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Intracellular pH Does Not Change during Phytochrome-Mediated Spore Germination in *Onoclea*

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Calcium ions may act as a "second messenger" in phytochrome-mediated spore germination in *Onoclea sensibilis* L. In this study we determine whether changes in intracellular pH also contribute to the signal transduction chain. Using ^{31}P nuclear magnetic resonance spectrometry, in which we are able to resolve changes as small as 0.2 pH units, we fail to observe any change in pH associated with red light irradiation. In addition artificially inducing an intracellular change in pH of greater than 1 pH unit (5.8–7.2) has no effect on germination. These data indicate that a sustained increase in intracellular pH does not contribute to the signal transduction chain in phytochrome-mediated fern spore germination.

INTRODUCTION

Calcium ions (Ca^{2+}) contribute to the signal transduction chain in the red light-stimulated activation of quiescent fern spores (Wayne and Hepler, 1984). Following the absorption of light by the photomorphogenetic pigment phytochrome, an increase in the rate of net calcium influx occurs (Wayne and Hepler, 1985a). The increase in the total intracellular calcium concentration is sufficient to account for a 100-fold increase in the concentration of intracellular free calcium from 0.1 to 10 μM. Inhibiting the red light-stimulated increase in calcium influx with far-red light or La^{3+} inhibits germination (Wayne and Hepler, 1985a) while artificially increasing the concentration of intracellular calcium with the ionophore A 23187 induces germination in the dark (Wayne and Hepler, 1984). These data indicate that calcium ions may act as a second messenger in phytochrome-mediated fern spore germination.

Fern spore activation is analogous to egg activation and oocyte maturation in animal cells where a rapid, transient increase in the concentration of intracellular calcium follows the stimulus (Jaffe, 1980; Moreau *et al.*, 1980). In eggs the increase in intracellular $[Ca^{2+}]$ triggers the cortical reaction, mitosis and cleavage, and, in oocytes, germinal vesicle breakdown. In both eggs and oocytes, a sustained increase in intracellular pH of approximately 0.3–0.6 pH units occurs a few minutes after the rise in calcium. The elevation of the intracellular pH may be an element of the signal transduction chain and has been implicated in the later events of egg activation and oocyte maturation including stimulation of DNA and protein synthesis as well as increased protein phosphorylation. The observations that artificially raising the intracellular pH with NH_4Cl or NH_4OH stimulates DNA and protein synthesis in sea urchin eggs while lowering the pH with weak acids prevents the stimulation (Houle and Wasserman, 1983; Mazia and Ruby, 1974; Winkler, 1982; Zucker *et al.*, 1978) support a role for pH in signal transduction. Further evidence that a change in pH contributes to signal transduction comes from studies which show that an increase in intracellular pH is responsible for the release from dormancy of bacterial (Setlow and Setlow, 1980) and yeast spores (Gillies *et al.*, 1981b) as well as shrimp cysts (Busa, 1982).

Because phytochrome, in many instances (Roux, 1983, 1984), and in *Onoclea* in particular (Wayne and Hepler, 1984), has been shown to utilize Ca^{2+} as a second messenger it is reasonable to ask whether pH changes accompany or postcede the Ca^{2+} influx. In several systems the far-red absorbing, physiologically active form of phytochrome has been shown to regulate proton extrusion (Brownlee and Kendrick, 1979; Lürssen, 1976; Pike and Richardson, 1977; Yunghans and Jaffe, 1972); such proton extrusion may give rise to an increase in intracellular pH. The purpose of this investigation is to test the hypothesis that a sustained increase in intracellular pH is part of the signal transduction chain in phytochrome-mediated fern spore germination.

