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INTRACELLULAR pH IN *DICTYOSTELIUM*: A ^{31}P NUCLEAR MAGNETIC RESONANCE STUDY OF ITS REGULATION AND POSSIBLE ROLE IN CONTROLLING CELL DIFFERENTIATION

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SUMMARY

Intracellular pH (pH_i) has been measured in *Dictyostelium discoideum* cells by ^{31}P nuclear magnetic resonance. Ax2 cells, newly harvested from growth medium, maintained a pH_i of 7·33 ± 0·04 (17) at an extracellular pH ranging from 3·5 to 6·5. Below pH 3·5 the cells tend to lyse, whereas at pH values above 6·5 their pH_i rises though they remain viable. pH_i regulation in acid medium is not dependent on external Na^+ or any other inorganic ion and so most probably involves the electrogenic plasma membrane proton pump.

No significant change in pH_i was detected during development through to the slug stage. Mature stalk cells gave a very acidic phosphate signal ($pH \leq 5{\cdot}5$) which was probably vacuolar in origin.

Indirect experiments had suggested that pH_i might regulate the development of *Dictyostelium* cells, with low pH_i favouring stalk cell and high pH_i favouring spore cell differentiation. In particular, two inhibitors of the plasma membrane proton pump, diethylstilbestrol and zearalenone, had been shown to be stalk cell inducers. In the present studies measurements of pH_i of cells exposed to these inducers failed to detect the expected drop in pH_i. In addition, DIF-1 (a low M_r factor), the natural inducer of stalk cell formation, caused, if anything, a slight alkalinization of the cells. Thus the original theory linking pH_i and cell differentiation is not supported by these results and therefore appears to require some modification.

Finally, extract experiments revealed the existence of two unidentified abundant phosphocompounds with resonant frequencies close to inorganic phosphate. The existence of these compounds can complicate the interpretation of spectra gained from living *Dictyostelium* cells.

INTRODUCTION

Intracellular pH (pH_i) is regarded as a potential regulator of the metabolism and behaviour of an increasing variety of cell types. Thus the activation of sea urchin oocytes, following fertilization, in part depends upon a rise in pH_i of about 0·5 of a pH unit (Shen & Steinhardt, 1978), *Artemia* cysts are activated by a similar though larger change in pH_i (see Nuccitelli & Deamer, 1982) and there is an intimate and widespread link between small changes in pH_i and progression through the cell cycle (Gerson & Burton, 1977; Schuldiner & Rozengurt, 1982). The exquisite pH sensitivity of purified phosphofructokinase (Trivedi & Danforth, 1966) and of

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proteins involved in cytoplasmic contractility (e.g. see Condeelis & Vahey, 1982) also hint at a possible involvement of pH_i in regulating glycolysis and cell motility.

A novel role for pH_i has been proposed in the development of *Dictyostelium discoideum* by Gross *et al.* (1983), namely to control whether an individual amoeba differentiates into a stalk or a spore cell. These authors made use of the fact that *Dictyostelium* amoebae can be induced to differentiate into either stalk or spore cells, while submerged as monolayers under a simple salts medium, containing cyclic AMP. It was found that incubation of the cells in conditions expected to increase pH_i, favoured spore cell differentiation (high extracellular pH, addition of weak bases) and, conversely, incubation in conditions expected to reduce pH_i favoured stalk cell differentiation (low pH, addition of weak acids). The most dramatic effects were obtained with diethylstilbestrol (DES) and zearalenone, which are known to be inhibitors of the *Dictyostelium* plasma membrane proton pump (Pogge-von Strandmann *et al.* 1984; R. Pogge-von Strandmann, personal communication) and as such would be expected to reduce pH_i by blocking proton export. These agents strongly favoured stalk cell differentiation. Finally, it was proposed that the natural inducer of stalk cell differentiation in *Dictyostelium*, a low M_r factor termed DIF (Kay *et al.* 1983), acted *via* a reduction in pH_i.

Although the evidence linking pH_i and cell differentiation in *Dictyostelium* is impressive in its variety, it is all indirect in the sense that none of the agents and treatments mentioned above has actually been shown to affect pH_i in this organism. In order to fill this gap and to acquire basic knowledge about the regulation of intracellular pH in *Dictyostelium*, we have measured pH_i in living cells using ^{31}P nuclear magnetic resonance. This method involves determining the ionization state of inorganic phosphate (P_i) from its chemical shift and has the great advantage of being non-invasive (Moon & Richards, 1973; Gadian *et al.* 1982; Gillies *et al.* 1982).

MATERIALS AND METHODS

Cell growth and development

Cells were grown and developed at 22°C. Strain Ax2 was grown in axenic medium containing 86 mM-glucose shaken at 180 rev. min^{-1} on an orbital shaker (Watts & Ashworth, 1970) and the cells were harvested at a density of 2×10^6 to 6×10^6 ml^{-1} by centrifugation (200 ***g***, 5 min). Cells were washed centrifugally twice more in NS (20 mM-KCl, 20 mM-NaCl, 1 mM-$CaCl_2$). Cells of strain V12M2 and HM44 were grown on agar nutrient plates in association with *Klebsiella aerogenes* and the cells freed of bacteria by differential centrifugation (Kay & Trevan, 1981).

In experiments investigating the effects of external pH on pH_i, washed cells were preincubated for 15–30 min in medium of the appropriate pH and composition at a density of 10^7 ml^{-1}, whilst being shaken at 180 rev. min^{-1}. They were then pelleted by centrifugation and resuspended in the same medium for n.m.r. spectroscopy. For normal development cells were allowed to develop at a density of 6×10^6 to 8×10^6 cm^{-2} on agar (1·5 % Oxoid L28 containing NS).

