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TEMPERATURE DEPENDENCE OF THE SODIUM–POTASSIUM PERMEABILITY RATIO OF A MOLLUSCAN NEURONE

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SUMMARY

1. The temperature dependence of the membrane potential of a molluscan giant neurone was examined under conditions which block the electrogenic activity of the Na–K exchange pump.

2. When the Na pump was blocked by ouabain or the removal of external K, the membrane potential depolarized as temperature was increased.

3. This depolarization was prevented by the replacement of external Na with impermeant cations, but was greater when Na was replaced with Li.

4. All observed effects of ouabain were attributable to inhibition of the Na pump. The depolarization in response to ouabain at warmer temperatures was completely reversible, and the rate of both onset and reversibility of the ouabain effect was dependent upon temperature.

5. Using a modified form of the constant field equation, the internal K concentration and the Na–K permeability ratio, P_{Na}/P_K, were calculated from the experimental data.

6. P_{Na}/P_K was found to increase from 0·028 at 4° C to 0·068 at 18° C. It is suggested that this increase is due primarily to a change in P_{Na}.

INTRODUCTION

The membrane potential of the gastro-oesophageal giant neurone (G cell) of the marine mollusc, *Anisodoris nobilis*, is dependent upon both ionic diffusion potentials and an electrogenic Na–K exchange pump (Gorman & Marmor, 1970). At any constant temperature and under conditions which block Na pump activity, the resting potential of the G cell can be

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predicted by a constant field equation (Hodgkin & Katz, 1949*a*) simplified to include only terms for Na and K and rewritten in exponential form (Moreton, 1968; Gorman & Marmor, 1970);

$$e^{VF/RT} = \frac{[K]_o}{[K]_i} + \frac{(P_{Na}/P_K)\,[Na]_o}{[K]_i}, \quad (1)$$

where V is the resting potential; P_K and P_{Na} represent the membrane permeability constants for K and Na; $[K]_o$, $[K]_i$, etc., are the external and internal concentrations of the ions present; T is the absolute temperature and R and F have their usual meanings. Our ability to predict membrane potential with eqn. (1) allows us to make inferences about the permeability ratio, P_{Na}/P_K, under a variety of conditions. Although direct evidence is lacking, several authors have proposed that the membrane permeability to Na and K may increase with temperature (Bernstein, 1902; Hodgkin & Katz, 1949*b*; Brading, Bülbring & Tomita, 1969; Latorre & Hidalgo, 1969; Sperelakis, 1969; Carpenter, 1970). Thus, it is important to evaluate the constancy of P_{Na}/P_K in the G cell and the possible effects of a variable permeability ratio on membrane potential. Our results suggest that P_{Na}/P_K varies significantly with temperature, and that this variability may, in part, determine the amount of electrogenic Na transport at different temperatures.

METHODS

The procedures used in the present experiments, and the notation for ions and solutions have been described in the previous paper (Gorman & Marmor, 1970).

RESULTS

The permeability ratio, P_{Na}/P_K

It is clear from eqn. (1) that deviation of the membrane potential from the behaviour of a K electrode is accounted for by Na entry ('leakage') into the cell. Fig. 1 shows the results of an experiment in which membrane potential was measured at 3° C over a wide range of $[K]_o$ in the presence and absence of external Na. For comparison, the data is plotted as both potential *vs.* log $[K]_o$, and as $e^{VF/RT}$ *vs.* $[K]_o$. At this temperature the electrogenic Na pump does not contribute to the potential (Gorman & Marmor, 1970); thus, the potential can be evaluated in terms of ionic concentrations and permeabilities. Clearly, the membrane potential in normal ASW deviates from the behaviour expected of a K electrode (e.g. the line E_K in Fig. 1*A*), but adheres quite closely to the predictions of eqn. (1) since the experimental points fall on a straight line in Fig. 1*B*. Knowing the value of $[Na]_o$, and obtaining $[K]_i$ from the slope of the line, P_{Na}/P_K can be calculated from the y-intercept. In ten experiments at

