Measurement of the Cytoplasmic pH of *Dictyostelium discoideum* Using a Low Light Level Microspectrofluorometer

Ruth Furukawa,[*‡] John E. Wampler,[*] and Marcus Fechheimer[‡]

*Department of Biochemistry and ‡Department of Zoology, University of Georgia, Athens, Georgia 30602

Abstract. Pyranine was employed as a sensitive pH indicator in a low light level microspectrofluorometer. The in vivo and in vitro standard curves of the 460/410-nm fluorescence excitation ratio of pyranine as a function of pH are identical. Therefore, pyranine is specifically sensitive to cytoplasmic pH in *Dictyostelium*. The cytoplasmic pH of single cells in a population of *Dictyostelium discoideum* amoebae was obtained for the first time. The median cytoplasmic pH of vegetative amoebae was 7.19. Carbonyl cyanide *m*-chlorophenylhydrazone, a mitochondrial uncoupler and a protonophore, lowered the median cytoplasmic pH to 6.12 when the extracellular pH was 6.1. This result is in accord with the protonophore activity of carbonyl cyanide *m*-chlorophenylhydrazone. Interest in the cytoplasmic pH of *Dictyostelium* has been greatly stimulated by the theory that cytoplasmic acidification promotes development of pre-stalk cells, while cytoplasmic alkalinization favors the pre-spore pathway (Gross, J. D., J. Bradbury, R. R. Kay, M. J. Peacey. 1983. *Nature (Lond.).* 303:244–245). The theory postulates that diethylstilbestrol (DES), an inducer of stalk cell differentiation and a plasma membrane proton translocating ATPase inhibitor, should cause acidification of the cytosol. Previous measurements of the effects of stalk cell inducers including DES on intracellular pH using ^{31}P nuclear magnetic resonance measurements have failed to confirm the predictions of the theory, and have suggested that significant modification of the model may be required. Using pyranine as the pH indicator, we find that the median cytoplasmic pH in cells treated with 10 μM DES dropped from 7.19 to pH 6.02. This effect is consistent with the pharmacological action of DES and with the proposal that DES, a stalk cell inducer, should acidify the cytosol. These results provide direct support for the theory that cytoplasmic pH is an essential regulator of the developmental pathway in *Dictyostelium*.

T HE significance of the regulation of pH both in the extracellular milieu and in intracellular spaces has been appreciated since the earliest days of biological experimentation. Cells expend energy to control the pH of distinct subcellular compartments at differing values. In addition, changes in the cytoplasmic pH (pH_i)[1] have been implicated in regulation of cellular metabolism, motility, the cell cycle, and of the activation and fate of cells during development (Roos and Boron, 1981; Busa and Nuccitelli, 1984).

The cellular slime mold *Dictyostelium discoideum* has a complex developmental life cycle involving differential gene expression, cell–cell interaction, morphogenetic movements, and terminal differentiation into a mature fruiting body containing spore and stalk cells. Our interest in the pH_i of *D. discoideum* is motivated by the following: (*a*) regulation of gelation and contraction in motile extracts of *D. discoideum* (Condeelis and Taylor, 1977; Taylor and Fechheimer, 1982) as well as purified actin binding proteins such as α-actinin

from *D. discoideum* (Fechheimer et al., 1982); (*b*) changes in pH_i during the cell cycle (Aerts et al., 1985); and (*c*) the potential role of pH_i as a regulator of differentiation in *Dictyostelium*. Specifically, it has been hypothesized that cytoplasmic acidification promotes the development of pre-stalk cells, while cytoplasmic alkalinization favors the pre-spore pathway (Gross et al., 1983). In addition, both ammonia and extracellular pH (pH_o) regulate slug migration, orientation, and the transition to the formation of the mature fruiting body (Schindler and Sussman, 1977; Williams et al., 1984; Bonner et al., 1986).

Measurements of pH_i can be classified in two distinct categories: (*a*) those based on methods that average pH from an entire cell or population of cells, and (*b*) those that permit spatial resolution of the measurement at the subcellular level. Roos and Boron (1981), and Nuccitelli and Deamer (1982) have reviewed the procedures and their relative merits. The pH_i of *Dictyostelium* has been measured by a number of methods in the first category: (*a*) ^{31}P nuclear magnetic resonance (Jentoft and Town, 1985; Satre and Martin, 1985; Satre et al., 1986; Kay et al., 1986; Town et al., 1987; Martin et al., 1987); (*b*) partitioning of a weak acid (Jentoft and Town, 1985); and (*c*) immersion of a pH electrode into a so-

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1. *Abbreviations used in this paper:* BCR, baseline corrected ratio; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DES, diethylstilbestrol; NMR, nuclear magnetic resonance; pH_i, cytoplasmic pH; pH_o, extracellular pH.

lution of digitonin lysed cells (Aerts et al., 1985, 1986, 1987). Perhaps the most useful technique with the potential for spatial resolution at the subcellular level is the use of pH-sensitive fluorescent dyes (Nuccitelli and Deamer, 1982). A number of groups have employed this approach in studies of *D. discoideum* pH_i using a variety of fluorophores including: (*a*) fluorescein (Jamieson et al., 1984; Ratner, 1986); (*b*) 6-carboxyfluorescein (Inouye, 1985); and (*c*) fluorescein- and rhodamine-labeled dextrans (Fechheimer et al., 1986). All of these studies have reported only the average pH of the population, and have not reported the pH_i of single cells in a population. The primary pitfalls in measurements of cytosolic pH of *Dictyostelium* with membrane permeant diacetate derivatives of fluorescein are rapid leakage of the free fluorophore out of the cell, delivery of acid to the cell upon cleavage of the acetate esters, poor sensitivity of fluorescein to pH at pH $\geqslant$ 7.0, and sensitivity of fluorescein fluorescence to unknown aspects of the intracellular environment leading to a large difference between in vitro and in vivo standard curves.

