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KINETICS OF SODIUM AND LITHIUM MOVEMENTS ACROSS THE BLOOD–BRAIN BARRIER OF AN INSECT

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SUMMARY

The electrical responses of axons were used to monitor the time-course of a change in the concentration of an ion species in the fluid bathing the axons in connectives of isolated cockroach nerve cords. Initial exposure of the connectives to sodium-deficient Ringer resulted in a depletion of extra-axonal sodium which was much slower than the restoration observed on return of the sodium Ringer. It is suggested that this asymmetry could result from a sodium reservoir which delays the initial decline. Subsequent net inward and outward movements of sodium ions were rapid and symmetrical. Unlike sodium ions, lithium ions were apparently unable to reach the axon surfaces following sodium depletion. In view of the similar properties of sodium and lithium ions in many biological systems it is therefore unlikely the the sodium movements were passive. Instead, the results support the idea of net sodium transport by the perineurial and/or glial elements.

INTRODUCTION

Ionic homeostasis of the brain microenvironment is of obvious importance in determining neuronal function. It is found in its most extreme form in some insects, where the ionic environment of the nerve cells can be constantly maintained in a balance that is quite different from that found in the body fluids. Such an extreme requirement is not found in other animal classes (see Abbott & Treherne, 1977). Insects are therefore convenient subjects for studies of ionic regulation of the neuronal environment.

It has been suggested that the ionic composition of the extracellular fluid in the insect central nervous system (C.N.S.) is regulated by the surrounding glial and perineurial cells (see Treherne, 1974, 1975). Such regulation would be augmented by the peripheral blood–brain barrier which limits passive intercellular exchanges with the blood (Treherne & Pichon, 1972). Homeostasis of the neuronal environment could be provided by the active transfer of ions across some components of the barrier system. The observation that there are rapid cation fluxes between the C.N.S. and the bathing medium (e.g. Treherne, 1965; Tucker & Pichon, 1972) lends some support to this idea.

In this study the kinetics of ion movements across the barrier have been studied by employing the electrical responses of insect axons as indicators of the ion levels in the

immediate vicinity of the axon surfaces under various experimental conditions (cf. Frankenhaeuser & Hodgkin, 1956). A primary objective was to provide a foundation for pharmacological studies on the regulatory system (preliminary report: Schofield & Treherne, 1975).

MATERIALS AND METHODS

Experimental procedure

The abdominal nerve cord of adult male cockroaches, *Periplaneta americana* L., was placed in a Perspex chamber. Both sucrose-gap and intracellular recordings were made from the penultimate pair of connectives. Action potentials (a.p.s) were evoked by electrical stimulation of the posterior end of the cord. 'Sucrose-gap' recording (employing mannitol rather than sucrose) was essentially as described in earlier investigations (e.g. Treherne, Schofield & Lane, 1973), giving an extracellular recording from the axons and enveloping tissue. Intracellular recording was with glass microelectrodes filled with 3 M-KCl, occasionally bevelled to assist penetration, and of 15–30 MΩ resistance. Axons were impaled by use of a Leitz micromanipulator. The two recording methods were sometimes used simultaneously with no modification to either, but when intracellular recordings alone were made, the mannitol was replaced by grease. For some experiments, the connective tissue sheath was first removed from the penultimate connectives by microsurgery before the cords were placed in the chamber. The de-sheathing damages the underlying perineurium (Lane & Treherne, 1970) and renders the axons more accessible to the bathing medium.

Ringer flowed continuously over the penultimate pair of connectives. With sheathed intact) connectives a period of at least 1 h in sodium Ringer was allowed for stabilization of action and d.c. potentials; with desheathed connectives, half an hour was allowed. The solution was then rapidly changed for another by operating a multi-way non-return valve (Holder & Sattelle, 1972).

Ringer solutions

The basic Ringer solution that was employed in these investigations was that devised as a mimic of extra-axonal fluid, for use with desheathed connectives, by Yamasaki & Narahashi (1959); it was of pH 7·2 and had the following composition (mM): Na, 214; K, 3·1; Ca, 1·8; Cl, 216·9; H_2PO_4, 0·2; HPO_4, 1·8.

To devise a Ringer suitable for use with intact connectives, as a blood substitute, is made difficult by dietary, diel and seasonal variations in blood ion levels (Pichon & Boistel, 1963; Pichon, 1970; Treherne, Buchan & Bennett, 1975; Lettau *et al.* 1977). Some experiments with intact connectives were made employing the 'blood' Ringer of Bennett, Buchan & Treherne (1975), but a.p. amplitude always declined while the preparations were in this Ringer during the period required for the experiments, whereas in Yamasaki & Narahashi's Ringer there was no such decline.

