Study of Polyphosphate Metabolism in Intact Cells by 31-P Nuclear Magnetic Resonance Spectroscopy

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1 Introduction

Inorganic polyphosphates (polyP) are naturally occurring linear polymers of orthophosphate that are found in microorganisms, lower eukaryotes such as yeast, and animals (Harold 1966, Kulaev and Vagabov 1983; Wood and Clark 1988). In certain cases, polyP can accumulate to more than 10% of total dry mass. The ubiquitous presence of polyP suggests that they may have important physiological functions. For example, polyP has been proposed to function as a high-energy reserve or a phosphate reserve and may play an important role in regulating the levels of ATP (Harold 1966). Due to their polyanionic nature, polyP can also serve as counter ions for cationic species such as Mg^{2+}, Mn^{2+}, basic amino acids, and polyamines. In this regard, they may function in counteracting the osmotic pressure exerted by basic amino acids and various cations accumulated in fungal vacuoles. In addition, the presence of polyP in nuclei and membrane in certain organisms would suggest that polyP may have other unknown functions. It has been difficult to study the metabolism, regulation, and function of polyP for the following reasons: (1) polyP does not possess chromophores in its chemical structures; (2) polyP cannot easily be derivatized with specific chromophore or fluorescent probe; and (3) polyP may exist as a mixture of polymers with varying chain length, ranging from 3 to 1000 residues. Several analytical methods have been developed to study polyP, including enzymatic assay, HPLC and electrophoresis (reviewed by Wood and Clark 1988). Among them, in vivo phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) remains, arguably, the unique one, being the least disruptive and quantitative (Roberts 1987).

In vivo NMR is a rapid and non-invasive technique suitable for studying metabolic processes in intact cells, tissues, and organelles. High resolution ^{31}P-NMR has provided valuable information on the identification and quantitation of phosphorus metabolites, on the intracellular pH and compartmentation, and on the kinetics and pathways of biochemical reactions. The

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use of phosphorus NMR to study polyP began almost a quarter of a century ago. The subject was reviewed more than a decade ago (Roberts 1987).Thus, this chapter will emphasize more on the work appearing in recent literature.

2 Phosphorus NMR

2.1 Basic Principles of NMR

Atomic nuclei with spin quantum number other than zero behave like a microscale magnetic bar. In the presence of external magnetic field, the nuclei with spin number of 1/2 can assume two orientations, each with associated quantum number of +1/2 or −1/2. The energy difference between these two states is proportional to the external field B_0 as shown by the following equation:

$$\Delta E = (\gamma/2\pi)hB_0,$$

where γ is gyromagnetic ratio of the nucleus, h is the Planck constant, and B_0 is the external field. Transistion can be induced by applying an oscillating magnetic field B_1, in the plane perendicular to the direction of B_0, with a frequency, υ_0, where υ_0 satisfies the following equation:

$$\upsilon_0 = (\gamma/2\pi)B_0.$$

Due to the nature of the chemical environment of the nuclei, the true effective field, B_{eff}, that the nucleus can experience may differ somewhat from B_0 as expressed with equation:

$$B_{eff} = B_0\ (1-\sigma),$$

where σ, shielding constant, is the small secondary field generated due to the electronic structures surrounding the nucleus.

The nuclear magnetic resonance (NMR) spectrum of a sample in a magnetic field can be generated by either sweeping the applied irradiation or by sweeping the external magnetic field (continuous-wave mode approach). The NMR spectrum thus generated appears as an array of resonance signals characteristic of the sample and reveals the chemical nature of the compounds containing the particular nuclei and the abundance of the nuclei.

This continuous-wave mode detection method has been replaced by the Fourier-transform (FT) NMR since 1966. In FT NMR, the radio frequency field is applied as pulses that cover sufficient range to excite all nuclei in the sample. The signal received after each pulse contains many frequency components and is called free induction decay (FID). This time-dependent signal is then converted to all its frequency-dependent components via a Fourier transform as shown by the equation:

$$F(\omega) = \int_{-\infty}^{+\infty} f(t)e^{iwt}dt,$$

where ω is frequency and equals 2πυ and i is the imaginary. The introduction of pulse-radio frequency, Fourier transform and improvement of superconducting magnets have greatly enhanced the sensitivity and power of NMR spectroscopy. There are three major types of application of NMR in biological systems. First, it can be used to study the structure of biological molecules such as proteins and nucleic acid in solution. It can also be used to examine the metabolism and native environment of biological molecules in the biological samples (e.g. intact cells). Finally, with additional magnetic field gradients, it can be used to generate images of biological samples.

2.2 Phosphorus NMR

The characteristics of ^{31}P-NMR are: (1) the ^{31}P nucleus is 100% naturally abundant, eliminating the need for enrichment; (2) the ^{31}P nucleus has a nuclear spin of 1/2 with a magnetic moment of 1.1305, and no nuclear quadrupole moment, thus eliminating the more complicated signal quadrapolar splitting: (3) the ^{31}P nucleus has moderate relaxation times, allowing relatively rapid signal averaging; (4) the ^{31}P-NMR chemical shifts of phosphorus compounds span over 600 ppm. The use of the multinuclear FT NMR Spectrometer (80–800 MHz, proton frequency) has also greatly alleviated the problem associated with low sensitivity of the ^{31}P nucleus that is only about 1/15 of that of ^{1}H at the same field. Phosphorus compounds of biological interest include phosphates, phosphonates, and various easters of phosphates and phosphonates. The chemical shift of ^{31}P in these biological compounds can span over a 30-ppm range, making ^{31}P-NMR an attractive tool for examining phosphorus metabolites in microorganisms, plants, and animal tissues. In addition, ^{31}P-NMR does not suffer from the problem of solvent suppression since no water signal will appear in the ^{31}P resonance region. Figure 1 shows schematically the chemical shifts of common biological compounds containing phosphorus. As can be seen, the phosphorus compounds can be grouped according to their chemical shifts. The simplicity of the ^{31}P spectrum, usually containing 8 to 12 resonances, is due to the fact that narrow signals are generated only from relatively mobile compounds. The insoluble or highly immobilized species such as membrane-bound phospholipids usually give very broad signals that are either NMR invisible or appear as broad components underlying the narrow metabolite signals.