In this study, we measure pH_i using an indicator fluorophore, pyranine, which has several advantageous features (Wolfbeis et al., 1983) lacking in the more commonly employed fluorophores such as fluorescein. The major problems outlined above have been circumvented. Using microspectrofluorometry, we have studied the intracellular pH of single cells within populations of *D. discoideum* amoebae and studied the effects of carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), a protonophore and mitochondrial uncoupler, and diethylstilbestrol (DES), an inhibitor of the plasma membrane proton pumping ATPase and an inducer of stalk cell differentiation of *Dictyostelium*, on the cytoplasmic pH. The results are consistent with the theory that pH_i may regulate the choice of differentiation pathway in *Dictyostelium*. Previous measurements of pH_i in *Dictyostelium* using either fluorescence (Ratner, 1986) or ³¹P NMR (Kay et al., 1986; Jentoft and Town, 1985; Town et al., 1987) have been inconsistent with the predictions of this theory with one exception (Inouye, 1985).

Materials and Methods

Cell Culture

Dictyostelium discoideum strain AX-3 was cultured axenically in HL-5 broth as previously described (Loomis, 1971). *D. discoideum* strain NC-4 were cultured on 2% nutrient agar in petri dishes in the presence of *Escherichia coli* B/r as described previously (Bonner, 1947). Cells were harvested after 24 h of vegetative growth before any clearing of the bacterial lawn could be detected.

Labeling of the Cells

Pyranine was introduced into the NC-4 amoebae by the sonication loading technique (Fechheimer et al., 1986; Fechheimer and Taylor, 1987). Briefly, the cells were washed in supplemented Sorensen's buffer containing 2 mM Na_2HPO_4, 15 mM KH_2PO_4, 1 mM $CaCl_2$, pH 6.1, supplemented with 10 mM glucose and 0.5 mg/ml each of tryptophan, phenylalanine, threonine, lysine, methionine, glycine, isoleucine, leucine, valine, arginine, and histidine as described previously (Marin, 1976). The addition of glucose and amino acids is thought to facilitate amoeba recovery from the shock of sonication and to help maintain the cells in a vegetative state. Approximately 30 million amoebae were suspended in 1.5 ml of 100 mM pyranine that was previously dissolved in supplemented Sorensen's buffer, sonicated at power one for 3 × 0.5 s using a sonicator (model 200; Branson Sonic Power Co.,

Danbury, CT), and diluted immediately into 8 ml of ice-cold supplemented Sorensen's buffer to prevent pinocytosis. The cells were washed in the supplemented Sorensen's buffer with 4 mM probenecid to remove the free fluorophore. The addition of probenecid (Sigma Chemical Co., St. Louis, MO) to the buffer helped to prevent the sequestration of the pyranine into vesicles and to prevent loss of the dye from the amoebae. Probenecid has been shown to inhibit clearance of anionic fluorescent dyes from the cytoplasm of macrophages (Steinberg et al., 1987). The fluorophore distribution was assessed by fluorescence microscopy. The pyranine was distributed in the cytosol with few or more frequently no fluorescent vesicles. The fluorophore did not leak from the cells detectably. In all cases, the measurements were completed within 4.5 h of the introduction of the fluorophore to the amoebae. At longer times, sequestration of the dye into vesicles and subsequent clearance from the cells was detectable even in the presence of probenecid. The sonication loaded cells are able to develop into mature fruiting bodies. Thus, neither the sonication nor the concentration of dye to which the cells are exposed are harmful to the health of the cells.

Approximately one million *D. discoideum* cells in 2 ml of supplemented Sorenson's buffer were allowed to adhere 15 min to the glass coverslip in a teflon holder (Bionique Laboratories Inc., Saranac Lake, NY) before measurements were taken while the remainder of the cells were placed on a rotating shaker. After the introduction of drugs, or the weak acid or base, the cells were allowed to stand an additional 15 min and measurements were performed for 30 min.

The cells were exposed to light only for the duration of the measurement, ∼15 s that was required to obtain one baseline corrected ratio (BCR) value. Each BCR value is an average of 10 measurements from both sample and reference locations at each of three excitation wavelengths, 460, 410, and 500 nm. Methods for microspectrofluorometry and measurement of the BCR are discussed below. The BCR measurement was repeated at least three times on each cell. The average BCR value was used in all subsequent calculations.

Drugs

DES (Calbiochem-Behring Corp., La Jolla, CA) and CCCP (Sigma Chemical Co., St. Louis, MO) were prepared fresh as stock solutions, 0.01 M and 0.2 mM, respectively, in ethanol and subsequently diluted into the supplemented Sorensen's buffer with probenecid to final concentrations of 10 and 5 μM, respectively. Cells treated with an equivalent dilution of solvent lacking drug were never detectably different in either morphology or pH_i from cells in supplemented Sorenson's buffer. The addition of probenecid did not inhibit the development of *D. discoideum* into mature fruiting bodies.

Standard Solutions

The fluorescence excitation properties of pyranine (Molecular Probes Inc., Eugene, OR) were measured as a function of pH in "standard" solutions containing 100 mM KCl, 2 mM $MgCl_2$, 40 mM Hepes, 40 mM Tris, 40 mM MES (2-[*N*-Morpholino]ethanesulfonic acid), and 50 μM pyranine and adjusted to the desired pH using either KOH or HCl. The fluorescence properties of these standard solutions was also measured in the presence of 10% (vol/vol) of lysed *Dictyostelium discoideum* strain AX-3 cells. The AX-3 cells were harvested by low speed sedimentation and exhaustively washed free of HL-5 with distilled, deionized water. The pellet of packed cells was lysed by freezing at −20°C. The frozen lysed cells were thawed and equilibrated to room temperature just before use.

