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Intracellular Gradients of Electrical Potential in the Epithelial Cells of the *Necturus* Gallbladder

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Summary. When single-barrelled electrodes (5–60 MΩ) were advanced under manual control from the mucosal side of the epithelium the mucosal membrane was on average indented by about 40 μm before the microelectrode penetrated the cell. Since this dimpling was comparable with the total depth of the cell, which recovered its original shape within 0.5 sec, the steady intracellular potential was recorded only about 14 μm from the basal (serosal) membrane. Fast recording of the associated change in potential revealed an abrupt drop to −26 mV at a mean rate of 84 V/sec, followed by a further slow drop to a steady value of about −50 mV at a mean rate of 0.28 V/sec. The initial level of −26 mV may be regarded as the potential difference across the mucosal membrane. This conclusion was confirmed by mounting the microelectrode on a piezoelectric probe, which delivered 3 μm jabs in less than 0.5 msec. With this device in operation to prevent dimpling, the mean potential difference across the mucosal membrane was recorded as −29 mV. In all cases the potential across the basal membrane was recorded as −52 mV. Manual advance of the microelectrode tip within the cytoplasm yielded an intracellular potential gradient of 0.6 mV/μm. The same potential profile and membrane potentials were demonstrated on penetrating the epithelium from the serosal side, and measurements with multibarrelled electrodes whose tips were staggered in depth gave roughly the same internal potential gradient. The resistivity of the cytoplasm was determined by a triple-barrelled microelectrode, and varied from 10 times that of *Necturus* saline at the mucosal end of the cell to 4 times in the middle and 6 times at the serosal end.

One of the problems of recording intracellular potentials with microelectrodes is the choice of criteria for a valid recording. In epithelial tissues no alternative methods have been used for recording these potentials, and the criteria used so far have been to some degree arbitrary. It is generally required as a first criterion that the penetration into the cell should be associated with an abrupt increase in potential, this being a sign that the cell potential has not changed from the value prior to penetration. However, the abruptness at entry has not been quantitatively considered. A second criterion is that the intracellularly recorded

Table 1.

Tissue	Intracellular potential (mV)	Author(s)
Rabbit ileum *in vitro*	−36	Rose & Schultz (1971)
	−10 to −15	Field & Curran (1968[a])
Rat jejunum *in vitro*	−10	Barry & Eggenton (1972)
	−10	Lyon & Sherrin (1971)
Bullfrog small intestine *in vitro*	−45	White & Armstrong (1971)
	−40	Lee & Armstrong (1972)
Rabbit gallbladder *in vitro*	−45	Frizzell, Dugas & Schultz (1975)
	−51	Cremaschi, Henin & Ferroni (1974)
	−70	Van Os (1974)
Tortoise small intestine *in vitro*	−40	Gilles-Baillien & Shoffenniels (1965)
	−8	Wright (1966)
Chironomus salivary gland	−26	Loewenstein, Nakes & Socolar (1967)
	−60	Rose & Loewenstein (1971)

[a] Unpublished observations, quoted by Schultz and Curran (1968).

potential should remain constant for a period typically more than 10 sec;[1] but the changes immediately after penetration, i.e. for times typically less than 100 msec, have not been investigated. A spectrum of values has been obtained from each tissue, but the limits and average values differ between various reports. Some researchers have argued (e.g. Rose & Schultz, 1971; Armstrong, 1975) that the recording of low average values of potential is due to inadequate impalements. Others state that it is the maximal value obtained that is correct (Van Os, 1974). This lack of agreement on choice and application of criteria is reflected in the values reported, e.g., from the intestine and gallbladder (Table 1).

Often the values in individual reports differ by up to 40 mV. In *Necturus*, for example, Frömter (1972) reported values for the gallbladder ranging between −40 and −80 mV, and Reuss and Finn (1975) reported values between −44 and −72 mV; in the surface cells of the gastric mucosa, Spenney, Shoemaker and Sachs (1974) obtained values between

1 Another criteria has been put forward by Lassen *et al.* (1971, 1974) for penetrations into blood cells and ascites tumor cells, which is that the potential measured within a period of less than 1–10 msec after penetration, should resemble the unperturbed intracellular potential.

−30 and −80 mV. In the *Necturus* gallbladder some of this variation was found in individual bladders; Frömter (1972) gives examples of 10 or 15 mV. However, it is difficult to see how individual cells in the same tissue could have different intracellular values of electrical potential when these cells are known to be electrically coupled via low resistance pathways (Loewenstein, Nakas & Socolar, 1967). The intracellular recordings depicted or described are usually multiphasic; immediately after the initial negative deflection on penetration the potential may drift towards more or less negative values. Superimposed on some recordings are small oscillations of the order of 1 mV and a frequency of 1 Hz, or stepwise changes of more than 5 mV (e.g. Cremaschi, Henin & Ferroni, Fig. 3, 1974; Frizzell, Dugas & Schultz, Fig. 2, 1975).

Recently, Zeuthen and Monge (1975) reported that in the epithelial cells of rabbit ileum *in vivo* a low negative potential (−6 mV) existed at the luminal end of the cells compared with −36 mV at the serosal end. Thus, the difference in reported values could be due to the existence of an intracellular gradient of electrical potential in the epithelial cells, and some workers may systematically record from deeper below the brush border than others.

This concept is supported by measurements in *Necturus* gallbladder epithelium (Zeuthen, 1976*a, b*). The present paper will deal with the recording of electrical potential and the mechanics of cell impalement. The rate of change in potential on cell penetration was studied by using a fast negative-capacitance amplifier, and the influence of tissue deformation by comparing the results obtained by advancing the electrode with a hand-driven micromanipulator to those obtained with a piezoelectric micromanipulator. Results of the same nature as those in the rabbit ileum *in vivo* were obtained. With the tissue bathed in Na^+-rich saline, a gradient of electrical potential was found in the epithelial cells, from −28 mV in the mucosal end to −53 mV in the serosal end. An obvious electrode artefact can be ruled out, by the fact that the direction of the gradient is independent of the direction of penetration, and because two electrodes located at different depths in the same cell recorded different potentials. The gradient is abolished when Na^+ in the bathing solution is replaced by K^+. The cytoplasmic specific resistivity was recorded by means of a triple-barrelled electrode, and considered in the light of the ultrastructure as seen in electron-micrographs.

A later paper[2] will deal with the gradients of the intracellular ion

2 T. Zeuthen. Intracellular gradients of ion activities in the epithelial cells of the *Necturus* gallbladder (*in preparation*).

activities. A discussion of the mechanisms that may underlie the intracellular gradients are reserved for this second paper.

Materials and Methods

Surgery and Mounting the Tissue

Necturus maculosus were obtained from Mogul-ED, the Mogul Corporation. Fifty animals were kept at 5 °C (Kaplan & Glazenski, 1965) and were used within 20 weeks. The animals were pithed and the gallbladder exposed by a longitudinal insertion in the abdominal wall. The bile was removed *in situ* and the inside of the bladder rinsed 10 times with saline *via* a fine hypodermic needle inserted through the bladder wall. The bladder was dissected and mounted in a modified Ussing-chamber only slightly different from that described by Frömter (1972); (Fig. 1). The epithelial cell layer formed a flat horizontal sheet with a total exposed area of 0.2 cm^2. The volume of the mucosal solution was about 1 ml. The tissue stretched to between 50 and 100% of the area occupied in the intact animal and was supported by a stainless steel mesh. The epithelial cell layer was facing upwards for electrode penetrations from the mucosal side, and downwards for electrode penetrations from the serosal side. In this latter case 60–90% of the connective tissue was dissected away from an area of about 1 mm^2 and the electrodes used were pulled from a thick glass described below. The serosal solution was continuously renewed

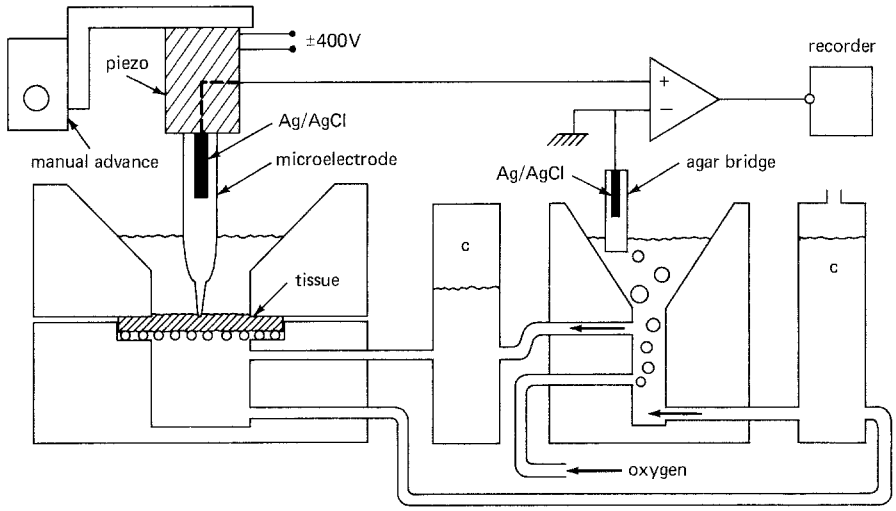

Fig. 1. Set-up used to record potentials from the *Necturus* gallbladder *in vitro*. The microelectrodes were advanced by a hand-driven micromanipulator. Single-barrelled electrodes could additionally be advanced in one step of 3 μm in about 0.1 msec by means of a piezoelectric crystal activated by a voltage pulse (±400 V). The recorded electrical potential was fed via an internal Ag/AgCl electrode into an amplifier and displayed on a chart recorder or oscilloscope. Potentials were referred to the serosal solution which was earthed via an agar bridge and an Ag/AgCl electrode. The serosal solution was circulated and oxygenated by means of a bubble lift. Two partially filled containers (*c*) were inserted in the circulation system in order to minimize tissue movement caused by the oxygen bubbles elevating the air-saline interface in the bubble lift. The drawing is not to scale

and oxygenated by means of a bubble-lift. Tissue movements caused by the creation of oxygen bubbles were minimized by passing the oxygenated saline through a partly filled closed reservoir (10 ml), and returning it *via* a similar open reservoir. Both the serosal and mucosal side of the tissue could be bathed in different salines: the Na^+-saline contained (in mM): 115.4 Na^+, 3 K^+, 2.7 Ca^{++}, 121.4 Cl^-, and 2.4 HCO_3^-; and the K^+-saline contained 118.4 K^+, 2.7 Ca^{++}, 121.4 Cl^-, and 2.4 HCO_3^-. The pH of the solutions was 7.5. When using tissues from animals kept for more than 8 weeks, 10 mM of glucose were added to the solutions. All reagents were of analytical quality.

