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Effects of pH and Light on the Membrane Conductance Measured in the Acid and Basic Zones of *Chara*

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Summary. The area-specific conductance of the membrane in the acid and basic zones (denoted by G_A and G_B, respectively) of *Chara* cells was measured in flowing solutions, containing 5 mM zwitterionic buffer, as a function of the external pH (denoted by pH^0). During illumination G_A was ~1 S/m² for pH^0 in the range 5 to 8.5, and increased markedly to 3 to 6 S/m² at higher pH^0. G_B, however, was always larger than G_A during illumination with a typical magnitude of 5 to 15 S/m² for pH^0 6 to 12. Thus under many experimental conditions it is possible that there is no single correct value for the membrane area-specific conductance. A flow of current in the external medium between the acid and basic regions was found to be associated with pH 'banding,' and also with G_B exceeding G_A. This current could be present in flowing solutions without added HCO_3^- over a wide range of pH^0 and at high (25 mM) buffer concentration. Combining measurements of G_A and G_B with measurements of the currents in the acid and basic zones (denoted by J_A and J_B, respectively), it was estimated that the resting (i.e. in the absence of net current flow) potential difference (PD) across the membranes within the individual zones (denoted by U_A and U_B) was -265 ± 20 and -183 ± 5 mV, respectively, during illumination. Upon the removal of illumination at $pH^0 = 7.5$, G_A, G_B and J_B were found to decrease rapidly during the initial few hundred seconds. During this period $(U_B - V_m)$ remained relatively constant. A transient hyperpolarization of V_m often occurred, the magnitude of which was correlated with the magnitude of J_B prior to the removal of illumination. After some 0.5 to 1 ksec of darkness, G_A and G_B had both decreased considerably and now $G_A \sim G_B$ and $U_A \sim U_B \sim V_m$. Eventually, after 2 to 8 ksec of darkness, the membrane conductance was effectively homogeneous with a much smaller magnitude (typically <0.2 S/m²) and V_m was depolarized by typically 5 to 15 mV.

Key Words conductance · *Chara* · banding · membrane · pH · light · current

Introduction

It is known that cells of *Chara* and *Nitella* can develop spatially distinct acid and basic regions in the external medium adjacent to the cell upon exposure to light (e.g. Spear, Barr & Barr, 1969; Lucas & Smith, 1973), and that large electric currents can flow between these regions (Walker & Smith, 1977). Recent experiments with *Chara* have shown that under some conditions, the membrane conductance in the basic zones can be much larger than in the acid zones[1]. This spatial inhomogeneity in membrane conductance has previously only been reported for cells bathed in stagnant, unbuffered solutions of pH ~5.5 (Smith & Walker, 1983), and also for cells suspended in air (Chilcott et al., 1983; Ogata, 1983; Ogata, Chilcott & Coster, 1983). The following are some reasons why it is of interest to study the membrane conductance within these individual zones over a wider range of conditions.

(a) Once the area-specific conductances of the acid and basic zones (denoted by G_A and G_B, respectively) differ, there is then obviously no correct single value for the membrane conductance. It is important to know when this situation might occur so that suitable measurement techniques can be employed.

(b) If the steady transverse currents through the membrane within each zone can be measured, it is then possible to calculate the contribution to the measured membrane PD[2] produced by these currents. The resting PD (i.e. for zero net current flow) within each individual zone can then be calculated.

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[1] In this paper acid or basic zones will refer to the regions of the cell membrane which, upon illumination and under appropriate experimental conditions, were found to produce, respectively, acid or basic regions in the external medium.

[2] *Abbreviations:* PD, potential difference; MES, 2[N-morpholino] ethanesulfonic acid; PIPES, piperazine-N,N′-bis(2-ethane)sulfonic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; TAPS, tris(hydroxymethyl)-methylaminopropanesulfonic acid; CHES, 2 [N-cyclohexylamino] ethanesulfonic acid; CAPS, cyclohexylaminopropanesulfonic acid.

(c) From measurements of the time dependence of the conductance and current within each zone during light/dark transitions it is possible to determine whether the resultant decrease in the current flowing between the zones is a consequence of changes in either the membrane conductance and/or changes in the resting PD of the individual zones.

In previous publications (Smith, 1983*a*; Smith & Walker, 1983) it was demonstrated how to evaluate the area-specific conductance of an individual membrane zone within a spatially inhomogeneous cylindrical cell. Using this approach we have estimated the separate area-specific conductances of the basic and acid zones in *Chara* while cells were bathed in a flowing, buffered, artificial pond water with pH in the range 5 to 12. We have also concomitantly measured the steady current flowing into the basic regions and hence estimated the membrane resting PD within individual zones. The time dependence of these parameters during changes of illumination was also studied.

Materials and Methods

Long (30 to 40 mm) internodal cells of *Chara australis* R.Br with diameters 0.6 to 0.9 mm were used. Cells with visible calcification were chosen to facilitate the desired positioning of the acid and basic zones within the perspex cell holder. This was divided into two or three electrically isolated sections by silicon grease or petroleum jelly packed tightly around the cell surface when blotted dry (*see* Smith & Walker, 1983). Unbuffered artificial pond water (APW: 0.1 mM KCl, 1.0 mM NaCl, 1.0 mM $CaCl_2$, pH ~ 5.5) was then added to each section. Location of the acid and basic regions was verified at this stage for each cell by the addition of a small amount of pH indicator (phenol red) during illumination. Measurements were performed at room temperature (22 ± 2°C) with illumination (~100 μEm^{-2} sec^{-1}) provided by a microscope light. The length of the cell within the measuring chamber was always sufficient to ensure that acid and basic zones were both always present within this chamber for each cell. During most measurements a buffered solution (APWB = APW + 5 mM buffer) flowed through the measuring section at ~0.2 ml/sec. The zwitterionic buffers used were MES (pH 5 to 6.5); PIPES (pH 6.7 to 7); HEPES (pH 7.5 to 8); TAPS (pH 8.5); CHES (pH 9 to 9.5) and CAPS (pH 10 to 11.7). After addition of the desired buffer the pH was adjusted by the addition of NaOH. The APWB in the other sections was maintained at pH^0 = 7.5 throughout experiments involving the variation of pH and/or light intensity.

Results are presented as mean ± standard error (number of samples).

Measurement of Membrane Conductance

The membrane conductance was measured at 1 Hz. This frequency is sufficiently low to ensure that the conductance calculated from the measured complex AC admittance Y^* reflects the DC membrane conductance, yet is sufficiently high to minimize any effects due to concentration changes in the unstirred layers (*see* Coster & Smith, 1977; Smith & Coster, 1980). A sinusoidal AC signal was generated by a computer (Cromemco Z2-D) and passed between a Ag/AgCl electrode located in an outer section and another such electrode located immediately adjacent to the edge of the measuring chamber (at $x = 0$) where the zone under investigation was located. The total AC current I_T so injected at $x = 0$ was derived from the resultant AC PD developed across a known impedance connected in series. The measured conductance G^* was then given by the real part of $Y^* = I_T/V(0)$, where $V(0)$ denotes the resultant AC membrane PD at $x = 0$ which was measured by two alternative techniques. The first determined the AC PD developed between two Ag/AgCl electrodes; one located in the measuring chamber adjacent to the cell surface at $x = 0$ and the other located in a separate intermediate section (*see* Smith & Walker, 1983). This technique could obviously not measure V_m, the resting membrane PD. In the second technique $V(0)$ and V_m were measured directly by a micropipette inserted into the vacuole at $x = 0$. The overall accuracy of the measurement of G^* was typically 1 to 2%.