Microspectrofluorometry

The microspectrofluorometer was designed in our laboratory (Rich and Wampler, 1981; Wampler, 1986) and is depicted in Fig. 1. Briefly, the optical components of this instrument consists of a 75-W xenon arc lamp (Carl Zeiss, Inc., Thornwood, NY), variable wavelength excitation and emission filters, and a Zeiss 40× microscope objective (NA = 1.0) in the inverted epifluorescence geometry. The excitation wavelength is changed by a stepping motor attached to the variable wavelength filter. The fluorescence emission is detected through a long pass filter with 50% transmission at 530 nm (Oriel 5130). The image detector involves two stages of microchannel plate image intensification, and detection by a SIT Vidicon. Detection with this system is essentially photon limited (Rich and Wampler, 1981).

In our instrument, we use the spatial resolution of the camera to obtain an effective double beam fluorescence measurement as a partial correction for stray light (Wampler et al., 1988). The signal from two pixel locations
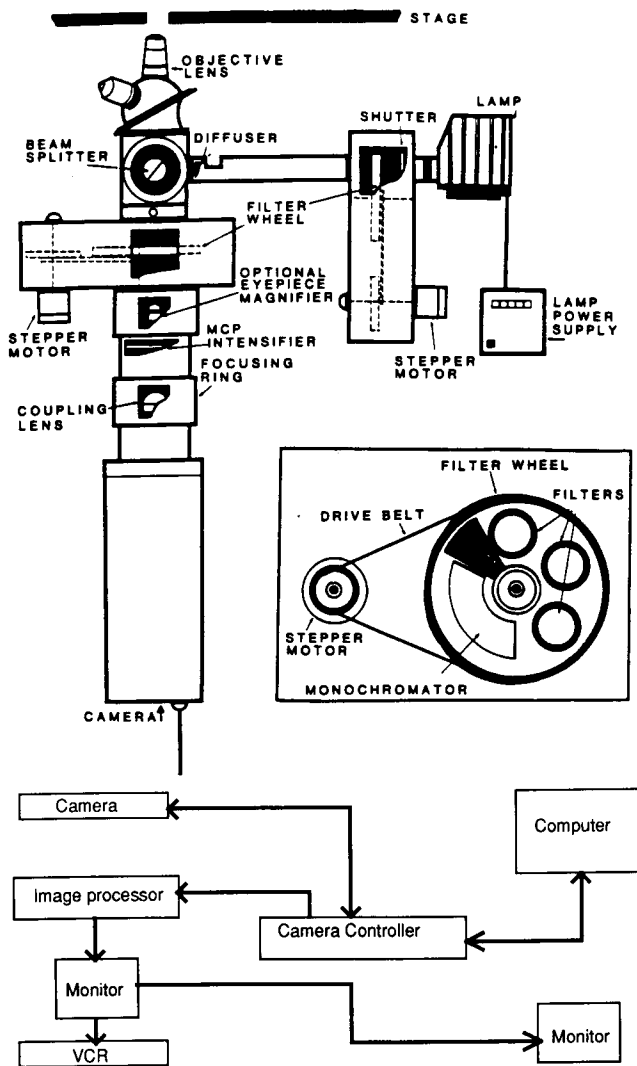
Figure 1. A schematic diagram of the microspectrofluorometer.

are measured. The first is within the fluorescent area of the sample and the second is from a location that is nonfluorescent. In addition, the fluorescence properties measured were found to be more reproducible if a signal at one wavelength on the spectrum is assigned as the arbitrary baseline point and subtracted from the signal at each of the other wavelengths. Thus, the pseudo-baseline corrected fluorescence excitation spectrum I_λ is determined as follows:

$$I_\lambda = [S_\lambda - S_b] - G[R_\lambda - R_b],$$

where S and R are the sample and reference pixels, respectively, λ and b are measurement and baseline wavelengths, respectively, and G is the spatial gain corection factor that compensates for the difference in sensitivity of the camera at the sample and reference positions. Fluorescence excitation spectra were measured from 370 to 500 nm using a baseline wavelength of 500 nm.

The fluorescence excitation ratio method was used to measure the pH-dependent spectral shape change. This method has the advantage of speed and corrects for the varying pathlength and concentration of the fluorophore inside living cells by taking the ratio of two signals from the spectrum. The baseline corrected fluorescence excitation ratio (BCR) was determined as follows:

$$BCR = \frac{[S_1 - S_b] - G[R_1 - R_b]}{[S_2 - S_b] - G[R_2 - R_b]}$$

where S and R are the sample and reference pixels, respectively, 1, 2, and

b are the wavelengths 460, 410, and 500 nm, respectively, and G is the spatial gain correction factor. pH measurements at any point in the microscope field reflect contributions from both in focus and out of focus sources of light.

In Vitro Standard Curve

The BCR of pyranine standard solutions was obtained by placing the solution in a cylindrical hole cut in a sheet of Sylgard, an optically clear ($n_D = 1.41$ at 77°F), silicone-based elastomer (Dow-Corning Corp., Midland, MI) that was previously glued with silicone adhesive to a microscope cover slip (No. 0 thickness). The top of the cylinder was coated with microscope oil (Carl Zeiss, Inc.) ($n_D = 1.53$) and subsequently covered with a microscope slide. The sample pixel was inside the fluorescent solution and the reference pixel was in the Sylgard. The in vitro standard curve refers to data taken with the pyranine standard solutions in the presence or absence of 10% (vol/vol) of lysed AX-3 cells. Data for standard curves were fit to a cubic polynomial using non-linear least squares (Bevington, 1969).

In Vivo Standard Curve

The internal pH of the *D. discoideum* amoebae was equilibrated to that of the extracellular pH by the partitioning effect of weak acids or weak bases (Roos and Boron, 1981). The ability to effectively clamp intracellular pH using high concentrations of weak acid or weak base has been documented previously (Heiple and Taylor, 1980; Pollard et al., 1979). The solutions consisted of 40 mM each of MES, morpholino propane sulfonic acid (MOPS), and Tris to which 40 mM of either sodium acetate or ammonium chloride was added. The pH was adjusted to the desired value with either KOH or HCl. A portion of the amoebae was incubated in the weak acid or base solution at the desired pH value for 15 min. After 15 min, the BCR values were measured for 30 min. The value of the fluorescence excitation ratio reaches a plateau value in <15 min after initial exposure to the weak acid or base solution under these conditions (Fechheimer et al., 1986). The in vivo standard curve refers to the data taken with NC-4 amoebae whose pH_i was equilibrated to the pH_o by the partitioning of weak acids or bases.

