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SODIUM AND LITHIUM MOVEMENTS AND AXONAL FUNCTION IN COCKROACH NERVE CORDS

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SUMMARY

Exposure to sodium-deficient (tris) saline caused an appreciable decline in the sodium content of intact connectives in the absence of equivalent reduction in the amplitude of the recorded action potentials. Return of sodium-depleted connectives to normal saline resulted in a rapid recovery of axonal function despite only a partial ($< 70\%$) recovery in sodium content. Replacement of sodium ions by those of lithium in the bathing medium resulted in a substantial accumulation of this cation. Lithium movements exhibited a marked asymetry, no significant decline in concentration being observed upon return to normal saline. These results are tentatively interpreted in terms of an exchangeable glial sodium fraction and are discussed in relation to extra-axonal sodium regulation.

INTRODUCTION

Insect nerve cells exist in a controlled chemical environment, the appropriate cation composition of the extra-axonal fluid being apparently maintained by the combination of an effective blood-brain barrier and a postulated perineurial-glial system which appears to be capable of achieving a net transport of sodium ions from the blood to the extra-axonal fluid (cf. Treherne & Pichon, 1972; Treherne, 1974). The existence of such a sodium transporting system has, for example, been adduced from recent observations on the rate of recovery of action potentials in sodium-depleted connectives (Schofield & Treherne, in preparation; Treherne, 1974) and on the effects of sodium transport inhibitors and cooling on the membrane potentials recorded in intact cockroach connectives (Pichon & Treherne, 1974).

The perineurial restriction to the intercellular diffusion of inorganic cations appears, rather unexpectedly, to be associated with rapid steady-state cation fluxes (Treherne, 1961*a*, *b*; 1962; Tucker & Pichon, 1972). The magnitude of the initial 'fast' component of ^{22}Na efflux (45% of exchangeable sodium) has led to the suggestion that it could, in part at least, be of glial origin (Tucker & Pichon, 1972). We have at the present time, however, little understanding of the functional significance of such a glial sodium fraction or knowledge of the processes involved in the transport of sodium ions from the blood, or bathing medium, to the extra-axonal fluid. The avail-

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able evidence suggests that an appreciable sodium transport is acheived by a mechanism which is sensitive to dilute dinitrophenol and ethacrynic acid, does not respond to externally applied ouabain and does not accept lithium ions (cf. Treherne, 1974). It is not known, however, at what level these effects are mediated in intact nervous connectives and it is, consequently, difficult to recognize the relative contributions of the perineurial and the glial membranes to extra-axonal sodium transport.

The present investigation was undertaken in an attempt to elucidate some of these unresolved questions. In these experiments non-steady state changes in sodium content, induced by alteration of the external sodium concentration, have been related to changes in the axonal responses recorded in intact connectives. The uptake of lithium ions has also been followed in an attempt to relate the rate of accumulation of this cation to some current research on the electrical responses induced by lithium saline in intact and experimental preparations (Schofield & Treherne, in preparation; Treherne, 1974).

METHODS AND MATERIALS

(a) *Flame photometry*

An integrating flame photometer, built by J. A. Ramsay (and kindly loaned to us by him) was used to analyse samples simultaneously either for Na^+ and K^+ or for Na^+ and Li^+. The flame photometer was built on principles described by Ramsay, Brown & Falloon (1953) and by Öberg, Ulffendahl & Wallin (1967). Samples of fluid were placed on a platinum wire, dried and burned in a clean hydrogen flame. Two photocells with appropriate interference filters were used to monitor the flame, a Niham amplifying and integrating unit displaying the output of the two cells. Within the ranges of concentrations burned (4×10^{-11} to 3×10^{-11} M for sodium and lithium ions; and twice that concentration for potassium ions), none of the cations interfered with each other, the interference of chloride and other anions being 'swamped' by the routine use of 2×10^{-8} M of $(NH_4)_2HPO_4$ which was burned with each sample. In practice, 2 μl of a sample or a standard solution plus 2 μl of 10 mM ammonium phosphate were placed on the wire, dried and burned, standard solutions containing appropriate amounts of the relevant ions being analyzed alternately with experimental samples. The results of three trials on each sample or standard solution were averaged. The standard solutions contained either NaCl and KCl or NaCl and LiCl in proportions and ranges similar to those found in experimental samples.

