

Cytoplasmic pH of *Dictyostelium discoideum* Amebae during Early Development: Identification of Two Cell Subpopulations before the Aggregation Stage

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Abstract. Development of the cellular slime mold *Dictyostelium discoideum* is initiated by the removal of nutrients, and results in formation of a mature fruiting body composed of two cell types, the stalk and spore cells. A considerable body of evidence supports the hypothesis that cytoplasmic pH may be an essential regulator of the choice to differentiate in either the prestalk or prespore pathway. We have devised methods for measurement and analysis of intracellular pH in developing *Dictyostelium* amebae in order to assess directly the potential role of cytoplasmic pH in regulating the pathway of differentiation. The intracellular pH of single *D. discoideum* amebae during development and in intact slugs has been measured using the pH-sensitive indicator pyranine in a low light level microspectrofluorometer. We have used the ATP-mediated loading method to introduce pyranine into these cells. Cells loaded by the ATP method appear healthy, have no detectable defects in development, and exhibit a similar population distribution of intracellular pH to

those loaded by sonication. The intracellular pH of populations comprised of single amebae was found to undergo a transient acidification during development resulting in a bimodal distribution of intracellular pH. The subpopulations were characterized by fitting two gaussian distributions to the data. The number of cells in the acidic intracellular pH subpopulation reached a maximum 4 h after initiation of development, and had returned to a low level by 7 h of development. In addition, a random sample of single amebae within a slug had a median intracellular pH of 7.2, nearly identical to the median pH (7.19) of similarly treated vegetative cells. No gradient of intracellular pH along the anterior to posterior axis of the slug was detected. Our data demonstrate the existence of two distinct subpopulations of cells before the aggregation stage of development in *Dictyostelium*, and offers support for the hypothesis that changes in intracellular pH contribute to development in *D. discoideum*.

THE cellular slime mold *Dictyostelium discoideum* is an excellent model system for the study of development and cellular differentiation. The developmental program of *Dictyostelium*, initiated by removal of nutrients, involves differential gene expression, cell-cell interaction, morphogenetic movements, and terminal differentiation into a mature fruiting body (Loomis, 1982). The experimental system is simplified by the presence of only two terminally differentiated cell types. A wealth of circumstantial evidence supports the hypothesis that intracellular pH (Gross et al., 1983) is a potential regulator of differentiation in *Dictyostelium*. For instance, low extracellular pH, weak acids such as acetic acid, and proton pump inhibitors such as diethylstilbestrol promote the development of prestalk cells, while high extracellular pH and weak bases such as ammonia favor the prespore pathway (Gross et al., 1983; Gross et al., 1988). In addition, weak acids such as propionate have been shown to promote expression of early prestalk specific genes (Town et al., 1987). Finally, both ammonia and extracellular pH (pH_o) regulate slug migration, orientation, and the

transition to formation of the mature fruiting body (Bonner et al., 1986; Schindler and Sussman, 1977; Williams et al., 1984).

The pH theory for control of the differentiation pathway has been experimentally tested by direct measurements of intracellular pH. The validity of the theory was seriously questioned by three observations. (a) The changes in cytoplasmic pH expected in cells treated with inducers of prestalk or prespore differentiation were not detected by ³¹P nuclear magnetic resonance (NMR)¹ (Kay et al., 1986; Satre et al., 1986; Satre and Martin, 1985; Town et al., 1987). (b) The pH_i of developing cells was found to be remarkably constant (Jentoft and Town, 1985; Kay et al., 1986; Satre et al., 1986; Satre and Martin, 1985; Town et al., 1987) with one exception (Jamieson et al., 1984). (c) No difference (Ratner, 1986) or only a small difference (Inouye, 1985) in the pH_i of prestalk and prespore cells was detected in studies of iso-

1. *Abbreviations used in this paper:* BCR, baseline corrected fluorescence excitation ratio; NMR, nuclear magnetic resonance.

lated cell populations separated by density gradient centrifugation.

We have developed reliable and sensitive methods for monitoring the pH_i in single vegetative amoebae of *Dictyostelium discoideum* using the pH-sensitive fluorophore pyranine and a low light level microspectrofluorometer (Furukawa et al., 1988). Microspectrofluorometric measurements using two different fluorophores and cell strains showed that inducers of stalk cell differentiation such as proton pump inhibitors and weak acids do cause significant acidification of the cytosol as predicted by the pH_i theory (Furukawa et al., 1988; Inoué, 1988).

In the present study, we have measured the intracellular pH of single cells within developing populations of *D. discoideum* amoebae. To perform these measurements, we have adapted the technique of ATP-mediated loading from mammalian cells (Steinberg et al., 1987b) to introduce pyranine into developing cells before aggregation, and to single cells in intact slugs. In addition, we have developed methods to analyze subpopulations within a data set obtained by single cell measurements with a high degree of accuracy. Our results indicate that a subset of developing amoebae exhibit a transient acidification of their cytosol. This subpopulation reaches a maximum size (~60% of the total cells examined) at ~4 h after starvation. These results support the hypothesis that intracellular pH changes contribute to the process of cell differentiation in *Dictyostelium*.

