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EXPERIMENTAL DISRUPTION OF THE BLOOD–BRAIN BARRIER SYSTEM IN AN INSECT (*PERIPLANETA AMERICANA*)

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INTRODUCTION

It is well established that the neurones of the insect central nervous system are protected from the rapid intercellular access of water-soluble ions and molecules in the haemolymph or bathing medium (see Treherne & Pichon, 1972). On the basis of the potassium-induced extraneuronal potentials (Treherne *et al.* 1970; Pichon & Treherne, 1970, 1971; Pichon, Moreton & Treherne, 1971) and the demonstration of the limited access of macro- and micro-peroxidase and of lanthanum (Lane & Treherne, 1969, 1970, 1972), it was proposed that intercellular diffusion was restricted by tight junctions in the insect perineurium (Lane & Treherne, 1972). It also appears that such a restricted net intercellular diffusion is associated with relatively rapid steady-state fluxes of radioactive sodium ions (Tucker & Pichon, 1972*a*, *b*). It has been tentatively proposed that the rapidly exchanging radio-cation components may represent a glial-perineurial fraction (see also Treherne & Pichon, 1972).

Previous attempts to disrupt the insect blood–brain barrier have involved mechanical techniques: namely, the surgical removal of the neural lamella and perineurium (Twarog & Roeder, 1956; Treherne & Maddrell, 1967; Treherne *et al.* 1970; Pichon, Sattelle & Lane, 1972) and stretching of the central nervous connectives of the cockroach (Treherne *et al.* 1970).

The perineurial damage involved in the former techniques has been shown, however, to result in extensive penetration of the glial cytoplasm, even by molecules as large as those of macroperoxidase (Lane & Treherne, 1969, 1970). Stretching of intact connectives has been found to produce deformation of the mesaxonal and other extracellular spaces which poses some difficulties in the interpretation of electrophysiological data (Lane & Treherne, 1970).

In this investigation an attempt has been made to disrupt the blood–brain barrier by transient and massive changes in the composition of the bathing medium. Such methods have been previously employed to produce apparent opening of the intercellular junctional complexes in the frog skin (Ussing, 1968) and disruption of the vertebrate blood–brain barrier system (Rapoport, Hori & Klatzo, 1972).

In the present study both electrophysiological and ultrastructural techniques have been used for characterizing the experimentally-induced changes in the blood–brain barrier system.

MATERIALS AND METHODS

Electrophysiological studies

The experiments in this investigation were carried out upon the abdominal nerve cord of the cockroach, *Periplaneta americana*. In the electrophysiological experiments, the sucrose-gap technique (Stämpfli, 1954) was used to record both DC potential changes and compound action potentials by employing modifications similar to those used by Pichon & Treherne (1970). In this modified technique the nerve cord is placed in a five-compartment nerve chamber cut into the surface of a Perspex block. The nerve cord lies in a groove linking the five parallel compartments and silicon grease seals are made around it where it lies between these compartments, to prevent mixing of the fluids contained in them.

Electrical potential changes in the penultimate pair of connectives, which lay in the central compartment of the nerve chamber, were measured with reference to the left-hand compartment which contained the crushed anterior end of the nerve cord in normal *Periplaneta* saline. Saline-agar bridges made the electrical connexion through KCl baths and chloride-coated silver wires to a high-impedance amplifier of unity voltage gain. Electrical insulation between the two compartments was provided by a mannitol solution, isotonic with normal saline and of low ionic content, flowing in the compartment between them. Continuous d.c. records were made using a Smiths Servoscribe RE511.20 chart recorder. Action potentials were displayed upon a Tektronix 561 oscilloscope and photographed at intervals with a Nihon–Kohden PC-21A camera.

Compound action potentials were produced by the electrical stimulation of the terminal region of the nerve cord, which lay in the right-hand pair of compartments. These were filled with normal *Periplaneta* saline and contained a pair of platinum wire stimulating electrodes. Stimulation was at low impedance from an earth-isolating unit, employing RF coupling to the output of a pulse generator, producing rectangular voltage pulses of 0·1 msec duration.

The electrical response of the penultimate pair of connectives to a change in the composition of the bathing medium was accomplished by employing a multi-way non-return valve (Holder & Sattelle, 1972) to rapidly change the solution flowing in the central compartments of the chamber.

The normal saline used was that devised for *Periplaneta* by Yamasaki & Narahashi (1959), and had the following composition: 214·0 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 HOP_4^{2-}. Sodium-free saline was made by replacing the sodium ions with tris (hydroxymethyl) methylammonium ions. In the high-potassium, sodium-free saline the sodium ions were replaced by potassium ions. Urea was added to the normal saline to make the 3·0M urea solution, and glucose was added to make the 3·0M glucose solution.

In these experiments the nerve cord was dissected from the cockroach under normal saline, and was then either placed directly in the nerve chamber as an intact preparation, or was de-sheathed. De-sheathing was performed upon the penultimate pair of connectives in saline upon a microscope slide using fine steel needles. During the removal of the connective tissue sheath by this procedure the underlying perineurium is also damaged (Lane & Treherne, 1970).

Ultrastructural studies

Abdominal nerve cords were dissected out, soaked in phosphate-free saline for 10–15 min, then treated with 3·0 M urea for 15, 60 or 180 sec. The tissues were then either placed directly in fixative, or left in the phosphate-free saline for 15 min and then fixed. Fixation was carried out in 2·5 % glutaraldehyde in 0·1 M phosphate buffer (pH 7·4) plus 0·2 M sucrose, for 1–2 h. The nerve cords were then washed in a phosphate buffer and sucrose solution, post-osmicated for 60 min at room temperature and stained *en bloc* in 2 % uranyl acetate in sodium hydrogen maleate buffer (Karnovsky, 1967). Dehydration through an ascending series of ethanols followed, with subsequent embedding in Araldite. Sections were cut on an LKB Ultrotome III, stained with uranyl acetate and lead citrate, and examined in a Philips EM 300.

