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# ELECTROPHYSIOLOGY OF THE MANTLE OF *ANODONTA CYGNEA*

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## Summary

1. When gassed with a $CO_2$-containing mixture, under short-circuit conditions, the isolated outer mantle epithelium (OME) of *Anodonta cygnea* generated a current which exhibited cyclic variations throughout the year.

2. The intracellular potential, under short-circuit conditions, had an average value of $-31 \pm 0{\cdot}5$ mV ($N = 65$). The potential was sensitive to changes in concentration of potassium and chloride on the haemolymph side of the preparation, but not on the shell side, and was insensitive to changes in sodium concentration on either side.

3. When the preparation was gassed with pure oxygen the current fell by $85 \pm 1$ % ($N = 8$). A similar fall in current ($88 \pm 2$ %, $N = 8$) was observed when the solution bathing the apical side of the epithelium was prepared without bicarbonate and gassed with 95 % $O_2$ + 5 % $CO_2$. If this same bathing solution was gassed with 100 % oxygen, the current fell by $67 \pm 2$ % ($N = 8$) at pH 7·2 and by $92 \pm 4$ % ($N = 8$) at pH 4·5.

4. The short-circuit current was inhibited by DIDS (0·5 mmol $l^{-1}$) and SITS (0·5 mmol $l^{-1}$) when these drugs were applied on the haemolymph side. The current was also inhibited by DNP (1 mmol $l^{-1}$), iodoacetamide (1 mmol $l^{-1}$) and diamox (1 mmol $l^{-1}$). Amiloride (1 mmol $l^{-1}$) blocked the current but only when applied on the haemolymph side. Ouabain (0·1 mmol $l^{-1}$) did not affect the current.

5. The net fluxes of rubidium (used as a tracer for potassium), chloride and calcium, measured with $^{86}Rb$, $^{36}Cl$ and $^{45}Ca$, respectively, were very small when compared with the short-circuit current. There was a small net flux of sodium (measured with $^{22}Na$) towards the haemolymph side. The net flux of bicarbonate (measured with [$^{14}C$]bicarbonate) was equal to the short-circuit current and was inhibited by DIDS. The permeability of the preparation to calcium was an order of magnitude higher than the permeability to sodium, potassium or chloride.

6. The intracellular concentrations of potassium and chloride measured with ion-sensitive microelectrodes were $26{\cdot}5 \pm 1{\cdot}1$ ($N = 16$) and $7{\cdot}9 \pm 0{\cdot}3$ ($N = 30$) mmol $l^{-1}$, respectively. When these concentrations were measured with chemical

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methods the values found were $29{\cdot}4 \pm 0{\cdot}4$ ($N = 20$) and $12{\cdot}9 \pm 0{\cdot}6$ ($N = 20$) $mmol\,l^{-1}$, respectively. The chemically measured intracellular concentration of sodium was $14{\cdot}0 \pm 0{\cdot}4$ ($N = 20$) $mmol\,l^{-1}$. When bicarbonate was removed from the haemolymph side the intracellular concentration of chloride increased.

7. The movements of calcium across the OME are by simple diffusion and the calcification of the shell probably results from the balance between a diffusion of calcium towards the extrapallial compartment when a favourable electrochemical gradient for this ion is created and the equally passive movement of calcium which results from the uphill transport of bicarbonate ions carried out by the epithelium. The balance between these two processes might undergo annual cyclic variations.

## Introduction

The mantle of lamellibranchs is a leaflet that covers the internal surface of the shell. Anatomically (Istin & Kirschner, 1968; Neff, 1972) it consists of two epithelia: the internal or cavity epithelium, which surrounds the body of the animal and is in contact with the external medium, and the outer epithelium facing the shell, from which it is separated by a thin layer of fluid, the extrapallial fluid. The two epithelia are joined by connective tissue which encompasses the haemolymph or blood compartment. They merge at the border of the mantle where this structure adheres to the most peripheral zone of the shell. The shell grows in area through its peripheral zone and in thickness at the remaining inner surface (Wilbur, 1983).

Shell formation is a regulated process of precipitation of calcium carbonate which becomes embedded in an organic matrix consisting largely of soluble and insoluble proteins (Crenshaw, 1972*a*; Wilbur & Manyak, 1984). A large amount of work has been done on the interactions between this matrix and the crystallization process (Wada, 1980; Wheeler *et al.* 1981; Weiner & Traub, 1981; Greenfield *et al.* 1984; Bernhardt *et al.* 1985). By its nature, the shell does not allow an easy interchange between the extrapallial fluid and the external environment. Thus both inorganic and organic components of the shell must be secreted first by the mantle into the extrapallial fluid. Wilbur (1972) showed that the cells of the outer mantle epithelium (OME) secrete the matrix proteins and studies with an isolated mantle–shell preparation of *Crassostrea virginica* have revealed that shell formation can take place without the participation of the digestive and circulatory systems (Hirata, 1953; Jodrey, 1953). These observations, together with the close proximity between mantle and shell, suggest that, at least in marine bivalves, the mantle is also involved in providing the mineral components of the shell.

The mantle is permeable to calcium (Istin & Maetz, 1964; Kirschner & Sorenson, 1964). Istin & Maetz (1964) reported that the calcium flux ratio measured in double-labelling experiments corresponds to a purely diffusional process. When the mantle is bathed on both sides by the same solution with a composition similar to that of the haemolymph the transmantle potential is very small. However, when the calcium concentration in the external solutions is reduced from $6\,mmol\,l^{-1}$ to much lower concentrations (around $1\,mmol\,l^{-1}$) the

mantle generates a potential which can be as high as 50 mV, shell side positive. Most of this potential is generated across the outer mantle epithelium (Istin & Kirschner, 1968). These observations indicate that the outer mantle epithelium has the polarity characteristic of epithelia in general and that it is capable of performing transcellular transport of ions.

In this work we report the first study of the electrophysiological properties of the isolated OME under short-circuit conditions. This study was undertaken to characterize the nature of the transepithelial potential and of the short-circuit current. It was also hoped that the transport properties of the OME could be related to the mechanism of shell formation.

## Materials and methods

Freshwater clams (*Anodonta cygnea*) were collected from the Lagoon of Mira in northern Portugal, and kept in aerated and dechlorinated water for up to 2 weeks. Preliminary experiments showed that they survived as well under these conditions as in water collected from their natural environment. Animals were considered healthy if the inner surface of the shell was smooth and shiny and if they closed their valves when disturbed.

The OME of both mantles were carefully dissected *in situ* and pieces, 1·2 cm in diameter, from the central area were mounted in Ussing-type chambers. Adequate mixing of the solutions in both half-chambers was achieved by bubbling carbogen (95 % $O_2$ + 5 % $CO_2$) through them. To avoid evaporation from the chambers the gas mixture was first saturated with water vapour. In some experiments, as indicated in the Results, oxygen saturated with water vapour was used for gassing. In most of the experiments the epithelium was continuously short-circuited by means of a simple electronic voltage-clamp system. The transepithelial conductance was measured by displacing the membrane potential by 10 mV for 15 s and recording the corresponding current pulse. In this preparation the I/V curve is linear at least between −100 and +100 mV.

### *Solutions*

Table 1 shows the composition of the haemolymph, extrapallial fluid and lagoon water, and of the control solution used in the experiments reported below. The composition of the haemolymph is similar to that of the extrapallial fluid (de Waele, 1930; Crenshaw, 1972*b*). Part of the difference between the total concentrations of cations and anions measured in the haemolymph and in the extrapallial fluid is probably due to bicarbonate. The calcium concentration of the control solution (1 mmol $l^{-1}$) was set lower than that (6 mmol $l^{-1}$) measured in the haemolymph or extrapallial fluid to expose the much higher currents and potentials that can be obtained at low calcium concentration, as demonstrated below. The relatively high concentration of potassium was used for the same reason. Choline was substituted for sodium or potassium as required. Sodium bicarbonate was replaced isotonically by mannitol plus 1–5 mmol $l^{-1}$ Tris-Cl

Table 1. *Ionic composition and osmolalities of haemolymph, extrapallial fluid, lagoon water and control solution*

| | *N* | Osmolality (mosmol $kg^{-1}$) | $Ca^{2+}$ (mmol $l^{-1}$) | $Mg^{2+}$ (mmol $l^{-1}$) | $Na^{+}$ (mmol $l^{-1}$) | $K^{+}$ (mmol $l^{-1}$) | $Cl^{-}$ (mmol $l^{-1}$) | $HCO_3^{-}$ (mmol $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|
| Haemolymph | 16 | 56·9 ± 0·55 | 5·58 ± 0·14 | 1·0 ± 0·05 | 20·8 ± 0·80 | 0·71 ± 0·03 | 15·2 ± 1·2 | |
| Extrapallial fluid | 16 | 54·7 ± 1·3 | 6·1 ± 0·31 | 1·0 ± 0·04 | 20·1 ± 0·82 | 0·84 ± 0·08 | 14·9 ± 1·2 | |
| Lagoon water | | 22·0 | 1·64 | 0·42 | 1·39 | 0·48 | 5·3 | |
| Control solution | | 55·0 | 1·0 | 0·5 | 18·0 | 7·0 | 18·0 | 10·0 |

Osmolalities were determined from the freezing point depression.
Calcium and magnesium concentrations were measured by atomic absorption.
Sodium and potassium concentrations were measured by flame photometry.
Chloride concentrations were measured by coulometry.

