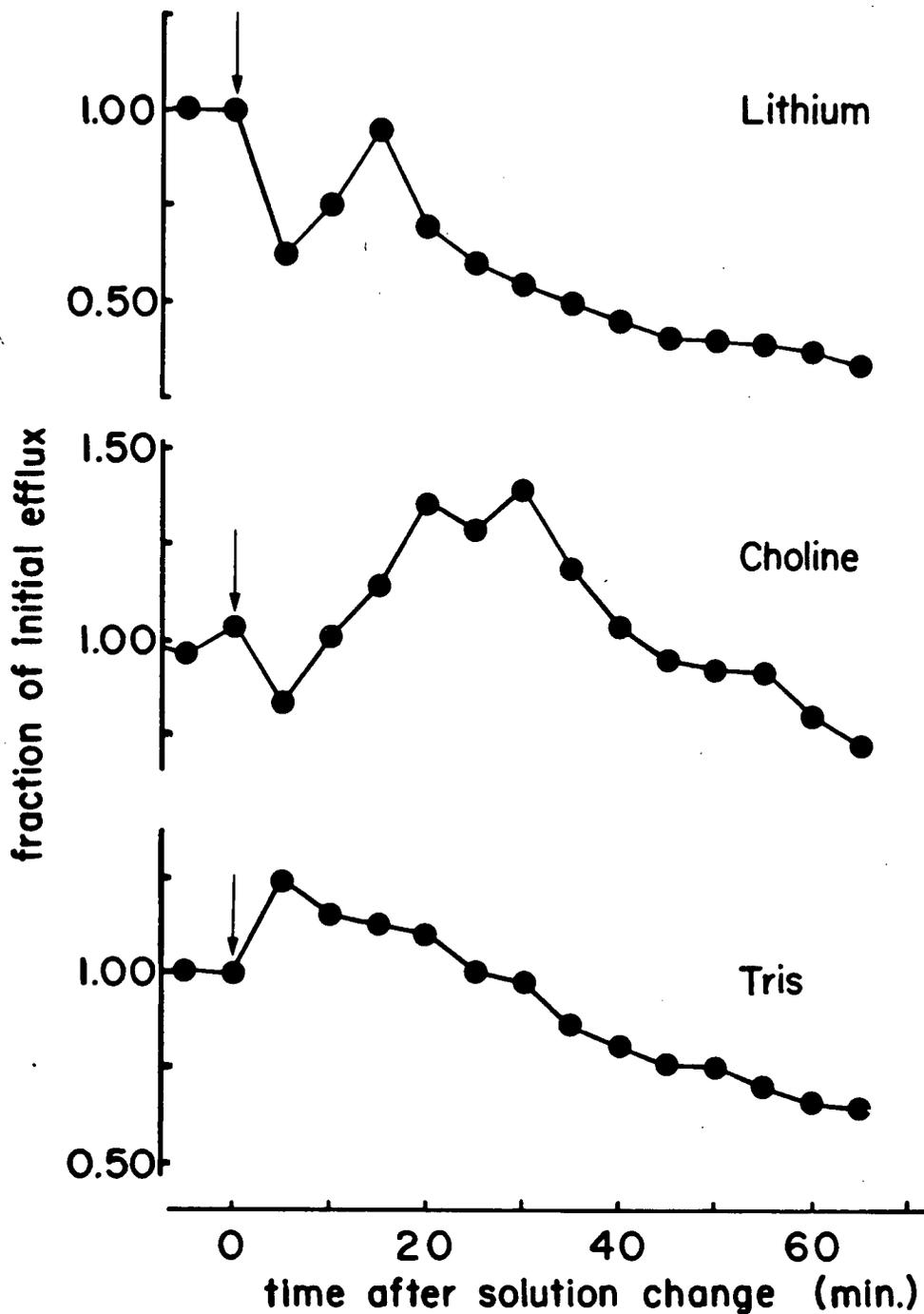
Figure 15. The effect on the sodium efflux of removal of sodium from the extracellular medium. At time zero minutes (arrow), the extracellular solution was changed from normal Ringer's solution to a sodium-free solution, substituted as indicated. Sodium efflux has been normalized to 1.0, so each cell serves as its own control. It can be seen from Fig. 16 that the cell-to-cell variation in the size of the efflux into normal Ringer's solution and into the various sodium-free solutions is so great that the different response to different sodium-free solutions is obscured.

stimulatory effects can be seen, and the transient effects appear to be different for the different substitute ions. They can be considered in turn.

Lithium. The replacement of external sodium by lithium caused a fall in the value of M_{Na} in 17 of 22 experiments. In the time immediately following the solution change, M_{Na} changed erratically, yet there was always an initial abrupt fall. The relative reduction was by 0.30 (SD = 0.17, n = 17). This almost always settled into a slow decline as $(a_{Na})_m$ fell.

It seemed likely that both a transient stimulatory and a sustained inhibitory effect resulted from the replacement of the external sodium by lithium. The portions of Fig. 15 which deal with the time period immediately after the change to sodium-free solution are only presented as qualitative results. The transients in the sodium efflux will be described separately in section 6. Only the sustained inhibitory effect will be considered here.

In one experiment, at very high $(a_{Na})_m$, the solution change was followed by an abrupt drop and then a marked increase in M_{Na}, plus a contraction of the cell, as was found by Brinley (1968). Return of the cell to normal Ringer's solution seemed to reduce the efflux of radiosodium, but M_{Na} could not be calculated because the contraction dislodged the electrodes. Baker, Blaustein, Hodgkin et al. (1969) have suggested that contractions in this situation might be due to an increased entry of calcium into the cell via the sodium-calcium exchange mechanism operating opposite to its usual manner due to the absence of external sodium. They observed such an effect in squid axon.

Choline. In five experiments where choline-substituted sodium-free solution was used, the initial behavior was again erratic. There was an abrupt drop in M_{Na}, followed by a rise to a level above the initial level (relative increase by 0.17, SD = 0.14, n = 5). Again, the sustained effect

was a slow decline of the efflux.

Tris. In two experiments where tris-substituted sodium-free solution was used, there was an abrupt rise in M_{Na} (relative increase by 0.36 on average), while again the sustained effect was a slow decline of the efflux.

Sucrose. In two experiments where sucrose-substituted sodium-free solution was used, the radiosodium efflux rose after the solution change and then declined slowly. Measurements of $(a_{Na})_m$ were not done in these two experiments. The qualitative behavior of the sodium efflux probably can be deduced from examination of the radiosodium efflux alone in this case.

As indicated in Fig. 15, M_{Na} seldom attained a low steady value after the removal of external sodium. It seems reasonable to attribute this slow decline to the fall of $(a_{Na})_m$ as cellular sodium is lost to the bathing solution. The size of the sodium efflux during this slow decline was estimated from equation (4) using the correction for Na^{*}_{cell}, and the results plotted in Fig. 16. The restrictions of this corrected calculation make it impossible to estimate M_{Na} at high values of $(a_{Na})_m$, as explained in sections 2.F and 4. The values at 30 and 35 mM are less certain than the others. Note, however, that the behavior is the same for lithium, choline, and tris solutions. This is the behavior after long immersion in sodium-free solution (beyond 30 minutes) while Fig. 15 shows in addition the behavior immediately after the change to sodium-free solution.

The variation of M_{Na} with $(a_{Na})_m$ can be presented over a slightly wider range of $(a_{Na})_m$ if equation (4) is employed without correction. In Fig. 17, the efflux of sodium into sodium-free lithium-substituted solution is presented as the change in M_{Na} of individual cells as sodium is lost from them into the sodium-free bathing solution. This is an approximate calcula-
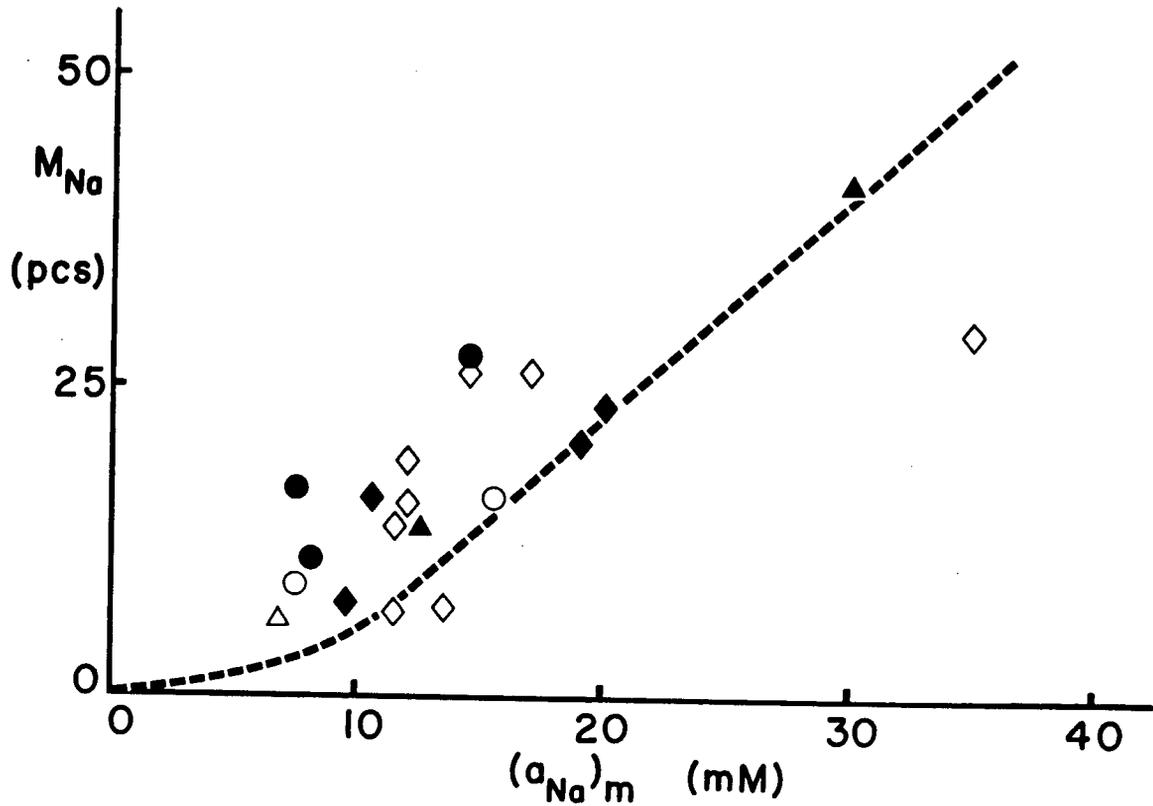
Figure 16. Sodium efflux from the cell into a sodium-free solution, calculated from equation (4) <u>with</u> correction for Na^*_{cell}, versus myoplasmic sodium activity at the time the efflux was calculated.

Diamonds: lithium-substituted solution. Circles: Tris-substituted solution. Open symbols: cells dissected on the day of the experiment. Closed symbols: cells dissected on the day before that of the experiment. Dashed line: efflux into normal Ringer's solution, from Fig. 12. Note: scale is different from Fig. 12.

tion because of the time constants involved and the use of Na^*_{cell}, as has

been discussed in section 2.F. Application of the correction for Na^*_{cell}

would raise the estimate of M_{Na}, especially at higher sodium content.

The range of $(a_{Na})_m$ remains just short of the region of most interest,

about 40 mM, at which the efflux into potassium-free solution exhibits a

shoulder. The efflux into sodium-free solution seems not to have a shoulder,

and so to be similar to the efflux into normal Ringer's solution, Fig. 12,

but unfortunately this cannot be alleged with certainty on the basis of

this data over the full range in which the efflux into normal Ringer's

solution has been measured. A similarity between the relationship of

$\frac{d(a_{Na})_i}{dt}$ to $(a_{Na})_i$ in sodium-free and sodium-containing solution has been

found in snail neurone by Thomas (1972b). The behavior found here for

barnacle muscle is quite similar to that found in snail neurone.

(d) Sodium efflux into solutions containing ouabain.

In the present series of experiments, only one concentration of ouabain

was tested on a particular cell. Exposure of the cell to ouabain causes a

continuing rise in $(a_{Na})_m$, as shown in Fig. 10, and it was not known how

the ouabain-insensitive sodium efflux depended on $(a_{Na})_m$. In the case of

submaximal inhibition, M_{Na} should increase as $(a_{Na})_m$ increases (Fig. 12).

As explained in Methods, a value for M_{Na} could be calculated only after the

initial rapid decline in M_{Na} was completed, so data at low concentrations

of $(a_{Na})_m$ could not be obtained.

In Fig. 18, the dependence of the sodium efflux on the myoplasmic

sodium activity in the presence of ouabain is presented. M_{Na} was calculated

from equation (4) with the correction for Na^*_{cell}. In the presence of 10^{-6} M

ouabain (open circles), the sodium efflux into normal Ringer's solution is

similar to but smaller than that into normal Ringer's solution which
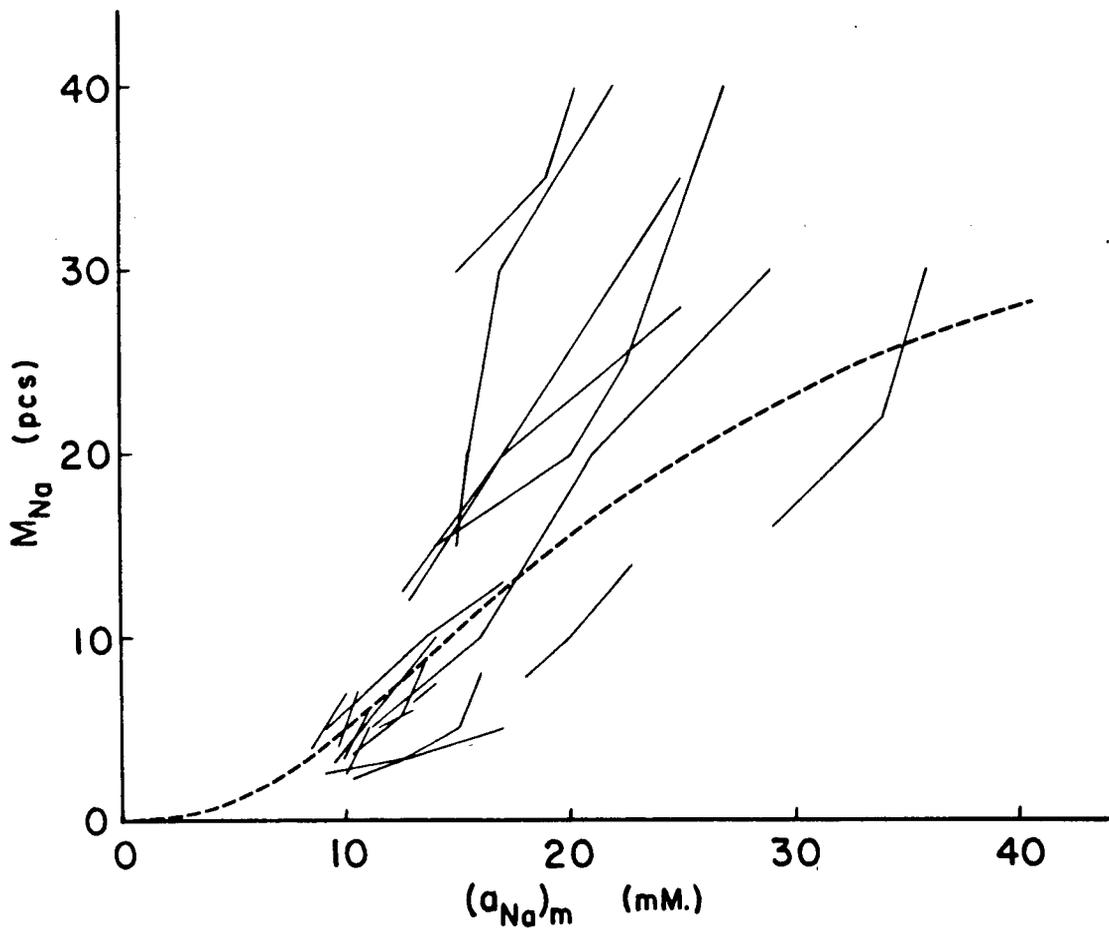
Figure 17. Sodium efflux from the cell into sodium-free lithium-substituted solution, calculated from equation (4) <u>without</u> correction for Na^{*}_{cell}, versus myoplasmic sodium activity. Each line represents a single cell, and the change in the sodium efflux as the myoplasmic sodium activity declined during immersion of the cell in the sodium-free solution. This is an approximate calculation, as explained in the text. The dashed line is the corresponding approximate calculation for efflux into normal Ringer's solution (Fig. 11).
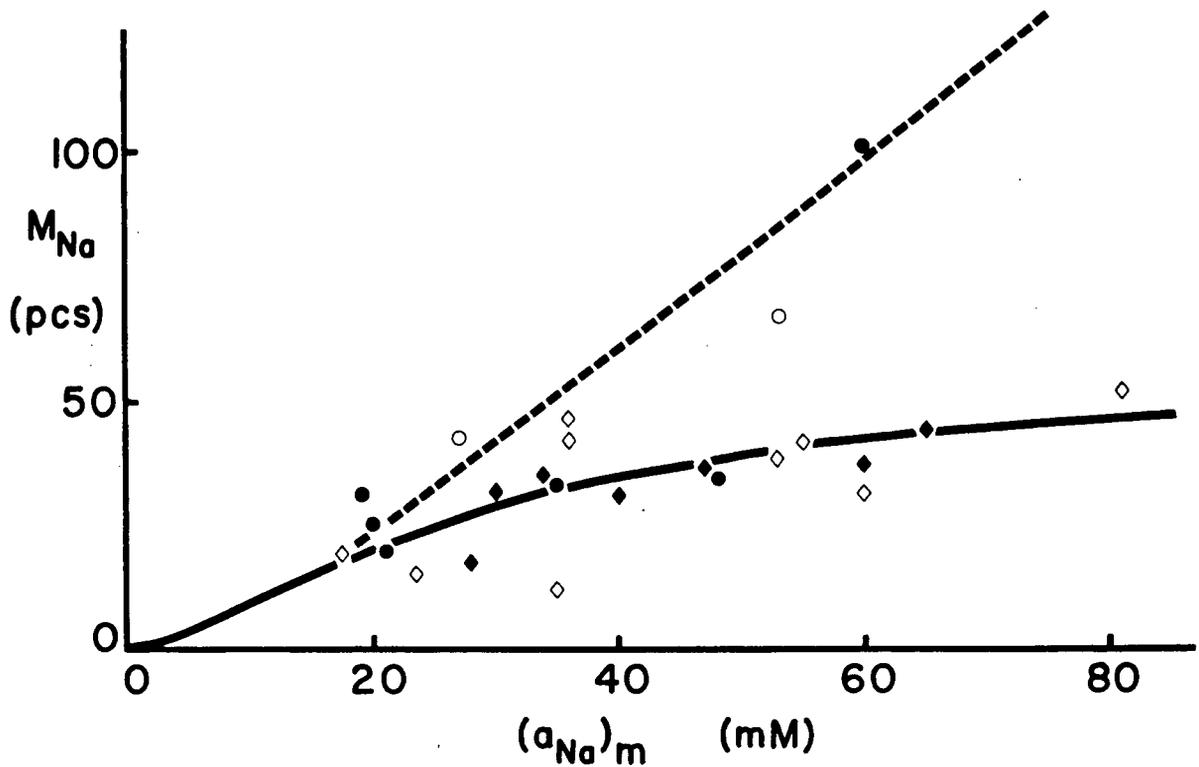
Figure 18. Sodium efflux from the cell into normal Ringer's solution to which had been added 10^{-6} M (circles) or 10^{-4} M (diamonds) ouabain, versus myoplasmic sodium activity. Open symbols represent cells dissected on the day of the experiment. Closed symbols represent cells dissected the day before that of the experiment. Solid line: kinetic model for three sodium ions binding successively to equivalent independent sites per cycle of the transport enzyme (k = 15 mM, M_{max} = 55 pcs). Dashed line: efflux into normal Ringer's solution, from Figure 12. Correction for Na^{*}_{cell} used in this instance is explained in the text.

contains no ouabain (Fig. 12 and broken line in Fig. 18). In the presence of 10^{-4} M ouabain (diamonds), the sodium efflux differs markedly, being much reduced at higher sodium concentrations.

The sodium efflux into normal Ringer's solution in the presence of 10^{-4} M ouabain shows only a weak dependence on $(a_{Na})_m$. It is very similar to the efflux into potassium-free solution (Fig. 14). If 10^{-4} M ouabain yields almost total inhibition of the (Na+K)ATPase, Fig. 18 shows the sodium efflux which is mediated by other transport mechanisms.

The construction of a dose-response curve is made difficult by the fact that the efflux depends quite strongly on $(a_{Na})_m$ at ouabain concentrations which yield submaximal inhibition, while even partial inhibition of the sodium transport system can result in appreciable increases in $(a_{Na})_m$. A dose-response curve could be constructed by comparing the efflux measured for a given concentration of ouabain with the efflux into normal Ringer's solution at the same value of $(a_{Na})_m$, via Fig. 12. The entire dose-response curve should represent cells of similar sodium content. Because there is so much scatter in the data, this endeavour was thwarted by the absence of a large number of experimental points in a small range of $(a_{Na})_m$.

Qualitative observations can be made. There was no effect of solutions containing 10^{-7}, 10^{-8}, or 10^{-9} M ouabain. The effect of 10^{-6} M ouabain is seen in Fig. 18 to be slight, while the effect of 10^{-4} M ouabain was marked. The binding of ouabain to an enzyme renders it unable to transport sodium, but the many enzymes which do not have ouabain bound to them constitute a very large functional reserve of sodium extrusion. Yet if the enzyme responded only to the myoplasmic sodium activity, the efflux at a given value of $(a_{Na})_m$ in the presence of enough ouabain to bind to the enzymes appreciably should result in a lower total sodium efflux. This appears to be the case in Fig. 18. In order to characterize the dose-response to ouabain

properly, more data for 10^{-6} M and 10^{-5} M ouabain must be obtained.

Brinley (1968) used the technique of exposing the cell to a series of solutions each with a greater concentration of strophanthidin than the preceding one. He also did experiments in which only a single concentration of strophanthidin was used on a cell, at the concentration judged to yield maximal inhibition (about 5×10^{-5} M). He found the fractional inhibition of the sodium efflux to be greater in cells of lower estimated sodium content $(Na)_i$. This is confirmed by Fig. 18. Brinley found that the dose for half-maximal inhibition of the sodium efflux by strophanthidin varied from about 1×10^{-7} M for cells of low sodium content to about 5×10^{-7} M for cells of higher sodium content. Bittar et al. (1973) reported a dose for half-maximal inhibition by ouabain of about 5×10^{-7} for barnacle muscle. Fig. 18 indicates that the actual dose of ouabain for half-maximal inhibition is greater than 10^{-6} M except at relatively low sodium content.

DISCUSSION

Microinjection.

It was noted in section 4 that microinjection causes only transient changes in the permeability, ion content, and transport properties of the barnacle muscle cell. An exception might be when concentrated solutions of NaCl are injected, as this often resulted in a sustained rise in $(a_{Na})_m$. A significant technical problem has been identified, in what appears to be longitudinal diffusion of injected radiosodium into noninjected regions of the cell. Mixing in the radial directions along the injected portion of the cell appears to be quite rapid.

