1
Introduction

Suppose a man has a tomato thrown at his head, and that he is able to take suitable evasive action. His reactions would involve changes in the activity of a very large number of cells in his body. First of all, the presence of a red object would be registered by the visual sensory cells in the eye, and these in turn would excite nerve cells leading into the brain via the optic nerve. A great deal of activity would then ensue in different varieties of nerve cell in the brain and, after a very short space of time, nerve impulses would pass from the brain to some of the muscles of the face and, indirectly, to muscles of the neck, legs and arms. The muscle cells there would themselves be excited by the nerve impulses reaching them, and would contract so as to move the body and so prevent the tomato having its desired effect. These movements would then result in excitation of numerous sensory endings in the muscles and joints of the body and in the organs of balance in the inner ear. The resulting impulses in sensory nerves would then cause further activity in the brain and spinal cord, possibly leading to further muscular activity.

A chain of events of this type involves the activity of a group of cell types which we can describe as 'excitable cells': a rather loose category which includes nerve cells, muscle cells, sensory cells and some others. An excitable cell, then, is a cell which readily and rapidly responds to suitable stimuli, and in which the response includes a fairly rapid electrical change at the cell membrane.

The study of excitable cells is, for a number of reasons, a fascinating one. These are the cells which are principally involved in the behavioural activities of animals: these are the cells with which we move and think. Yet just because their functioning must be examined at the cellular and subcellular levels of organization, the complexities that emerge from investigating them are not too great for adequate comprehension; it is frequently possible to pose specific questions as to their properties, and to elicit some of the answers to these questions by suitable experiments. It is perhaps for this reason that the subject has attracted some of the foremost physiologists of this century. As a consequence, the experimental evidence on which our knowledge of the physiology of excitable cells is based is often elegant, clearcut and intellectually exciting, and frequently provides an object lesson in the way a scientific investigation should be carried out. Nevertheless, there are very many investigations still to be done in this field, many questions which have yet to be answered, and undoubtedly very many which have not yet been asked.

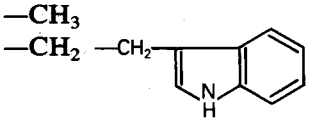
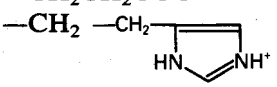
Most readers of this book will possess a considerable amount of information on basic ideas in the biological and physical sciences. But it may be as well in this introductory chapter to remind them, in a rather dogmatic fashion, of some of the background which is necessary for a more detailed study: to formulate, in fact, a few axioms.

The biological material

Cells

All large organisms are divided into a number of units called cells, and every cell is the progeny of another cell. This statement constitutes the cell theory. Every cell is bounded by a cell membrane and contains a nucleus in which the genetic material is found. The main part of the living matter of the cell is a highly organized system called the cytoplasm, which is concerned with the day-to-day activity of the cell. The cell membrane separates this highly organized system inside from the relative chaos that exists outside the cell. In order to maintain and increase its high degree of organization and in order to respond to and alter its environment, the cell requires a continual supply of energy. This energy must be ultimately derived

Table 1.1. *The amino acids found in proteins. They have the general formula R—CH(NH_2)COOH, where R is the side chain or residue. Proline is actually an imino acid. Cystine is two cysteines linked by a disulphide bridge. Abbreviations are given in three- and one-letter codes*

Type	Amino acid	Side chain	Abbreviations		Hydropathy index[a]
Nonpolar	Isoleucine	$-CH(CH_3)CH_2.CH_3$	Ile	I	4.5
	Valine	$-CH(CH_3)_2$	Val	V	4.2
	Leucine	$-CH_2.CH(CH_3)_2$	Leu	L	3.8
	Phenylalanine	$-CH_2.C_6H_5$	Phe	F	2.5
	Methionine	$-CH_2.CH_2.SCH_3$	Met	M	1.9
	Alanine	$-CH_3$	Ala	A	1.8
	Tryptophan		Trp	W	−0.9
	Proline	$-CH_2.CH_2.CH_2-$	Pro	P	−1.6
Uncharged Polar	Cysteine/cystine	$-CH_2.SH$	Cys	C	2.5
	Glycine	$-H$	Gly	G	−0.4
	Threonine	$-CH(OH)CH_3$	Thr	T	−0.7
	Serine	$-CH_2OH$	Ser	S	−0.8
	Glutamine	$-CH_2.CH_2.CO.NH_2$	Gln	Q	−3.5
	Asparagine	$-CH_2.CO.NH_2$	Asn	N	−3.5
Acidic	Aspartic acid	$-CH_2.COO^-$	Asp	D	−3.5
	Glutamic acid	$-CH_2CH_2COO^-$	Glu	E	−3.5
Basic	Histidine		His	H	−3.2
	Lysine	$-(CH_2)_3.NH_3^+$	Lys	K	−3.9
	Arginine	$-(CH_2)_3NH.C(NH_2)=NH_2^+$	Arg	R	−4.5

[a]The hydropathy index is from Kyte and Doolittle (1982), and referred to in chapter 3 and elsewhere.

from the environment, usually in the form of chemical energy such as can be extracted by the cell from glucose molecules. Thus, thermodynamically speaking, the cell is an open system maintained in a rather improbable steady state by the continual expenditure of energy. Its life is a continual battle against the second law of thermodynamics (which we may state without gross inaccuracy as 'things tend to get mixed up').

The cells of nervous systems are called *neurons*. Their primary function is the carriage of information in the form of changes in the electrical potential across the cell membrane, especially as unitary events known as nerve impulses or *action potentials*. The idea that the nervous system is composed of discrete cells is known as the neuron theory. This theory, which is merely a particular application of the cell theory, was developed during the nineteenth century, and is now generally accepted. The alternative proposal, that nervous systems are not divided into separate membrane-bounded entities (the reticular theory) was extremely difficult to reconcile with the observations of light microscopists, and seems to be conclusively refuted by the evidence of electron microscopy.

Proteins

So pervasive are the functions of proteins in cells that one way of defining living material is to say that it contains active proteins. Proteins are composed of chains made up from different combi-

nations of twenty different amino acids, and their properties depend critically upon the sequence in which these amino acids are arranged.

The protein's amino acid sequence is specified by the nucleotide base sequence in the DNA molecules which form the genetic material of the cell. This means that proteins are the products of evolution, so that present-day proteins largely represent stable and successful sequences. Animals whose cells produced too many unstable or non-functional sequences would have died before producing viable offspring, so the genes specifying those sequences have been largely eliminated.

The twenty amino acids in protein chains have different properties. Three of them are basic, with positive charges, two are acidic, with negative charges, seven are polar but uncharged, and eight are non-polar (table 1.1). The amino acid sequence determines the way in which the protein chain is folded. Non-polar residues tend to occur in the middle of the molecule or in association with lipids in the cell membrane. Polar and charged residues are more likely to be found on the outside of the molecule in contact with the aqueous environment. Hydrogen bonds, electrostatic interactions and disulphide links (between pairs of cysteine residues) all serve to hold the chain in its folded conformation.

The shape of many protein molecules changes when they react with smaller molecules or other proteins. Changes of this type underly much protein activity, such as enzymic hydrolysis, opening of membrane channels, and muscular contraction. The day to day activity of the cell can thus be largely described in terms of the actions of proteins; the reader of modern accounts of cell biology (such as those by Alberts *et al.*, 1983, and Darnell *et al.*, 1986) will find ample illustration of this statement.

Animals

Every animal has a history: every animal owes its existence to the success of its ancestors in combating the rigours of life, that is to say, in surviving the rigours of natural selection. Hence every animal is adapted to its way of life, and its organs, its tissues and it cells are adapted to performing their functions efficiently. There is an enormous variety in the form and functioning of animals, connected with a similar variety in ways of living. Nevertheless, it seems that in many cases there is only a limited number of ways in which animals can solve particular physiological problems. For instance, there is only a limited number of respiratory pigments, only a limited number of designs for hearing organs, and so on. Hence it is possible for us to detect certain principles in, and make certain generalizations about, the ways in which particular physiological problems are solved in different animals.

An animal is a remarkably stable entity. It is able to survive the impact of a variety of different environments and situations, and its cells and tissues are able to survive a variety of different demands upon their capacities. The main reason for this seems to be that an animal is a complex of self-regulating (homeostatic) systems. These systems are themselves coordinated and regulated so that the physiology and behaviour of the animal form an integrated whole.

Nervous systems

A nervous system is that part of an animal which is concerned with the rapid transfer of information through the body in the form of electrical signals. The activity of a nervous system is initiated partially by the input elements – the sense organs – and partially by endogenous activity arising in certain cells of the system. The output of the system is ultimately expressed via effector organs – muscles, glands, chromatophores, etc.

Primitive nervous systems consist of scattered but usually interconnected nerve cells, forming a nerve net, as in coelenterates. Increase in the complexity of responses is associated with the aggregation of nerve cell bodies to form ganglia, and when the ganglia themselves are collected and connected together, we speak of a *central nervous system.* The *peripheral nervous system* is then mainly composed of nerve fibres originating from the central nervous system. Peripheral nerves contain afferent (sensory) neurons taking information inwards into the central nervous system and efferent (motor) neurons taking information outwards; neurons confined to the central nervous system are known as interneurons. Ganglia which remain or arise outside the central nervous system, and the nerve fibres which lead to and arise from them to innervate the animal's viscera, are fre-

Table 1.2. *Some electrical units*

Quantity	SI unit	Symbol	SI equivalent
Current	ampere	A	
Charge	coulomb	C	A s
Potential difference	volt	V	$J C^{-1}$
Resistance	ohm	Ω	$V A^{-1}$
Conductance	siemens	S	Ω^{-1}
Capacitance	farad	F	$C V^{-1}$

Table 1.3. *Some prefixes for multiples of scientific units*

Multiple	Prefix	Symbol
10^{-2}	centi	c
10^{-3}	milli	m
10^{-6}	micro	μ
10^{-9}	nano	n
10^{-12}	pico	p
10^{-15}	femto	f
10^{3}	kilo	k
10^{6}	mega	M
10^{9}	giga	G

quently described as forming the *autonomic nervous system*.

One of the simplest, but possibly not one of the most primitive, modes of activity of a nervous system is the *reflex*, in which a relatively fixed output pattern is produced in response to a simple input. The stretch reflexes of mammalian limb muscles provide a well-known example (fig. 9.3). Stretching the muscle excites the endings of sensory nerve fibres attached to certain modified fibres (muscle spindles) of the muscle. Nerve impulses pass up the sensory fibres into the spinal cord where they meet motor nerve cells (the junctional regions are called *synapses*) and excite them. The nerve impulses so induced in the motor nerve fibres then pass out of the cord along peripheral nerves to the muscle, where their arrival causes the muscle to contract. Much more complicated interactions occur in the analysis of complex sensory inputs, the coordination of locomotion, the expression of the emotions and instinctive reactions, in learning and other 'higher functions'. These more complicated interactions are outside the scope of this book.

Electricity

Matter is composed of atoms, which consist of positively charged nuclei and negatively charged electrons. Static electricity is the accumulation of electric charge in some region, produced by the separation of electrons from their atoms. Current electricity is the flow of electric charge through a conductor. Current flows between two points connected by a conductor if there is a potential difference between them, just as heat will flow from a hot body to a cooler one placed in contact with it. The unit of potential difference is the *volt*. The current, i.e. the rate of flow of charge, is measured in *amperes*, and the quantity of charge transferred is measured in *coulombs*. Thus one coulomb is transferred by a current of one ampere flowing for one second.

In many cases it is found that the current (I) though a conductor is proportional to the potential difference (V) between its ends. This is *Ohm's law*. Thus if the constant of proportionality, the *resistance* (measure in *ohms*) is R, then

$$V = I \cdot R.$$

The specific resistance of a substance is the resistance of a 1 cm cube of the substance. The resistance of a wire of constant specific resistance is proportional to its length and inversely proportional to its cross-sectional area. The reciprocal of resistance is called *conductance* (G).

