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# QUANTITATIVE ANALYSIS OF RESTING MEMBRANE ELECTROGENESIS IN INSECT (DIPTERA) SKELETAL MUSCLE

## II. TESTING OF A MODEL INVOLVING CONTRIBUTIONS FROM POTASSIUM AND SODIUM IONS, AND THE ANOMALOUS EFFECT OF REDUCING EXTRACELLULAR SODIUM

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### Summary

A model of resting membrane electrogenesis in skeletal muscles of prepupal *Calliphora erythrocephala* was formulated. From experiments in which reversible effects of changing extracellular $K^+$ and $Na^+$ activities on the membrane potential ($E_M$) were measured, three different values of $\alpha$ (the ratio of the partial permeabilities of the membrane to $Na^+$ and $K^+$) were derived, each from a different range of extracellular $Na^+$ and $K^+$ activities. Two independent tests were carried out to determine the most realistic value of $\alpha$. Intracellular $K^+$ and $Na^+$ activities and $E_M$ values were measured in a population of cells, and the $E_M$ values predicted using the Goldman–Hodgkin–Katz equation for different values of $\alpha$. The best fit for the data was obtained for $\alpha = 0{\cdot}036$. In ionic substitution experiments, in which passive movements of $Cl^-$ were prevented or minimized, the changes in $E_M$ around the resting level could be explained with a high degree of accuracy by assuming again that $\alpha = 0{\cdot}036$. However, tests of the model by investigation of direct effects of reducing extracellular $Na^+$ concentration over a wide range of $E_M$ values gave an anomalous result. In low-$Na^+$ Ringer, $E_M$ values became more positive than the respective resting levels. The anomalous effect of low-$Na^+$ Ringer on $E_M$ did not involve a change in the $K^+$ equilibrium potential. Instead, it was probably due to a reduction in the $K^+$ permeability of the membrane. Possible mechanisms underlying this effect are discussed.

### Introduction

In the preceding paper (Dawson & Djamgoz, 1988), we showed that the $K^+$ equilibrium potential ($E_K$) of prepupal *Calliphora erythrocephala* skeletal muscle was significantly more *negative* than the prevailing membrane potential ($E_M$). We predicted, therefore, that at rest these muscles must be permeable to at least one

Key words: insect, muscle, membrane, electrogenesis.

ion other than $K^+$ with an equilibrium potential more positive than $E_M$. Furthermore, $Na^+$ was shown to meet this requirement, and $Cl^-$ was found not to be involved in resting membrane electrogenesis (Dawson & Djamgoz, 1988). In analogous muscles of *Drosophila melanogaster*, Jan & Jan (1976) suggested that the resting membrane has a significant permeability to $Na^+$, the ratio ($\alpha$) of the partial permeabilities to $Na^+$ and $K^+$ ($p_{Na}$ and $p_K$, respectively) being 0·23. To test this proposal directly, the effect of reducing extracellular $Na^+$ concentration on $E_M$ was tested using $Tris^+$ as the substitute. As extracellular $Na^+$ was decreased, the membrane potential initially hyperpolarized, as predicted. However, in salines containing up to one-half as much $Na^+$ as that found in normal saline, $E_M$ values surprisingly repolarized to levels more positive than normal. Comparable results were obtained by substituting sucrose or $MgCl_2$ for NaCl (Jan & Jan, 1976). The latter experiments were carried out by 'soaking' the muscles in modified salines, and impaling several cells in each preparation. In earlier experiments also, the dependence of membrane potentials on extracellular ion concentrations was tested in a similar way, sometimes by soaking or 'conditioning' muscles in modified salines for several hours (Wood, 1963, 1965; Huddart, 1966; Piek, 1975). It is not clear from these studies whether sufficient controls were carried out to ensure that the changes in membrane potentials seen were reversible or whether time-dependent loss of membrane potentials occurred. Importantly, Wareham, Duncan & Bowler (1973) demonstrated in cockroach coxal muscles that membrane potentials gradually decay if bicarbonate-free Ringer's solutions are used (possibly due to deterioration of intracellular pH; see Thomas, 1984, for a review of mechanisms of intracellular pH regulation). Most early Ringer's solutions (including that used by Jan & Jan, 1976) lacked bicarbonate. In the present study, we have used a modified Ringer and attempted to monitor additional membrane parameters during solution changes, as well as ensuring that the effects observed were reversible. Thus, the physiological integrity of the preparations and possible secondary effects of solution changes have been assessed. Preliminary reports of these findings have been given earlier (Dawson & Djamgoz, 1984*a*,*b*).

## Materials and methods

Experiments were carried out on prepupal *Calliphora erythrocephala* Meig (Diptera) at room temperature (18–20°C). Details of the preparations and ion-sensitive microelectrodes were as given in the preceding paper (Dawson & Djamgoz, 1988). Other details are as follows.

### *Microelectrodes*

Conventional microelectrodes were drawn from borosilicate glass capillaries (1 mm o.d., 0·6 mm i.d.) on a vertical puller. They were filled with 2·5 mol $l^{-1}$ KCl and had tip resistances (d.c.) in the range 10–20 MΩ. The tip potentials of these electrodes were <5 mV, as determined by breaking the tips and noting the

resulting difference in the potential reading in the bath (Adrian, 1956). The microelectrode was connected to a microprobe system (WPI M701) which had an inbuilt bridge circuit for current injection for measurement of membrane input resistance. Ion-sensitive microelectrodes were constructed and used as described previously (Djamgoz & Dawson, 1986; Dawson & Djamgoz, 1988).

### *Ionic substitution experiments*

Modified solutions were based on Rice's saline and were of constant ionic strength and osmolarity. In the $K^+$ substitution solutions, $Na^+$ was used as a substitute for $K^+$. $Na^+$ substitution experiments mostly involved a 100-fold reduction of $Na^+$ activity using a variety of substitutes for NaCl: choline chloride, glucose, tetramethylammonium chloride, magnesium chloride or *N*-methyl D-glucamine. The osmolarity of each solution was measured using a mini-osmometer (Roebling) and adjusted to normal (402 mosmol $l^{-1}$) using additional glucose, if necessary.

