An experimental approach to pH measurement in the intercellular free space of higher plant tissues

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Summary. An experimental approach to the measurement of pH in the free space of plant tissues was made, using both metal minielectrodes and H^+-sensitive resin glass microelectrodes inserted in small environments artificially created inside the tissue. Experiments were carried out on the parenchyma of higher plants (corn, potato, squash, *Senecio herraianus*), monitoring pH changes in the free space as a function of the pH of the external medium. The pH in the free space has been found to be different from that of the surrounding solution and its variation in time much slower than would be predicted by assuming that simple diffusion of H^+ ions took place. The results agree with the hypothesis that the free space behaves as a compartment distinct from both the cytosol and the surrounding medium.

Key words. pH; microelectrodes; free space; plants; parenchyma; apoplast.

Transport regulation and cell growth in higher plant tissues are influenced by pH levels in the intercellular free space[1]. H^+ concentration outside the cell membrane is mainly affected by the activity of a proton-extruding ATPase[2,3], which influences the movement of most solutes across the cell membrane[4,5]. Knowledge of pH profiles in the free space would therefore be useful for better investigations of elongation growth, and would help to discriminate between different pathways (apoplast or symplast) for salt and nutrient translocation in plants. Moreover, if the techniques of pH measurement in the free space could also be used to record the concentration of other ions, a better understanding of translocation phenomena could be achieved.

However, while recent powerful techniques allow us to record internal pH even in intact cells and tissues (weak acid/base distribution, NMR, optical probes)[6-8], little effort has been devoted to investigating pH values in the free space. Measurements of pH in the medium surrounding portions of tissue or plant organs are in fact often misleading, owing to the tissue morphology and the existence of Donnan barriers and unstirred layers around the cells.

Most of the recent techniques for pH measurement in microenvironments appear to be applicable in the free space as well. For some of them, however, there are serious drawbacks; in ^{31}P-NMR, for instance, determination of the free space pH is difficult because of the very similar value of the pH in the vacuole. Weak acids and optical probes have to be injected artificially into the free space, from which they would migrate into other compartments.

The use of H^+ sensitive microelectrodes appears to be promising for many reasons. It allows direct pH determinations; the same microelectrodes can be used also for intracellular records and, with minor additional features, for electric potential measurements; the same technique also applies to concentration measurements of other ionic species (by specific exchange resins).

Materials and methods

A) Microelectrodes

In our experiments two kind of electrodes were employed: platinum wire minielectrodes and H^+-sensitive resin glass microelectrodes.

Metal minielectrodes. The electrode utilized was the commercially available model MEPH-1 pH Beetrode (WP-Instruments) with a cylindrical Pt tip of ca 2 mm length and 100 µm diameter. The electrode, kept dipped in distilled water, was calibrated before each experiment and did not show maintenance problems. A typical calibration curve is reported in figure 1.

Glass microelectrodes. These electrodes were fabricated using a technique developed from that reported by Kurkdjian and Barbier-Brygoo[9]. Glass capillaries (Pyrex, Clark Electro Medical Instruments) of 1.5 mm diameter were cut into 5-cm segments the ends of which were rounded in a Bunsen flame. They were then kept in 50% HNO_3 for 1 h, washed, and boiled for 1 h in distilled water to remove organic residues left on the glass during handling, and finally dried in an oven at 30 °C overnight in covered petri dishes. Micropipettes were manufactured by using a puller M1 (Industrial Science Associates, Inc.) and kept in a covered glass vessel at 200 °C for 15 min in the presence of 20 µl TMSDMA (N-N-dimetyl-trimetyl sililamine, Fluka AG). The micropipettes can be stored for up to ten days provided they are kept in a desiccator for protection against dust and humidity. Electrodes fabricated in this way show an external tip diameter of 1–1.5 µm (internal 0.5 µm) and, once filled, have an impedance around 10^{12} Ohm. To reduce the electrical noise and the response delay the micropipette tips were broken under a microscope, to obtain an external diameter of 5–15 µm and a resistance of less then 10^{11} Ohm. All experiments were performed with these modified electrodes.

