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A REAPPRAISAL OF THE OIL-GAP TECHNIQUE FOR THE MEASUREMENT OF TRANSTUBULAR POTENTIALS IN INSECT EPITHELIA

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Summary

1. Transtubular potentials of insect Malpighian tubules and salivary glands are commonly measured in unperfused tubules in which the two ends are separated by means of an insulating oil gap. The validity of this method has been examined, using Malpighian tubules of a tenebrionid beetle, *Onymacris plana*.

2. The measured interelectrode potential was found to depend not only on the transtubular potential but also on potentials within the oil gap and at the open end of the tubule, and upon the core resistance, the length constant and a small but finite electrical leak beneath the insulating oil.

3. In perfused tubules of *O. plana*, the apparent transtubular potential recorded at the collecting end (analogous to the oil gap) was indistinguishable from that previously measured with the oil-gap technique. However, it was invariably lower than that recorded by an intraluminal pipette.

4. The results demonstrate that the interelectrode potential observed with the oil-gap technique is a poor measure of the true transtubular potential.

Introduction

The elegant technique designed by Ramsay (1954), for studying Malpighian tubules *in vitro*, has been modified and used extensively for measurement of transtubular potential in Malpighian tubules and salivary glands (O'Donnell & Maddrell, 1984; and see Fig. 1A). The modification of Berridge & Prince (1972) consists essentially of positioning the dissected tubule across three compartments: a central one filled with oil, and two lateral ones containing Ringer's solution; the electrodes are placed within the latter. The method is referred to in this paper as the 'oil-gap technique'.

An alternative method of measurement of transtubular potentials utilizes *in vitro* microperfusion of tubule segments (Burg *et al.* 1966). Here, the potential measured *via* an intraluminal probe at the perfusing end (VO) can be assumed to be the actual transtubular potential, whereas that measured by a probe within the

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collecting pipette, but distal to both the Sylgard insulation and the open end of the tubule (VL; see Fig. 3), may be considered analogous to the transtubular potential as measured in unperfused tubules by the oil-gap technique (see Fig. 1A). In essence, the difference in methodology is that in the perfusion technique the measuring probe lies within the lumen, with only the wall of the tubule interposed between it and the other electrode in the bath. In contrast, in the oil-gap technique the probe is outside an (open) tubule fragment, and separated from the remainder of the tubule and the other electrode by a layer of insulating oil. In the light of the discrepancies commonly observed between VO and VL (see below) and the differences in methodology, we have examined the oil-gap technique more closely.

Theory

The transtubular potential of unperfused insect tubular epithelia (salivary glands and Malpighian tubules) is customarily determined (Berridge & Prince, 1972; O'Donnell & Maddrell, 1984) as depicted in Fig. 1A. The two ends of the tubule, each bathed in Ringer's solution, are insulated from each other by a barrier of liquid paraffin or oil. The end of the longer segment (bath *B*) is closed, and the other end is open, exposing the tubule lumen to bath *L*. The electrode in bath *B* faces the basal surface of the tubule, whereas that in bath *L* is in continuity with the apical surface. Thus the interelectrode potential is apparently equal to the transtubular potential in bath *B*. Implicit in this argument are the assumptions that: the tubule is electrically homogeneous along its length; the insulated portion between the two baths is electrically silent; the values of the length constant and core resistance are immaterial; and the resistance of the open end of the tubule is so low as to effectively short-circuit such transtubular potentials as may be present in bath *L*.

Fig. 1B shows an equivalent electrical circuit for the oil-gap technique. ET and RT, the Thevenin equivalents of the transtubular electromotive force and its series resistance, respectively, can be assumed to be identical at both ends of an electrically homogeneous tubule. The shunt resistances represent the leak pathways for current flow through intact tissue at the closed end (RSh-B), and through intact tissue in parallel with the open orifice (RSh-L) at the other end. This circuit in turn is equivalent to two opposing batteries, EB and EL, joined in series (Fig. 1C). As RSh-B is greater than RSh-L, EB is necessarily a larger fraction of ET than is EL. It follows that the interelectrode potential reflects only the *difference* between EB and EL.