Materials and Methods

Cell Culture

Dictyostelium discoideum strain AX-3 was cultured axenically in HL-5 as previously described (Loomis, 1971). *D. discoideum* strain NC-4 was cultured on 2% nutrient agar in petri dishes in the presence of *E. 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. The cells were starved by washing three times with 17 mM phosphate buffer containing 2 mM Na_2HPO_4 and 15 mM KH_2PO_4 , pH 6.1. The cells were placed on 2% agar plates, and were harvested at the indicated times after initiation of development.

Introduction of Pyranine to Amoebae

Pyranine was introduced into the NC-4 amoebae by either the ATP-mediated loading or the sonication loading technique in the presence of probenecid. The method of sonication loading was previously described (Fechheimer et al., 1986; Fechheimer and Taylor, 1987; Furukawa et al., 1988). For ATP-mediated loading, $\sim 5 \times 10^7$ cells were washed off the agar plates with 17 mM phosphate buffer, pH 6.1, and held at 0°C for 15 min. The cells were collected by centrifugation for 5 min at 300 g. The cells were resuspended in 17 mM phosphate supplemented with 5 mM ATP, 5 mM probenecid (Sigma Chemical Co., St. Louis, MO), and 8 mM pyranine at pH 7.0 and held at 0°C for 10 min. The cells were washed free of fluorophore and ATP by washing with ice-cold 17 mM phosphate buffer supplemented with 5 mM probenecid, pH 7.0. After the free fluorophore was removed, the cells were washed at room temperature with 17 mM phosphate buffer supplemented with 5 mM probenecid, pH 6.1. ATP-mediated loading has been shown to be effective in introducing molecules with a molecular weight $< \sim 1,000$ D into the intracellular matrix (Steinberg et al., 1987b). Probenecid has been shown to inhibit clearance of anionic fluorescent dyes from the cytoplasm of macrophages (Steinberg et al., 1987a). The fluorescence distribution was assessed by fluorescence microscopy. The pyranine was distributed in the cytosol with few vesicles. Approximately 8% of the cells were loaded with pyranine in the cytosol. A greater percentage of cells can be loaded with prolonged exposure to the ATP-pyranine mixture.

Amoebae loaded with pyranine by the ATP-mediated method could subsequently develop on nonnutrient agar plates to form mature fruiting bodies.

In addition, cell physiology was assessed by observations of loaded cells during locomotion and by phagocytosis of *E. coli*. The presence of probenecid at the concentrations indicated has no apparent detrimental effects on cell morphology, development, locomotion, or cytokinesis of either loaded or unloaded cells. Cells having discernible fluorescent vesicles due either to pinocytosis or incomplete inhibition of vesiculation by the probenecid were not measured. The measurements were completed within 0.5 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.

Measurements of the pH_i of vegetative NC-4 cells using ATP-mediated loading was performed essentially as described above except that the 17 mM phosphate buffer was supplemented with 10 mM glucose and 0.5 mg/ml of essential amino acids (Marin, 1976) to delay the onset of development. Measurements of the pH_i of vegetative cells by sonication loading were performed as previously described (Furukawa et al., 1988).

Slugs (NC-4) were loaded with pyranine by a modification of the above protocol. Slugs were washed off the 2% agar plate with the pyranine-ATP-probenecid mixture at 0°C onto a microscope chamber (Bionique Laboratories, Saranac Lake, NY) and incubated for 5 min. The pyranine-ATP-probenecid mixture was removed. The slugs were gently washed two times with 17 mM phosphate buffer, pH 7.0, supplemented with 3 mM probenecid, followed by two washes with 17 mM phosphate buffer, pH 6.1, supplemented with 3 mM probenecid.

A subconfluent monolayer of *D. discoideum* cells (NC-4) or ~20 individual slugs in 17 mM phosphate supplemented with the appropriate concentration of probenecid, pH 6.1, were allowed to adhere 10 min to the glass coverslip in a microscope chamber (Bionique Laboratories) before measurements were taken while the remainder of the cells were developing on 2% agar.

Measurements and Microspectrofluorometry

The microspectrofluorometer and methodology employed was previously described (Furukawa et al., 1988; Rich and Wampler, 1981; Wampler, 1986; Wampler et al., 1988; Wampler et al., 1979). Briefly, the signal from two pixel locations is measured. The first is within the fluorescent area of the sample and the second is from a location which is nonfluorescent. In addition, the fluorescence properties measured are more reproducible if a third wavelength is used as an arbitrary baseline and subtracted from the signal at each of the other wavelengths. 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. In practice, the BCR measurement is taken simultaneously for an entire video line (255 pixels) so that some spatial as well as temporal averaging can be employed.

The cells were exposed to light only for the duration of the measurement, ~15 s which was required to obtain one 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 for a total of 30 measurements from both sample and reference pixels. The BCR measurement was repeated at least three times on each cell. The average BCR value was used in all subsequent calculations.

Standard Solutions and Standard Curves

The BCR values of pyranine (Molecular Probes, 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, and 50 μ M pyranine and adjusted to the desired pH using either KOH or KCl. The BCR values were also measured in the presence of 10% (vol/vol) of lysed *D. discoideum* AX-3 cells. The AX-3 cells were harvested by low-speed sedimentation and 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.