All errors quoted represent ± 1 standard error of the mean, as estimated from the spread of the results.

(b) *Preparation of the nerve cord samples for flame photometry*

Penultimate connectives of the abdominal nerve cord of adult male cockroaches were dipped very briefly (*ca.* 1 sec) in isotonic mannitol, blotted on filter paper and weighed on a 5 mg torsion balance to the nearest 5 μg. They were then placed in a small volume (100 or 200 μl) of de-ionized water in small titanium wells. Within 2 h the ion content of the water was maximal and the nerve cord contained no more ions than an equivalent volume of water in the well. Samples were thus taken for flame photometry between 4 and 24 h after initial immersion of the nerve cord in water. In initial trials,

Table 1. *Composition of experimental solutions, all of which has a pH of 7·20 and a concentration of 336·2 osmoles/l.*

	Normal (mM)	Lithium (sodium-free) (mM)	Sodium-free (Tris subst.) (mM)
NaCl	113·7	0	0
LiCl	0	120	0
KCl	25	18·7	18·7
$CaCl_2$	2	2	2
$MgCl_2$	2	2	2
Na_2HPO_4	1·8	0	0
NaH_2PO_4	0·2	0	0
$NaHCO_3$	2·5	0	0
K_2HPO_4	0	1·8	1·8
KH_2PO_4	0	0·2	0·2
$KHCO_3$	0	2·5	2·5
Tris chloride	17·0	17·0	130·25
Tris base	2·02	2·02	15·46

no sodium or potassium was detected in 200 μl. samples of isotonic mannitol in which a nerve cord had been dipped, but the rinse was used routinely to remove possible excess saline or haemolymph clinging to the cord.

(*c*) *Dextran experiments*

To distinguish between ions held at the surface and within the outermost connective tissue sheath and those of the underlying tissues, ligatured nerve cords were soaked for one hour in saline containing tritiated dextran (m.w. 77,500). The ligatured penultimate connectives were cut from the rest of the cord, blotted, weighed (in some experiments they were rinsed in isotonic mannitol) and analyzed in the usual way for sodium and potassium. In addition, 5 μl. samples of the soaking fluid were taken for analysis of tritium content.

(*d*) *Ringer solutions*

The solutions used are listed in Table 1. The levels of Na^+, K^+ and osmolarity are such that intact nerve cords did not change weight nor change in sodium or potassium concentrations after two hours immersion in normal Ringer. They did not change weight upon transfer to sodium-free (Tris) Ringer but did lose some potassium as well as losing considerable sodium (see text). The full *rationale* for the design of these Ringer solutions is given by Treherne, Buchan & Bennett (1975).

(*e*) *Recording of resting and action potentials*

Extracellular recordings were made in the penultimate connectives of the abdominal nerve cord using the modified 'sucrose-gap' technique (Pichon & Treherne, 1970). In these experiments isolated nerve cords were mounted across five parallel compartments in a small perspex chamber. The compartments were isolated from each other by silicone grease seals. The abdominal connectives were passed through two adjacent compartments containing normal saline. These were connected to a Farnell pulse-generating system *via* a photon-coupled isolating unit and platinum wires. The penultimate abdominal ganglion was buried in a grease seal, the penultimate connectives being contained in the middle compartment through which the experimental solutions

flowed at a regulated rate. The adjacent compartment contained flowing mannitol solution which was iso-osmotic with the physiological salines employed in this investigation (336·2 mM). The remaining compartment was filled with normal saline and was connected to a high-impedance negative-capacitance amplifier via an isotonic KCl-Agar bridge. The middle compartment, containing the experimental saline, was coupled to the indifferent electrode by an isotonic KCl-Agar bridge. The amplifier was connected to a Tektronix 561B oscilloscope and to a Servoscribe pen recorder. Action potentials were photographed using a Nihon–Kohden PC–2A oscilloscope camera.

