Intracellular pH in *Dictyostelium discoideum*: a $^{31}$P Nuclear Magnetic Resonance Study

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ABSTRACT We have used phosphorus-31 nuclear magnetic resonance to determine intracellular pH in the cellular slime mold *Dictyostelium discoideum*. We devised an air-lift circulator to maintain the dense cell suspensions in a well-oxygenated and well-stirred state while causing minimal perturbation to the sample flowing through the detector coils. Cells continued to develop normally in this set-up. Spectra acquired under these conditions typically show two peaks in the inorganic phosphate region corresponding to pH values of 7.16 ± 0.03 and 6.48 ± 0.02. These peaks are believed to represent the mitochondrial and cytosolic compartments respectively, based on a comparison of these values with published data and the collapse of the two compartments upon addition of the mitochondrial uncoupler carbonyl cyanide 4-( trifluoromethoxy)-phenylhydrazone. *Dictyostelium* cells show a remarkable degree of intracellular pH homeostasis. Both mitochondrial and cytosolic pH remained unchanged as extracellular pH was varied from 4.3 to 8.1. There was also no apparent change in the pH of either compartment after up to 13.5 hours' development in suspension.

The cellular slime mold *Dictyostelium discoideum* is a versatile model system which has been used for many experimental studies in cellular and developmental biology (1-3). The cells grow as individual amoebae feeding on bacteria. When the food supply is exhausted, the developmental phase of the life cycle is initiated. The separate amoebae aggregate chemotactically to form multicellular aggregates containing up to $10^5$ cells. These cell masses undergo a coordinated program of morphogenesis and cell differentiation to produce terminally differentiated fruiting structures consisting of a mass of spores supported by a cellulosic stalk. Execution of the developmental program requires the controlled expression of numerous genes in a well-ordered sequence. However, it has long been recognized that the developmental program can be strongly influenced by a variety of metabolic and environmental parameters, including nutritional status (4), temperature (5), illumination (6), ionic milieu (7), oxygenation (8), etc. Recent studies both from this laboratory and others have suggested that intracellular pH (pHi) may also influence the expression of the developmental program (9-11).

Phosphorus-31 nuclear magnetic resonance ($^{31}$P NMR) is one of several ways currently available to determine pHi. The $^{31}$P NMR method is non-invasive, using signals from endogenous phosphorus-containing compounds, and it is accurate. It can simultaneously provide useful information on the energetic status of the cell (ADP, ATP, sugar phosphate levels, etc.) (12, 13). To investigate the regulation and possible role of pHi in the developmental process, we have begun a $^{31}$P NMR study of *Dictyostelium discoideum*. In this paper, we report on a simple experimental set-up for the rapid acquisition of $^{31}$P NMR spectra, and on its application to the determination of pHi during development. The resulting spectra have enabled us to identify two intracellular peaks of inorganic phosphate (Pi) which we believe to be located in mitochondrial and cytosolic compartments, and to deduce the pH values of these compartments. We have also studied pH homeostasis as a function of extracellular pH. In addition, we have identified dihydroxyacetone phosphate in cell extracts and have observed an unusual major peak in the phosphodiester region.

MATERIALS AND METHODS

Strain, Growth, and Development Conditions

*Dictyostelium discoideum* strain V12 M2 was used for these studies. Cells were grown in shaken suspension on *Escherichia coli* B as previously described (11). For development, cells were washed free of bacteria and transferred to

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*Abbreviations used in this paper:* DMGH, a developmental buffer consisting of 15 mM 3, 3'dimethyl glutarate, 15 mM HEPES; FCCP, carbonyl cyanide 4-( trifluoromethoxy)-phenylhydrazone; MeP, methyl phosphonate; $^{31}$P NMR, phosphorus-31 nuclear magnetic resonance.
We therefore applied this approach to the NMR measurements. Previous work (17) has shown that Dictyostelium cells can be maintained healthy and at high cell density (~2 x 10^8 cells/ml) in suspension by bubbling O_2 to stir and aerate the culture. We therefore applied this approach to the NMR measurements. We experimented with flow rate, bubble size, cell density, and mode of data acquisition to determine the optimal conditions for our experiments. A high gas flow with large bubbles delivered through polyethylene capillary tubing gave a healthy, well-stirred cell suspension as judged by the β-ATP peak (peak VI in Fig. 2c), but broad lines and poor resolution in the 31P NMR spectra, especially in the Pi region. We reduced the gas flow and bubble size by using finer tubing, with an immediate improvement in resolution. However, flow rates compatible with good resolution were barely adequate to keep the cell suspension stirred and a sediment of cells often formed at the bottom of the tube. This problem could be overcome by reducing the cell density, but only at the expense of increased data acquisition time. In addition, the low gas flow rate may not adequately oxygenate the cells. We experimented with the number and position of the bubbling tubes and were often able to obtain good spectra, but were never able to prevent sedimentation and maintain a homogeneous cell suspension. Probably for this reason, spectra taken from successive samples or on different days showed some variability.