Let us apply Ohm's law to a simple calculation. In chapter 6 we shall see that under certain conditions small channels open to let sodium ions flow through. If we can measure this current flow and we know what the driving voltage is, we can calculate the conductance of the channel. Thus in one experiment the single channel current was 1.6 pA with a driving voltage of 90 mV. (Table 1.2 shows selected electrical units and table 1.3 gives prefix names for multiples and submultiples.) Applying Ohm's law, the conductance of the channel is given by

$$G = I/V$$

i.e.

$$\text{conductance (siemens)} = \frac{\text{current (amps)}}{\text{voltage (volts)}}$$

$$= \frac{1.6 \times 10^{-12}}{90 \times 10^{-3}}$$

$$= 17.8 \text{ pS}.$$

The total resistance of a number of resistive elements arranged in series is the sum of their individual resistances, whereas the total conductance of a number of elements in parallel is the sum of their conductances. A patch of membrane containing five channels each with a conductance of 17.8 pS, for example, will have a conductance of 89 pS if all the channels are open.

Two plates of conducting material separated by an insulator form a capacitor. If a potential difference V is applied across the capacitor, a quantity of charge Q, proportional to the potential difference, builds up on the plates of the capacitor. Thus

$$Q = V \cdot C$$

where C, the constant of proportionality, is the *capacitance* of the capacitor. When the voltage is changing, charge flows away from one plate and into the other, so that we can speak of current, I, through a capacitor, given by

$$I = C \cdot \mathrm{d}V/\mathrm{d}t$$

where $\mathrm{d}V/\mathrm{d}t$ is the rate of change of voltage with time. The capacitance of a capacitor is proportional to the area of the plates and the dielectric constant (a measure of the ease with which the molecules of a substance can be polarized) of the insulator between them, and inversely proportional to the distance between the plates. The total capacitance of capacitors in parallel is the sum of the individual capacitances, whereas the reciprocal of the total capacitance of capacitors in series is the sum of the reciprocals of their individual capacitances.

Scientific investigation

Science is concerned with the investigation and explanation of the phenomena of the natural world. Any particular investigation usually starts with an idea – a hypothesis – about the relations between some of the factors in the system to be studied. The hypothesis must then be tested by suitable observations or experiments. This business of testing the hypothesis is what distinguishes the scientific method from other attempts at the acquisition of knowledge, and hence it follows that a scientific hypothesis must be capable of being tested. We must therefore understand what is meant by 'testing' a hypothesis.

In mathematics and deductive logic it is frequently possible to prove, given a certain set of axioms, that a certain idea about a particular situation is true or not true. For instance, it is possible to prove absolutely conclusively that, in the system of Euclidean geometry, the angles of an equilateral triangle are all equal to one another. But this absolute proof of the truth of an idea is not possible in science. For example, consider the hypothesis 'No dinosaurs are alive today'. This statement would be generally accepted by biologists as being almost certainly true, but, of course, it is just possible that there are some dinosaurs alive which have never been seen. Some years ago the statement 'No coelacanths are alive today' would also have been accepted as almost certainly correct.

However, in many cases, it *is* possible to prove that a hypothesis is false. The hypothesis 'No coelacanths are alive today' has been proved, conclusively, to be false. If we were to find just one living dinosaur, the hypothesis 'No dinosaurs are alive today' would also have been shown to be false. It follows from this argument that in order to test a hypothesis it is necessary to attempt to disprove it. When a hypothesis has successfully survived a number of attempts at disproof, it seems more likely that it provides a correct description of the situation to which it applies (Popper, 1963).

If we can only test a hypothesis by attempting to disprove it, it follows that a scientific hypothesis must be formulated in such a way that it is open to disproof – so that we can think of an experiment or observation in which one of the possible results would disprove the hypothesis. Any idea which we cannot see how to disprove is not a scientific hypothesis.

But where do the ideas come from? Science is a progressive activity. Advances are usually made step by step. Ideas arise in a controlled imagination: the scientist usually starts from a generally accepted understanding of the situation and makes a small conjecture into the unknown. A high rate of progress follows two particular types of advance: ideas which provide a major reinterpretation of what we know, and new techniques. In 1954, for example, as we shall see in chapter 12, the study of muscular contraction entered a new and highly productive phase as the result of the formulation of

of the sliding filament theory, which itself arose in the context of advances in X-ray diffraction methods and electron microscopy. More recently, the advent of the patch clamp technique (chapter 6) has led to a great flowering of work on the ionic channels of cell membranes.

What implications does the nature of science have for learning about science? To be worth his salt, any student must get to grips with the intellectual credentials of his subject. For the science student, this implies that simply comprehending a proposition that we believe to be true is not enough. It is also necessary to understand why we believe it to be true, what the evidence for the proposition is, and hence what sort of evidence might lead us to revise our opinion about it.

It is for this reason that this book is much concerned with experiments and observations, and not simply with the understanding that has arisen from them. The conclusions from some of these experiments will stand the tests of further investigations in the future, those of others will have to be revised. The science student cannot hope to know everything about his subject, but if he understands just why he believes what he does, then he can look the future in the face.

2
Electrophysiological methods

Excitable cells can be and are studied by the great variety of techniques that are available for the study of cells in general. These include light and electron microscopy, X-ray diffraction measurements, experiments involving radioactive tracers, cell fractionation techniques, biochemical methods, and so on. We shall not deal with these methods here. The techniques which are particular to the study of excitable cells are those involving the measurement of rapid electrical events. Consequently, in order to understand the subject, it is necessary to have some idea of how these measurements are made. In this chapter we shall look briefly at some of the more general methods used. The powerful techniques of voltage clamping and patch clamping are introduced in later chapters. Duncan (1989) gives more detail on a variety of electrical measurement techniques.

Recording electrodes

If we wish to record the potential difference between two points, it is necessary to position electrodes at those points and connect them to a suitable instrument for measuring voltage. It is desirable that these electrodes should not be affected by the passage of small currents through them, i.e. that they should be non-polarizable. For many purposes fine silver wires are quite adequate. Slightly better electrodes are made from platinum wire or from silver wire that has been coated electrolytically with silver chloride. For very accurate measurements of steady potentials, calomel half-cells (mercury/mercuric chloride electrodes) may have to be used.

If the site we wish to record from is very small in size (such as occurs in extracellular recording from cells in the central nervous system), the electrode must have a very fine tip, and be insulated except at the end. Successful electrodes of this type have been made from tungsten wire which is sharpened by dipping it into a solution of sodium nitrate while current is being passed from the electrode into the solution; insulation is produced by coating all except the tip of the electrode with a suitable varnish.

The manufacture of a suitable electrode is rather more difficult if we wish to record the potential inside a cell. Apart from a few large cells such as squid giant axons, this necessitates the use of an electrode which is fine enough to penetrate the cell membrane without causing it any appreciable damage. The problem was solved with the development of glass capillary microelectrodes by Ling and Gerard in 1949. These are made on a suitable device which will heat a small section of a hard glass tube and then very rapidly extend it as it cools. The heated section gets thinner and cooler as the pulling proceeds, so that finally the pulling force exceeds the cohesive forces in the glass, and the tube breaks to give two micropipettes. If the machine designed to do this has been correctly adjusted, the outside diameter of the micropipette at its tip will be about 0.5 μm.

The micropipette now has to be filled with a strong electrolyte solution such as 3 M potassium chloride solution. This is most readily done if the glass tube from which the electrode is made has a fine filament of glass fused to its inner wall; the angle between the filament and the wall leads to high capillary forces, so that the pipette can be filled to its tip by injecting the solution into the barrel. The connection to the recording apparatus is made via a non-polarizing electrode such as a silver wire coated with silver chloride.

An electrode of this type has a very high resistance, from five to several hundred megohms; in fact the suitability of an electrode is usually tested by measuring its resistance, since the tip is too small to be examined satisfactorily by light microscopy. Further details of microelectrode methods are given in Standen *et al.* (1987).

Electronic amplification

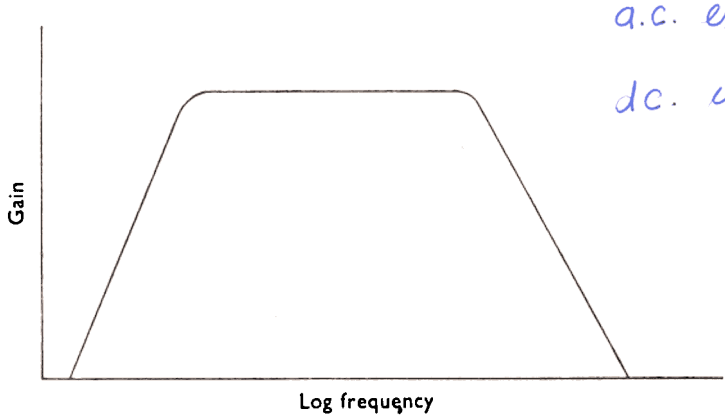
The potential differences which are measured in investigations on the activity of excitable cells vary in size from just over 0.1 V down to as little as 20 μV or so. Before being measured by a recording instrument, these voltages usually have to be amplified. This is done by means of suitable electronic circuits involving thermionic valves or transistors, the details of which need not concern us. However, there are three aspects of any amplifier used for electrophysiological recording purposes which we must consider: (1) the frequency response, (2) the noise level and the input resistance (3).

All amplifiers show a higher gain (the gain is the ratio of the output to the input voltages) at some frequencies than at others. A typical amplifier for use with extracellular electrodes might have a constant gain over the range 10 Hz–50 kHz, the gain falling at frequencies outside this range (fig. 2.1). An amplifier of this type is known as an a.c. amplifier, since it measures voltages produced by alternating current. A d.c. amplifier is one which can measure steady potential differences, i.e. its frequency response extends down to zero hertz. If we wish to measure steady potentials, or slow potential changes without distortion, then obviously we must use a d.c. amplifier. Amplifiers used with intracellular electrodes are usually d.c. amplifiers, so that the steady potential difference across the cell membrane can be measured, as well as the rapid (high-frequency) changes involved in its activity.

Any amplifier will produce small fluctuations in the output voltage even when there is no input signal. These fluctuations are known as *noise*, and are caused by random electrical activity in the amplifier. The existence of noise sets a lower limit to the signal voltage that can be measured, since it is difficult to distinguish very small signals from the noise. Consequently it is necessary to use an amplifier with a low noise level if we wish to measure signals of very small size.

If we connect a potential difference across the input terminals of an amplifier, a very small current flows between them, which is proportional to the potential difference. The proportionality factor is called the *input resistance* of the amplifier. It is determined by application of Ohm's law and is measured in ohms; for instance, the input resistance of a cathode ray oscilloscope amplifier is usually about 1 MΩ. Now suppose we connect an intracellular microelectrode whose resistance is, say, 10 MΩ to an amplifier with an input resistance of 1 MΩ. The equivalent circuit is shown in fig. 2.2*a*. The two resistances form a potential divider, so that the voltage input to the amplifier is only $10^6/(10^6 + 10^7)$ i.e. one-eleventh of the signal voltage. Obviously this is of little use for measuring the signal voltage. Hence, when using high-resitance electrodes, it is necessary to use an input stage with a very high input resistance, such as is given by the use of a junction field effect transistor. Suppose the input resistance of such a device is 1000 GΩ (fig. 2.2*b*); then the voltage recorded is $10^{12}/(10^{12} + 10^7)$, which is effectively equal to the signal voltage.

Fig. 2.1. The frequency response of an a.c. amplifier.



a.c. extracellular

d.c. intracellular

The cathode ray oscilloscope

We now have to consider how the voltage output from an amplifier is to be recorded and measured. If the voltage is steady, or only changing very slowly, we could use an ordinary voltmeter in which the current through a coil of wire placed between the poles of a magnet causes a pointer on which the coil is mounted to move. However, a device of this nature is no use for measuring rapid electrical changes since the inertia of the pointer is too great. What we need, in effect, is a voltmeter with a weightless pointer. This is provided by the beam of electrons in the cathode ray tube of a cathode ray oscilloscope.

