ELECTROPHYSIOLOGY OF THE GIANT NERVE CELL BODIES OF *LIMNAEA STAGNALIS* (L.) (GASTROPODA: PULMONATA)

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INTRODUCTION

The giant somata (cell bodies) of neurones in the central ganglia of *Limnaea stagnalis* (L.) have become material for several lines of neurobiological research. For example, the electrical responses of these cells have been employed in pharmacological studies (Vulfius & Zeimal, 1967; Vulfius *et al.* 1967; Zeimal & Vulfius, 1967; Jurchenko, Vulfius & Zeimal, 1973; Moreton & Gardner, 1974). Also, the changes in metabolic activity, including RNA synthesis, which accompany the electrical activity of these neurones have been investigated (D'Yakinova *et al.* 1965). In particular, their large size and relative accessibility have facilitated investigation by microelectrodes of the cellular and nuclear membrane potentials (Veprintsev & Sakharov, 1963; Gerasimov, Kostyuk & Maiskii, 1964; Maiskii & Gerasimov, 1964; Krishtal & Magura, 1970; Jerelova, Krasts & Veprintsev, 1971; Sattelle & Lane, 1972; Sattelle, 1973).

Early experiments on the giant neurones of *Limnaea* suggested some dependence of the resting potential on the external concentration of potassium ions and strongly implicated sodium ions in the generation of the action potential (Gerasimov *et al.* 1964; Maiskii & Gerasimov, 1964). In their ionic requirements for electrogenesis these cells appeared therefore to resemble conventional excitable cells such as the squid axon (Hodgkin, 1958, 1964). Studies on the resting membrane potential of other gastropod neurones reveal, in certain cases, marked deviations from the behaviour of an ideal potassium electrode (Gerasimov, Kostyuk & Maiskii, 1965b; Kerkut & Meech, 1967). A modification of the constant-field equation has been applied to data obtained for the soma membrane of neurones of *Helix aspersa* and accounts for its behaviour over a wide range (0–40 mM/l) of potassium concentrations (Moreton, 1968, 1972). The presence of an electrogenic sodium pump has also been demonstrated in the giant neurones of several gastropod molluscs, including *H. aspersa* (Kerkut & Thomas, 1965; Thomas, 1968, 1969), *H. pomatia* (Ayrapetyan, 1969a, b; Christoffersen, 1972), *Aplysia californica* (Carpenter & Alving, 1968) and *Anisodoris nobilis* (Marmor & Gorman, 1969, 1970; Gorman & Marmor, 1970). Moreton (1969) has described the resting potential in cells of *Helix aspersa* by a modified constant-field equation, including a term representing the effect of a potassium-sensitive electrogenic sodium pump. Recent preliminary studies on the potassium-dependence of the resting potential in neurones of *Limnaea* have revealed a considerable departure from the

Nernst relationship for an ideal potassium electrode (Sattelle, 1973; Sattelle & Lane, 1972). An aim of this report is to attempt to account for this behaviour and to investigate the possibility of a metabolic contribution to the resting potential.

In conventional excitable systems, the association of the action potential overshoot with a transient increase in sodium permeability has been shown, the active membrane behaving like a sodium electrode (Hodgkin, 1958, 1964). The effects of varying the external sodium concentration on the overshoot potential of gastropod giant neurones are complicated, however, by the demonstrated role of calcium as a charge carrier. For example, Oomura, Ozaki & Maeno (1961) concluded that the electrical activity of the giant neurones of *Onchidium verruculatum* depended only on calcium ions. In neurones of *Helix pomatia*, also, calcium ions appear to account completely for the inward current of the action potential in at least some neurones (Gerasimov, Kostyuk & Maiskii, 1964, 1965a, c; Meves, 1966, 1968; Krishtal & Magura, 1970). The lateral giant neurones of *Aplysia californica* have been shown to rely upon both sodium and calcium ions to carry the inward current component (Junge, 1967; Geduldig & Junge, 1968). A similar explanation accounts for observations on the giant cells of *Helix aspersa* (Kerkut & Gardner, 1967; Moreton, 1972). By contrast, action potentials in neurones of *Tritonia diomedia* appear to be sodium-dependent, but interpretation of the results is complicated in this case by the apparently restricted movement of sodium within the extracellular system (Magura & Gerasimov, 1966; Veprintsev *et al.* 1966). Such a summary of the general findings for different species would be misleading without reference to the fact that in almost every case cells with very different ionic mechanisms of excitability lie in close proximity within a single ganglion.

The simplest, apparently conventional sodium-dependent, system was reported by the first investigators of neurones of *Limnaea stagnalis* and *Planorbis corneus* (Gerasimov *et al.* 1964). Recently, however, an important calcium contribution to the inward current of the action potential has been found in neurones of *Limnaea* (Krishtal & Magura, 1970; Jerelova *et al.* 1971). In spite of this, cells in which calcium can support the action potential for extended periods, in the absence of sodium, have not been found in the central nervous system of *Limnaea*. Techniques used to establish the ionic dependence of the action potential of gastropod neurones include the application of tetrodotoxin and manganous ions, in addition to studying the effects of varying the concentrations of sodium and calcium ions in the bathing medium. The above techniques are employed in this investigation to determine the role of sodium and calcium in the generation of the action potential of *Limnaea* neurones.

METHODS

Specimens of *Limnaea stagnalis* were maintained in large aquaria containing tapwater under continuous flow conditions (cf. van der Steen, van den Hoven & Jager, 1969) until required for experimentation. Techniques for the dissection of the circumoesophageal ring of ganglia and details of the preparation chamber were as described in a previous paper (Sattelle, 1973). Experiments were performed on nerve cell bodies of the parietal and visceral ganglia. Impalement of the cells by microelectrodes was achieved without dissection of the thin ganglionic sheath. This condition has been referred to as *intact* in previous reports (Sattelle, 1973; Sattelle & Lane, 1972).

Intracellular recordings were obtained using glass microelectrodes filled with 3 M-KCl and with resistances of 10–30 MΩ. Membrane potentials were led to a unity-gain amplifier of 10^{10} Ω input impedance, with an input capacity of less than 1 pF and an input current of about 3 pA. The output of this device was connected to a Tektronix 502 or 561 oscilloscope, and potential changes were filmed on a Cossor or Nihon-Kohden oscilloscope camera. To evoke action potentials, rectangular de-polarizing current pulses were applied down the recording microelectrode via a 100 MΩ resistor. In some experiments two microelectrodes were inserted into a single cell. One of these was employed to pass the stimulating current, the other served to record transmembrane potential changes. Low-resistance microelectrodes filled with 1·0 M-NaCl were used for sodium injection experiments (cf. Kerkut & Thomas, 1965; Chiarandini & Stefani, 1967; Moreton, 1969).

