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Review

Molecular physiology of Na^+/H^+ antiporters, key transporters in circulation of Na^+ and H^+ in cells

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Contents

1. Introduction

Na^+/H^+ antiporters are ubiquitous membrane proteins found throughout the eukaryotic (both animal and plant) and prokaryotic kingdoms. They exchange Na^+ for H^+ across both cytoplasmic and organellar membranes. They therefore link the electrochemical potentials of sodium ($\Delta\tilde{\mu}_{Na^+}$) and hydrogen ($\Delta\tilde{\mu}_{H^+}$) ions, maintained across many of these biological membranes.

The existence of cation/H^+ antiporters was first postulated by Mitchell [1] and demonstrated in mitochondria by Mitchell and Moyle [2,3]. In bacteria, antiporter activity was first reported in *Streptococcus faecalis* [4]. In the cytoplasmic membrane of higher eukaryotes the Na^+/H^+ antiport was first demonstrated to function in vesicles from the brush borders of rabbit kidney and small intestine by Mürer et al. [5].

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Since then, the existence of this transporter has been described in many animal and plant cells.

A transmembrane ion cycle consists of two limbs: the first creates an electrochemical potential of a particular ion, driving the cycle; the second is a reaction(s) which recycles the ion back down its electrochemical

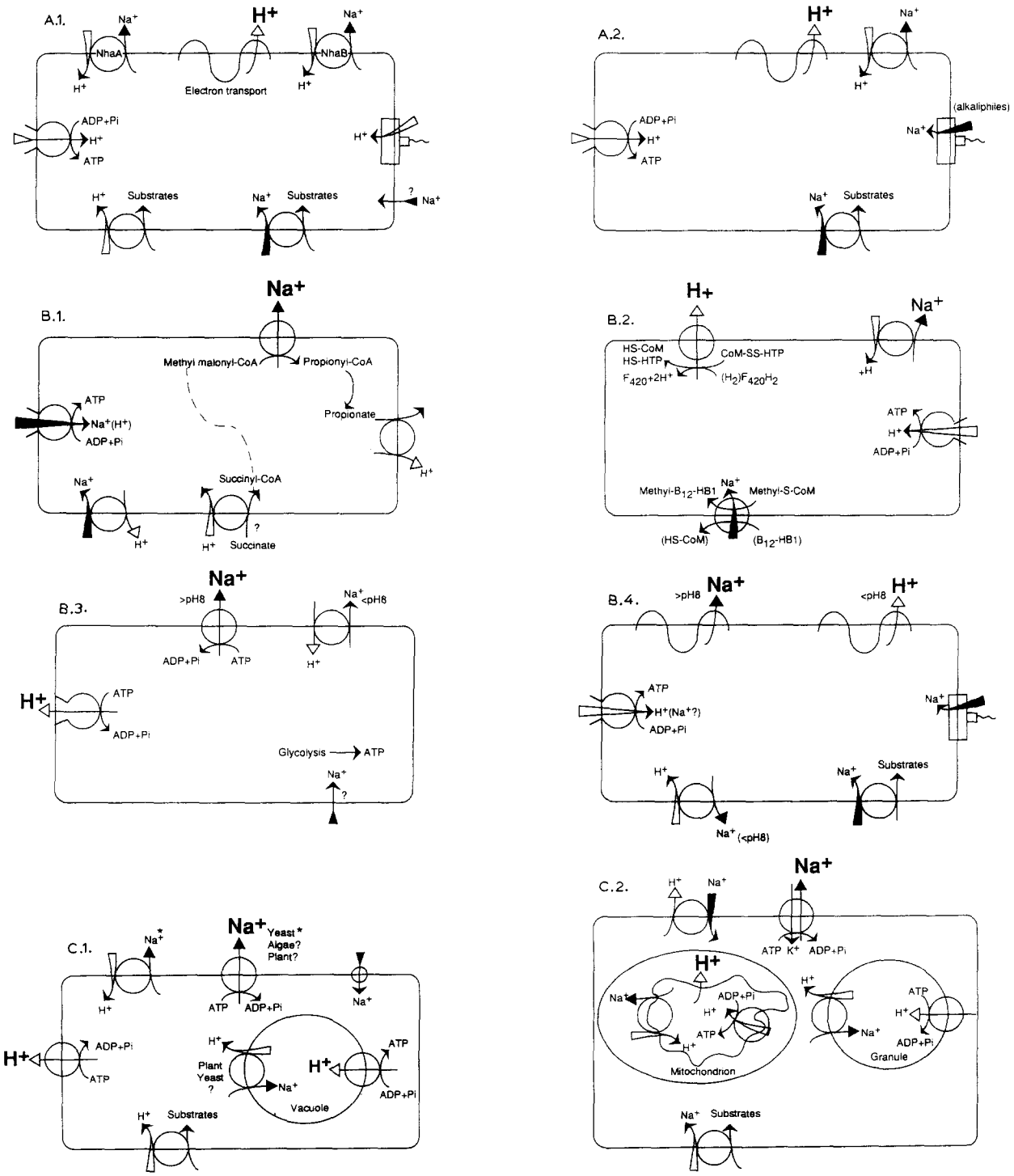

Fig. 1. Various patterns of H^+ and Na^+ cycles in cells. The main ion pump energizing the membrane is indicated by bold-face type. Uphill movement is depicted by ⍙ in the case of H^+ and ▲ in the case of Na^+, whereas downhill movements by ⍙ and ▲, respectively. * The conditions of operation and therefore the direction of ion fluxes via the antiporter are not yet clearly established. (A) Bacteria having secondard Na^+ cycles. (1) *E. coli* and similar neutrophilic bacteria. (2) Bacteria growing in extreme concentrations of Na^+ or H^+; extreme halophilic or alkaliphilic bacteria. (B) Bacteria having primary Na^+ cycles. (1) *Propionigenum modestum*-like. (2) *Methanosarcina barkeri*-like. (3) *Enterococcus hirae*-like. (4) *Vibrio alginolyticus*-like. (C) Eukaryotes. (1) Plant cells, algae, yeasts. (2) Animal cell.

gradient to complete the cycle. This cycle has been termed primary or secondary; the former when primary pumps maintain the driving force for the cycle, the latter when secondary transporters do so [6]. A Na^+/H^+ antiporter has the potential to maintain either a secondary Na^+ cycle, or a secondary proton cycle: in the former case the antiporters extrude Na^+ and recycle protons back to the cytoplasm; in the latter, they extrude protons and recycle sodium ions.

Thus, these universal devices which couple the cycles of the two most common ions in biology are most intriguing with respect to their biological roles, regulation and molecular mechanism. These properties of the Na^+/H^+ antiporters will be described in this review through a comparison of the antiporters in various cell types having different patterns of H^+ and Na^+ circulations. This is subsequent to the comprehensive reviews on the Na^+/H^+ antiporters of *Escherichia coli* which have recently been published [6–8].

2. Na^+/H^+ antiporters in bacteria having secondary Na^+ cycles

2.1. The E. coli paradigm of H^+ and Na^+ circulation

In most bacteria, including *E. coli*, [6] a primary H^+ cycle exists across the cytoplasmic membrane and the ensuing $\Delta\tilde{\mu}_{H^+}$ is the primary energetic currency (Fig. 1A.1). The proton cycle is initiated by a primary H^+ pump linked either to an electron transport, photochemical reaction, or ATP hydrolysis. In contrast, the Na^+ cycle is secondary; it is maintained by Na^+/H^+ antiporters, utilizing the preformed $\Delta\tilde{\mu}_{H^+}$ to drive Na^+ circulation.

The kinetics and bioenergetics of the Na^+/H^+ antiporters in *E. coli*, both in intact cells and isolated membrane vesicles have been most extensively studied and reviewed [7]. Although conditions for the solubilization and functional reconstitution of the antiporter activity have been advanced, attempts to identify and purify an antiporter protein with a straightforward biochemical approach were unsuccessful [9,10]. This was most probably due to the very low proportion of the antiporter proteins (0.1–0.2%) in the membranes [11]. The lack of molecular data regarding the antiporter activity has complicated any mechanistic interpretation since the measurements of the Na^+/H^+ exchange properties (electrogenicity, stoichiometry, pH dependence) in a membrane represent the overall membrane activity, without any clue as to how many individual processes contribute to the apparent activity. It was also impossible to directly test a model predicting the in vivo operation of two antiporters with different stoichiometries [12].

A genetic approach to study the Na^+/H^+ antiporter system in *E. coli* has been undertaken in several laboratories. Various mutants which have lost the Na^+/H^+ antiporter activity as well as the capacity to grow at alkaline pH have been isolated, implying the role of the antiporter in pH homeostasis [13–15]. Since the Na^+/H^+ antiporter excretes Na^+ and maintains an inwardly directed Na^+ gradient, the selections were based on the inability of the mutants to grow on carbon sources that require a sodium gradient for their uptake: a mutation in locus *phs* was obtained based on an inability to grow on both glutamate and melibiose [13]; Hit-1 could not grow on serine [14] and HS3051 was isolated as a strain that could not grow on melibiose in the presence of 100 mM NaCl [15]. Mapping of the *phs* mutation showed it to be an allele of *rpoA*, the gene encoding the α-subunit of the RNA polymerase [16–18]. This mutation causes a relatively selective transcription defect which affects several genes that seem to have a common regulatory mechanism [17]. Although the mutation lesion of Hit-1 has not yet been identified there are some indications that Hit-1 may carry more than one mutation [19,20].

