ACCUMULATION OF ppGpp AND pppGpp DURING NITROGEN DEPRIVATION OF THE CYANOPHYTE *ANABAENA CYLINDRICA*

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1. Introduction

An anomalous reaction of the ribosome promoted by lack of aminoacylated tRNA synthesizes 5′-diphosphate 3′-diphosphate guanosine (ppGpp) and 5′-triphosphate 3′-diphosphate guanosine (pppGpp) in heterotrophic bacteria [1]. A similar mechanism exists in the unicellular cyanophyte *Anacystis nidulans* [2], such that the mode of accumulation is comparable with that reported for heterotrophic bacteria [3–5]. Although little is known of the ability of other cyanophytes to accumulate ppGpp, one study reported no accumulation of ppGpp during incident light step-down of *Aphanocapsa 6308, Anabaena cylindrica* and *Anabaena catenula* or during nitrogen deprivation of *Anabaena cylindrica* [2].

In contrast, this communication reports on the ability of *A. cylindrica* to accumulate a nucleotide which incorporates radioactively labelled guanosine and co-migrates in paper, polyethyleneimine (PEI) cellulose thin-layer chromatography (TLC) and high pressure liquid chromatography (HPLC) systems with authentic ppGpp isolated from growth-inhibited *E. coli*. Accumulation of the nucleotide is short-lived and readily depressed in the presence of combined nitrogen, particularly ammonium ion.

2. Materials and methods

Ortho [^{32}P]phosphate was obtained from the Radiochemical Centre, Amersham, PEI cellulose TLC plates and 'orange ribbon C' chromatography paper were obtained from Schleicher and Schüll, Dassel.

2.1. *Organisms and growth conditions*

Anabaena cylindrica (Cambridge no. 1403/2a) was grown on the medium of [6] modified by reduction of phosphate concentration by a factor of 5×10^2 and addition of Hepes (0.4 g/l, pH 7.6). Cultures were incubated at 35 ± 0.5°C in an orbital incubator (80 rev./min) with continuous illumination (4800 lux) and gassing with air CO_2 (95:5, v/v). The basic medium was supplemented with 2 mM NH_4Cl or 20 mM KNO_3 to produce ammonium or nitrate medium. Nitrogen-free medium contained no source of combined nitrogen. In all experiments control and experimental cultures were derived from the same source culture by serial washing with nitrogen-free medium at least 3 times. The whole procedure was at 4°C. Control cultures were resuspended in prewarmed medium containing a combined nitrogen source and experimental cultures in prewarmed nitrogen-free medium.

2.2. *Extraction and identification of nucleoside polyphosphates*

Selected *A.cylindrica* cultures of A_{750} 0.15–0.2 were labelled with ortho [^{32}P]phosphate (100–300 μCi/ml) for at least one generation time before use in experiments to allow equilibration of exogenous and endogenous phosphate pools. The same exogenous concentration and specific radioactivity of ortho [^{32}P]phosphate was maintained throughout. Sampling and analysis were as in [4].

Analysis of extracts showed that the putative ppGpp and pppGpp nucleotides were readily labelled with [8-^{3}H]guanosine and comigrated with authentic nucleotides derived from Trimethoprim-treated *E.coli*

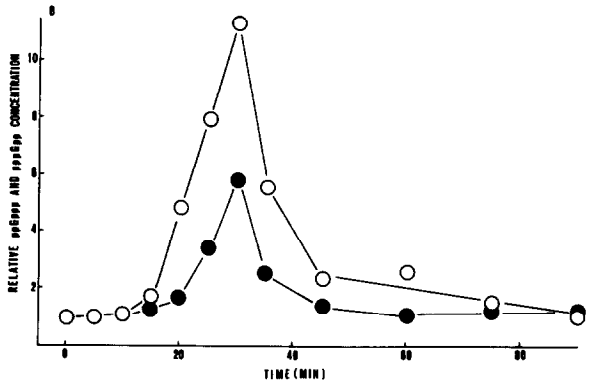
cultures on PEI cellulose TLC in a variety of phosphate and formate buffer, on 'orange ribbon C' chromatography paper eluted with 1.0 M Na_3PO_4 (pH 7.0):$(NH_4)_2SO_4$:*n*-propanol (10 ml:6 g:0.1 ml). In a HPLC system employing a partisil (O–5AX) 4.5 X 250 mm column eluted with linear and exponential gradients from 0–0.6 M KH_2PO_4 (pH 4.5) retention times were identical to that of the authentic nucleotides from *E.coli*, recovered from two dimensional fractionations on PEI cellulose plates.