The DIF-less mutant HM44 (Kopachik *et al.* 1983) was used for the induction of stalk cells in monolayers since it makes few if any stalk cells in the absence of added inducer. Washed HM44 cells were incubated in Sterilin tissue culture dishes at a density of 5×10^6 ml^{-1} (5×10^5 cells cm^{-2}) in 10 mM-MES, 10 mM-KCl, 2 mM-NaCl, 1 mM-$CaCl_2$, pH 6 (stalk salts) containing 100 μg ml^{-1} streptomycin sulphate, 15 μg ml^{-1} tetracyclin. Cyclic AMP (8 mM) was added at t_3 (3 h of starvation) and the appropriate inducers at t_8. After a further 3–8 h, cells were harvested from the plates using a silicone rubber scraper, centrifuged and washed centrifugally once more in fresh

incubation medium usually containing 0·5 mM-cyclic AMP and the appropriate inducer. The final pellets were resuspended in this same medium, so that spectra were usually taken in the presence of cyclic AMP and inducer. DIF-1 for these experiments was prepared from developing cells and purified through two steps of high-pressure liquid chromatography as described (Kay *et al.* 1983).

n.m.r. spectroscopy

Cells for spectroscopy were finally resuspended at a density of 2×10^8 to 3×10^8 ml^{-1} in about 2 ml of the media indicated in the text, with antifoam added to prevent frothing (≈1 μl ml^{-1} Sigma antifoam A) and were thermostatically controlled in the n.m.r. probe by an airstream at 22°C. Spectra of the living cells were recorded with bubbling of the sample during the interpulse delay. This was achieved by controlling oxygen delivery to the sample using a solenoidal valve that could be switched by pulses from the n.m.r. computer. Bubbling was switched on for 1 s immediately after each scan had been collected and a further 1 s was allowed for the bubbles to disperse completely before another n.m.r. pulse was applied so that the total interpulse delay was 2·1 s. A 66° pulse was applied and 4k data points were collected with a sweep width of 10 kHz without broadband proton decoupling; 200–500 such scans were collected for each spectrum. The samples were shimmed on the ^{1}H resonance of water and linewidths of 15–20 Hz obtained. As there is no suitable ^{31}P n.m.r. reference in the slime mould, the water resonance was set to the same absolute frequency by adjusting the static field for all experiments. This provides an effective chemical shift reference (Ackerman *et al.* 1981). Chemical shifts are expressed relative to a value for phosphocreatine of 0 parts per million (p.p.m.) at neutral pH. The data were processed using the convolution difference technique (Campbell *et al.* 1973; Ackerman *et al.* 1980) to remove the broad hump underlying the P_i region of the spectrum.

Extract spectra (200–500 scans) were recorded with 20° pulses repeated every 1 s and 4k data points collected in a sweep width of 8 kHz with broadband proton decoupling. This leads to differential saturation of the P_i and phosphoester peaks relative to ATP, for which the data have not been corrected but is the most efficient method of data collection. These spectra were resolution enhanced using the same convolution difference procedure as for the spectra of the intact cells.

All spectra were recorded at a frequency of 145·7 MHz on a Bruker AM 360 spectrometer with a 7·3 cm usable-bore 8·5 T magnet. The commercial 10 mm ^{31}P n.m.r. probe was used.

Extracts were prepared from continuously oxygenated cell suspensions by adding perchloracetic acid (PCA) to 6 %, vortex mixing and removing precipitated material by immediate centrifugation at 3000 ***g*** for 10 min. The supernatant was then decanted, brought to 10 mM-EDTA, neutralized with saturated $KHCO_3$ and the precipitated $KClO_4$ was removed by a second centrifugation. The resulting supernatant was filtered through a 0·45 μm filter and its n.m.r. spectrum was immediately taken. In this procedure we consider it important to spin down precipitated proteins and to add EDTA before neutralizing the extract, so as to minimize reactivation of phosphatases when the extract is subsequently neutralized. Neglect of some of these precautions may account for the high levels of P_i seen in the extracts prepared by other workers (Jentoft & Town, 1985).

In order to determine pH_i from the inorganic phosphate chemical shift from living cells, a titration curve of P_i was obtained in ionic conditions approximating the intracellular milieu (Maeda, 1983). A solution of 10 mM-KH_2PO_4, 10 mM-Na_2phosphocreatine (as chemical shift standard), 6·6 mM-KCl, 2 mM-$MgCl_2$, 1 mM-Na_2EDTA ('cytoplasmic salts') was used and the chemical shift of P_i was measured as a function of pH at 22°C.

Where indicated, chemical shift and pH_i measurements are given ±S.D. with the number of individual cell samples measured given in parenthesis.

RESULTS

^{31}P n.m.r. spectra from living cells and their interpretation

To obtain spectra within a reasonable time it is necessary to use a dense suspension of cells (2×10^8 to 3×10^8 ml^{-1}) in the n.m.r. tube and this in turn necessitates

bubbling with oxygen to keep the cells aerobic. Unfortunately the bubbles introduce field inhomogeneity, which lowers the spectral resolution, but by following Ogawa *et al.* (1978) this problem was overcome using an intermittent bubbler, controlled from the spectrometer, such that scans were only made in the intervals between periods of bubbling (see Materials and Methods). With this set up, high quality spectra could be acquired from living cells in 5–20 min. A typical example, obtained from Ax2 cells shortly after removal from growth medium, is shown in Fig. 1B. This spectrum shows six prominent ^{31}P resonances, of which three can be assigned to the α, β and γ phosphates of ATP (with contributions from other nucleotides), and three lie in the region occupied by P_i at different pH values. This latter region is our main concern, but it should be noted in passing that the high levels of ATP relative to P_i indicate that the cells remained well energized in the n.m.r. tube.

One of the peaks ($P_{i(ex)}$) in the P_i region can be assigned to extracellular P_i because it is still seen in medium from which the cells have been removed by centrifugation. By the same token, the other peaks are of cellular origin. PCA extracts were made from oxygenated cells and their spectrum immediately obtained (Fig. 1A). In the extract the signals from ATP are again prominent, together with three peaks in the P_i region. The major peak can be assigned to P_i (both cellular and extracellular in origin) on the basis of the pH dependence of its chemical shift (Fig. 2). We have not yet been able to identify either of the other two peaks, but the chemical shifts of both of them vary with the pH of the extract in the physiological range (Fig. 2), suggesting that they are phosphomonoesters. However, the compounds giving rise to peaks I and II would be unusual amongst phosphomonoesters, in that they resonate to the high field (i.e. right-hand side) of P_i at pH 7·0. Thus these compounds do not correspond in chemical shift to any of the phosphomonoesters commonly observed in biological systems; they cannot be any of the glycolytic intermediates, or AMP, IMP, phosphoryl choline, phosphoryl ethanolamine or serine phosphate (Gadian *et al.* 1979; Urgubil *et al.* 1979; Iles *et al.* 1982).