MATERIALS AND METHODS

Plant material and sterilization. Mature sporophylls of *Onoclea sensibilis* L. were collected in Amherst and Pelham, Massachusetts in January, 1981 and February

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1982 and stored in double plastic bags in a freezer at −15°C for at least 5 months to eliminate dark germination. Prior to an experiment, sporophylls were wetted with a 0.1% solution of Aerosol O. T. (Fisher Scientific Co., Pittsburgh, Pa.) and sterilized with 1000 ml of a 20% (v/v) solution of commercial bleach (5.25% NaOCl) in a manner modified from Stockwell and Miller (1974). Sterile spores were separated from the sporangia with the aid of vibrations from a vortex shaker.

Light treatments. All procedures were carried out under a dim green safelight which consisted of two "gold" fluorescent lamps (General Electric, Cleveland, Ohio; F40-T12-GO) and a green plexiglass filter (Rohm and Haas, Co., Philadelphia, Pa.). Red light was obtained by passing light from a Spaulding fixture (J. H. Spaulding Co., Cincinnati, Ohio) equipped with a 500 W Sylvania lamp (Sylvania Fused 3924) through one layer of a Roscolene medium red (No. 823) cellulose acetate filter (Capron Lighting Co., Needham, Mass.). Red light was applied with an energy fluence rate of 7.5 J m^{-2} sec^{-1} as measured with a Li-cor (Lincoln, Nebr.) quantum-radiometer-photometer (Model LI 185 B) with a Li-cor pyranometer sensor (LI-200SB). The energy fluence rate of the safelight could not be detected with this sensor. The spores were irradiated for 2 min with red light at 24°C to ensure saturation, except where stated otherwise.

Nuclear magnetic resonance experiments. Five hundred milligrams of dry, sterilized spores were floated on 250 ml of 10 m*M* Na^+-phosphate-buffered medium (pH 4.00–9.00) that included 1 m*M* $Ca(NO_3)_2$, 0.81 m*M* $MgSO_4$, and 3.45 m*M* KNO_3 in 100 mm diameter × 15 mm high polystyrene petri dishes. The spores remained on this medium for 24 hr in the dark at 23°C. The spores were then collected by filtration through a 5.0-μm Millipore filter (Millipore corporation, Bedford, Mass.), washed with distilled water, and placed in a 10-mm NMR tube, and rehydrated with just enough distilled water to moisten the spores but still allow air to pass through them so that they may respire. The tube of spores was placed in a Varian XL 300 Nuclear Magnetic Resonance Spectrometer and spun. Each 121 MHz ^{31}P-NMR spectrum represents the Fourier transform of 3000 free induction decays obtained with a sequence of 45° pulses and a 0.4 sec acquisition time at 23°C. The spectra were displayed with a line broadening of 10 Hz. Spectra were obtained with proton decoupling and without the field frequency lock employing D_2O except where otherwise specified. All NMR experiments were done under a dim green safelight.

Chemical shifts are referenced to 50 m*M* methylene diphosphonic acid (MDP) dissolved in 4 *M* Tris buffer and titrated to pH 8.90 with 12 *M* ultrapure HCl (Roberts *et al.*, 1980, 1981). The MDP was in a capillary placed coaxially in the sample tube. Standard pH buffer solutions were made by mixing 10 m*M* Na_3PO_4, Na_2HPO_4, and NaH_2PO_4 in the appropriate proportions. HCl (0.01 *N*) was added to the phosphate buffer to obtain the pH 4.00 buffer. pH measurements were made on a Beckman 3500 digital pH meter equipped with a Beckman 39505 combination electrode (Beckman Instruments, Fullerton, Calif.). The standard curve that relates the chemical shift of phosphate to pH is shown in Fig. 1.

Absolute pH measurements using NMR are only estimates because other parameters including ionic strength, temperature, and metal ions can also affect the chemical shift of P_i (Gadian, 1982). Presumably, after a stimulus these factors do not change over the levels that affect the P_i chemical shift. Thus very accurate measurements of changes in pH can be made. The sensitivity of the pH measurements depends on the pH measured. The P_i chemical shift is most sensitive to changes in pH near 6.9. Throughout the pH range measured in this study (5.50–7.73) we were able to resolve pH changes of at least 0.2 pH units. Our resolution is limited by the width of the peaks.

