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^{31}P NMR STUDIES OF METABOLISM IN *ACANTHAMOEBA CASTELLANII*: POLYPHOSPHATE RELEASE FROM ENCYSTED CELLS

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SUMMARY

^{31}P NMR studies of *Acanthamoeba castellanii* have shown that encysting cells release polyphosphate into the encystment medium. Mature cysts contain low levels of polyphosphate, as do vegetative cells. Young cysts (7 days) show detectable levels of nucleotide diphosphates and triphosphate similar to those observed in vegetative cells. Mature cysts (90 days) show only excreted polyphosphate as well as a component which has a chemical shift of a phosphodiester. The inorganic phosphate peak in the cyst shows that the cyst milieu is liquid-like and that the intracellular environment maintains a pH between 6 and 7.5 in the presence of extracellular values from 4 to 9.

INTRODUCTION

^{13}C and ^{31}P NMR studies of *Acanthamoeba castellanii* have shown that the transition from the trophozoite to the cyst is accompanied by profound structural (1) and metabolic (2) changes such as the cytoplasmic storage of phosphonic acid derivatives (1) and accumulation of α,α-trehalose (2). Herein we extend the earlier study to follow the levels of polyphosphate and nucleotide phosphates during the maturation of cysts, as well as the response of cysts to changes in the extracellular pH. The ^{31}P NMR technique provides valuable insight into the differences between immature and mature cysts; the latter have previously been found to excyst more efficiently (3).

MATERIALS AND METHODS

Acanthamoeba castellanii (Neff strain) was grown as described previously (1,2). Encystment was originated by harvesting 1 & cul-

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tures of 7 day old cells and resuspending the washed pellet in 1 ℓ encystment medium buffered to pH 9.0 (4). Alternatively, cysts were harvested from growth medium to which 50 mM $MgCl_2$ (5,6) had been added at 7 days. After 7 days under encystment conditions in shaken culture at 30°C, cysts were stored at 5°C, unshaken. Viability of cysts was determined using 0.125% (wt/vol) eosin (7) in distilled water.

^{31}P NMR spectra were obtained at 121.47 MHz, with proton decoupling, in 10 mm tubes at 30°C, using a Bruker CXP-300 spectrometer. The reference for ^{31}P chemical shifts was 85% H_3PO_4 in a coaxial melting point tube; positive shifts are to high field.

RESULTS

Figure 1 shows the ^{31}P NMR spectrum of trophozoites of *Acanthamoeba castellanii* obtained from a 7 day old culture by washing and resuspending in 0.1 M KCl, pH 5.9. In the low field region between -14 and -18 ppm are found phosphonic acids characteristic of *Acanthamoeba* (1). The inorganic phosphate peak at -1.6 ppm shows an intracellular pH of *ca.* 6.5 (8). Peaks at 5.4, 9.9 and 18.3 ppm are attributed to nucleotide diphosphates and triphosphates (9). At 23.6 ppm we find a peak assignable to central phosphate groups in polyphosphate (10,11). The absence of

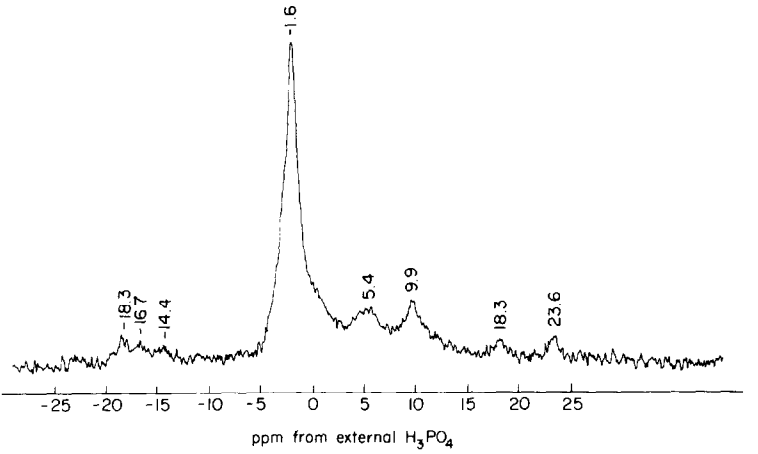

Figure 1. ^{31}P NMR spectrum obtained at 121.47 MHz of trophozoites of *Acanthamoeba castellanii*. Cells examined microscopically after experiment showed no lysis. Sweep width 25 KHz, 8 K data points, 12 μsec (90°C) pulse width, 5 μsec α-delay (delay between pulse and acquisition), 0.5 sec recycle time, 1,049 scans, 30°C, pH of suspending medium 5.9.

