J. Exp. Biol. (1972), 57, 513–541
With 1 plate and 17 text-figures
Printed in Great Britain

ELECTROPHYSIOLOGY AND IONIC MOVEMENTS IN THE CENTRAL NERVOUS SYSTEM OF THE SNAIL, *HELIX ASPERSA*

BY R. B. MORETON

A.R.C. Unit of Invertebrate Chemistry and Physiology, Department of Zoology, University of Cambridge

(*Received* 9 *March* 1972)

INTRODUCTION

The ionic mechanism of excitability in the nervous systems of gastropod molluscs has in recent years been the subject of some debate. Some species, such as the freshwater pulmonates *Lymnaea stagnalis* (Maiskii & Gerasimov, 1964; Sattelle & Lane, 1972) and *Planorbis corneus* (Gerasimov, Kostyuk & Maiskii, 1964; Maiskii & Gerasimov, 1964), have many neurones whose behaviour conforms to the 'conventional' sodium-potassium system, as typified by the squid giant axon (Hodgkin, 1958). Others, including the terrestrial pulmonates *Helix pomatia* (Gerasimov *et al.* 1964; Meves, 1968; Krishtal & Magura, 1970) and *Helix aspersa* (Kerkut & Gardner, 1967; Moreton, 1968*a*; Chamberlain & Kerkut, 1969), the marine opisthobranchs such as *Aplysia californica* (Junge, 1967; Geduldig & Junge, 1968) and *Tritonia diomedia* (Magura & Gerasimov, 1966; Veprintsev *et al.* 1966) and the freshwater lamellibranch *Anodonta cygnea* (Treherne *et al.* 1969; Carlson & Treherne, 1969), have many neurones whose action potentials appear to be wholly or partially independent of the concentration of sodium ions bathing the nervous system. A few giant cells in the abdominal ganglia of *Lymnaea* also exhibit this property (Sattelle & Lane, 1972).

In *Helix pomatia* the behaviour of the giant neurones under 'sodium-free' conditions can be accounted for by assuming that, as in the giant muscle fibre of the barnacle (Hagiwara, Chichibu & Naka, 1964; Hagiwara & Naka, 1964; Hagiwara, Hayashi & Takahashi, 1969), the inward current of the action potential is carried by influx of calcium ions, instead of sodium ions, into the cell. The overshoot of the action potential is linearly related to the logarithm of the calcium concentration, with a Nernst slope close to the theoretical value of 29 mV for a calcium electrode, and the cells are sensitive to externally applied manganous ions, but not to tetrodotoxin. In *Aplysia* (Geduldig & Junge, 1968) the neurones have been shown by similar experiments to be partially dependent on both sodium and calcium ions, both contributing jointly to the overshoot under 'normal' conditions.

In contrast to this, Carlson & Treherne (1969) have shown that conduction in the cerebro-visceral connectives of *Anodonta* is mediated by a 'conventional' sodium-dependent, tetrodotoxin-sensitive mechanism. Persistence of conduction under 'sodium-free' conditions is apparently due to the presence within the connective of a 'reservoir' of sodium ions, which can be mobilized as required to support the action potential of some of the axons. These stored sodium ions do not exchange readily

with sodium ions in the bathing medium, but can be made to do so if the connective is stimulated repetitively for long periods. Attempts to demonstrate the involvement of active transport processes in mobilizing the reserve were unsuccessful (Carlson & Treherne, 1969), and it was concluded that the store must be located extracellularly, possibly in association with acid mucopolysaccharide, which can be shown to be present in the intercellular clefts of the connectives. Recent experiments on *Tritonia* (Krasts & Veprintsev, 1972) suggest that in this species also conduction may be entirely sodium-dependent, the persistence of excitability under 'sodium-free' conditions being due to retention of sodium ions for a limited period in the tortuous narrow clefts which form the extracellular space in this nervous system.

In *Helix aspersa*, and in the cells of *Lymnaea* which are partially sodium-independent, the situation is confused. Although overshooting action potentials can be obtained for considerable periods under 'sodium-free' conditions (Kerkut & Gardner, 1967), long-term experiments (Moreton, 1968*a*) have shown that over periods of several hours there is a gradual decline in overshoot, leading in many cases to a complete loss of excitability. Restoring the sodium content of the bathing medium to normal results in a rapid recovery, the original value of the overshoot being restored within 2–4 min. Attempts to demonstrate directly the nature of the ions involved in generating the spike were only partially successful. Chamberlain & Kerkut (1969) showed that the cells are sensitive to cobaltous ions, suggesting that calcium ions are required to carry the inward current of the action potential. On the other hand, investigation of the calcium-dependence of the overshoot, both under 'normal' and under 'sodium-free' conditions, showed that although lowering the calcium concentration reduces the overshoot raising the concentration does not increase it. Also, even at low calcium concentrations, the Nernst slope is only about 18 mV, as compared with up to 29 mV for *Helix pomatia* neurones. The effects of calcium could thus be attributed to increasing stabilization of the membrane structure at higher concentrations, leading to improvement in the condition of the conductance mechanisms (cf. Frankenhaeuser & Hodgkin, 1957).

Experiments with the sodium-independent cells in *Lymnaea stagnalis* (Sattelle & Lane, 1972) have produced substantially similar results. In sodium-free solutions the action potential persists for a limited period, the decline in overshoot being somewhat faster than in *Helix aspersa*; sensitivity of the cells to manganous ions and to tetrodotoxin is variable, and experiments with calcium produced only partial slopes, of the order of 19 mV per decade concentration change.

It is thus possible that the excitability of giant neurones of *Helix aspersa* and *Lymnaea stagnalis* under 'sodium-free' conditions is due either to the use of other ions (such as calcium) in generating the rising phase of the action potential, or to regulation of the ionic environment of the neurones, presumably mediated by the glial system. Described below are the collected results of a long series of experiments on giant neurones of *Helix aspersa*, designed to distinguish between these two possibilities.

METHODS

Electrical activity of giant neurones in the isolated central nervous system of the snail *Helix aspersa* was recorded by the technique previously described (Moreton, 1968*b*, 1969). Plate 1 illustrates stages in the dissection procedure. In preparing this figure brains were dissected appropriately, fixed in Bouin's solution, dehydrated in alcohol and embedded in paraffin wax. Sections approximately 6 μm thick were stained with Azan. In some of the experiments on potassium movements preparations were used with the inner sheath intact (Plate 1 B, D). In all other experiments the preparation was de-sheathed, as shown in Plate 1 (C, E).

In the earlier experiments the volume of the experimental chamber was 5 ml; in later experiments, including those on the effect of tetrodotoxin, a smaller chamber with a volume of 0·5 ml was used. The preparation was secured by short, steel pins to a small slab of Polythene which formed the floor of the chamber.

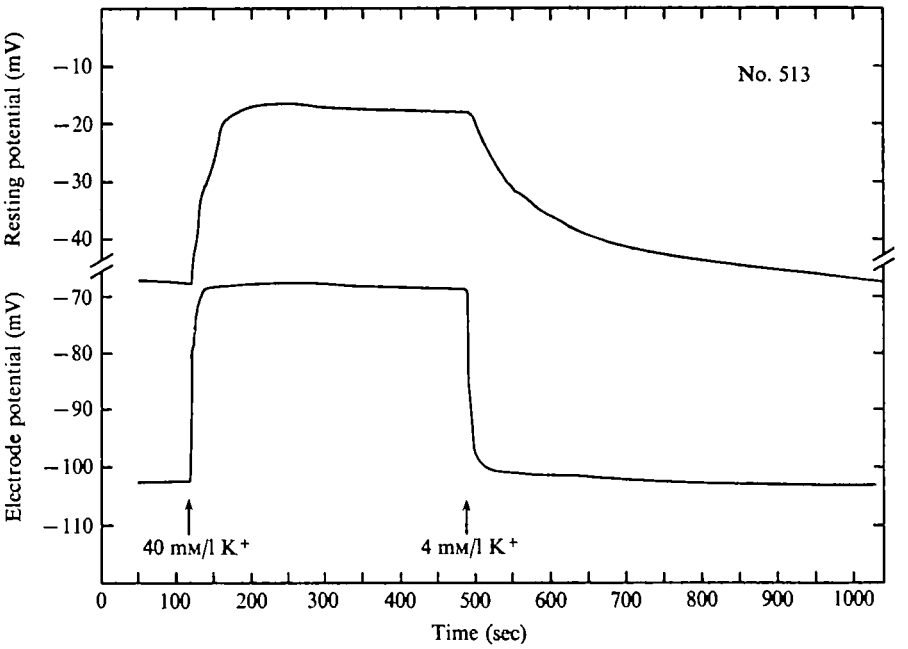
Mainly large (diameter >80 μm) cells in the left and right parietal ganglia were used, with a few from the visceral ganglion. Two glass microelectrodes, each filled with 3 M potassium chloride solution, and with a resistance of 8–25 MΩ, were inserted simultaneously into the cell body. Action potentials were evoked by passing single depolarizing current pulses down one electrode, via a 500 MΩ resistor. The recording electrode was connected by a short 3 M-KCl bridge to a chlorided silver wire, connected directly to the input of a high-impedance FET probe. To establish the zero level of the recording, the input to the probe could be connected to earth by means of a remotely controlled reed-switch placed close to the input. The silver wire, 3 M-KCl bridge and reed-switch were surrounded by a screen connected to the output of the probe, to reduce losses in bandwidth due to electrical capacity between input and earth. By this means, with a 20 MΩ microelectrode, the rise-time of the recording system was kept below 50 μsec. Since the rise-time of snail neurone action potentials is of the order of 2–10 msec, distortion due to limited bandwith of the recording system should be negligible.

Action potentials were photographed from an oscilloscope (Tektronix 502A or 565); in later experiments the use of a peak-memory circuit allowed the spike height to be recorded, together with the resting potential, on a chart recorder.

In experiments on potassium movements the potassium concentration in the solution immediately adjacent to the preparation was monitored by a potassium-selective microelectrode. This electrode was constructed from NAS_{27-4} glass (Eisenman, 1967) insulated with ceramic glaze (Carter *et al.* 1967), and was positioned adjacent to the recording electrode, and just above the cell.

The basic physiological saline used in the experiments had the composition (mM/l): NaCl, 80; KCl, 4; $CaCl_2$, 7; $MgCl_2$, 5; *Tris*/HCl, 5 (pH 8·0) (after Kerkut & Thomas, 1965). Sodium-free salines were made by replacing sodium chloride with equi-osmotic amounts of *Tris* chloride or sucrose. Other ions were varied by substitution with or for sodium chloride (or its substituent, in the case of sodium-free solutions).

Ouabain and potassium cyanide were made up freshly in Ringer for each experiment. In the case of cyanide it was necessary to readjust the pH by addition of a small amount of dilute hydrochloric acid.



Text-fig. 1. Effect of potassium ions on a giant neurone of *Helix aspersa*. At the points marked by arrows the solution in the experimental chamber was changed by rapidly flushing through a large volume (approximately 20 times the bath volume) of the new solution. The upper trace shows the resting potential of the cell; the lower trace shows the EMF of a potassium-selective glass microelectrode (see text), placed just outside the cell membrane.

RESULTS

Effects of potassium ions

Previous experiments (Kerkut & Meech, 1967; Moreton, 1968*b*) have shown that exposure of de-sheathed snail ganglia to high-potassium solutions results in rapid depolarization of the giant neurones, the half-time for depolarization being of the order of 1 min.

Text-fig. 1 shows the result of an experiment to measure the half-time for potassium depolarization of a giant neurone. The upper trace shows the resting potential of the cell, the lower trace the EMF of a potassium-selective microelectrode with an uninsulated length of 270 μm, placed just above the cell. Text-fig. 2 shows the 'steady-state' responses of cell and electrode to Ringer of different potassium concentrations, plotted using the method of Moreton (1968*b*). These relations can be used to calculate the effective concentrations of potassium ions (*a*) in the fluid adjacent to the cell membrane and (*b*) in the fluid just outside the preparation, at any given time.