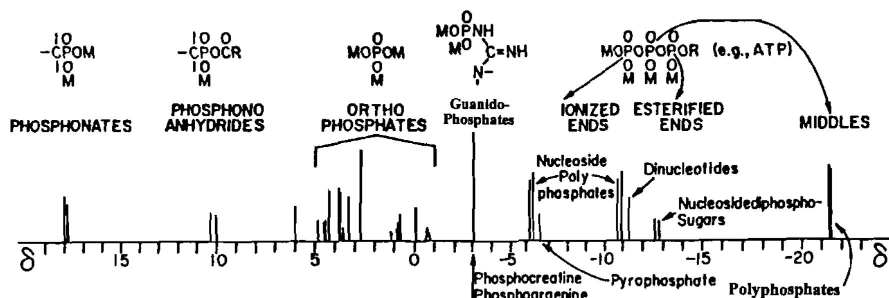


Fig. 1. Chemical shifts of biological phosphorus compounds at pH 10.0. (Adapted from Van Wazer and Ditchfield 1987)

3 Polyphosphates

3.1 Inorganic and Organic

A phosphate group can be defined as a molecular structure in which each phosphorus atom is surrounded by an approximately tetrahedral array of four oxygen atoms. Phosphates with one or more P-O-P linkages are formed by sharing of an oxygen between two different phosphate groups. Straight P-O-P chains are thermodynamically more preferred to branched chains so that linear and simple cyclic molecules are the common forms of phosphate molecules. Inorganic polyphosphate (polyP) refers to linear P-O-P chains containing more than three PO_4 units. In living systems, linear inorganic polyP can be as long as 1000 PO_4 units (Wood and Clark 1988). In general, polyP with a chain length of less than ~20 units is considered short-chain polyP whereas polyP with a chain length greater than 50 units is termed long-chain polyP. In vivo NMR only detects polyP, both short-chain and long-chain, that are sufficiently mobile. The term polyphosphate has also been used to refer to biological compounds such as diadenosine polyphosphates or inositol polyphosphates. This chapter is only concerned with phosphorus NMR study of linear inorganic polyphosphates.

3.2 Metabolism, Regulation, and Function

Most living microorganisms, from bacteria to yeast and algae, contain polyP to as much as 10~20 % of their dry weight (Kulaev and Vagabov 1983). The regulation and the function of polyP are of considerable interest in view of their high content in these microorganisms. PolyP is also present in higher eukaryotes, albeit at lower concentrations (Kumble and Kornberg 1995).

Several enzymes specifically involved in polyP metabolism have been purified or identified (Wood and Clark 1988). Some of these enzymes, including polyphosphate kinase and exopolyphosphatase from *E. coli* and yeast, have been cloned (Akiyama et al. 1993; Wurst et al. 1995), making it possible to employ genetic and molecular biological approach to study the polyP regulation.

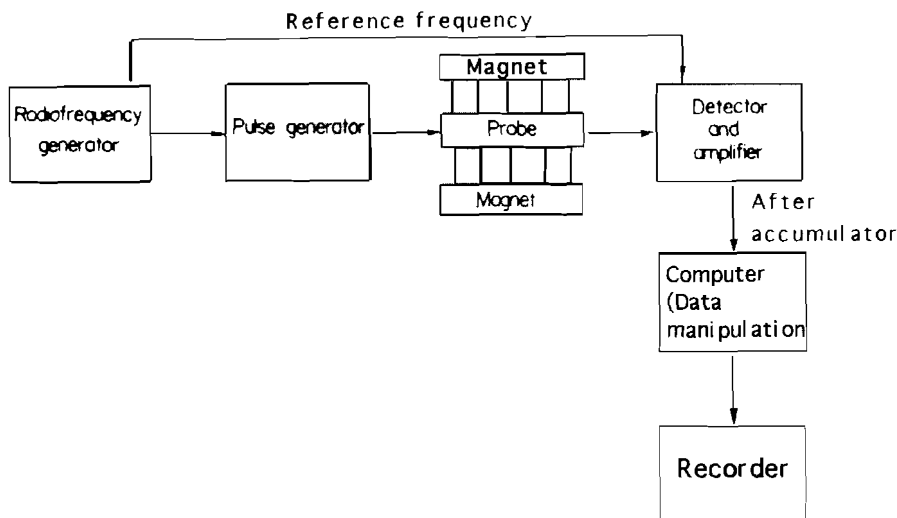
The ubiquitous occurrence of polyP, the wide range of polyP content in different organisms, and the subcellular distribution of polyP all suggest that polyP may have diverse functions depending on the cell types, organisms, and environments. So far polyP has been reported to function as (1) an energy storage source (Kulaev 1979), (2) a phosphate reserve (Kulaev 1979), (3) a substrate for glucokinase (Phillips et al. 1993), (4) a substrate for adenylate kinase (Bonting et al. 1991), (5) a buffer against pH stress (Pick et al. 1990) (6) a counterion to neutralize cationic species in vacuole (Cramer and Davis 1984), (7) a component of specific membrane channel for DNA entry (Reusch and Sadoff 1988), and (8) a regulator in response to environmental stress (Yang et al. 1993). It can be anticipated that, with the advancement of polyP research, additional functions will be discovered.

