Plant Physiol. (1977) 59, 664-666

Measurement of the Cytoplasmic pH in *Nitella translucens*

COMPARISON OF VALUES OBTAINED BY MICROELECTRODE AND WEAK ACID METHODS[1]

Received for publication August 27, 1976 and in revised form November 15, 1976

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ABSTRACT

A comparison has been made between the use of two types of pH microelectrode and the weak acid method for determining the cytoplasmic pH of *Nitella translucens* at an external pH of 6. There was good agreement between the value obtained with glass pH microelectrodes (7.54 ± 0.15 SE) and that obtained using the weak acid 5,5-dimethyloxazolidine-2,4-dione (7.42 ± 0.07 SE). Plastic-insulated antimony microelectrodes gave a significantly lower value (6.74 ± 0.15 SE) possibly due to disruption of the insulation by the cell wall. The addition of 1 mM NaN_3 rapidly reduced the pH recorded by the glass pH microelectrodes to about 5.3. A smaller change was observed using the weak acid method. The relevance of this observation to recent work on indoleacetic acid transport is discussed.

An estimate of the cytoplasmic pH of plant cells has recently become of great interest to those concerned with ion transport since it can be used to test certain aspects of hypotheses for anion-OH^- countertransport (4, 6) and the hydrogen ion pump at the plasmalemma (7). Two methods have been used extensively in animal physiology: pH microelectrodes and the distribution of weak acids or bases which are highly permeable in the associated form (10). The only microelectrode measurements of cytoplasmic pH in plant cells appear to be those of Davis (3) on *Phaeoceros laevis*. Walker and Smith (12) have recently used DMO,[2] a weak acid, to estimate the cytoplasmic pH of *Chara corallina*.

In this paper, we present the results of determinations of the cytoplasmic pH of *Nitella translucens* by both methods. We also use NaN_3 to show that changes in cytoplasmic pH may be a side effect of certain inhibitors and to provide a further test of the two methods.

MATERIALS AND METHODS

N. translucens was cultured in the laboratory (7). The experimental solution was an artificial pond water buffered at pH 6 (APW6) (7) to which 0.4 mM KCl was added. The experiments were performed at 20 C and the light intensity was 10 w m^{-2}.

pH Microelectrodes. Two types of pH microelectrode were used. Glass pH microelectrodes were obtained from Microelectrodes Inc. (Grenier Industrial Village, Londonderry, N.H.). The pH-sensitive region is about 50 μm in length and the tip is sealed. They were not designed for insertion through plant cell walls and therefore must be used with extreme caution to avoid breakage. We now prepuncture the cell wall with an empty perfusion pipette having a tip diameter of about 10 μm and attempt to insert the microelectrode at the same point.

Insertion of the 50-μm long tip of the microelectrode in the 10-μm thick layer of cytoplasm may be achieved in two ways. If the microelectrode is inserted very slowly, or in small steps, the cytoplasm may be carried inward without the tip of the microelectrode penetrating the tonoplast. Alternatively, the microelectrode may be inserted more rapidly until the junction with the borosilicate glass is just in the cytoplasm and the tip is in the vacuole. The cytoplasm will then stream around and completely envelop the electrode tip. However, this may take 1 to 2 hr.

Plastic-insulated antimony microelectrodes were obtained from Transidyne General Corp. (Ann Arbor, Mich.). Since the exposed tip length is nominally 5 μm, it should be easier to insert the tip in the cytoplasm. However, the cell wall presents a major problem since it frequently appears to push back the plastic insulation and gives low apparent values for the cytoplasmic pH.

The voltage generated by the pH microelectrode was measured against an ordinary microelectrode inserted in the vacuole using a Keithley 603 differential amplifier. The values obtained were corrected for the potential across the tonoplast which was measured using two microelectrodes as described previously (8). This avoided the problem of "sealing" (11) which occurs if a cytoplasmic microelectrode is used as a reference for the pH microelectrode. Sealing occurs when a new cell wall is formed around the electrode tip and the electrode is effectively excluded from the cell. This is accompanied by a precipitate change in the potential recorded by the microelectrode. Use of the vacuole microelectrode as a reference avoids repeated reinsertion of a cytoplasmic microelectrode and the accompanying disturbance of the cell.

Sealing of the pH microelectrode may also occur and gives rise to low apparent values for the cytoplasmic pH. This happened much less frequently than for an ordinary microelectrode, possibly due to the larger area of the tip.

At the end of each experiment, the electrodes were removed from the cell and calibrated in buffers at pH 6 and 8 containing concentrations of the major ions approximating those of the cell. The main purpose of this is to obtain a tip potential on the reference microelectrode equal to the value when it is in the cell.