An attempt was made to inject a long segment of the cell, and to collect radiosodium only along this injected region. This reduced the effect of longitudinal diffusion, but did not eliminate it.

A correction for the effect of longitudinal diffusion has been described. This involves no assumptions beyond those implicit in the statement of the relation between the flux of radiosodium and the flux of ^{23}Na, that is, the fundamental assumption of the tracer technique. It can only be applied in the form presented when the rate at which sodium is expelled from the cell is constant. Correction at other times requires a calculation of the rate of change of the sodium efflux.

Calculation of the sodium efflux was avoided during the intervals in which the sodium content of the cell was changing rapidly, such as immediately after certain changes in the composition of the external solution, because it was not clear whether the interior of the cell could be assumed to be well-mixed at such times. Similarly, the applicability of the flux model when the sodium efflux was very rapid was not known. It is thought that an 'unstirred layer' at the internal surface of the cell membrane could limit the sodium efflux in such circumstances.

In this connection, the data of Figs. 16 and 17 are of interest. The flux calculated at each instant during relatively rapid changes in $(a_{Na})_m$ (Fig. 17) appears if anything to exceed the corresponding flux calculated during steadier conditions (Fig. 16). If there were appreciable limitation of the efflux by an unstirred layer at the internal surface of the cell membrane, the former should be less than the latter. Of course, the measured value of $(a_{Na})_m$ should lag as well during film-controlled diffusion, and lithium is thought to be able to stimulate the sodium efflux as potassium does, but altogether it appears that radial mixing of ions inside the cell, which is diffusive, is not a great problem with the time resolution attain-

able in the present flux studies.

The implications of this, to continue this speculation further, is that the distance from any point in the interior of the cell to the cell membrane is small. It is known that the cleft system in barnacle muscle makes this so. Further, it is implied that the transport properties of the membrane lining the deep clefts are similar to those of the rest of the cell membrane. This seems reasonable from a functional point of view, but of course cannot be concluded with certainty from these considerations.

In practical terms, one could attempt to reduce the effects of longitudinal diffusion further by injecting a longer region of the cell and collecting isotope only at the centre of the injected region. This would make the technique of microinjection more difficult, and might not make the effect of longitudinal diffusion negligible. Overall, passive loading seems preferable for sodium efflux studies, even though it requires prolonged immersion of the dissected cells prior to the performance of the experiment. The correction devised here for the microinjection technique is as fully justified as the use of the tracer technique itself. However, as a general rule, it seems desirable to design experiments so that the least manipulation of the raw data must be done before an answer to the question under investigation can be obtained.

Aged cells.

The maintenance of dissected barnacle muscle cells in normal Ringer's solution has been shown to affect the cells but little. The cells tend to gain sodium and lose potassium, but the membrane potential and water content remain constant (eg. Table II). In the efflux experiments it has been found that the behavior of fresh and day-old cells is the same (Figs. 11; 14; 16 with less certainty; and 18). The difference in the response of

'fresh' and 'aged' frog skeletal muscle cells noted above is probably due to differences in the sodium content, as noted for example by Keynes and Steinhardt (1968).

Modes of sodium extrusion.

The dependence of the sodium efflux on the sodium content of the barnacle muscle, for efflux into normal Ringer's solution, is similar to that reported in snail neurone (Thomas 1972b) and in squid axon (Hodgkin & Keynes 1956; Sjodin & Beauge 1967; Brinley & Mullins 1968). It is different from that reported in red blood cells (Garay & Garrahan 1973) and in frog skeletal muscle (Harris 1965) in the failure to detect saturation even at relatively high levels of intracellular sodium content. It also differs from the results of Brinley (1968) and of Bittar et al. (1972) for barnacle muscle cells, almost certainly because of the problems involved in working with microinjection, as discussed at length above and in section 4.

The striking feature of the dependence is the apparent absence of saturation at myoplasmic sodium activities up to 50 mM and perhaps up to 70 mM. It is not clear why the barnacle muscle cell should have such a large functional reserve for sodium extrusion. Action potentials do not propagate in the barnacle muscle cell membrane under ordinary conditions, so this cannot be a large source of sodium influx in vivo. Perhaps a sodium-calcium exchange across the cell membrane is required for relaxation of the muscle, since its sarcoplasmic reticulum is so small. Frog striated muscle, by comparison, shows saturation at relatively low sodium content (shoulder at about 10 millimole sodium per kg tissue - Harris 1965).

The efflux into normal Ringer's solution is thought to be composed of several components. The dominant ones are thought to be sodium-potassium exchange via the (Na+K)ATPase and sodium-sodium exchange via some other

mechanism, as noted in the Introduction. A small contribution appears to be made by the sodium-sodium exchange mode of the (Na+K)ATPase in potassium-free solution and in energy-depleted cells, since a ouabain-sensitive sodium-sodium exchange has been reported in frog skeletal muscle under these conditions (Keynes & Steinhardt 1968; Kennedy & De Weer 1976). Thus the interpretation of Fig. 12 must be carried out by comparison with the corresponding plots when one or another of the sodium extrusion mechanisms is disabled. For this purpose, the curves fitted by eye to Figs. 12, 14, 16, and 18 are collected in Fig. 19.

The dependence of M_{Na} on $(a_{Na})_m$ in the absence of external potassium and that in the presence of 10^{-4} M ouabain are closely correlated, but differ from the dependence in normal Ringer's solution in that they show a definite saturation. This is consistent with the hypothesis that a dominant mode of sodium extrusion is the sodium-potassium exchange mode of the (Na+K)ATPase.

The efflux which remains in the presence of ouabain or in the absence of external potassium reflects the maximum contribution that other mechanisms can make to the total sodium efflux. This appears to be almost as great as the efflux into normal Ringer's solution over the 'physiological range'. However, it is widely held that this is mostly tightly-linked sodium-sodium exchange, and so ineffectual as far as sodium regulation is concerned. Further, even if this _were_ all effective sodium extrusion, it seems to fall short of what is needed under normal conditions to balance the influx. Thus exposure to ouabain or removal of external potassium will cause the sodium efflux to fall below the sodium influx. $(a_{Na})_m$ will rise steadily, and the specific activity of the myoplasm will be reduced. This will cause a reduction in the efflux of radiosodium.

This reduction in the efflux of radiosodium could be interpreted as representative of a fall in the sodium efflux, if the myoplasmic sodium
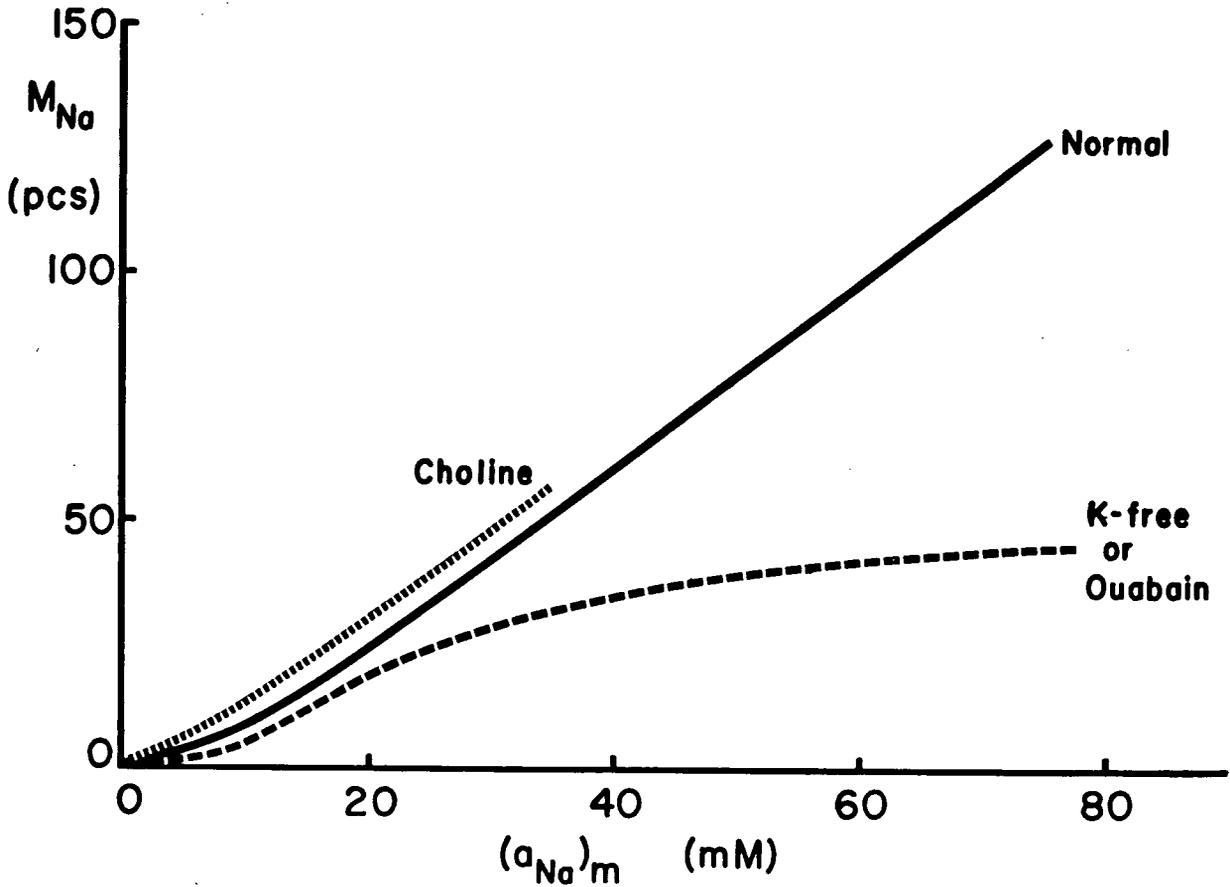
Figure 19. Summary of sodium efflux from the cell into various solutions, extracted from Figures 12 (normal Ringer's solution - solid curve), 14 (potassium-free solution - lower dashed curve), 16 (sodium-free solution - upper dashed curve, representing choline), and 18 (ouabain in normal Ringer's solution at 10^{-4} M - line as for potassium-free solution). The relative position of the different curves over the physiological range, (approx. 10 - 20 mM) was deduced form the behavior of a cell compared to itself as control, for a given test solution. The scatter of the grouped data does not permit the relative positions to be distinguished over this range. Thus: the efflux at a given value of $(a_{Na})_m$ increases when the external solution is changed from normal Ringer's solution to sodium-free choline-substituted solution, but decreases when it is changed to a potassium-free solution or to one containing ouabain. The curves for the latter two cases (with 10^{-4} M ouabain) are almost indistinguishable in the present series of experiments, and the efflux into potassium-free ouabain-containing solution was not examined. The curve for sodium-free lithium-substituted solution would be parallel to the choline curve, but below the normal curve.

activity is not monitored for the purpose of calculating the sodium efflux from radioisotope movement. That is, the fall in the sodium efflux due to inhibition of the pump might have been overestimated in the past.

If the mechanism which remains operational in the absence of external potassium and in the presence of ouabain does indeed represent sodium-sodium exchange, then it should be markedly reduced, if not eliminated, by the removal of sodium from the bathing solution. It has been found by other workers, however, that removal of external sodium and potassium, even when combined with exposure to ouabain, does not reduce the sodium efflux to the level expected if only passive fluxes are present (eg. Brinley 1968). Only when ATP is almost completely removed from the cell and inhibitors of metabolism are applied are the fluxes reduced to the passive rates, as discussed in section 2.

It appears from Fig. 19 that for barnacle muscle cells the efflux of sodium into sodium-free solution is very similar to the efflux into normal Ringer's solution. Further, this appears to be independent of the cation used in place of sodium in the bathing solution (Fig. 16). Is is conceivable but hardly likely that these cations could each substitute for sodium in a sodium-sodium exchange, or enter the cell by some other path, to yield the behavior observed. If it is assumed that the efflux into normal Ringer's solution reflects the normal operation of the cell, it appears that a sodium efflux which depends on external sodium contributes little to the normal flux in barnacle muscle cells.

Through the use of the intracellular sodium-specific microelectrode, the net reduction in the sodium efflux seen when the bathing solution is changed from one containing sodium to one not containing sodium has been revealed to be due to the consequent fall in $(a_{Na})_m$, over the 'physiological range' of $(a_{Na})_m$ and somewhat beyond. That the efflux into sodium-free solution seems to be slightly greater in magnitude than the efflux into sodium-containing solutions is consistent with the finding of other workers

that external sodium can inhibit the sodium efflux. The results with

lithium probably reflect in addition the ability of lithium to stimulate

like potassium (eg. Beauge 1975).

The existence of sodium-sodium exchange was first suggested by Ussing

(1949). The first evidence in favour of its existence in muscle cells was

presented by Keynes and Swan (1959) and their results demand closer considera-

tion. In their experiments, whole muscles of frogs were loaded with radio-

sodium (^{24}Na) by immersion for about 3.5 hours in normal frog Ringer's

solution which contained radiosodium. The muscle was then transferred at

5 or 10 minute intervals through a succession of test-tubes containing

inactive Ringer's or other solution. The amount of radioactivity leaving

the muscle during each interval (called Na^* in this thesis) was measured,

corrected for decay of this short-lived isotope, and plotted logarithmically

against time. Their estimate of changes in the rate of loss of sodium,

that is, of ^{23}Na, from the muscle cells were calculated from displacements

of this curve caused by changes in the ionic composition of the medium.

They found that the slope "$\underline{k_1}$" of the plot of ln Na^* versus time to be

constant during efflux into normal frog Ringer's solution. Likewise, the

slope "$\underline{k_1'}$" of the plot of ln Na^*_{cell} versus time was constant, where Na^*_{cell}

was calculated by back-addition as was done in the present series of

experiments on barnacle muscle (see Methods, section 4). The slope ratio

$\underline{k_1'}/\underline{k_1}$ was very close to unity, as expected for passively-loaded cells.

When the external solution was changed from normal frog Ringer's solu-

tion to sodium-free lithium-substituted solution, the value of Na^* decreased.

The plot of ln Na^* versus time assumed a steadier, almost linear slope

"$\underline{k_2'}$" after about 20 minutes. The slope of the plot of ln Na^*_{cell} versus

time exhibited a break, to a new smaller slope "$\underline{k_2}$" for sodium-free lithium-

substituted solution. Keynes and Swan found that $\underline{k_2'}/\underline{k_2}$ was close to 3.

It was this discrepancy that gave rise to their enquiry into different models for the nature of the sodium efflux. They considered the plot of Na^* versus Na^*_{cell} for efflux into sodium-free solution, which is like a power function with power three, and finally settled on a model in which sodium is transported out of the cell from a single homogeneous compartment, at a rate proportional to the cube of the sodium content of the cell.

In fact, the reason for the value of $\underline{k}'_2/\underline{k}_2$ being greater than unity is quite simple. The fundamental assumption of the tracer technique is that the tracer (^{22}Na or ^{24}Na) is in a well-mixed compartment, and behaves just as the ^{23}Na does. Then no matter what the mechanism by which sodium is transported out of the compartment, as long as the rate of sodium transport is steady the rate of efflux of tracer will be proportional only to the amount of tracer left in the compartment (assuming no backflux). Thus, $\frac{dNa^*}{dt}_{cell} = -k \times Na^*_{cell}$ where k is a positive constant. This equation defines the exponential function : $Na^*_{cell} = Na^*_{cell}(t=0) \cdot \exp(-kt)$. The 'rate constant' k for radiosodium efflux is determined by the rate of the sodium efflux. When this rate is steady, k is constant and the plot of $\ln Na^*_{cell}$ versus time is linear. This occurs for efflux into normal Ringer's solution for frog, as it does for barnacle muscle, but not for sodium-free solution.

If the rate of mixing in the compartment is always rapid compared to the rate of transport out of the compartment (which is an assumption of the tracer method), then a similar situation should occur at each instant of time even when the rate of sodium transport is changing:

$$Na^*_{cell}(t) = Na^*_{cell}(t=0) \cdot \exp(-k(t) \cdot t)$$

where now the 'rate constant' k is a function of time.

The rate of change of Na^*_{cell} must reflect both the rate of sodium

transport and the rate of change of the sodium transport, and this accounts

for the value of k'_2/k_2 found in frog.

More formally, if the total amount of radiosodium inside the cell is

Na^*_{cell}, and in interval Δt of time an amount of radiosodium Na^* is collected

in the bathing solution, then experimentally

$$\frac{dNa^*_{cell}}{dt} = -Na^*/\Delta t$$

where again the shorthand of 'pseudo-calculus' is employed. A plot of ln

Na^* versus time is a plot of ln $\frac{dNa^*_{cell}}{dt}$ versus time, and its slope is

$\frac{d}{dt} \ln \frac{dNa^*_{cell}}{dt}$; this is "$k'$" of Keynes and Swan. A plot of ln Na^*_{cell} versus

time has slope $\frac{d}{dt} \ln Na^*_{cell}$; this is "$k$" of Keynes and Swan. Straight-

forward differentiation of the above expression for $Na^*_{cell}(t)$ reveals:

$$k' = \frac{(k(t) + t\frac{dk}{dt})^2 - (2\frac{dk}{dt} + t\frac{d^2k}{dt^2})}{(k(t) + t\frac{dk}{dt})}$$

and

$$k = (k(t) + t\frac{dk}{dt}) .$$

When $k(t)$ is constant, as in normal Ringer's solution, where $(Na)_i$ is

steady, then $k' = k = k$ and $k'/k = 1$, as has been noted before.

However, when the muscle is in sodium-free solution, $(Na)_i$ will fall

roughly as an exponential function of time in frog muscle (White & Hinke

1976), and so $k(t)$ will fall. Then

$$k'/k = 1 - \frac{(2\frac{dk}{dt} + t\frac{d^2k}{dt^2})}{(k(t) + t\frac{dk}{dt})^2} .$$

It is not unreasonable to assume solely for the purposes of this discussion

that the decline of $k(t)$ is also describable by an exponential function:

$k(t) \sim k_0 \exp(-bt)$ where b is a positive constant, roughly equal to 0.01 $\min^{-1}$ after the first 20 minutes for frog muscle (White & Hinke 1976). Then

$$\underline{k}'/_{\underline{k}} \sim 1 - \frac{b\,(\,b\cdot t - 2\,)}{k\,(\,1 - b\cdot t\,)^2}$$

and $\underline{k}'/\underline{k}$ will exceed unity while $b\cdot t < 2$, which in this *very* rough, semiquantitative example is during the first 200 minutes.

The conclusion to be drawn, then, is that the "discrepancy" which led Keynes and Swan (1959) and Keynes and Steinhardt (1968) to postulate the existence of more than one intracellular compartment was a misinterpretation of the tracer data.

Similarily, the "plateaus" found by Mullins and Frumento (1963) for radiosodium efflux from frog skeletal muscle into sodium-free solution can be accounted for by the above expression for $\underline{k}'$, and the electrical coupling they proposed need not be invoked.

Further, all of the results on the sodium-free effect in frog muscle can be accounted for in terms of the changes in the rate of sodium efflux which occur as $(Na)_i$ changes. It is interesting that Keynes and Swan (1959) and Keynes and Steinhardt (1968) tried to account for their results by postulating that sodium-sodium exchange occurred at low values of $(Na)_i$ but not at high values. That is, they acknowledged the effect of their manipulations of the muscle cell on $(Na)_i$, and the dependence of the rate of sodium expulsion on $(Na)_i$, but did not take these features into account in their analysis of their tracer data. Apparently they did not feel that $(Na)_i$ could decline so precipitously in sodium-free solution (Keynes 1965). Recognition of this effect has come from measurements with sodium-specific intracellular microelectrodes (Thomas 1972b; White & Hinke 1976; Vaughan-Jones 1977).

The results imply that the behavior of the sodium transport in muscle is much more straightforward than previously had been thought. The linked sodium-sodium exchange which was supposed to be ouabain-insensitive and to comprise almost half of the sodium transport measured in muscle does not occur in barnacle muscle. Evidence previously presented for its existence in frog muscle has been shown to be incorrect. In this connection, it is interesting that when $(Na)_i$ was maintained by internal dialysis in squid axon, sodium-free solutions caused <u>no</u> reduction in the sodium efflux as long as ATP was included in the dialysis solution (Mullins & Brinley 1967).

The various effects on the sodium efflux of ouabain or changes in the ionic composition of the external solution, reported here for barnacle and by others for frog muscle, appear almost all to be explained by the curves of Fig. 19. Most of the results in nerve can be explained in like manner.

It almost seems that the overwhelming dominant mode of sodium transport in muscle and nerve, and perhaps in most cells, under normal conditions, is the sodium-potassium exchange mode of the (Na+K)ATPase. However, it would have to be proposed in addition that exposure of the cells to ouabain cannot inhibit all of the transport enzymes in the cell membrane, as suggested, for example, by Brinley (1968), and that 'recycling' of potassium which leaks out of the cell into potassium-free solution can occur, as suggested, for example, by Beauge (1975). The contribution of sodium transport modes involving calcium, amino acids, etc., should be relatively small normally. Certainly no proof of this hypothesis is claimed here, but these considerations seem to be a worthwhile basis on which to plan further experiments to find the mechanism of sodium expulsion into potassium-free and into ouabain-containing solutions.

<u>Kinetics</u>.

Much of the contribution of the (Na+K)ATPase to the sodium efflux can be eliminated selectively by exposure of the cells to ouabain. The remaining efflux can be assumed to be due mostly to one mechanism as a first approximation, and its kinetic characteristics examined. Of course, this might represent more than one mechanism, but there is no good reason to assume this now.

If a simple two-parameter model of the type discussed in section 2 is applied to the experimental data, a reasonable fit can be found for some values of the parameters. Selection of the most applicable model then depends on a knowledge of the values these parameters can assume, since each has a physical interpretation.

M_{max} is the maximum value the efflux can attain, and can be estimated quite well from the data at higher values of $(a_{Na})_m$ if saturation occurs. If saturation cannot be detected in the data, M_{max} cannot be estimated with any degree of assurance.

The value of k, the apparent dissociation constant of the enzyme-sodium complex when used in this context, reflects the binding energy. This energy can be quite high since the binding results in a slight change in the conformation of the enzyme, as discussed in section 2.

For a cyclical carrier system, the apparent dissociation constants are complicated functions of the rate constants which describe the various reactions in the cyclic sequence. They can be very different from the actual affinities (Caldwell 1969). It appears that the apparent dissociation constants at one surface of the membrane are independent of changes in the composition of the solution bathing the opposite surface under most conditions, even though such changes would alter some of the reaction rates in the cyclic sequence (Hoffman & Tosteson 1971; Garay & Garrahan 1973;

Chipperfield & Whittan 1976). It is likely, then, that the apparent affinities obtained via a good model will reflect the true affinities of the sodium sites.

From studies of the action of sodium in protecting the (Na+K)ATPase from inactivation by DCCD (dicyclohexylcarbodi-imide), Robinson (1974) was able to estimate the dissociation constant for the enzyme-sodium complex. In the absence of other ligands, the value was 2.3 mM. In the cell, potassium will compete with sodium for the sodium sites, and so the apparent dissociation constant will be too large. However, since the affinity of the sodium site for potassium is much less than that for sodium, the apparent value will probably be of the correct order of magnitude.