The BCR of pyranine standard solutions was obtained by placing the solution in a Sylgard cylinder (Dow-Corning Corp., Midland, MI) as previously described (Furukawa et al., 1988). The sample pixel was inside the fluorescent solution and the reference pixel was in the Sylgard. The in vitro standard curve refers to the data taken in the pyranine standard solutions in the presence or absence of 10% (vol/vol) of lysed AX-3 cells. As shown

previously, the *in vivo* and *in vitro* standard curves for pyranine in *D. discoideum* were indistinguishable (Furukawa et al., 1988). We have chosen to measure only the *in vitro* standard curve which is quickly and easily measured. Data for standard curves were fit with a cubic polynomial using nonlinear least squares (Bevington, 1969), and unknown values were determined from the fit curve.

Data Analysis

After transformation of the raw BCR measurements to pH from the standard curve equations, data were grouped and processed in several ways. Data taken during the developmental period were grouped into 1-h time intervals. Vegetative cell data were grouped into two categories, cells loaded by the sonication method (data previously published, Furukawa et al., 1988) and cells loaded by the ATP-mediated method described above. Measurements made on individual cells within slugs were grouped together. Data from each group were analyzed in four ways (a) the arithmetic mean pH and its standard deviation were calculated; (b) the median pH and the interquartile range were calculated; (c) the data were grouped according to the incremental pH ranges and analyzed as histograms; and (d) the data within each group were rank ordered and fit to integrated probability functions in order to extract quantitative information concerning the number of subpopulations represented and the descriptive parameters of these populations (see below).

Standard calculations were used to obtain the mean, standard deviation, median, and interquartile range of each data set. Histograms of pH were calculated by combining data over each 0.2-pH U increment from 5.5 to 8.1. All curve fitting was carried out using a nonlinear least squares routine derived from that described by Bevington (1969). This program utilizes the Levenberg-Marquardt algorithms (Levenberg, 1944; Marquardt, 1963). In all cases the nonlinear fitting algorithm terminated on the criteria of <0.01% change in Chi Square and multiple initial starting values of parameters were used to ensure that the global minimum was reached.

For analysis of distribution functions of the subpopulations for each group of pH measurement data, the procedures and approaches have been recently described elsewhere (Wampler, 1990). Each group of random pH measurements was sorted to obtain the empirical cumulative distribution. These data form an X, Y data set. When the axes are interchanged, this ranked data was treated as an X, Y data set for fitting where the X axis values were the pH and the Y values were the rank position (cell number) of each pH value. Random data presented in this way approximates the shape of the integrated probability distribution function of the sampled population normalized to the number of datum points in the sample. Three model distribution functions were fit: a single Gaussian distribution, and a linear combination of two or three Gaussian-shaped subpopulations. For each Gaussian component, the integrated normal distribution function was calculated using the four-term approximation formula discussed by Hastings (1955), and the calculation was scaled to the subpopulation sample size. Thus, the fitting parameters for each component were the mean pH, the standard deviation of the mean, and the size of the population or subpopulation that they represent. The reduced Chi Square (sum of the squares of the residuals divided by the number of datum values minus the number of fit parameters) was used to evaluate the "goodness of fit" and select between the models. The mean and standard deviation of the fits (square root of the reduced Chi Square) are reported here.

Results

ATP-mediated Loading of Pyranine

We have adapted the technique of ATP-mediated loading of exogenous molecules into mammalian cells (Steinberg et al., 1987b) to introduce pyranine to the cytosol of *D. discoideum*. The pyranine is excluded from numerous intracellular compartments and is located almost exclusively in the cytosol. A few fluorescent vesicles are sometimes noted. This morphology is similar to that described previously for amoebae loaded with fluorescent dextrans (Fechheimer et al., 1986) or fluorescent BSA (Fechheimer, 1987). ATP-loaded cells develop into normal mature fruiting bodies (data not shown). The pH_i of vegetative NC-4 amoebae loaded with the ATP-mediated loading method was measured, and the

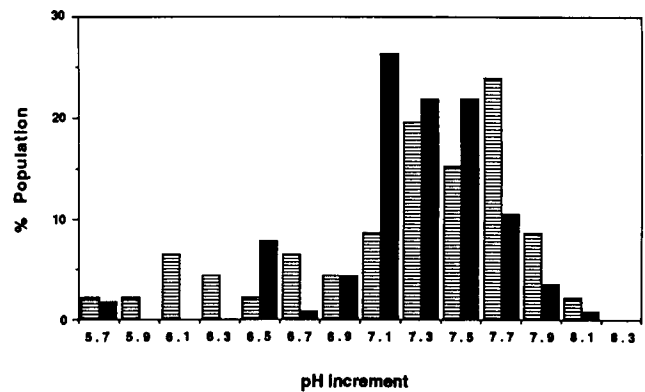


Figure 1. The distribution of pH_i of vegetative cells loaded with pyranine either by sonication loading or by ATP-mediated loading. The distribution of pH_i of cells loaded either by sonication (■), or by ATP-mediated loading (▨) is quite similar.

distribution of pH_i values obtained from ATP-loaded cells has been compared to that obtained for vegetative NC-4 amoebae loaded by the sonication technique (Fig. 1). From the histogram analysis, the distributions obtained by the two different loading methods are quite similar. The subset of cells with an acidic pH_i was more pronounced in the ATP-loaded cells. ATP-mediated loading does not require nutrients in the buffer to allow the cells to recover from the shock of sonication loading (Fechheimer et al., 1986). Thus, ATP-mediated loading is more appropriate for use in studying the development of cells which is initiated by starvation. We have successfully loaded the cytoplasm of individual amoebae while in the vegetative state, in preaggregation stages, and as late in development as the preculmination stage.

