Chapter 5

Regulation of intracellular ion content

Summary

Yeasts have minimal nutritional needs. Most species grow well in the presence of simple carbon and nitrogen compounds together with inorganic ions and a few growth factors. Inorganic ions are implicated in several cellular functions. Yeasts cells are not freely permeable to inorganic ions and these are taken up by the cell through different types of transporters. This chapter presents the intracellular concentrations of sodium and potassium in *C. halophila* cells cultivated and shocked by increased salt concentrations. In the absence of salt, *C. halophila* cells cultivated in MM with glucose, glycerol or mannitol as carbon sources presented intracellularly around 300 mM of potassium and 2 mM of sodium. When submitted to increasing salt concentrations, potassium and sodium ions maintained its intracellular values up to 2M NaCl. Above this salt concentration in the media, intracellular potassium concentration decreased steeply, while sodium increased abruptly to around 120 mM and remained stable up to 4.5M NaCl in the media. The observed decrease in intracellular potassium closely paralled the decrease in specific growth rate on glucose, which may suggest that, under salt stress, the maintenance of intracellular potassium ions might be an indispensable requirement for growth. *C. halophila* cells cultivated without salt were also shocked with 1, 2 and 3M NaCl during a 6 hours period. In these experiments potassium intracellular concentration increased. In the same period of time, sodium intracellular concentration also increased transiently during the 2 first hours, decreasing subsequently. This was not so in the presence of 3M NaCl, in which case the potassium intracellular concentration increase was only transient, while the sodium concentration increased and remained stable up to 6 hours incubation time. The amounts of intracellular sodium accumulated after 2 hours incubation varied linearly with the amount of NaCl the cells were subjected to. Results points to *C. halophila* being an efficient sodium excluder, able to maintain internal sodium concentrations below 150 mM.

5.1. Introduction

5.1.1. *Physiological role of ions in the life of microorganisms*

In order to grow microorganisms exhibit minimal and essential nutritional requirements, which are acquired from the growth environment (Chapter 1). Although microorganisms are extremely diverse in their nutritional requirements some compounds are commonly important in its life such as the carbon, nitrogen and phosphorus sources, and the less abundant elements, but as important to overall cell metabolism as the first, like magnesium, potassium, calcium and several other main elements required only in trace concentrations. In general cells take up inorganic cations for several reasons. These may involve: regulation of intracellular pH homeostasis and generation of proton motive force (in the case of H^+ transport) [Serrano, 1989]; osmoregulation and charge balancing (in the case of K^+) [Norkrans and Kylin, 1969; Rodríguez-Navarro *et al*., 1986]; enzyme cofactor functions (in the case of Mg^{2+} and Mn^{2+}) [Walker, 1994; Auling, 1994]; metalloenzyme structural functions (in the case of micronutrient divalent cations such as Fe^{2+}, Zn^{2+}, and Ni^{2+}) [Walker, 1998] and signal transduction second messenger functions (in the case of Ca^{2+}) [Iida *et al*., 1990]. In fact, one of the crucial and fundamental tasks of a living cell is to maintain the homeostasis of intracellular pH and ion concentrations. Homeostasis is even more critic when cells are faced with salt stress situations. Indeed, inorganic cations play an important role in the osmotic responses of bacteria [Csonka, 1989; Ventosa *et al*., 1998; Roeβler and Müller, 2001], *Archaea* [Grant *et al*., 1998; Oren, 1999; Martin *et al*., 1999; Roeβler and Müller, 2001; Roberts, 2004], algae [Hellebust, 1976], plants [Serrano, 1996; Rodríguez-Navarro, 2000; Serrano and Rodríguez-Navarro, 2001; Tester and Davenport, 2003] and fungi [Blomberg and Adler, 1992; Serrano, 1996; Serrano *et al*., 1997; Rodríguez-Navarro, 2000].

Sodium ions

Sodium is the most abundant free cation at earth superficies. However, yeast cells do not accumulate sodium ions under normal growth conditions and maintain very low cytosolic concentrations of this cation [Rodríguez-Navarro and Ortega, 1982]. In contrast to fungal cells, sodium is widely used in animal cells and in bacteria for the uptake of several solutes, *e.g*., aminoacids and carbon sources [Reizer *et al*., 1994]. However, a Na^+-P_i symport was reported in *N. crassa* [Mann *et al*., 1989; Versaw and Metzenberg, 1995] and in *S. cerevisiae* [Martínez and Persson, 1998], but its function is limited to alkaline conditions in the presence of both sodium and lower phosphate concentrations. Together, sodium ions and protons play primary roles in cell bioenergetics. When the concentration of these ions becomes too high or too low, they turn into strong stressors to all types of cells and hence every cell should have efficient homeostatic mechanisms for these ions. Sodium might be beneficial to the cell in situations where there is lower concentration of potassium available in the media. In normal situations sodium is present and then cells use, instead of protons, preferentially sodium ions for charge balance. In *S. cerevisiae*, Na^+ is not necessary when K^+ is not limiting [Camacho *et al*., 1981], but Na^+ restores the cell volume, cellular pH [Ramos *et al*., 1990], and growth [Camacho *et al*., 1981] when K^+ is limiting. For similar K^+ replacements, Na^+ is less toxic than H^+, nevertheless, sodium is toxic to cells at higher concentrations [Camacho *et al*., 1981], and this aspect will be discussed further ahead.

Potassium ions

Potassium is an obligatory mineral component of living cells [Camacho *et al*., 1981], which is essential for several cellular functions such as: enzyme activation [Bostian and Betts, 1978; Rosas *et al*., 1994],

protein biosynthesis [Pollard and Wyn-Jones, 1979], osmotic regulation [Norkrans and Kylin, 1969; Oren, 1986; Shen *et al*., 1999]; and intracellular pH and membrane potential regulation [Rodríguez-Navarro *et al*., 1986; Blatt and Slayman, 1987; Ramos *et al*., 1990; Calahora *et al*., 1998; Walker *et al*., 1996; Walker *et al*., 1998]. Potassium is also used and accumulated intracellularly at high concentrations by a specific group of prokaryotic microorganisms, the extremely halophilic *Archaea*, as osmolyte in salt stress conditions [Bayley and Morton, 1978; Kushner, 1985; Oren, 1986; 1999; Ventosa *et al*., 1998; Roeβler and Müller, 2001]. Because of the important functions played by K^+ in the cell, the decrease in the cellular K^+ content has deleterious effects. The first effect of K^+ deficiency, which may occur when the external K^+ concentration is too low to allow sufficient rate of uptake and other alkali cations are not available, is that a certain proportion of the normal K^+ content is replaced by H^+ because electrical neutrality must be satisfied in all cases. This replacement decreases the internal pH [Ramos *et al*., 1990; Walker *et al*., 1996; Walker *et al*., 1998], and K^+ deficiency may produce a detrimental effect by itself, by the pH decrease, or by concurrence of both factors. Thus, protein synthesis is inhibited only by the decrease of the cellular pH [Walker *et al*., 1998], whereas osmotic functions [Walker *et al*., 1996] may be affected by the decrease of the K^+ content.