To accommodate the competing demands of adequate aeration and stirring and minimal perturbation of the sample within the detector coils, we devised the bubbler-stirrer arrangement shown in Fig. 1, based upon the principle of air lift pumps used in some domestic aquaria and industrial fermenters. The cell suspension is vigorously bubbled with O_2 (100-150 ml/min), the bubbles being discharged within the inner tube outside the field of the detector. The rising bubbles coalesce and expand to fill the inner tube, and act like a piston to force the cell suspension up the inner tube and out.
through the outlet ports. This pumping action constantly circulates the cell suspension up the inner tube and back down the outer cylindrical annulus with a transit time of ~20 s. Under these conditions, no sedimented cells collect at the bottom of the tube and a well-oxygenated bubble-free suspension of cells flows gently through the detector region of the NMR probe. This set-up has significantly improved both the resolution and reproducibility of our data compared with the earlier samples using a simple capillary bubbler. A similar approach to keeping the main stream of bubbles outside the detector has been applied to yeast (13), although in that case a fine stream of bubbles from below was sufficient to keep the smaller yeast cells in suspension.

Typical spectra obtained by the evolving techniques are shown in Fig. 2. The resonance signals are assigned as shown in the figure legend. Fast bubbling produced well-energized cells, but gave poor resolution in the $P_i$ region of the spectrum (Fig. 2a). Bubbles from multiple fine capillaries at a reduced flow rate (~30 ml/min) gave the spectrum in Fig. 2b. The resolution of the spectrum is clearly improved. However, under these conditions, there was invariably some cell sediment, and we did not consistently obtain spectra of this quality. Fig. 2c shows a comparable spectrum obtained with the circulator-bubbler. Resolution and the signal:noise ratio are as good or better than that shown in Fig. 2b (see also Fig. 5). Using this arrangement there is never any sediment—the suspension is constantly and uniformly circulated—and we consistently obtain well-resolved spectra.

**Cell Development under NMR Conditions**

The magnitude of the $\beta$-ATP peaks in our spectra indicates that the cells are well energized under these high density experimental conditions. We also wished to determine whether developmental progress was normal. Cells were starved for 6 h at $10^7$ cells/ml as usual. At this time, one sample was left undisturbed, one sample was centrifuged and resuspended in fresh buffer of the same composition at the same cell density ($10^7$ cells/ml), while a third sample was resuspended at $2 \times 10^8$ cells/ml in buffer containing 10% $D_2O$, 1.5 mM MeP and a trace of antifoam, and placed in the circulator-bubbler. The first two samples were shaken gently at 120 rpm and the high density sample was bubbled with $O_2$ at 130-150 ml/min. At hourly intervals, samples were taken, frozen as cell pellets and subsequently assayed for the activity of three developmentally regulated enzymes, as described previously (11). The results are shown in Fig. 3. Expression of the aggregation stage-specific enzyme, cyclic AMP phosphodiesterase, continues in all three cultures and is slightly higher in the high density (NMR) culture (Fig. 3a). The post-aggregation enzyme glycogen phosphorylase, which begins to increase in amount at the onset of the multicellular stage, accumulates at comparable rates in all three cultures (Fig. 3b). The third enzyme, UDP galactose polysaccharide transferase, also a multicellular phase enzyme, accumulates in all three cultures, but is considerably higher in the control culture which was not disturbed by centrifugation and resuspension (Fig. 3c). Thus we conclude that development (as determined by the expression of these three enzymes) proceeds more or less normally under the conditions used for NMR analysis: high cell density, $O_2$, 10% $D_2O$, 1.5 mM MeP. The reduced or delayed expression of UDP gal transferase could well be due solely to the perturbations of transfer to fresh buffer.

**On the Multiple $P_i$ Peaks and the $pH_i$ Values They Indicate**

In the $P_i$ region of the spectrum (0.70-3.23 ppm), we consistently observe two major peaks. In some of our earlier spectra, a third peak or shoulder could be seen on the upfield side. We have not seen this using the circulator-bubbler,
suggesting that it may be an artifact due to variations in stirring and oxygenation within the cell suspension in the sample tube in our early experiments. The major aim of this study was to determine pH; hence we have attempted both to determine the pH value corresponding to the chemical shift of the two major peaks and also to assign the peaks to specific cellular compartments. Since we were unable to identify an appropriate endogenous reference standard and we were reluctant to use a capillary reference in our inhomogeneous, non-spun set-up, we added MeP to the cell suspension as a chemical shift reference (15, 16). In most experiments MeP was added just before each run, and presumably remained extracellular since (a) only a single peak was observed; (b) this peak was quenched by Mn$^{2+}$; and (c) at least in erythrocytes, 2–3 h is required for proper equilibration (16).