It was the application of the molecular biology approach which revealed the multicomponent nature of the Na^+/H^+ antiporter activity of *E. coli*, and permitted the cloning of the genes, the isolation of the encoded proteins, and the study of their regulation and their individual mechanism of activity.

2.2. Developing the molecular biology tools for the study of the Na^+/H^+ antiporters in E. coli

The molecular biology of the Na^+/H^+ antiporters was initiated by the study of a mutant which led to a strategy for cloning of antiporter genes by functional complementation. This mutant, with increased rather than decreased antiporter activity, was isolated by Tsuchiya, Wilson and collaborators based on its resistance to Li^+ [21]. Li^+ ions are toxic to *E. coli* cells due mostly to their effect on the cell pyruvate kinase [22]. When grown on melibiose, which is symported to the cells with Na^+, the toxicity is augmented due to Li^+ inhibition of the melibiose transporter. An *E. coli* mutant that tolerates Li^+ concentrations, otherwise toxic to the wild-type cells, has been isolated [21]. This mutant harbors at least two mutations responsible for the acquirement of the resistance. The first is in the *melB* allele (*melBLiD*), in which the replacement of proline at position 122 with serine brings about a modification in the melibiose transporter, so that it can now cotransport the sugar with both Li^+ and Na^+ [21,23]. The second mutation is in an additional locus, which causes an enhanced Na^+/H^+ antiporter activity capable of an increased excretion of the toxic ion

(which is also a substrate of the antiporter). We have already separated the two mutations and shown that the one which increases antiporter activity, *nha*Aup, (previously called *ant* up), is necessary to confer resistance to toxic levels of Li^+ ions [24]. The finding of this phenotype permitted the mapping of this mutation to about 0.3 min on the *E. coli* chromosome [24].

Taking advantage of the toxicity of Li^+ ions and the resistance associated with high activity of the antiporter, we cloned the wild-type *nha*A gene [24,25]. We assumed that when set in high copy number (plasmidic + chromosomal) the wild-type *nha*A would increase Na^+/H^+ antiporter activity and thereby confer Li^+ resistance to cells, i.e., an $NhaA^{up}$ phenotype. Scoring this phenotype in cells transformed with plasmids, containing inserts covering 15 kbp from *car* to *dnaJ* [26,27] that included the wild-type locus, yielded a plasmid bearing *nha*A, which confers Li^+ resistance and encodes an Na^+/H^+ antiporter (subsections 2.3 and 2.4).

To study the role of the antiporter and to assess whether *E. coli* has additional specific Na^+/H^+ antiporters, our strategy was to inactivate the chromosomal gene by replacing most, or all of it with a selectable marker [28]. The appropriate constructs were engineered in plasmids, taking care to include enough flanking sequences to allow for homologous recombination to take place. After selection and transduction into our isogenic strains, recombination in the desired locus was tested by southern hybridizations. We constructed *Δnha*A strains in which either two thirds [28] or the whole gene [29] was replaced with *kan* without disrupting any of the neighboring genes. The *Δnha*A strains obtained grow normally in low sodium medium, indicating that, at least under these conditions, *nha*A is not essential. *Δnha*A, on the other hand is markedly sensitive to Li^+ and Na^+ and its sensitivity to the latter ion increases with pH [28]. The *Δnha*A strain thus permitted the study of the involvement of *nha*A in the H^+ and Na^+ circulation of the cell (see below). Furthermore, although the level of the Na^+/H^+ antiporter activity in membrane vesicles isolated from *Δnha*A was reduced to 50% of the wild-type level, a detailed analysis of the remaining antiporter activity in the *Δnha*A strain revealed an additional Na^+/H^+ antiporter, designated NhaB. Its specific properties differ from those displayed by the NhaA protein: (I) The K_m for transport of Li^+ (0.86–1 mM) of NhaB is about 15-times higher, while the K_m for Na^+ (100 μM, pH 7.5) is 10-times lower than that of NhaA. (II) The activity of NhaB is practically independent of intracellular pH, while that of NhaA increases dramatically with increasing pH. Hence our results have demonstrated the presence of two different Na^+/H^+ antiporters in *E. coli*[28].

By cloning *nha*B [30] the generation of *Δnha*B and *Δnha*A*Δnha*B strains were made possible [19]. These mutants showed that there is no active additional specific Na^+/H^+ antiporters in *E. coli* and that the K^+/H^+ non-specific antiporter [31,32] is the remaining activity in *Δnha*A*Δnha*B.

Both *Δnha*A and *Δnha*A*Δnha*B are Na^+ and Li^+ sensitive. The sensitivity to Na^+ of the latter strain (> 10 mM) is even higher than that of the former (> 400 mM, pH 7). Therefore both strains provide us with a system for the cloning of genes, coding for other antiporters by functional complementation. Transformation of either of the deletion strains by multicopy plasmid carrying *nha*A, renders transformants resistant to the ions [19,28]. It was therefore anticipated that homologous, and even heterologous antiporter genes would be able to complement the deletion. Using this paradigm (with *Δnha*A), we succeeded in cloning three other genes: one derived from *Salmonella entiritidis* which is homologous to *nha*A [33], the *nha*B gene from *E. coli* [30] and in collaboration with the group of T.A. Krulwich, a novel gene from the alkaliphilic *Bacillus firmus* OF4, that codes for a putative antiporter [34]. For the cloning of *nha*B, it was essential to prepare the library from the *Δnha*A strain in order to prevent the recloning of *nha*A [30].

The higher Na^+ sensitivity of *Δnha*A*Δnha*B than that of *Δnha*A enables the application of even a wider range of selection pressures. Using this approach, we have recently cloned an *E. coli* gene which restores partial Na^+ resistance and membrane Na^+/H^+ antiporter activity to both *Δnha*A or *Δnha*A*Δnha*B strains [35]. The gene, designated *cha*A, maps at 27 min on the *E. coli* chromosome and predicts to encode an extremely hydrophobic protein with multiple membrane-spanning regions and a molecular weight of 39,200 daltons. A region in one of the predicted hydrophilic loops in the gene product structure possesses a strong sequence similarity to calsequestrin. The Ca^{2+}/H^+ antiporter activity of membranes from an *E. coli* transformant with this gene is enchanced and pH-independent. Mg^{2+} inhibits both the Na^+/H^+ and Ca^{2+}/H^+ antiporter activities conferred by the clone. We therefore propose that *cha*A is the structural gene for a non specific antiporter exchanging with H^+ both Ca^{2+} and Na^+.

The Na^+/H^+ antiporter deletion mutants are also most important for the study of structure function relationship of the antiporters. They enable the expression of various plasmidic mutated antiporter genes and the study of their phenotype without a background of the wild-type genes.

In summary, the antiporter genes and their respective deletion mutation has opened the way to the study of the properties, regulation, physiological role and even purificatiion and mechanistic studies of the antiporters (Sections 2.3–2.10).

2.3. Properties of nhaA and nhaB

The nucleotide sequences of the *nha*A and *nha*B genes have already been determined. They encode membrane proteins of molecular weight (M_r) 41,316 [11,25] and 55,543 [30] respectively. A hydropathic evaluation of the amino acid sequence of both proteins reveals the presence, respectively, of 11 and 12 putative transmembrane-spanning segments linked by hydrophilic segments of variable length. The proteins were specifically labeled using the T7 polymerase system [36], which was designed to label, in the intact cell, solely the product of genes cloned downstream of T7 promoter. In both cases, a protein which displays an apparent M_r in sodium dodecylsulfate polyacrylamide gel electrophoresis lower than that expected from the analysis of the sequence is labeled [33 and 45 kDa, respectively [11,30]]. This phenomenon has been described for many hydrophobic proteins. The label is associated with the membrane fraction even after undergoing washes with 5 M urea, a finding which corroborates that both proteins are integral membrane components. Both NhaA [11] and NhaB (Pinner E., Padan, E. and Schuldiner, S., unpublished results) have been purified in a functional state and shown to catalyse Na^+/H^+ exchange.

Sequencing of the genes allowed for an accurate mapping in the *E. coli* chromosome: *nha*A is located at 0.35 min distal to *dnaJ* and between two insertion sequences detected in various strains, IS186 and IS1; between IS186 and *nha*A there is *gef*, a member of a gene family encoding small toxic proteins of approximately 50 amino acids [37]; downstream of *nha*A, there is an additional open reading frame [27], which was recently found to be involved in regulation of expression of *nha*A and designated *nha*R [[38] and Section 2.10]; *rps*20 is located further downstream of IS1 [27]; *nha*B is located at 25.5 min between *fad*R and the *umuCD* operon [30]; downstream of *nha*B there is a small potential open reading frame, whose function is yet unknown [30].

