The salinity tolerance of freshwater cyanobacterium *Synechococcus* sp. PCC 7942 is determined by its ability for osmotic adjustment and presence of osmolyte sucrose

N P. LADAS and G.C. PAPAGEORGIOU

Institute of Biology, National Research Center Demokritos, Athens 153 10, Greece

Abstract

We investigated the factors that impose an upper limit of salinity tolerance to the unicellular freshwater cyanobacterium *Synechococcus* sp. PCC 7942. Above approx. 0.4 M NaCl, *Synechococcus* cells cease to proliferate, after having accumulated 0.3 M sucrose. Cells that pre-accumulated sucrose could tolerate up to 0.5 M NaCl, but not 0.6 M NaCl. After exposure to 0.5 M NaCl or higher, the cells were irreversibly modified becoming unable for osmotic volume adjustments.

Additional key words: absorption spectra; cell doubling; chlorophyll content and fluorescence; cytoplasmic osmolality; glucose; NaCl; saccharose; sugars.

Introduction

Living cells employ overlapping and complementary defences when challenged with abiotic stresses that cause cytoplasmic water deficits (Bray 1993, Potts 1994, Bohnert *et al.* 1995, Wood 1999). One defence mechanism is osmoregulation, which is achieved by synthesis, import, or both, of low molecular mass organic solutes (osmolytes). In plant cells, osmoregulation maintains turgor due to lower osmotic potential inside the cell than outside it which is important for the proliferation of plant cells (Whatmore and Reed 1990, Csonka and Hanson 1991).

Various species of cyanobacteria differ in salt tolerance and accumulate different compatible osmolytes. For example hypersaline species (upper salinity of 2.2-2.7 M NaCl) accumulate glycine betaine or glutamate betaine, while freshwater species (upper salinity 0.4-0.8 M NaCl) accumulate sucrose or trehalose (Blumwald *et al.* 1983b, Erdmann 1983, Mackay *et al.* 1984, Reed *et al.* 1986a). After genetic transformations, cells of freshwater *Synechococcus* sp. PCC 7942 acquire the capability of synthesising approx. 0.1 M glycine betaine (Deshnium *et al.* 1995, Nomura *et al.* 1995). Although the transformed cells proliferated faster in the presence of 0.3-0.4 M NaCl, they could not survive above 0.4 M NaCl.

In this work, we have investigated the causes that impose an upper salinity tolerance of this cyanobacterium.

Materials and methods

Cultures of cyanobacteria: *Synechococcus* sp. PCC 7942 cells were cultured photo-autotrophically at 31 °C, as in Papageorgiou *et al.* (1998). The culture medium was BG11 (Rippka *et al.* 1979). It was buffered at pH 7.5 with 20 mM Hepes NaOH (basal medium). Salt-grown cultures were initiated by inoculating NaCl-containing basal media with cells that were pre-cultured in basal medium (control cells).

Quantitation of soluble sugars: Low molecular mass sugars were extracted exhaustively from twice-washed *Synechococcus* cells with 80 % (v/v) ethanol in 25 mM Hepes-KOH, pH 7.1, at 80 °C (Avigad 1990). Pooled ethanol extracts were evaporated under reduced atmospheric pressure in a rotating evaporator and the solid residues were dissolved in 50 mM Na-acetate, pH 5.1. Total sugar content in the extracts was estimated by the

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Fax: +30 1 651 1767; e-mail: gcpap@mail.demokritos.gr

phenol-sulfuric acid method (Dubois *et al.* 1956) by reference to glucose standards. Glucose was determined enzymatically by oxidation to gluconic acid with glucose oxidase (*Sigma*, 20 units per cm^3) using an O_2 concentration electrode (*Rank Brothers*, Cambridge, UK). Saccharose was determined from the difference of glucose concentrations before and after inversion of the disaccharide with invertase (*Sigma*, 200 units per cm^3). Soluble sugars were detected and identified by thin layer chromatography (20×20 cm silica-gel; 0.2 mm thick; *Sigma*), elution with 10 : 65 : 15 : 10 v/v mixture of methanol : ethyl acetate : acetic acid : water, and development at 100 °C with a mixture containing 5 % sulfuric acid and 3 % phenol in ethanol.