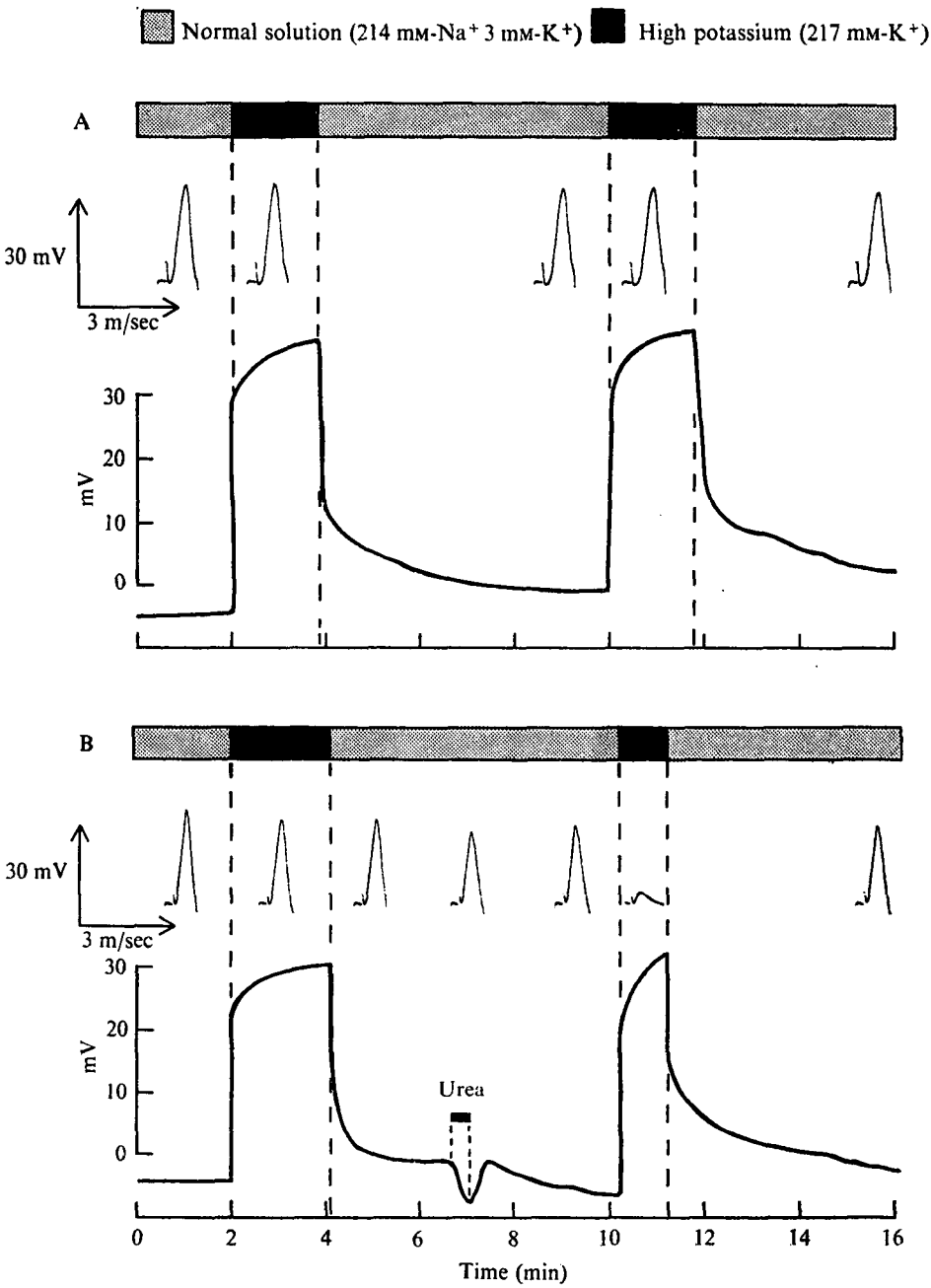
Isolated nerve cord preparations were also incubated in phosphate-free saline containing 1 mM lanthanum chloride at room temperature for 15 and 60 min periods, following treatment with 3·0 M urea for 15 sec. The lanthanum used in this investigation was thus primarily in the ionic form (Machen, Erlij & Wooding, 1972; Lane, 1972). This procedure differs from that of Revel & Karnovsky (1967) in which a colloidal suspension of lanthanum hydroxide in the fixative was used as an extracellular marker. The lower concentration of the ionic lanthanum was less likely to interfere with normal physiological processes and its smaller dimensions make it a more effective indicator of patent extracellular channels. Incubation in ionic lanthanum results in less dense accumulations of lanthanum in extracellular spaces, an effect which is presumed to result from subsequent washing out during fixation. The preparations were then fixed as described above, the phosphate buffer ensuring the precipitation of the ionic lanthanum present in solution. Controls were carried out by incubating preparations in saline to which no lanthanum had been added, for 15–60 min, both with and without prior treatment with 3·0 M urea for 15 sec.

Ventral nerve cords from *Periplaneta americana* were also treated with 3·0 M glucose in phosphate-free saline for 45 sec followed by fixation in 2·5 % glutaraldehyde in 0·1 M phosphate buffer (pH 7·4) plus 0·2 M sucrose at 4 °C, osmication, *en bloc* staining with uranyl acetate, dehydration and embedding. Other preparations treated with 3·0 M glucose were subsequently incubated for 15–60 min in phosphate-free saline with or without added 1 mM ionic lanthanum and then fixed. Further controls were incubated in saline without glucose, followed by fixation.