The effect of external pH on germination. Sterile spores (2 mg) were sown on 10 ml of 10 m*M* Na^+-phosphate-buffered medium (pH 4.00–9.00) that included 1 m*M* $Ca(NO_3)_2$, 0.81 m*M* $MgSO_4$, and 3.45 m*M* KNO_3. Spores were placed in the dark for 24 hr at 23°C. Spores were then irradiated with 5 min of broad-band red light at an energy fluence rate of 2.4 J m^{-2} sec^{-1} and again placed in the dark for 48 hr at 26.5°C. Spores were considered as germinated when two stained nuclei were observed

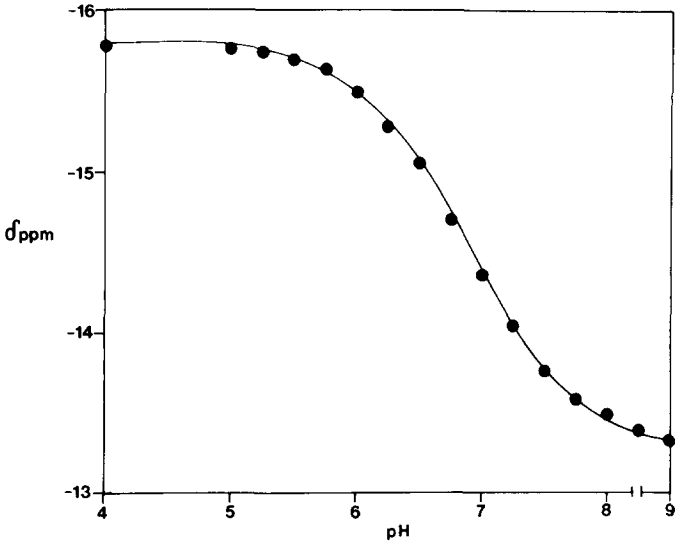

FIG. 1. The standard curve relating pH to the chemical shift of phosphate. Chemical shifts are referenced to 50 m*M* methylene diphosphonic acid (MDP) dissolved in 4 *M* Tris buffer and titrated to pH 8.90 with 12 *M* ultrapure HCl. The MDP was in a capillary placed coaxially in the sample tube. Standard pH buffer solutions were made by mixing 10 m*M* Na_3PO_4, Na_2HPO_4, and NaH_2PO_4 in the appropriate proportions. HCl (0.01 *N*) was added to the phosphate buffer to obtain the pH 4.00 buffer.

after staining with acetocarmine-chloral hydrate (Edwards and Miller, 1972). Two hundred spores per replicate were scored, and there were four replicates per treatment. All treatments were run at least twice. Data are expressed as the mean ± 2 SEM.

Measurement of proton efflux or influx. Spores (1–50 mg) were placed on 10 ml of 1 m*M* Na^+-phosphate-buffered medium (pH 4.00–9.00) that included 1 m*M* $Ca(NO_3)_2$, 0.81 m*M* $MgSO_4$, and 3.45 m*M* KNO_3 in polystyrene petri dishes 60 mm in diameter, 15 mm high or suspended in 3 ml of the same medium plus 0.01% Aerosol O. T. in test tubes. These spores were continuously aerated. They were left in the dark at 23°C for 24 hr. The spores that were in the petri dishes were then transferred to 35 mm diameter, 10 mm high petri dishes where the pH was continuously measured and recorded in the dark and during the irradiation with red light (energy fluence rate of 7.5 J m^{-2} sec^{-1}) with a Beckman Expandomatic SS-2 expanded scale pH meter (Beckman Instruments) equipped with either a Radiometer GK2391C microelectrode (Radiometer, Copenhagen, NV, Denmark) or a Beckman 39505 combination electrode (Beckman Instruments) connected to a Miniservo strip chart recorder (Esterline Angus, Indianapolis, Ind.). The media containing spores were back titrated with 5 m*M* NaOH to determine the amount of protons extruded.