Results

Pyranine Is a Specific and Sensitive Probe of pH_i in Dictyostelium

The fluorescence properties of most fluorophores are sensitive to their environment. It is essential to determine that the change in the fluorescence properties of the indicator dye are due to the property of interest and not due to nonspecific environmental changes. The property of interest in this study is the intracellular pH of *D. discoideum*. The fluorescence excitation spectra of pyranine were determined in the standard solutions at pH 8.75 and 5.25 in the presence and absence of 10% (vol/vol) lysed AX-3 cells as shown in Fig. 2. These measurements were performed on the standard solutions containing 10, 15, and 20% (vol/vol) lysed *D. discoideum* AX-3 cells. The fluorescence excitation spectra were identical within experimental error in the presence of 10, 15, and 20% (vol/vol) lysed AX-3 cells. We have chosen to use 10% (vol/vol) lysed AX-3 cells in all measurements since the solutions are extremely turbid at higher lysed cell concentrations. The fluorescence excitation spectra are distinctly different as a function of pH. In addition, the fluorescence excitation spectrum at a given pH is the same within experimental error in the presence or absence of the lysed amoebae.

The fluorescence spectral shape can be characterized analytically by the method of moments (Mulkerrin and Wampler, 1982). The first and second moments of the spectral distribution correspond to the mean wavelength position and dispersion, respectively. In Table I, the mean wavelength and
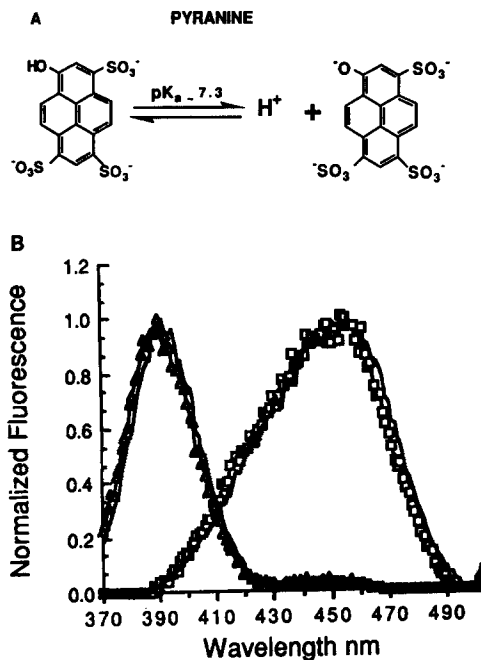
Figure 2. (A) The structure of pyranine. (B) The normalized fluorescence excitation spectra of pyranine in the standard solutions (see Materials and Methods) in the presence and absence of 10% (vol/vol) lysed AX-3 cells. The spectra were measured from 370 to 500 nm with a baseline wavelength of 500 nm. △ and □ are pyranine in pH 5.25 and 8.75 standard solutions, respectively. The solid lines corresponding to each curve are the fluorescence excitation spectra taken in the presence of 10% of lysed AX-3 cells. The difference between the mean wavelength values, 6 nm, was subtracted from the spectra at pH 5.25 in the presence of AX-3 cells to compare the spectral shape. This value is within the experimental value of the measurement.

dispersion of the fluorescence excitation spectra in the presence and absence of the lysed cells are given for a range of pH values. The relative error of these moments at high pH values is 1% and at low pH values 2%. The mean wavelength positions and dispersions of the spectra recorded at a variety of pH values are not significantly affected by the addition of

Table I. Effects of pH and Environment on the Fluorescence Excitation Spectrum of Pyranine

pH	Standard solutions		Standard solutions + AX-3	
	Mean	Dispersion	Mean	Dispersion
	nm	nm	nm	nm
8.75	444	21	446	21
8.25	449	20	446	20
7.75	448	20	444	24
7.25	438	25	439	26
6.75	426	30	427	30
6.25	403	25	412	28
5.75	397	14	404	20
5.25	394	11	400	13

The first spectral moment (mean wavelength) and second spectral moment (spectral dispersion) of the fluorescence excitation spectra of pyranine as a function of pH in the presence and absence of 10% (vol/vol) lysed *D. discoideum*.

10% lysed amoebae. These results indicate that the changes in the fluorescence excitation spectrum are due to differences in pH and not to the change in the local fluorophore environment due to the presence of cellular material. It is also noteworthy that the mean wavelength and dispersion of the spectral distribution for pyranine did not change within experimental error in the presence of either 10 μM DES or 5 μM CCCP (data not shown). Thus pyranine is a fluorophore that is insensitive to many environmental changes and is a sensitive pH indicator.

Measurement of intracellular pH by microspectrofluorometry requires calibration of the measured fluorescence excitation ratio or spectral shape change to pH. One problem that greatly complicates the relationship of the fluorescence properties to pH is that the in vivo and in vitro pH calibration curves are often not superimposeable (Heiple and Taylor, 1980). This leads to the question of which calibration curve to use when converting the data of interest to pH. In Fig. 3, the in vivo and in vitro calibration curves for pyranine as a function of pH are shown. The measurements of BCR with sodium acetate for pH > 7.0 and with ammonium chloride for pH < 6.5 were omitted, since the values were constant as expected (i.e., weak acids cannot equilibrate pH_o to pH_i when $pH_o \geqslant pH_i$ and weak bases cannot equilibrate pH_o to pH_i when $pH_o \leqslant pH_i$). The in vitro standard curves shown in Fig. 3 are pyranine in the presence or absence of lysed *D. discoideum* cells. The in vivo and in vitro BCR values as a function of pH are superimposeable within the error of these measurements. This result is unique to the authors' knowledge. It confirms the opinion of Wolfbeis et al. (1983) that pyranine is a pH-indicating dye near physiological pH values that is relatively insensitive to other environmental factors. There is no discrepancy between the in vivo and in vitro data. For this reason, we have chosen to compare the BCR obtained on live cells to the in vitro standard curves which are more easily and quickly measured.