Text-figs. 3 and 4 show the results of such calculations, averaged over 18 experiments on de-sheathed ganglia, and 11 experiments on ganglia with the inner capsule intact (Plate 1 B). Concentrations are plotted on a logarithmic scale, in the form used by Treherne *et al.* (1970); if the diffusion processes are 'first order', as would be the case, for example, in a system with no discrete diffusion barriers, such a plot should give rise to straight lines. As can be seen from the figures, the concentration in the external solution changes rapidly, with a half-time of $4{\cdot}8 \pm 0{\cdot}9$ sec (S.E.); 90% com-

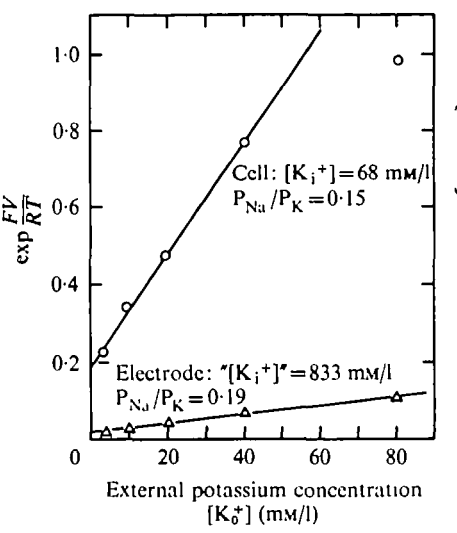


Fig. 2

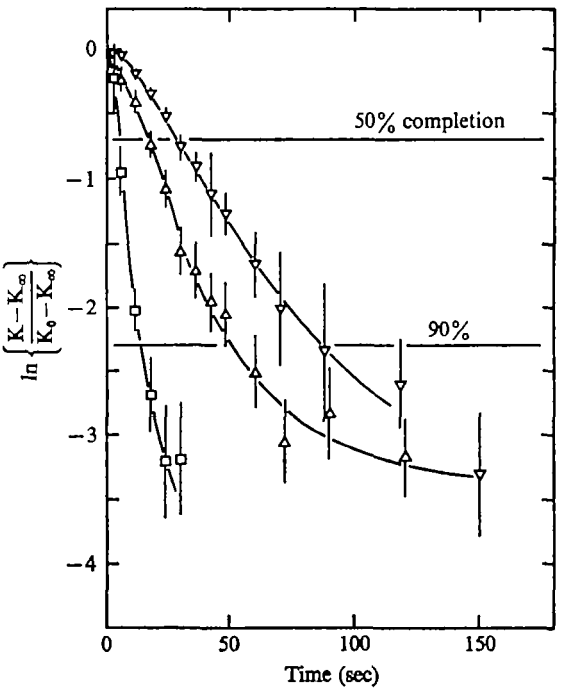


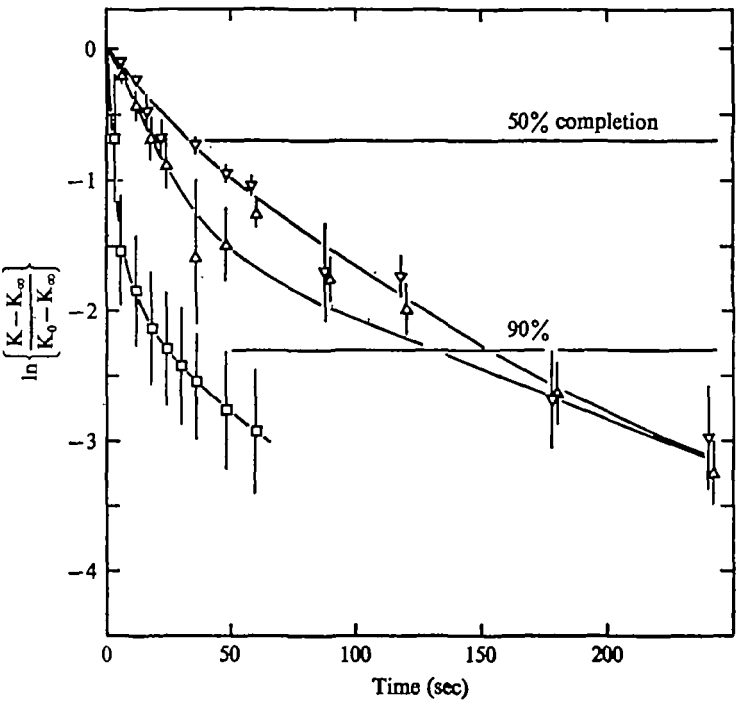
Fig. 3

Text-fig. 2. Potassium-dependence of the membrane potential (V) of the cell and the potassium electrode used in the experiment of Text-fig. 1. The effective internal potassium activity $[K_i^+]$ and selectivity P_{Na}/P_K were derived from the slope and intercept of each line (Moreton, 1968*b*).

Text-fig. 3. Time-dependence of the potassium concentration, derived from records such as Text-fig. 1. At time zero the concentration in the bulk solution is raised rapidly from K_0 to K_∞. The figure shows the concentration K (*a*) at the surface of the preparation (symbol □), (*b*) at the surface of the neurone in a de-sheathed preparation (△), and (*c*) at the surface of the neurone in a preparation with the inner capsule intact (▽). Each point is the mean of about 12 observations; the vertical bars show ± 1 S.E.M.

pletion is reached in approximately 15 sec. The later stages of the process are somewhat variable, particularly with decreasing concentrations (Text-fig. 4). Small errors in electrode potential become increasingly important as equilibrium is approached (the electrode potential could be read only to the nearest mV, and it is likely that at least part of the irregularity is due to this.)

Measurements of the extracellular potassium concentration are subject to errors due to changes in microelectrode tip potential, and long-term alterations in the properties of the cell. A simple, linear correction has been applied to minimize these. Text-figs. 3 and 4 show that the concentration at the cell surface in de-sheathed ganglia changes rather more slowly than that in the bathing medium, with a mean half-time of 19·2 ± 1·6 sec (S.E.). According to the formula given by Treherne *et al.* (1970), this corresponds to a diffusion path-length of 302 μm. The later stages of equilibration, particularly with decreasing concentrations, again showed long 'tails' in some cases. These could be due to a 'reservoir' effect caused by efflux of potassium ions from the glial cytoplasm, or to changes in cell membrane properties, or to

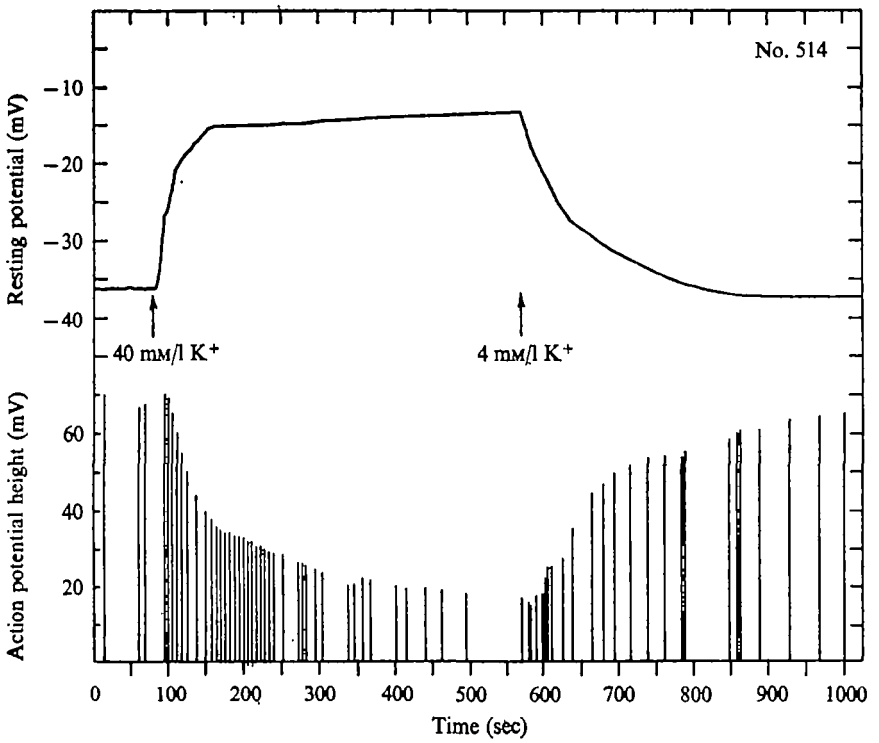


Text-fig. 4. Similar to Text-fig. 3, except that the potassium concentration was lowered at time zero (i.e. the data correspond to the repolarization phase of Text-fig. 1).

chloride movements. Their effect on the measured half-times was not serious, however, and their presence does not obscure the fact that changes in the potassium concentration in the bathing medium are very largely reflected at the neuronal surface within a period not greater than a few minutes.

That the 'depolarizations' observed in these experiments are real, and not due to the development of an extracellular potential difference, as was observed in the intact abdominal connective of the cockroach by Treherne *et al.* (1970), is shown by the experiment illustrated in Text-fig. 5. The action potential height was regularly monitored by means of a peak-memory circuit, and displayed as a series of vertical bars on the recorder chart. It will be seen that the time-course of the decline in action potential height is very similar to that of the change in resting potential.

Statistical analysis of variance of the individual half-times for potassium movement showed the following: (*a*) half-times for both increasing and decreasing concentrations are independent of the particular concentrations used, in the range 0–80 mM/l ($P > 0{\cdot}05$); (*b*) in de-sheathed preparations, there is a just significant difference between the half-times for increasing and decreasing concentrations, the latter being slightly longer ($P \doteqdot 0{\cdot}05$); (*c*) in sodium-free (*Tris*) Ringer, this asymmetry is not observed, both sets of half-times becoming indistinguishable from those observed for increasing concentrations in normal Ringer ($P > 0{\cdot}05$); (*d*) in 'intact' preparations, half-times for increasing and decreasing concentrations are similar ($P > 0{\cdot}05$), but both are significantly greater than any found in de-sheathed preparations ($P < 0{\cdot}001$). The mean half-time for 'intact' preparations is $32{\cdot}8 \pm 2{\cdot}2$ sec (S.E.).



Text-fig. 5. Effect of potassium ions on resting and action potentials of a giant neurone. The upper trace shows the resting potential; the vertical bars below indicate the height of the action potential (evoked by stimulation via a second intracellular microelectrode), recorded by a peak-memory circuit.

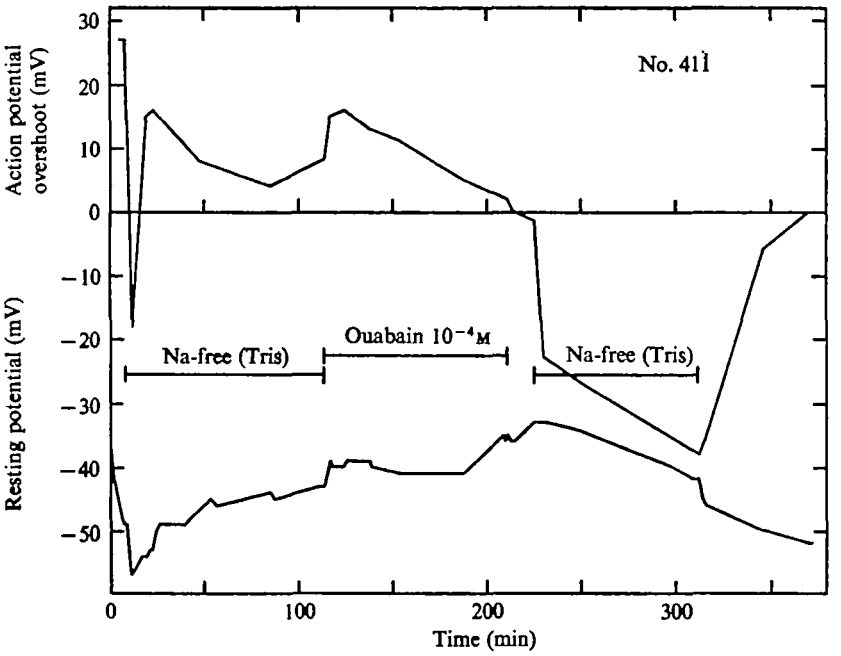
Table 1. *Half-times for potassium movement in* Helix aspersa *ganglia*

(All times are given in seconds, ±1 S.E., the number of results being given in parentheses.)

De-sheathed	Uptake	Efflux	Mean
Normal	19·4 ± 1·8 (1)	28·7 ± 3·0 (18)	24·0 ± 2·5 (36)
Na-free	17·8 ± 3·4 (4)	17·0 ± 1·5 (4)	17·4 ± 2·6 (8)
Mean	19·1 ± 2·2 (22)	27·5 ± 2·8 (22)	19·2 ± 1·6 (44)
Inner sheath intact	31·5 ± 2·2 (10)	36·3 ± 3·7 (10)	32·8 ± 2·2 (20)

The various half-times for potassium movement, measured in these experiments, are summarized in Table 1.