Potentiometric titration of the buffering capacity of cell homogenates. One hundred milligrams of spores were suspended in 1 ml of distilled water in a Vitro cell homogenizer and frozen in liquid nitrogen. Cells were then mechanically homogenized, and the pH was measured on a Beckman 3500 digital pH meter equipped with a Beckman 39505 combination electrode (Beckman Instruments). The samples were vigorously aerated to eliminate buffering due to dissolved CO_2. The homogenate was titrated with 0.1 *N* NaOH to greater than pH 9, back titrated to less that pH 4 with 0.1 *N* HCl, and again titrated with 0.1 *N* NaOH to above pH 9. The data presented in Fig. 5 are averages of these titrations and retitrations from two separate experiments.

Because pH is a log function, pH values were converted to $[H^+]$ before averaging and statistical analysis (Stevens, 1955). Proton concentrations were then transformed back to pH values and presented as the mean ± 2 SEM.

RESULTS

Red light does not induce a sustained change in the intracellular pH of *Onoclea* spores (Fig. 2, Table 1). This experiment was conducted 14 times with MDP as a reference and 14 times without MDP, but using the field frequency lock to ensure the stability of the static magnetic field, with supporting results. It is possible that there is a transient change in intracellular pH that is undetectable with NMR spectrometry. Although the signal to noise ratio is low after scanning for 1–4 min, it is possible to detect a phosphate peak that is indistinguishable in peak position from the dark control indicating that a transient change does not occur during this time.

The line width of the phosphate peak is relatively broad. In a spectrum obtained from a well-tuned spectrometer, in which the sample is rapidly spun, the line width is a consequence of biological factors, including

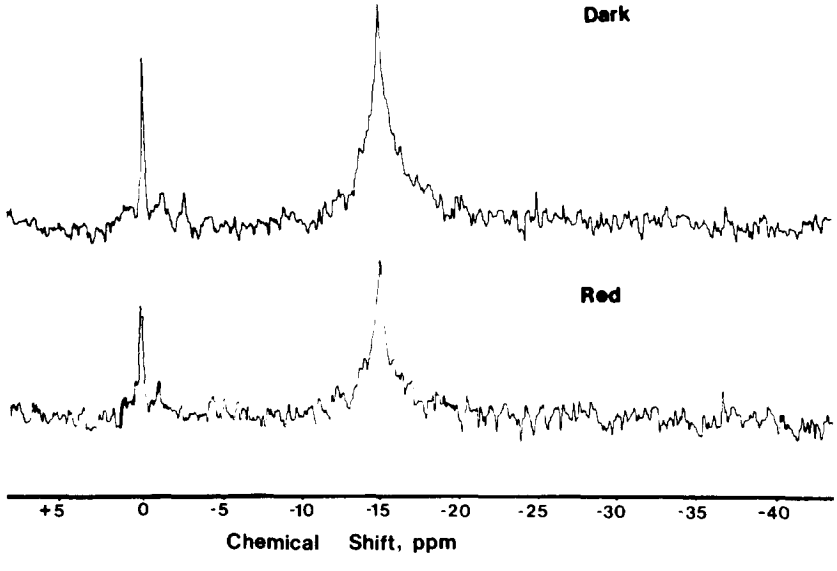

FIG. 2. ^{31}P-NMR spectra of dark and red treated cells. Before or after irradiation with red light (2.4 J m^{-2} sec^{-1}) for 2 min spores were collected by filtration, washed with distilled water, and placed in a 10-mm NMR tube, and rehydrated with just enough distilled water to moisten the spores. The tube of spores was placed in a Varian XL 300 Nuclear Magnetic Resonance Spectrometer and the 121 MHz ^{31}P-NMR spectrum was obtained as described under Materials and Methods.

