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Effects of osmotic stress and growth stage on cellular pH and polyphosphate metabolism in *Neurospora crassa* as studied by ^{31}P nuclear magnetic resonance spectroscopy

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High-resolution ^{31}P-NMR was employed to investigate the effects of growth stage and environmental osmolarity on changes of polyphosphate metabolism and intracellular pH in intact *Neurospora crassa* cells. Our study showed that changes of these parameters were growth-dependent. The ratio of polyphosphate to orthophosphate in vacuoles increased from 2.4 to 13.5 in *N. crassa* as cells grew from early log phase to stationary phase. Cytoplasmic pH and vacuolar pH changed, respectively, from 6.91 and 6.49 in early log phase cells to 7.25 and 6.84 in stationary phase cells. Hypoosmotic shock of *N. crassa* produced growth-dependent changes including: (i) a rapid hydrolysis of polyphosphate with a concomitant increase in the concentration of the cytoplasmic phosphate, (ii) an increase in cytoplasmic pH, and (iii) an increase in vacuolar pH. Early log phase cells produced the most dramatic response whereas the stationary phase cells appeared to be recalcitrant to the osmotic stress. Thus, 95% and 60% of polyphosphate in the early log phase and mid-log phase cells, respectively, disppeared in response to hypoosmotic shock, but little or no hydrolysis of polyphosphate occurred in stationary cells. The cytoplasmic pH and the vacuolar pH increased in response to hypoosmotic shock by 0.4 and 0.53 unit, respectively, in early log phase cells; and by 0.22 and 0.27 unit, respectively, in the mid-log phase cells. In contrast, hypoosmotic shock of the stationary phase cells did not cause any change in intracellular pH. The osmotic stress-induced polyphosphate hydrolysis and pH changes in early log and mid-log phase cells were reversible, suggesting that these changes were related environment osmolarity. Addition of polyamines or basic amino acids which are known to be sequestered in vacuoles did not affect polyphosphate metabolism.

Introduction

Polyphosphates (poly[P_i]) are naturally occurring inorganic polymer of orthophosphate (P_i) found in many organisms, from lower eukaryotes such as yeast to higher plants and animals [1]. It has been suggested that poly[P_i] plays important roles in regulating the levels of ATP and in functioning as either a high-energy reserve or a phosphate reserve via hydrolysis [2]. Due to their polyanionic nature, poly[P_i] can also serve as counter ions for specific cations (Mg^{2+}, Mn^{2+}, basic amino acids and polyamines) and thus function in reducing the osmotic pressure exerted by basic amino acids and various small ions accumulated in fungal vacuoles [1,3]. Despite these possible functions, the lack of chromophores in the chemical structure of poly[P_i] has made it difficult to study the metabolism of poly[P_i] by conventional biochemical methods. Nevertheless, the potential importance of poly[P_i] in various physiological processes has started to generate interest in developing novel assay procedures to quantitate and analyze poly[P_i] [1,2]. Among them, the least disruptive and most quantitative one is in vivo ^{31}P-NMR [4].

In vivo NMR is a rapid and non-invasive technique suitable for studying metabolic processes in intact cells, tissues and organelles [5–7]. High resolution ^{31}P-NMR has provided valuable information on the identification of phosphate metabolites, on the determination of their concentration, on the intracellular pH and compartmentation, and on the kinetics and pathways of biochemical reactions [8–10]. For example, in vivo ^{31}P-NMR has been employed to show that hydrolysis of poly[P_i] in yeast occurs to form pyrophosphate in response to external pH or under the condition of the phosphate starvation in the growth medium [11]. Re-

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Abbreviations: poly[P_i], polyphosphate; P_i^v, vacuolar phosphate; P_i^c, cytoplasmic phosphate.

cently, the degradation of poly[P_i] to form triphosphate and P_i was observed by the addition of ammonium ions to *Saccharomyces cerevisiae* cells [12].