With respect to the models discussed in section 2, for "n" ions binding before transport occurs, the value of k which gives a good fit to the data is lower for higher values of n.

The data for ouabain (Fig. 18) are almost identical to the data for potassium-free solution (Fig. 14). A linear regression of $1/(a_{Na})_m$ on $1/M_{Na}$ yields k = 105 mM, M_{max} = 116 pcs for the efflux in the presence of 10^{-4} M oubain, and k = 116 mM, M_{max} = 124 pcs for the efflux into potassium-free solution. These values for k are too large, however. For two ions binding successively, the best fit to the data, by trial and error, was with k = 20 mM, M_{max} = 60 pcs. For three ions it was k = 15 mM, M_{max} = 60 pcs. For four ions it was k = 15 mM, M_{max} = 55 pcs. On the basis of the above discussion, as a first approximation only, the data are fitted best by a model with at least three sodium ions binding successively to equivalent independent sites. The key to a more precise conclusion is good data at low levels of $(a_{Na})_m$, as discussed previously, plus extension of the data in normal Ringer's solution and sodium-free solution to the region of saturation.

Continuing in this rather speculative vein, it can be noted again that the efflux into sodium-free solution is very similar to the efflux into normal Ringer's solution (Fig. 19). If it is assumed that the dominant mode of sodium transport normally is sodium-potassium exchange, the two-parameter models can be applied. There is no good indication of M_{max}, but a good fit can be achieved for one ion binding with $k = 200$ mM, $M_{max} = 400$ pcs; for two ions with $k = 50$ mM, $M_{max} = 225$ pcs; for three ions binding with $k = 30$ mM, $M_{max} = 175$ pcs; and for four ions with $k = 27.5$ mM, $M_{max} = 200$ pcs. That is, the fit is better for higher numbers of ions binding successively.

Thus the two modes defined operationally here appear to differ in their kinetic characteristics in the context of the two-parameter models. For no value of n is it found that k is the same but M_{max} is lower for cells in which the (Na+K)ATPase is inhibited compared to cells in normal Ringer's solution. This suggests that the efflux observed in the presence of ouabain and in the absence of external potassium is not simply due to the operation of fewer transport enzymes. When more data at very low and very high levels of $(Na)_m$ can be obtained, it will be interesting to perform a more detailed kinetic analysis in terms of inhibition of different types.

SECTION 6.   COMPARISON OF SODIUM ELECTRODE AND RADIOSODIUM MEASUREMENTS

Two 'sodium-free' effects have been reported in sodium efflux studies
in muscle, as summarized in section 2.C. They differ in their time course.
The better-known effect is a sustained reduction in the sodium efflux when
sodium is removed from the bathing solution. In the last section, it was
reported that this effect appears to be due to the fall in the myoplasmic
sodium activity alone, at least in barnacle muscle cells.

The second 'sodium-free effect' is a large rapid fall in the intra-
cellular sodium activity measured with an intracellular sodium-specific
microelectrode. This effect has been observed in frog skeletal muscle
(White & Hinke 1976) and in crab striated muscle (Vaughan-Jones 1977). A
slightly different rapid fall was found in snail neurone (Thomas 1972b).

This rapid fall in $(a_{Na})_m$ coincides roughly with the transient stimula-
tion of the sodium efflux seen in Fig. 15 with choline and tris, and with
the 'biphasic transient' with lithium. The change from sodium-containing
to sodium-free solution is not instantaneous, and as noted in section 4,
the experimental apparatus was not designed to measure transient phenomena.
However, it was felt to be worthwhile to compare the information from the
intracellular microelectrode and the radioisotope, particularly during the
period where the transients occur.

In the experiments on frog muscle, chemical analysis indicated that
the fall in the myoplasmic sodium activity is due to movement of sodium ions
out of the cell (White & Hinke 1976). Studies of the efflux of radiosodium
from frog muscle into sodium-free solution have revealed some transient
rapid fluxes, but these were usually ascribed to the extracellular space
and ignored (eg. Hodgkin & Horowicz 1959).

Measurements with the intracellular microelectrode alone can only

reveal a loss of sodium from the major intracellular compartment, the myoplasm. They cannot reveal the fate of the lost sodium. A treatment of the data was thus devised in which microelectrode and radioisotope results are combined, so that changes in the sodium content of the myoplasm could be compared with the simultaneous loss of sodium from the cell. The experiments were conducted prior to the publication of the results for crab muscle.

It was found that a rapid fall in the myoplasmic sodium activity similar to that seen in snail neurone and in frog and crab muscle occurs in barnacle muscle under certain conditions, and that it is accompanied by a commensurate loss of sodium from the cell.

The transient changes in the sodium efflux which occur immediately after the change to sodium-free solution appear to reflect a direct effect on the transport mechanism, as well as an effect due to rapid changes in the sodium content of the cells.

METHODS

Dissection of single muscle cells, injection and collection of radiosodium, and measurements with microelectrodes have been described in previous sections. The fluid injected contained ^{22}NaCl and, sometimes, ^{23}NaCl, the latter in order to raise the myoplasmic sodium activity.

RESULTS

In Fig. 20 is presented the time course of the decline in $(a_{Na})_m$ for

three cells of different initial sodium content, during immersion in a

sodium-free lithium-substituted solution. The behavior is qualitatively

different for cells of different sodium content. The plot is semilogarith-

mic. A simple exponential fall of $(a_{Na})_m$ with time was seen in cells having

a lower initial value of $(a_{Na})_m$. A rapid initial fall of $(a_{Na})_m$, similar to

that seen in snail neurone (Thomas 1972b) but not unlike that seen in frog

skeletal muscle (White & Hinke 1976) and in crab striated muscle (Vaughan-

Jones 1977), was seen in cells having a higher initial value of $(a_{Na})_m$.

In frog and crab muscle, a large initial fall of $(a_{Na})_m$ was seen even in

cells having low initial values of $(a_{Na})_m$.

This behavior of barnacle muscle cells is different from that reported

by McLaughlin and Hinke (1968) and by Allen and Hinke (1971). However,

they had used a hypertonic sodium-free solution, and the initial behavior of

$(a_{Na})_m$ in their experiments included the effects of water movement, as

discussed in section 3.B.

The time course of the fall in $(a_{Na})_m$ in the cells having a high initial

sodium content could be fitted quite well by the sum of two exponentials,

but for the cells having a lower initial sodium content a single exponential

or occasionally even a linear function sufficed.

The hazards of 'curve peeling' were mentioned in section 4. There is

no reason to propose that the behavior of the cells of high initial sodium

content reflects the sum of two independent processes. It seems equally

likely that a single mechanism is operating but the rate constant is dis-

playing a dependence on the gradient of the chemical potential for sodium.

When rate constants for the rapid efflux were calculated by 'curve peeling',
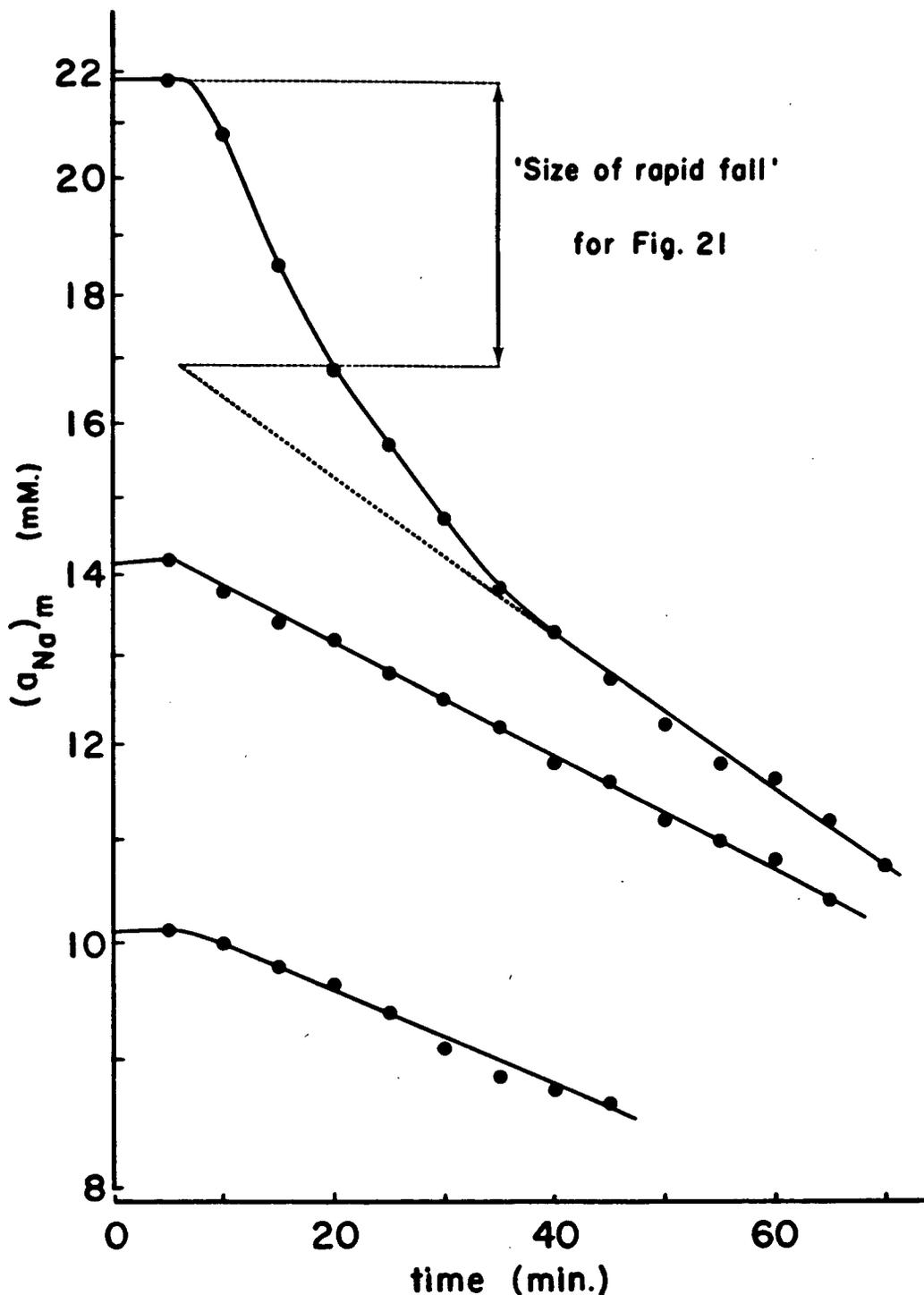
Figure 20. Fall of the myoplasmic sodium activity upon exposure of the cell to sodium-free lithium-substituted solution. Solution change from normal Ringer's solution occurred at time 5 minutes. Prior to that time, the steady value of the myoplasmic sodium activity of each cell in normal Ringer's solution is shown. The plot is semilogarithmic. For the cell which started with the highest sodium content (upper curve), it is shown how the 'size of the initial rapid fall' was calculated for Fig. 21, by extrapolation back to zero time of the linear tail of the curve.

the values were found to show considerable scatter and to be poorly corre-
lated with the initial value of $(a_{Na})_m$. It appeared that above a certain
threshold value of $(a_{Na})_m$ of about 15 mM the fast rate was present, while
below this threshold it was not. The mean value of the larger rate constant
was comparable to the value which describes the washout of the extracellular
space (0.15 min^{-1}, SD = 0.06 min^{-1} for 9 cells fitted by two exponentials; a
similar value was reported by White & Hinke 1976 for frog muscle). The
different significance of the rate constant in the two cases will be con-
sidered in the Discussion.

Vaughan-Jones (1977) noted that the change of $(a_{Na})_m$ during 15 minutes
of immersion of crab muscle in sodium-poor solution correlated well with the
initial value of $(a_{Na})_m$ in the cells. A similar result was found in
barnacle muscle, as shown in Fig. 21. The 'size of the rapid fall' in mM
was calculated by the method indicated in Fig. 20 top tracing. This really
just confirms the impression gained from Fig. 20 that, whatever the initial
value of $(a_{Na})_m$ above about 15 mM, the rapid fall is 'switched off' when
the value of $(a_{Na})_m$ falls to ca. 15 mM during immersion of the cell in the
sodium-free solution. The 'threshold' value of $(a_{Na})_m$ in crab muscle was
about 2 mM (Vaughan-Jones 1977).

A different and better approach is to measure the initial slope of the
tracings of $(a_{Na})_m$ versus time, to yield a single rate describing the rapid
fall. When this rate is plotted versus the value of $(a_{Na})_m$ at the start of
the fall, a correlation is seen (Fig. 22). The dependence is very similar,
even in the slight difference in slope for different cells at a given value
of $(a_{Na})_m$, to that displayed in Fig. 17 for the efflux M_{Na} (calculated from
equation (4) without correction for Na^*_{cell}) versus $(a_{Na})_m$. This in turn
is similar to the corrected value Fig. 16, as noted in section 5.

In theory, the relationship between the two is $V_m \cdot \frac{d(Na)}{dt} m = A \cdot M_{Na}$,
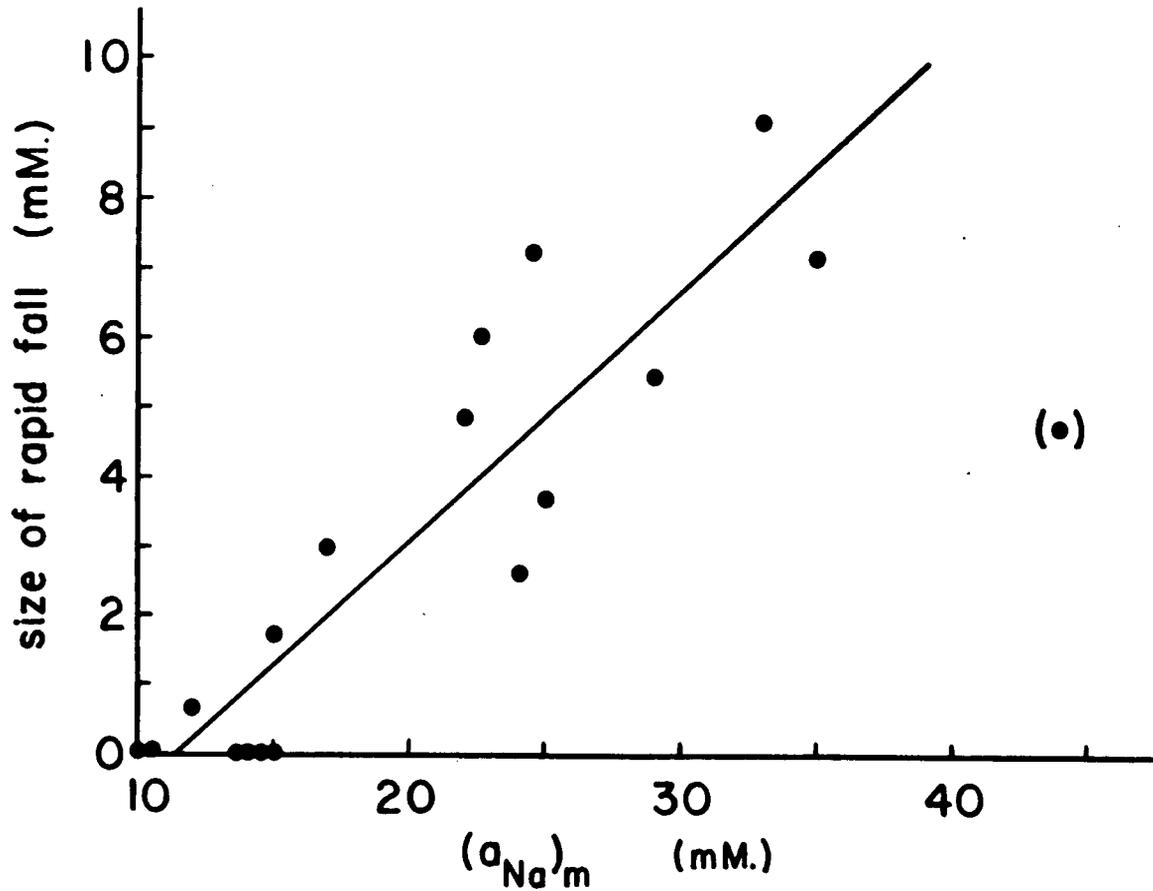
Figure 21. Size of the rapid fall in the myoplasmic sodium activity upon exposure of the cell to sodium-free lithium-substituted solution (see Fig. 20 and text), versus the steady value of the myoplasmic sodium activity in the cell prior to the change from normal Ringer's to sodium-free solution. One data point was excluded from the linear regression represented by the line, and is shown in parentheses.
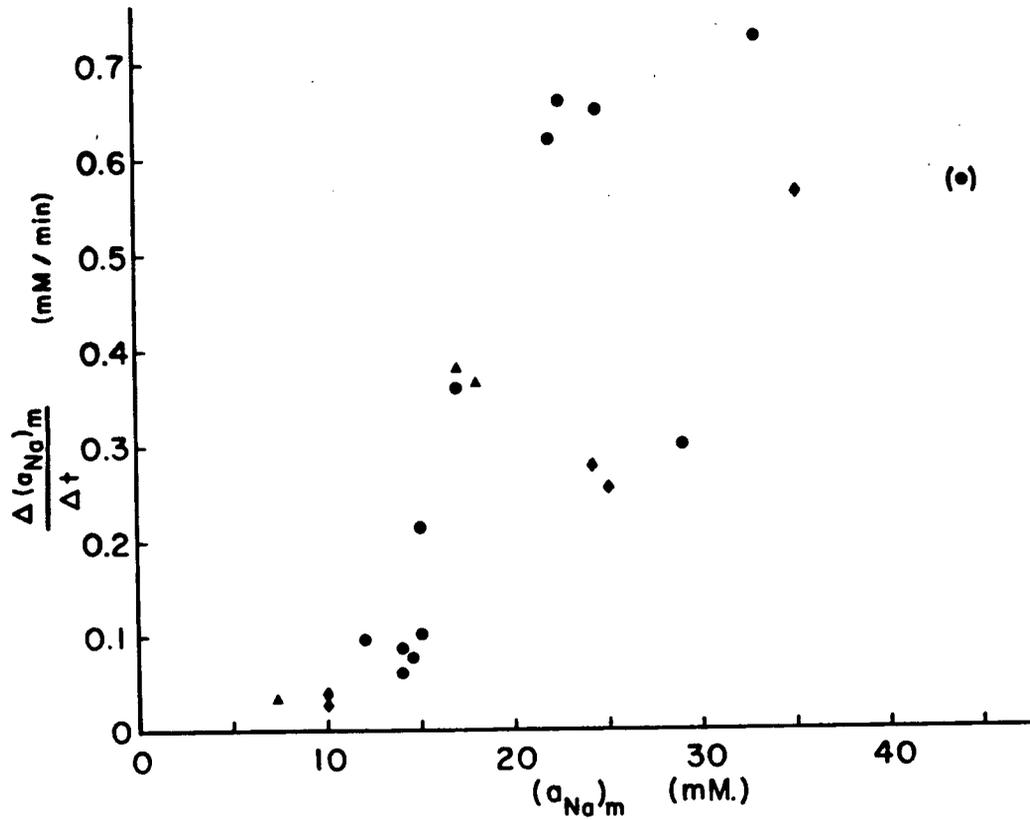
Figure 22. Rate of fall of the myoplasmic sodium activity immediately after exposure of the cell to sodium-free lithium-substituted solution, versus the steady value of the myoplasmic sodium activity in normal Ringer's solution prior to the change to sodium-free solution. The point in parentheses is from the cell excepted in Fig. 21. Circles: cells dissected on the day of the experiment. Diamonds: cells dissected on the day before that of the experiment. Triangles: cells dissected on the day of the experiment and subjected only to microelectrode experiments, not microinjection.

where A is the area of the surface enclosing V_m, and across which the efflux

of sodium occurs. The two are not completely independent, since measured

values of $(a_{Na})_m$ appear in both. However, M_{Na} is determined in addition

by the isotope efflux. A check of consistency is to eliminate $(a_{Na})_m$

between Figs. 17 and 22. The cells used were of about the same size. A

plot of M_{Na} vs. $\frac{d}{dt}(a_{Na})_m$ is linear, and the slope $V_m/\lambda A$ is 0.004 cm. This

should equal $\frac{0.68}{0.65}\frac{r}{20}$, so the mean value of the cell radii should be 0.0765

cm. The actual mean value of the cell radii for the experiments using

sodium-free solution is 0.066 cm (SD = 0.013 cm, 19 cells). This suggests

that the approximations used in arriving at equation (4) are quite good.

It also suggests that the rapid fall in $(a_{Na})_m$ is really due to transport

across the cell membrane, and further that the mechanism behaves just like

the mechanism which expels sodium from the cell into normal Ringer's

solution.

The relationship between the microelectrode measurements and the

radioisotope measurements of the efflux into sodium-free solution can be

examined in more detail, in light of the results of the preceding sections.

The injected radiosodium is deposited in solution in the myoplasm. It

very quickly equilibrates with the small pool of bound but rapidly-exchang-

ing sodium associated with the contractile proteins. Exchange between the

myoplasmic sodium and this loosely-bound sodium is much more rapid than

transport of sodium across the cell membrane, so this bound radiosodium plus

the free radiosodium constitute one compartment as far as the membrane

transport is concerned.

Radiosodium is continuously lost across the cell membrane to the

bathing solution, and via longitudinal diffusion to an effective intra-

cellular sink. These processes have been assumed to be independent.

The myoplasmic sodium activity $(a_{Na})_m$ is related to the sodium content

of the myoplasmic compartment Na_m as $\gamma_\pm Na_m/V_m = (a_{Na})_m$, by the definitions

of this model. A change in the sodium content of the myoplasmic compart-

ment in a given time is then

$$\Delta Na_m = \frac{V_m}{\gamma_\pm} \cdot \Delta(a_{Na})_m \quad . \qquad \qquad \ldots(9)$$

If it is assumed that the change in the sodium content of the myoplasm

which occurs when the cell is immersed in sodium-free solution is entirely

due to passage of sodium across the cell membrane and into the bath, then

$$\Delta Na_m = \frac{\Delta Na_m^*}{SA_m} \doteq \frac{Na^*}{Na_m^*} \cdot Na_m = \frac{Na^*}{Na_m^*} \frac{V_m}{\gamma_\pm} (a_{Na})_m \quad . \qquad \ldots(10)$$

SA_m actually includes free and loosely-bound sodium and radiosodium. The

electrode measures only free sodium. The exchangeable radiosodium (free

and loosely-bound) is given by the slope of the plot of $\ln Na^*$ versus time,

as explained in sections 2.F and 4. For this reason, (10) as written is

a slight underestimate of ΔNa_m. If Na_{cell}^* is used in place of Na_m^*, then

(10) underestimates ΔNa_m by a larger amount. The amount of the underesti-

mate due to this factor will increase with time, but should amount at most

to about 25% here, given the sodium content of these cells (Fig. 8). This

must be considered because the use of the instantaneous slope of the plot of

$\ln Na^*$ versus time to calculate Na^*/Na_m^* can only be done when a line can be

drawn through the data points, as explained in sections 2.F and 4, while

this plot is quite erratic just after the change to a sodium-free solution

(Fig. 15), since the rate constant for sodium transport is changing.