The regulated flow of the iso-osmotic mannitol and the experimental solutions, through the appropriate compartments in the perspex chamber, was achieved by a gravity-feed system from a series of elevated reservoirs. Rapid changes of solution were effected using a multiway, non-return, valve (Holder & Sattelle, 1972).

RESULTS

(a) *Experiments with radioactive dextran*

Compounds of large molecular weight (> 1900) have been shown to be unable to penetrate to the sub-perineurial extracellular spaces (Lane & Treherne, 1972). Radioactive dextran (m.w. 77500) was, therefore, used to estimate the proportion of the cations likely to be associated with the fluid contained in the superficial connective tissue sheath and on the surface of intact blotted connectives.

Penultimate connectives which had been ligatured and allowed to soak in normal saline containing tritiated dextran (25 mCi/g), for 30–60 min, yielded a dextran space of 16·0 ± 0·25% showing that a substantial amount of the sodium (84·0 ± 1·5%) was not dextran associated. Connectives which had been briefly dipped in isotonic mannitol before blotting and weighing also showed the same range of values.

(b) *Sodium levels in connectives exposed to sodium-free (tris-substitute) salines*

The experiments described in this and subsequent sections were performed during a period of several months (1 February 1974 to 1 August 1974). During this time the sodium content of the nerve cords changed gradually and significantly despite the provision of a standardized diet (Treherne *et al.* 1975). Thus for each set of experiments reported here the actual sodium content of control nerve cords are reported in the text, the experimental values being expressed as a proportion of these.

Intact connectives are known to exhibit variable electrical responses to changes in the cation composition of the bathing medium (cf. Tucker & Pichon, 1972). Such variations was also apparent in the present investigation in the responses of the recorded action potentials following exposure of intact connectives to sodium-free saline. In this study the electrical responses have been arbitrarily classified into those in which the action potentials were maintained at full amplitude for periods of one hour and those in which the action potentials showed a significant decline after 15 min exposure to sodium-free saline.

Fig. 1 illustrates the changes in the sodium content of connectives in which there was no significant decline in the amplitude of the action potentials for a period of at least 1 hr following exposure to sodium-free saline. It will be seen that despite the