3–5° C the mean value of $[K]_i$ in normal ASW was 235 mM. This is in close agreement with the value of 232 mM-$[K]_i$ found for *Aplysia* neurones by spectrophotometric analysis (Sato, Austin & Yai, 1967). The mean value of P_{Na}/P_K was 0·028 ± 0·002 (S.E. of mean); a value larger than that for squid axon, 0·01 (Hodgkin, 1958), or skeletal muscle, 0·01 (Hodgkin & Horowicz, 1959), but less than some estimates for other molluscan neurones, 0·03–0·10 (Kostyuk, 1968; Moreton, 1968).

Although the removal of external Cl alone had little effect on the membrane potential or the response to changing $[K]_o$, the removal of both external Na and external Cl caused the membrane potential to increase

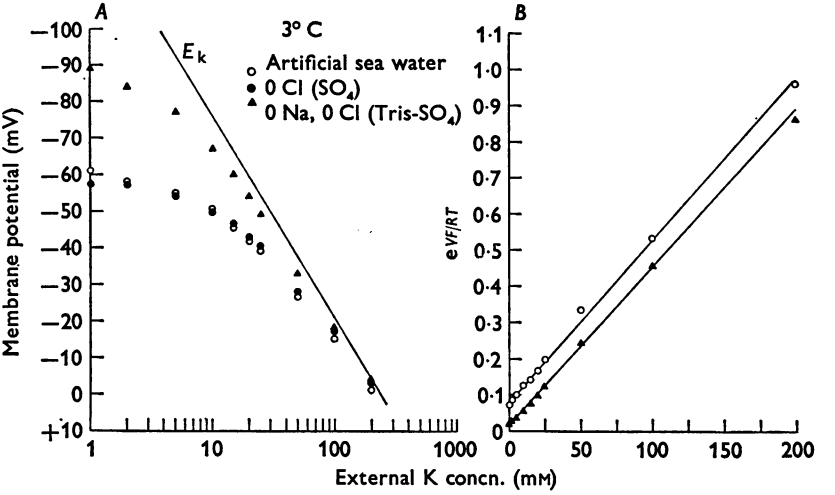

Fig. 1. Effects of external Na and external Cl removal on the membrane potential in different $[K]_o$. *A*, data from one experiment showing membrane potential *vs.* log $[K]_o$ before and after the replacement of external Cl and external Na with the impermeant ions Tris-SO_4. The line E_K has the slope predicted by the Nernst equation for a K electrode at 3° C. *B*, data from part *A* replotted with $e^{VF/RT}$ as the ordinate. The straight lines were drawn to fit the experimental data.

towards the level expected for a K electrode (Fig. 1*A*). On an $e^{VF/RT}$ plot (Fig. 1*B*) a K electrode would theoretically be represented by a straight line through the origin, parallel to the experimental points. It is striking that the experimental points in the absence of external Na fall on a straight line parallel to those in normal ASW despite the obvious failure to achieve the potential predicted by eqn. (1) when $[Na]_o = 0$. It is possible that $[Na]_o$ in the immediate extracellular space does not reach zero when external Na is removed from the bathing medium. Na may leak out of the cells or out of the suction system which holds the ganglion;

however, it is unlikely that enough external Na remained (approx. 140 mM) to account for the y-intercept in Fig. 1*B*. Possible differences in the activity coefficients for K inside and outside the cell (Mullins & Noda, 1963) probably do not account for a failure to achieve predictable potentials in very low $[K]_o$. The membrane may be slightly permeable to Tris or to other ions that might use the Na 'channel'. Alternatively, a continual slight leakage of K out of cells may make $[K]_o$ adjacent to the giant cell slightly higher than $[K]_o$ outside the ganglion, tending to shift the real y-axis of Fig. 1*B* towards the left so that an extrapolation of the 0 Na, 0 Cl (Tris-SO_4) ASW data would cross near the real origin.