The point of all of this is that (9) is what the electrode measures

and (10) is what the radioisotope measures. If the fall in $(a_{Na})_m$ in sodium-

free solution is solely or mostly due to passage of sodium out of the cell,

then the ratio of (9) to (10) should be close to unity. Since the only

value for (10) obtainable from the data under these circumstances is an underestimate, the ratio of (9) to (10) will be greater than unity by at most about 25%. If the fall in $(a_{Na})_m$ is due to an appreciable amount of sodium going elsewhere in the cell, then the ratio of (9) to (10) will be lower. What is 'appreciable' here is about 25% of the fall in $(a_{Na})_m$.

Of the 22 experiments with sodium-free lithium-substituted solution in which M_{Na} was measured, $(a_{Na})_m$ failed to fall in three, and a contracture interrupted one. For the remaining 18 experiments, the ratio of (9) to (10) was calculated:

$$\text{RATIO} = \frac{\dfrac{\Delta (a_{Na})_m}{\Delta t}}{\dfrac{1}{5 \text{ min}} \dfrac{Na^*}{Na^*_{cell}} \cdot (a_{Na})_m}$$

for each collection period from the moment of immersion in sodium-free solution. The results are shown in Fig. 23. The value of the RATIO rises to about 1.4, then declines to a value near 1.0 over the first 40 minutes in sodium-free solution.

Given the above considerations, it appears that almost all of the fall in $(a_{Na})_m$ seen in sodium-free solution is due to movement of sodium out of the cell, and not to a redistribution of sodium among intracellular compartments. The initial transient variations of M_{Na} for lithium-substituted solution might be an artifact of the solution change, but the stimulation in tris- and choline-containing solution is more difficult to explain. There was no transient in $(Na)_m$ after the change to sodium-free solution, just the onset of a well-behaved decline like those in Fig. 20. That is, the response of the transport mechanism to the substrate concentrations is different when tris or choline is present while external sodium is absent. At a given value of $(Na)_m$, compared to the efflux into normal Ringer's
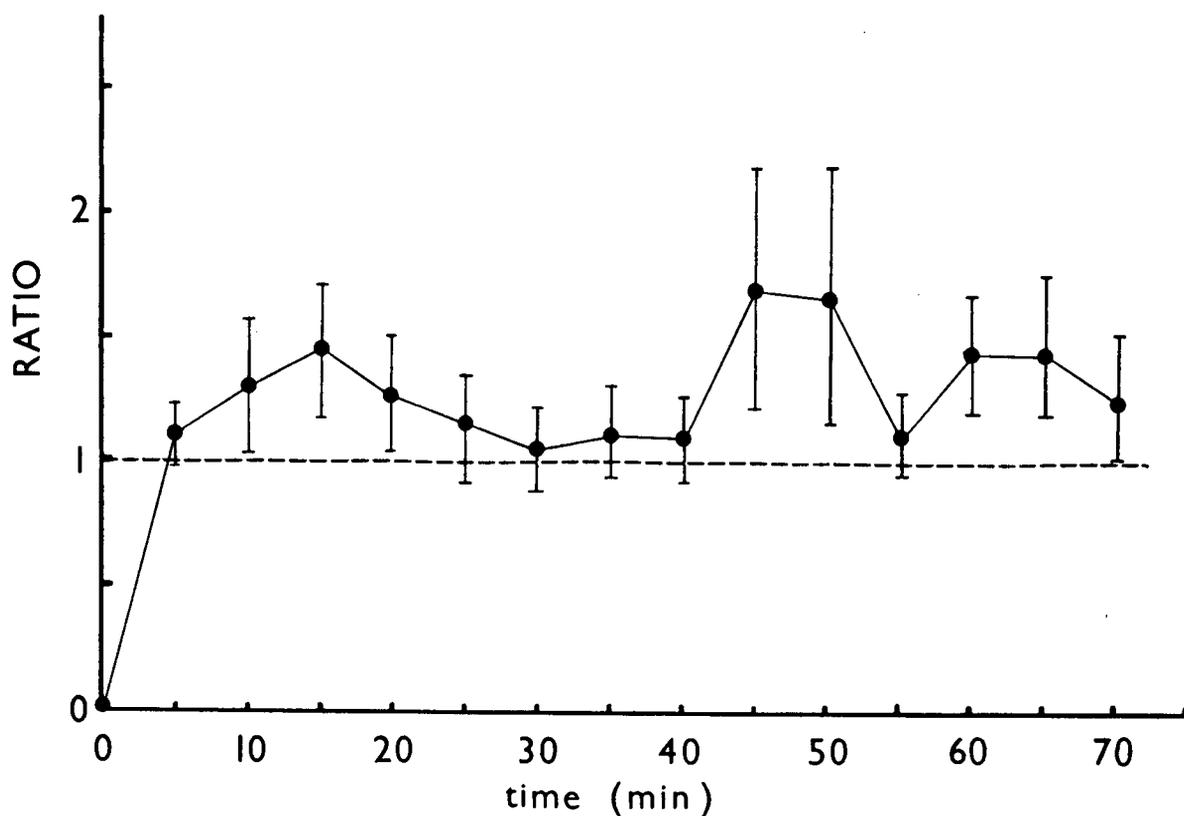
Figure 23. Value of the RATIO which represents the efflux of sodium from the myoplasm after exposure to sodium-free solution (time zero) as measured by the microelectrode relative to that measured by the efflux of radiosodium, as explained in the text. Solid line simply connects the data points. Each point represents the mean of the results for 17 - 18 cells (time 5 min to 40 min) or for 6 - 16 cells (time beyond 40 min), and the bars represent two standard deviations.

solution the sodium efflux appears to be slightly greater in the absence of external sodium with lithium as a substitute, and to be greater still with tris or choline as the substitute.

An hypothesis which could account for all of these cases is that external sodium inhibits sodium transport, that lithium removes some of this inhibition but as a small cation still acts much as external sodium does in the inhibition of sodium transport, and that the large cations choline and tris are much less able to act as external sodium does to inhibit sodium transport.

Then the M_{Na} versus $(a_{Na})_m$ characteristic would rise more steeply with lithium-substituted sodium-free solution than with normal Ringer's solution, and more steeply still for choline- or tris-substituted sodium-free solution. The characteristic curves for the three situations would be close together at low values of $(a_{Na})_m$, as found in Fig. 16, but farther apart at higher values of $(a_{Na})_m$. Then at a higher value of $(a_{Na})_m$, when the external solution is changed to one containing tris rather than sodium, the efflux will jump to the higher characteristic curve, and decline along this curve as $(a_{Na})_m$ falls. The initial effect will then be a stimulation of M_{Na} for cells of higher initial sodium content, with the decline of M_{Na} always occuring as $(a_{Na})_m$ fell, as found in Fig. 15. The biphasic response with lithium presumably reflects this summation of stimulatory and inhibitory effects, but cannot be explained in detail.

Membrane Potential.

In almost all experiments with lithium-substituted sodium-free solution, there was a depolarization of a few millivolts shortly after the change from normal Ringer's solution to sodium-free solution. No good correlation between the amount of depolarization and the rate of the rapid efflux could

be demonstrated. In the experiments with choline-substituted sodium-free solutions, there always was a hyperpolarization following the change to sodium-free solution. In the experiments with tris-substituted sodium-free solution, there was a less dramatic hyperpolarization following the solution change. These features will be discussed in section 7, which is concerned with the membrane potential and electrogenicity.

DISCUSSION

A rapid fall in $(a_{Na})_m$ in barnacle muscle cells occurs on immersion of the cells in sodium-free solution. The effect differs from that observed in frog muscle and crab muscle. First, it only occurs in barnacle muscle cells whose sodium content is elevated. The effect is seen even in frog and crab muscle cells of low sodium content. Second, the time course is different. The rapid efflux is complete after about 3 minutes in crab muscle, and after 5 to 20 minutes in frog muscle, but takes about 20 minutes in barnacle muscle. It seems likely that both features are due to differences in the M_{Na} versus $(a_{Na})_m$ characteristic curves for frog compared to barnacle (recall: in frog there is a steep rise of M_{Na} and saturation at low $(a_{Na})_m$ - Harris 1965). But crab is a crustacean like barnacle and should not behave so differently.

Other features of the electrode measurements in crab muscle are unusual. There is almost no change in $(a_{Na})_m$ during a 100 minute period of exposure of the cell to crab Ringer's solution to which had been added ouabain to 2×10^{-4} M (Vaughan-Jones 1977 - Fig. 5), or during a 55 minute period of exposure of the cell to potassium-free solution (Vaughan-Jones 1977 - Fig. 4).

This differs from the immediate and sustained rise of $(a_{Na})_m$ seen when snail neurone, frog skeletal muscle, or barnacle muscle cells are exposed to ouabain or potassium-free solution (Thomas 1972b; White & Hinke 1976; Fig. 10 of this thesis). Yet the results reported here for the response of barnacle muscle to sodium-free solutions show in general the same behavior as was seen in snail neurone and frog and crab muscle. For now, it will be assumed that a similar process yields the rapid fall in all four preparations.

The rapid fall of $(a_{Na})_m$ in barnacle muscle is associated with a commensurate efflux of sodium from the cell. Thomas (1972b) noted that the rate of fall of $(Na)_i$ was appropriate for the size of the unidirectional sodium efflux in the absence of sodium influx in snail neurone. Vaughan-Jones (1977) felt that intracellular redistribution of sodium could not be ruled out by his results. White and Hinke (1976) concluded on the basis of chemical analysis of the frog muscle that the fall of $(a_{Na})_m$ was associated with an efflux of sodium from the cell. They were mainly concerned with the microelectrode measurements but presented some radio-sodium measurements as well. The radiosodium measurements were relatively unsophisticated in that a passive loading period of four hours was used, on muscle bundles rather than single cells, and the large extracellular space was not cleared by immersion of the bundle in normal frog Ringer's solution before measurements in sodium-free solution were made. Any rapid efflux of sodium was thus masked by efflux from the extracellular space.

The rate constant describing the initial rapid fall of $(a_{Na})_m$, deduced by the curve peeling technique, is comparable in magnitude to the rate constant describing the efflux of radiosodium from the extracellular space. The comparison is deceptive, however, since in the case of the efflux of radiosodium from the extracellular space, the rate is the constant rate

determined by the diffusion of sodium in bulk solution. (In fact, the fundamental assumption of the tracer technique, that the rate of loss of tracer is proportional to the amount of tracer present, is not satisfied in the case of the loss of radiosodium from the extracellular space, since the process by which radiosodium leaves this compartment is the same as the process which mixes the compartment - diffusion in bulk solution.) In the case of the plot of ln Na^* versus time for radiosodium leaving the interior of the cell, the slope reflects not only the rate constant k but in addition, its rate of change $\frac{dk}{dt}$.

The point is, Na^*/Na_m^* never approaches the value of the slope of the semilog plot when that plot is not close to linear, and the size of the efflux never approaches the size of the efflux from the extracellular space, even though similar values for rate constants can be obtained by the imposition of the tracer analysis on the data.

In summary, the radioisotope and microelectrode measurements are consistent, and this indicates that the model for the states of the intracellular solvent and ions is consistent insofar as it was used in the interpretation of these measurements. The sodium-free effect as it is generally regarded, as the inhibition of a tightly-linked sodium-sodium exchange, has been found to be something quite different. The results in barnacle and most of the results in other muscles and in nerve concerning the stimulation of sodium efflux when the external sodium is removed can be viewed as reflecting an inhibition of sodium transport by external ions. The fall in the sodium efflux which follows removal of external sodium appears to be due to the consequent fall in the myoplasmic sodium activity.

SECTION 7. ELECTROGENIC SODIUM TRANSPORT IN BARNACLE MUSCLE

The principal mode of active transport of sodium in barnacle muscle cells appears to be the sodium-potassium exchange mode of the (Na+K)ATPase. If sodium ions are expelled from the cells at a rate greater than the rate at which potassium ions are taken up via this mechanism, the net efflux of cations which results will give rise to an electrical potential difference across the cell membrane. The mechanism by which this potential difference arises, as discussed in section 2.D, probably is similar to that by which the larger potential difference due to the passive movement of potassium ions out of the cell arises, as discussed in section 2.D. However, the small hyperpolarization of the cell directly due to the sodium pump will only be present when the sodium-potassium exchange is not one-for-one, while the larger potassium 'diffusion potential' will persist as long as the potassium ion gradient across the cell membrane is maintained.

An electrogenic effect is most easily detected in cells which have been loaded with sodium, as then the rate of active sodium extrusion is more rapid, but the effect can also be seen in cells with normal or low sodium content (Kerkut & Thomas 1965; Thomas 1972b). However, it has been detected in the course of the normal functioning of several cell types as well: post-tetanic hyperpolarization in nerve (eg. Rang & Ritchie 1968); post-synaptic potentials in nerve (eg. Libet & Kobayashi 1968); regulation of tension in vascular and gastrointestinal smooth muscle (eg. Prosser 1977); the response of pancreatic acinar cells to pancreozymin (Kanno 1975); and the response of pancreatic islet cells to glucose (Matthews & Sakamoto 1975; Atwater & Meissner 1975). The degree to which electrogenicity is necessary in the normal functioning of cells is not yet known.

An electrogenic contribution by the (Na+K)ATPase can be detected by

observation of the effect on E_m of alterations in the pump rate. Most specific is the observation of a depolarization of the cell when it is exposed to ouabain. An alternative method is to observe a hyperpolarization of the cell when the pump is stimulated by a sodium load, although this is not as specific as the use of ouabain (eg. DeWeer & Geduldig 1973).

A variation of these methods is to voltage clamp the cell, that is, to measure E_m with an intracellular electrode and pass a current across the membrane via a different electrode in order to keep E_m steady. Then changes in the amount of current which must be passed to maintain E_m in the face of some challenge such as a sodium load indicate the changes in the contribution of the pump to E_m which the challenge can elicit. In fact, this method bypasses the manifestation of electrogenic transport, the changes in E_m, and measures the unbalanced cation efflux by exactly opposing it (eg. Thomas 1969; Kostyuk et al. 1972).

None of the methods is ideal. Application of ouabain can inhibit much of the active sodium transport, but it is not known how the ouabain-insensitive transport changes in the face of this challenge. Voltage clamping involves the passage of a polyionic current across the membrane, and the composition of this current is not completely clear.

The experiments described in this section involve the measurement in a single cell of two quantities simultaneously: the membrane potential and the unidirectional sodium efflux. The response of these quantities to exposure of the cell to ouabain is compared. It is shown that an electrogenic effect is associated with the sodium efflux in barnacle muscle, and the correlation of the changes in E_m and M_{Na} is examined.

METHODS

The measurement of the sodium efflux has been described in sections 4 and 5. In this series of experiments, a single muscle cell was dissected, injected with radiosodium solution, and mounted in the perfusion chamber in the usual manner. The cell was perfused with normal Ringer's solution until a steady efflux was established. Then the perfusing solution was changed to normal Ringer's to which ouabain had been added, to 10^{-8} to 10^{-4} M in different experiments. The sodium efflux M_{Na} was calculated from equation (4), with correction for Na^{*}_{cell} since the best quantitative estimate was sought.

RESULTS

The change in the membrane potential E_m, recorded with a micropipette electrode, assumed a new steady value after exposure of the cell to ouabain, as indicated in Fig. 24. Similarily, the sodium efflux fell and, with 10^{-4} M ouabain, remained quite steady for 30 to 60 minutes despite a large increase in $(a_{Na})_m$. In order to apply the correction for longitudinal diffusion, the 'slope ratio' for efflux into normal Ringer's solution before the exposure of the cell to ouabain was used. This was necessary because the correction for Na^{*}_{cell} described previously cannot be applied when the efflux rate is changing.

Fig. 24 is a summary of more data from the experiment depicted in Fig. 10. Of particular interest here are the changes in the membrane potential E_m.

It can be seen that while the cell is being perfused with normal
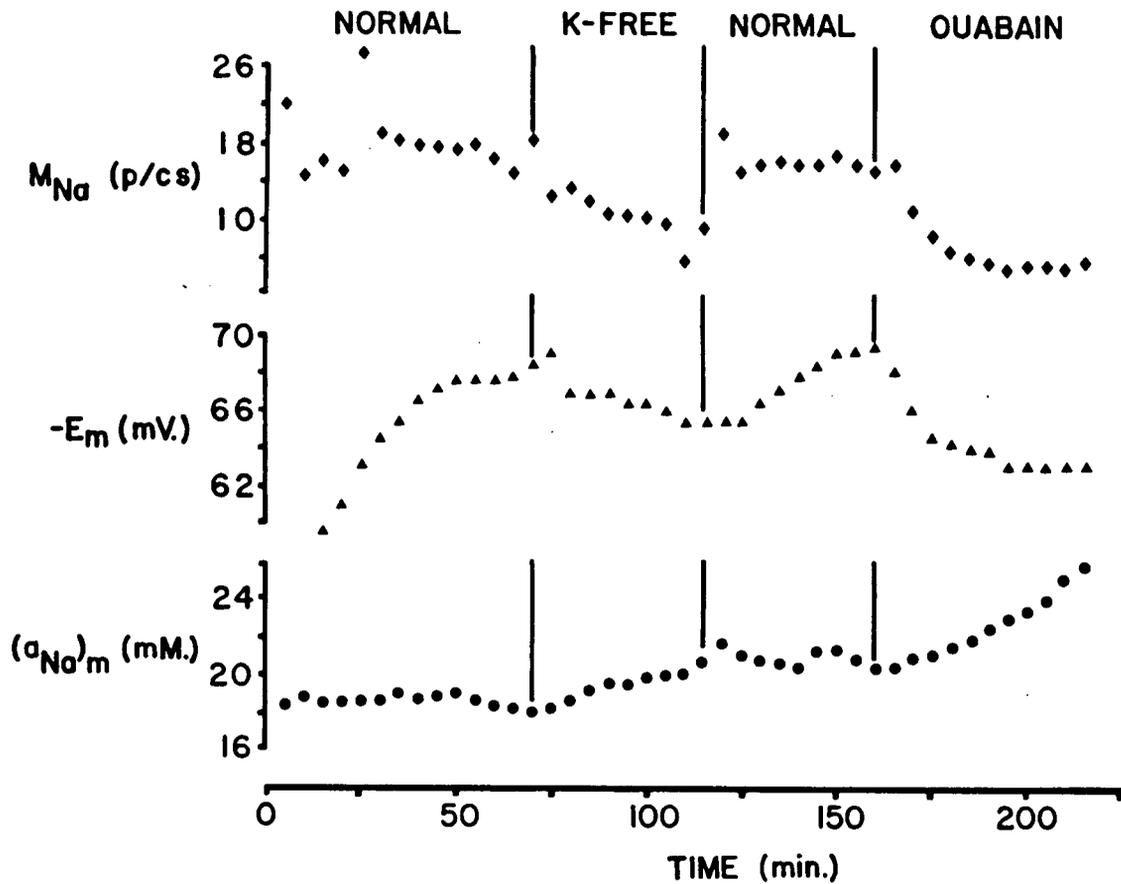
Figure 24. Change in the membrane potential which occurs on exposure of the cell to potassium-free or ouabain (10^{-4} M) containing solutions. Additional data from the same experiment is presented in Fig. 10. Top: sodium efflux calculated from equation (4) <u>without</u> correction for Na^{*}_{cell}. Middle: membrane potential measured with micropipette electrode. Bottom: myoplasmic sodium activity measured with a sodium-specific microelectrode.

Ringer's solution, $(a_{Na})_m$ was steady at about 18 mM, E_m became steady at about -68 mV, and M_{Na} was reasonably steady at about 18 pcs. As presented in this figure, M_{Na} is calculated directly from equation (4) without correction, to illustrate the detailed qualitative changes. ^{23}NaCl had been injected to raise $(a_{Na})_m$, and E_M recovered relatively slowly in this experiment, but attained a steady value.

Exposure to potassium-free solution is expected to slow the sodium-potassium exchange, and the membrane potential does fall slightly. The initial effect on E_m, a transient rise, probably reflects the effect of removal of external potassium on the membrane potential as expressed by the Goldman-Hodgkin-Katz equation, ie. the 'potassium diffusion potential'. The net effect seen should be the sum of a hyperpolarization due to the latter, and a depolarization due to inhibition of active sodium-potassium exchange. The decline of M_{Na} in potassium-free solution is seen in Fig. 24, as is the accompanying rise in $(a_{Na})_m$.

Restoration of the external potassium causes the sodium efflux to rise to its former level, and $(a_{Na})_m$ becomes steady. It would have been expected from Fig. 19 that M_{Na} would be slightly higher at the higher value of $(a_{Na})_m$. Apparently the effect is too small to be revealed in this uncorrected calculation of M_{Na}. The membrane potential rises, to a value somewhat higher than before. This cannot convincingly be ascribed to increased sodium pumping at the higher concentration of intracellular sodium, since the expected rise in the sodium efflux has not been demonstrated by comparison with its previous value in normal Ringer's solution. The extra rise in E_m might reflect a change in the sodium-potassium exchange ratio due to the sodium load brought about by the brief removal of external potassium, as will be discussed later in this section.

Exposure of the cell to normal Ringer's solution to which ouabain had

been added to 10^{-4} M is expected to slow the sodium extrusion considerably. Fig. 24 shows that M_{Na} falls quite abruptly, while $(a_{Na})_m$ commences a steady rise, and E_m falls abruptly with M_{Na}. This demonstrates that by the accepted criterion, there is an electrogenic contribution to the resting membrane potential in barnacle striated muscle, at least when it is loaded with sodium. This had not been shown before.

Fig. 25 is a summary of a number of experiments in which cells were exposed to ouabain. The change ΔE_m in the membrane potential upon exposure to ouabain is plotted versus the corresponding simultaneous change in the sodium efflux ΔM_{Na} quantitatively. The value of ΔE_m is expected to be accurate, since any uncertainties involved in the use of the micropipette electrode do not influence such a measured difference change in E_m. The value of ΔM_{Na} requires more attention. The approximations and uncertainties involved in the measurement of M_{Na} have been discussed at length in previous sections, and the approach taken is felt to yield the best possible estimate. The consistency of M_{Na} and $\frac{d}{dt}(a_{Na})_m$ presented in section 6 supports this assertion. Thus it seems justified to seek a quantitative description of the relationship between ΔE_m and ΔM_{Na}.