Almost all of poly[P_i] in *Neurospora crassa* is found in vacuoles, similar to other lower eukaryotes such as yeast [13]. In addition to poly[P_i], fungal vacuoles also sequester large pools of basic amino acids, such as arginine and ornithine, and polyamines [14,15]. Since both arginine and ornithine are precursors for polyamine biosynthesis in *N. crassa* [15,16], it has been speculated that metabolisms of poly[P_i] and polyamines in vacuoles may be interrelated [14–16]. We have been interested in the regulation of polyamine metabolism in *N. crassa* [21]. The key enzyme for polyamine biosynthesis, ornithine decarboxylase, is known to be sensitive to the osmotic and ionic characteristics of the cellular environment [17–20]. A large increase in ornithine decarboxylase activity can be induced in *N. crassa* by hypoosmotic shock (Maddatu, A. and Chen, K.Y., unpublished data). These studies, together with the notion that some cells could respond to the unfavorable stresses by hydrolyzing poly[P_i] to provide energy for maintaining cellular processes [11,12], prompted us to employ in vivo ^{31}P-NMR to examine whether osmotic stress may also affect poly[P_i] metabolism in *N. crassa*. We found that the hypoosmotic treatment of *Neurospora* cells caused a rapid hydrolysis of vacuolar poly[P_i] with the concomitant increases of intracellular P_i and pH. Moreover, the degree of the response of vacuolar poly[P_i] to osmolarity change was found to be dependent on the growth stage of the cell culture.

Materials and Methods

Strains and Neurospora culture. Wild-type strain 74A *N. crassa* was from the collection of R.H. Davis, University of California at Irvine. The conidial inoculum was grown as described [22]. Mycelia cells were grown exponentially from conidia (about 10^6 conidia per ml) in a 500 ml round-bottomed flask with aeration at 24°C. Vogel's minimal medium containing 5% sucrose was used as the growth medium. Cell cultures grown to an optical density (546 nm) of 0.8, 1.4 and 1.9, were designated as, respectively, early log, mid-log and stationary-phase cultures. In general, 200–300 ml of mycelia cultures at indicated growth stage were harvested for various treatment and NMR analysis. For hypoosmotic treatment, cells were washed once and then incubated in sterile water in a 500-ml round-bottomed flask for various time periods as indicated in the figure legends.

NMR measurements and data analysis. For the measurement of NMR spectroscopy, mycelia were collected by filtering through a Whatman filter paper No. 540 using a water aspirator, washed thoroughly with either Vogel's minimal medium or distilled water. The wet cell samples were resuspended in about 0.5 ml of Vogel's medium or distilled water containing 10% D_2O (to lock the magnetic field during measurements) and packed into a 5-mm NMR tube. Since mycelia were used in the experiment, the sample amount was measured in terms of either optical density or dry weight of mycelia per ml [22]. Each sample tube for NMR measurement contained about 20 to 50 mg dry weight of mycelia.

^{31}P-NMR measurements were performed with a Varian XL-400 spectrometer operated at 162 MHz with a sweep width of 20000 Hz. The free induction decay was performed with line broadening of 5.5 Hz. The flip angle used in all the measurements was calculated to be 0.61π radian. The RF pulse duration was 16 μs and the acquisition time was 0.398 s. All spectra were accumulated by the sum of free induction decays (760 to 1200) obtained within 4–10 min. Since all experiments were carried out at controlled temperature (10°C) and the duration of NMR measurement was short, we did not take extra measures to control extracellular conditions such as pH and oxygenation. Nevertheless, we did compare the medium pH before and after NMR measurements and found no difference. Chemical shifts were measured as ppm units using 85% phosphoric acid as an external standard and calibrated with glycerophosphocholine as an internal standard at $\delta = -0.49$ ppm [23]. Cytoplasmic and vacuolar pH values were measured from the chemical shifts of orthophosphate peaks using 50 mM sodium phosphate as a calibration curve as described by Moon and Richards [24]. No corrections were made to measure for the intracellular environment of ionic strength and metal-ion binding.

