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# Effect of Elicitation and Changes in Extracellular pH on the Cytoplasmic and Vacuolar pH of Suspension-Cultured Soybean Cells[1]

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## ABSTRACT

**We have employed both $^{31}P$ nuclear magnetic resonance spectroscopy and two intracellular fluorescent pH indicator dyes to monitor the pH of the vacuole and cytoplasm of suspension-cultured soybean cells (*Glycine max* Merr cv Kent). For the $^{31}P$ nuclear magnetic resonance studies, a flow cell was constructed that allowed perfusion of the cells in oxygenated growth medium throughout the experiment. When the perfusion medium was transiently adjusted to a pH higher than that of the ambient growth medium, a rapid elevation of vacuolar pH was observed followed by a slow (approximately 30 minute) return to near resting pH. In contrast, the concurrent pH changes in the cytoplasm were usually fourfold smaller. These data indicate that extracellular pH changes are rapidly communicated to the vacuole in soybean cells without significantly perturbing cytoplasmic pH. When elicitors were dissolved in a medium of altered pH and introduced into the cell suspension, the pH of the vacuole, as above, quickly reflected the pH of the added elicitor solution. In contrast, when the pH of either a polygalacturonic acid or *Verticillium dahliae* elicitor preparation was adjusted to the same pH as the ambient medium, no significant change in either vacuolar or cytoplasmic pH was observed during the 35 minute experiment. These results were confirmed in experiments with pH-sensitive fluorescent dyes. We conclude that suspension-cultured soybean cells do not respond to elicitation by significantly changing the pH of their vacuolar or cytoplasmic compartments.**

Rapid changes in cytoplasmic pH have been implicated as a mechanism of signal transduction in a wide variety of biological systems (4, 10, 13, 20, 21). Although the occurrence of such pH transitions under physiological conditions has been questioned recently in animal cells (6, 9, 30), there is still substantial evidence for their occurrence during the defense response in plants (4, 13, 20, 21, 27). This defense response, as it is currently understood, involves a rather global change in cell biochemistry encompassing transitions in pathways as diverse as phytoalexin synthesis (7), callose formation (5, 14), biosynthesis of lignin-like material (5, 22), hydroxyproline-rich protein synthesis (5), release of $H_2O_2$ (2), production of hydrolytic enzymes (5, 27), synthesis of protease inhibitors (5), and hypersensitive cell death (4). Because many of these pathways are biochemically unrelated, a pervasive signal such as a change in cytoplasmic pH would seem to be an efficient means of initiating them from a single stimulus. Therefore, it is not surprising that the possible involvement of a rapid intracellular pH change during elicitation has already received considerable attention (4, 13, 20, 21, 29).

In this study, we have attempted to confirm previous reports of elicitor-promoted pH transitions in the intracellular compartments of cultured plant cells. It was our intention to correlate the observed pH transitions kinetically with other signaling events currently under investigation in our laboratory (1, 2, 12, 17). However, when precautions were taken to avoid artifacts induced during sample manipulation, we were unable to detect any elicitor-stimulated pH change in either the vacuole or cytoplasm of cultured soybean cells using either a polygalacturonic acid or *Verticillium dahliae* elicitor. Instead, we have observed that any external displacement of culture medium pH is immediately transmitted to the vacuole, which in turn serves to protect the cytoplasm from the exogenous pH change. We have also observed that brief anoxia during cell handling can trigger a depression of cytoplasmic pH. The kinetics and magnitudes of these processes are described in this paper.

## MATERIALS AND METHODS

### Plant Material

Soybean (*Glycine max* Merr cv Kent) cell suspension cultures were maintained in W-38 medium and subcultured every 7 to 10 d as previously described (1, 17). W-38 medium is a modified Murashige and Skoog medium (Sigma catalog No. M6899) supplemented with sucrose, casein digest, 2,4-dichlorophenoxyacetic acid, and kinetin, as described elsewhere (11). Because soybean cells cultured in this medium contained too little phosphate (1.25 mM) to yield interpretable $^{31}P$ NMR spectra at rapid intervals (<7 min), a study was conducted to determine the appropriate level of phosphate supplementation necessary to obtain good spectral data. It was found that cells grown in 3.75 mM phosphate yielded well resolved spectra within the 7 min acquisition time interval we felt was necessary to obtain good kinetic data. Therefore, to prepare cells for NMR experiments, approximately 33 mL of cell suspension from a 7-d-old culture was transferred to 100 mL fresh W-38 medium containing 3.75 mM phosphate and

