J. Exp. Biol. (1971), **54**, 757–777
With 14 text-figures
Printed in Great Britain

A QUANTITATIVE STUDY OF THE IONIC BASIS OF EXTRANEURONAL POTENTIAL CHANGES IN THE CENTRAL NERVOUS SYSTEM OF THE COCKROACH (*PERIPLANETA AMERICANA* L.)

BY Y. PICHON,* R B. MORETON AND J. E. TREHERNE
A.R.C. Unit of Invertebrate Chemistry and Physiology, Department of Zoology, University of Cambridge

(*Received* 11 *November* 1970)

INTRODUCTION

Earlier studies carried out in this laboratory have demonstrated the existence of rapid and relatively large extraneuronal potential changes following elevation of the potassium concentration in the medium bathing intact cockroach connectives (Pichon & Treherne, 1970; Treherne *et al.* 1970). This phenomenon appeared to be associated with a restricted access of potassium ions to the fluid bathing the axon surfaces, for the extraneuronal positivation† observed with intracellularly located microelectrodes was not accompanied by an equivalent reduction in the amplitude of the recorded action potentials. It was also shown that this effect was reduced or abolished if tension was applied to the nerve or if the connectives were dried and briefly exposed to air (Pichon & Treherne, 1970). In such preparations only the continuous and relatively slow potential changes were observed, corresponding to the direct depolarization of the giant axons. It was concluded that this latter electrical response resulted from an increased access of potassium ions into the extracellular system, most probably as a result of the disruption of intercellular occlusions at the inner margin of the perineurium (Maddrell & Treherne, 1967; Lane & Treherne, 1970).

The previous investigations have not elucidated the nature of the extraneuronal potential changes resulting from increased potassium concentration in the bathing medium. Neither is it possible to predict the effects of other ions on these changes. The present study was therefore carried out in an attempt to throw some further light on the ionic basis of the extraneuronal potential changes in intact cockroach connectives. We have tested the effects of monovalent inorganic and organic cations (Li^+, Cs^+, Rb^+, K^+, TEA^+, tris and choline), divalent cations (Ca^{2+} and Mg^{2+}) and the effects of replacement of Cl^- by SO_4^{2-}. An attempt is made to elucidate the structural basis of the phenomena by comparison of the experimental results with those derived from four possible theoretical model systems.

* Senior Research Fellow of King's College, Cambridge.

† The terms 'positivation' and 'negativation' have been preferred to depolarization and hyperpolarization to distinguish the extraneuronal potential changes from the electrical events taking place at the axon membrane level.

METHODS

The potential changes described in this paper were measured using the 'sucrose gap' technique employed in a previous investigation (Pichon & Treherne, 1970). The penultimate connective was passed through petroleum jelly seals between three parallel compartments, the right-hand one being continuously perfused with the experimental solution. The central compartment contained flowing mannitol (483·0 mM/l) solution and the left-hand one contained normal Ringer solution. The potential changes resulting from alterations in the ionic concentration in the right-hand compartment were recorded via a saline-filled agar bridge connected to a high-impedance amplifier, the left-hand compartment being connected to the indifferent electrode via a second saline-agar bridge. Perfusion of the compartments was achieved using the gravity-fed system previously described.

The normal physiological solution used was that devised by Yamasaki & Narahashi (1959): 210·0 mM/l Na^+, 3·1 mM/l K^+, 1·8 mM/l Ca^{2+}, 216·9 mM/l Cl^-, 0·2 mM/l $H_2PO_4^-$ and 1·8 mM/l $H_2PO_4^{2-}$. Variation of the ionic composition of the experimental solutions was achieved by substituting various ions for those of sodium.

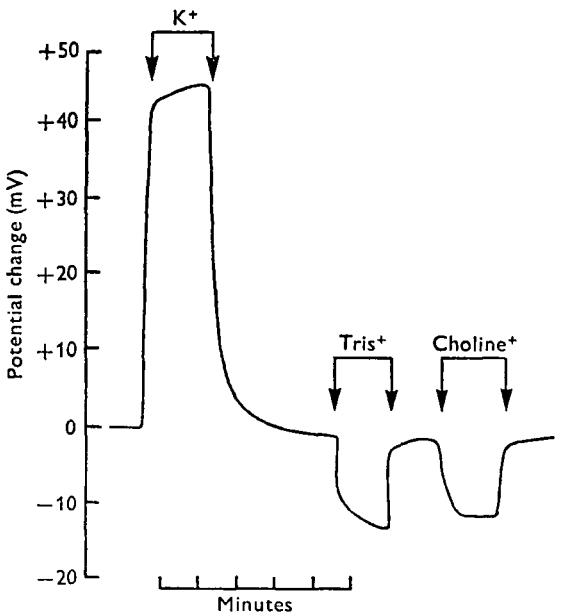

Fig. 1. The effects of substitution of tris and choline ions for those of sodium on the potentials measured using the 'sucrose-gap' technique. The potential changes produced by a high-potassium solution (214 M/l) are included for comparison.

RESULTS

Effects of choline and tris ions

Fig. 1 illustrates the effects of solutions in which the sodium ions were replaced by those of potassium and subsequently by those of choline or tris (2-Amino-2(hydroxymethyl)-propane-1,3-diol) on the potential changes measured using the sucrose-gap

technique in intact preparations. The rapid positivation produced by the high-potassium solution was followed by smaller negative-going potential changes in preparations exposed to solutions in which the normal concentration of sodium ions was replaced by the organic cations.

A somewhat variable response was obtained to the solutions in which sodium ions were replaced by those of choline or tris. The potential changes produced by choline solutions varied between 4·0 and 11·0 mV with a mean value of 7·0 ± 0·75 (S.E.) mV. These values are expressed relative to the potential changes produced by the high-potassium solution in Fig. 12. The potential changes produced by replacement of sodium ions with those of tris resulted in potential changes of between 5·0 and 15·0 mV, with a mean value of 9·3 ± 1·1 (S.E.) mV.

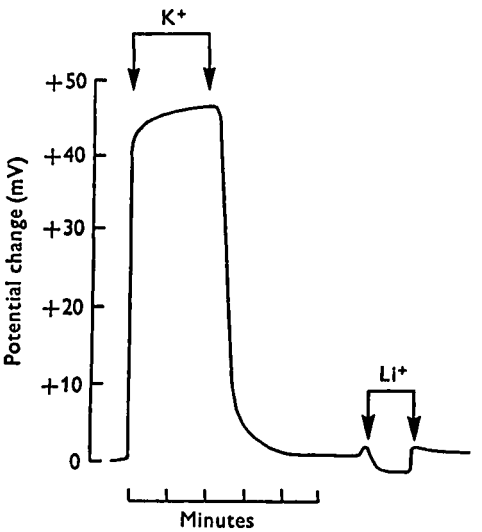

Fig. 2

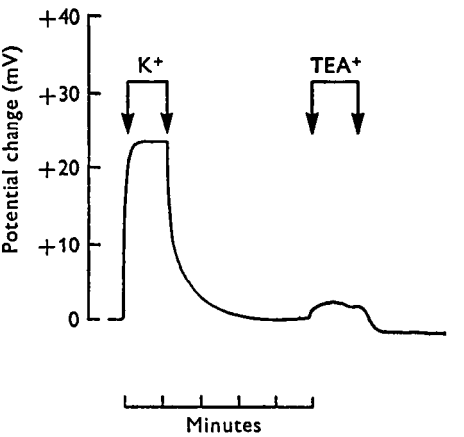

Fig. 3

Fig. 2. The effects of replacement of sodium ions by those of lithium and potassium on the potentials recorded using the 'sucrose-gap' technique.

Fig. 3. Potential changes resulting from substitution of potassium and TEA ions for those of sodium in the bathing solution.

Effects of lithium ions

Replacement of sodium ions in the bathing solution with those of lithium was found to produce only slight reversal of potential of the form illustrated in Fig. 2. The small and transient positivation obtained on change of solutions was a consistent feature of the results obtained with solutions in which sodium ions were replaced by those of lithium. The mean value of this change was 1·8 ± 0·37 (S.E.) mV.