Results

^{31}P-NMR spectrum of N. crassa at mid-log phase

Fig. 1 shows a representative ^{31}P-NMR spectrum of *Neurospora* cells grown at mid-log phase. The assignments of all peaks were made by comparing the ^{31}P-NMR spectrum of *Neurospora* with that of yeast and other microorganisms previously reported [8,11,12]. Three major resonance peaks with chemical shift of -22.43 ppm (peak 1), $+1.30$ ppm (peak 7) and $+1.80$ ppm (peak 8), were assigned, respectively, to poly[P_i] (non-terminal residues), vacuolar phosphate (P_i^v) and cytoplasmic phosphate (P_i^c). The phosphates at the α-position of nucleotide tri- and diphosphates were resonanced at -10.81 ppm, those at the β- and γ-positions of nucleotide triphosphate were at -19.50 ppm and -5.93 ppm, respectively. The peak at $+3.54$ ppm for sugar phosphate was also assigned. The chemical shift of P_i peak, representing the weighted average shift of monobasic and dibasic phosphates, can be use

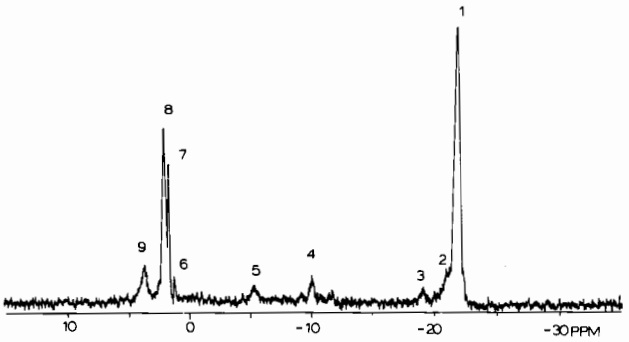

Fig. 1. ^{31}P-NMR spectra of *N. crassa* cells in the logarithmic phase of growth. Peaks assigned for various phosphorous compounds as follows: 1, polyphosphate (non-terminal phosphate residues); 2, polyphosphate (non-terminal phosphate residues); 3, penultimate residues of long chain polyphosphates; 4, diphosphates and sugar dinucleotides; 5, terminal residue of nucleotide triphosphates; 6, glycerophosphocholine or α-glycerol phosphoryl ethanolamine; 7, vacuolar orthophosphate; 8, cytosolic orthophosphate; 9, sugar monophosphates.

to estimate accurately the pH in various intracellular compartments in intact cells [24]. By using the P_i titration curve and the chemical shift of the P_i^c and P_i^v peak, we estimated the cytoplasmic pH and vacuolar pH in *N. crassa* at mid-log phase to be 7.03 and 6.63, respectively.

Effect of hypoosmotic shock on poly[P_i] hydrolysis

Fig. 2 shows the time-course of the effect of hypoosmotic shock on poly[P_i] metabolism in *N. crassa* (mid-log phase) as revealed by ^{31}P-NMR. 2 h after hypoosmotic treatment, the changes in the intensity of poly[P_i] peak and cytoplasmic P_i peak were striking (Fig. 2B vs. 2A). The decrease in poly[P_i] peak was accompanied by a concomitant increase in cytoplasmic P_i peak, suggesting a precursor-product relationship between these two phosphorous species. The remaining poly[P_i] in vacuoles appeared to be resistant to further hypoosmotic shock, since no further change in ^{31}P-NMR spectral pattern was observed when mycelia were maintained in water for 4 h (Fig. 2C vs. 2B). To examine whether the disappearance of poly[P_i] truly represents a physiological response or may be due to a permanent damage to the organism, hypoosmotically-treated cells were re-incubated in Vogel's medium for 30 min, and ^{31}P-NMR spectrum was taken. Fig. 2D shows that the poly[P_i] peak was fully recovered in hypoosmotically treated cells after transferring from water back to Vogel's medium. The recovery of the poly[P_i] peak was also accompanied by an increase in the ratio of poly[P_i] to P_i^c peak, again suggesting a product-precursor relationship between cytoplasmic P_i and poly[P_i].