Ringers were derived from the basic Ringer by completely or partly substituting sodium with either lithium, potassium, tris, choline or sucrose. These solutions were isotonic, with the exception of the tris Ringer which had a 22·6 mM chloride deficit to obtain the correct pH. A grossly hypertonic lithium Ringer was made by dissolving urea in lithium Ringer to give a urea concentration of 3·0 M.

RESULTS

Relationships between ion concentrations and action potential amplitude or resting potential, recorded from the penultimate connectives, are given in the first section below. These relationships are used in subsequent sections to monitor the time-course of a change in ion concentration at the level of the axon surface following a step-change in concentration in the medium bathing the connectives.

Characterization of axonal responses

Using desheathed preparations the effect of sodium upon action potential amplitude (Fig. 1) was determined by substitution of tris for sodium, and the effect of potassium upon resting potential (Fig. 2) was determined by substitution of potassium for sodium. The response to a given concentration was allowed to stabilize before it was measured.

There was rough agreement between the two techniques for the effects of potassium upon resting potential, but lowering the sodium concentration had less effect on action potential amplitude in the sucrose-gap recordings than in the intracellular recordings (Fig. 1). The deviation was most marked at low Na concentrations, where conduction block might be expected to influence the results.

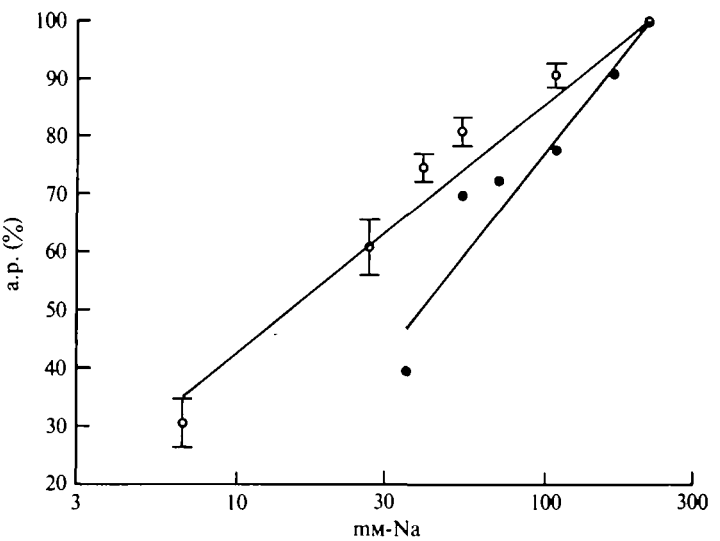

Fig. 1. Relationships between Na concentration and action potential amplitude in desheathed connectives. Intracellular recordings (●) obtained by Yamasaki & Narahashi (1959): mean amplitude at 100% a.p., 95 mV. Sucrose-gap recordings (○) from six preparations: mean amplitude at 100% a.p., 37 mV; 2 S.E. 6·6. The intracellularly recorded spikes show greater sensitivity to a change in Na concentration than the compound a.p.s. Both recording methods show a reasonable straight-line relationship between amplitude and logarithm of concentration. Lines fitted by linear regression on polar co-ordinates with origin at 214·0 mM-Na, 100% a.p. Vertical lines, ±2 S.E.