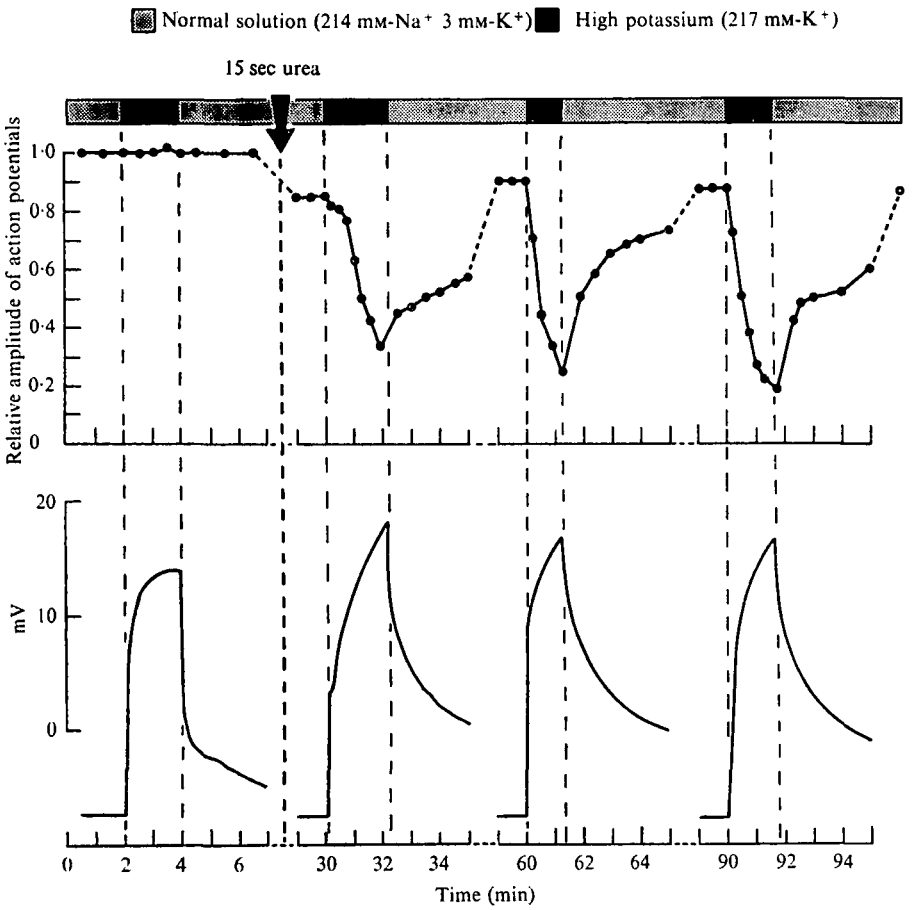
RESULTS

Electrophysiological observations

Brief exposures of intact connectives to a solution of 3·0 M urea were found to have a profound effect upon the electrical responses elicited by alterations of the cation concentration of the bathing medium. This is exemplified in Text-fig 1. With untreated preparations elevation of the external potassium concentration produced rapid and extensive d.c. potential changes. These have been postulated as being extraneuronal in origin and have been attributed to the effect of potassium ions in depolarizing the outwardly facing perineurial surfaces (Treherne *et al.* 1970; Pichon & Treherne, 1971; Pichon *et al.* 1971).



Text-fig. 1. Electrical responses recorded from intact connectives showing the effect of two exposures to an increased external potassium concentration. (A) Action potentials and extra-neuronal potentials show no appreciable alteration. (B) A 15 sec exposure to 3·0 M urea following the first exposure alters the response to the second exposure to high-potassium solution.

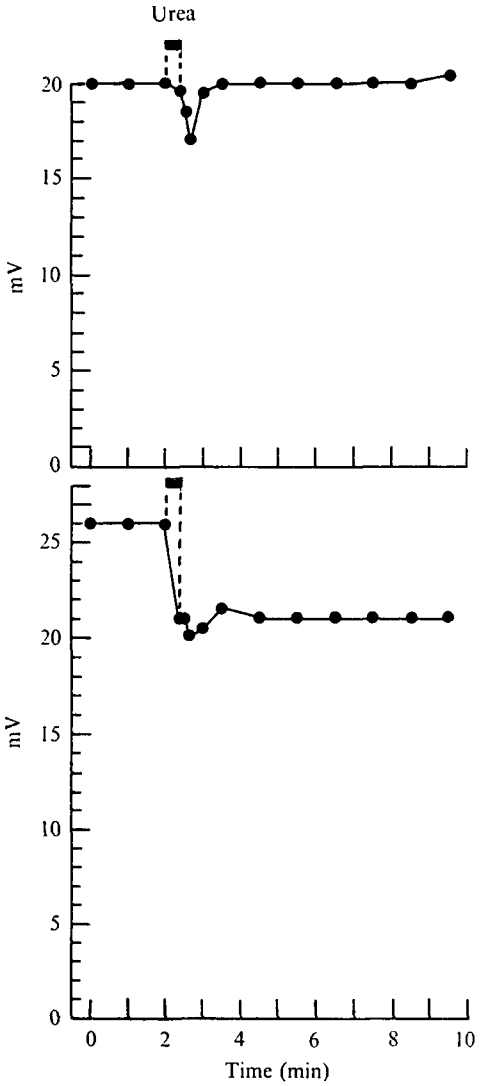


Text-fig. 2. Effects of successive pulses of elevated-potassium solution on extraneuronal potentials and action potentials following 15 sec exposure to hypertonic urea solution.

Under these conditions no appreciable reduction was observed in the amplitude of the recorded action potentials. A 15 sec exposure of an intact connective to urea, on the other hand, resulted in a relatively rapid decline in the action potentials following exposure to high-potassium, sodium-free saline, an effect which could indicate an increased access of potassium ions to the axon surfaces. Under these conditions it can reasonably be concluded that the d.c. potential changes produced by elevated potassium concentrations represent, in part at least, appreciable axonal depolarization.

Text-fig. 2 illustrates the effects of the application of successive pulses of high-potassium, sodium-free saline to urea-treated preparations. The potassium-induced decline in the amplitude of the action potentials, produced by a 15 sec exposure to the urea solution, was found to persist, indicating that the changes are not readily reversible.