Effects of growth stages of N. crassa on ^{31}P-NMR spectra

Intracellular pH and poly[P_i] turnover in yeast have been shown to be dependent on the growth stage [12,25]. A comparison of ^{31}P-NMR spectra of *N. crassa* at three different stages of growth, namely, early log phase, mid-log phase and stationary phase (Figs. 2A, 3A and 5A) revealed that growth stage of *N. crassa* significantly affected the following parameters: (i) cytopasmic pH and vacuolar pH, (ii) relative amount of poly[P_i] to vacuolar P_i, and (iii) relative amount of cytoplasmic P_i to vacuolar P_i. The effects of growth stage on these parameters are summarized in Table I. Of particular note is a marked increase in the ratio of poly[P_i] peak to P_i^v peak when mycelia grew from an early log phase to stationary phase. Thus, the majority of phosphorus in the stationary phase cells was in the form of poly[P_i] (Fig. 5A). The significant differences in ^{31}P-NMR spectra of *N. crassa* at different growth stage may allow us to identify the growth stage of *N. crassa* cultures based on their ^{31}P-NMR spectra.

Effects of the hypoosmotic shock of N. crassa at different stages of growth

The effects of hypoosmotic shock of *N. crassa* shown in Fig. 2 also appear to be functions of growth stage.

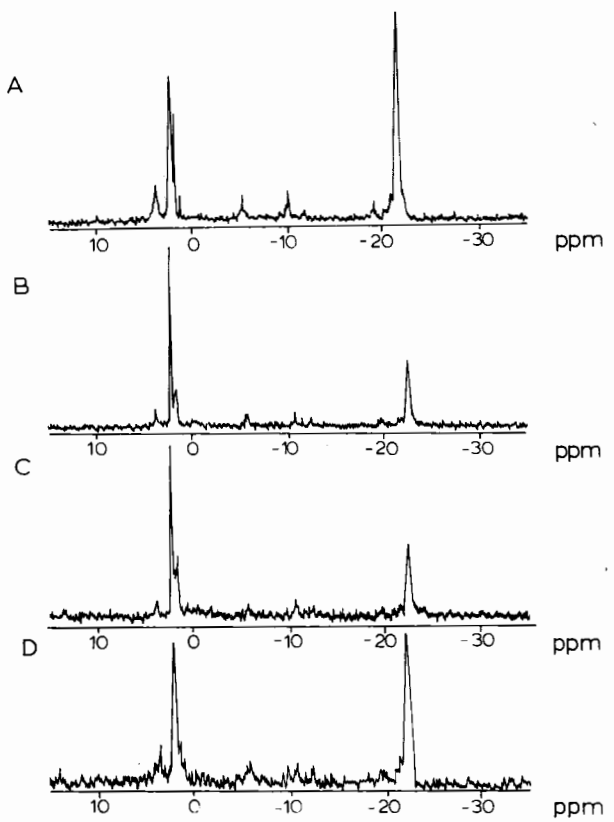

Fig. 2. ^{31}P-NMR spectra of *N. crassa* cells in the mid-logarithmic phase of growth. (A) untreated, (B) hypoosmotically treated for 2 h, (C) hypoosmotically treated for 4 h and (D) hypoosmotically treated for 2 h and then re-incubated in Vogel's medium for 30 min.

TABLE I

Effects of growth stages of N. crassa cells on cytoplasmic and vacuolar pH and phosphorus metabolites

Cytoplasmic and vacuolar pH in cells were estimated from the chemical shifts of P_i^c and P_i^v peaks using standard titration curve as described in Materials and Methods. The concentrations of poly[P_i] and free P_i in vacuole were estimated from the peak areas in ^{31}P-NMR spectra as determined by integration. The values presented represent an average of three separate measurements with errors less than 5%.

