INTRACELLULAR PHOSPHORUS POOLS IN INTACT ALGAL CELLS

^{31}P NMR and transmission electron microscopy studies

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1. Introduction

Increasing evidence points to the importance of intracellular phosphorus levels as a controlling factor in algal growth and development [1–4]. Intracellular storage pools have been partially characterized by chemical methods [4–8] and cytological inclusions ('metachromatic' or 'volutin' granules) have been shown to be deposits of phosphorus [5,9–11]. Chemical analyses indicated that in cells of high metachromatic activity, the storage compounds were mixtures of polyphosphates of high average chainlength, organized within 'polyphosphate bodies' [5–7]. These results, however, were obtained by destructive methods in extracts. The only instance when whole-cell studies were carried out was an X-ray energy dispersive analysis [12]. In this work on blue–green algae, phosphorus and calcium were shown to be the major elemental constituents of the metachromatic granules, but the phosphorus compounds have not been characterized. Morever, in bacteria [13] and *Tetrahymena* [14], granules were shown to be of chemical composition other than polyphosphates. Due to its potential metabolic significance [6], the direct characterization of the intracellular polyphosphate pool in intact cells is desirable.

The application of ^{31}P nuclear magnetic resonance spectroscopy (^{31}P-NMR)to complex biological systems [15–25] offers the only method available to achieve a non-destructive yet highly selective characterization of phosphorus compounds in intact cells.

Using this method, combined with transmission electron microscopy, we have investigated the intracellular storage pool of phosphorus in intact cells of the alga *Cosmarium* sp. We have found that the major intracellular phosphorus pool which is affected when orthophosphate is depleted from the growth medium are the polyphosphates. Upon addition of excess orthophosphate to the medium of exponentially-growing, phosphate-depleted *Cosmarium* cells, the polyphosphate peak reappears in the ^{31}P-NMR spectrum of the intact cells. The ^{31}P-NMR results in combination with a transmission electron microscopy study suggest that the main intracellular storage pool of phosphorus in *Cosmarium* cells consists mostly of long-chain polyphosphate molecules organized in compact aggregates.

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2. Methods

Cosmarium sp. was grown in an artificial culture medium [26], at 20°C. Light was provided by cool white fluorescent light bulbs with an intensity of 2.5 J. m^{-2}. s^{-1} and a 16:10LD photoperiod. The ambient orthophosphate concentration (P_o) was depleted over a number of growth cycles by successive transfers to lower P_o. Each growth cycle lasted 45–64 days with P_o of 2000, 200, 50, 5 and 0.6 μM.

For each ^{31}P-NMR measurement, ~2 × 10^8 cells/sample were harvested by several centrifugation steps at 4°C in Sorvall high speed centrifuge, and 10 mm diam. tubes were used with 10 mm sample height. An external reference of orthophosphoric acid, 2 mM in D_2O at pH 1.4 in a 4 mm diam. capillary at the center of the 10 mm tube was employed. The deuterium signal of the D_2O in the reference served for field lock. The ^{31}P spectra were recorded at 109.32 MHz observing frequency on a Bruker WH-270 NMR spectrometer operating in the Fourier Transform mode [29], using 16 K memory. The 'dead time' of the instrument was 30 μs and hence signals with linewidth of up to several thousand hertz could be reasonably measured. Typically several thousand scans were accumulated for each spectrum with a pulse-repetition time of 0.67 s and 90° flip angle with the spectral width varying between 5–12 kHz. No proton decoupling was employed throughout these experiments, hence none of the ^{31}P peak intensities should be affected by the nuclear Overhauser effect. Also a preliminary experiment was carried out to obtain an estimate of the T_1 values of the main signals. A T_1 of ~0.1 s of the polyphosphate phosphorus permitted us to use the above flip angle and repetition time and yet to expect the relative signal areas in the various spectra to represent a non-distorted measure of the phosphoric concentration in the cells. Assignment of the peaks to the various phosphate compounds was done in accordance with the ^{31}P-NMR literature, e.g. [19,30].

Total phosphorus and orthophosphate were determined by the method in [27] and [28], respectively, and cold trichloroacetic acid precipitation as in [6].

For electron microscopic examination, cell were fixed in 5% glutaraldehyde and postfixed in 2% osmium tetroxide (for 2 h, at 0°C). Fixatives were buffered with 0.1 M sodium cacodylate to pH 7.4. Dehydration was done with ethanol and the cells were embedded in Spurr's medium [31]. Sections were stained with uranyl acetate and lead citrate and observed in a Philips 300 electron microscope.