Effects of tetraethyl ammonium (TEA) ions

Fig. 3 illustrates the effect of a solution in which sodium ions were replaced by those of TEA. Incorporation of this organic cation in the bathing solution resulted in a slight positivation (1·9 ± 0·24 mV).

Effects of caesium ions

Replacement of sodium ions by those of caesium in the bathing solution resulted in an appreciable positivation, averaging 7·9 ± 0·24 (S.E.) mV (Fig. 4).

Effects of rubidium ions

Fig. 5 illustrates the effects of 214·0 mM Rb^+ in place of Na^+ in the bathing medium. It will be seen that rubidium produced a positivation which approached that recorded with the high-potassium solution. These results also show that application of a pulse

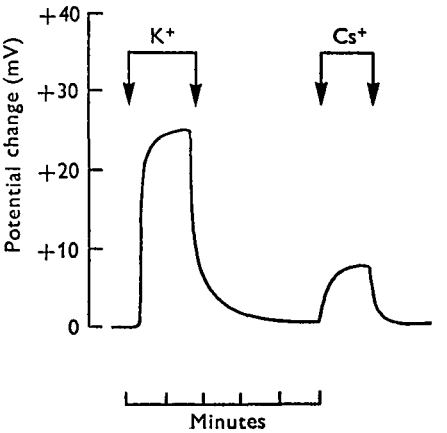

Fig. 4. Potential changes resulting from the exposure of connectives to experimental solutions in which sodium ions were replaced by those of potassium and caesium.

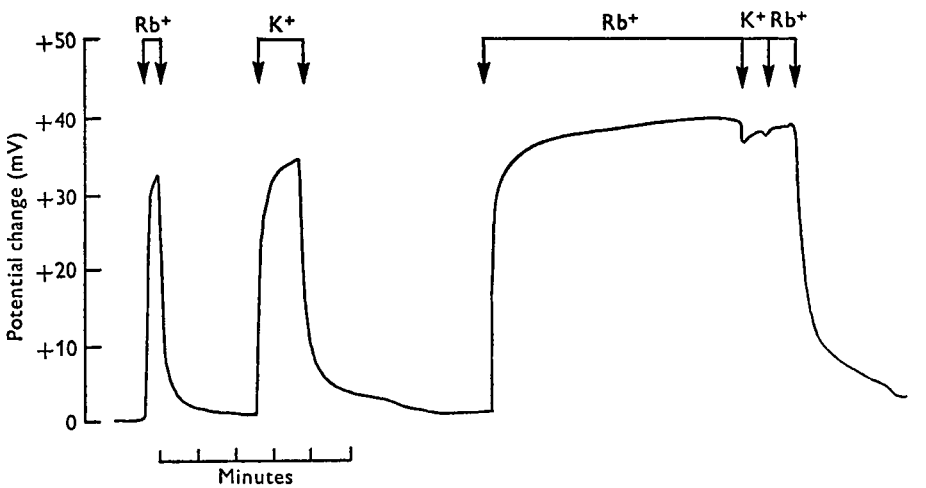

Fig. 5. Extraneuronal potentials produced by substitution of potassium and rubidium ions for those of sodium.

of potassium during an extended exposure to the rubidium solution or a pulse of rubidium during a prolonged exposure to high-potassium resulted in only small and transient potential changes. The positive potential change produced by the rubidium

solution averaged 29·1 ± 2·1 (S.E.) mV which, expressed as a fraction of the potassium positivation, corresponds to a relative change of +0·98 ± 0·07 (S.E.).

Effects of variations in the external potassium concentration

Fig. 6 shows the effects of pulses of solutions of various potassium concentrations on the potentials recorded in an intact connective. In these experiments sodium ions were replaced by those of choline, the concentrations being appropriately reduced

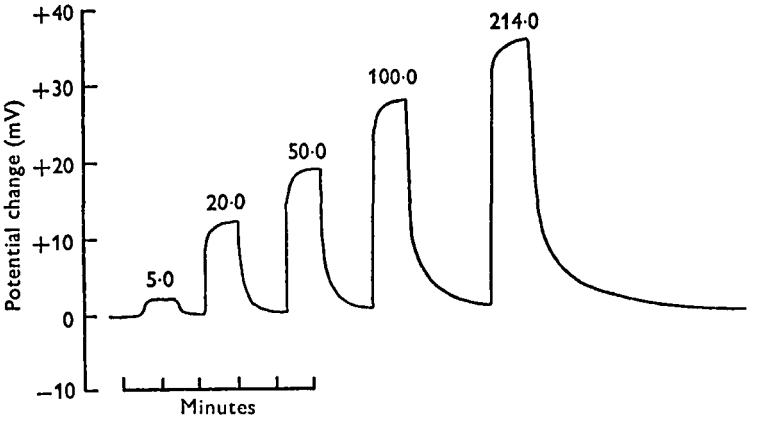

Fig. 6. The effects of concentration on the extraneuronal potential changes produced by potassium ions.

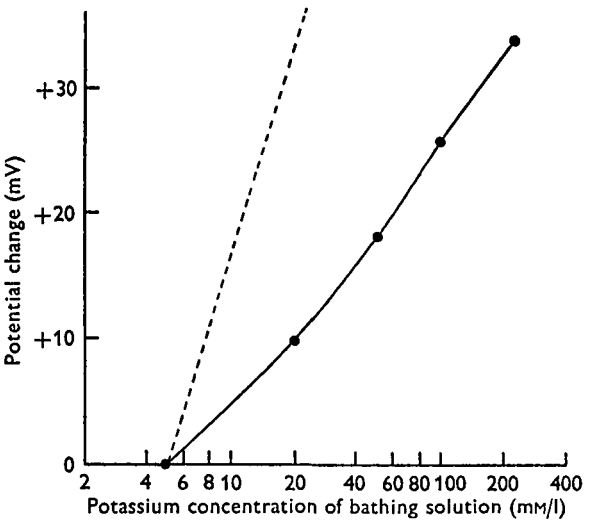

Fig. 7. The relation between extraneuronal potential changes and the potassium concentration of the bathing medium. The broken line illustrates the 57 mV slope for decade concentration change predicted by the Nernst equation.

with the increased potassium concentration. A semilogarithimic plot of the results is shown in Fig. 7. It will be seen that this relation shows a slight departure from linearity, an effect which was observed with all the preparations tested.

Effects of calcium and magnesium ions

Elevation of the concentration of these divalent cations (to replace those of sodium) in the bathing medium produced complex potential changes. Fig. 8 shows the potential changes recorded in an experiment in which the intact connective was exposed to two short pulses of a high-potassium solution, followed by a prolonged exposure to a high-calcium solution. The rapid positivations produced by the high-potassium solution were followed (with high-calcium solution) by a relatively rapid negative-going potential (1) which decayed rather more slowly (2) and then showed a slow negative component (3). Only a small positive potential change (4) was recorded on return to normal solution. A final pulse of high-potassium solution showed a change in the form of the recorded positivation which tended to develop more slowly.

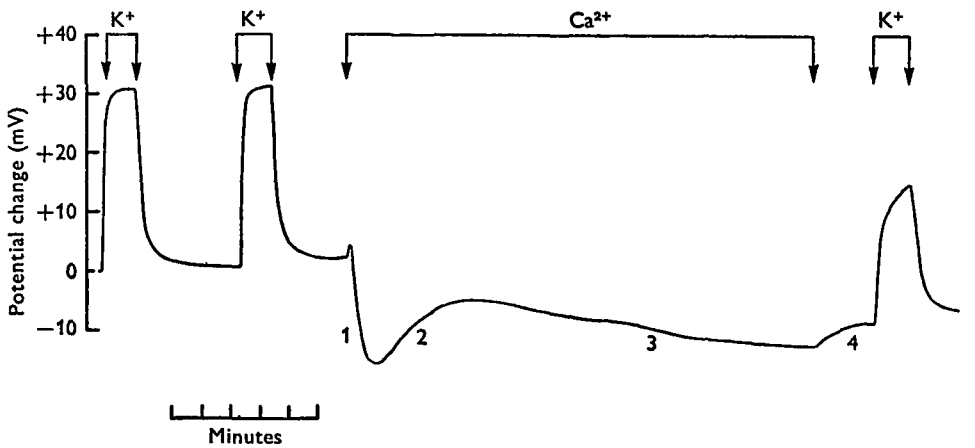

Fig. 8. Potential changes produced by substitution of sodium ions for those of calcium in the bathing medium, related to the effects produced by exposures to the high-potassium solution. The numbered components of the potential changes are referred to in the text.

