

## QUANTITATIVE ANALYSIS OF CELLULAR AND PARACELLULAR EFFECTS INVOLVED IN DISRUPTION OF THE BLOOD-BRAIN BARRIER OF AN INSECT BY HYPERTONIC UREA

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### SUMMARY

Brief exposure of central nervous connectives of the cockroach, *Periplaneta americana*, to 3 M-urea, a treatment known to increase the leak of cations across the blood-brain barrier system, caused a reduction in the resistance and potential difference (p.d.) across the perineurium, which forms the principal component of the barrier. There was also a reduction in the p.d. across the apical membrane of perineurial cells which might indicate a higher interstitial potassium level. There was no effect upon the p.d. recorded across the basolateral membrane of the cells, but there was some reduction in the ratio between the resistances of the basolateral and apical membranes. When the potassium level in the saline was raised, the depolarization of the basolateral membrane was less reduced by urea than the change in p.d. across the perineurium. During high K, the gradual depolarization of the perineurial membranes was speeded by urea treatment, as was the change in axon resting potential, as to be expected with more rapid entry of K into the interstitial system. Analysis of the initial effects of high K upon the electrical characteristics of the perineurium indicated that urea had reduced the resistance of the paracellular pathway by 73 % and the resistance of the basolateral membrane by 44 %. Most of the electrical events in urea-treated preparations could be simulated from the estimated parameters and an estimation of the rise in interstitial K level during the high K exposure. The study indicates that the increased leak across the perineurium caused by urea treatment results chiefly from a decrease in intercellular restriction.

### INTRODUCTION

If the insect nervous system is briefly exposed to hypertonic urea solution, a leak to the smaller cations is induced across the blood-brain barrier (Treherne, Schofield & Lane, 1973; Schofield & Treherne, 1978). In vertebrate epithelia, such treatment is thought to increase the paracellular leak, on the basis of an observed decrease in transepithelial resistance or change in flux (Ussing, 1968; Urakabe, Handler & Orloff,

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1970; Dibona & Civan, 1973; Finn & Bright, 1978), and from observations that the tight junctions change in their ultrastructure or ability to exclude tracer (Erlj & Martinez-Palomo, 1972; Dibona & Civan, 1973; Wade, Revel & DiScala, 1973; Dibona, 1978). The principal diffusion barrier in the insect blood-brain barrier system may be said to be formed by the superficial layer of neuroglial cells, the perineurium, from recordings made in the cockroach, *Periplaneta americana* (Schofield & Treherne, 1984). The only observed effect of urea treatment upon the ultrastructure of these cells is some intracellular damage; there is no effect upon the ultrastructure of the tight junctions, and no enhanced passage of lanthanum through the intercellular clefts (Treherne *et al.* 1973). It is not possible to measure the effect of urea upon the cation fluxes from perineurial cells (Treherne *et al.* 1982). From the available evidence, it may therefore be considered possible that urea treatment could increase the leak across the perineurium by damage to the cells, rather than by increasing the paracellular leak.

The damage that urea treatment causes to the perineurium of the cockroach is investigated in the present paper by measuring the effects upon the electrical characteristics of the perineurium (Schofield, Swales & Treherne, 1984; Schofield & Treherne, 1984), and analysis in terms of an electrical model (Schofield & Treherne, 1984).