3. Results and discussion

^{31}P-NMR spectra of intact *Cosmarium* cells, grown to stationary phase at low or high initial extracellular orthophosphate concentration (P_o) are shown in fig.1. *Cosmarium* cells grown at 0.6 μM P_o showed no phosphorus signal in contrast to cells grown at 2 mM P_o in which a large signal of non-terminal P of polyphosphates was detected (fig.1B). This signal of non-terminal P of polyphosphates has a relatively large linewidth [$\Delta\nu$ = 1500 Hz], thus, peaks of phosphate nuclei in polyphosphates of different chainlength, cannot be resolved. However, the fact that no terminal phosphate signal was detected indicated that in these cells most of the intracellular polyphosphates were of high chainlength (>25 phosphate units).

As *Cosmarium* cells were successively transferred to lower P_o the intracellular cold trichloroacetic acid-insoluble phosphorus pool markedly decreased. A similar trend was found in chemical determinations of cold trichloroacetic acid-insoluble P and ^{31}P-NMR estimations of polyphosphate P (table 1). This similarity is noteworthy although phosphorus fractions in cells grown at P_o < 200 μM gave rise to a low NMR signal-to-noise ratio. Thus, only crude estimates of the various intracellular P concentrations were obtained from NMR peak areas in these phosphorus-deficient cells.

The amount of free intracellular orthophosphate, as determined by ^{31}P-NMR did not change significantly as cells were transferred to lower P_o. The cytoplasmic pH, which was measured by the shift of the experimental orthophosphate peak in relation to the reference [19], remained constant at pH 6.5 ± 0.1 (table 1), a value similar to the intracellular pH measured in *Escherichia coli* cells under anaerobic conditions [24].

NMR spectra of cells harvested in exponential phase of growth in a low phosphate medium (P_o = 0.6 μM), showed only an orthophosphate peak and possibly phosphoenolpyruvate (fig.2A). When a large excess (2 mM) of orthophosphate was added to these exponentially growing *Cosmarium* cells 24 h before harvesting, a distinctive polyphosphate peak appeared in the ^{31}P-NMR spectrum of the cells (fig.2B). The size of the newly synthesized intracellular polyphosphate

