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AN INVESTIGATION OF THE ELECTROGENIC SODIUM PUMP IN SNAIL NEURONES, USING THE CONSTANT-FIELD THEORY

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INTRODUCTION

In a previous paper (Moreton, 1968) it was shown that the behaviour of the resting potentials of giant neurones of the common snail, *Helix aspersa*, can be described by an equation derived from the 'constant-field' theory (Goldman, 1943; Hodgkin & Katz, 1949). By fitting this equation to the experimental results, estimates of the intracellular potassium concentrations and of the ratios of sodium and potassium permeabilities of the cell membranes could be obtained. In deriving the equation it was assumed that the cell was in an approximately steady state, with no net current flowing across the cell membrane.

It is clear, however, that the assumption of a steady state can only be approximate, since the theory predicts that, for a cell membrane with an appreciable sodium permeability, external sodium ions will steadily leak into the cell, replacing the intracellular potassium. In a number of tissues, including nerve and muscle cells, the effect of this leakage is now known (e.g. Hodgkin & Keynes, 1954, 1955; Frazier & Keynes, 1959) to be rectified by the action of the sodium pump, a metabolically driven mechanism which exchanges intracellular sodium for extracellular potassium ions. If this exchange is strictly one-to-one, so that the pump causes no net transfer of electric charge across the cell membrane, then it will make no direct contribution to the resting potential of the cell. There is, however, an increasing body of evidence from studies on muscle (e.g. Frumento, 1965; Adrian & Slayman, 1966) and red blood cells (e.g. Post & Jolly, 1957), and in particular on molluscan giant neurones (Kerkut & Thomas, 1965; Thomas, 1968; Carpenter & Alving, 1968) that the number of sodium ions extruded by the pump constantly exceeds the number of potassium ions taken up. If this is the case, then, as has been pointed out by Geduldig (1968), the equations derived from the constant-field theory require modification, to allow for the current generated by the electrogenic pump.

The equation previously derived (Moreton, 1968) from the constant-field theory represents the resting potential, V, of a neurone, as a function of the extracellular concentration of potassium ions, as follows:

$$e^{FV/RT} = \frac{[K_o^+]}{[K_i^+]} + \frac{P_{Na}[Na_o^+]}{P_K[K_i^+]},$$

where the subscripts $_o$ and $_i$ refer to the extra- and intracellular ion concentrations, respectively, P_{Na} and P_K are the sodium and potassium permeabilities of the cell

membrane, and R, T and F have their usual meanings. This equation predicts a linear relationship between $e^{FV/RT}$ and $[K_o^+]$, from the slope and intercept of which estimates of the intracellular potassium concentration, and the relative sodium permeability of the cell membrane can be obtained. In the Appendix A to this paper, the effect is considered of a sodium pump which causes extrusion of positive ions from the cell at a net rate sM_a, where s is the surface area of the cell. It is shown that the equation should now include an extra term:

$$e^{FV/RT} = \frac{[K_o^+]}{[K_i^+]} + \frac{P_{Na}[Na_o^+]}{P_K[K_i^+]} + \frac{RTM_a}{FVP_K[K_i^+]}.$$

Provided that M_a remains constant, and that the alteration to the resting potential caused by the pump is not too large, this new equation still represents an approximately linear relationship between $e^{FV/RT}$ and $[K_o^+]$, with a slope equal to $1/[K_i^+]$. In particular, the case is considered of an electrogenic pump which operates at a constant rate, for extracellular potassium concentrations greater than a certain value, but which is completely inhibited when the potassium concentration falls to zero (as found by Glynn, 1956). The equation will then predict a relationship which is linear for high potassium concentrations, but deviates at low concentrations, in the direction of a decreasing resting potential. The slope of the linear portion can then be used to give the intracellular potassium concentration, while the deviation from the straight line at zero external potassium concentration gives the contribution to the resting potential, which is normally made by the pump.

The purpose of the experiments described here was to investigate the application of this modified equation to the behaviour of the giant neurones of the snail, *Helix aspersa*, and to attempt a quantitative comparison between the rate of extrusion of ions by the sodium pump, and the observed contribution of the pump to the cell's resting potential. The quantity of sodium ions pumped out by a single giant neurone over a reasonable period of time is not large enough to measure directly, using conventional tracer techniques, so it was necessary to measure the activity of the pump indirectly. If it is assumed that the total concentration of free ions in the intracellular fluid remains constant, then it is possible, as set out in the Appendix B, to establish a relationship between the intracellular concentrations of sodium and potassium ions. If the sodium pump is stimulated by injection of sodium ions into the cell at a constant rate (Kerkut & Thomas, 1965), determination of the intracellular potassium concentration at intervals, using the constant-field theory, should thus indicate to what extent the injected ions are accumulating in the cell, rather than being pumped out again. Subsequent inhibition of the sodium pump, for example by a metabolic poison, will result in a new rate of accumulation of the injected ions. Assuming the rates of entry by injection and by passive leakage through the cell membrane to be constant, the difference between the two rates of accumulation gives the rate at which the pump was acting, during the initial period of measurement.

Combining the rate of action of the pump with the membrane resistance of the cell, an estimate can be made of the contribution which an electrogenic pump, acting at the measured rate, would make to the cell's resting potential. This can be compared directly with the observed contribution, as obtained from the potassium-dependence of the resting potential.

METHOD

The method of dissection and recording from giant neurones was the same as that previously described (Moreton, 1968). Sodium ions were injected into the neurones by passive leakage from low-resistance microelectrodes (Kerkut & Thomas, 1965);

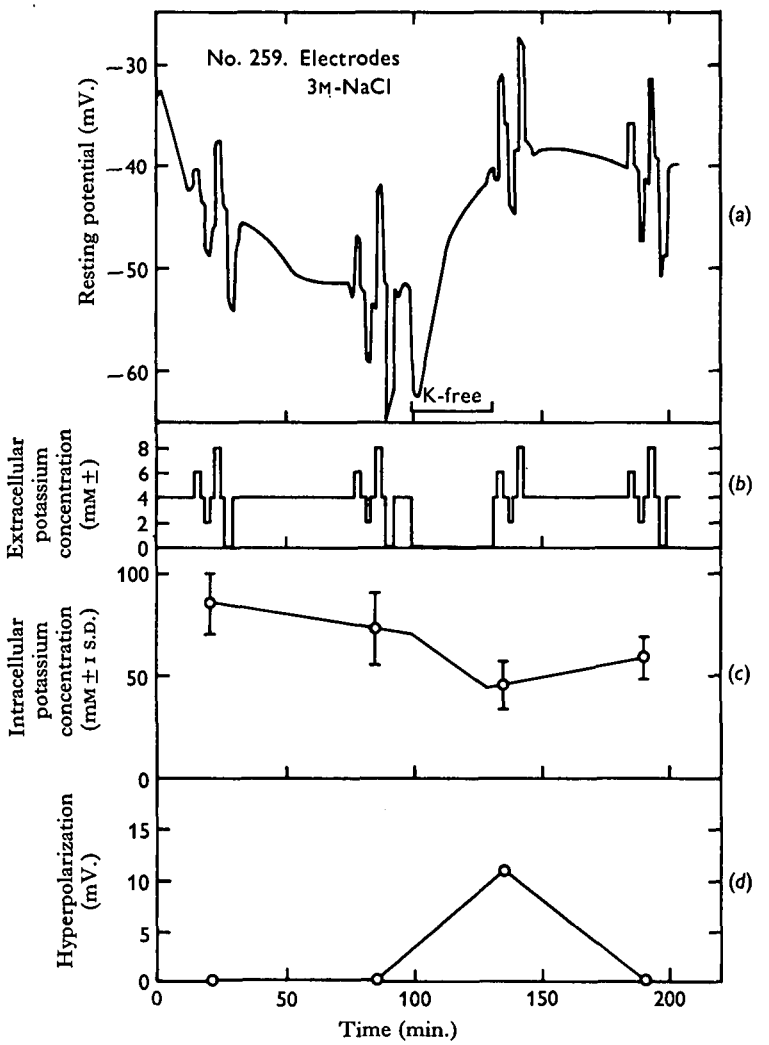

Fig. 1. Record of an experiment in which potassium-free solution was used to inhibit the sodium pump. Intracellular potassium concentrations (*c*) and contribution of the sodium pump (*d*) to the resting potential (*a*) were deduced from the behaviour of the latter as a function of the extracellular potassium concentration (*b*).