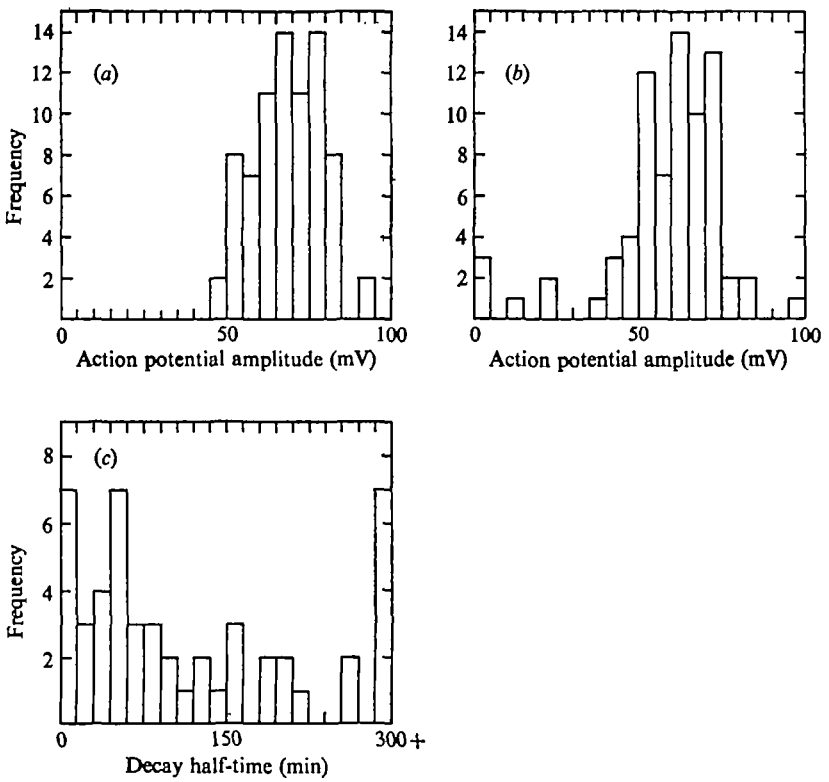
The experiments also provide measurements of the resting potential as a function of external potassium concentration, over a wide range, which may be compared with the results of Kerkut & Meech (1967) and Moreton (1968*b*). In general, the relationship between exp (*FV*/*RT*) and the potassium concentration was linear up to about 40 mM/l (Text-fig. 3), but deviated at higher concentrations, though in 5 out of 13 experiments a linear relationship was observed up to 80 mM/l. The mean P_{Na}/P_K, measured as in Moreton (1968*b*) from the linear part of the curve, was 0·169 ± 0·085 (S.E.M.), as compared with a value of 0·180 ± 0·015 obtained by the above.



Text-fig. 6. Record of an experiment to show the effects of sodium-free Ringer and of ouabain on resting and action potentials of a neurone. The upper trace shows the potential reached at the peak of the action potential and the lower shows the resting potential of the cell. Note the large, transient fall in the action potential on initial exposure to sodium-free solution. Exposure to ouabain in normal Ringer causes a gradual decline in overshoot followed by inability to produce an action potential in sodium-free solution. On returning to normal Ringer at the end of the experiment the action potential regains its original height, as observed before the final period of exposure to sodium-free solution, showing that the decline caused by the ouabain is not maintained under-sodium-free conditions.

Effects of sodium-free Ringer

The characteristic behaviour of snail neurones in sodium-free Ringer has been outlined by Kerkut & Gardner (1967), and the effects of prolonged exposure by Moreton (1968*a*). In the present work the initial transient fall in overshoot observed by the latter was seen in about 10% of experiments; in three experiments the action potential was abolished altogether, with an accompanying profound hyperpolarization (Text-fig. 6). Over a period of about 5 min the resting and action potentials recovered towards their initial values. In the remaining experiments an initial fall in overshoot, varying from only a few mV to almost complete abolition of the action potential, was followed by a more or less slow decline. The rate of decline was very variable from cell to cell, some cells maintaining action potentials for several hours, with only a small drop in overshoot, whereas others became inexcitable after only a few minutes. In three experiments action potentials were recorded from preparations which had been stored in sodium-free Ringer at 4 °C overnight. Text-fig. 7 illustrates the distribution of the results, expressed in terms of the amplitude of the action potential (A) in normal Ringer, (B) after 10 min in sodium-free Ringer and of the time required (C) in sodium-free Ringer for the amplitude of the action potential to decline to half the value shown in (B). (Values shown in (B) were obtained by extrapolation, where



Text-fig. 7. Histograms (based on results of 77 experiments) to show the distribution of action potential heights (*a*) in normal Ringer at the beginning of each experiment, (*b*) after 10 min exposure to sodium-free Ringer. (*c*) The distribution of the lengths of exposure to sodium-free Ringer required to reduce the height of the action potential to half its initial value, as shown in (*b*). The extreme right-hand column shows the number of experiments in which the action potential had still not declined to this extent after 5 h. These results are representative of the cells selected in the present experiments rather than of *Helix* neurones in general; in particular, in (*c*) results are shown only for those experiments in which the cell's behaviour was followed for a sufficient length of time.

there was an initial, transient fall in overshoot.) It should be noted that these distributions are not wholly typical of the behaviour of *Helix aspersa* neurones in general, since the cells were selected to some extent on a basis of their ability to generate action potentials in sodium-free solution. This is particularly true of the later experiments, since it was by that time possible to use certain identified cells, whose behaviour was more predictable than that of a cell selected at random.

Half-times for decline of the action potential are not significantly correlated with the magnitude of the initial decline on exposure to sodium-free solution ($r = -0{\cdot}108$; $P > 0{\cdot}25$).

On returning to normal (80 mM/l sodium), Ringer recovery was rapid, the full action potential height being regained within 2–3 min. The time required for recovery is thus comparable with that for potassium equilibration.

In three experiments large, non-transient reductions in action potential were observed on initial exposure to sodium-free solution. In these experiments the

Table 2. *Effects of sodium and calcium ions on the action potential*

(Each entry shows (*a*) the mean change in overshoot (mV) produced by substituting the concentration of sodium or calcium ions indicated for that present in normal Ringer; (*b*) the total range of effects observed (mV); and (*c*) the number of experiments. The table is divided into two sections: the main section shows the effect of varying the calcium concentration in the presence and absence of external sodium, and a smaller section shows, for comparison, the effect of sodium-free solution, derived from the same results as the histograms of Text-fig. 7.)

Calcium concentration (mM/l)	0	1	7	14	28
Normal sodium (80 mM/l)					
(*a*)	−14·0	−1·0	0	−0·3	−3·0*
(*b*)	−19 to −9	—	—	−5 to +2	—
(*c*)	4	1	—	4	1
Sodium-free					
(*a*)	−25·7	−11·1	0	+3·5†	−2·3†
(*b*)	−67 to −4	−21 to −4	—	−5 to +15	−24 to +12
(*c*)	15	7	—	11	8
Sodium concentration (mM/l)	0	80	.	.	.
Normal calcium (7 mM/l)					
(*a*)	−12·7	0	.	.	.
(*b*)	−84 to 0	—	.	.	.
(*c*)	71	—	.	.	.

* In one other experiment the cell became completely inexcitable, presumably because of the raised threshold in high calcium.

† In two other experiments the cell became completely inexcitable.

overshoot was roughly linearly related to the logarithm of the sodium concentration, the slope of the relationship varying between 30 and 43 mV for a tenfold concentration change.

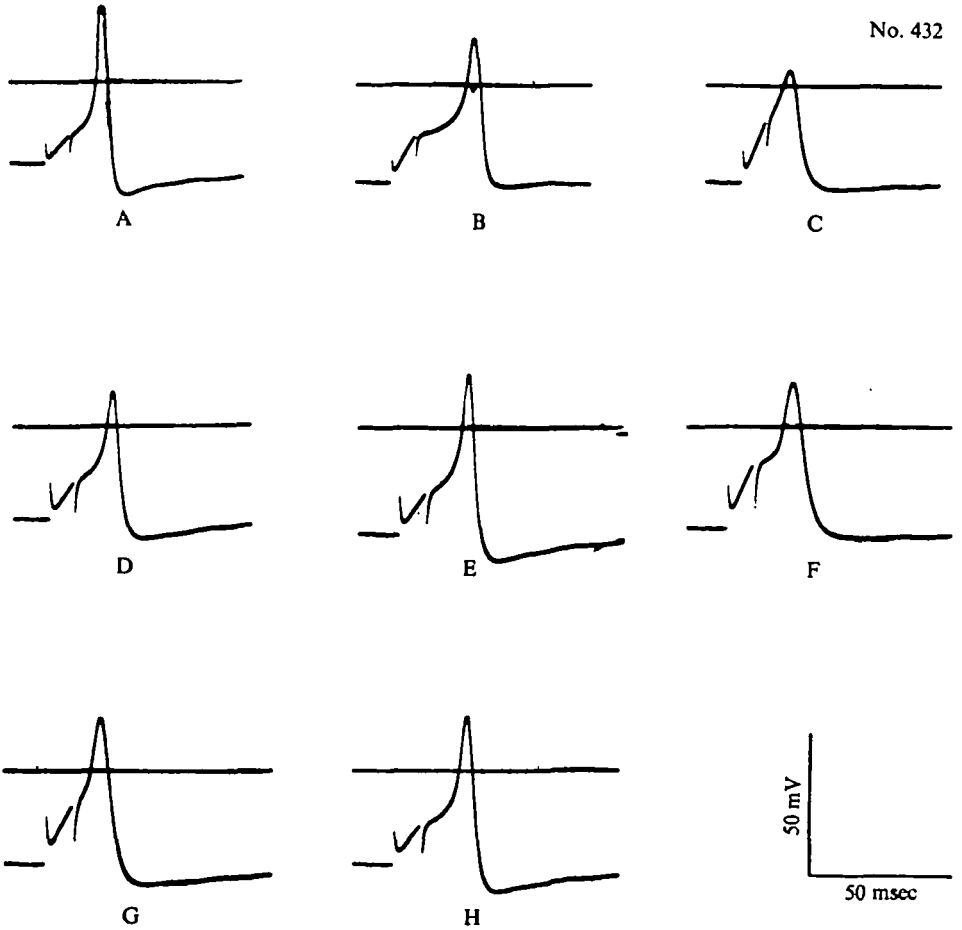
Effects of calcium ions

Kerkut & Gardner (1967) and Chamberlain & Kerkut (1969) showed that the action potentials of *Helix aspersa* neurones were reduced in height by lowered calcium concentrations, but not much affected by raised concentrations. Voltage-clamp experiments confirmed that the inward current in response to a depolarization behaved in a similar manner. The present experiments confirm this result in most cases, both in the presence and in the absence of external sodium. In only two experiments did raised calcium concentrations in the absence of external sodium give rise to an increased overshoot. 'Nernst' slopes for calcium, in the absence of sodium, varied between 18 and 25 mV (three experiments) as compared with the theoretical value of 29 mV per decade concentration change. Table 2 summarizes the range of results obtained.

Resting potentials were not consistently affected by variations in calcium concentration, except that calcium-free solutions generally caused a slight depolarization, averaging $5{\cdot}0 \pm 1{\cdot}1$ mV (S.E.). This effect was not dependent on the external sodium concentration ($P > 0{\cdot}25$).

Effects of manganous ions

The effect on the action potential of 10 mM/l manganous chloride added to normal Ringer was variable. Six cells showed only a small reduction in overshoot, with



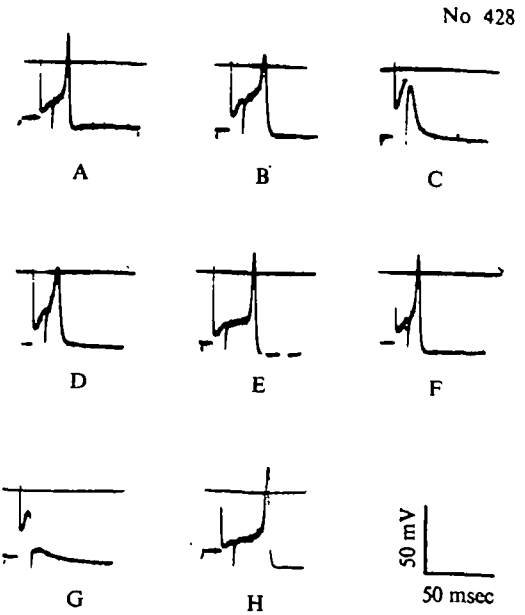
Text-fig. 8. Action potentials recorded from a cell which was apparently insensitive both to manganous ions and to sodium-free Ringer. (A) In normal Ringer (80 mM/l Na); (B, C) in sodium-free (Tris) Ringer, after 2 and 90 min, respectively; (D, E) 2 and 10 min after returning to normal Ringer; (F, G) 2 and 12 min after adding 10 mM/l Mn^{2+}; (H) 2 min after washing out the manganese.

lengthening of the rise-time of the action potential; of these, four also gave good action potentials in sodium-free solution (Text-fig. 8). Ten cells became inexcitable in 10 mM/l manganous chloride Ringer; of these, nine were excitable in sodium-free solution (Text-fig. 9).