4 Use of Phosphorus NMR to Study Polyphosphate in Intact Cells

4.1 NMR Apparatus and Sample Preparations

Figure 2 (above) shows the schematic block diagram of the pulse-Fourier-transform NMR spectrometer which uses high-power radio frequency pulses and Fourier transformation to achieve high sensitivity. In brief, very strong and homogeneous magnetic fields (2.0–18.9 Telsa) are produced by superconducting magnets where the sample will reside. A short, high-powered radio frequency pulse, lasting for microseconds, will be applied to the sample. The pulse is equivalent to a full range of frequencies for the particular nucleus examined. The response of the sample to the pulse is called time-domain signal, or free induction decay (FID), which is stored in the computer. Thousands of these accumulated time-domain signals are added and mathematically treated by Fourier transform which converts amplitude vs time signals into amplitude vs frequency signals. Figure 2 (below) illustrates that the Fourier transform of the FID gives NMR spectrum as a function of frequency.

Intact viable cells can be presented to NMR instrument in three types: (1) cells in concentrated suspension are transferred into NMR tube (Salhany et al. 1975); (2) cells or tissues, imbedded in agarose gel or beads, are trans-



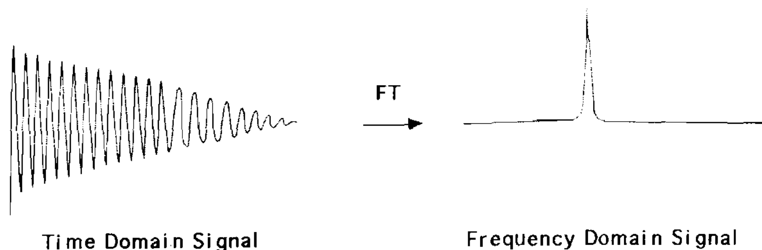


Fig. 2. *Above* Fourier-transform NMR spectroscopy. *Below* A time-domain FID is converted by Fourier transform (*FT*) to a frequency-domain signal

ferred into NMR tube that is equipped with perfusion device (e.g. Bental et al. 1990); and (3) cells are maintained in a cultivator which has radio frequency coil inserted (e.g. Mechan et al. 1992). There has been no rigorous and systematic study to compare these methods in order to determine whether the conditions of sample preparation may affect the polyP metabolism as monitored by NMR. Nevertheless, magnets with bore size up to 40 cm are available at 4.7 Telsa (200 MHz NMR frequency for ^{1}H). The increase in bore size would allow the use of larger sample size in an environment (e.g. cultivator) that bears more resemblance to the physiological conditions, not mentioning the possibility of generating NMR spectrum directly from live animals or plants.

4.2 Specific Information Gained on PolyP from Phosphorus NMR

PolyP generally gives three resonance peaks, terminal P (PP1) at about −7 ppm, penultimate P (PP2–PP3) at about −21.7 ppm, and internal P (PP4) at about −22.5 ppm. The chemical shift of PP1 may overlap with β-phosphorus of nucleotide disphosphate and γ-phosphorus of nucleotide triphosphate whereas PP2–3 may overlap with β-phosphorus of nucleotide triphosphate. The internal P of polyP, with a chemical shift around −21 to −24 ppm can be easily identified since only β-phosphorus of nucleotide triphosphate may show resonance close to polyP. In contrast, the terminal or penultimate P resonance occurs in regions where other resonance peaks cluster. PolyP that can be detected by NMR (i.e. is NMR visible) represents the more mobile fraction of total polyP. Thus, a lack of NMR-visible polyP signal does not necessarily indicate an absence of polyP in the sample. Figure 3 shows a typical ^{31}P-NMR spectrum for *Neurospora crassa*. Peak 1 represents *internal P of polyP. Peak 7 represents vacuolar* phosphate and peak 8 represents cytosolic phosphate. Since the chemical shift of orthophosphate peak is the weighted average of shifts of monobasic and dibasic phosphates, it can be used to directly estimate the precise pH of intracellular compartments.

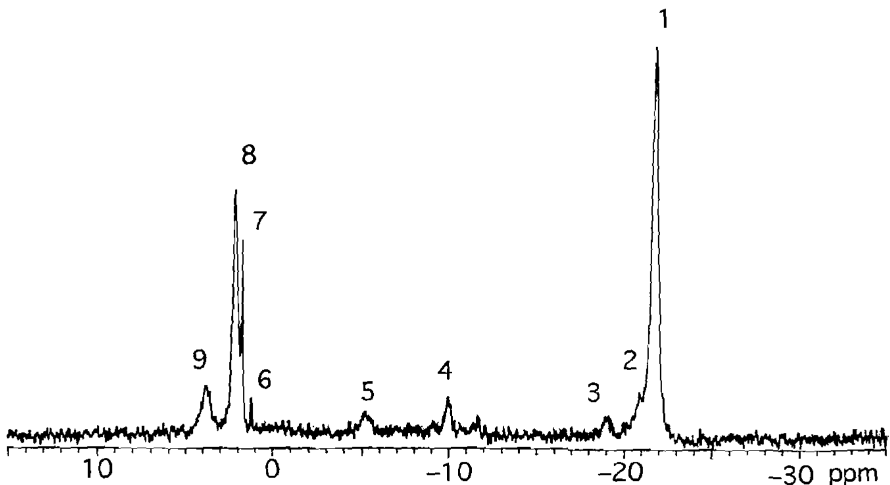


Fig. 3. *Phosphorus NMR spectrum of wild-type N. crassa* in logarithmic phase. Resonance peaks are assigned as follow: *1* polyP (inner phosphate); *2* polyP (penultimate phosphate); *3* β-phosphorus of nucleotide triphosphates; *4* diphosphates and sugar dinucleotides; *5* terminal residue of nucleotide triphosphates; *6* glycerophosphocholine or α-glycerol phosphoryl ethanolamine; *7* vacuolar orthophosphate; *8* cytosolic orthophosphate; *9* sugar monophosphates. (reprinted from Yang et al. 1993 with permission)

The ability to identify polyP signal and other phosphorus metabolites in the same spectrum makes the in vivo ^{31}P-NMR a powerful tool to monitor the fluctuation of polyP under various experimental conditions. The following parameters related to polyP metabolism and regulation in various organisms have been examined using phosphorus NMR technique: (1) compartmentation; (2) effects of growth conditions (e.g. energized state vs deenergized state, low phosphate vs high phosphate, logarithmic stage vs stationary phase; and (3) effects of environmental stress (e.g. alkalization, osmotic stress, heat, metal ions). Some of these studies are highlighted and discussed below.