The selective resin employed (cocktail) was prepared with bidistilled TDDA (tri-dodecyl-amine, Merck n. 821-160 90) (ligand), nitrophenyl-octyl-ether and sodium-tetra-phenyl-borate (Fluka n. 72020), left overnight in a CO_2-saturated atmosphere and then stored in the dark under CO_2 in a glass flask sealed with parafilm and a screw plug. In some experiments we used a commercially available 'cocktail' (Fluka n. 82500) highly selective for H^+. There was no detectable difference between the two cocktails.

The cocktail was injected into the electrode tip by a Hamilton microsyringe (5 µl) in an amount of ca 1–2 µl. Smaller amounts produce better electrical characteristics but decrease electrode life. After resin injection, the electrode barrel was filled by a standard syringe with a buffer solution composed of KH_2PO_4 40 mM and NaCl 13 mM, adjusted to pH 7 with NaOH 0.1 N.

Before in vivo measurements, electrodes were calibrated at different pH values in a buffer solution composed of mannitol 100 mM, KCl 3 mM, $CaSO_4$ 0.3 mM, MES buffer 5 mM, varying pH between 4 and 9 by addition of NaOH or HCl 0.1 N. Electrodes, the mean life of which is about 2–3 days, were stored in the dark with the tip dipped in a buffer solution of TRIS 25 mM and HCl at pH 7.

B) Experimental set up

The measurement electrode previously described was connected through an Ag-AgCl wire to one of the two input probes of a differential high-impedance amplifier (Dual Channel Differential Electrometer FD-223, WP-Instruments); its second input was connected to a calomel reference electrode (Radiometer mod. K4040) or to a KCl-filled microelectrode. Measurements could be read in mV on the amplifier digital display or directly on a pH scale once the calibration slope had been determined. The amplifier output was connected to an optical memory oscilloscope (Tektronix 5103 N) and/or a chart recorder (Omniscribe, Houston Instruments).

External pH (tissue perfusion solution) was monitored by a combined electrode (GK2401-C, Radiometer) connected to a pH-meter (pHM82, Radiometer Copenhagen).

Biological samples were placed in a rectangular lucite chamber perfused with an aerated solution recirculated by a peristaltic pump (2115 Multiperpex pump, LKB). The combined electrode, measuring the pH of the external solution, and the reference electrode for the free space pH measurements, were both immersed in the bathing medium. Insertion of the microelectrode inside the samples was performed under a Wild M3 stereomicroscope (Wild-Heerbrugg) using a Leitz micromanipulator.

The whole system formed by recording chamber, microelectrodes, microscope and micromanipulator was surrounded by a cage-screen to minimize electrical noise.

C) Electrode calibration

Before each experiment, resin microelectrodes or Pt minielectrodes were calibrated in a standard perfusion solution by varying the pH from 4 to 8 by addition of NaOH or HCl, and recording both external pH and, in parallel, the response of the measuring electrode. A calibration curve was obtained by fitting the observed values to a linear regression (fig. 1). Calibration was repeated at the end of the experiments and the data were discarded when the initial and final calibration curves differed by more than 5%.

D) Biological materials and their preparation

Parenchyma tissues of potato (tuber), squash (fruit), corn (root) and *Senecio herraianus* (leaf) were used. These materials were chosen because of the relative abundance of parenchyma in them: the size of the tissue samples and the depth of the holes were adapted to the characteristics of the different materials so as to obtain the maximal distance between holes and vascular bundles.