The cathode ray tube is an evacuated glass tube containing a number of electrodes and a screen, which is coated with phosphorus compounds so that it luminesces when and where it is bombarded by electrons, at one end (fig 2.3). The cathode is heated and made negative (by about 2000 V) to the anode; consequently electrons are emitted from the cathode and accelerate towards the anode. Since the anode has a hole in its centre, some of the electrons continue moving at a constant high velocity beyond it; these form the electron beam. The intensity and focusing of the beam can be controlled by other electrodes, not shown in fig 2.3, placed in the vicinity of the anode and cathode. When the electron beam hits the screen, it produces a spot of light at the point of impact. Between the anode and the screen, the beam passes between two pairs of plate electrodes placed at right angles to each other, known as the *X* and *Y* plates. If a potential difference is connected across one of these pairs, the electrons in the beam will move towards the positive plate. Thus when the electrons pass out of the electric field of the pair of plates, their direction will have been changed, and so the light spot on the screen will move, by an amount proportional to the potential difference between the plates. The *Y* plates are connected to the output of an amplifier whose input is the signal voltage to be measured; hence the signal voltage appears as a vertical deflection of the spot on the screen. The *X* plates are usually connected to a waveform generating circuit (the 'time-base' generator) which produces a sawtooth waveform. This sawtooth waveform thus moves the spot horizontally across the screen at a constant velocity, flying back and starting again at the end of each 'tooth'. As a consequence of these arrangements, the spot on the screen traces out a graph with signal voltage on the *y*-axis and time on the *x*-axis. By making the rise-time of the time-base sawtooth sufficiently fast, it is possible to measure the form of very rapid voltage changes.

Many oscilloscopes have tubes with two beams, each with separate *Y* plates and amplifiers, so that one can measure two signals at once. The time-base unit can frequently be arranged so that a single sawtooth wave, leading to a single sweep of the beam, can be initiated (or 'triggered') by some suitable electrical signal; this facility is essential for much electrophysiological work. In some cases it is possible to connect the *X* plates to another input amplifier, instead of to the time-base generator, so that a signal related to some quantity other than time is measured on the *x*-axis.

On the screen of a standard oscilloscope the waveform traced out by the electron beam persists for only a fraction of a second. A permanent record of the trace can be obtained by photographing it; many of the diagrams in this book are photographs of oscilloscope traces.

Storage oscilloscopes are devised so that the trace can be held on the screen for some time.

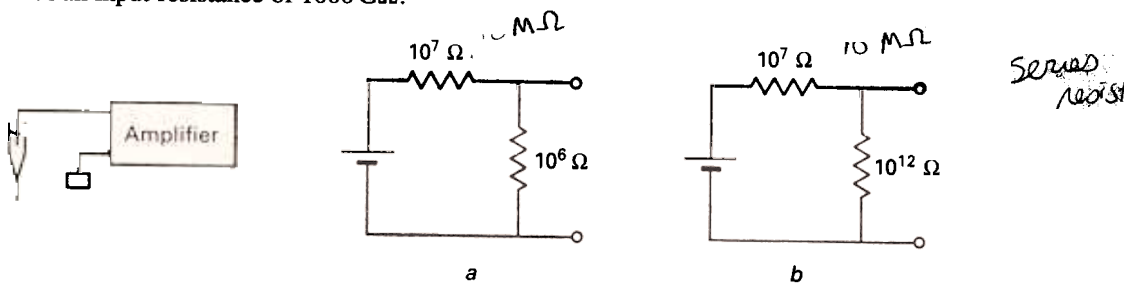
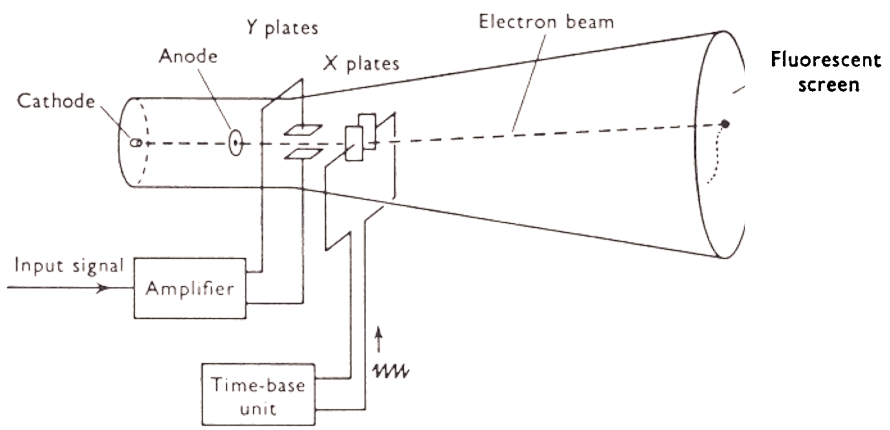


Fig. 2.2. Equivalent circuits of a glass capillary microelectrode whose resistance is 10 MΩ connected to *a*: an 'ordinary' amplifier with an input resistance of 1 MΩ, and *b*: an input stage with an input resistance of 1000 GΩ.

Analogue storage devices depend upon rather expensive modifications to the cathode ray tube. Digital storage oscilloscopes store the trace in memory, from which it is continually read out and displayed on the screen. An advantage of this type of instrument is that the memory can be read out into other devices, such as a chart recorder for hard-copy production.

Oscilloscopes are always easier to use if one can have a second or third look at what one wishes to display, so it is often useful and sometimes essential to be able to store the recorded signals in some permanent form. Such immediate data storage can be supplied by magnetic tape (using a frequency modulated tape recorder) or a computer memory.

Fig. 2.3. Simplified diagram to show the principal components of a cathode ray oscilloscope.



Electrical stimulation

An electrical stimulus must be applied via a pair of electrodes. Stimulating electrodes may be of any of the types previously described for recording purposes. The simplest way of providing a stimulating pulse is to connect the electrodes in series with a battery and a switch, but this is not satisfactory if brief pulses are required. In the past, stimulating pulses have been produced by such means as discharge of condensors or by using an induction coil, but nowadays most investigators use electronic stimulators which produce 'square' pulses of constant voltage, beginning and ending abruptly. A good stimulator unit will be able to produce pulses which can be varied in strength, duration and frequency. We need not be concerned here with the design of these instruments (see Young, 1973).

3

The resting cell membrane

If an intracellular microelectrode is inserted into a nerve or muscle cell, it is found that the inside of the cell is electrically negative to the outside by some tens of millivolts. This potential difference is known as the *resting potential.* If we slowly advance a microelectrode so that it penetrates the cell, the change in potential occurs suddenly and completely when the electrode tip is in the region of the cell membrane; thus the cell membrane is the site of the resting potential. In this chapter we shall consider some of the properties of the cell membrane that are associated with the production of the resting potential.

them, but not by the non-polar lipid chains in the middle of the membrane. X-ray diffraction studies (Wilkins *et al.*, 1971) fit well with the bilayer hypothesis, giving a distance of 45 Å between the polar head groups in erythrocyte membranes. This is about what we would expect from the dimensions of phospholipid molecules (fig. 3.2).

About this time it became clear that the Davson–Danielli model, with its thin layer of protein on each side of the lipid bilayer, did not really fit the facts. Wilkins *et al.* calculated that in red cell membranes there was insufficient lipid to account for the observed thickness of the membrane, and suggested that part of its area was occupied by protein. Enzymes which would hydrolyse phospholipids were able to attack cell membranes, suggesting that the phospholipids were not protected by an overlying layer of protein. But perhaps the most graphic evidence comes from a special technique of electron microscopy known as freeze-fracture. A portion of tissue is frozen and then broken with a sharp knife. The cell membranes then cleave along the middle so as to separate the inner and outer lipid leaflets. A replica of the fractured face is made, 'shadowed' with some electron dense material and then examined in the electron

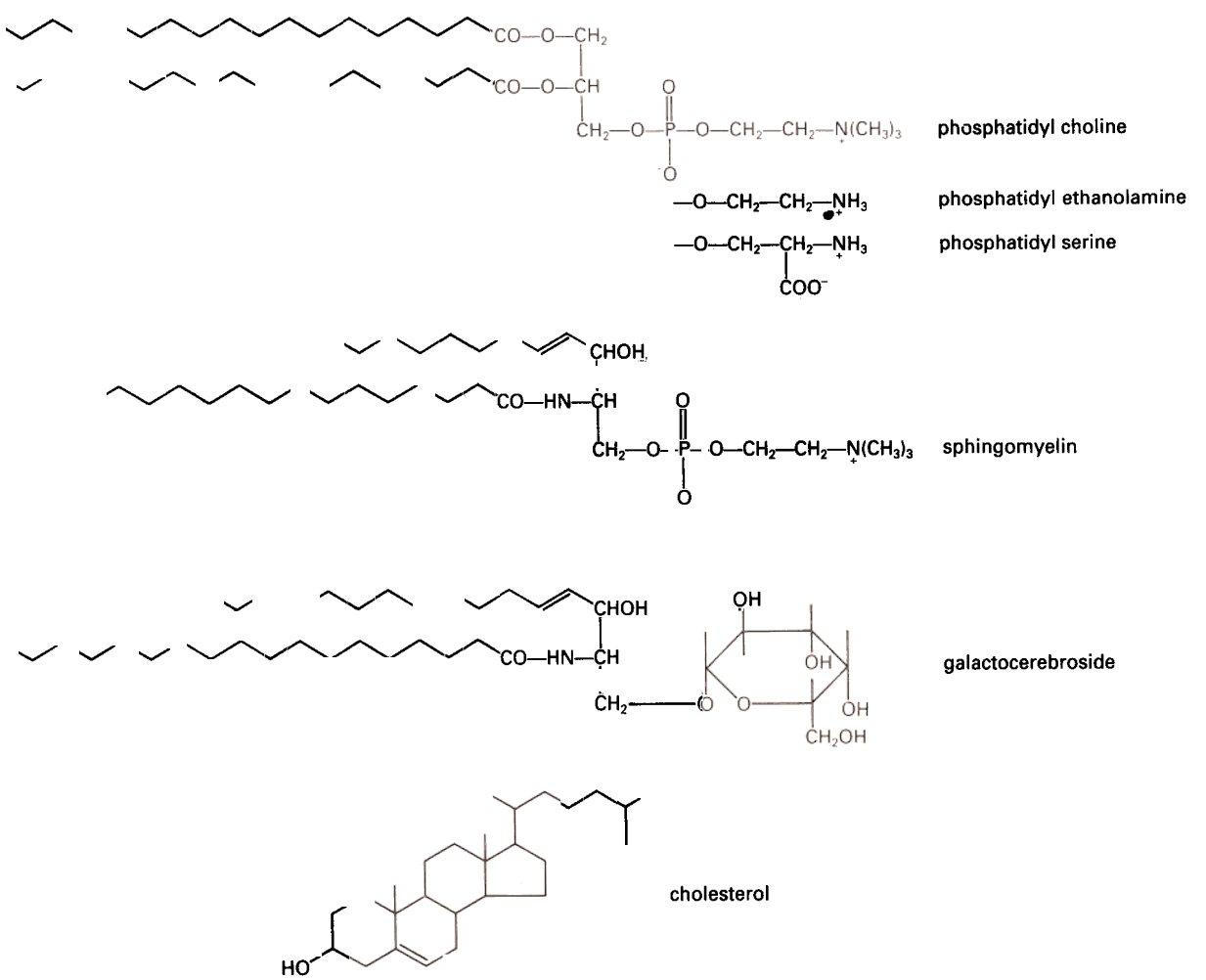


Fig. 3.1. Chemical formulae of some plasma membrane lipids. The fatty acid chains on the left of the formulae may be of variable length and may have one or more double bonds.

microscope. Small particles are seen projecting from both faces, but especially from the inner one; an example is shown in fig. 7.25*a*. If these are protein molecules (and it is difficult to see what else they could be) then they are clearly embedded within the lipid bilayer, rather than simply applied to its surface as in the Davson and Danielli model.

These results led to the conclusion that much of the protein of the plasma membrane penetrates the lipid bilayer, as is shown in fig. 3.3 (Singer and Nicolson, 1972; Bretscher, 1973). Singer and Nicolson suggested that the non-polar parts of these intrinsic membrane proteins are embedded in the non-polar environment formed by the hydrocarbon chains of the lipid molecules, whereas their polar sections project from the membrane into the

Fig. 3.2. The arrangement of phospholipid molecules in the cell membrane of the lipid bilayer. (Based on Griffith *et al.*, 1974, and Marsh, 1975).