It is thus apparent that the interelectrode potential will approximate to EB only if RSh-L is negligibly small relative to RT (and as RT is the same at both ends of the tubule, so also to RSh-B). If this is not the case, the larger the value of RSh-L, relative to RT or RSh-B, the smaller the interelectrode potential will be, relative to EB. It is apparent too that nonidentical values of ET and/or RT at the two ends of the tubule will further invalidate the interelectrode potential as a measure of EB; indeed in these circumstances the absolute length of tubule in bath *L* may also

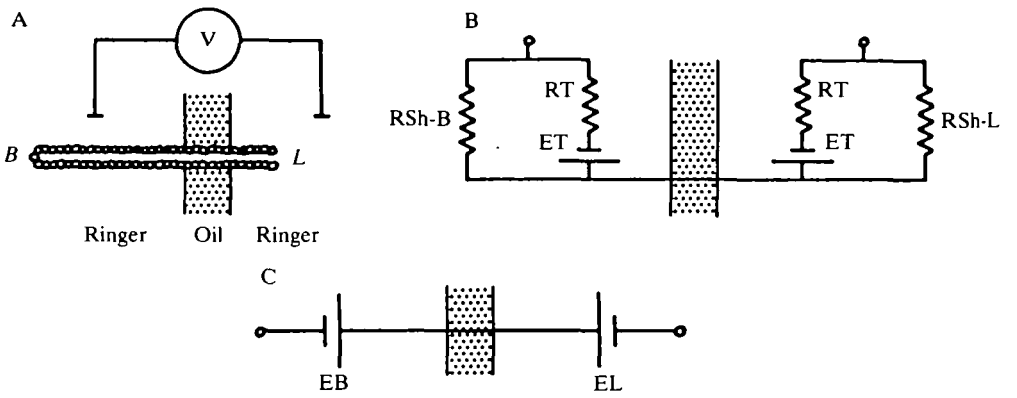

Fig. 1. Experimental arrangement and equivalent electrical circuits involved in the measurement of transtubular potential by means of the oil-gap technique. See text for an explanation of the abbreviations.

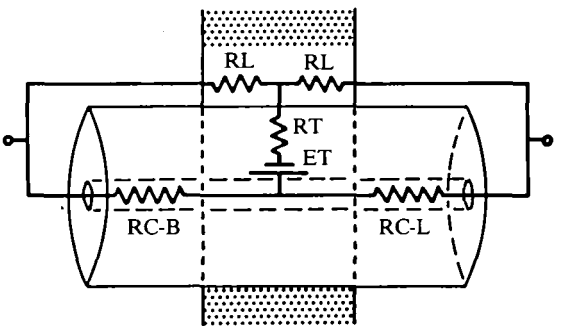

Fig. 2. Equivalent electrical circuit after addition of dinitrophenol to both baths; only the portion of tubule beneath the insulating oil remains electrically active. See text for an explanation of the abbreviations.

become critical. Should, for example, a distal portion of this end of the tubule be electrically silent (as might be expected consequent to the injury of dissection) and be several length constants long, then the core resistance of this electrically silent portion would further augment RSh-L, and so further reduce the apparent value of the transtubular potential in bath *B*. Finally, Fig. 1B,C assumes a total absence of electrical continuity between the two baths, other than through the tubule lumen, i.e. perfect application of the insulating oil to the outer aspect of the tubule. If, in practice, a thin layer of Ringer were trapped between the oil and the outside of the tubule, the resistance (RL) of this leak path between the two baths would reduce the interelectrode potential still further (Fig. 2).

We have explored the validity of the implicit assumptions listed above, by observing the changes induced in the interelectrode potential after manoeuvres designed to alter the relative values of individual components of the equivalent

electrical circuits in Figs 1B and 2. We have also examined the validity of the oil-gap technique directly, by comparison of the potentials measured simultaneously at the perfusing and collecting ends of isolated perfused tubules. These tubule segments were short enough to ensure electrical homogeneity along their length.

Materials and methods

All experiments were performed on the Malpighian tubules of adult female tenebrionid beetles, *Onymacris plana*. Beetles were collected by the Desert Ecological Research Unit at Gobabeb, Namibia, and maintained in the laboratory as described previously (Nicolson & Hanrahan, 1986). All experiments were carried out at room temperature (21–23 °C).