Elevation of the magnesium concentration in the bathing medium resulted in a pronounced potential change of opposite polarity to that produced by high-potassium solution (Fig. 9). Return to normal solution was associated with a relatively slow decrease in potential following a small transient positivation. A second exposure to high-magnesium resulted in a similar potential change to that observed in the first exposure, except that the absolute level attained was lower. This effect is more clearly seen in Fig. 10 when the successive pulses of high-magnesium were associated with roughly equivalent potential changes which showed a progressive decline in the absolute level attained. These results contrast with those for calcium in which, as will be seen from Fig. 10, there was a progressive decrease in the magnitude of the change but a return to the potential level initially obtained in normal solutions.

Effects of sulphate ions

To test the effect of the anion species present on the extraneuronal positivation produced by high external potassium concentrations, experiments were carried out in which sulphate was substituted for chloride in the bathing solutions, before replacing

the sodium ions by potassium. As will be seen from Fig. 11 there was very little effect of substitution of sulphate for chloride ions on the rapid initial positivation recorded with 214·0 mM/l potassium in the bathing solution.

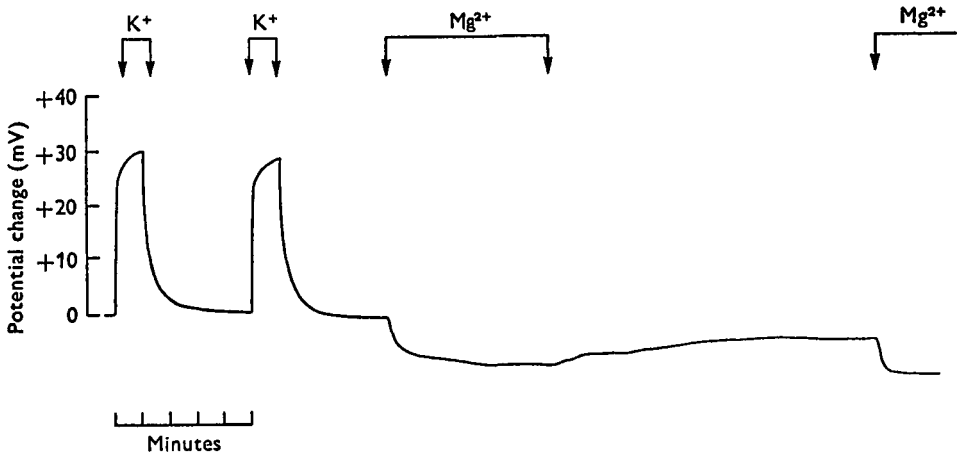

Fig. 9. Potential changes produced by substitution of magnesium ions for those of sodium in the bathing medium relative to those produced by exposure to high-potassium saline.

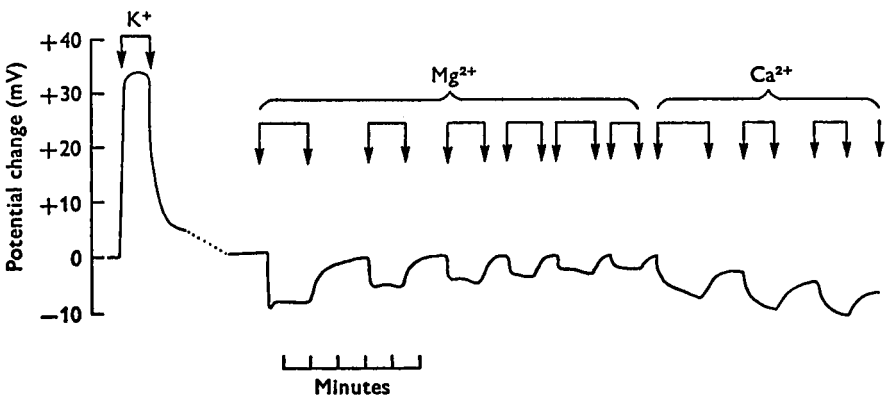

Fig. 10. Effects of successive exposures to pulses of high-magnesium and high-calcium solutions and the effect of high potassium concentration on potentials measured using the 'sucrose-gap' technique.

DISCUSSION

It has recently been established that the extraneuronal positivation (measured using microelectrodes or sucrose-gap technique), produced by elevation of the external concentration of potassium ions, is correlated with a restricted access of the cation to the fluid bathing the surfaces of the giant axons in intact cockroach connectives (Treherne *et al.* 1970; Pichon & Treherne, 1970). It was tentatively suggested, in earlier publications from this laboratory, that the observed restriction upon the intercellular movements of cations from the bathing medium was associated with occluded regions, containing tight junctions and septate desmosomes (Maddrell & Treherne, 1967), at the inner ends of the tortuous intercellular clefts which traverse the peri-

neurium, for the inward movement of peroxidase molecules has been shown to be restricted in this region (Lane & Treherne, 1970).

The postulation of a severe restriction upon the inward diffusion of cations at the inner end of the perineurial clefts provides a basis for the explanation of the extraneuronal potential changes produced by the substitution of potassium, and other cations, for sodium ions in the bathing medium. It can readily be envisaged, for example, that in the presence of high external concentrations of potassium ions there would be a depolarization of the outwardly directed perineurial membrane, the equivalent depolarization of the inwardly directed one being effectively prevented by the presence of the occluded regions at the inner ends of the perineurial clefts. Such a

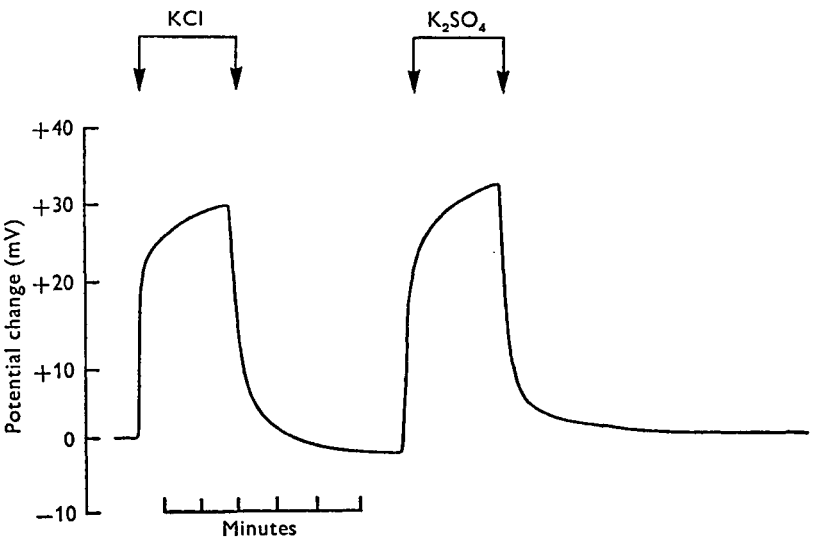

Fig. 11. Effects of substitution of sulphate ions for those of chloride on the extraneuronal potentials produced by substitution of potassium ions for those of sodium in the bathing medium.

system would produce rapid potential changes, measured using both extracellularly and intracellularly located microelectrodes, without an equivalent direct depolarization of the axonal membranes.

The various extraneuronal potential changes described above for other alkali metal and organic cations can also be interpreted in terms of their effects on the outer perineurial membrane. The appreciable negative potential change produced by tris and choline can, for example, be postulated to result from an efflux of extracellular sodium ions induced in the absence of this cation in the bathing medium. This effect would thus be analogous to the hyperpolarization produced by the replacement of sodium ions by those of tris in the solution bathing gastropod neurones (Moreton, 1968*a*; Sattelle, 1970). In the case of *Helix* neurones this effect can be reasonably inferred from application of the 'constant-field' theory which shows that the observed resting potential is considerably less negative than the potassium equilibrium potential largely as a result of the significant sodium permeability of the resting cell membrane (Moreton, 1968*b*). The appreciable extraneuronal positivation induced by externally

applied rubidium and caesium ions also parallels the effects of these cations on a variety of excitable cell membranes in which their action resembles, in varying degrees that of potassium ions (cf. Sjodin, 1959).