Of four cells tested in the absence of external sodium ions, all were made inexcitable by 10 mM/l manganous chloride; one (illustrated in Text-fig. 9) became inexcitable in the presence of only 1 mM/l manganous ions, whereas in the presence of external sodium, 10 mM/l manganous chloride was required to produce the same effect. All effects of manganous ions were reversible.

Effects of tetrodotoxin

Previously (Moreton, 1968*a*) it was reported that tetrodotoxin at a relatively high concentration (5×10^{-6} to 10^{-5} M) would abolish the action potentials of *Helix*



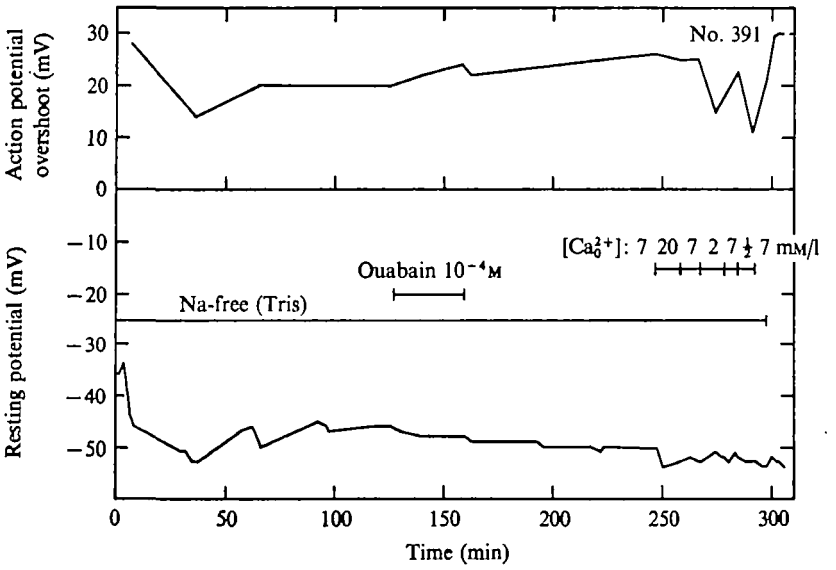
Text-fig. 9. Action potentials recorded from a cell which was sensitive to manganous ions but not to sodium-free Ringer. (A) In normal Ringer; (B) in sodium-free Ringer, after 32 min; (C) 2 min after adding 1 mM/l Mn^{2+}; (D) 59 min after washing out the manganese; still in sodium-free Ringer; (E) 11 min after returning to normal Ringer; (F) 9 min after adding 1 mM/l Mn^{2+}; (G) 2 min after increasing the Mn^{2+} concentration to 10 mM/l; (H) 4 min after washing out the manganese.

aspersa neurones. It has not been possible to repeat these results; at 10^{-5} M* in eight experiments effects ranged from none at all, to a reduction in overshoot of 19 mV, the mean reduction being 6·4 mV. In two of these experiments a transient disappearance of the action potential was observed, but the spike reappeared after 3–4 min, in the presence of the toxin, in one case recovering to its full initial amplitude.

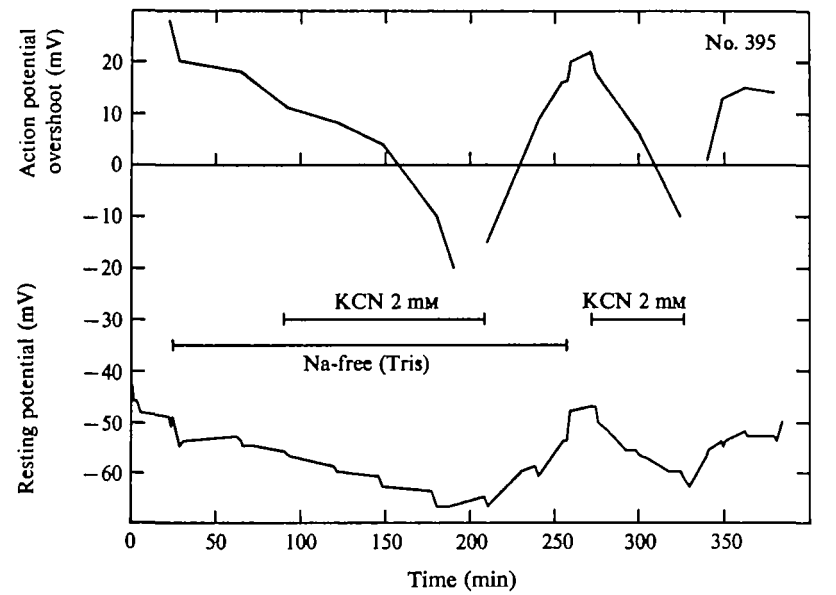
Effects of ouabain

In four experiments ouabain at 10^{-4} M was added to sodium-free Ringer, without effect on the action potential (Text-fig. 10); in two experiments brief (20 min) exposure to ouabain in normal Ringer was similarly without effect on the cell's behaviour on subsequent exposure to sodium-free Ringer. However, in four more experiments, prolonged (1½–3 h) exposure to normal Ringer, during or after exposure to ouabain caused a progressive decline in overshoot, followed by reversible failure of the action potential under sodium-free conditions (Text-fig. 6). The activity of the neuronal sodium pump is thus not directly necessary for maintenance of the action potential in sodium-free solution; but previous loading of the cell with sodium ions causes failure, on subsequent exposure to such conditions.

* Using a different batch of tetrodotoxin.



Text-fig. 10. Record of an experiment in which ouabain was applied under sodium-free conditions. The cell was impaled in sodium-free solution; ouabain applied under these conditions was without effect on the resting and action potentials. Also shown are the effects of various calcium concentrations on the overshoot in sodium-free solution. Reduced concentrations caused reversible reductions in overshoot, but raising the concentration to 20 mM/l was without effect. Changing to normal Ringer at the end of the experiment produced a small increase in overshoot.



Text-fig. 11. Record of an experiment in which 2 mM/l cyanide was applied to the preparation, both in the presence and in the absence of external sodium. In both cases a reversible decline in action potential overshoot was observed, leading to eventual abolition of the spike. Under 'normal' conditions this was accompanied by a decline in resting potential, but under sodium-free conditions the resting potential was not affected.

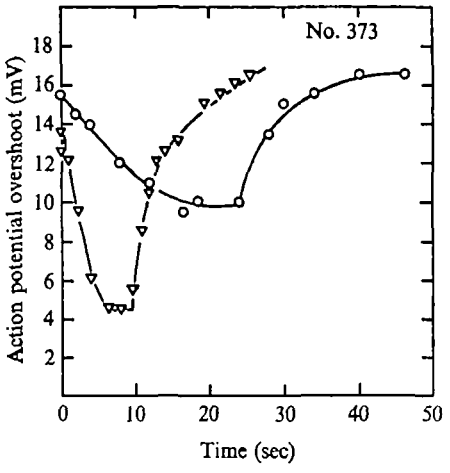


Fig. 12

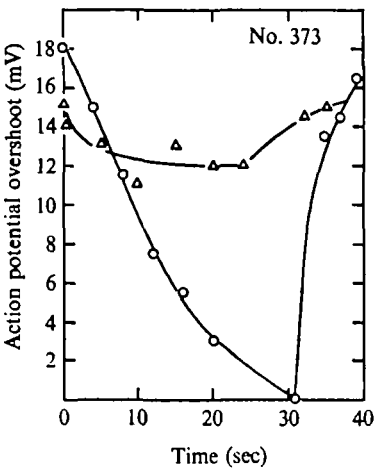


Fig. 13

Text-fig. 12. Behaviour of the action potential overshoot during and after rapid, repetitive stimulation of the neurone. The frequencies of stimulation were: 5/sec, reduced to 0·5/sec during recovery (○); 10/sec, reduced to 1/sec during recovery (▽).

Text-fig. 13. The same experiment as Text-fig. 12, but carried out under sodium-free conditions. Frequencies of stimulation were: 3/sec, reduced to 0·3/sec during recovery (△); 5/sec, reduced to 0·5/sec during recovery (○).

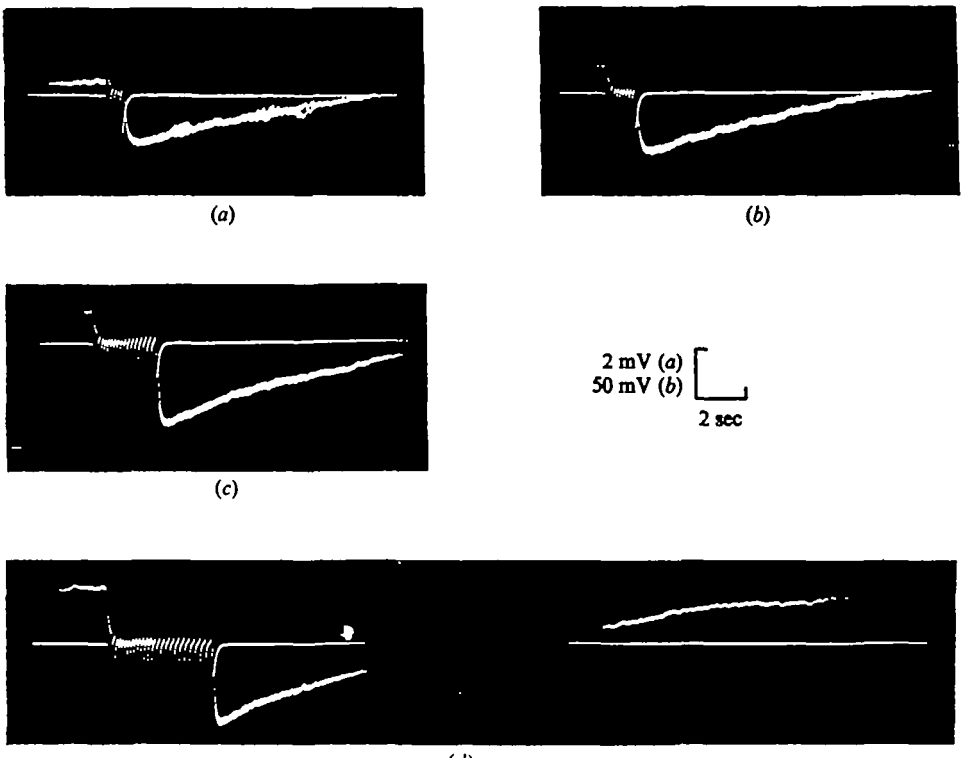
Effects of cyanide

The effect of 2 mM potassium cyanide in sodium-free Ringer was to hasten the slow decline in overshoot normally observed. This effect was seen in four experiments, and was reversible on washing out the cyanide, without further access to external sodium ions (Text-fig. 11). In two of the experiments (Text-fig. 11) the effect of cyanide in normal Ringer was also tested and was found to be similar.

Effects of stimulation

The effects of stimulation on the resting and action potentials of the neurones depend strongly on the frequency of stimulation. At high frequencies (greater than 3/sec) the action potential overshoot declines rapidly (Text-fig. 12), reaching a steady level which depends on the frequency. On reducing the rate by a factor of 10 the action potential gradually recovers its original overshoot, over a period of about 15 sec. Under sodium-free conditions, the decline in overshoot is more marked and occurs at lower frequencies of stimulation (Text-fig. 13), but the time required for recovery is similar.