Calculation of the Membrane Area-Specific Conductance

The local membrane area-specific conductance G at the point of current injection was calculated in the following fashion. In a previous report (Smith & Walker, 1983) we measured for each cell not only G^*, but also λ, the local characteristic length for the decay of transverse membrane PD. Thus an effective area of membrane through which the injected current flowed could be estimated and G consequently calculated from G^*. The relationship (as determined from measurements of the AC membrane PD at two points on the cell), between the measured λ and the measured membrane impedance times unit length (z) derived from G^* was found to be in good agreement with the theoretical relationship for a one-dimensional cable, i.e.

$$\lambda = (z/(r_o + r_i))^{1/2} \tag{1}$$

where the parameters r_o and r_i denote the longitudinal resistances per unit length of the external solution and the vacuole, respectively. This agreement is evident in Figs. 7–9 of Smith and Walker (1983) and reveals a degree of correlation such that it was not felt to be necessary to independently measure λ in the experiments reported herein. This simplified the experiments and removed the possibility of any membrane damage produced by two micropipettes inserted in close proximity.

For measurements of G in the acid zones the membrane was usually treated as homogeneous and conventional cable theory applied. This is a reasonable approximation if the basic zones are distant by approximately the cable length in the acid regions (*see* Smith, 1983*a*). Thus the area-specific conductance G_A of the acid zones was calculated from G^* via the equation

$$G_A^{1/2} \tanh(G_A L^2 \pi d(r_o + r_i))^{1/2} = G^*(r_o + r_i)^{1/2}/(\pi d)^{1/2} \tag{2}$$

where d and L, respectively, denote the diameter and length of the cell section under study. The value of $(r_o + r_i)$ was determined as described previously (Smith & Walker, 1983).

If current was injected into a basic zone of width in excess of its cable length, the membrane could be considered homogeneous with little error. However, in other situations an iterative procedure employing the theory of an inhomogeneous cable was necessary to correctly evaluate G_B (Smith 1983*a*). This model is

relatively insensitive to the properties of the acid zones and so average values of $G_A(pH^0)$ from a series of experiments on other cells were used. The width of each basic zone was estimated from the boundary revealed by phenol red. For highly conductive basic zones the possible error in G_B associated with the correction for cable properties might be as high as ~20%.

Experimental Protocol for Dependence of G upon pH^0

The experimental procedure involved leaving the cell for at least one hour at pH^0 7.5 (pH^0 denotes the bulk pH of the bathing solution) after mounting in the experimental chamber and confirming that each cell did exhibit pH banding in APW while illuminated. When the bathing solution was taken to a new pH^0 value the conductance was continuously monitored until it became steady (usually after a few minutes). The bathing solution was then usually taken back to pH^0 7.5 and this pH maintained until the conductance was similar to that measured prior to the pH change. Thus the cell was exposed to pH^0 values other than 7.5 for the minimum time possible, with the objective of minimizing the magnitude of any changes in the cytoplasmic pH (*see* Bisson & Walker, 1981, 1982*a*). Towards this end pH^0 changes were also made in alternate directions, wherever convenient.

Measurement of Local DC Current

The transverse DC current flowing through the membrane was derived from the transverse electric field adjacent to the cell surface. This was measured by the following apparatus, which is a simple adaption of the 'vibrating probe' (e.g. Jaffe & Nuccitelli, 1974). The computer generated a sinusoidal current at 8 Hz which was amplified and connected to a loudspeaker. Attached to the center of the loudspeaker cone was a long Teflon®-coated silver wire (300 μm diameter) constrained within a narrow length of glass tubing (internal diameter ~1 mm). This reduced flexing of the silver wire and ensured that its tip, which was bent at a right angle and coated with AgCl, oscillated transversely and adjacent to the cell surface with an amplitude similar to that of the loudspeaker voice coil. The tip was positioned slightly above the horizontal plane through the middle of the cell and located on the side of the cell opposite to the vacuolar micropipette. The PD so generated between this tip oscillating in the steady electric field close to the cell surface and a stationary reference electrode was fed into an amplifier with a gain that was strongly peaked in the frequency range 1 to 10 Hz. The output of this amplifier, V_{PROBE}, was connected to one channel of an analogue-to-digital converter (ADC) and the voltage present across the speaker voice-coil, V_{SPKR}, was connected to another channel. The generation of the sinusoidal current, the concomitant sampling of V_{PROBE} and V_{SPKR} and the analysis of the data were performed by the same software as was used to measure the membrane admittance. Thus the ratio A_P ($=V_{PROBE}/V_{SPKR}$), which should be proportional to the magnitude of the transverse current, was calculated. The direction of the current flow was indicated by the relative phase of V_{PROBE} with respect to V_{SPKR}. As with the admittance measurements, the relative magnitude of any noise or nonlinearity present could be estimated from the correlation index for each fit to a general sinusoidal function. The tip of the silver wire was located 250 mm from the loudspeaker voice-coil, obviating the possibility of significant cross-coupling between V_{PROBE} and V_{SPKR}.

To provide an absolute calibration for the current density at the cell surface, the following procedure was adopted. The approach used involved maintaining the apparatus in the same configuration as would be employed for a 'banding' cell, but substituting a cell with homogeneous electrical properties to produce a known current density through the cell surface. As explained below this should substantially reduce the contribution of several sources of error. Thus a young internodal cell without visible calcification was mounted in the experimental chamber and left in the dark for ~8 ksec. Previous measurements (Smith & Walker, 1983) have shown that the membrane conductance under such conditions will be essentially spatially uniform and sufficiently low in magnitude to ensure a uniform current density at the cell surface within the measuring chamber, i.e. λ will be large in comparison with the length L of the cell in the measuring chamber (L was typically 8 mm during the calibration procedure). A DC current of known magnitude was then passed through the cell section in the measuring chamber and the surface current density J calculated using the measured surface area of this cell section. During both calibration and experimental measurements the probe tip was kept approximately the same distance from the cell surface (300 to 500 μm) and in the same relative position. This should have minimized any errors due to a distortion of the current flow produced by the presence of the tip itself, and also reduced any errors due to components of the motion of the probe tip that were not perfectly perpendicular to the cell surface. It should have also corrected to a reasonable extent for the decrease in current density with increasing radial distance from the cell (*see* Walker & Smith, 1977; Lucas & Nuccitelli, 1980). The measured relationship between A_P and J was found to be linear and the constant of proportionality derived from the slope. This constant was found to be similar at each end of the cell section, confirming that the current density through the cell surface during calibration was relatively uniform. Tests were also made to ensure that the ratio A_P was essentially independent of the magnitude of V_{SPKR} over the range used, and that the proportionality constant decreased linearly with the conductivity of the bathing solution.

Because the probe tip is Ag/AgCl, it will be sensitive to the local Cl-gradient. However, V_{PROBE} was found to be relatively small if (a) no DC current was passed during calibration and (b) after several hours of darkness for a previously banding cell. Thus steady gradients of extracellular Cl^- appeared not to be significant, which is consistent with the known magnitudes of Cl^- currents (e.g. Beilby & Walker, 1981).

The precision of relative measurements of the DC current was typically better than 2%. However, errors in estimating the absolute magnitude of the DC current density flowing across the membrane could easily be as large as 20%. An inward current flow will be expressed as a positive value of J.