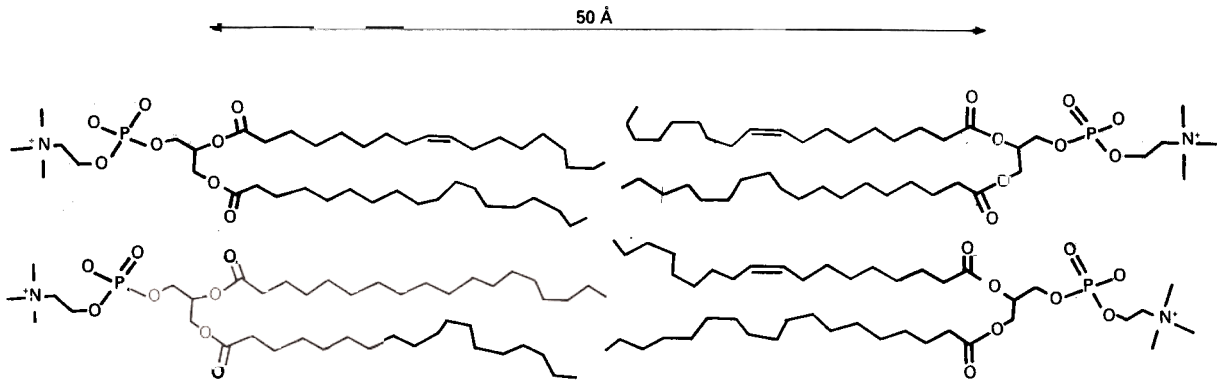
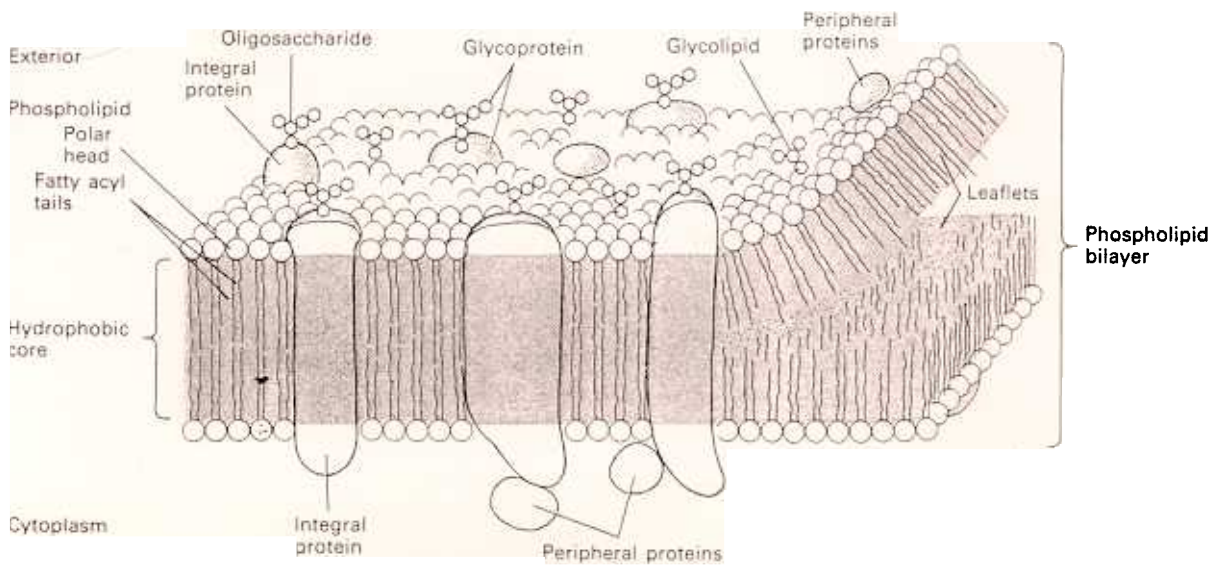


Fig. 3.3. Fluid mosaic model of the cell membrane. The phospholipid bilayer is shown split into two leaflets (as it might be by the freeze fracture technique) at the right. Integral proteins traverse the bilayer, peripheral proteins sit on its surface. Oligosaccharides occur on the outer surface of the membrane, attached mainly to proteins (forming glycoproteins) but also to lipids. (From Darnell *et al.*, 1986.)



polar environment provided by the aqueous media on each side of the membrane.

Singer and Nicolson view the membrane as a mosaic in which a variety of protein molecules serve different functions. There is evidence that some of the protein molecules are able to move in the plane of the membrane (rather like icebergs in the surface waters of some arctic sea) and hence the structure illustrated in fig. 3.3 has become known as the fluid mosaic model. The model has been refined since its original formulation, to include peripheral proteins, which are attached to the membrane but not embedded in it, and to recognize that many of the proteins may have their movements restricted by attachment to each other, to peripheral proteins or to proteins of the cytoskeleton (Nicolson, 1976).

Plasma membranes are highly asymmetrical structures (Bretscher, 1973; Rothman and Lenard, 1977). Phospholipids are differentially distributed in the two leaflets of the bilayer, so that there is more sphingomyelin and phosphatidylcholine in the outer leaflet and more phosphatidylethanolamine and phosphatidylserine in the inner one (Verkleij *et al.*, 1973). Proteins show an absolute asymmetry: they are nearly all positioned in their own particular way with respect to the inner or outer surface of the membrane. Thus, for example, transporting enzymes always have their ATP-binding sites on the inner (cytoplasmic) surface, glycoproteins have their sugar residues on the outer surface, and the peripheral proteins on the outer surface have quite different functions from those on the cytoplasmic surface.

We shall see later that the cell membrane, largely because of the impermeability of its lipid bilayer, acts as a barrier separating the contents of the cell from the outside world. But the proteins embedded in the membrane may act as doors in the barrier through which particular information or specific substances can be transferred from one side to the other. We shall see that there are many different doors and many different keys to open them.

Concentration cells

Consider the system shown in fig. 3.4. The two compartments contain different concentrations of an electrolyte XY in aqueous solution, and are separated by a membrane which is permeable to the cation X^+ but impermeable to the anion Y^-. The concentration in compartment 1 is higher than that in compartment 2. Obviously X^+ will tend to move down its concentration gradient, so that a small number of cations will move from compartment 1 to compartment 2, carrying positive charge with them. This movement of charges causes a potential difference to be set up between the two compartments. The higher the potential gets, the harder it is for the X^+ ions to move against the electrical gradient. Hence an equilibrium position is reached at which the electrical gradient (tending to move X^+ from 2 to 1) just balances the chemical or concentration gradient (tending to move X^+ from 1 to 2). Since the potential difference at equilibrium (known as the *equilibrium potential* for X^+) arises from the difference in the concentration of X^+ in the two compartments, the system is known as a *concentration cell*.

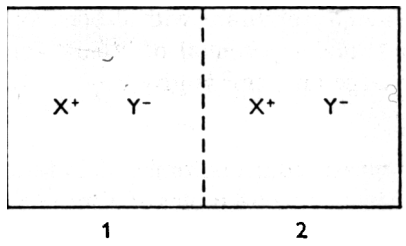
What is the value of this potential difference? Suppose a small quantity, δn moles, of X is to be moved across the membrane, up the concentration gradient, from compartment 2 to compartment 1. Then, applying elementary thermodynamics, the work required to do this, δW_c, is given by

$$\delta W_c = \delta n \cdot RT \log_e \frac{[X]_1}{[X]_2}$$

where R is the gas constant (8.314 J deg^{-1} $mole^{-1}$), T is the absolute temperature, and $[X]_1$ and $[X]_2$ are the molar concentrations* of X in compartments 1 and 2 respectively. Now consider the electrical work, δW_e, required to move δn moles of X against the electrical gradient, i.e.

* Or, more strictly, the activities of X in the two compartments. The simplification given here is valid as long as the activity coefficient of X is the same in each compartment.

Fig. 3.4. Concentration cell. See text for details.



from compartment 1 to compartment 2. This is given by

$$\delta W_e = \delta n \cdot zFE$$

where z is the charge on the ion, F is Faraday's constant (96 500 coulombs mole^{-1}) and E is the potential difference in volts between the two compartments (measured as the potential of compartment 2 with respect to compartment 1). Now, at equilibrium, there is no net movement of X, and therefore

$$\delta W_e = \delta W_c$$

or $$\delta n \cdot zFE = \delta n \cdot RT \log_e \frac{[X]_1}{[X]_2}$$

i.e. $$E = \frac{RT}{zF} \log_e \frac{[X]_1}{[X]_2}. \qquad (3.1)$$

[illegible] the Nernst equation after [illegible]

[illegible] (3.2)

or $$E = \frac{58}{z} \log_{10} \frac{[X]_1}{[X]_2} \qquad (3.3)$$

where E is now given in millivolts. For instance, in fig. 3.4, suppose $[X]_1$ were ten times greater than $[X]_2$, then E would be +58 mV if X were K^+ and +29 mV if X were Ca^{2+}; if the membrane were permeable to Y and not to X, then E would be −58 mV if Y were Cl^-, and −29 mV if Y were SO_4^{2-}.

How many X ions have to cross the membrane to set up the potential? The answer depends upon the valency of the ion, the value of the potential set up and the capacitance of the membrane. Let us consider a membrane with a capacitance (C) of 1 μF cm^{-2} and a potential of 70 mV across it. Then the charge on 1 cm^2 is given by

$$Q = CV,$$

where Q is measured in coulombs, C in farads and V in volts. The number of moles of X moved will be CV/zF, where z is the charge on the ion and F is Faraday's constant. In this case, assuming that X is monovalent,

$$\frac{CV}{zF} = \frac{10^{-6} \times 7 \times 10^{-2}}{96\,500}$$
$$= 6.8 \times 10^{-13} \text{ moles cm}^{-2}$$

which is a very small quantity. For instance (to anticipate a little), supposing we are dealing with a squid giant axon, diameter 1 mm, with a membrane capacitance of 1 μF cm^{-2}, and a membrane potential of 70 mV set up primarily by a potassium ion concentration cell: 1 cm^2 of membrane will enclose a volume containing about 3×10^{-5} moles of potassium ions, and thus a loss of 6.8×10^{-13} moles would be undetectable. In this case the difference between the numbers of positive and negative charges inside the axon would be about 0.000 002 %.

It is important to realize that the movements of ions from one compartment to another which we have been discussing are *net* movements. At the equilibrium position, although there is now no net ionic movement, X ions still move across the membrane, but the rate of movement (the *flux*) is equal in each direction. Before the equilibrium position is reached, the flux in one direction will be greater than that in the other. Fluxes can only be measured by using isotopic tracer methods. In describing fluxes across cell membranes, movement of ions into the cell is called the *influx*, and movement out, the *efflux*.

Ionic concentrations in the cytoplasm

Various microchemical methods are available for determining the quantities of ions present in a small mass of tissue. These include various microtitration techniques, flame photometry (in which the quantity of an element is determined from the intensity of light emission at a particular wavelength from a flame into which it is injected) and activation analysis (in which the element is converted into a radioactive isotope by prolonged irradiation in an atomic pile). All estimations made on a mass of tissue must include corrections made for the fluid present in the extracellular space. The size of the extracellular space is usually estimated by measuring the concentration in the tissue of a substance which is thought not to penetrate the cell membrane, such as inulin. Table 3.1 gives a simplified balance sheet of the ionic concentrations in

Table 3.1. *Ionic concentrations in frog muscle fibres and in plasma*

	Concentration in muscle fibres (mM)	Concentration in plasma (mM)
K	124	2.25
Na	10.4	109
Cl	1.5	77.5
Ca	4.9	2.1
Mg	14.0	1.25
HCO_3	12.4	26.6
Organic anions	*ca.* 74	*ca.* 13

Simplified after Conway, 1957.