Test solutions were delivered to the preparation chamber from elevated reservoirs via a multiway non-return valve (Holder & Sattelle, 1972). The normal Ringer solution consisted of: 50·0 mM/l NaCl; 1·6 mM/l KCl; 2·0 mM/l MgCl₂; 4·0 mM/l CaCl₂; pH 7·4. When a high-potassium Ringer was required, the concentration of potassium chloride was increased at the expense of sodium chloride. A low-potassium Ringer was produced by omitting potassium chloride without substitution. For low-sodium Ringer, tris chloride was added to replace all or part of the sodium chloride. When concentrations of sodium in excess of that in normal Ringer were required, the sodium level was simply elevated. In some experiments the concentrations of potassium and calcium were varied over a narrow range without substitution. Experiments were performed at room temperature (18–23 °C), but in any single experiment the temperature did not vary by more than 0·5 °C.

<div align="center">

RESULTS

A. *The resting potential*
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Potassium dependence

It has been demonstrated in previous reports that the cell membranes of giant neurones in an *intact* ganglion of *Limnaea* are readily accessible to potassium ions in the bathing medium (Sattelle, 1973; Sattelle & Lane, 1972). Resting potentials were, therefore, recorded following a short (4 min) exposure of the preparation to a new potassium concentration. It was not found necessary to bathe the cells in normal Ringer following the application of each new test solution. Resting potentials were determined for different concentrations of potassium (between 0·5 mM/l and 50·0 mM/l) in the bathing medium. The relationship of the resting potential to the logarithm of the external potassium concentration [K⁺ₒ] is shown for a single neurone in Fig. 1 A. Only at potassium concentrations higher than 5 mM/l was a straight-line relationship obtained, where a tenfold change in [K⁺ₒ] corresponded to a 34 mV change of resting potential. In the fifteen cells investigated in this way, the slopes of the straight-line sections of the graphs were recorded. Changes of 28–43 mV were observed in the resting potential accompanying decade changes in [K⁺ₒ].

In another series of experiments, resting potentials were recorded over a narrow range of [K⁺ₒ] without adjustment of the sodium concentration. The constant-field
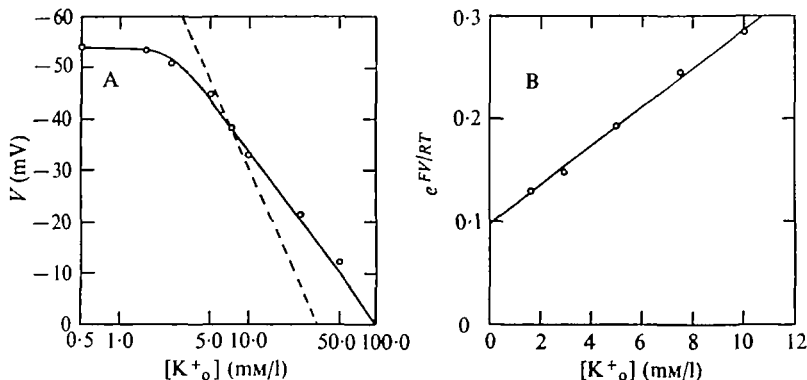
Fig. 1. Potassium dependence of the resting potential. Graph A shows the relationship between the resting potential (V) and the logarithm of the external concentration of potassium ions [K^+_o] for a single neurone. The broken line indicates the slope for a membrane behaving as an ideal potassium electrode. Graph B shows the relationship between $e^{FV/RT}$ and [K^+_o] plotted linearly for a single cell.

equation of Hodgkin & Katz (1949) was applied to these data following the method of Moreton (1968). This author has derived the following equation, which describes the resting potential V of a neurone as a function of the extracellular concentration of potassium ions [K^+_o] as follows:

$$e^{FV/RT} = \frac{[K^+_o]}{[K^+_i]} + \frac{P_{Na}[Na^+_o]}{P_K[K^+_i]}.$$

The equation predicts a straight-line relationship between $e^{FV/RT}$ and [K^+_o] from the slope and intercept of which the intracellular potassium concentration [K^+_i] and the relative sodium permeability (P_{Na}/P_K) of the cell membrane can be estimated. A more complete description of the membrane potential is achieved when a term is included to account for the effects of the sodium pump (cf. Moreton, 1969), but provided the net efflux of monovalent cations remains constant and the change in the resting potential induced by the pump is not too great, an approximately linear relationship between $e^{FV/RT}$ and [K^+_o] is obtained.

At concentrations between 0·5 and 10·0 mM/l an approximately straight-line relationship was obtained between $e^{FV/RT}$ and [K^+_o]. Data for a single cell are illustrated in Fig. 1B. Similar graphs were drawn for 23 neurones from which the results summarized in Fig. 2 were derived. Intracellular potassium concentrations of between 34·5 and 74·1 mM/l with a mean of 56·2 (S.D. ± 9·0) mM/l were estimated. It was also noted that resting potentials measured at 1·6 mM/l [K^+_o] ranged from −49·0 to −60·0 mV, the mean value being −53·3 (S.D. ± 2·7) mV. Values for the permeability ratio (P_{Na}/P_K) were determined for each neurone and were found to vary between 0·060 and 0·172, with a mean of 0·117 (S.D. ± 0·028). In this connexion, it is of interest to note that an initial hyperpolarization of the soma membrane almost always accompanied the removal of sodium from the Ringer solution. This usually persisted for 1–4 min and was followed by a rather slower recovery and not infrequently by a slow depolarization of the membrane (Figs. 4, 5).