A special set of modified Ringer's solutions was also prepared in which the product $(aK_o + 0{\cdot}036aNa_o) \times aCl_o$ was kept constant. These solutions were constructed such that the following pair of simultaneous equations was satisfied:

$$\text{(i)} \qquad (aK_o + 0{\cdot}036aNa_o) \times aCl_o = \text{constant } [(1946\,\text{mmol}\,l^{-1})^2] ,$$

$$\text{(ii)} \qquad Cl_{KCl} + Cl_{NaCl} = Cl_{Total} ,$$

where $aK_o$, $aNa_o$, $aCl_o$ are $K^+$, $Na^+$ and $Cl^-$ activities in the solutions, respectively; $Cl_{KCl}$ is the $Cl^-$ concentration present as KCl, $Cl_{NaCl}$ is the $Cl^-$ concentration present as NaCl, and $Cl_{Total}$ is the total $Cl^-$ content of normal Ringer (i.e. 194·3 mmol $l^{-1}$). The osmolarity of these solutions also was kept normal with additional glucose. Throughout the text ionic 'activity' rather than 'concentration' has been used for consistency. However, since all modified solutions were of constant ionic strength, $K^+$ and $Na^+$ activities can be converted to concentrations by dividing by the activity coefficient of the saline (i.e. 0·72; see Dawson & Djamgoz, 1988).

### *$K^+$ pulsation experiments*

A micropipette was pulled from 1·5 mm o.d. glass capillary and its tip broken to 100–200 $\mu$m diameter. It was attached to a microelectrode with sealing wax such that the tip of the broken pipette was around 0·5 mm behind the tip of the microelectrode, whilst at right angles to their axes the two were separated by about 1 mm. The broken pipette was filled with Ringer containing 97·2 mmol $l^{-1}$ $K^+$ (substituted for $Na^+$). Constant-sized (around 4 $\mu$l) droplets could be ejected from the pipette by applying air pulses of 1 s duration from an air cylinder set to 10–30 Pa *via* a solenoid-activated valve driven by a physiological stimulator. The assembly of pipettes was positioned in the recording chamber such that the broken pipette was 'upstream' of the recording microelectrode to ensure that the ejected pulses of the high-$K^+$ Ringer were washed onto the impaled cell and away from it.

## Results

### *Effect of varying extracellular $K^+$ and $Na^+$ activities on the $E_M$*

A typical recording of the changes in the resting potential ($E_M$) resulting from varying the extracellular $K^+$ activity ($aK_o$) between 0·97 and 97·2, using $Na^+$ as the substitute, is shown in Fig. 1. During the solution changes, the cell was twice returned to normal Rice's saline to ensure that the $E_M$ changes recorded were reversible, and that the cell remained healthy throughout the experiment, which could last up to 4 h. Sustained changes in $E_M$ were found to result from alterations in $aK_o$ and these were fastest when $aK_o$ was reduced (see Fig. 1). For example, when $aK_o$ was reduced from 9·7 to 3·6 mmol $l^{-1}$, the $E_M$ reached a new, steady level within 15 min; when $aK_o$ was increased from 36 to 97·2 mmol $l^{-1}$, the $E_M$ did not reach a steady value for more than 30 min. Such effects were not related to the age of the preparation, since 'slow' responses were observed even at the start of an experiment. Data similar to Fig. 1 were obtained from 11 cells (each from a different preparation). The $E_M$ depended on the logarithm of $aK_o$ more or less linearly, with the maximum slope of the line giving only 31 mV per 10-fold change in $aK_o$, however (not illustrated). This is considerably less than the 58 mV per 10-fold change in $aK_o$ expected for a membrane that is permeable only to $K^+$, and agrees with our conclusion in the preceding paper that $K^+$ is not likely to be the only ion involved in resting membrane electrogenesis in these muscles.

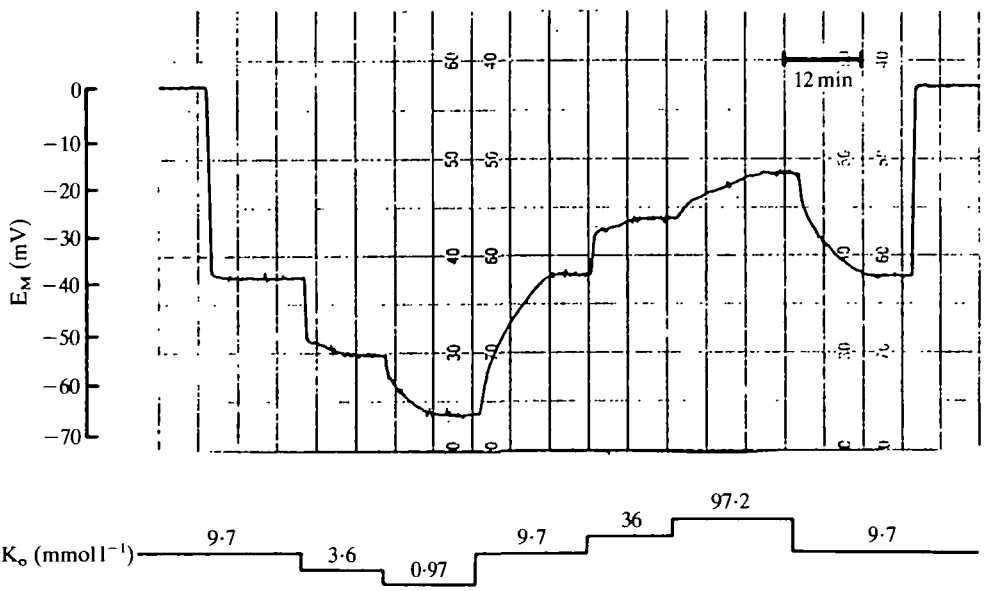


Fig. 1. Chart recording of a $K^+$ substitution experiment on a *Calliphora* prepupal muscle cell. The bath $K^+$ activity, $aK_o$, is indicated on the lowermost trace ($Na^+$ was used as the substitute). Twice during the experiment the preparation was returned to normal Ringer ($aK_o = 9{\cdot}7$ mmol $l^{-1}$) to ensure that the cell returned to its original resting potential.

To eliminate the possibility that there might be some time-dependent deterioration of the preparations, a number of shorter substitution experiments involving only three solution changes were also performed. A cell was impaled in normal Ringer ($aK_o = 9{\cdot}7\,mmol\,l^{-1}$) and a steady $E_M$ recorded. Low-$K^+$ Ringer ($aK_o = 0{\cdot}97\,mmol\,l^{-1}$) was then introduced and the membrane potential allowed to equilibrate. This was followed by high-$K^+$ Ringer ($aK_o = 97{\cdot}2\,mmol\,l^{-1}$), and finally the cell was returned to normal Ringer to ensure that the original $E_M$ was obtained. Using this protocol, a maximum change of 30 mV was recorded for $aK_o$ values between 9·7 and 97 (cf. 31 mV for the same step in full substitution experiments), and a maximum change of 24 mV was recorded for $aK_o$ values between 0·97 and $9{\cdot}7\,mmol\,l^{-1}$ (cf. 23 mV from the full substitution experiments). Thus, the slopes obtained for a given $aK_o$ range using the two types of protocol were not significantly different, providing further evidence that the membrane properties of the cells did not change during the full substitution experiments.