Potato. Into rectangular blocks of tissue (20 × 20 × 30 mm) holes 10 mm deep, 1.5 mm in diameter were bored by mean of a Pasteur pipette, 2–5 mm apart and 5 mm from the surface of contact with the bathing solution. Before pH measurements, the block of tissue was washed with distilled water to remove cellular debris produced by the cuts, and kept aerated in distilled water for 1 h. Holes were then filled by a syringe with a solution of 100 mM mannitol, 3 mM KCl and 0.3 mM $CaSO_4$. The tissue was finally immersed into the perfusion solution composed of 100 mM mannitol, 3 mM KCl, 0.3 mM $CaSO_4$ and 5 mM MES buffer, pH 6.5. Perfusion medium (agitated by a magnetic stirrer) and tissue were both located inside a lucite chamber (dimensions: 120 × 50 × 28 mm).

In some experiments a pH gradient between the opposite sides of the tissue block was created by sealing the contact surfaces between the tissue and the lateral faces of the chamber with silicon grease and adjusting the pH of the two compartments to the desired value.

Squash. From the parenchyma of squash fruit a cylindrical block was cut (dimensions: height 20 mm, base diameter 8 mm). A hole was dug in the center of the cylinder along its longitudinal axis (diameter 1.5 mm, depth 10 mm) by mean of a Pasteur pipette. Sample location and perfusion solutions were the same as for potato tissue.

Corn. Zea mays hybrid seeds (cultivar XL 72 A) were germinated in the dark for three days on filter paper soaked in 0.5 mM $CaSO_4$. Seeds with a main root 70–80 mm long were chosen and blotted. From the root the central cylinder was removed by gentle twisting and traction, thus obtaining a tubular structure consisting of the cortical parenchyma and the epidermis. This was cut at 20 mm from the apical end. The internal lumen had a

diameter of 0.6 mm, and the root external diameter was 1.5–2.0 mm. The root was mounted vertically on a silicon base located on the bottom of the recording chamber.

Senecio herraianus. The leaf, spherical in shape with a diameter of about 8 mm, was peeled and a hole 1 mm wide and 5 mm deep was bored by mean of a Pasteur pipette. Since vascular bundles are peripheral and symmetrical, hole orientation is not relevant. The leaf was then immersed for 15 min into an aerated solution of 0.15 mM $CaSO_4$, 0.25 mM KCl and 50 mM mannitol. The same solution was placed inside the hole. All the experimental arrangements were the same as for potato tissue. A difference from the measurements on the other (non-photosynthetic) materials was that in the case of *Senecio* leaves, where transport also occurs at the level of chlorophyll parenchyma, light conditions were taken into account.

All the experiments reported in the following section were replicated at least three times without any significant variation.

Results and discussion

A) Characteristics of microelectrodes

Figure 1 shows the calibration curves of the three kinds of electrodes described in 'Materials and methods' (Pt metal, H^+ sensitive resin cocktail or commercial). Pt electrodes exhibit a remarkable stability and linearity in the pH range of our experiments (4–8) with a Nernstian slope of 55–57 mV/pH unit at room temperature. The chemical composition of the medium does not influence the slope but, to a slight degree, its intercept on the ordinate axis. The response of this kind of electrode is very rapid (less than a second) and its low impedance also makes it possible to connect it to a standard pH meter. Resin microelectrodes show good linearity in the pH range 5–8 with slight deviations in the regions 4–5 and 8–9. Nernstian slopes are between 60 and 65 mV/pH unit (T = 25 °C) and remain constant for several hours. A drift in electric potential observable in some cases is probably due to partial occlusion of the electrode tip. Calibration is therefore recommended during long-lasting experiments. High impedance (10^{11}–10^{12} Ohm) makes the response for resin electrodes slower than that of metal electrodes, but the response time never exceeded 10 s.

B) pH measurements in parenchymal tissue

Plant materials rich in extended areas of non-vascular parenchyma were chosen for a first approach to pH measurements in the free space. Small holes were bored in the tissue and, once washed out and freed from cellular residues, they were left to equilibrate with the intercellular free space of adjacent cells. pH was monitored by inserting an electrode into the holes.

A preliminary investigation was carried out in order to determine the time for equilibration inside the hole as a function of its localization in the tissue and its distance from the perfusion medium. Figure 2 shows a pH time course in potato tissue prepared as described in 'Methods'. Approximately 20 min were required to reach a stable pH value lasting more than one hour. The location of the hole in the tissue did not influence the equilibration time. The initial pH value was lower than at equilibrium, probably due to the relatively low pH of the washing solution.