	Early log	Mid-log	Stationary
Cytoplasmic pH	6.91	7.03	7.25
Vacuolar pH	6.49	6.63	6.84
Cytoplasmic/vacuolar pH gradient	0.42	0.40	0.41
Poly[P_i]/P_i^v	2.38	3.26	13.5

Fig. 3 shows that, within 2 h of hypoosmotic shock, the poly[P_i] peak in early log phase cells disappeared almost completely (Fig. 3B vs. 3A). Concomitant with the disappearance of poly[P_i] peak, there was a large increase in cytoplasmic P_i peak, suggesting that the two processes were related. Fig. 4 shows that such a product-precursor relationship appears to be regulated by medium osmolarity. After 2 h incubation under hyperosmotic condition, almost all phosphorus in log-phase cells appeared in the poly[P_i] peak (Fig. 4C vs. 4B). However, under hypoosmotic condition, majority of phosphorus appeared in cytoplasmic P_i peak (Fig. 4D vs. 4B). In contrast, such an osmolarity-dependent

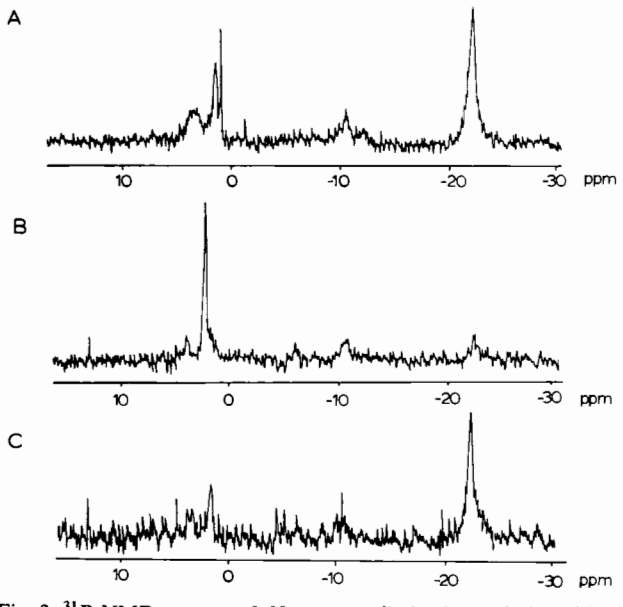

Fig. 3. ^{31}P-NMR spectra of *N. crassa* cells in the early logarithmic phase of growth. (A) Untreated, (B) hypoosmotically treated with water for 2 h, (C) hypoosmotically treated for 2 h and then re-incubated in Vogel's medium for 30 min before NMR spectroscopy.

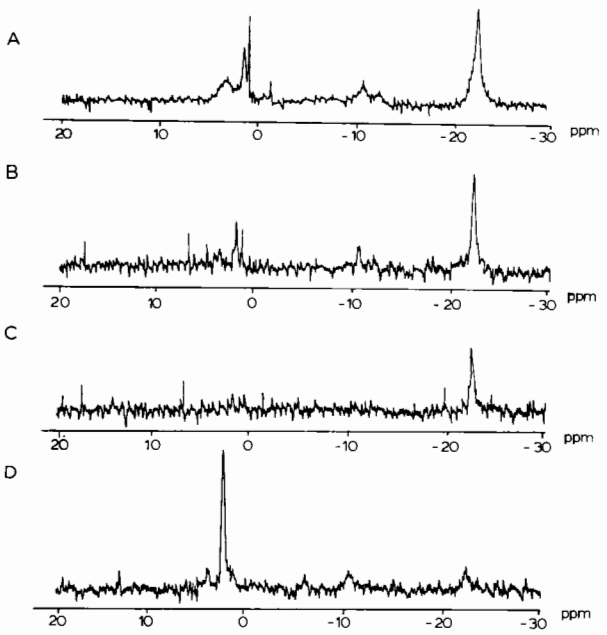

Fig. 4. ^{31}P-NMR spectra of *N. crassa* cells in the logarithmic phase of growth. (A) Cells were harvested at the logarithmic phase of growth; (B) cells were incubated in fresh Vogel's minimal medium for 30 min; (C) cells were incubated in fresh Vogel's minimal medium containing 1 M sorbitol; (D) cells were incubated in distilled water.