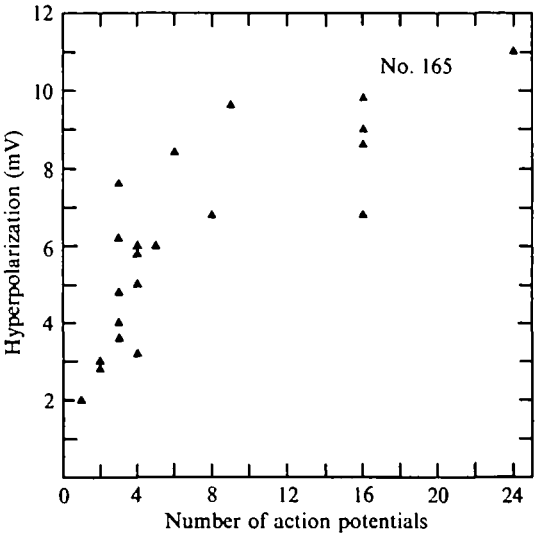
In some experiments the decline in overshoot was accompanied by a marked hyperpolarization of the resting cell membrane (Text-fig. 14). The extent of this depended on the number of spikes elicited (Text-fig. 15) and also on the external potassium concentration (Text-fig. 16). It could thus be due to an increase in the cell's potassium permeability, or to depletion of the extracellular sodium concentration, allowing the membrane potential to approach more closely the potassium equilibrium potential (cf. Moreton, 1968*b*). After stimulation the resting potential returned to its original value with a half-time which was independent of the number of spikes.



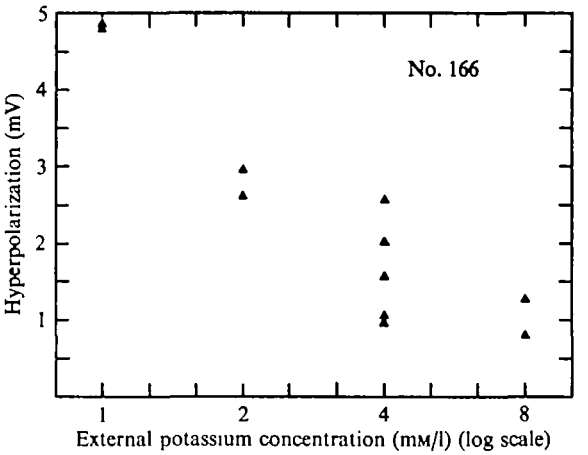
Text-fig. 14. Effect of rapid stimulation on the resting potential. The cell was stimulated by passing a long-duration depolarizing pulse through the stimulating electrode, the number of action potentials elicited being determined by the length of the pulse. The membrane potential was recorded simultaneously on two traces (*a*) at 2 mV/cm d.c., and (*b*) at 50 mV/cm a.c., to show the number of spikes: 4 in A, 9 in B, 17 in C and 25 in D. D is a composite mount of two consecutive sweeps to show the completion of the recovery phase.

Mean half-times varied between 0·3 and 9·6 sec, with an overall mean of $5{\cdot}6 \pm 0{\cdot}6$ sec (S.E.).

Experiments were also carried out on the effect of prolonged periods of stimulation at lower frequencies, under sodium-free conditions. After the initial, small decline in overshoot expected from the effects of high-frequency stimulation, little effect was observed on the behaviour of the action potential. In the experiment of Text-fig. 17 the cell was stimulated at 2/sec for 133 min – a total of nearly 16000 action potentials – without diminution in overshoot, although the resting potential declined slowly during this time. If the maintenance of excitability in sodium-free solution is due to the existence of a sodium reservoir in the ganglion, the reservoir must either be very large, or must be replenished by the activity of a sodium pump sufficiently rapidly to sustain the production of action potentials at this frequency indefinitely.



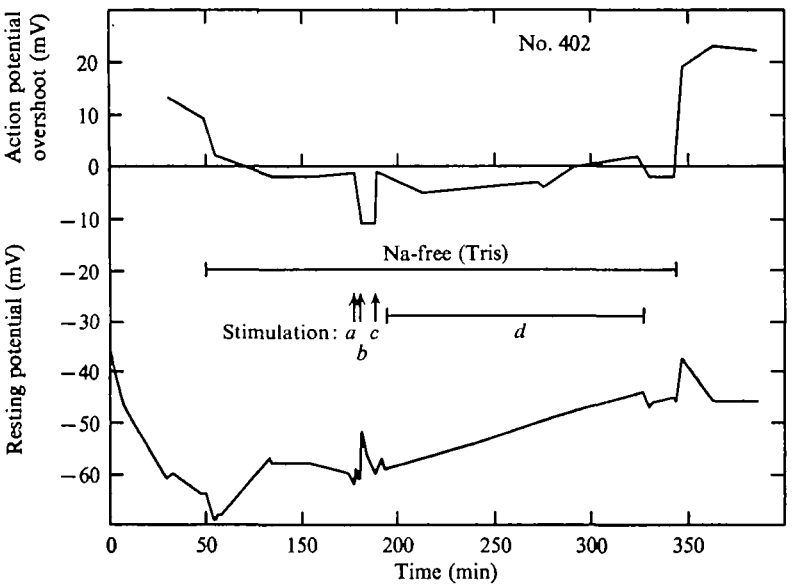
Text-fig. 15. Summary of results from the experiment of Text-fig. 14, to show the dependence of the observed hyperpolarization on the total number of spikes elicited. Each point represents one observation.



Text-fig. 16. Results from a similar experiment to that of Text-figs. 14 and 15, showing the effect of external potassium concentration on the hyperpolarization observed after repetitive firing of the cell. Each point represents one observation; the number of action potentials elicited in each case was 4.

DISCUSSION

The experiments described above fall into three groups: pharmacological experiments bearing on the ionic mechanism of the action potential; dynamic experiments giving information on the accessibility of the neuronal surface to small ions and molecules from the bathing medium; and experiments on the role of active transport in maintaining the ionic balance of the neurones and their environment. Inevitably, the significance of results in each of the three classes depends on both the others; pharmacological experiments can be interpreted only by assuming that the agents



Text-fig. 17. Long-term effects of stimulation at various frequencies, under sodium-free conditions. The neurone was stimulated as follows: (*a*) 10/sec for 20 sec; (*b*) 10/sec for 60 sec; (*c*) 10/sec for 60 sec; (*d*) 2/sec for 133 min. No long-term effects on the overshoot were observed, although the high-frequency stimulation caused a tansient decline.

used to modify neuronal behaviour can penetrate reasonably rapidly to the cells, and the same is true of experiments on metabolic inhibitors. Similarly, experiments on accessibility can only be interpreted if the effects of the tracer substance on the neurones can be characterized and to some extent quantified. Thus in discussing the results of any one experiment, the results and interpretation of a number of others must be taken for granted. The self-consistency and completeness of the overall synthesis must provide the justification for the various assumptions made.

Experiments on accessibility

Experiments on movements of potassium and sodium ions in the abdominal ganglia of *Helix* will be discussed first since, although their interpretation is indirect, it was the apparently anomalous results of this type of experiment which first led to the suspicion that neuronal action potentials in *Helix* were generated and maintained by a mechanism different from that found in 'conventional' excitable tissues, and hence to the investigations which followed.

The resting potential of giant neurones of *Helix aspersa* is determined principally by the external concentration of postassium ions (Kerkut & Meech, 1967; Moreton, 1968*b*). If the resting potential is denoted by V mV, the 'steady-state' relationship between $\exp(FV/RT)$ and the potassium concentration was shown by Moreton (1968*b*) to be linear, for concentrations between 0 and 16 mM/l. The present experiments show that this relationship holds up to 40 mM/l, though at still higher concentrations substantial deviations were found, the change in resting potential being smaller than expected (Text-fig. 2).

It should be noted that, at high potassium concentrations, the amount of sodium in the bathing medium is reduced. If the sum of sodium and potassium concentrations is kept constant, so that

$$[K_0^+] + [Na_0^+] = Y, \tag{1}$$

then equation (3) of Moreton (1968*b*) becomes

$$\exp\frac{FV}{RT} = \frac{(1 - P_{Na}/P_K)[K_0^+] + (P_{Na}/P_K)\,Y}{[K_i^+]}, \tag{2}$$

the linear relationship with $[K_0^+]$ being preserved.

This result may be compared with that of Kerkut & Meech (1967), who found that the variation of V with $\log_{10}[K_0^+]$ was relatively slight up to 20 mM/l, the curve becoming steeper at higher potassium concentrations, and approaching a 58 mV decade slope. However, these authors found considerable variations, both between cells and with the time of year. It is possible that the more marked dependence of the resting potential on the potassium concentration, which was found here at low concentrations, may be accounted for by such factors. The precise form of the resting potential/potassium concentration curve does not, of course, affect the calculations of extracellular potassium concentrations, provided it is adequately known. A linear relationship does, however, simplify the computation.

The early portion of the time course of potassium movement into the extracellular space surrounding the giant neurones is very similar to that calculated by Treherne *et al.* (1970) for movements in the long, narrow channel formed by the mesaxon spiral of the cockroach giant axon; a brief transient phase (of duration short compared with the half-time) is followed by a simple exponential approach to equilibrium. As plotted in Text-figs. 3 and 4, an initial, curved portion is followed by a linear section of the curve in each case. During the early stages of equilibration it thus appears that potassium movements are taking place in a relatively simple system, unrestricted by the presence of any discrete diffusion barriers. The path length of 302 μm, calculated from the mean half-time given in Table 1, probably consists partly of the 'unstirred layer' of solution adjacent to the preparation, and partly of the narrow cleft between the cell and adjacent glial processes. Measurements with the potassium-selective electrode show that the time required to change the bath concentration is small; but the dimensions and location of the electrode do not allow concentration changes to be followed directly at a distance less than about 100 μm from the preparation.

Electron-microscopy of 'de-sheathed' preparations (Moreton & Lane, unpublished) shows that, as suggested by the light micrograph of Plate 1 (E), the neurones are still tightly invested by one or more layers of very fine (< 1 μm) glial processes, which cover their surface almost entirely. Direct access to the 100–200 Å cleft between neurone and glia is only possible at a few junctions between adjacent processes. In order to achieve equilibration of potassium ions over the whole neuronal surface (which would be necessary in order to achieve a genuine steady-state resting potential) diffusion must therefore occur round a substantial fraction of the circumference of the neurone, which is of the order of 250–600 μm. Potassium diffusion times

in the de-sheathed preparation may thus reasonably be accounted for purely on a basis of the length of the diffusion pathway.

The degree of asymmetry between rates of depolarization and repolarization in de-sheathed preparations (see section 1 of Results), and also the departure of the curves in Text-figs. 3 and 4 from linearity at large values of the time, may possibly be explained by experimental error. At low potassium concentrations the membrane potential is less sensitive to the potassium concentration, so that the error in the latter, produced by a given error in potential, is larger. During depolarization the concentration and the membrane potential are both changing rapidly while the concentration is low; at times close to and longer than the half-time the concentration has already risen sufficiently to reach the region where the variation in potential is steeper. Errors in concentration thus become smaller as equilibrium is approached. During repolarization with decreasing concentrations of potassium, the steep part of the potential/concentration curve is mostly covered while the concentration is changing very rapidly, and readings during the later part of the process become very inaccurate.

A further complication is introduced by the presence of long-term changes in the properties of the cell. The effect of a slow drift in membrane potential is to alter the 'aiming potential' during the equilibration process. In the early stages of equilibration, when the membrane potential is in any case considerably different from its final value, this is of small importance. During the later stages, however, when the membrane potential has already approached its 'aiming' value quite closely, errors in the latter become important; again, the effect is to introduce uncertainty into the later parts of the potassium uptake and efflux curves, and again the efflux curves will be most affected since the dependence of potential upon concentration is least steep during the affected portion of the process. Although in analysing the results some attempt has been made to correct for long-term changes in membrane potential, it is evidently impossible to do so with much accuracy, since it would be necessary to know how the entire resting potential/concentration curve was affected at every time during the experiment.

The origin of such slow changes is uncertain. They could result simply from changes in the relative ionic permeabilities of the cell membrane (cf. the effects of repetitive stimulation, as discussed below), or from movements of potassium chloride across the cell membrane (Boyle & Conway, 1941). Increasing the potassium concentration, at a constant chloride level, leads to a gradual uptake of potassium chloride. If the chloride permeability of the cell is high enough, as is the case with frog muscle fibres (Hodgkin & Horowicz, 1959), this will result in a second, slow phase of depolarization, following the initial rapid change (cf. Text-figs. 1, 5). On returning to a lower potassium concentration these changes are reversed. Whether the chloride permeability of snail neurones is sufficient for these effects to be significant is doubtful (Kerkut & Thomas, 1964; Kerkut & Meech, 1966).

The reason for the disappearance of the asymmetry between depolarization and repolarization times when the experiments are carried out in the absence of external sodium is not known; the variability of the results is such, however (Table 1), that it is not considered profitable to discuss them in greater detail.