#### METHODS

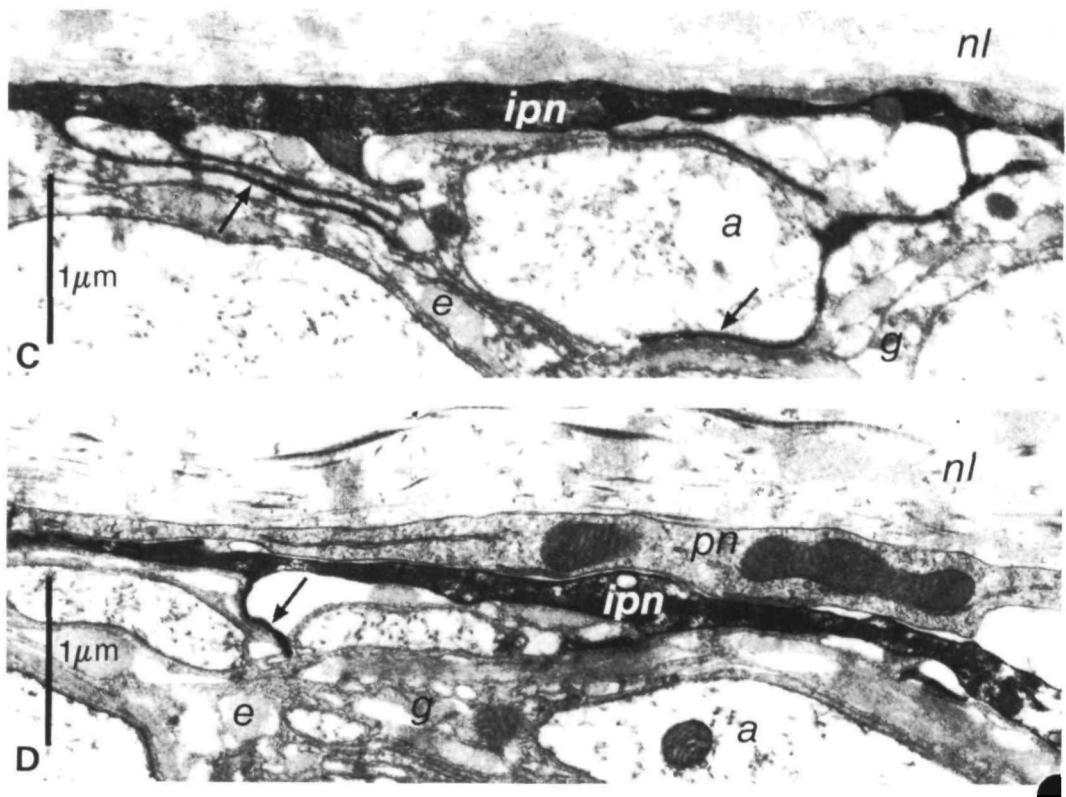
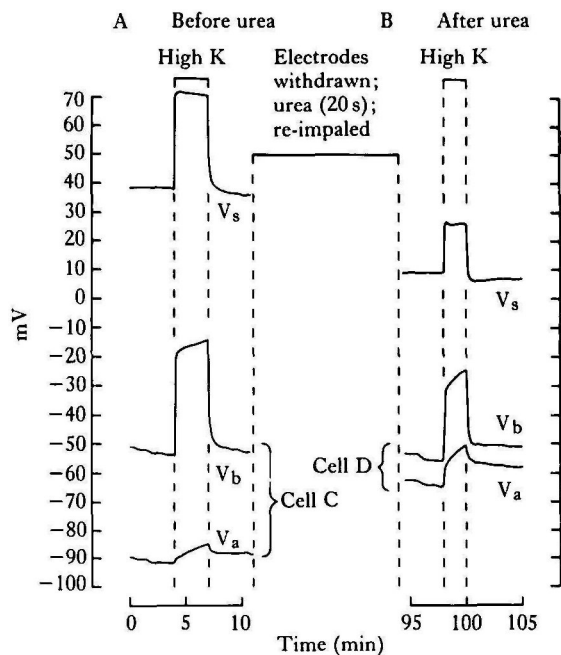
Adult male cockroaches, *Periplaneta americana*, were obtained from laboratory culture. The abdominal nerve cord was isolated, then sealed with petroleum grease into a Perspex chamber, and covered in saline (Treherne *et al.* 1973). In the compartment containing the penultimate connectives, the saline was flowing and its composition could be rapidly varied. Flow was stopped when the 3 M-urea solution was pipetted in. Experiments were at room temperature (18–26°C).

Recordings were made with microelectrodes inserted into the penultimate connectives as described earlier (Schofield *et al.* 1984). Extracellular stimulation was applied near the terminal ganglion to evoke action potentials. Resistances were measured by injecting current pulses (1–10 s,  $-1\mu\text{A}$ ) through a low resistance microelectrode, with the tip in an interstitial position, as in the previous paper (Schofield & Treherne, 1984).