It was observed that a decline in the amplitude of the recorded action potentials occurred (in preparations bathed in normal physiological saline) following treatment with hypertonic urea solutions. Brief exposure of de-sheathed preparations to 3·0M

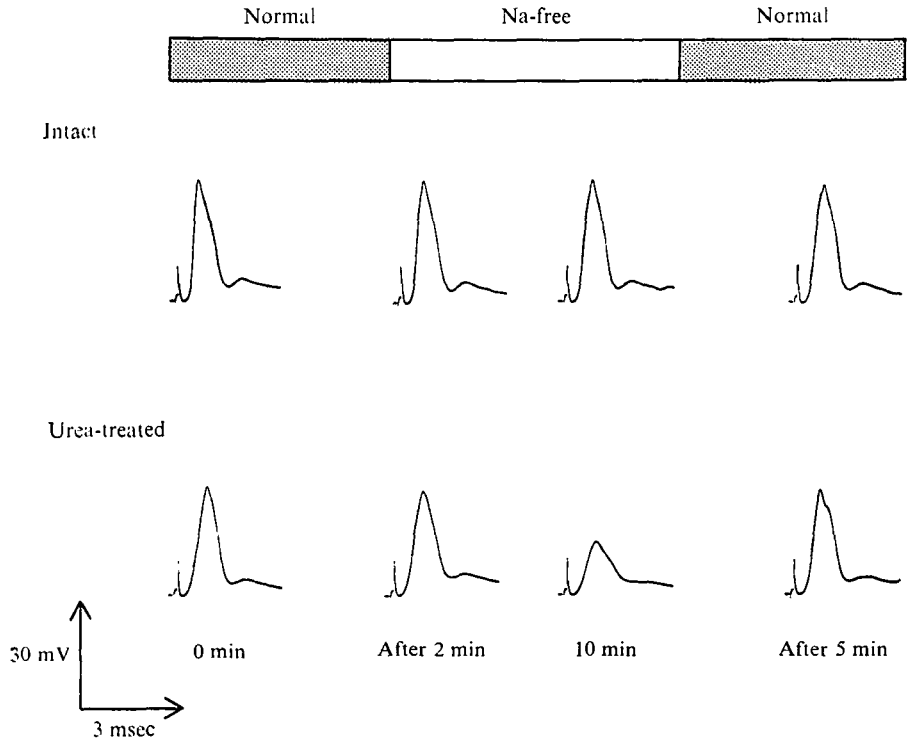


Text-fig. 3. Effects of 15 sec exposure to hypertonic urea solution on the amplitude of action potentials recorded in de-sheathed and intact connectives.

urea did not result in a sustained decline in the amplitude of the action potentials (Text-fig. 3).

Exposure of urea-treated preparations to sodium-free solutions resulted in a decline in the amplitude of the recorded action potential (Text-fig. 4). This response contrasts with those recorded in intact, untreated preparations, but was slower than those observed with de-sheathed preparations (Text-fig. 5).

Exposure of intact connectives to 3·0 M glucose solution induced rather complex d.c. potential changes, both on return to normal saline and on subsequent exposure to high-potassium, sodium-free saline (Text-fig. 6). The potassium-induced potential changes recorded in such preparations frequently exhibited an initial transient phase of depolarization. This type of response was also occasionally encountered in un-

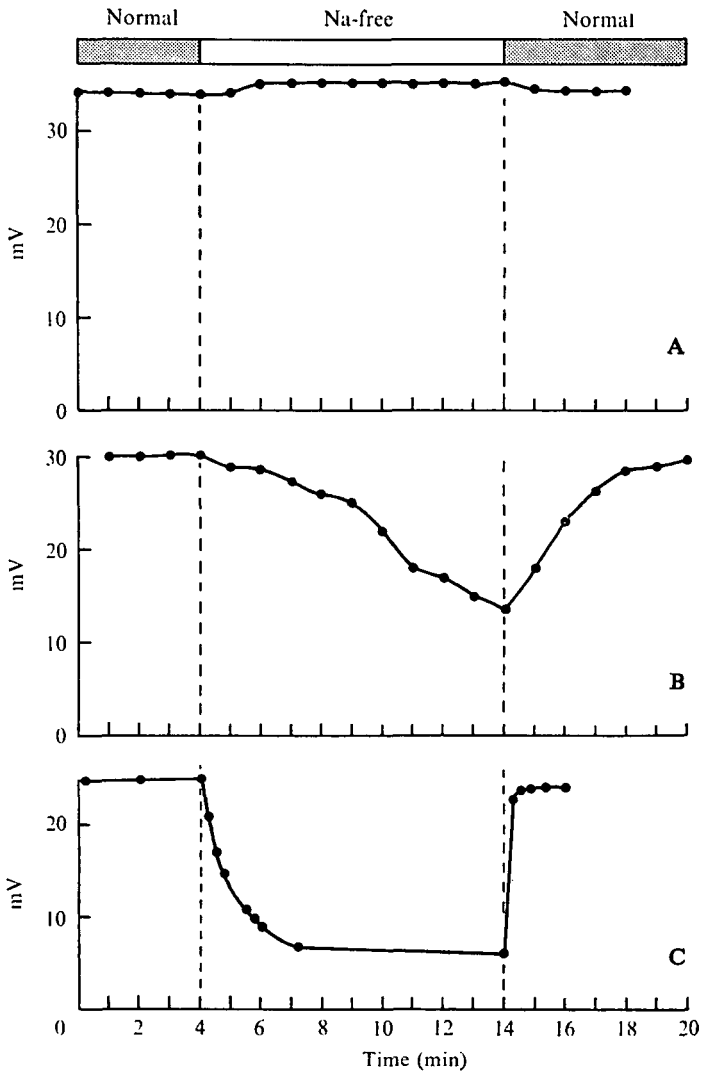


Text-fig. 4. Effects of sodium-free solution on action potentials recorded in an intact preparation and one treated with hypertonic urea solution for 15 sec.

treated preparations. No decay in the amplitude of the action potentials, equivalent to that observed with urea-treated preparations, occurred during exposure to high-potassium, sodium-free saline in preparations treated with 3·0M glucose solution.