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allowed to grow for 7 d. The above cells were then transferred again to fresh W-38 medium containing 3.75 mM phosphate and grown an additional 36 h. This transfer schedule yielded cells capable of a normal defense response as indicated by the stimulation of a rapid burst of $H_2O_2$ upon treatment of the suspension with elicitors (3). Directly before each experiment, the cells were harvested via filtration through a glass fiber filter. The cells were then washed three times with an equal volume of phosphate-free W-38 cell culture medium to remove external phosphate, suspended in 25 mL of phosphate-free W-38 cell culture medium, and placed in the NMR tube flow cell apparatus. These cells were then kept oxygenated and metabolically active by perfusion with 2 L of oxygenated phosphate-free W-38 medium throughout the time course of the experiment.

### Elicitor Preparation

The oligogalacturonide fraction (degree of polymerization, approximately 12) used as an elicitor was prepared from citrus pectin as previously described (19). A typical preparation contained 500 μg galacturonic acid equivalents/mL. The *Verticillium dahliae* 277 elicitor was prepared as described previously (1, 17). A typical elicitor preparation from this pathogen contained 750 μg protein and 500 μg glucose equivalents/mL.

The above isolation procedures resulted in the production of highly active elicitor molecules dissolved in their storage buffer at neutral pH. However, when desired, this storage buffer was either exchanged for the circulating culture medium or simply adjusted to the precise pH of the growth medium before initiation of an elicitation study. This was done by dialysis or direct titration with HCl or NaOH, respectively. As will be shown below, these adjustments prevented pH changes in the medium from promoting pH changes in the vacuole and cytoplasm.

### $^{31}$P NMR Spectroscopy

The NMR flow cell/perfusion system used in these experiments was essentially the same as constructed in ref. 8 with the exception that peristaltic pumps, instead of air pressure, was used to circulate the growth medium employed in the experiments.

$^{31}$P NMR spectra were obtained with a Nicolet NT-200 NMR spectrometer using a 20 mm diameter selective $^{31}$P frequency probe. The NMR spectra were accumulated with a pulse width (90°) of 30 μs, an acquisition time of 0.536 s, and a recycle time of 0.200 s. Using this scheme, a block of 500 scans took 6.13 min to acquire. Chemical shifts were measured relative to the signal from an internal capillary containing a 2% (v/v) aqueous solution of methylenediphosphonic acid.

The cytoplasmic and vacuolar pH values were determined from chemical shifts of the cytoplasmic and vacuolar Pi resonances using calibration curves obtained from Pi solutions of different pH values containing 10 mM KCl, 10 mM $MgCl_2$, and 5 mM citrate, *i.e.* solution conditions that mimic cytoplasmic/vacuolar ionic conditions (18).

After loading the cells into the flow cell/perfusion apparatus, the cells were allowed to equilibrate for 20 min to ensure both a stable internal pH and a steady metabolic rate. If this equilibration was not performed, decreases in compartment pH, probably due to the brief anaerobiosis during cell transfer, were invariably observed. After equilibration, either 4.5 mg of a polygalacturonic acid elicitor, 10 mg of a *V. dahliae* elicitor, or 20 mL of a buffer solution of the desired pH was injected at the bubble trap and spectra were acquired for the desired time.