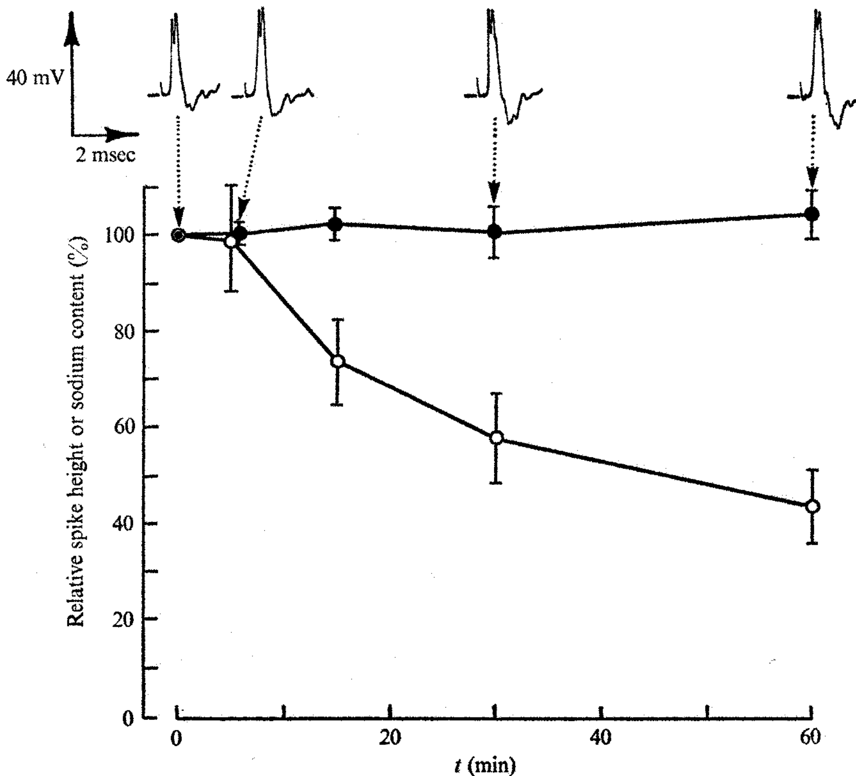

Fig. 1. The effects of exposure to sodium-free, tris, saline on the relative sodium content (open circles) of intact connectives which showed no significant decline in the amplitude of action potentials (closed circles). In this and all subsequent figures the vertical lines indicate the extent of the standard error on the mean, the number of connectives analysed (n) for each value were as follows: 25 (0 min); 8 (5 min); 11 (15 min); 7 (30 min) and 6 (60 min).

relative constancy in the amplitude of the recorded action potentials there was, nevertheless, a substantial decline in the sodium content of the connectives, which after 1 h had declined to 44% of the initial average value (from 69·0 ± 4·5 to 30·0 ± 5·4 m-mole/kg).

With 'leaky' preparations (in which the action potentials showed some decrease after 15 min exposure to sodium-free conditions) the sodium content also exhibited a more precipitate decline than the amplitude of the recorded action potentials (Fig. 2). Thus at 15 min, before any significant decrease in the amplitude of the action potentials was apparent, the relative sodium content of the connectives had nevertheless declined to 73·6 ± 9·0%.

(*c*) *Changes in sodium content associated with recovery of action potentials in sodium-depleted connectives*

In another series of experiments 36 preparations were exposed to sodium-free (tris-substituted) saline until the amplitudes of the action potentials recorded in the penultimate connectives, had fallen to 50%. The connectives were then exposed to normal saline for varying periods, the recovery of the action potentials being moni-

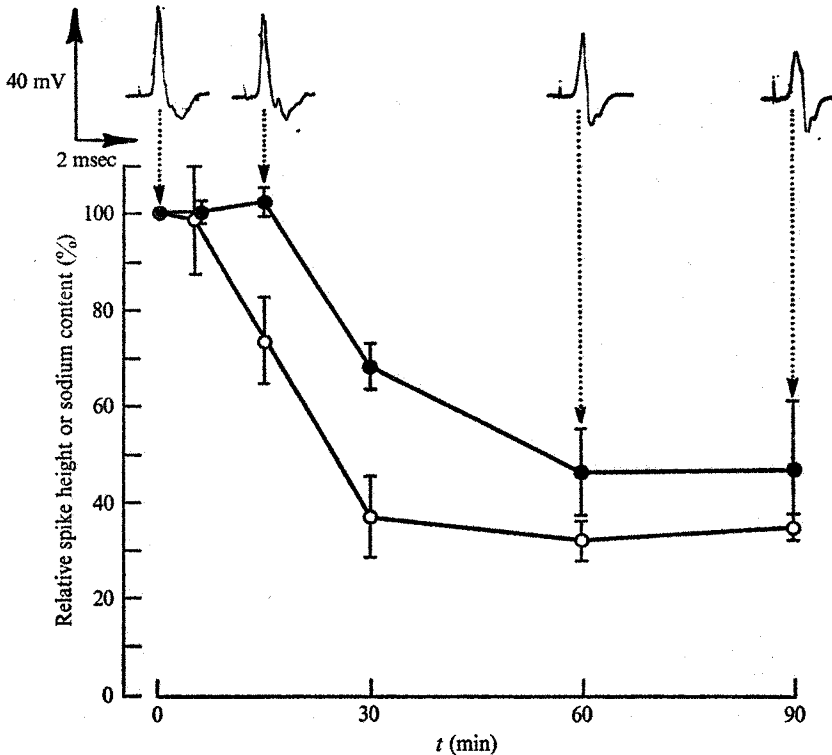

Fig. 2. The effects of exposure to sodium-free, tris, saline on the relative sodium content (open circles) of intact connectives in which the amplitude of the action potentials (closed circles) declined after 15 min. $n = 25$ (0 min), 8 (5 min), 11 (15 min), 6 (30 min), 7 (60 min) and 4 (90 min).