Microelectrodes

Single-barrelled electrodes were made from borosilicate glass capillaries. Two wall thicknesses were used: o.d. 1.0 mm, i.d. 0.4 mm, or o.d. 2.0 mm, i.d. 1.0 mm. The capillaries were provided with an internal glass fiber (Tasaki, Tsukahara, Ito, Wagner & Yu, 1968; supplied by Clark Electromedical Instruments) whereby the electrode could be filled immediately after pulling. The electrodes were filled with 2 M NH_4NO_3, or in a few experiments with either 1 M Na_2SO_4 or 2 M KCl, and stored for less than 2 hr in air in order to reduce hydration and thereby the conductivity of the glass wall. The electrodes had an impedance of 5–60 MΩ when measured in Na^+-saline. The conductivity of the electrode wall was assessed by storing the electrode for up to 2 hr in Na^+-saline, whereafter the tip was advanced into a drop of silicone-grease (Edwards®) placed at the bottom of a Na^+-saline-filled container, while simultaneously monitoring the impedance. The impedance increased by a factor of 50–100 if the advance into the nonconducting grease was less than 2 μm. When the tip was advanced 5 μm the impedance increased more than a hundredfold. As electrodes were used for less than 1 hr, the shunting of a cell potential through the glass wall would be less than 2%. The electrodes had a tip potential that varied less than 3 mV when the concentration of a NaCl or KCl test solution was varied between 100 and 200 mM, and in which up to 50 mM of the Cl^- was replaced by HCO_3^-. The length of the shanks of the electrodes was 0.5 cm, and the taper of the tips pulled from the thin glass was 2–3°, and from the thick, 4–6°.

Triple-barrelled electrodes were made from one double-barrelled and one single-barrelled electrode. The two electrodes were held in micromanipulators with their tapered shanks parallel at a distance of 2–5 μm and fast-setting Araldite® was applied at the thicker parts of their shanks. The tip of the single-barrelled electrode was positioned 3–10 μm in front of or behind the tip of the double-barrelled electrode (Fig. 2). The double-barrelled electrode (for reference on manufacture of double-barrelled electrodes, *see* Frank & Becker, 1964) was made from one thin-walled and one thick-walled glass capillary, and had a tip diameter less than 0.6 μm. The impedance of the large barrel, which was used for current-injecting, was 6–10 MΩ when filled with saturated Na^+ or K^+-citrate, or 2 M NH_4NO_3; the impedance of the smaller barrel, which was used for recording potentials, was 10–50 MΩ when filled with 2 M NH_4NO_3. The other single-barrelled electrode was pulled from a thin-walled glass capillary, and also recorded potential. Thus, when inserted into a cell this triple-barrelled electrode recorded the electrical potential simultaneously at two different points. Furthermore, when current pulses were passed through the large barrel of the double-barrelled electrode to a large external electrode the specific resistivity of the cytoplasm could be assessed. If the potentials evoked intracellularly by this current were V_{c1} at the double-barrelled tip and V_{c2} at the single-barrelled tip, then assuming spherical symmetry:

$$V_{c1} = \frac{\rho_c i}{2\pi r_1} + V_m \tag{1}$$

$$V_{c2} = \frac{\rho_c i}{2\pi r_2} + V_m \tag{2}$$

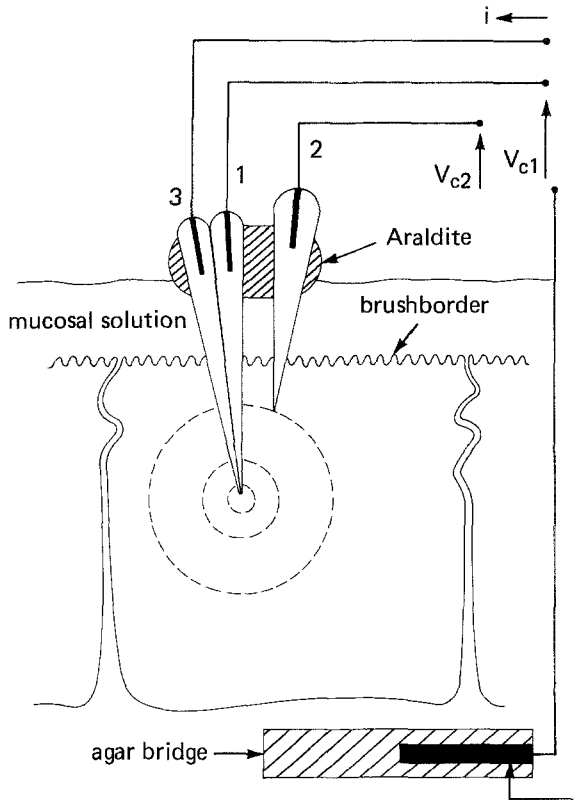

Fig. 2. A triple-barrelled electrode inserted into a cell of the epithelium of the gallbladder of *Necturus*. The electrode consists of one double-barrelled electrode and one single-barrelled electrode held together with a drop of Araldite®. The distance between the double-barrelled and the single-barrelled tip is less than 10 μm, with one tip in front of the other. One barrel of the double-barrelled electrode (No. 1) is used for recording the electrical potential, the other (No. 3) for current injection. The single-barrelled electrode (No. 2) is used for recording the electrical potential. Barrel No. 1 will record the intracellular potential deeper below the brush border (*b*) than barrel No. 2. When current pulses are injected via barrel No. 3 and the large external electrode, the evoked potential will to a good approximation have a spheric symmetry around the double-barrelled tip. Equipotential surfaces are indicated by a broken line. The attenuation (V_{c1}–V_{c2}) of the evoked potential inside the cell is measured between barrels No. 1 and No. 2. When compared to the same attenuation obtained in the mucosal solution the specific resistivity of the cytoplasm can be determined as described in the text. All potentials are referred to the fourth electrode which consisted of an agar bridge (*ab*) and a large Ag/AgCl-electrode (*a*) placed in the serosal solution

where ρ_c was the specific resistivity of the cytoplasm, and V_m was the potential drop caused by the current i passing across the cell boundaries, and r_1 and r_2 were the distances from the current-injecting tip to the potential-recording tips. When these values were compared with the potentials obtained in the mucosal bathing solution:

$$V_{s1} = \frac{\rho_s i}{2\pi r_1} \tag{3}$$

$$V_{s2} = \frac{\rho_s i}{2\pi r_2} \tag{4}$$

Where ρ_s was the specific resistivity of the Na-saline, ρ_c could be obtained as

$$\rho_c = \rho_s \frac{V_{c1} - V_{c2}}{V_{s1} - V_{s2}}. \tag{5}$$

Any contribution to the evoked potential from the cell boundary could be assessed from the equation,

$$V_m = V_{c1} \frac{\frac{V_{s2}}{V_{s1}} - \frac{V_{c2}}{V_{c1}}}{\frac{V_{s2}}{V_{s1}} - 1}. \tag{6}$$

Spherical symmetry will be disrupted when the current-injecting tip is close to the serosal membrane, in which case one of the potential-sensing electrodes will be close to this membrane as well. The other potential-sensing electrode would be about 3 μm away from the membrane. The effect of this distortion on the measured resistivity can be assessed from a model experiment in which the insulating cell wall was represented by a surface of insulating silicone grease immersed in saline. From this it was seen that only if the current-injecting electrode was as close as 1 μm to the insulating surface did the triple-barrelled electrode overestimate the resistivity of the saline by 20%. As the cells are 20 μm wide and 40 μm long, the current injecting tip must have been more than 1 μm away from the membrane in the vast majority of the cases. The influence of the cell walls on the recorded resistivity must therefore have been negligible.

Recording

Ag/AgCl electrodes were sealed into the shafts of the microelectrodes by means of a drop of fast-setting Araldite® or self-hardening silicone rubber (Dow-Corning). If the electrode was filled with 2 M NH_4NO_3 or 1 M Na_2SO_4, 200 mM KCl was added to the solution in the shaft. The signal from the Ag/AgCl electrodes was fed into high impedance amplifiers (Analog Devices 311 K, input impedance 10^{14} Ω) arranged to have an amplification of 1 and a response time of 0.1 sec. After amplification (for details of the electronics *see* Zeuthen, Hiam & Silver, 1974), the signals were displayed on a chart recorder (Bryans 3000). A fast resolution of the electrical potential changes was achieved by feeding the signal from the electrodes into a negative capacitance amplifier (W-P Instruments, Model 701) with a response time of less than 10 μsec, and displaying the potential on a storage oscilloscope (Tectronix type 564). All potentials were referred to the serosal solution, which was earthed by means of an agar bridge containing 2 M KCl (Figs. 1 and 2). Both for recording the impedance of the electrodes and to inject current, negative or positive rectangular current pulses of 10^{-9} amps lasting 6 sec were applied every 12 sec. All measurements were done inside a Faraday cage. Intracellularly recorded potentials were accepted for evaluation if they changed less than 10% over a period not shorter than 10 sec. All numbers are given with the standard error of the mean (SEM).

