

CONTRIBUTIONS OF THE  
SODIUM PUMP AND IONIC GRADIENTS TO THE MEMBRANE  
POTENTIAL OF A MOLLUSCAN NEURONE

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SUMMARY

1. The membrane potential of the gastro-oesophageal giant neurone of the marine mollusc, *Anisodoris nobilis*, was examined during changes of temperature and of the ionic medium.

2. The response of the membrane potential to rapid changes in the external K concentration was prompt, stable, and reversible up to 200 mM-K, and was independent of the external Cl concentration.

3. Warming the cell produced a prompt hyperpolarization that was approximately 10 times greater than predicted by the Nernst or constant field equations. Electrogenic activity of the Na–K exchange pump was shown to be responsible for this effect.

4. At temperatures below 5° C, the relationship between the membrane potential and the external K concentration could be predicted by a constant field equation.

5. At temperatures above 5° C, the membrane potential could not be predicted by the constant field equation except after inhibition of the electrogenic Na pump with ouabain or the reduction of internal Na.

6. Inhibition of the electrogenic Na pump by low external K concentrations was dependent upon the external Na concentration.

7. It is concluded that the membrane potential is the sum of ionic and metabolic components, and that the behaviour of the ionic component can be predicted by a constant field type equation.

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## INTRODUCTION

For cell membranes that are selectively permeable to Na, K and Cl, the resting membrane potential may be described by an equation derived from constant field theory (Goldman, 1943; Hodgkin & Katz, 1949)

$$V = \frac{RT}{F} \ln \frac{P_K [K]_o + P_{Na} [Na]_o + P_{Cl} [Cl]_i}{P_K [K]_i + P_{Na} [Na]_i + P_{Cl} [Cl]_o}, \quad (1)$$

where  $V$  is the resting potential;  $P_K$ ,  $P_{Na}$  and  $P_{Cl}$  represent the membrane permeability constants for each ion;  $[K]_o$ ,  $[K]_i$  etc. are the external and internal concentrations of the ions present; and  $R$ ,  $T$  and  $F$  have their usual meanings. Predictions from eqn. (1) are only accurate if the permeability constants do not vary with temperature or ionic concentrations, and if active processes do not contribute to the membrane potential. To some degree these conditions are met in the squid axon, to which eqn. (1) has been applied with reasonable accuracy (Hodgkin & Katz, 1949). This is not the case in muscle fibres, in which the membrane permeability to K may vary as a function of  $[K]_o$  (Hodgkin & Horowitz, 1959; Noble, 1965), nor for a variety of nerve cells (Blackman, Ginsborg & Ray, 1963; Gerasimov, Kostyuk & Maiskii, 1965; Kerkut & Meech, 1967; Carpenter & Alving, 1968), some of which derive a portion of their resting potential from electrogenic Na transport (Kerkut & Thomas, 1965; Carpenter & Alving, 1968; Marmor & Gorman, 1970). In this paper we show that in a molluscan neurone, whose Na-K exchange pump is electrogenic, the resting potential can be predicted by a constant field equation only under conditions which inhibit the electrogenic pump. Preliminary reports of these data have been published elsewhere (Marmor & Gorman, 1969, 1970).

## METHODS

The giant neurone (G cell) of the gastro-oesophageal ganglion of the marine mollusc, *Anisodoris nobilis* (MacFarland), was used in all experiments. Animals were obtained from Dr Rimmon Fay (Pacific Bio-marine Supply Co., Venice, California) and were usually gathered from waters at temperatures of 10–18° C, although they may be found in colder waters as well (R. Fay, personal communication). Specimens were generally kept for 1 to 2 weeks in a sea-water tank at 11–13° C before use. Basic anatomical and physiological features of this preparation have been described elsewhere (Gorman & Mirolli, 1969). The ganglion is small (300–500  $\mu$  diam.), and nearly 50% of its contents are occupied by the readily identifiable G cell (200–400  $\mu$  diam.). At 11–13° C this cell usually has a resting potential of 50–60 mV and an action potential of 100–120 mV. Cells which did not meet these values were excluded from study. The G cell is particularly suitable for studies on the resting potential since it lacks spontaneous synaptic or pace-maker potentials (Gorman & Mirolli, 1969). It has the additional advantage that seasonal variations in membrane potential do not occur (unpublished observations on fifty-two cells over one year) as they do in neurones from terrestrial molluscs (Kerkut & Meech, 1967; Moreton, 1968).

The ganglion and approximately 1 cm of the medial nerve containing the G cell axon was removed from the animal and mounted in an experimental chamber (Fig. 1) where it was continually perfused with artificial sea water. The ganglion was held by gentle suction through a  $150\ \mu$  opening in the floor of the chamber, and the cut end of the nerve was drawn into a sea-water filled pipette for stimulation (Gorman & Mirolli, 1969). The possibility that sea water from the suction system might contaminate the chamber during changes of fluid is small since the suction opening touched less than 5% of the ganglion surface, and the suction would cause test fluids to enter the cell holder rather than the other way around. Moreover, the continuous flow of bathing fluid through the linear chamber prevented any accumulation of previous solutions. Test solutions were switched into the chamber with a four-way valve and flowed through a flattened coil (volume less than 0.1 ml.) which was separated from a thermo-electric cooling device (Cambion Electronics, Cambridge, Massachusetts) by a thin plastic film. The small volume of the coil, and the close approximation to the cooling unit, allowed solutions to be changed in less than 10 sec while maintaining any temperature from 1 to 25° C. The temperature could be changed rapidly by varying the current through the thermo-electric device, and was monitored with a small thermistor probe near the cell.

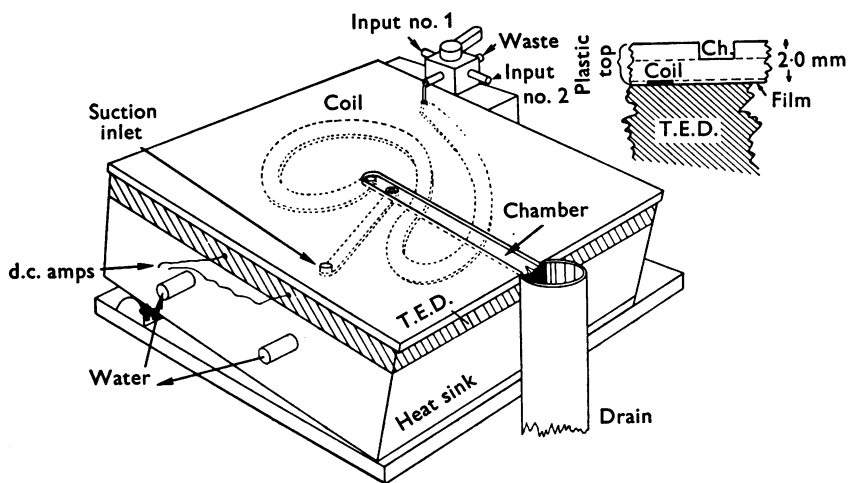


Fig. 1. Schematic diagram of the experimental chamber. The inset shows a cross-section of the chamber and thermoelectric device (T.E.D.).