tored and the connectives analysed for sodium content. The results showed that although return to normal external sodium conditions typically resulted in relatively rapid recovery of the action potentials there was no equivalent recovery in the sodium content of these connectives, there being only a modest increase to between 50 and 60% of the initial sodium content (which during the period of these experiments varied between 52 and 63 m-mole/kg) (Fig. 3). No correlation was observed between the sodium level attained, following return to normal saline, and the preceding period of exposure to sodium-free conditions.

Effects of lithium and tris-substituted salines on the sodium content of isolated connectives

In these experiments the penultimate connectives were isolated with hair ligatures and placed in small dishes of sodium-free saline (either Li^+ or tris-substituted) for a period of 1 h. They were then returned to normal saline. Connectives were monitored after varying periods so as to follow the succesive changes in sodium content associated with the alteration of the ionic composition of the bathing solutions.

As with the preparations maintained under 'mannitol-gap' conditions it was found that connectives bathed in sodium-free, tris, saline showed only a partial recovery (from 53% to about 70%) on return to normal saline (Fig. 4). In the presence of

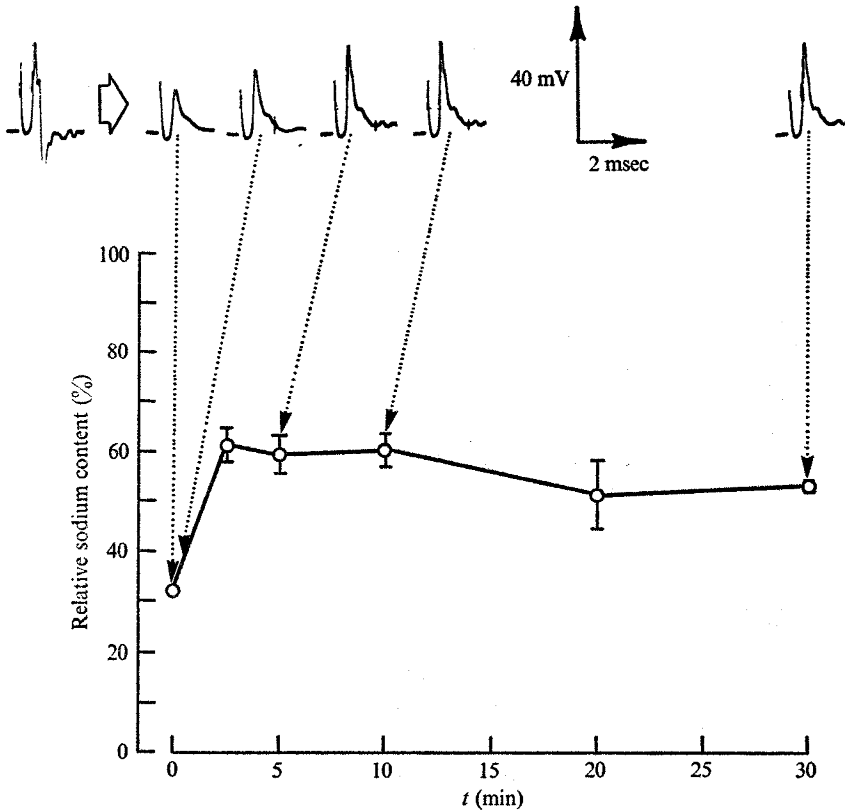

Fig. 3. Recovery of sodium content and action potentials in sodium-depleted connectives on returning to normal saline. In these connectives the action potentials were reduced to half-amplitude by exposure to sodium-free, tris, saline (arrow), for between averages of 49–70 min; before return to normal saline. $n = 6$ (2·5 min), 7 (5 min), 12 (10 min), 6 (20 min) and 4 (30 min).

lithium ions, on the other hand, the sodium content decreased more rapidly and also showed a more complete recovery than that observed in the presence of tris ions. The sodium content of freshly-dissected connectives averaged 48 ± 3·0 m-mole/kg ($n = 8$) during these tests, a value which was taken to represent the 100% level in the data presented in Fig. 4.