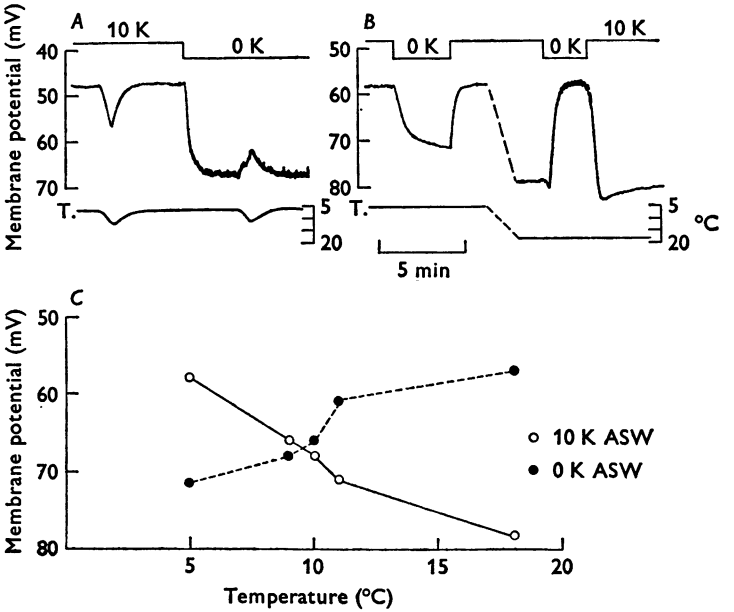

Fig. 2. Effects of external K removal on the temperature dependence of the membrane potential. *A*, penwriter record of the potential showing the response to briefly warming the cell in the presence and absence of external K. *B*, record showing the response of the membrane potential at 5 and 18° C to removing K_o. *C*, plot of membrane potential *vs.* temperature in 10 K ASW and 0 K ASW (same cell as in *B*).

The temperature dependence of the permeability ratio, P_{Na}/P_K

The constant field equation predicts that the membrane should hyperpolarize slightly with increasing temperature (approx. 0·2 mV/° C). This cannot be tested when the electrogenic Na pump is active, but should be testable when the Na pump is blocked. Blockage of the pump can be achieved by lowering $[K]_o$ or $[Na]_i$ (Keynes & Swan, 1959; Brinley & Mullins, 1968), or by exposing the cell to ouabain (Glynn, 1964). When the

Na pump of the G cell was inhibited by the removal of external K (Fig. 2*A*), rapid warming of the cell produced a prompt depolarization instead of the expected hyperpolarization. Similarly, the removal of external K in the warm produced a large depolarization (Fig. 2*B*). Fig. 2*C* shows that the depolarizing effect of 0 K ASW increased with increasing temperature. A depolarizing response to warming was also observed when the Na pump was blocked with 5×10^{-4} M ouabain (Fig. 3). However, depolarization on warming did not occur in the absence of external Na, regardless of whether the Na pump had been blocked with ouabain (Fig. 3) or depletion of internal Na by prolonged exposure to 0 Na ASW. These observations suggest that the depolarization which occurs in the absence of Na pump activity requires Na influx.

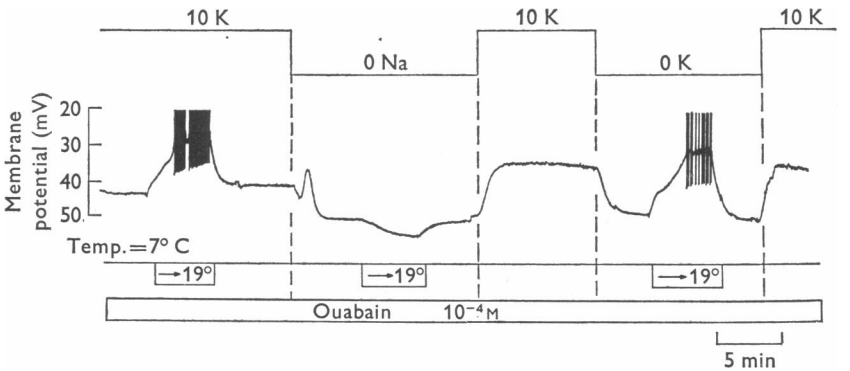

Fig. 3. The effects of external Na and external K removal on the temperature dependence of the membrane potential in ouabain. Penwriter record during perfusion with 5×10^{-4} M ouabain, showing the response to briefly warming the cell. External Na was replaced with Tris where indicated.