Membrane potentials were recorded differentially between an intracellular micro-electrode and one placed in the extracellular fluid. The intracellular electrode was inserted through the intact ganglion sheath. All electrodes were filled with 3 M-KCl, had tip potentials of less than 5 mV, and had resistances of 5–10 M $\Omega$ . The potentials measured with both electrodes placed extracellularly were unaffected by changes in temperature between 0 and 25° C. Potentials measured in this manner varied by less than 1 mV between solutions (Table 1) containing Na, K, Li, or Tris as the major cation. The variation was as much as 5 mV between solutions containing Cl, SO<sub>4</sub>, or propionate as the major anion, and was larger when sucrose was used as a replacement. Since these potentials were either small or not critical to our results, no corrections were made.

TABLE I. Composition of artificial sea-water (ASW) solutions (mm/l.).

	Na	K	Li	Cho- line	Tris	Ca	Mg	Cl	SO <sub>4</sub>	HCO <sub>3</sub>	Propionate	Sucrose
ASW	480	10.2	0	0	0	10.5	54.7	560	28.8	2.43	0	0
0 K ASW	490	0	0	0	0	10.5	54.7	560	28.8	2.43	0	0
490 K ASW	0	490	0	0	0	10.5	54.7	560	28.8	2.43	0	0
0 Na, (Tris) ASW	0	10.2	0	0	525	10.5	54.7	441	28.8	0	0	0
0 Na, 0 K (Tris) ASW	0	0	0	0	536	10.5	54.7	438	28.8	0	0	0
0 Na, (Li) ASW	0	10.2	480	0	0	10.5	54.7	560	28.8	2.43	0	0
0 Na, (choline) ASW*	0	10.2	0	480	0	10.5	54.7	560	28.8	2.43	0	0
0 Na, 0 K (Sucrose) ASW	2.43	0	0	0	0	10.5	54.7	73	28.8	2.43	0	976
0 Cl (Propionate) ASW†	480	10.2	0	0	0	10.5	54.7	0	54.7	2.43	509	0
0 Cl (Tris-SO <sub>4</sub> ) ASW†	480	10.2	0	0	228	10.5	54.7	0	494	0	0	0
0 Na, 0 Cl (Tris-SO <sub>4</sub> ) ASW	0	10.2	0	0	800	10.5	54.7	0	695	0	0	0
0 Na, 0 K, 0 Cl (Tris-SO <sub>4</sub> ) ASW	0	0	0	0	825	10.5	54.7	0	695	0	0	0

\* Contains D-tubocurarine,  $5 \times 10^{-4}$  g/ml., since choline alone has a depolarizing acetylcholine-like effect on the G cell.

† Stock solutions also made up with 0 K and 490 K by exchanging K and Na as in the normal Cl stock solutions.

Electrical connexion to the micro-electrodes was made by stable Ag-AgCl pellet-type electrodes and fed to a pair of solid state, differentially balanced, unity gain electrometers (Instrumentation Laboratories, Watertown, Massachusetts). The differential signal was amplified and displayed simultaneously on an oscilloscope and on a rectilinear pen recorder (Mark 280, Clevite Instrument Co., Cleveland, Ohio). Total d.c. drift in the recording system averaged 2–3 mV over an 8 hr period.

The blood of the doridacean nudibranchs has essentially the same ionic composition as sea water (McCance & Masters, 1937). Artificial sea water was prepared according to the Woods Hole Marine Biological Laboratory formula (Cavanaugh, 1956) and corrected for the salinity of the region of the Pacific Ocean where the animals were collected (35%). All solutions were calculated and tested to have an osmolarity of 975–1025 m-osmole and a pH of 7.8–8.0. The stock test solutions are shown in Table 1. Intermediate concentration of the major ions were obtained by mixing appropriate quantities of these stock solutions. Thus, high K concentrations were made by replacing Na with K. The notation used to describe different artificial sea water (ASW) solutions is similar to that of Baker & Connelly (1966). For example, '15 K ASW' contains 15 mM-K, and 0 Na (Tris) ASW' has all Na replaced with Tris(hydroxymethyl)amino-methane. The extracellular (o) and intracellular (i) concentrations of major ions will be referred to by the ion symbol in square brackets with the appropriate subscript (e.g.  $[K]_o$  and  $[K]_i$ ).

Ouabain (Mann Pharmaceuticals) was dissolved directly in the test solutions.

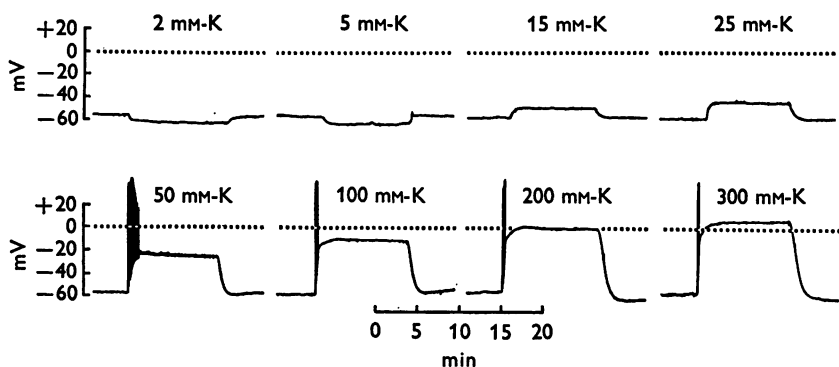


Fig. 2. Membrane response to different  $[K]_o$  at 11° C (penwriter record). Each change of  $[K]_o$  in the bathing medium was complete within 30 sec. The dotted lines show the level of zero membrane potential.