The changes in the lithium content of ligatured penultimate connectives during exposure to sodium-free, lithium, saline and, subsequently, to normal saline are summarized in Fig. 5. It will be seen that the decline in sodium content was accompanied by a substantial uptake of lithium ions. During the subsequent periods of exposure to normal saline the uptake of sodium ions was not accompanied by an equivalent decline in the lithium content (Fig. 5).

DISCUSSION

The results indicate that substantial changes in the sodium content of intact connectives can occur in the absence of significant alterations in the amplitude of the recorded action potentials. This was most dramatically seen in the case of prepara-

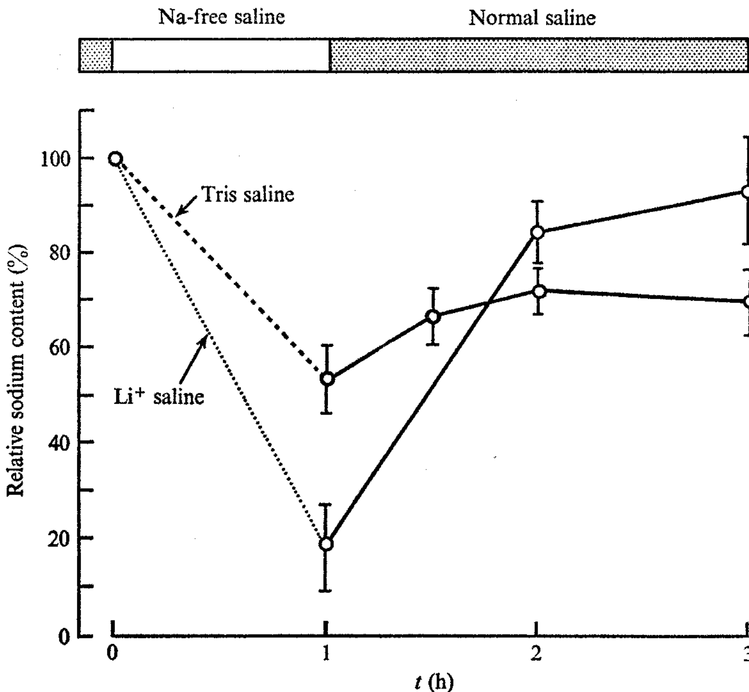

Fig. 4. Changes in the relative sodium content of intact, ligatured, connectives on exposure to sodium-free salines and subsequent return to normal saline. Tris and lithium were used as substitutes in the sodium-free salines. For tris-treated connectives, $n = 8$ (1 h), 4 (1·5 h), 7 (2 h) and 7 (3 h). For Li^+, $n = 4$ (1 h), 5 (2 h) and 7 (3 h).

tions which showed no significant decline in the amplitude of the action potentials during a period of 1 h, for after this time it was found that the sodium content had declined to 44% of the initial value. As the inwardly-directed component of the action current is largely carried by sodium ions (cf. Narahashi, 1963; Pichon, 1974) it can be reasonably concluded that a significant proportion of the sodium was lost without appreciable alteration of the gradient of this cation across the axonal membranes. The lack of effect of sodium-free saline on the extra-axonal level of this cation for appreciable periods, in intact preparations, could be predicted from the presence of the perineurial intercellular occlusions (Maddrell & Treherne, 1967) which would clearly restrict net efflux from the extracellular fluid (cf. Treherne & Pichon, 1972; Treherne, 1974). The magnitude of the sodium fraction lost following exposure of intact connectives to sodium-free, tris, saline is equivalent to the 'rapidly-exchanging' fraction demonstrated in previous experiments on radio-sodium efflux (Treherne, 1961, 1962; Tucker & Pichon, 1972). The present observations yield a value of about 40%, for the total sodium lost after 1 hr exposure to sodium-free conditions (when corrected for that contained on the surface and in the neural lamella) which is of similar magnitude to the initial 'fast' component of ^{22}Na efflux observed by Tucker & Pichon (1972) under conditions of maximal peripheral restriction to intercellular diffusion. As with the rapidly-exchanging radio-sodium fraction (Tucker & Pichon, 1972) the 40% lost during exposure to sodium-free conditions can be reasonably