For some cells, plots of resting potential against the external potassium concentration revealed a slight inflexion at very low values of [K^+_o] (Fig. 3A). Since similar
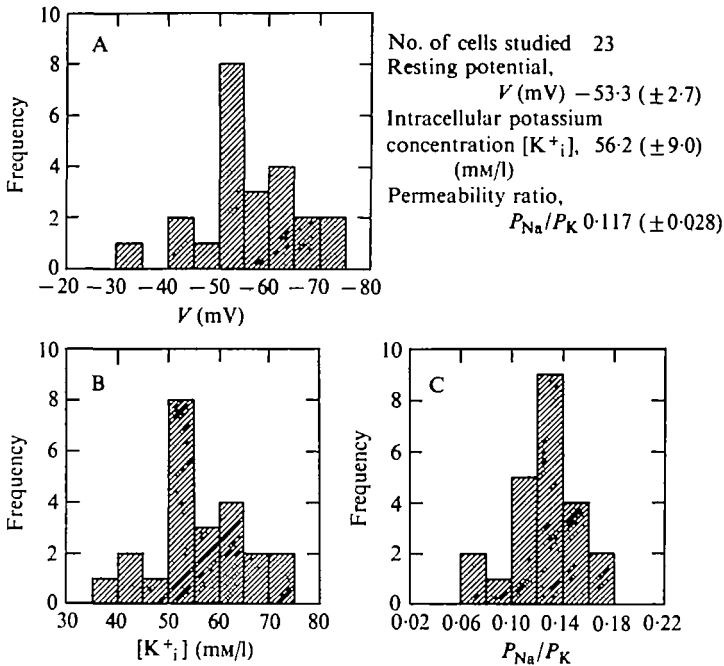
Fig. 2. Some properties of giant neurones. A summary, for 23 cells, of the mean values and distribution of: (A) resting potential (V); (B) intracellular potassium concentration $[K^+_i]$; (C) permeability ratio (P_{Na}/P_K). The standard deviation is quoted in brackets following the mean values.

findings in the giant neurones of *Anisodoris nobilis* were attributed to the action of an electrogenic sodium pump (Marmor & Gorman, 1970), a number of experiments were performed to test for the existence of such a pump in neurones of *Limnaea*.

Electrogenic sodium pump

In those cells in which inflexions in the $V \times [K^+_o]$ curves were noted at low values of $[K^+_o]$, the experiments were repeated in the presence of 10^{-4} M ouabain. Under these conditions the inflexion was abolished (Fig. 3A) and the curve reverted to the type illustrated in Fig. 1A. When ouabain was added to normal Ringer at concentrations of 10^{-4} M a rapid depolarization of the cell membrane (5–7 mV) typically ensued. Two of the fourteen cells investigated exhibited much larger (20–25 mV) depolarizations in response to the same concentration of ouabain, but these changes were only partially recoverable. These findings suggested the presence of an electrogenic sodium pump in certain cells of *Limnaea*.

A few experiments were performed in which sodium ions were allowed to diffuse into neurones from low-resistance electrodes filled with 1·0 M sodium chloride. This produced a hyperpolarization of the membrane which could be blocked by exposing the preparation to potassium-free Ringer (Fig. 3B). Control experiments were conducted with low-resistance electrodes filled with potassium chloride solutions.
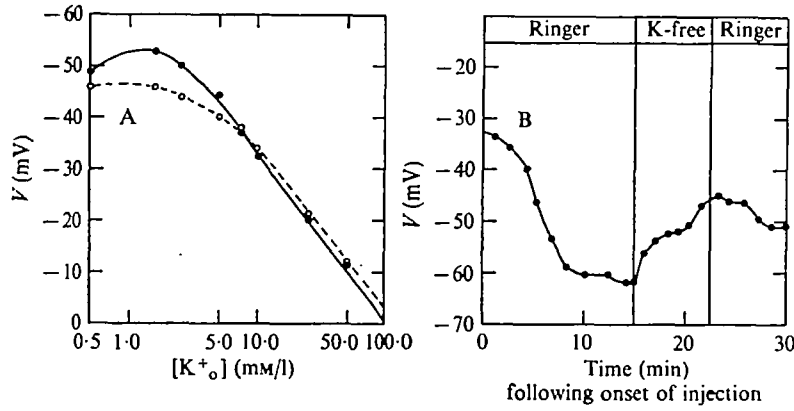
Fig. 3. Electrogenic sodium pump. A shows the inflexion sometimes observed in $V \times [K^+_o]$ curves at low values of $[K^+_o]$ (closed circles) and its abolition in the presence of 10^{-4} M ouabain (open circles). B illustrates the hyperpolarization induced by sodium injection and its reversal by the application of potassium-free Ringer.
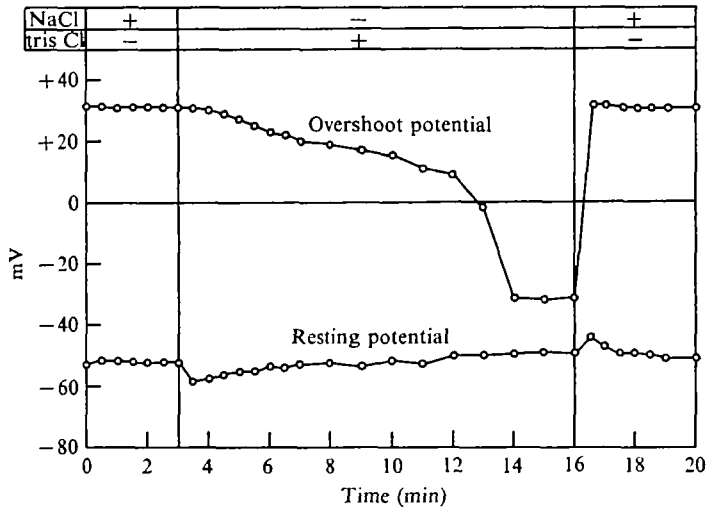

Fig. 4. Changes in overshoot potential and resting potential of a sodium-sensitive cell during exposure to sodium-free Ringer.

B. The action potential

Effects of sodium-free Ringer

For most cells, a change from normal Ringer to sodium-free (tris) Ringer resulted in a cessation of the activity of 'spontaneously' firing neurones within 3–5 min. Under similar conditions evoked action potentials could be recorded for slightly longer periods, an 8–10 min exposure being required for the abolition of the overshoot potential (Fig. 4). Such neurones are hereafter referred to as sodium-sensitive cells.

A few cells were found from which action potentials could be recorded during pro-longed exposure to sodium-free (tris) Ringer. Most of these cells ceased firing 'spontaneously', but the active membrane response could be evoked under sodium-free
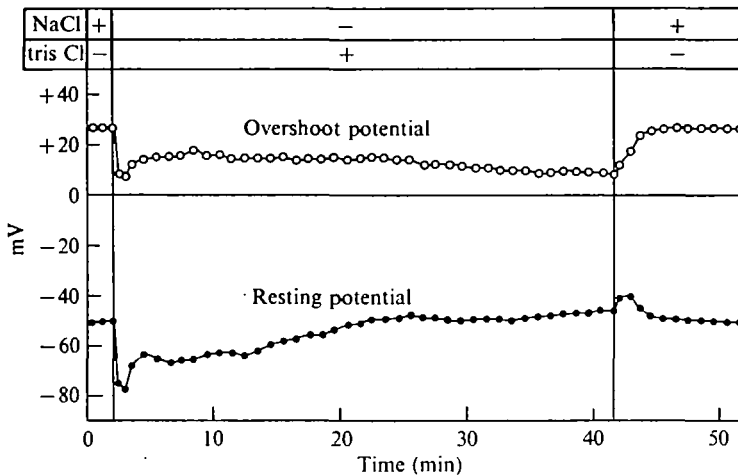
Fig. 5. Changes in overshoot potential and resting potential of a sodium-insensitive neurone during exposure to sodium-free Ringer.

conditions for periods of about an hour. In such cases the spike amplitude declined slowly throughout the period of exposure to sodium-free Ringer (Fig. 5). On rare occasions, cells were observed to fire 'spontaneously' in the absence of sodium for about 30 min. As the period of exposure to sodium-free Ringer increased the duration of the action potential became prolonged and a tendency for plateau formation during the falling phase was discernible. These neurones are hereafter referred to as sodium-insensitive cells.