Individual amoebae have also been successfully loaded in the intact slug. This obviates the need for any physiological insult to mechanically dissociate the slug in order to study the prespore and prestalk cells. Comparison of differential interference contrast and epifluorescence images of a slug loaded with pyranine using the ATP-mediated method clearly illustrates introduction of the dye to a small and apparently random subpopulation of the cells (Fig. 2, a and b).

ATP-mediated loading must produce transient pores in the plasma membrane in order to introduce molecules into the cytosol. In macrophages, the largest molecules introduced were ~1,000 D (Steinberg et al., 1987a). Human neutrophils have been successfully loaded with aequorin (Ozaki and Kume, 1988). We have partially characterized the pore size of the ATP-mediated loading method in *Dictyostelium*. With NC-4 amoebae, the percentage of cells loaded with 4,000 and 19,000 D fluorescein-labeled dextrans was lower than pyranine loaded by the ATP method with the percentage of loaded cells decreasing with increasing molecular weight. The presence of probenecid (Steinberg et al., 1987a) did not prevent rapid vesiculation and subsequent clearance of the fluorescent dextran from the cytoplasm.

The axenic strain AX-3 was also loaded with pyranine by the ATP-mediated loading technique. The cytosol was loaded with pyranine, but the fluorophore was rapidly vesiculated. Within 10 min, the fluorophore was observed mainly in vesicles and cleared from the cytoplasm, even in the presence of a high concentration (8 mM) of probenecid. AX-3 amoebae

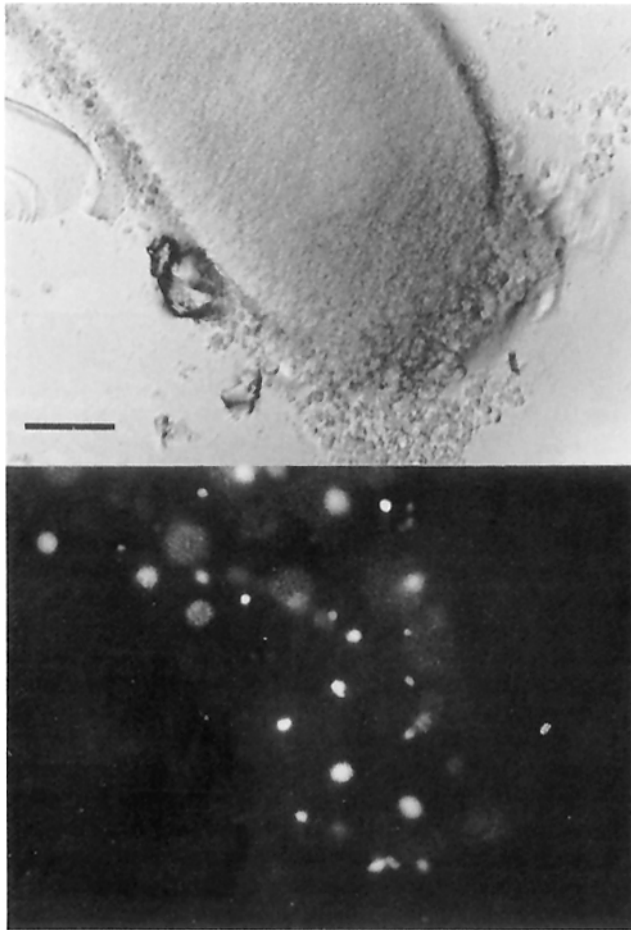


Figure 2. ATP-mediated loading of amebae with pyranine in intact slugs. The top picture is a portion of a *Dictyostelium* slug loaded with pyranine using the ATP loading technique as viewed through differential interference optics in a light microscope. The bottom picture is the same slug viewed through fluorescence optics. The bright images scattered randomly throughout the picture are individual amebae loaded with pyranine. Cells above or below the focal plane appear to have a larger diameter than cells in focus in the fluorescence micrograph. Bar, 100 μm .

ingest nutrients mainly by fluid pinocytosis versus phagocytosis by NC-4. The rapid vesiculation of the dye in AX-3 could be related to the high rate of pinocytosis. Therefore, all experiments were performed with NC-4 amebae, and no measurements of pH_i of the AX-3 strain were attempted.

Intracellular pH of Developing D. discoideum

We have measured the intracellular pH of single *D. discoideum* amebae during development. The histograms of intracellular pH were constructed for 1-h time intervals. They suggest that the distribution of intracellular pH values during development is bimodal (Fig. 3 A). This result is in contrast with the result that the distribution of the intracellular pH of the vegetative cells appeared unimodal (Fig. 1). Within the first hour of development, there appear to be two subpopulations with distinct intracellular pH distributions. The population of cells with an acidic intracellular pH appears most prominent after 4 h of development (Fig. 3 A), and subse-

quently decreases with time. 7 h after starvation, the intracellular pH returns to a more neutral pH distribution with a few percent of cells with acidic pH.

The intracellular pH of individual amebae in slugs was also measured. The position of the individual amebae in the slugs was random over the length of the slug. The distribution of the intracellular pH is centered around a neutral pH with a small acidic subpopulation (Fig. 3 B).