Electrode Advance

The electrodes were advanced by means of a hand-driven micromanipulator of the Huxley type, which enabled a continuous advance to be made with an accuracy of 0.2 μm in three mutually perpendicular directions. The electrodes were attached to the manipulator by means of a Teflon holder, and advanced in steps of 1–10 μm complete in 0.5 sec.

Single-barrelled electrodes could also be advanced in one step of 3 μm complete in less than 0.1 msec by means of a piezoelectric device similar to that described by Lassen *et al.* (1974). Briefly, the electrode holder was attached to the end of a 5 cm long piezoelectric crystal. The crystal was shaped as a tube (o.d. 15 mm, i.d. 5 mm), with a metal coating on the inner and outer surfaces. When a potential of 400 V was applied between these two coatings, the piezoelectric tube expanded within 0.1 msec by 3 μm in length. The crystal was placed inside a shielding steel tube, and the piezodrive was attached to the hand-driven micromanipulator. Thus, the electrode could be advanced by a combination of hand-driven steps and one piezodriven step (Fig. 1).

Table

The micromanipulator and the Ussing chamber were fixed onto a $2 \times 50 \times 50$ cm aluminum plate which was placed on a $5 \times 60 \times 60$ cm paving stone. This was laid on top of a 200 liter dustbin (British Standard) filled with sand. The dustbin stood on the floor upon a 1 cm thick plate of polystyrene.

Results

I. Tissues Bathed in Na^+-Saline (Table 2)

Single-barrelled electrodes advanced from the mucosal side. Electrodes were advanced by the hand-driven micromanipulator through the mucosal solution in steps of 1–10 μm until the tip just touched the tissue. When this point was reached, the impedance of the electrodes increased by about 10% and the recorded electrical potential changed by about 1 mV (*see* Figs. 5 and 4). The position of the micromanipulator was then noted, and the advance continued in either of two ways: (*a*) The stepwise advance was continued by hand across the mucosal membrane, cell interior and serosal membrane, after which the electrode was with-

Table 2. Intracellular electrical potentials of the *Necturus* gallbladder epithelium when bathed in physiological saline (Na^+-saline)

Mode of advance		Electrical potential (mV) just inside the:		No. of observations/ animals
		Mucosal membrane	Basal serosal membrane	
From the mucosal side:	Stepwise by hand	-26 ± 1.2	-54 ± 1.8	24/2
	By piezo and stepwise by hand	-29 ± 1.5	-52 ± 2.5	22/9
From the serosal side:	Stepwise by hand	-28 ± 4.3	-53 ± 3.5	9/6

drawn into the mucosal solution. (*b*) The piezodrive was activated and the electrode advanced 3 μm across the mucosal membrane into the cell. The advance was then continued by hand.

When the entry was made and continued by hand on additional advance of 37 ± 1.4 μm ($n=64$, 10 animals) was required before the electrode penetrated into the cell (Fig. 3*a*). The dimpling was independent of electrode impedances in the range 5–60 MΩ. A further advance of only 14 ± 1.0 μm ($n=38$, 10 animals) caused the electrode to emerge through the serosal membrane as judged by the abrupt return of the potential to near zero values. The potential change during such a penetration into the cell from the mucosal solution was biphasic (Fig. 4*b*). Initially the potential decreased to a value of -26 ± 1.2 mV ($n=24$, 4 animals) at a fast rate between 3 and 280 V/sec [average 84 ± 15.8 V/sec ($n=23$, 4 animals)]. This was followed by a slow and roughly exponential decrease to -53 ± 1.8 mV ($n=24$, 4 animals) at an average rate of 0.28 ± 0.06 V/sec ($n=24$, 4 animals). When the electrode recorded a steady potential intracellularly, each additional advance towards the serosal membrane was associated with a stepwise change in potential of the order of 0.5 mV/μm towards more negative values, (Fig. 4*a*). The electrodes recorded the largest intracellular potential of -54 ± 1.8 mV ($n=27$, 4 animals) immediately before penetrating the serosal membrane.

If the mucosal membrane was penetrated in a 3-μm step by means of the piezodrive, an additional advance of 42 ± 1.9 μm ($n=26$, 10 animals) was required before the tip penetrated the serosal membrane (Fig. 3*c*). The intracellular potential was -29 ± 1.5 mV ($n=22$, 9 animals) immediately behind the mucosal membrane (Figs. 5 and 6). Each additional stepwise advance made with the hand-driven manipulator towards the serosal membrane was associated with a stepwise change in potential towards a more negative value. Each steady potential value could be maintained for a period up to 15 min. Once intracellular, the electrode could also be retracted by one step of 2–5 μm, whereupon it recorded a stepwise change in potential of the order of 2 mV towards a less negative value. The electrodes again recorded the largest negative potential, -52 ± 2.5 mV ($n=12$, 9 animals), immediately before penetrating the serosal membrane. The results were the same whether the electrode was filled by 2 M NH_4NO_3, 2 M KCl or 1 M Na_2SO_4. The recorded electrical potential shown as a function of the distance advanced for 11 different penetrations in Fig. 6; on average, the potential became 0.6 mV more negative for each 1 μm advanced, although the gradient tended to be smaller in the middle of the cell (compare Figs. 5*b* and

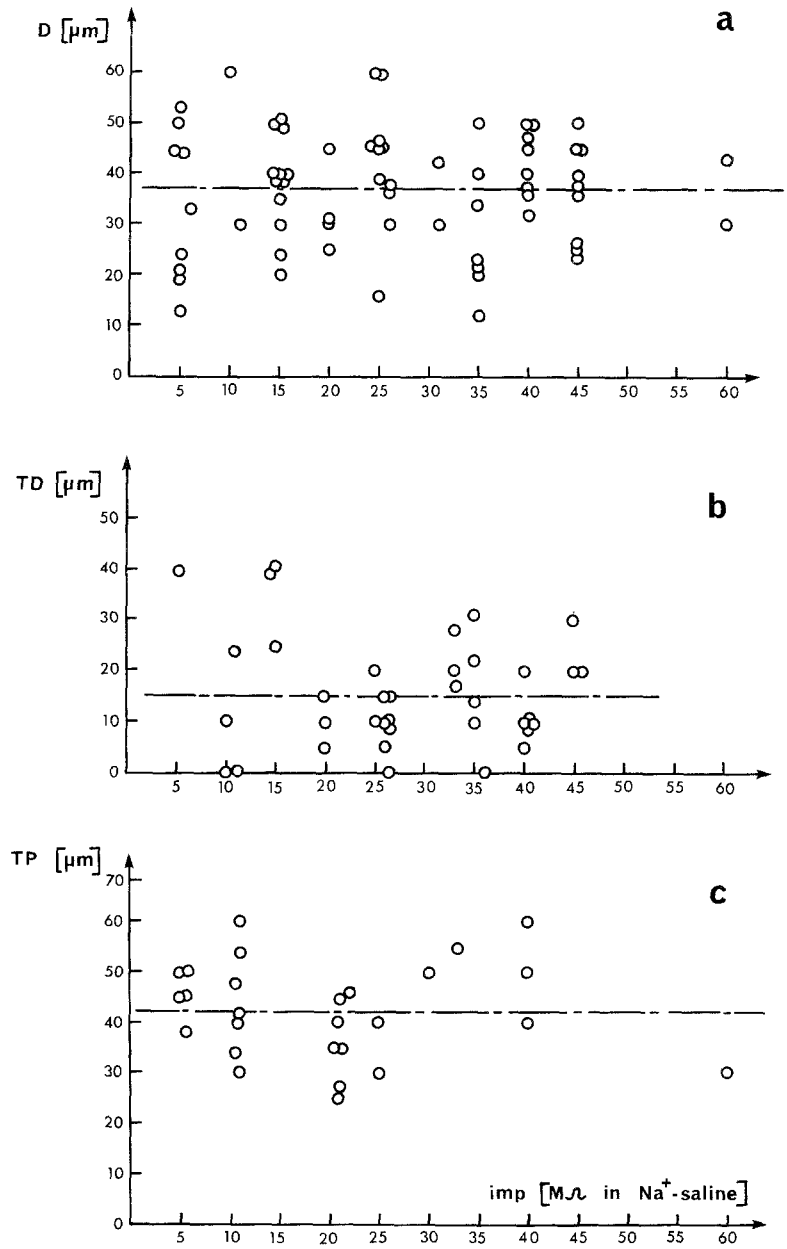

Fig. 3. (*a*) Dimpling (D) in µm as a function of the impedance of the electrode. The electrode was advanced by the hand-driven micromanipulator from the mucosal side. The dimpling was calculated as the amount of additional advance needed from the moment when the electrode just touched the mucosal membrane until it penetrated into the cell. (*b*) After this penetration an additional advance of TD (µm) caused the tip to cross the serosal membrane into the subserosa. (*c*) Alternatively, the mucosal membrane was penetrated without dimpling, by advancing the electrode by a piezoelectrical tranducer. In this case an additional advance of TP (µm) caused the tip to cross the serosal membrane