Table 3.2. *Ionic concentrations in squid axoplasm and blood*

	Concentration in axoplasm (mM)	Concentration in blood (mM)
K	400	20
Na	50	440
Cl	40–150	560
Ca	0.4	10
Mg	10	54
Isethionate	250	—
Other organic anions	*ca.* 110	—

Simplified after Hodgkin, 1958.

frog muscle determined in this way. In the giant axons of squids, which may be up to 1 mm in diameter, the situation is much more favourable, since the axoplasm can be squeezed out of an axon like toothpaste out of a tube; table 3.2 shows the concentrations of the principal constituents.

The main features of the ionic distribution between the cytoplasm and the external medium are similar in both the squid axon and the frog muscle fibre. In each case the intracellular potassium concentration is much greater than that of the blood, whereas the reverse is true for sodium and chloride. Moreover, the cytoplasm contains an appreciable concentration of organic anions. These features seem to be common to all excitable cells. As we shall see, these inequalities in ionic distribution are essential to the electrical activity of nerve and muscle cells. They are dependent upon active transport processes which are driven by metabolic energy.

Active transport of ions

Sodium

The Nernst potential for any particular ion species shows what the cell membrane potential would be if the distribution of that ion across the membrane had reached equilibrium. If the actual membrane potential is not equal to the Nernst potential, then the ion is not in equilibrium and will tend to flow down its electrochemical gradient if the membrane is permeable to it.

The resting potential of frog muscle cells is usually around −90 to −100 mV, and that for squid axon is typically about −60 mV. Table 3.3 shows the Nernst potentials for the major monovalent ions in these cells. Clearly the distribution of potassium and chloride ions is fairly close to equilibrium in both cases, since their Nernst potentials are not far from the resting potential. Sodium ions, however, have a Nernst potential which is more than 100 mV more positive than the resting potential, and hence there is a large electrochemical gradient for sodium ions, tending to drive them into the cell.

One explanation for this situation might assume that the cell membrane is impermeable to sodium ions. However, this is not so; resting nerve and muscle cells do take up radioactive sodium ions (for example, the resting influx of sodium into giant axons of the cuttlefish *Sepia* is about 35 pmoles cm^{-2} s^{-1}). If nothing were done about this influx, the system would in time run down, so as to produce equal concentrations of each ion on both sides of the membrane. In fact, the cell prevents this by means of a continuous extrusion of sodium ions (frequently known as the 'sodium pump'). Since such extrusion must occur against an electrochemical gradient, we would expect this process to be an active one, involving the consumption of metabolic energy.

Let us first consider some experiments by Hodgkin and Keynes (1955*a*) on the extrusion of sodium from *Sepia* giant axons. An axon was

Table 3.3. *Application of the Nernst equation to nerve and muscle cells*

	Ion	Ionic concentrations (mM) External	Internal	Nernst potential (mV)
Frog muscle	K	2.25	124	−101
	Na	109	10.4	+59
	Cl	77.5	1.5	−99
Squid axon	K	20	400	−75
	Na	440	50	+55
	Cl	560	108	−41

placed in sea water containing radioactive sodium ions and stimulated repetitively for some time, so that (as we shall see in chapter 5) the interior of the axon became loaded with radioactive sodium. It was then placed in a capillary tube through which nonradioactive sea water could be drawn (fig. 3.5), and the efflux of sodium determined by measuring the radioactivity of samples of this sea water at intervals. This particular arrangement has two advantages: the efflux from the cut ends of the axon is not measured, and the measured efflux occurs from a known length of axon into a known volume of sea water. It was found that the relation between the logarithm of the efflux and time is a straight line of negative slope (as is shown in the first section of the graph in fig. 3.6), indicating that the efflux of radioactive sodium falls exponentially with time. This is just what we would expect to see if both the internal sodium ion concentration and the rate of extrusion of sodium ions are constant, since under these conditions a constant proportion of the radioactive sodium inside the axon will be removed in successive equal time intervals.

When 2:4-dinitrophenol (DNP) was added to the sea water, the sodium efflux fell markedly, and then recovered when the DNP was washed away (fig. 3.6). Now DNP is an inhibitor of metabolic activity, and probably acts by uncoupling the formation of the energy-rich compound adenosine triphosphate (ATP) from the electron chain in aerobic respiration, hence this experiment implies that the extrusion of sodium is probably dependent on metabolic energy supplied directly or indirectly in the form of ATP. More conclusive evidence for this view is provided by a series of experiments by Caldwell *et al.* (1960), one of which is shown in

Fig. 3.5. Apparatus used to measure the efflux of radioactive sodium from *Sepia* giant axons. (From Hodgkin and Keynes, 1955*a*.)

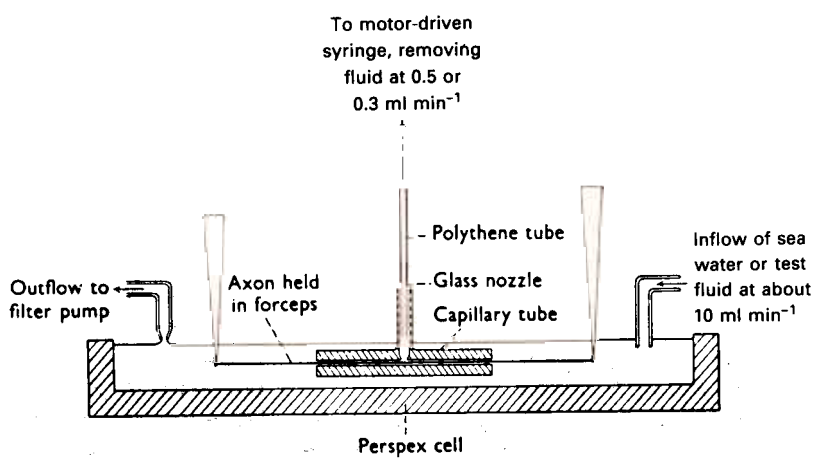


fig. 3.7. A solution containing radioactive sodium ions was injected into a giant axon of the squid *Loligo* by means of a very fine glass tube inserted longitudinally into the axon, and the efflux was then measured. Soon after the start of the experiment the axon was poisoned with cyanide. Cyanide ions interfere with the energy-producing processes in aerobic respiration (by inhibiting the action of cytochrome oxidase), and so the efflux of sodium fell to a low level. Injections of ATP, arginine phosphate or phosphoenol pyruvate into the axon produced transient increases in the rate of sodium extrusion, so confirming the view that the sodium pump is driven by the energy derived from the breakdown of energy-rich phosphate compounds.

Hodgkin and Keynes also showed that the

Fig. 3.6. The effect of the metabolic inhibitor 2:4-dinitrophenol (DNP) on the efflux of radioactive sodium from a *Sepia* giant axon. (From Hodgkin and Keynes, 1955*a*.)

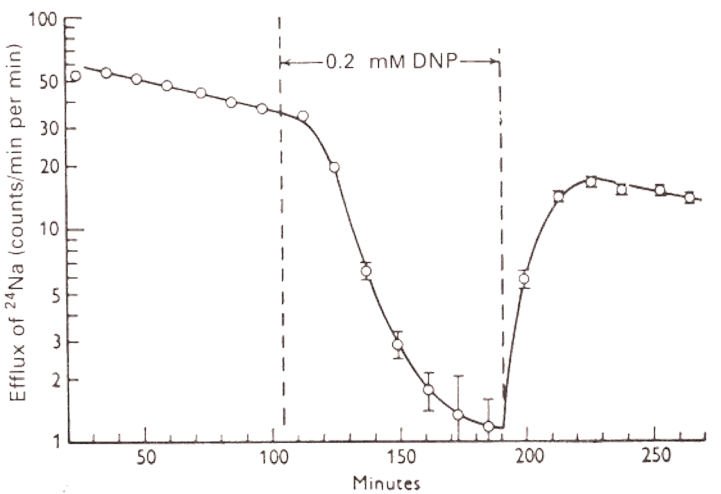
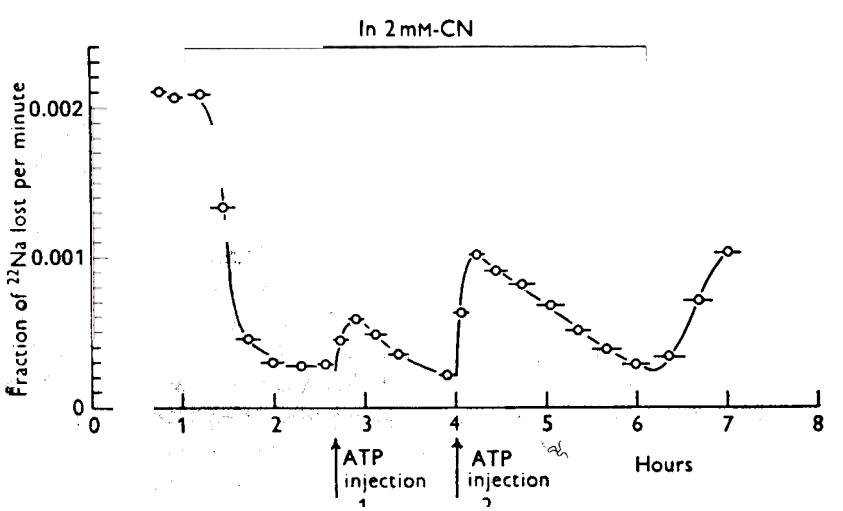


Fig. 3.7. The effect of ATP injection on the sodium efflux from a squid giant axon poisoned with cyanide. More ATP was introduced with the second injection than with the first. (From Caldwell *et al.*, 1960.)



sodium efflux from *Sepia* axons is dependent upon the external potassium ion concentration; potassium-free sea water reduced the efflux to about a third of its normal value. This implies that the sodium extrusion process is coupled to the uptake of potassium.

In terms of the fluid mosaic model of the cell membrane (fig. 3.3), we may suppose that sodium ions are pumped at particular sites each consisting of a particular protein molecule or group of molecules. How many of these sites are there in any particular area of membrane? The problem has been approached by measuring the binding of the drug ouabain to nerve axons. Ouabain is a glycoside found in certain plants and used as an arrow poison in parts of Africa. It acts by inhibiting the sodium pump of cell membranes. By measuring the uptake of radioactive oubain it is possible to estimate the number of sodium-pumping sites in a system, assuming that each site binds one molecule of ouabain. In squid giant axons, Baker and Willis (1972) estimate that there are 1000–5000 sites per square micrometre of cell membrane, and Ritchie and Straub (1975) estimate that for garfish olfactory nerve there are 350 sites per square micrometre.

More information about the sodium pump came from experiments on human red blood cells. Thus Post and Jolly (1957) measured the ratio of sodium ions to potassium ions moved by the pump. They stored red cells for two days at 2 °C; this would stop the activity of the pump and allow sodium entry and potassium loss by passive fluxes. On raising the temperature to 37 °C the pump became active again and so after a few hours it was possible to measure the change in the amounts of sodium and potassium ions in the cells. The results showed that two potassium ions were taken up for every three sodium ions extruded. In similar experiments with resealed red cell ghosts (red cells which have had their haemoglobin removed), Garrahan and Glynn (1967) found that one ATP molecule was split for every three sodium ions extruded.

Our understanding of the sodium pump was much enhanced when Skou (1957) isolated an ATPase that was stimulated by sodium and potassium ions from crab nerves. An ATPase is an enzyme which splits ATP, usually with the object of utilizing the energy of the ATP molecule for some energy-demanding function of the cell. Later work showed that the enzyme is a dimeric protein consisting of an α subunit (molecular weight 100 kDa) which contains the ATPase activity and the ouabain-binding site, and a β subunit (molecular weight 55 kDa).

The structure of the α subunit has been determined using complementary DNA cloning techniques. The base sequence of the complementary DNA was determined, and from this the amino acid sequence of the protein could be deduced. Details of such methods are given by Watson *et al.* (1983), for example, and we shall look at their application to the acetylcholine receptor (one of the first large membrane proteins to be examined with these techniques) in chapter 8.

The sequences of the Na,K-ATPase from sheep kidney (Shull *et al.*, 1985) and from the electric organ of the electric ray Torpedo (Kawakami *et al.*, 1985) are very similar, with over 85% of the sequence identical in the two enzymes. The α chain in the sheep kidney enzyme consists of a sequence of 1016 amino acids. From this sequence it is possible to make some deductions about the shape of the molecule.