Experimental Protocol for Quasi-Simultaneous Measurements of J, G, and V_m

In these experiments a micropipette was inserted into the vacuole at $x = 0$. The current probe was manipulated so that its tip was typically 300 to 500 μm distant from the cell surface at a longitudinal position estimated to be at the center of the region under study. Previous measurements using a current probe of higher resolution have shown that the current density is relatively uniform within each basic region (Lucas & Nuccitelli, 1980) and so the behavior of this current should adequately re-

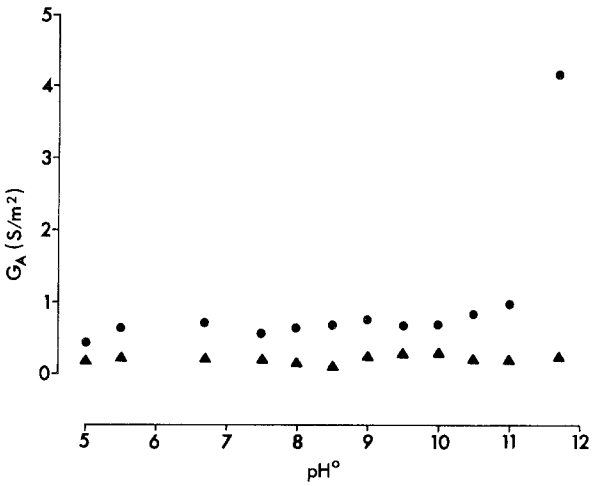

Fig. 1. Dependence upon pH^0 of the area-specific conductance G_A of an acid zone measured via an inserted micropipette. Data points denoted by (●) were obtained in bright illumination and those denoted by (▲) after 5 ksec of darkness. All measurements were on a single cell bathed in flowing APWB (5 mM buffer)

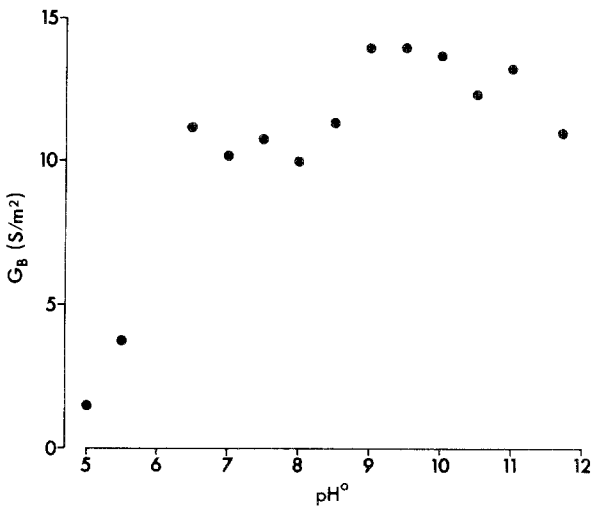

Fig. 2. Dependence upon pH^0 of the area-specific conductance G_B of an illuminated basic zone measured via an inserted micropipette. The cell was bathed in flowing APWB (5 mM buffer)

flect that for the entire zone. The amplitude of vibration of the probe tip was occasionally adjusted during experiments to as small a value as possible consistent with a reasonable signal-to-noise ratio for V_{PROBE}. The amplitude was, however, always <100 μm.

For each set of quasi-simultaneous measurements of J, G and V_m, the following sequence of operations was produced under computer control. Firstly V_m was sampled via one channel of the ADC. The output of a digital-to-analogue converter was then temporarily connected by a relay to the current electrodes and 4 cycles of a 1 Hz sinusoidal current injected into the cell. $V(0)$ and I_T were measured by the computer during the last cycle and G calculated. The probe tip was then oscillated at 8 Hz for 4 cycles. During the last cycle V_{PROBE} and V_{SPKR} were sampled by the computer, which then calculated A_P and hence J. This overall procedure lasted 15 sec, and thus G, J and V_m were each measured every 15 sec. The 5-sec delay which occurred between the injection of AC current to measure G and the sampling of the steady current should have ensured that there was no interference between these measurements. In between measurements of J, the probe tip was stationary to minimize stirring of the external medium adjacent to the cell surface.

During these measurements, I_T, V_m, V_{PROBE}, and V_{SPKR} were displayed on an oscilloscope and V_m was also monitored by a chart recorder. The bathing solution was stagnant during experiments involving the measurement of J, but was replaced regularly.

Possible Errors in Determining G

It should be noted that in the measurements reported herein the membrane conductance refers to that of the plasmalemma and tonoplast in series. In an unbuffered solution of $pH^0 \sim 5.5$ the tonoplast conductance of whorl cells is typically 3 to 5 times that of the plasmalemma (e.g. Smith, 1983*b*). Thus the plasmalemma conductance could be significantly higher than that reported herein for the 'membrane,' but as yet we are ignorant of how the tonoplast conductance depends upon pH^0.

The theory used to determine G_B from G^* assumed that abrupt transitions between G_A and G_B occurred at the boundaries indicated by phenol red. However, experiments performed with cells suspended in air (Chilcott et al., 1983; Ogata, 1983; Ogata et al., 1983) indicate that under these conditions the measured transitions in conductance can occur over distances of several mm (to some extent this will be a consequence of the cell cable properties). A theoretical treatment of the properties of cells with nonabrupt transitions in conductance, presented as an Appendix, indicates that any errors in our values for G_B produced by either the assumption of an abrupt boundary or the incorrect location of that boundary will be relatively small (*see* Fig. 8).

A possibility that should also be considered is that the presence of the 'vibrating probe' might induce enough local stirring to affect the membrane electrical properties, particularly as such an effect has been reported previously (Lucas & Nuccitelli, 1980). To minimize such stirring a very low frequency (8 Hz) of vibration was chosen and the tip was only vibrating when a measurement of J was to be made. Thus the tip was actually vibrating for only 3% of the time. Measurements of G were found to be unaffected by both vibration of the tip and its removal from the bathing solution. These results suggest that any disturbing effect of the probe on the electrical properties of the membrane should be slight.

Results

Variation of Conductance with pH^0

The measured dependences upon pH^0 of the area-specific conductances G_A and G_B of the acid and basic zones in two representative cells bathed in flowing APWB (5 mM buffer) are shown in Figs. 1 and 2, respectively. Similar results were obtained with and without an inserted micropipette, which suggests that the insertion of a micropipette was not significantly damaging the membrane. For illumi-

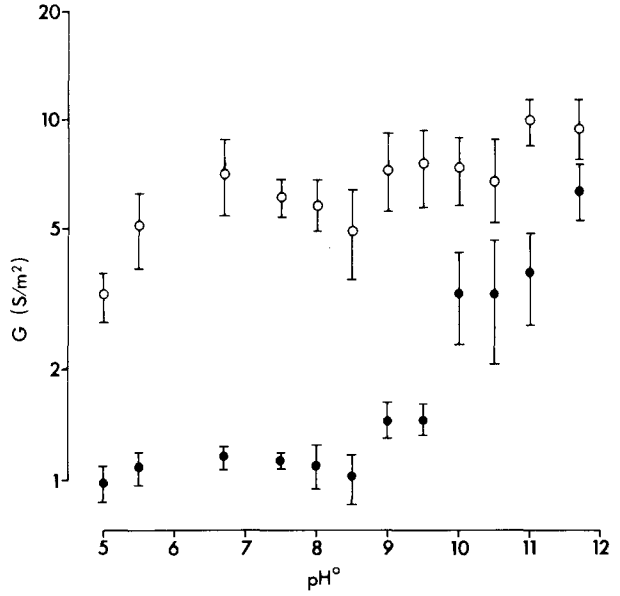

Fig. 3. Measured dependence upon pH^0 of the area-specific conductance G of illuiminated acid zones (● – 8 cells) and basic zones (○ – 6 cells). Data is presented as mean ± standard error. Cells were bathed in flowing APWB (5 mM buffer). Note that G is plotted on a logarithmic scale