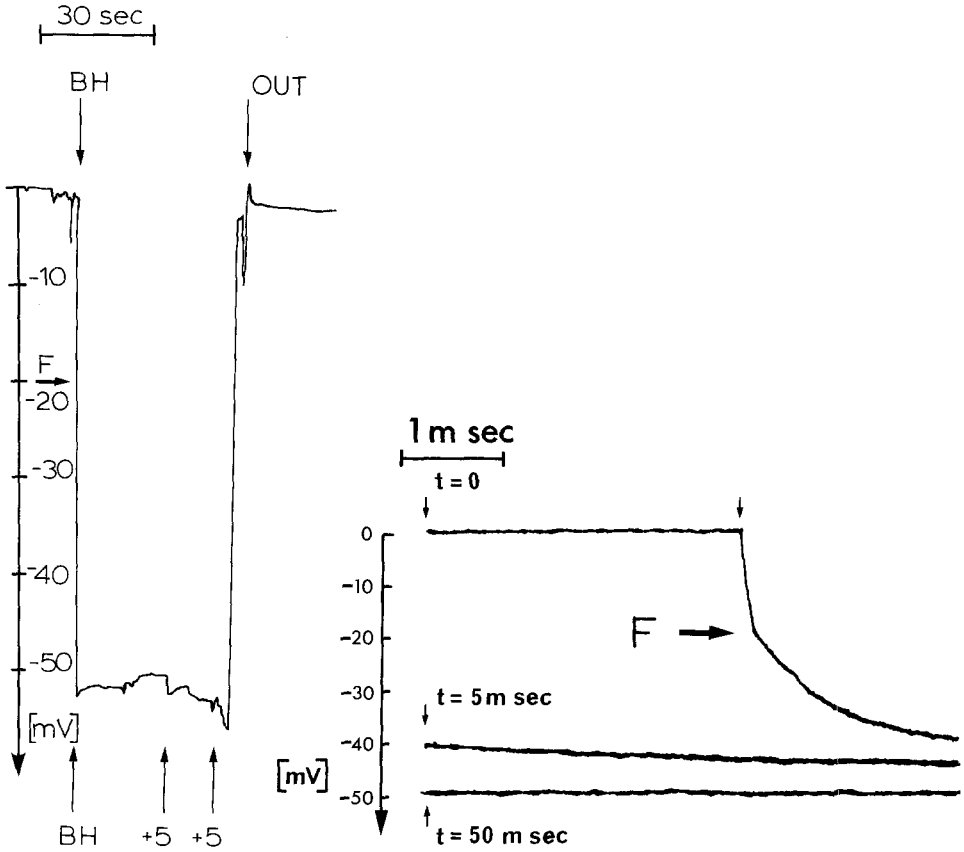

Fig. 4. (*a*) Intracellular recording achieved by advancing the electrode by the hand-driven micromanipulator displayed on a chart recorder with a rise-time of 0.5 sec. Na^+-saline in the lumen. When the electrode just touched the mucosal membrane, indicated by a change of a few mV, an additional advance of 40 μm caused the electrode to penetrate the mucosal membrane, at *BH*. An additional advance of two steps of 5 μm each, caused the electrode to penetrate the serosal membrane. The electrode was pulled back into the mucosal solution at *out*. (*b*) The entrance phase *BH* resolved on a smaller time scale by means of a negative-capacitance amplifier (resolution time 10 μsec) and displayed on a storage oscilloscope. The change of potential on penetration is biphasic, consisting of a fast linear phase taking the potential up to about −20 mV (indicated by *F*) and a slow exponential phase lasting for more than 100 msec. The change in potential is shown at $t=0$ sec (arbitrary time) and after 5 and 50 msec

6). In a few penetrations Ca^{++} was removed from the Na^+-saline, but this did not affect the results.

In experiments *a* and *b* taken together the potential just behind the cells, in the sub-serosa, was recorded as -2.7 ± 0.6 mV ($n=64$, 9 animals). This was associated with a temporary increase in electrode impedance of about twofold.

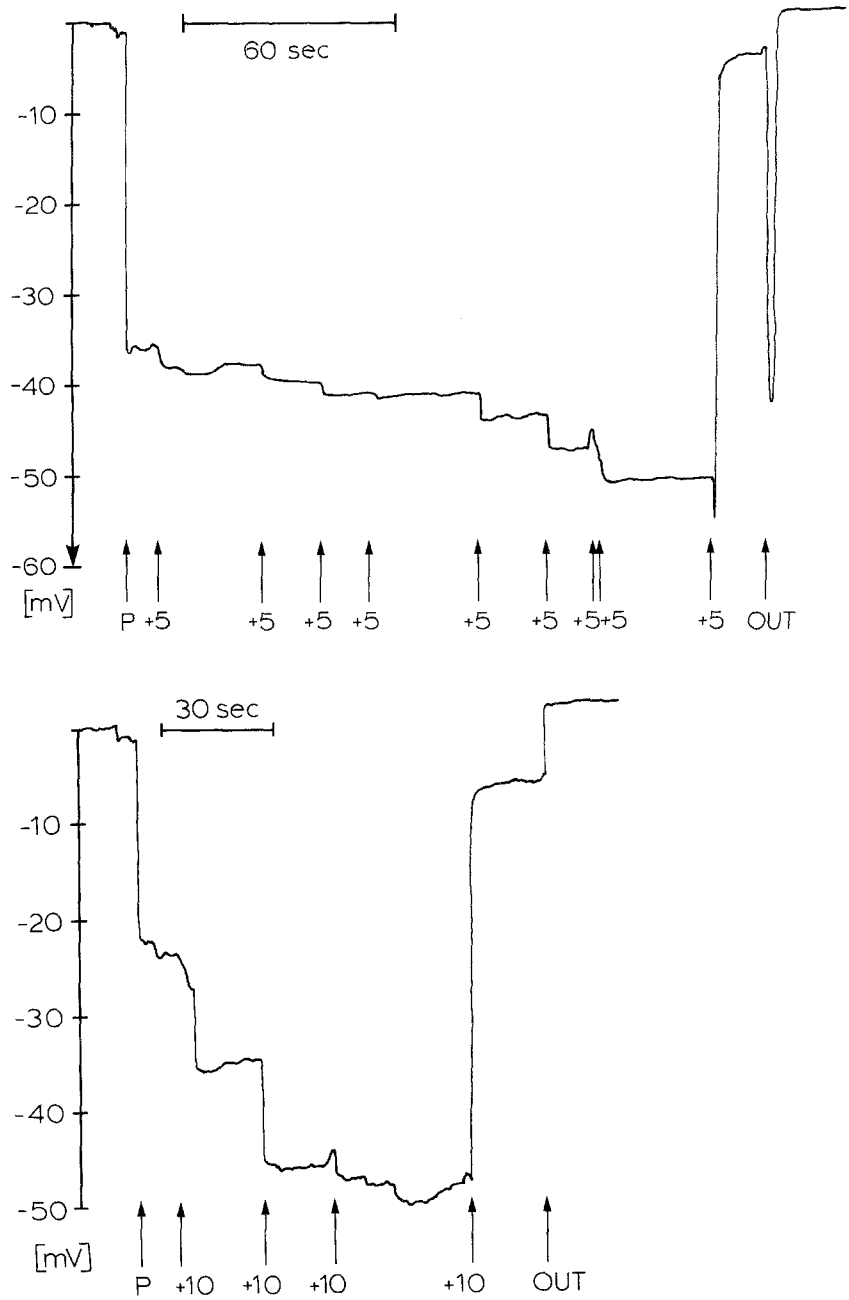

Fig. 5. Two examples of the profile of electrical potential across the epithelial cells when both the mucosal and the serosal solution is Na^+-saline, and the mucosal membrane was penetrated by advancing the electrode by the piezoelectrical micromanipulator. The advance of the electrode through the cell layer was continued by the hand-driven micromanipulator in steps of 5–10 μm. The electrode was pulled back into the mucosal solution at *out*

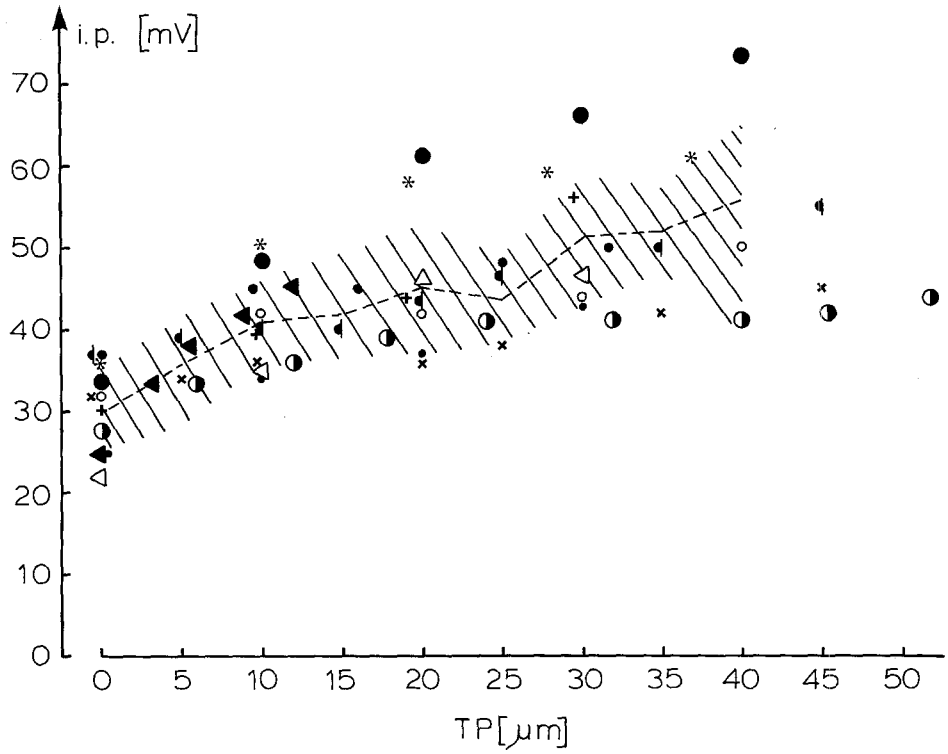

Fig. 6. The intracellular electrical potential (*i.p.*) as a function of depth of advance of the electrode below the mucosal membrane. The mucosal membrane was penetrated by advancing the electrode by means of the piezoelectric device. Both the mucosal and serosal solution were Na^+-saline. The results of 11 individual penetrations are shown by 11 different symbols. The area between the upper and lower limit of the standard error of the mean is shown shaded. The mean and standard error of the mean were calculated from the values pooled from an interval of 10 µm

In about 5% of all experiments the potential changed in two steps on entering the cell. The first step was about -10 mV and the second about -20 mV. These recordings were rejected (*see* footnote 2). 10% of the recordings had superimposed oscillations of about 1 mV with a frequency of about 1 Hz. In about 2% of the animals, large (maximum 5 mV) sinusoidal oscillations were observed with a frequency of the order 0.1 Hz. In about 5% of the recordings, the electrical potential was recorded as about 5 mV less negative at the extreme serosal end when compared to the value obtained 25 µm inside the cell.