In some experiments the pH of the external medium was changed in order to study possible pH variations in the free space as a response to H^+ diffusion between medium and holes. In these experiments the distance between the

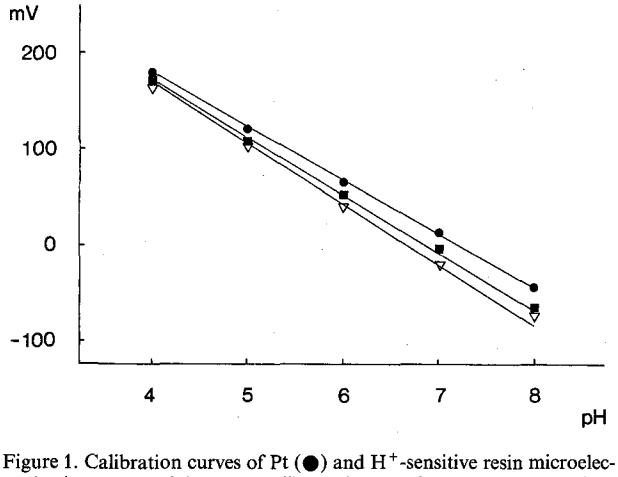

Figure 1. Calibration curves of Pt (●) and H^+-sensitive resin microelectrodes (■ commercial, ▽ cocktail). Testing solution: 100 mM mannitol, 3 mM KCl, 0.3 mM $CaSO_4$, 5 mM MES buffer. pH adjusted between 4 and 8 with 0.1 N NaOH or HCl. Linear regression parameters: Pt, slope = −55.1 mV/pH unit, r = 0.99; commercial resin, slope = −61.2 mV/pH unit, r = 0.99; 'cocktail' resin, slope = −63.0 mV/pH unit, r = 0.99.

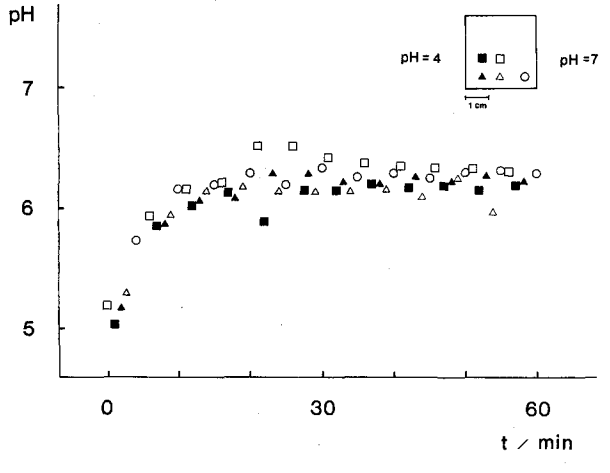

Figure 2. pH time-course in potato tissue free space (Pt electrode); symbols refer to measurements performed inside the various holes dug in the tissue block; positions of holes and pH on both sides of the sample is shown in the insert. Bathing solution composition is described in 'Materials and methods'.

holes and the tissue boundary facing the solution was reduced to less than 3 mm. At such distances and in the presence of pH gradients higher than 1 unit, the diffusion of H^+ ions into the free space according to Fick's laws should produce an appreciable pH change after few min. In fact, no variation in free space pH was detected for some hours. When the external pH was decreased from 7.5 to 4.5 after 1 h of tissue pre-equilibration, the pH inside the hole remained stable at 6.4 and no effect of external pH change was detected during the following 90 min (data not shown).

Also in the squash fruit parenchyma the pH inside the holes reached a constant value stable for several hours; the time constant of transient pH change was of the same order of magnitude as was observed in potato parenchyma (less than 1 h).