Lack of asymmetry in experiments with the inner sheath intact is presumably due to masking by the general increase in diffusion times. With the inner sheath intact,

diffusion times are increased by about 70%. This does not necessarily imply any specific restriction by the inner sheath, since in this condition the number of glial folds overlying the neurones is considerably larger (Treherne & Moreton, 1970), so that the path length for diffusion is increased. Pentreath & Cottrell (1970) have shown that the connective tissue surrounding *Helix aspersa* ganglia is freely permeable even to such large molecules as ferritin. The situation in *Helix* thus appears to differ from that in the cockroach (Treherne *et al.* 1970; Pichon, Moreton & Treherne, 1971), where a considerable degree of restriction to diffusion of potassium ions apparently occurs at the perineurium, peripheral to the central nervous tissues. Molluscan ganglia have no equivalent of the cellular perineurium (Treherne & Moreton, 1970).

Experiments on movements of ions other than potassium are subject to uncertainty in that their quantitative effects on the neurones are not known. The initial, rapid effect of sodium-free solution on the action potential, and the rapid recovery on returning to normal Ringer (Text-fig. 6; cf. Moreton, 1968*a*), suggest that the movement of sodium ions is relatively unrestricted. The same is true for calcium (Text-fig. 10; cf. Kerkut & Gardner, 1967; Chamberlain & Kerkut, 1969), and for manganous ions (Text-figs. 8, 9). However, if it is assumed that the action potential mechanism is purely sodium and potassium-dependent, then it must also be assumed that in many experiments the extracellular sodium concentration, although rapidly reduced on initial exposure to sodium-free solution, does not fall to zero, but reaches a state of quasi-equilibrium with some kind of reservoir. The transient fall and subsequent recovery observed in some experiments (Text-fig. 16) can be accounted for by assuming that processes responsible for replenishing or making available the reservoir can increase their rate in response to a fall in external sodium concentration, but do so only after a delay. This might easily be the case if active transport were involved.

The experiments on repetitive stimulation also have a bearing on the movements of sodium ions. The rapid fall in overshoot during stimulation at high frequencies can be attributed to depletion of ions in the narrow intercellular cleft which forms the ionic environment of the cell. The net inward movement of ions required to generate the rising phase of a single action potential can be estimated roughly from the product of the potential change and the membrane capacity. The latter is given for *Helix pomatia* by Maiskii (1964) as 20 μF cm^{-2}, which is probably an over-estimate, owing to the considerable folding of the neuronal cell membrane (Treherne & Moreton, 1970). Taking a value of 10 μF cm^{-2} as roughly corrected for folding, and a potential displacement of 70 mV (cf. Text-fig. 7), the minimum net influx of monovalent ions required per impulse is 7×10^{-12} M cm^{-2} (cf. the value of $3{\cdot}99 \times 10^{-12}$ M cm^{-2} calculated by Hodgkin & Huxley (1952) for squid axons at 18·5 °C). At a concentration of 80 mM/l a cleft 200 Å wide contains about 10^{-10} M cm^{-2}, so that a single action potential would reduce the concentration by about 7%. Recovery after stimulation could take place by diffusion of sodium ions from the bathing medium, assisted by active extrusion from neurones and glia; the time-course of recovery is consistent with this (cf. potassium half-times, as discussed above).

Hyperpolarization of the resting cell membrane after high-frequency stimulation is presumably due to some specific change in membrane properties. Depletion of extracellular sodium ions could produce a hyperpolarization, since the membrane potential is normally considerably less negative than the potassium equilibrium

potential (Moreton, 1968*b*), but such depletion, while it apparently does occur, would be expected to be accompanied by a comparable increase in the extracellular concentration of potassium (cf. Frankenhaeuser & Hodgkin, 1956; Orkand, Nicholls & Kuffler, 1966). As the cell membrane is more permeable to potassium ions than to sodium ions, the net result would be a depolarization. Examination of Text-fig. 14 shows moreover that (*a*) appreciable hyperpolarization is developed after a number of action potentials too small to cause any significant reduction in overshoot (cf. Text-fig. 12); (*b*) the maximum hyperpolarization is developed a short time after the last action potential of the series, rather than progressively during stimulation; (*c*) the time required for recovery is too short to be accounted for by diffusion of ions towards or away from the cell membrane.

Hyperpolarization of the cell membrane after stimulation could also be produced as a result of stimulation of the electrogenic sodium pump by a local rise in intracellular sodium concentration, caused by influx of sodium ions during the action potential (cf. Kerkut, Brown & Walker, 1969). The potassium-dependence of the effect, however (Text-fig. 16) suggests that it is due to an increase in potassium permeability similar to, but more long-lasting than, that responsible for the 'negative after-potential'; it could perhaps be regarded as a summation of the residual effects of a number of such after-potentials. Similar 'post-tetanic hyperpolarization' has been observed in stretch-receptor neurones of the crayfish (Nakajima & Takahashi, 1966; Sokolove & Cooke, 1971) and in mammalian central neurones (Kuno, Miyahara & Weakly, 1970). In these cases the effect was attributed to stimulation of the electrogenic sodium pump, but Gage & Hubbard (1966) attributed post-tetanic hyperpolarization in motor nerve terminals of the rat to an increase in potasium permeability of the cell membrane. It could also be responsible for the spontaneous behaviour of 'bursting pacemaker' neurones in *Aplysia* (cf. Chen, von Baumgarten & Takeda, 1971).

Increased potassium permeability could be the result of calcium ions entering the cell during the action potential (Meech, 1972).

If the cell's resting potential is assumed to be given by the equation

$$\exp\left[\frac{FV}{RT}\right] = \frac{[K_0^+]}{[K_i^+]} + \frac{P_{Na}[Na_0^+]}{P_K[K_i^+]} \tag{3}$$

(Moreton, 1968*b*), then it can be shown that a change in the selectivity of the cell membrane $\Delta(P_{Na}/P_K)$ will cause a change in membrane potential ΔV, given by

$$\frac{F}{RT}\exp\left(\frac{FV}{RT}\right)\Delta V = \frac{[Na_0^+]}{[K_i^+]}\Delta\left(\frac{P_{Na}}{P_K}\right). \tag{4}$$

The product $\Delta V \exp(FV/RT)$ should thus have a constant value, independently of the external potassium concentration. Examination of the data shown in Text-fig. 16 shows that this is approximately true, except for $[K_0^+] = 1$ mM/l, when the resting potential of this particular cell in any case departed considerably from the value expected from equation (3). The product has the value $-0{\cdot}047 \pm 0{\cdot}006$ (S.E.); using the intracellular potassium concentration of 89 ± 13 mM/l, derived from equation (3), it can be deduced that, in this experiment, the permeability ratio P_{Na}/P_K has the

value 0·165, falling by an amount $\Delta(P_{Na}/P_K) = -0{\cdot}053 \pm 0{\cdot}011$ (S.E.), after stimulation.

At lower frequencies of stimulation (2/sec), the action potential overshoot remains constant over long periods, even under 'sodium-free' conditions (Text-fig. 17). This is, however, consistent with the replacement of ions by active extrusion from the cells, since the rate required for 2 imp./sec is only 14×10^{-12} M cm^{-2} sec^{-1}, which is within the capabilities of the neuronal sodium pump (Moreton, 1969; Thomas, 1972). Under 'sodium-free' conditions, assuming that sodium ions are still required to generate action potentials, this rate must be increased to allow for loss of ions from the extracellular space to the bathing medium. The rate of loss, assuming diffusion of sodium ions at a similar rate to that for potassium ions, from a cleft 200 Å wide, is about 15×10^{-12} M cm^{-2} sec^{-1}, so that the net rate of active transport required is about 30×10^{-12} M cm^{-2} sec^{-1} – still within the capability of the sodium pump.

The rate of loss to the bathing medium can also be used to calculate the size of the reservoir needed to maintain activity for the observed periods under 'sodium-free' conditions. If the intracellular concentration is 20 mM/l (the maximum neuronal value which would still allow a sufficient concentration gradient across the cell membrane to generate the action potential), a glial process 1 μm thick contains enough sodium ions to last for 3 h; the neurones, which are mainly 40–200 μm in diameter, presumably contain much greater reserves (but compare the relatively low intra-cellular sodium activities reported by Thomas (1972) for the neurones). No data is available on direct measurements of sodium loss from *Helix* ganglia. It is possible that the slow decline in overshoot during prolonged exposure to sodium-free solution is due to the gradual exhaustion of an intracellular sodium store.

The behaviour of the resting potential during exposure to sodium-free solution is difficult to interpret. There is an initial hyperpolarization, though this is not as large as would be expected from the constant-field theory (see equation (3) above), and is seldom maintained; the resting potential frequently drifts back towards the original level (cf. Text-figs. 6, 10, 11, 17). Return to normal Ringer usually, though not always, causes a depolarization, which is again often only transient. The initial, transient hyperpolarization appears to be correlated with the transient fall in action potential overshoot (cf. Text-fig. 6), and could thus be due to a temporary depletion of extracellular sodium ions. However, the subsequent, slow decline in overshoot is not accompanied by any hyperpolarization, so it must be presumed that substantial readjustments of membrane selectivity (and possibly also of intracellular ion concentrations) occur. It is not possible to draw any definite conclusions from the long-term behaviour of the resting potential.

Pharmacological experiments

The experiments on the effects of calcium ions, manganous ions and tetrodotoxin are merely repetitions and elaborations of similar experiments previously carried out on *Helix aspersa* (Kerkut & Gardner, 1967; Moreton, 1968*a*; Chamberlain & Kerkut, 1969) and *Helix pomatia* (Gerasimov *et al.* 1964; Meves, 1968). For this reason, and because the results are inconclusive, it is not proposed to discuss them in great detail. Interpretation of the effects of calcium on the action potential is subject to the complication that this ion plays an important part in determining the time-courses and

amplitudes of the conductance changes occurring during the action potential (Frankenhaeuser & Hodgkin, 1957), quite apart from any direct contribution it may make to the inward current. By assigning appropriate characteristics to the sodium and potassium 'channels' the observed calcium response could be explained purely on this basis. Alternatively, it could be assumed that, under 'sodium-free' conditions, calcium is the ion responsible for carrying the inward current, the lack of effect of high concentrations being simply due to an increase in the threshold of activation, so that the inward current-carrying channel is never fully 'open'. This would also explain the marked increase in threshold of the action potential, which occurs when the calcium concentration is raised (Gerasimov *et al.* 1964; Kerkut & Gardner, 1967). It should be noted, too, that the intracellular calcium activity is probably very low (less than 10^{-6} M, Meech & Strumwasser, 1970; Meech, 1972), whereas the extracellular activity is of the order of 5 mM/l. Thus the electrochemical gradient for calcium ions across the membrane is large, whereas the number of ions available to carry the current is limited by the relatively low extracellular concentration. It is therefore unlikely that the membrane potential at the peak of the spike could approach the calcium equilibrium potential at all closely, so that even if the spike were purely calcium-generated, the slope of the overshoot/concentration curve might fall considerably short of the theoretical value for a calcium electrode.*

Manganous ions have been shown to exert a blocking action on membrane current-carrying systems using calcium (Hagiwara & Nakajima, 1966), although recently their effect has been shown not to be entirely specific in all cases (Besseau, Lenfant & Mironneau, 1971). They are effective in blocking the action potential in *Helix pomatia* neurones (Meves, 1968) and also in some neurones of *Lymnaea stagnalis* (Sattelle & Lane, 1972). The present results with manganous ions, together with the variable effect of tetrodotoxin, could be interpreted as indicating that *Helix aspersa* neurones possess inward current channels capable of using both sodium and calcium ions, depending to a variable extent more on one species than on the other. The correlation between the effects of these agents and that of exposure to sodium-free solution is not as good as would be expected on this hypothesis; again, it is possible that manganous ions interact with calcium binding sites, affecting membrane stability, as well as with possible calcium-specific inward current channels.

Metabolic inhibitors

Experiments on potassium movements and on the effects of stimulation over long periods have shown that, if regulation of the sodium concentration in the extraneuronal environment does occur, the reservoir required is too large to be extracellular. Since active transport processes would be required to make available an intracellular reserve of sodium ions, the application of sodium-pump inhibitors such as ouabain (Kerkut & Thomas, 1965; Moreton, 1969) or cyanide (Hodgkin & Keynes, 1955; Moreton, 1969) would be expected to accelerate the decline in action

* Hagiwara & Takahashi (1967) have suggested that apparent 'saturation' of the overshoot/concentration curve in barnacle muscle fibres at high concentrations could be due to partial regulation of the extracellular calcium concentration by binding of calcium to the cell membrane. Cf. also Hagiwara *et al.* (1969).

potential overshoot, under 'sodium-free' conditions. Under 'normal' conditions a gradual decline would also be expected, due to passive influx of sodium into the neurones. The normal rate of activity of the sodium pump is not great, however (Moreton, 1969; Thomas, 1972), so that the expected rate of decline is slow.