The qualitative relationship between ΔE_m and ΔM_{Na} is clear from Fig. 25. The line drawn is really just a visual aid. A quantitative expression of the relationship between ΔE_m and ΔM_{Na} in the context of the Goldman-Hodgkin-Katz theory has been developed in section 2.F. The passive trans-membrane fluxes of ions are treated in the constant field approximation, and the net active cation efflux of electrogenic transport is included phenomenologically. That is, the passive fluxes are assumed to arise from a specified driving force, while the net active cation efflux is just added into the current balance equation. The result is
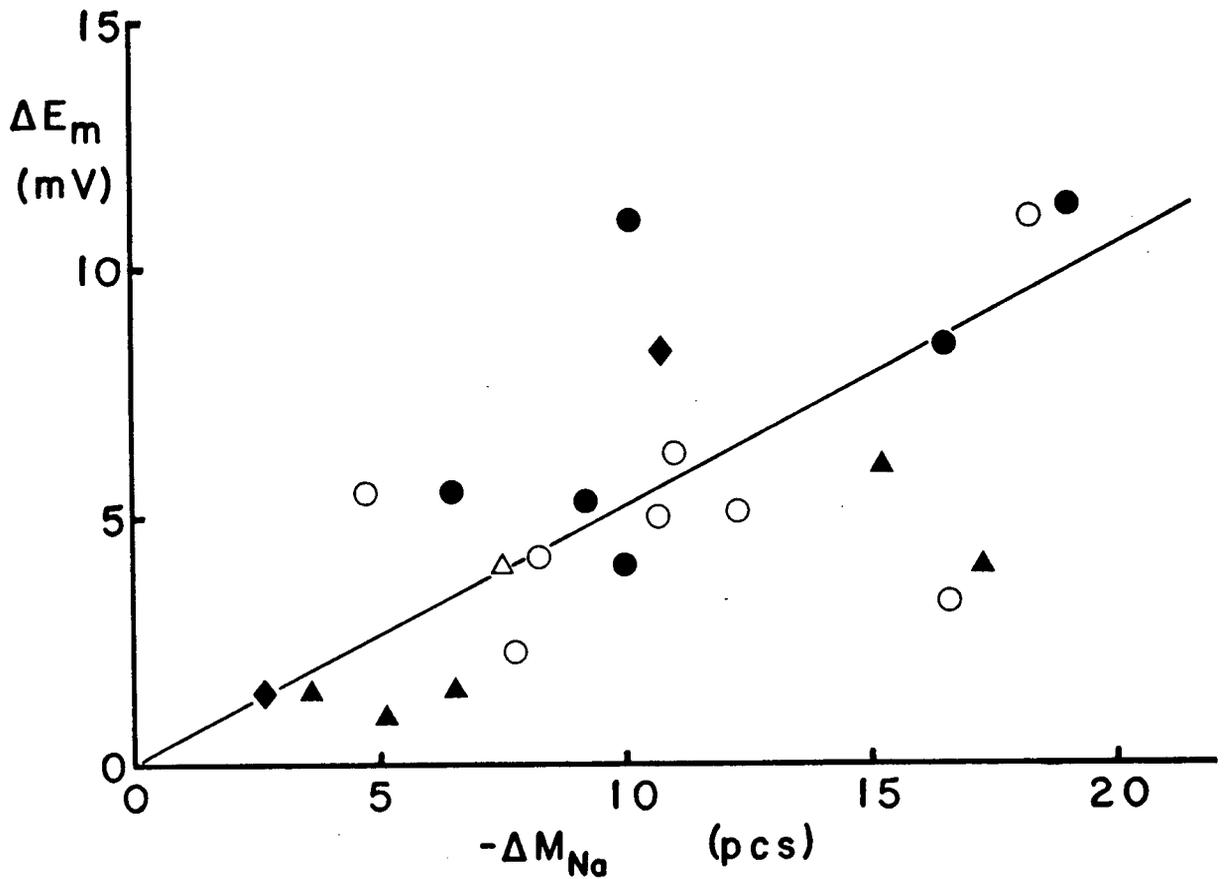
Figure 25. Change in the membrane potential ΔE_m which occurs upon exposure of the cell to ouabain, plotted versus the corresponding simultaneous change in the sodium efflux $-\Delta M_{Na}$ calculated from equation (4), <u>with</u> correction for Na^*_{cell}. Circles: 10^{-4} M. Diamonds: 10^{-5} M. Triangles: 10^{-6} M. Open symbols represent cells dissected on the day of the experiment. Closed symbols represent cells dissected on the day before that of the experiment.

$$\Delta E_m \doteq \left(\frac{R\,T}{F}\right)^2 \cdot \frac{1}{U\,E_m}\left(1 - \frac{1}{\mathcal{R}}\right) \cdot \Delta M_{Na} \cdot \qquad \qquad \ldots(8)$$

where R, T, and F have their usual significance, $U = P_K(K)_o + P_{Na}(Na)_o + P_{Cl}(Cl)_i$. P_j is the permeability of the membrane to species j, $(K)_o$ and $(Na)_o$ are the external concentrations, $(Cl)_i$ is the intracellular concentration, and $\mathcal{R} \equiv -M_{Na}/M_K > 0$ is the coupling ratio for sodium-potassium exchange, assumed here to be almost entirely active.

Fig. 25 exhibits a great deal of scatter, but if it is interpreted in terms of (8), it seems that $\mathcal{R}$ is greater than unity at smaller values of ΔM_{Na}, but closer to unity at higher values. Interpretation of (8) and Fig. 25 will be discussed in detail later in this section.

In Fig. 26 are presented the measured steady values of the membrane potential E_m for cells of different sodium content. The sodium content of the cells was raised by injection of NaCl. Several effects were anticipated. The sodium load should stimulate active sodium expulsion, and any electrogenic effect should become more prominent. On the other hand, the injection of chloride will yield a depolarization, as can be seen from the Goldman-Hodgkin-Katz equation. The injection of sodium tends to yield a hyperpolarization in a similar manner, but P_{Na} is small compared to P_{Cl} so the effect will also be small. Also, the injection of concentrated solutions of NaCl might change the permeability of the cell membrane. The net effect is seen in Fig. 26 to be slight.
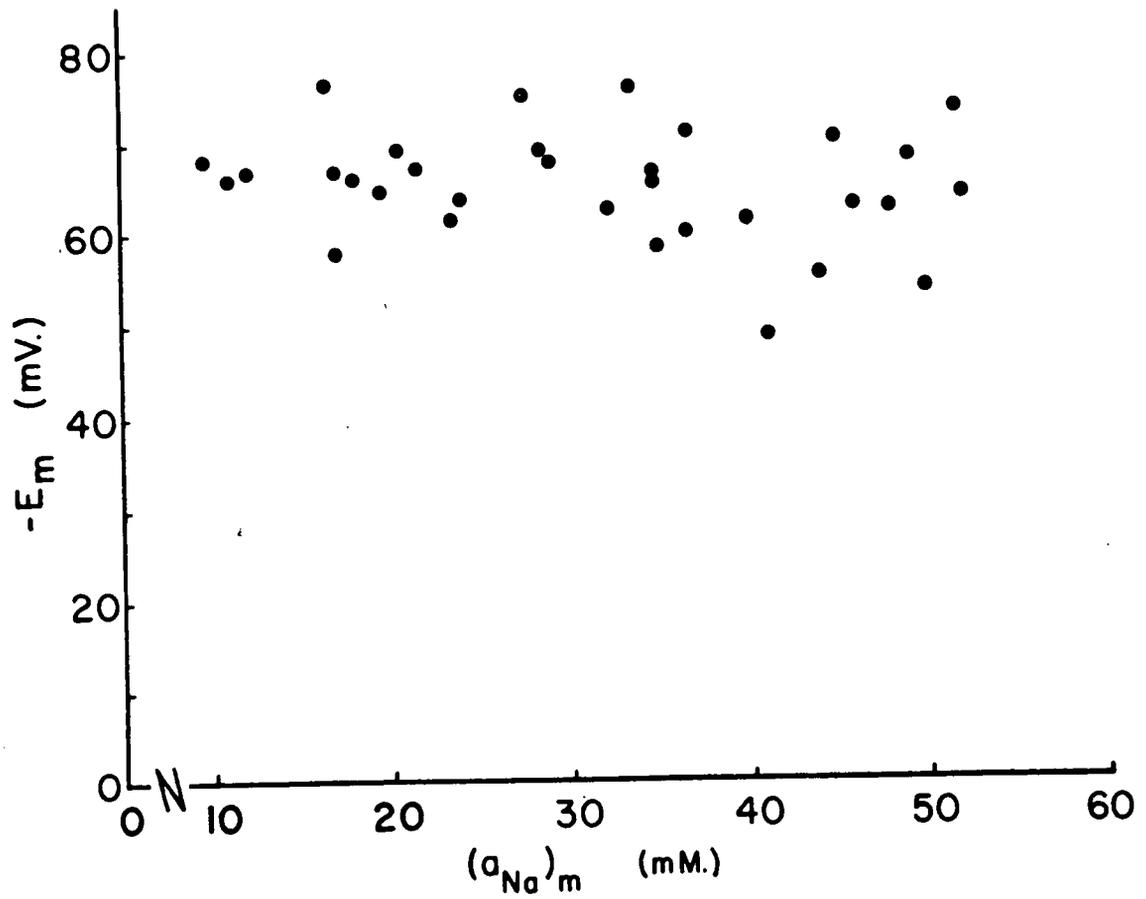
Figure 26. Resting membrane potential for cells in normal Ringer's solution at approximately 23°C, after microinjection, versus the steady value of the myoplasmic sodium activity.

DISCUSSION

<u>Nonelectrogenic membrane potential</u>.

In the GHK equation for the resting membrane potential, the sodium, potassium, and chloride concentrations appear. Typically, P_{Na} is small compared to P_K and P_{Cl}. It was noted that E_m shows little dependence on $(a_{Na})_m$ overall (Fig. 26), and that this could be due to the cancellation of several different effects of injection of NaCl. In section 6, it was noted that in experiments with lithium-substituted sodium-free solution there was a depolarization of a few millivolts shortly after the change from normal Ringer's solution to sodium-free solution, while there was a hyperpolarization after the change from normal Ringer's solution to choline- or tris-substituted sodium-free solution. It was noted above that there was a transient hyperpolarization and a sustained depolarization after the change from normal Ringer's solution to potassium-free solution.

Choline and tris will pass the cell membrane at a much slower rate than will the small inorganic cations. The GHK equation predicts that removal of external sodium should cause a slight hyperpolarization, slight because P_{Na} is small relative to P_K and P_{Cl}. The rate of the sodium efflux into sodium-free solution is not very different from that into normal Ringer's solution (Fig. 19), but the initial stimulation of the sodium efflux in choline- and tris-substituted solutions might cause an increase in the electrogenic hyperpolarization of the cell. The coupling ratio $\mathcal{R}$ of the (Na+K)ATPase might be changed by these manipulations, but there is no basis on which to speculate about such an effect. It might be that the permeability of the membrane to lithium is greater than that to sodium, so that the switch from normal Ringer's solution to lithium-substituted solution yields a depolarization as lithium ions enter the cell.

Thus the net depolarization in sodium-free lithium-substituted solution can be accounted for as the sum of two passive effects: small hyperpolarization due to the reversal of the sodium concentration gradient, and larger depolarization due to the creation of a large cation (lithium) gradient opposing the potassium gradient. On the other hand, the net depolarization in potassium-free solution can be accounted for as the sum of a passive and an electrogenic effect: a hyperpolarization due to an increase in the potassium concentration gradient, and, in the case of Fig. 25, a larger depolarization due to inhibition of the electrogenic sodium-potassium exchange when external potassium is removed. The size of the latter effect is indicated by the larger depolarization which occurs when the electrogenic transport is inhibited with ouabain. In this case, the potassium concentration gradient does not change abruptly.

Electrogenic sodium transport.

The relation between ΔE_m and ΔM_{Na} will be discussed in terms of equation (8) of section 2.F, which was reproduced above. The unbalanced cation efflux is $(1 - 1/\mathcal{R}) \cdot \Delta M_{Na}$ by definition, and as it increases the size of the membrane potential should increase. The proportionality factor between ΔE_m and $(1 - 1/\mathcal{R}) \cdot \Delta M_{Na}$ was discussed in section 2.F.

It has been assumed implicitly in the derivation of this expression that the coupling ratio $\mathcal{R}$ is unchanged by the application of ouabain. That is, it has been assumed that a certain number of the transport enzymes are transporting more sodium than potassium, all with the same ratio. Exposure to ouabain has been assumed to halt the operation of some of these enzymes, but to leave the others unchanged, since in isolated (Na+K)ATPase preparations all but a few partial reactions are halted by the binding of ouabain. This would be so if the transport enzymes were independent, and

if the rate for each enzyme were governed only by the concentration of substrates, in particular by $(Na)_m$. Such an assumption was avoided in the examination of the kinetic characteristics of sodium transport in section 5, but will be made here as the simplest case for the purposes of discussion.

Thus it is assumed that for each data point, $\mathcal{R}$ was unchanged by the manipulation which brought about ΔE_m and ΔM_{Na}. Differences from linearity in Fig. 26 are then due to different values of $\mathcal{R}$ for different experiments, although some of the scatter is probably due to differences in the value of $\dot{E}_m$ for different experiments. The latter can cause variations of 10 to 15% in the proportionality factor in equation (8) (see Fig. 26).

Attempts to calculate the value of $\mathcal{R}$ for each experiment were complicated by the fact that the calculation involves the ratio of small differences between large quantities which are subject to experimental uncertainty. A consistent description can be formulated, however. The slope of the best line drawn by eye in Fig. 25 is 0.52 mV/pcs. The value of U can be estimated from published data to be 10 pcs ($(Na)_o$ = 450 mM, $(K)_o$ = 8 mM, where $\gamma_\pm$ = 0.7; $(a_{Cl})_m$ = 23 mM (Hinke, Caille, & Gayton 1973); P_{Cl}/P_K = 0.04, (Hagiwara et al. 1968); it is assumed that P_K = 1 x 10^{-6} cm/sec and P_{Na}/P_K = 0.01 by analogy with nerve and vertebrate striated muscle). Then $\mathcal{R} \doteq 2.1$, or somewhat greater than the value of 1.5 found in red blood cells. For squid axon the value of $\mathcal{R}$ has been reported to vary with the sodium content (Mullins & Brinley 1969). No claim can be made here beyond consistency of the model and data, but consistency is found.

The assumption that $\mathcal{R}$ is constant is consistent with the results of Thomas (1969) for snail neurone. He found that the feedback current in a voltage clamp was directly proportional to the sodium efflux stimulated by a sodium load, and speculated that the ATP:Na:K ratio of 1:3:2 might be universal. The result in squid axon indicates otherwise.

Further, Kostyuk et al. (1972) found that the feedback current for a
voltage clamped snail neurone was exquisitely sensitive to the value of the
membrane potential. It appeared that M_{Na} was relatively unaffected by
small hyperpolarizations, while M_K was greatly increased. The net effect
was suggested to be a decrease in the pump ratio $\mathcal{R}$ toward unity as the
cell was hyperpolarized. The experiment on which this conclusion is based
appears to show that the pump ratio responds almost instantaneously to
changes in the membrane potential.

It should be mentioned here that Glitsch (1972) presented for cardiac
muscle a plot similar to Fig. 25. He found almost perfect correlation
between M_{Na} and E_m during recovery of the cardiac muscle from incubation in
the cold. However, in his calculation of the unidirectional sodium efflux
as the difference between the net change in sodium content of the tissue as
measured by flame photometry, and the estimated change in the passive sodium
influx in the constant field theory, he has imposed on his data exactly the
functional dependence he claims to find. It seems likely that electrogenic
sodium transport does occur in cardiac muscle, but his methods cannot be
used to obtain accurate measurements of this phenomenon.

In summary, it has been demonstrated that there is a depolarization of
the barnacle muscle cell when the (Na+K)ATPase is inhibited by ouabain.
The size of this depolarization is correlated in size and in time course
with the accompanying decrease in the unidirectional sodium efflux measured
with radiosodium and a sodium-specific microelectrode. These results
establish the existence of electrogenic sodium transport in barnacle muscle.
The response of the membrane potential to potassium-free and to sodium-free
solutions can be explained as the sum of an effect on the passive and the
active contributions.

It was not possible to calculate the coupling ratio for active transport

for the individual experiments, because the calculation involves the ratio of small differences in large quantities and the scatter of the data is quite large. In addition, the permeability of the barnacle muscle cell membrane to the major inorganic cations has not been measured accurately. The results of the present experiments can be interpreted consistently in terms of a phenomenological extension of the constant field model, with a coupling ratio of about two sodium ions transported out per potassium ion transported in, presumably via the (Na+K)ATPase. It was not possible to detect a dependence of the coupling ratio on the membrane potential or on the rate of sodium transport, but such a dependence cannot be ruled out.

This approach to the measurement of the electrogenic potential could be quite fruitful. A specific mechanism for the active cation efflux, such as the assumption of a driving force involving the affinity of the hydrolysis of ATP and the transmembrane difference in the electrochemical potential for sodium, potassium, and chloride, could be used in the derivation of an expression for the total membrane potential. A great deal of preliminary study of the passive permeabilities and the composition of the transmembrane ionic currents will have to be carried out before detailed conclusions can be drawn from radioisotope and voltage clamp experiments, but the findings of such study would be of interest in other contexts as well.

SECTION 8. DISTRIBUTION OF HYDROGEN IONS DURING STEADY CONDITIONS

It has been known for some time that the normal intracellular pH is higher than would be the case if hydrogen ions were distributed passively across the cell membrane (eg. Caldwell 1956; Waddell & Bates 1969). It has been concluded that some process dependent on metabolism and equivalent to the expulsion of hydrogen ions from the cell must exist, but until very recently nothing more could be said.

As summarized in section 2.A, evidence has been found for the involvement of several processes in the active control of the intracellular pH. The electron transport system of aerobic metabolism appears to be able to control the destination of the hydrogen ions it produces. There appears to be in the cell membrane an ATPase which is able to transport hydrogen ions. The extracellular sodium concentration has been found to influence the internal pH and this has been interpreted as sodium-hydrogen exchange. An association between anion fluxes and the internal pH has been found and interpreted as chloride-bicarbonate exchange. Finally, a bacterial membrane protein which can translocate hydrogen ions when exposed to light has been isolated.

The use of tracer techniques like those described in the preceding sections in connection with the sodium flux cannot be used to study hydrogen fluxes. The experimental study of hydrogen ion transport can only be carried out by indirect methods, all of which involve the measurement of changes in the intracellular pH. It thus is very important to ascertain the accuracy and reliability of the methods currently used to measure the intracellular pH. A direct comparison of the two most commonly used methods for measuring the intracellular pH thus was carried out, and the results are presented in section 9 of this thesis.

During these experiments, it was noticed that the transmembrane difference in pH measured with a pH-specific intracellular microelectrode (which agreed with the results of an indicator method), showed a correlation with the size of the membrane potential E_m. Such a correlation is predicted by the current theory of the passive movement of ions across the cell membrane, and has been sought by other workers, but has not been found (Caldwell 1954, 1958; Kostyuk & Sorokina 1961; Aickin & Thomas 1975; Thomas 1976b).

One worker did find a correlation, but of a different sort from that predicted by theory (Carter et al. 1967; Carter 1972). It appears that these results were due to some problem with the measuring techniques, however (Paillard 1972; Lev & Armstrong 1975).

In this section are presented new experimental results pertaining to the relationship between the membrane potential and the transmembrane difference in pH. These results are analyzed in terms of the transmembrane cation distribution predicted by current theory.

METHODS

Single muscle cells of the giant barnacle were dissected as described in section 3. The composition of the special solutions used to cause changes in the intracellular pH was as follows. To raise the intracellular pH: normal barnacle Ringer's solution without tris chloride but with 5mM $(NH_4)_2SO_4$, pH = 8.2 (referred to as "NH_4 Ringer's solution"). To lower the intracellular pH: normal barnacle Ringer's solution without tris chloride but with 1 mM $NaHCO_3$, through which is bubbled continuously at a

constant rate a 95% O_2 -. 5% CO_2 gas mixture to maintain a pH as close as possible to 6.2 (referred to as "CO_2 Ringer's solution"). The composition of normal Ringer's solution without tris chloride is Na 450 mM, K 8 mM, Ca 20 mM, Mg 10 mM, Cl 518 mM. Normal barnacle Ringer's solution contains, in addition, 25 mM tris chloride and is titrated to pH 7.6.

The pH of the bathing solutions was monitored throughout the exposure of the cells to them, with a conventional pH meter (Instrumentation Laboratories). Standard buffer solutions for the calibration of the pH meter and the pH-specific glass microelectrodes were prepared accoring to Robinson (1967). These agreed exactly with commercially made buffer solutions, so the two sets were combined to give five calibration points over the pH range investigated here.

pH-specific glass microelectrodes were constructed by the method of Hinke (1969). Micropipette electrodes for the measurement of membrane potential were constructed by conventional techniques (eg. Hinke 1969), and tested for small tip potential as described in section 3. The technique for making measurements with these microelectrodes was as described in section 3 for sodium-specific electrodes. Occasionally the pH-specific microelectrode was referred directly to the intracellular micropipette electrode as a test of consistency. (The use of an indicator to measure the intracellular pH of identically-prepared companion cells is described in section 9.)

At the end of each microelectrode measurement, the impaled cell and a companion cell were removed for analysis for sodium, potassium, and water content. The electrolyte and water contents of the impaled and non-impaled cells were not significantly different. Furthermore, their mean values (not corrected for the extracellular space) were not different from the mean values of cells used in the experiments involving the pH indicator DMO

(section 9).

RESULTS

Viability of isolated cells.

As in all of the work in barnacle muscle cells reported in this thesis,

if an impaled cell showed any sign of damage the data from it was discarded.

The impaled cells, judged to be damage-free after prolonged immersion in a

test solution, showed resting potentials and electrolyte composition similar

to those reported by others (Tables III and IV).

No dependencies were detected between the sodium, potassium, or water

content of each cell, on the one hand, and the intracellular pH or the

transmembrane pH gradient, on the other hand. Mean values of cell sodium,

potassium, and water content are presented in Table IV in order to provide

an indication of the physiological state of the cells under the three

experimental conditions. Most of the variation demonstrated here probably

is due to animal variations (related to size, age, season, and nutrition)

rather than to experimental conditions. However, the cells equilibrated

in CO_2 Ringer's solution had a larger inulin space (14% of cell water) than

did the cells equilibrated in normal Ringer's solution (8.2%) or in NH_4

Ringer's solution (7.9%) and this would account for the larger total sodium

content of these cells (Table IV).

The resting membrane potential of the cells during the various steady

conditions ranged from -45 to -90 mV (as indicated in Fig. 29), although

the mean value was -72 mV (Table III). Usually, the membrane potential is

used as an indicator of the viability of the cell, as noted in previous

TABLE III

INTRACELLULAR pH AND MEMBRANE POTENTIAL IN CRUSTACEAN MUSCLE

Species	Temp.	Mean E_m	pH_e	Mean pH_i	Reference
A) Maia squinado	20°C	-62 mV	7.55	7.15	Caldwell (1958)
B) Carcinus maenus	20	-40	7.8	7.20	Caldwell (1958)
C) C. maenus	23	-60	7.65	6.91	Paillard (1972)
D) C. maenus	20	-65	7.5	7.27	Aickin & Thomas (1975)
E) Balanus nubilus	23	-72	7.6	7.32	McLaughlin & Hinke (1968)
F) B. nubilus	15	-50	7.8	7.34	Boron & Roos (1976)
G) B. nubilus	22	-59	7.5	7.31	Boron (1977)
H) B. nubilus	23	-72	7.8	7.23	This study

TABLE IV

MEAN WATER AND ELECTROLYTE CONTENT OF TEST CELLS

Equilibration bath	No. of cells	Cell water (%)	Cell (Na) (mmole/kg cell water)	Cell (K) (mmole/kg cell water)
Normal Ringer's	73	74.56 0.11*	42.9 1.3	172.4 1.6
CO_2 Ringer's	55	75.10 0.15	50.4 2.3	182.3 2.3
NH_4 Ringer's	16	73.90 0.28	36.6 3.4	181.0 2.1

Extracellular space contents are included in the analyses.