Dependence of the overshoot potential upon sodium

For cells in which the action potential was rapidly blocked in the absence of sodium, the concentration of this ion in the bathing medium was varied and the subsequent changes in overshoot potential were noted. The results for a single cell are presented in Fig. 6. A straight-line relationship emerged between the overshoot potential and $[Na^+_0]$ plotted on a logarithmic scale. Over the range of sodium concentrations from 5 to 60 mM/l, a tenfold change in the external sodium concentration corresponded to a 31 mV change in the overshoot potential. Eight neurones investigated in this way produced sodium slopes of 29–37 mV for a decade change in $[Na^+_0]$. These data appear to implicate sodium ions in the generation of the action potential, but the relationship described deviates considerably from the potential change predicted for a membrane behaving as a perfect sodium electrode.

The puffer-fish poison tetrodotoxin (TTX) blocks action potentials in neurones by abolishing the influx of sodium ions (Narahashi, Moore & Scott, 1964; Nakamura, Nakajima & Grundfest, 1965). Sodium-sensitive neurones of *Limnaea* were relatively insensitive to the effects of TTX applied at low concentrations in normal Ringer. For example, at a concentration of 5×10^{-6} M spikes were reduced in amplitude only after relatively long exposures to TTX. At 5×10^{-5} M, however, the toxin blocked action potentials in the majority of these cells (Fig. 7).
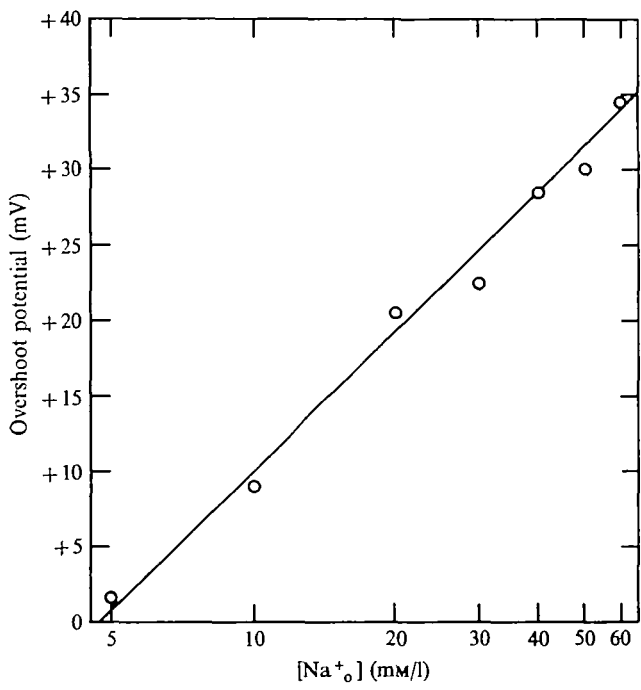
Fig. 6. The relationship between the size of the overshoot of the action potential and the logarithm of the external sodium concentration $[Na^+_o]$ for a sodium-sensitive cell.
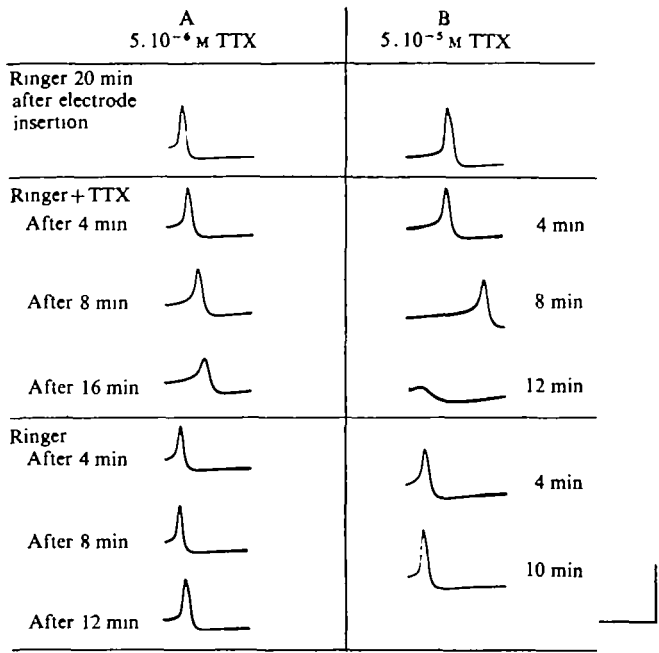

Fig. 7. The effects of tetrodotoxin (TTX) applied at concentrations of 5×10^{-6} M (A) and 5×10^{-5} M (B) on action potentials of sodium-sensitive cells. Apart from the evoked response recorded after 12 min of exposure to TTX in B, all records are of spontaneous action potentials. Scale bars represent 80 mV (vertically) and 100 msec (horizontally).
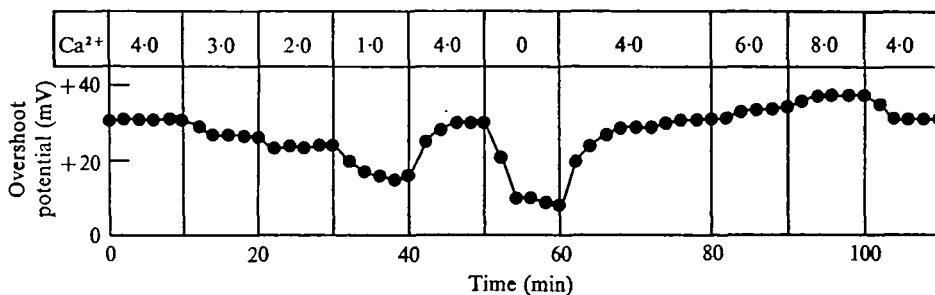
Ca²⁺	4·0	3·0	2·0	1·0	4·0	0	4·0	6·0	8·0	4·0