TABLE 1
THE EFFECT OF RED LIGHT ON INTRACELLULAR pH

Using MDP as a reference			Using D_2O as a reference		
Dark	Red	ΔpH	Dark	Red	ΔpH
5.50	5.50	0.00	6.24	6.20	−0.04
5.85	5.25	−0.60	6.76	6.76	0.00
6.00	5.85	−0.15	6.76	6.76	0.00
6.12	6.12	0.00	6.76	6.76	0.00
6.20	6.20	0.00	6.76	6.76	0.00
6.35	5.98	−0.37	6.76	6.76	0.00
6.45	6.50	+0.05	6.76	6.76	0.00
6.45	6.45	0.00	6.76	6.76	0.00
6.45	6.35	−0.10	6.76	6.76	0.00
6.45	6.45	0.00	6.76	6.76	0.00
6.45	6.60	+0.15	6.76	6.76	0.00
6.80	6.35	−0.45	6.76	6.76	0.00
7.00	6.50	−0.50	6.80	6.80	0.00
7.73	7.73	0.00	6.89	6.84	−0.05

Note. Spores were collected by filtration, washed with distilled water, and placed in a 10-mm NMR tube, and rehydrated with distilled water. The tube of spores was placed in a Varian XL 300 Nuclear Magnetic Resonance Spectrometer and spun. Each 121 MHz ^{31}P-NMR spectrum represents the Fourier transform of 3000 free induction decays obtained with a sequence of 45° pulses and a 0.4 second acquisition time at 23°C. The spectra were displayed with a line broadening of 10 Hz. Spectra were obtained with proton decoupling and without the lock when MDP was used as a reference. When D_2O was used as a reference, spores were either rehydrated in 1 m*M* $Ca(NO_3)_2$, 0.81 m*M* $MgSO_4$, or 3.45 m*M* KNO_3 (pH 5.2) to which D_2O was added or spores were rehydrated in H_2O and D_2O was added to a coaxial capillary.

pH or ionic heterogeneity within or between spores, the presence of paramagnetic ions (e.g., Mn^{2+}), or the viscosity of the cytoplasm. In order to test whether the broad peak is composed of a few narrow peaks which represent compartments in the spore or different populations of spores, we performed a two-pulse experiment and varied the time between pulses. In these experiments we found that the broad phosphate peak is not composed of a few narrow peaks and therefore there is only one resolvable compartment and population of spores. However, the line width may be due to a normal distribution of pH within or among the spores. It is unlikely that the line width is a result of the presence of paramagnetic ions because the concentrations of these ions are nanomolar (Wayne and Hepler, 1985b) and probably too low to cause a broadening of the peak. We cannot eliminate the possibility that the line width is a consequence of the viscosity of the cytosol/cytogel.

The pH of spores briefly hydrated in distilled H_2O is 6.51 (6.46–6.58, $n = 3$) as measured by ^{31}P-NMR spectroscopy and 6.51 (6.28–7.08, $n = 4$) when the spores are homogenized and measured polarometrically. The congruence reveals that ^{31}P-NMR spectroscopy is an effective indicator of intracellular pH in fern spores. Electron microscopic observation shows there is no vacuolar compartment in fern spores that would mix upon homogenation (Bassel *et al.*, 1981). The lack of a large vacuolar compartment is confirmed by the ^{31}P-NMR spectra which has only a single phosphate peak. Two peaks, corresponding to the vacuolar and cytoplasmic phosphate, are found in spectra of vacuolate *Onoclea* leaves (data not shown). The NMR protocol does not harm the spores (Table 2). Even spores irradiated in the tube, spun for 8 hr in the dark, removed, and placed on medium in the dark will still germinate. The above protocol does not stimulate dark germination of spores.

Because we did not observe a red light-induced change in intracellular pH, we tested whether artificially inducing a change in intracellular pH would prevent germination in the light or stimulate germination in the dark. By sowing the spores in media buffered between pH 4.00 and 9.00, the intracellular pH can be manipulated by about 1 pH unit (Fig. 3). Although the intracellular pH can be varied in magnitude greater than it would be expected to change if it were acting as an intracellular signal, germination of *Onoclea* spores in calcium-sufficient media is independent of intracellular pH in this range (Fig. 4).