RNA and protein estimations were made from duplicate 10 ml samples treated as in [3]. The product of the NaOH digest was used to estimate RNA content by the method in [7] and protein by the method in [8].

3. Results and discussion

Removal of ammonium or nitrate as the sole source of combined nitrogen supporting steady state exponential growth of *A.cylindrica* culture elicited the accumulation of ppGpp and pppGpp (fig.1). The ability of the nitrate-grown culture to accumulate the nucleotides was repeatedly greater than that of ammonium-grown cultures. In nitrate-grown cultures an ~20-fold increase in ppGpp and 15-fold increase in pppGpp was routinely observed. In comparison, ammonium-grown cultures accumulated routinely 12- and 8-fold increases, respectively. Residual low concentrations of ammonium ions may account for the difference as the ability of a culture to accumulate ppGpp increased with the thoroughness of the washing procedure. Re-addition of trace ammonium concentrations (2 μM) significantly depressed ppGpp and especially pppGpp concentrations.

The kinetics of accumulation differed significantly from those of heterotrophic bacteria both ppGpp and pppGpp accumulation were short-lived, lasting some 15 min. In nitrate, but not ammonium grown cultures, a second smaller though equally transient accumula-



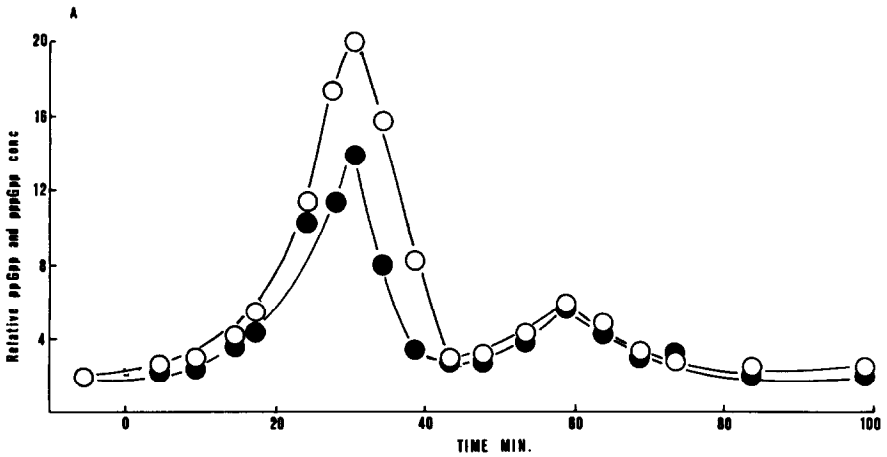


Fig.1. Accumulation of ppGpp and pppGpp in nitrogen-deprived *A.cylindrica* cultures. (A) Nitrate-grown culture. (B) Ammonium-grown culture. (○) Relative ppGpp concentration: basal concentration, nitrate, 0.11 nmol A_{750}^{-1}; ammonium, 0.53 nmol A_{750}^{-1}. (●) Relative pppGpp concentration: basal concentration nitrate, 0.04 nmol A_{750}^{-1}; ammonium, 0.15 nmol A_{750}^{-1}.

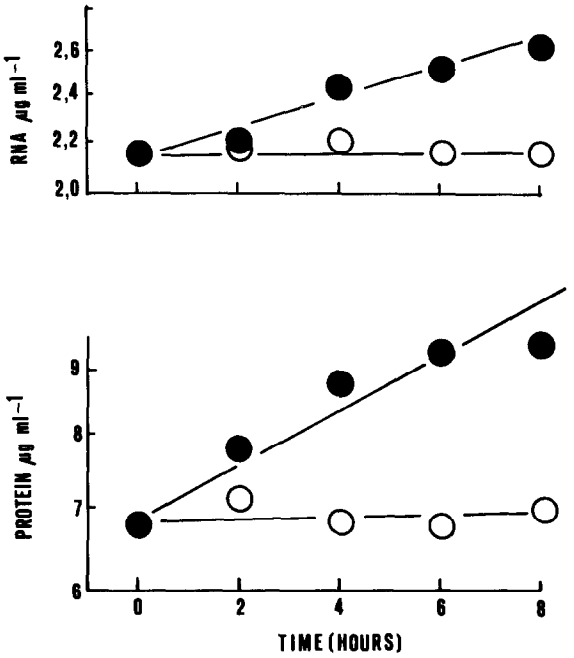


Fig.2. RNA and protein contents of *A.cylindrica* nitrate-grown culture deprived of nitrogen. (A) RNA content. (B) Protein content. (○) Inhibited culture; (●) growing control culture.