Unperfused tubules

The experimental arrangement was essentially identical to that depicted in Fig. 1A, and has been described in full elsewhere (Nicolson & Isaacson, 1987). The length of tubule immersed in Ringer was 5–7 cm at the closed end, and about 1 cm at the open end, these being insulated from each other by a 2-mm barrier of silicone oil. The beetle's cryptonephric system necessitated severing the tubule near the hindgut and ligaturing it with silk thread, this end corresponding to the closed end of Fig. 1A. The bath solution, based upon the haemolymph composition of *Onymacris plana*, was similar to that used previously (Nicolson & Isaacson, 1987) and contained (in $mmol\,l^{-1}$): NaCl, 125; KCl, 15; $MgCl_2$, 5; $CaCl_2$, 2; $KHCO_3$, 6; KH_2PO_4, 4; glycine, 10; proline, 10; serine, 10; histidine, 10; glutamine, 10; and glucose, 50.

After a stable value of interelectrode potential had been obtained, usually after some 20–30 min, the effects of subjecting the tubule segment in bath *L* to one or more of the following treatments were observed: (a) addition of $1\,mmol\,l^{-1}$ 2′,4′-dinitrophenol (DNP, British Drug Houses); (b) replacement of the bath solution by high-K^+ Ringer ($20\,mmol\,l^{-1}$ NaCl, $120\,mmol\,l^{-1}$ KCl, other constituents unchanged); or (c) addition of diuretic hormone (DH) extract, prepared by homogenizing the brain and corpora cardiaca of the beetle (Nicolson & Hanrahan, 1986). Additional treatments included (d) adding DNP to bath *B* as well as to bath *L*; and (e) reducing the length of the tubule segments in either or both of the two baths, both in the absence and in the presence of DNP.

Perfused tubules

Isolated segments (0·7–1·5 mm exposed to the bath) from the midportion of *Onymacris* Malpighian tubules were perfused according to the technique of Burg *et al.* (1966). Usually only one tubule was used from any one beetle. The bath solution was the same as that employed for the unperfused tubules (see above). The perfusate contained $20\,mmol\,l^{-1}$ NaCl and $120\,mmol\,l^{-1}$ KCl, but was otherwise identical to the bath solution. The height of the reservoir of perfusion fluid was held constant at some 12 cm above the bath. A layer of unpolymerized

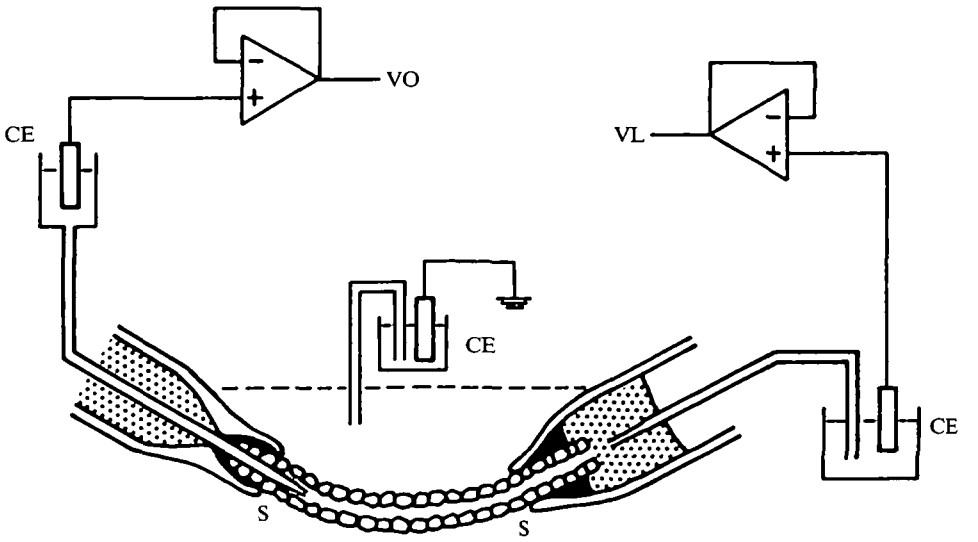

Fig. 3. Electrical circuit and pipette system for perfused tubules. CE, calomel electrode; S, Sylgard seal; other notation is explained in the text.

Sylgard (Dow Corning) within the orifices of both perfusing and collecting pipettes (Fig. 3) ensured electrical isolation of the tubule lumen from the bath. The far (open) end of the tubule, distal to the Sylgard in the collecting pipette, was 100–200 μm in length. The tubules were perfused for 2–5 h. In some instances DH (prepared as above) or 1 mmol l^{-1} cyclic AMP (Sigma) was added to the bath.