Fluorescence measurements: Kinetics of Chl *a* fluorescence were measured in DCMU-pretreated (20 μM) *Synechococcus* cell suspensions [20 g(Chl) m^{-3}] with a *PAM* fluorometer (*H. Walz*, Effeltrich, Germany) which was operated in the continuous mode. Fluorescence signals were generated by periodic 1 μs excitation flashes (0.012 μmol m^{-2} s^{-1}; 1.6 kHz; 650 nm; $\Delta\lambda$ = 25 nm), and were sampled and displayed by the *DA-100* software. The exciting beam was sufficient to reduce all Q_A, the primary electron acceptor of photosystem 2, but too weak to acclimate the cells to irradiance (state 2 to state 1 transition; see Papageorgiou *et al.* 1998).

Other methods: Cell suspension densities were quantified in terms of the 730 nm turbidity (A_{730}) which below 0.4 varied linearly with the cell number per unit culture volume. Cell population doublings (n) were calculated from the expression $n = [(\log A_{730})_t - (\log A_{730})_0]/\log 2$, where subscripts $_0$ and $_t$ specify the culturing time interval. The relative content of Chl *a* per cell was estimated in terms of the quantity g(Chl) m^{-3} $(A_{730})^{-1}$. Chl *a* concentrations in *Synechococcus* cell suspensions were determined in N,N'-dimethylformamide extracts according to Moran (1982). Absorbances in a single spectral band were measured in an *Uvidec-610* (*Jasco*, Tokyo, Japan) spectrophotometer. Absorption spectra of cell suspensions were measured with a model *557* (*Hitachi*, Tokyo, Japan) dual wavelength spectrophotometer, under conditions that minimise radiation scattering. Irradiances were measured with a Quantum Radiometer (*Li-Cor*, Lincoln, NE, USA).

Results and discussion

We standardised the cells used in this research by reference to culture proliferation characteristics and to Chl *a* synthesis (Fig. 1). Cells pre-cultured in basal medium (38.04 mM Na^+ and 0.25 mM Cl^-) were sub-cultured in basal medium or in basal medium with 0.2 or 0.4 M NaCl added. Initially, in basal medium and in the 0.2 M NaCl-containing medium, the cell population doubled every 17 h, while in the 0.4 M NaCl-containing medium it doubled every 117 h. After 5 d, the culture growth dropped to 1 doubling every 68 h in the first two media, while in the 0.4 M NaCl medium it accelerated to one doubling every 34 h (Fig. 1*A*).

Exposure to 0.4 M NaCl had a severe impact on the Chl *a* content of the cells (Fig. 1*B*). It declined by approx. 80 % in the first 5 d, but then it rose again. Characteristically, the onset of accelerated Chl *a* synthesis coincided with the onset of rapid cell proliferation (Fig. 1*A*). By contrast, in basal medium and in medium enriched with 0.2 M NaCl, Chl *a* rose by approx. 30 % in the first two days after inoculation and remained fairly constant thereafter. In the 0.4 M NaCl cultures, these results indicate an initial phase during which Chl *a* holochromes are degraded, and a second phase during which net Chl *a* synthesis and holochrome assembly take place. In our conditions, the initial phase during which cells did not divide and their population remained practically constant lasted 4-5 d. Cell division was resumed and the cell population increased during the second phase. In the basal medium culture and

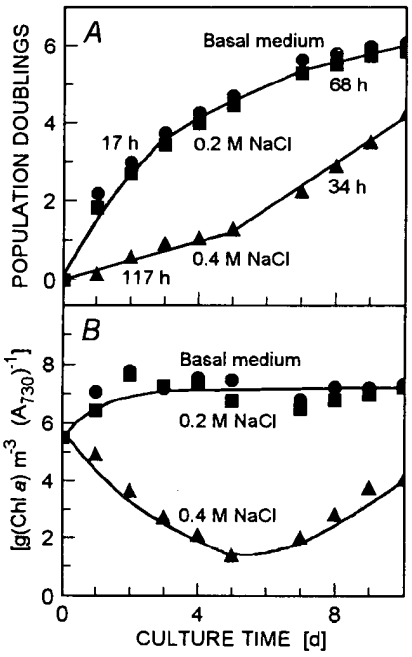

Fig. 1. Cell population doublings (*A*) and chlorophyll (Chl) *a* content per cell (*B*) of *Synechococcus* sp. PCC7942 cultured in basal medium (BG11 plus 0.02 M Hepes NaOH, pH 7.5, *circles*), basal medium plus 0.2 M NaCl (*squares*), and basal medium plus 0.4 M NaCl (*triangles*). Cell numbers per cm^3 culture were set proportional to A_{730}; Chl *a* content per cell was set proportional to g(Chl *a*) m^{-3}(culture) $(A_{730})^{-1}$.

in the 0.2 M NaCl culture,the cell cycle evolved without interference. Cells multiplied without delay, and the Chl *a* content remained virtually constant.