Ultrastructural observations

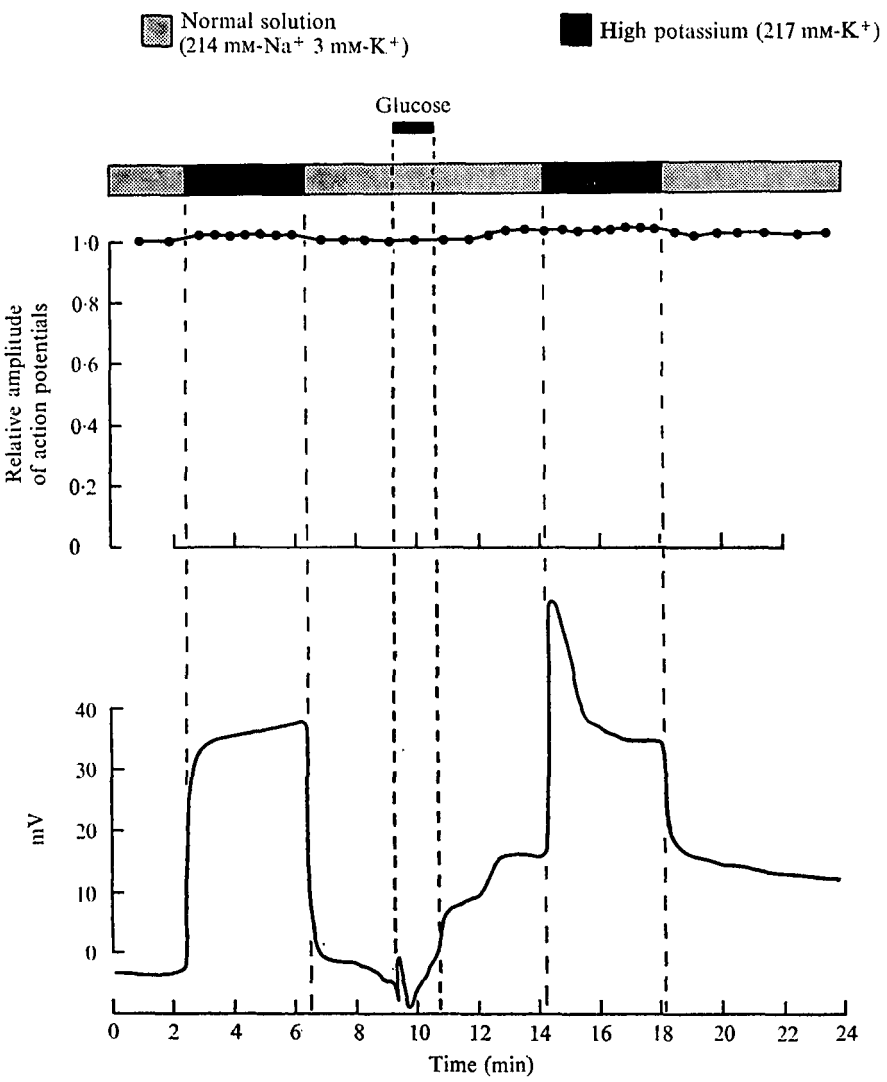
The fine structural details of cockroach nerve connectives have been dealt with in detail elsewhere (Treherne *et al.* 1970) where it has been shown that beneath the acellular neural lamella lies a modified glial layer called the perineurium. The cells of this layer interdigitate with one another in a highly complex fashion and the intercellular clefts formed between their lateral borders are kept to a minimum at the basal region of the cells by septate desomosomes, gap junctions and tight junctions (Lane & Treherne, 1972). When cockroach nerve cords are examined ultrastructurally in this study after being treated only with fixative, the perineurium displays the usual occasional lacuna or space between the lateral borders of adjacent cells (Plate 1, fig. 1). The glial cells and axons which lie beneath display no empty spaces between cells; those extracellular spaces which exist between glial cells always contain an electron-opaque material (Plate 1, fig. 1). Control preparations incubated in normal saline before fixation display lacunae between perineurial cells (Plate 1, fig. 2) and also at times some actual cell damage (see * in Plate 1, fig. 2). When nerve cords are treated with urea for 15 sec and then fixed, spaces between perineurial cells also occur (Plate 2, fig. 3); but these seem not much more extensive than those observed in



Text-fig. 5. Effects of sodium-free solution on the action potentials recorded in intact (A), urea-treated (B) and de-sheathed (C) preparations.

saline-incubated controls (Plate 1, fig. 2). No damage seems to be inflicted on the underlying axons and glia. When urea treatment is prolonged for as much as 60 or 180 sec, further disruption occurs to the edge of the perineurial cells bordering on the neural lamella and a subsequent 15 min in saline leads to much greater vacuolization of the perineurial layer.

Preparations incubated in ionic lanthanum in saline also display some disruption of membranes, presumably caused (as the controls indicate) by the saline alone (Plate 2, fig. 4). The lanthanum can be seen to penetrate the neural lamella, the perineurial clefts (Plate 2, fig. 4) and ultimately the septate desomosomes and gap junctions (Plate 2, fig. 4) found between adjacent perineurial cell membranes. How-



Text-fig. 6. Effects of exposure to 3·0 M glucose solution on the action potentials and d.c. potentials recorded in an intact connective.

ever, the lanthanum appears to proceed no further than this level, never being found in the glial and axonal mass beneath (Plate 2, fig. 4).

When nerve cords were treated with 3·0M glucose followed directly by fixation, some slightly enlarged extracellular perineurial spaces become evident, but these are no more extensive than those observed in the saline-incubated controls. In the latter, in fact, some cytoplasmic damage is evident, especially after more prolonged incubation times. Material incubated in ionic lanthanum subsequent to the 3·0M glucose treatment shows deposits of lanthanum present in the perineurial clefts, but not extending beyond to the glial-axonal mass lying beneath.