5.1.2. *Intracellular concentrations of monovalent cations and chloride anion in microorganisms*

Potassium

The intracellular potassium content varies according to growth conditions, ranging in fungal and plant cells from 50 to 250 mM [Serrano *et al*., 1997; Rodríguez-Navarro, 2000; Serrano and Rodríguez-Navarro, 2001]. In yeasts, potassium is the most prevalent cation in the cytoplasm (about 250 to 350 mM of potassium at external concentrations around 10 mM) [Serrano, 1994; García *et al*., 1997; Prista *et al*., 1997; Thomé-Ortiz *et al*., 1998]. In most of the environments, potassium concentrations lie in the micromolar range. Therefore, K^+ concentration ratios across the plasma membrane may be 10^3 or higher, and K^+ uptake must be carried out against a steep concentration gradient. K^+ is also accumulated intracellularly to high values, as osmolyte under high salt concentrations, in the following prokaryotes: the red-pigmented extreme halophilic *Archaea* (members of the family *Halobacteriaceae,* the only family in the order *Halobacteriales*) [Grant and Larsen, 1989; Grant *et al*., 1998; Martin *et al*., 1999]; the moderately halophilic methanogens [Matheson, 1985] and in two bacteria groups: (i) the halophilic anaerobic members of *Haloanaerobiaceae* [Oren *et al*., 1984] and (ii) the halophilic sulphate-reducers [Caumette *et al.,* 1991; Olliver *et al*., 1991]. Even the non-halophilic *Archaea* maintain relatively high intracellular concentrations of K^+ (>0.5 M) [Sprott and Jarrel, 1981; Martin *et al*., 1999].

Sodium

Under normal growth conditions, bacteria [Ventosa *et al*., 1998], plants [Serrano and Rodríguez-Navarro, 2001; Tester and Davenport, 2003] and yeasts cells [Rodríguez-Navarro and Ortega, 1982] excrete continuously Na^+ in order to maintain intracellular low levels. Intracellular Na^+ content varies greatly from microorganism to microorganism, both among different species and within the same species [Ventosa *et al*., 1998]. Generally, Na^+ concentration is, at different extents, lower inside the cells than outside, in cells submitted to different levels of salt stress. This is accomplished via several transporters which will be further ahead discussed. Apparently sodium intracellular content increases

with increasing external NaCl concentration, but this occurs between different species in a nonlinear fashion [Ventosa *et al*., 1998; Prista *et al*., 1997; García *et al*., 1997]. Thus, external Na^+ concentrations of 3 M may implicate, in the moderately halophilic bacteria, intracellular values from ± 0.5 to > 3M, depending on the species and growth conditions [Ventosa *et al*., 1998]. Furthermore it has been reported that intracellular ion concentrations are determined using different experimental methodologies and these may account greatly for the differences obtained for the same organism under similar conditions and also justify the high values reported for extreme halophilic species [Ventosa *et al*., 1998]. In the case of yeasts that are more sensitive to sodium, the highest concentration reported intracellularly was 0.8 M in *D. hansenii*, observed at an external salt concentration of 2M [Prista *et al*., 1997]. *D. hansenii* cells showed to accumulate intracellularly sodium in a linear relation with the NaCl in the medium [Prista *et al*., 1997]. The same did not happen to *S. cerevisiae,* neither to *Candida tropicalis* [García *et al*., 1997]. In the case of *S. cerevisiae*, Na^+ intracellular content increased in the presence of 0.5 M NaCl and remained stable for higher salt concentrations, at least up to 1 M [García *et al*., 1997]. As an opposite example, *C. tropicalis* did not increase significantly its intracellular sodium content up to 1M NaCl in the medium [García *et al*., 1997].

Chloride

Estimations of intracellular chloride concentrations in cells are scarce in literature. Reports on moderately halophilic bacteria exist but results are greatly variable, from relatively low values (55 and 139 mM in *Halomonas halodenitrificans* and *Salinivibrio costicola*, respectively, grown at 1M NaCl) to values as high as 0.7 to 1M in *Pseudomonas halosaccharolytica* grown at NaCl concentrations between 1 and 3M [Ventosa *et al*., 1998]. Apparently the intracellular chloride concentration accompanies the internal Na^+ and K^+ concentration. The assumption that Cl^- is the main counterion for the intracellular cations in moderate halophiles is not necessarily so. Since no other ions have been detected at high concentrations inside the cells, it has been speculated that most of the cellular cations may be associated with negative charges present on proteins, cell envelopes, and other macromolecules [Kushner, 1988]. Recently was reported that the moderately halophilic, aerobic bacterium *Halobacillus halophilus* requires chloride for growth and salt adaptation [Roeβler and Müller, 1998]. Because *H. halophilus* accumulates compatible solutes but not KCl to counterbalance the external salt concentration, a function of Cl^- solely as an intracellular anionic osmolyte is excluded [Roeβler and Müller, 2002]. In addition to growth, endospore germination, activation of transport of the compatible solute glycine betaine, mobility and flagellum production have been identified as Cl^- dependent processes [Roeβler and Müller, 1998]. A very high requirement for chloride was also demonstrated in two groups of Bacteria that accumulate inorganic salts intracellularly rather than using organic osmotic solutes: the anaerobic Haloanaerobiales and the aerobic extremely halophilic *Salinibacter rubber* [Oren *et al*., 2002; Müller and Oren, 2003]. It is thus becoming clear that chloride has specific functions in haloadaptation in different groups of halophilic microorganisms.

As far we know, estimation of intracellular chloride content in yeasts was only reported in *Z. rouxii* cells submitted to a salt shock during 3 hours with varying salt concentrations [Yagi and Nishi, 1993]. *Z. rouxii* cells accumulated both sodium and chloride at similar values in a linear relation with the NaCl in the medium. However it remains to be known if this behavior is maintained in cells adapted to salt, as well as if it is common to other yeasts.