for this purpose, electrodes filled with 3 M solutions of sodium chloride or sodium acetate, and with resistances of 3–8 MΩ were used. Control experiments on the effects of potassium injection were carried out with electrodes of similar resistance, filled with 3 M potassium chloride solution. In order to measure the membrane resistance of the cell, it was necessary to use two microelectrodes. These were inserted simultaneously,

a special holder being devised for this purpose. Membrane resistances could then be measured at intervals throughout each experiment, by passing a direct current down one of the electrodes; hyperpolarizing currents were used, as this is the direction in which the membrane potential is driven by an electrogenic sodium pump.

The potassium-dependence of the resting potential was investigated as described previously (Moreton, 1968). In order to follow the behaviour of the sodium pump, this procedure was repeated at intervals throughout each experiment, with periods of approximately 30 min. in between, during which the preparation was left undisturbed. In a typical experiment (Figs. 1–3), the first of these periods would be in normal Ringer, of the composition used by Kerkut & Thomas (1965); during the second (and third, in the case of cyanide) the sodium pump would be inhibited, and finally a period in normal Ringer was allowed for possible recovery of the pump.

Three methods were used to inhibit the sodium pump: (1) exposure to potassium-free Ringer; (2) exposure to the cardiac glycoside ouabain; (3) exposure to cyanide ions. Ouabain was used at a concentration of 10^{-4} M (78·3 mg./l.), made up in normal Ringer. Sodium cyanide was used at 2 mM, made up in Ringer buffered with 5 mM sodium bicarbonate instead of *Tris*, as the latter was found to form a complex with the cyanide, which had a depolarizing effect on the neurones, causing a large fall in their membrane resistance. Experiments were carried out at room temperature (18–20° C.).

The results gave estimates of the intracellular potassium concentration, and the contribution made by the sodium pump to the resting potential, at intervals throughout each experiment, as illustrated in Figs. 1–3. The rate of action of the sodium pump was determined from the intracellular potassium concentration, according to the equations derived in Appendix B.

RESULTS

(1) *Potassium-dependence of the resting potential.* The presence of low-resistance microelectrodes containing 3 M solutions of sodium chloride or acetate had a considerable hyperpolarizing effect on the neurones, as was found by Kerkut & Thomas (1965). Table 1 shows the mean values of the resting potential, recorded immediately after impalement with each type of microelectrode, and also the mean values of the steady level reached after a 20 min equilibration period, with standard errors calcu-

Table 1. *Resting potential measurements*

(All errors are given as one standard deviation, calculated from the spread of the results. Figures in parentheses are the number of results in each group.)

	Microelectrode sloution		
	3M-$NaOCOCH_3$ (25)	NaCl (29)	KCl (18)
Resting potential (mV.)			
Initial	$-36{\cdot}7 \pm 1{\cdot}6$	$-33{\cdot}1 \pm 1{\cdot}3$	$-37{\cdot}4 \pm 2{\cdot}0$
Steady level	$-61{\cdot}5 \pm 2{\cdot}9$	$-53{\cdot}9 \pm 1{\cdot}6$	$-44{\cdot}1 \pm 2{\cdot}2$
Hyperpolarization (mV.)	$3{\cdot}9 \pm 4{\cdot}0$	$8{\cdot}7 \pm 7{\cdot}0$	$0{\cdot}8 \pm 1{\cdot}5$
Intracellular potassium concentration (mM)	$147{\cdot}3 \pm 12{\cdot}0$	$86{\cdot}4 \pm 5{\cdot}2$	$99{\cdot}0 \pm 7{\cdot}5$
Permeability ratio P_{Na}/P_K	$0{\cdot}13 \pm 0{\cdot}02$	$0{\cdot}09 \pm 0{\cdot}01$	$0{\cdot}18 \pm 0{\cdot}03$

lated from the spread of the results. With sodium chloride-filled and acetate-filled electrodes the resting potential increased by an average of 16·9 ± 1·1 mV. over this period.

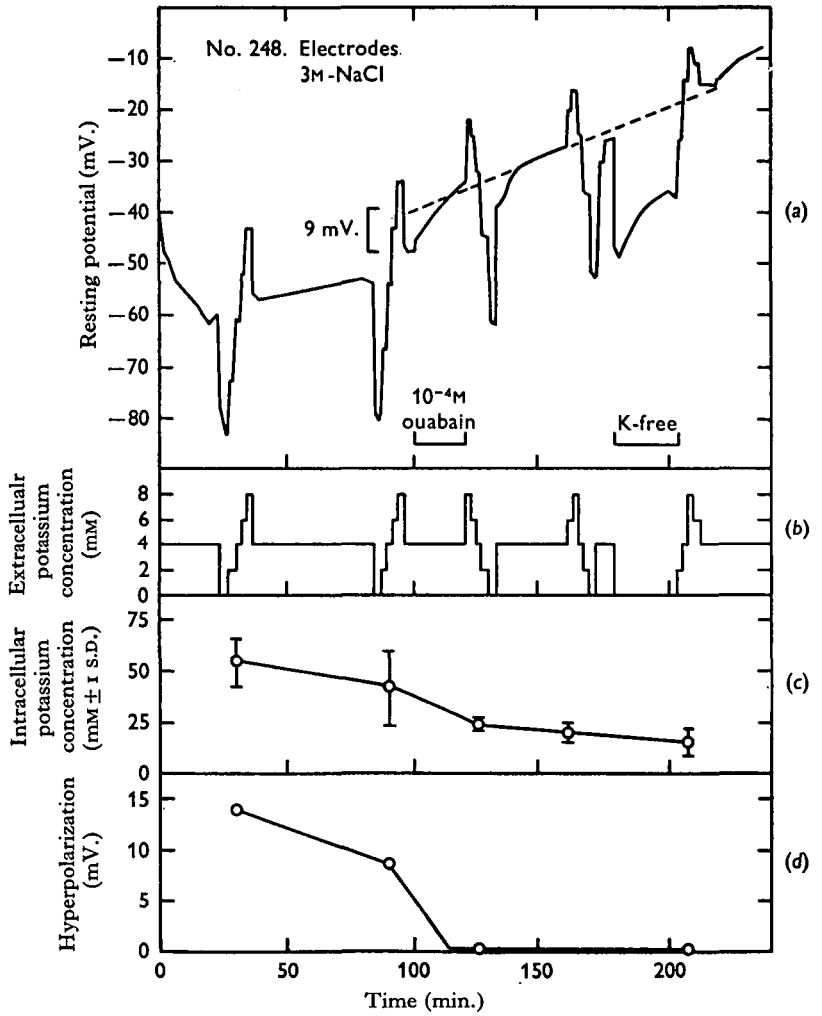

Fig. 2. Record of an experiment in which ouabain was used to inhibit the sodium pump. Layout similar to Fig. 1; the broken line in (*a*) shows the extrapolation used to estimate the direct effect of the ouabain on the resting potential. Subsequent exposure to K-free Ringer produced no further evidence of pumping.