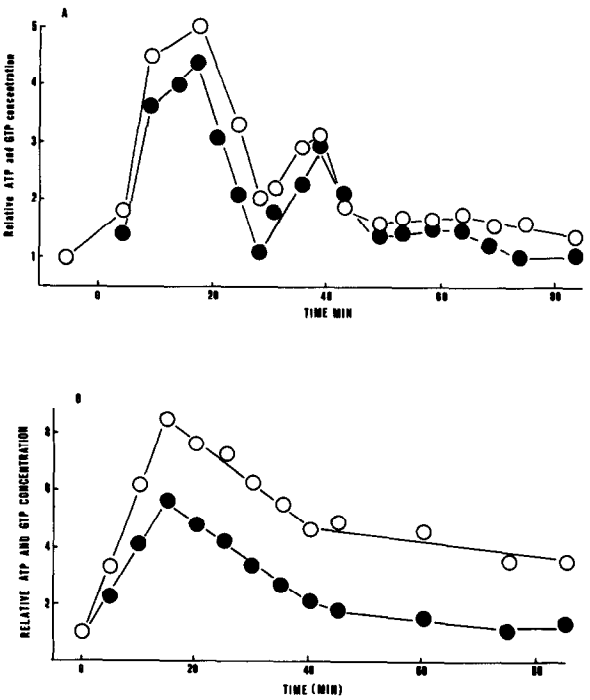


Fig.3. Accumulation of purine nucleoside triphosphates in nitrogen-deprived *A.cylindrica*. (A) Nitrate-grown culture. (B) Ammonium-grown culture. (○) Relative ATP concentration: basal concentration nitrate, 5.7 nmol A_{750}^{-1}; ammonium, 7.3 nmol A_{750}^{-1}. (●) Relative GTP concentration: basal concentration nitrate, 1.3 nmol A_{750}^{-1}; ammonium, 2.2 nmol A_{750}^{-1}.

tion occurred. In both nitrate and ammonium grown cultures the time of peaking of ppGpp and pppGpp accumulation was remarkably consistent despite the use of a procedure which is not immediately effective. Analysis of RNA and protein contents of nitrogen deprived cultures indicate a cessation of macromolecular synthesis within ~10–20 min of resuspension (fig.2).

In accordance with [3] and unlike the situation in heterotrophic bacteria [9], the guanosine triphosphate pool did not decline on accumulation of ppGpp and pppGpp. Both purine ribonucleoside triphosphate pools in nitrogen deprived nitrate grown cultures of *A.cylindrica* increased ~5-fold. Such increases were short-lived, having a duration similar to that of ppGpp and pppGpp and occurred immediately prior to those accumulations. Again a second smaller increase was observed in nitrate, but not ammonium grown cultures, fig.3.

The synthesis of ppGpp in *A.cylindrica* is most probably a ribosome-mediated reaction [2]. In support of this concept translational inhibitors such as chlortetracycline and chloramphenicol prevent, or rapidly deplete, ppGpp accumulation [11]. Nitrogen deprivation presumably promotes ppGpp synthesis by curtailing amino acid biosynthesis through lack of combined nitrogen. Consequent shrinkage of the amino acid pools would deplete aminoacylated tRNA and stimulate ribosome mediated ppGpp synthesis. However, the cellular nitrogen economy of *A.cylindrica* and other cyanophytes differs from that of heterotrophic bacteria due to their ability to develop nitrogen fixing capabilities and heterocysts which is triggered by nitrogen deprivation. This developmental programme requires new protein synthesis [12]. The amino acid supply required is presumably drawn from the mobilization of stored proteinaceous material. Nitrogen deprivation of *A.cylindrica* reduces protein

synthesis [13] and marked changes in protein metabolism occur, in particular, rapid degradation of storage proteins [13,14].

Any improvement in amino acid availability would remove the stimulus for ppGpp synthesis. The inhibition of protein and stable RNA accumulation would also be relieved. We would suggest that the kinetics of accumulation of ppGpp and purine nucleoside triphosphates reflect the oscillations of a regulatory mechanism governing degradation of storage proteins. Depletion of endogenous amino acid pools would promote amino acid mobilization. This flux, swelling endogenous pools, may curtail further degradation of storage protein until renewed translation again depletes them, triggering further degradation. Such fluctuations of the endogenous amino acid pools would account for the observed accumulation of ppGpp which would reach peak concentration during transient periods of low amino acid concentrations. Similarly amino acid lack would promote inhibition of protein and RNA synthesis resulting in accumulation of nucleoside triphosphates. Subsequent relief of inhibition of macromolecular synthesis, as amino acid pools are endogenously supplemented, would allow renewed usage and thus depletion of the accumulated nucleotides.

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