An additional problem that is inherent in microspectrofluorometry is that the optical components such as the lamp or monochromators are not of the quality and stability often found in conventional research fluorometers. This problem is due to the spatial constraints in instrumental geometry. One consequence of this problem is that the BCR of in vitro standard solutions was not reproducible for more than a period of 2 wk. This problem has been noted previously (Bright et al., 1987). Thus values of pH were calculated from the in vitro standard data taken within a few days (with a maximum of 1 wk) of a given experiment.

pH_i of Dictyostelium

The cytoplasmic pH of single vegetative *D. discoideum* amoeba are shown in Fig. 4. The median pH for these cells given in Table II is 7.19. The typical standard deviation of any individual value of the pH is ~3%. Thus, the distribution of the values of pH shown reflects the heterogeneity of cytoplasmic pH in the population rather than the dispersion of the measurements. 50% of the cells had a cytoplasmic pH between 6.99 and 7.38 (the first and third quartiles; Table II). In this case the distribution function appears Gaussian. In Table III our median pH value is compared to results from other workers. A cytoplasmic pH of 7.19 is in accord with results obtained using carboxyfluorescein (Inouye, 1985)
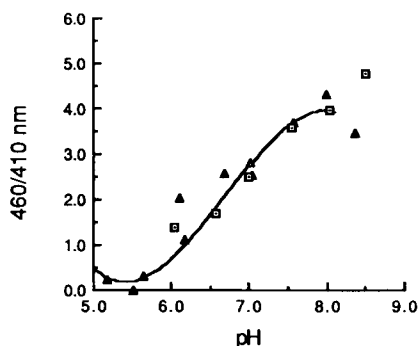
Figure 3. The BCR of pyranine measured in vitro and in vivo as a function of pH. The BCR values of pyranine measured in vivo in the presence of either 40 mM sodium acetate or 40 mM ammonium chloride are given by △ and □, respectively. ▲ is the average BCR value of pyranine in the presence or absence of 10% (vol/vol) lysed AX-3 cells. The solid line through the in vitro BCR values is an example of the result of a cubic polynomial equation fit of the data (see text for further details).

and fluorescein (Ratner, 1986), by the null-point method (Aerts et al., 1985), weak acid distribution (Jentoft and Town, 1985), and by ^{31}P NMR (Kay et al., 1986; Martin et al., 1987).

Effect of DES and CCCP on pH_i of Dictyostelium

The median pH_i of the vegetative *D. discoideum* amoebae in the presence of 10 µM DES is 6.02 (Table II). The pH_i is shifted remarkably towards acidic values of pH as shown in Fig. 6. The typical standard deviation for any individual value of pH is ~3%. The distribution of the values of pH is remarkably small and perhaps can be characterized by a Gaussian distribution. This result is in accordance with the data which shows that DES is an inhibitor of the plasma membrane proton pump in *D. discoideum* (Pogge-von Strandmann et al., 1984; Serrano, et al., 1985).

The median pH_i of vegetative *D. discoideum* amoebae in the presence of 5 µM CCCP with an external pH of 6.1 is 6.12 (Table II). The pH_i values are shifted to acidic values of pH_i as shown in Fig. 5. The values of pH_i cannot be described by a Gaussian distribution. The drug CCCP is known to be an uncoupler of mitochondrial ATP production as well
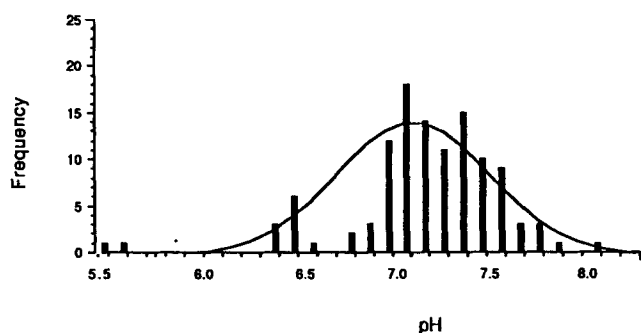

Figure 4. The cytoplasmic pH in single vegetative *D. discoideum* amoebae. The histogram interval is the total number of cells in a 0.1 pH unit increment. The continuous curve plotted is a fit of the mean and standard deviation of the data to a Gaussian distribution.

Table II. Median and Quartile Range of Intracellular pH of Dictyostelium discoideum

Drug	Q_1*	Median	Q_3‡	n§
DES	5.87	6.02	6.14	94
none	6.99	7.19	7.38	114
CCCP	6.04	6.12	6.23	65

* Q_1 is the first quartile, i.e., the value of the pH where $n = 25\%$.
‡ Q_3 is the third quartile, i.e., the value of the pH where $n = 75\%$.
§ n is the number of cells measured.

as a protonophore (Liberman and Topaly, 1968; Finkelstein, 1970). The proton transport efficiency of most weak acid protonophores depends on their pK_a (Liberman and Topaly, 1968). The proton transport efficiency decreases when pH_o deviates from the pK_a. The pK_a of CCCP is 6.0 in 57% ethanol (Buckingham, 1987). The external pH of the media was varied in the presence of 5 µM CCCP and the BCR was measured (Table IV). The mean pH of the *D. discoideum* cells increased as a function of increasing external pH but did not attain the value of the external media. This could be due to kinetic effects accompanying the decreased proton transport efficiency.

Discussion

Comparison of Measurements of Intracellular pH of Dictyostelium

The pH_i value characteristic of a population of cells is a weighted average. With measurements involving groups of cells this weighting is a complex function including influences of all cells regardless of their health or life cycle stage. In addition, measurements based on fluorescence (and probably those from other methods as well) suffer from nonlinear weighting due to the pH dependence of signal strength and the complex, nonlinear functional form of the measurement calibration curve (e.g., Fig. 3). With fluorescein-based measurements, for example, higher pH values give much stronger signals thus giving biased weighting to high pH individuals within the population. At the same time, the lack of pH resolution in the high pH region (pH > 7.0) with this dye makes it difficult to assign values with precision in the physiological range. The result is that measurements performed on suspensions of cells will be heavily influenced by any subgroup of cells having high pH. In addition, changes in the population pH will be masked to the extent that all cells within the population do not respond uniformly to a stimulus.