For membrane proteins it is particularly useful to know which parts of the molecule are embedded in the membrane and which parts are in contact with the cytoplasm or the extracellular space. Different amino acid side chains have different affinities for aqueous and lipid environments; thus valine and leucine, for example, are hydrophobic (they will tend to be found preferentially in a lipid environment) and glutamic and aspartic acids, lysine and arginine are hydrophilic and so more likely to be found in an aqueous environment.

Kyte and Doolittle (1982) arranged the twenty amino acids commonly found in proteins on a scale of hydropathicity (also known as hydropathy or hydrophobicity) which measures their affinity for nonpolar environments. The scale runs from a hydropathicity index from +4.5 for the hydrophobic isoleucine to −4.5 for the hydrophilic arginine; the values for the different amino acids are shown in table 1.1. The scale can be used to tell us something about the structure of a protein whose amino acid sequence is known. For an amino acid at position i, the average hydropathicity index of all the amino acids from position $i - n$ to

position $i + n$ (where n is 7 or 9, for example) is plotted. The result is a hydropathicity profile whose peaks show hydrophobic sections of the protein chain and whose valleys show hydrophilic sections. If the hydrophobic sections are long enough, we may suspect that they are embedded in the lipid environment of the membrane and cross from one side of it to the other.

Application of the Kyte–Doolittle analysis to the Na,K-ATPase sequence (fig. 3.8) shows that there are at least six and perhaps eight major hydrophobic sequences, suggesting that the protein chain traverses the membrane six or eight times (fig. 3.9). ATP hydrolysis seems to involve binding to a lysine residue at position 501 and phosphorylation of an aspartic acid residue at 369; the chain is probably coiled so as to bring these two residues together. Binding of ouabain probably occurs at the tryptophan residue at position 310 in the short external portion between the third and fourth hydrophobic sequences.

Jorgensen (1975, 1982, 1985) found that the digestive enzyme trypsin cleaves the α chain at different sites according to whether sodium or potassium ions are present. This implies that the molecule changes shape when combined with these two different ions; it seems probable that this shape change is involved in the pumping activity.

Chloride

In squid giant axons, Keynes (1963) showed that, besides the potassium-linked sodium pump, there is another active transport system, concerned with the inward movement of chloride ions. If chloride were passively distributed across the axonal membrane, then the internal chloride concentration would be given by

$$E = \frac{RT}{-F} \log_e \frac{[\mathrm{Cl}]_o}{[\mathrm{Cl}]_i}$$

(where E is the resting potential), or, in isolated axons,

$$-60 = -58 \log_{10} \frac{560}{[\mathrm{Cl}]_i}$$

i.e. $[\mathrm{Cl}]_i = 55$ mM.

In fact, however, the average internal chloride concentration in Keynes's experiments was 108 mM, or about twice what one would expect. There are two possible explanations for this situation: either about half of the internal chloride was bound in non-ionic form, or there was an active

Fig. 3.8. Hydropathy plot of the amino acid sequence of sheep kidney Na,K-ATPase. Hydrophobic regions are above the x axis and hydrophilic ones below it. The major hydrophobic regions 1 to 8 are probably places where the chain crosses the membrane. (From Shull *et al.*, 1985).

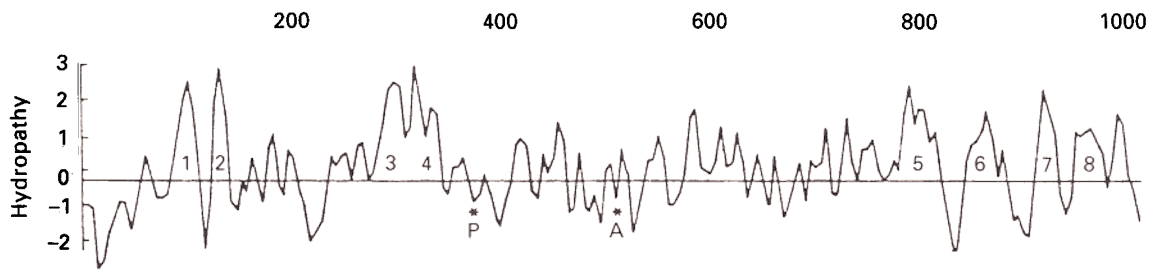
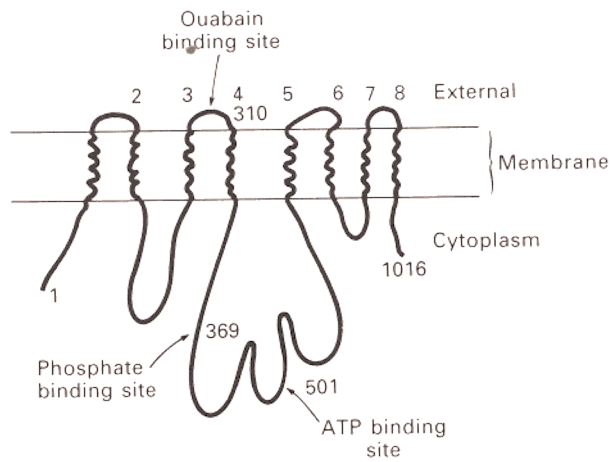


Fig. 3.9. A two-dimensional impression of the structure of the Na,K-ATPase molecule. In fact the molecule is probably folded so that the hydrophobic regions 1 to 8 are closer together.



inward transport of chloride. Measurements on extruded axoplasm with a chloride-sensitive electrode (a silver wire coated with silver chloride) showed that the activity coefficient of chloride in the axoplasm was much the same as that in free solution, which indicates that very little of the chloride can be bound. The chloride influx was halved by treating the axon with DNP, thus confirming the conclusion that there is some active uptake of chloride ions. The functional importance of this 'chloride pump' is obscure; Russell (1984) suggests that it may be important in the regulation of intracellular pH.

Fig. 3.10 summarizes the conclusions reached in this and the previous sections.

Calcium

The total concentration of calcium in axoplasm is low, usually in the range 50–200 μM (Baker and Dipolo, 1984).

If a small quantity of radioactive calcium ions is injected into a squid axon, the resulting patch of radioactivity does not spread out by diffusion or move in a longitudinal electric field (in contrast to potassium, for example – see fig. 3.12). This suggests that nearly all the calcium is bound in some way, and that only a small proportion is in free solution in the ionic form (Hodgkin and Keynes, 1957; Baker and Crawford, 1972). By using the protein aequorin, which emits light in the presence of calcium ions, it is possible to show that the concentration of ionized calcium is as low as 0.1 μM. Similar results are obtained using a calcium-sensitive microelectrode (Dipolo *et al.*, 1983).

The calcium is bound in a number of different ways by the axoplasm (Baker and Schlaepfer, 1978; Brinley, 1978). The mitochondria split ATP to take up an appreciable quantity of calcium. There is also an energy-independent binding capacity, part of which is accounted for by the calcium-binding protein calmodulin.

With an internal concentration around 0.1 μM, there is thus clearly a very great concentration gradient of calcium ions across the membrane, and it is therefore not surprising that there is a continual influx of calcium ions. This influx is normally balanced by a corresponding efflux in which the calcium ions are being moved up their electrochemical gradient, a process which must consume energy.

There are two components to this efflux. Firstly, there is an ATP-dependent 'calcium pump' which is a membrane-bound Ca-ATPase, active at low internal calcium ion concentrations. The enzyme has a molecular weight of 138 kDa and is activated by calmodulin. One ATP molecule is split for each calcium ion transported, and (in mammalian cells at 30 °C) 25–100 calcium ions are transported per second at each site (Carafoli and Zurini, 1982; Carafoli *et al.*, 1986).

The second system is one on which internal calcium ions are exchanged for external sodium ions. It comes into action when the internal calcium ion concentration is somewhat raised, and it is driven by sodium ions moving down their concentration gradient (Blaustein and Hodgkin, 1969; Dipolo *et al.*, 1983; Baker, 1986). Under suitable conditions a similar exchange working in the oppo-

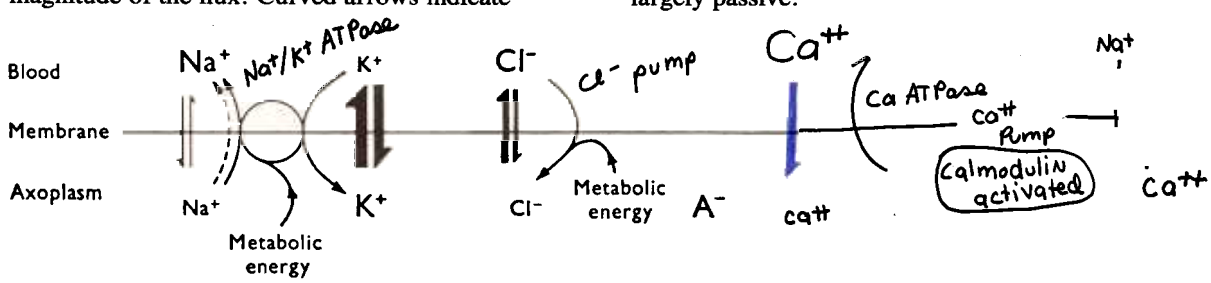


Fig. 3.10. A schematic representation of the movements of the major monovalent ions across the squid giant axon membrane in the resting condition. Passive fluxes are represented by straight arrows whose thickness suggests the magnitude of the flux. Curved arrows indicate active transport. Concentrations are suggested by the size of the chemical symbols. A^- represents indiffusible organic anions. In frog muscle cells the chloride fluxes are relatively larger, and chloride movement seems to be largely passive.

site direction can be detected; this requires ATP for its activity (Baker *et al.*, 1969; Baker, 1986).

Baker pointed out that the very low intracellular concentration of ionized calcium allows calcium ions to be used as a potent physiological trigger. Thus release of 1% of the bound calcium in squid axoplasm would increase the free ionic concentration forty-fold, from 0.1 to 4 μM. We shall see in later chapters that such trigger actions are fundamental to the release of synaptic transmitter substances and the initiation of muscular contraction.

The resting potential

The potassium electrode hypothesis

At the beginning of this century, Bernstein produced his 'membrane theory' of the resting potential. At that time there was some doubt as to whether there actually was any resting potential in the intact, uninjured cell. Bernstein held that there was such a potential, and that it arose as a result of the selective permeability of the cell membrane to potassium ions: he was the first, it seems, to apply the Nernst equation to the living cell.

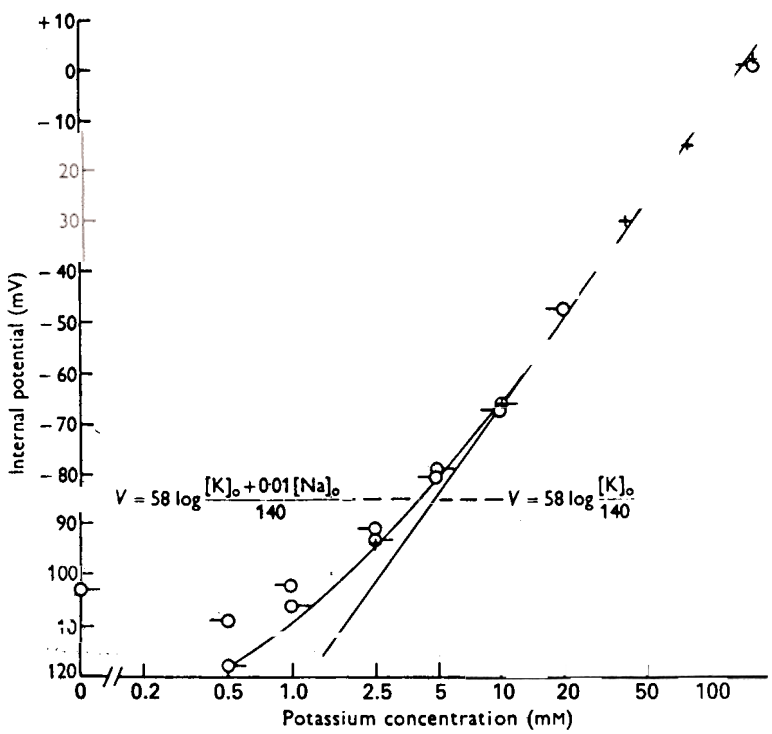
If it is correct that the resting membrane is selectively permeable to potassium ions, then we would expect the membrane potential to be given by the Nernst equation for potassium ions, which my be written, at 18 °C, as

$$E = 58 \log_{10} \frac{[K]_o}{[K]_i} \qquad (3.4)$$

The best method of readily testing this hypothesis is to vary the external potassium concentration and observe the change in membrane potential; (3.4) predicts that the relation should be a straight line with a slope of 58 mV per unit increase in $\log_{10}[K]_o$.