A mutant, Hit1, which cannot grow on serine as a carbon source in a Na^+-dependent fashion was isolated [14]. Since this mutant showed impaired Na^+ extrusion capacity and a lack of growth at alkaline pH (with glycerol as a carbon source) it was concluded that the mutation affects the Na^+/H^+ antiporter activity. Recently [39], the mutation was mapped at 25.6 min on the *E. coli* chromosome suggesting that *hit*1 resides in *nha*B locus. However, we could not complement Hit1 by the cloned *nha*B for growth on serine at neutral pH or for growth on glycerol at alkaline pH. The phenotype of Hit1 also differs from *Δnha*B and suggests that the strain contains additional mutation(s) [19,20]. However, it is also possible that Hit1 is a dominant mutation of *nha*B, which cannot be repaired by multicopy *nha*B. A molecular characterization of the *hit*1 mutation is therefore required.

The sequences of the two antiporters NhaA and NhaB, show very little similarity. Their overall identity as determined with the algorithm of Smith and Waterman [40] is 20%.

2.4. Overproduction and purification of NhaA and NhaB

In order to prove that a certain gene codes for a protein that catalyzes a given activity, one must purify the protein, show that it is responsible for the function and that the amino-acid sequence of the isolated protein is, at least in part, identical with that deduced from the nucleotide sequence of the gene. With membrane proteins that only catalyze vectorial reactions, this is no simple task. There are at least three reasons for the scarcity of membrane proteins that have been purified in a functional state: (1) the biochemistry of membrane proteins is more difficult than that of soluble proteins and necessitates the choice of proper detergents to keep the protein in solution; (2) the techniques for reconstitution are not always simple and may be somewhat cumbersome and laborious for a quick assessment of purifications; and (3) the proteins of interest are not always abundant, so large amounts of material must be processed.

In the case of the Na^+/H^+ antiporter, successful reconstitution has been already reported [9,10]. In both reports, octyl glucoside was used as a detergent, the driving force was a ΔpH, generated either by the reconstituted respiratory chain [10] or by an ammonium gradient [9]. Since the transporter could be labeled specifically using the T7 polymerase system [25], we were able to follow its course rapidly and efficiently during the various purification steps [11]. Probably the most important aid in the purification process was the 200-fold overproduction of the antiporter. In order to overproduce the *nha*A gene product, a plasmid was constructed (pEP3T) in which the promoterless gene was cloned downstream of the strong inducible *tac* promoter. When cells carrying pEP3T are induced with isopropylthiogalactoside (IPTG) and the membrane proteins resolved by electrophoresis (SDA-PAGE) a diffuse protein band of about 33 kDa becomes evident after 30 min, and induction is maximal after 90 to 120 min. As expected from our estimation that under these conditions NhaA represents only 0.1–0.2% of the membrane protein, the band is undetectable before induction. However, by the end of the induction period it becomes the most abundant protein in the membrane. Essentially, all NhaA appears to be membrane-associated. Cell growth declines with kinetics similar to NhaA induction, demonstrating the detrimental effect of NhaA overproduction on growth. Overproduction of other membrane proteins, including transporters [41],

have similar effect on growth, which have been related to impairment of membrane functions. In dodecyl maltoside extracts of membranes prepared from *nha*A-induced cells, the antiporter is about 20% of the total protein, about 100–200-fold more abundant than in extracts from wild-type cells. When membranes produced from such induced cells are solubilized and reconstituted, the observed Na^+/H^+ antiport activity is about 100-fold higher than the activity measured in proteoliposomes prepared from membranes derived from wild-type cells. This implies that most, if not all, of the overproduced protein is catalytically active. An additional purification of only 4-fold on 2-diethylaminoethanol (DEAE) and hydroxyapatite columns is sufficient to yield a highly purified fraction [11].

When comparing the activity of the purified protein (600 nmol/min per mg protein) to the activity of reconstituted total membrane extract (120 nmol/min per mg protein), the 5-fold rise in specific activity is similar to the purification fold calculated from the ^{35}S labelling of the protein. Hence, the overall purification obtained, including the overproduction step, is about 400–500-fold.

When reconstituted into liposomes, this protein catalyzes practically all the modes of action that were previously documented in intact cells as well as in membrane preparations [11,42–48]. Thus, proteoliposomes reconstituted with NhaA, accumulate Na^+ ions against their concentration gradient upon imposition of a pH gradient generated by ammonium diffusion. This activity displays an apparent K_m of 110 μM (pH 8.6).

Another mode of catalysis which has been measured in the NhaA proteoliposomes is downhill transport of $^{22}Na^+$ in the presence or in the absence of imposed $\Delta\tilde{\mu}_{H^+}$. The most striking property of this reaction is that the antiporter is virtually shut down when the reconstituted proteoliposomes are loaded with potassium acetate and $^{22}Na^+$ at pH 6.5 and diluted into a medium of identical composition containing valinomycin and devoid of Na^+. The rate of efflux increases upon imposition of either a proton gradient or an electrical potential across the membrane. Thus, in proteoliposomes diluted into media containing choline acetate and valinomycin, the membrane potential, generated by the outwardly directed K^+ gradient, accelerates sodium efflux. Upon dilution of the proteoliposomes into media containing potassium gluconate, the proton gradient formed by the outwardly directed acetate gradient accelerates the efflux many fold [11,48].

The downhill movement of Na^+ ions is coupled to the movement of H^+ ions against their concentration gradient. This was tested in a series of experiments in which proteoliposomes, reconstituted with NhaA, were loaded with 10 mM NaCl and with the pH indicator pyranine. Upon dilution of the proteliposomes into a medium devoid of Na^+ a rapid acidification of the internal milieu is observed, as indicated by the changes in the fluorescence of the trapped pyranine. As expected, this acidification is prevented by and reversed, upon addition of ammonium salts or nigericin [11,48].

Using the functional purification method of NhaA, we have already obtained a highly enriched active preparation of NhaB (Pinner, E., Padan, E. and Schuldiner, S., unpublished results). The purification of the antiporters and their reconstitution in an active form in proteoliposomes enabled us to obtain data which otherwise could not have been obtained, especially in a cell that as we now know contains more than one antiporter. These include the H^+/Na^+ stoichiometry of the antiporters, their pH sensitivity, and their sensitivity to various inhibitors.

2.5. The H^+/Na^+ stoichiometry of the NhaA antiporter is 2

The issue of whether or not the antiporter is electrogenic has been a matter of controversy over the years. Although it was first suggested that the exchange is electroneutral [49], further evidence indicated an electrogenic exchange (H^+: $Na^+ > 1$) [43–47,50,51]. It was also proposed that below a certain pH_{out}, the antiporter is electroneutral, while above it, it is electrogenic [45]. This question was addresed in an extensive and careful study by Macnab and colleagues [12,46,47,50]. In these studies, steady-state values of $\Delta\tilde{\mu}_{H^+}$ and $\Delta\tilde{\mu}_{Na^+}$ were measured under various conditions in endogenously respiring *E. coli* using $^{23}Na^+$- and ^{31}P-NMR spectroscopy. Na^+ extrusion and maintenance of a low intracellular Na^+ concentration were found to correlate with the development and maintenance of $\Delta\tilde{\mu}_{H^+}$. At pH 6.7 a concentration ratio ($[Na^+]_{out}/[Na^+]_{in}$) of about 25 was observed; this was independent of extracellular Na^+ concentrations over the measured range of 4 to 285 mM, indicating that intracellular Na^+ concentration is not regulated. When measured at various pH values, it was found that in the acidic to neutral pH range the Na^+ chemical potential followed the proton chemical potential quite closely, always slightly exceeding. Above pH 7.4, there was a progressive divergence between the two values. Thus, whereas the ΔpH continued to decrease, reached zero at pH 7.5, and changed signs (pH_{in} becoming more acidic than pH_{out}), ΔpNa [$\Delta pNa = (-\log[Na^+]_{in}/[Na^+]_{out})$] practically leveled off at a value of 25 to 40 mV, corresponding to an Na^+ concentration gradient of 2.5- to 5-fold at the alkaline pH values. As a consequence, the apparent overall stoichiometry changes from 1.1 at $pH_{out} = 6.5$ to 1.4 at $pH_{out} = 8.5$ [50].

It was suggested that this change in apparent overall stoichiometry might reflect a change in the relative

rates of two antiporters with different stoichiometries, rather than a change in the stoichiometry of a single protein [12]. Our studies indeed show that there are two antiporters and that at least one of them, NhaA, is electrogenic. As described above, the downhill movement of ^{22}Na catalyzed by purified NhaA is stimulated by the imposition of a membrane potential (negative inside), even at pH values as low as 6.5 [11]. The downhill movement of Na^+ via NhaA generates a membrane potential, as suggested by the fact that in the absence of valinomycin, the efflux rate of $^{22}Na^+$ is severalfold lower than in its presence. The direct measurement of a membrane potential with Oxonol VI further supports the rehogenic nature of the antiporter [48].