The bath was held at ground potential by a saturated KCl calomel electrode, immersed in bath fluid and joined to the bath by a short 3 % agar-saturated KCl bridge. Similar electrodes detected the transtubular electrical potential differences at both the perfusing (VO) and collecting (VL) ends of the tubule. The electrode at the perfusing end was placed within the perfusion reservoir, whereas that at the collecting end, bathed in perfusion fluid, was joined to the luminal fluid within the collecting pipette by a 3 % agar-saturated KCl bridge. The calomel electrodes sensing VO and VL were joined to high input impedance ($10^{13}\,\Omega$) unity-gain voltage followers. On mounting the tubule, the perfusing pipette was advanced down the lumen until VO evinced no further increase. VO and VL were displayed on digital millivoltmeters, of 0·1 mV maximal resolution, and recorded at 5–10 min intervals. Potentials are expressed with reference to the bathing solution. All results are expressed as means ± S.D.

Results

Unperfused tubules

(a) Addition of DNP to bath *L*, on 22 occasions in 12 tubules, immediately increased the interelectrode potential from 7·5 ± 16·4 to 35·2 ± 15·6 mV, an increase of 28·7 ± 14·5 mV.

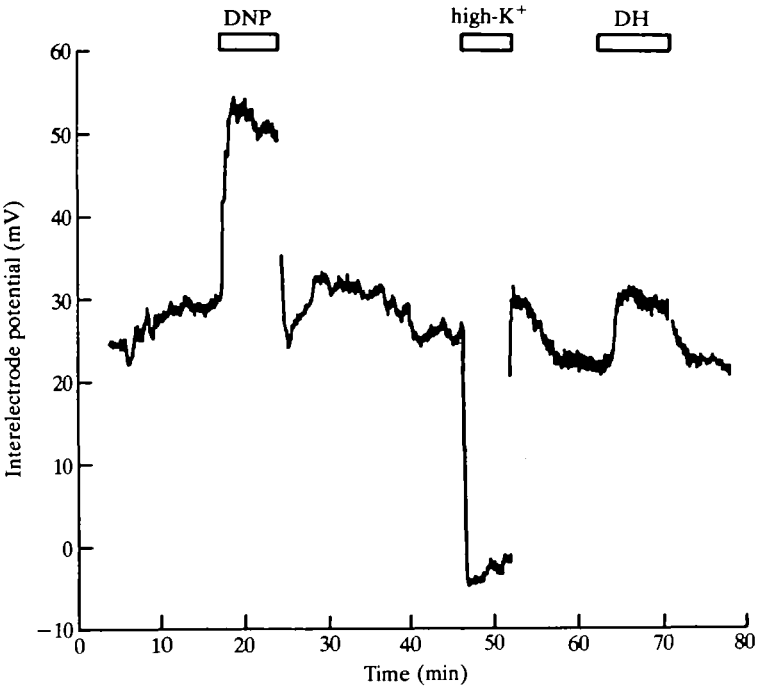

Fig. 4. Changes in interelectrode potential brought about by the sequential addition of dinitrophenol (DNP), high-K^+ saline and diuretic hormone (DH) to bath *L*. The oscillations superimposed on the PD in this trace are due to contractions of muscle strands which are associated with the tubules of *Onymacris plana* (Nicolson & Isaacson, 1987).

(b) Raising the K^+ concentration of bath *L* from 25–130 mmol l^{-1}, in seven tubules, promptly lowered the interelectrode potential from 12·3 ± 9·7 to −9·1 ± 8·5 mV, a decrease of 21·4 ± 9·5 mV.

(c) Addition of DH to bath *L* increased the interelectrode potential, in seven tubules, from 11·0 ± 10·1 to 28·7 ± 11·5 mV, a rise of 17·7 ± 9·8 mV. All the above effects could be obtained in succession in any one tubule (Fig. 4).

(d) Addition of DNP to bath *B* alone reduced the interelectrode potential from 19·3 ± 18·8 to −12·4 ± 19·8 mV ($N = 9$). This mean difference of 31·7 mV is very similar to the 28·7 mV change observed on addition of DNP to bath *L* (see above). When initial values of the interelectrode potential were low (range 2–7 mV), final values in the presence of DNP were uniformly negative (−12 to −56 mV). Adding DNP to both baths *B* and *L*, in eight tubules, reduced the initial interelectrode potential of 9·3 ± 9·4 mV, not to zero, but to 4·3 ± 7·9 mV (range −6 to +18 mV). In any individual tubule, the opposing changes in interelectrode potential caused by adding DNP first to one bath, then to the other, were strongly correlated (Fig. 5).