Single-barrelled electrodes advanced from the serosal side. At the serosal end of the cell the electrical potential was -53 ± 3.5 mV ($n=9$, 6 animals) and just inside the mucosal membrane it was -28 ± 4.3 mV

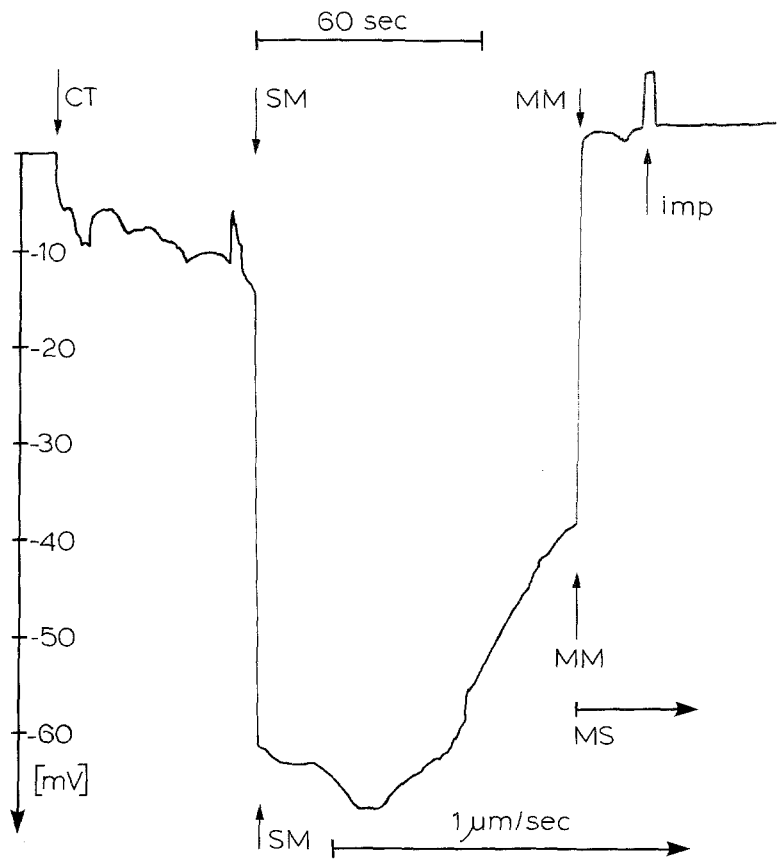

Fig. 7. The profile of electrical potential across the epithelial cell layer obtained when the electrode was advanced from the serosal side. Both the serosal and the mucosal solution was Na^+-saline. At *CT* the electrode entered the connective tissue and a negative potential was recorded concurrently with a temporary increase in impedance up to 10-fold; the advance was 1 µm/sec. At *SM* the electrode entered the cell and the advance was stopped. After about 20 sec the advance was continued at a rate of about 1 µm/sec. At *MM* the electrode penetrated the mucosal membrane and entered into the mucosal solutions (MS). Further advance left the potential unchanged. At *imp* the electrode impedance was tested

($n=9$, 6 animals) (*see* Fig. 7). The difference between the two ends was 25 ± 3.1 mV ($n=9$, 6 animals). The serosal membrane was assumed to be penetrated when the electrical potential changed within less than 5 msec, by more than 20 mV. The mucosal membrane was assumed to be penetrated when the potential returned to near zero values within 5 msec, and when a further advance did not cause any change in the recorded potential. When the electrode was advanced through the connective tissue a potential of -5 to -10 mV was recorded concurrently with a temporary increase in the impedance of the electrode by more than a factor of ten.

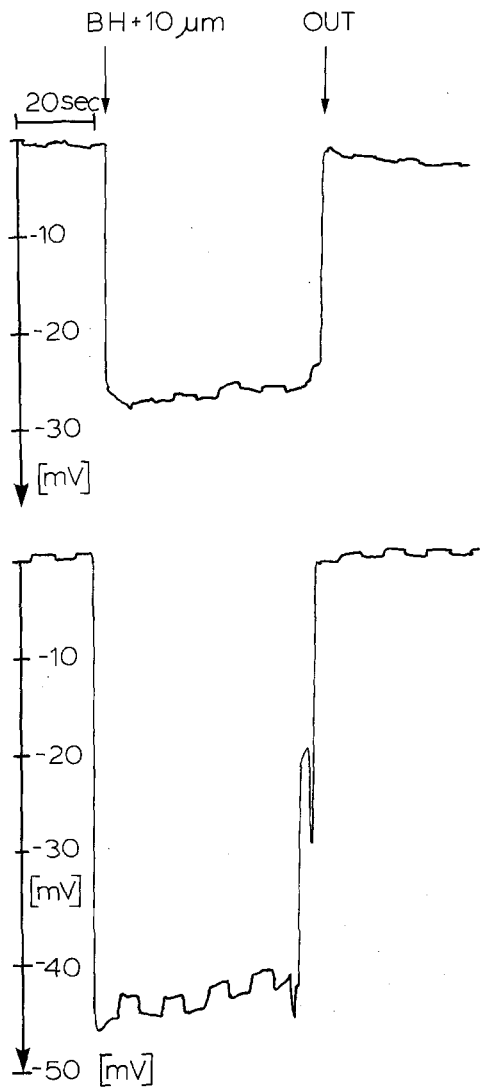

Fig. 8. Recording by the triple-barrelled electrode (*see* Materials and Methods and Fig. 2) of the intracellular potentials and specific resistivity of the cytoplasm when the tissue is bathed in Na^+-saline. When the leading barrel, which in this case was the double-barrelled tip, touched the mucosal membrane the electrode was advanced 10 µm into the cell by the hand-driven micromanipulator (*BH*+ 10 µm). The electrode was pulled back into the mucosal solution at *out*. The trailing single-barrelled electrode was placed 5 µm behind the leading barrel in the direction of movement and 5 µm to the side in the direction parallel to the plane of the tissue. A square pulse of current (-10^{-9} amps) was continuously passed *via* one barrel of the double-barrelled tip and a large external electrode. From the difference in evoked potentials between the mucosal solution and the cytoplasm the specific resistiviy could be determined (*see* text)

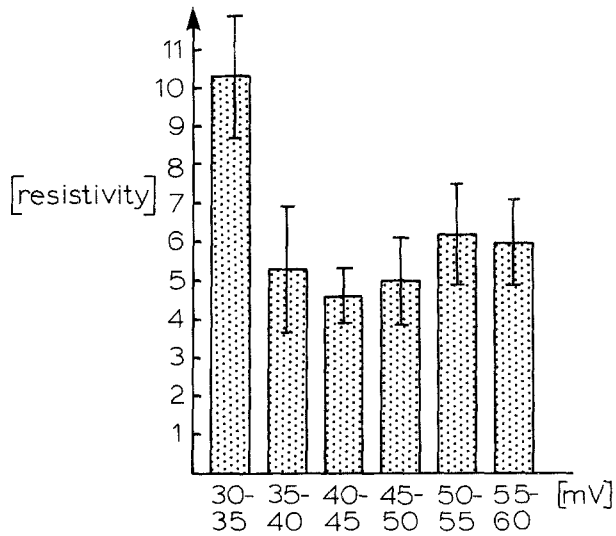

Fig. 9. Specific resistivity of the cytoplasm (relative to Na^+-saline) recorded by the triple-barrelled electrode, as a function of the intracellular potential; this can be transformed to a function of depth below the mucosal membrane by using the relation between potential and depth shown in Fig. 6. Only the resistivity obtained for potentials between -30 and -35 mV is significantly different from any of the others ($p<0.01$)

Triple-barrelled electrodes advanced from the mucosal side. These measurements had three purposes: (*a*) to measure simultaneously the intracellular electrical potential at two different points in the same cell, (*b*) to estimate the specific resistivity of the cytoplasm, and (*c*) to estimate the impedance of the cell boundaries.

(*a*) That tip which was deepest below the mucosal membrane recorded the largest negative potential (Figs. 2 and 8). The difference in potential between the two tips divided by their separation in the direction of advance gave an intracellular gradient of 0.8 ± 0.16 mV/μm ($n=59$, 5 animals). In six of these recordings the potentials were equal at the two tips. In nine recordings the leading barrel recorded a potential which was about 1 mV less negative than the trailing tip. The two adjacent tips of the double-barrelled electrodes always recorded the same intracellular potential.