DISCUSSION

The results indicate that brief exposure of the intact connectives to hypertonic urea resulted in an increased access of sodium and potassium ions to the axonal surfaces. This was manifested by the relatively rapid decline in the amplitude of the action potentials recorded, in urea-treated preparations, during exposure to high-potassium and to sodium-free salines.

The standard, 15 sec, exposure to hypertonic urea solution did not appear to induce appreciable ultrastructural changes to the perineurium as compared with those observed in saline-incubated controls. The intercellular penetration of ionic lanthanum from the bathing medium was also found not to be enhanced by the urea treatment. As in normal preparations, the lanthanum was confined to the perineurial clefts, it being observed to pass through both septate desmosomes and gap junctions.

The ultrastructural observations on the penetration of ionic lanthanum do not, therefore, provide evidence of an increased access to the axonal surfaces via the perineurial clefts. It has previously been suggested that the peripheral restriction to intercellular diffusion results from the presence of the perineurial tight junctions (Lane & Treherne, 1972), for, as has also been shown here, both septate desmosomes and gap junctions can be passed by ionic lanthanum. However, it should be borne in mind that at 2·0 mM/l lanthanum has an ionic diameter (calculated from diffusion of $LaCl_3$) of 27·8 Å (Stern & Amis, 1959). It could be thus envisaged that the effective width of 'opened' tight junctions might still restrict the passage of ionic lanthanum, whilst allowing an increased intercellular access of sodium and potassium ions. This situation would thus differ from that in naturally 'leaky' epithelia in which ionic lanthanum has been demonstrated to pass through 'tight' junctions (Whittembury & Rawlins, 1971; Machen *et al.* 1972).

The above interpretation accords with that proposed for the frog skin in which it has been suggested that hypertonic urea solutions cause an opening of the zonulae occludentes (tight junctions) thus creating a non-selective, extracellular shunt path for sodium chloride through the skin (Ussing, 1968). As with the insect perineurium, electron-microscopical observations have not apparently revealed any ultrastructural basis for this supposition (see Discussion in Ussing, 1968). The possibility cannot, therefore, be eliminated that the increased leak of sodium and potassium ions to the axonal surfaces in urea-treated insect connectives might result from effects on the perineurial membranes, thus contributing to an increased intracellular leak channel. Since there is no obvious mechanical disruption of the perineurial membranes or passage of ionic lanthanum it would, however, be necessary to postulate some unspecified effects upon the cation permeability of these membranes. The lack of effect of hypertonic urea on the resting and active membrane responses of axons, in desheathed preparations, could represent circumstantial evidence which does not accord with the latter hypothesis.

It appears, then, that exposure to hypertonic urea, but not to glucose, causes a disruption of the blood–brain barrier system, an effect which is not associated with appreciable damage to the perineurial membrane. In these circumstances it was of interest to observe that the amplitude of action potentials was reduced in urea-treated preparations as compared with intact connectives when bathed in normal

cockroach saline. It was originally observed by Pichon and Boistel (1967) that lower axonal resting potentials (58·1 ± 5·4 mV) were recorded in intact as compared with desheathed cockroach connectives (67·4 ± 6·2 mV); the action potentials in intact preparations were also found to exceed those recorded from desheathed ones 85·9 ± 4·6 mV). It has been suggested that the estimated difference of 19·7 mV between the overshoot (measured in giant axons in desheathed and intact preparations) could result from the maintenance of a fluid environment at the axon surface of different ionic composition to that of the bathing medium (Treherne and Moreton, 1970). If the effects were due to sodium ions alone, for example, the 48 mV increase in the active membrane potential for decade change in external concentration (Yamasaki and Narahashi, 1959) would imply that the concentration of this cation exceeded that in the bathing medium (210·2 mM/l) by a factor of 2·3.

As emphasized by Treherne and Moreton (1970) there are considerable uncertainties involved in the above interpretation. In particular, the possibility cannot be eliminated that the differences between the electrical responses recorded in intact and desheathed connectives resulted from axonal damage produced by the desheathing procedure. The observation that an equivalent reduction in spike amplitude can occur following urea treatment would tend to minimize this possibility, since application of hypertonic urea solution to desheathed preparations produced no detectable change in the action potentials following return to normal saline. The correlation between the disruption of the blood-brain barrier, and the decline in amplitude of the action potentials would thus accord with the hypothesis that the ionic composition of the extra-axonal fluid differs from that of the bathing medium. Such a difference could be maintained by a glial-mediated sodium regulation as provisionally proposed by Treherne & Moreton (1970) and Treherne & Pichon (1972). The nature of ion-regulating systems in the insect nervous system is currently being investigated in this laboratory.

SUMMARY

1. The effects of hypertonic urea and hypertonic glucose solutions upon the 'blood–brain barrier' in the isolated abdominal nerve cord of the cockroach have been studied.

2. Electrophysiological studies showed that a hypertonic solution of urea, but not of glucose, was effective in reducing the barrier to the entry of potassium and the loss of sodium.

3. Electron-microscopic studies revealed no significant ultrastructural changes, and no disruption of the perineurial barrier to the entry of ionic lanthanum, following comparable exposure to the hypertonic solutions.

4. It is suggested that this alteration of the blood–brain barrier may result from a selective change in permeability of the perineurial membranes or tight junctions.