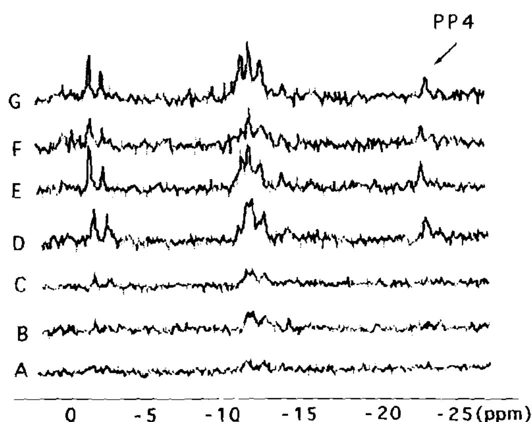
4.3 Eubacteria

4.3.1 *Escherichia coli*

The genes involved in polyP metabolism in *Escherichia coli*, polyP kinase (*ppk*) and polyphosphatase (*ppx*), have been cloned (Akiyama et al. 1992, 1993). Both genes have been placed behind inducible promoters and could be overexpressed, thus allowing tight regulation of polyP content in *E. coli* (Van Dien et al. 1997). For example, when *ppk* is induced early in growth, overproducing PPK could lead to an accumulation of large amounts of polyP of up to 80 μmol in phosphate monomer units per g of dry cell weight. In constrast, the induction of the *ppx* gene subsequently could cause polyP degradation with a half-life of 210 min. By controlling the concentration of inducer in the medium, the steady-state polyP level can be precisely controlled. The availability of these molecular tools and genetic information should make *E. coli* an ideal system to study polyP regulation and function by the combined use of NMR and molecular biology. Sharfstein and Keasling (1994) have compared the difference in polyP biosynthesis between wild type *E. coli* and *ppx* defect mutants. As can be seen in Fig. 4 the accumulation of polyP occurs in wild type *E. coli*, but not in mutant *E. coli* that has a defect in the *ppx* gene after phosphate-shift. In contrast, polyP is constitutively present in the mutants due to the lack of PPX activity.

Phosphate is clearly stored as polyP in *E. coli*. However, under phosphate starvation, polyP with a chain length of up to 100 can be transported from the medium into periplasm of *E. coli* and utilized as the sole phosphorus source. This process has been demonstrated with the use of ^{31}P-NMR (Rao and Torriani 1988). Furthermore, with the use of NMR, the same group was able to show that porins PhoE and OmpF facilitate a higher permeability for poly-P100 than porin OmpC does.

It has been shown that polyP may be involved in DNA uptake in competent *E. coli*. Reusch and Sadoff (1988) reported that polyP forms a complex



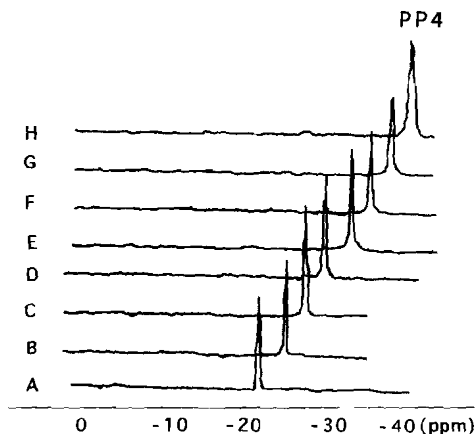


Fig. 4. PolyP in *E. coli* W3110 (*top*) and polyP in *E. coli* CA38/pBC29 (*bottom*) during phosphate shift. *A* Phosphate-starved; *B* 30 min after phosphate shift; *C* 60 min after shift; *D* 95 min after shift; *E* 130 min after shift; *G* 255 min after shift. (Adapted from Sharfstein and Keasling 1994)

with polyhydroxybutyrate (PHB), and Ca^{2+}, and such complex may act as the membrane component responsible for DNA entry in *E. coli.* Such polyP-containing membrane-bound complex may also behave as voltage-activated calcium channels (Huang and Reusch 1996). However, this interesting aspect of polyP function may be difficult to study by NMR since polyP in the complex is not NMR visible.

4.3.2
Propionibacterium acnes

The cells of light-sensitive skin bacterium *Propionibacterium acnes* have been examined by ^{31}P-NMR. The spectra show a large accumulation of polyP when grown on Eagles medium. Addition of glucose to the cell suspension gives rise to a change in the pH gradient across the cell membrane and a

decrease in the polyP peak. A lethal dose of broad-band near-ultraviolet light (corresponding to a 10% survival in a survival test) increases the amount of polyP visible in the NMR spectra. The increase is likely due to an equilibrium between long-chain invisible polyP in granules and short-chain polyP in cytoplasm (Kjeldstad and Johnsson 1987). When the cells were exposed to temperatures from 15 to 45 °C, the amount of polyP increases with increasing temperature (see Fig. 5). Similar to UV irradiation, this increase is due to equilibrium between NMR-invisible long-chain polyP in granules and free short-chain polyP in cytoplasm. In both cases, there are no UV- or temperature-induced changes in the other phosphorous components seen in the spectra, except a decrease in ATP for higher temperatures (Kjeldstad et al. 1989). The physiological significance of the observation is not clear. However, it is of interest to note that the short-chain polyphosphates such as tetra- or penta-polyphosphate have been proposed to serve as alarmones for the production of heat-response proteins (Lee et al. 1983).