(e) Shortening the tubule segment in bath *L*, in five tubules, by severing it as close as possible (1–2 mm) to the oil barrier, increased the interelectrode potential

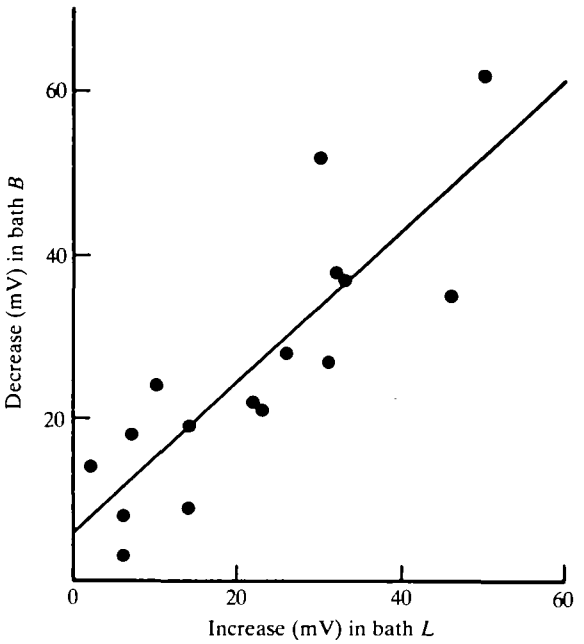

Fig. 5. Correlation between the potential changes caused by adding dinitrophenol (DNP) first to one end, then to the other, of individual tubules. The regression line is $y = 5{\cdot}70 + 0{\cdot}93x$ ($r = 0{\cdot}85$; $P < 0{\cdot}001$).

by $2{\cdot}7 \pm 1{\cdot}0$ mV (from $4{\cdot}2 \pm 7{\cdot}2$ to $6{\cdot}9 \pm 7{\cdot}3$ mV). Removal of the silk ligature from the long tubule segment in bath *B* had no effect on the interelectrode potential. Progressive shortening of this segment, until it was cut to within about 1 cm of the oil barrier, had no effect; at this point the interelectrode potential began to fall. When the segments in both baths were about 2 mm in length, the interelectrode potential was $2{\cdot}3 \pm 2{\cdot}2$ mV ($N = 4$; initial value $6{\cdot}4 \pm 3{\cdot}1$ mV).

Repeating this procedure in eight tubules, but with DNP in both baths, yielded essentially the same results. The initial interelectrode potential with normal lengths of tubule bathed in DNP was $3{\cdot}4 \pm 9{\cdot}7$ mV. This value was raised by shortening the segment in bath *L* to less than 1 cm and lowered by shortening that in bath *B*. However, even when the two segments were cut as short as possible, there frequently remained an appreciable residual voltage ($0{\cdot}9 \pm 7{\cdot}3$ mV; range -10 to $+15$ mV).

Perfused tubules

The transtubular potentials recorded at the perfusing and collecting ends of the tubule were almost never identical. On initiating perfusion, VO was invariably positive; VL was always less than VO and, at low values of VO, sometimes a few millivolts negative relative to the bath. During the first 30 min of perfusion, VO usually fell, more or less steeply, while there was relatively little change in VL. In 16 tubules, VL was recorded at frequent intervals during the first 30–45 min of

perfusion. At 9 ± 6 min (range 2–23 min), VO was 33 ± 22 mV (range 11–84 mV), while VL was 14 ± 11 mV (range 2–43 mV). By 32 ± 6 min (range 19–42 min), VO had fallen to 23 ± 13 mV (range 9–57 mV), while VL was 11 ± 7 mV (range 0–24 mV). Thus VL differed substantially from VO, both initially ($P < 0.01$; paired t-test) and after 30 min of 'equilibration' ($P < 0.01$). This pattern persisted during the remaining 2–5 h of perfusion. VL was, with but one or two exceptions (in which it transiently approximated to VO), always markedly less than VO.

Increases in VO induced by adding 1 mmol l^{-1} cyclic AMP to the bath ($N = 18$ applications in 13 tubules), were accompanied by parallel, but significantly smaller, increases in VL (21·9 ± 15·1 and 14·3 ± 9·2 mV, respectively; $P < 0.01$, paired t-test). Similarly, nine applications of DH to eight tubules resulted in decreases of 15·7 ± 14·1 mV in VO and 10·0 ± 11·3 mV in VL ($P < 0.05$). In seven of the 13 tubules treated with cyclic AMP and in three of the eight tubules treated with DH, however, the changes in VL and VO were almost identical.