The behaviour of the resting potential as a function of the extracellular potassium concentration is illustrated in Fig. 4 (*a*), (*b*). At concentrations of 4 mM and above, the function $e^{FV/RT}$ was found to vary linearly. At concentrations below 4 mM, deviations were often observed, the resting potential being less negative than the value required for a linear relationship. Significant deviations of this kind were found in 30 cases out of 53. If it is assumed that, under these conditions, the behaviour of the resting potential is described by an equation such as equation (9) of Appendix A, then the deviations from linearity can be explained in terms of variations in the rate of action

of the electrogenic sodium pump. On this basis it is possible to calculate the contribution made by the pump to the resting potential as a function of the extracellular potassium concentration. Figure 4(*c*) shows the result of such a calcluation; at potassium concentrations above 4 mM the contribution from the pump is constant, but

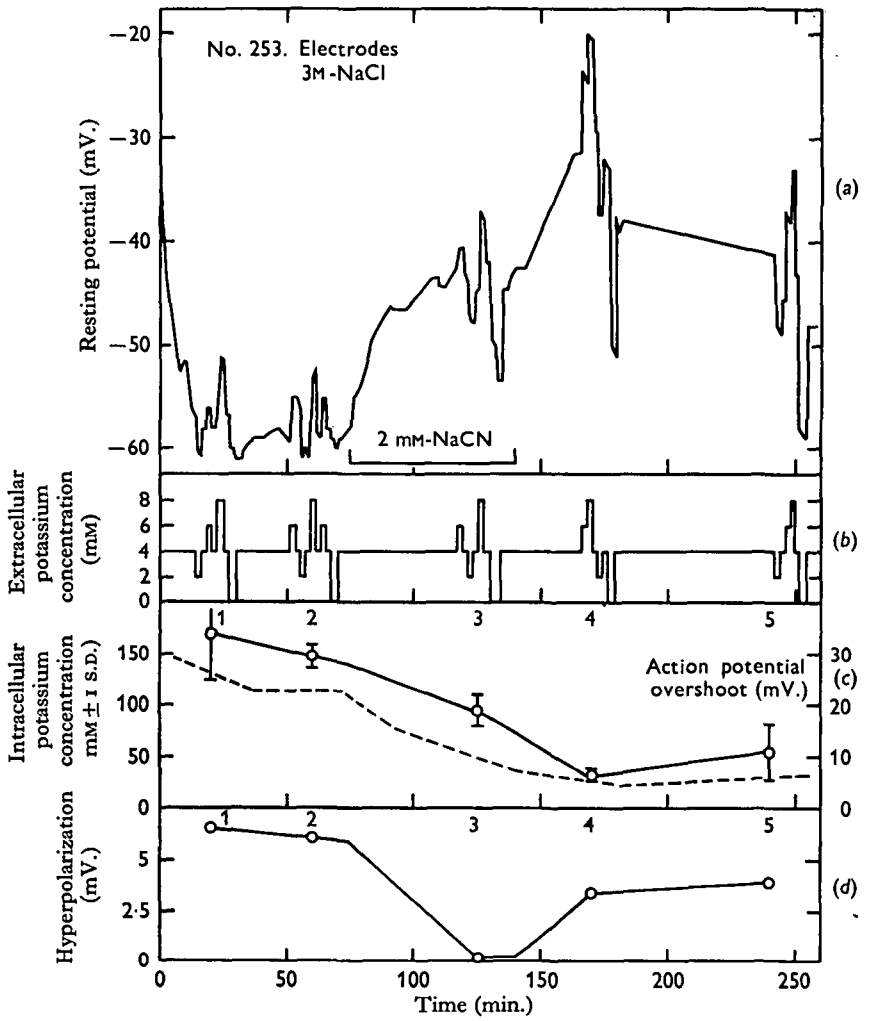

Fig. 3. Record of an experiment in which cyanide was used to inhibit the sodium pump. Layout similar to Figs. 1 and 2, except that the behaviour of the action potential overshoot is also shown (broken curve in (*c*)). The numbers in (*c*) and (*d*) refer to Fig. 6.

below 4 mM it falls off increasingly rapidly, and is assumed to drop to zero when the cell is exposed to potassium-free Ringer. The dotted line in Fig. 4 (*b*) is obtained by subtracting from each value of the resting potential the contribution due to the pump.

Table 1 shows the average of all the observed values of the contribution made by the pump, at a potassium concentration of 4 mM or above; the figures are arranged separately, according to the type of microelectrodes used to make the measurement.

The linear portion of graphs such as Fig. 4 (*b*) can be used, according to equation (9), to give estimates of the intracellular potassium concentration, and the relative sodium

permeability of the cell membrane. Table 1 shows the mean results, obtained from measurements made near the beginning of each experiment. The results obtained with sodium chloride-filled and potassium chloride-filled electrodes are similar to those obtained in previous experiments (Moreton, 1968); those obtained with sodium acetate-filled electrodes show some significant differences, which will be discussed below.

(2) *Effect of inhibition of the sodium pump by ouabain, cyanide or potassium-free Ringer.* The application of ouabain at 10^{-4} M caused a rapid fall in the resting potential, the effect being complete within 20 min. (Fig. 2). At the end of this time the net reduction in resting potential averaged 5·9 mV., from 10 experiments; in the same experiments, the average calculated contribution (as in Fig. 4 (*c*)) made by the pump to the resting potential was 4·4 mV., immediately before application of the ouabain. When the ouabain action was complete, investigation of the potassium-dependence of the resting potential showed that $e^{FV/RT}$ now varied linearly at all concentrations, as illustrated in Fig. 5. The value of the intracellular potassium concentration given by the two graphs of Fig. 5 is the same, so that the initial action of ouabain can be described as the abolition of the contribution apparently made by the sodium pump to the resting potential. The effect was not reversible.

The action of cyanide on the neurones was much slower than that of ouabain, it being necessary to expose the preparation to 2 mM sodium cyanide for 30–60 min. before the full effect was seen. It was therefore not possible to measure any direct effect on the resting potential; the effect on the potassium-dependence of the resting potential was, however, the same as that of ouabain: any deviation from linearity, such as that illustrated in Fig. 4 (*b*), was abolished. The effect was partly reversible, about 30 min. being required after washing out the cyanide. Figure 6 shows the results obtained from the experiment of Fig. 3, in which five successive measurements of the potassium-dependence of the resting potential were made, during progressive inhibition and recovery of the sodium pump. The points in Fig. 3 (*c*), (*d*), calculated from Fig. 6, illustrate the correlation between the hyperpolarization attributed to the sodium pump, and the behaviour of the intracellular potassium concentration, which is an index of the rate of sodium extrusion.

The action of potassium-free solution on the pump was apparently almost immediate; the initial change in resting potential occurred with the same time-course as the change caused by exposure to any potassium concentration higher than the normal value (Fig. 1). In most cases the resting potential in potassium-free solution was slightly more negative than in 4 mM potassium, though in some cases the contribution due to the pump was so large in normal Ringer than the reverse was the case, as found by Kerkut & Thomas (1965) (see Fig. 4 (*a*)). In no experiment, however, did the resting potential in normal Ringer exceed the potassium equilibrium potential, as calculated from the estimated intracellular potassium concentration.