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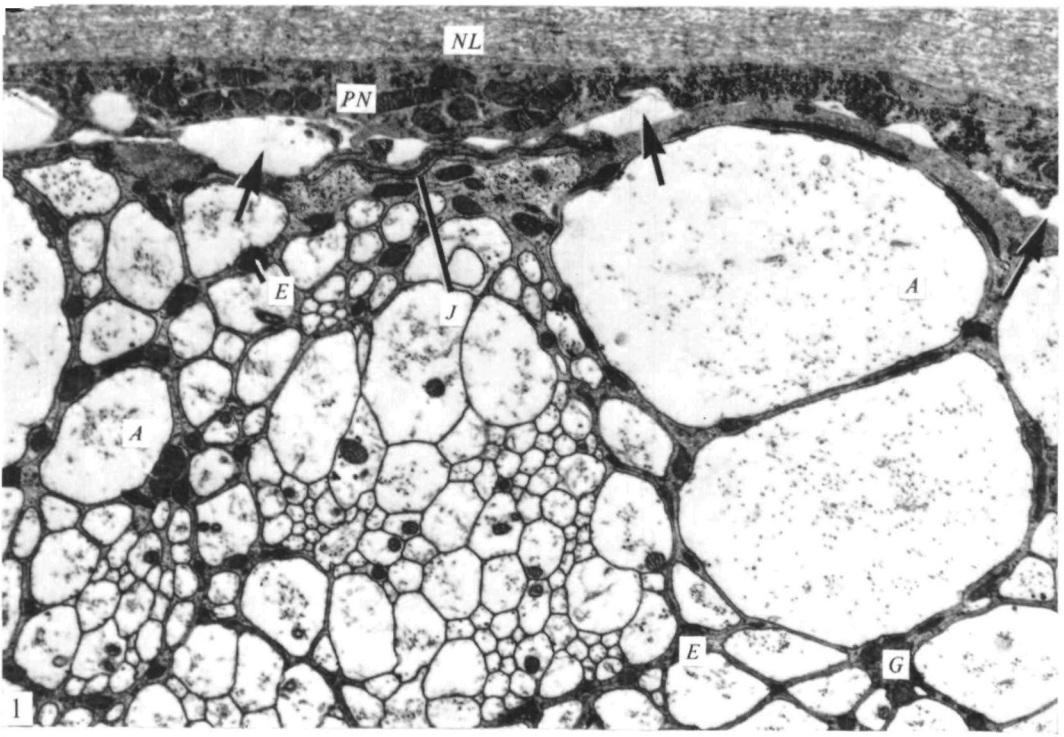
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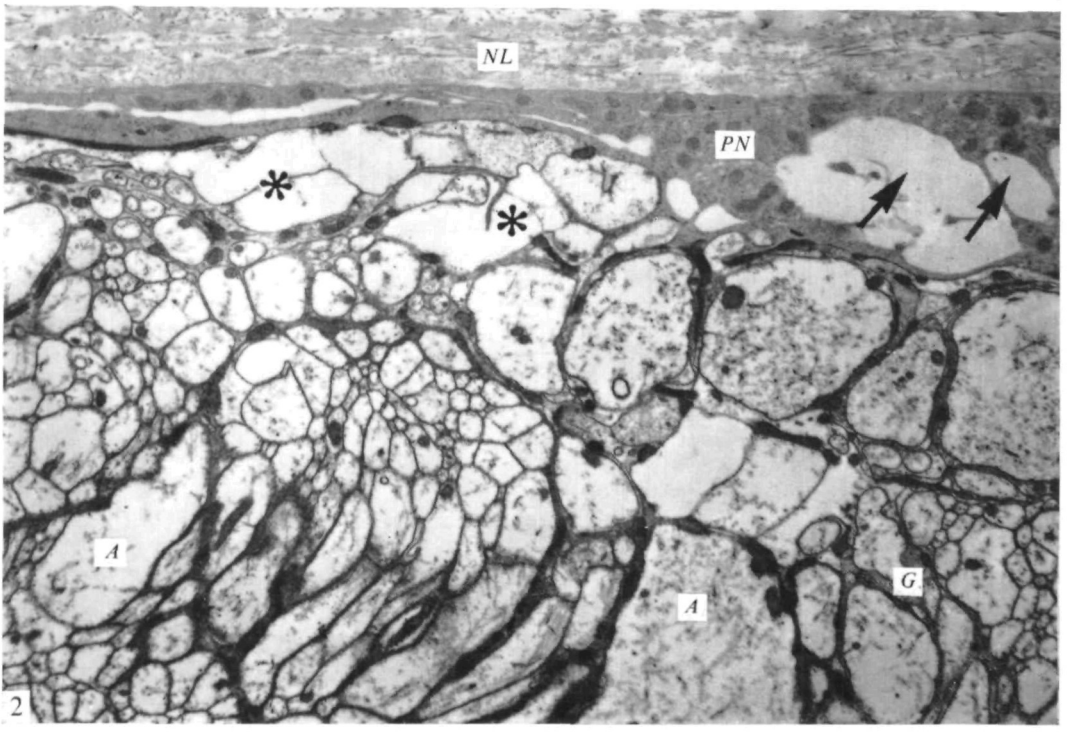
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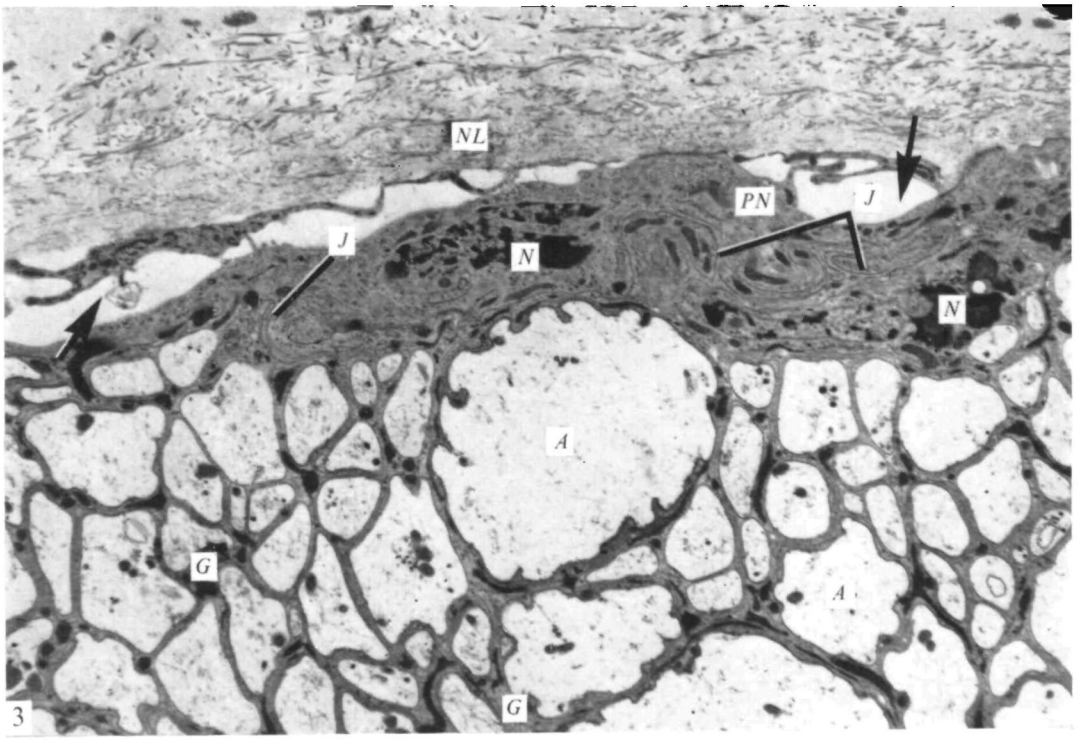
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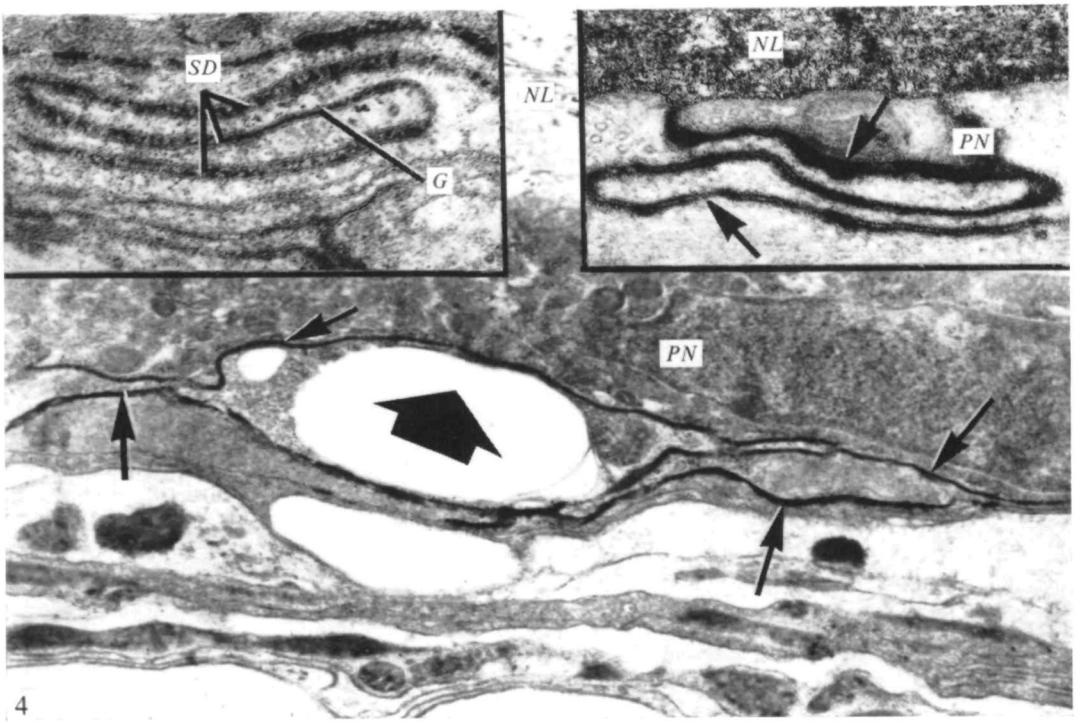
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EXPLANATION OF PLATES