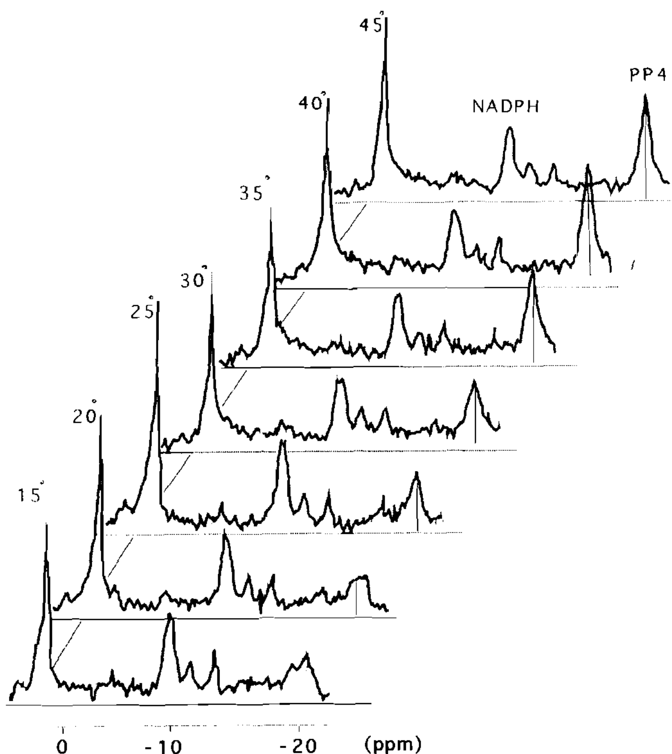


Fig. 5. Effect of temperature on polyP in the bacterium *P. acnes*. Cells were suspended in buffer with 30–40% cell volume for 15 min at indicated temperatures (°C) and then spectra were recorded at 22 °C. PP4 is the resonance peak of polyP. (Adapted from Kjieldstad et al. 1989)

4.3.3
Acinetobacter johnsonii

Activated sludge in wastewater treatment plants is enriched with polyP-accumulating bacteria such as *Acinetobacter*. The strictly aerobic, polyP-accumulating *Acinetobacter johnsonii* strain 210A degrades its polyP when oxidative phosphorylation is impaired. The end products of this degradation, divalent metal ions and inorganic phosphate, are excreted as a neutral metal-phosphate via the electrogenic symport system of the organism. In vivo ^{31}P-NMR studies of polyP degradation in anaerobic cell suspensions reveal the presence of a considerable outwardly directed phosphate gradient across the cytoplasmic membrane corresponding to a gradient of at least 100 mV. Thus, energy recycling by metal phosphate efflux contributes significantly to the overall production of metabolic energy in *A. johnsonii* 210A (van Veen et al. 1994).

4.4
Lower Eukaryotes

4.4.1
Yeast

Salhany et al. (1975) first applied high-resolution ^{31}P-NMR to study phosphorus metabolites in intact yeast cells. Since then, yeast has become a popular system for intact cell NMR research (Gillies et al. 1981; Grimmecke et al. 1981; Reidl et al. 1989). Figure 6 shows that the added orthophosphate is rapidly accumulated by the cells and stored mainly in a stable pool of polyP with an average chain length of about 200 units during phosphate-shift experiments (Bourne 1990). The average chain length is estimated from the ratio of the area of internal P peak to that of the terminal P peak. Figure 7 shows the ^{31}P-NMR spectra of yeast-form cells at early stationary phase of growth, as well as germ tubes and hyphae. The intensity of most signals, as measured relative to that of Pi, is clearly modulated both at the different phases of growth and during yeast-to-mycelium conversion. In particular, the intensity of the polyP signal is high in exponentially growing cells, then progressively declines in the stationary phase, is very low in germ tubes and, finally, becomes undetectable in hyphae (Cassone et al. 1983). However, Greenfield et al. (1987) did not observe significant difference in total polyP between log phase and stationary phase yeast cells.

A recent ^{31}P-NMR study by Shirahama et al. (1996) shows that under phosphate starvation, wild-type yeast cells continue to grow for two to three generations, implying that wild-type cells can use polyP to sustain the growth. In contrast, delta slp1 cells, which are defective in the vacuolar compartment and lack polyP, cease their growth immediately under phos-

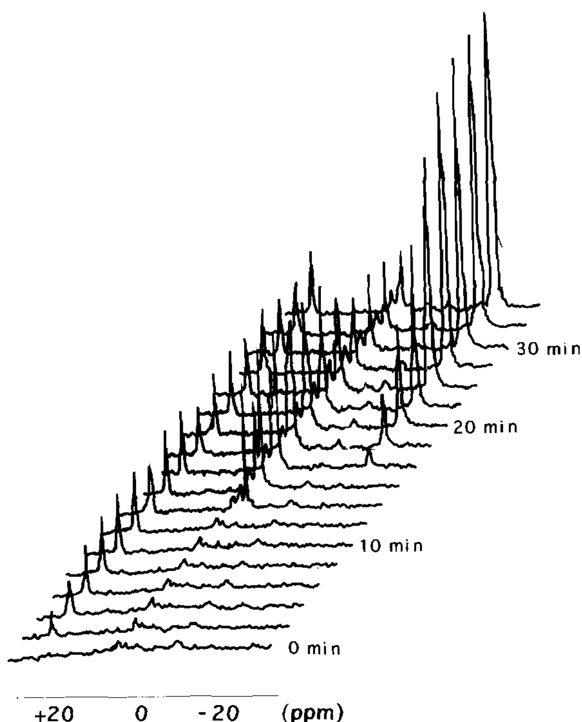


Fig. 6. Phosphate-shift experiments with yeast *C. utilis*. At time 2 min methylphosphonate (200 μmol) was added to the culture. At time 15 min and 38 min orthophosphate (800 μmol) was added to the culture. (reprinted from Bourne 1990 with permission)