Although the presence of two subpopulations of cells during early development is detectable by visual inspection of the histograms in Fig. 3 A, quantitative analysis of the distributions required modeling of the data using the three approaches described in Materials and Methods. Table I lists the median pH and interquartile range, the mean pH, and standard deviation of the mean for each of these groups of data. If the data are assumed to represent measurements sampling cells comprising a single, uniform distribution, the results show a trend of transient change during development, but the variation between values also shows some seemingly random fluctuations (see below). The median pH decreases from the vegetative value to a minimum of 6.1 at 4 h of development. After 7 h, the median pH value increases to 7.22, and is not significantly different from that measured in the slugs.

The assumption of a single, uniform distribution function is challenged by the histograms of Fig. 3 A. It seems clear from these plots that at least two subpopulations are being sampled in the developing cells and that the contributions of these subpopulations to the total may be changing with time. It is important to realize that considerable information is lost when this data is combined to generate histograms, since values from 50 to 100 cells are combined to yield a single measurement parameter in this method of data analysis. Thus, a detailed analysis of the shape of the histograms is not possible. However, nonlinear fitting of complex integrated distribution function models to the ranked data sets reveal both the number and the characteristics of the subpopulations. The results of these fits are tabulated in Table II.

This analysis indicates that a major change in the population distribution occurs during development. If a single normal distribution is forced to fit the data over the entire time course of development, the fits are characterized by a transient pH change with widely varying mean pH and dispersion values, and deterioration in the "goodness of fit" as shown in Table II. Examination of these fits, particularly at the intermediate times, show them to be poor models of the data with a strong pattern of bias in the residuals. An example of a fit using the 3–4-h data set is shown in Fig. 4. Application of the model with two subpopulations clearly gives a better fit than a single distribution function. As summarized in Table II, the second model gives better "goodness of fit" values, stable population parameters over most of the time course, and a clear transient variation in the population distribution of the cells during development. The more complicated three function model which seems to be suggested by the residuals plots in some cases does not improve Chi Square (not shown). Thus, the level of uncertainty in the datum values and the sample sizes do not warrant more complex analysis.

The results of the fits using the two Gaussian distribution models are summarized by the graphs of Fig. 5. They suggest a hypothesis involving a transient increase in an acidic sub-

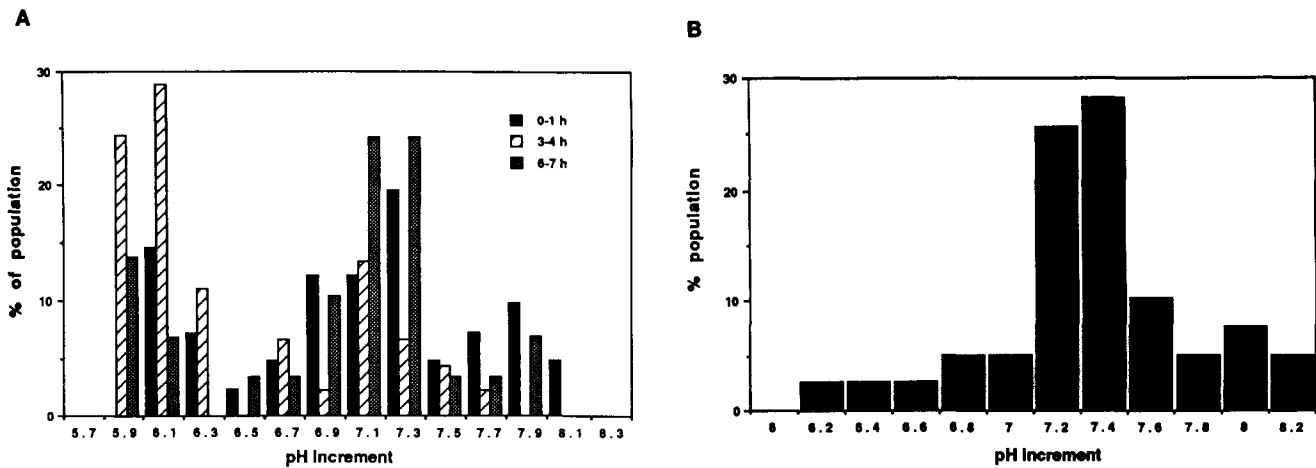


Figure 3. Changes in intracellular pH of *D. discoideum* during development. (A) The distribution of pH_i of developing amebae is distinctly bimodal in contrast to the distribution of vegetative cells. A subpopulation of the cells with acidic pH_i is detectable as soon as 1 h after starvation, and reaches a maximum number after 4 h. The number of cells in this subpopulation decreases with time. (B) The distribution of pH_i of individual amebae in slugs. Amebae in slugs exhibit a pH_i distribution with a smaller dispersion of the neutral pH cells than observed in vegetative cells, with a few percent of acidic pH_i cells.

population which has a mean cytoplasmic pH of 6.0 and a narrow dispersion ($SD = 0.1$). This subpopulation appears to be present at low levels even in the vegetative cells and the slug (see Table II). The second population has a mean pH_i of 7.2 with a broader dispersion ($SD = 0.5$). It is interesting to note that during the 3–4-h time frame after initiation of development, over 60% of the amebae are found in the acidic subpopulation.

Discussion

The pH Theory of Cell Differentiation in *Dictyostelium*

Dictyostelium discoideum grow vegetatively as single cells in the presence of a food source, and initiate development

upon removal of nutrients. Marked changes in gene expression characterize the preaggregation phase of development before any morphological manifestation is apparent. Between 8 and 12 h of development, cells aggregate into streams and mounds mediated by chemotaxis to extracellular cAMP. These aggregates transform into a multicellular slug at ~ 16 h of development. After 24 h, terminal differentiation to form a mature fruiting body containing two differentiated cell types, the stalk and spore cells, is complete (Loomis, 1982).