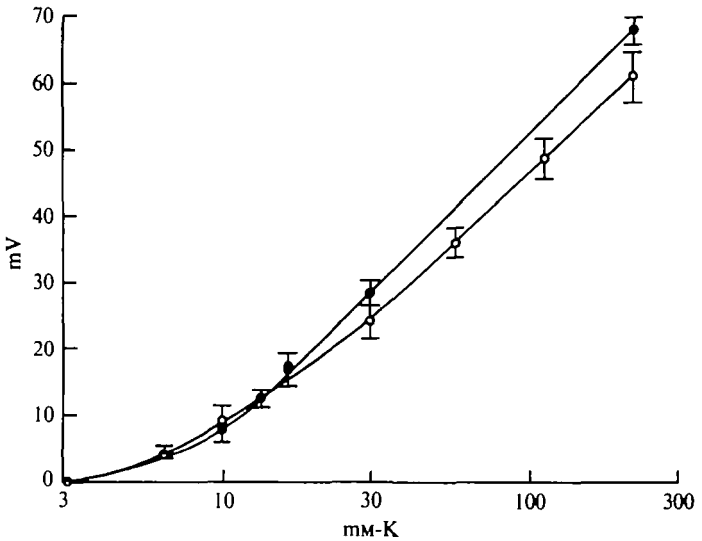

Fig. 2. Relationships between K concentration and resting potential. Intracellular recordings (●) from six preparations: mean r.p. at 3·1 mM-K, −79 mV; 2 S.E., 4·0. Sucrose-gap recordings (○) from six preparations. Where there is a straight-line relationship, voltage change per decade concentration change is 46 mV for intracellular and 43 mV for sucrose-gap. Lines fitted by eye through mean values; vertical bars ±2 S.E.

Estimated sodium movements in intact preparations

Initial exposure of intact preparations to sodium-deficient (tris or choline substituted) Ringer could cause a decline in action potential amplitude. The decline occurred after a variable period (frequently of 20 min or more) and sometimes, after a transient, paradoxical increase in amplitude. A relatively prompt recovery could be obtained by returning the sodium Ringer. In some preparations no appreciable decline was observed over periods as long as 100 min.

Changes in action potential amplitude were interpreted as net changes in extra-axonal sodium concentration only when depletion was achieved with complete and not partial substitution of Ringer sodium. The technique did not assume a value for extra-axonal sodium under equilibrium conditions (an assumption which would almost certainly be false in the presence of active regulation). Instead, concentration was expressed in relative terms (C') with a value of 1·0 when the preparation was in the sodium Ringer (e.g. Fig. 3). The restoration of extra-axonal sodium was always seen to be faster and less variable in rate than the loss: in eight sucrose-gap preparations the mean half-time for recovery was 68 s (S.E. 10). (Where action potential amplitude at the origin of the decline differed from that at the end of the recovery, the former was taken as reference for the decline, the latter for the recovery. Rates obtained in this way were not compared if the amplitude differed by more than 5%.) The asymmetry was seen with both intracellular and sucrose-gap recordings from intact or stretched preparations.

The possibility exists that cockroach axons can adapt to reduced extracellular sodium concentration during sodium depletion of intact connectives, either by change