Saline for the first section of the Results was that of Treherne *et al.* (1982), to aid comparison with results in that study, and had the following composition: 120 mM-Na, 10 mM-K, 2 mM-Ca, 2 mM-Mg, 55 mM-mannitol, 5 mM-trehalose, 131.7 mM-Cl, 2.5 mM- $\text{HCO}_3$ , 1.8 mM- $\text{HPO}_4$ , 0.2 mM- $\text{H}_2\text{PO}_4$  (pH 7.8). For subsequent sections, a filtered (0.45  $\mu\text{m}$  Millipore) 3 mM-K saline was used as in the previous paper to try to reduce variation in interstitial K level (Schofield & Treherne, 1984): 127 mM-Na, 3 mM-K, 2 mM-Ca, 2 mM-Mg, 50 mM-mannitol, 5 mM-trehalose, 135 mM-Cl, 3 mM-OH, 8.6 mM-HEPES (pH 7.2). High K saline (130 mM) was made by substitution of K for Na. Hypertonic urea solution was a solution of 3 M-urea in saline.

Microelectrodes for the first section of the Results, involving injection of horseradish peroxidase (HRP; Sigma type VI), were as used previously (Schofield *et al.* 1984). In other sections, electrodes were as in the preceding paper (Schofield & Treherne, 1984).





Cells injected with HRP could be seen under the light microscope after development, and material was prepared for examination under a Phillips EM300 as described previously (Schofield *et al.* 1984).

Probabilities of differences between values were calculated by Mann-Whitney U-test. All tests were two-tailed.

## RESULTS

### *Effect of urea upon p.d.s associated with the blood-brain barrier*

Simultaneous recordings from apparent perineurial cells and interstitial system were made from preparations that had been exposed to 3 M-urea saline for 20 s (5 cells in 4 preparations) and were compared with recordings obtained from untreated preparations (10 cells in 10 preparations). Urea-treated preparations were exposed to high K saline after an average period of 43 min (s.e. 12.5). One of the pairs of recordings made before urea treatment (Fig. 1A) was made from the same preparation as a pair made after treatment (Fig. 1B), and the cells were identified morphologically (Fig. 1C, D). In other impalements, the cell was injected with peroxidase and had the appearance under the light microscope of a perineurial cell (Schofield *et al.* 1984).

Urea treatment did not affect the p.d. across the basolateral membrane ( $V_b$ ) in the 10 mM-K saline (Fig. 1A, B). In untreated preparations,  $-56$  mV (s.e. 1.6) was recorded, whereas a value of  $-53$  mV (s.e. 1.3) was obtained after treatment ( $P > 0.10$ ). The interstitial p.d. ( $V_s$ ) was much reduced by urea (Fig. 1A, B), having a value of 24 mV (s.e. 2.3) without treatment, and 9 mV (s.e. 2.4) after it ( $P < 0.02$ ). Hence, the p.d. across the apical membrane ( $V_a$ ) was reduced, from  $-80$  mV (s.e. 2.4) to  $-62$  mV (s.e. 3.2) ( $P < 0.02$ ).

Upon raising the K level in the saline to 130 mM, there was a greater depolarization of the basolateral membrane than change in interstitial p.d. in urea-treated as well as untreated preparations. But the changes were larger for the untreated preparations (Fig. 1A) than for urea-treated ones (Fig. 1B), and the ratio between the changes was altered. The depolarization of the basolateral membrane was 31 mV (s.e. 2.8) without treatment, and 19 mV (s.e. 2.6) after treatment ( $P = 0.020$ ), while the change in  $V_s$  was reduced from 27 mV (s.e. 2.5) to 10 mV (s.e. 1.9) ( $P < 0.02$ ). Thus the ratio between the change in  $V_s$  to the change in  $V_b$  was reduced from 0.85:1 (s.e. 0.025) to 0.6:1 (s.e. 0.11) ( $P < 0.02$ ), principally by a reduction of the change in  $V_s$ . The change in  $V_a$  was 4.6 mV (s.e. 0.86) in untreated preparations, and 9 mV (s.e. 2.9) after treatment ( $P > 0.10$ ). These effects indicate a reduction in the ratio between shunt resistance and apical resistance, as shown in the next section of the Results.