### *A model of resting membrane electrogenesis*

The dependence of $E_M$ on $aK_o$ could be explained better by also taking into account the variations in extracellular $Na^+$ activity and assuming that $\alpha$ (see Introduction) had a value of 0·086. This value of $\alpha$ was obtained empirically by fitting a straight line of slope 58 mV per 10-fold change in $aK_o + \alpha aNa_o$, to give the highest possible correlation coefficient (0·94). Thus, according to this analysis, $E_M$ for any bathing solution would be given by the simplified Goldman–Hodgkin–Katz equation (Goldman, 1943; Hodgkin & Katz, 1949):

$$E_M = 58\log\left(\frac{aK_o + 0{\cdot}086aNa_o}{aK_i + 0{\cdot}086aNa_i}\right)(mV)\,. \qquad (1)$$

Rearranging equation 1 and substituting values of constants for *normal* Ringer (Rice's saline):

$$E_M = 76 - 58\log(aK_i + 0{\cdot}086aNa_i)\,(mV)\,. \qquad (2)$$

The graph of $E_M$ against the logarithm of $(aK_o + 0{\cdot}086aNa_o)$ shows that although all points fall close to a line with a slope of 58 mV per 10-fold change in $aK_o + 0{\cdot}086aNa_o$ (Fig. 2), some data points still show a surprisingly wide deviation from the straight-line fit. Since the low-$Na^+$ media were found to have anomalous effects on the membrane potential (see later), the two sets of $E_M$ data obtained in Ringer's solutions on either side of the normal (i.e. high $aK_o$/low $aNa_o$ and low $aK_o$/high $aNa_o$) were also considered separately, and a different value of $\alpha$ was calculated for each set (Table 1).

In the rest of the paper, experiments designed to test this model of resting membrane electrogenesis and critically determine the best value of $\alpha$ are described and discussed.

### *Effects of varying extracellular $Na^+$ activity alone on the $E_M$*

These experiments were carried out to test *directly* whether $Na^+$ had any effect on $E_M$. Fig. 3 shows a typical recording of the effect on $E_M$ of reducing

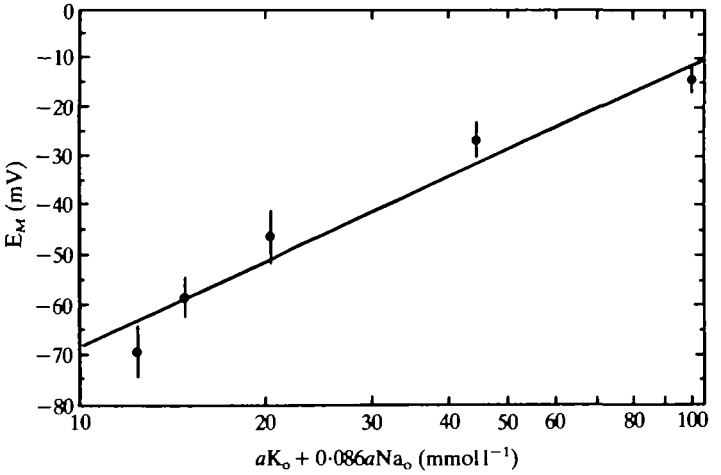


Fig. 2. Graph showing the relationship between the resting membrane potential ($E_M$) and the logarithm of ($aK_o + 0{\cdot}086aNa_o$), where $aK_o$ and $aNa_o$ are the extracellular (bath) $K^+$ and $Na^+$ activities, respectively. Data points denote means ± S.E. and can be fitted with a straight line (as shown) with a slope of 58 mV per 10-fold change in the abscissa (correlation coefficient, 0·94).

extracellular $Na^+$ activity from 124·6 to 1·25 mmol $l^{-1}$ (using $Mg^{2+}$ as the substitute). Immediately after the introduction of the low-$Na^+$ Ringer into the recording chamber, $E_M$ showed an initial hyperpolarization of some 8 mV from its resting level of −50 mV. This was followed after less than 1 min by a sustained *depolarization* of some 18 mV to a steady $E_M$ level of −40 mV which was reached 10 min after the introduction of the low-$Na^+$ Ringer. This effect reversed completely within 5 min of the return to normal Ringer.

To test that this result was not in some way due to the unsuitability of $Mg^{2+}$ as a $Na^+$ substitute, this type of experiment was repeated using a variety of other substitutes: choline, *N*-methyl D-glucamine, tetramethylammonium and glucose. Typical traces are shown in Fig. 4. In all cases, when low-$Na^+$ was substituted for normal Ringer, an initial hyperpolarization of 2–7 mV was seen, and this was followed by a sustained phase leading to $E_M$ levels 2–8 mV more positive than the original resting potentials.

Two possible causes of the unexpected depolarization of the $E_M$ in low-$Na^+$ Ringers were considered: (a) a decrease in $aK_i$ and a corresponding positive shift in $E_K$, which would account for the change in $E_M$, since $K^+$ has been shown to be

Table 1. *Values of $\alpha$ calculated from $E_M$ data obtained in Ringer's solutions of varying compositions*

| Ringer composition | $\alpha$ | Correlation coefficient |
|---|---|---|
| High $aK_o$/low $aNa_o$ | 0·169 | 0·92 |
| Low $aK_o$/high $aNa_o$ | 0·036 | 0·99 |
| All $aK_o$ and $aNa_o$ | 0·086 | 0·94 |

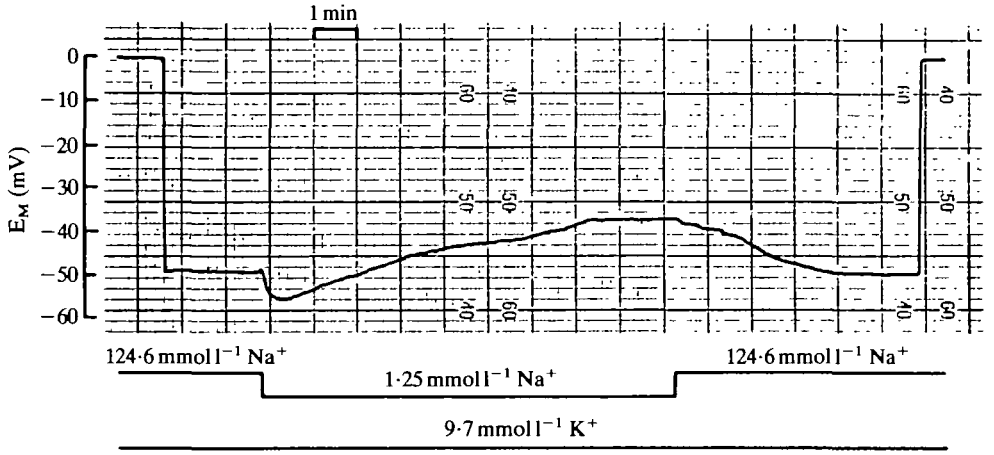


Fig. 3. Chart recording of a $Na^+$ substitution experiment on a *Calliphora* prepupal muscle cell, using $Mg^{2+}$ as a substitute. The extracellular $Na^+$ activity was lowered 100-fold (from 124·6 to 1·25 mmol $l^{-1}$) while $K^+$ was kept constant at 9·7 mmol $l^{-1}$, as indicated on the bottom lines.