Single cell measurements also have potential problems. While individual cells may be selected for measurements, the selection process can be biased to cells with bright signal or of a particular morphological class. Even with good random sampling, the characteristic value obtained for a population of individually measured cells involves distinctly different weighting than the value obtained from measurements involving a group. Typically with the individual measurements, the measured value for each cell is converted into a pH before it is combined with the value for other cells. Thus, the weighting of signal strength and nonlinear calibration curves are different in the two cases.

A population of vegetative *Dictyostelium* amoebae is het-

Table III. Intracellular pH of Dictyostelium discoideum Amoeba

D. discoideum strain	External pH	Cytosolic pH	Method	Reference
AX-3	6.2	6.2 ± 0.1	Fluorescein	Jamieson et al., 1984.
AX-3	5.0-7.5	6.93 ± 0.21	Fluorescein	Ratner, 1986.
V12 M2	6.0	7.1	Carboxyfluorescein	Inouye, 1985.
NC-4	6.1	6.7	FITC-dextran	Fechheimer et al., 1986.
AX-2	6.6-7.4	7.3	null-point	Aerts et al., 1985.
V12 M2	5.2, 7.5	6.6, 6.9	weak acid	Jentoft and Town, 1985.
V12 M2	4.5-8.0	6.48 ± 0.02*	^{31}P NMR	Jentoft and Town, 1985.
AX-2	5.0-7.5	6.7 ± 0.1*	^{31}P NMR	Satre and Martin, 1985.
AX-3	–	6.7	^{31}P NMR	Ratner, 1986.
AX-2	3.5-7.0	7.33 ± 0.04	^{31}P NMR	Kay et al., 1986.
V12 M2	3.5-7.0	7.48	^{31}P NMR	Kay et al., 1986.
HM 29	4.3-7.8	6.5*	^{31}P NMR	Town et al., 1987.
AX-2	6.3	7.4 ± 0.2	^{31}P NMR	Martin et al., 1987.
AX-2	ND	7.3-8.0	null-point	Aerts, 1988.
NC-4	6.1	7.19	Pyranine	This study.

* The values cited in the table are those reported in the original manuscripts. These values may be revised due to reinterpretation of the assignments of the peaks in the ^{31}P NMR spectra of *Dictyostelium discoideum* (see Martin et al., 1987 and the text).

erogeneous. The cells present represent different stages in the life cycle, different metabolic states, and different degrees of loading with the indicator dye. At least one of these variables, the cell cycle, is thought to influence intracellular pH (Aerts et al., 1985; Aerts et al., 1987). Thus, we have no a priori expectation concerning the shape or breadth of the distribution function that characterizes pH within such a population. We can not expect it to be a normal distribution, or even an unimodal distribution. In such cases, it is good practice to use the median value as the location parameter to represent the characteristic pH for the population rather than the mean. Similarly, the interquartile range (the range containing the central 50% of the population) is used as a measure of dispersion rather than the standard deviation (Downie and Heath, 1974).

In this study, we have measured single cells in a microspectrofluorometer using pyranine as the indicator dye. Pyranine is a very sensitive indicator of pH_i, but is relatively insensitive to other aspects of the intracellular environ-

ment. The dynamic range of the pyranine BCR is large, with an ~15-fold change from the acidic to basic pH values studied here (Fig. 3). This result confirms the study of Giuliano and Gillies (Giuliano and Gillies, 1987) that pyranine is a sensitive pH_i indicator that is appropriate for use in the physiological range. In addition, we have determined with our cells and instrument that the in vitro and in vivo standard curves are identical which is a unique result (Fig. 3). There is no discrepancy between the standardization methods. These results indicate both that the weak acid and base treatments effectively fix the cytoplasmic pH at values equal to the extracellular pH, and that the response of the intracellular fluorophore is specifically sensitive for pH and is not affected by extraneous aspects of the local environment. Thus, a number of the major problems inherent in measurements of cytoplasmic pH using fluorescein are avoided with pyranine.

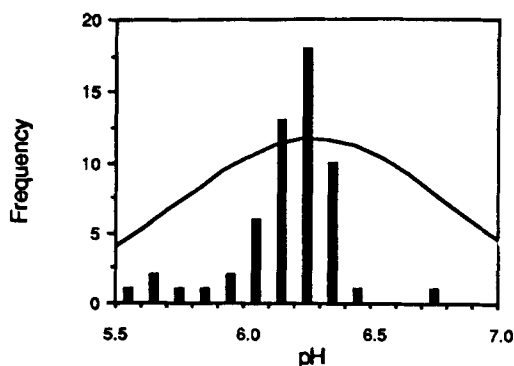The median cytosolic pH of 7.19 for vegetative *D. discoideum* amoebae measured in this study is in accord with

Figure 5. The pH_i of vegetative *D. discoideum* amoebae in the presence of 5 µM CCCP at an external pH of 6.1. The histogram interval is the total number of cells in a 0.1 pH increment. The continuous curve plotted is a fit of the mean and standard deviation of the data to a Gaussian distribution. Note the acidic shift of the pH_i values in the presence of CCCP.
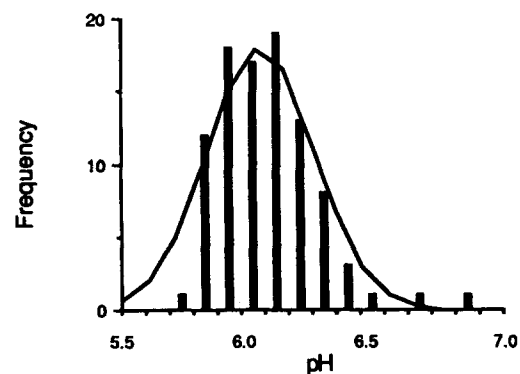

Figure 6. The pH_i of single vegetative *D. discoideum* amoebae in the presence of 10 µM DES. The histogram interval is the total number of cells in a 0.1 pH increment. The continuous curve plotted is a Gaussian distribution. Note the acidic shift of the pH_i values in the presence of DES.