Thomas (1972) has shown that the average intracellular sodium activity in snail neurones is about 3·6 mM/l, increasing by 0·54 mM l^{-1} min^{-1} if the sodium pump is inhibited by ouabain. This value for the intracellular sodium activity corresponds to a sodium equilibrium potential of about +72 mV. The action potential overshoot falls considerably short of this value (Text-figs. 8, 9), so that it is difficult to estimate the rate of decline expected from a particular rate of rise of $[Na_i^+]$. At the rate given, however, the sodium gradient across the cell membrane would fall to zero in about 140 min.

The results show, however, that ouabain applied during or immediately before exposure to sodium-free solution is without effect on the subsequent behaviour of the action potential (Text-fig. 10).* But when ouabain is applied some considerable time before the exposure to sodium-free solution, the expected slow decline in overshoot is followed by a dramatic and reversible fall in action potential height when the external sodium concentration is reduced (Text-fig. 6). The latter effect can be explained by assuming that, during exposure to normal Ringer after ouabain treatment, the neurones become loaded with sodium ions. Any subsequent fall in extracellular sodium concentration then reduces the sodium equilibrium potential to a more negative level than would have been the case had the intracellular concentration been at its original, low value. The lack of effect of ouabain under 'sodium-free' conditions suggests that, if active transport is involved in maintaining the extraneuronal environment, it is not by the 'conventional' ouabain-sensitive sodium pump. The lack of even a slow decline in overshoot under 'sodium-free 'conditions in Text-fig. 10, together with the complete recovery of the overshoot to its original level on returning to normal Ringer, suggest that under 'sodium-free' conditions little or no loading of the cells with sodium occurs, even after ouabain treatment. This in turn suggests that the extracellular level of sodium is considerably below normal under these conditions.

Poisoning with cyanide causes or accelerates decline in action potential overshoot both in the presence and absence of external sodium. When cyanide is removed, recovery occurs, without further access to sodium ions from the bathing medium. Maintenance of the ionic gradients necessary for the action potential thus depends on two mechanisms: one operates only under 'normal' conditions, and is sensitive to ouabain (and presumably to cyanide); the other operates also under 'sodium-free' conditions, and is sensitive only to cyanide. The ouabain-sensitive system must include the neuronal sodium pump, and may also include the glial sodium pump, although there is no direct evidence for this. It is clearly not involved in any relevant aspect of extracellular ionic regulation.

The ouabain-insensitive mechanism could be a glial sodium pump, biochemically different from the neuronal sodium pump, and concerned with ionic regulation; alternatively, if maintenance of the action potential under 'sodium-free' conditions is considered to be due to the use of calcium ions as inward current carriers, the ouabain-

* Note that the action of ouabain is irreversible (Kerkut & Thomas (1965)).

[redacted]nsensitive mechanism could be the neuronal calcium pump (whose existence must, of course, be postulated under such conditions). The decline in overshoot following application of cyanide under 'sodium-free' conditions would then be explained in terms of progressive loading of the cells with calcium; on removal of cyanide the calcium is gradually pumped out again, restoring the concentration gradient so that the action potential recovers.

The effects of metabolic inhibitors can thus be interpreted in terms either of ionic regulation and a 'conventional' action potential mechanism, or of a 'mixed' system, in which ionic regulation does not occur, but the inward current during the action potential can be carried either by sodium or by calcium ions. The latter hypothesis is supported by the fact that ouabain applied under 'sodium-free' conditions does not affect the action potential, which suggests that the extracellular sodium concentration under these conditions is low.

Experiments with tetrodotoxin and manganous ions have failed to provide evidence for an exclusively sodium-dependent inward current during the action potential. Experiments on potassium movements, together with the relatively rapid actions of other small ions and molecules, suggest that the neuronal surface is readily accessible from the bathing medium. This fact, together with the lack of effect of long-term stimulation, suggests strongly that regulation of the extra-neuronal ionic environment does not occur to any great extent in *Helix aspersa* ganglia.

The following hypothesis is therefore proposed, on the grounds that while it is by no means unique or conclusively proved it accounts quite simply for the wide variety of confusing and apparently contradictory results obtained.

Giant neurones in the abdominal ganglia of *Helix aspersa* can use both sodium and calcium ions to carry the inward current during the action potential. The relative extent to which calcium ions can be used varies from cell to cell. If either ion species is removed, or its use is prevented (e.g. calcium channels are blocked by manganous ions), the overshoot is determined by the extent to which the other can be used. When both are present, the overshoot is determined jointly by their equilibrium potentials, and by their relative abilities to cross the cell membrane.

Potassium, sodium and calcium ions move readily between the extra-neuronal space and the bathing medium, so that their concentrations at the two locations can differ only for short periods of time, immediately following abrupt changes in the environment of the nervous system.

When sodium ions are removed from outside the cell, their concentration gradient across the cell membrane is initially reversed, since the intracellular concentration is appreciable. If sodium ions are able to cross the membrane to any great extent during the action potential, an outward sodium current will flow, tending to counteract any inward calcium current which is present. But under these circumstances, with no passive sodium influx into the cell, the neuronal sodium pump will be able to reduce the intracellular sodium concentration to a very low level. The action potential overshoot will thus fall rapidly at first, due to the reversed sodium current, but will recover subsequently as the intracellular sodium concentration falls – as is observed in some experiments. Recovery may also be assisted by an increase in calcium permeability, triggered off by the fall in external sodium concentration, so that the membrane 'switches over' from one type of system to the other. This could also explain

the gradual disappearance of the initial hyperpolarization during prolonged exposure to sodium-free solution.

Pre-treatment with ouabain accentuates the initial drop in overshoot by loading the cells with sodium, and prevents recovery by irreversibly inhibiting the sodium pump.

The neurones must also possess an active transport mechanism to extrude calcium, and maintain the concentration gradient necessary for excitability. If this pump is of the calcium–sodium exchange type found in squid axons (Blaustein & Hodgkin, 1969) it will be unable to function fully under sodium-free conditions. The cells will thus gradually become loaded with calcium, which explains the gradual decline in overshoot observed during prolonged exposure to sodium-free solution. Restoration of the external sodium concentration to its 'normal' level restores a favourable sodium gradient, allowing immediate recovery of the action potential using sodium ions as the current carrier.

The variable length of time for which excitability is maintained in sodium-free solution could be a function either of the extent of passive inward leakage of calcium, or of the extent to which residual amounts of sodium leaking from neurones and glia are able to maintain the activity of the calcium pump. In some cases calcium-pump activity must be assumed to remain, since recovery after cyanide poisoning is possible without access to external sodium. The extent of coupling between calcium efflux and external sodium concentration may also be variable. The squid-axon calcium pump is insensitive to ouabain (Blaustein & Hodgkin, 1969), which would explain the lack of effect of this substance when applied under sodium-free conditions.

SUMMARY

1. Experiments were carried out on giant neurones in the abdominal ganglia of *Helix aspersa* in an attempt to determine (*a*) the relationship between the ionic composition of the extra-neuronal environment and that of the bathing medium, and (*b*) the identity of the ions responsible for carrying the inward current of the action potential.

2. Potassium ions diffuse readily between the bathing medium and the extra-neuronal fluid, the mean half-time for equilibration being $19{\cdot}2 \pm 1{\cdot}6$ sec (S.E.). With the inner connective tissue sheath intact, the mean half-time is increased to $32{\cdot}8 \pm 2{\cdot}2$ sec. Both half-times can be accounted for purely by the geometry of the extra cellular system.

3. Although their effects on the nerve membrane are not quantifiable, it seems likely from the rapidity of their action that other ions, such as sodium, calcium and manganese, are able to move at similar rates between the bathing medium and the surface of the neurones.

4. Repetitive stimulation at frequencies above 2/sec causes a reversible decline in action potential overshoot. The time course of this effect and of the recovery suggests that it is due to depletion of ions in the restricted extracellular space, with subsequent replacement by active transport, and by diffusion from the bathing medium. Some cells also show a hyperpolarization, which is probably due to a change in ionic permeability of the resting cell membrane.

5. Although many cells can give action potentials in sodium-free solution, the overshoot declines gradually over a period of some hours. This decline is reversibly accelerated by 2 mM/l cyanide, but is unaffected by 10^{-4} M ouabain. But if ouabain is applied in normal Ringer the overshoot declines slowly, and after a time the ability to give action potentials in sodium-free solution is irreversibly lost.

6. Experiments with 10 mM/l manganous ions gave variable results, some cells being made inexcitable, others little affected; correlation with ability to give action potentials in sodium-free solution was not good. Tetrodotoxin at 10^{-5} M caused only small reductions in overshoot.

7. It was concluded that the extra-neuronal environment in *Helix aspersa* is not subject to ionic regulation. The behaviour of the action potential can best be explained by assuming that both sodium and calcium ions contribute to the inward current. The neuronal calcium pump probably resembles the calcium/sodium exchange pump found in squid axons. Under low-sodium conditions, this pump is unable to operate fully, so that the overshoot declines slowly due to loading of the cells with calcium. Poisoning with cyanide accentuates this effect. Ouabain affects only the sodium pump; in sodium-free solution there is no passive sodium influx, so the overshoot is unaffected. In normal Ringer the cells become loaded with sodium, so that subsequent exposure to sodium-free solution reverses the sodium gradient across the cell membrane, counteracting the inward calcium current, and making the cells inexcitable. Restoration of a favourable sodium gradient causes rapid recovery of the action potential in all cases.

REFERENCES

Besseau, A., Lenfant, J. & Mironneau, J. (1971). Etude comparée des effets inhibiteurs du manganèse et du lanthane sur le courant lent des membranes myocardiques. *J. Physiol., Paris* **63**, 13–14A.

Blaustein, M. P. & Hodgkin, A. L. (1969). The effect of cyanide on the efflux of calcium from squid axons. *J. Physiol., Lond.* **200**, 497–527.

Boyle, P. J. & Conway, E. J. (1941). Potassium accumulation in muscle and associated changes. *J. Physiol., Lond.* **100**, 1–63.

Carlson, A. D. & Treherne, J. E. (1969). The ionic basis of the fast action potentials in the isolated cerebro-visceral connective of *Anodonta cygnea*. *J. exp. Biol.* **51**, 297–318.

Carter, N. W., Rector, F. C., Campion, D. S. & Seldin, D. W. (1967). Measurement of intracellular pH with glass microelectrodes. *Fedn Proc. Fedn Am. Socs exp. Biol.* **26**, 1322–6.

Chamberlain, S. G. & Kerkut, G. A. (1969). Voltage clamp analysis of the sodium and calcium inward currents in snail neurones. *Comp. Biochem. Physiol.* **28**, 787–80.

Chen, C. F., von Baumgarten, R. & Takeda, R. (1971). Pacemaker properties of completely isolated neurones in *Aplysia californica*. *Nature New Biology* **233**, 27–9.

Eisenman, G. (1967). *Glass Electrodes for Hydrogen and Other Cations*, ch. 5. New York: Marcel Dekker.

Frankenhaeuser, B. & Hodgkin, A. L. (1956). The after-effects of impulses in the giant nerve fibres of *Loligo*. *J. Physiol., Lond.* **131**, 341–76.

Frankenhaeuser, B. & Hodgkin, A. L. (1957). The action of calcium on the electrical properties of squid axons. *J. Physiol., Lond.* **137**, 218–44.

Cage, P. W. & Hubbard, J. I. (1966). The origin of the post-tetanic hyperpolarization of mammalian motor nerve terminals. *J. Physiol., Lond.* **184**, 335–52.

Geduldig, D. & Junge, D. (1968). Sodium and calcium components of action potentials in the *Aplysia* giant neurone. *J. Physiol., Lond.* **199**, 347–65.

Gerasimov, V. D., Kostyuk, P. G. & Maiskii, V. A. (1964). Excitability of the giant nerve cells of various lunged molluscs (*Helix pomatia*, *Limnea stagnalis* and *Planorbis corneus*) in solutions free from sodium ions. *Bull. exp. Biol. Med.* **58**, 1013–16.