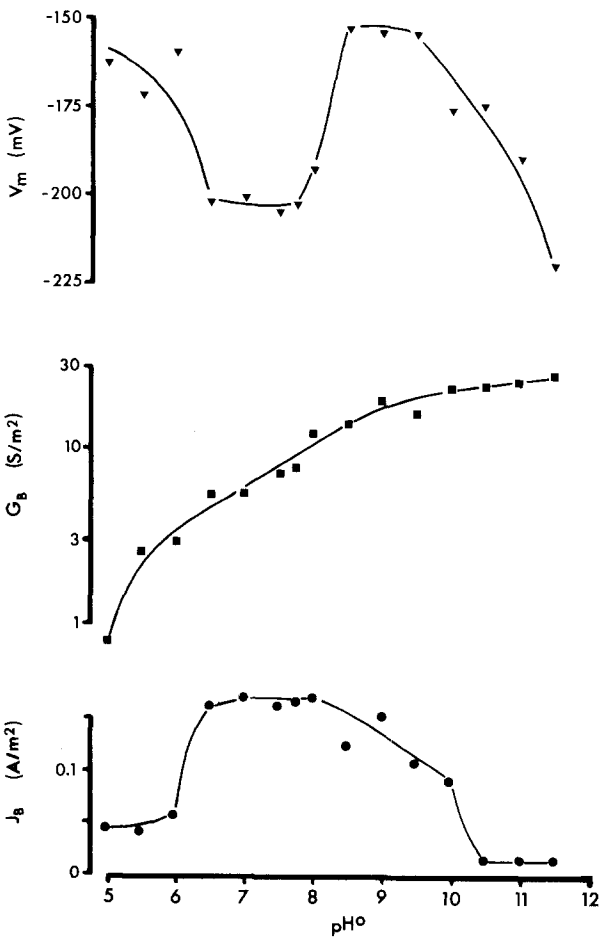

Fig. 4. The dependence upon pH^0 of the electrical parameters of an illuminated basic zone bathed in stagnant APWB (5 mM buffer). All measurements are from a single cell. G_B denotes the area-specific conductance and J_B denotes the inward current density in this basic zone. V_m denotes the membrane resting PD. The continuous lines are drawn through the data points for clarity. The magnitudes of G_B and J_B were found to be reproducible when the cell was returned to $pH^0 = 7.5$ from both high or low pH^0. After 2 ksec of darkness G_B was 0.19 S/m² at $pH^0 = 7.5$ and 0.26 S/m² at $pH^0 = 11.5$

nated acid zones G_A was always found to increase considerably with increasing pH^0 when a threshold pH^0 value in the range 10 to 12 was reached, in a similar fashion to that reported previously on unspecified regions of the cell (Bisson & Walker, 1980, 1981, 1982*a*). The transition to this high conductance state was reversible, and could be produced severally on the same cell[3]. For illuminated basic zones the area-specific conductance G_B was found to be relatively independent of pH^0 in the range 6 to 12. In this range G_B was found to be always much higher than G_A measured at $pH^0 < 9$, but G_B was found on the average to be similar in magnitude to that of G_A measured at pH^0 11 to 12. Figure 3 shows the average pH^0 dependence of G_A and G_B determined from experiments on 14 cells in flowing APWB. An example of the pH^0 dependence of G_B in stagnant APWB is shown in Fig. 4.

Variation of V_m with pH^0

In the six experiments in the series of measurements in flowing APWB where micropipettes were inserted into the vacuole, V_m was found to be maximally hyperpolarized (to ~−190 mV) at pH^0 7 to 8, and then to depolarize with increasing pH^0 to ~−160 mV until hyperpolarization began again at pH^0 9 to 10. This overall dependence of V_m upon pH^0 is similar to that reported previously by Bisson and Walker (1981, 1982*a*). Similarly it was found that the exact nature of the dependence varied with the history of the cell. The dependence of V_m upon pH^0 in stagnant (*see* Fig. 4) and flowing APWB were found to be similarly shaped, but with V_m typically depolarized by ~10 mV in flowing solutions, probably as a consequence of traces of NH_3^+ ions (*see* Walker, Beilby & Smith, 1979).

[3] Sometimes it was found that prolonged exposure to high pH^0 could produce a temporary inhibition of these high values of G_A. However, if the cell was returned to pH^0 7.5 and left for a period, the ability of G_A to increase at high pH^0 returned. This inhibition is possibly a consequence of a protection mechanism which intervenes to prevent changes in cytoplasmic pH which might otherwise produce permanent damage.

Variation of J_B with pH^0

An example of the pH^0 dependences of V_m, G_B and J_B (the current density through the basic zone), measured quasi-simultaneously in stagnant APWB, is shown in Fig. 4. It is apparent that J_B has a large magnitude which is relatively independent of the value of pH^0 until the extreme values of $pH^0 < 6$ or $pH^0 > 10$ are reached.

Relationship between G_B and J_B at $pH^0 = 7.5$

To investigate further whether high values of G_B (i.e. $>G_A$) are associated with the presence of large current flows between the acid and basic regions, and hence presumably also linked with pH banding, the following measurements were made. Cells were bathed in APWB at $pH^0 = 7.5$, but unlike in the experiments described above, they were not necessarily allowed to attain their long-term, maximal degree of 'banding.' This was achieved by making measurements either relatively soon after electrode insertion or after a period of darkness. From 11 cells so studied it was found that G_B was in the range 0.6 to 9.5 S/m^2 and J_B was in the range 0 to 0.18 A/m^2. High values of G_B were found to be well correlated with high values of J_B. From 19 measurements on these 11 cells it was found that $J_B > 0.04$ A/m^2 if and only if $G_B > 3$ S/m^2. The straight line of best fit to this data was described by the relationship $J_B = -0.014 + 0.019\ G_B$ with a correlation coefficient of 0.84. The integral probability of this data deriving from an uncorrelated parent population is $<0.001\%$.

In most of the reported experiments, J_A, the average current density through the acid zone, was not explicitly measured. It could be estimated however, via the equation $J_A = -J_B A_B / A_A$, which will hold in the steady state where A_A and A_B refer, respectively, to the surface areas of the acid and basic zones. Thus J_A was estimated to be in the range 0 to -0.15 A/m^2. These values for J_A and J_B are larger than those determined in this laboratory from the measured extracellular variation of PD using the Laplace equation (Walker & Smith, 1977; Cathers, 1979), but are slightly less than those determined elsewhere using a probe vibrating at a higher frequency (Lucas & Nuccitelli, 1980; Lucas, 1982). Some of the latter difference presumably is a consequence of the different compositions of bathing solution (our APW contained no added HCO_3^-) and the greater manipulation of the cell required for our experiments. These results confirm other observations (Walker & Smith, 1977; Lucas, 1982) that large extracellular currents can still flow in a bathing solution containing 5 mM zwitterionic buffer (*see also below*).