Fig. 3.11 shows the membrane potential of isolated frog muscle fibres in solutions containing different potassium ion concentrations (Hodgkin and Horowicz, 1959). It is evident that (3.4) fits the results very well at potassium concentrations

Fig. 3.11. The effect of the external potassium ion concentration on the membrane potential of isolated frog muscle fibres. The external solutions were chloride-free, the principle anion being sulphate. (From Hodgkin and Horowicz, 1959.)



above about 10 mM, but below this value the membrane potential is rather less than one would expect. Similar results have been obtained in squid axons (Curtis and Cole, 1942; Hodgkin and Keynes, 1955*b*) and many other cells (Hodgkin, 1951).

What is the reason for the departure from the simple potassium electrode hypothesis at low external potassium ion concentrations? We have seen that sodium ions are distributed across the cell membrane so as to produce the potentiality of a concentration cell with the inside positive, and since the membrane is not completely impermeable to sodium ions, we might expect this concentration cell to play some part in determining the membrane potential. Just how this effect will make itself felt will depend upon the properties of the membrane. A useful approach, known as the 'constant field theory' has been developed by Goldman (1943). This theory assumes that ions move in the membrane under the influence of electrical fields and concentration gradients just as they do in free solution, that the concentrations of ions in the membrane at its edges are proportional to those in the aqueous solutions in contact with it, and that the electrical potential gradient across the membrane is constant. From these assumptions it is possible to show (see Hodgkin and Katz, 1949) that, when there is no current flowing through the membrane, the membrane potential is given by

$$E = \frac{RT}{F} \log_e \frac{P_K[K]_o + P_{Na}[Na]_o + P_{Cl}[Cl]_i}{P_K[K]_i + P_{Na}[Na]_i + P_{Cl}[Cl]_o}, \quad (3.5)$$

where P_K, P_{Na} and P_{Cl} are permeability coefficients

Equation (3.5) is often known as the Goldman–Hodgkin–Katz equation. The permeability coefficients are measured in centimetres per second and defined as $u\beta RT/aF$, where u is the mobility of the ion in the membrane, β is the partition coefficient between the membrane and aqueous solution, a is the thickness of the membrane, and R, T and F have their usual significance. Actual values for the permeability coefficients must be determined by measuring the fluxes of the different ions through the membrane, but their ratios (such as P_K/P_{Na}) can be estimated from membrane potential measurements.

If chloride ions are omitted from the system, or if they are assumed to be in equilibrium so that the equilibrium potential for chloride is equal to the membrane potential, (3.5) becomes

$$E = \frac{RT}{F} \log_e \frac{[K]_o + \alpha[Na]_o}{[K]_i + \alpha[Na]_i}, \quad (3.6)$$

where α is equal to P_{Na}/P_K. Equation (3.6) is plotted in fig. 3.11, assuming α to be 0.01; it is clear that it provides a good fit for the experimental results. Results similar to those shown in fig. 3.11 have also been obtained from observations on the membrane potentials of nerve axons (Hodgkin and Katz, 1949; Huxley and Stämpfli, 1951). We can therefore conclude that sodium ions play some small part in determining the membrane potential of nerve and muscle cells, their effect being greater when the external potassium ion concentration is low.

One further piece of information is required before we can accept the hypothesis that the resting potential is determined mainly by the Nernst equation for potassium ions. It is necessary to show that the internal concentration of free potassium ions (which is involved in (3.4)–(3.6)) is the same, or almost the same, as the total internal potassium concentration (which is what can be measured by chemical methods of analysis). For instance, if half the internal potassium were found in non-ionic form, the predicted values for membrane potentials would have to be reduced by nearly 20 mV. This question was investigated by Hodgkin and Keynes (1953) on *Sepia* giant axons. They placed an axion in oil, but with a short length of it passing through a drop of sea water containing radioactive potassium ions. Thus, after a time, this short length of axon contained radioactive potassium. It was then placed in a longitudinal electric field, so that the potassium ions would move along the axon towards the cathode if they were free to do so, and the longitudinal distribution of radioactivity was measured at intervals by means of a Geiger counter masked by a piece of brass with a thin slit in it.

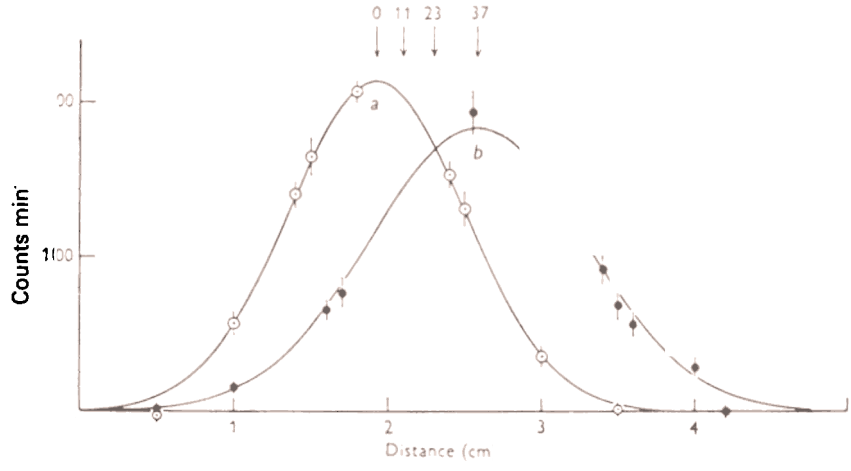
The result of one of these experiments is shown in fig. 3.12, from which it is clear that the internal radioactive potassium ions are free to move in an electric field. From the rate of movement, it was possible to calculate the ionic mobility and diffusion coefficient of radioactive potassium ions in the axon: the values were found to be close to those in a 0.5 M solution of potassium chloride. Thus the radioactive potassium is present inside the

axon in a free, ionic form. Since Keynes and Lewis (1951) had previously shown that radioactive potassium exchanges with at least 97% of the potassium present in crab axons, it seemed very reasonable to conclude that almost all the potassium in the axoplasm is effectively in free solution and so can contribute to the production of the resting potential.

The electrogenic nature of the sodium pump

Consider a membrane ion pump which is concerned solely with the active transport of one species of ion in one direction. There would then be a current flow across the membrane and a change in membrane potential caused by the depletion of charge on one side of the membrane. There would be similar consequences from a pump which coupled inflow of one ion to outflow of another but in which the numbers of ions transported in the two directions were not equal. A pump in which such a net transfer of charge does take place is described

Fig. 3.12. Movement of radioactive potassium in a longitudinal electric field in a *Sepia* giant axon. The two curves show the distribution of radioactivity immediately before (*a*) and 37 minutes after (*b*) application of the longitudinal current. Arrows at 11 and 23 minutes show the positions of peak radioactivity at these times. (From Hodgkin and Keynes, 1953.)



as being electrogenic. In mammalian red blood corpuscles, for example, there is very clear evidence that two potassium ions are taken up for every three sodium ions extruded (Post and Jolly, 1957); there must thus be a net flow of charge equal to one third of the sodium ion flow.

The sodium pump of nerve axons was once thought to be electrically neutral, because of a supposed one-for-one exchange of sodium for potassium ions. Later experiments on a variety of cells indicated that this is not so and that the pump is electrogenic (Ritchie, 1971; Glynn, 1984). Some of the best evidence for this conclusion comes from experiments by Thomas (1969, 1972) on snail neurons; let us examine them.

The essence of Thomas's method was to inject sodium ions into the cell body of a neuron and observe the subsequent changes in membrane potential. In order to perform the injection two microelectrodes filled with a solution of sodium acetate were inserted into the cell and current passed between them; sodium ions would thus be carried out of the cathodal electrode into the cytoplasm, so raising the internal sodium concentration. A third microelectrode was used to record the membrane potential.

Thomas found that injection of sodium ions into the cell is followed by a hyperpolarization of up to 15 mV, after which the membrane potential returned to normal over a period of about 10 min, as is shown in the first parts of the traces in fig. 3.13. Fig. 3.13*a* shows that ouabain greatly reduces this hyperpolarization, suggesting that it is connected with an active sodium extrusion. In fig. 3.13*b*, the second injection of sodium ions occurs while the cell is in a potassium-free environment, when there is no hyperpolarization, suggesting that sodium extrusion is coupled to potassium

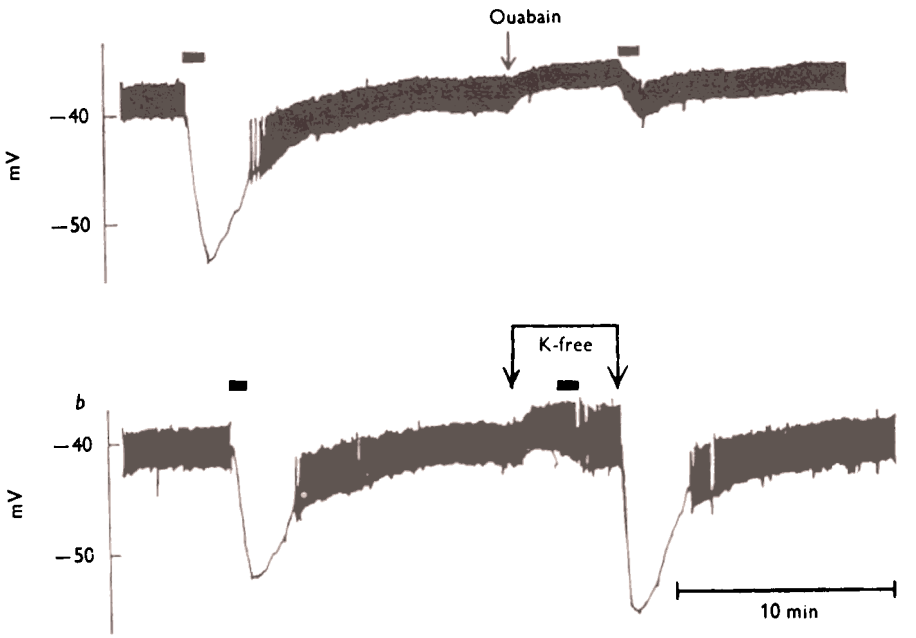


Fig. 3.13. Responses of snail neurons to injections of sodium ions, demonstrating the electrogenic nature of the sodium pump. The records show pen recordings of the membrane potential; black bars indicate the duration of the injecting currents. The first response in each trace shows the hyperpolarization which normally follows sodium injection. After treatment with ouabain (*a*) this is greatly reduced. In a potassium-free external solution (*b*), the hyperpolarization does not occur until external potassium is replaced, indicating that sodium extrusion is coupled to potassium uptake. (The thickness of the trace at less negative membrane potentials is caused by spontaneous activity in the neuron, the individual action potentials being too rapid to be registered fully by the pen recorder.) (From Thomas, 1969.)

uptake. (Incidentally this experiment also eliminates the hypothesis that the hyperpolarization is caused by a local reduction in potassium ion concentration at the outer surface of the cell, caused by such a coupled uptake of potassium.) Injections of potassium or lithium ions produced no membrane hyperpolarization.

These observations suggest that, while sodium extrusion is coupled to potassium uptake, the number of sodium ions moved is greater than the number of potassium ions. There is thus a net flow of positive charge outward during the action of the pump, seen as a hyperpolarization of the membrane. Is it possible to estimate the ratio of sodium ion to potassium ion movements? Thomas attacked this problem by using an ingenious method known as the voltage-clamp technique. We shall examine the principle of this method in chapter 5; suffice it to say here that in involves a negative feedback control system whereby the membrane potential is maintained at a constant level ('clamped') by passing current through it via another electrode. Membrane currents can thus be measured at constant membrane potentials. Thomas found that sodium injection was followed by an outwardly directed current which took about 10 min to fall to zero.