(3) *Measurement of pumping rate.* Following any initial direct effect, inhibition of the sodium pump during continuous injection of sodium ions into the cell was generally found to cause a gradual decline in the resting potential, amounting typically to some 10–20 mV. over a half-hour period. When potassium-free Ringer was used, the decline began immediately, and was immediately reversed on returning to normal Ringer; in the case of ouabain, the decline was observable after about 20 min.

exposure, and was irreversible; but with cyanide there was a delay, corresponding with the delayed effect on the potassium-dependence of the resting potential, and the effect was partly reversible. It was thus possible, from the rate of change of the intracellular potassium concentration, to estimate the rate at which sodium extrusion had been occurring, prior to inhibition of the pump, by using equation (15) of Appendix B. The results are summarized in Table 2, expressed as sodium efflux per unit area of

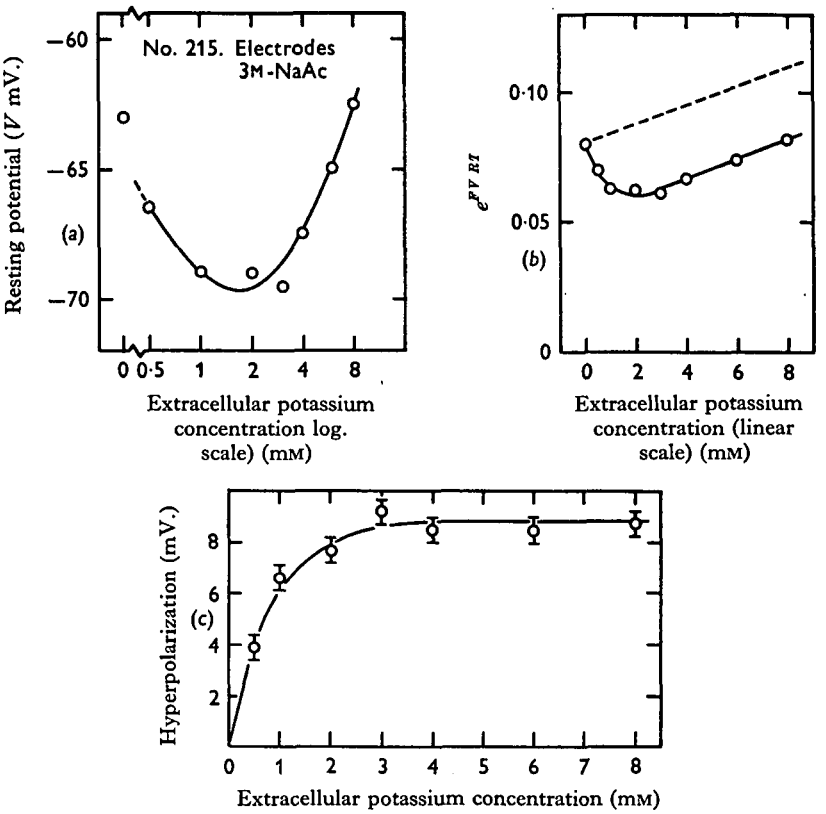

Fig. 4. Potassium-dependence of the resting potential of a neurone, during sodium injection, plotted on logarithmic (*a*) and linear (*b*) scales. Broken line in (*b*) shows the theoretical behaviour, after subtracting the hyperpolarization due to the sodium pump, the behaviour of which is shown in (*c*) (cf. Glynn (1956)).

cell membrane, assuming the cells to be spherical. The mean pumping rate from all the experiments is $23{\cdot}0 \pm 4{\cdot}0$ pmole cm.$^{-2}$ sec.$^{-1}$ (error $\pm$ 1 S.D.), corresponding to a rate of change of concentration of 0·82 mM min.$^{-1}$ in a cell of diameter 100 μ. In the control experiments, using potassium chloride-filled electrodes, the mean pumping rate was much smaller, being represented by the figure of $-0{\cdot}4 \pm 4{\cdot}8$ pmole cm.$^{-2}$ sec.$^{-1}$. The passive influx of sodium ions through the cell membrane of a cell is thus small in comparison with the rate at which ions are injected by low-resistance microelectrodes.

The errors indicated in Table 2 are the standard deviations derived from the regression analysis used to estimate the intracellular potassium concentration; unfortunately, three separate estimations are required to determine each pumping rate

(see equation (15)), so that the errors in individual results are bound to be large. The results are 'best linear estimates' of the mean pumping rate, as determined using each method of inhibiting the sodium pump. Agreement between the figures is reasonable, although the cyanide results tend to be higher than the others; however they are also less reliable, chiefly owing to the greater duration of the cyanide experiments. The average results obtained with sodium chloride-filled or acetate-filled electrodes are the same.

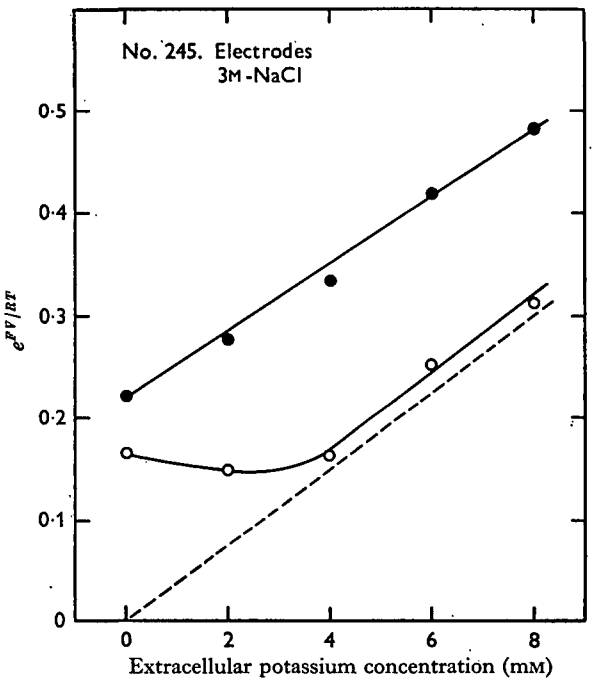

Fig. 5. Potassium-dependence of the resting potential of a neurone, during sodium injection, immediately before (open symbols) and 20 min. after (filled symbols) exposure to ouabain. The parameters of the cell were, before: $K_i^+ = 26{\cdot}8 \pm 12{\cdot}6$ mM; $P_{Na}/P_K = 0{\cdot}05$; hyperpolarization = 22·8 mV; after: $K_i^+ = 30{\cdot}7 \pm 7{\cdot}6$ mM; $P_{Na}/P_K = 0{\cdot}085$; hyperpolarization = 0. Before exposure to ouabain, the resting potential in normal Ringer almost reached, but did not quite exceed, the potassium equilibrium potential, as indicated by the broken line.

(4) *Membrane resistance.* The input resistance of the cell was determined, before and after each series of readings of the resting potential, by passing direct current down the second microelectrode. The resulting potential change was found to be proportional to the current, as measured by the potential difference across a 1 MΩ resistor in series with the current source, for hyperpolarizations up to 20 mV. The resistances of the neurones varied widely, in the range of 1–10 MΩ. On average, the resistances of the smaller neurones, with diameters up to 120 μ, were apparently independent of diameter, suggesting that the surface area of the cells was considerably modified by folding of the cell-membrane. Cells with diameters of 40–120 μ had a mean resistance of $4{\cdot}5 \pm 0{\cdot}3$ MΩ; above this diameter, the resistance decreased with increasing size in the manner expected for spherical cells, so that neurones of diameter 200 μ had a resistance of only $1{\cdot}5 \pm 0{\cdot}2$ MΩ.