From Table 1 it is apparent that the polarity of the extraneuronal potential can be related to the radii, either crystal or hydrated, of the inorganic monovalent species, only those of smaller hydrated radii than Na^+ producing a positivation. The very small positivation produced by TEA^+ also accords with the observation that this ion can partly replace sodium in nerve membranes (Binstock & Lecar, 1969; Y. Pichon, unpublished result) and is similar enough to potassium with a single shell to be accepted by a potassium 'site' but unsymmetrical enough to block the 'channel' (Armstrong, 1966).

Table 1. *Summary of experimental results showing polarity and extent of extraneuronal potential change resulting from the replacement of sodium by various cations in the bathing medium*

(Values for crystal ionic radii taken from Ussing (1960) and those for hydrated radii from conductivity and ionic mobility measurements quoted by Stern & Amis (1959).)

Ion species	Crystal radius Å	Hydrated radius Å	Polarity of extraneuronal potential	Extent of extraneuronal potential (mV mean ±S.E.)
Li^+	0·60	3·40	−	7·8±0·37
Na^+	0·95	2·76	0	0
K^+	1·33	2·32	+	30·0±2·4
Rb^+	1·48	2·28	+	29·1±2·1
Cs^+	1·69	2·28	+	7·9±0·24
TEA^+	−	2·76	+	1·9±0·34
$Tris^+$	−	−	−	9·3±1·1
$Choline^+$	−	−	−	7·0±0·75
Mg^{2+}	0·66	4·65	−	Variable
Ca^{2+}	0·99	3·21	−	Variable

On the basis of the measured extraneuronal potential changes it is possible to arrange the monovalent cations in the following sequence of effectiveness:

$$K^+ > Rb^+ > Cs^+ > TEA^+ > Na^+ > Li^+ > choline^+ > tris^+$$

(Fig. 12). This order corresponds to sequence IV in the classification devised by Diamond & Wright (1969) to describe the relative potencies of alkali cations in biological systems, i.e. $K^+ > Rb^+ > Cs^+ > Na^+ > Li^+$. The sequence for the extraneuronal potential changes approximates to that deduced from the measurements of the potential changes produced at the inner surface of the frog skin by Lindley & Hoshiko (1964), i.e. $K^+ > Rb^+ > Cs^+ > Li^+ > Na^+$, which differs from sequence IV of Diamond & Wright only in the greater potency of Li^+ relative to Na^+. The system proposed to account for the observed extraneuronal potential changes in intact cockroach connectives does, in fact, exhibit other points of apparent similarity with the model advanced to account for the ionic basis of the potential changes in the frog skin (cf. Ussing, 1965; Keynes, 1969). In both these cellular layers, for example, it is envisaged that the ionic changes in the bathing medium affect only one of two membrane surfaces. In the insect perineurium, as in the frog skin, access between the inner and outer epithelial surfaces is restricted by intercellular occlusions in the form of lateral tight junctions (Maddrell & Treherne, 1967; Lane & Treherne, 1970).

The perineurial system proposed here differs, however, from the frog skin in the small extent of the potential changes produced by alteration in the potassium concentration of the bathing medium. Koefed-Johnsen & Ussing (1958) showed that the inner epidermal surface of the frog skin approximated to a potassium electrode. It is apparent from Fig. 7 that the measured extraneuronal potentials in the cockroach connective depart very markedly from the 57 mV slope for decade change in external potassium concentration, the equivalent slope being only about 17 mV.

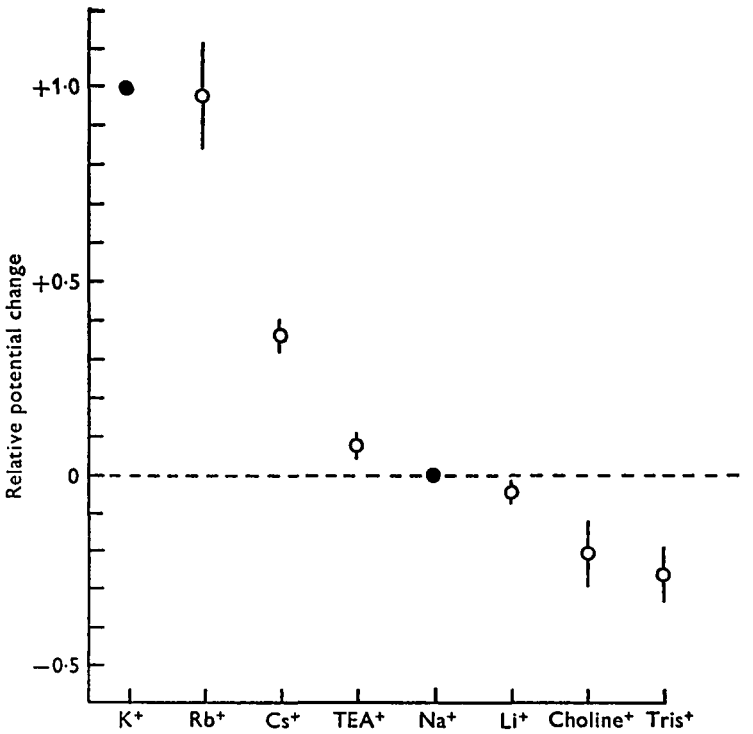

Fig. 12. The extent of the potential changes produced by substitution of various monovalent ions for those of sodium in the bathing medium. The potential changes are expressed as a fraction of the positivation produced by potassium ions. The vertical lines indicate the extent of twice the standard error of the mean.

It could be envisaged that this extreme departure from the relationship predicted by the conventional Nernst equation might result from the appreciable permeability of the outward-facing perineurial surface to other ion species. It is presumably not due to attenuation of the potentials as measured by the sucrose gap technique, since the potassium-dependence of the resting potential of the axons in de-sheathed preparations was found to show the same 42 mV slope, when measured by the sucrose-gap technique, as when measured directly with an intracellular microelectrode (Schofield & Treherne, unpublished observations). Such a system might, therefore, reasonably be supposed to be described by constant-field equations (Goldman, 1943; Hodgkin & Katz, 1949).

The theoretical section of this paper describes an attempt to analyse the situation on the basis of the constant-field theory, using the method developed by Moreton (1968*b*),

It is concluded that the potassium-dependence of the extraneuronal positive potential similar to the theoretical behaviour of a model system, consisting of a peripheral diffusion barrier (presumably located in the perineurium) in series with a long, narrow channel, representing the system of intercellular clefts connecting the inward-facing surface of the perineurium with the space surrounding the giant axon (cf. Treherne *et al.* 1970). The diffusion barrier is selectively permeable to potassium ions, its relative permeabilities to sodium and choline ions being expressed by the ratios

$$P_K:P_{Na}:P_{Ch} = 1:0{\cdot}19:0{\cdot}11.$$

Its potassium permeability is such that changes in the external potassium concentration give rise to corresponding, but smaller, changes in the concentration at the inner face. The absolute value of the permeability is not determined, however, as insufficient evidence is available about the geometry of the real system. A theoretical analysis of the diffusion of ions in the model system, using the constant-field theory to give the flux of ions across the barrier, shows that the behaviour of the concentration at the inner face of the barrier agrees quite well with that required to account for the behaviour of the potential. The experimental results are thus in accord with the supposition that the access of ions to the interior of the unstretched connective is restricted by the existence of a peripheral, ion-selective barrier. It is not possible to draw any firm conclusions regarding the morphological nature of the barrier. However, as the neural lamella appears to be relatively permeable to ions and molecules (Treherne, 1962; Lane & Treherne, 1969), it seems reasonable to suppose that the barrier is formed at least in part by the tight junctions observed at the base of the intercellular clefts between perineurial cells (Maddrell & Treherne, 1967). The passage of ions into the system would then occur either through the tight junctions or by penetration through the membranes and cytoplasm of the perineurial cells. Presumably, passage across the tight junctions accounts for at least part of the ionic movements, since even relatively large ions such as choline are evidently able to pass to a considerable extent (see p. 772).