## RESULTS

### *Effects of changes in $[K]_o$ on membrane potential at 11° C*

Fig. 2 shows the responses of the membrane potential to changes in  $[K]_o$  at 11° C, a temperature at which the animal is found and at which it is kept in our laboratory. The potential usually reached a steady-state level within 1–2 min after each change and the new level was maintained for periods up to 30 min. The 1–2 min latency was invariably present despite the fact that a complete exchange of fluids in the experimental chamber occurred in less than 15 sec. It has been shown that the region

between the G cell and its deeply invaginating glial covering is continuous with the extracellular and extraganglionic spaces (J. Crayton & M. Miralli, in preparation); however, diffusion of ions into these areas must take time and may account for a major part of this delay. The time course of recovery, on return to normal (10 K) ASW, was usually more prolonged than that of onset, particularly when cells were left in high  $[K]_o$ . However, in contrast to results obtained from neurones in other molluscs (Kerkut & Meech, 1967) recovery occurred even when the membrane potential was

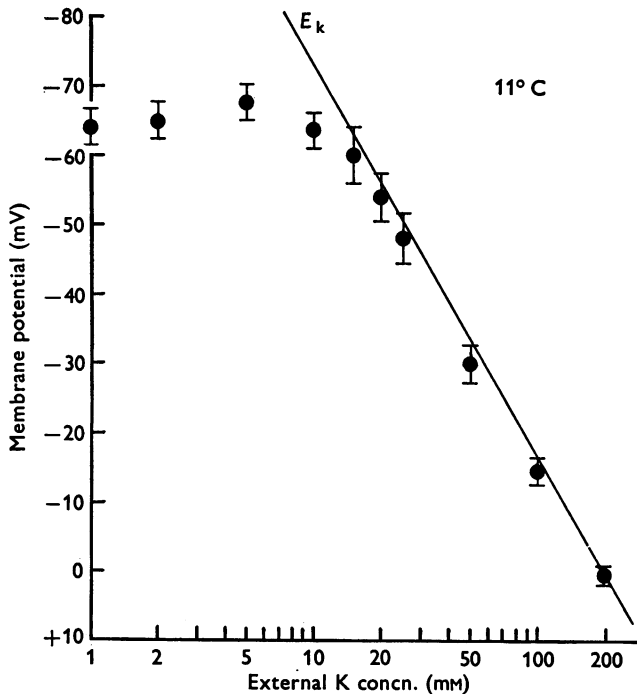


Fig. 3. Plot of membrane potential *vs.*  $\log [K]_o$  at  $11^\circ\text{C}$ . Each point represents an average from five experiments, with the s.e. of the mean shown. The line  $E_K$  has the slope predicted by the Nernst equation for a K electrode at  $11^\circ\text{C}$ .

made positive by K concentrations greater than 200 mM (Fig. 2). Because the responses to changing  $[K]_o$  were stable and reversible,  $[K]_o$  was varied sequentially in many of the experiments described below. There was no difference between results obtained in this manner and those obtained by returning to 10 K ASW between each change of solution.

The combined results of five experiments in which  $[K]_o$  was varied at  $11^\circ\text{C}$  are shown in Fig. 3. For  $[K]_o$  greater than 15 mM, the membrane

potential decreased by 54.4 mV/tenfold increase in  $[K]_o$ . This is in close agreement with the theoretical slope of 56.6 predicted by the Nernst equation

$$E_K = \frac{RT}{F} \ln \frac{[K]_o}{[K]_i} \quad (2)$$

for a K electrode and shown by line  $E_K$  in Fig. 3. Below 15 K ASW the membrane potential deviated from the Nernst equation. Between 5 and 15 K ASW this deviation might be predicted by eqn. (1) if the membrane were slightly permeable to ions other than K (Hodgkin & Katz, 1949). As  $[K]_o$  was reduced below 5 mM, however, the cell depolarized. This behaviour cannot be accounted for by the constant-field equation, if the membrane permeabilities remain constant.

#### *The Cl permeability of the G cell*

Most of the previous data concerning the Cl permeability of molluscan nerve cells (Gerasimov *et al.* 1965; Kerkut & Meech, 1967) suggests that the role of Cl in maintaining the resting potential is small. If Cl is an important determinant of membrane potential, its complete removal should produce a significant depolarization (Hodgkin & Horowitz, 1959), but in the G cell replacement of external Cl with either propionate or  $SO_4$  had only a small effect (less than  $\pm 5$  mV). Moreover, the membrane response to varying  $[K]_o$  was essentially the same in time course, magnitude and reversibility whether  $[Cl]_o$  was present or absent or a constant  $[K]_o \times [Cl]_o$  was maintained (see Fig. 1, Gorman & Marmor, 1970).

#### *Effects of temperature on the resting potential and its response to $[K]_o$*

The response of the G cell to warming was often a prompt hyperpolarization of 1–2 mV/ $^{\circ}C$ . Some cells did not show such a large hyperpolarization when initially warmed, but their response increased after cooling to 2–5 $^{\circ}C$  for 15–60 min. Further cooling for periods up to 2 hr did not improve this response to temperature. Unless otherwise noted, experiments in this paper were begun only after a significant thermal sensitivity of the cell had been demonstrated.

In thirty-five cells, warming from 7 to 17 $^{\circ}C$  produced an average hyperpolarization of  $20 \pm 1$  mV (s.e. of mean) which was maintained for 45–60 min. Beyond this period, the hyperpolarization gradually declined to approximately 25% of its initial magnitude, and remained stable at that level for several hours. The magnitude of the initial change in potential is approximately 10 times greater than predicted by eqns. (1) or (2). These results are consistent with observations on other molluscan neurones (Kerkut & Ridge, 1962; Murray, 1966). The response to temperature was

not linear (Fig. 4*A*). Between 5 and 14–18° C the potential increased with increasing temperature, but below 5° C and above 18° C the effects of temperature were minimal or in the opposite direction. Above 23° C the cell depolarized. If the cell was warmed rapidly, the changes in potential were nearly simultaneous with those in temperature (Fig. 4*B*) suggesting that changes in the internal ionic concentrations do not account for the hyperpolarization.