A large number of environmental factors have been shown to influence the development of *Dictyostelium discoideum* amebae along either the prespore or the prestalk differentiation pathway. Inducers of stalk cell differentiation include: low extracellular pH, a variety of proton pump inhibitors including diethylstilbestrol, diffusible inducing factor (the nat-

Table I. The Mean and Median Intracellular pH of *Dictyostelium discoideum* Amebae during Development

Developmental time	1st quartile \ddagger	Median \S	3rd quartile \parallel	n \uparrow	Mean	SD
h^*						
0**	6.98	7.40	7.73		7.27	0.66
0 \ddagger	6.99	7.19	7.39		7.15	0.40
1	6.41	7.08	7.38	41	6.98	0.61
2	6.00	6.55	6.94	63	6.52	0.53
3	5.89	6.76	7.28	52	6.74	0.81
4	5.91	6.11	6.86	48	6.36	0.56
5	6.11	6.97	7.14	55	6.76	0.58
6	6.00	6.53	7.02	48	6.52	0.51
7	6.54	7.07	7.29	31	6.94	0.58
slug	7.04	7.20	7.43	50	7.22	0.44

* The data were collected over 1-h intervals. The time indicated is the time at the end of the interval.

\ddagger The first quartile is the pH at which 25% of the cells are below.

\S The median is the pH at which 50% of the cells are below.

\parallel The third quartile is the pH at which 75% of the cells are below.

\uparrow n is the number of cells.

** Vegetative cells loaded by sonication, data from Furukawa et al., 1988.

$\ddagger\ddagger$ Vegetative cells loaded with ATP.

Table II. The Population Distribution of the Intracellular pH in *D. discoideum*

Time	Model 1			Model 2					
	χ^2	X	σ	χ^2	n_1	X_1	σ_1	X_2	σ_2
h									
0*	0.256	7.19	0.31	0.196	6.0	5.81	0.39	7.21	0.28
0 \ddagger	0.369	7.09	0.98	0.208	19.6	6.10	0.37	7.61	0.37
1	0.239	7.13	0.78	0.171	19.0	6.02	0.12	7.16	0.44
2	0.402	6.57	0.69	0.177	31.6	5.93	0.10	6.91	0.42
3	0.468	6.76	1.04	0.195	35.5	5.88	0.12	7.21	0.65
4	0.607	6.19	0.54	0.154	61.6	5.93	0.15	6.94	0.27
5	0.334	7.04	0.86	0.192	35.0	6.07	0.25	7.11	0.27
6	0.351	6.67	0.74	0.211	16.2	5.96	0.12	7.32	0.77
7	0.368	7.41	0.85	0.230	28.8	6.04	0.38	7.18	0.22
slug	0.294	7.18	0.29	0.286	12.7	6.28	0.86	7.20	0.20

* Vegetative cells loaded by sonication, data from Furukawa et al., 1988.

\ddagger Vegetative cells loaded with ATP.

Model 1 and 2 are calculated fits for 1 and 2 Gaussian distributions, respectively, using the Levenberg-Marquardt algorithm for a nonlinear least squares fit. χ^2 is the "goodness of fit" parameter. X and σ are the mean and the standard deviation, respectively, of the Gaussian distribution(s). n_1 is the percentage of subpopulation 1 for the two function model.

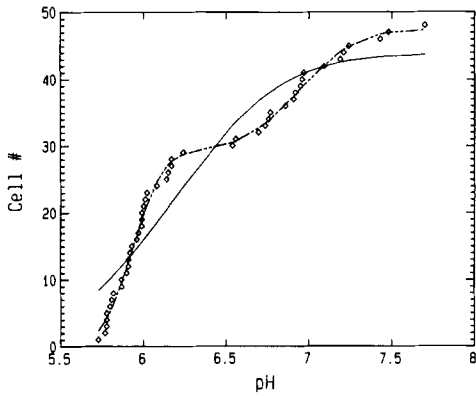


Figure 4. Sample fits of Gaussian distribution functions for the data set of intracellular pH values from developing cells between 3 and 4 h after starvation. The data have been sorted in ascending order with the X ordinate representing the pH value and the Y ordinate representing the rank position of the datum. The solid line is the best fit to a scaled, integrated single Gaussian distribution. The broken line is the best fit to a model using two Gaussian distribution functions. The data were fit using the equations and procedures described in the text.

ural inducer of stalk cell differentiation), and weak acids such as acetate and propionate (Brookman et al., 1987; Dominov and Town, 1986; Gross et al., 1983; Gross et al., 1988; Kay et al., 1986; Morris et al., 1987; Town et al., 1987). Inducers of spore cell differentiation include high extracellular pH and weak bases such as ammonia. The fact that treatments inducing spore cell differentiation were expected to alkalize the cytosol, while treatments inducing stalk cell differentiation were expected to acidify the cytosol led to the proposal that the cytoplasmic pH is a regulator of differentiation in *Dictyostelium* (Gross et al., 1983).

The pH theory has been tested by direct measurements of cytoplasmic pH using a number of analytical methods with markedly varying results. These experiments can be grouped into three categories (a) measurements of pH_i in the presence of inducers of spore or stalk cell differentiation; (b) measurements of the pH_i of prestalk and prespore cells; and (c) measurements of pH_i during early development.