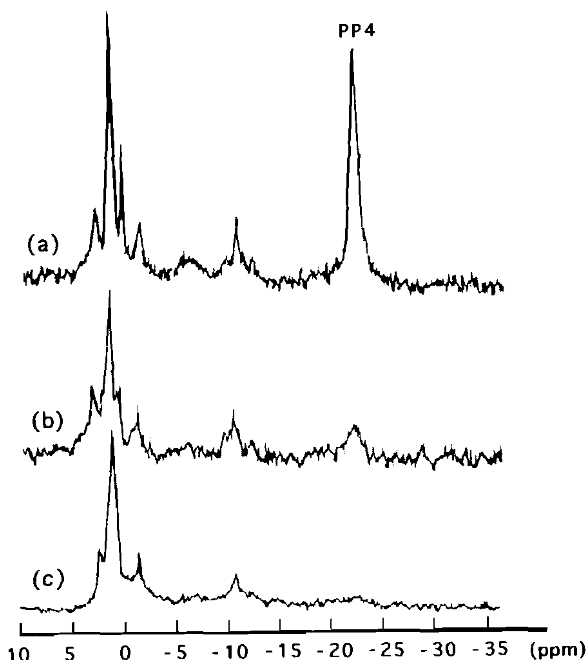


Fig. 7. Phosphorus NMR spectra of yeast *C. albicans* at different stages of growth. **a** Early stationary phase. **b** Germ tubes. **c** Hyphae. (Adapted from Cassone et al. 1983)

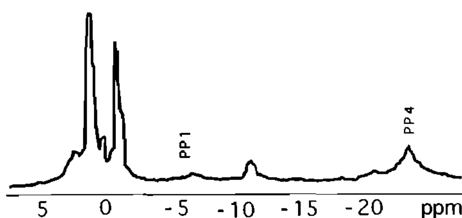
phate starvation. This study provided strong evidence that vacuolar polyP represent an active pool for phosphate and is mobilized to cytosol during phosphate starvation and sustained cell growth for a couple of rounds of cell cycle.

The proposed pH buffering and phosphogenic functions of polyP are investigated by subjecting chemostat-cultivated *Saccharomyces cerevisiae* to alkalization by NaOH addition and anaerobiosis (Castro et al. 1995). The subsequent changes in intracellular phosphate-containing species were observed by ^{31}P-NMR. The alkalization increases cytosolic pH and causes rapid polyP degradation to short chains. The pH changes and extent of polyP degradation depend inversely on initial polyP content. In contrast, anaerobiosis results in the complete hydrolysis of polyP to orthophosphates as opposed to short-chain polyP. The bulk of NMR-visible polyP (vacuolar) degradation to short polymers conceivably contributes to neutralizing added alkalinity. During anaerobiosis, however, polyP degradation may serve other functions, such as phosphorylation potential regulation.

Loureiro-Dias and Santos (1989) have shown that 2-deoxyglucose also causes a significant decrease in the polyP level and a downshift of cytosolic pH by 0.4 pH units as monitored by ^{31}P-NMR. Beauvoit et al. (1991) reported that a rapid hydrolysis of polyP occurs in yeast cells in the presence of either uncoupler CCCP or a vacuolar membrane ATPase specific inhibitor, bafilomycin A1, as monitored by ^{31}P-NMR. These studies support the notion that polyP is coupled to the energy state of the organism. The vacuolar polyP content appears to depend on two factors: vacuolar pH, strictly linked to the vacuolar ATPase activity, and inorganic phosphate concentration. Thus, polyP is totally absent in a null vacuolar ATPase activity mutant as indicated by ^{31}P-NMR. However, it is not clear whether these mutants may contain polyP in the NMR-invisible form (e.g. membrane or DNA bound). These ATPase mutants will be useful for probing polyP functions.

Possible involvement of polyP in the cellular repair of ionization damage has been studied with ^{31}P-NMR. Using a novel NMR spectroscopy probe which incorporated a bioreactor, a radiation source, and a radio frequency detection circuit tunable between 100 and 300 MHz for in vivo NMR spectroscopy of ^{23}Na, ^{13}C, and ^{31}P at 11.7 Tesla, it has been shown that a rapid decrease in adenosine triphosphate (ATP) and polyP occurs at the onset of irradiation (8 Gy/h) followed by a slow recovery of polyP (Magness and McFarland 1997). Similar study has been made by Holahan et al. (1988). They employed ^{31}P-NMR spectroscopy to examine alterations in phosphate pools during cellular recovery from radiation damage in yeast cells. They suggest that the polyP is hydrolyzed as a source of phosphates for repair of radiation damage.

The thermotolerant yeast *Hansenula polymorpha* is able to grow on vanadate concentrations (>96 mM) that are toxic to other organisms. *Hansenula polymorpha* cells growing on a vanadate-containing medium undergo a sig-



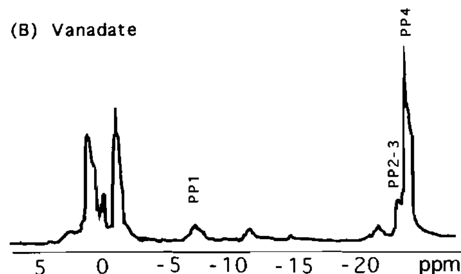


Fig. 8. Effect of vanadium on polyP in *H. polymorpha* cells. **A** Control cells. **B** Cells cultured in presence of 50 mM sodium orthovanadate. (reprinted from Mannazzu et al. 1997 with permission)

nificant increase in cell vacuoles and a thickening of the cell wall. The presence of small cytoplasmic vesicles and an increase in cristae at the level of the plasma membrane were also observed. These ultrastructural modifications were accompanied by a large increase in the intracellular polyP level, as detected by in vivo ^{31}P-NMR (Fig. 8). The increases in vacuoles and polyP in vacuoles suggest that these changes may be involved in vanadium detoxification (Mannazzu et al. 1997). However, more quantitative analysis is needed to assess the role of polyP in metal detoxification.

4.4.2
Neurospora crassa

Greenfield et al. (1988) used ^{31}P-NMR to study the effect of insulin on phosphorus metabolites in wall-less *Neurospora crassa* mutant. The spectra show millimolar levels of intracellular inorganic phosphate (Pi), phosphodiesters, and diphosphates including sugar diphosphates and polyP. Although insulin affects the growth rate of *N. crassa* mutants, it apparently has no effect on polyP level.