involved in membrane electrogenesis (Fig. 1); and (b) a decrease in the partial permeability of the membrane to $K^+$, which would again lead to a depolarization of $E_M$, without a change in $E_K$. The following experiments were carried out to distinguish between these two possibilities.

### *Lack of effect of low-$Na^+$ Ringer on $aK_i$*

A typical chart recording of an experiment in which the $aK_i$ and $E_M$ of a cell were measured using a potassium-sensitive microelectrode, and the effect of a low-$Na^+$ Ringer on these parameters, is shown in Fig. 5. The upper trace gives the membrane potential of the cell (initially −38 mV) and the lower one gives $aK_i$ (= 80 mmol $l^{-1}$). When low-$Na^+$ Ringer was applied to the preparation, the $E_M$ changed, as noted earlier. However, there was no significant change in $aK_i$. We concluded, therefore, that the sustained depolarization of $E_M$ seen in low-$Na^+$ Ringer's solutions could not be due to a change in the $E_K$.

### *The effect of low-$Na^+$ Ringer on membrane input resistance*

Any change in the relative permeability of the cell membrane to $K^+$ and $Na^+$ might be reflected as a net change in the input resistance of the membrane. In this group of experiments, the effect of lowering extracellular $Na^+$ activity on the input resistances of the cell membranes was investigated using intracellular current injection by bridge circuitry. A typical recording from one such experiment is shown in Fig. 6. The upper trace shows the membrane potential (resting level, −42 mV) and its response to the application of the current pulses. The lower trace is the current monitor. Clearly, the delayed depolarizing phase of the $E_M$ change is accompanied by a sustained increase in the input impedance of the cell membrane.

The value of the input resistance in normal Ringer was $96 \pm 9\,k\Omega$, and this increased by an average of 26% in low-$Na^+$ Ringer to give $121 \pm 10\,k\Omega$ ($N = 7$).

*The effect of low-$Na^+$ Ringer on the partial permeability of the membrane to $K^+$*

To examine whether the membrane input resistance increase observed when the cell was exposed to low-$Na^+$ Ringer was due to a reduction in the $K^+$ permeability of the membrane, $K^+$ pulsation experiments were carried out. This type of

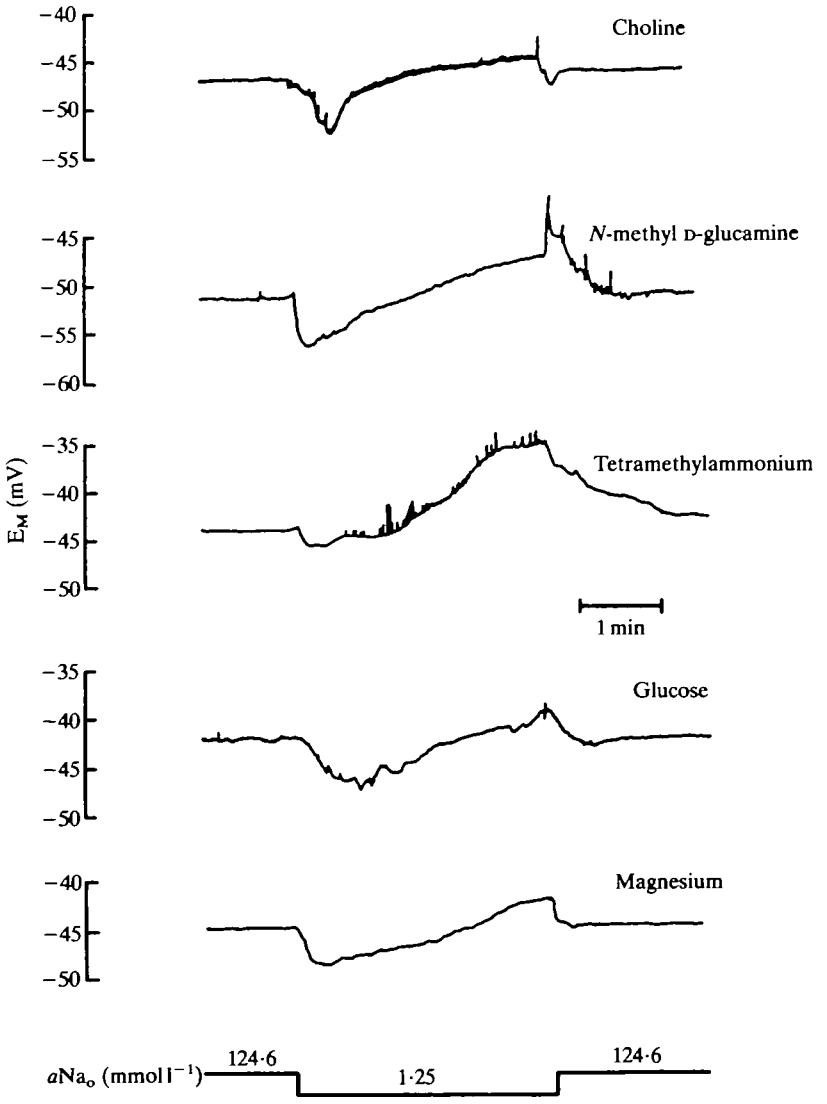


Fig. 4. Chart recordings of $Na^+$ substitution experiments on *Calliphora* prepupal muscle cells, using a variety of $Na^+$ substitutes (listed to the right of each trace). The extracellular $Na^+$ activity ($aNa_o$) was lowered from 124·6 to 1·25 mmol $l^{-1}$, as indicated on the bottom line.