In later experiments, cells were allowed to develop for 6–9 h in the presence of 3 mM MeP. This concentration of MeP had no effect on development as judged by the expression of two developmentally regulated enzymes, cyclic AMP phosphodiesterase and glycogen phosphorylase (data not shown). However, even after a 6–9-h incubation with 3 mM MeP, we were unable to detect a MeP signal from washed amoebae under conditions where both intra- and extracellular signals should have been resolved. Hence we could not use MeP peak-splitting as a method for calculating pH, and routinely use MeP simply as a reference standard.

For each spectrum, we have calculated the pH value corresponding to the two major P$_i$ peaks. The results of analyzing many spectra collected at different external pH values and at times from 3–13.5 h of development are shown in Fig. 4. Peak I corresponds to a pH value of 7.16 ± 0.03 and peak II to a value of 6.48 ± 0.02. In Fig. 4a, all the data are plotted as pH vs. pH$_o$, regardless of the developmental age of the cells. In Fig. 4b, the same data are replotted as pH vs. developmental age (from the initiation of starvation) regardless of pH$_o$. From these results it can be seen that the pH values of the two peaks are independent both of pH$_o$ (between 4.3 and 8.1) and of the developmental age of the cells (between 3 and 13.5 h). Both peaks appear to be intracellular since (a) the buffer is initially phosphate free; (b) in many cases neither
of the calculated pH values corresponded to the extracellular pH for that run; and (c) re-analysis of the supernatant buffer after removal of cells by centrifugation showed levels of P~ which were barely detectable under the conditions used to acquire the cellular spectra.

**Effect of the Uncoupler FCCP**

To test the hypothesis that the two major peaks represented mitochondrial and cytoplasmic P~, we examined the effect of the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (FCCP) on our spectra. In each case, addition of FCCP (30–100 µM) just before a run resulted in the collapse of the two peaks into a single peak of intermediate pH and the loss of the peaks due to ATP and ADP at -5, -10 and -18 ppm. An example is shown in Fig. 4. After acquiring the control spectrum in a, 30 µM FCCP was added and the cells returned to the spectrometer for two additional 20-min acquisition periods. The gradual collapse of two P~ peaks and loss of high energy phosphates can be clearly seen.

**Spectra from Cell Extracts**

The broad and complex peak(s) in the -5 to +5 ppm region of the spectra from live cells presumably reflects both the variety of compounds present (phosphomonoesters including AMP and sugar phosphates, 4.5 to 2.6 ppm; inorganic phosphate, 3.23 and 0.70 ppm; and phosphodiesters, 1 to -1 ppm) and the heterogeneity of the cellular environment. To examine the major components, we made cell extracts and collected spectra from them. A typical extract spectrum is shown in Fig. 5. The main features of this spectrum are (a) a group of sugar phosphate peaks around 4 ppm; (b) a single sharp P~ peak at +2.6 ppm, possibly with a satellite on the downfield side; (c) a large broad peak around -0.5 ppm, with some satellites; and (d) the nucleotide peaks at -5, -10, and -18 ppm. By adding authentic standards, we have identified one of the sugar phosphates as dihydroxyacetone phosphate, previously shown to be present in significant amounts in *Dictyostelium* (18). However, we have not yet identified the major peak around -0.5 ppm, although it does not coincide with either creatine phosphate or glycerol phosphoryl choline. The width of this peak suggests that it is actually composed of several closely related species.

**DISCUSSION**

We have described a simple circulator-bubbler device to keep dense suspensions of *Dictyostelium* amoebae well aerated and stirred. This device may also be useful for NMR studies on other eukaryotic cells which can tolerate vigorous bubbling in suspension. Using this arrangement, we have collected and analyzed 31P NMR spectra from *Dictyostelium* cells during the first 13.5 h of development. In these spectra we consistently identify two well-resolved P~ peaks (peak I [pH 7.16 ± 0.03] and peak II [pH 6.48 ± 0.02]; values are ± SEM, n = 18). We believe that peak I is mitochondrial based on the correspondence of our pH value to the results reported for isolated mitochondria and for the analogous peak in other eukaryotic cells (12) and that peak II is from the second major P~ pool in the cell, the cytosol. The assignment of the two major peaks to inorganic phosphate in the mitochondria and cytosol respectively is also supported by the collapse of these two compartments to a single pH value after the addition of the mitochondrial uncoupler FCCP. By taking NMR spectra over a wide range of pH values, we have also shown that both the mitochondrial and cytosolic pH values show a remarkable degree of homeostasis under these experimental conditions, remaining constant over an extracellular pH range from 4.3 to 8.1. A similar degree of pH homeostasis has also been reported for *E. coli* (15). We also find that intracellular pH does not change significantly in the first 13.5 h of development in suspension.