(pH 7·2). Chloride was replaced by sulphate plus mannitol. The osmolality of the solutions was measured with a Knaub Halbmikro osmometer. The pH of the solutions was 7·1–7·2 except when solutions prepared with bicarbonate were equilibrated with pure oxygen or when solutions prepared without bicarbonate were equilibrated with carbogen. Calcium and magnesium were measured by atomic absorption (Varian AA175 atomic absorption spectrophotometer), sodium and potassium by flame photometry (Eppendorf B700 flame photometer), and chloride with the Aminco–Cotlove chloridometer. When chloride was replaced by sulphate or gluconate the free calcium concentration, monitored with a calcium electrode, was adjusted if necessary by addition of calcium sulphate or calcium gluconate, respectively.

### *Measurement of isotope fluxes*

Radioisotope fluxes of $^{45}Ca$, $^{36}Cl$, [$^{14}C$]bicarbonate, $^{86}Rb$ and $^{22}Na$ were measured using the same basic procedure. The 'hot' solutions were prepared with a specific activity of 0·01 $\mu$Ci $\mu$mol$^{-1}$. The two mantles from the same animal were used each time, one for the measurement of haemolymph to shell-side fluxes and the other for the measurement of the fluxes in the opposite direction. An equilibration period of at least 1 h was allowed before sampling started. Three measurement periods of 20–30 min were used for each condition. At the end of each period the 'cold' chamber was completely emptied for counting and a small sample taken from the hot side to measure the specific activity. In the case of bicarbonate, at the beginning of each period both half-chambers were filled with control solution pre-equilibrated with carbogen. The hot solution was prepared by adding the isotope after equilibration with carbogen. The chambers were then stoppered without a gas phase. $^{45}Ca$, $^{36}Cl$ and [$^{14}C$]bicarbonate were measured by liquid scintillation counting (Beckman LS 8100) in Bray's solution and $^{86}Rb$ and $^{22}Na$ by gamma counting (Picker Pace 1). All samples were counted to a minimum of $10^4$ counts.

### *Chemical determination of water and ion contents*

The isolated OME was incubated for 1 h in control solution gassed with carbogen and containing 0·2 $\mu$Ci ml$^{-1}$ [$^{14}C$]inulin. The preparation was then blotted with filter paper and placed on a tared aluminium box, weighed, dried to constant mass at 100 °C and then transferred to a vial containing suitable amounts of 0·1 mol l$^{-1}$ $HNO_3$. The vials were subjected to slow agitation at room temperature for 24 h to allow for the extraction of inulin, sodium, potassium and chloride. [$^{14}C$]Inulin was counted by scintillation in Bray's solution. Sodium and potassium were determined by flame photometry and chloride by coulometry. The total water content was obtained from the difference between wet and dry masses, and the extracellular space was calculated assuming that inulin equilibrates in the extracellular space. Ion concentrations are expressed as mmol kg$^{-1}$ cell water.

*Microelectrodes*

Conventional microelectrodes were made from 1·2 mm o.d. filamented borosilicate glass tubing filled with 0·5 mol $l^{-1}$ potassium acetate and 10 mmol $l^{-1}$ KCl. The electrodes had resistances greater than 300 MΩ. Impalements were considered satisfactory when there was: a sharp change in potential upon penetration of the cell; stable readings for at least 20 min; a change of electrode resistance of less than 30 % upon penetration of the cell; and return to the same potential (within 5 mV) when the electrode was removed from the cell. Most impalements were performed from the haemolymph side because fewer failures due to tip breakage occurred than when the cells were impaled from the shell side. Identical results were obtained in each case.

Ion-sensitive microelectrodes were prepared from the same glass. Double-barrelled micropipettes were first made and one of the barrels was silanized by vapour treatment with dimethyldichlorosilane (simple room-temperature method, Thomas, 1978) for around 1·5 min and oven-baked at 120°C for 1 h. Slight variations of this technique had to be introduced occasionally as a result of changes in atmospheric humidity. The shank of the silanized micropipette was then partially filled with Cl-lix Corning 477913 or with K-lix Corning 477317 and backfilled with 100 mmol $l^{-1}$ KCl. The other pipette was filled with 0·5 mol $l^{-1}$ potassium acetate and 10 mmol $l^{-1}$ KCl. When the reference electrode was filled with 2 mol $l^{-1}$ NaCl similar intracellular potassium concentration readings were obtained. Measurements made with sodium-sensitive microelectrodes are not reported here since it was found that there was strong interference from calcium.

Calibration curves in pure KCl solutions were used to select ion-sensitive microelectrodes with response curves greater than 50 mV per tenfold change in concentration. In the case of the $Cl^-$-sensitive microelectrodes, the selected microelectrodes were then calibrated in KCl solutions containing 10 mmol $l^{-1}$ sodium bicarbonate. Two-point calibration curves (standards above and below the measured intracellular concentration) were obtained several times in the course of each experiment to evaluate the stability of the ion-sensitive microelectrodes. To find out if there was any interference when the ion-sensitive microelectrode was inside the cell, we tested the effect on the intracellular concentration of removal of the ion from the external solution. When chloride was removed from the external baths the chloride-sensitive microelectrode reading fell in less than 5 min to a value corresponding to a concentration that was 5 % of the control value. In the case of potassium, and after 10 min, the microelectrode reading corresponded to less than 10 % of the control concentration.

The microelectrode measurements were performed in a specially designed chamber in which the preparation was mounted as a horizontal diaphragm of 0·79 $cm^2$ placed on a nylon mesh. The preparation was continuously short-circuited. The membrane conductance was measured by displacing the membrane potential by 10 mV for 15–30 s and recording the corresponding current pulse. The displacement of the intracellular potential was also recorded and used to compute the ratio between apical (and basolateral) resistance and total transcellular

resistance. The value of this ratio provided a further indication of whether the microelectrode was correctly placed.

The solution bathing the haemolymph side of the epithelium will be referred to as the 'haemolymph solution' and the solution bathing the shell side of the preparation as the 'shell solution'.

We use the terms 'apical membrane' or 'apical barrier' to designate the part of the membrane of the epithelial cells that separates the intracellular compartment from the shell solution. The remainder of the membrane, which separates the cell compartment from the haemolymph solution, will be called 'basolateral membrane' or 'basolateral barrier'.

### *Statistical methods*

All statistical tests were *t*-tests (Hald, 1952). The hypothesis tested in each case was that the mean value was different from zero. The *P* values given correspond to errors of the second kind.

## Results

The OME is bathed *in vivo* by the heamolymph and the extrapallial fluid. The composition of these fluids was very similar (Table 1), confirming earlier observations (de Waele, 1930; Crenshaw, 1972).

When the OME is mounted in Ussing type chambers and bathed on both sides by the same solution it generates a spontaneous transepithelial electrical potential. Under short-circuit conditions it generates a current corresponding to the flow of positive current from the haemolymph to the shell side. Fig. 1 reports measurements of short-circuit current and conductance performed in two groups of eight pairs of OME, each pair taken from one animal. The preparations were bathed in control solution. One group was studied in February and the other in November. In both groups there was an initial current transient followed by a steady period which lasted several hours. The average conductance followed a similar time course. However, the two groups showed the following differences. In February the initial transient was a decrease in both current and conductance and since the fall in current was more pronounced there was also a fall in open-circuit voltage (not plotted in Fig. 1). In November there was an initial, albeit small, increase in current and voltage (not shown), the conductance changing very little.

Fig. 2 shows the average values of the short-circuit current, open-circuit voltage and conductance obtained in mantles collected from groups of 24 animals each studied over a period of 3 years. It can be seen that these variables underwent a cyclic variation throughout the year. The lowest currents and voltages were measured in March–April ($13{\cdot}6 \pm 1{\cdot}9\,\mu A\,cm^{-2}$ and $23 \pm 2{\cdot}7$ mV, respectively) and the highest values in November ($37{\cdot}9 \pm 2{\cdot}3\,\mu A\,cm^{-2}$ and $40{\cdot}9 \pm 2{\cdot}2$ mV, respectively). From August to December relatively high and stable currents and voltages were obtained. Most of the experiments reported below were performed during this period.