### Measurement of Intracellular pH during Elicitation Using Fluorescent Probes

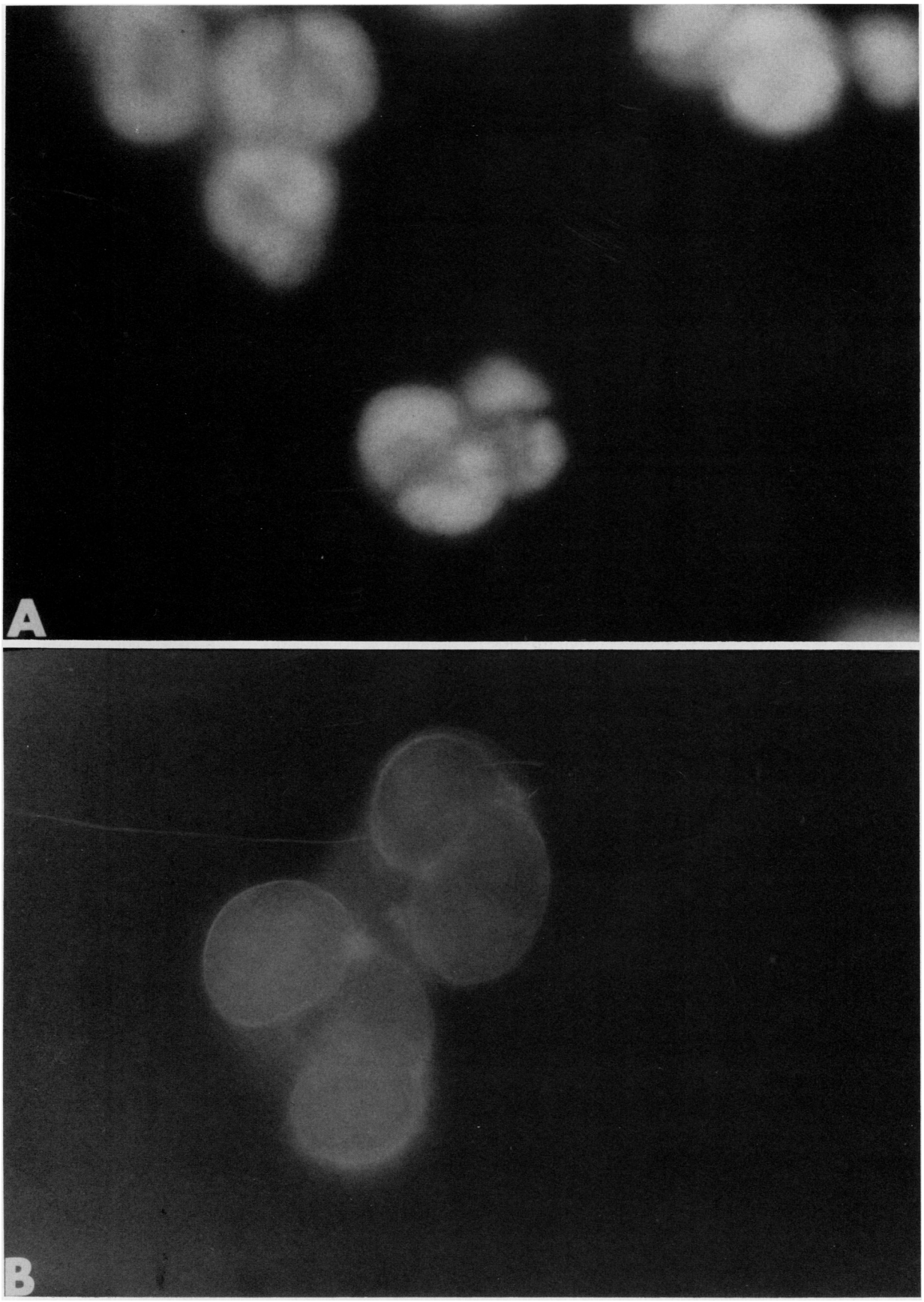
To determine the pH changes in the internal compartments of the soybean cells by fluorescence spectroscopy, a dye had to be chosen whose fluorescence was dependent on its ionization state and whose $pK_a$ was at or near the pH of the internal compartment of interest. This ensured that the fluorescence of the dye would change when the pH of the compartment of interest changed. Furthermore, a dye had to be selected that was not oxidized by the elicitor-induced oxidative burst (2). Therefore, the dyes that were used in this study were DCMF,[2] whose $pK_a$ (7.0) was suitable for measuring cytoplasmic pH but largely insensitive to changes in vacuolar pH, and DCCF ($pK_a$ = 5.1), which was more suitable for measuring vacuolar pH changes. The dyes were loaded into the soybean cells by adding 15 μL of a 1 mg/mL solution (in DMSO) of the permeant acetate derivative of the dye to 15 mL of a 36-h-old soybean cell suspension culture in W-38 medium and incubating for 20 min. The cells were then filtered, washed with 30 mL of growth medium that was previously removed via filtration from the same cell culture, and suspended in 15 mL of the same partially "spent" growth medium. To confirm the intracellular localization of our chosen pH-sensitive fluorescent dyes, a small sample of the loaded cells was observed under an Olympus BHT fluorescent microscope. Figure 1 illustrates that the fluorescence of DCCF and DCMF is largely confined to the vacuole and cytoplasm, respectively. Autofluorescence of the same cells was less than 0.001 the intensity of the labeled cells, and this autofluorescence did not change upon elicitation. After resuspending the loaded and washed cells, 1.5 mL of the cell suspension culture was then placed in a quartz cuvette equipped with a stir bar and the fluorescence was continuously monitored using a Perkin-Elmer MPF-44A fluorescence spectrophotometer (for DCCF, $\lambda_{ex}$ = 504 nm, $\lambda_{em}$ = 535 nm; for DCMF, $\lambda_{ex}$ = 504 nm, $\lambda_{em}$ = 529 nm). After brief equilibration, the cells were treated with either 25 μg polygalacturonic acid elicitor or 50 μg *V. dahliae* elicitor and the fluorescence was monitored continuously as a function of time. For both dyes, an increase in fluorescence corresponds to an elevation of intracellular pH.