5.1.3. *Ion toxicity in yeasts and its targets*

Sodium chloride has two distinct effects on cells: one is osmotic and the other is the intrinsic sodium toxicity. The osmotic effect results when high sodium concentrations enter the cell and causes the immediate and extensive loss of cytoplasmic water. To overcome this situation cells activate the extrusion of sodium and accumulate compatible solutes. However, in the yeasts *S. cerevisiae* [Gaxiola *et al*, 1992; Gläser *et al*., 1993] and *Z. rouxii* [Rodríguez-Navarro, 1971], growing on glucose media, NaCl is much more toxic than equivalent osmotic concentrations of KCl or sorbitol. This situation is, although, different in *A. nidulans* [Beever and Laracy, 1986], *Candida tropicalis* [García *et al*., 1997] and *D. hansenii* [Neves *et al*., 1997], and even *S. cerevisiae* growing in galactose as carbon source [García *et al*., 1997], in which cases it has been shown that NaCl, KCl and sorbitol have similar toxicities. It seems, apparently, that with glucose as the carbon source osmotic adjustment is more effective. Therefore, sodium toxicity is apparently the major problem raised by salinity. Metabolic toxicity of Na^+ is largely a result of its ability to compete with K^+ for binding sites essential for cellular function [Serrano *et al*., 1997]. In addition, sodium could also interfere with magnesium and calcium sites [Serrano *et al*., 1997]. More than 50 enzymes are activated by K^+, and Na^+ cannot substitute in this role [Bhandal and Malik, 1988]. Low salt concentrations (50-100 mM) have positive effects on many enzymes because this is the ionic strength of the cytoplasm of normal organisms and protein hydration is maximal under these conditions. On the other hand, high levels of Na^+, or high Na^+/K^+ ratios are toxic because they can disrupt various enzymatic processes in the cytoplasm by disturbing the hydrophobic-electrostatic balance between the forces maintaining protein structure [Wyn Jones and Pollard, 1983; Serrano, 1996]. Moreover, protein synthesis requires high concentrations of K^+, relying on the K^+ requirement for the binding of tRNA to ribosomes [Blaha *et al*., 2000] and probably other aspects of ribosome function [Wyn Jones *et al*., 1979]. In yeasts, the ability to maintain high intracellular levels of potassium in the presence of high Na^+ concentrations, allows the maintaining of high K^+/Na^+ ratios and thus to reduce the specific toxicity of Na^+ [Gaxiola *et al*., 1992; Haro *et al*., 1993; Gómez *et al*., 1996]. The toxicity of salt on cells is observed at intracellular concentrations of Na^+ and Cl^- of 50-100 mM, which is much lower than the levels required for ionic strength effects [Serrano *et al*., 1997]. The specific cationic effects on protein binding sites are more important for salt toxicity than non-specific ionic strength effects operating at high salt concentrations [Serrano *et al*., 1997].

Chloride toxicity has not yet been observed in yeasts. Thus, it is not known if this is due to either limited uptake of the anion, very active extrusion (due to the negative-inside membrane potential [Serrano, 1991]) or chloride–resistant enzymes [Serrano *et al*., 1997]. However, it was reported several years ago that *S. cerevisiae* seems to exclude Cl^- [Conway and Armstrong, 1961], while other fungi allow it to varying extents [Shere and Jacobson, 1970; Luard, 1982a,b; Wethered and Jennings, 1985; Wethered *et al*., 1985].

Cellular targets of salt toxicity include protein synthesis and some reactions of sugar metabolism, which are salt-sensitive *in vitro* [Wyn Jones and Pollard, 1983]. The product of the yeast *HAL2* gene (Hal2p) is up to now the only *in vivo* target of sodium and lithium toxicity [Gläser *et al*., 1993; Murguía *et al*., 1995, 1996; Albert *et al*., 2000], and its expression improves salt tolerance in yeasts [Gläser *et al*., 1993] and plants [Arrillaga *et al*., 1998]. Hal2p is a nucleotidase that requires magnesium for catalysis and is inhibited by low concentrations of both lithium and sodium. Inhibition of Hal2p during salt stress results in the accumulation of PAP (3'-phosphoadenosine-5´-phosphate) in the cell, which has the potential to produce a variety of toxic effects such as inhibition of sulphotransferases [Roth *et al*., 1982]

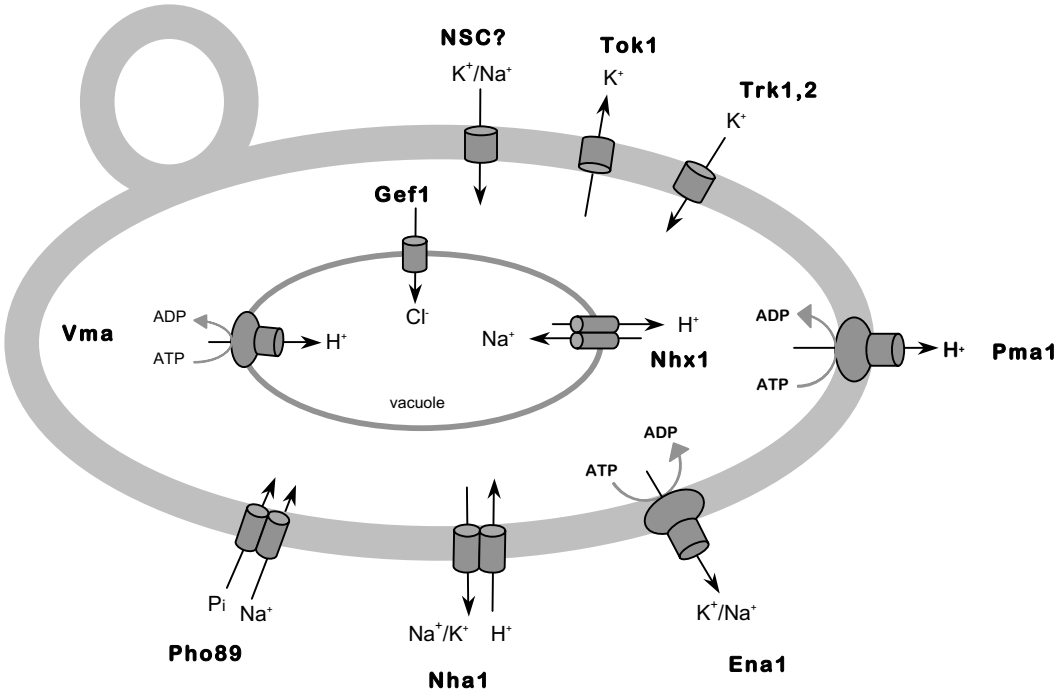
and RNA processing enzymes [Dichtl *et al*., 1997]. In yeast PAP accumulation also blocks the sulphur assimilation pathway [Murguía *et al*., 1995, 1996].

5.1.4. *Transport of ions*

In primitive unicellular organisms, devoid of rigid cell walls, ion transport mechanisms most probably solved several basic problems. In addition to pH regulation there was a problem of osmotic swelling for which salt extrusion could be the only defense [Serrano *et al*., 1997]. Thus, the capability for salt extrusion must have been a very early property of cells because of the need to control cellular osmolarity. However, the appearance of NaCl as a stress factor is considered a recent event in the evolution of living organisms, since the concentration of NaCl in the ancestral sea was eventually relatively low (150 mM) as compared with nowadays (500 mM) [Serrano *et al*., 1997].

Yeast uses several systems for translocating ions across cell membranes (Figure 5.1). These include: active transport via carrier permeases, counter-transport with protons or extrusion via ATPases and passive transport through ion channels.

Figure 5.1. Transporters of K^+ and Na^+ in the yeast *S. cerevisiae*. Ena1-P-type Na+/K+ pumping ATPase; Gef1- chloride channel; NCS non-selective channels; Nha1- electroneutral antiport; Nhx1-vacuolar antiporter; Pma1-P-type H^+ pumping ATPase; Trk1- potassium high affinity uptake system; Tok-potassium efflux channel; Vma-vacuolar V-type H^+-pumping ATPase.