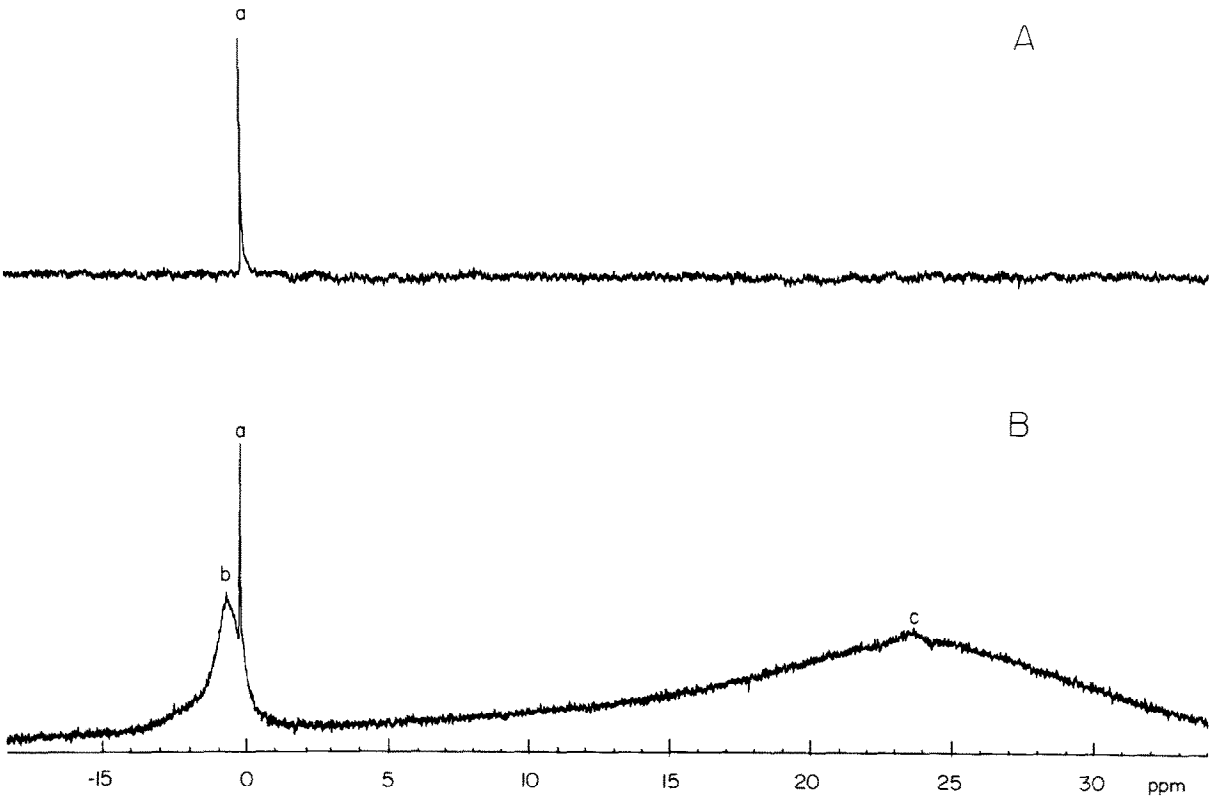

Fig.1. ^{31}P-NMR spectra of intact stationary phase *Cosmarium* sp. cells grown at 0.6 μM (A) and 2 mM (B) P_o in a standard growth medium: (a) Phosphoric acid external reference (2 mM, pH 1.4); (b) intracellular orthophosphate (pH 6.5); (c) non-terminal phosphate of polyphosphates. ^{31}P shifts in this and other NMR spectra are relative to the position of 85% H_3PO_4.

Table 1
Intracellular phosphorus pools in *cosmarium* sp.

Measured by chemical analysis			Estimated by ^{31}P-NMR		
P_o (μM)	Total cellular P (μmol P/100 mg dry wt algae)	Cold trichloroacetic acid-insoluble P	Cellular orthophosphate (arbitrary units)	Non-terminal P of poly(P)	pH
2000	164 ± 18	152 ± 18	4.9 ± 0.3	63.6 ± 9.0	6.5
200	96 ± 5	90 ± 5	5.0 ± 0.6	44.0 ± 1.8	6.4
50	77 ± 7	70 ± 10	7.2 ± 5.9	28.6 ± 16.3	6.6
5	25 ± 5	21 ± 5	3.1 ± 2.6	Undetected	6.5
0.6	6 ± 1	0.2 ± 1	Undetected	Undetected	

Cosmarium sp. was grown in an artificial culture medium and successively transferred to media with diminishing initial orthophosphate concentrations (P_o). The results presented here were obtained from cells harvested 45 days after the transfers. The calculated means ± SD are based on combined results of 3–6 expt. The size of the P-pools was also estimated from the areas under the ^{31}P-NMR signals and is given in each case normalized to the external reference and to the cell packing factor of the sample