Table IV. *Cytoplasmic pH of D. discoideum in the Presence of 5 μM CCCP as a Function of Extracellular pH*

pH$_o$	pH$_i$
5.5	5.89 ± 0.20
6.1	6.24 ± 0.51
6.5	6.31 ± 0.21
7.6	6.67 ± 0.29
8.5	6.61 ± 0.07

that obtained by the fluorescence excitation ratio method using carboxyfluorescein (Inouye, 1985) and fluorescein (Ratner, 1986), by the null-point method (Aerts et al., 1985, 1987), weak acid distribution (Jentoft and Town, 1985), and by ^{31}P NMR (Kay et al., 1986; Martin et al., 1987). These comparisons are summarized in Table III. In our study, the range of cytoplasmic vegetative pH values observed is broad (5.6 to 8.1), and the distribution function appears to be unimodal, symmetric and, perhaps, Gaussian (Fig. 4). The interquartile range is 6.99 to 7.38 (Table II). Treating these data as if they are from a normal distribution gives a mean of 7.14 and a standard deviation of 0.40. The heterogeneity seen is greater than the measurement error ($\sim$ ±0.2 at pH 7.2), larger than the range of pH oscillations (7.2–7.45) reported for the cell cycle in *Dictyostelium* (Aerts et al., 1985), and larger than the range from pH 7.23 to 7.43 that was estimated from the broadening of the line width of intracellular signals in ^{31}P NMR (Kay et al., 1986).

A number of other more acidic values have also been reported as summarized in Table III. The low values measured using fluorescein diacetate (Jamieson et al., 1984) may be caused at least in part by acid loading from cleavage of the diacetate within the cells. This problem may not have been noted by other workers using the diacetate analogues of fluorescein due to lack of in vivo calibration procedures (Ratner, 1986; Inouye, 1985). A significant shift in the effective pK_a toward more acidic values is seen for the in vivo standard curve for fluorescein-labeled dextran introduced into *D. discoideum* by sonication loading (Pruett, 1987). Similar shifts of the in vivo pH dependence of fluorescein spectra have been reported using other fluorescein analogues and methods for their introduction into cells (Heiple and Taylor, 1980; White, R. A., D. W. McCurdy, A. C. Harmon, P. L. Pruett, L. H. Pratt, M. Fechheimer, and J. E. Wampler, manuscript submitted for publication).

The only other study of the pH$_i$ of vegetative *D. discoideum* amoebae involving single cell measurements used flow cytometry (Fechheimer et al., 1986). While the population distribution of pH$_i$ was not reported, the average value of cytoplasmic pH measured in this study was 6.7. Fechheimer et al. (1986) used sonication loading to introduce fluorescein- and rhodamine-labeled dextrans into the cells. The presence of high concentrations of dextran may have deleterious effects on cells, and perhaps influence the pH$_i$. Use of high concentrations of fluorescein-labeled dextran (>100 mg/ml) for sonication loading results in a decrease in the recovery of loaded cells, and a median pH$_i$ well below 7.0 (Pruett, 1987).

The differences in cytosolic pH determined by ^{31}P NMR have been ascribed to an uncertainty in assignment of the peaks in the NMR spectra to cytoplasmic and mitochondrial compartments (Jentoft and Town, 1985; Town et al., 1987; Satre and Martin, 1985; Satre et al., 1986; Kay et al., 1986). Kay et al. (1986) assign the peak corresponding to a pH of 7.3 to the cytosol, and assert that the pH of the mitochondrial compartment cannot be detected in the ^{31}P NMR spectrum of *D. discoideum*. The groups of Town and Satre assign the peak corresponding to a pH of 6.7 to the cytosol, and the peak at 7.3 to the mitochondria. The Satre group has recently revised their interpretation of the NMR spectra. The chemical shift that had been assigned to a phosphate peak corresponding to a pH of 6.7 and assigned to the cytosol was identified as emanating from phytic acid (Martin et al., 1987). The new interpretation is that the mitochondrial compartment is not visible in *Dictyostelium* by NMR, and that the value of 7.3 is the pH of the cytosol. This interpretation agrees with that of Kay et al. (1986). This value of cytoplasmic pH also agrees well with our measurements using pyranine.

Intracellular pH of Dictyostelium in the Presence of the Mitochondrial Uncoupler CCCP

CCCP, a mitochondrial uncoupler, has been used in these studies by the Satre and Town groups to assign particular ^{31}P NMR chemical shifts to the cytosolic and mitochondrial compartments of the cells. The spectra recorded in the presence of CCCP show a collapse between the resonances initially assigned at pH 6.7 and 7.3, to a single broad peak corresponding to a pH of 6.9. These data were used to assign the higher pH compartment to the mitochondrion. However, the results of Martin et al. (1987) indicate that the peak at pH 7.3 emanates from phosphate in the cytosol (see above). The mitochondrial ATPase uncoupling induced by CCCP is probably due to the transport of protons by CCCP that dissipates the H$^+$ gradient in the mitochondria. Our measurements using pyranine show that the cytoplasmic pH is easily equilibrated to the external pH of 6.1 in the presence of CCCP (Fig. 5; Table II). Presumably, this effect is optimal because 6.1 is the pH value at which CCCP is most efficient at transporting protons. The intracellular pH was not entirely equilibrated to the external pH when pH$_o$ did not equal 6.1. The change in the ^{31}P NMR spectra upon the addition of CCCP is probably due to the approach of all cellular compartments to pH equilibrium, not just the mitochondria. Therefore, CCCP is not appropriate for use in assigning a particular ^{31}P NMR chemical shift to the mitochondrial compartment.