Measurements of pH_i of *Dictyostelium* in the presence of the inducers of stalk and spore cell differentiation have yielded surprisingly disparate results. No change in pH_i by ³¹P NMR was detected when H⁺-ATPase inhibitors (Kay et al., 1986) and weak acids (Town et al., 1987) which are expected to acidify the cytoplasm are added to *Dictyostelium*. By contrast, cytoplasmic alkalization in the presence of ammonia and acidification in the presence of either acetate or the proton pump inhibitor diethylstilbestrol, which were predicted by the pH theory for differentiation (Gross et al., 1983), are observed by microspectrofluorometry (Furukawa et al., 1988). Similarly, acidification of the cytosol upon addition of the protonophore carbonyl-cyanide *m*-chlorophenylhydrazine in the presence of a low extracellular pH was detected by microspectrofluorometry, but not by ³¹P NMR (see Furukawa et al., 1988). In addition, slight acidification of the cytoplasm in the presence of diethylstilbestrol and in the presence of weak acid has been verified in an independent study employing carboxyfluorescein as the pH indicator (Inouye, 1988). Finally, the action of weak acids and weak bases on pH_i in *Dictyostelium* have been confirmed by measurements of cell homogenates using a pH electrode (Aerts et al., 1987).

Previous measurements of the intracellular pH of amoebae in slugs have also yielded mixed results. Ratner (1986) measured the intracellular pH of prespore and prestalk cells using fluorescein diacetate, and obtained pH values of 6.11 ± 0.11 and 6.16 ± 0.04 for the prespore and prestalk cells, respectively. Inouye (1985) also measured the intracellular

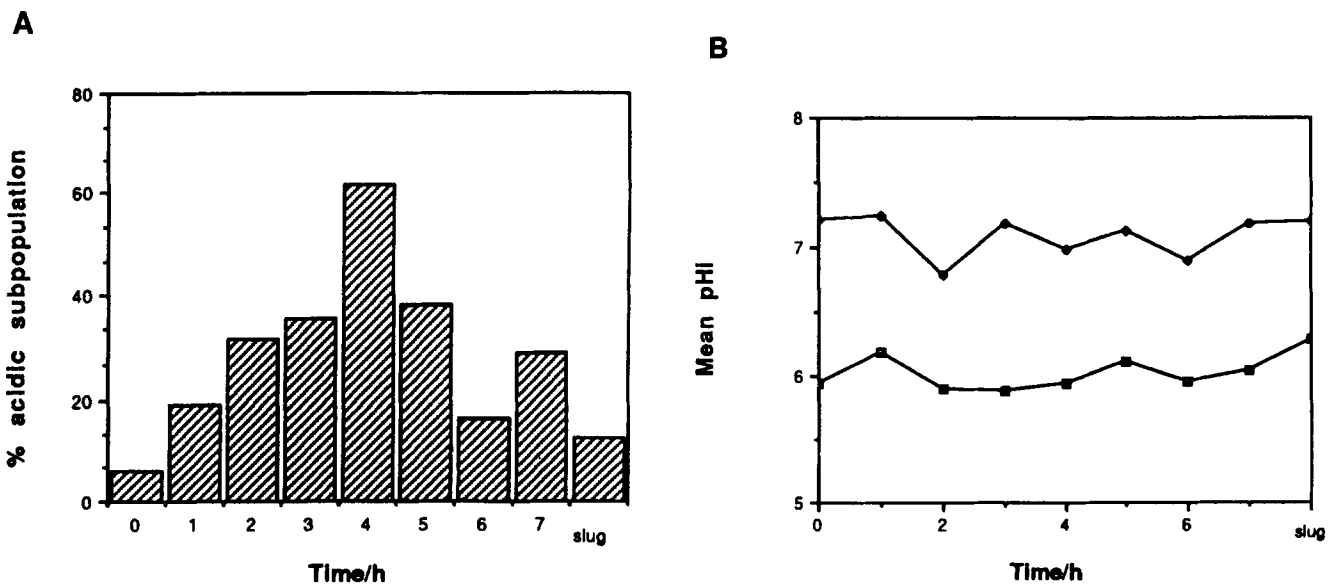


Figure 5. Two subpopulations of cells during early development of *Dictyostelium*. (A) The calculated percentage of the acidic subpopulation as a function of the time of development. A transient increase in the size of the acidic subpopulation is apparent. (B) The mean pH_i for the two subpopulations during development. The mean pH_i of each of the subpopulations is relatively constant as a function of development. These parameters were calculated as described in Data Analysis.

pH of prespore and prestalk cells utilizing the fluorescent probe, carboxy fluorescein dibutyrate, and reported pH_i values of 6.96 ± 0.19 and 7.25 ± 0.09 with a p*H*_o of 6.5 for the prestalk and prespore cells, respectively. More recently, a similar approach was used to measure an intracellular pH of 7.38 ± 0.09 in prestalk and 7.49 ± 0.03 in prespore cells (Inouye, 1988). In addition, a null point method has been effectively employed to demonstrate a difference of 0.2 pH U in the average intracellular pH of cell populations enriched for either prestalk or prespore cells (Aerts, 1988). In this study, we have measured pH_i of random cells in intact slugs (Fig. 3 B). We detected the presence of a small subpopulation of cells with an acidic pH_i and a subpopulation with a neutral pH_i (see Table II). We observe no direct correlation between pH_i and position along the length of the slug. Thus, the results reported to date agree with the consensus that there is no difference or at best only a small difference in intracellular pH between prespore and prestalk cells at the slug stage.