Cells had been immersed for more than 2 hours in the specified solution.

* S.E. of mean.

sections. Here, cells with membrane potentials which were relatively low (yet as it turned out, still within the normal range reported by other workers) were accepted if no damage was apparent on visual inspection after immersion for 2 hours in the chosen solution, and the sodium, potassium, and water content was within the normal range established by previous work. This was done to see if the relation between membrane potential and pH found in the higher range extended into the lower range.

<u>Intracellular pH from microelectrodes.</u>

Figures 27 and 28 provide examples of changes in the intracellular pH value (pH_i), extracellular pH value (pH_e), and membrane potential E_m with time following a change in the bathing solution. These plots were constructed from data taken every 5 to 15 minutes (where necessary) from original continuous recordings. In Fig. 27, cell A was allowed to equilibrate for 105 minutes after impalement. During this period, the intracellular pH was very steady even though the membrane potential increased from -75 to -81.5 mV and the external pH decreased from 7.69 to 7.62. The 'acid injury' and the slow recovery following electrode penetration as described by Thomas (1974) and Aickin and Thomas (1975) using the recessed-tip pH-specific microelectrode on smaller cells (snail neurone) was not observed here with the spear-type pH-specific microelectrode and barnacle muscle cells.

Following the bath change to CO_2 Ringer's solution the intracellular pH of cell A decreased from 7.31 to 6.94 in about 30 minutes, remained relatively stable for the next 30 minutes, then began to decrease for no apparent reason. Such behavior by a cell has not been explained but was considered undesirable and hence, the few cells showing it were not used in any analysis related to the membrane potential (or to DMO - next section).
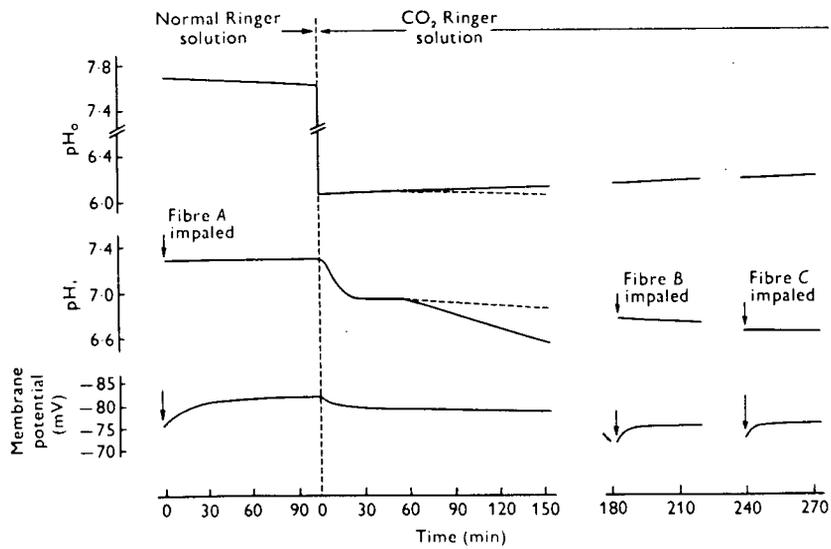
Figure 27. Re-constructed tracings of intracellular pH, pH_i, and membrane potential, E_m, from three cells of a muscle which was subjected to a bath change from normal Ringer's solution to CO_2 Ringer's solution. Vertical arrows indicate when each cell was impaled. pH_o is the extracellular pH.
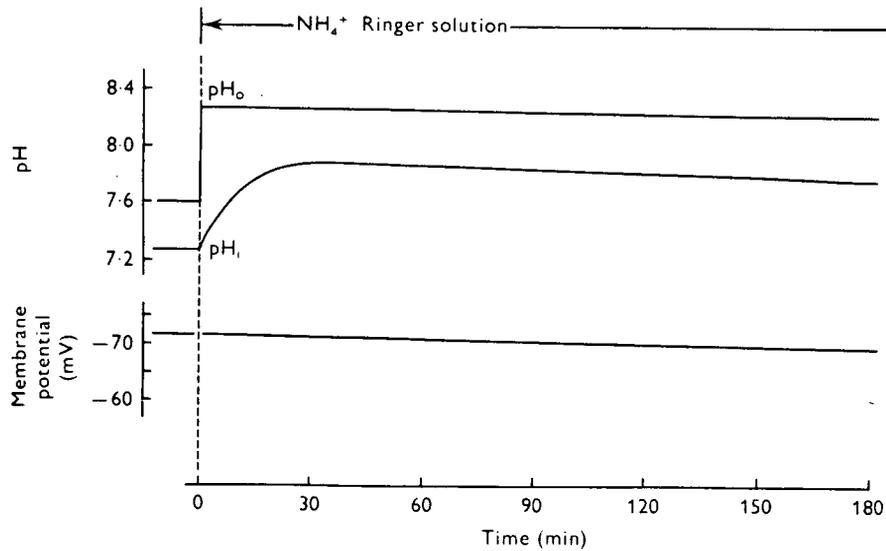
Figure 28. Re-constructed tracings of pH_i and membrane potential E_m from one cell of a muscle which was subjected to a bath change from normal Ringer's solution to NH_4 Ringer's solution. Not shown are the complete tracings following electrode impalement which occurred 90 minutes before zero time. pH_o is the extracellular pH.

Also shown in Fig. 27 are the results from two companion cells with a history of exposure to test solutions identical to that of cell A. Cell B was impaled after 180 minutes and cell C was impaled after 240 minutes of equilibration in CO_2 Ringer's solution.

Fig. 28 shows a typical tracing from a cell which was allowed to equilibrate for more than 60 minutes in normal Ringer's solution (not shown) then exposed to NH_4 Ringer's solution for about 200 minutes.

It was noted that no transients in the intracellular pH occurred when the cell was exposed to CO_2 Ringer's or NH_4 Ringer's solutions. Transients were reported during similar experiments on snail neurone (Thomas 1974). The transients consisted of a slow decline of the intracellular pH during immersion of the cells in NH_4 solution and comparable to the NH_4 Ringer's solution used in the present work, and a slow rise of the intracellular pH during immersion of the cells in HCO_3 solution comparable to CO_2 Ringer's solution. Such effects had not been seen in the preparation used in the studies described here, which were performed prior to the publication of the results in snail neurone. After the results in snail neurone were published, measurements of the type shown in Figs. 27 and 28 were repeated on barnacle muscle cells from a different batch, and it was confirmed that such transients do not occur in the preparation used in these studies.

Subsequently, pH transients similar to those seen in snail neurone were reported in squid axon (Boron & DeWeer 1976), and in barnacle muscle cells (Boron 1977). The preparation used by Boron (1977) was a barnacle muscle cell cut from the basis and cannulated at both ends. The "barnacle sea water" used by Boron was slightly different from but, on the whole, quite similar to barnacle Ringer's solution, aside from the use of 5 mM (N-((2-hydroxy-ethyl)piperazine)-N'-2-ethyl sulfonic acid (HEPES) as buffer rather than tris. However, the cells had to be immersed in calcium-free

solution during the cannulation procedure to prevent the occurrence of "serious damage." This might have changed the permeability properties of the membrane. It was noted that the membrane potential of this preparation was relatively low (Table III). The sodium, potassium, and water content of the cells were not reported.

Membrane potential and the transmembrane gradient of pH.

In Fig. 29 is plotted the transmembrane difference in pH, (pH_e - pH_i), measured by pH-specific electrodes, versus the corresponding membrane potential measured by a conventional micropipette electrode. Only one value of pH_i and E_m were taken from each cell. The cells had been immersed in one of the three test solutions for at least 2 hours before measurements were made. Acceptable values were those which varied no more than 0.04 pH units and ± 1mV over a 30 minute period. Almost all cells tested were acceptable by this criterion.

The mean values of (pH_e - pH_i) and E_m for the different cell types listed in Table III are plotted as letters A to G for comparison.

In Fig. 29, from above downward, the continuous line represents the Gibbs-Donnan relation for hydrogen: pH_e - pH_i = $-(FE_m)/(2.3RT)$; the upper dashed line is the linear regression of the data for cells in normal Ringer's solution; and the lower dashed line is the linear regression for cells in CO_2 Ringer's solution. The linear regression for cells in NH_4 Ringer's solution was similar to that for cells in normal Ringer's solution and is not shown. The slope and y-intercept for each regression plot are listed in Table V.

Fig. 29 shows that the steady conditions for each test solution do not conform to a Donnan equilibrium for hydrogen ions across the cell membrane, but they do show a dependency of (pH_e - pH_i) on E_m with a Donnan-type
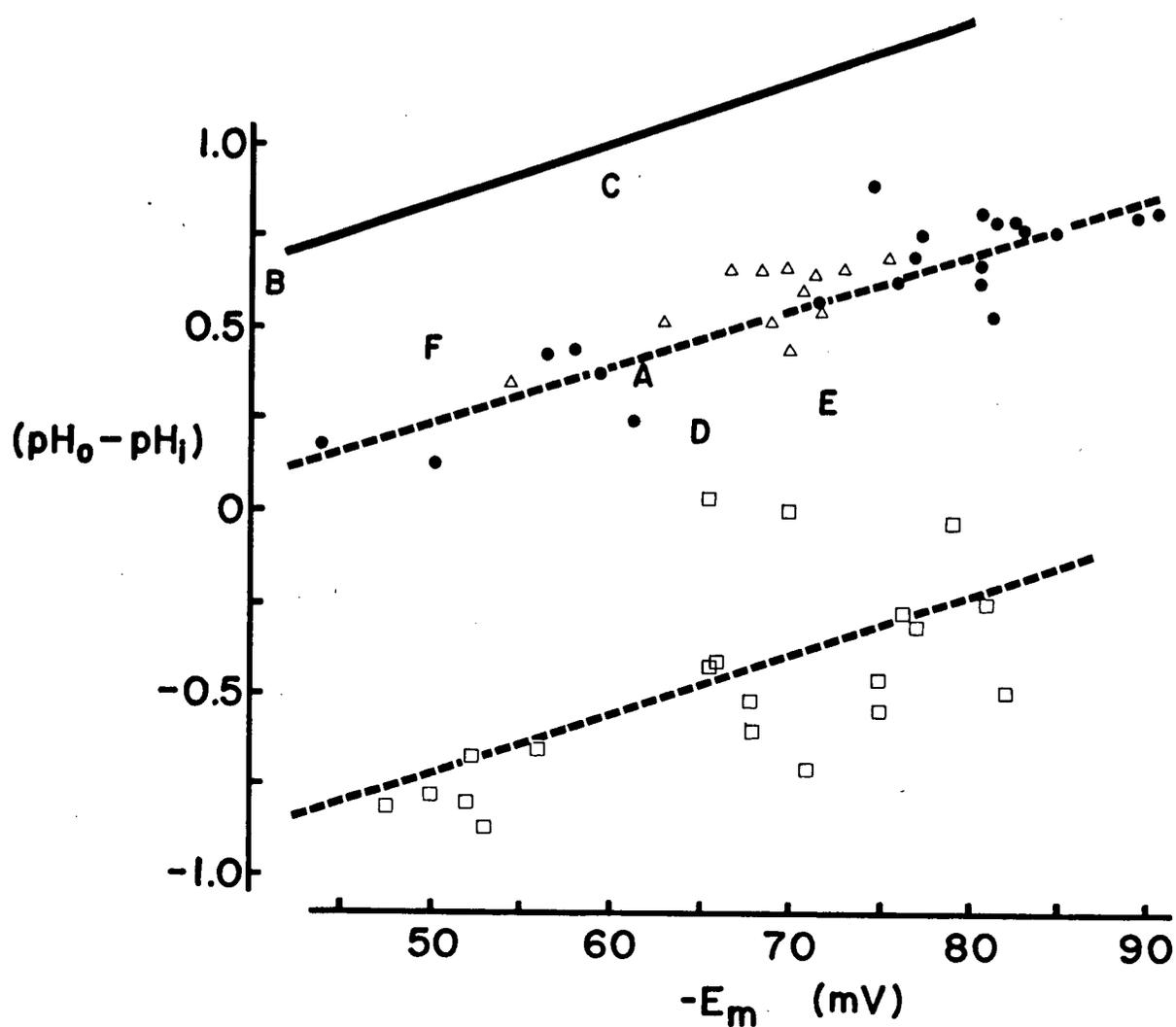
Figure 29. Relationship between the $\log_{10}$ of the transmembrane hydrogen ion activity gradient and the resting membrane potential from single cells equilibrated for more than 2 hours in one of three solutions. Filled circles: normal Ringer's solution. Open triangles: NH_4 Ringer's solution. Open squares: CO_2 Ringer's solution. The two dashed lines are the regression lines for the normal Ringer's data and the CO_2 Ringer's data. The line for the NH_4 Ringer's data is not shown. The upper continuous line represents the Gibbs-Donnan relation. Note that a cell is only represented once in this plot and no cell was included if its internal pH was found to vary by more than 0.04 in 30 minutes after a 2 hour incubation. Cell membrane potential and bath pH always varied less than pH_i. Letters A - F represent data from other workers summarized in Table III.

slope $(-F/RT)$.

TABLE V

CALCULATION OF FLUX j^m FROM DATA OF FIG. 29

Solution	NH_4	Normal	CO_2
No. of experiments	12	21	20
Mean pH_e	8.18	7.84	6.19
S.E.	0.007	0.03	0.016
Slope (mV^{-1})	-0.0140	-0.0155	-0.0165
S.E.	0.004	0.002	0.005
y-intercept*	-0.37	-0.52	-1.55
S.E.	0.30	0.14	0.32
j^m/P_H (mole-cm^{-3} at $E_m = -70mV$)	1.1×10^{-11}	3.0×10^{-11}	1.8×10^{-9}

* y-intercept $= \log_{10}\left[1 - \dfrac{j^m}{P_H(H)_e}\left(\dfrac{1 - \exp((FE_m)/(RT))}{(FE_m)/(RT)}\right)\right]$

DISCUSSION

Absence of pH transients.

Transient changes of pH_i have been found in preparations of snail

neurone (Thomas 1974), squid axon (Boron & DeWeer 1976b), and barnacle

muscle (Boron 1977) upon exposure of these cells to NH_4 and CO_2 solutions

similar to NH_4 Ringer's and CO_2 Ringer's solution. On the other hand, no

such transient changes of pH_i were found in the barnacle muscle cell

preparation used here. The properties of the cell membrane in nerve might

be different from those in muscle. The barnacle muscle cell preparation used by Boron (1977) was cut from the basis, exposed to calcium-free solution, and cannulated at both ends. It seems likely that this treatment and perhaps the slightly different solutions used have altered the permeability properties of the cell membrane. For example, the membrane potential is rather low in Boron's preparation.

The technique of 'pH transients' has revealed several new properties of the mechanism which regulates pH_i, notably the influence of anion fluxes and extracellular sodium. Because of the disagreement of the results in intact cells exposed only to physiological solutions before testing, and the preparation of Boron (1977), it is not certain to what extent the pH transients detected reflect the normal functioning of the cell.

It seems imperative that an attempt be made to clarify this situation, so the results obtained with this potentially very useful new technique can be interpreted with confidence in muscle cells.

$(pH_e - pH_i)$ versus E_m.

The steady value of $(pH_e - pH_i)$ from cells in three different conditions of acidity show a linear dependence on the membrane potential with a slope near $F/(2.3RT)$, (Table V), the slope of the Donnan line. Both Caldwell (1954, 1958) and Kostyuk and Sorokina (1961) searched for a relation between pH_i and E_m but were unable to establish one.

Carter and co-workers (1967) observed a linear relation between $(pH_e - pH_i)$ and E_m but their relation was different in that all their data points followed the Donnan line. Furthermore, Carter reported that whenever the membrane potential was altered the intracellular pH changed almost instantaneously to an appropriate value along the Donnan line. Although no

deliberate attempt to change E_m was made in the present experiments, these experiments provide numerous examples (eg. Figs. 27 & 28) which indicate that pH_i and E_m often varied independently on a short time scale. Aickin and Thomas (1975) observed little change in pH_i for snail neurone when the membrane potential was increased for 10 minutes following modest changes in the potassium content of the external solution. Similarly, examination of the results on the sodium distribution in the cells on which sodium flux studies reported in the preceding sections were done revealed little dependence of $(Na)_m$ on E_m. Recall that the sodium content of the cells had been changed artificially to obtain a range of values of $(Na)_m$.

The reason that different workers have sought a relationship between $(pH_e - pH_i)$ and E_m is that a dependency between the two is predicted by the accepted theory for ion fluxes across membranes. Active ion transport also occurs, however, so the dependency might not be apparent in the whole cell. A formal derivation of the relationship between the steady transmembrane distribution of cations and the membrane potential is presented in section 2.F(ii). The result for hydrogen ions is

$$pH_e - pH_i = \log_{10}\left[1 - \frac{M}{RT(H)_e}\right] - \frac{F}{2.3RT}E_m \qquad \ldots (6)$$

where F, R, and T have their usual significance, $(H)_e = 10^{-pH_e}$, and M is a definite integral which contains the active hydrogen ion transport (or its equivalent). No assumption has been made except that the driving force for passive cation flux is given by the Nernst-Planck equation and is independent of the active flux. The result (6) has been cast in a form which corresponds to the equation for the lines in Fig. 29 to emphasize that quite elementary principles seem to predict such a relationship.

If now the constant field assumption is adopted and the active flux

j^m is assumed to be constant, the integral M can be evaluated and (6) becomes

$$pH_e - pH_i = \log_{10}\left[1 - \frac{j^m}{P_H(H)_e}\left(\frac{1 - \exp(FE_m/(RT))}{FE_m/(RT)}\right)\right] - \frac{F}{2.3RT}E_m \quad \ldots(11)$$

where P_H is the permeability of the membrane to hydrogen ions. The term in square brackets corresponds to the y-intercept listed in Table V.

The term in square brackets in (11) cannot be expected to assume the same value during all steady conditions in a given test solution, since it contains E_m and because j^m/P_H might vary with E_m. However, since the slope of the plot for each test condition appears to be relatively constant, and nearly equal to $F/(2.3RT)$, (Fig. 29), it follows that the y-intercept for a given test condition must be relatively constant, at least for the range of membrane potential in question (-45 to -90 mV). It also follows that j^m/P_H must vary with E_m at about an equal and opposite rate as the round bracket term varies, if the constant field approximation is taken at face value for the purposes of discussion.

The calculation of parameters via the approximate equation (11) is semiquantitative, but it provides a means of estimating the size of j^m in the three test situations. In Table V are shown the values of j^m/P_H for each situation when E_m is -70 mV. An estimate for P_H of 5×10^{-4} cm/sec has been provided by Woodbury (1971). The values in Table V indicate that the hydrogen ion transport system is required to function about two orders of magnitude faster when the intracellular pH is reduced from 7.2 to about 6.7 by immersion of the cell in CO_2 Ringer's solution. They also indicate that the hydrogen ion transport system is not challenged when the intracellular pH is raised to 7.6 by immersion of the cell in NH_4 Ringer's solution.

In summary, it has been found that following prolonged immersion of

the cells in the various test solutions, the transmembrane hydrogen ion distribution is related to the membrane potential but not in accordance with a simple Gibbs-Donnan distribution. A model which recognizes the existence of two independent net fluxes of hydrogen ions across the cell membrane was developed to account for the results. One of the fluxes represents passive movement and the other represents active hydrogen ion transport or an equivalent process. Semiquantitative estimates from the model yield an increase in the active hydrogen transport by two orders of magnitude when pH_i is reduced from 7.2 to 6.7. In the absence of more information about the actual mechanisms which regulate the intracellular pH, and about the passive permeability of the membrane to hydrogen ions, nothing further can be gained from these results.

SECTION 9.   COMPARISON OF THE INTRACELLULAR pH

MEASURED BY DMO AND BY MICROELECTRODES

The experimental study of hydrogen ion transport can only be carried

out by indirect methods, all of which involve the measurement of changes in

the intracellular pH, (pH_i) (eg. Waddell & Bates 1969). Use of an intra-

cellular pH-specific microelectrode is probably the method of choice,

especially for the measurement of the recently-discovered transient changes

in pH_i which seem to be due to an active process which regulates pH_i (Thomas

1974; Boron & DeWeer 1976b; Boron 1977).

Of the several methods employed or proposed for measuring pH_i (Waddell

& Bates 1969; Rose 1968; Moon & Richards 1973), the one most often used is

the 'weak electrolyte distribution' method, with the weak acid 5,5-dimethyl-

oxazolidine-2,4-dione (DMO) as the indicator. Indicator methods yield an

estimate for pH_i which reflects the pH of all compartments in the cell which

are accessible to the indicator, and not all of these have the same pH.

For example, mitochondria behave as if they have a relatively high pH

(Garthwaite 1977). An intracellular pH-specific microelectrode will measure

the pH in the intracellular solution which surrounds the sensitive tip, but

it is technically very difficult to construct such electrodes with sensitive

tips sufficiently small for use in most cellular systems of interest.

Waddell and Bates (1969) noted that some of the uncertainties of the

weak electrolyte method could be clarified by a study which combines the

weak electrolyte and microelectrode methods in the same cellular system.

The results of such a study (accounts of which have already been published -

Menard, Nee, & Hinke 1975; Hinke & Menard 1976, 1978) are presented in this

section.

A similar study using barnacle muscle cells was carried out indepen-

dently by Boron and Roos (1976), and will be discussed. A comparison of the DMO and electrolyte methods as applied to cells of the plant <u>Nitella translucens</u> has also been published (Spanswich & Miller 1977). It suffers from problems with ensuring that the sensitive tip of the electrode is intact and entirely within the intracellular region of interest, and with determining the cytoplasmic volume. It will not be discussed further.

The results from the studies using barnacle cells indicate that the DMO method can yield a value for the pH inside the cell during steady conditions which is not too different from the microelectrode result. However, some new difficulties with the DMO method were found.

METHODS

Two muscle bundles from a single barnacle were dissected as described in section 3. Both were then immersed in one of three test solutions (normal Ringers, CO_2 Ringer's, or NH_4 Ringer's solutions) for 2 hours at 23° C before measurements were begun. One bundle was used for microelectrode measurements and one for a DMO uptake experiment. At the start of the DMO experiment, the bathing solution was altered only by the addition of radioisotopes.

Measurements with pH-specific microelectrodes and chemical analysis of the cells were described in section 8. The DMO technique will be described in detail here.