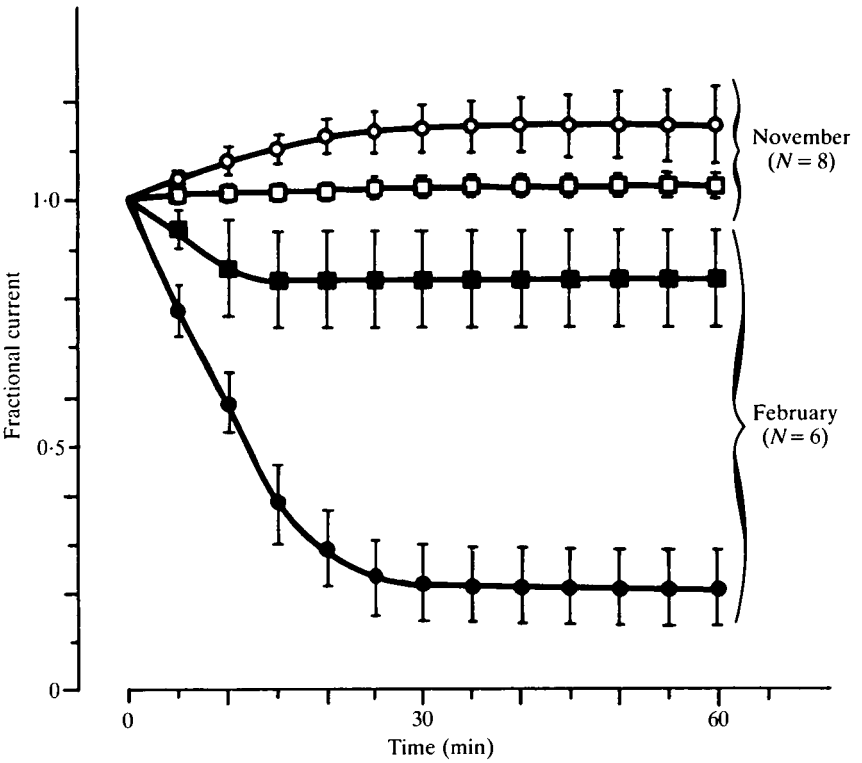


Fig. 1. Initial transients of short-circuit currents (circles) and conductances (squares). The values plotted are the mean ± S.E.M. of the measurements taken at time t divided by the values at time zero. Filled symbols correspond to preparations studied in February ($N = 18$) and open symbols correspond to preparations studied in November ($N = 18$). At time zero mean currents for the November and February measurements were 41·3 and 23·4 $\mu$A cm$^{-2}$, respectively.

*Ion substitutions and effect of carbon dioxide*

Microelectrode studies have shown that the relative permeability of both apical and basolateral membranes to sodium is small (Sorenson *et al.* 1980). Fig. 3 shows the effect on the short-circuit current and conductance of replacing sodium or potassium with choline, and chloride with sulphate, in the bathing fluids. The current showed a fall of about 13 % ($P < 0{\cdot}01$) when choline was substituted for sodium in the haemolymph solution, a fall of 8 % ($P < 0{\cdot}01$) when the change was made on the shell side, and no change when the substitution was made on both sides. In the same experiments the total conductance of the preparation did not change when sodium was removed from the shell side and there was a fall of 10 % when sodium was removed from both sides ($P < 0{\cdot}01$) or from the haemolymph side ($P < 0{\cdot}01$). These results show that the contribution of a net flux of sodium to the short-circuit current was probably very small and that the relative permeability of the preparation to sodium was also very small.

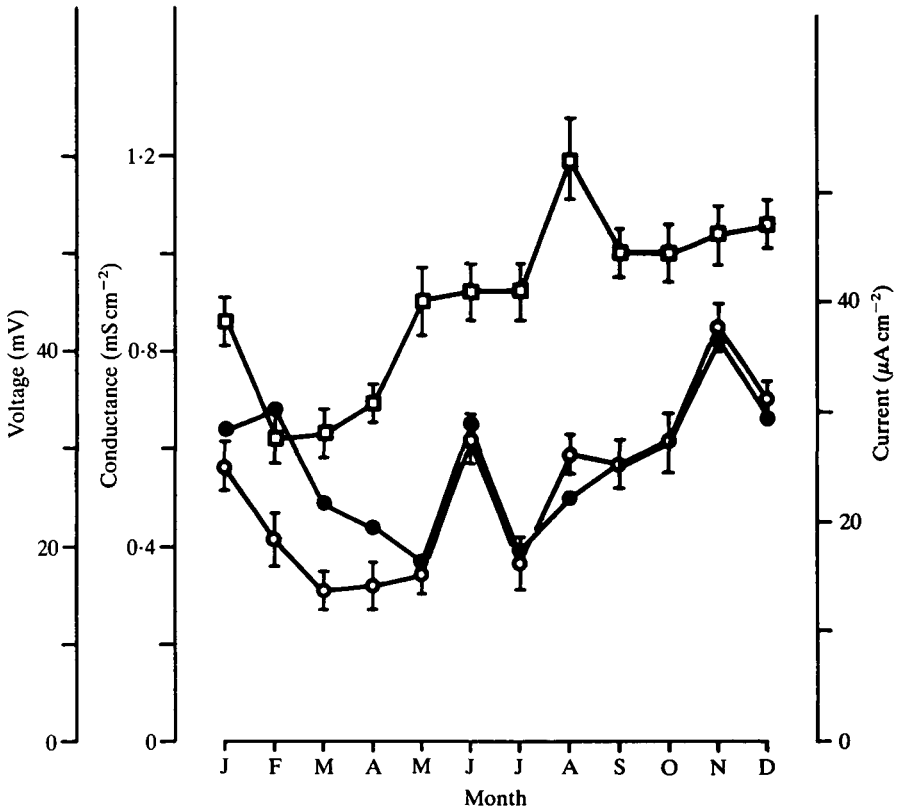


Fig. 2. Monthly means ± S.E.M. of the currents (○), conductances (□) and voltages (●) measured in groups of 24 animals over a period of 3 years.

Substitution of propionate for chloride on the shell side, under open-circuit conditions, is known to cause a transient depolarization of the apical membrane (Sorenson *et al.* 1980). In the present experiments (under short-circuit conditions), replacement of chloride with sulphate on the haemolymph side reduced the current by 36% ($P < 0{\cdot}01$, Fig. 3) and the total conductance decreased by 15% ($P < 0{\cdot}01$). When chloride was replaced on the shell side both current and conductance decreased by less than 5% ($P < 0{\cdot}1$ in both cases) and the replacement of chloride on both sides caused a fall in conductance of about 21% ($P < 0{\cdot}01$) and a decrease in current of 13% ($P < 0{\cdot}01$). These observations suggest that the basolateral membrane is relatively permeable to chloride. However, the fall in current observed upon removal of chloride from the haemolymph side cannot be due to a diffusional current (resulting from the gradient of chloride concentration across the preparation created by the removal of chloride) since a net diffusional flux of chloride towards the haemolymph side would cause an increase in short-circuit current. The short-circuit current does not seem to be due to a net flux of chloride (from shell to haemolymph side) since removal of chloride from the shell solution did not cause a reduction of the short-circuit current.

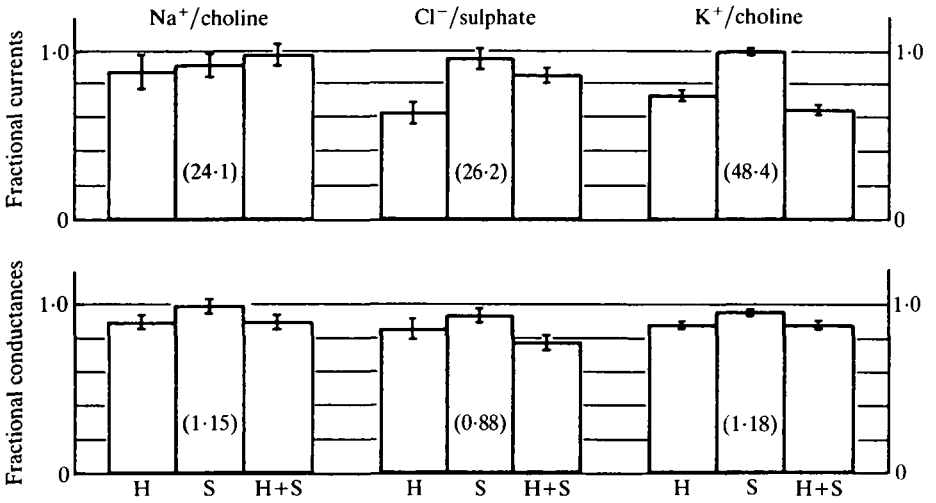


Fig. 3. Dependence of the short-circuit current and conductance on sodium, chloride and potassium. Each group of three bars (for currents and conductances) represents a type of experiment (replacement of $Na^+$, $Cl^-$ or $K^+$) performed on 16 mantles (from eight animals). The preparation was first set up in control solution. When the current was constant the ion substitution was performed. After a new plateau had been reached control solution was reintroduced to the chamber. The readings from the two control periods were averaged and the reading from the experimental period divided by this average to give a ratio. The sequence of changes was different in each experiment. Choline was substituted for $Na^+$ or $K^+$, and sulphate + mannitol for chloride. The proportional changes are plotted as means ± S.E.M. The values in brackets are absolute averages of the control values for each group of experiments. Currents are expressed in $\mu A\,cm^{-2}$ and conductances in $mS\,cm^{-2}$. H, replacement on haemolymph side; S, replacement on shell side; H + S, replacement on both sides.