The effects of temperature on the membrane potential were explored over a range of $[K]_o$, under conditions (cold temperature and ouabain) which should inhibit the Na pump (Fig. 4). The potential was more depolarized at 11° C with ouabain than at 4° C (Fig. 4*A*), although the basic agreement with eqn. (1) and the slopes of the lines (i.e. $[K]_i$) were not significantly altered. It is unlikely that this depolarization is due to a direct effect of ouabain on membrane permeability since ouabain had no effect in the cold (Fig. 4*B*). Since the only difference between the lines in Fig. 4*A* is the y-intercept, eqn. (1) requires either that the $\{(P_{Na}/P_K)[Na]_o\}/[K]_i$ term change with temperature, or that an additional term be added which varies with temperature but not with $[K]_o$. An additional term might result from metabolic processes such as an ionic pump not involving K; however, in the absence of direct evidence for such a process,

it seems reasonable to ascribe the changes in the y-intercept to changes in the existing terms of the equation. Since $[Na]_o$ is held constant, and the parallel slopes at 4 and 11° C imply that $[K]_i$ is constant, we are led to the hypothesis that the permeability ratio, P_{Na}/P_K, increases with increasing temperature. Since the second term of eqn. (1) should be eliminated when $[Na]_o = 0$, a small hyperpolarization, consistent with a dependence on absolute temperature, would be expected on warming in the absence of both Na_o and Na pump activity. Warming the G cell under these conditions produced a hyperpolarization (Fig. 3), although its magnitude was sometimes larger than predicted.

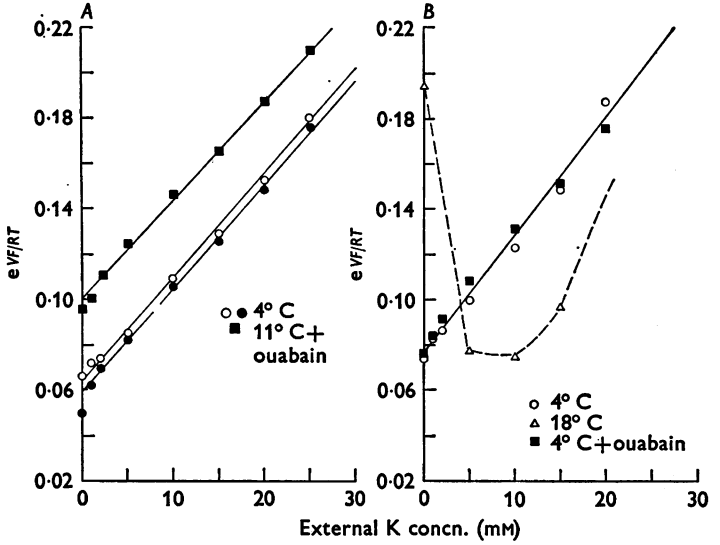

Fig. 4. Temperature dependence of the membrane potential in the presence of ouabain. *A*, plot showing $e^{VF/RT}$ *vs.* $[K]_o$ at 4° C before warming (○), at 11° C in the presence of 5×10^{-4} M ouabain (■), and at 4° C after the ouabain was washed out (●). The straight lines were drawn for best fit through the experimental points. *B*, data from a different experiment showing $e^{VF/RT}$ *vs.* $[K]_o$ at 4° C in the presence and absence of 5×10^{-4} M ouabain: the experimental points fall on a single straight line. Data at 18° C, without ouabain, is shown for comparison.