Fig. 1. P.d.s in and across an identified perineurial cell in 10 mM-K saline, and effects of high K, (A) before urea treatment, and (B) for another cell in the same preparation after treatment. (C) The injected perineurial cell (*ipn*) identified for recording (A) shown in thin transverse section of connective ( $\times 22\,400$ ). The cell lies directly beneath the neural lamella (*nl*). Processes (arrows) lie among axons (*a*) and glia (*g*). Interstitial system (*e*). (D) The injected perineurial cell (*ipn*) identified for recording (B) shown in a further section of the connective ( $\times 22\,400$ ) lies beneath another perineurial cell (*pn*). Other symbols as above.

After the initial changes in p.d. induced by high K, the difference between basolateral and interstitial p.d.s diverged more rapidly in the urea-treated preparations (Fig. 1B) than in the untreated condition (Fig. 1A), indicating a more rapid depolarization of the apical membrane.

### *Characteristics of the perineurium after urea-treatment*

The effect of urea upon the perineurium was investigated further by recording from six preparations treated with 3 M-urea for 30 s, and bathed in 3 mM-K saline, for analysis and comparison with data obtained for untreated preparations in the preceding paper (Schofield & Treherne, 1984). Time between urea treatment and exposure to high K was 54 min (s.e. 5.2). P.d.s were recorded simultaneously in an apparent perineurial cell, the interstitial system, and an axon, while current was pulsed across the perineurium from a nearby interstitial electrode. From the initial changes produced by raising the saline K level to 130 mM, several parameters were calculated, as in the preceding paper (Schofield & Treherne, 1984).

The results (Table 1, Fig. 2) show that the effect of urea upon the recorded p.d.s was similar to that in the preceding section (Fig. 1). In addition, the axon shows a fast depolarization during the high K exposure (compare Fig. 2 with Fig. 2 of Schofield & Treherne, 1984). Trans-perineurial resistance ( $R_t$ ) was reduced, and there was some reduction in the ratio between resistances of basolateral and apical membranes ( $R_b:R_a$ ). From the changes in p.d. produced by high K, the ratio of shunt resistance to apical resistance ( $R_s:R_a$ ) was seen to be much decreased. The resistance data indicate that there was a fall in  $R_s$  of about 73 % and in  $R_b$  by about 44 %, with no change in  $R_a$ . The ratio of shunt resistance to cell resistance ( $R_s:R_b + R_a$ ) was not significantly different from that for untreated preparations. The electromotive force

Table 1. *Comparison between electrical parameters obtained previously in untreated preparations (Schofield & Treherne 1984) with those obtained after urea-treatment*

Parameter	Untreated	Urea-treated	Significance ( <i>P</i> value)
Axon resting potential (mV)	-75 ± 2.3	-67 ± 2.9	> 0.10
Time for 5 mV depolarization (s)	169 ± 33	15 ± 3.4	< 0.002
$V_a$ (mV)	15 ± 1.4	3 ± 1.7	< 0.002
$V_b$ (mV)	-60 ± 1.3	-61 ± 1.5	> 0.10
$V_s$ (mV)	-76 ± 1.9	-64 ± 2.5	< 0.02
K effect on resting potential (mV)	-0.2 ± 0.5	2.2 ± 0.7	> 0.10
K effect on $V_a$ (mV)	26 ± 1.9	8 ± 1.2	< 0.002
K effect on $V_b$ (mV)	30 ± 2.1	12 ± 1.5	< 0.002
K effect on $V_s$ (mV)	4.6 ± 0.8	4.1 ± 1.0	> 0.10
$R_t$ (k $\Omega$ )	72 ± 3.0	29 ± 4.1	< 0.002
$R_b:R_a$	11 ± 1.3	5.8 ± 0.5	< 0.05
$R_s:R_a$	9 ± 1.8	2.2 ± 0.4	< 0.02
$R_s$ (k $\Omega$ )	146 ± 29	40 ± 7.1	< 0.002
$R_b$ (k $\Omega$ )	192 ± 21	108 ± 10	< 0.02
$R_a$ (k $\Omega$ )	21 ± 2.9	19 ± 1.6	> 0.10
$R_t:R_b + R_a$	0.9 ± 0.28	0.33 ± 0.06	> 0.10
$E_b$ (mV)	-31 ± 6.7	-53 ± 7.2	< 0.05
$E_s$ (mV)	-78 ± 2.1	-65 ± 3.6	< 0.02
K effect on $E_b$ (mV)	56 ± 7.1	30 ± 5.0	< 0.02