Yang et al. (1993) used high-resolution ^{31}P-NMR to investigate the effects of growth stage and environmental osmolarity on changes of polyP metabolism and intracellular pH in wild type *Neurospora crassa* cells. They found that both polyP and cytosolic pH are growth-dependent. As shown in Fig. 9, the ratio of polyP to orthophosphate in vacuoles increases from 2.4 to 13.5 in *N. crassa* as cells grew from early log phase to stationary phase. This

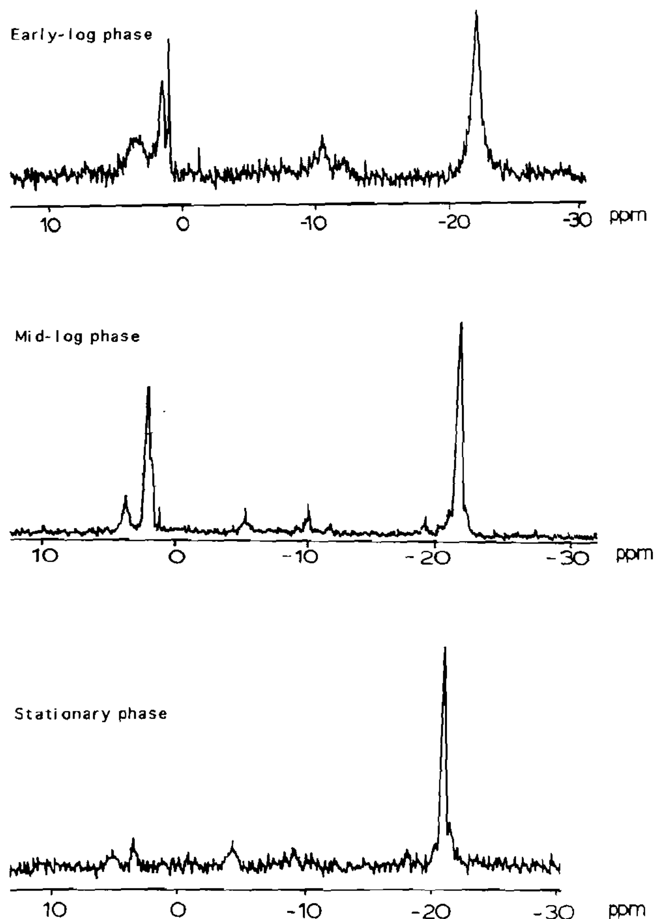


Fig. 9. Phosphorus NMR spectra of *N. crassa* at different stages of growth

observation is consistent with the notion that polyP serves as a phosphate reserve when growth rate slows down. From the same ^{31}P-NMR spectrum, it can be shown that cytosolic pH increases from 6.91 to 7.25 and vacuolar pH from 6.49 to 6.84 in *N. crassa* from early log phase to stationary phase. They also reported that hypoosmotic shock of *N. crassa* produces growth-dependent changes including: (1) a rapid hydrolysis of polyP with a concomitant increase in the concentration of the cytoplasmic phosphate; (2) an increase in cytoplasmic pH: and (3) an increase in vacuolar pH. Early log phase cells produced the most dramatic response (Fig. 10) 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, disappeared in response to hypoosmotic shock, but little or no hydrolysis of polyP occurrend in stationary cells. The cytosolic pH and the

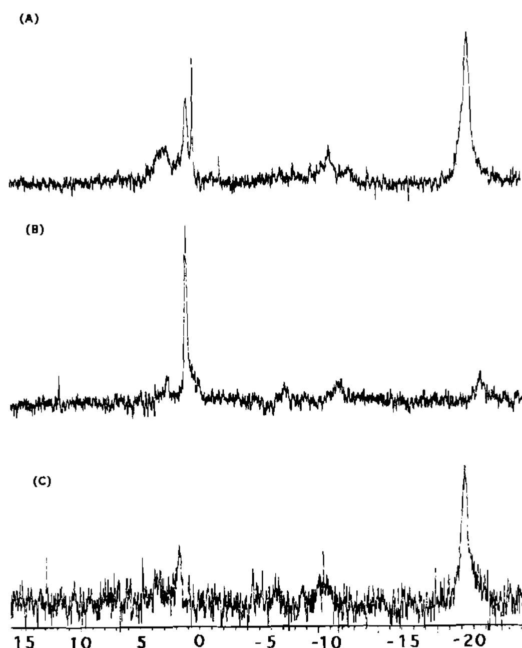


Fig. 10. Effect of hypoosmotic stress on polyP in *N. crassa* cells at early logarithmic phase of growth. A control. B Hypoosmotically treated with water for 2 h. C Hypoosmotically treated for 2 h and then re-incubated in Vogel's medium for 30 min. (reprinted from Yang et al. 1993 with permission)

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 are reversible, suggesting that these changes are related to environment osmolarity (Yang et al. 1993).

4.4.3 *Physarum polycephalum*

^{31}P-NMR spectroscopic analysis of the polyP pool in cellular and nuclear extracts of *Physarum polycephalum* demonstrates that plasmodia and cysts contain inorganic polyP with an average chain length of about 100 phosphates. However, long-chain polyP is degraded to short-chain polyP with an average chain length of about 10 phosphates only during sporulation. Since the degradation of polyP occurs even in the presence of a sufficiently large pool of inorganic phosphate, Pilatus et al. (1989) concluded that the degradation of polyP serves in supplying energy for biosynthesis during sporulation rather than in increasing the availability of phosphate. Their results, based on NMR analysis, show that 25 % of total polyP resides in nuclei. The data as presented, however, do not clearly indicate whether the degradation

occurs in the nuclei or in cytoplasm. Additional study is also needed to confirm the conversion of polyP to ATP through the action of polyP:ADP phosphotransferase activity.