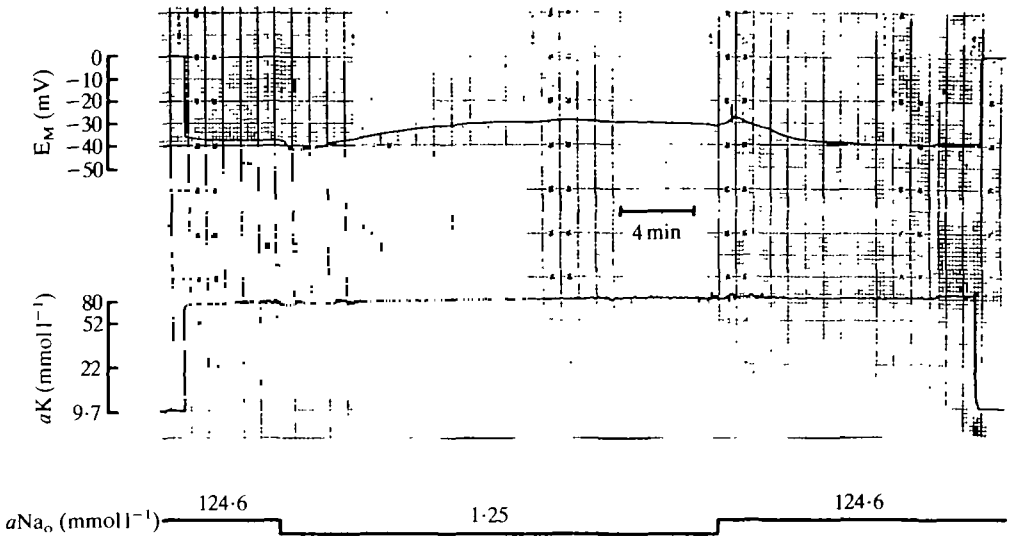


Fig. 5. Chart recording of an experiment in which the intracellular $K^+$ activity and membrane potential ($E_M$) were monitored using a double-barrelled $K^+$-sensitive microelectrode while the extracellular $Na^+$ activity ($aNa_o$) was reduced from normal (124·6 mmol l$^{-1}$) to 1·25 mmol l$^{-1}$ (as indicated on the bottom line).

experiment is illustrated in Fig. 7A. The uppermost trace gives membrane potential, including the transient depolarizing responses resulting from high-$K^+$ pulses, the applications of which are denoted by short bars beneath the $E_M$ trace. In normal Ringer (125 mmol l$^{-1}$ $Na^+$) the pulses depolarized the $E_M$ by some 15 mV. In low-$Na^+$ Ringer, however, the membrane responses were reduced to around 3 mV. The effect was completely reversible. The reduction in membrane responses started during the initial hyperpolarization of the membrane and persisted into the delayed depolarization phase, indicating a delay between the apparent reduction of partial $K^+$ permeability and membrane response, similar to the situation in $K^+$ substitution experiments (see Fig. 1). Average data are shown in Fig. 7B. Clearly, in the low-$Na^+$ Ringer, the responsiveness of the cell membrane to high-$K^+$ pulses is significantly decreased, a 100-fold reduction in $aNa_o$ leading to an average reduction in membrane response to 4 % of its original value. Thus, the partial permeability of the cell membrane to $K^+$ decreases substantially in a low-$Na^+$ medium, giving rise to the anomalous effect of reduced extracellular $Na^+$ on membrane potential. The value of $\alpha$ is not fixed, therefore, and this might explain the slight deviation of the data points from the straight line in Fig. 2.

### *Measurement of both intracellular $K^+$ and $Na^+$ activities in the same population of cells: a test of the model without involving any solution change*

According to the model for resting membrane electrogenesis based on the simplified version of the Goldman–Hodgkin–Katz equation (equation 2), by

taking into account the transmembrane gradients of $K^+$ and $Na^+$ it should be possible to predict the $E_M$ of a cell when bathed in *normal* Ringer, from a knowledge of the intracellular $K^+$ and $Na^+$ activities and a given value of $\alpha$, as follows:

$$E_M = 58\log\left(\frac{9{\cdot}7 + \alpha 124{\cdot}6}{aK_i + \alpha aNa_i}\right). \quad (3)$$

We were attracted to this type of test of the model, in spite of the technical difficulty involved, since it avoided any solution change. Thus, $aK_i$ values, $aNa_i$ values and $E_M$ values were measured in a group of 18 cells, and $E_M$ values calculated using the above equation for the three different values of $\alpha$ given in

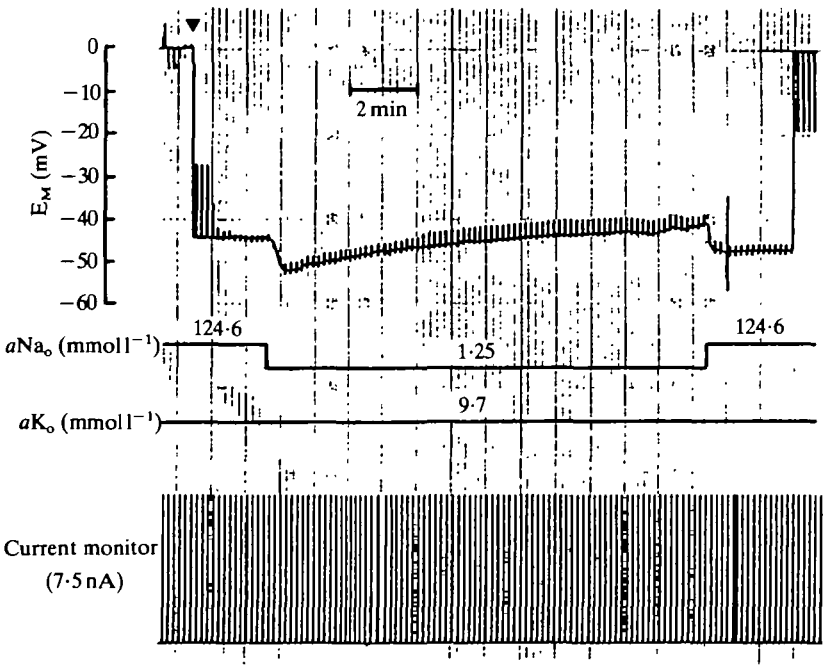


Fig. 6. Chart recording of a $Na^+$ substitution experiment on a *Calliphora* prepupal muscle cell during which membrane input resistance was monitored by repeated application of constant-sized (7·5 nA) current pulses using bridge circuitry. Before impaling the cell (marked by the arrowhead) the microelectrode resistance was balanced. When the cell was subsequently impaled, voltage pulses corresponding to the input resistance of the cell were recorded. After noting these, the bridge was rebalanced, and the extracellular $Na^+$ activity reduced from normal (124·6 mmol l$^{-1}$) to 1·25 mmol l$^{-1}$ while keeping a constant $K^+$ activity of 9·7 mmol l$^{-1}$ (middle lines). During the sustained depolarizing phase of resting membrane potential ($E_M$) change, voltage pulses reappeared on the $E_M$ trace, indicating a clear increase in the input resistance of the cell membrane. All effects were reversible by washing with normal Ringer. When the microelectrode was finally withdrawn from the cell, voltage pulses equal and opposite to those seen upon the initial impalement of the cell were recorded, indicating that the microelectrode resistance remained steady during the entire recording. The lowermost trace denotes the application of current pulses.