Discussion

As the perfused region of the tubule was only 0·7–1·5 mm long, it was presumably electrically homogeneous along its length. Yet the apparent transtubular potential (VL) sensed by the probe within the collecting pipette – beyond the Sylgard insulation and distal to the end of the tubule – was consistently and markedly less than that recorded by the intraluminal probe at the perfusing (proximal) end (VO). This observation is not new. A similar discrepancy was noted many years ago in perfused rabbit renal tubules (Helman *et al.* 1971); although not investigating it experimentally, these workers suggested that 'a possible reason for this difference is that the short segment of tubule extending through the Sylgard 184 into the collecting pipet is electrically active and introduces an electrical potential in series between the tubule lumen and collecting pipet electrode'. That the cause of this phenomenon is localized to the segment within or distal to the insulation is evident from the observation that VL becomes identical to VO if the distal probe is advanced into the tubule lumen, beyond the Sylgard seal (Grantham *et al.* 1970).

There is also some evidence of this phenomenon in studies on insect tubular epithelia. Berridge *et al.* (1975), for example, inserted a microelectrode into the lumen of the *Calliphora* salivary gland to measure transtubular potential; the value obtained was about 11 mV greater than that measured by their oil-gap technique (Berridge & Prince, 1972). Similarly, Williams & Beyenbach (1984) measured a transtubular PD in perfused mosquito Malpighian tubules of 53 ± 3 mV (mean ± S.E.; $N = 110$). Identical values (54 ± 6 mV; $N = 3$) were obtained by advancing microelectrodes into the lumen of unperfused tubules (Sawyer & Beyenbach, 1985), but preliminary use of the oil-gap method (Williams & Beyenbach, 1984) gave readings which were much lower (15–30 mV; $N = 4$).

Our findings on perfused Malpighian tubules of *Onymacris* confirm and extend

these earlier observations. Although VL was almost invariably less than VO, the relationship was not fixed, but varied with time. After about 30 min of equilibration, VL was indistinguishable from the apparent transtubular PD of 13 ± 14 mV ($N = 36$) measured with the oil-gap technique (Nicolson & Isaacson, 1987), but still some 12 mV less than VO. At low levels of VO, VL was occasionally negative, but negative values of VO were never seen. These results are consistent with a potential source, within the collecting pipette, of smaller magnitude but opposite polarity to that within the proximal part of the tubule.

Furthermore, the addition to the bath of agents (cyclic AMP, DH) known to raise or lower the transtubular potential usually resulted in disproportionately larger responses in VO than in VL, suggesting an electrical shunt between the collecting pipette and the bath. As VL is entirely analogous to the apparent transtubular potential as measured by the oil-gap technique, our studies on unperfused tubules were designed to explore these potential sources of error in the latter method more closely.

In the unperfused tubules, the interelectrode potential increased on the addition of DNP or DH to bath *L*, but fell when the K^+ concentration in this bath was raised. [The basal membrane of *Onymacris* tubules is highly permeable to K^+ but impermeable to Na^+ (Nicolson & Isaacson, 1987), so that the simultaneous change in bath Na^+ concentration is irrelevant.] These potential changes were in opposite directions to those observed on adding these agents to bath *B* (Nicolson & Isaacson, 1987), which is consistent with the suggestion (Fig. 1C) that the interelectrode potential is not a measure of the transtubular potential, but merely the resultant of two potentials, of opposing polarities, connected in series. Moreover, these effects not only confirm the expected (Fig. 1B) presence of transtubular sources of potential in bath *L*, but also reveal as erroneous the assumption that the low shunt resistance at the open end of the tubule renders any such potentials immeasurably small.

Still further light on the nature of the shunt resistance in bath *L* is found in the results obtained on addition of DNP to individual tubules, first to one bath and then to the other; the absolute magnitudes of the resultant (oppositely directed) changes in potential were closely correlated (Fig. 5). This suggests that the shunt resistances of the tubule segments in the two baths were not dissimilar. Yet one end of the tubule was closed and the other was open to the bath. This apparent anomaly can be resolved if the length constant of the tubule is considerably less than the lengths (1–7 cm) of the segments in the two baths. Fig. 5 further suggests that electrogenicity had been abolished only in that segment exposed to DNP, an impression corroborated by preliminary experiments on perfused tubules, in which addition of DNP to the bath reduced the transtubular potential to zero.