Fig. 8. The effects of variations in the external concentration of calcium $[Ca^{2+}_o]$ on the overshoot of the action potential of a sodium-sensitive cell. During this experiment the resting potential did not change by more than 3 mV.
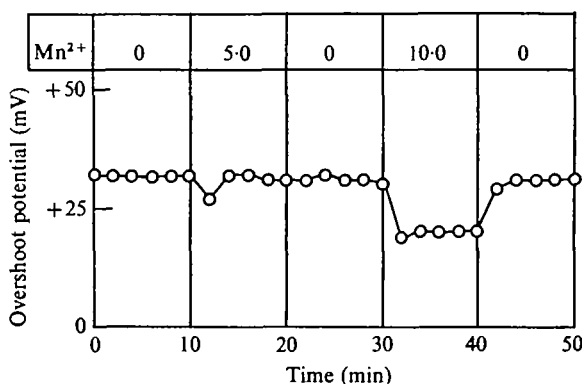
Mn²⁺	0	5·0	0	10·0	0

Fig. 9. The effects of manganous chloride on the overshoot of the action potential of a sodium-sensitive cell.

Dependence of the overshoot potential upon calcium

The calcium-dependence of the overshoot of the action potential was investigated in sodium-sensitive cells. Complete removal of calcium from the bathing medium resulted in a marked reduction of the overshoot potential. The external calcium concentration was varied over the range 1–10 mM/l and the effects on the overshoot potential were noted. In these experiments, no attempt was made to replace calcium by any substitute as only a relatively narrow range of calcium concentrations was used. The results for a single cell are presented in Fig. 8 and correspond to an overshoot potential change of 18 mV per decade change in $[Ca^{2+}_o]$. Experiments on six neurones produced similar slopes.

Manganous ions are thought to block membrane currents carried by calcium ions (Hagiwara & Nakajima, 1966). When manganous chloride (5–10 mM/l) was added to sodium-sensitive cells only a slight reduction (5–10 mV) of the overshoot potential ensued in the four cells investigated (Fig. 9).

The calcium-dependence of the overshoot potential was also studied in those cells from which action potentials could be evoked for extended periods in sodium-free Ringer. The calcium concentration was varied over the range 1–10 mM/l. Results obtained for cells in the presence and absence of sodium have been summarized in
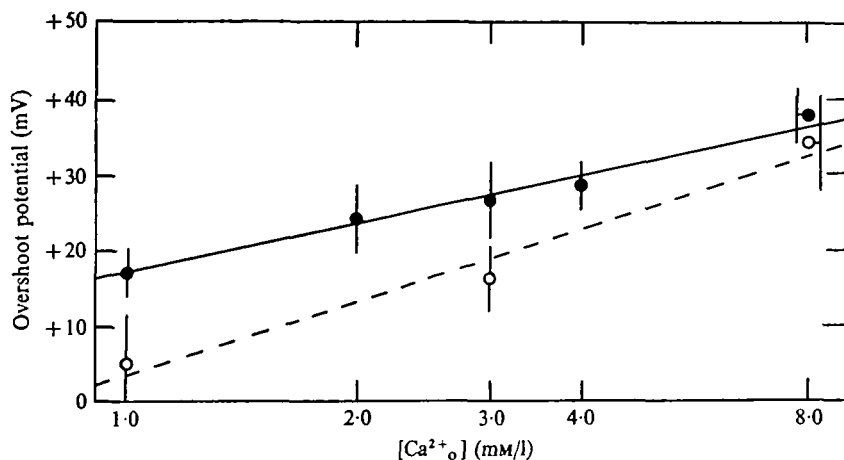
Fig. 10. Calcium-dependence of the overshoot of the action potential of sodium-insensitive neurones in the presence (closed circles) and absence (open circles) of sodium (50 mM/l) in the Ringer.
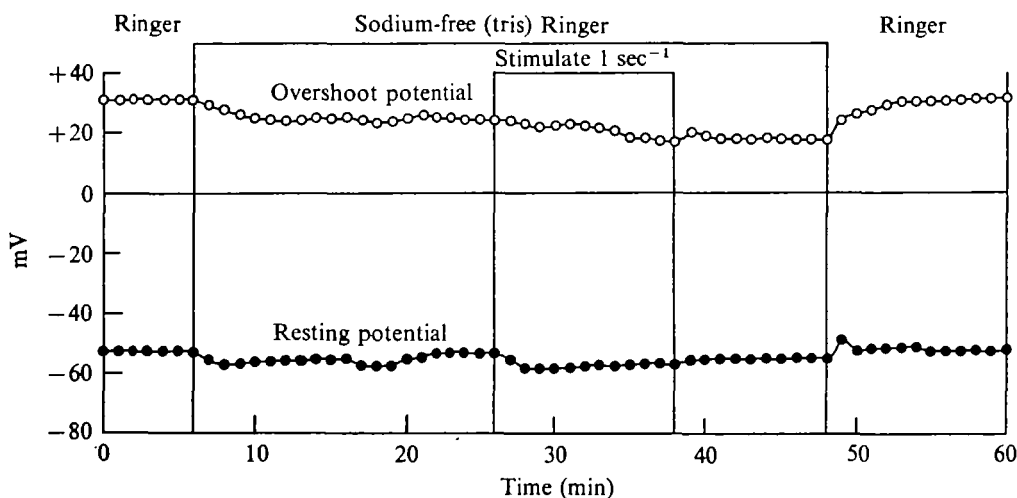

Fig. 11. Effects of stimulation (1 sec^{-1} for 12 min) on the overshoot of the action potential of a sodium-insensitive neurone during exposure to sodium-free Ringer.

Fig. 10. In the presence of sodium (50 mM/l), the change in the overshoot potential accompanying a tenfold change in $[Ca^{2+}_o]$ was 18 mV. In sodium-free Ringer, this potential change increased to 29 mV, close to the calculated potential change under conditions in which calcium alone accounted for the inward current of the action potential (cf. Findlay, 1962; Hagiwara & Naka, 1964).