Comparing the spectra of living cells with those of their extract, the $P_{i(int)}$ from living cells is of a size appropriate for the P_i peak from the extract (after allowing for $P_{i(ex)}$) and its chemical shift of about 5·1 is well within the range of P_i but outside the ranges of the unknown compounds. This peak is therefore assigned to (cytoplasmic) P_i and its chemical shift of $5{\cdot}082 \pm 0{\cdot}035$ (17) indicates an intracellular pH of $7{\cdot}33 \pm 0{\cdot}04$ for cells incubated at extracellular pH (pH_o) between 4 and 6 (see Fig. 3) and using the P_i titration curve obtained in cytoplasmic salts (Fig. 2 and see Materials and Methods). The pK of P_i in cytoplasmic salts is about 0·25 of a pH unit more alkaline than that of P_i in the extract, due to the higher ionic strength of the extract (Fig. 2). Thus, although we believe that calibration in cytoplasmic salts gives the best representation of the intracellular environment of phosphate, if this assumption is incorrect there would be a slight systematic error in all of our pH_i values. However, such an error would not affect any of our main conclusions.

The P_i signal from living cells is 0·5 p.p.m. wide at half-maximal height (measured without any resolution enhancement) and is consistently broader than the extracellular P_i signal by about 0·3 p.p.m. Part of this increase is attributable to the