Replacement of potassium with choline (Fig. 3) on the haemolymph side caused the short-circuit current to fall by 24% ($P < 0{\cdot}01>$) while the conductance fell by 11% ($P < 0{\cdot}01$). A similar effect was obtained when potassium was replaced on both sides (34% and 12%, respectively). Removal of potassium from the shell side did not affect the current and had a very small effect on the conductance.

The dependence of the short-circuit current on bicarbonate cannot be studied in isolation since a solution prepared without bicarbonate and equilibrated with carbogen has a pH of around 5. Istin & Kirschner (1968) observed that the removal of carbon dioxide from both sides, while leaving bicarbonate in the solutions, induced a collapse of the open-circuit potential. We studied the effect of the removal of carbon dioxide from the gas mixture and of the removal of bicarbonate separately or simultaneously and the results are shown in Fig. 4 using the same method of plotting as for Fig. 3. Removal of $CO_2$, bicarbonate or both from the haemolymph side had no effect on either current or conductance. Removal of $CO_2$ from the shell side caused a fall in current of 30% but had little effect on the conductance, whereas the removal of bicarbonate alone from the shell side caused

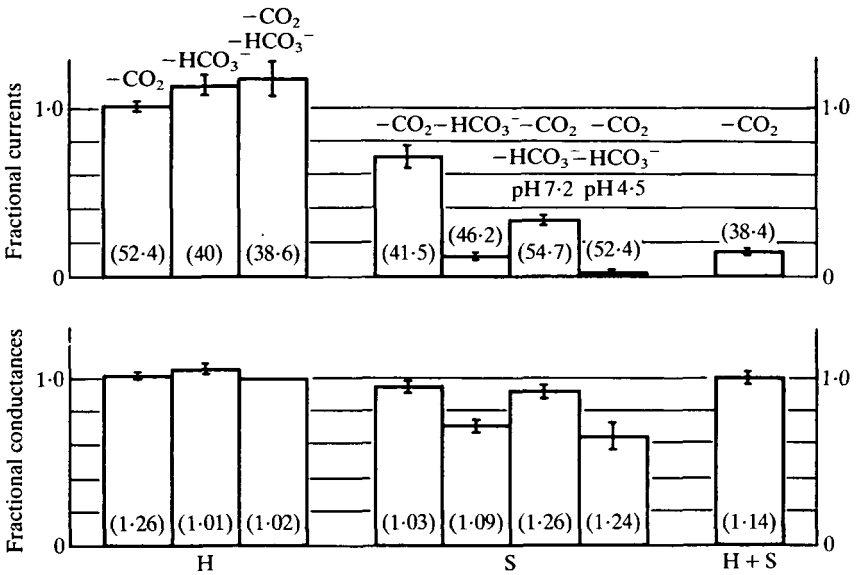


Fig. 4. Dependence of the short-circuit current and conductance on bicarbonate and carbon dioxide. Mantles of 12 animals were used. The design of the experiments and the method of plotting are described in Fig. 3. Sodium bicarbonate was replaced by mannitol + Tris-Cl (pH 7·2). The average control values of currents (in $\mu A\,cm^{-2}$) and conductances (in $mS\,cm^{-2}$) are given in brackets at the bottom of each bar. The first three bars of both currents and conductances show the effects of removing $CO_2$, bicarbonate or both from the haemolymph side (H). The next four bars show identical changes performed on the shell side (S). The effect of removing both $CO_2$ and bicarbonate from the shell side was studied at two pH values (7·2, 4·5). The last bar shows the effect of removing $CO_2$ from both sides (H + S).

a fall in current of 88 % and a decrease in conductance of 30 %. This effect may be due, at least in part, to the decrease in pH (from 7·2 to around 5) in the shell solution. The effect of pH can also be seen in Fig. 4. When both $CO_2$ and bicarbonate were removed from the shell solution and the pH was set at 7·2 with $5\,mmol\,l^{-1}$ Tris-Cl the current fell by 67 % but the conductance fell by only 8 %. The removal of carbon dioxide and bicarbonate at pH 4·5 reduced the current by 92 % and the conductance by 33 %. The removal of carbon dioxide from both sides reduced the current by 85 % but the conductance did not change.

### *Effect of inhibitors*

Several inhibitors were found to affect the current (Fig. 5). 4,4-diisothiocyanostilbene 2,2-disulphonic acid (DIDS) (Fig. 5A) and 4-acetamido-4′-isothiocyamatostilbene-2,2′-disulphonic acid (SITS) (Fig. 5B), both at $0{\cdot}5\,mmol\,l^{-1}$, were ineffective from the shell side but when added to the haemolymph side induced a rapid fall followed by a progressive inhibition which, in the case of DIDS, was almost complete after 1 h.

Istin & Kirschner (1968) reported that in the absence of $CO_2$ the transepithelial electrical potential was increased by dinitrophenol (DNP). As can be seen from