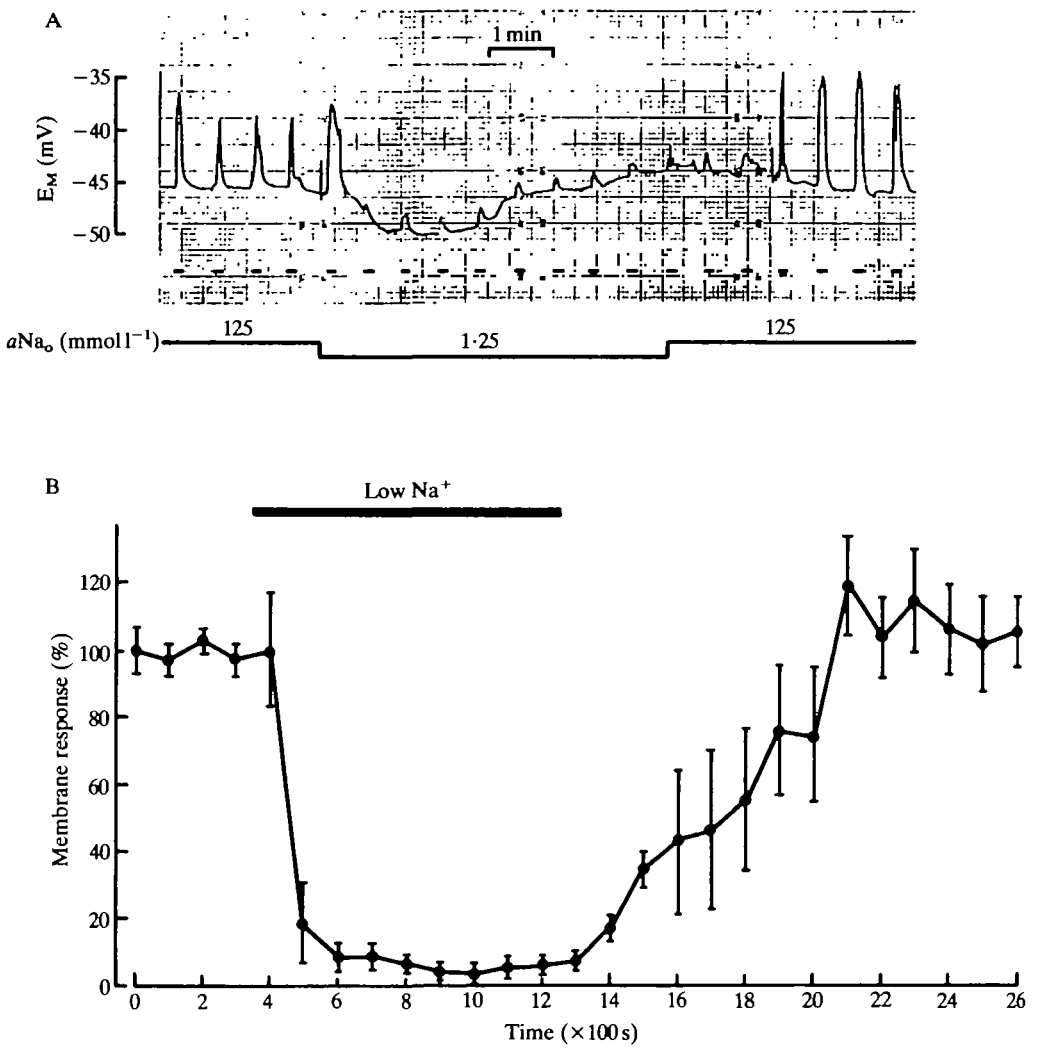


Fig. 7. (A) Chart recording of a $K^+$ pulsation experiment on a *Calliphora* prepupal muscle cell under steady perfusion with Ringer. This shows that the response of the cell membrane potential ($E_M$) to constant-sized pulses of high-$K^+$ (97·2 mmol $l^{-1}$) Ringer was markedly reduced when the cell was exposed to low-$Na^+$ Ringer. The extracellular $Na^+$ activity ($aNa_o$) is indicated on the bottom line. Applications of high-$K^+$ pulses are indicated by short bars beneath the $E_M$ trace. (B) Graph illustrating the change in the magnitude of the membrane response to constant-sized pulses of high-$K^+$ Ringer when the cells were exposed to low-$Na^+$ (1·25 mmol $l^{-1}$) Ringer (duration indicated by thick horizontal bar). This shows the marked reduction in the membrane response (assumed to reflect the partial $K^+$ permeability of the membrane, $p_K$) which occurred in the presence of low levels of extracellular $Na^+$. Data points denote means + S.E. ($N = 7$; each cell from a different preparation).

Table 2. *Comparison of $E_M$ values measured directly and those predicted from equation 2 for the three different values of $\alpha$ (see also Table 1)*

| | $\alpha$ | | |
|---|---|---|---|
| | 0·169 | 0·086 | 0·036 |
| $E^*_M$ (mV) | −27 ± 1 | −39 ± 1 | −48 ± 1 |
| $E_M$ (mV) | −48 ± 1 | −48 ± 1 | −48 ± 1 |
| $E^*_M - E_M$ (mV) | 21 ± 1 | 9 ± 1 | 0 |
| $P$ | 0·001 | 0·001 | |

Data are given as means ± S.E. ($N = 18$).

$P$ denotes the significance level of the difference between predicted and measured values (Student's $t$-test).

$E_M$, measured values; $E^*_M$, predicted values.

Table 2. Resulting data are illustrated in Fig. 8, showing the relationships between predicted and measured values of $E_M$, and also expressed in Table 2. Clearly, the best prediction for the data is obtained for the value of $\alpha$ (0·036) derived from $K^+$ substitution experiments involving solution changes in which $Na^+$ activity was kept equal to or greater than the normal value.