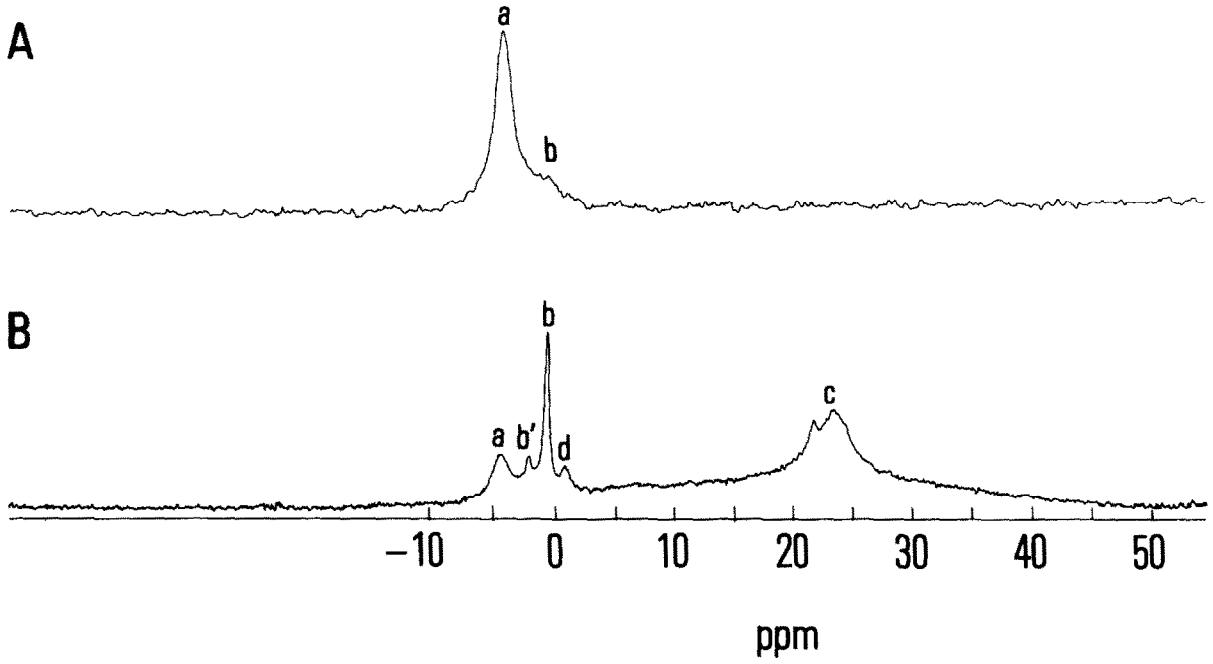

Fig.2. ^{31}P-NMR spectra of intact exponential phase *Cosmarium* sp. cells grown in low phosphate (0.6 μM) medium (10 000 scans) (A) and (B) phosphate-depleted cells from A to which 2 mM orthophosphate was added 24 h before harvesting (4500 scans): (a) Glycerol phosphate external reference (1.13 mM; pH 9); (b') intracellular orthophosphate; (b) phosphoenol pyruvate; (c) non-terminal phosphate of polyphosphate; (d) tRNA (?). The reference signal was artificially broadened by a high pH aqueous relaxation reagent [32] to avoid saturation by high pulsing rate.

pool, as estimated from the peak areas is comparable to that in *Cosmarium* cells grown to stationary phase in 2 mM orthophosphate. Other minor peaks, corresponding to phosphoenol pyruvate and perhaps to tRNA were also observed in these cells.

The broad NMR signal of polyphosphates could have been caused by several relaxation processes like P–P dipolar, chemical shift anisotropy and also fast-exchanging paramagnetic metal cations and a more careful examination is needed to clarify this point. This large linewidth might be indicative of a slow molecular reorientation rate caused either by a very high molecular weight of these polyphosphates or by their organization in some rigid supramolecular aggregate. However, it seems that a T_1 of the order of 0.1 s excludes the possibility of solid-like state.

We further attempted to locate the site of the newly formed polyphosphate fraction within cells using transmission electron microscopy. Whereas only a few electron dense granules were observed in the P-starved cells (fig.3A,B) many granules of 600 Å av. diam. appeared in these cells, 24 h after the addition of orthophosphate to the medium (figs. 3C,D). Similar granules were observed in cells grown to stationary phase at 2 mM P_o (fig.3E).

When *Cosmarium* was grown in orthophosphate rich medium ($P_o > 5$ μM), up to 90% of the total cellular phosphorus had been associated with the cold

Fig.3. Electron-micrographs of phosphate-depleted and phosphate-rich *Cosmarium* sp.: C, chloroplast; N, nucleus; P, polyphosphate granules; PS, partly-sectioned polyphosphate granule; S, starch granule; W, cell wall. (A) Phosphate-starved cell grown at low initial ambient orthophosphate concentration, 0.6 μM (× 11 050). (B) Detail of a phosphate-starved cell (× 23 800). (C) Cell from the same culture as above to which 2 mM orthophosphate was added 24 h before fixation (× 9350). (D) A magnified portion of the cell in (C) Note the 'mushroom' appearance of the polyphosphate granules (P) attaching to starch granules (S) (× 38 250). (E) Section through a cell grown for 2 months at 2 mM initial ambient orthophosphate concentration. (× 11 050).