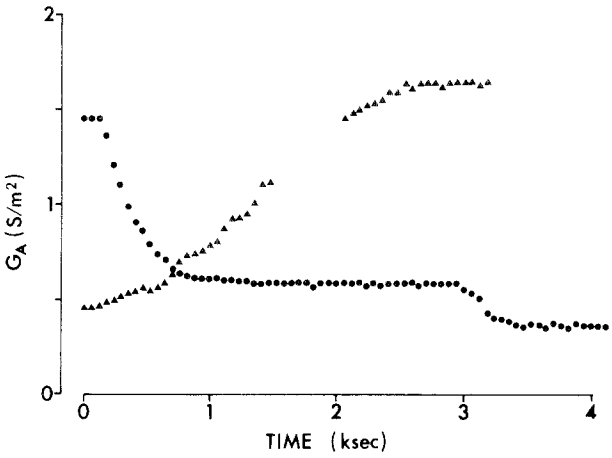

Fig. 5. The time dependence of the area-specific conductance G_A of an acid zone bathed in flowing APWB at $pH^0 = 7.5$ during changes in illumination. Data points denoted by (●) were measured after illumination was removed at time = 0. Data points denoted by (▲) were measured on the same cell after a series of measurements on the pH^0 dependence in the dark had been performed which lasted ~5 ksec. These latter points have been shown displaced to the left, i.e. illumination is also shown as being restored at time = 0

Effect of Varying Buffer Strength

To examine how buffering might affect the inhomogeneity of membrane conductance and the associated current flow between the acid and basic zones, the magnitude of G_B and J_B was examined in stagnant solutions of unbuffered APW (subscript$_U$), APW + 5 mM HEPES (subscript$_5$), APW + 25 mM HEPES (subscript$_{25}$), and APW with a 'buffer cocktail' composed of 1 mM each of MES, HEPES, TAPS, CHES and CAPS (subscript$_Q$). The pH of the last three solutions was adjusted to pH 7.5 with NaOH. From measurements on five cells it was found that the ratios G_5/G_U, G_{25}/G_U and G_Q/G_U were 0.50 ± 0.06, 0.47 ± 0.07 and 0.43 ± 0.07, respectively. Measurements also indicated that large magnitudes of J_B could remain during these increases in buffering capacity, and values in excess of 0.05 A/m^2 were still observed in 25 mM buffer, the 'buffer cocktail' or unbuffered APW. It was also observed that V_m hyperpolarized by 42 ± 7 mV when a buffered solution at $pH^0 = 7.5$ replaced the unbuffered APW ($pH^0 \sim 5.5$).

Dependence of Conductance upon Illumination

The area-specific conductance of both acid and basic zones was found to decrease considerably upon the removal of illumination at all values of pH^0 studied. To examine this in more detail a series of quasi-

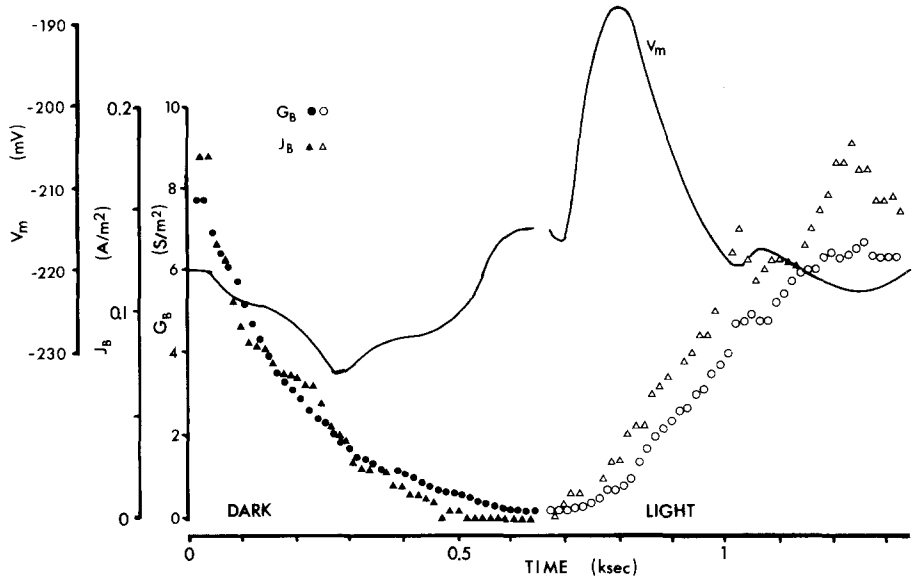

Fig. 6. The behavior of the electrical parameters measured in a basic zone as a function of time for a light/dark and a dark/light transition. The area-specific conductance G_B is indicated by (○) in the light and (●) in the dark. The current-density flowing into the basic zone J_B is indicated by (△) in the light and (▲) in the dark. The continuous line indicates the behavior of the membrane resting PD V_m which was taken from the chart recorder. The cell was bathed in stagnant APWB at pH^0 = 7.5. Illumination was removed at time = 0 and restored at time = 0.65 ksec

simultaneous measurements of J_B, G_B and V_m were made in APWB at pH^0 = 7.5 during light/dark transitions. The major part of the conductance change usually occurred in the first 300 to 500 sec after the removal of illumination. After this period a temporary 'plateau' was often observed (e.g. *see* Fig. 6 of Smith & Walker, 1983). Representative behavior for an acid zone is shown in Fig. 5. It was generally found that G reached a steady value in the dark (typically <0.2 S/m² for both acid and basic zones) only after 2 to 8 ksec had elapsed. Upon the restoration of illumination, G was always found to increase and usually returned to a final value similar to that measured previously during illumination. However, a temporary overshoot in G often occurred.

After long periods in the dark the large increase in G_A at high pH^0 no longer occurred (e.g. *see* Fig. 1) although a small increase in G_B at high pH^0 was sometimes evident. G_B was, however, always less than 0.5 S/m².

Dependence of Extracellular Current upon Illumination

From experiments on six cells it was found that the initial time dependence of J_B and G_B were similar (e.g. Fig. 6), although for some cells J_B decreased proportionally more slowly than G_B. The time taken for the magnitude of J_B to decrease below the resolution of the current probe (~1 mA/m²) was 450 to 600 sec (mean value = 500 ± 30(6) sec). This decay time is similar to that measured elsewhere (Walker & Smith, 1977; Lucas & Nuccitelli, 1980). Plots of G_B against J_B were made to further investigate their correlation during the initial few hundred seconds of light/dark/light transitions. An example is shown in Fig. 7. Their inter-relationship was found to be reasonably linear, with slopes ranging from 20 to 35 mV (mean = 26 ± 2(11) mV).