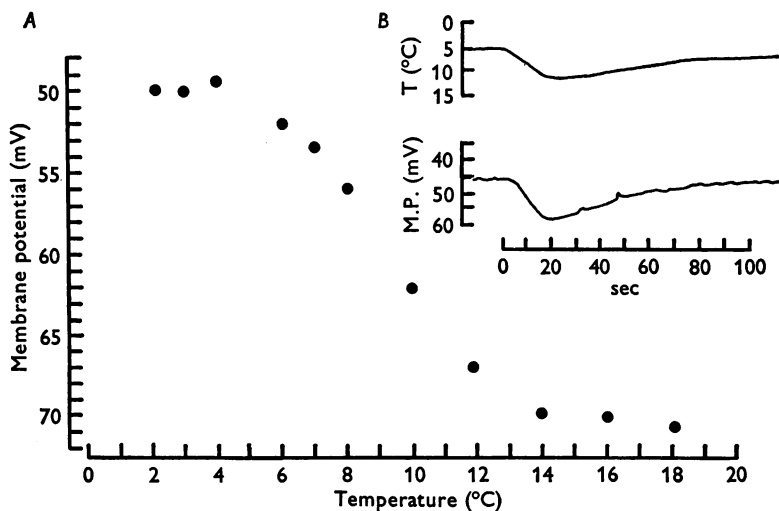


Fig. 4. The relationship between temperature ( $T$ ) and membrane potential (M.P.). *A*, steady-state potential at different temperatures. *B*, penwriter record of membrane potential and temperature showing the response of the cell to brief warming.

Since the hyperpolarizing response to warming has been associated with an electrogenic component of the Na–K exchange pump in molluscan cells (Carpenter & Alving, 1968), it is reasonable to inquire whether the behaviour of the potential at 11° C in response to  $[K]_o$  reflects the activity of the pump. To test this hypothesis, experiments were done at temperatures which would be expected to reduce ( $< 5^\circ C$ ) or enhance ( $> 15^\circ C$ ) pump activity. Fig. 5*A* shows the combined results of five experiments in which  $[K]_o$  was varied at cold and warm temperatures. It is apparent that in normal ASW the cell is considerably more hyperpolarized at 17° C than at 4° C, and that the depolarizing effect of low  $[K]_o$  is accentuated at 17° C but is eliminated at 4° C. In addition, the slope of a line through the 20–200 mM- $[K]_o$  experimental points is 45 mV/tenfold change in  $[K]_o$  at 4° C compared to 57 mV at 17° C. (The Nernst equation would predict slopes of 55 and 56.5 mV respectively for these temperatures.) One may



also note an inflexion in the experimental curve between 20 and 100 mM- $[K]_o$  which is more pronounced at 17° C than at 4° C (see Fig. 6). Repeating these experiments in the absence of external Cl gave essentially identical results.

If  $[K]_i$  changes significantly during changes of  $[K]_o$  or temperature the data shown above would be difficult to evaluate. Several observations argue against this possibility. First, it is unlikely that  $[K]_i$  could change rapidly enough to account for the prompt response of the G cell to changing  $[K]_o$  or temperature, particularly in the absence of Cl movement. Secondly, the

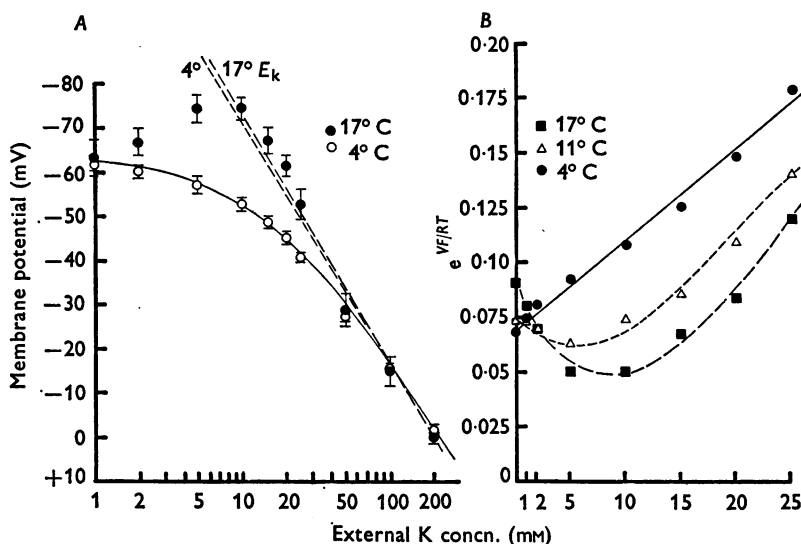


Fig. 5. Effects of temperature on the relationship between membrane potential and  $[K]_o$ . A, plot of membrane potential *vs.*  $\log [K]_o$  at 4 and 17° C. Each point represents an average from five experiments with s.e. of mean shown. The dashed lines,  $E_K$ , have the slopes predicted by the Nernst equation for K electrodes at the temperatures indicated. The smooth curve through the experimental points at 4° C was drawn from the constant field equation (see text for explanation). B, data from Figs. 3 and 5A replotted with  $eVF/RT$  on the ordinate and a linear scale of  $[K]_o$  on the abscissa. The straight line through the points at 4° C is equivalent to the curved line in A.

potential remained essentially stable for at least one half hour at any  $[K]_o$  up to 200 mM and at any temperature up to 18° C, making it doubtful that  $[K]_i$  was changing. Thirdly, the value of  $[K]_o$  at which the membrane potential became zero was reproducible in successive tests on a given cell, suggesting that  $[K]_i$  is not a labile quantity.

It is important to determine whether the behaviour of the membrane potential can be adequately described by a constant field equation at any

temperature. Since Cl does not contribute significantly to the resting potential, and since the large negative resting potential requires that  $P_{\text{Na}}[\text{Na}]_i \ll P_{\text{K}}[\text{K}]_i$ , eqn. (1) can be simplified (Hodgkin & Horowitz, 1959) to

$$V = \frac{RT}{F} \ln \frac{[\text{K}]_o + (P_{\text{Na}}/P_{\text{K}}) [\text{Na}]_o}{[\text{K}]_i} \quad (3)$$

Fig. 5*A* shows that a line obtained from eqn. (3) by choosing values for  $[\text{K}]_i$  (235 mM) and  $P_{\text{Na}}/P_{\text{K}}$  (0.028) can fit all of the experimental points at 4° C.

Agreement or disagreement with eqn. (3) can be seen more clearly if eqn. (3) is rewritten in exponential form (Moreton, 1968), so that,  $e^{VF/RT}$  is a linear function of  $[\text{K}]_o$ :

$$e^{VF/RT} = \frac{[\text{K}]_o}{[\text{K}]_i} + \frac{(P_{\text{Na}}/P_{\text{K}}) [\text{Na}]_o}{[\text{K}]_i} \quad (4)$$

If data are plotted in this manner, points satisfying the constant field equation will fall along a straight line whose slope allows an estimate of  $[\text{K}]_i$ , and whose  $y$ -intercept allows an estimate of  $P_{\text{Na}}/P_{\text{K}}$ .