Determination of the rates of H^+ and Na^+ fluxes permitted the calculation of the H^+/Na^+ stoichiometry of NhaA [48]. H^+ movements were followed by the pH indicator, pyranine, and Na^+ movements by monitoring either $^{22}Na^+$ or changes in fluorescence of a novel sodium indicator, SBFI (sodium-binding benzofuran isophtalate). Some of the problems which we faced in these experiments illustrated important properties of the antiporter, i.e., measurable movements of Na^+ could be detected only under conditions in which the formation of $\Delta\tilde{\mu}_{H^+}$ by the antiporter was prevented, or when preformed gradients were collapsed. Thus, proteoliposomes loaded with Na^+ lose only minute amounts of the ion, while a pH gradient is formed and reaches its maximal value as soon as it can be measured. The addition of 10 mM methylamine, transiently alkalinizes the internal milieu and enables the escape of some of the Na^+ ions. The addition of three identical aliquots of methylamine are necessary to release most of the remaining internal Na^+. If the generation of $\Delta\tilde{\mu}_{H^+}$ is prevented by performing the experiment in the presence of potassium acetate and valinomycin, half of the internal Na^+ is lost after about 60 s [48]. A stoichiometry of about 2 was estimated for the NhaA antiporter in these experiments.

A stoichiometry of $2H^+/Na^+$ was also estimated in experiments using a thermodynamic, rather than a kinetic approach. The size of the $\Delta\tilde{\mu}_{H^+}$ generated by an Na^+ gradient could be predicted from the equilibrium conditions ($\Delta\tilde{\mu}_{Na^+} = -n\Delta\tilde{\mu}^+_H$), which means that $\Delta pNa^+ = n\Delta pH + (n-1)\Delta\Psi$.

We measured the size of the $\Delta\Psi$ generated at various ΔpNa^+ with Oxonol VI, in the presence of nigericin, which allowed for an electroneutral exchange of K^+ and H^+ and thereby discharges the ΔpH. The magnitude of the $\Delta\Psi$ generated at various pH values (7.1 to 8.2) was consistent with a stoichiometry of 2 [48].

Our results suggest that the apparent changes in stoichiometry measured in the intact cell [46,47,50] and membrane vesicles [45] at alkaline pH are not due to a change in stoichiometry of NhaA, but rather, as was predicted [12], to its relative contribution to the Na^+ cycle. As yet, the H^+/Na^+ stoichiometry of NhaB is not known.

2.6. Sensitivity of the antiporter to inhibitors

Amiloride has been reported as an inhibitor of many antiporters (see Section 6). However, there is some controversy in the literature as to the effect of amiloride-like compounds on *E. coli* antiporter activity. Amiloride was claimed to inhibit Na^+-dependent changes in pH gradients in everted vesicles ($K_i = 40$ μM) [52]. These data were interpreted as an effect on the antiporter; however, results both in our laboratory and in others [53] indicated a potent uncoupler activity of amiloride. We were unable to detect any significant inhibition of downhill sodium efflux catalyzed by purified NhaA, with either amiloride, or several of its derivatives [11].

Of a long list of chemical modifiers tested (reviewed in [53]), the only one found to inhibit the antiporter was the histidyl reagent diethylpyrocarbonate (DEPC). It inhibits Na^+ efflux in a specific way, since hydroxylamine reverses the inactivation. DEPC also inhibits Na^+ efflux by purified NhaA [54].

2.7. The pH sensor in NhaA-structure and function relationship

The proposed role of the antiporter in pH_{in} regulation, i.e., the acidification of the cytoplasm at alkaline extracellular pH, implies that the activity should be dependent on, and/or regulated by, pH, so that the higher the pH the higher the activity [55]. Studies of Na^+ effects on lactose-dependent H^+ circulation have suggested that the Na^+/H^+ antiporter is more active at alkaline than at neutral pH [56]. Studies in right-side-out membrane vesicles [43,44] have shown that the activity of the antiporter is extremely dependent on pH_{in}. In these studies, right-side-out membrane vesicles were loaded with $^{22}Na^+$ at various pH values and the rate of the downhill efflux (V_{Na+}) was monitored. The authors found that imposition of $\Delta\tilde{\mu}_{H^+}$ stimulated Na^+ efflux at all external pH values between 5.5 and 7.5. The contributions of the electrical ($\Delta\Psi$) and chemical (ΔpH) potential to the acceleration mechanism were studied by their selective dissipation with valinomycin and nigericin in respiring vesicles, and by the imposition of artificial gradients. $\Delta\Psi$ even stimulated the efflux at acid pH (5.5), provided the internal pH was increased by the imposition of a pH gradient, suggesting that at this pH the antiporter is also electrogenic. The effect of ΔpH on V_{Na+} is dependent on the external pH value; at low pH the relation is nonlinear and indicates the existence of an apparent threshold.

This threshold progressively decreases as the pH rises and at pH 7 and above it disappears completely. These variations in behavior can be accounted for by variations in pH_{in}. The authors proposed that the high internal H^+ concentration inhibits Na^+ efflux by competition, as suggested by the change in the apparent K_m for Na^+ from 40 mM at pH_{in} 6.8 to 3.5 mM at pH_{in} 7.7. These important conclusions were complicated somewhat by several factors which could not be controlled at the time: (1) 'nonstimulated' passive leaks also increased with pH and, above pH 7.5, were too high to ignore or to correct for; (2) the existence of more than one transport system for Na^+ was then unknown, therefore, the relative contributions of each system could not be analyzed; and (3) the changes in pH_{in} always necessitated changes in ΔpH as well, and therefore the two factors could not be isolated from one another. The first two problems do not exist in proteoliposomes reconstituted with pure NhaA.

As described above, when the efflux of $^{22}Na^+$ from proteoliposomes is monitored, it is stimulated upon imposition of ΔpH, a stimulation which can partly be ascribed to the change in pH_{in}. The efflux has also been measured under conditions where the only driving force is the gradient of Na^+ generated upon dilution. In these experiments, the generation of $\Delta\Psi$ or ΔpH by the action of the antiporter is prevented by the presence of valinomycin, high concentrations of K^+, inside and outside the proteliposomes, and the penetrating weak acid acetate which rapidly short-circuits any pH gradient generated. Under these conditions, we have measured a stimulation of up to 2000-fold in the efflux rate from proteoliposomes reconstituted with NhaA upon the increase of the pH from 6.5 to 8.5 [11]. This stimulation of the purified NhaA is solely a kinetic effect since no H^+ ion gradient is generated under the conditions tested. Moreover, the stimulation reflects a direct effect solely on NhaA, without background activity of the other Na^+/H^+ antiporter(s) (NhaB). In the experiments described above, both internal and external pH were modified; we do not yet know whether a change in the *cis* side alone is sufficient to bring about the stimulation. We also must still determine how the kinetic parameters are affected by pH. The downhill efflux rates are very high, increasing up to 2.2 mmol/min/mg at pH 8.5 (Na^+_{in} = 15 mM), a value which corresponds to a turnover number of $10^3\,s^{-1}$. This turnover number is one of the highest reported thus far for an ion-coupled transport system, and is only 10-times lower than the turnover number of the erythrocyte anion exchanger [57].

We suggest that the steep pH dependence of NhaA defines a 'set point' for the activity, such that NhaA is practically inactive at pH values below the intracellular homeostatic one [7.6 to 7.8 suggested by Padan et al. [55]]. When the pH increases, the antiporter is activated so that it can acidify the cytoplasm back to the 'resting pH_{in}' in a self-regulated mechanism. This self-controlled mechanism based on a pH meter and titrator in the same molecule seems to be quite a successful one since it is built in completely different molecules (see Section 6).

The identification of the 'pH sensor' on such pH-regulated proteins and the study of whether this H^+-sensing and the ion-transporting sites are identical, overlapping or different are most intriguing. It is conceivable that residues involved in pH sensing or H^+ transport undergo protonation at the physiological pH range of activity. Histidines (pK 6.0 in solution) have been implicated in the mechanism of H^+ transport: in the lactose carrier [58–60], in the photosynthetic reaction center [61], as well as in the Na^+/H^+ antiporter activity of *E. coli* [62].

We therefore deleted, or mutated by site-directed mutagenesis, the histidines of NhaA, and found that none of the eight histidines of NhaA are necessary for activity, while His-226 is essential for the response of the protein to pH [63]. As revealed by analysis of the Na^+/H^+ antiporter activity of membrane vesicles, the replacement of His-226 by Arg markedly changes the pH dependence of the antiporter. Whereas, the activation of the wild-type NhaA occurs between pH 7 and pH 8, that of H226R antiporter occurs to the same extent, but at a pH range between 6.5 and 7.5. Furthermore, while the wild-type antiporter remains fully active at least up to pH 8.5, H226R is reversibly inactivated above pH 7.5, reaching 10–20% of the maximal activity at pH 8.5. We suggest that His-226 is part of the 'pH sensor', the pH-sensitive site that regulates the activity of NhaA [63].

A comparison of the phenotypes conferred by plasmids bearing the various mutations to the phenotype of the wild type upon transformation of strains Δ*nha*A or Δ*nha*AΔ*nha*B demonstrates that this 'pH sensor' is physiologically essential. Similar to the wild-type gene, all mutants, except H226R, confer Na^+ resistance up to pH 8.5 as well as Li^+ resistance. However, H226R cannot grow at alkaline pH in the presence of Na^+, but is as competent as the wild type at the neutral or acidic pH range [63].

Although His-226 is part of the 'pH sensor' of NhaA, it is evident that other amino acids are also involved in this sensor since the mutated protein still reacts to pH albeit abnormally. If protonation of His-226 or Arg-226 is involved in the reactivity of the protein to pH, we must conclude that either one, or both, has a pK in the protein which is different from its pH in solution. It is also possible that a change in conformation caused by H226R reflects part of the pH sensing mechanism of NhaA.