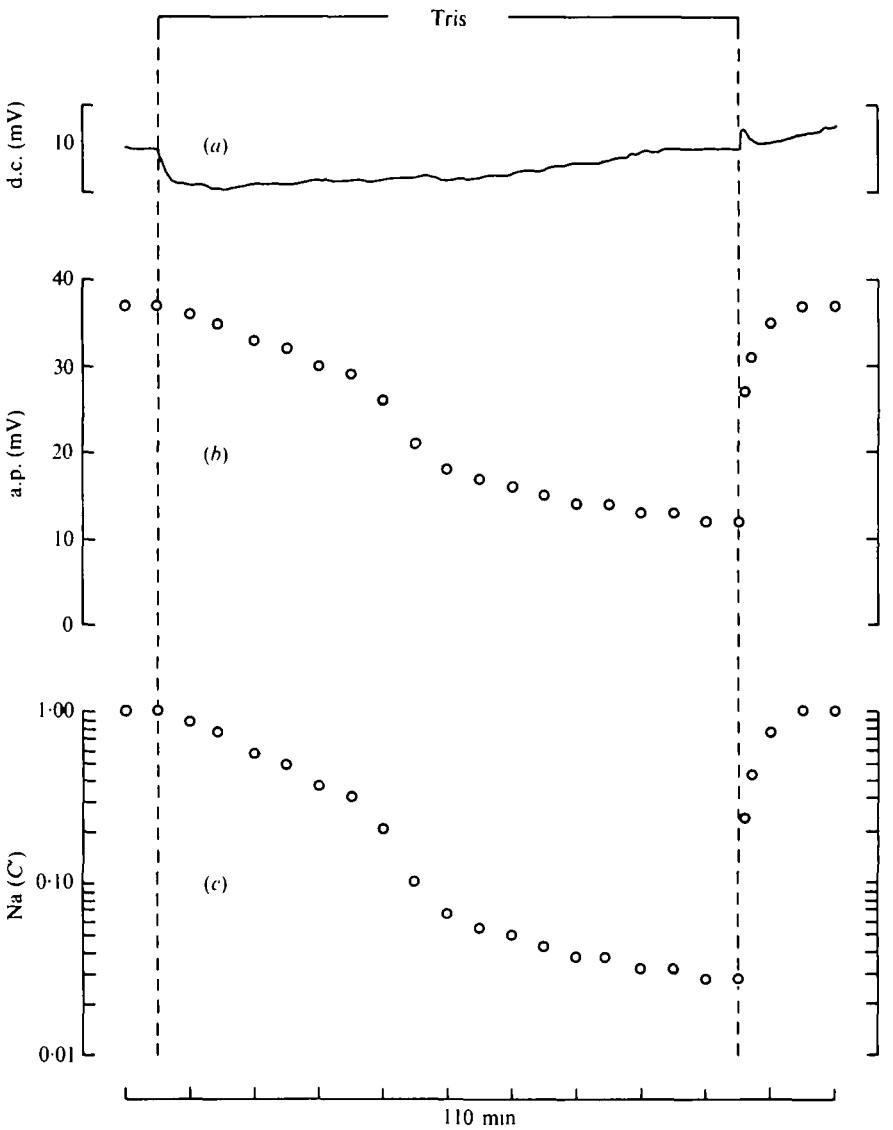

Fig. 3. Estimation from a sucrose-gap recording (*a*, *b*) of the change of extra-axonal sodium concentration (*c*) in an intact preparation in response to substitution of tris for sodium in the bathing Ringer. Relative Na concentration is given as C' values, which were derived from the action potential amplitudes as described in the text.

in active membrane characteristics or change in $[Na^+]_i$ and thus counter a reduction in action potential amplitude. To test this, the axons of desheathed connectives were exposed to 53·5 mM-Na (tris-substituted) Ringer for periods of up to 40 min. A.p. amplitude was then reduced by around 20% (cf. Fig. 1). Sucrose-gap recorded action potentials showed no sign of recovery, indicating that the axons could not adapt and were therefore unlikely to adapt in intact preparations.