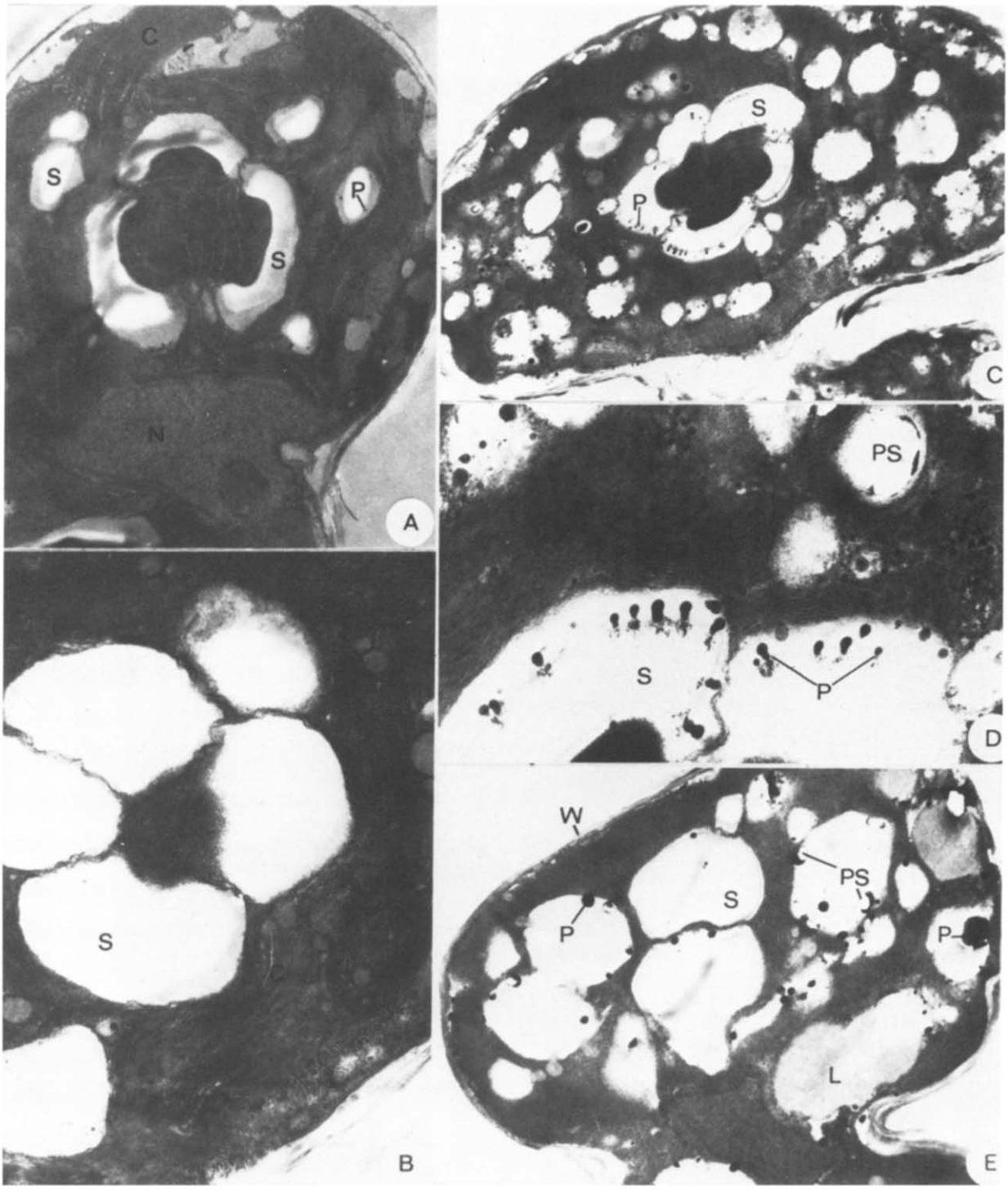

Fig.3

trichloroacetic acid-insoluble fraction [26]. This intracellular pool had decreased to low levels when the cultures had become phosphorus depleted. Biomass yield and the cold trichloroacetic acid-insoluble phosphorus fraction had been correlated. This study strongly suggests that this intracellular phosphate accumulation pool consists mostly of long chain ($n > 25$) polyphosphates.

The parallel emergence of the electron dense bodies and of the polyphosphate ^{31}P-NMR signal upon addition of orthophosphate to phosphorus depleted cells is consistent with the suggestion that at least in *Cosmarium* sp., the so-called 'volutin granules' are compact polyphosphate aggregates. Metal cations, some probably paramagnetic, could serve to neutralize negative electric charge. These, together with basic proteins that would also restrict intramolecular motion of the polyphosphate phosphates, could bring about the broad NMR lines.

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