(*b*) In the cytoplasm of the epithelial cells of the gallbladder the specific resistivity varied with depth beyond the mucosal membrane from 10 times that of the Na^+-saline in the mucosal end of the cell to 4.5 times in the middle and 6.2 times in the serosal end. In Fig. 9 the resistivity is presented as a function of the intracellular potential recorded by the double-barrelled tip of the electrode. Only at the mucosal end was the

value significantly different from that found elsewhere in the cell ($p < 0.01$).

As a comparative test of the method the specific resistivity of *Myxicola* axoplasm was recorded as 2.2 ± 0.3 ($n=6$, 2 animals) times that of seawater. This is comparable with the value of 2.7 times seawater obtained by other methods (Carpenter, Hovey & Bak, 1975).

(*c*) The contribution of the cell boundary to the intracellularly evoked potential [V_m Eq. (6)] was not significantly different from zero.

II. Tissues Bathed in K^+-Saline (Table 3)

Single-barrelled electrodes were advanced from the mucosal solution through the epithelial cell layer in steps of 1–10 μm by means of the hand-driven micromanipulator, after the mucosal membrane had been penetrated by means of the piezodrive. The cell layer was transversed in a total of 36.0 ± 2.2 μm ($n=21$, 4 animals).

Two types of experiments were done. In (*a*) with K^+-saline on both sides, the intracellular potential just inside the mucosal membrane was -5.2 mV (Fig. 10*a*). The same potential was recorded until the electrode had transversed to a depth equivalent to 75% of the thickness of the epithelial cell layer. In the rest of the cell each additional stepwise advance of the tip towards the basal-serosal membrane caused a slightly more negative potential to be recorded, with a maximum of -8.4 mV just before this membrane was penetrated. In (*b*) initially both sides of the

Table 3. Intracellular electrical potentials of the *Necturus* gallbladder epithelium when bathed in K^+-saline and Na^+-saline

	Time after application of solutions (sec)	Potential (mV) in the mucosal part (% of total excursion) of the cell		Potential just inside the basal serosal membrane (mV)	No. of observations/ animals
		mV	%		
Mucosal and Serosal solution K^+-saline	100–3000	-5.2 ± 1.8	75 ± 7.1	-8.4 ± 1.6	10/3
Mucosal solution: K^+-saline	< 500	-10.4 ± 1.7	70 ± 7.8	-18.7 ± 2.9	7/3
Serosal solution: Na^+-saline	500–3000	-15.6 ± 2.1	51 ± 14.0	-20.4 ± 1.0	4/3

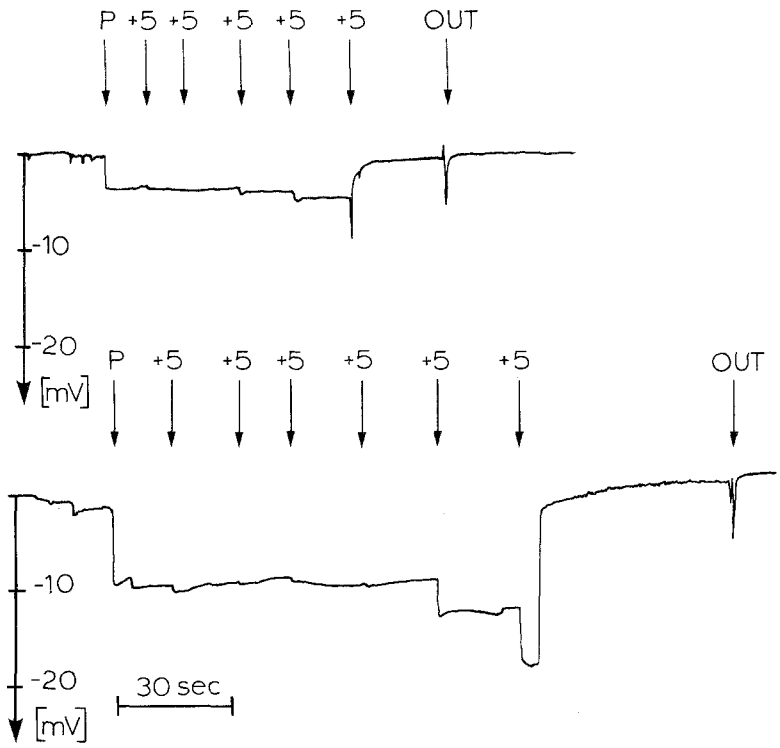

Fig. 10. Examples of the profile of electrical potential across the epithelial cells when (*a*) both the mucosal and the serosal solution were K^+-saline and (*b*) when the mucosal solution was K^+-saline but the serosal solution was Na^+-saline. The mucosal membrane was penetrated by advancing the electrode by the piezoelectric device, and the advance of the electrode through the cell layer was continued by the hand-driven micromanipulator in steps of 5 μm. The electrode was pulled back into the mucosal solution at *out*

tissue were bathed in Na^+-saline. Only the mucosal solution was then changed to K^+-saline and the transmucosal potential changed to a new steady value between -10 and -20 mV within 1 min (serosa positive). The mucosal K^+-saline was now renewed and measurements commenced. For times less than 500 sec the pattern was the same as in (*a*) although the potentials were more negative; averaging a constant -10 mV in the mucosal 70% of the cell and a maximum of -19 mV just inside the serosal membrane (Fig. 10*b*). For times larger than 500 sec the potentials were even more negative, being consistently -16 mV in the mucosal half of the cell, with a maximum of -20 mV just inside the serosal membrane.

Discussion

The results suggest the following hypothesis: A gradient of electrical potential exists within the epithelial cells of the *Necturus* gallbladder when these cells are bathed in a saline similar to the blood plasma (Na^+-saline). The potential just inside the mucosal membrane is about -28 mV, rising to -53 mV just inside the serosal membrane. On average the gradient is approximately linear, averaging -0.6 mV/μm in the direction perpendicular to the mucosal surface. The gradient is largely abolished when the Na^+ in the mucosal solution is changed for K^+ (K^+-saline).

When the electrode was advanced by the hand-driven micromanipulator from the mucosal side to penetrate the mucosal membrane, the tissue moved an average of 37 μm before the electrode penetrated into the cell. In the 200-msec period immediately after penetration the tissue returned to its original position, in effect causing the tip to penetrate deep into the cell. An additional advance of only 14 μm therefore caused the electrode to penetrate the serosal membrane, compared with 42 μm if the mucosal membrane initially was penetrated by means of the piezodrive. The dimpling of the mucosal membrane was independent of the electrode impedence and therefore the sharpness of the tip, indicating that the major source of friction between the tissue and the electrode is between the glass wall of the tip and the glycocalix and microvilli on the mucosal cell surface. This pattern of tissue movement was also reflected in the recorded electrical potentials. As the electrode penetrated the mucosal membrane and part of the cytoplasm, the recorded potential changed to -26 mV within about 0.5 msec, and by an additional -25 mV within the next 200 msec. The first abrupt increase in potential can be identified as the potential difference across the mucosal membrane, as it was identical to that recorded when electrode was advanced on by means of the piezodrive. The slow additional increase in potential was evidently due to the tip moving through the cytoplasm as the tissue returned towards its original position. Similar potentials were obtained when the electrode was advanced from the serosal side.

The simultaneous recording of two different potentials at two different points in the same cell by means of the triple-barrelled electrode confirms that a gradient of electrical potential exists in the penetrated cell. This gradient of -0.8 mV/μm was larger than that of -0.6 mV/μm recorded during the advance of single-barrelled electrodes from mucosa to serosa.

However, the measurements with the triple-barrelled electrodes are not directly superimposable on the measurements with the single-

barrelled electrodes. The two tips of the triple-barrelled electrode were separated by 3–10 μm in the direction of movement (vertical to the tissue), but they were also separated by 2–5 μm perpendicular to this direction. Firstly, the two tips penetrate at different points of the mucosal membrane and might not always dimple this membrane to the same degree. The depth below the mucosal membrane might therefore be slightly different to that expected. Secondly, this way of measuring the electrical gradient differs from that where only one electrode is advanced in that any horizontal component of the electrical gradient will be measured as well. This could cause a larger dispersion of the results obtained, a different mean value and in a few cases the recording of a "reversed" gradient as compared to the values obtained with the single-barrelled electrodes. Thirdly, the gradient, as obtained with the single-barrelled electrodes was based on the total advance needed to transverse the cell 42 μm, which could have been larger than the actual height of the cell due to dimpling of the serosal membrane.

Electrode Artefacts

The next question is whether the recorded gradient could be an artefact due to the presence of electrodes. Four electrode artefacts are possible: (*a*) an incomplete seal between membrane and electrode; (*b*) low electrical resistance of the glass wall of the electrode; (*c*) changes in the tip potential; (*d*) leakage of salt solution out of the tip of the electrode.

(*a*) An incomplete seal between the electrode and membrane at the point of penetration cannot have caused the observed gradient. When the cells are impaled from either the serosal side or from the mucosal side do the electrical potential record about 18 mV more negative in the serosal end of the cell than in the mucosal end (Table 1). Should the intracellular gradient be due to a leak, one would expect the recorded electrical potential to be less negative in the serosal end when one penetrates from the serosal end. That is so, because one must expect an eventual leakage current to pass that membrane in which the eventual leak is produced by the impalement. Even the results obtained with penetrations from the mucosal side alone, render such a leak improbable. Assuming that the intracellular potential was constant (e.g. −55 mV) throughout the cell interior in the unperturbed cell, then the electrode should record this potential instantaneously on penetrating the mucosal membrane.