Fig. 5*B* shows data from Figs. 3 and 5*A*, in the lower ranges of  $[\text{K}]_o$ , plotted on a linear scale with  $e^{VF/RT}$  as the ordinate. It is apparent that the experimental points at 4° C fall on a straight line as predicted by eqn. (4), while those at 11 and 17° C do not. Fig. 6 shows the behaviour of the potential over a full range of  $[\text{K}]_o$ . Although at 18° C the membrane was more hyperpolarized between 5 and 25 mM- $[\text{K}]_o$  than at 5° C, the potential at 18° C fell off at higher  $[\text{K}]_o$  (the 'inflection' between 20 and 100 mM- $[\text{K}]_o$  which was noted earlier). At 5° C the plot is linear over the entire range of  $[\text{K}]_o$ .

#### *Effects of ouabain and $[\text{Na}]_i$ on the membrane response to $[\text{K}]_o$*

The non-linear response of the membrane potential to changing  $[\text{K}]_o$  might be accounted for by a dependence of  $P_{\text{K}}$  on  $[\text{K}]_o$  (Hodgkin & Horowitz, 1959; Noble, 1965). However, this mechanism would not account for hyperpolarization of the membrane as the cell is warmed, or the disappearance of the non-linear behaviour at cold temperatures. These phenomena could be explained by an electrogenic Na pump whose effects are inhibited by cold temperatures and the removal of  $[\text{K}]_o$  (Baker, Blaustein, Keynes, Manil, Shaw & Steinhardt, 1969*b*). To explore this possibility, experiments were done under other conditions known to affect the Na pump.

The most specific inhibitors of the Na transport process are the cardiac glycosides, such as ouabain (Glynn, 1964). The addition of  $5 \times 10^{-4}$  M ouabain to ASW had no effect on the membrane potential at temperatures

< 5° C, but blocked the ability of the G cell to hyperpolarize when warmed. Fig. 7A shows an  $e^{VF/RT}$  plot of the membrane response to changes in  $[K]_o$  at 11° C in the presence and absence of ouabain. The addition of ouabain depolarized the cell and abolished the non-linear behaviour of the membrane potential. This experiment cannot easily be performed at higher temperatures (> 13° C) since the depolarization in ouabain leads to spontaneous cell discharge (see Fig. 3, Gorman & Marmor, 1970). The removal of  $K_o$  at 11° C does not depolarize the cell in the

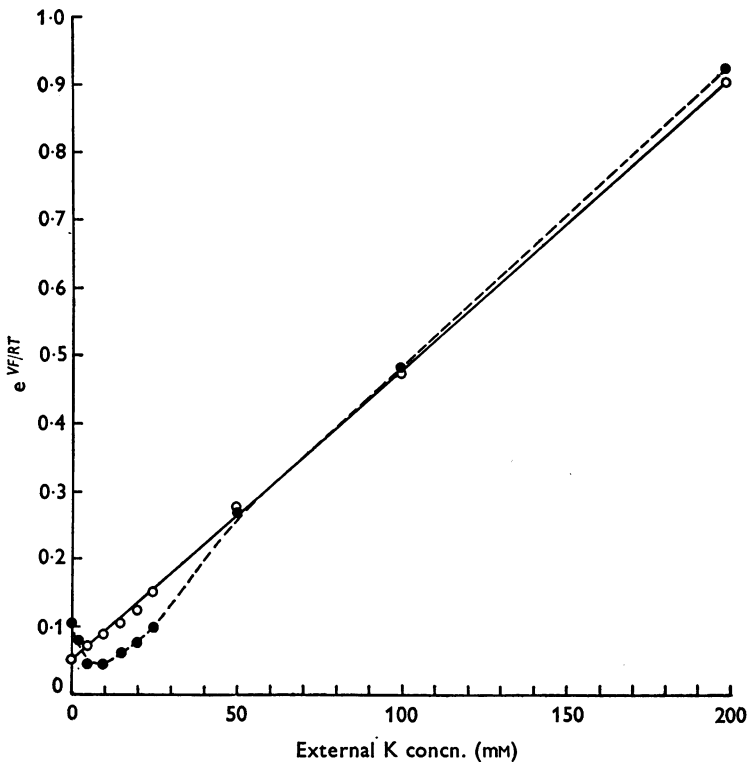


Fig. 6. The relationship between  $e^{VF/RT}$  and  $[K]_o$  at 5 and 18° C (filled circles). The data is from one of the experiments shown in Fig. 5. The straight line is drawn through the experimental points at 5° C (open circles).

presence of ouabain, and conversely, there is little response to adding ouabain once external K is removed. This would suggest that both ouabain and the removal of external K act upon the same process.

Lowering  $[Na]_i$  should also diminish the amount of metabolic Na efflux (Keynes & Swan, 1959; Brinley & Mullins, 1968) and consequently decrease the contribution of an electrogenic Na pump to membrane potential. The

hyperpolarization in response to warming, and the depolarization produced by ouabain at warm temperatures, can be used as indices of electrogenic Na transport. Both of these responses are present immediately after replacing external Na with Tris, but disappear after 15–30 min exposure to 0 Na (Tris) ASW at temperatures above 11° C, presumably as a function of depleting  $[Na]_i$ . After 30 min in 0 Na (Tris) ASW the non-linear behaviour of the membrane potential in response to changing  $[K]_o$  at 18° C was eliminated (Fig. 7B). These results are consistent with the observa-

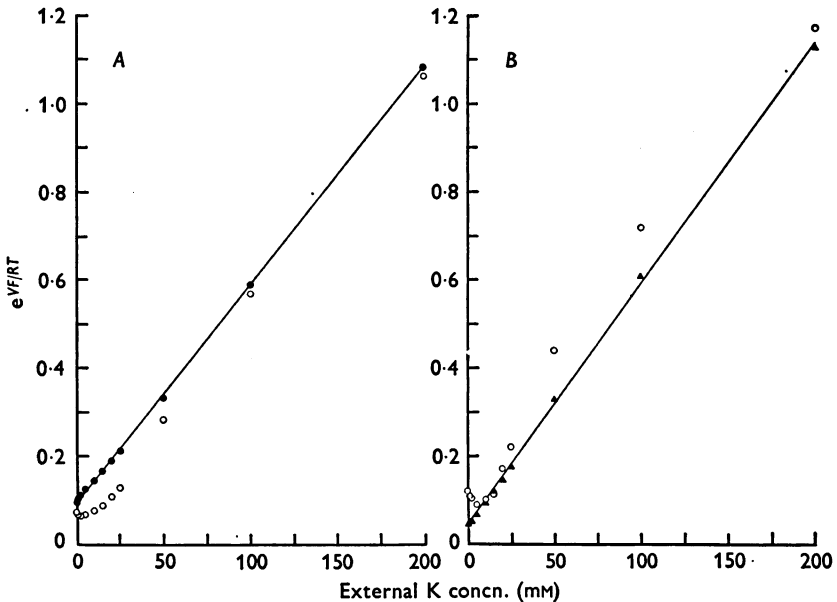


Fig. 7. Dependence of  $eV_F/RT$  on  $[K]_o$  after the addition of ouabain or internal Na depletion. *A*, data at 11° C before (○) and after (●) the addition of  $5 \times 10^{-4}$  M ouabain. *B*, data at 17° C before (○) and 30 min after (●) the replacement of external Na with the impermeant cation, Tris. Straight lines are drawn through the experimental points after ouabain in *A* and after Na replacement in *B*.