We compared cells from 7-d-old cultures. According to Fig. 1, these are late logarithmic phase cells for the basal medium and the 0.2 M NaCl cultures, and beginning logarithmic phase cells for the 0.4 M NaCl cultures.

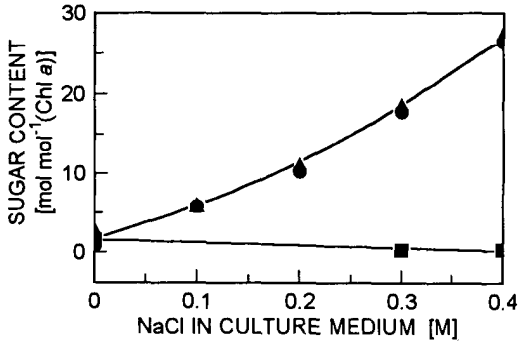

Fig. 2. Effect NaCl, added to basal culture medium, on the glucose-to-Chl *a* and saccharose-to-Chl *a* molar ratios [expressed as hexose (Chl *a*)$^{-1}$] of 7-d-old *Synechococcus* sp. PCC 7942 cultures. Total sugars (*open circles*), glucose (*open squares*), and saccharose (*open triangles*).

Blumwald *et al.* (1983b) reported that in freshwater *Synechococcus* cytoplasmic saccharose content rises with the salinity. In our experiment, saccharose was virtually absent in basal medium cells. Its content increased with the culture medium salinity and at 0.4 M NaCl it reached approx. 26 hexose equivalents per Chl *a* (sucrose:Chl *a* = 13 mol mol^{-1}). Only traces of glucose were detected, and a test for trehalose (a glucose-glucose osmolyte) proved negative.

Mehlhorn and Sullivan (1988) determined the Chl *a* concentration of *Anacystis nidulans* cells on the basis of

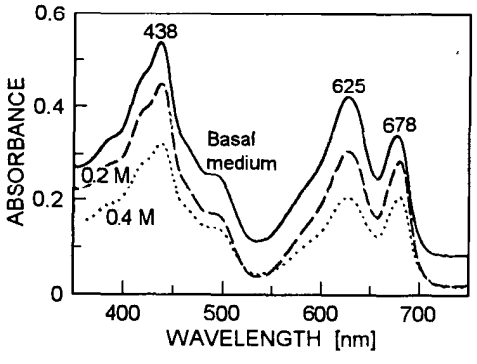

Fig. 3. Absorption spectra of 7-d-old *Synechococcus* sp. PCC7942 cultures in basal medium and basal medium plus 0.2 or 0.4 M NaCl. The spectrum in basal medium was displaced in the ordinate for clarity.

osmotic cell volume measurements with ESR spin probes. Cells cultured in buffered BG11 contained 38 mM Chl *a*, while those cultured in the presence of 0.5 M NaCl contained 20 mM Chl. Interpolating from these values, we estimated that *Synechococcus* sp. PCC 7942 (*A. nidulans* R-2) cells contained 24 mM Chl *a* and approx. 300 mM saccharose, when they were cultured in 0.4 M NaCl. This suggests that saccharose is the main (and most likely the only) compatible organic osmolyte with which this organism counteracts unfavourable external salinity.