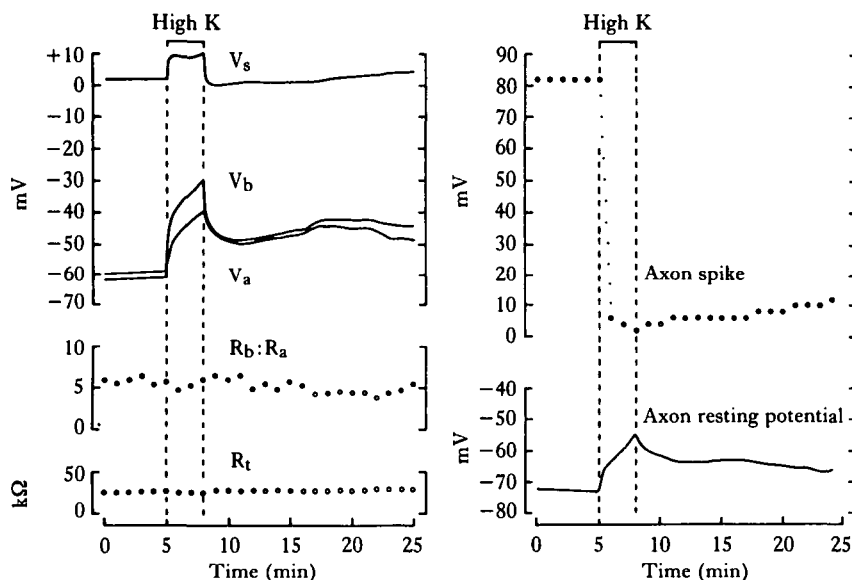


Fig. 2. Effect of high K upon simultaneous measurements of p.d. and resistance associated with the perineurium, and the resting and action potentials of an axon, in a urea-treated preparation in 3 mM-K saline. Deflections in p.d. produced by current injection are not shown, for clarity.

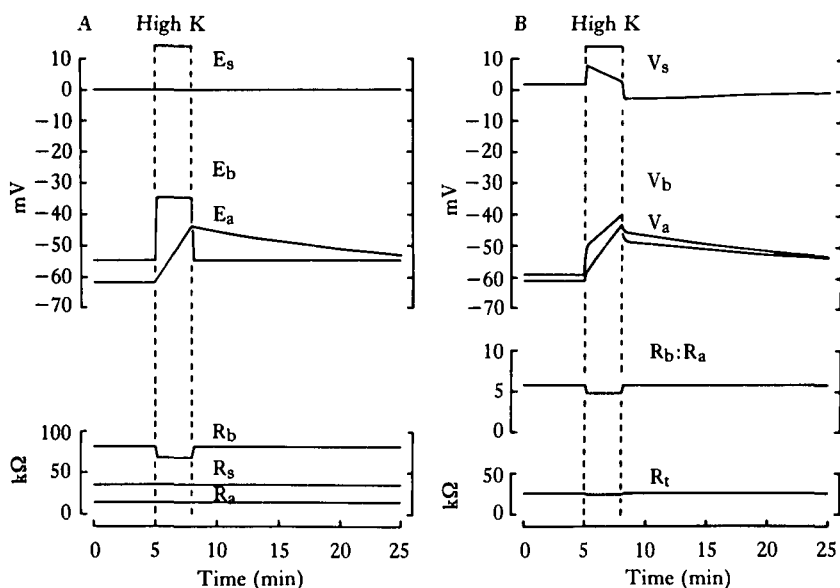


Fig. 3. (A) Calculated effects of a change in saline K concentration from 3 to 130 mM upon e.m.f. sources and resistances in the perineurium in a urea-treated preparation, using starting values and change in interstitial K derived from the recording shown in Fig. 2. (B) P.d. and resistance parameters calculated from the values shown in (A).