4.4.4
Aspergillus terreus

Lyngstad and Grasdalen (1993) have used a 10-mm-diameter airlift bioreactor for in vivo NMR studies of *Aspergillus terreus*, another mycelial/pellet forming organism, grown in suspension. Signals were observed for intra- and extracellular orthophosphate, glycerol-3-phosphorylethanolamine, glycerol-3-phosphorylcholine, sugar phosphates and polyP. They also showed that polyP signals disappeared when the respiratory gas was exchanged for pure N_2 and the intracellular pH was estimated at 6.2 from the spectra.

4.4.5
Algae

Microorganisms including unicellular algae can be trapped inside agarose beads (3% w/v) at a concentration of around 7×10^8 cells per ml beads (Bental et al. 1990). During in vivo NMR experiments, the cells in the beads can be perfused continuously with the desired medium at a rate of 1 ml/min. They have used this set-up to examine the effect of osmotic shock on polyP in algae. In addition, by perfusing phosphate-depleted algal cells trapped inside agarose beads with orthophosphate-containing medium, the authors were able to follow the process of polyP synthesis in whole, living cells (Bental et al. 1991). The results suggest that, in *Dunaliella*, low molecular weight, probably cyclic, polyP intermediates are synthesized from Pi, and are then condensed to high molecular weight polymers. Studies of the intracellular organization of the polyP by electron microscopy and NMR techniques indicate that most of these polymers are stored in the cell in a soluble form, and not in solid-like structures (Bental et al. 1991).

Pick et al. (1990) carried out in vivo ^{31}P-NMR studies in the unicellular-alga *Dunaliella salina*, and demonstrated that the cytoplasmic alkalization as induced by ammonium ions (20 mM) induces hydrolysis of polyP, which is correlated kinetically with the recovery of cytoplasmic pH. Analysis of acid extracts of the cells indicates that long-chain polyP is hydrolyzed mainly to tripolyphosphate. The results suggest that the hydrolysis of polyP provides a pH-stat mechanism to counterbalance alkaline stress (Pick et al. 1990). Similar obsersation has also been reported by Greenfield et al. (1987) with yeast.

The phosphate metabolism of *Platymonas subcordifirmis* was investigated by ^{31}P-NMR over a wide range of external pH (Kugel et al. 1987). Under unaerobic condition, Kugel et al. found that the polyP chain starts to disinte-

grate under high pH conditions. Since polyP responds to cytoplasmic pH change, the authors concluded that polyP may be fairly accessible to cytoplasm.

4.5
Plant and Animal Cells

PolyP can be detected in cultured cells by radiolabeling and polyacrylamide gel electrophoresis (Cowling and Birnboin 1994). The estimated level of polyP is about 0.02 fmol/cell compared to 0.5–3 fmol ATP/cell, much lower than that in microorganisms. Kumble and Kornberg (1995) then unambiguously demonstrated the presence of polyP in mammalian tissues and cells and in various subcellular fractions. They used pure polyP kinase to convert ATP to polyP and hydrolysis of polyP to orthophosphate by a pure exopolyphosphatase as a means to estimate the amount and the size of polyP. PolyP in various rat tissues has a chain length of about 50–800 residues and a concentration range of 20–100 μM, which is roughly 0.2–1 % that of DNA when expressed as nucleotides. It has been shown that aging for rats or apoptosis in HL-60 cells may perturb polyP pools (Lorenz et al. 1997). Despite these elegant studies, no serious attempt has been made to examine whether phosphorus NMR can be adopted to study polyP in animal or plant tissues and cells.

5
Conclusions

High-resolution ^{31}P-NMR provides the only means to monitor polyP at real time in intact tissues and cells that are maintained in various physiological states. Phosphorus NMR spectra cover a wide chemical shifts range and reveal most biological phosphorus compounds in a snapshot. Thus, one can get a dynamic picture of the interconversion of almost all the phosphorus metabolites, including the turnover of polyP and the equilibrium between NMR-visible and -invisible polyP. From the area of the resonance peak, one can estimate the concentration of intracellular polyP on the basis of phosphate unit. By comparing the area of inner P resonance peak and terminal P resonance peak, one can estimate the average length of polyP. The major limitation of using NMR in polyP research is its inability to detect insoluble or immobile polyP due to line broadening. In addition, it remains a technical challenge to bring the detection sensitivity down to micromolar range. Despite these limitations, the potential of phosphorus NMR as a research tool for probing the metabolism and functions of polyP has yet to be fully explored.

Most of the studies discussed above relied on ^{31}P-NMR as a convenient tool to quantitative the soluble polyP in intact cells under different physio-

logical conditions. Such comparison is based on the assumption that the chemical environments of polyP remain the same so that the signal intensities can be used directly to compare polyP levels. Line broadening can occur when polyP is complexed with paramagnetic ions or when the viscosity of the environment increases and polyP becomes less mobile. In this context, it is worth noting that Sianoudis et al. (1986) found that addition of EDTA or NaOH to *Chlorella fusca* cell culture causes a sharpening and an increase of the polyP signals, indicating that polyP is probably membrane bound. Further, they showed that intracellular alkalization induces the conversion of NMR-invisible polyP to NMR-visible polyP. Using non-penetrating cations, like UO_2^{2+} and Eu^{3+}, Tijssen and Steveninck (1984) showed that 20% of polyP is membrane bound.

As can be seen from this review, the techniques and strategies of using ^{31}P-NMR to study polyP have not changed very much over the past 15 years. Sophisticated multiple pulse approach including two-dimensional and multiple quantum spectroscopy has not been employed by investigators yet in polyP research. In the past, there is also a lack of systematic comparison of polyP metabolism and function in different organisms using phosphorus NMR. In the future, phosphorus NMR study of intact cells should perhaps emphasize not only the in-depth study of a particular organism, in conjunction with biochemical and molecular biological tools, but also the broader comparative studies of different organisms, particularly other well-established experimental multicellular organisms such als *C. elegans* and *Drosophila*.

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