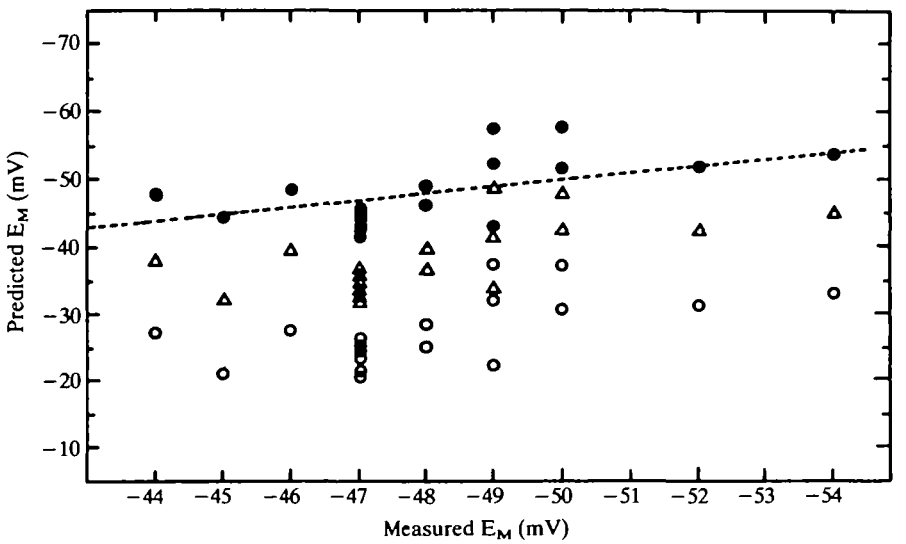


Fig. 8. Graph showing the relationships between the resting membrane potential values measured and predicted from the Goldman–Hodgkin–Katz equation (see equation 3 in text) for a population of 18 cells assuming different values of $\alpha$. Open circles, $\alpha = 0{\cdot}169$; filled circles, $\alpha = 0{\cdot}036$; open triangles, $\alpha = 0{\cdot}086$. Dashed line denotes equality, i.e. predicted $E_M$ = measured $E_M$.

*The effect of varying extracellular $K^+$ and $Na^+$ activities on $E_M$, whilst keeping $[(aK_o+0{\cdot}036aNa_o)\times aCl_o]$ constant*

The purpose of this set of experiments was to test whether the most realistic value of $\alpha$ (0·036) deduced in the first part of this paper would explain membrane electrogenesis in a restricted range of ionic concentrations around normal. The product was kept constant so as to prevent passive movements of intracellular $Cl^-$. Substitution solutions were constructed as described in Materials and methods. A typical result is shown in Fig. 9. As expected, all $E_M$ changes were quick and reversible, occurring within less than 1 min of the completion of the solution change (cf. Fig. 1). The changes in $E_M$ with changes in $(aK_o+0{\cdot}036aNa_o)$ are represented graphically in Fig. 10. The latter illustrates the linear relationship between $E_M$ and the logarithm of $(aK_o+0{\cdot}036aNa_o)$. The slope of the line is 58 mV per 10-fold change in $aK_o+0{\cdot}036aNa_o$ with an extremely high correlation coefficient (0·996). Thus, this confirms that the value of 0·036 for $\alpha$ accurately predicts the prevailing resting membrane potential in a cell.

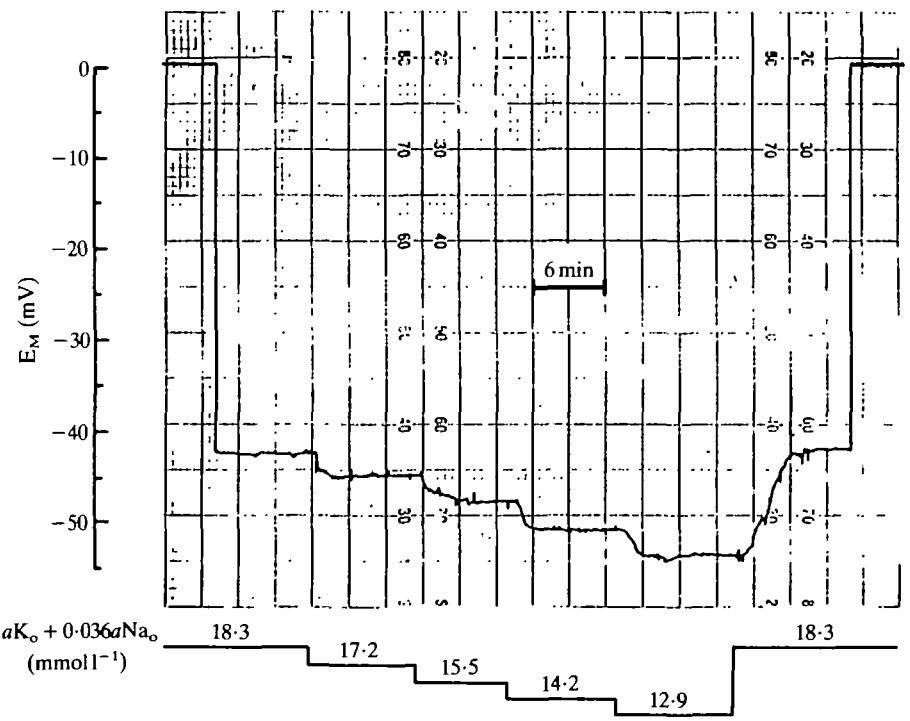


Fig. 9. Chart recording showing a substitution experiment on a *Calliphora* prepupal muscle cell in which the extracellular $K^+$, $Na^+$ and $Cl^-$ activities ($aK_o$, $aNa_o$ and $aCl_o$, respectively) were varied while $(aK_o+0{\cdot}036aNa_o)\times aCl_o$ was kept constant. It illustrates the rapid and sustained nature of the resting membrane potential ($E_M$) changes.

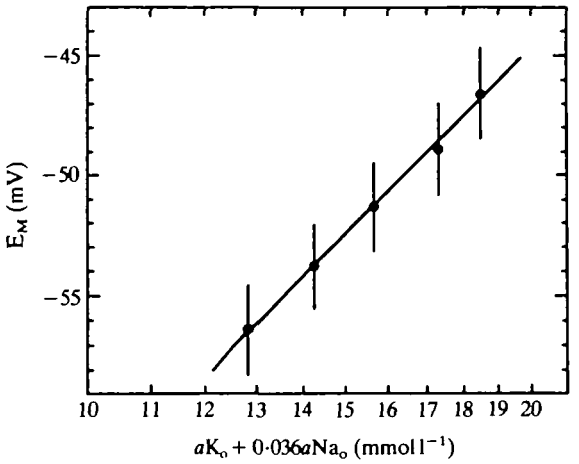


Fig. 10. Graph illustrating the relationship between the resting membrane potential ($E_M$) and the logarithm of ($aK_o + 0{\cdot}036aNa_o$), as defined in the legend of Fig. 9. All points are given as means ± S.E. ($N = 7$) and these fall extremely close to a line with a slope of 58 mV per 10-fold change in ($aK_o + 0{\cdot}036aNa_o$) (correlation coefficient, 0·996).