Combining the input resistance of the neurone with the estimated rate of extrusion of ions by the sodium pump, it is possible to calculate the contribution which the pump might be expected to make to the cell's resting potential, if it were electrogenic. Unfortunately, a quantitative comparison involves a number of theoretical and practi-

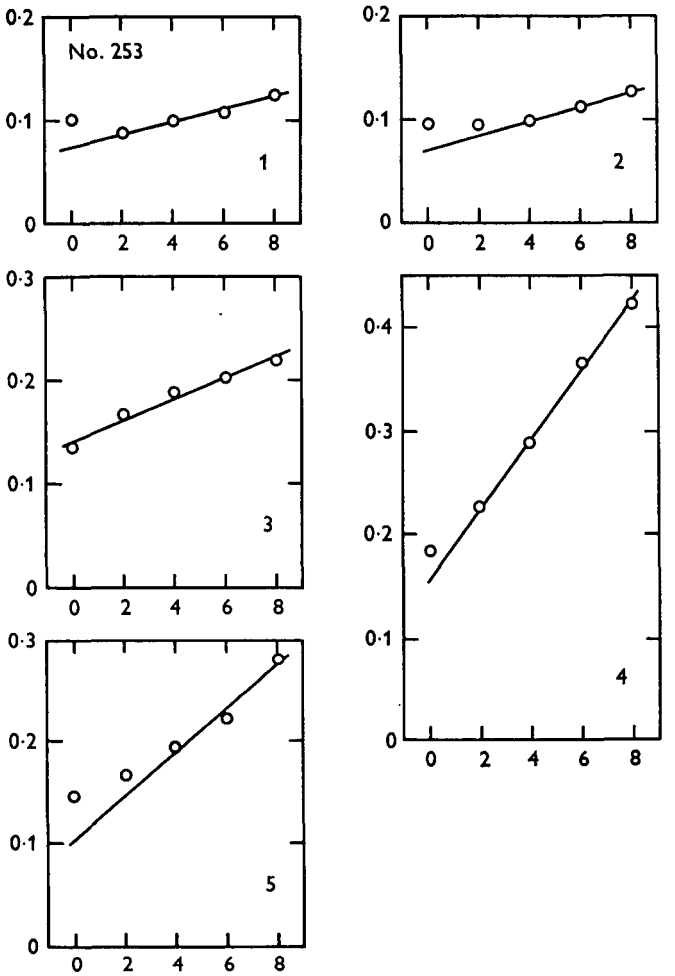

Fig. 6. Potassium-dependence of the resting potential, from the experiment of Fig. 3 (effect of cyanide). Ordinate: $e^{FV/RT}$; Abscissa: extracellular potassium concentration in mM. The numbers refer to the corresponding points in Fig. 3 (*c*), (*d*).

cal difficulties, the nature of which is discussed below, so that it is not reasonable to expect more than an order-of-magnitude agreement. The large standard errors and variability of individual results make any sort of statistical analysis difficult. In Table 3 the experiments have been divided into three groups, according to the magnitude of the observed hyperpolarization attributable to the sodium pump, and the mean values of the observed and expected contributions have been calculated. The results are in agreement as to order of magnitude, but do not permit any further quantitative conclusions to be reached. Examination of individual figures shows a positive correlation between 'observed' and 'expected' values (r = 0·43 on 26 results; 0·01 < P < 0·05).

(5) *Control experiments.* Control experiments were carried out with potassium chloride-filled electrodes, using all three methods of inhibiting the sodium pump. The behaviour of the resting potential under these conditions was as previously described (Moreton, 1968), showing no evidence of electrogenic pumping. The results are summarized in the last columns of Tables 1 and 2; neither the resting potential nor the intracellular potassium concentration was significantly affected by inhibition of the pump.

Table 2. *Rates of sodium extrusion* (M_a')

(All figures are in pmole cm.$^{-2}$ sec.$^{-1}$, with an error of one standard deviation.)

	Microelectrode solution			
Inhibitor	3M-$NaOCOCH_3$	NaCl	Mean	KCl
Potassium-free solution	43·2 ± 14·0	13·9 ± 8·9	22·3 ± 7·5	'−6·5' ± 6·4
Ouabain	20·5 ± 5·2	16·6 ± 14·4	22·2 ± 5·1	8·0 ± 8·4
Cyanide	72·8 ± 25·3	52·8 ± 23·9	63·9 ± 17·4	5·7 ± 14·5
Mean	25·1 ± 4·8	18·1 ± 7·2	**23·0 ± 4·0**	'−0·4' ± 4·8

Table 3. *'Observed' and 'expected' hyperpolarizations*

(All figures are in mV., with an error of one standard deviation. Figures in parentheses give the range of values recorded in each group.)

Group	'Observed' hyperpolarization	'Expected' hyperpolarization
1	0·5 ± 1·0 (0–5)	1·7 ± 2·0 (0–4·5)
2	7·8 ± 1·1 (5–10)	5·3 ± 5·6 (2–11)
3	12·3 ± 1·6 (10–15)	3·5 ± 3·2 (0·5–8)

DISCUSSION

The experiments described in this paper represent an attempt to study quantitatively the behaviour of the sodium pump in single giant neurones of the snail, when stimulated by continuous injection of sodium ions through low-resistance microelectrodes. The application of the 'constant-field' theory to the resting potential (Moreton, 1968) has been extended to cover the effects of an electrogenic sodium-potassium exchange pump of the type found in other nerve and muscle cells (e.g. Adrian & Slayman, 1966; Hodgkin & Keynes, 1955, 1956) and in red blood cells (e.g. Glynn, 1962; Post & Jolly, 1957). This gives information about the pump in two ways: first, the direct electrogenic effect of the pump can be estimated from the potassium-dependence of the resting potential; and second, the rate of extrusion of ions can be measured from the behaviour of the intracellular potassium concentration, when the pump is stopped by applying various inhibitors. Comparison of the results obtained in these two ways can be used to investigate the hypothesis that the observed behaviour of the resting potential is in fact due to the operation of an electrogenic sodium pump.

(1) *The resting potential.* The resting potentials of snail neurones increase considerably, following the insertion of sodium chloride-filled or acetate-filled microelectrodes (see Table 1). Part of this increase can be attributed to recovery of the cell

from damage caused by the puncture—a burst of rapid firing is often observed on impalement, suggesting some transient depolarization—and some increase is also seen after insertion of potassium chloride-filled electrodes. Part of it seems, however, to be caused specifically by the presence of the sodium salt in the electrodes. It is possible that the results could be affected by changes in the tip-potentials of the electrodes themselves, though this is regarded as unlikely, since the resting potential measured immediately after impalement is independent of the type of electrodes used (Table 1).

The hyperpolarization of the cells caused by the insertion of sodium-containing microelectrodes is thus presumably due to some effect of the ions leaking from the electrodes into the cell. Examination of the potassium-dependence of the resting potential shows that at least part of the hyperpolarization can be abolished by exposing the preparation to a solution deficient in potassium ions, as was found by Kerkut & Thomas (1965) (Fig. 4*a*, *b*). The behaviour of the resting potential can thus be described by equations such as (9) and (10) of the Appendix A, suggesting that the resting potential is partly determined by the activity of an electrogenic sodium-potassium exchange pump, which requires extracellular potassium ions for its operation, as depicted in Fig. 4(*c*). If the microelectrodes contain potassium chloride, injection of which would not be expected to stimulate the sodium pump (e.g. Glynn, 1962; Hodgkin & Keynes, 1956), this effect is not seen.