The setpoint of the pH sensor of NhaA is around pH 7.5, which is the homeostatic value of the intra-

cellular pH of *E. coli* [55], and the setpoint of Nhe1 is pH 6.5 [64]. It will be most interesting to compare the 'pH sensors' of these proteins (Section 6).

2.8. The role of NhaA and NhaB antiporters in halotolerance in E. coli

The most compelling evidence for the major role of Na^+/H^+ antiporter activity in the Na^+ cycle of bacteria is provided by our work with *E. coli*. Analysis of the phenotype of a *Δnha*A mutant reveals that Na^+ has a specific toxic effect on the cells, and that the sensitivity of the cells to Na^+ is pH-dependent, which markedly increases with increasing pH [28]. Thus, *Δnha*A cannot adapt to the high sodium concentrations which have no effect on the wild type (0.7 M NaCl at pH 6.8). The Na^+ sensitivity of *Δnha*A is pH-dependent, increasing at alkaline pH (0.1 M NaCl at pH 8.5). Nor can the *Δnha*A strains challenge the toxic effects of Li^+ ions (0.1M), a substrate of the Na^+/H^+ antiporter system. It is thus concluded that *nha*A is indispensable for adaptation to high salinity, for challenging Li^+ toxicity, and for growth at alkaline pH (in the presence of Na^+).

The *Δnha*B strain shows no impairment in its ability to adapt to high salt or alkaline pH nor in its resistance to Li^+ [19]. These findings suggest that NhaA alone can cope with the salt and pH stress, having adequate capacity for these functions. Also, the expression of *nha*A is highly regulated and increases significantly under the conditions in which it is essential: high salt, alkaline pH (in the presence of Na^+ ions), and the presence of toxic Li^+ ions [[65] and section 2.10].

The double mutant, *Δnha*A*Δnha*B, grows very poorly in the presence of Na^+ concentrations as low as 15–20 mM. At concentrations of 100 mM Na^+ (pH 7.5), growth is completely arrested. Yet in the absence of added Na^+ (contaminating levels of 10 mM) it grows at the entire pH range, pH 6.5–pH 8.4. Analysis of the antiporter activity in membranes prepared from the *Δnha*A*Δnha*B strain shows no residual activity of Na^+/H^+ antiporter [19].

Based on these results we can tentatively conclude that, in relation to *nha*B, *nha*A is more flexible and capable of handling a higher load of Na^+ within the entire pH range. Thus, it can support growth in the absence of *nha*B even at high salinity and pH [6,28]. On the other hand, *nha*B can support growth only at relatively low sodium concentrations. In addition, *nha*B shows a higher affinity for Na^+ than NhaA [7,8], suggesting that the recurrent theme described for many other transport systems might also hold for the systems handling Na^+ and H^+: a low-affinity, high-capacity system (*nha*A) and another high-affinity, low-capacity system (*nha*B) are required to cope with adaptation to a wide range of concentrations [7]. Interestingly, and in contrast to other chromosomally encoded mineral transport systems [66], the high-capacity system is regulated in the case of the Na^+/H^+ antiporters [65]. However, very little is known thus far about the regulation of *nha*B or its kinetic properties.

Although it confers with resistance to Li^+, *nha*A does not increase the limits of the pH or salt that wild-type *E. coli* can cope with, suggesting that factors other than *nha*A are active in setting the upper limits of tolerance. Although not proven, osmotolerance may be a crucial factor in these upper limits of growth.

2.9. The role of Na^+/H^+ antiporters in pH homeostasis

One of the major roles assigned to the antiporter is in the regulation of intracellular pH (pH_{in}) mainly at alkaline extracellular pH [55,67,68]. In *E. coli*, pH_{in} has been shown to be clamped at around 7.8, despite huge changes in the extracellular medium pH [55,67–69]. When the pH of the external medium is rapidly lowered or raised by over one unit, the *E. coli* internal pH shifts slightly, then recovers [70,71]. During anaerobic growth, cells maintain a constant internal pH of 7.4 at external pH 6.6–7.0 [72]. Many bacteria as well as eukaryotic cells have since been shown to strictly maintain a constant cytoplasmic pH at around neutrality [50,55,67,68,73–76]. Relatively small increases in pH_{in} halt cell division and activate the expression of specific genes [77,78] and of regulons [79,80]. It is therefore not surprising that both eukaryotic and prokaryotic cells have evolved several pH_{in} regulative mechanisms to eliminate metabolically induced changes in pH_{in} or to counter extreme environmental conditions [28,64,67, 68,74,81–84].

The elucidation of the mechanisms of pH homeostasis in neutrophilic bacteria, including *E. coli*, have proven to be remarkably elusive [6,67–69,80]. A well-documented system is that of *Enterococcus hirae (Streptococcus faecalis)*, in which the proton-translocating ATPase regulates internal pH by excreting H^+ [85,86]. However, this cell is limited in its pH range of growth in the absence of carbonate (pH 6.5–7.9) [[86,87] and Section 4]. In *E. coli, unc* mutants regulate internal pH normally [88].

We proposed that Na^+/H^+ antiporters in conjunction with the primary H^+ pumps are responsible for homeostasis of intracellular pH in *E. coli* [55,67]. This suggestion had its most compelling experimental validation in akaliphiles, in which it was shown that Na^+ ions are required for acidification of the cytoplasm and for growth ([89,90,91] and Section 3). In neutrophiles, such as *E. coli*, there is no direct evidence that supports this contention, since it is not clearly established that Na^+ is required for growth at alkaline pH. Nor is the requirement for Na^+ easy to demonstrate in some alkaliphiles, presumably due to a very high affinity for

Na^+ (as low as 0.5 mM), such that the contamination present in most media suffices to support growth [92]. McMorrow et al. [15] have taken special precautions to reduce Na^+ to very low levels (5 to 15 μM) and reported a strict requirement for Na^+ (saturable at 100 μM) for growth of *E. coli* at pH 8.5. This range of concentrations of Na^+ required for growth is well within the range of the K_m of the NhaB system (40 to 70 μM) [30].

Revealing that the activity of two antiporters comprises the Na^+/H^+ antiporter activity of the cell and developing the tools for the measurement of their activity, separately and in combination, permitted the assessment of possible simultaneous involvment of the Na^+/H^+ antiporters in pH homeostasis and in Na^+ extrusion.

The primary means of H^+ extrusion under aerobic conditions is the electron transport chain, which in the absence of permeable ions develop a negligible ΔpH and a large $\Delta\Psi$. Apparently Na^+ and K^+ are involved in the modulation of $\Delta\tilde{\mu}_{H^+}$ components: an electrogenic uptake of K^+, with or without H^+, compensates for charge extrusion and thus permits the development of ΔpH [67,68,93]; an exchange of Na^+ and H^+ has been implied in the generation of an inverted ΔpH at alkaline pH [55]. We know that NhaA is electrogenic, with a stoichiometry of two ([48] and Section 2.5) and that it is a highly active system: the maximal value of NhaA mediated downhill Na^+ efflux at pH 8.5 is about 400 nmol/min/mg cell protein, and it can increase even more when fully induced [6]. Since the rate of H^+ extrusion through the respiratory chain is about 1000 nmol/min per mg cell protein, and the rate of K^+ transport through the Trk systems is about 500 [94], our measurements reveal that NhaA can, indeed, quantitatively account for a rapid response to changes in ion content. A quantitative measurement of NhaB activity and study of *nha*B regulation will no doubt provide a more detailed understanding of the Na^+ and H^+ cycles in *E. coli*.

One approach to study the involvement of the Na^+/H^+ antiporters in pH homeostasis is the analysis of the phenotypes of the various antiporter mutants and the deduction of the role of the antiporters by a comparison to the wild type phenotype under different conditions pertaining to Na^+ and H^+. As long as Na^+ is withheld from the medium, *Δnha*A*Δnha*B, and the two respective single mutants grow at the entire pH range of growth [19,28]. Hence, the simplistic assumption that cells lacking the antiporters are pH-sensitive has been disproved. One possibility to explain these results is that although the antiporters are involved in pH regulation, pH-homeostasis is not required in the absence of Na^+. On the other hand, assuming that pH-homeostasis is an absolute requirement for growth [55,76], these results suggest that neither NhaA nor NhaB participate in pH homeostasis, or that in their absence, *kha*A [31,95] or another as yet unidentified system regulates intracellular pH. A mutation, Hit1, which is closely linked to *nha*B has recently been proposed to affect pH homeostasis, based on its growth inhibitory effect at alkaline pH [39]. Since a deletion in *nha*B does not yield similar phenotype, we conclude that Hit1 contains an additional unidentified mutation, or that *hit*1 modifies NhaB so that the aberrant protein inhibits growth at alkaline pH [19]. The role of NhaB as that of NhaA in pH-regulation is therefore still unclear.

As described above, the addition of Na^+ dramatically affects the growth of *Δnha*A, while its sensitivity to Na^+ intensifies with increasing pH [28]. Whether or not the inhibitory effect of Na^+ is through amelioration of pH homeostasis is still not known. Alternatively, the increase in the detrimental effect of Na^+ on *Δnha*A with pH, may be explained by either the observed increase in intracellular Na^+ with pH [50] and/or an increase in Na^+ toxicity in increasingly alkalinizing cytoplasm. The possibility that Na^+ may compete for H^+ in the active sites of many essential systems has recently been raised (for review see Padan and Schuldiner, [6]).