Effects of stimulation

Experiments were performed to determine the effects of high-frequency stimulation on the ability of the sodium-insensitive cells to maintain action potentials in the absence of this cation. The application of suprathreshold depolarizing pulses at a
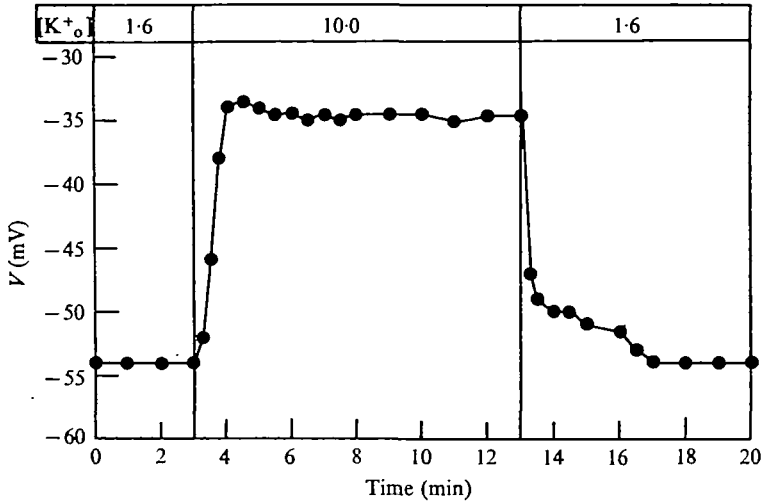
Fig. 12. The time-course of depolarization and repolarization of a sodium-insensitive cell during exposure to high-potassium Ringer (10·0 mM/l K$^+$) and subsequent return to normal Ringer (1·6 mM/l K$^+$).

frequency of about 1 sec^{-1} for periods of between 10 and 15 min produced some reduction of the overshoot potential in sodium-free Ringer in the five cells investigated, but the magnitude of this effect was somewhat variable (Fig. 11). In all cases, however, when the stimulation ceased a partial but rapid recovery of the action potential was observed.

Accessibility of neurones to ions

In previous studies (Sattelle & Lane, 1972; Sattelle, 1973) it has been shown that neurones of *Limnaea* are readily accessible to potassium ions in the bathing medium. The changes in membrane potential of a sodium-insensitive cell were followed during exposure of the ganglion to a high-potassium (10 mM/l K$^+$) Ringer. This change in the composition of the Ringer was rapidly reflected by a depolarization of the soma membrane (Fig. 12). It was also noted that a change from sodium-free Ringer to normal Ringer produced a rapid recovery of the action potential in both sodium-sensitive and sodium-insensitive cells.

Activity patterns and the shape of the action potential

Using microelectrodes filled with 3 M-KCl, various types of activity were recorded from neurones of *Limnaea* (Fig. 13). Some cells fired continuously, either at a constant rate (Fig. 13, record A), or irregularly (Fig. 13, record D), whilst others produced intermittent bursts of activity (Fig. 13, records B, C, E). One type of bursting activity consisted of a group of spikes followed by a silent period in which the membrane became hyperpolarized (Fig. 13, records B, C). Another type of bursting activity was observed during which periodic oscillation of the membrane potential eventually reached a sufficient level of depolarization to trigger a spike or a group of spikes. The categories of activity recorded correspond to those designated by Tauc (1966).
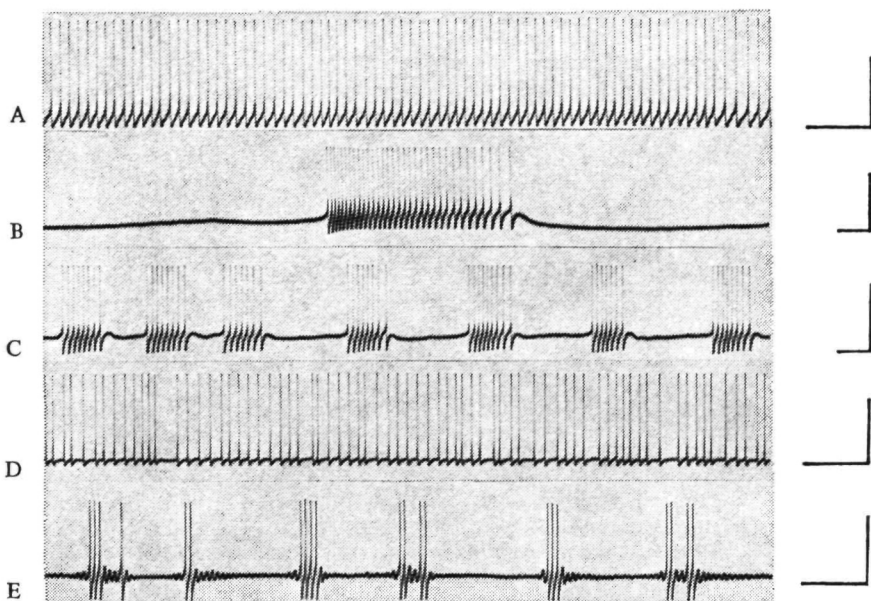
Fig. 13. The types of activity patterns recorded from nerve cell bodies of the parietal and visceral ganglia. The scale bars represent 50 mV (vertically) and 5 sec (horizontally).
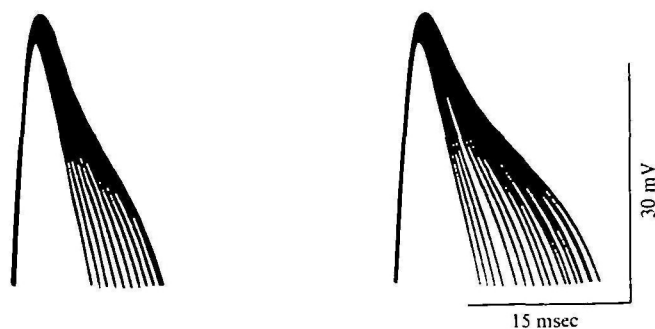

Fig. 14. Changes in spike shape during bursts of activity. In both cases the rising phase of the action potential has been used to trigger the sweep. Superimposed, successive sweeps show the tendency for plateau formation during the falling phase of the action potential.

An increased tendency for plateau formation in the falling phase of the action potential was observed during the activity of burster cells (Fig. 14), each burst of which was accompanied by a depolarization of several millivolts. Such results were consistently obtained and did not appear to be attributable to faulty impalements. Increments of depolarizing current and the replacement of the calcium in normal Ringer by barium produced extended action potentials with a plateau phase in neurones of *Helix aspersa* (Kerkut & Gardner, 1967). The changes in shape of the action potential appear, therefore, to be linked to the depolarization of the membrane that takes place during a burst of activity. During the activity of burster cells of *Limnaea*, the spike interval decreased to a minimum in *intact* neurones after approximately one quarter of the duration of the burst and thereafter increased, often to well
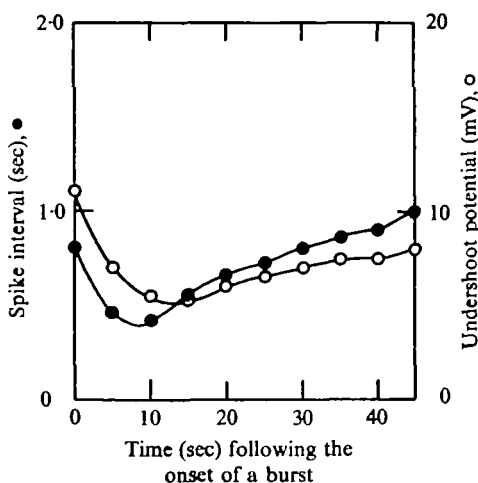
Fig. 15. Changes in the spike interval and undershoot potential
during a single burst of activity of a burster neurone.