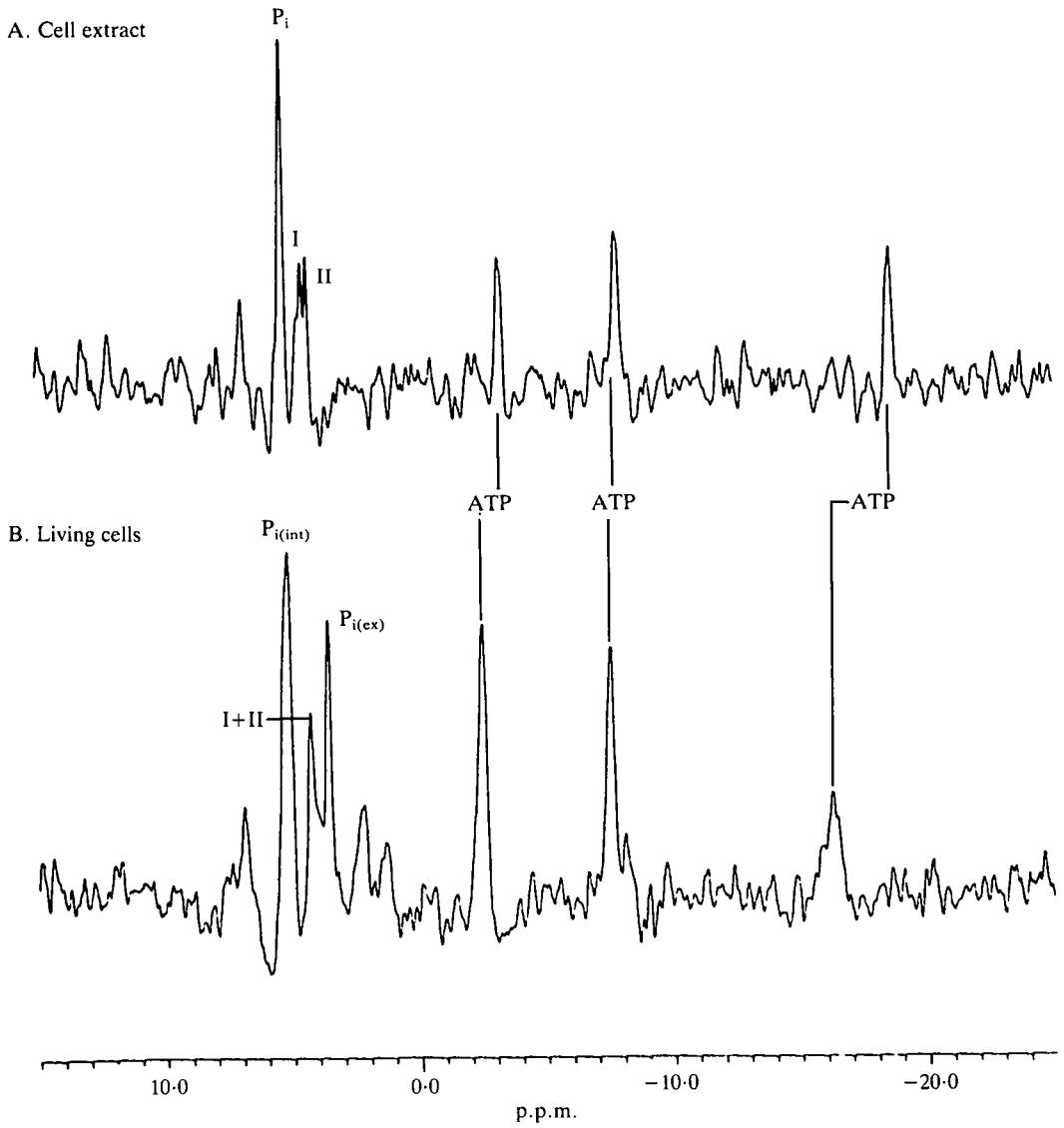

Fig. 1. ^{31}P n.m.r. spectra of living cells (B) and of the extract made from them (A). Cells of strain Ax2 were harvested during logarithmic growth and shaken in suspension for 20 min in 40 mM-Tris (citrate), pH 6·0, plus NS. they were then spun down, resuspended in the same medium at 2×10^8 to 3×10^8 ml^{-1}, n.m.r. spectroscopy was performed and a PCA extract prepared, all as described in Materials and Methods. The extract spectrum was taken at pH 7·21, close to the intracellular pH of 7·38 indicated for the living cells by the chemical shift of $P_{i(int)}$. The three peaks of ATP do not line up exactly between living cells and their extract because the Mg^{2+} in the extract has been chelated with an excess of EDTA: hence ATP is being compared with MgATP. The signals due to extracellular and intracellular phosphate ($P_{i(ex)}$ and $P_{i(int)}$) from the living cell spectrum are combined in the extract spectrum, and the peak I+II in the living cells is believed to be derived from the unknown phospho-compounds I and II seen in the extract. Chemical shifts are given relative to phosphocreatine at 0 p.p.m. in cytoplasmic salts at neutral pH and 22°C. This reference was recorded with the static field adjusted to the same value as for all the experiments (see Materials and Methods).

broader natural line-width of the intracellular signal and this can be estimated from measurements of relaxation times to contribute about 0·1 p.p.m. to the broadening of the $P_{i(int)}$ signal. We are inclined to ascribe the rest of the broadening to heterogeneity in pH_i, and it is worth noting in this respect that a broadening of 0·2 p.p.m. would correspond to about 0·2 of a pH unit. Thus, although the mean pH_i of cells is very reproducible at 7·33 it is possible that the population contains cells of pH_i varying between, say, 7·23 and 7·43.

The peak designated (I+II) from living cells is presumed to be a combination corresponding to peaks I and II in the extract. Occasionally, two peaks can be resolved in the spectra from living cells suggesting that both compounds are present. The mean chemical shift of peak (I+II) is 4·26 ± 0·06 (15), which corresponds to pH values of 6·9 and 6·8 using the extract titration curves of I and II. This is to be compared with a pH of 7·1 reported for $P_{i(int)}$ if the extract titration curve rather than the curve measured in cytoplasmic salts is used. Given the uncertainty as to whether the curves for P_i and the unidentified compounds will show similar shifts in pK between extract and living cell, it is quite possible that the unknown compounds are reporting the same pH from living cells as is P_i.

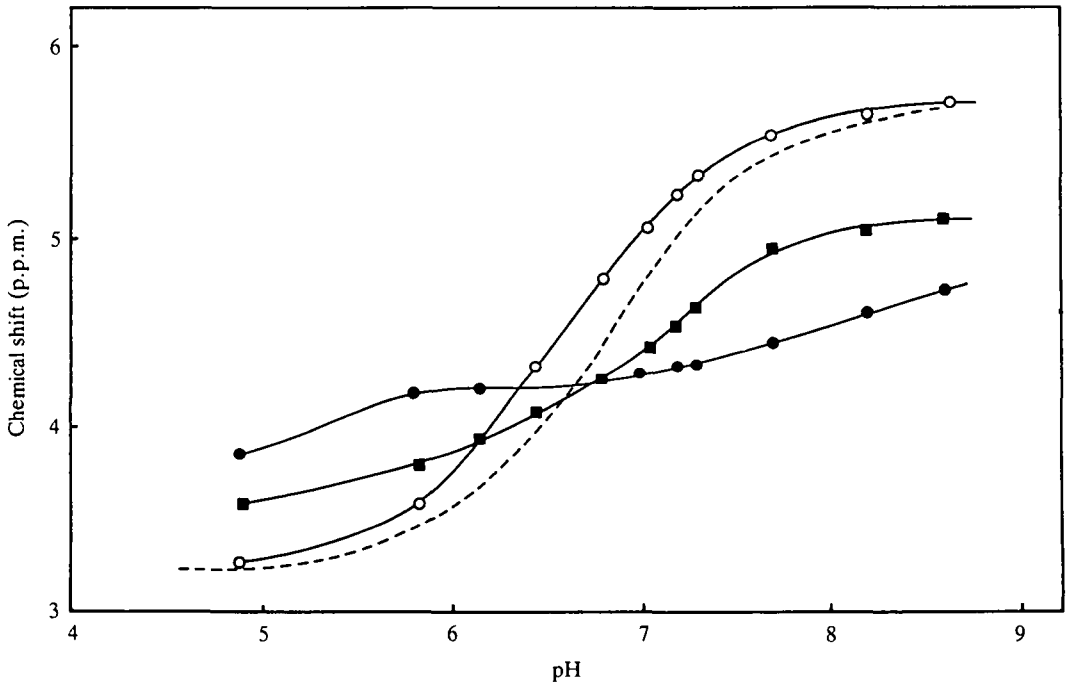

Fig. 2. Titration curve of inorganic phosphate and the unknown compounds I and II. The titrations shown by continuous lines were obtained using the extract from Ax2 cells shown in Fig. 1A; that shown by a broken line was obtained in cytoplasmic salts (see Materials and Methods), approximating the intracellular milieu and was used to obtain pH_i values given elsewhere. (■——■) Unknown phospho-compound I; (●——●) unknown phospho-compound II; (○——○) inorganic phosphate in the extract; (----) inorganic phosphate in cytoplasmic salts.