Dependence of V_m upon Illumination

The behavior of V_m after the removal of illumination was found to be complicated. In some cells a small, rapid depolarization of ~5 mV which lasted 50 to 100 sec occurred immediately after the removal of illumination. At longer times it was found that V_m could either depolarize by up to 15 mV, remain unchanged, or exhibit a temporary hyperpolarization of up to 30 mV. This hyperpolarization appears to be similar to that reported previously by Volkov (1973). When present, the hyperpolarization reached a maximum value some 250 to 300 sec after the removal of illumination (mean = 350 ± 60(10) sec), and at the time of this maximum J_B had dropped to 10 to 40% of its value in the light (mean = 30 ± 6(10)%). After 2 to 5 ksec had elapsed in darkness, V_m was found to be depolarized by 5 to 15 mV from its value in the light. When the magnitude of the transient change in V_m in the initial few hundred seconds (denoted by ΔV_m and measured with respect to the value of V_m after long periods of darkness), was studied as a function of the value of J_B measured immediately prior to the removal of illumination, a reasonably linear correlation was evident. Thus only small transients were apparent for cells that were not 'banding' (i.e. J_B = 0) and the largest hyperpolarizations occurred for cells with large external currents. The line of best fit to the data was given by

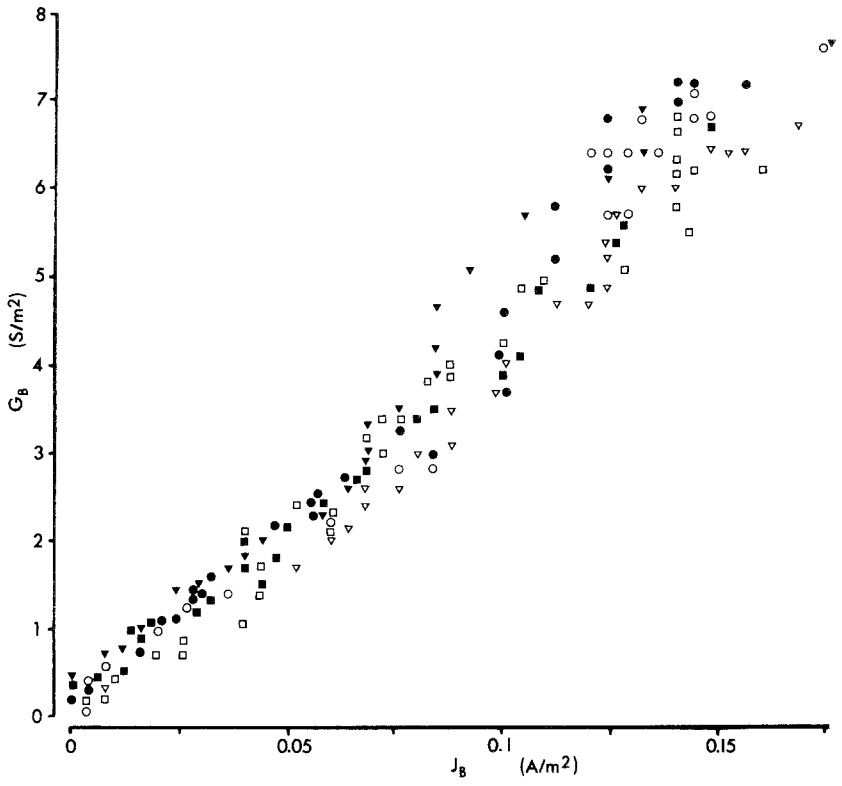

Fig. 7. The measured relationship between J_B, the inward current density in a basic zone, and G_B, its area-specific conductance, during three complete light/dark/light cycles. Data points are all from the same cell bathed in stagnant APWB at pH^0 = 7.5 and were measured in the order (●), (○), (▼), (▽), (■), (□). The open and solid symbols refer to measurements in the light and dark, respectively. The slope of the relationship (as judged by eye) was 22 mV

$$\Delta V_m = (0.7 \pm 3.2) \times 10^{-3} - (0.19 \pm 0.04) J_B$$

with a correlation coefficient of 0.75. The integral probability that the data came from an uncorrelated parent population was 2.5×10^{-4}.

When illumination was restored, a large transient depolarization in V_m was sometimes observed (*see* Fig. 6). This was then followed by a slow hyperpolarization of 5 to 15 mV.

Discussion

Dependence of G_A and G_B upon pH^0

An obvious and important feature of the results presented is that G_B was found to be significantly larger than G_A over a wide range of pH^0 (typically 5.5 to 10—*see* Fig. 3) for illuminated cells in a flowing, buffered (5 mM) solution. The electrical properties of the *Chara* membrane are thus shown to be spatially quite inhomogeneous under these conditions (as predicted by Bisson & Walker, 1980). This can significantly alter the interpretation of conductance measurements made via point current injection (*see* Smith, 1983*a*) because, in a wide range of commonly used experimental situations, there is not necessarily a single correct value for the membrane area-specific conductance. With increasingly high pH^0 (10 to 12) the membrane appeared to become increasingly homogeneous until at pH^0 11.7 the measured difference between G_A and G_B approached the experimental error.

The increase in G_A with increasing pH^0 described herein is similar to that reported previously for the overall conductance determined by point current injection into an unspecified region (Bisson & Walker, 1980, 1981, 1982*a*), although the possibility of different conductances in the acid and basic zones was not allowed for in the latter experiments. This neglect is presumably responsible for some of the differences at $pH^0 < 11$. The values obtained for pH^0 11 to 11.7 are similar, presumably because the electrical properties of the membrane at this very high pH^0 become effectively spatially uniform.

What is the pH at the Membrane Surface?

Our measurements show that the shapes of the dependences of G_A and G_B upon pH^0 are quite different. This appears to rule out the possibility that the difference in magnitude between G_B and G_A arises solely because the ratio of membrane area to cell surface area is higher in the basic zones, as might occur, for example, if the charasome density was different. One possible explanation of the different dependences is that the intrinsic behavior of the

membrane in the acid and basic regions is simply quite different in its dependence upon the local pH at the membrane surface (denoted by pH_A and pH_B, respectively). However, the observed capability of the basic regions to replace acid regions (Lucas & Dainty, 1977) under various conditions seems to invalidate this explanation. A simpler explanation is that the intrinsic behavior of the membrane is similar in the acid and basic zones, and that G_A and G_B differ simply because pH_A and pH_B differ.[4] In terms of this model, when $pH^0 = 7.5$ the high value of G_B would then be explained by the assumption that pH_B has a high value (i.e. a value similar to the value of pH_A at which the value of G_A would be similar to G_B). If the relationship between pH^0 and pH_A can be estimated, pH_B could then be derived (or vice versa).

Experiments with pH electrodes located immediately adjacent to the cell surface in stagnant media indicate a pH of 5.5 to 7 and a pH of 10 to 10.5 in the acid and basic zones, respectively (e.g. Lucas & Smith, 1973; Lucas, Ferrier & Dainty, 1977; Cathers, 1979; Lucas & Nuccitelli, 1980) for a bathing solution of $pH^0 \sim 7$ to 8. It is, however, virtually impossible to directly measure the pH which presumably influences the local value of G, i.e. the pH at the membrane surface inside the cell wall. Our conductance measurements show that G_B does not strongly depend upon pH^0 over the range 6 to 12 and this suggests that during illumination pH_B might remain at a value as high as 10. Such a high pH might be maintained partly as a consequence of the dissociation of the $CaCO_3$ crystals in and on the cell wall.[5] Figure 3 shows that G_A, however, does vary considerably with pH^0 and this suggests that pH_A might also vary considerably with pH^0. Again this will prove difficult to determine directly, but it does not appear unreasonable to suggest that pH_A might be acid with respect to pH^0 by perhaps up to one pH unit. Under some conditions it might even be possible to use the measured values of G as 'indicators' of the local pH at the membrane surface which is, at present, inaccessible to pH microelectrodes.