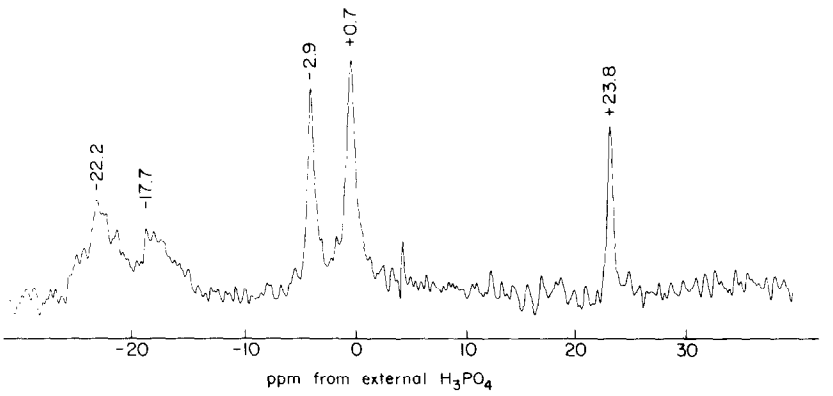

Figure 2. ^{31}P NMR spectrum obtained at 121.47 MHz of 90 day old cysts of Acanthamoeba castellanii suspended in encystment medium (see Materials and Methods) at pH 8.4. Cells were concentrated from the medium by centrifugation prior to preparation of NMR sample. Conditions are as in Figure 1. The number of scans was 2,204.

detectable resonances for end groups indicates a very large proportion of non-terminal residues in the polymer. Cells maintained in 0.1 M KCl for 24 hrs show no evidence of cyst formation from ^{31}P NMR spectra as characterized by the appearance of broad peaks from phosphonic acid derivatives at ca.-22 ppm (1). However levels of ATP and polyphosphate are greatly reduced in the washed starved cells.

Figure 2 shows the ^{31}P NMR spectrum of mature cysts (90 days) (3) in encystment medium at pH 8.4. A peak at -22.2 ppm is characteristic of the encysted state. In the high field region an intense resonance (23.8 ppm) is attributed to polyphosphate. No nucleotide phosphates are observable. The pH of the cyst, as judged from the inorganic phosphate peak at -2.9 ppm, is ca. 7.5. The peak at 0.7 ppm is not identified, but occurs in the region for phosphodiesters such as in phospholipid (12) or nucleic acid (13). Washed cysts, obtained by addition of $MgCl_2$ to growth medium, yielded qualitatively similar spectra; however, the levels of polyphosphate were much reduced. Similarly, washing cysts obtained

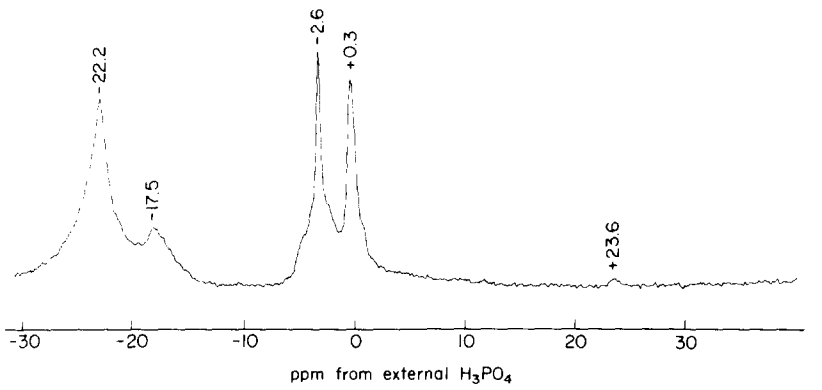

Figure 3. ^{31}P NMR spectrum at 121.47 MHz of cysts of Acanthamoeba castellanii obtained from encystment medium, washed and resuspended in sodium carbonate-bicarbonate buffer at pH 9.0. Conditions are as in Figure 1. The number of scans was 11,219.

from encystment medium in 0.1 M KCl at pH 6.0 yielded spectra which showed much reduced levels of polyphosphate (Figure 3). This could be due to activation of pH-dependent polyphosphatase such as seen in Dictyostelium discoideum (14). However, examination of the supernatant from centrifugation of cells in encystment medium showed that the polyphosphate was highly concentrated in the medium. Dialysis of the medium showed that the polyphosphate was non-dialysable, therefore of high-molecular weight, which would explain the absence of detectable terminal residues. Thus, it would appear that the encystment process, which is known to result in excretion of numerous cellular components (15), also results in expulsion of polyphosphate. Variation of pH between 9.0 and 4.0 of the polyphosphate-containing extracellular fluid did not show any hydrolysis of the polyphosphate, indicating that the medium does not contain any active pH-dependent polyphosphate.