complementary behavior of poly[P_i] and cytoplasmic P_i appears to be absent in stationary cells. Fig. 5 shows that the poly[P_i] peak of stationary phase cells remained prominent with little or no reduction of intensity after an exposure to water for 2 h (Fig. 5B vs. 5A). Further hypoosmotic treatment of the stationary phase cells had no effect on the intensity of the poly[P_i] peak (data not shown).

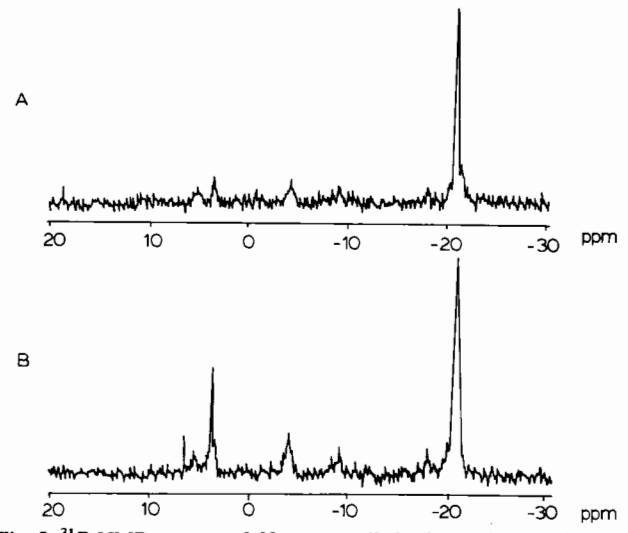

Fig. 5. ^{31}P-NMR spectra of *N. crassa* cells in the stationary phase of growth. (A) Untreated control; (B) hypoosmotically treated for 2 h.

Effects of hypoosmotic shock on intracellular pH

In addition to poly[P_i] metabolism, ^{31}P-NMR spectra also revealed other changes resulting from hypoosmotic shock including a downfield shift of P_i^c and P_i^v peak, indicating increases in cytoplasmic and vacuolar pH. Table II summarizes the effects of growth stage on these changes as calculated from ^{31}P-NMR spectra shown in Figs. 2, 3, and 5. In response to hypoosmotic shock, early log phase cells exhibited the largest increase in cytoplasmic and vacuolar pH, by 0.4 and 0.53 unit, respectively. For mid-log phase cells, hypoosmotic shock caused pH increases in both cytoplasmic and vacuolar compartments, by 0.22 and 0.27 unit, respectively. In contrast, little or no change in either cytoplasmic or vacuolar pH could be observed in stationary cells following 2 h of hypoosmotic shock. Since the magnitudes of pH changes induced by osmotic stress appeared to be propotional to the extent of poly[P_i] hydrolysis in *N. crassa* at different stages of growth, these two events may be either coupled or similarly coordinated.

Effects of polyamines and amino acids on poly[P_i] metabolism

The vacuole in *N. crassa* has been considered as an amine- and poly[P_i]-storage organelle which contributes significantly to the control of cytosolic basic amino-acid concentrations [3]. About one third of polyamines in *N. crassa* is found to be sequestered in vacuole [14]. It has been suggested that poly[P_i] may serve as counter ion in reducing the osmotic pressure exerted by basic amino acids and various small ions accumulated in vacuoles of yeast and *N. crassa* [1,3]. It is therefore of interest to examine whether perturbation of amino-acid and polyamine pools in *N. crassa* will affect poly[P_i] metabolism. Fig. 6 shows that an addition of alanine, arginine or putrescine, up to 10 mM, to *N. crassa* did not significantly alter the ^{31}P-NMR spectra. We have also examined the effects of lysine, ornithine and polyamines (spermidine and spermine), on poly[P_i] metabolism and found that none of them affected ^{31}P-NMR spectra (data not shown), indicating that poly[P_i] metabolism was not affected by the presence of basic amino acids or polyamines.