As in the previous case, we interpret this transient change as being due to an exchange between the solution injected into the hole and that in the free space. The time course of pH change inside the holes during equilibration and its final value (7.8) were not influenced by lowering the external pH from 7 to 4.5 and then raising it again from 4.5 to 6.5 (fig. 3).

The experiments on maize roots (the central cylinder of which had been removed as described in 'Methods') aimed at studying transport between the external environment and the root lumen. In this case the two compartments were separated only by a few tens of cell layers. A stable value of the pH in the lumen was reached (around 7.0) and variations of external pH (up to 2 units) did not modify this value during more than 1 h. As shown in figure 4, a resin electrode, kept for more than 4 h inside the lumen of a maize root, recorded a pH value that was essentially constant and independent of pH values in the perfusion medium ranging from 7 (beginning of the experiment) to 4.5 (end).

Figure 5 illustrates the time course of pH change inside a hole in the parenchyma of a *Senecio herraianus* leaf,

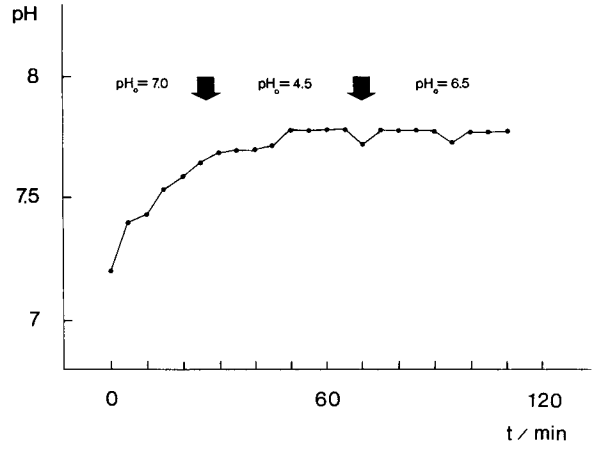

Figure 3. pH time-course in squash parenchyma free space (resin microelectrode); pH of bathing solution was varied during the experiment, as indicated in the figure. Solution composition is described in Methods.

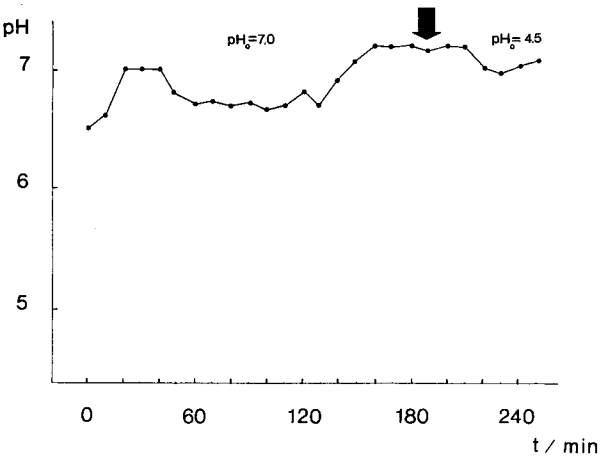

Figure 4. pH time-course in maize root parenchyma free space (resin microelectrode); pH of bathing solution was varied during the experiment as indicated in the figure. Solution composition is described in Methods.

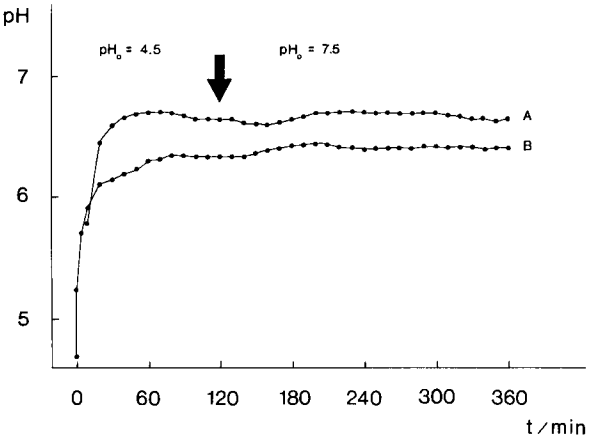

Figure 5. pH time-course in chlorophyll parenchyma free space of *Senecio herraianus* leaf (Pt electrode); the experiment was carried out in the light (A) and in the dark (B) and the pH of the bathing solution was varied as indicated in the figure. Solution composition is described in Methods.