Weak Acid Method. The procedure was basically similar to that described by Walker and Smith (12). DMO labeled with ^{14}C was obtained from New England Nuclear as a solution in ethyl acetate. The solvent was evaporated and the DMO dissolved in 20 ml APW6 + 0.4 mM KCl to give a final concentration of about 30 μM. Cells were separated, measured, and stored for 16 to 18 hr in the dark. They were then pretreated for 3 hr in the light in CO_2-free APW6 + 0.4 mM KCl before immersion in the labeled solution. After the appropriate time, they were removed singly and rinsed for 2 to 3 sec to remove the radioactivity from the exterior. One end was then cut off and a measured sample of

[1] This research was supported by National Science Foundation Grants GB 28124X and BMS-75-15277.

[2] Abbreviations: DMO: 5,5-dimethyloxazolidine-2,4-dione; NPA: N-1-napthylphthalamic acid; APW: artificial pond water.

clear vacuolar sap obtained. This was placed in one vial of scintillation fluid and the remainder of the cell in another. The degree of quenching in each sample was determined by recounting after the addition of 100 μl of solution containing 40,000 cpm ^{14}C-DMO. Assuming that the vacuole occupies 95% of the cell volume (12), and knowing the total radioactivity in the cell, the amount of radioactivity in the cytoplasm may be calculated. Samples of the external solution were also counted and the cytoplasmic pH was calculated from the equation

$$pH_c = pK + \log [(C_c/C_o)(1 + 10^{(pH_o - pK)}) - 1]$$

using a value of 6.39 for pK (1). C_c and C_o are the DMO activities per unit volume of cytoplasm and external solution, respectively.

Although the cytoplasm occupies only 5% of the total cell volume, the amount of DMO accumulated relative to the vacuole is large enough to allow reasonable estimation. In a typical experiment, involving eight cells, the vacuole contained an average of 4,680 cpm and the cytoplasm contained 2,683 cpm. The value of C_c/C_o was 9.14.

Results are expressed in the form: mean ± SE (number of cells).

RESULTS

Microelectrode Measurements. The mean value for the cytoplasmic pH recorded during successful impalements with glass pH microelectrodes was 7.54 ± 0.15 (8). Lack of success was usually due to breakage of the electrode tip which rendered it insensitive to changes in pH. It should be noted that incomplete insertion of the electrode into the cytoplasm, insertion into the vacuole (pH about 5.5), or sealing of the microelectrode all give apparent values for the pH that are lower than 6. Thus, it was difficult to account for values in the range 6 to 8 other than as readings corresponding to the pH of the cytoplasm. This was supported by visual observation of the envelopment of the microelectrode tip by cytoplasm when these readings were obtained. Although interpretation of individual values must be made critically, we are confident that this technique provides a reliable measure of the cytoplasmic pH.

With the plastic-insulated microelectrodes, on the other hand, the results are more ambiguous. The value obtained was 6.74 ± 0.15 (6) which was 0.8 units lower than the value obtained using the glass microelectrodes. Even when it has not been possible to make a "successful" insertion, the microelectrode will still respond normally to the calibration buffers and it is assumed that the cell wall has pushed back the insulation, exposing a greater length of the tip. Under these circumstances, the sensitive region outside the cytoplasm appears to control the observed pH since only a transient rise is recorded on insertion. Even when high values for the pH are observed, it is impossible to be certain that the insulation beyond the plasmalemma is completely intact. The lower values observed with this type of electrode may be due to exposure of a small fraction of the sensitive tip to the exterior of the cell. This would result in a lower apparent pH due to both the potential across the plasmalemma and the lower external pH.

Weak Acid Method. The value obtained for the cytoplasmic pH using DMO was 7.42 ± 0.07 (7) which was close to that obtained with glass microelectrodes and was obtained after immersion of the cells in the radioactive solution for only 1 hr. This is possible because the apparent value of the cytoplasmic pH does not increase significantly after 30 min even though the activity in the vacuole appears to increase between 30 min and 4 hr (Fig. 1).

Effect of Azide on the Cytoplasmic pH. We have found that 1 mM NaN_3 produces a very rapid, large and reversible effect on the cytoplasmic pH when observed using pH microelectrodes.