Reducing the length of tubule in bath *L*, from about 1 cm to 1–2 mm, raised the interelectrode potential by some 3 mV. In the light of the above, the PD of the 'battery' in bath *L* must have decreased accordingly. Although this could be ascribed to the trauma of dissection, leaving only damaged tissue (reduced ET; Fig. 1B) in bath *L*, it is also consistent with reduction of the tubule length to within

a few length constants of the oil barrier, so giving more effective short-circuiting (reduced RSh-L) at the tubular orifice. Support for the latter suggestion can be found in the results of shortening the segment in bath *B*. Here, removal of the ligature had no effect on the interelectrode potential, which did not begin to fall until the tubule was cut back to within about 1 cm of the oil barrier, as would be expected were the length constant some small fraction of a centimetre. We have recently measured a length constant of approximately 300 μm in *Onymacris* tubules (L. Isaacson & S. Nicolson, in preparation). When the tubule segments in both baths *B* and *L* were cut to within 1–2 mm of the oil gap, the interelectrode potential fell to $2{\cdot}3 \pm 2{\cdot}2$ mV. The variation can be ascribed to slightly different values of RSh-B and RSh-L (it was not possible to cut the two segments to precisely equal lengths). The mean near-zero PD is consistent either with virtual removal of all potential sources, or with two 'batteries' of opposing polarities but similar potentials connected in series, as might be present if the tubule segments within the oil barrier were still electrically active. Evidence for the latter is provided by the following.

Addition of DNP to both baths, followed by shortening of both tubule segments, produced a perhaps surprising result: the interelectrode PD in different tubules, far from approximating to zero, ranged from -10 to $+15$ mV. The magnitude and polarity of this potential was found to be entirely dependent upon the relative lengths of the tubule segments in baths *B* and *L*; if that in bath *B* was longer, the potential was positive, and *vice versa*. The greater the difference in tubule segment lengths, the greater the potential observed. These results are consistent with the expression of a transtubular potential still present within the segment of tubule lying within the oil barrier (Fig. 2). This would be shunted, in each bath, by the core resistance of the electrically inactive tubule segment in series with the resistance at the tubule orifice; the longer the segment, the greater the core (and therefore the shunt) resistance, and so the larger the voltage of the 'battery' in that particular bath.

A measurable transtubular potential within the oil barrier implies a leak path of conducting fluid (Ringer) between the oil and the outer wall of the tubule (Fig. 2). Direct evidence for this was found in the perfused tubule studies (see above), in which increments or decrements in VO, induced by addition of cyclic AMP or DH to the bath, were accompanied in many instances by non-identical changes in VL. In view of the complex basal architecture of Malpighian tubules (Hanrahan & Nicolson, 1987), the persistence of such a film of Ringer beneath the oil seems highly probable. For perfused tubules we used only a conventional layer of Sylgard at the collecting end, so that the measurements would be analogous to those made with the oil-gap technique. Lapointe *et al.* (1984) have shown that a longer Sylgard seal is necessary for near perfect insulation; the difference between the values of VO and VL may then, however, be considerable.

Our results are thus consistent with the analysis presented in Figs 1 and 2. Essentially, the oil-gap technique appears to measure just the difference in potential between those parts of the tubule within a few length constants either

side of the gap, as modified by their respective shunt resistances, and partially attenuated by a residual leak path beneath the oil.

We conclude that the implicit assumptions underlying the use of the oil-gap technique to determine the transtubular potential in unperfused tubules (Fig. 1A) are of more than doubtful validity. Even if electrical homogeneity along the length of the tubule pertains (unlikely in view of the drift that occurs in prolonged experiments and the regional variation in morphology and physiology seen in many insect tubules), it is impossible to so shorten the tubule segment in bath *L* as to ensure reduction of RSh-L (and so EL) to zero. Nor will abolition of electrical activity in the tubule segment in bath *L* (with DNP or another inhibitor) ensure a zero voltage here. The latter treatment merely gives expression to the transtubular potential still present in the segment of tubule within the oil barrier, while increasing the shunt resistance by incorporation of the core resistance within the now 'silent' segment. Perhaps the only situation in which the use of the oil-gap technique can be justified is the measurement of short-lived relative changes in the measured interelectrode potential, as observed during the action of stimulants (Berridge, 1981).

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