Dependence of 'Banding' upon pH^0

In terms of the proposed model, the measured decrease in G_B for $pH^0 < 6$ would indicate a decrease in pH_B associated with an inhibition of pH banding. This is supported by the observed decrease in J_B at $pH^0 < 6$ (*see* Fig. 4). It is interesting to note that measurements of pH_B (Lucas, 1979) and measurements of the external PD between the acid and basic regions, made in this laboratory, also suggest that pH banding ceases around pH^0 5 to 5.5 (*see also* Mercier et al., 1980). Our data also show that G_A and G_B become similar, and hence in terms of this model pH_B might approach pH_A and consequently pH banding might decreases, at pH^0 around 10.5 (*see* Fig. 3). This is again consistent with our measurements of J_B (*see* Fig. 4) and agrees with the value suggested by measurements of the external PD. There thus appears to be a reasonable correlation between the presence of high values of G_B (i.e. $>G_A$), the flow of current between the acid and basic zones, and the presence of pH banding. This view is further supported by the observed correlation between the magnitudes of G_B and J_B at $pH^0 = 7.5$ (*see* Results).

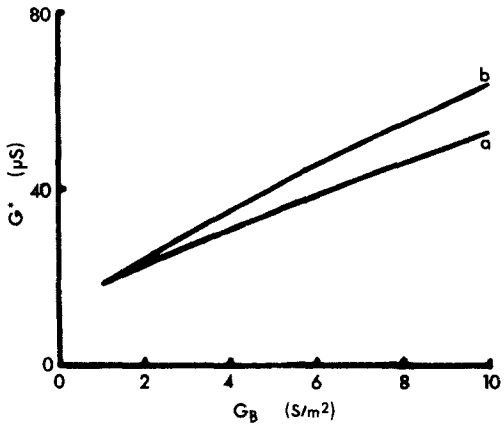

Fig. 8. Theoretical relationships (calculated as described in the Appendix) between the measured conductance G^* (at 1 Hz) and the area-specific conductance G_B of a basic zone into which current was injected at $x = 0$. The curve (*a*) shows the relationship for an abrupt transition between G_A and G_B at $x = 2$ mm. The relationship for a graded transition in G in between $x_1 = 1$ mm and $x_2 = 3$ mm was found to be indistinguishable from (*a*) on the scale shown. Curve (*b*) was calculated for a graded transition between $x_1 = 1$ mm and $x_2 = 5$ mm. Other parameters had the following values: $G_A = 1$ S/m², $L = 10$ mm, $d = 0.7$ mm, $N = 1000$, $C_A = C_B = 10$ mF/m², $\omega = 2\pi$ rads/sec, $r_T = 2.5$ MΩ/m

Efficacy of Buffering

Our results show that an increase of zwitterionic buffer concentration from 5 to 25 mM at $pH^0 = 7.5$ only slightly reduced the magnitudes of G_B and J_B, which suggests that pH_B, and also pH banding, are only slightly reduced. The inability of high concen-

[4] It is also possible that the pH of the cytoplasm will affect the conductance. However, in the absence of definite information this possibility will be neglected at present.

[5] Subsequent measurements with a recessed tip pH microelectrode have found that the pH at the cell surface in the basic regions in close proximity to crystals of $CaCO_3$ is ~10 for $pH^0 > 6$.

trations of buffer to totally inhibit pH banding might be due in part to restricted diffusion of the buffer to the membrane surface through the external unstirred layer and cell wall. The presence of $CaCO_3$ crystals will also decrease the efficacy of buffers.

It should also be noted that any observed decrease in banding with increasing buffer concentration need not be necessarily a consequence of the enhanced buffering capacity itself, because in many situations the ionic strength and conductivity of the bathing solution are also concomitantly increased. It is not impossible that the increased ionic strength will itself alter the membrane electrical properties. Also if the conductivity of the bathing solution is increased, this can reduce the local characteristic or cable length for current flow through the membrane. Thus the acid and basic zones might no longer be in their optimum locations and consequently 'banding' could be reduced.

Dependence of V_m upon the Current Between Acid and Basic Regions

As mentioned previously by Walker and Smith (1977), the passage of a current between the acid and basic zones necessitates some re-assessment of the interpretation of V_m in terms of ionic fluxes. Towards this end we now present a brief, simplified treatment which assumes that the electrical properties of the membrane alter abruptly at the junction of the acid and basic regions, and that the transverse current and membrane PD are both effectively homogeneous within each region. The equivalent circuit assumed for each individual zone is a common one whereby each ionic transport process is represented by a conductive element and an EMF connected in series (e.g. Spanswick, 1974; Smith & Beilby, 1983). In the absence of detailed information otherwise, each conductance element has been assumed linear over the small voltage range involved (the measured *I-V* curves suggest that this approximation is not too unreasonable—*see* Bisson & Walker, 1981).

When the net current density J_A through the acid zone is zero, its equilibrium PD will be related solely to those of the individual equivalent EMF's present in the acid zone, the contribution of each being proportional to the equivalent series conductance of that process. This resting PD for $J_A = 0$ is defined as U_A. Similar definitions apply to J_B and U_B. When cells are bathed in a conductive ionic solution the potential appears to differ only slightly (typically <5 mV) over the cell surface (e.g. Walker & Smith, 1977; Cathers, 1979). Unless there are unexpectedly large variations in the cytoplasmic potential, this implies that the actual membrane PD in the acid and basic zones (defined as V_A and V_B, respectively) is similar, and hence the difference $V_{AB} = V_A - V_B$ is small in comparison with V_A and V_B.

When a current J flows through each zone an additional PD will be produced equal to J/G (from Ohm's Law). Hence

$$V = U - J/G. \tag{3}$$

Assuming $V_{AB} \sim 0$ the following approximate relationship might be expected to hold

$$V_m = U_A - J_A/G_A = U_B - J_B/G_B$$
$$= (A_A G_A U_A + A_B G_B U_B)/(A_A G_A + A_B G_B). \tag{4}$$

Thus in a banding cell, the flow of current from the acid to the basic zones will, respectively, depolarize and hyperpolarize these zones from the PD that would be evident for an isolated zone in the absence of an external current. V_m will then be intermediate to U_A and U_B. The macroscopic separation of U_A and U_B in banding charophytes allows measurement of G_A, G_B, V_m, J_A and J_B, and then U_A and U_B can be calculated via Eq. (4).

From our quasi-simultaneous measurements of J_B and G_B on six cells when $J_B > 30$ mA/m^2 it was possible to calculate that the difference between V_m and U_B ($=J_B/G_B$) was $-17 \pm 2(14)$ mV. V_m was $-200 \pm 5(14)$ mV and U_B was $-183 \pm 5(14)$ mV. Interestingly this value of U_B is similar to that observed for V_m at $pH^0 = 10.5$ (*see* Fig. 4), when the behavior of the whole cell presumably resembles that of a basic zone.

With the present apparatus it was not possible to measure G_A and J_A simultaneously and accurately for the cells studied with the vibrating probe. This was because cells with large basic regions were chosen to give strong extracellular currents, and in such cells the conductance of the basic zones made a sufficiently large contribution to the conductance measured in the acid zones to make accurate estimation of G_A difficult. If G_A for these cells was similar to that measured for other cells (~1.1 S/m^2, *see* Fig. 3) then $V_m - U_A$ was $70 \pm 15(14)$ mV and U_A was calculated to be $-265 \pm 20(14)$ mV. It must be stressed that this value for U_A is derived from averaged parameters for the acid zones measured on different cells. As measurements indicate that the surface pH, J_A and G_A can vary within each acid region (e.g. Lucas & Nuccitelli, 1980; Chilcott et al., 1983; Ogata, 1983; Ogata et al., 1983), it is quite possible that U_A in some positions could be significantly hyperpolarized beyond the average value quoted here.