(2) *Effect of sodium-pump inhibitors.* Exposure of the cell membrane to a potassium-free solution is known to have an inhibitory effect on the sodium pump in nerve and muscle cells (Keynes, 1954; Hodgkin & Keynes, 1955), and in red blood cells (Glynn, 1956). The pump is also known to be inhibited quite specifically by the cardiac glycoside ouabain (e.g. Schatzmann, 1953; Caldwell & Keynes, 1959; Baker, 1964; Glynn, 1957; Treherne, 1966) and by more general metabolic inhibitors, such as cyanide (e.g. Hodgkin & Keynes, 1955). The action of ouabain and cyanide on snail neurones was to abolish the potassium-dependent hyperpolarization induced by injection of sodium ions. In the case of ouabain, the resting potential was simply reduced by an amount corresponding to the degree of hyperpolarization originally present (Figs. 2, 5); the action of cyanide was too slow for its direct effect on the resting potential to be observed, but the effect on the potassium-dependence of the potential was the same as that of ouabain (Fig. 6). It is thus clear that the potassium-dependent hyperpolarization of snail neurones, caused by insertion of low-resistance microelectrodes filled with solutions of sodium salts, is abolished whenever the sodium pump in the neurones is inhibited.

(3). *Behaviour of the intracellular potassium concentration.* Equation (9) of Appendix A gives estimates of the intracellular potassium concentration and the ratio of sodium and potassium permeabilities of the cell membrane. The values obtained (see Table 1) with all three types of microelectrode are similar, except that in the experiments with sodium acetate-filled electrodes the resting potential and the mean intracellular potassium concentration tend to be higher than in the other experiments. This could be due to accumulation of the injected anions in the cell (Coombs, Eccles & Fatt, 1955; Araki, Ito & Oscarsson, 1961) requiring extra potassium ions to balance their charge; injected chloride ions would be able to some extent, though probably only partially, to escape through the cell membrane (Kerkut & Thomas, 1964).

During the initial period, when the sodium pump was not inhibited, the intracellular potassium concentrations of the majority of cells examined remained approximately constant (Figs. 1–3). It was assumed that, where sodium ions were injected, the pump was able to extrude them as fast as they entered the cell. In a few cases this was evidently not so, as the potassium concentration was observed to fall.

Following the inhibition of the sodium pump by any of the three means employed, the intracellular potassium concentration was generally observed to fall; the results were variable, but from the results of Table 2 it is clear that, on average, inhibition of the sodium pump during continuous injection of sodium ions has the effect of causing an increased decline of the intracellular potassium concentration. Having accepted this, it follows that the intracellular potassium ions must be replaced by some other cations, sodium ions being the only ones present in sufficient quantity. A rise in the intracellular sodium concentration could be caused either by injection from the microelectrodes, or by increased influx through the cell membrane. In the latter case, no inference could be drawn about the activity of the sodium pump; however, there was no evidence of any marked effect of ouabain or cyanide on the passive electrical properties of the cell membrane (e.g. Fig. 6), so it is unlikely that the sodium influx would be very much increased. In potassium-free solution the resting potentials of the cells were often higher than in normal Ringer, which could cause a rise in the sodium influx, but on the other hand the control experiments showed that the behaviour of the intracellular potassium concentration under conditions of potassium chloride injection is very little, if at all, affected by inhibition of the sodium pump (Table 2), suggesting that the sodium influx through the cell membrane is in any case small compared with the rate of injection from sodium chloride-filled or acetate-filled electrodes. There is some direct evidence of increased intracellular sodium concentration, in that the overshoot of the action potential was measured in some experiments, and found to decrease in the expected manner (see Fig. 3).

It is concluded that the increased decline of intracellular potassium concentration after inhibition of the sodium pump occurs because sodium ions leaking from the electrodes are no longer pumped out of the cell; it is assumed, for want of evidence to the contrary, that the rate of injection from the electrodes is constant*, though there is little doubt that some of the variability in the apparent rates of sodium extrusion arises from variations in the rate of injection of ions from the electrodes, due for example to partial blockage occurring during an experiment. This is especially true in the experiments with cyanide, which were generally longer than those with ouabain or potassium-free Ringer.

The rate of sodium extrusion can then be inferred, assuming that the cell remains in osmotic equilibrium with the surrounding solution, from the equations of Appendix B.

(3) *Electrical resistance of the cell membrane.* The input resistances of the neurones are similar to those of other molluscan neurones of comparable size (e.g. Maiskii, 1964). Assuming the cells to be smooth spheres, as they appear under a low-powered

* Nastuk & Hodgkin (1950) calculate that leakage of ions from a microelectrode tip immersed in water rapidly reaches a steady rate, limited by diffusion down the shank. The quantity of electrolyte present in the electrodes used in the present experiments is sufficient to maintain leakage at the observed rate for many hours, without noticeable depletion.

microscope (Tauc & Gerschenfeld, 1962), the resistance of around 4·5 MΩ for a cell of diameter 100 μ corresponds to a specific resistance of the cell membrane of around 1500 Ω cm.^{2}, which is not very different from the specific resistance of other excitable membranes (e.g. Frank & Fuortes, 1956; Fessard & Tauc, 1956). The relation between resistance and size is, however, unexpected—for a smooth spherical cell the input resistance should vary inversely as the square of the diameter. Above 120 μ this is roughly true, but for cells up to this size the resistance of the whole cell is nearly constant, suggesting either that the apparent specific resistance of the cell membrane is genuinely a function of cell size, or that the surface area of the cell is not the same as that of a sphere of the measured diameter. The former could be the case if, for example, the resistance of the cell were determined partly by the presence of surrounding structures, such as glial folds; modifications to the surface area of the cell could result from infoldings of the cell membrane, not visible under the conditions of the experiments. There is some evidence (author's unpublished electron micrographs) for both possibilities.

(4) *Quantitative comparison of results.* The results discussed so far have shown that injection of sodium ions into snail giant neurones, by passive leakage from low-resistance microelectrodes, causes hyperpolarization of the cells. This hyperpolarization, in its dependence on the extracellular potassium concentration, and its abolition by ouabain and cyanide, appears to result from stimulation of an electrogenic sodium-potassium exchange pump located in the cell membrane. Inhibition of the sodium pump, while sodium ions are continuously injected, results in a decline of the intracellular potassium concentration. This is not observed if potassium ions are injected, and is assumed to be due to replacement of the intracellular potassium ions by injected sodium, which is no longer being extruded from the cell by the sodium pump. The results thus give a quantitative measure of the rate at which the sodium pump operates, when stimulated by sodium injection. By combining this with the measured membrane resistance of the cell, it is possible in principle to calculate the size of the contribution which an electrogenic pump, operating at the observed rate, would make to the resting potential of the cell; this 'expected' contribution may be compared directly with the 'observed' contribution, calculated from the potassium-dependence of the resting potential. There are, however, three main difficulties in the way of this comparison.

First, in order to convert the rate of decline of the intracellular potassium concentration into the actual rate of extrusion of sodium ions from the cell (Appendix B, equation (15)), it is necessary to know the cell's volume. The volume can be estimated visually reasonably well by using a micrometer eyepiece, if the cell is assumed to be spherical, but this may only be an approximation. In particular, it is difficult to allow for the contribution made by the axon and dendrites, which Maiskii (1964) assesses from conductivity data to be up to 30% of the total effective volume, in spite of the relatively small size of the axon (Hanneforth, 1965). Also, the effect of swelling of the cell, due to accumulation of injected anions, is difficult to assess. In the case of acetate injection, where it is assumed that all the injected anions remain in the cell, some allowance can be made (equation (16)), but the extent to which injected chloride ions are able to escape from the cell is largely unknown, although some accumulation undoubtedly does occur (Kerkut & Thomas, 1964). The results have been corrected on the basis that all the injected anions remain in the cell.

