Buffer and Salt Damage to the Filamentous Cyanobacterium *Anabaena cylindrica*

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The commonly used buffer HEPES was found to cause severe disintegration of the cyanobacterium *Anabaena cylindrica*, as measured by filament breakage, cell disruption, phycocyanin release and nitrogenase inhibition. The effect became increasingly severe as the buffer concentration was increased above 10 mM. The observed cell damage does not appear to be unique to HEPES, similar observations being made with Tris/HCl, sodium phosphate, sodium sulphate and sodium chloride. It appears that the cells are very sensitive to ionic strength.

INTRODUCTION

Growth and metabolism of cyanobacteria in closed systems frequently results in pH changes, thus necessitating the addition of buffers. The requirements of the buffer are simply that it has an ionizing functional group with a pK in the pH range required. Ideally the buffer should be inert as far as the biological system is concerned and fulfil certain other criteria also (Good *et al.*, 1966; Ferguson *et al.*, 1980). Relatively few buffers have been available for the pH range 6–8 and, of those tested, HEPES, introduced by Good *et al.* (1966), has become one of the most successful and widely used (Ferguson *et al.*, 1980). A perusal of the recent cyanobacterial literature reveals that HEPES is overwhelmingly the most widely used buffer, both for experiments with intact cells and those with cell-free extracts. Buffer concentrations in the range 10–50 mM are commonly used. We report here that, although *A. cylindrica* can be grown in 10 mM-HEPES buffer, higher concentrations were damaging to cell structure and nitrogenase activity. Tris and phosphate buffers at 50 mM were also damaging, as were similar concentrations of other salts. It is suggested that this organism is very sensitive to elevated ionic strengths.

METHODS

Organisms and growth. *Anabaena cylindrica* ATCC 27899 was obtained from the American Type Culture Collection and grown as described previously (Daday *et al.*, 1977), but with the growth medium (initial pH 7.5) at $\frac{1}{8}$ strength with respect to all components except phosphate. Cells were harvested by centrifugation (6000 g, 10 min) at a concentration in the range 120–150 Klett units as measured by a Klett–Summerson colorimeter, where 100 Klett units were equivalent to 0.23 mg dry weight ml⁻¹. Buffers were at pH 7.5 unless otherwise stated.

Nitrogenase assay. Nitrogenase activity was measured by the reduction of acetylene to ethylene, as described previously (Lambert & Smith, 1980). All assays were performed in air mixed with 10% acetylene at atmospheric pressure.

Pigment measurement. Phycocyanin levels were measured from the absorbance at 620 nm (Myers & Kratz, 1955).

Dry weight determination. This was done by washing 10 ml volumes of cells with water and drying overnight at 85 °C.

Chemicals. Chemicals were of the highest available purity. HEPES was obtained either from Sigma (lot no. 71F-5041) or Calbiochem-Behring (lot no. 210127). The results were not dependent on the source of this chemical.

RESULTS AND DISCUSSION

Effect of buffers and salts on cell structure and nitrogenase activity

In an experiment involving the assay of nitrogenase activity in 50 mM-HEPES buffer, pH 7.5, we were surprised to find zero activity and severe filament disruption. Nitrogenase activity (Fig. 1a) and phycocyanin release (Fig. 2a) were subsequently measured as a function of buffer concentration. Both parameters reflected increasing cell damage with increasing buffer concentrations. The observations correlated with a microscopic visual inspection of the cells: increasing buffer concentrations led to progressively more damage to the organism as seen by filament and cell disruption. A search for alternative buffers revealed that Tris and sodium phosphate produced the same trends. These observations were made both at pH 7.5 and 6.8 and with cells transferred from pH 6.8 medium to pH 7.5 HEPES buffer; qualitatively, it can be said that to this extent they were not pH-dependent.

It appeared from these results that the cell damage and consequent nitrogenase inhibition were not due to specific buffer effects, but to ionic effects, and similar results were obtained with other salts, including sodium sulphate and sodium chloride (Figs 1 and 2). The results were in no way due to osmotic effects: sorbitol, at concentrations up to 50 mM, produced no visible cell damage nor phycocyanin release (Fig. 2a). Interestingly, increasing sorbitol concentrations progressively inhibited nitrogenase activity (results not shown), a result for which we offer no explanation. Most of the damage was progressive with time and hence Figs 1 and 2 are arbitrary in this sense. The time course of phycocyanin release induced by sodium sulphate and HEPES (Fig. 3) was to some extent variable from one batch of cells to another and also from one lot of cells to another within a given population, as observed microscopically. The susceptibility to these ionic effects seemed to be somewhat dependent on the age and concentration of the cultures, and also with the amount of mucilage excreted by the cells. In the reported experiments the various buffers and salts were simply made up in water (at pH 7.5), but similar results were obtained in the presence of the medium (results not shown).

The ionic strength of a salt solution, I , is defined as $I = \frac{1}{2} \sum m\mu^2$, where m = molar ionic concentration and μ = ionic charge. When the abscissa scales of Figs 1(a) and 2(a) were normalized to ionic strength (Figs 1b and 2b) the curves became much closer together, indicating that a large part of the observed effects was due simply to ionic strength. However, the curves do not completely superimpose and hence it can be assumed that specific ions also influenced the results to a greater or lesser degree. It is well known, for example, that di- and trivalent ions bind more readily to proteins than do uni-univalent salts.

It may be concluded from these results that using HEPES or Tris buffers at concentrations

Fig. 1. Nitrogenase inhibition by HEPES (●), NaCl (○), sodium phosphate (□) and sodium sulphate (▲). Results are expressed relative to a control without added buffers or salts and are given as a function of the molar concentration (a) and ionic strength (b). Activity was measured as an initial rate 5 h after the addition of the salts. Cell concentrations were in the range 120–150 Klett units.

Fig. 2. Phycocyanin release as a function of the buffer or salt concentration (a) or as a function of ionic strength (b), for HEPES (●), NaCl (○), sodium phosphate (□), sodium sulphate (▲) and sorbitol (■). Measurements were made 5 h after adding the salts. Cell concentrations were in the range 120–150 Klett units.

Fig. 3. Time course of phycocyanin release after suspending cells to 120 Klett units in sodium sulphate (○) or HEPES buffer (●) (both 50 mM).

above 10 mM with certain filamentous cyanobacteria is hazardous and for such organisms phosphate buffers should not be used even at this concentration. This is likely to create problems in many experiments where such a concentration provides insufficient buffering capacity. However, cyanobacteria clearly show different susceptibilities to ionic strength. For example, HEPES at 20 mM had no significant effect on the growth rate of *Oscillatoria redekei* and *Aphanizomenon flos-aquae* (Smith & Foy, 1974). Also marine organisms have presumably adapted to higher ionic strengths; *Anabaena* sp. CA, for example, grows optimally at 75 mM-NaCl (Stacey *et al.*, 1977).

We are not the first to report buffer effects on cyanobacteria. Bothe *et al.* (1977) reported total vegetative cell destruction at high (100 mM) concentrations of phosphate and Ferguson *et al.* (1980) reported growth inhibition of *Anabaena variabilis* by HEPES and other buffers, although the growth rate was still 50% of the control even in 100 mM-HEPES. Bridges & Ward (1976) reported inhibition of photosynthetic oxygen evolution from *Agmenellum quadruplicatum* by phosphate, TES and Tris buffers. The conclusion of these workers, that inhibition occurs at the level of photosystem II, seems unnecessarily restrictive in view of the general nature of the damage caused by buffers and other salts reported herein.

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