Depending on the capacitance of the cell membrane and the resistance

of the leak the cell would subsequently depolarize. In similar sized *Amphiuma* red cells and Ehrlich ascites tumor cells, this depolarization time was of the order of several msec (Lassen *et al.*, 1971, 1974), amply within the resolution time of the recording system employed in this study (10 μsec).

(*b*) Boiling or storage for a long period in solution causes hydration and consequent low electrical impedance of glass (Holland, 1964). This will cause shunting of an intracellularly recorded electrical potential. As the leakage is largest in the thin glass wall towards the tip, the shunting would be less the deeper the electrode was advanced into the cell and more negative potentials would be recorded.

Whereas this effect could partly account for the recordings achieved with penetration from the mucosal side, they would not explain why a reversed gradient was observed with penetrations from the serosal side. Furthermore, the electrodes were made and used within 1 hr, while the impedance of the glass wall was still at least 50 times that of the tip of the electrode (*see* p. 285). Shunting of an intracellularly recorded potential was therefore always below 2%.

(*c*) In order to explain the present results as a change in tip potential, one has to assume the presence of an intracellular gradient of some substance able to cause changes in tip potentials when Na^+-saline is in the lumen of the gallbladder. The gradient of this substance should largely disappear when Na^+ in the mucosal solution is replaced by K^+. Furthermore, this substance must react with the tip of the electrode in times of less than 10 μsec, or else it would be detected when the electrode penetrated the mucosal membrane as a transient change in potential. As the two tips of the double-barrelled electrode in the triple-barrelled assembly always recorded the same potential, the substance should affect tips of different diameters equally.

However, the effect of substances known to affect tip potentials depends on the tip diameter (Agin & Holzmann, 1966). Furthermore, selecting electodes with tip potentials less than 3 mV yielded correct values for the intracellular potential in muscle fibers (Adrian, 1956). Finally a gradual change in tip potential cannot explain the observed gradient of Na^+ (Zeuthen, 1976*a*) as will be explained in a subsequent paper on gradients of ionic activity.[2]

(*d*) Any effect on the recorded potential due to diffusion of the filling solution out of the electrode can be ruled out. The recordings were independent of the tip diameter of the electrode or of whether the electrodes were filled with NH_4NO_3, KCl or Na_2SO_4.

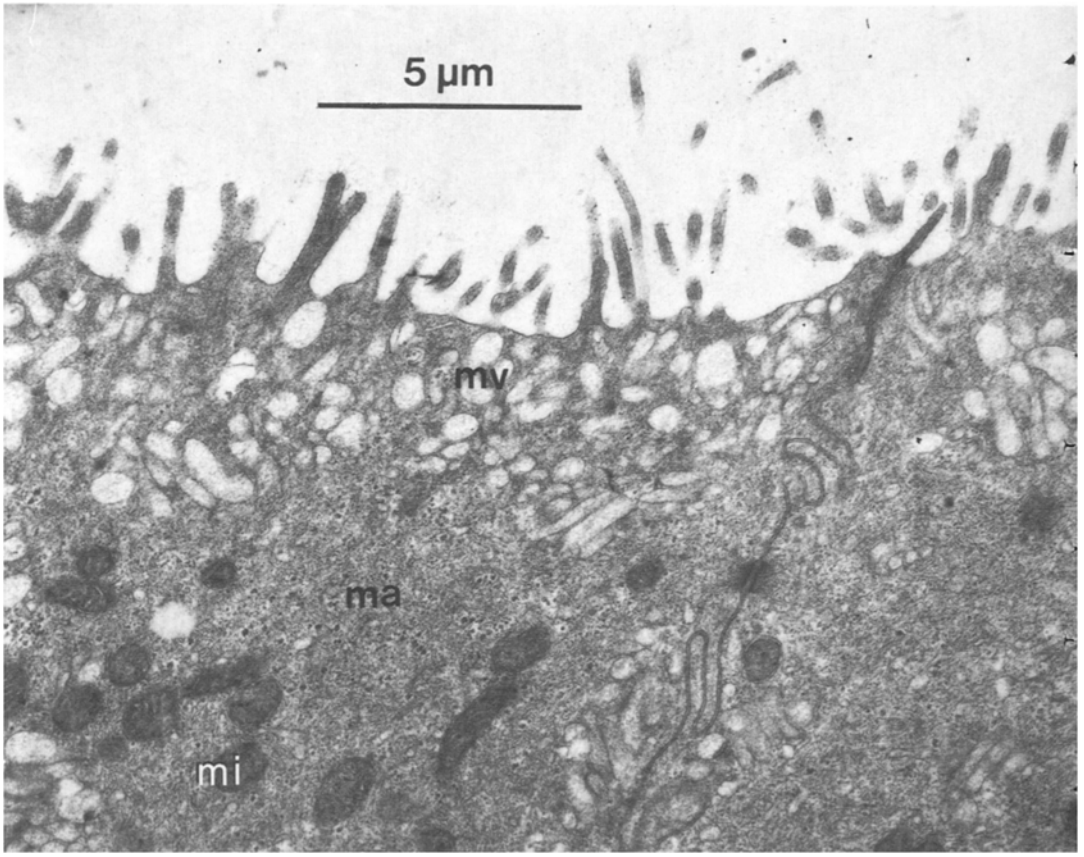

Fig. 11. Electron-micrographs of *Necturus* gallbladder epithelium, courtesy of Dr. B.S. Hill. *mv*, mucus-filled vesicles; *mi*, mithochondria; *ma*, matrix; *n*, nucleus; *bm*, basement membrane; *c*, connective tissue. The influence of these structures on the resistivity of the cytoplasm is discussed in the test

Specific Resistivity of the Cytoplasm

The main features of the cytoplasm revealed in electron-micrographs (Fig. 11*a* & *b*) were a high density of membrane-bound mucus-filled vesicles and mitochondria at the mucosal end of the cell, a large nucleus in the middle, and a dense, occasionally planar matrix throughout the cytoplasm. Thus, obviously, the resistivity must vary over very short distances, say from within the mucus-filled vesicles to the adherent cytoplasm. However, the measurements with the triple-barrelled electrode were based on the induced potential drop between two electrode tips separated by 3–10 µm. The recorded resistivity was therefore an average of the values from within 3–10 µm. The average resistivity of the cyto-

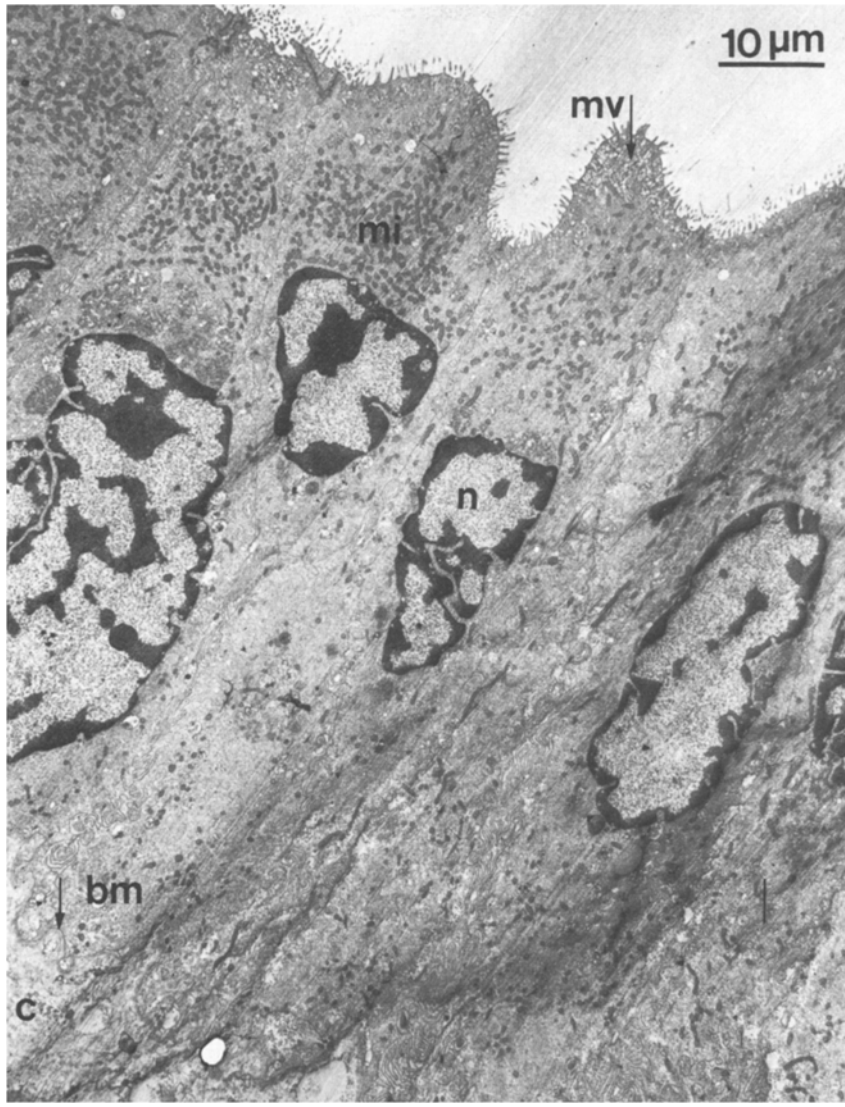

Fig. 11

plasm was high, ranging from 10 times that of the Na-saline at the mucosal end of the cell to 4 times in the middle and 6 times at the serosal end of the cell. As the sum of the activities of Na^+, K^+ and Cl^- (Zeuthen, 1976*a*) was approximately constant at 200 mM throughout the cell and is close to that of the saline, the high resistance must be due to a steric effect. The presence of micro-organelles would not only reduce the volume accessible to current flow, but would also increase

the tortuosity of the current pathway (Ogston, 1966; Parsons & Boyd, 1972). The high internal resistivity could be explained assuming free diffusion of Na^+, K^+ and Cl^- outside the mucus vesicles and the mitochondria, and between the matrix, but supposing that these structures themselves have a high resistivity. The relatively low value of resistivity in the center of the cell could be due to the presence of the large nucleus.