Spores were homogenized and titrated with a strong acid and base to determine if the capacity of the spores to buffer themselves *in vivo* resulted from a passive buffering capacity of the intracellular medium. It takes an average of 6.20 μmole H^+/100 mg spores to change the pH by 1 unit (calculated from pH 3.87 to 9.03) compared to the 0.54 μmole H^+ it takes to change the equivalent volume of water by 1 pH unit. The titration curve of the cell homogenate plateaus twice; once at a pH less than 4 and again at a pH greater than 9 (Fig. 5). The *in vitro* cell homogenates are least buffered around the normal pH of the dry spore (pH 6.5).

In order to complement our experiments on intracellular pH, we investigated the effect of red light on proton efflux. Initially, using a Radiometer electrode, we found

TABLE 2
THE EFFECT OF THE NMR PROTOCOL ON GERMINATION

	Percentage germination	
	−NMR protocol	+NMR protocol
Dark	0.0 ± 0.0	0.1 ± 0.0
Red light	96.9 ± 1.5	96.3 ± 1.2

Note. Spores were sown in the dark on 10 m*M* Na ± phosphate-buffered medium including 1 m*M* $Ca(NO_3)_2$, 0.81 m*M* $MgSO_4$, and 3.45 m*M* KNO_3 (pH 4–9). After 24 hr, the spores were either kept in the dark or irradiated with red light (7.5 J m^{-2} sec^{-1}) for 2 min. Spores were either placed in the dark at 26.5°C or subjected to the NMR protocol (see Materials and Methods) and then removed and kept in the dark at 26.5°C. Percent germination was scored 48 hr after irradiation. Data are expressed as the mean ± 2 SEM.

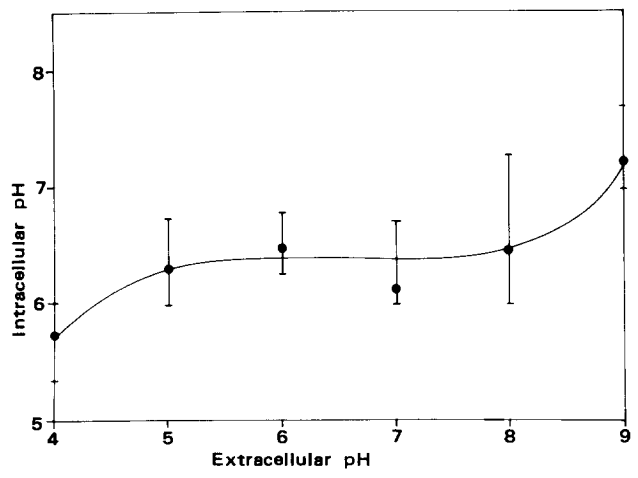

FIG. 3. The effect of external pH on intracellular pH. Five hundred milligrams of dry, sterilized spores were floated on phosphate-buffered medium (pH 4.00-9.00) as described under Materials and Methods. After 24 hr in the dark at 23°C the spores were collected by filtration, washed with distilled water, and placed in a 10-mm NMR tube, and rehydrated with just enough distilled water to moisten the spores. ^{31}P-NMR spectra (121 MHz) were obtained as described under Materials and Methods. Data are expressed as the mean ± 2 SEM.

that red light-stimulated proton efflux in the spores of *O. sensibilis* with a lag time of less than 1 min. However, this result was due to the electrode's sensitivity to light. Using a Beckman 39505 combination electrode which does not respond to light, we have been unable to detect proton efflux from *Onoclea* spores (spore density = 1–17 mg dry weight/ml) following red irradiation (Wayne, 1985).