## RESULTS

### Normal $^{31}$P NMR Spectra of Soybean Suspension Culture Cells

Figure 2 displays a typical $^{31}$P NMR spectrum of a live soybean cell suspension culture. Resonances were assigned

[2] Abbreviations: DCMF, 5-(and 6-) carboxy-4′,5′-dimethylfluorescein; DCCF, 5-(and 6-)-carboxy-2′,7′-dichlorofluorescein.


A
B

using literature values (20, 25, 28) and confirmed in experiments using either imposed anaerobiosis or the nonmetabolizable glucose analog, 2-deoxyglucose, as described elsewhere (20, 28). From the intensities of the resonances in Figure 2, it can be seen that the cells were aerobic and metabolically normal. Thus, the vacuolar pH (5.43 ± 0.55, $n$ = 15 experiments) and cytoplasmic pH (7.29 ± 0.17) were within the expected range (8), and the ADP content was significantly lower than the ATP content. Furthermore, the high level of glucose-6-phosphate indicates that there was sufficient substrate available for further ATP production. Therefore, we conclude that our flow cell/perfusion apparatus was effective in providing adequate substrates and oxygen for maintaining metabolism during the entire NMR experiment.

### Effects of Perturbing the Medium pH on Intracellular pH Determined by $^{31}$P NMR

Figure 3 shows the effects of briefly altering the pH of the external perfusion medium on the vacuolar and cytoplasmic pH values of cells grown in growth medium equilibrated at pH 5.4. The first treatment (arrow 1) was an injection of 20 mL phosphate-free W-38 growth medium adjusted to pH 7.0. This treatment resulted in a rapid increase (4 min) of the medium pH to 6.7, measured at the in-line pH meter, followed by a fast reequilibration (11 min) of the pH back to pH 5.5. This rapid return of the circulating medium pH is a consequence of the mixing of the 20 mL aliquot of pH 7 medium with the medium in the reservoir. Importantly, the large change in external pH promoted a rapid (6 min) increase in vacuolar and cytoplasmic pH of 1.4 units and 0.3 unit, respectively, followed by a return to basal pH levels after 30 min. The second treatment (arrow 2) was an additional injection of 20 mL phosphate-free W-38 growth medium adjusted to pH 8.0. As before, this perturbation also induced a rapid increase (4 min) of the cell pack medium pH to 7.25 followed by a reequilibration (11 min) of the medium pH back to 5.7. This manipulation of external pH also caused rapid increases in vacuolar and cytoplasmic pH values of 1.3 and 0.3 units, respectively, followed by return to basal pH levels after 30 min. These data suggest that the pH values of intracellular compartments of soybean cells will be transiently changed by the buffer pH of any solution added to the cell suspension. Similar conclusions have been drawn by other workers investigating analogous external pH insults, and these are reviewed in ref. 15.

### $^{31}$P NMR Measurement of Intracellular pH after Treatment with Elicitor Solutions Adjusted to Medium pH

To determine whether elicitors alone might stimulate pH transitions in the cytoplasm or vacuole of cultured soybean cells, experiments were performed in which elicitor solutions were adjusted to a pH identical to that of the circulating growth medium. Figure 4A and B illustrates the effect of addition of either 10 mg *V. dahliae* elicitor dissolved in 15 mL phosphate-free W-38 growth medium adjusted to the pH of the circulating medium (usually 5.4) or 4.5 mg polygalacturonic acid elicitor dissolved in the same volume of growth medium. Importantly, even after dilution (approximately 70-fold) into the medium reservoir, these elicitor concentrations were sufficient to achieve a maximum defense response as monitored by pyranine oxidation (polygalacturonic acid elicitor) or phytoalexin accumulation (*V. dahliae* elicitor). However, as the figures illustrate, the injections resulted in no major changes in intracellular pH. Several additional experiments were also performed with higher/lower elicitor concentrations and the same results were obtained (data not shown).