The potential changes resulting from elevated concentrations of monovalent cations other than sodium or potassium are similarly interpretable in terms of the selective permeabilities of the perineurial barrier. The extraneuronal potentials resulting from elevated concentrations of divalent ions, however, were complex in form and are consequently more difficult to interpret. It would seem reasonable to suppose that the initial rapid negative potential changes produced by high concentrations of calcium and magnesium ions were similar in nature to those produced by choline and tris, that is, they resulted from a significant outward movement of sodium ions across the outer perineurial membrane in the presence of relatively non-penetrant ions in the bathing medium. The subsequent potential changes in the presence of high external concentrations of calcium ions might then reasonably be expected to result from the specific effects of this cation, either on the external perineurial membrane, or on the tight junctions.

Similarly the progressive reduction in the potential level observed in successive exposures to pulses of high-magnesium solution could result from a progressive decrease in the sodium permeability of the barrier, so that the sodium efflux during successive exposures to a high concentration of magnesium is gradually reduced.

THEORETICAL SECTION

This section describes an attempt to analyse the situation in the intact connective theoretically, using the constant-field theory, as developed by Moreton (1968*b*). Assuming that the behaviour of the extracellular potential changes observed in the experiments are due to the presence in the intact, unstretched connective of a peripheral diffusion barrier, the appropriate model system must consist of such a barrier, in series with a long, narrow channel, at the inner end of which is the giant axon (Treherne *et al.* 1970). The barrier will then presumably represent either the cell membranes of the perineurial cells, or the tight junctions at the base of the perineurium, or a combination of the two. The long channel will represent in a simplified form the mesaxon channel, in series with the network of intercellular spaces between the glial cells. In the first instance, it is assumed that the system of channels is closed (i.e. that significant exchange of ions with the intracellular compartment does not occur). This simplification is probably not justified – the effects of exchange with the intracellular compartment will be discussed below – but it forms a useful starting point for the investigation. Clearly, the detailed behaviour of such a grossly simplified model can resemble only qualitively that of the real tissue; nevertheless, varying degrees of elaboration of the model are likely to produce only quantitative changes in its theoretical behaviour, so that the analysis of its behaviour may be expected to throw at least some light on the mechanisms responsible for the behaviour of the extraneuronal potential.

The first stage in the analysis is to investigate the electrical and permeability properties which must be attributed to the perineurial barrier, in order to reproduce the experimental behaviour of the extraneuronal potential. These properties can then be used to investigate the movements of ions in the model system, occurring in response to changes in the composition of the bathing medium, and hence to predict some aspects of the behaviour of the giant axons under these conditions. The most complete set of results available is that shown in Fig. 7, relating the extraneuronal potential to the concentration of potassium ions in the external medium. These results will be used as the principal criterion with which to test the suitability of the chosen parameters of the barrier.

Application of the equations of Hodgkin & Katz (1949) to the potential difference across a single diffusion barrier, governed by the constant-field theory, leads to the equation

$$\exp(FV/RT) = \frac{[K_0^+]+\delta[Ch_0^+]+\beta[Cl_i^-]}{[K_i^+]+\delta[Ch_i^+]+\beta(Cl_0^-]}, \qquad (1)$$

where the symbols have the meanings listed on p. 775. In order to produce data comparable with those of Fig. 7, the external medium has been taken as containing potassium, choline and chloride as the only monovalent ions. It has also been assumed that, in the relatively prolonged exposure to sodium-free solution which is implied in this experiment, all the extracellular sodium in the connective has been washed out. In any case, the presence of a constant amount of sodium in the extracellular compartment will not affect the form of equation (1), since it will affect only the denominator on the right-hand side. Since the potassium concentration in the external medium varies

widely, it follows that $[Ch_0^+]$ also varies; in fact, the solutions were made up to be isosmotic, so that

$$[K_0^+]+[Ch_0^+] = Y, \tag{2}$$

where Y is a constant. Equation (1) may thus be written as

$$\exp(FV/RT) = \frac{(1-\delta)[K_0^+]+\delta Y+\beta[Cl_i^-]}{[K_i^+]+\delta[Ch_i^+]+\beta[Cl_0^-]}. \tag{3}$$

All terms on the right-hand side of equation (3) are now constant, so that the equation predicts a linear relationship between $\exp(FV/RT)$ and $[K_0^+]$. Fig. 13 (*a*) shows the results of Fig. 7 plotted in this form; it is clear that equation (3), as it stands, is insufficient to account for the behaviour of the extraneuronal potential.

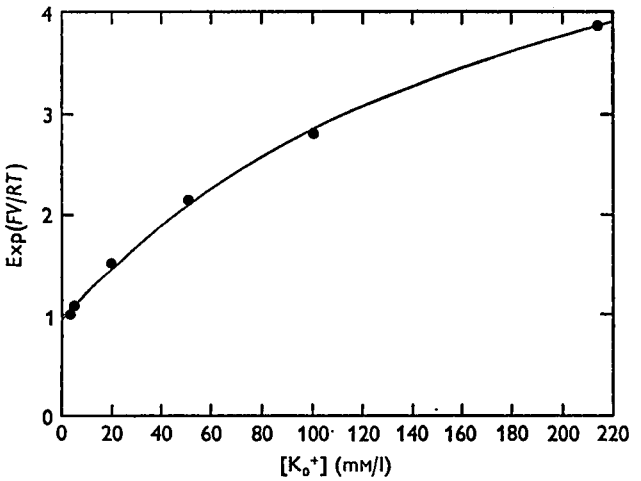

Fig. 13. The relationship between exp (*FV/RT*) and the external potassium concentration, from the data of Fig. 7. From the constant-field theory, for a simple, single-barrier system, this relationship would be expected to be linear. The continuous curve represents a 'least-squares' fit of equation (9) to the experimental results, using the values of the parameters given in the text.

A single barrier in parallel with a leakage channel of arbitrary ion-selectivity, such as might be formed by cell membranes of the perineurial cells in parallel with an intercellular pathway, the latter partially restricted by tight junctions between cells, is similarly insufficient. This can readily be seen, since equation (1) is derived by addition of the individual ionic currents across the barrier, without reference to the morphological location of the 'channels' through which they move. Provided, that the concentrations of the ions are uniform over each of the two faces of the system, addition of the currents will always produce an equation of the form (1), in which the effective permeability to each ion is the sum of the permeabilities of the individual 'channels' through which it can pass.

The consideration of the passage of ions across the tight junctions between cells does, however, raise the possibility of contribution by divalent ions (calcium and magnesium) to the extraneuronal potential. The equation for the flux of a divalent ion across a constant-field barrier has the form

$$M_{Ca} = (2FV/RT)P_{Ca}\frac{[Ca_0^{2+}]-[Ca_i^{2+}]\exp(2FV/RT)}{1-\exp(2FV/RT)}, \tag{4}$$

so that, when the fluxes of mono- and divalent ions are combined, the resulting expression for the potential difference across the barrier will in general be quadratic in $\exp(FV/RT)$. However, while a quadratic expression can be made to fit the experimental results in Fig. 13 quite well, there are two serious objections to the theory of involvement of divalent ions. First, equation (4) will only contain quadratic terms in $\exp(FV/RT)$ if the concentrations of the divalent ion concerned on the two sides of the barrier are unequal; there is no evidence of this in the cockroach connective. Second, the considerable departure from linearity of the results in Fig. 13 indicates that a comparatively large contribution from divalent ions would be required to account for the behaviour of the extraneuronal potential. Exposure of the preparation to high concentrations of calcium or magnesium would then be expected to cause a large influx of these ions, and consequently the development of a positive extraneuronal potential, in contrast to the negative potentials observed experimentally (Figs. 9, 10). The results show that any contribution from divalent ions to the potential, if it exists at all, is so small as to be completely masked by the efflux of sodium ions, resulting from the replacement of sodium in the bathing medium by a high concentration of divalent cations.