Further evidence that P_{Na}/P_K changes with temperature was obtained by plotting $e^{VF/RT}$ in 0 K ASW *vs.* temperature, using data from thirty-four experiments (Fig. 5*A*). Since eqn. (1) reduces to

$$e^{VF/RT} = \frac{(P_{Na}/P_K)\,[Na]_o}{[K]_i} \quad \text{when } [K]_o = 0,$$

the increase in $e^{VF/RT}$ with temperature can be accounted for by a rise in P_{Na}/P_K, assuming that $[Na]_o$ and $[K]_o$ are constant. $[Na]_o$ was held con-

stant experimentally, and although $[K]_i$ may have changed slightly during the few minutes used to warm the cell (less than 10 min for most of these experiments) it is unlikely to have changed enough to account for the two to threefold change in $e^{VF/RT}$. In ten of these experiments a full plot of $e^{VF/RT}$ *vs.* $[K]_o$ was obtained at temperatures $< 5°$ C to give a numerical value for $[K]_i$. Using this value, P_{Na}/P_K was calculated in three ranges of temperatures for each experiment. Fig. 5*B* shows that P_{Na}/P_K increased from 0·028 to 0·068 as the temperature rose from 4 to 18° C.

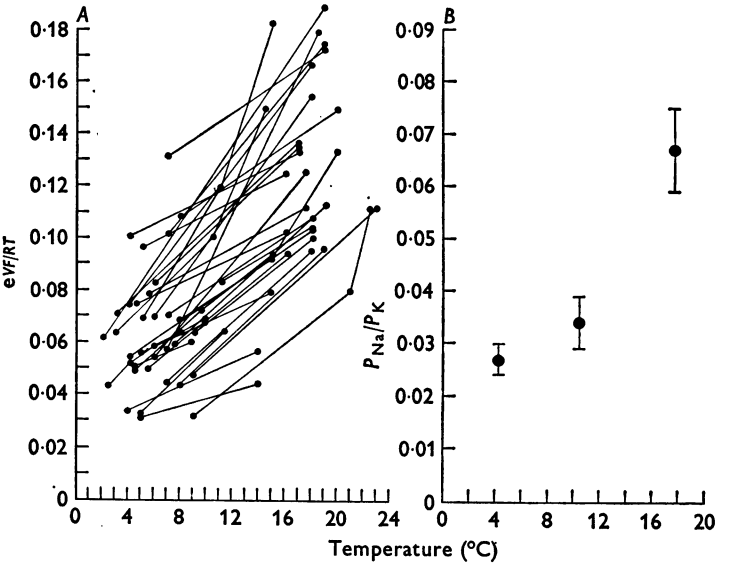

Fig. 5. Temperature dependence of the permeability ratio, $P_{Na}P_K$. *A*, combined data from thirty-four experiments showing the relationship between $e^{VF/RT}$, and temperature in 0 K ASW. *B*, the permeability ratio P_{Na}/P_K plotted as a function of temperature (see text for method of calculating the ratio). Data is averaged from ten experiments with one S.E. of mean shown.

Effects of ouabain on membrane permeability

It is important to clarify whether ouabain has any effects on the G cel other than inhibition of the Na pump.

First, the effects of ouabain were fully reversible in the G cell (Fig. 6*A*). After washout of ouabain the membrane potential gradually returned to its original level and manifestations of Na pump activity (sensitivity to temperature, removal of external K, ouabain, etc.) were restored. These observations were valid over a wide range of $[K]_o$ and rule out the possibility that ouabain caused permanent damage to the membrane. The dose–response curve for the ouabain depolarization (Fig. 6*B*) shows that

between 10^{-5} and 10^{-4} M ouabain was required for a maximal effect; hence the concentrations used ($1–5 \times 10^{-4}$M) were not excessive.