By integrating this current with respect to time it was possible to estimate the amount of charge transferred. Thomas found that this was always much less than the quantity of charge injected as sodium ions. And yet he also found that all of the injected sodium was extruded during the period of membrane current flow; he did this by using an intracellular sodium-sensitive microelectrode (one which produces an electrical signal proportional to the sodium ion concentration in its environment). This means that the sodium outflow must be partly balanced by an inflow of some other cation, for which the obvious candidate is potassium. Thomas calculated that the net charge transfer in the pump was about 27% of the sodium ion flow. This figure is fairly near to the 33% that would be expected from a system like that in red blood cells, where three sodium ions move for every two potsssium ions.

The Donnan equilibrium system in muscle

In the system shown in fig. 3.14, two potassium chloride solutions are separated by a membrane which is permeable to both potassium and chloride ions. The system is at constant volume so that, although the membrane is permeable to water, no net flow of water can occur between compartments i and o. Let us assume that the two potassium chloride solutions are initially at the same concentration. A quantity of a potassium salt KA is now added to compartment i, and it dissolves to give K^+ and A^- ions; we shall assume that the anion A^- cannot pass through the membrane. The concentration of potassium ions in compartment i, $[K]_i$ is now greater than in compartment o, $[K]_o$ so that potassium ions move from i to o. Since the total number of positive and negative charges in i must be approximately* equal (the same applies to o, of course), chloride ions move from i to o also. Now since $[K]_i \neq [K]_o$ and $[Cl]_i \neq [Cl]_o$, a concentration cell potential must arise between the two compartments in each case, such that, applying the Nernst equation,

$$E_K = \frac{RT}{F} \log_e \frac{[K]_o}{[K]_i} \tag{3.7}$$

and

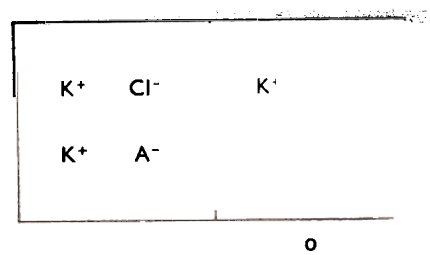
$$E_{Cl} = \frac{RT}{-F} \log_e \frac{[Cl]_o}{[Cl]_i} \tag{3.8}$$

where E_K is as the potassium equilibrium potential and E_{Cl} is the chloride equilibrium potential. But there can only be a single potential difference across the membrane; hence potassium chloride will continue to move from i to o until $E_K = E_{Cl}$.

When this stage is reached, the system is in

* See p. 15 for what is meant by 'approximately' in this context.

Fig. 3.14. Donnan equilibrium system. See text for details.



equilibrium and it follows that

$$\frac{RT}{F}\log_e\frac{[K]_o}{[K]_i}=\frac{RT}{-F}\log_e\frac{[Cl]_o}{[Cl]_i}$$

Therefore

$$[K]_o/[K]_i=[Cl]_i/[Cl]_o$$

or

$$[K]_i\times[Cl]_i=[K]_o\times[Cl]_o \qquad (3.9)$$

Equation (3.9) is the Donnan rule, which states that the product of the concentrations of the diffusible ions in one compartment is equal to the product of the concentrations of the diffusible ions in the other compartment, at equilibrium.

Thus the presence of the indiffusible anions in compartment i results in an inequality of distribution of the diffusible ions, potassium and chloride. However, we cannot apply this simple system to an animal cell, because an animal cell is not a constant volume system, and therefore the osmotic concentration must be the same on each side of the membrane. In the simple system of fig 3.14 this is not so, as the following analysis shows. Applying the condition for approximate electrical neutrality in each compartment,

$$[K]_o=[Cl]_o$$

and

$$[K]_i>[Cl]_i.$$

Now

$$[K]_i\times[Cl]_i=[K]_o\times[Cl]_o,$$

therefore

$$[K]_i+[Cl]_i>[K]_o+[Cl]_o,$$

therefore

$$[K]_i+[Cl]_i+[A]>[K]_o+[Cl]_o,$$

i.e. the osmotic concentration in i is greater than that in o. Thus if the constant-volume constraint were removed from the system, water would move from compartment o to compartment i. This would change the ionic concentrations in the two compartments and so disturb the Donnan equilibrium. Hence potassium chloride would move from o to i, again upsetting the osmotic equilibrium. These processes would continue until the concentration of A in i was infinitesimal and the concentrations of potassium and chloride were equal throughout the system.

Now consider a similar system (fig. 3.15) in which the membrane is effectively impermeable to sodium ions and some sodium chloride is added to compartment o. Potassium and chloride will move until the Donnan equilibrium is established, i.e. until

$$[K]_i\times[Cl]_i=[K]_o\times[Cl]_o$$

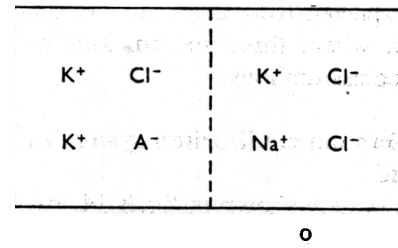
But now $[K]_o$ is less than $[Cl]_o$, so that if the constant volume constraint is removed, it *is* possible for the system to be in Donnan equilibrium and osmotic equilibrium at the same time. In effect, the osmotic effect of the indiffusible anion in i is balanced by that of the effectively indiffusible cation (sodium) in o.

We must conclude that a system similar to that shown in fig. 3.15 might account for the inequalities of ionic distribution seen in nerve and muscle cells. If this is so, then it should be possible to show that the [K][Cl] product is equal inside and outside after equilibrium has been reached in various conditions. This experiment was performed by Boyle and Conway (1941), who developed the theory outlined above. They soaked frog muscles in solutions containing various potassium chloride concentrations for 24 h, and then determined the intracellular potassium and chloride concentrations. The results conformed to the Donnan equilibrium hypothesis at all external potassium concentrations above about 10 mM.

Non-equilibrium conditions

We must now consider the contribution of chloride ions to the resting potential in muscle. This, together with some related problems, was extensively investigated by Hodgkin and Horowicz (1959) on isolated frog muscle fibres. The great advantage of using single fibres is that, with a suitable

Fig. 3.15. Double Donnan equilibrium system. See text for details.



apparatus, it is possible to change the ionic concentrations at the cell surface within a fraction of a second, so eliminating the inevitable diffusion delays involved in work with whole muscles. When the external solution was changed for one with different potassium and chloride concentrations but with the same $[K]_o[Cl]_o$ product, the new membrane potential (given by (3.7) and (3.8)) was reached within two or three seconds, and thereafter remained constant. However, if the chloride concentration was changed without altering the potassium concentration, the membrane potential jumped rapidly to a new value, but this transient effect gradually decayed over the next few minutes and the potential returned to very nearly its original value (fig. 3.16). The explanation of this effect is based on the Donnan equilibrium hypothesis. The chloride equilibrium potential is given by (3.4), or, at 18 °C,

$$E_{Cl} = -58 \log_{10} \frac{[Cl]_o}{[Cl]_i} \qquad (3.10)$$

At the start of the experiment shown in fig. 3.16 (when $[Cl]_o$ is 120 mM) we assume that chloride is in equilibrium, i.e. that E_{Cl} is equal to the membrane potential of −98.5 mV; this gives a value of 2.4 mM for $[Cl]_i$. When $[Cl]_o$ is reduced to 30 mM, E_{Cl} will change by 35 mV to −63.5 mV. The membrane potential changes to −77 mV, which is intermediate between E_K and E_{Cl}, indicating that the membrane is permeable to both potassium and chloride ions. Then, in order to restore the Donnan equilibrium, potassium chloride moves out of the cell until E_{Cl} is equal to E_K. This point is reached when $[Cl]_i$ has fallen to about 0.6 mM and, since there is also some movement of water out of the cell in order to maintain osmotic equilibrium, $[K]_i$ is practically unchanged. Hence the new steady potential (reached after 10–15 min in fig. 3.16) is the same as it originally was. When $[Cl]_o$ is returned to 120 mM, there is a similar transient

Fig. 3.16. The effect of a sudden reduction in the external chloride concentration on the membrane potential of an isolated frog muscle fibre. (From Hodgkin and Horowicz, 1959.)

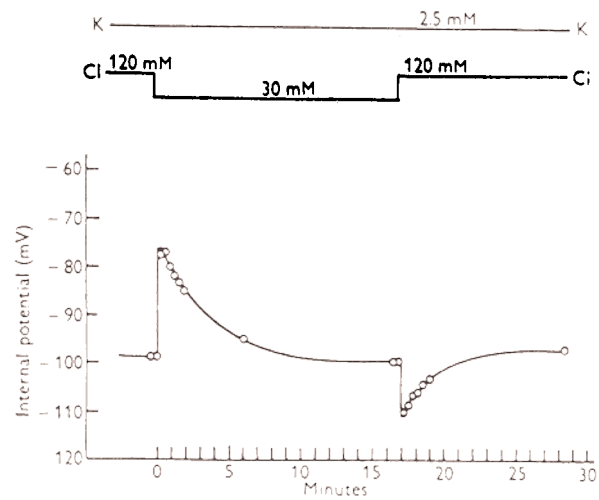
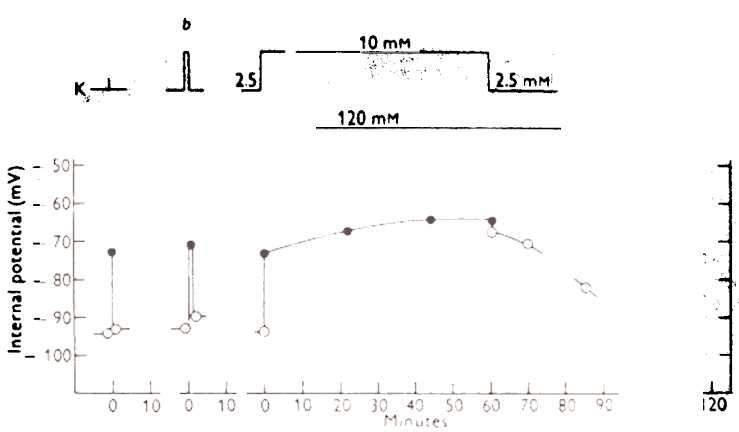


Fig. 3.17. The effect of changes in the external potassium ion concentration on the membrane potential of an isolated frog muscle fibre. (From Hodgkin and Horowicz, 1959.)



membrance potential change in the opposite direction, and equilibrium is then restored by movement of potassium chloride and water into the fibre. The fact that these experiments can be so readily interpreted in this way provides further evidence in favour of Boyle and Conway's Donnan equilibrium hypothesis.

Fig. 3.17*c* shows the results of a similar experiment in which $[K]_o$ is changed from 2.5 to 10 mM. This will change E_K by 35 mV, and so the membrane potential jumps to a new value intermediate between E_K and E_{Cl}. Equilibrium is then reached by entry of potassium chloride (and water) into the fibre, so that the membrane potential gradually moves from −73 to −65 mV. On returning $[K]_o$ to 2.5 mM, there is a small instantaneous repolarization, and then equilibrium (and the normal resting potential) is slowly restored by loss of potassium chloride from the fibre. Notice that, in this case, the fourfold increase in $[K]_o$ causes an instantaneous depolarization of 21 mV, whereas the later fourfold decrease causes an instantaneous repolarization of only 3 mV. This must indicate that there is some rectification process in the potassium pathway, so that potassium ions can move inwards very easily but outwards with much more difficulty. The larger repolarizations in figs. 3.17*a* and *b* are caused by the fact that the internal chloride concentration has not had time to change much from its normal level, so that E_{Cl} is still near the normal resting potential. Hodgkin and Horowicz calculated that P_K is about 8×10^{-6} cm s^{-1} for inward current but may be as little as 0.05×10^{-6} cm s^{-1} for outward current, whereas P_{Cl} remains at about 4×10^{-6} cm s^{-1} irrespective of the direction of the current flow.