When intact preparations were repeatedly exposed to Na-free solutions, asymmetry

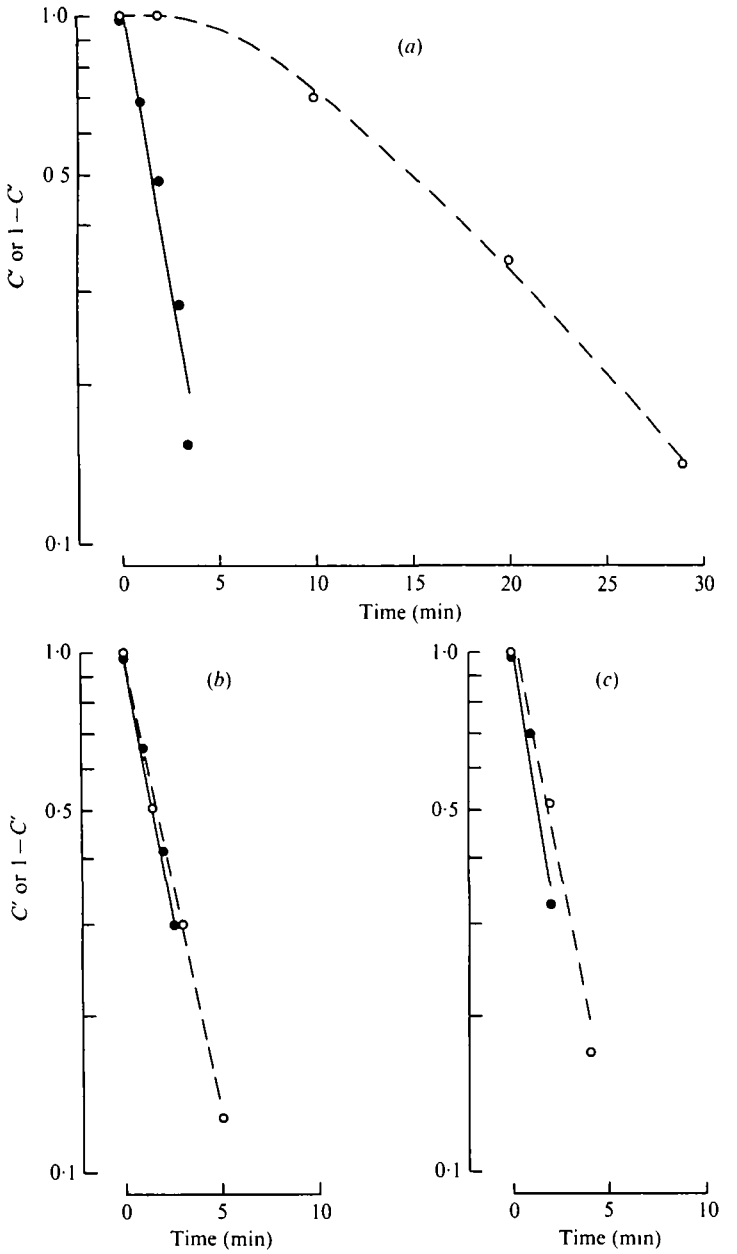

Fig. 4. Changes in extra-axonal sodium concentration in an intact preparation, resulting from three successive exposures to sodium-free (sucrose-substituted) Ringer. 5 min in basic Ringer was allowed between exposures (*a*) and (*b*); 40 min between exposures (*b*) and (*c*). Estimation from a sucrose-gap recording: depletion of Na expressed as C' values (○); recovery expressed as $1-C'$ values (●) to simplify comparison with depletion. Half-times for decline: (*b*), 98 s; (*c*), 113 s. Half-times for recovery: (*a*), 95 s; (*b*), 90 s; (*c*), 81 s. Lines fitted by linear regression on rectangular co-ordinates except for the broken line in (*a*) which was fitted by eye.

was not seen for the second and subsequent exposures even after allowing 60 min between exposures. Since the tris or choline could have affected the preparation, the experiments were repeated with sucrose as a substitute, with the same results (Fig. 4).

Recovery of sodium appeared to be largely due to a first-order process, as indicated by the straightness of the plots of log $(1-C')$ against time. Loss of sodium during initial depletion appeared to be a complex process, whereas loss in subsequent exposure did not (Fig. 4).

Estimated sodium and potassium movements in desheathed preparations

In desheathed preparations, rates of Na movements were estimated essentially as in intact preparations, by tris substitution of Na and observation of changes in a.p. amplitude. Rates of K movements were estimated by substituting Ringer Na with K and observing changes in resting potential. Unlike in experiments with intact preparations, however, substitution of Na was only partial, since complete replacement produced changes that were too rapid to follow accurately and was not necessary with the method of analysis that was used. This method assumes that the concentration of the studied ion is the same as the concentration in the Ringer when equilibrium is reached, which was thought a reasonable assumption in view of the relatively leaky nature of desheathed preparations (cf. Treherne *et al.* 1970). The formula was $(C_t-C_\infty)/(C_0-C_\infty)$, where C_t was the concentration at any time, t; C_0 was the concentration at the start of the change; and C_∞ was the concentration when the change

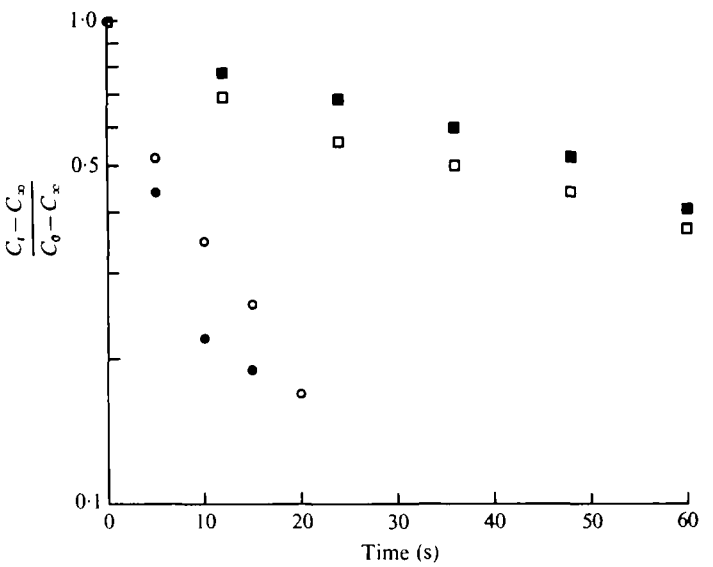

Fig. 5. Changes in extra-axonal sodium and potassium concentration in a desheathed preparation, estimated from a sucrose-gap recording, according to the formula $(C_t-C_\infty)/(C_0-C_\infty)$ as described in the text. Exposure to a Ringer sodium concentration of 40·1 mM (tris substituted), resulted in a depletion of extra-axonal Na (○), which was followed in basic Ringer by a recovery (●); as indicated by action potential amplitude. Substitution of Ringer sodium by potassium, to produce a potassium concentration of 16·5 mM resulted in an elevation of extra-axonal K (■), which was followed in basic Ringer by a decline (□); as indicated by resting potential.

was complete. (Rates during exposure were not compared with rates during recovery if reference a.p. amplitude differed by more than 5% or if reference d.c. levels differed by more than 5 mV.)