tions of Moreton (1969) who found that injection of Na into *Helix* neurones produced ouabain-sensitive non-linear behaviour of the membrane potential similar to the behaviour we observe at warm temperatures.

The effect of replacing  $[Na]_o$  with Tris was usually a prompt and monophasic hyperpolarization, consistent with the cessation of Na leakage (Gorman & Marmor, 1970). However, at temperatures  $> 5^\circ$  C the response in some cells was bi- or tri-phasic, especially on the initial trial. All results reported in 0 Na (Tris) ASW were obtained on a stable hyperpolarized base line.

After inhibition of the Na pump with ouabain, the cell reached a stable level of potential which was maintained for several hours. This suggests that some mechanisms for maintaining ionic equilibrium remain in the absence of the ouabain-sensitive Na-K exchange pump (Baker, Blaustein, Hodgkin & Steinhardt, 1969*a*).

*Effect of increasing  $[K]_o$  after exposure to K-free ASW*

The interpretation that an electrogenic Na pump is responsible for the non-linear behaviour of the membrane potential is indirectly supported by a second set of experiments. At intermediate temperatures (9–11° C) the

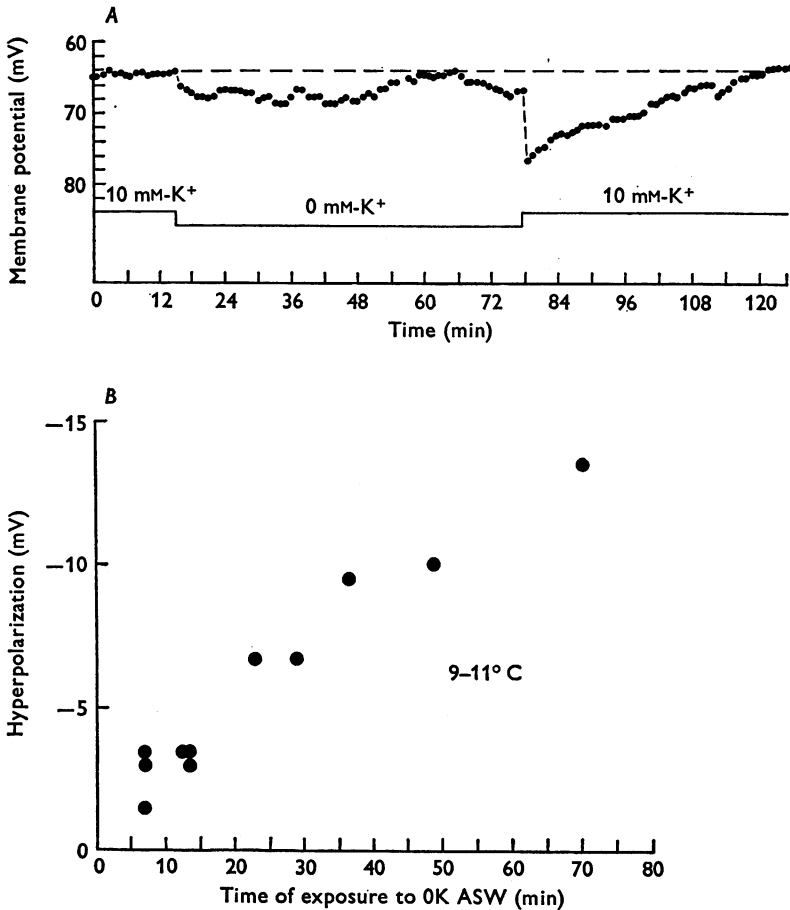


Fig. 8. Hyperpolarizing response to raising  $[K]_o$  after prolonged exposure to K-free solution at 9–11° C. *A*, plot of the membrane potential during and after 65 min exposure to 0 K ASW. *B*, data from seven experiments showing the relationship between the time of exposure to 0 K ASW and the magnitude of the hyperpolarization on returning to normal (10 K) ASW.

membrane response to 0 K ASW was either a slight depolarization or hyperpolarization. Upon returning to 10 K ASW, however, the initial response was always a hyperpolarization (Fig. 8 A) after which the potential returned to the control level over a period of minutes. The magnitude of the hyperpolarization was directly related to the length of exposure to 0 K ASW (Fig. 8 B), and was abolished by ouabain. The initial response to restoring  $[K]_o$  was directly opposite to the predictions of eqns. (2) or (3) for a decrease in the K concentration gradient. In addition, the hyperpolarization persisted long after  $[K]_o$  had reached a stable value eliminating the possibility (Noble, 1965) that the effect was produced

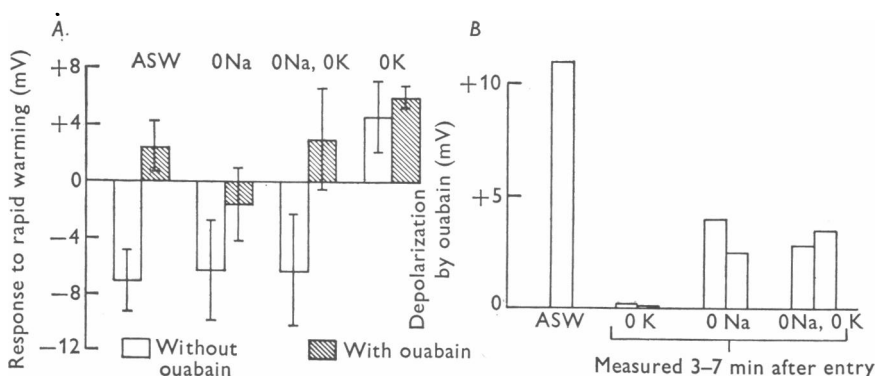


Fig. 9. Indices of electrogenic Na pump activity in the presence and absence of external K and external Na. *A*, response of the membrane potential to a brief warming of the cell. Each bar shows the mean data from three to six cells with 1 s.d. indicated. The initial temperature was 5–7° C in each case and the cells were warmed 6–8° C within 1 min. *B*, the depolarizing response to ouabain. Results from a single experiment at 16° C in which  $5 \times 10^{-4}$  M ouabain was added to the bathing medium shortly after removal of external K or external Na, or both.