Secondly, ouabain had no obvious effect in the cold (Fig. 4*B*), and had little or no effect in the warm after inhibition of the Na pump by removing external K or lowering $[Na]_i$ (Gorman & Marmor, 1970). Moreover, depolarization of the cell with acetylcholine (3×10^{-6} g/ml.) to the same level as produced by ouabain did not block a further depolarization by 0 K ASW at 16° C (Fig. 7). This suggests that the effects of ouabain were not simply a result of membrane depolarization.

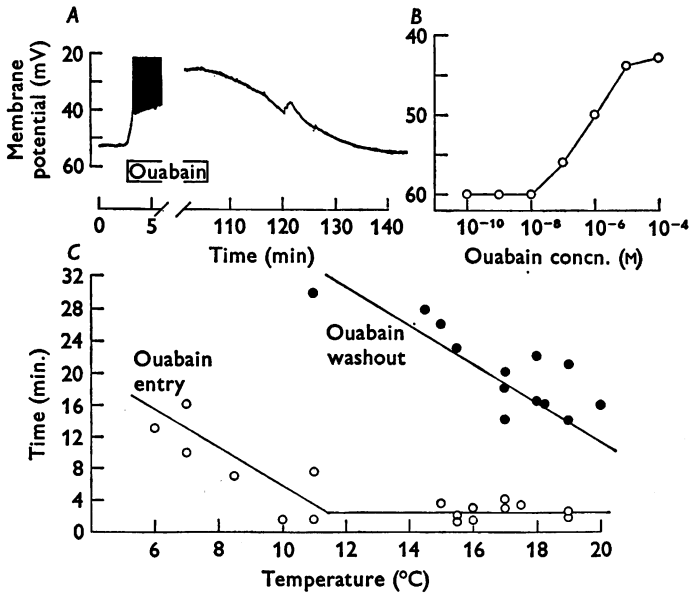

Fig. 6. Effects of ouabain on the membrane potential. *A*, penwriter record at 19° C showing the prompt depolarization in response to 10^{-4} M ouabain, and the time course of recovery after ouabain was removed from the bathing medium. *B*, dose–response curve showing the effects on membrane potential at 18° C of different concentrations of ouabain (different cell from *A*). *C*, data from several cells showing the time required, at different temperatures, for $1–5 \times 10^{-4}$ M ouabain to depolarize the cell to a stable base line, and for the cell to recover its original base line after ouabain was removed.

Finally, it has been suggested (Baker, 1968) that the effectiveness of cardiac glycosides as inhibitors of the Na pump depends upon the level of metabolic activity in the cell. In agreement with this hypothesis, we have found that the time required for the cell to reach a stable depolarization in response to 5×10^{-4} M ouabain was dependent upon temperature (Fig. 5*C*). A minimum time was required at temperatures where pump activity was

high (> 13° C). It is interesting that the time for recovery of the potential to its original base line, after ouabain was removed, was also dependent upon temperature. This suggests that the manner in which ouabain blocks or binds to the pump mechanism is such that metabolic activity is also required to reverse the process.

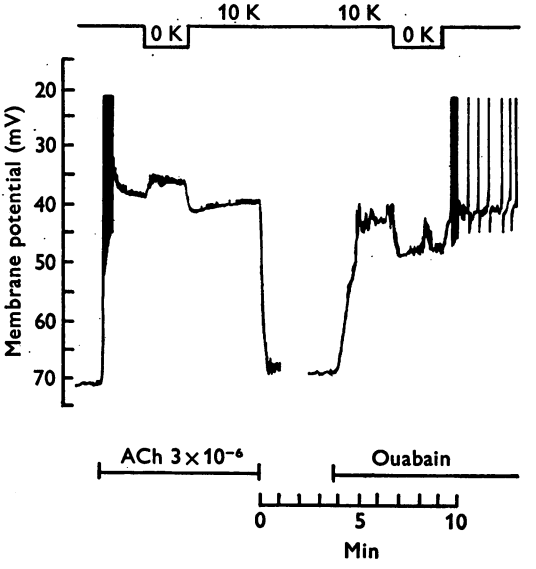

Fig. 7. Comparison of effects of acetylcholine and ouabain on the membrane potential. Penwriter record at 16° C showing the response to 0 K ASW during depolarization produced by acetylcholine, 3×10^{-6} g/ml., and a comparable depolarization produced by 5×10^{-4} M ouabain.