Similar results were obtained with both recording methods. In each preparation, the loss and subsequent recovery of extra-axonal sodium appeared to be first order processes, and had similar half-times (Fig. 5). Similar findings were made for the entry and exit of potassium ions, but the movements were slower than the sodium movements (Fig. 5). For eight desheathed preparations, recorded under sucrose-gap, mean half-times for sodium loss and recovery were 12 s (S.E. 3·2) and 8 s (S.E. 2·1) respectively, and the mean half-time for potassium loss and entry were 32 s (S.E. 4·3) and 36 s (S.E. 5·2) respectively. The half-time for potassium entry is similar to the value of 24 s (S.E. 5·2) observed by Treherne *et al.* (1970).

When preparations were exposed successively to different magnitudes of change in concentration of an ion in the bathing Ringer, the rates of change in extra-axonal concentration were seen to be similar when comparison was made between the successive exposures.

Estimated lithium movements in desheathed and intact preparations

Lithium ions are known to substitute for sodium ions in carrying the inward current of the action potential in cockroach giant axons (Narahashi, 1963), so a.p. amplitude was used to determine the rate of access of lithium ions to the axon surfaces. Tris Ringer was applied to preparations, to deplete them of extra-axonal sodium, and was then replaced by lithium Ringer. The sucrose-gap was adopted as the recording technique for these and further experiments because it was difficult to maintain impalements of the axons for the time that was required to deplete intact connectives of sodium.

In desheathed preparations application of the lithium caused a near recovery of action potential amplitude. If allowance was made for the incomplete nature of the recovery, its rate appeared similar to that of the recovery obtained by applying sodium Ringer. The lithium-mediated action potentials then declined in amplitude, to about 60% of full amplitude within 15 min. This effect is presumed to result from the accumulation of lithium within the smaller axons. Restoration of Ringer sodium produced a slow recovery of these action potentials to about 90% of their original amplitude.

With intact preparations, lithium was never seen to restore the action potential amplitude after a decline caused by exposure to tris Ringer, although a slight temporary increase was occasionally seen (Fig. 6). The recovery that was obtained by subsequent exposure to sodium Ringer was slower than that recorded with no lithium treatment. For example, the half-time for the recovery illustrated in Fig. 6*a* is 400 s, which may be compared with the half-time of 68 s for intact preparations given above.