The H^+-pumping ATPases of tonoplast and plasma membrane, the K^+ transporters (Trk-HKT and HAK), the Na^+ (K^+)/H^+ antiporters (Nha1, SOS1, Nhx1), the outward-rectifying K^+ channels (Tok1-ORK) and the Cl^- channels (Gef1-CLC) were reported to be homologous in fungi and plants [Serrano *et al*., 1999; Serrano and Rodríguez-Navarro, 2001]. The major differences identified up to now indicate that fungi have neither inward-rectifying K^+ channels (IRK) nor vacuolar H^+-pumping pyrophosphatases (Vmp), whereas plants have no Na^+-extrusion ATPases (Ena1) [Serrano and Rodríguez-Navarro, 2001]. The major monovalent ion transport systems of *S. cerevisiae* are depicted in Figure 5.1 and will be discussed below.

5.1.4.1 *Hydrogen*

Since cell membranes are not freely permeable to hydrogen ions, transmembrane proton gradients are established by active proton-pumping mechanisms like the H^+-ATPase. As said before in Chapter 2, due to their characteristics, plasma membrane H^+-ATPase is described as a master enzyme because it controls cell pH, nutrient and ion transport and overall cell growth. In yeasts the H^+-ATPase is a major constituent of the plasma membrane, comprising as much as 50% of total membrane protein [van der Rest *et al*., 1995]. Besides the P-type H^+-ATPase localized in the plasma membrane, *S. cerevisiae* possess a F-type H^+-ATPase in the mitochondrial inner membrane and a V-type H^+-ATPase in the vacuolar membrane. In contrast to the plasma membrane H^+-ATPase (encoded in *S. cerevisiae* by the *PMA1* gene), the mitochondrial vacuolar ATPases are composed of multiple different subunits and also differs in the proton/ATP stoicheiometry, which may be related with each specific function at different organelles [van der Rest *et al*., 1995]. With regard to pH and ion homeostasis in yeast, the H^+-ATPase is instrumental in modulating both intra- and extracellular pH and the internal ion composition. The plasma membrane H^+-ATPase has the capability of generating a 10,000-fold difference between the concentration of protons on either side of the membrane and therefore is inextricably linked with yeast growth. The magnitude of the gradient in yeast depends on the presence of other cations, notably K^+, which is exchanged for H^+ in a 1:1 stoicheiometry, being H^+-ATPase closely linked to ion homeostasis. Actually H^+-ATPases belong to the group of ATP-driven transporters within the category of cation translocating (subfamily P-type ATPase), which suggests that originally H^+-ATPases had a broad specificity for cations and then, according to each microorganism needs and intrinsic cellular characteristics, evolved to specific cation-pumps.

5.1.4.2. *Potassium*

Potassium is an obligatory component of yeasts, and all of them depend on K^+ uptake, and also eventually on K^+ efflux, to grow and to keep themselves alive, since most of the time they accumulate intracellularly high concentrations of K^+, higher than in the surrounding environment [Rodríguez-Navarro, 2000]. Potassium transport in fungi can be divided roughly in two classes: the K^+ uptake systems and the K^+ efflux systems. K^+ uptake systems comprises three distinct families: TRK (***Tr***ansporter of ***K***$^+$) whose first representative was found in *S. cerevisiae* [Gaber *et al*., 1988], HAK (***H***igh ***A***ffinity ***K***$^+$ transport), whose first member was found in *S. occidentalis* [Bañuelos *et al*., 1995a], and K^+ channels, originally cloned from *A. thaliana* [Anderson *et al*., 1992; Sentenac *et al*., 1992]. K^+ efflux systems include the K^+/H^+ antiporters and the K^+-ATPases.

The TRK transporter family represent high affinity potassium transporters which have been

identified in *C. albicans* [Genbank Accession nº AF267125, Miranda, M. unpublished results], *D. hansenii* [reviewed in Rodríguez-Navarro, 2000], *K. lactis (KlTrk1)* [Miranda *et al*., 2002], *N. crassa* [Haro *et al*., 1999], *S. cerevisiae* (*TRK1,2*) [Gaber *et al*., 1988; Ko *et al*., 1990; Ko and Gaber, 1991], *S. occidentalis* (*TRK*) [Bañuelos *et al*., 2000], and *S. pombe* (*TRK1,2*) [Soldatenkov *et al*., 1995; Lichtenberg-Fraté *et al*., 1996; Calero *et al*., 2000]. The most remarkable characteristic of the Trk1p of *S. cerevisiae* (ScTrk1p) is the variability of its K^+ uptake affinity, according to the K^+ status of the cell. In K^+-starved cells, the K^+ transport has high affinity. This allows a strong discrimination between K^+ and Na^+ [Ramos *et al*., 1985], and a moderate capacity to discriminate between K^+ and Rb^+ [Rodríguez-Navarro and Ramos, 1984]. The discriminatory capacity between K^+, Rb^+ and Na^+ of the low affinity ScTrk1p is much lower [Rodríguez-Navarro and Ramos, 1984; Ramos *et al*., 1985]. Due to the complex kinetic responses of alkali cation transport in *S. cerevisiae* [Armstrong and Rothstein, 1964; Borst-Pauwels, 1981], it is possible that ScTrk1p can transport only K^+, or Na^+, K^+ and Na^+, or K^+ and H^+, depending on the medium conditions [Rodríguez-Navarro, 2000].

The HAK transporter of *S. occidentalis* (SoHakp) was characterized as showing high-affinity for K^+, no discrimination between K^+ and Rb^+, poor discrimination between K^+ and Cs^+, strong discrimination against Na^+ and Li^+ and a high concentrative capacity (10^7) that suggested it mediated "active " K^+ uptake [Bañuelos *et al*., 1995a]. Transporters with homology to the SoHakp transporter have been found in *D. hansenii* [reviewed in Rodríguez-Navarro, 2000] and in *N. crassa* [Haro *et al*., 1999]. Although HAK transporters are not fully characterized, they are, with basis on the high-affinity K^+-H^+ symport characterized in *N. crassa* [Rodríguez-Navarro *et al*., 1986], tentatively assumed to be K^+-H^+ symporters [Haro *et al*., 1999].

Potassium channels catalyse the permeation of K^+ ions across cellular membranes. So far, in contrast to plants [Rodríguez-Navarro, 2000], no genes encoding inward-rectifying K^+ channels in fungi have been identified. In fungi two different K^+ channels have been identified by patch-clamp assays: an outward-rectifying K^+ channel and an inward **N**on-**S**pecific **C**ation channel (NSC). The first is a plasma membrane channel and conducts K^+ outward at depolarising membrane potentials. This channel can also take up K^+ into cells under some conditions [Fairman *et al*., 1999]. This channel was first recorded in *S. cerevisiae* by Gustin and collaborators (1986) as *ScTOK1* (also known as *DUK1*, *YCK1* or *YORK*) [Ketchum *et al*., 1995; Zhou *et al*., 1995; Lesage *et al*., 1996]. Recently, an homologous of the *ScTOK1* gene has been cloned and characterized in *N. crassa* (*NcTOKA*) [Roberts, 2003]. In contrast to animal and plant cells, very little is known of ion function in fungal physiology. Although high-affinity K^+ uptake in *N. crassa* is via a K^+-H^+ symporter (NcHak1p) [Haro *et al*., 1999], the K^+ uptake via NcTokap is energetically less expensive allowing to acquire K^+ passively [Roberts, 2003]. Furthermore, NcHak1p is pH dependent, and thus NcTokap may be advantageous in suboptimal conditions for the symporter.