Effects of Li on membrane permeability

The results in the preceding sections imply that P_{Na}/P_K increases with temperature, but give no clear information about independent changes in P_{Na} and P_K. Experiments in which Li was used as a replacement for Na suggest that the temperature dependence of the permeability ratio at least involves changes in P_{Na}. In a number of tissues, Li and Na appear to use common 'channels' for passive movement across the membrane (for references see Schou, 1957). This is true for the G cell as well, since Li supported the action potential whereas Tris or sucrose did not (unpublished observations). The membrane potential was measured at different temperatures in the absence of Na pump activity, with Na or Li as the major external cations (Fig. 8). The cell was depolarized at higher temperatures in the presence of both Na and Li, consistent with a temperature dependence of P_{Na}/P_K and presumably P_{Li}/P_K. However, the depolarization was greater in $[Li]_o$ than in $[Na]_o$. This suggests that either

P_{Li} *or* P_{Na} varies with temperature. If the assumption is correct that Li and Na use common 'channels' through the membrane, then it is probable that both P_{Na} *and* P_{Li} vary with temperature.

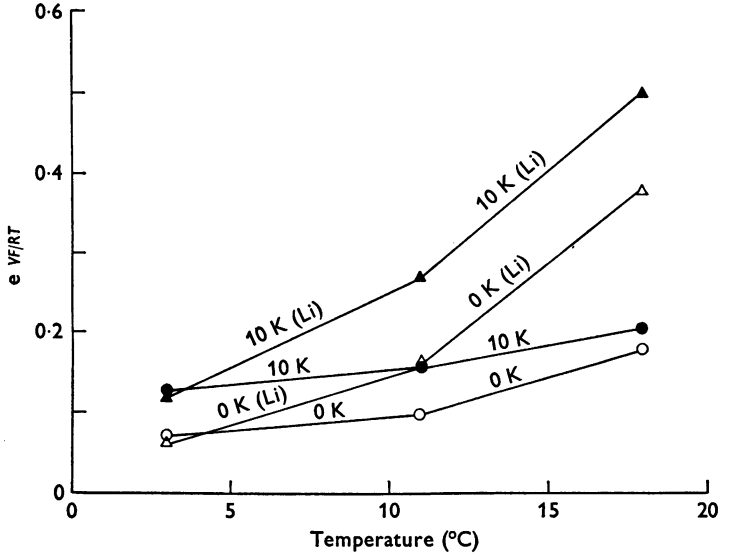

Fig. 8. Effect of Li on the temperature dependence of the membrane potential. The data is combined from two experiments in which 5×10^{-4} M ouabain was added to the bath at all temperatures above 5° C, and in which external Na was completely replaced by Li where indicated.

DISCUSSION

Eqn. (1) predicts that membrane potential should be proportional to the absolute temperature, assuming that ionic permeabilities remain constant and there are no metabolic or active processes contributing to the potential. This prediction has not been confirmed experimentally. In the squid axon, to which the constant field equation has been successfully applied at constant temperature (Hodgkin & Katz, 1949*a*), warming either caused a depolarization (Latorre & Hidalgo, 1969) or had little effect (Hodgkin & Katz, 1949*b*). The report that muscle cells followed the temperature-dependence of eqn. (1) (Ling & Woodbury, 1949) has been challenged by more recent studies (Apter & Koketsu, 1960). Our results suggest that changes in the permeability ratio, P_{Na}/P_K, are largely responsible for the inability of eqn. (1) to predict the temperature dependence of the membrane potential under conditions where metabolic processes are blocked. This conclusion is in general agreement with recent results on *Aplysia* neurones (Carpenter, 1970) and the squid axon (Latorre