These results are in generally good agreement with data obtained from measurements on weak acid partitioning in
In this laboratory (Town, C. D., unpublished data), using dimethyloxazolidine 1,4 dione, we calculate $p_{H_i}$ to be 6.6 at an extracellular pH of 5.2, rising to pH 6.9 at an extracellular pH of 7.5. From less extensive benzoate data, we observe no effect of extracellular pH on $p_{H_i}$, and calculate $p_{H_i}$ to be 6.86 ± 0.10. Weak acid measurements are thought to represent a volume-weighted harmonic mean of all cellular compartments (19) irrespective of their $P_i$ content, and should therefore correspond quite closely to cytosolic pH. Weak acid partitioning is, however, subject to potential artifacts such as anion permeability and membrane potential effects, which may account for the difference between the weak acid results and the NMR data. The NMR analysis allows us to identify phosphorus-containing peaks whose intensity is proportional to the amount of $P_i$ present. Hence, we have detected and resolved two major pools of intracellular $P_i$, whose average pH values differ by more than the line-width of our spectra (~0.1 pH units). We would not resolve separate compartments whose pH was close to these average values, nor would we detect compartments which contained significantly less $P_i$ either because of their size or $P_i$ concentration. Such peaks would fall within the broad envelope of the $P_i$ region. However, we believe that our assignments of peaks I and II are correct and that peak II represents a mass-weighted average of major $P_i$ pools excluding the mitochondria. This is expected to constitute the bulk of the cytosol and to include the nucleus, since $P_i$ should pass freely through the nuclear pores. However, vacuolar systems constituting only a fraction of the cell's volume would go undetected. The precision of our calculated pH values depends upon the validity of the chemical shift parameters used. These were experimentally determined on cell-free extracts at approximately the concentration of the intracellular milieu, and should be reasonably accurate. Any error in these values would cause small but systematic changes in all the pH values and thus would not affect our conclusions concerning pH homeostasis or time-independence of pH. Based on the known effects of ionic strength, etc. on the chemical shift parameters, we feel that it is unlikely that the absolute values of our pH measurements are in error by more than 0.1 pH units. With these qualifications, we feel that our NMR studies have provided one of the first accurate measurements of both cytosolic and mitochondrial pH in *Dictyostelium* and demonstrated their invariance with respect to both extracellular pH and developmental age under these experimental conditions. While this work was under review, measurements of $p_{H_i}$ in *Dictyostelium* using fluorescein fluorescence were reported (20). In this study, cells of the axenic strain Ax-3 were loaded with fluorescein, then allowed to attach to slides prior to fluorescence measurements. During early development (0-2 h), $p_{H_i}$ was 6.23 ± 0.1. At around 2 h, there was a transient alkalinization of ~0.9 pH units of around 15 min duration after which $p_{H_i}$ fell to 6.3-6.4 and was not further measured. Our measurements are on strain V12M2 under different conditions of growth and development. We could not have observed the transient alkalinization reported by Jamieson et al. (20) because our measurements began later and in any event our temporal resolution (~17 min per scan) is too low. Our NMR pH, value of 6.48 at 3 h is 0.2 pH units higher than their fluorescein measurement. Without comparative measurements, we cannot tell how much of this apparent $p_{H_i}$ difference is due to the different strains, growth conditions, and developmental buffers. Once hydrolyzed to fluorescein, the fluorescent probe can pass freely across intracellular membranes. Although not evident microscopically, it is possible that part of their fluorescein signal came from lower pH compartments, not detected by NMR. It is equally possible that part of our "cytosolic" $P_i$ signal came from parts of the cell less accessible to fluorescein. In any event, their measurements and ours set lower and upper limits on current values for cytosolic pH in *Dictyostelium* cells which are on average considerably lower than those of higher eukaryotes (21, 22).

It has been postulated that intracellular pH in *Dictyostelium* may regulate not developmental progression per se, but the
selection of the alternate pathways leading to stalk cell and spore formation at later stages of development (10, 11). Our results demonstrating pH invariance with respect to pH₇ and developmental age in a homogeneous cell suspension do not directly address this hypothesis. However, with this method established we now plan to measure intracellular pH in cells exposed to weak acids, weak bases, proton pump inhibitors, and other reagents which have been shown to selectively modulate stalk and spore gene expression.

In the spectra of cell extracts we find a massive peak around -0.5 ppm. We have not yet characterized or identified the compound(s) responsible for this peak. They appear to be macromolecular, since they can be removed from extracts by Centricon ultrafiltration. Thus they may represent phosphorylated sugars in glycoproteins or polysaccharides which are perchloric acid soluble. In any event these molecules probably contribute, at least in part, to the broad lines and poor resolution in the P₁-phosphodiester part of the spectrum from live cells. It will be interesting to determine the nature and functional significance of these compounds.

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