If alternative mechanisms do exist for pH homeostasis operating in the absence of the antiporters (see above), they may be inefficient in the face of an increased Na^+ load at alkaline pH, increasing the sensitivity of *Δnha*A at alkaline pH in the presence of Na^+. There is also the last possibility, that the antiporters are involved only in Na^+ extrusion, and their role becomes more prominent at alkaline pH for the reasons described above.

Recently, *E. coli* has been shown to grow with negligible proton motive force, in the presence of carbonyl cyanide m-chlorophenylhydrazone (CCCP), both at alkaline pH [20,96] and at neutral pH [97]. Since the Na^+/H^+ antiporter cannot operate and pH homeostasis is not maintained under these conditions these results imply that neither pH homeostasis nor the operation of the Na^+/H^+ antiporters are essential under these conditions. Since the media used in these experiments contained low Na^+ these results coroborate our results with *Δnha*A*Δnha*B conducted in the absence of added Na^+ (see above). Thus, the above discussion of the impact on the understanding of the mechanism of pH homeostasis of the mutant phenotype, also applies to the energy uncoupled cells. It is hence apparent that in order to further understand the mechanism of pH homeostasis, its relation to the Na^+ cycle and the role of the Na^+/H^+ antiporters in these processes, we must directly measure the capacity of pH homeostasis and Na^+ circulation, i.e., pH_{in} and $[Na^+]_{in}$, in the wild-type, and the antiporter mutants under various loads of Na^+ and pH stress. These

experiments are currently being conducted in our laboratory.

These data together with the parameters regarding the regulation of activity and expression of the antiporters will eventually permit us to construct a model, simulating the integrative activity of the antiporters in the H^+ and Na^+ cycles of the cells. Understanding these cycles will provide important clues for our comprehension of the process of adaptation to extreme pH and salt environments.

2.10. Regulation of expression of the antiporters

Since the fluxes of Na^+ and H^+ are coupled through the two antiporters, knowledge of the regulation of activity and expression of each of the individual elements is crucial for the understanding of both Na^+ and H^+ circulation of the cells, and the integrative operation of the antiporter in these circulations. The only regulation of expression thus far studied has been that of *nha*A, where we have mapped two promoters by primer extension in the 5′ upstream region [65]. In addition, a quite extensive putative secondary structure in the RNA can be predicted in the 5′ end of the gene [25], and the first codon is GTG rather than ATG [11]. GTG has been found to mediate the initiation of translation in about 8% of the documented *E. coli* proteins [98], and it is believed to be used in mRNA's that are poorly translated. Also the codon usage in *nha*A is typical for poorly expressed proteins [33]. We estimate that under the optimal growth conditions (L broth adjusted to pH 7.5 in which the Na^+ is replaced with K^+ and the contamination levels of Na^+ are around 10 mM, or minimal salt medium to which sodium is not added), NhaA is a minor component of the membrane (less than 0.2%, or an equivalent of less than 500 copies per cell [11]. The expression with an exogenous promoter (*tac*) is much higher when the regulatory sequences of *nha*A are deleted [11], implying that at least under some conditions, the upstream region has an inhibitory effect on expression of *nha*A.

We have constructed a chromosomal translation fusion between *nha*A and *lac*Z (*nha*A′-′*lac*Z) and found that the levels of expression are very low unless Na^+ or Li^+ are added. Na^+ and Li^+ ions increase expression in a time-and concentration-dependent manner [65]: the maximal increase is detected when the cells are exposed to 50 to 100 mM of either ion for a period of 2 h. This effect is specific to the nature of the cation and is not related to a change in osmolarity. Alkaline pH potentiates the effect of the ions. The pattern of regulation of *nha*A thus reflects its role in the adaptation to high salinity and alkaline pH in *E. coli* [6,28]. It also implicates the involvement of a novel regulatory gene, in addition to *nha*A and *nha*B.

This is *nha*R, previously known as *ant*O [99], or 28 kDa protein [27]. In addition to its location downstream of *nha*A, and due to the fact that there are no conspicuous consensus sequences of either terminators or promoters between the two genes, *nha*R and *nha*A seem to form an operon. In addition, the expression with foreign promoters cloned upstream of *nha*A brings about an expression of *nha*A as well as *nha*R [25].

A multiple dose of *nha*R enhances the Na^+ dependent induction of the *nha*A′-′*lac*Z fusion. The fact that the dose level affects the induction by Na^+, but not the basic level of expression, suggests that the Na^+ induction involves *nha*R, either directly or indirectly, and that NhaR exerts its effect in *trans*. Furthermore, extracts derived from cells overexpressing *nha*R, exhibit DNA binding capacity specific to the upstream sequences of *nha*A, as observed by gel retardation assays [38]. Inactivation of the chromosomal *nha*R by insertion unveils a phenotype of sensitivity to Li^+ higher than that displayed by the wild type [38]. A change of tolerance towards Na^+ in these cells becomes apparent only at pH 8.5, under conditions in which the load seems to be more pronounced, as suggested by the phenotype of the Δ*nha*A strain and by the pattern of regulation of *nha*A. Both phenotypes are corrected by *nha*R in *trans* [38].

On the basis of the above results, it is now proposed that NhaR is a positive regulator of *nha*A. This suggestion is in accordance with the fact that NhaR belongs to the OxyR-LysR family of positive regulators first described by Henikoff et al. [99] and also studied by Christman et. al., [100]. All the proteins in this group have a conserved helix-turn-helix domain in their N-terminus, which is supposed to bind to DNA. Several of these proteins such as OxyR, which is essential for the resistance of the organism to oxidative stress [100,101], are involved in the response of the organism to stress. We therefore suggest that NhaR and its effector *nha*A are the first example of a signal transduction of a specific adaptation to Na^+, and possibly to alkaline stress as well, unrelated to the stress of osmolarity or ionic strength. Indeed, extending our studies to extreme stress conditions of pH and Na^+, under which the cells do not divide but survive, show that in the absence of NaCl, the wild type stops growing at pH 8.7 and lyses at pH 9.6. Between pH 8.8 and 9.6, it survives for periods which depend on the time of exposure, as well as the combination of the pH and Na^+ stresses, the higher the pH, the higher the sensitivity to salt concentration. The reason for the extreme stress caused by the combination of alkaline pH and Na^+ is not clear, however, NhaA and its Na^+ induced regulation via *nha*R are essential for survival under this extreme Na^+/pH stress [28,38,65].

The molecular details of the mechanism by which cells sense environmental adversity and then transduce the stress signal into a change in gene expression is

known only for a limited number of responses: MerR, a regulator of mercury resistance in *E. coli*, is activated to induce the expression of mercuric reductase upon binding mercury [102,103] and OxyR, a regulator of genes involved in resistance to oxidative stress, transduces an oxidative stress signal to RNA polymerase upon its own oxidation [104]. For many other environmental stresses including heat, a transcriptional regulator has been characterized, but little is known about how the environmental signal is transmitted to it [104,105].

Na^+ and Li^+ are extracellular inducers of *nha*A and alkaline pH markedly potentiates the inducibility by Na^+. Although K^+, ionic strength and osmolarity were excluded from being inducers [65], the existence of other inducers cannot be ruled out, yet a large body of evidence suggests that the immediate signal for induction of *nha*A is the intracellular, rather than the extracellular level of Na^+ [6]. Thus, the effectiveness of extracellular Na^+ ($[Na^+]_{out}$) in inducing *nha*A increases under conditions at which the intracellular concentration ($[Na^+]_{in}$) rises (at alkaline pH [50], in antiporter defective strains [6] or upon addition of uncouplers (Kotler, Y., Rimon, A., Schuldiner, S. and Padan, E., unpublished results)). In addition, large variations in $[Na^+]_{out}$ have practically no effect on *nha*A induction in strains carrying either multicopy *nha*A or *nha*B and which display a higher extrusion capability [6,38].

Thus, our working hypothesis is that a change in extracellular Na^+, the extracellular signal, is conveyed into the cell as a change in the intracellular Na^+. NhaR itself transduces this information and induces expression of *nha*A. It will be most intriguing to delineate this novel signal transduction pathway which is essential for halotolerance.

3. Na^+H^+ antiporters in bacteria growing in extreme concentrations of Na^+ and H^+: halophilic and alkaliphilic bacteria

All known extreme halophilic and alkaliphilic bacteria maintain a primary proton cycle and a secondary Na^+ cycle across the cytoplasmic membrane by electron transport linked primary H^+ pumps and an Na^+/H^+ antiporter activity [Fig. 1A.2 and [75,106–109]]. Na^+ reenters the cells in both groups via solute transporters, which are Na^+ coupled [106,110]. Hence these bacteria require Na^+/H^+ antiport activity to extrude the Na^+ that is translocated inward during growth, and to maintain an Na^+ gradient directed inward. In the case of the alkaliphiles, the flagellar motor is also Na^+ dependent [111,112]. These secondary Na^+ cycles operate against the heaviest load of extracellular Na^+ (> 3.5 M NaCl) [106,113], or with the lowest $\Delta\tilde{\mu}_{H^+}$ (−25 to −50 mV) respectively [[108–110,114]; but see also 115]]. Interestingly, primary Na^+ pumps have not yet been discovered in either extreme halophiles or extreme alkaliphiles (non-marine non-halotolerant).