& Hidalgo, 1969). As we emphasized earlier, our data is most economically interpreted in terms of a temperature-sensitive permeability ratio, but we cannot rule out the possibility that other mechanisms in the membrane produce the observed results. For example, an ion pump that does not involve K, such as a Na–Ca exchange mechanism (Baker, Blaustein, Hodgkin & Steinhardt, 1969), might account for our data if it was capable of depolarizing the cell and was inhibited by cold temperatures and low $[Na]_o$.

The specific mechanism by which the Na/K permeability might change with temperature is not clear. Small increases in both P_{Na} and P_K with temperature would be expected on thermodynamic grounds in a system where ions move passively. However, considering that the mobility of ions may not be the only factor controlling their movement through a biological membrane (Krogh, 1946; Mullins, 1961) it is not surprising that thermodynamic considerations alone do not account for the temperature sensitivity of the permeability ratio. Our results could be explained if the predominant effect of temperature on membrane permeability was either to raise P_{Na} or decrease P_K. We believe it is more likely that P_{Na} increases with temperature, consistent with the proposals of a number of authors (Bernstein, 1902; Hodgkin & Katz, 1949*a*; Latorre & Hildago, 1969; Carpenter, 1970). First, there is some evidence in other tissues that the K permeability of the membrane rises rather than falls with warming (Brading *et al.* 1969; Sperelakis, 1969). Secondly, our experiments with Li suggest that P_{Na}, considered independently of P_K, is not constant with temperature. This latter data must be interpreted with caution, however, since Li has been shown to have effects on ouabain-insensitive ion fluxes (Baker *et al.* 1969).

There has been debate whether the electrogenic Na pump can generate a portion of the steady-state resting potential (Thomas, 1969). It has been well established that the Na pump can be electrogenic after experimental Na loading of a variety of cells (Kernan, 1962; Kerkut & Thomas, 1965; Rang & Ritchie, 1968) including the G cell (Gorman & Marmor, 1970). Evidence for electrogenic pumping under steady-state conditions has been presented for *Aplysia* neurones in which P_{Na} appears to increase with warming (Carpenter, 1970). We have noted previously (Gorman & Marmor, 1970) that at a warm temperature the G cell will maintain a steady resting potential greater (more negative) than that in the cold for several hours. Since P_{Na}/P_K is 2–3 times as large at 18 as at 4° C, the Na leakage must be considerably greater in the warm than in the cold. Although other explanations are possible, this Na leakage may stimulate the electrogenic Na pump, thus providing a mechanism to maintain a large steady-state potential at warm temperatures.

The excellent fit between the experimental data and eqn. (1), under conditions where the electrogenic pump was eliminated at different temperatures, provides sufficient justification for the use of this equation as an empirical description of the data. The question is raised, however, whether the constant field equation has any theoretical validity when the original conditions (which include constant permeabilities) are not met. Fortunately, an equation of similar form can be derived without the specific assumption of a constant field and constant ionic mobilities (Sollner, Dray, Grim & Neihof, 1955; Patlak, 1960). Thus, the constant field equation may apply to a variety of excitable membranes in which ionic diffusion is responsible for the potential, but in which the membrane permeability to different ions varies predictably with experimental conditions. For example, in the absence of metabolic pumps, and given that P_{Na}/P_K is a function of temperature, the constant field equation predicts the resting potential of the G cell. Similarly, it predicts the potential of the muscle fibre given that P_K is a predictable function of $[K]_o$ (Noble, 1965). Although these observations lend support to the general concept that ionic diffusion potentials are primarily responsible for the membrane potential of excitable cells, our success with the use of an empirical equation does not imply it is applicable as a physical model. The validity of electrodiffusion theory as an explanation for physical processes within the complex biological membrane still requires experimental verification.

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