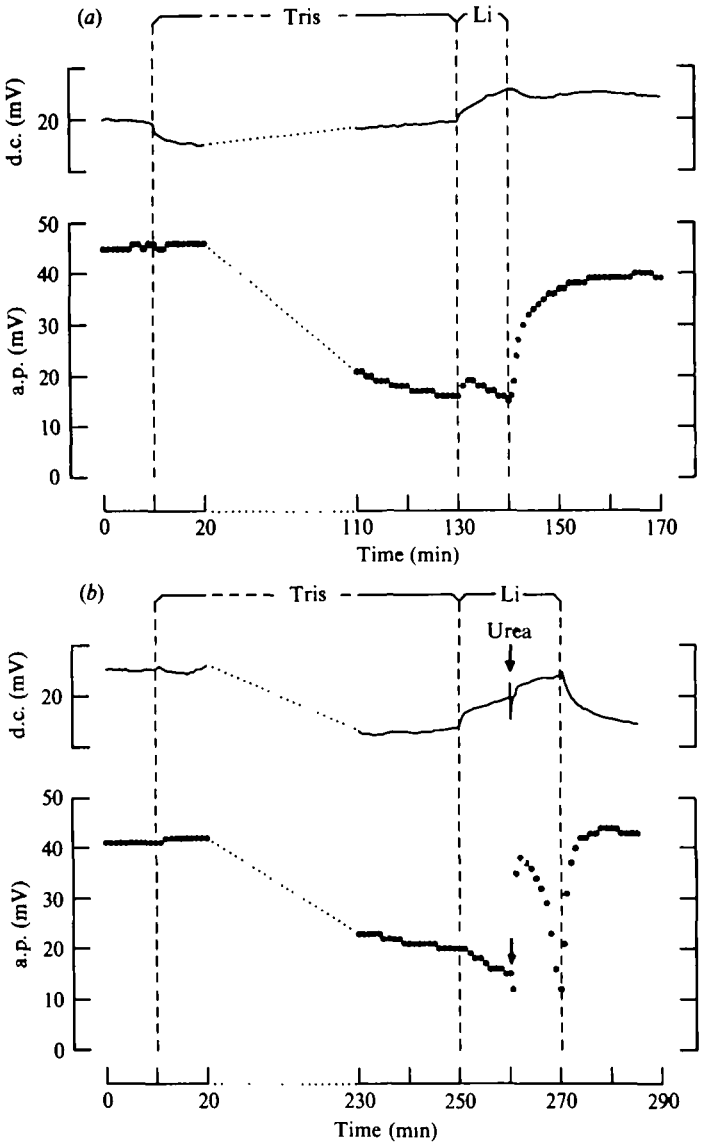

Fig. 6. Effects upon intact preparations of replacing tris with lithium following a decline in action potential amplitude in tris Ringer, recorded with the sucrose-gap. In (*a*), action potential amplitude does not recover until the lithium is replaced with sodium. In (*b*) action, potential amplitude is recovered temporarily in the lithium Ringer by raising the osmotic pressure of the Ringer by 3·0 M urea for 30 s.

Estimated lithium movements in urea-treated preparations

The contrast between the recovery of action potentials caused by lithium in desheathed as compared with intact preparations suggests that lithium ions were unable to cross the blood–brain barrier of the intact connectives. To test this, intact

preparations were briefly exposed to a high concentration of urea, a procedure known to disrupt the blood–brain barrier (Treherne *et al.* 1973). During exposure to lithium Ringer, after depletion of extra-axonal sodium in tris Ringer, the Ringer was made hypertonic with urea by 3·0 M for 15 or 30 s. Following this, action potential amplitude was nearly, but only temporarily, restored in the lithium Ringer (Fig. 6*b*). It thus appears that lithium ions do not gain access to the axons of intact preparations because of the blood–brain barrier, and that, if access is provided, lithium ions can transiently support the action potentials in these preparations, as in desheathed ones.

Extraneuronal potentials

The changes in d.c. potential that were recorded in intact preparations on exposure to tris or lithium Ringer could occur without corresponding change in action potential amplitude and so can be presumed to be of extraneuronal origin. Such potential changes have been postulated to arise from diffusional potentials across the perineurium (Treherne *et al.* 1970; Pichon, Moreton & Treherne, 1971).

DISCUSSION

Insects possess two features, apparently unique in the animal kingdom, which necessitate ionic homeostasis of the fluid environment of their central nerve cells.

First, a blood–brain barrier entirely restricts the extraneuronal fluid. In vertebrates, the brain cells are not structurally sequestered in this fashion because, although there is a blood–brain barrier, the fluid between the brain cells is confluent with a large reservoir (the cerebro-spinal fluid). In invertebrates other than insects there appears to be a relatively free movement of small water-soluble ions and molecules between the blood and the neuronal surfaces (Abbott & Treherne, 1977). Insect nerve cells are, therefore, contained in a very restricted fluid environment, formed by the closely applied glial and neuronal membranes; the intercellular channels are frequently only a few (ten) nm in width. This aggravates a need for ionic homeostasis, at least in the short term, for in the absence of such a mechanism the intercellular fluid in the insect central nervous system would be liable to an accumulation or depletion of ions during sustained nervous activity. Even the steady-state passive leakage of potassium from glial cells into clefts 20 nm in width could change the intercellular potassium concentration by 1 mM/s (Treherne *et al.* 1970).

Second, the ionic composition of the blood of some insects is an unsuitable extraneuronal medium. In two phytophagous insects the concentration of sodium relative to that of potassium is too low to allow action potential generation (Treherne & Maddrell, 1967*a*, *b*; Weidler & Diecke, 1969; Pichon, Sattelle & Lane, 1972). In the cockroach (an omnivorous insect with a relatively high blood sodium level) axonal depolarization sufficient to significantly increase apparent sodium inactivation is induced in desheathed preparations by potassium activities equivalent to those of the blood (Thomas & Treherne, 1975). In these species and many others (unless they possess novel neuronal conduction mechanisms), ionic concentration gradients have to be maintained between the fluid at the neuronal surfaces and the blood; there is thus a need for long-term ionic homeostasis of the brain microenvironment.