above the initial spike interval (Fig. 13, record B). The undershoot of the action
potential fell to a minimum at about the point where the neurone was firing at its
greatest rate (Fig. 15). This phase of the action potential is most sensitive to potassium,
since it approaches the potassium equilibrium potential E_K. It may be, therefore, that
at fast firing rates potassium ions extruded from the active neurone accumulate in the
immediate extracellular spaces. As the rate of firing decreases, less accumulation will
occur. The result is undoubtedly complicated by changes in the resting potential, but
the undershoot potential has been shown to increase as the resting potential decreases
(Coombs, Eccles & Fatt, 1955) which would tend to obscure rather than enhance the
result obtained.

DISCUSSION

This report describes studies on the cationic dependence of the resting potentials
and action potentials in giant neurones from the parietal and visceral ganglia of
Limnaea stagnalis (L.). A mean resting potential (V) of $-53 \cdot 3$ (S.D. $\pm 2 \cdot 7$) mV has been
obtained in studies on 23 *intact* cells. This closely resembles the value of $-53 \cdot 5$
(S.D. $\pm 1 \cdot 9$) mV obtained from 28 *intact* cells of the right parietal ganglion (Sattelle,
1973). These figures are much lower than the equilibrium potential (E_K) for the
membrane behaving as an ideal potassium electrode. A mean value for E_K has been
calculated to be -89 mV, based on data presented in this report. Similar values of
resting potential have been obtained for other gastropod molluscs (cf. Tauc, 1955;
Kerkut & Walker, 1961; Maiskii, 1964; Maiskii & Gerasimov, 1964; Kerkut &
Thomas, 1965; Kerkut & Meech, 1967; Moreton, 1968, 1969). The resting potential
is relatively insensitive to changes in $[K^+_0]$ at concentrations close to the physio-
logical level. At potassium concentrations above 5 mM/l the resting potential varies
linearly with the logarithm of the external potassium concentration. Over this section
of the graph, changes in the resting potential of 28–43 mV accompany a decade
change of $[K^+_0]$. There is thus a considerable departure from the 58 mV per decade

slope for a membrane behaving as an ideal potassium-selective electrode (cf. Fig. 1). It is, nevertheless, clear that the distribution of potassium ions between the neuro- plasm and the fluid bathing the surfaces of the nerve cells accounts for a substantial proportion of the resting potential.

Plots of $e^{FV/RT}$ against $[K^+_o]$ produce straight lines when the potassium concen- tration is varied between 0·5 and 10 mM/l. It appears that over this range of $[K^+_o]$, the modified constant-field equation of Moreton (1968, 1969) provides a better description of the membrane properties of *Limnaea* cells than does the Nernst equation. From these plots a mean value for $[K^+_i]$ of 56·2 (S.D. ±9·0) mM/l has been estimated (this figure has been used in the calculation of E_K – see above). Information on the selective permeability of the cell membrane to potassium ions is also provided by these graphs in terms of the permeability ratio P_{Na}/P_K. The mean value of 0·117 (S.D. ±0·028) for this ratio is high compared to the figure of 0·04 found for squid axons (Hodgkin & Katz, 1949) but comparable to the P_{Na}/P_K ratio of 0·180 (S.D. ±0·015) determined for nerve cell bodies in *Helix aspersa* (Moreton, 1968). Such a high resting permeability to sodium ions may account for some of the changes in the resting potential during exposure to sodium-free (tris) Ringer. The initial hyperpolarization may be attribut- able, at least in part, to the efflux of sodium ions following this reversal in direction of the sodium gradient. In this connexion it is interesting to note the observation of R. W. Meech (personal communication), that during the early part of this sodium- free hyperpolarization in cells of *Helix aspersa* there is no change in membrane resistance, which accords with the above explanation. As this hyperpolarization proceeds in *Helix* cells a fall in membrane resistance is observed suggesting that the leakage of sodium out of the cell does not fully account for this phenomenon. The subsequent recovery of the membrane potential and depolarization observed in *Limnaea* neurones is more difficult to explain, presumably involving changes in selectivity of the cell membrane and intracellular ionic concentrations. It is, never- theless, clear that the membranes of these gastropod nerve cell bodies are much less selective for potassium ions than is the classical axonal preparation.

There is an increasing body of evidence for a metabolic contribution to the resting potential of nerve and muscle cells via an electrogenic (asymmetric) sodium- potassium exchange pump, a good deal of which derives from studies on gastropod giant neurones (cf. Kerkut & York, 1971; Thomas, 1972). The demonstration of an electrogenic pump involves a study of the effects on resting potentials of low $[K^+_o]$, high $[Na^+_i]$ and pump inhibitors such as the cardiac glycoside ouabain. The $V \times [K^+_o]$ curves for *Limnaea* neurones depart from a straight-line relationship at low external concentrations of potassium. This usually takes the form of a 'flattening off', but in some cells amounts to an inflexion and in these cases a reduction of $[K^+_o]$ produces a depolarization of the membrane. The observation that the hyperpolarization induced by sodium injection is abolished by exposure to potassium-free Ringer provides confirmatory evidence for the existence of such a pump. The depolarization of the cell membrane induced by the application of 10^{-4} M ouabain in normal Ringer and the abolition of the inflexions in the $V \times [K^+_o]$ curves by the same concentration of this cardiac glycoside suggest that, at least in some cells, the pump is active under steady-state conditions.

Results of experiments designed to assess the ionic basis of action-potential pro-

duction are discussed separately for those categories of cells which can be loosely described as sodium-sensitive and sodium-insensitive. For the first group of cells the evidence for the sodium-dependence of the action potential can be summarized as follows:

(a) Sodium-free (tris) Ringer blocks action potentials.

(b) A straight-line relationship is observed between the overshoot of the action potential and log $[Na^+_o]$ over a range of external sodium concentrations from 5 to 60 mM/l. Within these limits, overshoot potential changes of 29–37 mV accompany decade changes in $[Na^+_o]$.