NSC1 is an inward calcium-blocked non-specific cation channel mediating the main component of the low-affinity K^+ uptake in *S. cerevisiae* [Bihler *et al*., 1998, 2002; Roberts *et al*., 1999]. NSC1 is inhibited by divalent cations and is Trk1,2p and Tok1p independent [Bihler *et al*., 1998, Roberts *et al*., 1999]. The gene(s) encoding for NSC1 were not yet identified.

Early research on K^+ transport in *S. cerevisiae* suggested that this participates in the control of the cellular pH and K^+ content, achieved by the steady state between influx and efflux [Rodríguez-Navarro, 2000]. K^+/H^+ antiporters and K^+-ATPases have been identified in fungi as mechanisms of K^+ efflux, but are not specific for K^+. All of them were isolated as Na^+ efflux systems able to use additionally potassium. For this reason they will be discussed in sodium transport section. Because K^+ availability and pH values vary in natural environments, free-living organisms cannot keep a permanent transmembrane ΔpH relying only in K^+/H^+ antiporters, as do mitochondria. Mitochondria

is the best example of the indispensability of K^+ efflux [Nicholls and Ferguson, 1997], which is mediated by an electroneutral K^+/H^+ antiporter [Garlid, 1996], whose existence was predicted by Mitchell 40 years ago [Mitchell, 1961]. Fungal cells are furnished with a K^+/H^+ antiporter [Camarasa *et al*., 1996; Bañuelos *et al*., 1998; Ramírez *et al*., 1998; Sychrová *et al*., 1999], but its function cannot be permanent, because of the variability of the environmental pH. Furthermore, this antiport is not specific for K^+ as will be further described. Nevertheless, the *NHA1* gene encodes an alkali cation/H^+ antiporter in *S. cerevisiae* [Prior *et al*., 1996], which works at acidic pHs to maintain the steady state of K^+ in the cells, through the K^+ efflux through the plasma membrane in high potassium environment [Bañuelos *et al*., 1998; Klincová *et al*., 2001b]. As a result, Nha1p may participate in the regulation of the internal pH, cell volume and osmotic pressure [Csonka, 1989; Csonka and Hansen, 1991; Sychrová *et al*., 1999]. Recently, Benito and collaborators (2002) showed that *S. pombe CTA3* gene, which were assigned earlier to be a Ca^{2+}-ATPase is, in fact, a genuine K^+-efflux ATPase. In the same study, it was concluded that fungal K^+- or Na^+-ATPases evolved from an ancestral K^+-ATPase, through processes of gene duplication [Benito *et al*., 2002]. In hemiascomycetes, this type of molecular evolution associated process seems to be involved in the generation of bifunctional ATPases, while in *Neurospora*, and eventually in other euascomycetes, they have been suggested to potenciate the origin of specialized ATPases [Benito *et al*., 2002].

5.1.4.3. *Sodium*

There is no specific Na^+ uptake systems described in literature, being the members of the HKT family of potassium transporters, which exhibit different degrees of Na^+ transport activity [Rubio *et al.,* 1999; Rodríguez-Navarro, 2000], and NSC channels, the main candidates to mediate Na^+ uptake, namely when cells are exposed to salt stress. Additionally it has been suggested that the product of the gene *PHO89* [Martínez and Persson, 19998], a high affinity Na^+-Pi symport, might also mediate Na^+ uptake. However, as presented above in section 5.1.1, the characteristics of this transporter do not support that suggestion. Due to the toxicity of Na^+ at high concentrations, cells must have active extrusion systems for Na^+ detoxification. Two different types of transport systems mediating active sodium efflux have been characterized in fungi plasma membranes: Na^+/H^+ antiporters and Na^+-ATPases.

Na^+/H^+ antiporters are membrane proteins that play a major role in the regulation of intracellular pH and cell cation homeostasis throughout the biological kingdom, from bacteria to humans and higher plants [Padan *et al*., 2001; Sychrová, 2004]. So far, genes encoding specific plasma membrane Na^+/H^+ antiporters have been identified and characterized in have been characterized in yeasts: *NHA1* in *S. cerevisiae* (**N**a^+/**H**$^+$ antiporter) [Prior *et al*., 1996], *sod2* in *S. pombe* [Jia *et al*., 1992], *ZSOD2, ZSOD22* [Watanabe *et al*., 1995; Iwaki *et al*., 1998] and *ZrSOD2* [Kinclová *et al*., 2002] in *Z. rouxii*, *CNH1* in *C. albicans* [Soong *et al*., 2000; Kinclová *et al*., 2001a], and *PsNHA1,2* in *P. sorbitophila* [Bañuelos *et al*., 2002]. Na^+/H^+ antiporters are membrane proteins that may mediate the efflux not only of Na^+ and Li^+, but also K^+ and Rb^+ [Bañuelos *et al*., 1998; Kinclová *et al*., 2001a,b]. In spite of their high sequence homology, the family of yeast Na^+/H^+ antiporters can be divided, in what regards substrate specificity and probably cell function, into two distinct subfamilies: (i) the subfamily with substrate specificity only for Na^+ and Li^+ (*S. pombe* and *Z. rouxii* antiporters) and with a primary detoxification function in cells and (ii) the subfamily (*S. cerevisiae*, *P. sorbitophila* and *C. albicans* antiporters) mediating transport of all alkali metal cations that, besides elimination of toxic cations, probably has a role in other cell functions such as regulation of intracellular K^+ concentration, pH and cell volume [Kinclová *et*

al., 2002; Bañuelos *et al*., 2002]. The cation-transport activity of Na^+/H^+ antiporters is governed by the electrochemical gradient of protons across the plasma membrane [Niu *et al*., 1995; Rodríguez-Navarro *et al*., 1994], and thus cation efflux is higher at acidic external pH values.

Yeast cells accumulate metabolically important compounds and ions in the acidic organelle vacuole. The acidic pH is required for organelle functions and is mediated by a proton pump, vacuolar H^+-ATPase (V-ATPase), and could be regulated by ion transport systems [Hirata *et al*., 2002]. In fact, V-ATPase was found to be required specifically for NaCl tolerance in *S. cerevisiae* [Hamilton *et al*., 2002], which highlights the importance of the vacuole in salt resistance. Besides the Na^+/H^+ antiporters identified in the plasma membrane, it has been also reported in *S. cerevisiae* the existence of a low-affinity electroneutral Na^+/H^+ antiporter in the prevacuolar compartment [Nass and Rao, 1998; Darley *et al*., 2000], encoded by the Sc*NHX1* gene [Nass *et al*., 1997], which mediates the sequestration of Na^+ within the intracellular compartment. The combination of the ScNHX1 and a sulphate transport was suggested more recently to establish the electrochemical proton gradient and the acidic pH in vacuoles [Hirata *et al*., 2002].