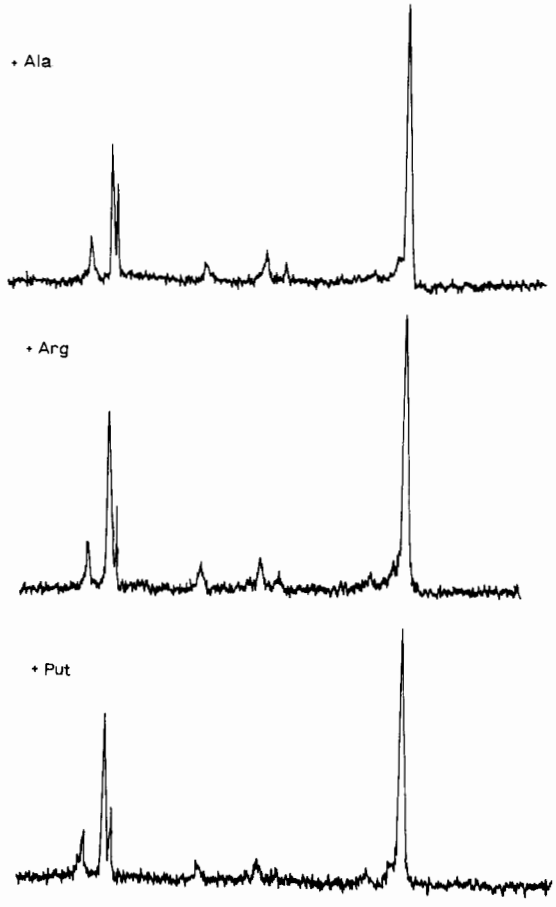

Fig. 6. Effects of amino acids and polyamines on the ^{31}P-NMR spectra of *N. crassa* cells at logarithmic phase. (A) 10 mM alanine, (B) 10 mM arginine, (C) 10 mM putrescine. Exogenous amino acid or polyamine was added to the cultures grew at mid-logarithmic phase to a final concentration of 10 mM and incubated for 60 min before NMR spectroscopy.

TABLE II

Effects of hypoosmotic treatment on polyphosphate metabolism and intracellular pH

ΔpH^c, the difference of cytoplasmic pH in hypoosmotically-treated cells relative to that in the control cells; ΔpHv, the difference of vacuolar pH in hypoosmotically-treated cells relative to that in the control cells; Δpoly[P_i], percentage of poly[P_i] hydrolysis after hypoosmotic shock. The peak area of poly[P_i] in each spectrum was determined by integration. The estimated error from three separate measurements was less than 5%.

	ΔpH^c	ΔpH^v	Δpoly[P_i]
Early logarithmic phase	+0.4	+0.53	−95%
Mid-logarithmic phase	+0.22	+0.27	−60%
Stationary phase	0	0	0

Discussion

^{31}P-NMR spectroscopy allows one to obtain a dynamic picture of the status of phosphorus metabolites in living cells under various experimental conditions. The ^{31}P-NMR spectrum of *N. crassa* at mid-log phase shows that poly[P_i] and P_i are two major phosphorus-containing components in cells (Fig. 1), similar to that reported for other fungal systems [11,12]. The present study, however, further showed that the ^{31}P-NMR spectra of *N. crassa* at different growth stage appear to be strikingly different (Figs. 2A, 3A and 5A). The most distinct feature is the ratio of poly[P_i] to P_i^v (Table I). This finding should permit one to use ^{31}P-NMR spectra to predict the growth stage of *N. crassa* cultures.