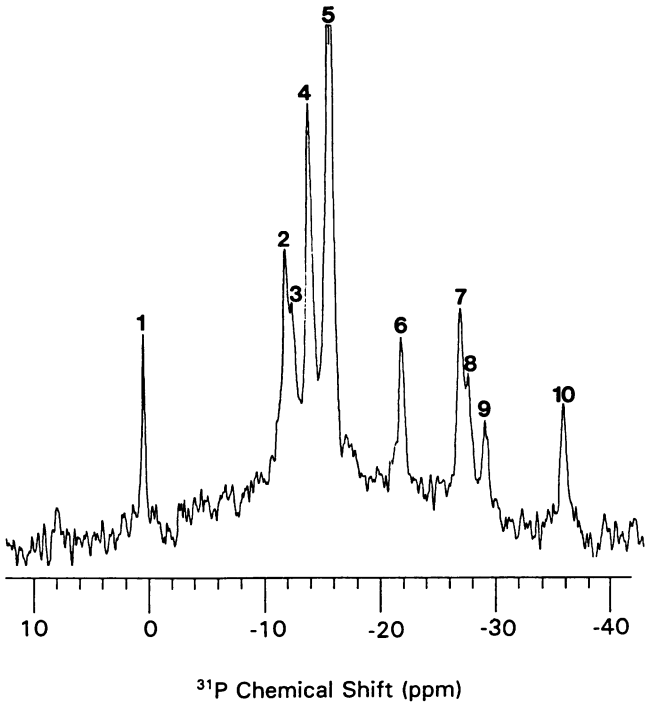



**Figure 2.** Representative $^{31}$P NMR spectrum of 36-h-old soybean suspension culture cells grown in W-38 medium containing 3.75 mM phosphate. The labeled resonances are: 1, methylenediphosphonic acid; 2, glucose-6-phosphate; 3, fructose-6-phosphate; 4, cytoplasmic Pi; 5, vacuolar Pi; 6, γ-phosphate of adenosine triphosphate; 7, α-phosphate of adenosine triphosphate; 8, NADP; 9, UDP-glucose; 10, β-phosphate of adenosine triphosphate. The β-phosphate of ADP would normally be seen near resonance 6, but because of its small size it is obscured.

**Figure 1.** Localization of the intracellular fluorescence of the pH-sensitive fluorescent probes, DCCF and DCMF. Cells suspended in W-38 growth medium at pH 5.4 were loaded with the permeable diacetate forms of DCCF and DCMF, as indicated in "Materials and Methods." After loading, the cells were observed at 40× magnification with an Olympus BHT fluorescence microscope equipped with a DM-500 (0–515) dichroic mirror and an EY-455 excitation filter and G-520 emission filter. Under these conditions, excitation wavelengths from 440 to 495 nm and emission wavelengths from 500 to 550 nm were allowed. Distinct vacuolar staining is seen in the cells loaded with DCCF (A) with reduced fluorescence in the nucleus and cytoplasm. Cells loaded with DCMF (B) displayed a fluorescence pattern characteristic of cytoplasmic staining, *i.e.* diffuse fluorescence throughout the cell with increased intensity near the periphery.

In contrast, if the elicitor solutions were not adjusted to precisely the pH of the circulating medium, pH transitions of the sort seen in Figure 3 were observed.

### Measurement of Intracellular pH Changes in Soybean Cells with Fluorescent Probes after Elicitation of the Plant Defense Response

To confirm the changes in intracellular pH measured by $^{31}P$ NMR, another pH detection method, namely the use of fluorescent pH indicators, was employed to follow changes in intracellular pH. As previously mentioned, the ionization characteristics of the fluorescein-derived probes, DCCF and DCMF, make these dyes particularly suitable for measuring small pH changes in vacuolar and cytoplasmic compartments, respectively (see Fig. 1). Figure 5A and B illustrates that treatment of soybean cells with elicitors dissolved in W-38 growth medium adjusted to pH 7.0 results in the expected transient increases in the vacuolar and cytoplasmic pH values seen in the $^{31}P$ NMR experiments (Fig. 3). Thus, a virtually instantaneous rise in fluorescence was invariably followed by a gradual decline to near prestimulation levels. The same series of experiments also confirm that the above pH changes arose solely from perturbation of the medium pH, because no change in cytoplasmic or vacuolar pH occurred after addition of elicitor solutions adjusted to the pH of the culture medium (Fig. 5A, B). Based on these and the $^{31}P$ NMR results, we conclude that cultured soybean cells do not exhibit a major intracellular pH transition in response to elicitation.