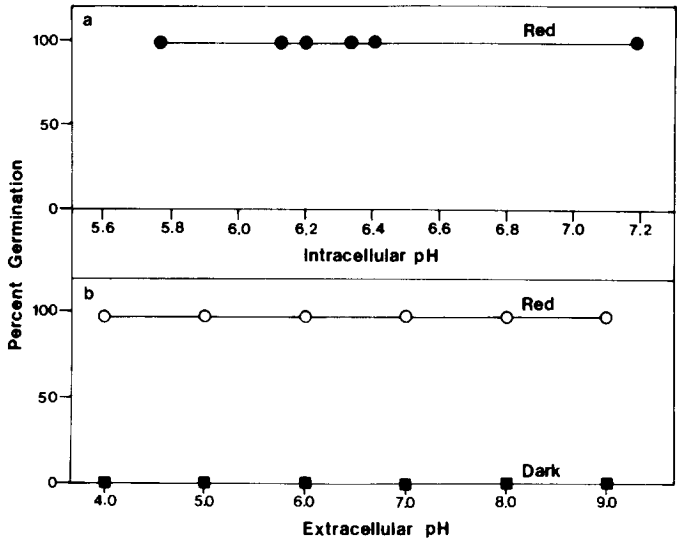

FIG. 4. The effect of intracellular and extracellular pH on germination. Sterile spores (2 mg) were sown on 10 ml of 10 m*M* Na-phosphate-buffered medium (pH 4.00-9.00) in the dark for 24 hr at 23°C. Spores were then irradiated with 5 min of broad-band red light at an energy fluence rate of 2.4 J m^{-2} sec^{-1} and again placed in the dark for 48 hr at 26.5°C and the percentage germination was determined. Two standard error bars are smaller than the symbols.

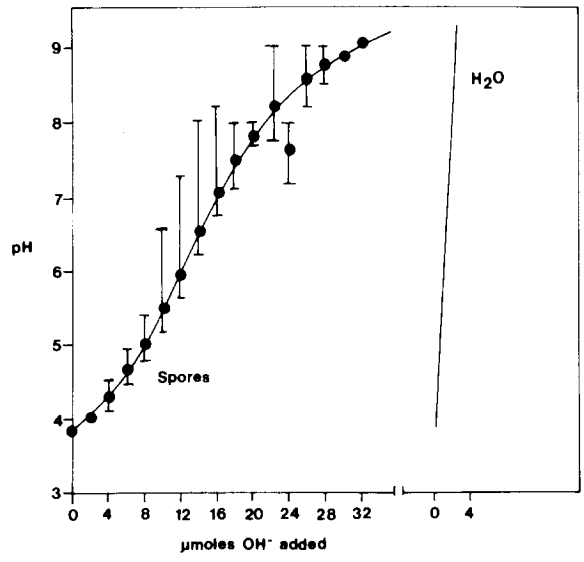

FIG. 5. Potentiometric titration curve of *Onoclea* spore cell homogenates. Frozen spores were mechanically homogenized and the pH was measured. The samples were vigorously aerated to eliminate buffering from dissolved CO_2. The homogenate was titrated with 0.1 *N* NaOH to greater than pH 9, back titrated to less than pH 4 with 0.1 *N* HCl, and again titrated with 0.1 *N* NaOH to above pH 9. Data are expressed as the mean ± 2 SEM.