In fungi, a P-type ATPase, which extrudes Na^+ probably in exchange for H^+, was initially characterized and their genes cloned in the following yeasts: *ENA1-4* (***E****fflux of* ***Na****trium*) in *S. cerevisiae* [Haro *et al*., 1991], *DhENA1,2* in *D. hansenii* [Almagro *et al*., 2001], *SoENA1,2* in *S. occidentalis* [Bañuelos and Rodríguez-Navarro, 1998], *ZrENA1* in *Z. rouxii* [Watanabe *et al*., 1999] and in *N. crassa* [Benito *et al*., 2000]. More recently, Benito and collaborators (2002) after a search in several databases using the *ScENA1* sequence, found that *ENA* genes exist in many different fungi, besides those reported before. These were: *C. albicans, Pichia angusta, S. exiguous, K. lactis, Kluyveromyces thermotolerans, C. tropicalis, Y. lipolytica, S. bayanus, Fusarium solani, Gigaspora rosea, A. nidulans* and *Pleurotus ostreatus* [Benito *et al*., 2002]. The same authors also found that *ENA*-ATPases are complex K^+ or Na^+-ATPases, *i.e*., some *ENA*-ATPases may keep mixed activity as K^+- and Na^+-ATPases, whereas other may be Na^+ or K^+-ATPases. In fact it was demonstrated that some *ENA*-like ATPases very important for Na^+ tolerance also exhibited a significant affinity for K^+ ions, suggesting that in these cases Na^+ extrusion might bring about an unnecessary K^+ efflux, at least under a certain threshold of intracellular sodium concentration [Benito *et al*., 2002]. *S. cerevisiae* genes are among all the genes cloned the best studied. *ScENA1* seems to be the most important gene of the four cloned in terms of Na^+ extrusion and its expression is induced at high pH in the presence of Na^+ or Li^+ [Garciadeblás *et al*., 1993], by osmotic shock, by glucose limitation and by regulation of the activity of the proteic phosphatases PPZ [Posas *et al*., 1995; Yenush *et al*., 2002].

5.1.4.4. *Chloride*

Chloride channels are widely distributed in prokaryotic and eukaryotic cells and belong to several families [Flis *et al.,* 2002]. They play a key role in major functions of the cell and tissues such as regulation of cell volume, cell excitability and acidification of intracellular organelles [Jentsch *et al*., 1999, 2002]. Conway and Downey (1950) concluded that the plasma membrane of *S. cerevisiae* is virtually impermeable to chloride and the potent inhibition of yeast transcription *in vitro* by chloride provides indirect evidence that internal chloride should be absent [Lue and Kornberg, 1987]. However, the existence of low affinity chloride uptake into isolated yeast vacuoles was reported [Wada *et al*., 1992; Hirata *et al*., 2002]. Furthermore, two putative chloride channels in yeast are encoded in the *S. cerevisiae* genome. One gene is *GEF1* [Huang *et al*., 1994], whose product is localized to the Golgi apparatus

[Schwappach *et al*., 1998] and is homologous to the CLC chloride channels [Jentsch and Gunther, 1997]. As to the other open reading frame, to date no functional studies were undertaken. *S. cerevisiae* do not uptake significantly chloride [Coury *et al*., 1999], being attributed to the Gef1 protein two roles: its participation in the high-affinity iron transport system and its function in cation detoxification [Gaxiola *et al*., 1998]. Gef1 was suggested to be required as an anion channel to provide the couterbalance charge that will permit cation compartmentalization into organelles or vesicles with acidic interiors [Gaxiola *et al*., 1998; Flis *et al*., 2002].

5.1.5. *Ion homeostasis strategies for salt stress tolerance*

To maintain an optimum cytoplasmic concentration of K^+ and a stable high intracellular K^+/Na^+ ratio, cells employ three distinct strategies: (i) strict discrimination among alkali metal cations at the level of influx (higher affinity of transporters for potassium than for sodium), (ii) efficient efflux of toxic cations from cells, and (iii) selective sequestration (compartmentation) of cations in organelles [Latterich and Watson, 1991; Niu *et al*., 1995; Haro *et al*., 1993; Mager and Varela, 1993; Nass *et al*., 1997; Rodríguez-Navarro, 2000; Kinclová *et al*., 2002; Sychrová, 2004]. Considering the fact that no specific sodium uptake systems have been reported, sodium-potassium selectivity at influx level and increased K^+ uptake, are assigned as important factors in salt tolerance, specially in plants, since it avoids sodium entry and allows a high internal K^+ concentration, and consequently a high K^+/Na^+ ratio compatible with the functioning of intracellular systems, namely ribosomal protein synthesis [Serrano, 1996; Schachtman and Liu, 1999]. In fact, it is known that intracellular Na^+/K^+ ratios above 0.5 are toxic to yeast cells [Camacho *et al*., 1981; Gaxiola *et al*., 1992]. For instance, it has been reported that *S. cerevisiae* increased this ratio up to a value of 1.1 during growth in the presence of 1M NaCl, while *C. tropicalis* maintained the same ratio below 0.5 during growth at salt concentrations ranging from 0 to 1M NaCl [García *et al*., 1997]. These ratios correlated with a higher resistance to salt in *C. tropicalis* than *S. cerevisiae* [García *et al*., 1997]. Several authors have determined the intracellular concentrations of Na^+ and K^+ in the halotolerant yeast *D. hansenii*, but the values differ to some extent [Larsson *et al*., 1990, Neves *et al*., 1997; Prista *et al*., 1997; Thomé-Ortiz *et al*., 1998; González-Hernández *et al*., 2004], which may have their explanation in the methodologies used for cation and intracellular volume measurements. *D. hansenii* was compared with *S. cerevisiae* and showed to have stronger sodium extrusion mechanisms and to be less inhibited by high external salt concentrations [Norkrans and Kylin, 1969]. Additionally, the results obtained for intracellular cation concentrations also suggested that *D. hansenii* was able to cope with higher intracellular cation concentrations (higher Na^+/K^+ ratios) than other yeasts [Prista *et al*., 1997] and thus might have an intrinsic resistance to the toxic effects of sodium not observed, particularly, in *S. cerevisiae* [González-Hernández *et al*., 2004]. For this reason *D. hansenii* was classified as an *includer* and *S. cerevisiae* as an *excluder* [Ramos, 1999].

When yeast cells are exposed to salt stress, Na^+ enters the cell as a low-affinity substrate through several cation-transporting systems, and cells must cope with the increased cytoplasmic concentration of toxic cation. In *S. cerevisiae,* the three mechanisms described above function cooperatively to prevent sodium accumulation. The most efficient sodium-eliminating system operating in *S. cerevisiae* is a Na^+-ATPase, (*ENA1-4*) [Wieland *et al*., 1995], while the Na^+/H^+ antiporter (*NHA1*) plays a minor role in salt tolerance of this yeast [Bañuelos *et al*., 1998]. On the other hand, tolerance to high external concentrations of Na^+ and Li^+, in *S. pombe* and *Z. rouxii*, relies essentially in Na^+/H^+ antiporters [Jia *et al*., 1992; Watanabe *et al*., 1999]. Although yeasts are being reported to possess

different mechanisms driving K^+ and Na^+ in and out of the cell, the important differences in Na^+ tolerance among them cannot be explained exclusively on the basis of differences in ion fluxes [Ramos, 1999].