(c) Tetrodotoxin blocks action potentials in most cells albeit at rather high concentrations (5×10^{-6} M).

(d) Action potentials in these cells are, nevertheless, sensitive to changes in $[Ca^{2+}_o]$ and over the range of external calcium concentrations of 1–10 mM/l a straight-line relationship is observed between the overshoot of the action potential and log $[Ca^{2+}_o]$ with a slope of approximately 18 mV per decade change in $[Ca^{2+}_o]$.

(e) The overshoot potential is relatively insensitive to manganous ions, even when applied at concentrations of 10 mM/l.

The above findings provide compelling evidence of a role for sodium ions as charge carriers during the inward current of the action potential of this group of *Limnaea* neurones. It is, however, clear that the behaviour of the active membrane departs from that of an ideal sodium electrode. It could be postulated, on the basis of the sensitivity of the overshoot potential to $[Ca^{2+}_o]$, that calcium ions play a major role as charge carriers. Such a view is difficult to reconcile with the complete abolition of the action potential by the removal of sodium (in the presence of calcium) and by the application of tetrodotoxin. It seems likely, therefore, that the effects of calcium on the action potentials of these neurones are best interpreted as an effect of this cation on the sodium-conductance mechanism (Magura & Gerasimov, 1966; Chamberlain & Kerkut, 1967, 1969).

The so-called sodium-insensitive cells can produce action potentials for extended periods in sodium-free Ringer during which the overshoot potential slowly declines. A number of possible explanations can be invoked to explain this behaviour. Under sodium-free conditions, for example, a sodium-dependent spike could be maintained for some time using a source of sodium ions trapped in the extracellular spaces. Such a view has been proposed to account for the behaviour of neurones of *Tritonia diomedia* in sodium-free Ringer (Krasts & Veprintsev, 1972), but it demands the existence of a barrier to free movement of ions between the fluid bathing the surfaces of the cells and the external medium. The ready accessibility of these *Limnaea* neurones to potassium ions in the bathing medium argues against this hypothesis. A second possible explanation is that a sodium-dependent spike is maintained by an intracellular (e.g. glial) source of sodium which can be made available only to certain cells within the ganglion. Such a mechanism has been suggested to explain the maintenance of activity under sodium-free conditions of the larger axons within the central nervous connectives of *Anodonta cygnea* (Treherne, Carlson & Gupta, 1969). It has been shown for *Anodonta* that electrical stimulation of these fast-conducting axons in sucrose–Ringer leads to a substantial depletion of the sodium-reservoir (Carlson & Treherne, 1969). By contrast, stimulation at high frequency of the sodium-

insensitive cells of *Limnaea* in sodium-free Ringer results in only a very small decline in the overshoot potential. A third possible explanation of the behaviour of these cells in the absence of external sodium is that another cation, such as calcium, takes on the role of charge carrier. This explanation obviates the need to postulate a diffusion barrier and accounts for the minimal effects of stimulation on the overshoot potential when the cell is bathed in a sodium-free Ringer which contains calcium. The evidence that the change in overshoot potential per decade change in $[Ca^{2+}_0]$ increases from 18 mV in the presence of sodium to 29 mV under sodium-free conditions is strong evidence in favour of this hypothesis.

Most of the conclusions of this study of the ionic mechanisms involved in action potential production are in general agreement with the results obtained for neurones of *Helix aspersa* (Moreton, 1972). It remains to reconcile the findings presented here with the apparently conflicting results of earlier workers on the ionic basis of excitation in the giant neurones of *Limnaea stagnalis*. Gerasimov *et al.* (1964) suggested an important role for sodium ions in action potential generation. This has been substantiated for certain neurones. Jerelova *et al.* (1971) discount a major role for sodium, based largely on the difficulty of obtaining clear results with tetrodotoxin and the strong calcium-dependence of the overshoot potential in most cells investigated. These latter authors employed a normal Ringer containing 8 mM/l magnesium, whereas the experiments reported here were conducted in the presence of only 2 mM/l magnesium (in both cases the calcium concentration was 4 mM/l). It is of interest to note, therefore, that R. W. Meech & N. B. Standen (unpublished observations) have recently shown in neurones of *Helix aspersa* that tetrodotoxin reversibly reduces action potentials at low calcium concentrations only when the magnesium level is very low. This influence of magnesium on TTX action in snail neurones might well explain the uncertain effects of the toxin on *Limnaea* neurones reported by other investigators (Jerelova *et al.* 1971). The major conclusion of these authors that the overshoot potential is determined mainly by calcium ions is substantiated in the case of the sodium-insensitive cells described in this report.

It thus appears that sodium and calcium ions can act as charge carriers in the generation of action potentials in neurones of *Limnaea stagnalis* and that their relative contribution varies from cell to cell. This latter observation may account for the earlier conflicting reports concerning the ionic dependence of excitability in these cells.

SUMMARY

1. A mean resting potential of $-53\cdot3$ (S.D. $\pm 2\cdot7$) mV has been obtained for 23 neurones of the parietal and visceral ganglia of *Limnaea stagnalis* (L.). Changes in the resting potential of between 28 and 43 mV accompany tenfold changes in $[K^+_0]$. A modified constant-field equation accounts for the behaviour of most cells over the range of external potassium concentrations from 0·5 to 10·0 mM/l. Mean values have been estimated for $[K^+_i]$, 56·2 (S.D. $\pm 9\cdot0$) mM/l and P_{Na}/P_K, 0·117 (S.D. $\pm 0\cdot028$).

2. Investigations on the ionic basis of action potential generation have revealed two cell types which can be distinguished according to the behaviour of their action potentials in sodium-free Ringer. Sodium-sensitive cells are unable to support action potentials for more than 8–10 min in the absence of sodium. Sodium slopes of between

29 and 37 mV per decade change in $[Na^+_0]$ have been found for these cells. Tetro-dotoxin (5×10^{-5} M) usually blocks action potentials in these neurones. Calcium-free Ringer produces a marked reduction in the overshoot potential and calcium slopes of about 18 mV per decade change in $[Ca^{2+}_0]$ are found. Manganous chloride only partially reduces the action potential overshoot in these cells at concentrations of 10 mM/l.

3. Sodium-insensitive neurones maintain action potentials in the absence of external sodium. Stimulation only slightly reduces the amplitude of the action potential under these conditions and such cells are readily accessible to potassium ions in the bathing medium. A calcium-slope of 29 mV per decade change in $[Ca^{2+}_0]$ has been observed in these cells in the absence of external sodium.

4. It is concluded that both sodium and calcium ions can be involved in the generation of the action potential in neurones of *Limnaea stagnalis*, their relative contribution varying in different cells.

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