by a change in  $P_K$  as  $[K]_o$  was changing. These data can be accounted for if the removal of external K blocks a Na pump and allows  $[Na]_i$  to increase so that electrogenic Na transport is stimulated when the pump is reactivated with external K. This response would be analogous to the hyperpolarization that occurs after tetanization or Na injection in other tissues such as mammalian non-myelinated nerve fibres (Rang & Ritchie, 1968), the crayfish stretch receptor (Nakajima & Takahashi, 1966) and *Helix* neurones (Kerkut & Thomas, 1965; Thomas, 1969).

#### *Interaction of $[Na]_o$ and $[K]_o$ in affecting Na pump activity*

It has recently been shown in crab nerve (Baker & Connelly, 1966) and squid axon (Baker *et al.* 1969*b*) that the ability of low  $[K]_o$  to inhibit the

ouabain-sensitive Na efflux is dependent upon  $[\text{Na}]_o$ . As shown above, in normal  $[\text{Na}]_o$ , the removal of external K abolished the hyperpolarizing response to warming the cell (Fig. 5B) and the depolarizing response to ouabain in the warm (Fig. 7A). However, these responses were both present in 0 K, 0 Na (Tris) ASW (Fig. 9), indicating that a reduction of  $[\text{Na}]_o$  diminished the ability of low  $[\text{K}]_o$  to inhibit the electrogenic Na pump.

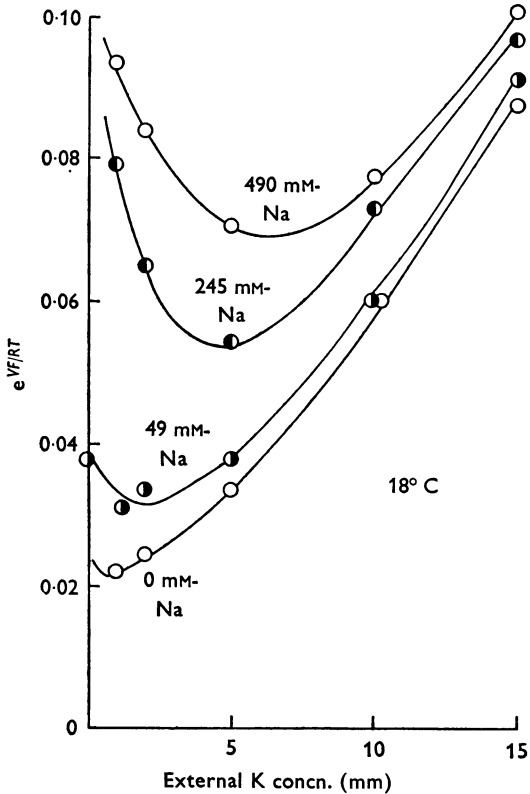


Fig. 10. The effects of different  $[\text{Na}]_o$  and  $[\text{K}]_o$  on  $e^{VF/RT}$  at a warm temperature. Data from an experiment at 18° C in which precautions were taken to avoid depletion of internal Na (see text for further explanation).

This result does not depend upon the use of Tris as a replacement for external Na since the temperature sensitivity of the G cell was the same whether external Na was replaced with Tris, choline, or sucrose. It may be noted in Fig. 9B that the depolarization produced by ouabain is considerably larger in normal ASW than in 0 Na ASW or 0 K, 0 Na ASW measured 3–7 min after removing external Na. Internal Na would not be depleted in this short a time, and the diminution in the response to

ouabain probably reflects the loss of Na leakage which normally accounts for part of the depolarization in ouabain and acts as a stimulant for the Na pump (Mullins & Noda, 1963; Gorman & Marmor, 1970).

Fig. 10 shows the effect of intermediate concentrations of  $\text{Na}_o$  and  $\text{K}_o$  upon  $e^{V_F/RT}$  at 18° C. Values were obtained 2 min after entering each solution, and to prevent internal Na depletion the cell was never left in reduced  $[\text{Na}]_o$  for more than 5 min. The progressive straightening of these curves as  $[\text{Na}]_o$  was reduced is consistent with a progressive diminution in the effects of  $[\text{K}]_o$  on the Na pump; however, this is not the only explanation since the removal of Na leakage affects the membrane potential and the Na pump in other ways as well.

#### DISCUSSION

Our results show that the resting potential of the *Anisodoris* G cell can be experimentally separated into at least two components which, for the sake of discussion, we will refer to as the metabolic and ionic components. The former is sensitive to temperature, ouabain and low  $[\text{K}]_o$ , and is interpreted as depending upon electrogenic Na transport. The latter depends upon ionic concentration gradients and membrane permeabilities in accordance with the constant field equation. A similar division of the membrane potential has been postulated (Grundfest, 1955; Huddard, 1967; Carpenter & Alving, 1968), but has not been experimentally demonstrated over a full range of  $[\text{K}]_o$  and temperature.

The observations that warming hyperpolarizes the cell, that a reduction of  $[\text{K}]_o$  or  $[\text{Na}]_i$  blocks this hyperpolarization, and that ouabain depolarizes the warmed cell suggest strongly, when taken together, that the activity of the Na pump can directly contribute to the *Anisodoris* G cell membrane potential. This is consistent with results in other molluscan neurones (Kerkut & Thomas, 1965; Carpenter & Alving, 1968; Moreton, 1969; Thomas, 1969). The marked hyperpolarization on raising  $[\text{K}]_o$  after exposure to 0 K ASW at 9–11° C is additional evidence that a process other than an electrodiffusion potential can generate a potential in this cell. Other explanations for these phenomena must be considered. Alterations in internal ionic concentrations by diffusion or by non-electrogenic pumping are unlikely to account for these results since the responses to changing temperature or the external medium can be very rapid and of too short latency. Moreover, the depolarizing effect of low  $[\text{K}]_o$  is difficult to explain in terms of concentration gradients, although it could be accounted for if the membrane permeability to K decreased as  $[\text{K}]_o$  decreased (Hodgkin & Horowicz, 1959; Noble, 1965). However, this latter explanation would not account for the sensitivity of the cell to temperature,



$[Na]_i$ , and ouabain. Ouabain might cause permeability changes in addition to its effect on the Na pump (Brinley & Mullins, 1968), but these changes would have to be temperature sensitive since ouabain had no effect at temperatures less than 5° C. The ability of cold, low  $[K]_o$ , internal Na depletion, and ouabain to block each other's effect on the membrane potential adds to the argument that they act on a common process.