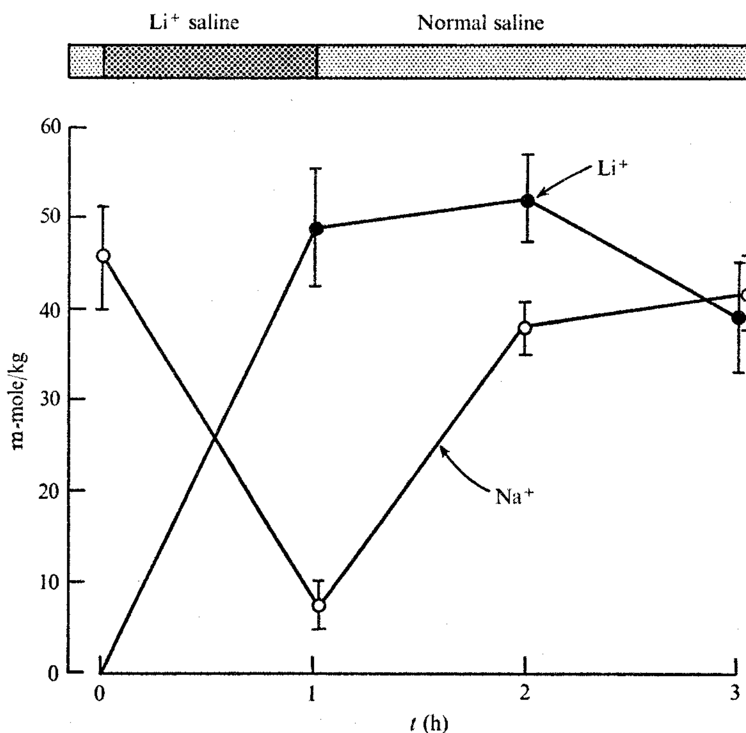

Fig. 5. Changes in lithium (closed circles) and sodium content (open circles) of ligatured intact connectives during exposure to sodium-free, lithium, saline and subsequent return to normal saline. n = 7 (0 h), 4 (1 h), 5 (2 h) and 6 (3 h).

and provisionally identified as being of glial origin. It is, otherwise, difficult to accommodate a sodium fraction of this magnitude in the absence of evidence for its location in the nerve sheath, extracellular or axonal elements.

The above interpretation invokes the perineurial restriction to intercellular diffusion and the existence of an appreciable exchangeable glial sodium fraction to explain the substantial decline in sodium content which can be observed in the absence of equivalent decline in the magnitude of the recorded action potentials. Such a system by itself, however, cannot account for the relatively rapid recovery of the action potentials observed on return of sodium-depleted connectives to normal saline, for this response was associated with only a partial recovery of the sodium content (in preparations previously exposed to tris-substituted saline).