both in the light and in the dark. A constant value ranging between 6.7 and 7.0 was reached in about 30 min. The pH was then stable for periods up to 6 h and it was not influenced even after 4 h by variation of the external pH from 4.5 to 7.5 and vice versa. In figure 5A the pH of the external medium surrounding a leaf sample in the light had been kept for 2 h at 4.5 and then raised at 7.5 for a period of 4 h without any detectable pH variation inside the hole.

The results of experiments in the dark are presented in figure 5B (other conditions unchanged). The transition from light to dark did not affect either the equilibration time or the stability of the final pH value even when the external pH was varied by three units; however, the pH value at equilibrium was 0.3 units lower than in the light, probably because of the higher CO_2 level due to the absence of photosynthesis.

The presence of K^+ in the perfusion medium did not influence the phenomena previously described. With a potassium concentration varying from 0.1 to 10 mM, the pH in the free space behaved as in the previous experiments and changes in external pH were not able to modify the equilibrium pH values (data not shown).

It should be noticed that the *Senecio herraianus* leaves had been deprived of the epidermis, and that the tissue morphology had made it possible to operate in regions of the sample well separated from vascularized parts.

Our data show that it is possible to monitor pH in the free space and that its value can be considerably different from that of the external medium. It is usually assumed that external pH is very close to that in the free space [10, 11]. On the contrary the present results indicate that, even when a certain equilibrium between the external fluid and the free space has been reached, a difference is likely to persist. Furthermore, even important changes of external pH do not seem to influence the free space pH significantly, which suggests that H^+ movement into the tissue cannot be explained by simple diffusion between free space and external environment.

In order to verify this hypothesis, we have applied Fick's second law of diffusion for H^+ ions along the gradient between the external and the free space compartments, making the assumption of that ion diffusion only takes place across the free space region. The geometry of the system has been inferred from the typical parenchyma morphology, assuming the free space volume to be about 10% of the total. Diffusion models and physical constants have been taken from Yam et al. [12] and Crank [13]. According to these theoretical predictions, significant pH variations should have been detected, within the parameter values of our experiments, after less than one hour (table). Actually no appreciable change was recorded even after several hours. This suggests the presence of mechanisms of active pH regulation operating by ion exchange at plasmalemma level and able to mask diffusion phenomena.

Discussion is still open about the relationships between H^+ pump activity, pH in the free space and elongation growth in plant tissues [14–16]. Our results suggest that the determination of pH in the free space requires more theoretical and experimental work. Further investigations therefore seem necessary to correlate physico-chemical conditions in the free space and important physiological phenomena such as elongation growth or apoplast transport.

H^+ ion diffusion in a dilute aqueous solution as a function of time (h) and distance from the source (x). T = 20 °C. H^+ concentration C/C_o has been obtained from the Fick's second law as $C = C_o$ erf c $(x/2\sqrt{Dt})$ where $D = 9 \times 10^{-5}$ cm²/s.

X (cm)	1	2	3	4	5	6 h
0.1	0.91	0.92	0.94	0.95	0.96	0.98
0.2	0.80	0.87	0.88	0.90	0.91	0.96
0.3	0.72	0.80	0.83	0.86	0.87	0.88
0.4	0.62	0.70	0.77	0.80	0.83	0.85
0.5	0.55	0.67	0.72	0.77	0.78	0.80
0.6	0.47	0.64	0.67	0.70	0.74	0.76
0.7	0.39	0.53	0.62	0.67	0.70	0.72
0.8	0.32	0.46	0.57	0.62	0.67	0.69
0.9	0.24	0.43	0.52	0.58	0.62	0.65
1.0	0.21	0.37	0.47	0.54	0.58	0.62

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