The extreme halophiles seem to tolerate a high cytoplasmic concentration of Na^+ (up to 1M) [116]. It appears therefore that they compromise with an Na^+ gradient lower than 10, which can be easily produced by the Na^+/H^+ antiporter in spite of the heavy Na^+ load imposed on cell energetics.

The properties of this antiporter appear to be unique; it requires a gating potential of 100 mV and restricts Na^+ back-flow even when $\Delta\tilde{\mu}_{H^+}$ is reversed [117]. Furthermore, it is the only known prokaryotic antiporter inhibited by DCCD [117–119]. Lanyi et al. [106,120] initially studied the Na^+/H^+ exchange in halobacterial envelope vesicles, compared the $\Delta\tilde{\mu}_{H^+}$ to $\Delta\tilde{\mu}_{Na^+}$ during illumination and concluded that the antiporter is electrogenic with a H^+/Na^+ stoichiometry of 2. A later study implied that the antiporter is not necessarily electrogenic [121]. A more recent investigation estimated the DCCD-sensitive changes in $\Delta\tilde{\mu}_{H^+}$ and $\Delta\tilde{\mu}_{Na^+}$ during steady-state illumination reported an Na^+/H^+ stoichiometry of 1 [122]. This discrepancy emphasizes the difficulty in determining Na^+/H^+ stoichiometry in membrane preparations (see subsections 2.4 and 2.5).

The Na^+/H^+ antiporter activity of *H. halobium* was found to be highly sensitive to pH [117,123]. pH-sensitive sites have been suggested both at the cytoplasmic and medium face of the membrane with mechanistic implications. However, the effect of non relevant pH-dependent leaks and transport system cannot be ruled out in membrane preparation. Although functional purification has not yet been achieved, solubilization and functional reconstitution of the non-purified *Halobacterium* Na^+/H^+ antiporter has been obtained [118].

In the extreme alkaliphiles, a low $\Delta\tilde{\mu}_{H^+}$ is maintained across the membrane, the exact value of which is still debatable [109,115]. This low $\Delta\tilde{\mu}_{H^+}$ is due to the Na^+/H^+ antiporter which generates a secondary Na^+ cycle with a reversed ΔpH (3 units acidic inside, Fig. 1A.2). Compelling evidence shows that in addition to Na^+ extrusion, the antiporter activity has a crucial role in the homeostasis of cytoplasmic pH of the extreme alkaliphiles; the resulting secondary Na^+ cycle facing values of extracellular pH up to 11, maintains intracellular constant at about pH 8.5 [75,109,124]. Although growing at neutral pH, the mutants devoid of antiporter activity lost the capacity to grow above pH 9 [125–127]. Alkaliphilic cells subjected to an upward shift in pH (pH 8.5 ⇒ 10.5), maintain their cytoplasmic pH at pH 8.5 when adequate Na^+ is present. A sustained maintenance of pH_i below pH 8.5 is observed in such experiments when Na^+ and a solute that is co-transported with Na^+ are both present. If, on the other

hand, Na^+ is absent during the upward pH shift, the pH_i immediately rises to 10.5. Accordingly, most alkaliphilic *Bacillus* species can easily be shown to require Na^+ for growth [89]. In some other cases the Na^+ requirement for growth can only be demonstrated when special care is taken to reduce the inevitable contaminating Na^+ [114].

The Na^+/H^+ antiport activity has been measured in cells, vesicles and proteoliposomes that were reconstituted with crude membrane extracts (review in 110,128,129). These studies suggest that it is electrogenic, competitively inhibited by Li^+ and regulated by intracellular pH being dramatically inhibited upon increasing proton concentration in the cytoplasm (pH_{in} = 7) [110,127–130] [114,127].

The question whether the antiporters functioning in the alkaliphiles have unusual kinetic properties and stoichiometries, await the purification and functional reconstitution of the antiporters in proteoliposomes. Similarly, the study of the physiology and regulation of these antiporters await the development of molecular genetic tools in the alkaliphiles [131]. Nevertheless, by utilizing the advances made in the molecular biology study of the Na^+/H^+ antiporters of *E. coli*, a gene for an apparent antiporter (NhaC) from *B. firmus* OF4, has recently been cloned in *E. coli* [[34] and section 2.2]. A DNA library from the alkaliphilic bacteria was transformed into *Δnha*A of *E. coli* and a clone which reversed the ion sensitive growth phenotype of *Δnha*A was isolated.

The sequence of the cloned gene predicted to encode a putative hydrophobic protein with ten α helixes spanning the membrane consistent with many known transporters [34]. Utilizing *Δnha*A of *E. coli* and the same selection procedure, additional alkaliphilic inserts were found to restore certain Na^+ resistance to the *E. coli* mutant and some were even found to enhance Na^+/H^+ exchange activity. Interestingly, some of these inserts show an amino acid sequence similarity with putative ion binding sites of various ion transport systems [132] including *cad*C of *Staphylococcus aureus* cadmium resistance plasmid (pI258; [132]).

4. Na^+/H^+ antiporters in bacteria having primary Na^+ cycles

The dependence of the Na^+/H^+ antiporters on $\Delta\tilde{\mu}_{H^+}$ for Na^+ extrusion suggests that this form of Na^+ export is inefficient when $\Delta\tilde{\mu}_{H^+}$ limits and/or when the Na^+ concentration is high and imposes a heavy load against the $\Delta\tilde{\mu}_{H^+}$. Whereas the extreme halophiles and alkaliphiles do not fall into this category (see Section 3), alternative systems initiating primary Na^+ cycles exist in anaerobic (Fig. 1B.1, 1B.2, 1B.3) and marine bacteria (Fig. 1B.4). Decarboxylases utilizing energy from decarboxylation reactions serve as primary Na^+ pumps in many anaerobes [Fig. 1B.1 and [133]]. Several anaerobes also possess Na^+-ATPase [Fig. 1B.1; 1B.3 and [133–137]]. In methanogens, several metabolic reactions are coupled to the formation and utilization of the Na^+ gradient [Fig. 1B.2 and [138,139]]. In marine bacteria [Fig. 1B.4 and [140–142]] and other halotolerant and alkaline-tolerant bacteria (for classification of alkaliphiles and alkaline-tolerant bacteria see [110]) primary Na^+ pumps are linked to electron transport [143,144]. The latter organisms may also possess Na^+-ATPase [Fig. 1B.4 and [145–147]]. It has been suggested that stress caused by low $\Delta\tilde{\mu}_{H^+}$ induces a primary Na^+ pump even in organisms such as *Escherichia coli* [148–151].

In addition to the primary Na^+ cycle, all these bacteria have Na^+/H^+ antiporters. The aerobic marine bacteria represented by *Vibrio alginolyticus* [Fig. 1B.4 and [140,152]] and moderate halophiles [143,144] have a primary H^+ cycle in addition to the primary Na^+ cycle. The Na^+/H^+ antiporter of this organism maintains a secondary Na^+ cycle which is observed under conditions in which the Na^+ pump activity is low, i.e., below pH 8 [140]. Thus, the extrusion of Na^+ against its concentration gradient at acidic pH is preformed by the Na^+/H^+ antiporter and inhibited by CCCP. Na^+ extrusion at alkaline pH is resistant to CCCP and dependent on respiration. Accordingly, growth is inhibited by the uncoupler at acidic pH but not at alkaline pH and so are all $\Delta\tilde{\mu}_{Na^+}$ requiring processes, i.e., active transport and flagellar motility (Fig. 1B.4).

The pH profile of CCCP-resistant growth does not simply reflect the pH dependence of the primary Na^+ pump, which is still functional at acidic pH. In fact, the overacidification it creates accounts for the growth inhibition in the presence of the uncoupler at acidic pH. Whether the primary Na^+ pumps are expressed and active together with the secondary Na^+ cycle thus having a role at acidic pH in the non inhibited cells, is not yet clear. However in mutants defective in the primary Na^+ pumps, the secondary Na^+ cycle is observed even at alkaline pH [140].

Since at alkaline pH, $\Delta\Psi$ is the only product of the electron transport linked primary H^+ pumps, it is implied that the Na^+/H^+ antiporter activity of *V. alginolyticus* is electrogenic. Provided that the stoichiometry of cation/e^- at the electron transport segment (NADH: quinone oxidoreductase) is identical between the Na^+ pump and the H^+ pump, a secondary Na^+ cycle dependent on electrogenic antiporter must consume more NADH than does the Na^+ pump for the generation of the same magnitude of $\Delta\tilde{\mu}_{Na^+}$ at alkaline pH (when $\Delta\Psi = \Delta\tilde{\mu}_{H^+}$). It has been suggested that, for this difference in energy economy, the primary Na^+ pump is widely distributed among marine bacteria [153].

It is possible that at alkaline pH, the Na^+/H^+ antiporter activity of *V. alginolyticus* is important for pH homeostasis. However, K^+/H^+ antiporter, which is activated at alkaline cytoplasm, has been implied in the regulation of cytoplasmic pH in this organism [154].