The following micrographs are transverse sections through connectives of the ventral nerve cord of the cockroach, *Periplaneta americana*. *NL*, neural lamella; *PN*, perineurium; *A*, axons; *G*, glia; *J*, junctions between interdigitating lateral perineurial cell borders.

PLATE 1

Fig. 1. Connective from ventral nerve cord fixed directly in glutaraldehyde in phosphate buffer plus sucrose with no treatment prior to fixation. Note that the clefts between adjacent perineurial cells may be fairly extensive (arrows). Extracellular spaces between the underlying glial cells contain an electron-opaque material (*E*). × 11 850.

Fig. 2. Connective from ventral nerve cord incubated in phosphate-free saline for 15 min prior to fixation in a glutaraldehyde solution similar to that used for the preparation in Fig. 1. The effects of incubation in saline appear to include the production of increased spaces between the perineurial cells (arrows) as well as some cytoplasmic damage (*) immediately beneath them. × 9570.

PLATE 2

Fig. 3. Connective from nerve cord treated with 3·0 M urea for 15 sec and then fixed in a glutaraldehyde solution similar to that used in the controls. Note that although the clefts (arrows) between perineurial cells appear somewhat larger than the control in Fig. 1 there is no underlying cytoplasmic damage. The perineurial junctions appear to be intact. *N*, nuclei of perineurial cells. × 7800.

Fig. 4. Connective from nerve cord treated with 3 M urea for 15 sec, and then incubated in saline containing 1 mM ionic lanthanum for 60 min at room temperature. The tissue was then fixed exactly as were the control preparations. There are some spaces in the perineurial cells (large arrow) due presumably to the prolonged incubation times. Ionic lanthanum has penetrated the clefts between adjacent perineurial cells (arrows), but has not reached the glial and axonal mass beneath. × 23 950.

The insert on the right shows considerable accumulations of lanthanum both in the neural lamella and in the extracellular cleft (arrows) between perineurial cells which leads directly from the lamella. × 79 800.

The insert on the left shows that lanthanum can pass through the septate desmosomes (*SD*) that are found between the membranes of adjacent perineurial cells, as well as the gap junctions (*G*). × 84 130.