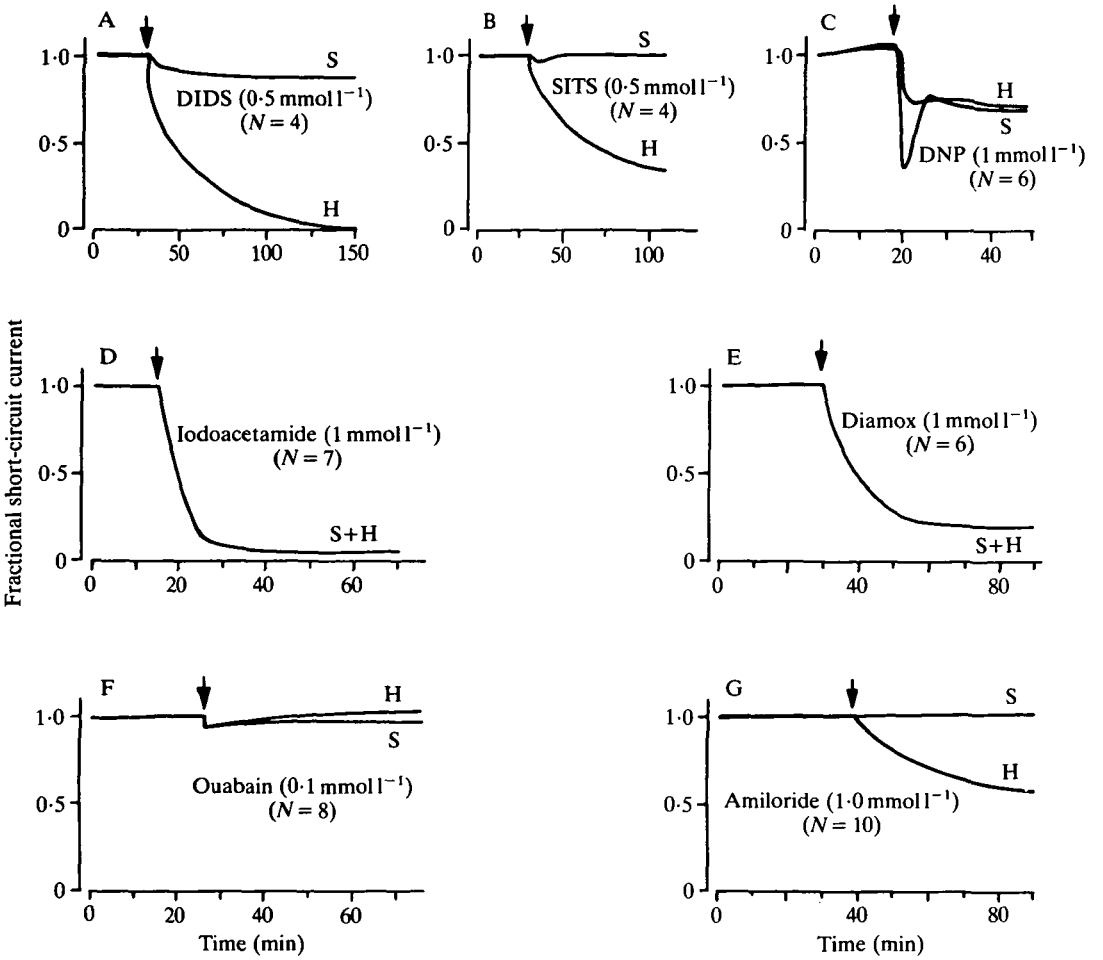


Fig. 5. Effect of metabolic and transport inhibitors. The effect of each inhibitor was studied on pairs of mantles (number in brackets). The preparations were set up in control solution. The values plotted are the averages of the ratios between the values measured at time t and those measured immediately before adding the inhibitor. H, inhibitor added to the haemolymph side; S, inhibitor added to the shell side; H + S, inhibitor added to both sides. The largest standard errors of the mean were (A) DIDS, 0·07; (B) SITS, 0·03; (C) DNP, 0·06; (D) iodoacetamide, 0·05; (E) diamox, 0·04; (F) ouabain, 0·04; (G) amiloride, 0·06.

Fig. 5C, under short-circuit conditions and in the presence of $CO_2$, DNP (1 mmol $l^{-1}$) had the same final effect when added from either side. The current fell to 70 % of its control value. However, when the drug was added from the shell side, the initial transient consisted of a sharp fall to less than 40 % of the initial value followed by an equally sharp rise to around 70 %, whereas adding it to the haemolymph side caused a slightly slower fall to 70 % of the control value.

Iodoacetamide (Fig. 5D) at 1 mmol $l^{-1}$, acted equally well from either side. It

caused an almost complete inhibition of the short-circuit current in less than 15 min. Diamox (Fig. 5E), also at 1 mmol $l^{-1}$, caused a progressive inhibition of the short-circuit current which fell to 20 % of the control value after 1 h. This effect is consistent with earlier observations that the open-circuit potential was inhibited by this drug (Istin & Kirschner, 1968). When ouabain (Fig. 5F) was added to a concentration of 0·1 mmol $l^{-1}$ to either of the two sides the current remained almost constant. Amiloride (Fig. 5G) at 1·0 mmol $l^{-1}$ had no effect from the shell side but produced a progressive and slow inhibition from the haemolymph side. The current fell to 50 % after 1 h.

In conclusion, the effect of DIDS would seem to implicate an anion exchanger (Boron, 1983) and the amiloride inhibition points to the involvement of a proton/$Na^+$ exchanger (Benos, 1982), both on the basolateral membrane

### *Ion fluxes*

Ion fluxes were measured under short-circuit conditions (Table 2). The unidirectional fluxes of calcium, at calcium concentrations of 1 mmol $l^{-1}$, were small (less than 16 % of the short-circuit current) and the net flux was around 4 % of the short-circuit current. The fluxes of chloride and rubidium (used as a tracer for potassium) were also small. Table 2 also shows that there was a small (15 % of the short-circuit current), but statistically significant ($P < 0{\cdot}01$), net flux of sodium towards the haemolymph side.

If the unidirectional fluxes of $^{14}C$ correspond to movements of bicarbonate, the net flux would account entirely for the short-circuit current. The net flux of $^{14}C$ was almost completely inhibited when DIDS (0·5 mmol $l^{-1}$) was added to the haemolymph compartment. Since in the presence of DIDS (0·5 mmol $l^{-1}$) the flux from haemolymph to shell side was not affected (it increased slightly), it is unlikely that this flux is mediated through an anion exchange mechanism. The large unidirectional haemolymph to shell side fluxes of $^{14}C$ observed in the presence of DIDS may be due to the diffusion of labelled carbon dioxide as observed previously in amphibian colonic mucosa (Cooperstein & Hogben, 1959; Carlinsky & Lew, 1970).

### *Microelectrode studies*

To analyse the mechanisms responsible for the membrane potentials, intracellular recordings were made (Table 3). The short-circuit currents and open-circuit voltages recorded in the chamber used for these experiments were considerably lower than those measured in the Ussing-type chambers, because we could not maintain adequate $CO_2$ tensions in the microelectrode chamber.

For an average transepithelial open-circuit potential of $-11{\cdot}1 \pm 1{\cdot}5$ mV ($N = 42$), shell side positive, the average intracellular potential under short circuit was $-31 \pm 0{\cdot}5$ mV ($N = 65$) in relation to either haemolymph or shell side. The resistance of the apical membrane accounted for 80 % of the transcellular resistance ($0{\cdot}8 \pm 0{\cdot}02$, $N = 48$).

To detect relative differences of permeability of the apical and basolateral barriers to sodium, potassium and chloride these ions were replaced by choline

Table 2. *Ion fluxes under short-circuit conditions*

| | Haemolymph to shell side | | | Shell to haemolymph side | | | |
|---|---|---|---|---|---|---|---|
| Ion (*N*) | $I_{sc}$ | $J_{H\rightarrow S}$ | $I_{sc}/J_{H\rightarrow S}$ | $I_{sc}$ | $J_{S\rightarrow H}$ | $J_{S\rightarrow H}/I_{sc}$ | J/I net |
| $Ca^{2+}$ (12) | 6·09 ± 0·31 | 0·32 ± 0·01 | 0·05 ± 0·00 | 5·02 ± 0·16 | 0·37 ± 0·02 | 0·07 ± 0·01 | 0·02 ± 0·01 |
| $Cl^-$ (15) | 4·91 ± 0·06 | 0·61 ± 0·12 | 0·13 ± 0·02 | 5·99 ± 0·21 | 0·76 ± 0·04 | 0·13 ± 0·01 | 0·001 ± 0·03 |
| $Rb^+$ (15) | 3·49 ± 0·43 | 0·30 ± 0·04 | 0·15 ± 0·04 | 3·48 ± 0·29 | 0·43 ± 0·06 | 0·13 ± 0·02 | −0·03 ± 0·04 |
| $Na^+$ (12) | 4·03 ± 0·36 | 0·45 ± 0·05 | 0·12 ± 0·01 | 3·48 ± 0·32 | 0·75 ± 0·12 | 0·27 ± 0·06 | 0·15 ± 0·05 |
| $HCO_3^-$ (13) | 6·45 ± 0·34 | 6·12 ± 0·52 | 1·01 ± 0·11 | 6·56 ± 0·31 | 13·17 ± 0·91 | 2·03 ± 0·13 | 1·02 ± 0·10 |
| +DIDS (0·5 mmol $l^{-1}$) | 0·06 ± 0·04 | 6·65 ± 0·39 | | 0·00 ± 0·02 | 5·6 ± 0·38 | | |

Fluxes and short-circuit currents ($I_{sc}$) are expressed in mol $cm^{-2}$ $s^{-1}$ × $10^{10}$.
Isotopes used were $^{45}Ca$, $^{36}Cl$, $^{86}Rb$, $^{22}Na$ and $[^{14}C]HCO_3$.
H → S, fluxes from haemolymph to shell side.
S → H, fluxes from shell to haemolymph side.

Table 3. *Intracellular potentials and ion concentrations under short-circuit conditions*

| $[Cl^-]$ | $[Cl^-]/[Cl^-]_{eq}$ | $[K^+]$ | $[K^+]/[K^+]_{eq}$ | $[Na^+]$ | Method |
|---|---|---|---|---|---|
| 7·9 ± 0·3 (30) | 1·3 ± 0·07 (30) | 26·5 ± 1·1 (16) | 1·16 ± 0·05 (16) | | Ion-sensitive microelectrode |
| 12·9 ± 0·61 (20) | | 29·4 ± 0·4 (20) | | 14 ± 0·4 (20) | Flame photometry |

| $V_{ic}$ | $I_{sc}$ | $g_T$ | Rs/Rs + Rh | $V_{oc}$ |
|---|---|---|---|---|
| −31 ± 0·5 (65) | 11·5 ± 0·9 (46) | 1·4 ± 0·13 (42) | 0·8 ± 0·02 (48) | −11·1 ± 1·5 (42) |

Values of short-circuit currents, open-circuit voltages and epithelial conductances obtained in the same preparations are also given.

$[Cl^-]_{eq}$ and $[K^+]_{eq}$ are the intracellular equilibrium concentrations of $Cl^-$ and $K^+$ computed from the intracellular potential and the corresponding extracellular concentrations. All concentrations in mmol $l^{-1}$ cell water.