A further possibility, raised by the consideration of passage of ions through the perineurial cells, is that the extraneuronal potential difference may be developed across a system of two diffusion barriers in series, such as would be formed by the inner and outer cell membranes. The behaviour of such a system, which is formally analogous to the isolated frog skin (Ussing, 1965), may be analysed very simply, by addition of the potential differences across the two membranes:

$$\begin{aligned} V = V_1 + V_2 &= (RT/F)\ln\left\{\frac{[K_0^+]+\delta[Ch_0^+]+\beta[Cl_c^-]}{[K_c^+]+\alpha[Na_c^+]+\beta[Cl_0^-]} \times \frac{[K_c^+]+\alpha'[Na_c^+]+\beta'[Cl_i^-]}{[K_i^+]+\delta'[Ch_i^+]+\beta'[Cl_c^-]}\right\} \\ &= (RT/F)\ln\left\{\frac{[K_0^+]+\delta[Ch_0^+]+\beta[Cl_c^-]}{[K_i^+]+\delta'[Ch_i^+]+\beta'[Cl_c^-]} \times \frac{[K_c^+]+\alpha'[Na_c^+]+\beta'[Cl_i^-]}{[K_c^+]+\alpha[Na_c^+]+\beta[Cl_0^-]}\right\}. \end{aligned} \tag{5}$$

Clearly, if both the concentrations of the ions in the (intracellular) compartment between the two barriers (represented by symbols with the subscript c) and the extracellular chloride concentrations remain constant, then the second factor in the logarithm of equation (5) is simply a constant multiplier, and the equation reduces to the form (3), with a linear relationship between $\exp(FV/RT)$ and the external potassium concentration.

It is thus clear that a simple combination of ion-selective diffusion barriers, governed by the constant-field equation, and with constant concentrations of the contributing ions at its inner face, does not have properties consistent with the potassium-dependence of the extraneuronal potential. The non-linearity of the results when plotted as in Fig. 13 can, however, be accounted for if it is assumed that the concentration of potassium ions at the inner face of the barrier is not constant but changes in response to changes in the potassium concentration in the external medium. The simplest model embodying this concept consists of a single, ion-selective barrier; the potassium concentration at the inner face undergoes changes proportional to, but smaller than, the changes in the bathing medium, so that

$$[K_i^+] = [K_i^+]^0 + \mu([K_0^+] - [K_0^+]^0) = (1-\mu)[K_0^+]^0 + \mu[K_0^+], \tag{6}$$

where μ is a number less than unity, and the superscript '0' is used to denote the values of the concentrations in the 'undisturbed' system, which are assumed to be uniform throughout, so that $V = 0$. If it is assumed in view of the small effect on the electrical properties of replacing chloride ions by sulphate (see Fig. 11), that chloride movements are small, then potassium entering the inner compartment must replace an equal amount of choline, so that

$$[Ch_i^+] = [Ch_i^+]^0 - \mu([K_0^+] - [K_0^+]^0). \tag{7}$$

Substituting the values of $[K_i^+]$ and $[Ch_i^+]$ from equations (6) and (7) into equation (3), we thus have, for the potential difference across the barrier:

$$\exp(FV/RT) = \frac{(1-\delta)[K_0^+] + \delta Y + \beta[Cl_i^-]}{\mu(1-\delta)[K_0^+] + (1-\mu)(1-\delta)[K_0^+]^0 + \delta Y + \beta[Cl_0^-]} \tag{8}$$

$$= \frac{a[K_0^+] + c}{[K_0^+] + b}, \tag{9}$$

where

$$a = \frac{1}{\mu} \qquad (= 6{\cdot}42), \tag{10}$$

$$b = \frac{(1-\mu)(1-\delta)[K_0^+]^0 + \delta Y + \beta[Cl_0^-]}{\mu(1-\delta)} \qquad (= 190{\cdot}2), \tag{11}$$

$$c = \frac{\delta Y + \beta[Cl_i^-]}{\mu(1-\delta)} \qquad (= 173{\cdot}0). \tag{12}$$

In an experiment in which the only change made in the bathing solution is to replace choline ions by an equivalent quantity of potassium ions, the behaviour of the potential difference across the barrier will thus be given by an equation of the form (9), in which the three coefficients, a, b and c are constant. The continuous curve in Fig. 13 shows that an equation of this form can be fitted quite satisfactorily to the experimentally observed behaviour of the potential. The best values of the coefficients are given by the figures in brackets above.

Equation (10) gives the value of μ directly; from equations (11) and (12)

$$\mu(b-c) = (1-\mu)[K_0^+]^0 + \frac{\beta([Cl_0^-] - [Cl_i^-])}{1-\delta}, \tag{13}$$

so that $$\beta([Cl_0^-] - [Cl_i^-]) = 0{\cdot}059(1-\delta) \doteqdot 0{\cdot}059 \text{ if } \delta \text{ is small.} \tag{14}$$

Eliminating δ between equations (11) and (12) then leads to the simple result

$$\beta[Cl_i^-] \doteqdot \beta[Cl_0^-] = \mu c = 26{\cdot}95, \tag{15}$$

whence the relative chloride permeability of the barrier is

$$\beta = 0{\cdot}124, \tag{16}$$

the chloride concentrations on the two sides of the barrier being almost the same.

Alternatively, if the chloride concentrations on the two sides are regarded as identical, then the value of $\beta[Cl_i^-] = \beta[Cl_0^-]$ cancels out from equations (11) and (12), so that it is not determined. In either case, the result for δ, the relative permeability of the barrier to choline, is given by

$$\delta = \frac{\mu c - \beta[Cl_i^-]}{\mu c + Y}. \tag{17}$$

Clearly, if the value of $\beta[Cl_i^-]$ is that given by (15), then $\delta \doteqdot 0$, so that the only ions crossing the barrier are potassium and chloride, which is contrary to the original assumption that the ions contributing to the potential differences were chiefly potassium and choline. On the other hand, if the chloride concentrations on the two sides of the barrier are equal, then we can regard the chloride contribution as small, and

$$\delta \doteqdot \frac{\mu c}{\mu c + Y} = 0{\cdot}110. \tag{18}$$

The experimental results thus appear to be consistent with a model in which the peripheral diffusion barrier has a choline permeability approximately one-tenth of its potassium permeability, and allows the passage of potassium ions at such a rate, in relation to the structure of the underlying tissues, that the potassium concentration at its inner face changes, in response to a change in the external solution, but only to about one-sixth of the extent of the external change ($\mu = 0{\cdot}156$).

Analysis of diffusion

The values of the parameters of the barrier, as deduced above, can be used to predict the time course of the uptake of potassium ions from a high-potassium solution, and of efflux of sodium ions into a solution of choline or tris chloride. Diffusion of ions in the extracellular system is considered as a one-dimensional problem, as in the analysis of the stretched connective (Treherne *et al.* 1970), with boundary conditions governed by the flux across the peripheral barrier. A solution is to be found to the diffusion equation

$$D\frac{\partial^2 C}{\partial x^2} = \frac{\partial C}{\partial t}, \tag{19}$$

in the region $0 < x < l$, with boundary conditions

$$\partial C/\partial x = 0 \quad \text{at} \quad x = l, \tag{20}$$

$$-D(\partial C/\partial x) = M_K(C) \quad \text{at} \quad x = 0, \tag{21}$$

where $M_K(C)$ is the flux across the barrier, given by the constant-field theory as

$$M_K = (FV/RT)\,P_K \frac{[K_0^+] - [K_i^+]\exp(FV/RT)}{1-\exp(FV/RT)} = \delta'' Y P_K \ln\left\{\frac{C_0 + \delta'' Y + \beta''[Cl_i^-]}{C_i + \delta'' Y + \beta''[Cl_0^-]}\right\}, \tag{22}$$

from equation (3), writing $\delta'' = \frac{\delta}{1-\delta}$, $\beta'' = \frac{\beta}{1-\delta}$, and $C_i = C(0, t)$, the concentration at $x = 0$. Since the boundary condition expressed by equations (21) and (22) is non-linear, a functional solution to the diffusion equation is not available; the following numerical method (Carslaw & Jaeger, 1959) was used. The variables were divided into small increments,

$$x = m\epsilon \,(m = 0, 1, 2, \ldots, 5); \quad t = n\tau \quad (n = 0, 1, 2, \ldots).$$

The diffusion equation was replaced by the corresponding finite difference equation

$$\frac{D}{\epsilon^2}(C_{m+1,n} - 2C_{m,n} + C_{m-1,n}) = \frac{1}{\tau}(C_{m,n+1} - C_{m,n}), \tag{23}$$

which gives the value of the concentration at $x = m\epsilon$, $t = (n+1)\tau$, in terms of the concentrations at $x = (m-1)\epsilon$, $m\epsilon$ and $(m+1)\epsilon$, at time $t = n\tau$. If the concentration

profile at time $t = 0$ is known, concentrations at all subsequent times $t = n\tau$ can be calculated by repeated application of equation (23).