(e.m.f.) generated by the apical membrane ( $E_a$ ) was reduced, while that developed by the basolateral membrane ( $E_b$ ) was enhanced.

### Simulation

Changes in recorded values were simulated from the estimated parameters, and from an estimated time course for the accumulation of K in the interstitial system. In untreated preparations the initial K concentration was assumed to be 3 mM (Thomas & Treherne, 1975). The level in urea-treated preparations may have been higher since  $E_a$  was reduced (Table 1). If we assume that the mean value of axon resting potential reflects such a difference, then the K level in urea-treated preparations can be estimated to be 9.9 mM (Fig. 2 of Schofield & Treherne, 1978). The method employed in the preceding paper for untreated preparations (Schofield *et al.* 1984) was then used to calculate change in K concentration, and effects upon  $E_a$  and  $R_a$ . (The change in  $R_b:R_a$  was used only to provide a value for  $R_b$  during the K exposure, and any inaccuracy would affect little except the value of  $V_b$ .)

As in untreated preparations (Schofield *et al.* 1984), parameters estimated from a recording (Fig. 2) gave reasonable simulation of most recorded values (Fig. 3).

From the average parameters derived for untreated and urea-treated preparations (Table 1), the average effect of the urea treatment could be simulated (Fig. 4), and the simulated changes in p.d. and resistance (Fig. 4B) may be compared with recordings (Figs 1, 2).

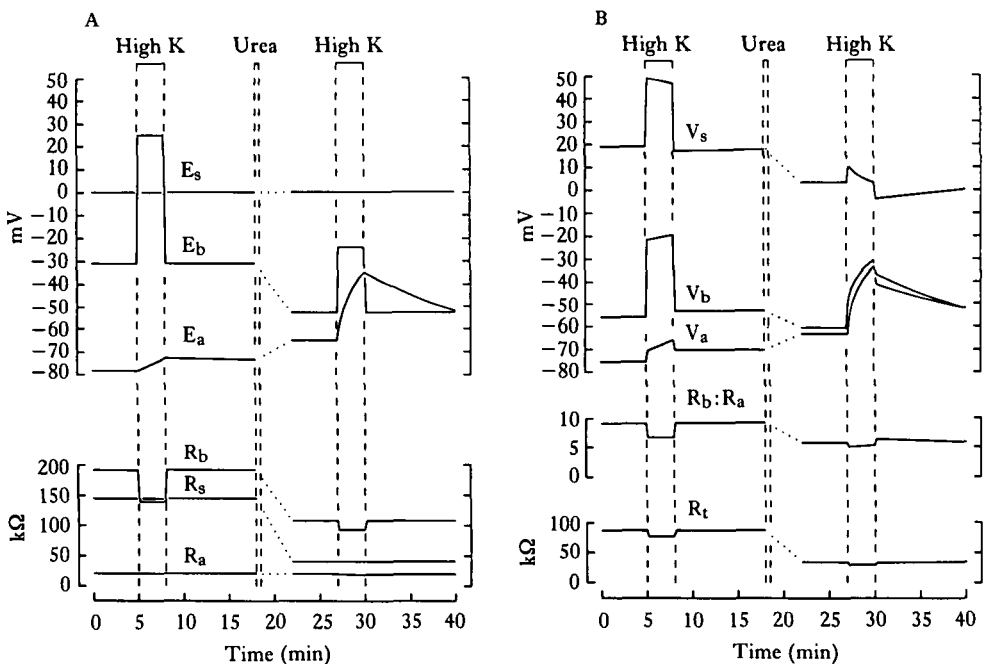


Fig. 4. (A) Calculated effects of a change in saline K concentration from 3 to 130 mM upon average values of e.m.f.s and resistances in the perineurium before and after urea-treatment, using average changes in interstitial K. (B) P.d. and resistance parameters calculated from the values shown in (A).