Secondly, the term representing the contribution of the sodium pump to the resting potential in equation (9) (Appendix A) depends on the potassium permeability of the cell membrane. Calculation of this from the total membrane conductivity is difficult, since the chloride permeablity is not known. Potassium permeabilities have been calculated on the assumption that the chloride permeability is small (Moreton, 1968), and that the membrane conductance can be represented in terms of the ionic permeabilities by the equation derived from the 'constant-field' theory by Hodgkin & Katz (1949), although there is evidence that the constant-field theory is not so successful in describing the conductivity of some excitable cell membranes, as it is in describing the behaviour of the resting potential (Hodgkin & Horowicz, 1959, 1960; Adrian & Freygang, 1962). In particular, if the chloride permeability of the cell membrane is of the same order as the potassium permeability, the value of the latter will have been over-estimated by nearly 100% by the method used.

Thirdly, it is necessary to assume that the treatment used to inhibit the sodium pump is completely effective. Tracer studies on the sodium efflux from squid giant axons show that ouabain (Caldwell & Keynes, 1959) and cyanide (Hodgkin & Keynes, 1955) reduce the sodium efflux to at most $\frac{1}{8}$ of its former value, but potassium-free solution is rather less effective (Hodgkin & Keynes, 1955), and ouabain is not so effective in other preparations (e.g. Baker, 1964; Glynn, 1957; Treherne, 1966). The residual sodium efflux may be attributable to passive leakage, or to exchange-diffusion, however, in which case it may be ignored for the present purpose.

In view of these considerations, and of the experimental errors, it is concluded that an order-of-magnitude agreement is the best that can be expected from the results. Table 3 shows the relevant figures, expressed in terms of the 'observed' and 'expected' contributions by the pump to the resting potential. It should be noted that the 'expected' contributions have been calculated on the basis of a 'purely electrogenic' pump, i.e. one which extrudes sodium ions, without any coupled uptake of potassium ions ($M_a = M_a'$ on p. 200), and are therefore the largest which could be caused by a pump operating at the observed rate. In fact, it is more likely that the pump exchanges at least some potassium ions for the extruded sodium (e.g. Hodgkin & Keynes, 1955; Post & Jolly, 1957), in which case its contribution to the resting potential would be less than that calculated ($M_a < M_a'$). It is thus clear from Table 3 that the rate of sodium extrusion by the pump, as measured in the present experiments, although agreeing perhaps as well as can be expected with that required to produce the observed hyperpolarizations, tends to be too small. It is not, therefore, possible to draw any quantitative conclusions regarding the stoichiometry of the pump. More accurate measurements of the pumping rate are required, and must wait on the development of more direct techniques for evaluating the intracellular ion activities, such as the use of cation-sensitive microelectrodes.

SUMMARY

1. Sodium ions injected into giant neurones of *Helix aspersa* by diffusion from low-resistance microelectrodes caused hyperpolarization of the cells. Under these conditions the behaviour of the resting potential could be described by a modified

'constant-field' equation, including a term representing the effect of a potassium-sensitive, electrogenic sodium pump.

2. Exposure to potassium-free solution, ouabain or cyanide abolished the hyperpolarization, and caused a gradual fall in the intracellular potassium concentration, as estimated from the constant-field equation.

3. Assuming that this fall was due to replacement of intracellular potassium by injected sodium ions, it was possible to calculate the rates of injection and pumping of sodium ions, and, using the measured membrane resistance of the cell, the hyperpolarization which the sodium pump could cause, if it were electrogenic.

4. This was related to the observed hyperpolarization, supporting the view that the latter was caused by stimulation of the electrogenic sodium pump.

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APPENDIX A

Potassium-dependence of the resting potential

The purpose of this section is to derive an equation for the resting potential of a neurone by using the 'constant-field' theory in the same way as in the previous paper on the subject (Moreton, 1968), but making allowance for the effect of activity of an electrogenic sodium-potassium exchange pump. The notation used will be essentially the same as was used previously, square brackets being used to denote ionic activities in solution, and the subscripts $_o$ and $_i$ to denote outside and inside the cell, respectively.

The starting-point is the familiar 'constant-field' equation

$$V = \frac{RT}{F} \ln \left\{ \frac{P_{\mathrm{K}}[\mathrm{K}_o^+] + P_{\mathrm{Na}}[\mathrm{Na}_o^+] + P_{\mathrm{Cl}}[\mathrm{Cl}_i^-]}{P_{\mathrm{K}}[\mathrm{K}_i^+] + P_{\mathrm{Na}}[\mathrm{Na}_i^+] + P_{\mathrm{Cl}}[\mathrm{Cl}_o^-]} \right\}, \tag{1}$$

where V is the resting potential of the cell, P_{K}, etc., are the respective ionic permeabilities of the cell membrane, and R, T and F have their usual meanings. This equation is derived (Hodgkin & Katz, 1949) from three separate equations, themselves derived from the constant-field theory of Goldman (1943), for the net fluxes of the individual ions through the cell membrane. The fluxes per unit area are represented respectively as M_{K}, etc., the direction from outside to inside the cell being considered as positive:

$$M_{\mathrm{K}} = -\frac{RT}{F} u_{\mathrm{K}} \frac{d[\mathrm{K}^+]}{dx} - [\mathrm{K}^+] u_{\mathrm{K}} \frac{d\psi}{dx}, \tag{2}$$

$$M_{\mathrm{Na}} = -\frac{RT}{F} u_{\mathrm{Na}} \frac{d[\mathrm{Na}^+]}{dx} - [\mathrm{Na}^+] u_{\mathrm{Na}} \frac{d\psi}{dx}, \tag{3}$$

$$M_{\mathrm{Cl}} = -\frac{RT}{F} u_{\mathrm{Cl}} \frac{d[\mathrm{Cl}^-]}{dx} + [\mathrm{Cl}^-] u_{\mathrm{Cl}} \frac{d\psi}{dx}, \tag{4}$$

where $[\mathrm{K}^+]$, etc., represent the local concentrations of the ions within the cell membrane, u_K, etc., are their ionic mobilities in the membrane phase and ψ is the local

electric potential. x is a distance co-ordinate, measured inwards from the outer surface of the cell membrane. In the derivation of equation (1) these three equations are combined with the assumptions that the electric field within the membrane is constant, so that

$$\frac{d\psi}{dx} = \frac{V}{a}, \tag{5}$$

where a is the membrane thickness, and that the cell's contents must remain electrically neutral, which implies, in the absence of any electrogenic pump, that the total ionic current across the cell membrane must be zero:

$$0 = M_{\mathrm{K}} + M_{\mathrm{Na}} - M_{\mathrm{Cl}}. \tag{6}$$

Equations (2)–(4) can then be integrated, to yield equation (1).

In the presence of an electrogenic sodium pump, however the assumption of no net passive ionic current across the cell membrane will no longer be correct; the passive fluxes of ions must now combine to produce a current balancing the flow of charge caused by the pump. Thus if the pump effectively causes a net *efflux* of monovalent cations from the cell at the rate M_a, the condition for electroneutrality becomes

$$M_a = M_{\mathrm{K}} + M_{\mathrm{Na}} - M_{\mathrm{Cl}} \tag{7}$$

instead of (6). Combining (7) with (2), (3) and (4) and integrating, the new equation for the resting potential is

$$V = \frac{RT}{F} \ln \left\{ \frac{P_{\mathrm{K}}[\mathrm{K}_o^+] + P_{\mathrm{Na}}[\mathrm{Na}_o^+] + P_{\mathrm{Cl}}[\mathrm{Cl}_i^-] + RTM_a/FV}{P_{\mathrm{K}}[\mathrm{K}_i^+] + P_{\mathrm{Na}}[\mathrm{Na}_i^+] + P_{\mathrm{Cl}}[\mathrm{Cl}_o^-] + RTM_a/FV} \right\} \tag{8}$$

differing from equation (1) in the presence of the term RTM_a/FV, which represents the effect of the electrogenic sodium pump on the resting potential.