The method of measuring resistivity by means of the triple-barrelled electrode might be criticized for systematically underestimating the resistivity if the cytoplasmic structures were damaged in the vicinity of the double-barrelled electrode and gave a less steric hindrance to the current flow. This phenomenon may account for the halving of the recorded resistivity on withdrawing the electrode about 1 μm. The method might also be criticized for recording too high a resistivity when the current-injecting tip is near a cell boundary. However, with an inter-electrode separation of 0.3 μm in the double-barrelled tip the distance to the boundary would need to be less than 1 μm in order for the resistivity of the cytoplasm to be overestimated by 20% at this point (*see* Materials and Methods).

Compatibility with Other Studies

The entrance of the electrode into the cell should be associated with an abrupt change in potential, but this should be complete in less than 5 msec. Changes due to tissue movements, as described here, or depolarization due to inefficient sealing between membrane and electrode will occur during the next 200 msec. These changes will remain undetected if the recording system has a time constant of 500 msec, which applies to most chart recorders. The recorded potential should remain stable long enough for the potential to be taken as being constant, which with the level of noise and oscillation in this study was easily within 10 sec. Requirements of stability for longer periods (e.g. Armstrong, 1975) would not add information on the intracellular potential, only on the endurance of the cell.

The existence of an intracellular gradient of electrical potential would explain the large variation in reported values of the intracellular potential and also the drifts and oscillations often observed in this potential (for references *see* p. 282). Some authors may systematically record closer to the serosal membrane than others, and therefore observe more negative potentials. Due to tissue movement, as discussed above, the electrode

will move to a certain depth on penetrating the mucosal membrane, and this depth will depend on the shape of the electrode and the friction between glass and tissue. This friction will depend on the degree of hydration of the glass, and therefore on how the electrode has been stored and filled. Many authors have noted that the recorded potential changed after the positioning of the electrode. As the tissue to some degree follows the electrode on advance, the tissue will move to its original position when the advance of the electrode is stopped and in effect the electrode moves deeper into the tissue. This will cause a drift to more negative potentials. Again depending on the friction between glass and tissue and on the mode of advance, this drift will be fast or slow. Muscle activity in the *sub-serosa* will cause a movement of the impaled cell, and hence change the position of the tip. Thus a spontaneous sinusoidal contraction (1/10 Hz) of the muscles could produce the sinusoidal change in recorded electrical potential occasionally observed in this study and by Frömter (1972). Similarly, the small and fast (1 Hz, 1 mV) oscillations in potential could be due to small (1 μm) movements of the tip relative to the tissue. Such oscillation could also be artificially produced by moving the electrode forwards and backwards ± 2 μm.

Reuss and Finn (1974) did not observe any gradient when advancing an electrode across the toad urinary bladder epithelium. But because of the friction between glass and tissue it is difficult to advance the tip of an electrode continuously through a cell with a height of only 5 μm. It is likely that the tissue follows the electrode advance for distances of 5 μm or more, and the resulting movement of the tip relative to the tissue will be in steps of 5 μm. Chowdhury and Snell (1965, 1966) did report a gradient in frog skin and in toad urinary bladder, but they minimized tissue movements by advancing the electrode by a piezoelectric crystal. By isolating the epithelium of isolated toad skin from the chorium it has been possible (Rawlins, Mateu, Fragachan & Whittembury, 1970) to advance microelectrodes from the serosal side. These authors achieved results similar to those of Chowdhury and Snell (1965, 1966) in some cases, but on the whole a wide spectrum of different electrical potentials was obtained, in disagreement with the view that the potential across these skins increases in two discrete steps (e.g. Engbaek & Hoshiko, 1957).

Reuss and Finn (1975) measured the "input impedance" of the impaled cell by passing current pulses of 10^{-9} amps through the electrode. Similarly, in the experiments with the triple-barreled electrodes,

the increase in evoked potential at the tip of the current-injecting electrode [V_{c1} in Eq. (1)] at entry into the cell was a measure of the "input impedance" (*see* Fig. 8). The ratio of increase obtained here was comparable to the ratio of increase obtained by Reuss and Finn (1975, Fig. 1). However, I am in a more favorable position than these authors to interpret the results as I have two electrodes inside the same cell to monitor the evoked potential. The evoked potentials recorded from the two different points inside the cell were not the same [V_{c1} and V_{c2}, Eqs. (1) and (2)]; the further away the recording electrode was from the current-injecting tip, the smaller was the evoked potential. The finding is therefore that the "input impedance" is not only a measure of the resistivity of the cell boundaries but largely a measure of the resistivity of the cytoplasm.

Tissue Models

When current is injected into a cell by an intracellular electrode the current spreads radially in the vicinity of the tip and cylindrically from the cell *via* the junctional shunts. If the epithelial cell layer is equated to a flat conducting sheet bound by two membranes, the evoked potential can be divided into two parts (Eisenberg & Johnson, 1970), one proportional to a Bessel-function for points further away from the tip than the thickness of the cell layer, and one proportional to the sum of a spherical function and the Bessel function for points close to the tip. It is the factor to and space constant of the Bessel-function alone that should be used in determining the size of the paracellular shunt and the impedances of the cell membranes. This model has been used by Frömter (1972) and Reuss and Finn (1975) to determine the electrical parameters of the *Necturus* gallbladder; by Spenney, Shoemaker and Sachs (1974) in the *Necturus* gastric mucosa and by Reuss and Finn (1974) in the toad urinary bladder. The model was used with at least two assumptions: (*a*) that the cytoplasmic resistance was much less than the membrane resistance and (*b*) that the three-resistor model is a realistic model of the tissue. This last assumption means that the lateral spaces can be represented by one resistor only, that is to say that the transmurally induced current that enters the lateral spaces at the leaky junctions proceeds entirely in the lateral spaces and does not enter the cell across the serosal membranes. The implications of these assumptions have been discussed in detail by Zeuthen (1976*c*) and the results will only be summarized here:

(*a*) It is not enough to assume that the resistance of the cell cytoplasm is lower than the resistance of the membranes. One must also assume that the resistivity of the cytoplasm is similar to that of saline. If, for example, the resistivity of the cell cytoplasm was as high as 4–10 times that of saline as reported here, then the spherical potential attenuation around the current-injecting tip cannot be ignored. The recorded value of the evoked potential in the current-injected cell would therefore depend on where the potential-sensing electrode was placed in this cell. Ultimately the determination of the factor and space constant (the space constant in the horizontal direction of the tissue) of the Bessel function would depend on which weight the point obtained inside the current-injected cell was given when the points were fitted to the curve.

(*b*) Frömter (1972) determined the impedances of the intercellular spaces as 200 Ωcm^2 which he then used to determine the width of these spaces as between 100–300 Å when there was no tortuosity of the spaces. The impedance of the serosal membrane was determined as 2880 Ωcm^2, similar to the value obtained by Reuss and Finn (1975). Using these values the length constant of the lateral spaces in the direction perpendicular to the plane of the tissue (not the length constant in the direction parallel to the tissue) can be estimated as only 2–5 times the thickness of the tissue.[3] Thus an error is probably involved in assuming that the lateral spaces can be represented by a single resistor.

In the three-compartment model of absorptive epithelia (Rose & Schultz, 1971; White & Armstrong, 1971) all of the serosal membrane is assumed to have the same electrical and ionic environment. From the present results this is seen to be an oversimplification.

3 The impedance of the serosal membrane r_s was estimated as 2880 Ω cm^2 (cm^2 of apparent area). If we further assume the tortuosity τ to be of a kind that linearly increases the length l of the lateral intercellular spaces this length can be expressed as τh where h is the height of the cell. The width of the spaces can then be expressed as $a_o \tau$ where a_o is 100–300 Å. With a cubic cell the area of the serosal membrane would be about five times larger than the apparent mucosal area if there was no tortuosity; therefore the true impedance per cm^2 of serosal membrane is $\tau r_s 5$ where r_s is 2880 Ωcm^2. The ratio between the length constant λ and the actual length of the lateral space (l) will then calculate as:

$$\frac{\lambda}{l}=\frac{\lambda}{h\tau}=\frac{1}{h\tau}\sqrt{\frac{5\tau r_s a_0 \tau}{2R}}=\sqrt{\frac{5\cdot 2880\cdot(100-300)\cdot 10^{-8}}{2(30\cdot 10^{-4})^2\cdot 100}}=2\text{to }5 \tag{7}$$

where the formulae for λ is taken from Katz (1966, p. 75). R is the resistivity of the solution in the spaces (100 Ωcm^2).

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Note Added in Proof

It should be emphasized that the perfusion fluids used in this study were equilibrated with O_2. Presence of gaseous CO_2 in the perfusion fluids diminished the magnitude of the intracellular gradients of electrical potential.

The integrity of the cytoplasm is probably a necessary condition for the maintenance of the electrical gradient. Thus the gradient was not systematically observed when the electrode was retracted across the cell *via* the track formed by the preceding advance from the mucosal solution into the subserosa; on retraction the cytoplasmic resistivity was also reduced by a factor of at least two.

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