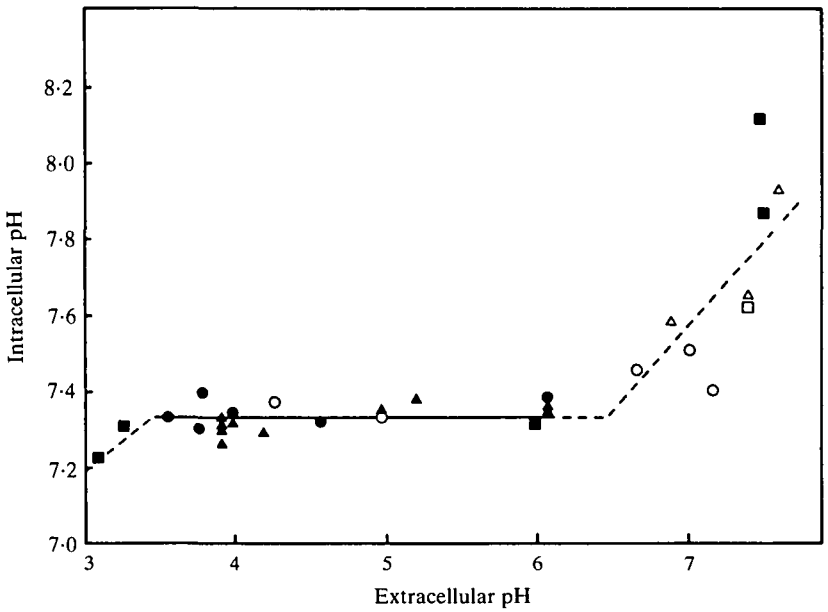

Fig. 3. Dependence of intracellular pH on extracellular pH. Freshly harvested Ax2 cells were preincubated for 15–30 min in the appropriate buffer containing NS before being spun down and subjected to n.m.r. in the same buffer. To cover a large pH range and to check for any potentially deleterious effects of the buffer, a range of buffers was used as follows: (●) 20 or 40 mM each of Tris and citric acid (KOH); (▲) 40 mM-citrate (Tris); (■) 100 mM-citrate (Tris); (△) 40 mM-HEPES (Tris); (□) 40 mM-bis-Tris-propane (citrate); (○) 100 mM-Tris (citrate). The substance in parenthesis was used to adjust the pH of the buffer. Extracellular pH was determined at the end of the n.m.r. run.

Regulation of intracellular pH

It is apparent from Fig. 3 that pH_i of Ax2 cells is essentially unaffected by incubation at pH_o values varying between 3·5 and 6. Below pH_o of 3·5 the cells become acid, ATP levels are reduced and the cells tend to lyse. At $pH_o > 6{\cdot}5$ pH_i rises and the $P_{i(int)}$ and $P_{i(ex)}$ signals cannot always be resolved from each other in the cell suspension. However, the contribution from $P_{i(ex)}$ was estimated by taking a spectrum from the cell-free supernatant after the cells had been removed by centrifugation. Cells at alkaline pH maintain high ATP levels (Fig. 4) and remain perfectly viable. Amoebae of strain V12M2 behave in a similar fashion to Ax2 at acid pH, but unlike Ax2 they are little affected by alkaline pH_o up to at least pH_o 7·50 (not shown) and they maintain a somewhat higher pH_i of 7·48.

In cells of many higher organisms, acid loads are resisted by the excretion of protons in an Na^+/H^+ exchange mechanism driven by the Na^+ electrochemical gradient (Schuldiner & Rozengurt, 1982; Roos & Boron, 1981). However, yeast and fungi have an electrogenic proton pump for pH_i regulation, which is independent of external ions (Goffeau & Slayman, 1981). Since *Dictyostelium* cells possess a fungal-type proton pump, it has been suggested that like fungi they may use this to resist acid loads (Pogge-von Strandmann *et al.* 1984). This idea is supported by the fact

that pH_i is unaffected by incubation of cells in a Tris/citrate buffer lacking Na^+ and all other added inorganic ions (Fig. 4).

pH_i during normal development

Cells of strains Ax2 or V12M2 were allowed to develop on agar, harvested at 3-h intervals and ^{31}P spectra were obtained. In neither strain was there a detectable