Finally, measurements of intracellular pH during early development of *Dictyostelium* have also been performed using multiple analytical methods with mixed results. No change of cytoplasmic pH during development of whole cell populations of *Dictyostelium* amoebae has been measured by ³¹P NMR by three different groups of investigators (Jentoft and Town, 1985; Kay et al., 1986; Satre et al., 1986; Town et al., 1987). By contrast, our measurements reveal a transient acidification in pH_i of *D. discoideum* amoebae during the first few hours of development (Figs. 3 A and 5; Tables I and II). We have observed that the distribution of pH_i values of developing cells appears transiently bimodal due to the presence of a subpopulation with an acidic pH_i. This observation was made quantitative by fits of the data to models in which either one or two Gaussian subpopulations are assumed to exist. The mean values and dispersion of the two populations were remarkably stable over the entire time course of development. This fact supports the suggestion that the two subpopulations are real entities. A clear increase and subsequent decrease in the size of the acidic subpopulation is revealed by this analysis (Model 2, Table II; Fig. 5).

A possible reason why no change in pH_i has been observed with NMR, is that NMR gives an average value for the whole population and the standard deviation in this case is reflected in the width of the NMR peak. If the assumption is made that the sample measured by microspectrofluorometry is representative of the entire population of cells that is measured by NMR, a comparison of the mean pH_i and standard deviation can be made. The mean pH_i and standard deviation of the data obtained by microspectrofluorometry is shown in Table I. The mean pH_i values indicate that a maximum change of 0.60 pH U could be measured between 1 and 4 h of development, but with a large standard deviation due to the bimodal nature of the distribution. The large standard deviations indicate the uncertainties that must exist in employing a measurement technique in which all of the cells in a population are averaged. The standard deviation is so large that a change in pH_i could be obscured. Thus, the measurement of pH_i from single cells yields information that could be missed using techniques that average over the entire population. In fact, a hypothesis involving changes in vacuolar pH and cytoplasmic calcium has been advanced to explain the earlier reports in which no change in cytoplasmic pH of developing cells was detected (Gross et al., 1988).

Significance of the Transient Change in Intracellular pH_i for the Development of *Dictyostelium*

We have described accumulation of an acidic subpopulation of cells during development of *Dictyostelium* reaching a maximum number at ~4 h of development, recovering to a more neutral pH_i by ~7 h of development. This process occurs entirely within the preaggregative phase of development. We postulate that this cytoplasmic acidification may be related either to progression through the stages of preaggregation development and changes in transcription and translation of developmentally regulated genes, and/or to an effect of the cell cycle on the pathway of differentiation in *Dictyostelium*.

Major changes in gene expression have been described during the early phases of differentiation in *Dictyostelium* (Mehdy et al., 1983; Cardelli et al., 1984, 1985; Finney et al., 1985; Singleton et al., 1988; Mehdy and Firtel, 1985; Datta and Firtel, 1987; Kessin, 1988; Kimmel, 1984, 1987; Mann et al., 1988a, b; Mann and Firtel, 1989). The increases and decreases in expression of these genes are occurring in the time frame of the increase and decrease in the size of the acidic subpopulation of cells described in this communication. We consider it possible that our observed transient alteration in intracellular pH is related to the multitude of changes in gene expression that occur in this period. Elegant experiments have revealed that addition of propionic acid does induce an increase in late prestalk gene expression (Town et al., 1987), while no correlation of cytoplasmic alkalization and prespore gene expression has been seen (Van Lookeren Campagne et al., 1989). Additional studies of the kinetics of the transient change in pH_i in individual cells and of the changes in gene expression and developmental fate of those cells are required to resolve this hypothesis.

Intriguing relationships between the cell cycle, intracellular pH, and early development are also quite pertinent to the potential role of intracellular pH in the development of *Dictyostelium*. Oscillation of intracellular pH during the vegetative cell cycle of *Dictyostelium* AX-2 has been demonstrated directly (Aerts et al., 1985). The average intracellular pH of *Dictyostelium* decreases to a minimum of 7.2 for ~1.5 h around the time of mitosis (Aerts et al., 1985). The average intracellular pH then increases to a maximum of 7.45 and remains elevated through early G₂. A number of groups have related the selection of either prestalk or prespore differentiation pathways to the stage of the cell cycle at the time of initiation of development. Cells starved at or within an hour after mitosis differentiate in the prestalk pathway, while cells starved late in the cell cycle exhibit a preference for the prespore pathway (Weijer et al., 1984; McDonald and Durston, 1984; Ohmori and Maeda, 1987; Gomer and Firtel, 1987). Thus, studies of the effects of intracellular pH and the cell cycle on development of *Dictyostelium* are consistent, and may be probing interrelated regulatory mechanisms.

A complete description of the mechanisms of development in *Dictyostelium* will require integration of the facts obtained at different levels of investigation, and unraveling of the regulatory networks at the molecular level. The observation that the intracellular pH of developing cells is not constant, but exhibits a transient acidification, offers support for the theory that intracellular pH is important for development in *Dictyostelium*.

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