Whether the increase in poly[P_i] accumulation during growth is due to an increase in biosynthesis or a decrease in degradation, or both, remains to be investigated. Since poly[P_i] has been suggested to be a reserve for P_i and energy, it is possible that such a growth-dependent change in poly[P_i] may reflect a built-in strategy for *N. crassa* under conditions of nutrient deprivation. Nevertheless, it can be noted that such a growth-dependent change in poly[P_i] in vacuoles is not observed in yeast [12].

In addition to the ratio of poly[P_i] to P_i^v, the cytoplasmic pH and vacuolar pH were also found to be growth-dependent (Table I), both values increased by about 0.35 pH units when *N. crassa* grew from early logarithmic phase to stationary phase. Whether these changes in intracellular pH can be related to poly[P_i] metabolism is not clear. Growth-dependent changes of intracellular pH has been observed by ^{31}P-NMR in *S. cerevisiae* [12] and *Candida albicans* [26]. In both cases, cytoplasmic pH increases but vacuolar pH decreases during growth, resulting in an increase in cytoplasmic/vacuolar pH gradient, presumably maintained by a proton-translocating ATPase [27]. Whether such an ATPase exists in *Neurospora* cells and whether it plays a role in maintaining the pH gradient remain to be investigated.

^{31}P-NMR spectra of logarithmic phase cells under hypoosmotic conditions revealed a rapid disappearance of poly[P_i] peak (Figs. 2 and 3). This observation could be due to (i) an immobilization of soluble poly[P_i] and the reduction of the degree of NMR visibility, (ii) a breakdown of poly[P_i] due to hydrolysis, or (iii) both. Since the disappearance of poly[P_i] peak is accompanied by a concomitant rise of P_i^c peak (Fig. 3B vs. 3A), we proposed that poly[P_i] hydrolysis in *N. crassa* is a likely cause for the disappearance of poly[P_i] peak after hypoosmotic shock. The almost quantitative conversion of poly[P_i] to cytoplasmic P_i as shown in Fig. 3 also supports this notion. That poly[P_i] hydrolysis may indeed be tightly coupled to cellular osmoregulation is further suggested by the following observations: (i) The hydrolysis of poly[P_i] is reversible once the hypotonically-treated cells were re-incubated in normal Vogel's medium (e.g., Fig. 3C vs. 3B) and (ii) The relative peak intensity of poly[P_i] can be manipulated by osmolarity in medium (Fig. 4). Surprisingly, hypoosmotic shock-induced hydrolysis of poly[P_i] was observed only in early and mid-log phase cells but not in stationary phase-cells (Fig. 5 vs. Figs. 2 and 3). If indeed poly[P_i] hydrolysis represents a protective mechanism against osmotic stress, stationary cells under hypoosmotic conditions may have to evoke a protective mechanism other than poly[P_i] hydrolysis. In this regard, it is of interest to note that osmotic stress also induces ornithine decarboxylase activity and caused a large increase in putrescine accumulation in both prokaryotes and eukaryotes, and that putrescine has been suggested to play a role in cellular osmoregulation [19,20]. Since our data demonstrated a different intracellular milieu in *N. crassa* cells at different growth stages (Table I), it is tempting to speculate that intracellular milieu (e.g., poly[P_i]) may affect cellular osmoregulation. For example, the osmotic stress-induced pH shift is observed only in logarithmic-phase cells but not in stationary cells (Table II).

Osmoregulatory processes are important to almost all living organisms for the stabilization of the intracellular milieu against environmental fluctuation of water and ionic activities. In the present study, we were able to monitor changes of the composition of intracellular milieu under stress conditions using an in vivo NMR technique. Our data indicated that osmotic stress induces hydrolysis of poly[P_i] and increases of pH in both cytoplasmic and vacuolar compartments. Moreover, our data showed that all these changes are tightly coupled to the growth stage of *N. crassa* in cultures.

Acknowledgements

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