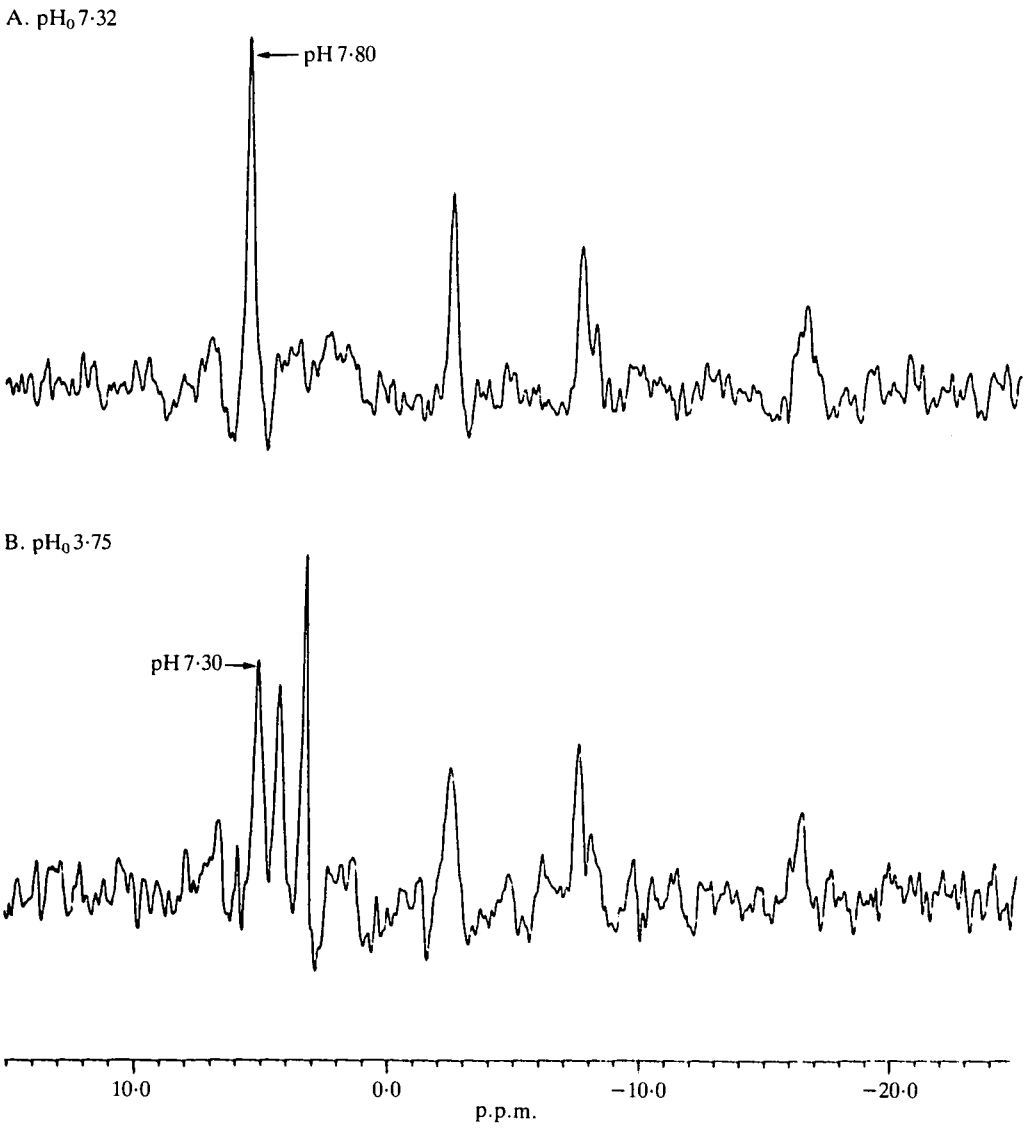

Fig. 4. ^{31}P n.m.r. spectra of Ax2 cells incubated at alkaline pH (A) or at acidic pH in the absence of added inorganic ions (B). Cells were preincubated for 15–30 min in the appropriate buffer before taking the spectra (Materials and Methods). In A this buffer was 40 mM-Tris (citrate), pH 8·2, plus NS and at the end of the n.m.r. run the pH was reduced to 7·32. In B 40 mM-citrate (Tris), pH 3·75, without any other added ions was used and the pH change by the end of the run was less than 0·3 of a pH unit. The P_i signal in A is the sum of $P_{i(int)}$ and $P_{i(ex)}$ (see Results).

Table 1. *Effects of stalk-cell inducers on intracellular pH*

Conditions	pH_i	Stalk cells (final %)
Expt 1		
Control	7·58	0
+DIF-1	7·61 (2)	>70
Expts 2, 3, 4		
Control	7·41 ± 0·09 (6)	0
+DIF-1	7·48 (2)	80
+DES	7·42 ± 0·07 (5)	59
+zearalenone	7·32 (2)	29
+tamoxifen	7·34 ± 0·18 (3)	0·3

Amoebae of strain HM44 were allowed to differentiate in tissue culture dishes for 8 h submerged in a simple salts medium containing 8 mM-cyclic AMP, as described in Materials and Methods. DIF-1 (2000 units ml^{-1}), DES (12 μM), zearalenone (60 μM) or tamoxifen (5 μM) were then added as indicated and pH_i was measured between 3 and 8 h after addition of the inducers. In experiment 1, pH_i was measured in the absence of cyclic AMP and in experiments 2, 3 and 4 it was measured in the presence of 0·5 mM-cyclic AMP. In all experiments the appropriate inducer was also present during measurement of pH_i. Stalk cells were scored by phase-contrast microscopy in undisturbed dishes after 36 h. Amoebae whose pH_i had been measured differentiated into stalk cells, when restored to their original incubation conditions, with the same efficiency as those in undisturbed dishes.

change in pH_i during development through to the slug stage (not shown). Spectra were also obtained from terminally differentiated stalk cells of the mature fruiting body, which showed a single massive signal from P_i at the acid end of its titration curve, indicating a $pH \leq 5{\cdot}5$ (not shown). This signal is most probably of vacuolar origin, as the vacuole is by far the major cellular compartment of the mature stalk cell. The low pH_i indicated is in line with that seen in plant cell vacuoles (Roberts *et al.* 1980).

pH_i and the mechanism of stalk cell induction

A specific prediction of the model relating pH_i and cell differentiation in *Dictyostelium* is that DIF, the physiological inducer of stalk cell differentiation, and the non-physiological inducers DES and zearalenone should act to lower pH_i (Gross *et al.* 1983). To test this prediction, pH_i was measured in cells that had developed in monolayers with cyclic AMP, in the presence of the various inducers. In order to minimize the effects of endogenous DIF, a 'DIF-less' mutant was used (Kopachik *et al.* 1983) and the inducers were added after 8 h of development, at which time other experiments have shown that they have an immediate effect (for instance, specific mRNAs are induced within 2 h; J. Williams, K. Jermyn, M. Berks & R. Kay, unpublished data). Removal experiments also showed that the presence of the inducer was required for at least 5 h for it to have a full effect (R. Kay, unpublished data). Thus the inducers must be having some immediate and sustained effect on the cells but, as shown in Table 1 and Fig. 5, they did not produce a systematic or large drop in pH_i. In passing it should also be noted that cells of strain