The base plate of the muscle bundle was anchored to a glass bar and the short tendon of each cell was anchored to another glass bar by means of silk threads during incubation. The cells were more or less parallel to

one another and approximately at their in situ length. This assembly was then immersed in a continuously stirred 100 ml bath of Ringer's solution (one of the three above) to which was added (^{14}C)DMO (to 300 disintegrations per minute (dpm)/μl, SA = 8.8 mc/mole) and either (^{3}H)inulin (to 1000 dpm/μl, SA = 122 mc/g) or (^{3}H)sorbitol (to 1000 dpm/μl, SA = 200 mc/mmole). At various times over the next 3 hours, single cells were cut away from the assembly, rinsed for 30 seconds in isotonic sucrose wash solution (Table I), blotted on filter paper and placed in pre-weighed stoppered liquid scintillation vials. After recording the wet weight and constant dry weight, the amounts of ^{14}C and ^{3}H in each cell were determined on two channels of the Mark II Liquid Scintillation Counting System (Nuclear-Chicago, Searle). A manually adjustable window was used for the ^{3}H channel and a pre-set double-label window was used for the ^{14}C channel to maximize single channel counting efficiencies and to minimize channels overlap. Efficiencies were determined by the external standards ratio method. The sixteen quenched standards (Amersham-Searle) contained ^{3}H or ^{14}C dissolved in the same scintillation medium as the unknown samples. Every sample was counted for at least 20 minutes and all sample counts were stable. Some cells were wet ashed and analysed for sodium and potassium content by flame photometry to check on the physiological state of the cells. The bath was routinely analysed for ^{14}C, ^{3}H, sodium, and potassium at the beginning and end of each experiment. The pH of the bath was monitored continuously using commercial electrodes and a conventional pH meter.

Calculations.

Fig. 30 summarizes how DMO is expected to cross the membrane and, once across, how it is expected to behave. It is assumed that the non-ionized form of the weak acid (HDMO) crosses the membrane readily and passively,
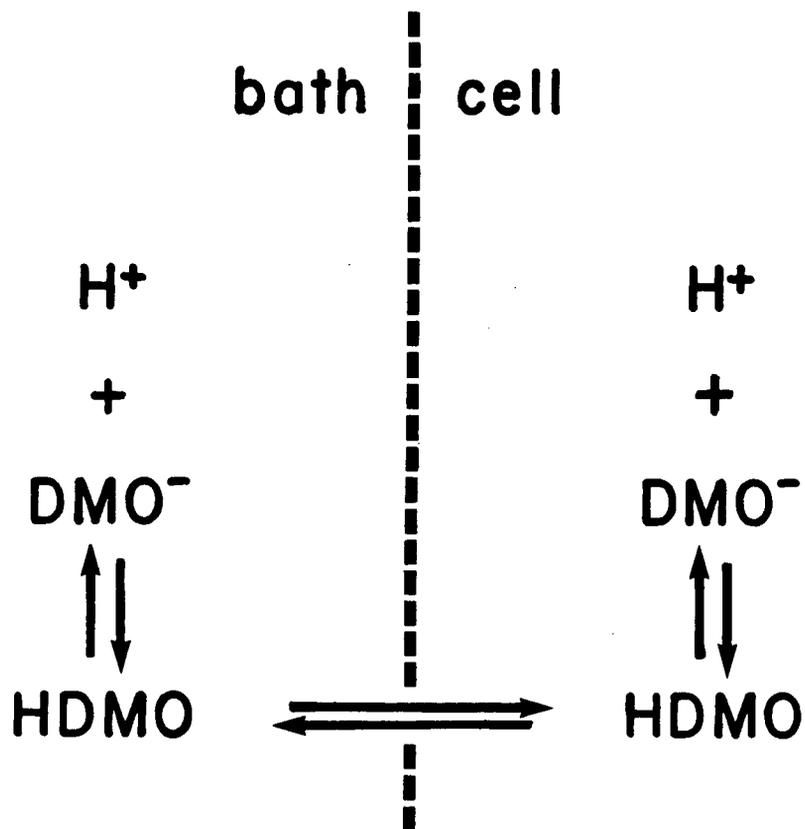
Figure 30. Diagram illustrating the cellular permeation, equilibration, and dissociation of a weak acid (HDMO), assuming only the non-ionized form of the acid passes the membrane.

and that a steady state is eventually achieved when $(HDMO)_i$ equals $(HDMO)_e$.
In fact, of course, the ionized form of DMO will cross the membrane but
only at a relatively very slow rate. It is also assumed that the two aqueous
phases are homogeneous and that HDMO is able to distribute itself uniformly
in each phase. Finally, it must also be assumed that the dissociation
constant, K', for HDMO is similar in the two aqueous phases.

With these assumptions, Waddell and Butler (1959) proposed the
existence of two distinct ionization equilibria for HDMO, one inside and
the other outside the cell (Fig. 30). They combined the two mass law
equations defining the two equilibria to obtain the following expression
for pH_i, "pH_{DMO}":

$$pH_i = pK' + \log_{10}\left\{\left[\frac{C_t}{C_e}\left(1 + \frac{V_e}{V_i}\right) - \frac{V_e}{V_i}\right]\left[10^{(pH - pK')} + 1\right] - 1\right\} \quad \ldots(12)$$

where pH_e is the pH of the bath, V_i is the analysed cell water, V_e is the
extracellular water of the cell, $V_i = V_t - V_e$, C_t is the total DMO content
of the cell, and C_e is the total extracellular DMO of the cell.

Waddell and Butler examined the partial derivative of the ratio C_t/C_e
with respect to pH_e as an index of precision when calculating pH_i from the
weak electrolyte distribution method. They recommended the use of DMO
partly because of its inertness intracellularly and partly because the pK'
of DMO was such as to yield a high value for the partial derivative
$$\frac{\partial (C_t/C_e)}{\partial pH_e}.$$

In the present experiments where two radioactive labels were used
simultaneously, C_t and C_e are of less interest than the seven parameters
which were actually measured to obtain pH_{DMO}. The Waddell and Butler
equation was therefore rewritten as follows:

$$pH_{DMO} = pK' + \log_{10}\left\{ \left(\frac{[B_H] \, V_t}{D_H} - 1 \right)^{-1} \left(\frac{[B_H] \, D_e}{[B_C] \, D_H} - 1 \right) \left[10^{(pH_e - pK')} + 1 \right] - 1 \right\} \quad ..(13)$$

where $B_H = ((^3H)\text{inulin})_e$ in dpm per unit bath volume, $B_C = ((^{14}C)\text{DMO})_e$ in dpm per unit bath volume, D_H is total ^3H (dpm) in a blotted fibre, and D_C is total ^{14}C (dpm) in a blotted fibre. This was the equation used to calculate a pH_{DMO} value for every cell exposed to the two isotopes regardless of how long the cell was in the equilibrating medium.

pK' for DMO.

The $(^{14}C)\text{DMO}$, $(^3H)\text{inulin}$, and $(^3H)\text{sorbitol}$ were obtained from New England Nuclear at the specific activities stated above. Non-radioactive DMO was obtained from Eastman Organic Chemicals. The latter was used to determine a pK' value for DMO at 23° C and in the presence of half-molar NaCl, corresponding to the uptake experiment. NaOH (CO_2-free) was used to titrate the acid form of DMO at two concentrations (0.5 and 1.0 mM), both in the absence and in the presence of 0.573 M NaCl. The NaOH (CO_2-free) was obtained by treating a freshly prepared AgOH precipitate with a purged NaCl solution: the final concentration was tested by titration of potassium hydrogen phthalate. The conductimetric end point of the DMO-NaOH titration in the absence of NaCl was determined first and then the titration curve for the same volume of solution, containing the same amount of DMO, plus NaCl to 0.573 M was obtained, using a Radiometer Titrigraph. The resulting mean pK' values are 6.170 (SD of an observation = 0.032, n = 4) in the presence of 0.573 M NaCl and 6.443 (SD of an observation = 0.051, n = 4) in the absence of NaCl. The former value was used in equation (13) for all pH(DMO) calculations. For comparison, Boron and Roos (1976) give a pK' value of 6.18 at 25° C for DMO in 'barnacle sea water' and a pK' value of 6.33 at 25° C for DMO in water.

<u>RESULTS</u>

<u>Course of the uptake of DMO and inulin.</u>

The time course of penetration for the extracellular space marker
((^{3}H)inulin) into the single cell can be expressed as the ratio (V_e/V_t)
of the calculated volume of the extracellular space to the total cell water
content (assuming water density is 1 g/cm^3 in both cases). This quantity
is suitable for comparison between different cells. The same calculation
can be done for the (^{14}C)DMO marker, to yield a space which has no signifi-
cance as a volume but which is useful as a cell-specific index for compari-
son of the DMO content in different cells.

Ideally, when the uptake of (^{14}C)DMO or (^{3}H)inulin is calculated in
this manner and plotted against time of exposure to the isotopes, the
resulting curve for each isotope should show a fast initial rise from zero
to a value which remains constant with time. Actually, what one finds is
a fast initial rise followed by a slow sustained rise which is nearly
linear with time. In Fig. 31, for example, are shown the curves for the
uptake of (^{14}C)DMO and (^{3}H)inulin by the cells of one bundle (after 2 hours
of immersion in non-radioactive normal Ringer's solution) placed at time
zero into the normal Ringer's solution containing the two isotopes. The
slow linear rise in both these curves is usually interpreted as the result
of some non-saturable non-specific adsorption and not as slow entry of the
isotopes into regions to which access is difficult. This interpretation
is supported by the finding that the addition of 1 mM unlabelled DMO (more
than 65 times the concentration of (^{14}C)DMO) to the Ringer's solution,
both before and during uptake, did not produce any change in the slopes of
the 'plateau' of the (^{14}C)DMO or (^{3}H)inulin uptake curves. The slow uptake
for (^{14}C)DMO and (^{3}H)inulin, as in Fig. 31, was rather similar in all ten
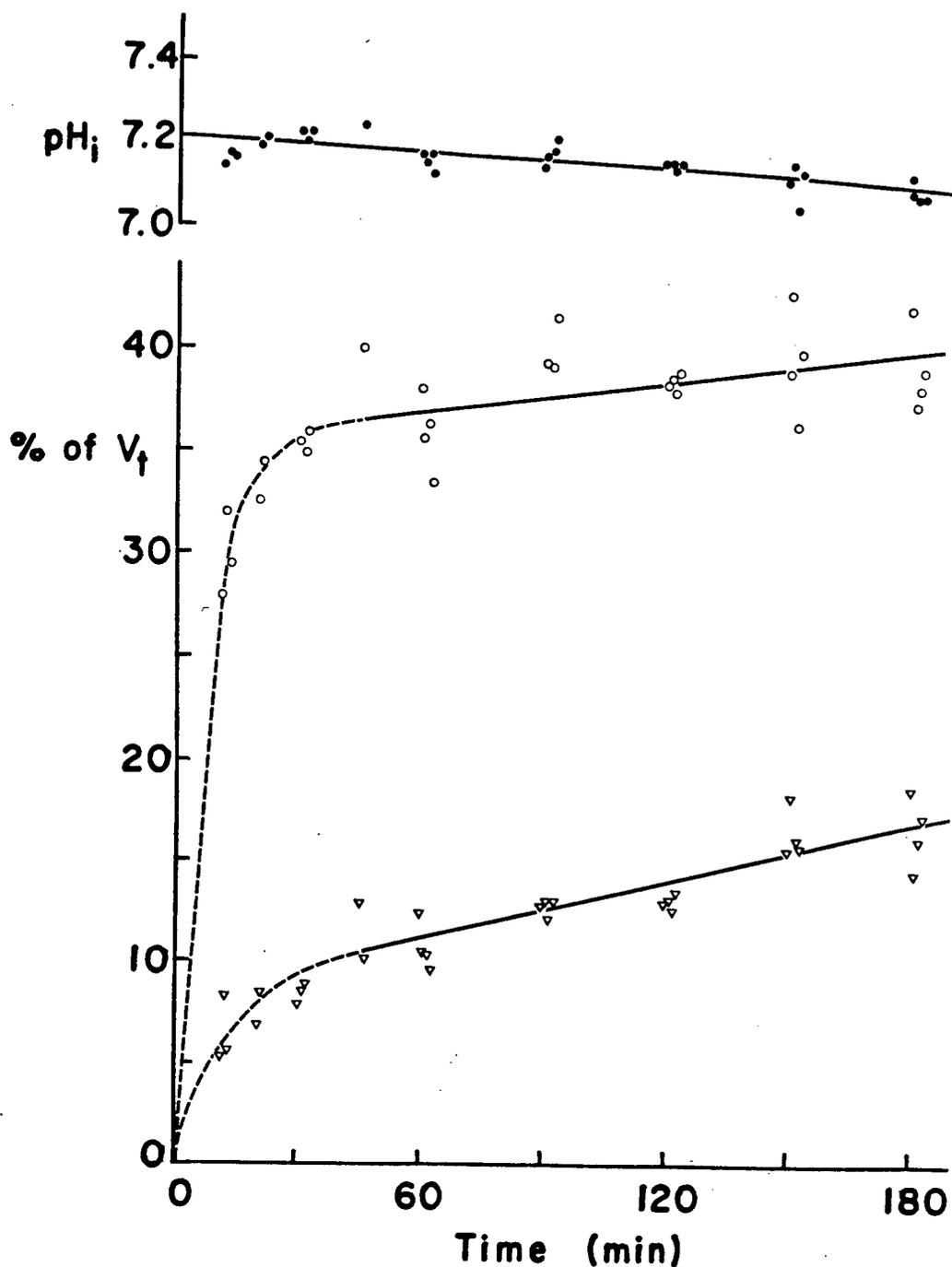
Figure 31. Uptake of the indicator compounds with time by cells in normal
Ringer's solution after 2 hours of pre-incubation in non-radioactive Ringer's
solution. The data are from one muscle and each entry is from a separate
cell. Triangles: inulin. Open circles: DMO. Filled circles: calculated pH_i
for each cell, from equation (13). Continuous lines are plotted from a
linear regression and dashed lines are drawn by eye.

experiments but an inspection of Figs. 32 and 33 shows that the 'plateau' slopes for (^{14}C)DMO and (^{3}H)inulin can be opposite to one another. This indicates that the adsorptions of DMO and inulin are quite independent of one another.

Intracellular pH from DMO.

The double isotope technique permitted the calculation of a pH(DMO) for every cell removed from the bath (using equation (13)). For example, the intracellular pH(DMO) points in the upper curve of Fig. 31 were obtained from the (^{14}C)DMO and (^{3}H)inulin points of the two uptake curves of the same figure. It can be noted that all of the pH(DMO) values are rather similar, i.e. they change very little with uptake time. Even during the first 10 minutes when (^{14}C)DMO and (^{3}H)inulin are taken up rather rapidly, the calculated pH(DMO) is similar to the pH(DMO) values for the slow rise period (times greater than 45 minutes). The pH of the bath for this experiment remained constant at 7.79.

An example of double isotope uptake during low pH conditions is shown in Fig. 32, and an example during high pH conditions is shown in Fig. 33. For all three conditions the pH(DMO) curve consistently declined linearly with time and it does not appear to be related to the uptake behavior of either one of the two isotopes.

All the (^{14}C)DMO uptake points in Fig. 32 were divided by a factor of 5.5 in order to present the data of the low pH experiment in a more compact form. The fact that it was necessary to do this illustrates that a great deal more neutral DMO is available for intracellular penetration under low pH conditions than under normal or high pH bath conditions. For example, the concentration of neutral DMO (pK = 6.17) should increase sixteen times when the bath pH is lowered from 7.66 to 6.20.
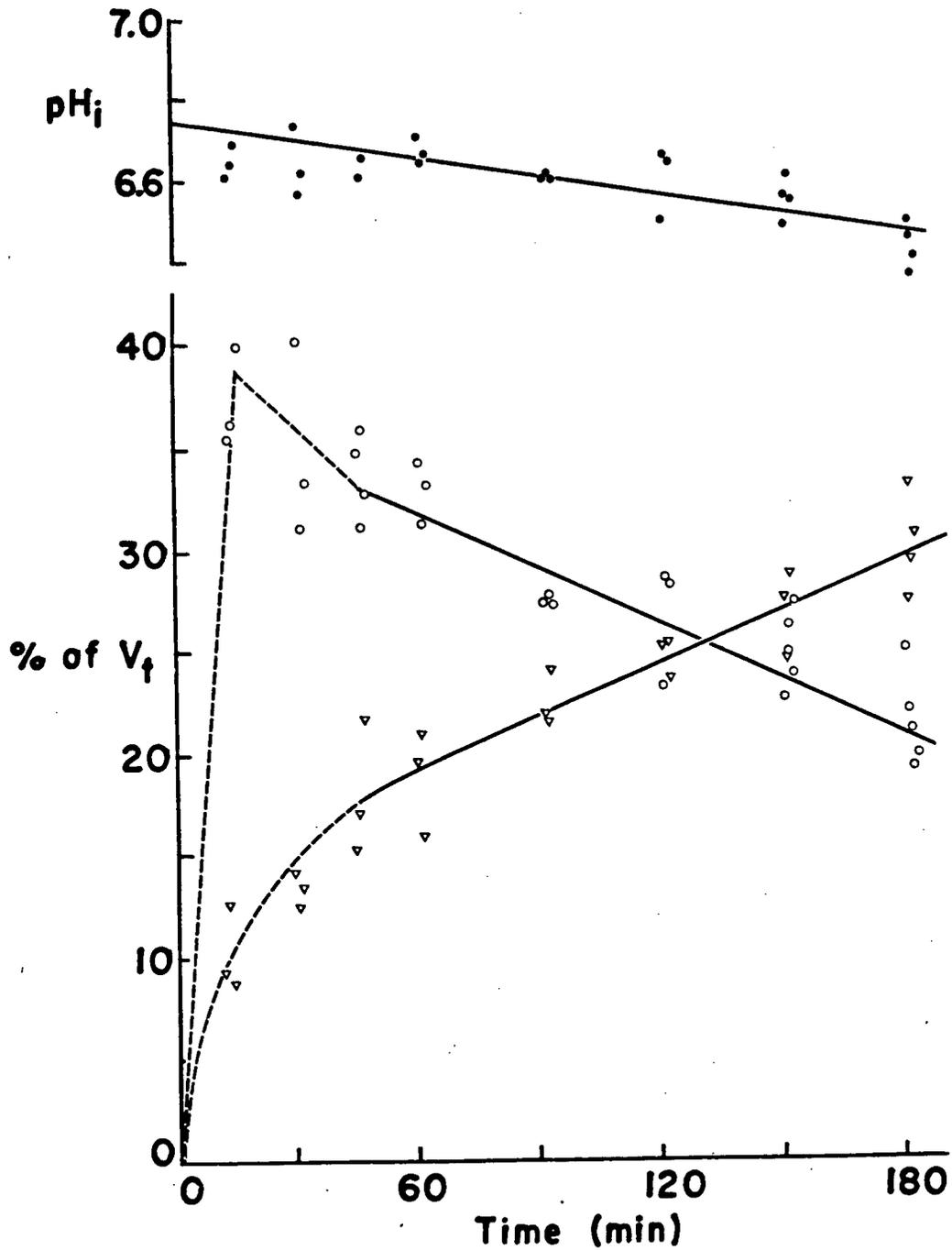
Figure 32. Uptake of indicator compounds with time by cells in CO_2 Ringer's solution after 2 hours of pre-incubation in non-radioactive CO_2 Ringer's solution. Symbols are as in Fig. 31. Note: all of the DMO uptake values (open circles) were divided by 5.5 to facilitate plotting.
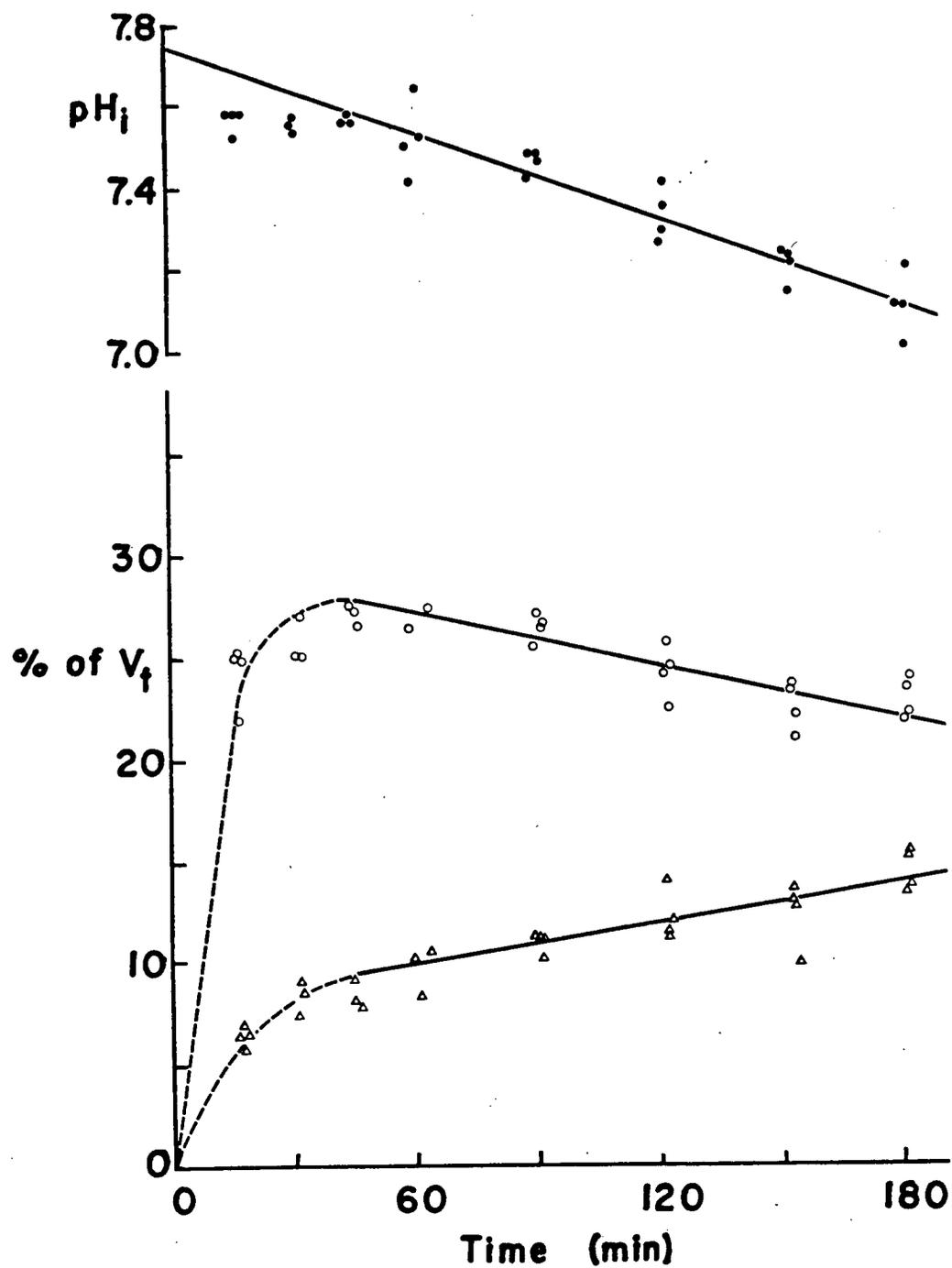
Figure 33. Uptake of indicator compounds with time by cells in NH_4 Ringer's solution after 2 hours of pre-incubation in non-radioactive NH_4 Ringer's solution. Symbols are as in Fig. 31.

Intracellular pH from microelectrodes.

It was shown in the preceding section that the intracellular pH measured with the microelectrode was very steady after two hours of immersion in any of the three test solutions (Figs. 28 & 29).

Comparison of pH(DMO) and pH_i results.