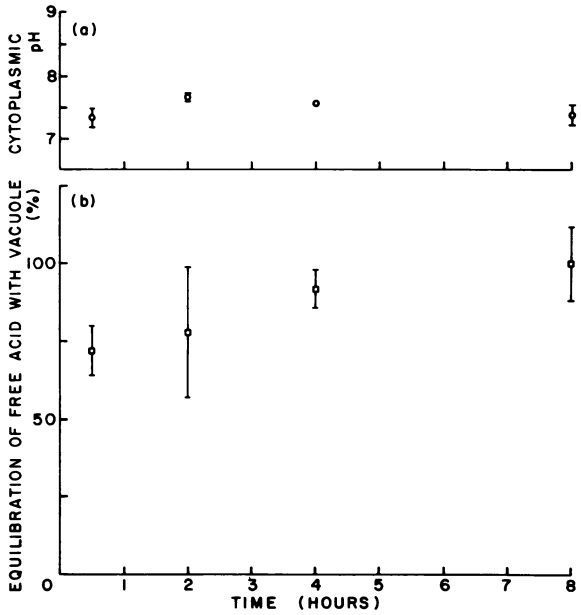

FIG. 1. Time courses for (a) the apparent cytoplasmic pH using the DMO method and (b) the concentration of DMO in the vacuole as a percentage of the free acid in the external solution.

The mean steady value for three cells was 5.32. Since the accuracy of the weak acid method depends on the accumulation of a large amount of the anion in the cytoplasm, and hence a high cytoplasmic pH, it was thought that acidification of the cytoplasm with azide would provide a test of the ability of the method to measure such changes. Azide reduced the apparent pH to 6.52 ± 0.19 (8) from the control value of 7.42 given above.

DISCUSSION

There is reasonable agreement between the values for the cytoplasmic pH measured using the glass microelectrode and DMO methods at an external pH of 6. The value is slightly lower (0.2–0.3 pH units) than that obtained by Walker and Smith (12) for *C. corallina* using the DMO method. The value for the cytoplasmic pH obtained using the plastic-insulated pH microelectrodes is significantly lower than that obtained using the glass microelectrodes but is similar to the values (6.6–6.8) obtained by Davis (3) using microelectrodes of the same type. As mentioned above, the low values may be due to imperfections in the insulation in the region of the microelectrode outside the cell. Even when the value obtained for the pH appears to be reasonable, it is not possible to be certain that the insulation is completely intact. Thus, the glass microelectrodes, in which this source of uncertainty is eliminated, are considered more suitable for the measurement of pH in plant cells.

An advantage of the glass microelectrode over the DMO method is that it may be assumed that it records a pH corresponding more closely to that of the "ground cytoplasm." Also, it is possible to record the time course of pH changes. A disadvantage is that the technique is difficult and time-consuming. It is possible that the recessed tip type of microelectrode described by Thomas (9) would be more suitable.

The advantage of the DMO method is that it is possible to make a large number of determinations in a relatively short time. Even the long incubations used by Walker and Smith (12) are not necessary if it is only the cytoplasmic pH that is of interest. One source of error is the cytoplasmic volume which cannot, in practice, be measured for each cell. Fortunately, the estimate of pH under normal conditions is rather insensitive to the exact

value used for the volume; a decrease of the cytoplasmic volume from 5 to 3% of the total cell volume increases the calculated pH by only 0.2 pH units. This is also true when azide is present and cannot account for the high apparent pH recorded by the DMO method. A possible explanation would be that a compartment within the cytoplasm (the chloroplast stroma?) remains alkaline in the presence of azide.

Walker and Smith (12) have already discussed the implications of the observed cytoplasmic pH with regard to the H^+ pump and the Cl^- influx. We shall deal with these problems in greater detail in a subsequent paper.

The effect of azide on the cytoplasmic pH deserves further comment. Since other treatments which appear to inhibit the hydrogen ion pump do not produce such large changes in pH (Spanswick, unpublished), it is possible that the decrease in pH is brought about by the penetration of hydrazoic acid (pK = 4.74) in the associated form and its subsequent dissociation. The cytoplasm may not be as well buffered as it is generally assumed and changes in cytoplasmic pH may be an undesirable side effect of this and similar inhibitors. The decrease in cytoplasmic pH may also provide an alternative explanation for the observed side effects of azide on IAA uptake and loss from maize coleoptiles made recently by Cande and Ray (2). According to Rubery and Sheldrake (5), the influx of IAA occurs primarily by passive permeation of the associated molecule IAAH. Since the cytoplasmic pH is high, the IAA would accumulate as the anion. To explain the observed efflux, a carrier for the anion (IAA^-) has been postulated which can be inhibited by inhibitors of polar transport such as NPA. In the presence of NPA, Cande and Ray (2) observed that addition of azide produced an increase in the efflux of IAA. In the absence of NPA, azide decreased the total accumulation of IAA. They suggested that the interaction of NPA with the IAA^- carrier depends upon energy metabolism. The results can be explained more simply if azide also reduces the cytoplasmic pH in maize coleoptiles. This would result in an increase in the efflux of IAAH, giving an apparent stimulation of the IAA efflux when added in the presence of NPA. It would also abolish the accumulation of IAA^- in the cytoplasm which is normally the result of the pH gradient. It is not necessary to make any further assumptions about the nature of the IAA carrier.

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