The above observations could be explained by postulating that exposure to sodium-free, tris, saline induces an increase in passive leakage through the intercellular channels of the perineurium. Under these circumstances return to normal saline could result in an increased intercellular access of sodium ions to the axonal surfaces, so as to rapidly restore the action potentials, with a slower and only partial increase in the glial sodium content. This hypothesis is, however, difficult to sustain in the face of the observation that lithium ions fail to induce a restoration of axonal function in intact sodium-depleted connectives (Schofield & Treherne, in preparation; Treherne, 1974) for this cation substitutes for those of sodium in passive ion movements in other biological systems (cf. Ussing *et al.* 1960)

An alternative and, apparently, more satisfactory hypothesis would be that the rapid restoration of axonal function observed in sodium-depleted connectives, on return to normal saline, results from a glial-mediated transport of sodium ions into the extra-axonal fluid. Such a glial transport system could cause a restoration of axonal function in the absence of an equivalent increase in the sodium content of the connectives: the increase in the extra-axonal sodium concentration being largely determined by the rate of sodium transport which accompanies the relatively slow and partial increase in concentration of this cation in the glial cytoplasm.

The postulation of a glial-mediated sodium transport accords with concepts derived from other recent investigations. In particular, the effects of dilute dinitrophenol and ethacrynic acid and the inability of lithium ions to restore axonal function in intact, sodium-depleted, preparations have been interpreted in terms of an extra-axonal transport of sodium ions (Schofield & Treherne, in preparation; Treherne 1974). The latter effect contrasts with that obtained in preparations in which the blood-brain barrier was disrupted with hypertonic urea, for in this case the rapid intercellular access of lithium ions resulted in a rapid recovery of action potentials in sodium-depleted preparations. It has also been recently demonstrated that the axonal sodium pump is pharmacologically separable from that associated with the perineurial and/or glial membranes: the former being inhibited by cardiac glycosides and the latter by ethacrynic acid (Pichon & Treherne, 1974). These recent observations have led to the conclusion that sodium ions are transported across the perineurial and glial elements by a system which is sensitive to dinitrophenol and ethacrynic acid but does not accept lithium ions. It is not known, however, at what level these effects ai e exerted. Ethacrynic acid could, for example, inhibit sodium transport at the outer perineurial or at the glial membranes. Similarly, it is not known whether lithium ions are excluded by the outer perineurial membrane or whether they penetrate into the glial cytoplasm (via presumed low-resistance connexions with the perineurial membranes) and are not accepted by the sodium pump situated on the glial membranes.

The present data suggests that the latter possibility is the more likely, for it has been shown that lithium ions can exchange rapidly with those of sodium and accumulate within the tissues of the intact connectives (Fig. 5). The inability of lithium ions to restore axonal function in intact sodium-depleted connectives clearly suggests that this cation was unable to penetrate to the extracellular fluid and, thus, to reach the axons. It seems most probable, therefore, that the appreciable accumulation of lithium ions occurred within the perineurial and glial cytoplasm. This interpretation implies that lithium ions can penetrate the outer perineurial membrane, so as to accumulate within the glial cells, but do not readily cross the glial membranes to gain access to the fluid bathing the axon surfaces.

We have, as yet, very little knowledge of the mechanisms involved in the uptake of sodium ions by the outwardly-directed perineurial surfaces and, consequently, it is difficult to speculate on the processes involved in lithium accumulation. The extra-neuronal potential changes induced by alteration of the cation composition of the bathing medium indicate that the outer perineurial membrane is relatively permeable to potassium, with only a limited passive permeability to sodium and lithium ions (Treherne *et al.* 1970; Pichon, Morton & Treherne 1971). The possibility cannot be eliminated, however, that the absolute sodium permeability could be sufficient to

enable this cation passively to diffuse down an electrochemical gradient into the perineurial cells. Alternatively the cockroach perineurium could be similar to the avian salt gland, the sodium ions crossing the outer membrane by an active mechanism (Treherne, 1974). This possibility would accord with the present observations. In particular the apparent asymmetry in net uptake and loss of lithium ions could imply that both this cation and sodium are accumulated by an inwardly-directed mechanism which accepts both cations. This interpretation would imply that sodium extrusion from the perineurium is acheived by a separate transport mechanism which does not accept lithium ions. Such a discrimination has been postulated for the sodium pump in crab nerve cells (Baker, 1965). It is hoped that future investigations will elucidate these possibilities.

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