Two methods were adopted to compare the pH(DMO) values with the pH_i (microelectrode) values in the three test conditions. In one (Fig. 34a), the pH(DMO) value at zero time (obtained by extrapolation to zero time of the regression line through the points after 45 minutes of the pH(DMO) curve) is compared to the mean pH_i for microelectrode measurements on companion cells selected as described above. In the second method (Fig. 34b), the mean of all pH(DMO) values after 45 minutes is compared to the same mean pH_i. The 'extrapolated' pH(DMO) value in Fig. 34a should be independent of artifacts due to non-specific adsorption of the two indicators. The second method is similar to the common practice of assuming that DMO uptake reaches a single constant value after 1 hour. Only complete experiments are represented in Fig. 34a and 34b, i.e. a full double isotope uptake experiment combined with successful microelectrode impalements of at least three and usually five cells.

It can be noted that the 'extrapolated' pH(DMO) value is nearly always higher than the mean pH_i value. For example, the regression line (continuous) through the points predicts a pH(DMO) of 7.18 when pH_i is 7.00. Since the regression line is nearly parallel to the identity line (dashed), one can expect the 'extrapolated' pH(DMO) values to yield correctly the change in intracellular pH. It can be noted from Fig. 34b, however, that the regression line for the 'plateau' pH(DMO) values crosses the identity line. It is unlikely, therefore, that measured changes in 'plateau' pH(DMO) would
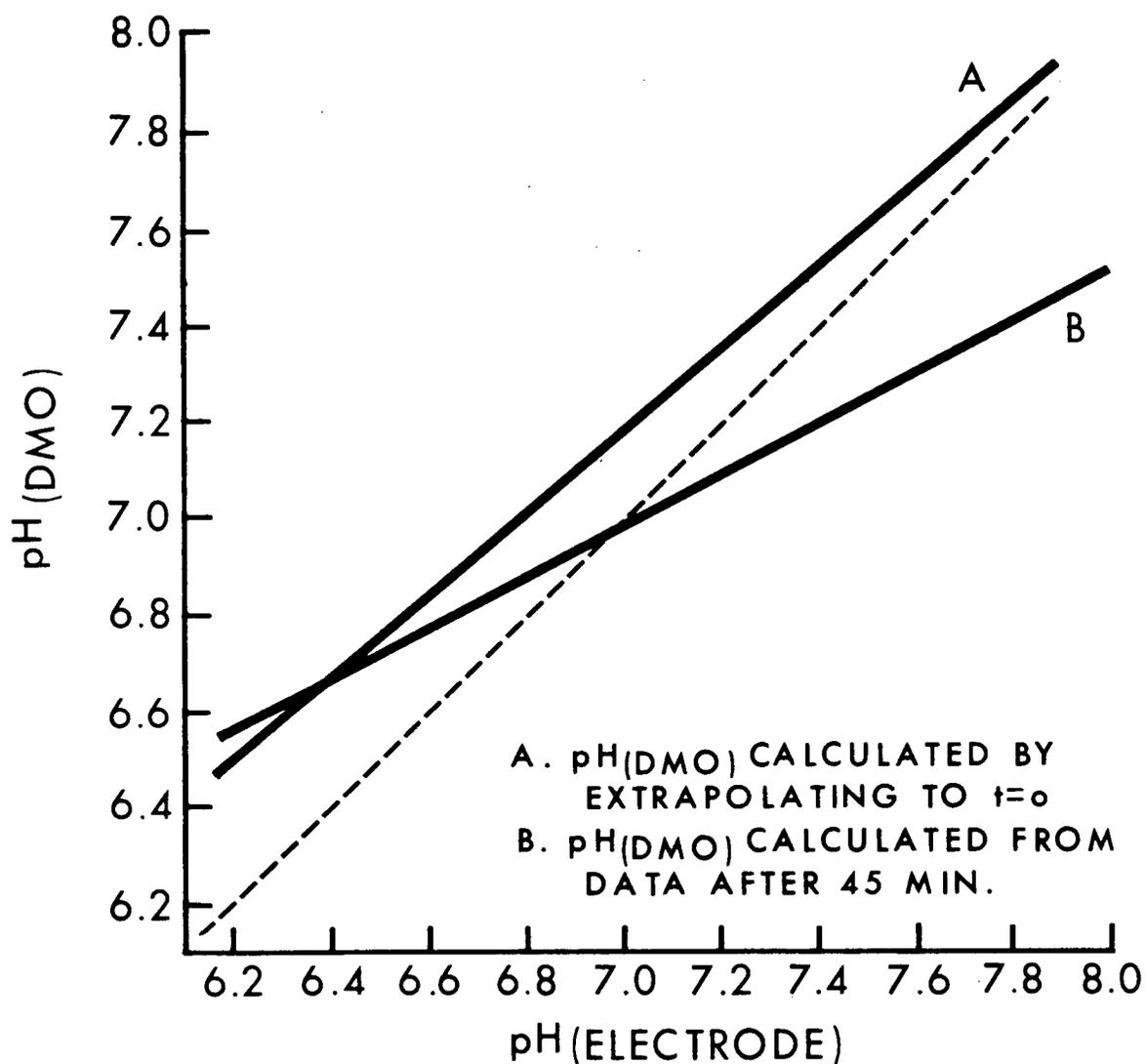
Figure 34. Correlation lines between pH(DMO) and pH(electrode) from cells on which pH_i was measured by the DMO and the pH microelectrode methods. For line A, the pH(DMO) was calculated by extrapolation to zero time of data taken after 45 minutes. For line B, the pH(DMO) was calculated as the mean of all points after 45 minutes. The dashed line indicates equality.

correctly reflect the real changes in intracellular pH.

DISCUSSION

DMO technique.

Fig. 31 revealed that the calculated values of pH(DMO) are similar on magnitude even though the amount of labelled indicator varied considerably with time. In equation (13) (or (12)), there are seven parameters each of which is measured with a certain degree of uncertainty, yet the calculated pH_{DMO} values do not reflect the degrees of uncertainty of the seven parameters in an obvious way.

To obtain a rough indication of how a change in each of the seven parameters might affect pH_{DMO}, the partial derivative of pH_{DMO} with respect to each parameter X_i at a typical point in the 'plateau' region (time greater than 45 minutes) for cells under normal, acid, and alkaline conditions was evaluated. The change in pH_{DMO}, δpH_{DMO}, due to changes in the X_i parameters, δX_i, can be estimated to first order from the expression:

$$\delta pH_{DMO} \doteq \sum_{i=1}^{7} \frac{\partial pH_{DMO}}{\partial X_i} \cdot \delta X_i \qquad \ldots (14)$$

The sensitivity of pH_{DMO} to a change in X_i is indicated by $\partial(pH_{DMO})/\partial X_i$, but it is more informative to consider the fractional change, $\delta X_i / X_i$, of each parameter which would produce a small but important change in pH_{DMO}, i.e. an increase of 0.05. The results of this exercise are summarized in Table VI. Also included, as the first column of this table, are the estimations of maximum uncertainty in the measurement of the seven

TABLE VI

SENSITIVITY OF pH(DMO) TO ERRORS IN MEASUREMENT

	Estd. uncertainty	Normal		Acidified		Alkalinized	
		∂X_i	$\dfrac{\partial X_i}{X_i}$	∂X_i	$\dfrac{\partial X_i}{X_i}$	∂X_i	$\dfrac{\partial X_i}{X_i}$
V_t (mg)	0.7%	-0.95	8%	-0.52	5%	-0.87	8%
pH_E	0.2%	0.04	0.5%	0.07	1%	0.04	0.5%
pK	0.5%	0.35	6%	-0.20	3%	0.53	9%
D_H (dpm)	1.5%	-354	24%	919	36%	-168	14%
D_C (dpm)	1.5%	77	6%	272	6%	38	5%
B_H (dpm/λ)	2-3%	-175	19%	-200	25%	-147	15%
B_C (dpm/λ)	2-3%	-17	6%	-16	6%	-14	5%

Notes:

(a) The density of water was taken to be 1 g/ml. Therefore, 1 mg = 1λ

(b) The values of the seven parameters in a given calculation were taken from the 15th fibre of each experiment. For example, the values of the parameters for the normal fibre were: V_t = 12.035 mg, pH_e = 7.73, pK' = 6.17, D_H = 1456 cpm, D_C = 1293 cpm, B_H = 937 cpm/λ, B_C = 285 cpm/λ.

parameters. The most reliable parameter is pH_e. Notice, however, that pH_e need only change by 0.5% to effect an increase of 0.05 in pH_{DMO}. In contrast, there can be a 24% change in D_H, which is a measure of the extracellular space of the cell, before pH_{DMO} changes by 0.05. This finding suggests that reliable extimations of pH_{DMO} can be expected in experiments were mean extracellular space determinations are made, rather than 'individual' determinations as in this study. Since pH_{DMO} is so sensitive to pH_e, the latter should be monitored continuously in an experi-

ment to enable the experimenter to maintain a chosen value or to match the value of pH_e at a given time to the other parameters at that time for a given calculation.

The data in Table VI also show that pK' can increase by 6% in normal Ringer's solution before an increase in pH_{DMO} of 0.05 occurs. This estimate is, of course, based on the assumption that the two pK' entries in equation (13) are identical, or, put another way, that the internal and external pK' values for DMO are identical. If pK' (DMO) were 6.5 or greater, its precise value would have to be taken more seriously (although the other parameters would be different as well). DMO was recommended as an indicator for pH_i partly because of its pK' (Waddell & Butler 1959).

The more difficult question, whether $pK'_i = pK'_e$ remains unanswered although there is some evidence which suggests that pK_i is very close to pK'_e. First, Boron and Roos (1976) have shown that pK(DMO) is not seriously affected by ionic strength or by a change in electrolyte from NaCl to KCL. Second, Hinke (1970) has shown that most of the intracellular water in muscle behaves like extracellular bulk water in its solvent properties and that the activity coefficient for cations in the intracellular aqueous phase is similar in magnitude to the ionic activity coefficient in barnacle Ringer's solution. The barnacle cell like other muscle cells does, of course, contain contractile protein filaments with a number of fixed negative charge sites which can act as a proton sink, but it is difficult to imagine how such negative charge sites could alter the DMO dissociation process in a way other than how one might imagine an alteration in DMO dissociation to be caused by a buffer with a reasonable capacity, in the external medium. Finally, it is inconsistent to worry about possible false activities of HDMO and DMO in myoplasm which might produce a different pK'_i without also worrying about the true value of V for DMO which should be

used in equations (12) and (13) rather than V_t (Hinke 1970).

Two more points can be made from the data in Table VI. First, the relative weight of each parameter for the calculation of pH_{DMO} does not change when the cell is under acid or alkaline conditions. Second, the partial derivatives of pH_{DMO} with respect to some of the parameters have negative signs. Of particular interest are the opposite signs of the partial derivatives with respect to D_H and D_C because these are most relevant to the uptake curves of Fig. 31. During the first 30 minutes of uptake, both D_H and D_C are increasing appreciably, yet the calculated values of pH_{DMO} in this period are all similar in magnitude and close to pH_{DMO} values obtained after 45 minutes, when the changes in D_H and D_C are smaller. It appears that the parameters D_H and D_C in equation (13) are so placed as to stabilize the pH_{DMO} calculation during the double-isotope experiments. If this reasoning is valid, then it may be used as a good reason to advocate the use of the double-isotope technique even though, as was pointed out earlier, the technique does not seem warranted if one looks only at the relatively weak dependence of pH_{DMO} on D_H (Table VI).

An ideal volume marker should produce an uptake curve which rises rapidly at first and then quickly bends to a horizontal plateau. In Fig. 31 it is shown that neither (^{14}C)DMO nor (^{3}H)inulin behaved in such an ideal manner. Instead, both markers produced slightly inclined plateaus after 45 minutes and up to 180 minutes, which suggests that either an equilibrium is not attained, or that each marker is involved in a non-saturable non-specific adsorption process. To obtain an independent measure of the rate of passage of (^{14}C)DMO across the cell membrane, (^{14}C)DMO was microinjected into the myoplasm of an intact cell. The efflux of the radioisotope was observed to occur with a rate constant of 0.09 min^{-1}. With such a rapid transmembrane flux rate, it can readily be calculated that DMO should reach

a steady state across the membrane within minutes. Thus, the slowly rising linear plateau of the (^{14}C)DMO uptake curve cannot be attributed to a failure to achieve a steady state. (^{3}H)inulin was also injected into a cell and it was observed that the radioisotope did not cross the membrane. From this result one can conclude (a) that the rapid efflux of (^{14}C)DMO was not due to injury to the membrane following microinjection, and (b) that the slowly rising linear plateau of the (^{3}H)inulin uptake curve could not be explained by a slow penetration of (^{3}H)inulin into the cell. To investigate the slow rise further, several muscle cells were preincubated in a bath containing a relatively high concentration of non-radioactive DMO (more than 65 times that of (^{14}C)DMO). In these experiments, no change in the slope of the slowly rising plateau was observed on either the (^{14}C)DMO curve or on the (^{3}H)inulin curve. In other experiments, the uptake curves of (^{3}H)sorbitol with and without preincubation in unlabelled sorbitol (5 mM) were examined and in both cases a slow uptake was observed, just as with inulin.

It has not been possible to explain the uptake curves for cells which were treated with CO_2 or NH_4 Ringer's solution. They do not reflect actual changes in pH_i, since the microelectrode measurements reveal that pH_i was steady under the conditions of the DMO experiment. It is not likely that the DMO was transported or metabolized by the cell. It bears repeating that the use of CO_2 and NH_4 solutions to alter pH_i is a commonly used technique.

pH(DMO) versus microelectrode measurements.

The value for the intracellular pH obtained with the pH-specific glass microelectrode is assumed to be the best measurement of this quantity. It reflects the pH in the intracellular solution which surrounds the sensitive

tip of the microelectrode. In the barnacle muscle cell, there are few intracellular compartments, as discussed in section 2.B. The distribution of the indicator DMO can reasonably be expected to be dominated by the properties of the large myoplasmic compartment. Since it is the properties of this compartment that the microelectrode measures, the accuracy of pH(DMO) in barnacle muscle cells is revealed by the closeness of its value to the value measured with the microelectrode.

It has been found that the best value obtained from the DMO distribution, the 'extrapolated' pH(DMO), is consistently 0.1 to 0.2 units higher than pH_i (i.e. pH(microelectrode)). As discussed in section 2, this might at first glance be taken to reflect the existence of relatively alkaline intracellular compartments.

By comparison, Boron and Roos (1976), using similar electrodes but the 'plateau' value of pH(DMO), reported that pH(DMO) was smaller by about 0.05 than the values of 7.26 to 7.35 measured with the microelectrode for the cells when in 'barnacle sea water'. (In the present study, mean pH_i for the cells when in normal Ringer's solution was 7.23 - Table III.) They suggested three explanations for the small values of pH(DMO): (i) the existence of a low intracellular pK' value for DMO; (ii) an appreciable permeability of the cell membrane to ionized DMO; (iii) the presence of an acidic intracellular compartment. Actually, the use of the 'plateau' pH(DMO) could by itself account for the slight discrepancy between their results and those reported here.

In light of the preceding discussion, however, it is difficult to ascribe much significance to the small difference between the best values for pH(DMO) and pH(microelectrode). Uncertainties in the DMO method involving the neglect of 'nonsolvent water', the assumption of equality of pK_i' and pK_e', the possible permeability of the cell membrane to ionized DMO,

and the non-ideal uptake behavior of the indicators would have to be resolved before such a discrepancy could profitably be examined in detail.

The most important conclusion to be drawn from these studies is a practical one. pH(DMO) can give an estimate of the intracellular pH accurate to about 0.2 units, and a rather better estimate of the difference in the value of pH_i during different steady conditions, if 'extrapolated' pH(DMO) is evaluated. The DMO technique should be of little use in the measurement of transient changes in pH, although its use for such a purpose has been reported (Roos & Boron 1978).

SECTION 10. SIGNIFICANCE OF THE RESULTS AND SUGGESTIONS FOR FUTURE WORK

The work on sodium fluxes reported in this thesis falls into three parts: the formulation of a model for measuring sodium efflux, the testing of the model, and the application of the model to two specific problems.

A practical model for the calculation of the unidirectional efflux of sodium from the myoplasm was devised. The myoplasm bathes the inner surface of the cell membrane, and the sodium activity in it can be measured directly with a sodium-specific glass microelectrode. The new result which made a simple formulation of the model possible was that the nonmyoplasmic sodium does not appear to exchange appreciably with the myoplasmic sodium, at least during the washout of cellular sodium into a lithium-substituted sodium-free solution. It is not easy to reconcile this result with the existence of a great deal of 'loosely bound' sodium associated with the cellular proteins, although there is good experimental evidence that such sodium exists. Further work should be done on the size and the location of the mobile and immobile fractions of cellular sodium.

In any case the results of the present experiments can be interpreted consistently in the model that: some cellular sodium is free in solution in the myoplasm, as envisaged in the early models; a relatively small amount is 'bound loosely' to fixed charges inside the cell, as envisaged in more recent models; some is highly mobile and outside the cell membrane, a fraction acknowledged but not quantified previously; and finally some is nonparticipatory as far as these experiments are concerned, and probably is purely 'structural' in the sense that it has little to do with the ion movements which are so vital to the moment-to-moment life of the cell. Most of the latter fraction is probably located outside the cell membrane, in the glycocalyx. Each of the fractions will probably be found to be heterogeneous when characterized in terms other than just mobility.

The rationale for the new technique (the combination of sodium-specific microelectrode and radiosodium measurements) was straightforward. The fruits of it are better measurements of the 'true' flux. Just as the use of a rotating frame of reference in mechanics gives rise to complicated fictitious forces, so errors such as incorrect estimation of the myoplasmic activities or neglect of the longitudinal diffusion of an injected marker substance give rise to 'fictitious fluxes' and complicated models.

Once the fictitious fluxes were eliminated from the sodium efflux measurements for barnacle muscle cells, it was found that the notion of compartments which exchange ions in a complicated fashion via many different transport systems need not be invoked yet. There is still progress to be made by using simple models correctly.

The kinetic characteristics of the sodium efflux were interpreted in the usual manner, as reflecting a chemical reaction mediated by a membrane-bound protein-lipid enzyme complex. Some of the efflux can be ascribed to the (Na+K)ATPase, on the basis of the response to ouabain. Further, the similarity of the response to ouabain and to potassium-free solution implies that it is only via the sodium-potassium exchange mode that the (Na+K)ATPase makes an appreciable contribution to the active sodium efflux in barnacle muscle cells. If this were so, a different mechanism for active sodium transport, insensitive to ouabain and (perhaps) not requiring external potassium, would have to be postulated. What seem better working hypotheses are that not all of the in situ (Na+K)ATPase can be affected by ouabain, or that the binding of ouabain to an enzyme in situ does not stop it from transporting ions, contrary to the case with the isolated enzyme. Thus, the kinetic characteristics of the sodium efflux in the presence of different concentrations of ouabain could be analyzed in the context of the established models for the various possible actions of inhibitors of enzyme-mediated reactions.

The results obtained with ouabain are at once intriguing and frustrating.

Because so much ground had to be covered in order to make the complicated new technique for flux measurement practical, because the time available to do the experiments was limited by the constraints of the M.D.-Ph.D. program, and because the thesis supervisor moved to a different university, it was not possible to do as many experiments as were desired. A major part of future work will be on the characterization of the ouabain-sensitive and -insensitive components of the efflux.

A complication which appears to have been resolved is the effect of extracellular sodium on the sodium efflux. It had been known that replacement of the sodium in the extracellular solution with some other species could cause an increase in the sodium efflux from nerve or muscle cells of high sodium content. A sodium-sodium exchange not mediated by the (Na+K)ATPase (or at least, not sensitive to ouabain) and prominent only when the intra-cellular sodium concentration was low, was postulated. The results reported here for barnacle muscle cells are consistent with a much simpler explanation. The rate of active sodium transport is governed almost entirely by the intra-cellular sodium concentration under normal conditions. Removal of external sodium causes a decline in the intracellular sodium concentration, and consequently a decline in the sodium efflux. In addition, extracellular sodium appears to be able to modify slightly the response of the sodium transport mechanism to the intracellular sodium concentration. At a given intracellular sodium concentration the sodium efflux is greater when the external sodium is replaced by a large cation. This is a synthesis of hypotheses put forward by other workers in a similar context.

This explanation is consistent with the results reported by other workers for nerve and muscle cells. An incorrect interpretation of radio-sodium flux experiments has been shown here to be the cause of what appears to be a longstanding misconception concerning "sodium-sodium exchange". The use of the new technique reported here, in which care is taken to determine

the actual magnitude of the sodium flux, revealed the true behavior of the sodium efflux, although in retrospect the result could have been deduced from the microelectrode experiments published by other workers.

It is not claimed that sodium-sodium exchange does not occur, but rather that the contribution it makes to the sodium efflux in muscle cells is much smaller than had been thought. The new results lead to a refinement of the current models concerning the sodium distribution and movements in cells.

The new technique also yielded a nice demonstration that electrogenic sodium transport occurs in barnacle muscle cells. This certainly is not an unexpected finding, but it had not been shown convincingly before. More important are the quantitative results of these experiments. Measurement of the absolute value of the sodium efflux is essential if a quantitative correlation between the changes in sodium efflux and membrane potential is to be obtained. Such a correlation is important for the evaluation of mechanistic models of ion movement, as it can provide an independent measurement of parameters. A model was derived here, by making the simplest possible extension from the current successful model for the resting membrane potential. The derivation presented was intended to express the basic physical process which appears to give rise to the electrogenic potential. Approximations were employed in order to obtain a simplicity of form, because the data obtained so far do not admit to detailed analysis. (As it turned out, however, the quantitative results were quite good.) The way is indicated for an investigation of the detailed correlation between the flux and the membrane potential. This will permit modelling of the actual mechanism of the transport process to be done and tested.

The work on the hydrogen ion reported here, which actually was done as a brief project before the work on sodium, yields a practical result and presents two puzzles. One puzzle concerns the DMO technique. How can it occur that when the cell is acidified with CO_2 or alkalinized with NH_3, the DMO is first

taken up but then is expelled? The intracellular pH measured by the micro-electrode remains quite stable in these situations. The very slow acidification following the alkalinization by NH_3 is in agreement with Roos' model of NH_4^+ penetration (Roos 1965), and the absence of alkalinization following the acidification by CO_2 is probably due to the low concentration of HCO_3^- used. The cells do not gain or lose water. The indicator is not metabolized. That the effect occurs in both acid and alkaline conditions implies that it is due to the techniques rather than to some physicochemical process, yet only the standard techniques were employed. This question demands further investigation.

The second puzzle concerns the relationship between the transmembrane pH difference and the membrane potential. As was discussed in section 8, an elementary model predicts the effect seen, and analysis of the techniques used revealed no source of error. The fact remains, however, that other workers have sought and failed to find this effect for pH. Further investigation of this effect, for other ions as well as for hydrogen, is certainly indicated. It might be that the pH-sensitive potential difference measurable in glycerinated cells is involved in this effect.

The practical result is the demonstration that careful use of the weak-electrolyte (DMO) method can yield accurate measurements of changes in the intracellular pH between states of the cell in each of which conditions are steady. This result, and the analysis of the effect which variation in each of the parameters has on the final result, is of particular utility to those who work with very small cells or with multicellular systems, for which the use of microelectrodes is not possible at present.

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