## DISCUSSION

Damage to the blood-brain barrier system by brief exposure to 3 M-urea was evident as an increase in the rate of depolarization of axons during exposure to high K saline, as in a previous investigation (Treherne *et al.* 1973). The interstitial K level in preparations bathed in 3mM-K saline may also have been higher, since the calculated e.m.f. generated by the apical membrane was smaller. The higher e.m.f. produced by the basolateral membrane shows that the perineurial cells were still functioning. It might also indicate that the K level at the surface of the basolateral membrane has become lower, as a result of damage to the neural lamella – which might contain a higher K concentration than in the saline (see Discussion of preceding paper, Schofield & Treherne, 1984). After urea-treatment, the neural lamella does appear more fragmented than in untreated preparations (compare Fig. 1C, D with Figs 3B, C, 4A and 5A in Schofield *et al.* 1984; and Fig. 1 with Fig. 3 in Treherne *et al.* 1973).

The main effect of urea upon the electrical characteristics of the perineurium was indicated to be a reduction in shunt resistance, by about 73%. This was primarily responsible for the large reduction in trans-perineurial resistance (Table 1). The shunt may thus be expected to afford less restriction to diffusion, and the increased access to the interstitial system could account for the more rapid penetration of K, as well as an increase in size of a fast fraction of cation efflux (Treherne *et al.* 1982). There was also a reduction in  $R_b$ , by about 44 %, indicating damage to this membrane. Some intracellular damage has been observed previously (Treherne *et al.* 1973), and could explain a decrease in the half-time for slow efflux of sodium (Treherne *et al.* 1982), following urea treatment.

The decrease in resistance would lead to an increase in current flow across the cells and back through the shunt (Schofield & Treherne, 1984). The change in current would be insufficient to sustain the interstitial p.d. across the lower  $R_s$ , and so the p.d. would fall in value. This decrease in 'sheath' potential is good evidence that the p.d. is not an artefact generated at the electrode tip, as was once suggested (Pichon & Boistel, 1967). During high K, the decreased shunt resistance also means that less change would be induced in  $V_s$ , as observed previously (Treherne *et al.* 1973). The current flow during high K would be higher than in untreated preparations, leading to a greater offset of the depolarization of  $E_b$ , and hence a smaller positive shift in  $V_b$ .

The effects of 3 M-urea upon the recordings could be simulated by employing the method of the preceding paper (Schofield & Treherne, 1984). Again the simulation gave a reasonable prediction of most values, but could predict somewhat less change in  $V_b$ , and greater change in  $V_s$  (Fig. 3), than was observed (Fig. 2). Since  $R_t$  could increase (data not shown), it appears that  $R_s$  may have been increasing. This might be a result of swelling, i.e. the reverse effect of hypertonic solutions (Urakabe *et al.* 1970; Dibona & Civan, 1973), which might be expected if potassium entered the cell (Boyle & Conway, 1941) especially if Cl was also permeant, as in muscle cells (Hodgkin & Horowitz, 1959). (However, there is no measurable Cl permeability of the basolateral face, as indicated by the effects of substitution of Cl: Schofield *et al.* 1984.) Or it might result from a change in cAMP level (Duffey, Hainau, Ho & Bentzel, 1981).

The effect of brief exposure to 3 M-urea, to increase the leak of cations through the insect blood-brain barrier, and change the p.d.s associated with the barrier, are thus indicated to be caused by an increase in diffusion between the perineurial cells more than by a change to the cells themselves. This analysis could be made despite wide variation in parameters between preparations. Where comparison between recordings made in the same preparation can be achieved, it should be possible to study more subtle issues. Study of the pharmacology of the perineurial glial cells should then be possible.

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