Hagiwara, S., Chichibu, S. & Naka, K-I. (1964). The effects of various ions on resting and spike potentials of barnacle muscle fibres. *J. gen. Physiol.* **48**, 163–79.

Hagiwara, S., Hayashi, H. & Takahashi, K. (1969). Calcium and potassium currents of the membrane of a barnacle muscle fibre in relation to the calcium spike. *J. Physiol., Lond.* **205**, 115–29.

HAGIWARA, S. & NAKA, K-I. (1964). The initiation of spike potential in barnacle muscle fibres under low intracellular Ca^{++}. *J. gen. Physiol.* **48**, 141–62.

HAGIWARA, S. & NAKAJIMA, S. (1966). Differences in Na and Ca spikes as examined by application of tetrodotoxin, procaine and manganese ions. *J. gen. Physiol.* **49**, 793–806.

HAGIWARA, S. & TAKAHASHI, K. (1967). Surface density of calcium ions and calcium spikes in the barnacle muscle fibre membrane. *J. gen. Physiol.* **50**, 583–601.

HODGKIN, A. L. (1958). Ionic movements and electrical activity in giant nerve fibres. *Proc. Roy. Soc.* B **148**, 1–37.

HODGKIN, A. L. & HOROWICZ, P. (1959). The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J. Physiol., Lond.* **148**, 127–60.

HODGKIN, A. L. & HUXLEY, A. F. (1952). A quantitative description of membrane current and its application to conduction and excitation in nerve. *J. Physiol., Lond.* **117**, 500–44.

HODGKIN, A. L. & KEYNES, R. D. (1955). Active transport of cations in giant axons from *Sepia* and *Loligo*. *J. Physiol., Lond.* **128**, 28–60.

JUNGE, D. (1967). Multi-ionic action potentials in molluscan giant neurones. *Nature, Lond.* **215**, 546–8.

KERKUT, G. A., BROWN, L. C. & WALKER, R. J. (1969). Cholinergic IPSP by stimulation of the electrogenic sodium pump. *Nature, Lond.* **223**, 864–5.

KERKUT, G. A. & GARDNER, D. R. (1967). The role of calcium ions in the action potentials of *Helix aspersa* neurones. *Comp. Biochem. Physiol.* **20**, 147–62.

KERKUT, G. A. & MEECH, R. W. (1966). The internal chloride concentration of H and D cells in the snail brain. *Comp. Biochem. Physiol.* **19**, 819–32.

KERKUT, G. A. & MEECH, R. W. (1967). The effect of ions on the membrane potential of snail neurones. *Comp. Biochem. Physiol.* **20**, 411–29.

KERKUT, G. A. & THOMAS, R. C. (1964). The effect of anion injection and changes in the external potassium and chloride concentration on the reversal potentials of the IPSP and acetylcholine. *Comp. Biochem. Physiol.* **11**, 199–213.

KERKUT, G. A. & THOMAS, R. C. (1965). An electrogenic sodium pump in snail nerve cells. *Comp. Biochem. Physiol.* **14**, 167–83.

KRASTS, I. V. & VEPRINTSEV, B. N. (1972). The giant neurone of *Tritonia*: its electric properties and the ionic dependence of the action potential. *Comp. Biochem. Physiol.* **41**A, 289–96.

KRISHTAL, O. A. & MAGURA, I. S. (1970). Calcium ions as inward current carriers in mollusc neurones. *Comp. Biochem. Physiol.* **35**, 857–66.

KUNO, M., MIYAHARA, J. T. & WEAKLY, J. N. (1970). Post-tetanic hyperpolarization produced by an electrogenic pump in dorsal spinocerebellar tract neurones of the cat. *J. Physiol., Lond.* **210**, 839–55.

MAGURA, I. S. & GERASIMOV, V. D. (1966). The effect of calcium ions on electrical activity of the giant neurones of the mollusc *Tritonia diomedia*. *Zh. Evol. Biochim. Physiol. SSSR* **2**, 5–11.

MAISKII, V. A. (1964). Electrical characteristics of surface membrane of the giant nerve cells of *Helix pomatia*. *Fedn Proc. Fedn Am. Socs exp. Biol.* **23**, T 1173–6.

MAISKII, V. A. & GERASIMOV, V. D. (1964). An electrophysiological investigation of the giant neurons of certain pulmonate molluscs. *Bull. exp. Biol. Med.* **58**, 1402–5.

MEECH, R. W. (1972). Intracellular calcium injection causes increased potassium conductance in *Aplysia* nerve cells. *Comp. Biochem. Physiol.* **42**A, 493–9.

MEECH, R. W. & STRUMWASSER, F. (1970). Intracellular calcium injection activates potassium conductance in *Aplysia* nerve cells. *Fedn Proc. Fedn Am. Socs exp. Biol.* **29**, 834.

MEVES, H. (1968). The ionic requirements for the production of action potentials in *Helix pomatia* neurones. *Pflügers Arch.* **304**, 215–41.

MORETON, R. B. (1968*a*). Ionic mechanism of the action potentials of giant neurones of *Helix aspersa*. *Nature, Lond.* **219**, 70–1.

MORETON, R. B. (1968*b*). An application of the constant-field theory to the behaviour of giant neurones of the snail, *Helix aspersa*. *J. exp. Biol.* **48**, 611–24.

MORETON, R. B. (1969). An investigation of the electrogenic sodium pump in snail neurones, using the constant-field theory. *J. exp. Biol.* **51**, 181–201.

NAKAJIMA, S. & TAKAHASHI, K. (1966). Post-tetanic hyperpolarization and electrogenic Na pump in stretch receptor neurone of crayfish. *J. Physiol., Lond.* **187**, 105–27.

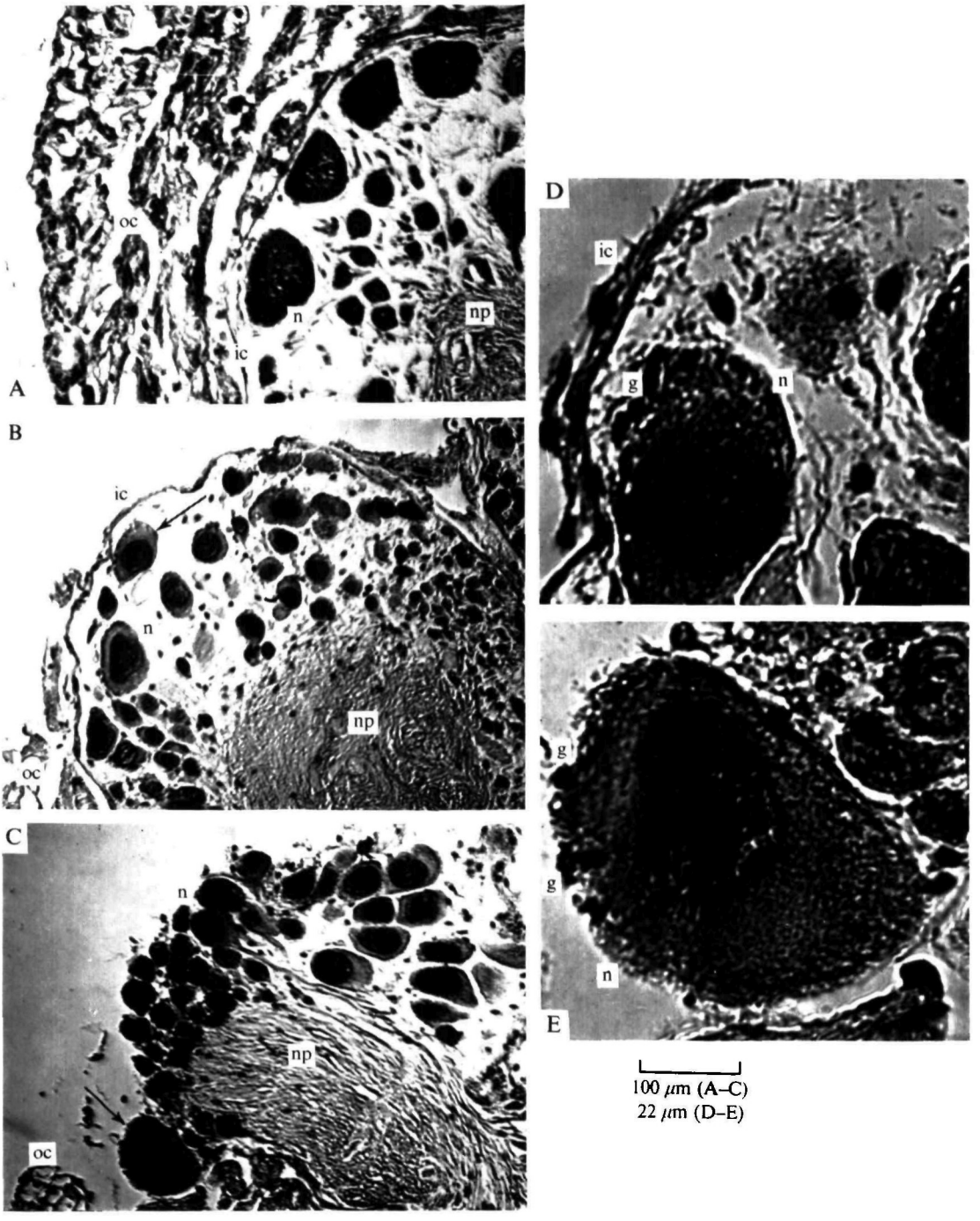
ORKAND, R. K., NICHOLLS, J. G. & KUFFLER, S. W. (1966). Effect of nerve impulses on the membrane potential of glial cells in the central nervous system of amphibia. *J. Neurophysiol.* **29**, 788–806.

PENTREATH, V. W. & COTTRELL, G. A. (1970). The blood supply to the central nervous system of *Helix pomatia*. *Z. Zellforsch. mikrosk. Anat.* **111**, 160–78.

PICHON, Y., MORETON, R. B. & TREHERNE, J. E. (1971). A quantitative study of the ionic basis of extraneuronal potential changes in the central nervous system of the cockroach (*Periplaneta americana* L.). *J. exp. Biol.* **54**, 757–77.

SATTELLE, D. B. & LANE, N. J. (1972). Architecture of gastropod central nervous tissues in relation to ionic movements. *Tissue & Cell* **4** (in the Press).

SOKOLOVE, P. G. & COOKE, I. M. (1971). Inhibition of impulse activity in a sensory neurone by an electrogenic pump. *J. gen. Physiol.* **57**, 125–63.


A
oc
n
np
ic
B
ic
n
np
oc
C
n
np
oc
D
ic
g
n
E
g
g
n
100 μm (A–C)
22 μm (D–E)

THOMAS, R. C. (1972). Intracellular sodium activity and the sodium pump in snail neurones. *J. Physiol., Lond.* **220**, 55–71.

TREHERNE, J. E., DEFOREST MELLON, JR. & CARLSON, A. D. (1969). The ionic basis of axonal conduction in the central nervous system of *Anodonta cygnea* (Mollusca: Eulamellibranchia). *J. exp. Biol.* **50**, 711–22.

TREHERNE, J. E., LANE, N. J., MORETON, R. B. & PICHON, Y. (1970). A quantitative study of potassium movements in the central nervous system of *Periplaneta americana*. *J. exp. Biol.* **53**, 109–36.

TREHERNE, J. E. & MORETON, R. B. (1970). The environment and function of invertebrate nerve cells. *Int. Rev. Cytol.* **28**, 45–88.

VEPRINTSEV, B. N., GERASIMOV, V. D., KRASTS, I. V. & MAGURA, I. S. (1966). An investigation of the mechanism of the action potential in giant neurones of the nudibranch mollusc *Tritonia diomedia*. *Biofizika* **11**, 1000–6.

EXPLANATION OF PLATE

Stages in the dissection of the brain of *Helix aspersa*.

(A) Part of the left parietal ganglion before removal of the connective tissue, showing the loose, spongy outer capsule (*oc*) and the thin, continuous inner capsule (*ic*) overlying the neurones (*n*), which form a layer round the central neuropile (*np*).

(B) Right parietal ganglion, after removal of the outer capsule. The inner capsule is still intact.

(C) Right parietal ganglion, after removal of the inner capsule ('de-sheathed'). The neurones appear to be exposed directly to the outside medium, though at higher manification (E) glial cells (*g*) can be seen still adhering to them.

(D) Higher magnification of one neurone (arrowed) in (B). Several glial cells (*g*) are associated with the neurone, lying between it and the inner capsule (*ic*).