## Discussion

Measurements of the intracellular $K^+$ activities ($aK_i$) and calculations of the $K^+$ equilibrium potentials ($E_K$) presented in the preceding paper indicated strongly that in addition to $K^+$, the resting membrane of prepupal *Calliphora* skeletal muscle was permeable to an ion with an equilibrium potential more positive than the $E_M$. This has been confirmed in the present study. Thus, the generation of the $E_M$ has been shown to be explicable in terms of $K^+$ and $Na^+$ permeabilities. From experiments involving combined $K^+$ and $Na^+$ substitutions, the value of $\alpha$ was initially deduced to be 0·086. Jan & Jan (1976) reached a qualitatively similar conclusion in analogous muscles of *Drosophila*, although they estimated $\alpha$ to be 0·23. In the skeletal muscles of *Locusta*, Hoyle (1953) originally showed that a 10-fold change in the extracellular $K^+$ concentration resulted in an $E_M$ change of only 50 mV, thereby indicating that $K^+$ was not the only ion contributing to $E_M$. A comparable result was obtained by Janiszewski, Olszewska & Borkowska (1976) in *Acheta*. The situation in Orthoptera has been clarified by Leech (1986), who has demonstrated that, as in Diptera, resting membrane electrogenesis in the skeletal muscles of *Schistocerca* is due to $K^+$ and $Na^+$ permeabilities, $\alpha$ having a value of 0·016.

Attempts to test the *Calliphora* model by performing $Na^+$ substitution experiments to determine directly the effect of $Na^+$ on $E_M$ were only partially successful, however. When the extracellular $Na^+$ concentration was reduced, the $E_M$ hyperpolarized, as expected if $Na^+$ were involved in generating the $E_M$. This initial hyperpolarization was followed by an unexpected sustained depolarizing phase, during which the $E_M$ became more positive than the resting level. Broadly

similar effects have been reported in a variety of other insects. Jan & Jan (1976) obtained a comparable result in *Drosophila*. The role of $Na^+$ in resting membrane electrogenesis in insect skeletal muscles was studied in *Periplaneta*, *Carausius* and *Locusta* by Wood (1957, 1961, 1963) and in *Schistocerca* by Lea & Usherwood (1973), and consistent results were obtained in all four species: $E_M$ values were found to be relatively depolarized in salines containing steadily decreasing $Na^+$ levels. A similar effect was also shown for leech neuropile glial cells (Walz & Schlue, 1982). Wood (1963) found that as the extracellular $Na^+$ concentration was reduced, the intracellular $Na^+$ concentration (measured by flame photometry) decreased in parallel, thereby indicating again a finite permeability of the membranes to $Na^+$. When $aNa_i$ drops, one might expect that the activity of an electrogenic $Na^+/K^+$ pump would be slowed (Thomas, 1972) and that this might indirectly affect the $E_M$. This possibility can be ruled out, however, since such a secondary effect would also affect the intracellular $K^+$ activity, which is clearly not the case (Fig. 4). Djamgoz (1986, 1987) has presented evidence to suggest that in insects with 'conventional' haemolymph ion levels the $Na^+/K^+$ pump does not directly generate a part of the $E_M$.

The response of *Calliphora* skeletal muscles to lowered extracellular $Na^+$ involves a significant increase in the membrane input impedance (Fig. 5) and a fall in the partial permeability to $K^+$ ($p_K$) (Figs 6, 7). The mechanism of the reduction in $p_K$ is not clear. It has been shown that skeletal muscles of Diptera possess three types of $K^+$ conductance mechanism (Salkoff & Wyman, 1983). (i) The classical delayed, rectifying $K^+$ conductance of the kind described in detail in the squid giant axon by Hodgkin & Huxley (1952). Lowering the extracellular $Na^+$ concentration has been shown to have no effect on this conductance, however (Hodgkin & Huxley, 1952). (ii) A fast, transient $K^+$ conductance, the time course of which could not explain the sustained change in $E_M$. (iii) A $Ca^{2+}$-activated $K^+$ conductance ($g_{KCa}$) of the type found in snail neurones (Meech & Standen, 1975) and a variety of other cell types (see Meech, 1978 for a review). Reducing extracellular $Na^+$ concentration might be expected to have an indirect effect on $g_{KCa}$. For example, in sheep heart Purkinje fibres, a reduction in $aNa_o$ causes an increase in the intracellular $Ca^{2+}$ activity ($aCa_i$) by inhibiting the $Na^+/Ca^{2+}$ exchange mechanism (Bers & Ellis, 1982), and similarly in snail neurones (Partridge & Thomas, 1976). Such a rise in $aCa_i$ would activate $g_{KCa}$ and lead to an increase in $p_K$ and a hyperpolarization in $E_M$, which is in fact observed. This scheme does not, however, explain the sustained depolarizing change in $E_M$. The depolarization might, however, be explained if reducing $aNa_o$ led to a reduction in $aCa_i$. Such an effect has been reported in leech sensory neurones (Deitmer & Schlue, 1984), but it is not known if it also exists in *Calliphora* muscles. Interestingly, Jan & Jan (1976) found that the anomalous effect of reducing $aNa_o$ on $E_M$ could be rectified by increasing the external $Ca^{2+}$ concentration. Thus, it appears that there is a link between the transmembrane $Na^+$ gradient and/or $aCa_i$ (not involving conventional $Na^+/Ca^{2+}$ exchange) and the $K^+$ permeability of the membranes, but further work is required to clarify the situation.

In conclusion, the available data suggest that the value of $\alpha$ obtained from the high-$Na^+$ end of the $K^+$ substitution experiment (Fig. 2; Table 1), i.e. $\alpha = 0{\cdot}036$, is a realistic estimate. At rest therefore, nearly 97 % of the membrane permeability is to $K^+$ and some 3 % to $Na^+$. These figures have been confirmed by experiments on resting muscles involving no change of bathing media. Further evidence for the correctness of this value of $\alpha$ was obtained from experiments in which the effect of varying $aK_o$ and $aNa_o$ in a narrow range on $E_M$ whilst keeping $(aK_o + 0{\cdot}036aNa_o) \times aCl_o$ constant was tested. The latter experiments also showed that the $E_M$ changes induced by these solutions were rapid, equilibrating within a few minutes, as opposed to several tens of minutes experienced in the earlier $K^+$ substitution experiments (Fig. 1), thereby also providing additional evidence for the passive distribution of $Cl^-$ in these muscles.

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