Rs/Rs + Rh is the ratio of the potential drop across the apical barrier and the potential drop across the whole epithelium when a pulse of 10 mV is applied across the preparation.

Currents are given in $\mu A\,cm^{-2}$; conductances in $mS\,cm^{-2}$; voltages in mV; number of experiments is given in parentheses.

$V_{ic}$, intracellular electrical potential (in mV) in relation to the electrical potential of the bathing solutions.

$V_{oc}$, transepithelial electrical potential (in mV) under open-circuit conditions, that is when the transepithelial current is zero. Shell side used as reference.

(cations) and sulphate or gluconate (chloride) in the bathing solutions. Intracellular potentials were measured under short-circuit conditions.

Removal of sodium, potassium or chloride from the haemolymph solution produced small hyperpolarizations: −2·86 ± 0·55 ($N = 7$), −1·88 ± 0·33 ($N = 4$) and −7·19 ± 0·26 mV ($N = 20$), respectively. Similar substitutions performed on the shell side caused hyperpolarizations, respectively, of −3·8 ± 0·38 mV ($N = 4$) after sodium removal, −0·33 ± 0·33 mV ($N = 6$) after potassium removal and −1·58 ± 0·02 mV ($N = 6$) after chloride removal. When calcium was raised to 6 mmol $l^{-1}$ in the haemolymph solution the intracellular potential rose by 13·9 ± 1·1 mV ($N = 7$). Identical changes in the shell solution effected a smaller depolarization of 3·7 ± 0·9 mV ($N = 4$).

Intracellular concentrations of sodium, potassium and chloride were determined using ion-selective electrodes and chemical analysis (Table 3). The intracellular chloride concentration measured with $Cl^-$-sensitive microelectrodes was 7·9 ± 0·3 mmol $l^{-1}$ ($N = 30$). This value was 30 % higher than the average equilibrium value calculated from the membrane potential and the external chloride concentration. The average value obtained by chemical analysis was 12·9 ± 0·6 mmol $l^{-1}$ ($N = 20$). The intracellular potassium concentration determined with potassium-sensitive microelectrodes was 26·5 ± 1·1 mmol $l^{-1}$ ($N = 16$). This value was 10 % higher than the equilibrium value. The average intracellular concentration determined by flame photometry was 29·4 ± 0·4 mmol $l^{-1}$ ($N = 20$). Recordings were also made with sodium-sensitive microelectrodes but results are not given in Table 3 since it was found that at the sodium concentrations of the

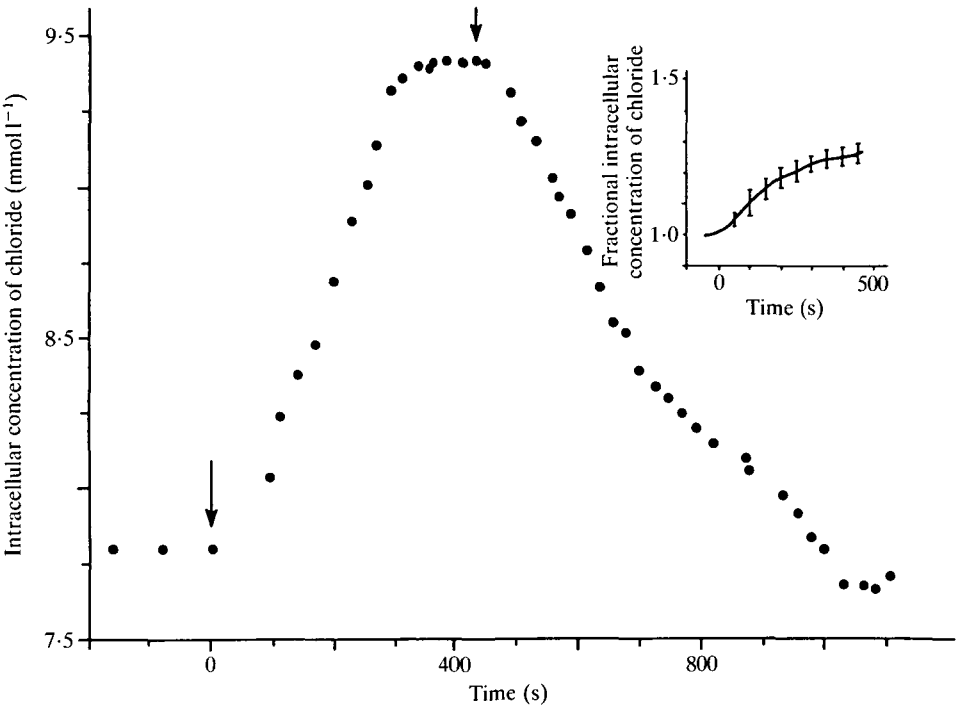


Fig. 6. Dependence of intracellular chloride concentration on bicarbonate in the haemolymph solution. Removal of bicarbonate is indicated by first arrow and reintroduction by the second arrow. Intracellular concentrations were measured with $Cl^-$-sensitive microelectrodes. The inset shows the results obtained with four preparations. For each preparation the value at time t was divided by the value at time zero. Means ± S.E.M. are plotted.

control solution there was strong interference from calcium. The average value obtained by flame photometry was $14 \pm 0{\cdot}4\,\text{mmol}\,l^{-1}$ ($N = 20$).

To examine whether there was any coupling between the fluxes of bicarbonate and chloride, the change in intracellular chloride concentration with time was measured upon removal of bicarbonate from the bathing fluids. When bicarbonate was removed from the shell side, the intracellular concentration of chloride remained constant. Fig. 6 shows an experiment in which bicarbonate was removed from and then reintroduced into the haemolymph solution. In this experiment removal of bicarbonate from the haemolymph solution caused a slow increase in intracellular chloride concentration at rate of about $0{\cdot}3\,\text{mmol}\,l^{-1}\,s^{-1}$. Similar results were obtained in four mantles, and the relative change in intracellular chloride concentration is plotted in the inset in Fig. 6.

*Calcium-dependence of the short-circuit current and open-circuit voltage*

As mentioned above our control solution contained less calcium ($1\,\text{mmol}\,l^{-1}$ instead of $6\,\text{mmol}\,l^{-1}$) and more potassium ($7\,\text{mmol}\,l^{-1}$ instead of $0{\cdot}7\,\text{mmol}\,l^{-1}$) than the haemolymph or extrapallial fluid. The effect of raising the calcium

concentration to 6 mmol $l^{-1}$ (substituting for mannitol) is presented in Table 4. The elevation caused a small decrease in short-circuit current (7 %), a large increase in conductance (50 %) and a fall in open-circuit potential (37 %). When, in addition to an increase in calcium, potassium was lowered from 7 to 1 mmol $l^{-1}$ by replacing it with choline the current fell by 33 %, the conductance increased by 25 %, and the potential fell by 47 %. It can be seen from Table 4 that changing the concentration of calcium in the bathing solutions did not affect the net calcium fluxes, which remained negligibly small at high and low calcium concentrations. The effect of calcium on the short-circuit current must therefore be through the transport of other ions. Table 4 also shows that the preparation was still able to generate a current and an open-circuit potential in the absence of calcium.

## Discussion

Shell formation and growth entail at least two distinct but interrelated processes: the production of an organic matrix consisting mainly of protein and the deposition of calcium carbonate in crystal form. The precipitation of calcium carbonate from the extrapallial fluid depends on calcium and carbonate achieving concentrations in this fluid above their solubility product and the most likely entry pathway for these ions into the extrapallial cavity is through the OME.

As originally shown by Istin & Maetz (1964), Istin & Kirschner (1968) and Sorenson *et al.* (1980), in the absence of ion concentration gradients the mantle generates a spontaneous potential *in vitro*. The orientation of this potential (shell side positive) might indicate an active transport of calcium towards the extrapallial fluid which, *in vivo*, might drive bicarbonate towards the same compartment through the electrical coupling provided by the membrane potential. This hypothesis is untenable since the movements of calcium across the mantle are purely diffusional, an observation later confirmed by other authors (Istin & Maetz, 1964; Istin & Kirschner, 1968; Sorenson *et al.* 1980; and the present work).

### *The nature of the short-circuit current*

This paper reports the first results obtained with the short-circuited outer mantle epithelium. Fig. 7 is a pictorial summary of our findings. The ion-substitution experiments show that the current is not due to a net transepithelial flux of sodium, potassium or chloride and that, although these ions account for most of the ionic content of the bathing solutions, their contribution to the transepithelial conductance is probably less than 30 % (around 10 % each).