Salinity-stressed *Synechococcus* cells mobilise metabolic reserves and divert the products to the synthesis of saccharose. These processes account for the initial lag in culture proliferation (Fig. 1*A*) and the initial degradation of Chl *a* holochromes (Fig. 1*B*). Absorption spectra showed a massive degradation of C-phycocyanin, the principal phycobiliprotein of freshwater *Synechococcus* (Fig. 3). The peak absorption ratio of the C-phycocyanin to Chl *a* (A_{625}:A_{678}) decreased from 1.32 to 1.10, and to 1.00, as NaCl concentration increased from 0 to 0.2 M, and to 0.4 M. At the same time, the Chl *a* absorption band maximum of cells cultured in 0.2 M NaCl was shifted by 1-2 nm toward the red region of the spectrum, and by 4-5 nm in cells cultured in 0.4 M NaCl.

In the following experiment, cells pre-cultured for 7 d in the presence of 0.4 M NaCl were sub-cultured in fresh

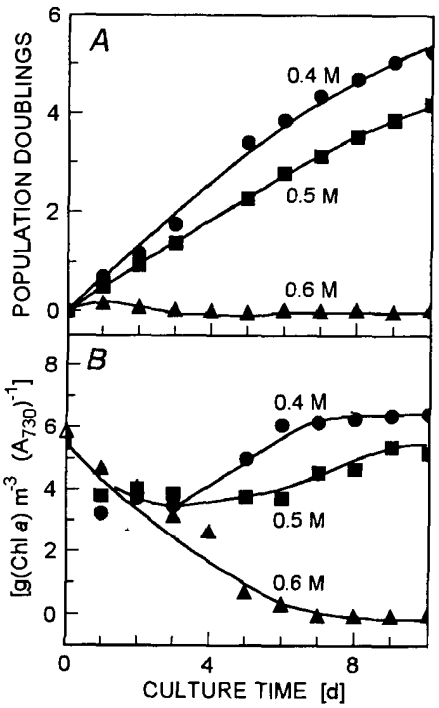

Fig. 4. Cell population doublings (*A*) and relative chlorophyll (Chl) *a* content (*B*) of *Synechococcus* sp. PCC7942 cells, precultured for 6 d in basal medium plus 0.4 M NaCl, and subcultured in basal medium plus 0.4 (*circles*), 0.5 (*squares*), or 0.6 (*triangles*) M NaCl.

basal media containing 0.4, 0.5, and 0.6 M NaCl (Fig. 4). Cells sub-cultured in 0.4 and 0.5 M NaCl proliferated without delay, with population doublings every 37 and 50 h, respectively. These rates contrast with the culture proliferation lag observed when basal medium cells were sub-cultured in 0.4 M NaCl (Fig. 1*B*). We conclude that cells pre-cultured in the presence of NaCl accumulated sufficient saccharose to enable them to proliferate without delay when they were transferred to media containing 0.4 and 0.5 M NaCl. However, cells pre-cultured for 6 d in 0.4 and 0.5 M NaCl ceased to divide when they were transferred to 0.6 M NaCl.