Presently a considerable amount of knowledge has been accumulated on the molecular regulation of these genes by signal transduction pathways. At least three are known to be involved: the HOG pathway [Márquez and Serrano, 1996; Gustin *et al*., 1998; Posas *et al*., 1998, 2000; Rep *et al*., 2000], the calcineurin pathway [Mendoza *et al*., 1994; Hirata *et al*., 1995; Wieland *et al*., 1995; Mendoza *et al*., 1996] and the Ca^{2+}-calmodulin-calcineurin independent pathway [Wieland *et al*., 1995] addressed in Chapter 1. Nevertheless, and in spite of all this knowledge, ion fluxes regulation is a rather complex matter, which cannot be fully understood if regarded exclusively from the molecular point of view.

5.2. Results and discussion

5.2.1. *Internal Na^+ and K^+ concentration during salt stress in* Candida halophila

According to results from the literature [Barnett *et al*., 2000; van der Sluis *et al*., 2001] and from the previous chapters *C. halophila* appears to be an yeast able to maintain a very stable ion homeostasis, even in the presence of high ionic strength. For this reason, we decided to measure intracellular Na^+ and K^+ ions in *C. halophila* cells. In view of the discrepancies observed in the literature mentioned concerning the intracellular concentrations of ions (Section 5.1.5), we decided to use three experimental approaches: AAS, ICP and NMR (Material and Methods). We present results from AAS, since no significant differences were observed between results obtained using the other technological approaches. Intracellular Na^+ and K^+ concentrations were firstly determined in *C. halophila* cells growing in MM supplemented with three different carbon sources (Table 5.1). As can be seen in table 5.1 internal ion content does not vary significantly with the different carbon sources tested. MM concentrations of K^+ and Na^+ were respectively ± 500mM and ± 7mM. This corresponds to an in/out ratio below 1 and very similar for both ions. Since potassium uptake requires active metabolism, either respiratory or fermentative [Walker, 1998], it is expected that exponentially growing cells present equal $[K^+]_{in}$ regardless to the carbon source.

Table 5.1. Intracellular sodium and potassium concentrations in *C. halophila* cells cultivated on MM with 2% (w/v) of each carbon source and collected in mid-exponential growth phase. Means are the result of at least 4 independent assays.

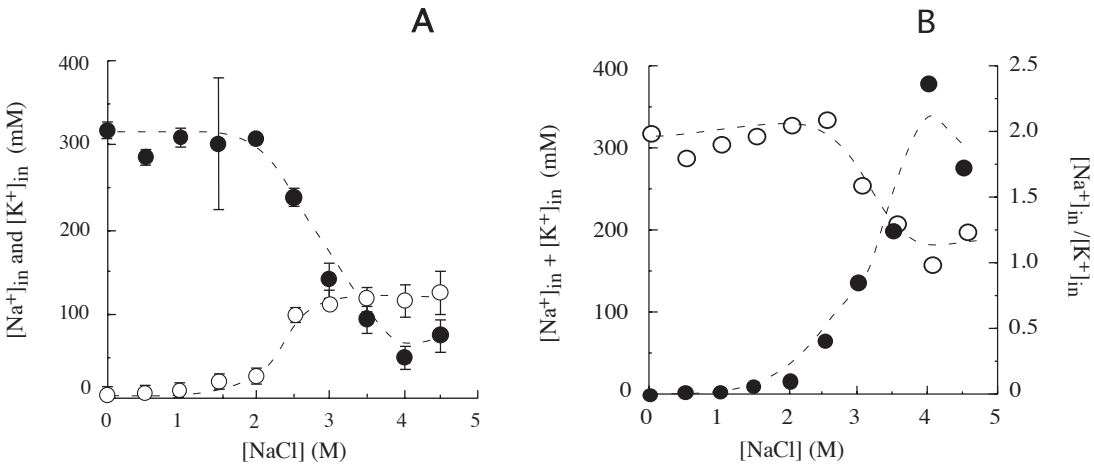
Carbon source	$[Na^+]_{in}$ (mM)	$[K^+]_{in}$ (mM)
Glucose	4.05 ± 2.23	317.73 ± 1.76
Glycerol	2.46 ± 2.16	280.68 ± 3.52
Mannitol	4.29 ± 2.88	251.30 ± 2.42

C. halophila cells were cultivated in MM supplemented with increasing NaCl concentrations up to 4M, with glucose as carbon source and its intracellular content of Na^+ and K^+ were measured

(Figure 5.2). As can be seen in Figure 5.2, potassium concentration maintained approximately constant, around 300 mM, up to ± 2 M NaCl. This potassium internal concentration has been frequently referred as normal in other yeasts in the absence of salt [Neves *et al*., 1997; García *et al*., 1997; Prista *et al*., 1997; Thomé-Ortiz *et al*., 1998].

In the same cells, sodium concentrations were also maintained constant, but surprisingly low, around 30 mM. Above 2 M NaCl, a turning point, sodium increased to another level (± 150 mM), while potassium decreased progressively reaching a minimum of ± 50 mM at the highest salt concentrations. Considering the total amount of the two ions, sodium plus potassium (Figure 5.2-B), these changes vary from ± 300 to ± 200 mM, from cells grown in the absence of salt to cells cultivated at 4.5 M: a decrease of 1/3 in total ion amount. Accordingly, Na^+/K^+ ratios above 0.5, *i.e.*, the value reported in literature as toxic for cells [Camacho *et al*., 1981; Gaxiola *et al*., 1992] were only verified for external salt concentrations higher than 3M.

Figure 5.2. A- Intracellular sodium (○) and potassium (●) concentrations in *C. halophila* cells cultivated on MM with 2% glucose (w/v) supplemented salt and collected at mid-exponential growth phase. **B**- Na^+/K^+ (●) ratios and total intracellular cation concentration ($Na^+ + K^+$) (○).



The decrease observed in intracellular potassium concentrations closely accompanied specific growth rate at the same conditions (Figure 5.3), reason why we can suppose that the maintenance of high potassium intracellular levels, and consequently lower Na^+/K^+ ratios, is an indispensable requirement for growth at high salt concentrations as postulated before for other yeasts [Camacho *et al*., 1981]. *D. hansenii* might represent a slightly different case since apparently and though published results vary according to each author [Prista *et al*., 1997; Neves *et al*., 1997], its internal Na^+ and K^+ concentrations under salt stress is higher than in *S. cerevisiae* [García *et al*., 1997], in *C. tropicalis* [García *et al*., 1997] and presently in *C. halophila*. Furthermore, *D. hansenii* is able to grow in the absence of K^+ in the media, by substituting K^+ by Na^+ [Prista *et al*., 1997].