It can be shown that for a diffusional process across a membrane and in the absence of an electrochemical gradient the unidirectional flux ($J_i$) of an ion i, measured with radioisotopes, is given by:

$$J_i = P_i C_i \,, \tag{1}$$

where $P_i$ is the permeability of the epithelium to the ion and $C_i$ the concentration of the ion. Using this expression and from the fluxes reported in Table 2 we can

Table 4. *Calcium- and potassium-dependence of short-circuit current, epithelial conductance, open-circuit voltage and unidirectional calcium fluxes*

| $N$ | $Ca^{2+}$ | $K^+$ | $I_{sc}$ | $g_T$ | $V_{oc}$ |
|---|---|---|---|---|---|
| 7 | 1 | 7 | 53·7 ± 4·7 | 1·39 ± 0·05 | 38·8 ± 0·80 |
| | 6 | 7 | 49·7 ± 4·0 | 2·06 ± 0·08 | 24·3 ± 2·10 |
| 7 | 1 | 7 | 53·3 ± 5·1 | 1·34 ± 0·05 | 40·1 ± 4·20 |
| | 6 | 1 | 36·1 ± 4·6 | 1·68 ± 0·06 | 21·7 ± 2·80 |
| 11 | 0 | 7 | 23·3 ± 1·3 | 0·55 ± 0·05 | 45·5 ± 3·90 |
| | 6 | 7 | 18·0 ± 1·4 | 1·75 ± 0·05 | 16·0 ± 1·40 |

Results of three groups of experiments.
$N$ is the number of pairs of mantles in each group of experiments.
Calcium and potassium concentrations are in mmol $l^{-1}$.
$I_{sc}$ is in $\mu A\,cm^{-2}$; $g_T$ in $mS\,cm^{-2}$; $V_{oc}$ in ml.

| $[Ca^{2+}]$ (mmol $l^{-1}$) | $N$ | Haemolymph to shell side | | | Shell to haemolymph side | | |
|---|---|---|---|---|---|---|---|
| | | $J_{H\rightarrow S}$ | $I_{sc}$ | $J_{H\rightarrow S}/I_{sc}$ | $J_{S\rightarrow H}$ | $I_{sc}$ | $J_{S\rightarrow H}/I_{sc}$ |
| 1 | 4 | 0·32 ± 0·12 | 6·08 ± 0·81 | 0·051 ± 0·02 | 0·32 ± 0·03 | 6·05 ± 0·51 | 0·052 ± 0·002 |
| 6 | 4 | 0·32 ± 0·10 | 3·56 ± 0·51 | 0·092 ± 0·03 | 0·49 ± 0·19 | 3·25 ± 0·25 | 0·156 ± 0·002 |

Calcium fluxes and short-circuit currents are expressed in mol $cm^{-2}\,s^{-1} \times 10^{10}$.
H → S, unidirectional fluxes from haemolymph to shell side.
S → H, unidirectional fluxes from shell to haemolymph side.

estimate the overall epithelial permeabilities to sodium, potassium, chloride, calcium and bicarbonate. Using the average of the two unidirectional fluxes the values obtained for calcium, chloride and potassium are, respectively: $P_{Ca} = 3{\cdot}5\times10^{-5}$, $P_{Cl} = 3{\cdot}3\times10^{-6}$ and $P_K = 5{\cdot}3\times10^{-6}$ cm s$^{-1}$. From the haemolymph to shell side flux of sodium a $P_{Na}$ of $2{\cdot}5\times10^{-6}$ cm s$^{-1}$ is obtained. These computations show that the OME is almost 10 times more permeable to calcium than to sodium, potassium or chloride. This is probably the cause of the fall in transepithelial potential and the increase in membrane conductance when the calcium concentration on both sides is raised from 1 to 6 mmol l$^{-1}$. If we extrapolate these observations to the *in vivo* situation, where the concentration of calcium is high (6 mmol l$^{-1}$ or higher) in the haemolymph and in the extrapallial fluid, the transepithelial potential should be very small. The high permeability to calcium could also explain why the calcium concentration is so similar in the haemolymph and the extrapallial fluid (Table 1; de Waele, 1930).

It can also be shown that the slope conductance ($g_i$) of an ion (i) which moves across a membrane by simple diffusion when that ion is at the same electrochemical potential on both sides of the membrane is given by:

$$g_i = [(zF)^2/\boldsymbol{R}T]P_iC_i = [(zF)^2/\boldsymbol{R}T]J_i\,, \qquad (2)$$

where $F$ is the Faraday (96 500 C equiv$^{-1}$), z is the valence (equiv mol$^{-1}$), $\boldsymbol{R}$ is the ideal gas constant (8·315 J degree$^{-1}$ mol$^{-1}$) and $T$ the absolute temperature (293 K). With this expression we can compute the conductances for the different ions and obtain the following values: $g_{Na} = 0{\cdot}17$, $g_K = 0{\cdot}14$, $g_{Cl} = 0{\cdot}26$ and $g_{Ca} = 0{\cdot}52$ mS cm$^{-2}$. Adding the conductances of sodium, potassium, chloride and calcium we obtain a value of 1·09 mS cm$^{-2}$, and the total conductance of the preparation measured electrically ranges between 0·86 and 1·2 mS cm$^{-2}$. Thus, the conductance due to these four ions accounts for almost all of the total conductance, the contribution of the calcium conductance being 50 % of the total. The bicarbonate conductance estimated from the flux (from haemolymph to shell side) of $^{14}C$, assuming that it corresponds to a diffusional flux of bicarbonate, is higher (1·3 mS cm$^{-2}$) than the total conductance of the preparation. This means either that bicarbonate is moving across the preparation in exchange for another anion, for example chloride, or that the $^{14}C$ flux reflects the movement of labelled $CO_2$. This last explanation seems more likely for two reasons: DIDS, which abolishes completely the short-circuit current, does not affect the flux of $^{14}C$ from haemolymph to shell side, and the unidirectional fluxes of chloride are very small.

The only sizeable net fluxes detected with radioisotopes in the absence of electrochemical gradients were those of sodium and bicarbonate. The sodium net flux towards the haemolymph side is so small that it may not be detectable in ion substitution experiments (Fig. 3). The effects of DIDS and amiloride indicate that both a bicarbonate/chloride exchange (Boron, 1983; A in Fig. 7) and a proton/sodium exchange (Benos, 1982; B in Fig. 7) are involved in the generation of the short-circuit current. The slowness of the effects of the two inhibitors indicates

that the role of both exchange mechanisms is probably to maintain ionic gradients, the gradual fall in current corresponding to the slow dissipation of these gradients.

*The sodium pump*

The insensitivity of the short-circuit current to ouabain is not conclusive evidence that the $Na^+/K^+$ pump is not present in this epithelium. A $Na^+,K^+$-ATPase has been found in microsomal fractions of the mantle of several bivalves (Deaton, 1982). The values of the intracellular concentrations of sodium and potassium reported in this work are consistent with its presence in the mantle of *Anodonta cygnea*. The concentration of intracellular sodium determined by flame photometry ($14\,mmol\,l^{-1}$) is clearly below the equilibrium value assuming a membrane potential of $-31\,mV$. Since the membrane potential was not measured