However, the recent reinterpretation of the ^{31}P NMR spectra (Martin et al., 1987) cannot explain the large difference between the cytoplasmic pH in the presence of CCCP measured either by ^{31}P NMR or by pyranine. For instance, in the presence of 5 μM CCCP we measure cytoplasmic pH values of 5.9 and 6.2 at extracellular pH values of 5.5 and 6.1, respectively (Table IV). By contrast, a cytoplasmic pH of 6.9 at an extracellular pH of 5.5–5.7 was measured in the presence of CCCP by ^{31}P NMR (Satre and Martin, 1985; Satre et al., 1986). The values differ by nearly a full pH unit which is greater than the measurement error estimated for any of the methods.

Intracellular pH of Dictyostelium in the Presence of DES, an Inhibitor of the Proton-translocating ATPase and an Inducer of Stalk Cell Differentiation

DES is an inhibitor of fungal types of plasma membrane

proton-translocating ATPases (Bowman et al., 1978). DES was found to be an effective inhibitor of the *Dictyostelium* enzyme (Pogge-von Strandmann et al., 1984; Serrano et al., 1985). Our results indicate that the cytoplasmic pH of *Dictyostelium* amoebae in the presence of DES is ~6.02 or more than a full pH unit less than that for untreated cells (Fig. 6; Table II). This result is in accord with the ability of DES to inhibit the plasma membrane proton pump in *D. discoideum* (Pogge von-Strandmann et al., 1984; Serrano et al., 1985) so that protons accumulate in the cytosol, lowering pH_i. We suggest from these results that the plasma membrane proton pump is important in the regulation of intracellular pH of *D. discoideum*. This interpretation agrees with that of Kay et al. (1986) who found no evidence for sodium-proton antiport or for sodium dependent bicarbonate-chloride exchange in maintenance of the cytoplasmic pH of *Dictyostelium*.

The effect of DES on the cytoplasmic pH of *Dictyostelium* is also significant to the development of *Dictyostelium*, because DES promotes the pre-stalk differentiation pathway in *Dictyostelium* (Gross et al., 1983). It has been hypothesized that the intracellular pH of *D. discoideum* during development determines the differentiation of the amoebae into pre-spore and prestalk cells (Gross et al., 1983). Specifically, agents such as weak acids (i.e., acetic acid), DES, or other inhibitors of the plasma membrane proton pump that are expected to lower pH_i were found to induce differentiation of the pre-stalk pathway, while agents such as ammonia and other weak bases expected to increase the intracellular pH favored differentiation to the pre-spore pathway (Gross et al., 1983). Therefore, the result of the present paper that DES induces a marked cytoplasmic acidification supports the cytoplasmic pH theory for the selection of a differentiation pathway in *Dictyostelium*. In addition, the effects of acetate and ammonia on intracellular pH (Fig. 3) confirm the expected effects of weak acid and weak base on cytoplasmic pH that are predicted by the pH theory for selection of a differentiation pathway.

By contrast, similar direct tests of this hypothesis from other laboratories have offered little or no support for the concept that intracellular pH plays a role in the selection of a differentiation pathway. For instance, the acidification of cytosol expected of a weak acid was not observed by ^{31}P NMR using concentrations of propionic acid that are sufficient to stimulate pre-stalk differentiation (Town et al., 1987). These authors observed a broadening of the phosphate peak in ^{31}P NMR upon treatment with propionate, but no shift of the peak indicative of acidification. In a separate study, it was reported that 10 mM sodium proprionate had no effect on cytoplasmic pH, but caused a decrease in mitochondrial pH (Satre et al., 1986). This result can now be interpreted as a change in cytoplasmic pH, since the assignment of the peaks has recently been revised (Martin et al., 1987). However, the cytosolic pH in the presence of a weak acid could not be measured in this study, since it was no longer resolved in the presence of 10 mM propionate (Satre et al., 1986). No change in the pH_i of *D. discoideum* in the presence of DES was detected using ^{31}P NMR (Kay et al., 1986). Similarly, other agents shown to stimulate differentiation in the pre-spore pathway induced no detectable change in cytoplasmic pH as assessed by ^{31}P NMR (Kay et al., 1986).

The primary direct discrepancy between our results and those from the Kay laboratory involve the effect of DES on cytoplasmic pH. We observe significant cytoplasmic acidification, while no effect is detected in their experiments. There are three main variables that could account for the results. First, completely different analytical methods were used to measure cytoplasmic pH in the two studies. Second, our measurements were performed on vegetative cells, while the ^{31}P NMR results were obtained on cells between 5 and 8 h of development. Third, there is a large difference in the time the cells were incubated in the presence of DES before the measurement of pH_i. The measurement of pH_i in this study commenced 15 min after the exposure of the amoeba to DES and was performed for 30 min afterward. DES was immediately effective in lowering the value of pH_i. The measurement of pH_i by ^{31}P NMR was performed 5 h after the initial exposure of the *Dictyostelium* cells to DES (Kay et al., 1986). Perhaps the amoebae have an efficient means of counteracting the initial effects of DES using a proton transporting channel or pump that is distinct from the DES-sensitive ATPase.

Development of *D. discoideum* cells into pre-spore and pre-stalk cells has also been shown to depend on the phase of the cell cycles in which the amoeba are starved (Gomer and Firtel, 1987). It is interesting to note that the cytoplasmic pH of *Dictyostelium* oscillates during the cell cycle (Aerts et al., 1985). Thus, the intracellular pH and cell cycle hypotheses are not mutually exclusive (Aerts et al., 1987). Rather, intracellular pH or the cell cycle or both may contribute to selection of a differentiation pathway in *Dictyostelium*. The study by Aerts (1988) published during our final revision of this manuscript also supports the idea that pH_i may influence the pathway of differentiation in *Dictyostelium*. We suggest that the pH theory for selection of a differentiation pathway remains viable, and must be rigorously tested using a variety of analytical methods until the many discrepancies in the experimental results have been resolved.

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