The present study provides kinetic evidence that the regulation of extraneuronal sodium levels in the cockroach is mediated, at least in part, by membrane transport mechanisms. This contrasts with the situation in the relatively 'leaky' nervous connectives of decapod crustaceans (the only other invertebrates in which regulation of the neuronal ionic environment has been studied) where limited, short-term, ionic homeostasis has been postulated to result from essentially passive mechanisms (Abbott, Moreton & Pichon, 1975; Abbott & Pichon, 1976; Abbott, Pichon & Lane, 1977).

The results show that during initial exposure of intact connectives to sodium-deficient Ringer, there was a decline in extra-axonal sodium concentration that was slower than the recovery observed on return of the sodium Ringer. With subsequent exposures, the decline was as rapid as the recovery (Figs. 3, 4). A simple explanation for this initial asymmetry and its subsequent loss, is that there was an increase in the passive intercellular permeability of the peripheral blood–brain barrier. However, it seems unlikely that this occurred because no comparable permeability to Li was indicated at the end of an initial sodium depletion period. A more plausible explanation is that the initial decline in extra-axonal sodium was delayed by sodium from a reservoir. It might also be that there was a change in the rate of active transport of sodium to and from the axon surfaces. Previous study indicates the presence of a sodium reservoir, for the net loss of sodium from intact connectives during first exposure to sodium-free Ringer has been demonstrated to be greater than that lost from the extra-axonal fluid: around 50% net loss has been observed without reduction in a.p. amplitude (Bennett *et al.* 1975). If this reservoir could not easily be re-filled following extreme sodium depletion, this would explain why the rate of initial loss was seen, in the present study, to be slower than subsequent losses. Evidence that there is a reservoir that is not easily re-filled under these conditions is provided by Bennett *et al.* (1975) who found that around 40% of the initial sodium content of connectives was not replaced. The effect of the reservoir on the rate of recovery of extra-axonal sodium would depend on the nature of the communication between the reservoir and the extra-axonal fluid. The reservoir might be intracellular (such as has been suggested to explain asymmetrical cation movements in crustacean nervous connectives: Abbott *et al.* 1977) or might be extracellular, perhaps involving the negative charges of the hyaluronic acid known to be found extracellularly in the cockroach C.N.S. (Ashhurst & Costin, 1971). Net exchanges of free sodium ions with fixed anion groups could occur asymmetrically, and thus give slow re-filling, depending upon the nature of the cations which displaced sodium ions.

In desheathed preparations, ions moving between the extra-axonal fluid and the medium bathing the preparations, through the disrupted perineurial and glial system, follow a route that is believed to be largely intracellular (Treherne *et al.* 1970). In the present investigation, sodium ions were found to move along this route more quickly than potassium ions: the opposite to that to be predicted from either the aqueous diffusion rates of the two ion species or the passive permeability properties of glial membranes (cf. Kuffler & Potter, 1964; Kuffler, Nicholls & Orkand, 1966). The difference in rate might be determined by some factor affecting diffusion, such as fixed charges, or by active pumping, perhaps by the relatively undamaged glial membranes nearest the axons.

The ability of sodium but not lithium ions to reach the axon surfaces in intact preparations can be explained if one step of the route is provided by sodium/potassium pumps which, as in frog muscle (Keynes & Swan, 1959) and crab neurones (Baker, 1965) do not accept Li ions. Active transport of sodium to the axons is also supported by the observation that sodium entry is slowed by ethacrynic acid, a sodium transport inhibitor, and by dinitrophenol (Schofield & Treherne, 1975). It is also supported by the speed of the recovery, for the mean half-time of 68 s is faster than that of around 200 s that can be predicted for movement due to intercellular aqueous diffusion using the model of Treherne *et al.* (1970). Since extra-axonal sodium losses subsequent to the initial loss are as fast as sodium entry it appears that they also could be following a route that contains an active component. This could explain why ethacrynic acid apparently slows sodium loss as well as entry (Schofield & Treherne, 1975). Sodium pumping from the bathing medium could be by pumps on the outer face of the perineurium – which might also accept lithium as proposed by Bennett *et al.* (1975) – and pumping from the perineurial/glial system (i.e. in both directions) could be by the Na/K pumps which are found on the surfaces of most cells. The dynamic control of extra-axonal sodium that would result from pumps working in both directions could provide a speedy means of achieving homeostasis.

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