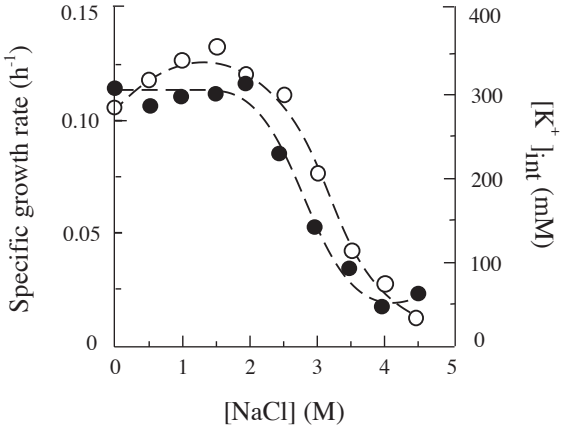


Figure 5.3. Variation of specific growth rate (○) and intracellular potassium concentration (●) with salt in the growth medium of *C. halophila*.

Substituting potassium by sodium in growth medium of *C. halophila*, using H_3PO_4 instead of KH_2PO_4 in MM composition, as described by Prista and collaborators (1997), and adding 0.5 % NaCl, growth was impaired (not shown). Following the classification introduced by Ramos (2000) we may consider *C. halophila* as an efficient *excluder*.

We compared the above results concerning intracellular ion concentrations obtained in cells cultivated under salt-stress with cells cultivated without salt and submitted to a sudden shock using 1, 2 and 3 M NaCl (Figure 5.4). During the time period of the experiment, 5 hours, an increase of $[K^+]^{in}$ was observed, which, at 3M NaCl, was only transient. In the same period of time, $[Na^+]^{in}$ also increased transiently, although at 3M NaCl, the posterior decrease was much slower than at 2 or 1M. On the other hand, the highest amount of sodium ions achieved during this transient increase, was higher for higher salt concentrations: ± 18 mM Na^+ at 1 M NaCl; ± 150 mM Na^+ at 2 M and ± 250 mM Na^+ at 3 M. This increase followed a linear variation with extracellular salt concentration (correlation 0.997). Furthermore, this transient maximum value was achieved approximately between 1 to 2 hours incubation time, after which it decreased. Some equilibrium has to, eventually, take place further ahead. But the recovery from shock, in order to attain values identical to the ones obtained in cells adapted to stress during growth, must take a longer period of time. Although this does not resume the extent of lag phases mentioned previously in Chapter 2, it most probably contributes to their length. This is a situation rather similar to the one described for intracellular volume adjustment (Chapter 3).

This behavior has been already reported for *C. tropicalis* and *S. cerevisiae*, that when shocked with 1M NaCl exhibited both a marked decrease in K^+ internal content during a 2 hour incubation period (almost 50%) and an increase in internal Na^+ concentration [García *et al*., 1997]. The difference between these two yeasts was that *S. cerevisiae* did not recover its internal normal values of Na^+ and K^+ when adapted to grow in the presence of 1M, while *C. tropicalis* was able to recover the normal K^+ content present in cells without stress, and to extrude the Na^+ accumulated in the meanwhile. It was also observed a similar marked decrease in $[K^+]_{in}$ and an increase in $[Na^+]_{in}$ *D. hansenii*, but this reaction happened both in shocked and adapted cells at salt concentration up to 1M NaCl [González-Hernández *et al*., 2004]. Whether this behaviour maintains for higher salt concentrations remains to be experimented not only in *D. hansenii*, but also in other halotolerant yeasts.

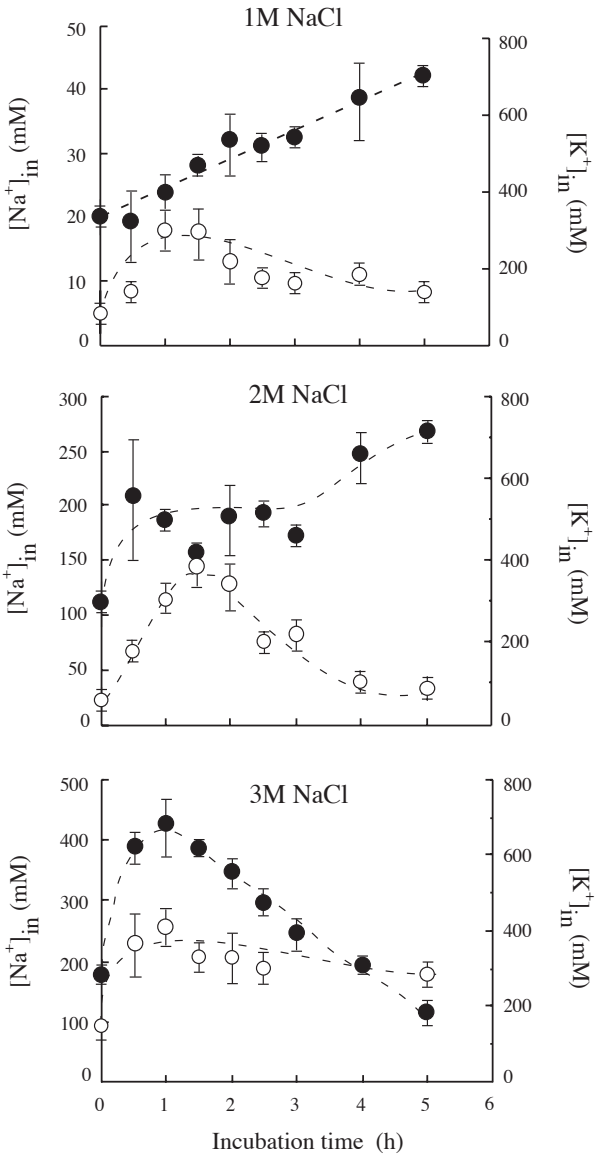


Figure 5.4. Variation of intracellular sodium (○) and potassium (●) concentrations in *C. halophila* cells cultivated on MM with 2% glucose (w/v), collected at mid-exponential growth phase and shocked with 1, 2 and 3 M NaCl during 5 hours of incubation period.

Na^+ intracellular concentration determined in *C. halophila* cells under salt stress corroborate the results obtained before in Chapter 4 for enzyme activity sensitivity to salt.

5.2. Concluding remarks

As main comments to the results in this chapter, we stress the following:

- *C. halophila* cells growing under salt stress were able to maintain intracellular high K^+ concentrations and low Na^+ concentrations for external salt concentrations up to 2M.

Above this concentration internal K^+ concentration decreased steeply while Na^+ increased and stabilized for external salt up to 4.5M.

- K^+ intracellular concentration decrease with increasing external salt concentration accompany the behavior of growth rate in the same conditions, suggesting that potassium intracellular concentrations may be a requirement for growth at high salt concentrations.

- *C. halophila* cells submitted to salt shock of different degrees, showed transient fluctuation in Na^+ and K^+ internal concentrations, suggesting that recover of steady-state internal ion concentration is longer according to the severity of the salt pulse.

- According to the internal ion response of *C. halophila* we may postulate that this yeast is an efficient *excluder* and thus must have very efficient transporters to extrude Na^+. In addition it might also possess efficient transporters for K^+ uptake and/or good discrimination between the uptake of both ions.