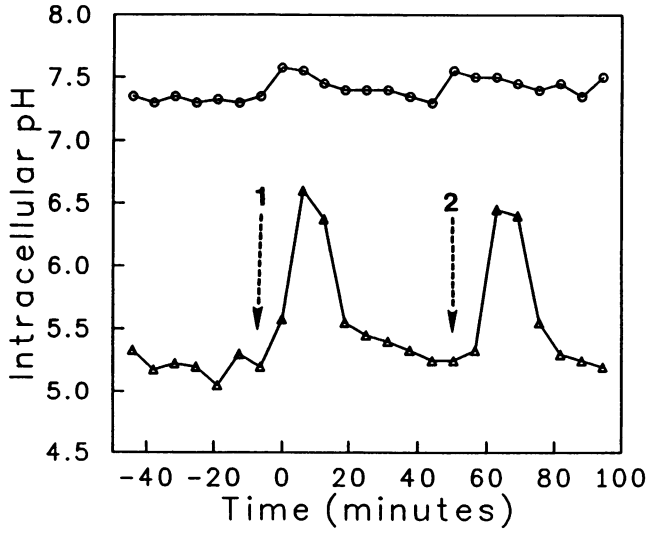



**Figure 3.** Effect of external pH perturbations on the cytoplasmic pH (O) and vacuolar pH (Δ) of suspension-cultured soybean cells measured by $^{31}P$ NMR. The first treatment (arrow 1) involved addition of 20 mL phosphate-free W-38 growth medium adjusted to pH 7.0. This treatment resulted in a fast (4 min) increase in the cell pack medium pH to 6.7, followed by a return to pH 5.5 after 11 min. The second treatment consisted of the addition of the same medium adjusted to pH 8.0. The arrow indicates the time when the extracellular pH change entered the cell pack.

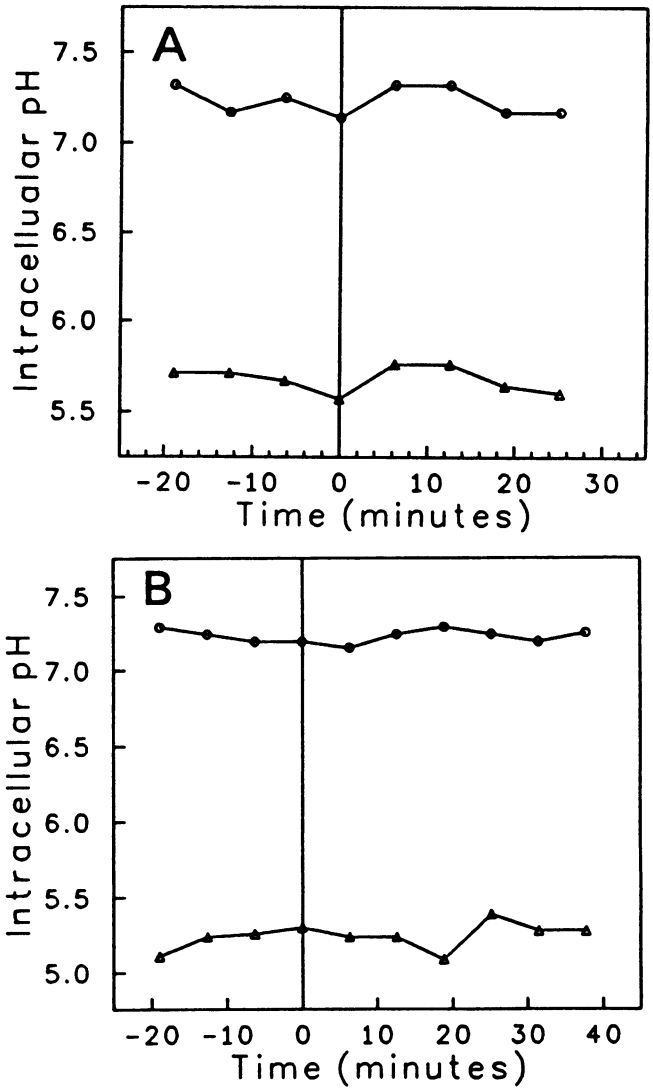



**Figure 4.** The effect on cytoplasmic pH (O) and vacuolar pH (Δ) of addition of elicitors suspended in growth medium of the same pH as the circulating medium. In panel A, 4.5 mg polygalacturonic acid elicitor dissolved in phosphate-free W-38 growth medium adjusted to the same pH as the circulating growth medium (usually 5.4) was added at time 0 through the injection port of the flow system. In panel B, 10 mg *V. dahliae* elicitor dissolved in the same medium and readjusted to the pH of the circulating medium was introduced in the same manner.