As in the treatment previously applied to the 'constant-field' equation (Moreton, 1968), equation (8) can be simplified by making some assumptions about the properties of the cell. First, for reasons discussed in the previous paper, the terms depending upon the intracellular sodium concentration, and upon the chloride permeability, are neglected. Secondly, it is noted that the denominator of the argument of the logarithm in equation (8) is likely to be large, compared with the numerator (corresponding to the fact that the resting potential is large and negative). If it is assumed that the sodium pump does not contribute more than a moderate fraction (say 20%) of the resting potential, the term in M_a will thus make up a significant fraction of the value of the numerator, but not of the denominator, from which it can be omitted. Thirdly, the logarithm is removed.

Dividing the right-hand side through by P_{K}, the simplified equation becomes

$$e^{FV/RT} = \frac{[\mathrm{K}_o^+]}{[\mathrm{K}_i^+]} + \frac{P_{\mathrm{Na}}[\mathrm{Na}_o^+]}{P_{\mathrm{K}}[\mathrm{K}_i^+]} + \frac{RTM_a}{FVP_{\mathrm{K}}[\mathrm{K}_i^+]}, \tag{9}$$

which is the same as equation (8) of Moreton (1968), except for the additional term, which will be referred to as the 'sodium pump term'.

In the interpretation of equation (9) two further points should be noted. First, the

sodium pump term is negative, since V is negative; its presence therefore has the effect of making $e^{FV/RT}$ smaller, i.e. of hyperpolarizing the cell. Secondly, although the sodium pump term depends on V, the latter is found experimentally to vary only slowly with $[K_o^+]$. It can easily be shown by differentiation that, in a typical experiment, the sodium pump term can without serious error be regarded merely as a constant addition to the right-hand side of the equation, provided that the flux M_a remains constant. If it is assumed, as is justified by experiment, that the behaviour of M_a with varying extracellular potassium concentration is represented by the relation

$$M_a = \text{constant } ([K_o^+] \geqslant 4 \text{ mM}); \; M_a \to 0 \text{ as } [K_o^+] \to 0, \tag{10}$$

then $e^{FV/RT}$ will vary linearly with $[K_o^+]$ when the latter is greater than 4 mM, but will deviate in the direction of lower resting potentials, when $[K_o^+]$ falls below this value. The slope of the linear portion of the relationship can be used to determine the intracellular potassium concentration, and the intercept can be used to find the ratio of permeabilities, P_{Na}/P_K.

Note that equation (9) contains no term depending explicitly on the rate of injection of ions from the microelectrodes. This is because it was derived entirely by reference to the ionic currents through the cell membrane. The electrodes are regarded as passing zero net current, since they are connected to the stimulating and recording apparatus only through very high impedances, so that injection of ions from them affects the resting potential only indirectly, through alteration of the intracellular ion concentrations, and stimulation of the sodium pump.

APPENDIX B

Osmotic balance of the cell

The purpose of this section is to relate the behaviour of the intracellular potassium concentration, as evaluated from the equations derived above, to the rates of injection and pumping of sodium ions in the experiments. It is assumed that the total ionic strength of the intracellular fluid remains constant throughout the experiment, and that the cell's complement of anions consists of a fixed number of non-penetrating anions, together with any further anions injected from the electrodes and unable to escape through the cell membrane. Two extreme cases will be considered, in which the injected anions either escape completely from the cell, or remain entirely trapped within it.

(*a*) *Cell membrane permeable to anions.* If the cell membrane is sufficiently permeable to the injected anions so that they are able to escape completely from the cell (as may be the case when chloride ions are injected), then the cell's volume and total complement of cations will remain constant. If sodium ions are injected at a rate X, the intracellular potassium concentration will therefore be determined by

$$\frac{d[K_i^+]}{dt} = -\frac{X}{v} + \frac{sM_a'}{v}, \tag{11}$$

where v is the cell's volume, s is its surface area, and M_a' is the rate of extrusion of *sodium* ions by the pump. The net efflux of *cations* due to the pump, M_a, used in the

previous section, is clearly related to, and not greater than, M_a'. If injection continues at a constant rate, while the sodium pump is progressively inhibited, then the value of M_a' can be inferred from the difference between the rates of change of the intracellular potassium concentration, since

$$\frac{sM_a'}{v} = \left[\frac{d[K_i^+]}{dt}\right]_{\text{pump}} - \left[\frac{d[K_i^+]}{dt}\right]_{\text{no pump}} \tag{12}$$

(*b*) *Cell membrane impermeable to anions.* If the cell membrane does not allow the escape of any of the injected anions (as will presumably be the case when sodium acetate is injected) then accumulation of the injected sodium salt into the cell will cause its volume to increase, by uptake of water from the surrounding solution. The intracellular potassium concentration will thus fall, as the potassium ions in the cell are diluted, although there will not be any increase in the actual efflux of potassium ions from the cell. The intracellular ion concentrations will be determined by

$$\frac{d}{d}(v[K_i^+]) + \frac{d}{dt}(v[Na_i^+]) = X - sM_a, \tag{13}$$

$$\frac{d}{dt}(v[An_i^-]) = X, \tag{14}$$

where An^- is the injected anion. By combining these two equations, and considering the two conditions $M_a = 0$ (pump inhibited) and $M_a > 0$ (pump active), it can be shown that

$$\frac{sM_a'}{v} \cdot \frac{v(0)}{v} = \left[\frac{d[K_i^+]}{dt}\right]_{\text{pump}} - \left[\frac{d[K_i^+]}{dt}\right]_{\text{no pump}}, \tag{15}$$

where $v(0)$ is the volume of the cell at time $t = 0$, and v is the volume at the time t, when the measurements are made; the relationship between v and $v(0)$ is

$$v = v(0) + 2Xt/B, \tag{16}$$

where B is the total osmolarity of the Ringer (in the present experiments $B = 213$ mM).

The application of equations (15) and (16) to the experimental results requires a technique of successive approximations, the factor $[v(0)]/v$ in equation (15) being first neglected, to get an estimate of the flux M_a', and hence, from the initial behaviour of $[K_i^+]$, of the rate of injection, X. The value of X can then be used to find the appropriate value of $[v(0)]/v$. The experimental results have been corrected roughly on this basis, since the evidence seems to favour at least partial anion impermeability of the cell membrane, though there are evidently too many other sources of error in the quantitative interpretations for an accurate correction to be worthwhile. The factor $v/[v(0)]$ was found in the experiments to have values ranging from 0·7–3·0.

Note that both equations (12) and (15) give estimates of the quantity sM_a'/v. In relating this to the magnitude of the 'sodium pump term' in equation (9), it is necessary to know the cell's volume, but not its surface area, since the value of the potassium permeability, P_K, inferred from the cell's resistance is inversely proportional to the area. The connexion between M_a (equation (9)) and M_a' (equations (12) and (15))

depends on the stoichiometry of the sodium-potassium exchange mediated by the pump. Clearly M_a (net cation extrusion) cannot be greater than M_a' (net sodium extrusion), so that the values of the 'expected' contribution to the resting potential, which have been inferred directly from M_a', are the largest possible.

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