The best simple method of applying the boundary conditions is to calculate the concentrations at the fictitious points $m = -1$ and $m = 6$. These must be such that the corresponding concentration gradients at $m = 0$ and $m = 5$ would cause the same fluxes as those specified by the boundary conditions. If the flux at $x = 0$ is given by $M_K = Df(C_{0,n})$, where $f(C_{0,n})$ is obtained from (22), this condition is expressed by

$$C_{-1,n} = C_{1,n} + 2\epsilon\left\{f(C_{0,n}) + \frac{1}{2}(C_{0,n+1} - C_{0,n})\left[\frac{\partial f}{\partial C}\right]_{0,n}\right\}. \tag{24}$$

Combining this expression with (23), $C_{0,n+1}$ can be calculated.

At $x = l$ ($m = 5$), there is no flux at the termination of the channel, so that

$$C_{6,n} = C_{5,n}. \tag{25}$$

Repetitive calculations were made using a Hewlett–Packard 9100B desk calculator and $X-Y$ Plotter, using $l = 800\,\mu m$ ($\epsilon = 0{\cdot}016$ cm) (cf. Treherne *et al.* 1970) and $\tau = 5$ s. Fig. 14 shows the behaviour of the potassium concentrations (*a*) at the peripheral and (*b*) at the axonal end of the intercellular cleft, following a step change in the

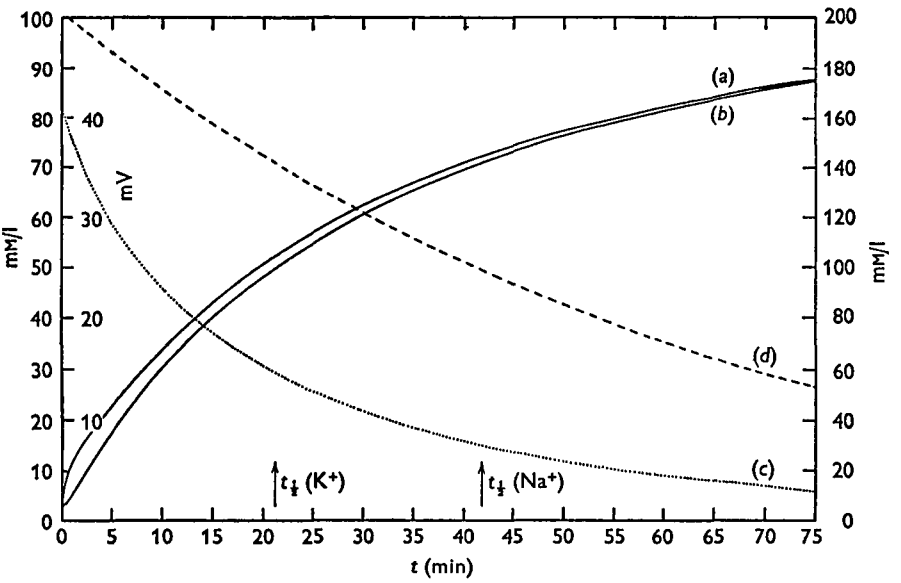

Fig. 14. Movements of ions in the model system. The external concentration is changed instantaneously at time $t = 0$. (*a*) and (*b*) Potassium concentrations at peripheral and axonal ends respectively of the extracellular system, following an increase of the external concentration from 3·1 to 100 mM/l. (*c*) Corresponding behaviour of the potential difference across the peripheral barrier. (*d*) Sodium concentration at the axonal end of the system, following a decrease of the external concentration from 200 mM/l to zero, sodium ions being replaced by those of choline.

The left-hand scale of ordinates in mM/l refers to curves (*a*) and (*b*); the scale of mV to curve (*c*); and the right-hand scale of mM/l to curve (*d*). The arrows indicate half-times for potassium uptake and sodium efflux.

external potassium concentration from 3·1 to 100 mM, the initial concentration in the system being regarded as everywhere 3·1 mM. The value of the potassium permeability of the barrier was chosen arbitrarily as 8×10^{-5} cm s^{-1}. Unfortunately, it is very

difficult to give a quantitative interpretation of the results of this stage in the investigation. As has already been mentioned, the model system provides only a highly simplified representation of the extracellular diffusion pathways within the connective. Thus, the figure given above for the potassium permeability of the peripheral barrier is of quite arbitrary significance, since it includes the ratio of the area of barrier available for the passage of ions to the total cross-sectional area of the diffusion pathway which is effectively in series with it. In reality, the perineurium overlies a mosaic of glial cells and intercellular clefts, the exact analysis of which would be extremely difficult, even if sufficient electron-microscopical data could be accumulated.

Qualitatively, Fig. 14 shows that the concentration of potassium ions immediately inside the barrier, curve (*a*) is expected to rise rapidly at first, subsequently increasing more slowly, towards the value at the outer face. The potential difference across the barrier, as derived from equation (3), curve (*c*) thus falls rapidly at first, from an initially high value, and then more slowly. The initial high value would be unlikely to be attained in practice, since the raised concentration of ions at the outer surface of the barrier would not be established instantaneously owing to the presence of unstirred layers in the bathing medium. The value reached by the potential when it has begun to fall more slowly may be interpreted as representing the extraneuronal potential level observed in the experiments. Curve (*b*) in Fig. 14 shows the corresponding behaviour of the potassium concentration at the inner, axonal end of the extracellular cleft, which rises initially more slowly, subsequently following a parallel course to that at the outer end, but with a lag time comparable with that required for diffusion in the stretched connective (Treherne et al. 1970).

The detailed behaviour of the concentration is likely to be affected significantly by exchange of ions with the intracellular (glial) compartment. This is in contrast with the situation in the stretched connective (Treherne *et al.* 1970), where movements of ions in the extracellular spaces were shown to be much more rapid than exchange across the cell membranes, so that the latter could be neglected. In the present case, the flux of ions across the peripheral barrier is comparatively slow, so that it can be shown, for example, that movement in the extracellular system will be significantly affected by exchange across the glial cell membranes, even if the latter have a potassium permeability as low as 10^{-8} cm s^{-1}. Since the rate of uptake by the glia would presumably increase with the extracellular potassium concentration, the effect would be to accentuate the difference between the initial rapid rise and the subsequent slow approach to equilibrium (Fig. 14). This is in accord with the experimental results, which show that the extraneuronal potential does not decline appreciably during several minutes exposure to a high-potassium solution, showing that the concentration at the inner face of the barrier has not increased significantly beyond the initial, rapidly established level during this time. It would also explain the observations of Treherne *et al.* (1970), that the axonal action potential is not appreciably attenuated after 10 min exposure to a high-potassium solution, showing that the extra-axonal concentration increases only very slowly under these conditions. Diffusion in the extracellular system is thus presumably much retarded by uptake of potassium ions by the glia.

The potassium permeability, used to obtain curves (*a*) and (*b*) in Fig. 14, was chosen to give a concentration of 15·6 mM/l at the inner face of the barrier after 5 min

The calculated potential difference across the barrier at this time will thus agree with the experimental figure of 26 mV. Corresponding calculations for other values of the external potassium concentration produce results in broad agreement with the experimental figures, although in view of the considerations discussed above it is unreasonable to expect more than a qualitative agreement.

Effects of other ions

The effect on the potential difference across the barrier of replacing sodium in the bathing medium by other cations is clearly dependent on the ability of the latter to penetrate the barrier. Ions which penetrate more readily than sodium (potassium, rubidium, caesium, TEA) cause a positive-going potential change; ions which penetrate less readily than sodium cause a negative-going change, since the principal effect is to cause sodium efflux from the system. In the case of divalent ions, particularly calcium, there appears also to be a secondary effect, which could be interpreted in terms of changes in the permeability properties of the barrier. Thus, the initial, transient negative potential caused by high calcium (Fig. 8) is presumably due to sodium efflux, which is subsequently reduced, possibly owing to a fall in the sodium permeability of the barrier, caused by the high concentration of calcium ions. There is, however, insufficient evidence to form a basis for more detailed investigation.