## DISCUSSION

A number of studies have examined the influence of an external pH change on the pH values of the vacuolar and cytoplasmic compartments in a wide variety of cell types, including mung bean root-tips (31), barley root-tips (23), cultured tobacco cells (32), *Chara corallina* (24), and cultured carrot and oil palm cells (8). These studies all conclude that both the cytoplasmic and vacuolar compartments are highly buffered and, therefore, resistant to internal pH changes arising from external pH insults (8, 23, 24, 31, 32). The studies performed in mung bean and barley root-tip cells (23, 31),

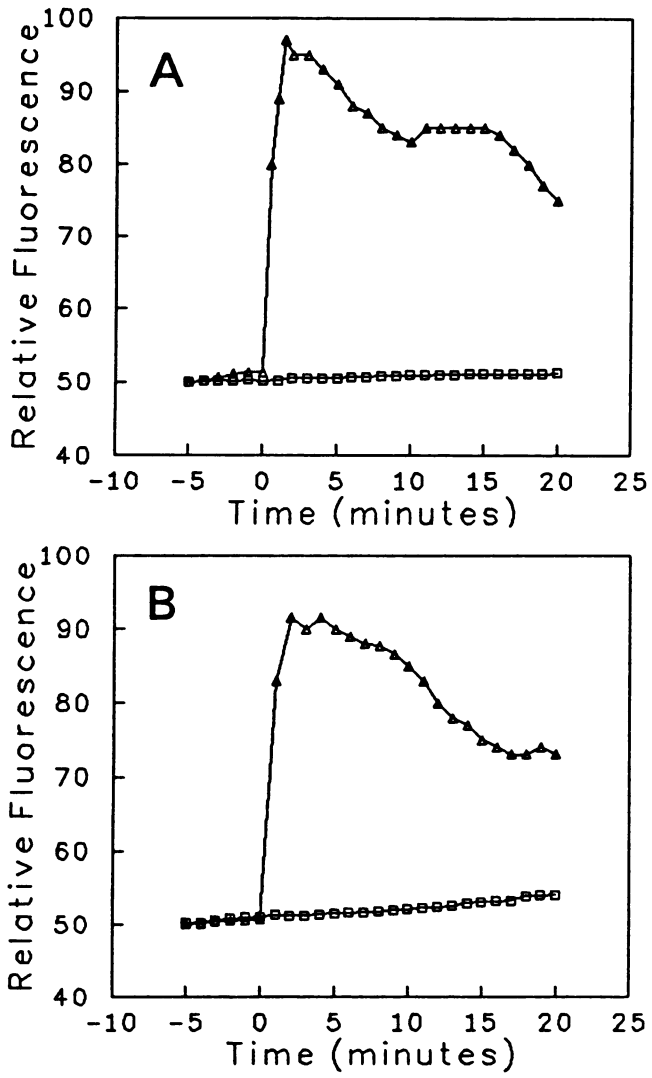



**Figure 5.** Effect of elicitors suspended in W-38 growth medium adjusted to the ambient pH of the suspension culture medium (usually pH 5.4; □) or adjusted to pH 7.0 (△) on the fluorescence of DCCF (A) or DCMF (B) intracellular pH indicator dyes. After DCCF or DCMF loading, 1.5 mL of the loaded cell suspension was placed in a quartz cuvette and the fluorescence was constantly monitored before, during, and after treatment with 25 μg polygalacturonic acid elicitor. Results with *V. dahliae* elicitor were similar. The growth medium pH usually rose from 5.4 to 6.0 upon addition of the nonadjusted elicitor solution. The fluorescence of both pH indicator dyes increases as pH is raised.

cultured tobacco cells (32), and *C. corallina* (24) further report that the compartmental pH changes are in the same direction as the external pH changes, whereas the experiments on oil date palm and carrot cells indicate that the internal pH changes occur in an opposite direction from the external perturbation (8). Although the results for the different systems seem to be somewhat different, all systems report some type of efficient intracellular pH regulation to counteract an external pH change. An excellent recent review of the mechanisms employed by plant cells to resist a physically or biologically stimulated change in intracellular pH is provided elsewhere (15).