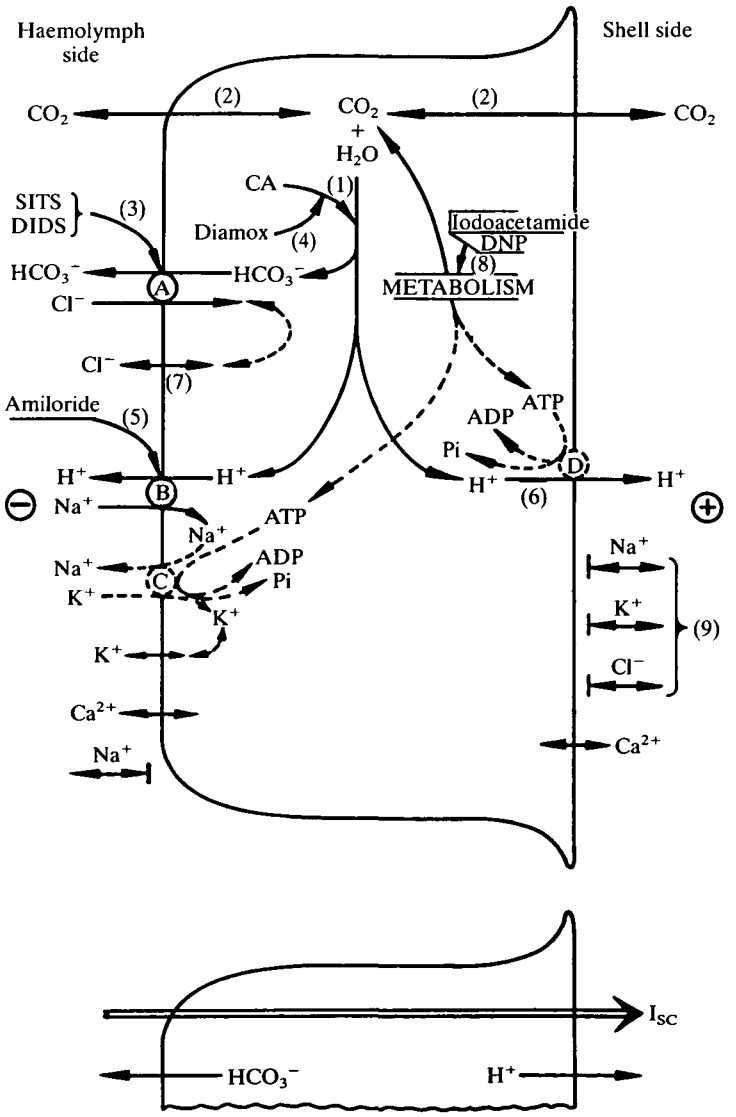


Fig. 7

simultaneously this statement requires confirmation. We were able to measure the intracellular electrochemical and electrical potentials for potassium simultaneously and we found that potassium is at equilibrium or slightly above equilibrium. Despite the fact that we could not demonstrate an effect of ouabain, the presence of a sodium pump in the basolateral barrier is represented in Fig. 7 (pump C) since it could provide a route for the recirculation of the sodium taken up through the sodium/proton exchanger (pump B).

### *The role of $CO_2$*

The inhibition of the short-circuit current by diamox shown in Fig. 5 is an indication that part of the bicarbonate transport depends on the supply of this ion *via* the catalysed hydration of $CO_2$ (1, Fig. 7) which takes place inside the cells. When the bathing solutions are equilibrated with 5 % carbon dioxide (around 40 mmHg partial pressure; 1 mmHg = 133·3 Pa), and assuming a solubility of around 0·05 mmol $mmHg^{-1}$ at 20 °C (Edsall & Wyman, 1958), the concentration of dissolved $CO_2$ in the bathing solutions and in the cytoplasmic water is at most 2 mmol $l^{-1}$. Assuming that the thickness of the epithelium is 15 $\mu$m and that 70 % of its volume is occupied by intracellular water there will be around 2 nmol of $CO_2$ per square centimetre of epithelium. At a rate of hydration of 0·038 $s^{-1}$ (Maren, 1967) the maximum rate of production of carbonic acid and bicarbonate will be 76 pmol $s^{-1}$ $cm^{-2}$ of epithelium, which is equivalent to 7·3 $\mu$A $cm^{-2}$. Yet, we were able to measure short-circuit currents up to 70 $\mu$A $cm^{-2}$. These rough calculations, which are probably overestimates of bicarbonate production, show that, under our experimental conditions, the uncatalysed hydration of carbon dioxide is unable to support fully the measured short-circuit current.

The mechanism responsible for the short-circuit current seems thus to involve the diffusion of carbon dioxide into the cytoplasm (2, Fig. 7), its hydration catalysed by carbonic anhydrase and the extrusion of at least part of the

Fig. 7. Electrophysiology of the outer mantle epithelium. The figure summarizes the relevant findings. The short-circuit current ($I_{sc}$) is due to the transport of bicarbonate to the haemolymph side and of protons towards the shell side (lower part of the figure). Protons and bicarbonate ions are generated mainly through the catalysed hydration of $CO_2$ (by carbonic anhydrase, CA, and 1) since most of the current is inhibited by Diamox (4). When $CO_2$ is not present in the gas mixture the current falls to very low values. This indicates that the main sources of $CO_2$ are the bathing fluids (2). Cell bicarbonate is transported across the basolateral barrier in exchange for chloride (A) and this exchange is blocked by SITS and DIDS (3). There is a recirculation of chloride across the same barrier. Some of the protons generated intracellularly are excreted towards the haemolymph side in exchange for sodium (B). This process is inhibited by amiloride (5). A sodium pump in the basolateral barrier is postulated, which provides a recirculation route for sodium. The potassium taken up by this pump diffuses out through the same barrier. An electrogenic proton pump (D) across the apical barrier is also postulated. The effects of DNP and iodoacetamide (8) can be explained by a fall in the concentration of intracellular ATP. The permeability of the apical barrier to sodium, potassium and chloride is very small (9). The permeability of the basolateral barrier to sodium is also very small. Values for the intracellular electrical potential and intracellular concentrations of sodium, potassium and chloride are given in Table 3.

bicarbonate produced intracellularly into the haemolymph compartment. Such a mechanism would explain the effect of DIDS (3, Fig. 7) when added to the haemolymph solution, the effect of diamox (4, Fig. 7) and the increase in intracellular chloride concentration when bicarbonate is removed from the haemolymph solution. We cannot exclude the possibility that some bicarbonate might enter the cell through the apical barrier.

To regulate intracellular pH, a number of protons equal to the number of bicarbonate ions excreted must leave the cell. The effect of amiloride (5, Fig. 7) indicates that some of these protons may also be excreted across the basolateral membrane in exchange for sodium (pump B, Fig. 7); the remainder are probably excreted across the apical membrane (6, Fig. 7). Although the chloride/bicarbonate exchange is electrically neutral, it can build up an electrochemical gradient for chloride between the cytoplasm and the external compartments which can drive a diffusional efflux across the basolateral membrane (7, Fig. 7) that will cancel the influx of this ion through the chloride/bicarbonate exchanger. The overall result would be an efflux of bicarbonate towards the haemolymph solution and a recirculation of chloride across the basolateral membrane. Under short-circuit conditions the intracellular potential is sensitive to the removal of chloride from the haemolymph solution but the effect observed is a hyperpolarization and not a depolarization as might be expected from a diffusional effect.

Of all the inhibitors used, iodoacetamide and DNP had the most dramatic effects on the short-circuit current. The metabolic effect of iodoacetamide, a thiol inhibitor (Dixon & Webb, 1964), is not fully characterized. It inhibits glycolysis almost completely and it also blocks the triosephosphate dehydrogenase system causing a fall in the level of intracellular ATP (Pon, 1964). Thus both DNP (initially) and iodoacetamide cause a decrease in ATP production (8, Fig. 7). If the fall in short-circuit current reflects this effect it indicates a close link between intracellular ATP concentration and net charge transport across the epithelium. The sodium pump is not this link inasmuch as the effect of ouabain was very small and slow. As reasoned above, the extrusion of bicarbonate across the basolateral barrier implies an identical extrusion of protons across the apical membrane. Preliminary experiments performed in our laboratories showed that when poorly buffered solutions were used, the epithelium alkalinized the haemolymph solution and acidified the shell solution. The short-circuit current was insensitive to amiloride added to the shell solution and the intracellular potential did not change when sodium, potassium or chloride was removed from the same solution (9, Fig. 7). This indicates not only that the permeability of the apical membrane to these ions is, relatively, very small but also that the extrusion of protons is not by a proton/sodium exchange. However, when the proton concentration was raised in the shell solution there was a marked inhibition of the short-circuit current (Fig. 4). These considerations suggest the possibility of an electrogenic proton pump (pump D, Fig. 7) at the apical barrier. Electrogenic proton pumps have been identified in the urinary bladders of the turtle *Pseudemys scripta* (Steinmetz *et al.* 1967) and the toad *Bufo marinus* (Ludens & Fanestil, 1972), in the collecting

duct of the mammalian kidney (Stoner *et al.* 1974) and in frog skin (Ehrenfeld *et al.* 1985).

### *The role of calcium: shell formation*

Although our results, and those of other workers quoted above, do not elucidate the role of the outer mantle epithelium in the calcification of the shell, the hypothesis that such a role exists is difficult to disclaim. The structure is highly permeable to calcium and a slight gradient of calcium (towards the shell side) may generate a small but sufficient net calcium flux towards the shell side. If the behaviour *in vitro* of the OME reflects the way it functions *in vivo*, the role of the central part of the mantle is to prevent the calcification of the shell, because the transport of bicarbonate towards the haemolymph, the open-circuit potential and the transport of protons towards the extrapallial fluid all oppose the creation of conditions favouring calcium bicarbonate precipitation.

The calcification of the shell may thus be the result of a balance between the movements of calcium across the OME resulting from the electrochemical gradient of this ion towards the extrapallial fluid, created by processes that raise its concentration in the haemolymph, and the metabolically dependent transport of bicarbonate and protons, which will move calcium in the opposite direction. However, the function of the mantle must be modulated by other factors, since shell growth is not constant and the electrical behaviour of the OME undergoes cyclic variations throughout the year, probably related to the reproductive cycle.

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