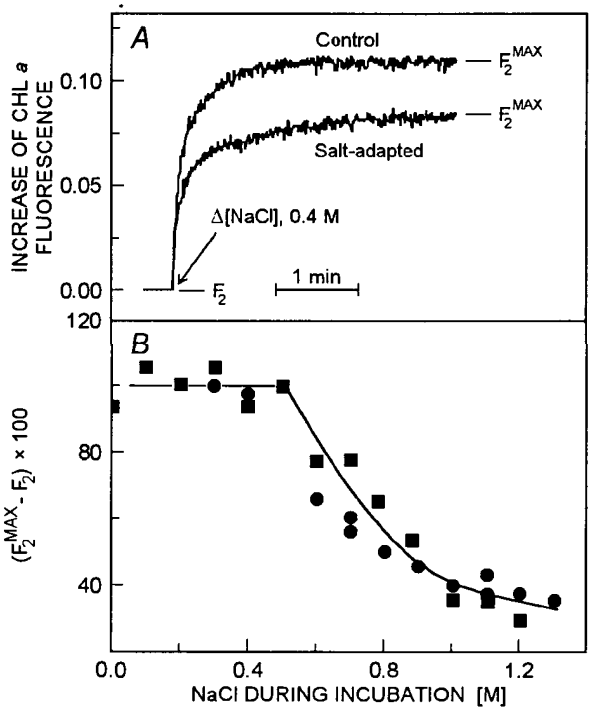

Fig. 5. (*A*) Chlorophyll (Chl) *a* fluorescence rise of *Synechococcus* sp. PCC7942 cells induced by increasing the NaCl concentration in the suspension by 0.4 M (Δ[NaCl]); cells cultured in BG11 and suspended in BG11 (*control*), and cells cultured in BG11 plus 0.3 M NaCl and suspended in BG11 plus 0.3 M NaCl are compared. (*B*) The amplitude of Chl *a* fluorescence rise, induced by 0.4 M NaCl, as a function of the NaCl concentration with which the cells had been pre-treated for 20 min; cells cultured in BG11 and suspended in BG11 (*open circles*) and cells cultured in BG11 plus 0.3 M NaCl and suspended in BG11 plus 0.3 M NaCl (*closed circles*) are compared. At the end of the 20 min treatment, the cells were returned to the respective suspension media, and the NaCl-induced (0.4 M) rise of Chl *a* fluorescence was recorded similarly as in *A*.

In the 0.4 M NaCl subculture, Chl *a* content decreased initially by 25-30 %, but after the 3rd d the process was reversed and after 6 d it fully recovered. In the 0.5 M NaCl sub-culture, Chl *a* also decreased by 25-30 %, but the recovery was delayed until after the 6th d. Finally, in the 0.6 M NaCl sub-culture all Chl *a* disappeared from the cells within 5-6 d. Hence the limit of salinity tolerance of *Synechococcus* sp. PCC 7942 cells is between 0.5 and 0.6 M NaCl, regardless of the presence of saccharose in the cytoplasm.

A characteristic trait of freshwater *Synechococcus* is the rapid passive uptake of NaCl (Stamatakis *et al.* 1999). Osmotically driven water influx accompanies the NaCl influx. As a result, the cell volume increases to maximum. Such volume changes have been detected and quantified with ESR spectrometry (Blumwald *et al.* 1983a, Allakhverdiev *et al.* 2000), particle size analysis (Reed *et al.* 1986b), H-NMR spectrometry (Nitschmann and Packer 1992), and Chl *a* fluorometry (Stamatakis *et al.* 1999).

The principle of fluorimetric monitoring of cell volume changes in *Synechococcus* sp. PCC 7942 cells is illustrated in Fig. 5*A* with basal medium (control) cells and cells adapted to 0.3 M NaCl. Control cells contained virtually no saccharose, while salt-adapted cells contained 9 mol(saccharose) mol^{-1}(Chl *a*) (see Fig. 2). The cells were dark-acclimated for 4 min, and then they were excited with the weak modulated radiation of the *PAM* fluorometer. The excitation generated a low steady Chl *a* fluorescence (F_2; set equal to zero in Fig. 5*A*) but it was too weak for a PAR-acclimative transition (state 2 to state 1 transition). Injection of NaCl solution, in order to raise the suspension NaCl concentration to 0.4 M, drove Chl *a* fluorescence to higher levels (F_2^{MAX}). The difference ($\Delta F = F_2^{MAX} - F_2$) is a measure of cell volume swelling (ΔV) which is caused by the influxes of NaCl and water (Stamatakis *et al.* 1999).

Further, cells cultured for 6 d in basal medium were first incubated for 20 min in the presence of various NaCl concentrations, and then they were resuspended in NaCl-free basal medium (Fig. 5*B*). Likewise, NaCl-adapted cells were first incubated for 20 min in their culture medium (containing 0.3 M NaCl) plus additional NaCl, and then they were resuspended in their culture medium. During the 20 min incubations, the cells were loaded with progressively increasing amounts of NaCl, which were determined by the NaCl concentrations of the suspension media. NaCl-adapted cells contained also saccharose; control cells did not.

The NaCl-induced Chl *a* fluorescence rise of the latter cells was recorded (Fig. 5*A*) and the normalised amplitudes $[(F_2^{MAX} - F_2) \times 100]$ were plotted against the NaCl concentration of the suspension media in which the cells were incubated for 20 min. According to Fig. 5*B*, the quantity $[(F_2^{MAX} - F_2) \times 100]$ decreased sharply above approx. 0.5 M NaCl, both in the control cells and in the NaCl-adapted cells. These results indicate progressive, NaCl-dependent loss of the ability *Synechococcus* cells for osmotic volume adjustments (see also Stamatakis *et al.* 1999), which

occurred regardless of the presence of sucrose in the cytoplasm.