By two independent techniques, fluorescence spectroscopy and $^{31}P$ NMR, we have demonstrated that cultured soybean cells experience (a) a rapid change in vacuolar pH concurrent with a smaller change in cytoplasmic pH whenever the pH of their suspension medium is modified, and (b) no measurable change in cytoplasmic or vacuolar pH when stimulated by polygalacturonic acid or *V. dahliae* elicitors. Although the former observation is consistent with the findings of other investigators for other cell types (8, 23, 24, 31, 32), the latter observation disagrees with four previous reports. Thus, suspension cultures of *Phaeseolis vulgaris* (20), *Petroselinum hortense* (13, 29), and *Nicotiana tabacum* (4) have been observed to respond to elicitation with substantial changes in intracellular pH. In *P. vulgaris* (20), a rapid acidification of both the cytoplasm and vacuole occurred within 10 min of elicitation, followed by a long-term alkalinization of both subcellular compartments (>1 h). The responses of *P. hortense* to elicitors were examined by two different groups with apparently different results. Strasser *et al.* (29) reported a 0.5 pH unit drop in vacuolar pH upon treatment with an elicitor from *Alternaria carthami,* but they made no mention of a change in cytoplasmic pH. Kneusel *et al.* (13), on the other hand, observed a 0.25 pH unit decrease in cytoplasmic pH upon stimulation with a *Phytophthora megasperma* extract, but they report no acidification of the vacuole. Finally, in *N. tabacum,* treatment with a bacterial elicitor led to a slow (60–90 min) decrease in the average cellular pH of 0.75 pH units (4).

The discrepancies among these various results could actually have arisen from several sources. First, it should be noted that different species as well as different elicitors were employed in the several elicitation studies (4, 13, 20, 29), and it has not yet been established whether all elicitors initiate the same signal transduction pathway in all cells. Second, the compositions of the growth media in the various studies were also not identical. In the case of cultured animal cells, the simple inclusion of low concentrations of $HCO_3^-$ in the growth medium causes the disappearance of growth factor-stimulated intracellular pH transitions (6, 9, 10, 30). Based on these observations, the hormone-stimulated pH changes in animal cells now are believed to be more metabolically related than involved in signal transduction pathways (6, 9, 10, 30). Third, it is conceivable that minor pH transitions were, in fact, elicited in our cell suspension and that they were simply too small to detect above the normal random pH fluctuations of the suspension. Even in resting cells, changes in cytoplasmic or vacuolar pH of <0.2 pH units were commonly seen and disregarded as either experimental noise or normal metabolic fluctuations. However, pH changes of larger magnitudes than this would not have escaped our attention, and we can confidently say that in our system they did not occur when the elicitor pH was adjusted to the same pH as the growth medium. Finally, although unlikely, the difference between our results and those of other groups could have arisen from aeration problems or possibly external pH perturbations. Whenever our soybean cells were deprived of $O_2$, their vacuolar pH rapidly decreased (data not shown). Furthermore, as documented above, elicitor solutions of any pH

different from the pH of the suspension medium invariably induced a pH change in the intracellular compartments.

In contrast with earlier studies (4, 13, 20, 29), we did not attempt to monitor cellular pH transitions longer than 37 min postelicitation. Callose production (14), mRNA synthesis (16, 26), and the oxidative burst (2) are all well underway by this time. In fact, in our soybean cell culture the oxidative burst occurs synchronously in >95% of the cells within 5 min of elicitor addition, the precise lag period depending on the amount of elicitor added. Therefore, we have assumed that most signals that were to be transduced across the membrane will already have been sent by this time, and that subsequent changes in cytoplasmic/vacuolar pH are more related to elicitor-stimulated metabolic changes than to signal transduction. Examination of the kinetics of other signal transduction processes should help decide whether this assumption is correct.

In conclusion, we believe the question of whether elicitors promote a transient change in cytoplasmic or vacuolar pH is still unanswered. Additional studies incorporating precautions to guard against potential artifacts inherent in *in vivo* intracellular pH measurements will have to be conducted to fully resolve this issue. Even if pH transitions are eventually detected in some cells, further studies will then have to be designed to evaluate whether the transitions are a cause or simply a consequence of the elicited metabolic changes.

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