Incubation of cysts under varying pH conditions (between 4 and 9) showed that cysts regulate their internal pH in a narrower range than that of the environment (Figure 4), as has been observed in yeast spores (16). The narrow resonance from inorganic phosphate shows the internal environment of the cyst to be liquid-like.

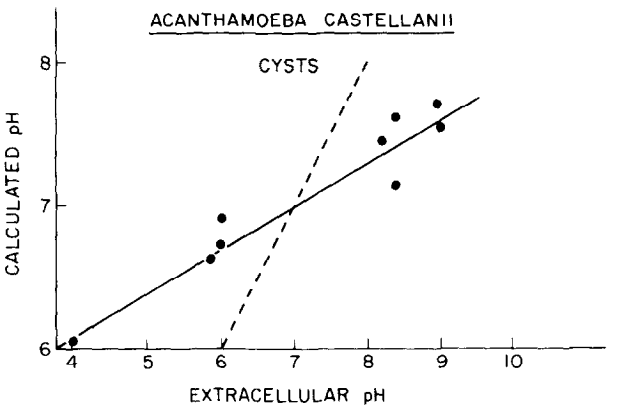

Figure 4. Plot of pH measured from ^{31}P NMR chemical shifts of inorganic phosphate peak from cysts of Acanthamoeba castellanii vs. measured pH of bathing medium.

DISCUSSION

The presence of inorganic polyphosphates in bacteria, yeast and fungi has been extensively documented (17,19). Higher organisms, are less well studied. Among the myxomycetes Dictyostelium discoideum has been shown to possess polyphosphate levels which vary in concentration according to developmental state (14,20). The protozoan Chaos chaos has been reported to contain polyphosphates (21). It is consistent with known facts on prokaryotes and fungi that polyphosphate accumulates when the organism is placed in conditions unfavourable to growth. Although the encystment medium used to generate cysts of Acanthamoeba contains no phosphate at the outset, polyphosphate is found in the medium in the course of formation and maturation of the cysts. Hence, this must occur at the expense of cellular phosphate rather than by uptake from the medium. In contrast, increased polyphosphate synthesis is observed in yeast in high phosphate-containing media (10). Our findings are consistent with the concept of an antagonistic relationship between polyphosphate accumulation and nucleic acid metabolism (17,22). Polyphosphate accumulation is thought to act as a buffer

against large changes in inorganic phosphate and pH which could occur in the course of catabolic activity of the cell. Furthermore, accumulation of polyphosphate would minimize disturbance of the osmotic equilibrium of the cell (17). The existence of extracellular polyphosphate is compatible with the observation that encystment results in loss of large amounts of cellular material, including membranes. It has been shown (23) that inorganic polyphosphate can bind polymers of galactosamine. Acanthamoeba possesses a galactosamine-containing lipophosphonoglycan (24) which spans the membrane (25); loss of membrane during encystment could entail loss of cytoplasmic polyphosphate as well as inorganic phosphate bound to membrane.

The appearance of most of the NMR-observable polyphosphate in the medium does not preclude the presence of low levels of inorganic polyphosphate in the cyst (Figure 3). It is believed that inorganic polyphosphate plays a crucial role in synthesis of m-RNA, ribosomes and metabolic intermediates necessary for the initial steps in spore germination (17). Inorganic polyphosphate of very high molecular weight may well be unobservable under high-resolution NMR operating conditions, as are DNA and RNA in cells. The peak observed at ca. +1.0 ppm in cysts could well result from low molecular weight fragments of nucleic acids or micelles of phospholipids, both of which are unobservable in their normal macromolecular arrangements in the cell.

The ^{31}P NMR spectra of young (14-21 days) cysts (1) and mature cysts (70-100 days) demonstrate the utility of the technique in developmental studies of organisms.

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