DISCUSSION

Activation of many quiescent cells, including eggs and oocytes, results from an increase in the intracellular free [Ca^{2+}] (Cuthbertson *et al.*, 1981; Gilkey *et al.*, 1978; Jaffe, 1980, 1983; Johnson *et al.*, 1976; Moreau *et al.*, 1980; Ridgway *et al.*, 1977; Steinhardt *et al.*, 1977). This increase is followed by an acid efflux (Epel, 1979, 1980; Gilles *et al.*, 1981a; Ii and Rebhun, 1979; Mehl and Swann, 1976; Paul, 1975) and a subsequent rise in the intracellular pH (Iwamatsu, 1984; Johnson *et al.*, 1976; Lee and Steinhardt, 1981a, 1981b; Nuccitelli *et al.*, 1981; Shen and Steinhardt, 1978, 1979; Webb and Nuccitelli, 1981, 1982). The increase in intracellular pH stimulates DNA and protein synthesis in sea urchin eggs while blocking the natural increase in intracellular pH prevents the stimulation (Grainger *et al.*, 1979; Mazia and Ruby, 1974; Winkler *et al.*, 1980, 1985). These data have led to the hypothesis that an increase in pH is part of the signal transduction chain in the activation of animal eggs. However, this theory does not receive unanimous support (Gilkey, 1983; Jaffe, 1980). For example, there is not a particularly good correlation between the time courses and magnitudes of induced pH increase and change in the rate of protein synthesis following fertilization or ammonia treatment. Additionally, altering the intracellular pH affects the rate of protein synthesis in starfish eggs and oocytes, even though changes in the rates of protein synthesis following fertilization and application of the hormone 1-methyl-adenine, respectively, are not accompanied by a change in intracellular pH (Johnson and Epel, 1982).

Furthermore, an increase in pH is not necessary for the stimulation of protein synthesis, increased phosphorylation, or germinal vesicle breakdown in frog eggs (Stith and Maller, 1985), and finally, the prevention of an increase in intracellular pH in medaka (Gilkey, 1983) and frog (Lee and Steinhardt, 1981a) eggs does not inhibit activation. The increase in intracellular pH that occurs after fertilization may contribute to the signal transduction chain in sea urchin eggs, but it certainly is not a universal element in the activation of quiescent cells.

We are unable to find any evidence that a red light-induced change in intracellular pH or proton efflux contributes to the signal transduction chain in the activation of the germination response in *Onoclea* spores. Indeed, we present evidence that germination is independent of intracellular pH in the range of 5.8 to 7.2. Dark sown spores of *Onoclea* are metabolically active even though they will not germinate (Ikuma and Chen, 1983). Perhaps an increase in intracellular pH only contributes to the activation of metabolically dormant cells (Johnson and Epel, 1982). It is also possible that red light induces a rapid, transient change in intracellular pH which is not detected by NMR spectroscopy.

The cytoplasm of fern spores is highly buffered. The titration curve is nearly identical to the titration curve of nerve axoplasm (Spyropoulos, 1960). Interestingly, the ranges of maximum passive buffering capacity *in vitro* do not correspond to the range of maximum buffering *in vivo*. This illustrates the importance of compartmentation and active sequestering and/or efflux to the regulation of intracellular pH (Smith and Raven, 1979; Sze, 1985). Further support for the role of proton pumping comes from the observation that DCCD, an inhibitor of the proton pump, prevents the regulation of intracellular pH (Torimitsu *et al.*, 1984). Observations on the effect of external pH on vacuolate root tip cells of mung bean show that the cytoplasm of root cells is highly buffered while the pH of the vacuole varies widely. The large vacuole in root cells may serve as a source or sink for "cytoplasmic protons" thereby allowing the cytoplasmic pH to remain around neutrality (Torimitsu *et al.*, 1984). This mechanism of cytoplasmic buffering may not be possible in *Onoclea* spores which do not possess a large vacuolar compartment.

Further support for the lack of correlation between plant membrane effectors and intracellular pH comes from the work of Roberts *et al.* (1980, 1981, 1982). Both the fungal metabolite fusicoccin and 25 m*M* K_2SO_4 induce a massive increase in proton efflux in corn roots that does not result in a detectable change in intracellular pH. However, changes in cytoplasmic pH are involved in the regulation of pyruvate decarboxylase activity which contributes to the survival of the maize roots under hypoxia (Roberts *et al.*, 1984).

In conclusion, red light does not cause a sustained increase in intracellular pH in fern spores. Thus a sustained change in pH does not contribute to the signal transduction chain in phytochrome-mediated fern spore germination. A comparison of the involvement of Ca^{2+} and intracellular pH in the activation of a variety of cell types seems to indicate that calcium ions but not protons (or hydroxyls) are the common activators of quiescent cells (Jaffe, 1980; Steinhardt *et al.*, 1974).

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