It has been suggested that the Na pump may act as a current source over a wide range of  $[K]_o$  (Moreton, 1969), in parallel with the passive membrane resistances (Frumonto, 1965; Rapoport, 1970). The effects of the pump could be described by modifying, or adding to the 'Na leakage' term of eqn. (4) (Mullins & Noda, 1963; Moreton, 1969), and one would then expect the points at 18° C on an  $e^{VF/RT}$  plot to fall below, but parallel to, those at 5° C for  $[K]_o > 10$  mM (see Fig. 6). This analysis seems to hold within a limited range of  $[K]_o$  in both the G cell (10–25 mM) and *Helix* neurones (3–8 mM, Moreton, 1969). However, at higher  $[K]_o$  the apparent contribution of the Na pump diminishes between 20 and 100 mM- $[K]_o$  (Figs. 5, 6, 7) illustrating some of the difficulty in drawing conclusions from data in a limited range of  $[K]_o$ .

Our data indicate that 0 Na ASW blocks the inhibitory action of 0 K ASW on the Na pump. However, the ouabain-sensitive Na efflux should be inhibited in 0 Na ASW, if  $[K]_o$  reaches true zero (Baker & Connelly, 1966; Baker *et al.* 1969*b*). It is possible that low residual levels of  $[K]_o$  exist in the immediate vicinity of the G cell membrane, secondary to K leakage from the G cell and those cells adjacent to it, or to slight diffusion of K from the ASW in the cell suction holder. Estimates of  $[K]_o$  in the immediate extracellular space of other cells during perfusion of the tissue with K-free solution have ranged from 0.22 mM (Mullins & Noda, 1963) to as much as 2 mM (Baker & Connelly, 1966; Baker *et al.* 1969*b*). The presence of a variable quantity of external K adjacent to the cell would also explain our finding that on some occasions in the warm, 0 K ASW alone had less of a depolarizing effect on membrane potential than 0 K ASW plus ouabain. The basic conclusions of this paper would not be changed if the true extracellular  $[K]_o$  were slightly higher than  $[K]_o$  in the bathing medium; one would simply conclude that the Na pump is inhibited by a smaller reduction of  $[K]_o$  than might have been thought.

The contribution of the electrogenic Na pump to membrane potential is influenced by the preceding treatment of the cell. For example, the length of time a G cell was kept in 0 K ASW at 9–11° C determined the magnitude of the hyperpolarization on returning to 10 K ASW. Similarly, exposure to temperatures below 5° C for periods of 15–60 min can enhance the hyperpolarization we observe in response to warming. Both these conditions stimulate electrogenic activity by increasing  $[Na]_i$ . However,

in both cases, the duration of Na loading was much less than that needed to demonstrate electrogenic Na transport in skeletal muscle fibres (Kernan, 1962; Frumento, 1965). It should be pointed out that the hyperpolarization on returning to 10 K ASW is a transitory phenomenon, after which the membrane potential returns to its original base line, whereas the hyperpolarization in the warm has both a transitory and a maintained effect. This difference may reflect the fact that raising the temperature not only activates the Na pump, but also increases the membrane permeability to Na (Gorman & Marmor, 1970), so that the Na pump may remain electrogenic under steady-state conditions in the warm.

The large electrogenic effect of the Na pump in giant molluscan neurones may be more a function of their high membrane resistance (Tauc, 1966) than of some unique properties of the Na pump in these cells. The Na pump is unequally coupled in the squid axon (Baker *et al.* 1969*b*) but little electrogenic effect of the Na pump is measured, presumably because the membrane resistance is relatively low (Hodgkin & Keynes, 1955). Baker *et al.* (1969*a*) and others have shown that active Na efflux occurs through several mechanisms which differ in their sensitivity to inhibitors and their sensitivity to external ions. Our results show that the ouabain-sensitive component of the Na pump contributes directly to the membrane potential, but we have no evidence on whether the other components of the Na efflux are electrogenic. The fact that the cell can maintain a steady potential after ouabain suggests that non-ouabain-sensitive components of the Na pump must have an important role, although not necessarily an electrogenic function, in maintaining membrane potential.

The importance of the Na pump in maintaining the Na and K concentration gradients across the cell membrane has been recognized for a number of years. More recently, electrogenic activity of the pump has been implicated in a variety of neuronal responses, including post-tetanic hyperpolarization (Nakijima & Takahashi, 1966; Rang & Ritchie, 1968), post-synaptic potentials (Nishi & Koketsu, 1968; Kerkut *et al.* 1969; Pinsker & Kandel, 1969) and photoreceptor potentials (Smith, Stell, Brown, Freeman & Murray, 1968). Our results emphasize that the resting characteristics of the membrane potential may also depend upon electrogenic Na transport, thereby modifying the sensitivity of the neurone to changes in temperature and local ionic environment. It is interesting that the electrogenic pump is operative over the range of temperatures *Anisodoris* normally encounters (5–20° C) and outside this range makes very little contribution to potential.

The resting potential of many cells besides the G cell is not adequately described by eqn. (1). Our results suggest that a separation of the membrane potential into metabolic and ionic components may succeed in

explaining some of the behaviour of the potential in these tissues. For example, a depolarization occurs when  $[K]_o$  is lowered near zero in other molluscan neurones (Kerkut & Meech, 1967; Carpenter & Alving, 1968), mammalian sympathetic ganglion cells (Blackman *et al.* 1963), smooth muscle (del Castillo *et al.* 1964; Kuriyama, 1963), heart muscle (Noble, 1965) and skeletal muscle (Hodgkin & Horowicz, 1959). Although the Na pump has been shown to be electrogenic under conditions of Na loading in several of these tissues, the contribution of metabolic processes to the resting potential has not been explored over a full range of both  $[K]_o$  and temperature in any of these tissues. It has been pointed out that the resting potential may arise from multiple mechanisms in a heterogeneous membrane (Grundfest, 1966). Our results support this interpretation and suggest two general hypotheses which may be applicable to a variety of excitable cells; first, metabolic processes may directly generate a portion of the membrane potential; and secondly, in the absence of metabolic pumps, the membrane potential may be predicted by a constant field type equation providing that the ionic permeability factors are either constant or predictable.

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