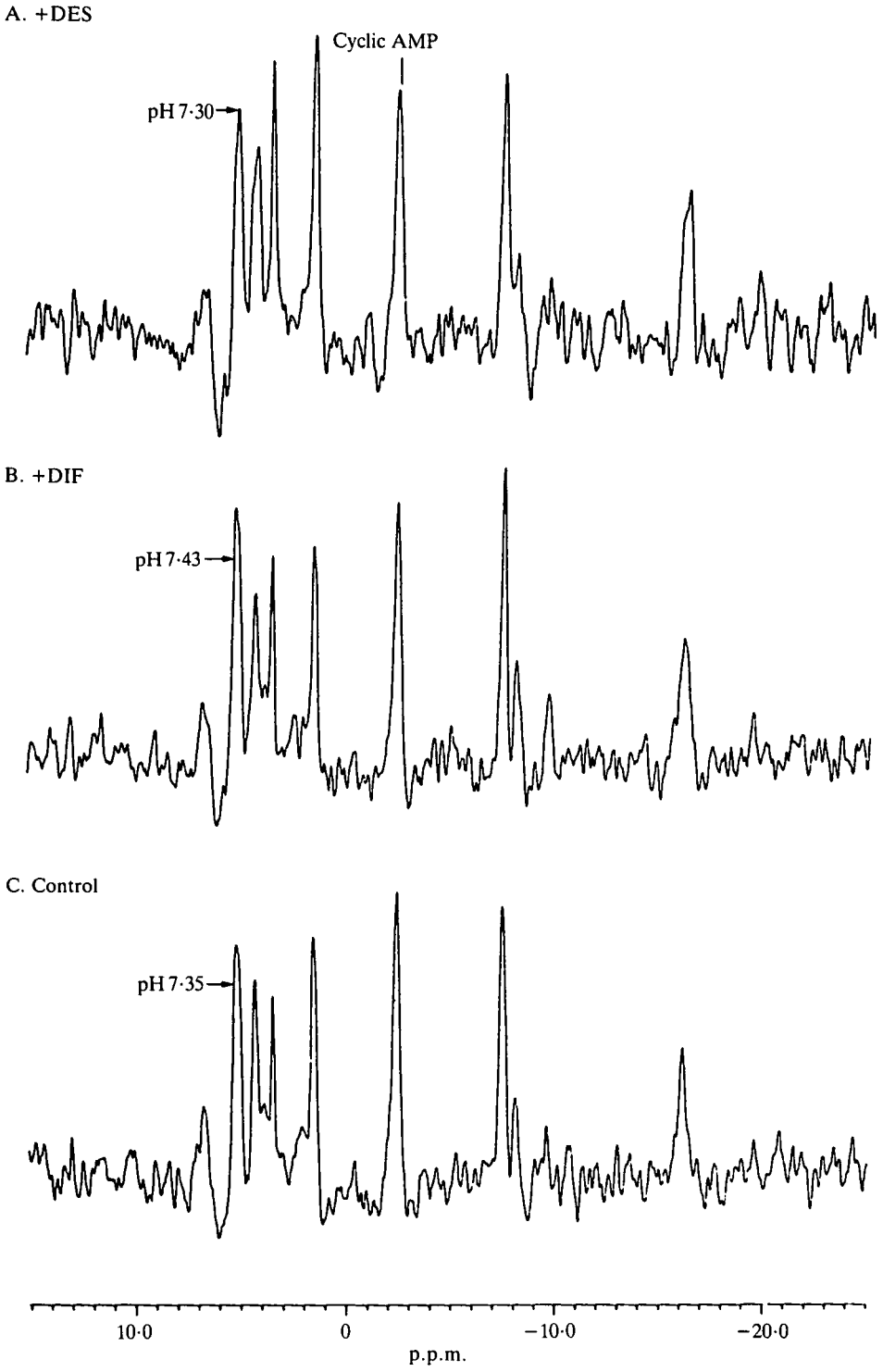
A. +DES
Cyclic AMP
pH 7·30
B. +DIF
pH 7·43
C. Control
pH 7·35
10·0
0
−10·0
−20·0
p.p.m.

Fig. 5

HM44 (like their parent V12M2) maintain a somewhat higher pH_i than do Ax2 cells. DES did not have any significant effect on pH_i, though it efficiently induced stalk cell formation, while zearalenone produced a slight acidification but was less efficient as a stalk cell inducer. Tamoxifen, a more potent proton pump inhibitor than DES (J. Gross, M. Peacey & R. Pogge-von Strandmann, unpublished data), produced a similar drop in pH_i to that induced by zearalenone but induced hardly any stalk cells. Finally, DIF-1 itself produced, if anything, a slight alkalinization of the cell, rather than the expected acidification. In further experiments with DIF-1, it was found that a very acidic P_i signal appears after about 6 h of treatment, at about the time that microscopic observation first shows vacuoles to be forming within the amoebae. The relative intensity of the acidic signal increased as mature stalk cells differentiated, until the spectrum came to resemble that of stalks separated from mature fruiting bodies (not shown). In any case the acidic shift is not an early consequence of DIF addition and so cannot be a candidate for the initial effects of DIF on cells.

DISCUSSION

High quality ^{31}P n.m.r. spectra can easily be obtained from dense suspensions of *Dictyostelium* cells, oxygenated intermittently using a gated bubbler. The cells remain healthy and are well energized in the n.m.r. tube as judged from their ATP levels. Under most conditions the spectra show two prominent cellular signals in the inorganic phosphate region. Comparing these spectra with those of freshly prepared cell extracts, we assign the more downfield peak (i.e. left hand) from living cells to P_i and the other peak to a combination of two unidentified phospho-compounds, possibly phosphomonoesters. The chemical shift of the P_i peak then indicates an intracellular pH of $7{\cdot}33 \pm 0{\cdot}04$, this value being assumed to reflect the cytoplasmic pH, since the cytoplasm should be the major cellular location of inorganic phosphate. The precise pH value indicated by a particular phosphate chemical shift depends to some extent upon the chemical milieu and, although we obtained our phosphate calibration curve in a medium approximating the intracellular one (Maeda, 1983), it is possible that there is a systematic error of about $\pm 0{\cdot}1$ of a pH unit in the absolute values obtained. It should also be borne in mind that the breadth of the P_i signal indicates a possible heterogeneity in cellular pH_i values of up to 0·1 of a pH unit either side of the mean.

In a parallel study, Jentoft & Town (1985) have obtained ^{31}P n.m.r. spectra similar to ours from living *Dictyostelium* cells, but they interpreted them rather

Fig. 5. Effect of stalk-cell inducing agents on intracellular pH. Cells of strain HM44 were allowed to develop in submerged culture for 8 h with 8 mM-cyclic AMP before addition of the inducer as indicated. At 1–5 h after this cells were harvested and resuspended in the same medium containing 0·5 mM-cyclic AMP and the appropriate inducer for spectroscopy (Materials and Methods). The new peak falling between the P_i region and γ-ATP is due to the added cyclic AMP. In undisturbed plates of cells stalk, cells were scored at 36 h by phase-contrast microscopy: controls (C) $< 0{\cdot}5$ %; +DES (A) 55 %; +DIF (B) 86 %. Cells restored to plates in their initial incubation conditions after spectroscopy differentiated with an efficiency similar to that of the undisturbed ones.