Movements of other ions in the system can, of course, be investigated by a method analogous to that used for potassium, provided that the permeability of the barrier to them is known. An interesting result is obtained in the case of sodium; if an intact connective is placed in sodium-free, choline solution, the resulting potential differences across the barrier will be given by

$$\exp(FV/RT) = \frac{[K_0^+]+\delta[Ch_0^+]+\beta[Cl_i^-]}{[K_i^+]+\alpha[Na_i^+]+\beta[Cl_0^-]}. \qquad (26)$$

Experimentally, $V = -11$ mV (Fig. 1) from which it can be calculated that α, the relative sodium permeability of the barrier, has the value 0·190. The subsequent time course of sodium efflux, calculated by the method described above, is shown as curve (*d*) in Fig. 14. The half-time for decline of the extraaxonal sodium concentration is 42 min, as compared with a predicted 21 min for potassium uptake. The model thus predicts a degree of asymmetry between sodium efflux and potassium uptake, resulting from the different permeabilities of the peripheral barrier to these ions.

List of Symbols

$[K_0^+]$, $[Ch_0^+]$, $[Ca_0^{2+}]$, $[Cl_0^-]$ = Concentrations* of potassium, choline, calcium and chloride ions in the external medium (mM/l).

$[K_i^+]$, etc. = Concentrations of the respective ions at the inner face of the peripheral diffusion barrier (mM/l).

P_K, etc. = Permeabilities of the barrier to the respective ions (cm s^{-1}).

V = Potential difference across the barrier (mV).
= 'Extraneuronal potential.'

* Strictly, the equations for the potential difference should be derived from the activities of the ions concerned. It is assumed, for convenience, that these are approximately equal to their concentrations. If the activity coefficients on the two sides of the barrier are similar, the effect of this approximation will not be serious (cf. Moreton, 1968*b*).

F = The Faraday (coulombs mole^{-1}).
R = Gas constant (joules mole^{-1} °K^{-1}).
T = Absolute temperature (°K).
M_K, etc. = Inward net fluxes of the respective ions across the barrier (mole cm^{-2} s^{-1}).
$\delta = P_{Ch}/P_K$.
$\alpha = P_{Na}/P_K$.
$\beta = P_{Cl}/P_K$.
$Y = [K_0^+] + [Ch_0^+]$. (mM/l).
μ = (Change in $[K_i^+]$/Change in $[K_0^+]$.)
D = Diffusivity of potassium ions ($= 1{\cdot}8 \times 10^{-5}$ cm^2 s^{-1}).*
C = Local concentration of potassium ions in the extracellular system (mM/l).
C_0 = Concentration of potassium ions in the bathing medium (mM/l).
x = Distance along the extracellular pathway, measured from the peripheral barrier (cm).
l = Total length of extracellular pathway, from peripheral barrier to axon (cm).
t = Time, measured from the time when the bathing solution is changed (s).
Other symbols are as defined in the text.

SUMMARY

1. Measurements have been made of the extraneuronal potential changes produced by replacement of sodium ions with other organic and inorganic cations in the solution bathing isolated abdominal connectives.

2. On the basis of the observed extraneuronal potential changes it is possible to arrange monovalent cations in the following sequence of effectiveness:

$$K^+ > Rb^+ > Cs^+ > TEA^+ > Na^+ > Li^+ > choline^+ > tris^+.$$

3. It is concluded that the ionic dependence of the extraneuronal potentials is similar to that of a theoretical model system consisting of a perineurial diffusion barrier in series with a long, narrow channel representing the system of intercellular clefts connecting the inwardly facing surface of the perineurium with the extraaxonal fluid.

4. On the basis of this model the effect of high external concentrations of, for example, potassium ions would be to depolarize the outwardly facing perineurial cell membranes. The considerable departure of the observed potentials from the values predicted by the Nernst equation can be accounted for in terms of the short-circuiting effect, due to the finite ionic permeability of the tight junctions between perineurial cells. Qualitative predictions can also be made concerning the rates of movement of potassium and sodium ions in the extracellular system.

We gratefully acknowledge the expert technical assistance of Mr P. K. Schofield and the help of Mr J. Rodford in preparing the diagrams.

* It is assumed that diffusion in the extracellular system is unrestricted (cf. Treherne *et al.* 1970).

REFERENCES

ARMSTRONG, C. M. (1966). Time course of TEA^+ induced anomalous rectification in squid giant axons. *J. gen. Physiol.* **50**, 491–503.

BINSTOCK, L. & LECAR, L. (1969). Ammonium ion currents in the squid giant axon. *J. gen. Physiol.* **53**, 342–61.

CARSLAW, H. S. & JAEGER, J. C. (1959). *Conduction of Heat in Solids*, 2nd edition, p. 469. Oxford University Press.

DIAMOND, J. M. & WRIGHT, E. M. (1969). Biological membranes: the physical basis of ion and non-electrolyte selectivity. *A. Rev. Physiol.* **31**, 581–646.

GOLDMAN, D. E. (1943). Potential, impedance and rectification in membranes. *J. gen. Physiol.* **27**, 37–60.

HODGKIN, A. L. & KATZ, B. (1949). The effect of sodium ions on the electrical activity of the giant axon of the squid. *J. Physiol.* **108**, 37–77.

KEYNES, R. D. (1969). From frog skin to sheep rumen: a survey of transport of salts and water across multicellular structures. *Q. Rev. Biophys.* **2**, 177–281.

KOEFOED-JOHNSEN, V. & USSING, H. H. (1958). The nature of the frog skin potential. *Acta physiol. scand.* **42**, 298–308.

LANE, N. J. & TREHERNE, J. E. (1969). Peroxidase uptake by glial cells in desheathed ganglia of the cockroach. *Nature, Lond.* **223**, 861–2.

LANE, N. J. & TREHERNE, J. E. (1970). Uptake of peroxidase by the cockroach central nervous system. *Tissue and Cell* **2**, 413–25.

LINDLEY, B. D. & HOSHIKO, T. (1964). The effects of alkali metal cations and common anions on the frog skin potential. *J. gen. Physiol.* **47**, 749–71.

MADDRELL, S. H. P. & TREHERNE, J. E. (1967). The ultrastructure of the perineurium in two insect species (*Carausius morosus* and *Periplaneta americana*). *J. Cell Sci.* **2**, 119–28.

MORETON, R. B. (1968*a*). Ionic mechanism of the action potentials of giant neurones of *Helix aspersa*, *Nature Lond.* **219**, 70–1.

MORETON, R. B. (1968*b*). An application of the constant-field theory to the behaviour of giant neurones of the snail, *Helix aspersa*. *J. exp. Biol.* **48**, 611–23.

PICHON, Y. & TREHERNE, J. E. (1970). Extraneuronal potentials and potassium depolarization in cockroach giant axons. *J. exp. Biol.* **53**, 485–93.

SATTELLE, D. B. (1970). Neuronal function in freshwater gastropods. Ph.D. Thesis: University of Cambridge.

SJODIN, R. A. (1959). Rubidium and caesium fluxes in muscle as related to the membrane potential. *J. gen. Physiol.* **42**, 983–1003.

STERN, K. H. & AMIS, E. S. (1959). Ionic Size. *Chem. Rev.* **59**, 1–64.

TREHERNE, J. E. (1962). The distribution and exchange of some ions and molecules in the central nervous system of *Periplaneta americana*. *J. exp. Biol.* **39**, 193–217.

TREHERNE, J. E., LANE, N. J., MORETON, R. B. & PICHON, Y. (1970). A quantitative study of potassium movements in the central nervous system of *Periplaneta americana*. *J. exp. Biol.* **53**, 109–36.

USSING, H. H. (1960). *The Alkali Metal Ions in Biology*. Springer Verlag.

USSING, H. H. (1965). Transport of electrolytes and water across epithelia *Harvey Lect.* **59**, 1–30.

YAMASAKI, T. & NARAHASHI, T. (1959). The effects of potassium and sodium ions on the resting and action potentials of the giant axon of the cockroach. *J. Insect. Physiol.* **3**, 146–58.