It is encouraging that measurements upon *Chara* cells suspended in air, when both J_A and J_B are quite small (~5 to 7 mA/m^2) and hence V_m measured in the acid and basic zones should be approximately equal to U_A and U_B, respectively, reveal a maximum difference in V_m (i.e. $U_A - U_B$) of approx. −(50 to 70) mV (Ogata, 1983; Ogata et al., 1983). This is slightly less than the value of $(U_A - U_B) = -87 \pm 15$ mV reported herein for cells bathed in APWB, but is sufficiently close to suggest that our approach is reasonably valid. However, it will be necessary to wait until J and G can be measured simultaneously and accurately on the same cell bathed in APW as a function of longitudinal distance, before the detailed behavior of U_A and U_B under various conditions can be determined.

The average value of U_A quoted above is not inconsistent with the membrane properties of the acid zones being dominated by an electrogenic proton pump (e.g. Spanswick, 1972, 1974), and the average value of U_A is close to what is thought to be the most hyperpolarized value of V_m possible for a $2H^+$/ATPase (*see* Walker & Smith, 1975; Reid & Walker, 1983). However, some individual measured values for U_A considerably exceeded this limit and it would also be necessary to check that the electrical properties of the acid zones are indeed dominated by the pump before firm conclusions can be drawn about the pump stoichiometry. Towards this end a more accurate method of measuring U_A is under development. The values of U_B are consistent with the presence of a passive proton (or hydroxyl) uniport in the basic zones (*see* Bisson & Walker, 1980, 1981, 1982*a*,*b*).

Removal of Illumination

A decrease in the overall conductance of charophytes placed in the dark has often been reported previously (e.g. Hope, 1965; Nishizaki, 1968; Spanswick, 1972; Volkov, 1973; Walker & Smith, 1977). Our experiments show that, upon the removal of illumination, G_A (*see* Fig. 5) and G_B (*see* Fig. 6) both initially decrease at approximately the same rate as J_B (and presumably also J_A). This suggests that the driving force on the current flow, i.e. $U_A - U_B$, is not changing rapidly during the initial 500 sec. Plots of G_B against J_B, measured after the removal of illumination, showed that the ratio J_B/G_B, and hence $(U_B - V_m)$, is essentially constant for the initial few hundred seconds (*see* Fig. 7). The variation in U_B during this period is therefore similar to the variation of V_m. Thus U_B does not vary by a large amount and this suggests that pH_B might remain reasonably high during the initial period of darkness. This is in agreement with the measured behavior of the pH at the cell surface in the basic zones (e.g. Lucas et al., 1977; Ferrier & Lucas, 1979).[6] The $CaCO_3$ crystals on the cells used by us would also help to maintain this pH at a high value. The measured variations in G_A and G_B indicate that their values are not dependent solely upon the pH at the membrane surface, but also upon some other 'signal' which varies with the level of illumination.

At long times after the removal of illumination, J_A and J_B become very small. Then $(U_A - U_B)$ must be small and consequently $U_B \sim U_A \sim V_m$. We have no further information on their behavior at this time because J_A and J_B are then beyond the present limit of resolution of our current measurements. At this stage the conductance of the cell is fairly homogeneous. There then occurs a slow decrease in G_A and G_B over several ksec until a limiting value, which is typically <0.2 S/m^2, is reached.

As described in the Results, the behavior of V_m is complicated during light/dark transitions. This is presumably a consequence of V_m being determined by at least four parameters (G_A, G_B, U_A, U_B—*see* Eq. 4) which can vary individually with changes in illumination. The slow and small depolarization observed for cells that were not 'banding' (i.e. $J_B \sim 0$) is presumably a consequence of the operation of various long-term control mechanisms (e.g. Hansen, 1978). For 'banding' cells the behavior of V_m will depend upon the relative rates at which U_A, U_B, G_A and G_B vary. The transient change in V_m, which was always observed for banding cells with a magnitude correlated with that of J_B, could be a consequence of G_A decreasing less rapidly than G_B and/or U_A or U_B being transiently hyperpolarized. Again, better measurements of U_A will be required to distinguish between these possibilities. Our results do, however, help to explain the variable nature of the results of different experimenters investigating the behavior of V_m during light/dark transitions. This is because the degree of 'banding' appears to be a possible important 'hidden parameter.' Indeed for a strongly banding cell it might be possible for G_B to decrease much more rapidly than G_A and then it would be possible for V_m to transiently approach U_A. This could be what is happening during the very large transient hyperpolarizations observed by Lucas (1982). The complicated behavior of V_m during dark/light transitions is presumably also explicable in terms of the above approach.

[6] Subsequent measurements with a recessed tip pH microelectrode found that the pH at the cell surface in the basic regions typically took 3 to 4 ksec to reach its new value after the removal of illumination.

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Appendix

Effects of Graded Transitions in Conductance Between Acid and Basic Zones

The electrical properties of a radially symmetric, cylindrical cell of length L and diameter d are now considered. AC current at an angular frequency ω is injected at $x = 0$ where the resultant AC change in the membrane PD is measured. The membrane admittance Y is assumed to vary longitudinally along the cell (i.e. with varying x) in the following fashion:

$$Y(x) = Y_A \quad \text{for } x_2 < x < L \tag{A1}$$

$$Y(x) = Y_B \quad \text{for } 0 < x < x_1 \tag{A2}$$

$$Y(x) = Y_A + (Y_B - Y_A)(x_2 - x)/(x_2 - x_1) \quad \text{for } x_1 < x < x_2. \tag{A3}$$

This corresponds to the injection of current into a basic region extending from 0 to x_1 with area-specific capacitance and conductance given by C_B and G_B, respectively. Thus $Y_B = G_B + j\omega C_B$ where $j = (-1)^{1/2}$. An acid region with area-specific capacitance C_A and conductance G_A extends from x_2 to L and here $Y_A = G_A + j\omega C_A$. The region intermediate to the acid and basic zones is assumed to possess parameters that are linearly graded between these two extremes.

If the membrane between $x = 0$ and $x = L$ is assumed to be composed of N annular elements, each of width $\Delta x = L/N$, then the admittance Y_i of the ith element located at position x is given by

$$Y_i = \pi d \Delta x Y(x). \tag{A4}$$

The longitudinal resistances per unit length of the external and internal media are denoted by r_o and r_i, respectively. The total longitudinal resistance r_T for each annular element is thus

$$r_T = (r_o + r_i)\,\Delta x. \tag{A5}$$

A recurrence relationship for Y_i^*, the measured admittance at the ith annular element due to the elements with greater or equal i, is then

$$Y_i^* = Y_i + 1/(r_T + 1/Y_{i+1}^*) \tag{A6}$$

where $Y_N^* = Y_N$. Equation (A6) can then be solved iteratively to yield Y_1^*, the admittance of the cell as measured at $x = 0$.

Some examples of the theoretical effects of graded transitions in conductance are shown in Fig. 8. Generally it was found that the presence of graded rather than abrupt transitions would make little difference to the interpretation of the experimental data.