differently. In the cell extract prepared by Jentoft & Town (1985) P_i was by far the predominant signal and was much larger than the signal corresponding to our unknown compounds I and II. This led them to propose that both the major peaks in the P_i region of the spectrum from living cells must derive from P_i and therefore that there are two major compartments of different pH containing P_i in the cell. The acid signal (pH 6·48) was proposed to arise from the cytoplasm and the basic one (pH 7·16, corresponding to our cytoplasmic signal) from the mitochondria. We believe that the assignment of peaks by Jentoft & Town (1985) is incorrect and can be explained by hydrolysis of phospho-compounds in their cell extracts. The P_i/ATP ratio in our freshly prepared extracts is very similar to that in the living cells (see Fig. 1), allowing for the fact that in the extract both $P_{i(int)}$ and $P_{i(ex)}$ are combined. However, the spectrum of the extract, published by Jentoft & Town (1985), has a P_i/ATP ratio some fivefold higher than that from living cells. So even if compounds I and II have not been completely hydrolysed in the extract their contribution to the 'acidic-P_i' peak can be easily overlooked because of the overwhelming size of the P_i signal. Finally, although we regard the above arguments as conclusive, we are unaware of any other observation of clearly resolved mitochondrial and cytosolic P_i signals from unmanipulated biological systems. In isolated liver cells, where a separate signal has been attributed to mitochondria, this can only be clearly resolved from the cytoplasmic signal after treatment with valinomycin (Cohen *et al.* 1978). In the well studied case of heart muscle, no discrete mitochondrial signal is normally observed, even though this tissue is rich in mitochondria (Bailey *et al.* 1981; Garlick *et al.* 1979). A possible mitochondrial signal has been detected in isolated perfused heart (Garlick *et al.* 1983), but again this is present only as a small shoulder to the cytosolic peak.

Assuming our interpretation of the spectra to be correct, then the P_i signal from the spectra of cells of strain V12M2 obtained by Jentoft & Town (1985) indicates a pH_i of 7·10 using our phosphate titration curve and is still significantly different from our value of 7·44 for that strain and may reflect a difference in growth conditions. Estimates of the pH_i of growing Ax2 cells by the 'null point' cell lysis method (pH_i 7·2–7·45; Aerts *et al.* 1985) and of slug stage V12M2 cells by a fluorescent dye method (pH_i 6·96–7·28; Inouye, 1985) are also in good agreement with the n.m.r. results. However, the much lower values for Ax3 cells (pH_i 6·2–6·3), also obtained with a fluorescent dye method (Jamieson *et al.* 1984) are not readily reconciled with the above results.

In any event, the pH_i reported here for *Dictyostelium* cells fits comfortably within the range of values reported for other active eukaryotic cells: *Tetrahymena*, pH_i 7·25–7·55; *Saccharomyces cerevisiae*, pH_i 7·0–7·3 (Gillies, 1982); *Physarum*, pH_i 7·0–7·5 (Steinhardt & Morisawa, 1982), where in each case the range indicates the variation of pH_i during the cell cycle. Most measurements of pH_i in vertebrate cells also indicate a pH_i of 7·0–7·5 (Roos & Boron, 1981).

The pH_i of Ax2 cells is unaffected by incubation of the amoebae at an extracellular pH of 3·5, implying that the cells must possess an efficient means of exporting protons. As pH_i is maintained in buffers lacking external Na^+ and other inorganic

ions, the proton extrusion cannot be driven by an Na^+ gradient *via* an Na^+/H^+ antiport, as in many types of higher cells (Schuldiner & Rozengurt, 1982; Moolenaar *et al.* 1983). Rather these results strongly support the suggestion by Pogge-von Strandmann *et al.* (1984) that pH_i is primarily regulated by the plasma membrane proton pump in *Dictyostelium*, as in many other free-living eukaryotes (see Goffeau & Slayman, 1981). The fact that Ax2 cells cannot resist an alkaline load indicates that they lack an effective means of importing protons, though the same may not be true for cells of strain V12M2.

The idea that pH_i regulates the choice between stalk and spore cell differentiation in *Dictyostelium* (with low pH_i favouring stalk cell formation) resulted in a number of accurate predictions, most notably that inhibitors of the proton pump would be stalk cell inducers (Gross *et al.* 1983; Town, 1984; J. Gross, M. Peacey & R. Pogge-von Strandmann, unpublished data). Unfortunately the results described here are not encouraging for the hypothesis in its simplest form and suggest that some modification is required. Firstly, no significant change in pH_i was detected up to the slug stage of normal development, when the precursors of the stalk and spore cells have already differentiated. Nor was the P_i signal from slug cells split into two signals, which might correspond to prestalk and prespore cells (but see Inouye, 1985, who reported that separated prestalk and prespore cells differed in pH_i by about 0·3 of a pH unit). Secondly, in the *in vitro* conditions for cell differentiation, inducers of stalk cell differentiation did not produce the expected cytoplasmic acidification. DES, the most potent non-physiological inducer, did not produce a detectable change in pH_i, and DIF-1, the natural inducer, produced if anything a slight alkalinization.

There are several ways in which the present results might be reconciled with the original hypothesis linking pH_i and cell differentiation. At the technical level, it could be that our method of measuring pH_i lacks the necessary sensitivity or that the conditions of measurement (dense, bubbled cells) abolish the differences in pH_i that we are seeking, but we have no reason to suspect that either possibility is true. A more serious possibility is that the relevant pH changes are either too transitory to detect in our experimental protocol or are limited to a small cellular compartment, such as an endocytotic vesicle (Forgac *et al.* 1983), the signal from which is not detectable by ^{31}P n.m.r. Perhaps the most likely possibility is that the manipulations of proton flux across the plasma membrane (which will have been the effect of the treatments described by Gross *et al.* 1983) have a secondary effect on the movement of some other ion into or out of the cytoplasm and that this ion in turn controls cell differentiation. At the moment we are attracted to the idea that this ion might be Ca^{2+}.

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