Freshwater cyanobacteria tolerate between 0.5-0.7 M NaCl (Mackay *et al.* 1984). *Synechococcus* 6311 (*A. nidulans*) grows in 0.6 M NaCl (Blumwald *et al.* 1983a,b, 1984), while *Synechococcus* sp. PCC 7942 (*A. nidulans* R-2) and its glycine betaine-synthesizing transformants could not be cultured above 0.4 M NaCl (Deshnium *et al.* 1995, Nomura *et al.* 1995). We demonstrated that the tolerance of *Synechococcus* sp. PCC 7942 cells, which pre-accumulated saccharose, can be extended to 0.5 M NaCl, but not to 0.6 M NaCl. The ability of cells for osmotic volume adjustments was irreversibly impaired when they were exposed to 0.5 M NaCl, or more.

Metabolically active cells, suspended in BG11, contain approx. 29 mM Na^+ in the dark and 11 mM Na^+ in the light (Ritchie 1992). After hyper-saline shock, the cell shrinks osmotically in less than 10 ms (Papageorgiou *et al.* 1998). Immediately after, it begins importing NaCl and water at high rate ($t_{1/2} \approx 1.8$ s for Δ[NaCl] = 0.4 M; Stamatakis *et al.* 1999). Within 1-2 min, internal Na^+ rises to a few hundred mM, and the cell volume is maximised (Blumwald *et al.* 1983a, Nitschmann and Packer 1992). Na^+, at high concentration, is a denaturing cation and cells expel it energetically (Dewar and Barber 1973, Paschinger 1977), *via* Na^+/H^+ antiporters (Blumwald *et al.* 1984) and Na^+-ATPase pumps (Ritchie 1992) that are located in the plasma membrane. As a result, within 10 min or so, internal Na^+ drops to a steady value in the range 11-29 mM (Blumwald *et al.* 1983a, Ritchie 1992), and cell volume adjusts to a lower value (Nitschmann and Packer 1992, Papageorgiou, G.C. and Alygizaki-Zorba, A., unpublished). Na^+ extrusion and cell volume adjustments are prevented in energetically-depleted cells (*e.g.*, in the presence of protonophoric uncouplers; Nitschmann and Packer 1992, Stamatakis *et al.* 1999), or in the presence of water channel and Na^+ channel blockers (Allakhverdiev *et al.* 2000).

Since the freshwater *Synechococcus* cell is unable to prevent the massive invasion of NaCl during a salinity upshift episode, it resorts to active extrusion of Na^+. To this task it commits readily tangible energy resources, such as existing ATP pools and trans-membrane ion gradients, which are replenished both by photosynthesis and by catabolic reactions (*e.g.*, degradation of pigment holochromes; see Figs. 1*B* and 3). Supplementary defences, such as gene expression and protein and osmolyte biosyntheses are too slow in bacteria (1 h or more; Wood 1999). A hyper-saline shock would have killed *Synechococcus* cells before they could be activated, were it not for their ability to expel internal Na^+ in relatively short time. The osmolyte saccharose is accumulated in the cytoplasm in response to the salinity dose (NaCl concentration×time; Fig. 2) which the cells experience. For 6 d-old cells cultured in 0.4 M NaCl, internal sucrose concentration was 0.3 M.

Saccharose is a protein structure stabiliser, but at high concentrations it can interfere with enzyme activities through viscosity effects and co-solvent effects. The relative viscosity of 0.3 M saccharose solution (1.333 after Wolf *et al.* 1984) can slow down diffusion-controlled reactions (Jakim *et al.* 1992). As co-solvent, saccharose could also replace water at macromolecular surfaces, dehydrate macromolecular clefts, and induce macromolecules to crowd (Wood 1999, and cited references).

It is likely, however, that high internal saccharose content is not the determinant factor that limits the salinity tolerance of *Synechococcus* sp. PCC 7942 to less than 0.5 M NaCl. Related *Synechococcus* 6311 tolerates 0.6 M NaCl, or more. In view of the Fig. 5, the significant factor may be the inability of cells that had been exposed to 0.5 M NaCl, or more, for osmotic volume adjustment. Excessive salinity impairs the cell envelope (plasma membrane and cell wall) of this cyanobacterium irreversibly, and so the cells cannot expand, or deform, elastically in response to osmolality changes.

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