In anaerobic bacteria represented by *Propionigenum modestum*, there is a primary Na^+ cycle, consisting of a primary Na^+ pump linked to decarboxylation reaction such as methylmalonyl-coA decarboxylase [Fig. 1B.1 and [134]], and F_1F_0 type ATPase which can pump H^+ but under certain physiological conditions, uses only Na^+ [134,155]. Nevertheless, the existence of Na^+/H^+ antiporters in *P. modestum* are suggestive since the coupling ion in their secondary solute transport process is supposed to be H^+ [156].

Since $\Delta\tilde{\mu}_{Na^+}$ (directed inward) is the energetic currency of this organism, the Na^+/H^+ antiporter would only be able to excrete H^+ in this bacterium at the expense of the $\Delta\tilde{\mu}_{Na^+}$. This can be important for the pH homeostasis in this organism, which usually grows at neutral or acidic pH conditions. The formed $\Delta\tilde{\mu}_{H^+}$ could also possibly be utilized for $\Delta\tilde{\mu}_{H^+}$ coupled reactions (Fig. 1B.1). However, there is no information regarding the composition and properties of the antiporters in this or similar bacteria.

Methanogens have primary H^+ pumps linked to electron transport reactions which maintain a primary cycle completed by an H^+/ATPase (Fig. 1B.2). They also have a secondary Na^+ cycle via an Na^+/H^+ antiporter. This cycle is completed by a reversible primary Na^+ pump, which is not an Na^+/ATPase [Fig. 1B.2 and [157]]. Methyl-tetrahydromethanopterin coenzyme M methyltransferase [158] and formyl-MFR dehydrogenase [138] were shown to operate as reversible Na^+ pumps. Depending on the substrates, $\Delta\tilde{\mu}_{Na^+}$ can be either consumed or generated by these pumps. In the former case the required $\Delta\tilde{\mu}_{Na^+}$ is generated by the Na^+/H^+ antiporter (Fig. 1B.2), whereas in the latter, $\Delta\tilde{\mu}_{H^+}$ is formed by the antiporter for ATP synthesis via an H^+/ATPase [138]. The latter mode of the antiporter in H^+ extrusion is similar to the animal eukaryotic mode (Section 6).

Interestingly, the Na^+/H^+ antiporter activity of the methanogens is inhibited by the specific inhibitors of the eukaryotic Na^+/H^+ exchanger, harmaline and amiloride, and it exchanges H^+ for Li^+ and Na^+ [138]. However unlike the eukaryotic system, which is electroneutral that of the methanogens is electrogenic. A stoichiometry of 1.5 H^+/1 Na^+ has been determined in intact cells [138].

In fermenting anaerobes such as *Enterococcus hirae* (Fig. 1B.3), ATP is produced by substrate level phosphorylation. A primary H^+ cycle is initiated by an F_1F_0 type H^+ ATPase, which functions to excrete H^+, maintains a $\Delta\tilde{\mu}_{H^+}$ and regulates intracellular pH [86,87,159,160]. This organism has a Na^+/H^+ antiporter as well as an Na^+ inducible primary pump, Na^+/ATPase, which is similar to the vacuolar type (archaebacterial) H^+/ATPase [137,161]. Both systems have been implied mainly in Na^+ excretion, the Na^+/H^+ antiporter at neutral and acidic pH where $\Delta\tilde{\mu}_{H^+}$ is sufficient whereas the Na^+ ATPase at alkaline pH where $\Delta\tilde{\mu}_{H^+}$ is limiting [162].

The Na^+/H^+ antiporter gene of *E. hirae* has been cloned by an approach similar to that used for cloning of *E. coli* antiporters (subsections 2.1 and 2.2) using a complementation of an Na^+ sensitive *E. hirae* mutant defective in both, ATP-driven Na^+-extrusion and the Na^+/H^+ antiporter [163]. The gene termed *nap*A enhances Na^+/H^+ antiporter activity. It codes for a hydrophobic protein composed of 383 amino acids likely to form 12 transmembraneous helices. The disruption of the *nap*A gene leads to the loss of Na^+/H^+ exchange activity as measured in whole cells or membrane vesicles. The NapA antiporter shows significant homology to KefC a putative K^+/H^+ antiporter in *E. coli* [164]. Only very small homology was found with NhaA and NhaB [6].

Clostridium fervidus an anaerobic thermophilic bacterium has recently been shown to possess an Na^+/ATPase dependent primary Na^+ cycle and to lack Na^+/H^+ antiporter [165]. Corroborating the need of the antiporter in pH homeostasis, this bacterium grows at a very limited pH range.

5. Na^+/H^+ antiporters of plant cells and eukaryotic microorganisms

Membrane research of plant cells and eukaryotic microorganisms over the past decade has established that the cytoplasmic membrane of these cells maintain a primary proton cycle initiated by a P-type H^+/ATPase excreting H^+ [Fig. 1C.1 and for a comprehensive review see [166]]. The proton uptake limb of this cycle is most probably formed by solute/proton symporters [Fig. 1C.1 and [167]].

There are some indications of a primary Na^+ cycle in the extreme halophile *Dunaliella salina* [168] and a Na^+/ATPase has recently been cloned from yeast cells [Fig. 1C.1 and [169]]. On the other hand, Na^+/H^+ antiporters and thus secondary Na^+ cycles are widely spread in the cytoplasmic membranes of eukaryotic microorganism as well as in plant cells (Fig. 1C.1).

The Na^+/H^+ antiporter activity was followed in membrane vesicles of *Dunaliella* by either the sodium-dependent change in vesicular ΔpH, as monitored by absorbance changes of acridine orange, or by the ΔpH-dependent uptake of ^{22}Na into the intravesicular space. These membranes catalyze Na^+/H^+ antiport in a manner that is highly specific for Na^+ (apparent K_m 16 mM). No other ion tested (K^+, Li^+, Cs^+, $(CH_3)_4N^+$,

choline$^+$, Tris$^+$) served as substrate. Li^+ and amiloride inhibit the process competitively with apparent K_i values of 30 μM and 25 μM, respectively [170,171]. Sulfhydryl reagents such as p-chloromercurisulfonate, $HgCl_2$ or the H^+/ATPase inhibitor, N',N'-dicyclohexylcarbodiimide, had no effect.

The effect of the medium pH on the activity of isolated membrane vesicles shows a strong increase between pH 7 to an optimum at pH 8.3, and then a decrease between 8.3 and pH 9. This dependency may reflect a strong reliance on either pH or on ΔpH, or both,since in these experiments both pH and ΔpH were varied. Since the orientation of the vesicles with respect to the cytoplasmic membrane has not been identified, it is still unclear whether the effective pH is at the cytoplasmic or external face of the membrane.

The Na^+/H^+ antiporter activity was solubilized by extraction with triton (or cholate) and reconstituted into phospholipids/cholesterol liposomes [171]. The reconstituted activity was greatly stimulated by the presence of valinomycin and KCl, suggesting that the exchange is electrogenic, presumably exchanging more than one proton for each Na^+ ion. Interestingly, the electrogenicity was not identified in intact membrane vesicles, suggesting that the plasma membrane is sufficiently permeable to ions so as to make imperceptible the effects of the added permeability to K^+ induced by valinomycin. In addition, the reconstituted antiporter was much less sensitive to inhibitors by amiloride and Li^+ in comparison to the membranes. This may relate to its mode of association with the artificial membrane or to the relative solubility of the inhibitors in the new matrix.

The Na^+/H^+ antiporter of *Dunaliella* has been implied in the adaptation to high salinity and in pH homeostasis [168,172]. Weak acid-induced intracellular acidification was used to follow the activity of the plasma membrane Na^+/H^+ antiporter in vivo. By monitoring the changes in both intracellular pH and Na^+ and the effect of inhibitors, it was shown that the Na^+/H^+ antiporter is involved in intracellular pH homeostasis [168,172]; intracellular acidification due to the addition of weak acids at appropriate external pH elicited a dramatic increase in intracellular Na^+ which then decreased. Whereas Li^+ inhibited the Na^+ influx phase, vanadate inhibited its Na^+ efflux phase, implying the involvement of Na^+/H^+ antiporter activity in the former process and an ATPase (most probably an Na^+/ATPase) in the latter [168].

The effect of different growth conditions on the activity of the Na^+/H^+ antiporter in *Dunaliella salina* has also been investigated [172]. Studying the adaptation of the algal cells to ammonia at alkaline pH (interpreted as acidification of the cytoplasm) or to high NaCl concentrations, increased the Na^+/H^+ exchange activity of the plasma membrane. The enhanced activity was manifested both in vivo (by stimulation of Na^+ influx into intact cells in response to internal acidification) and in vitro (by a larger ^{22}Na accumulation in plasma membrane vesicles in response to an induced pH gradient). Kinetic analysis revealed that the stimulation does not result from a change of the K_m for Na^+, but rather from an increase in the V_{max}. These results suggest that adaptation to ammonia or to high salinity induces overproduction of the plasma membrane Na^+/H^+ antiporter in *Dunaliella*, showing the importance of the antiporter in these adaptations [172].

A possible correlation between certain membrane polypeptides and the Na^+/H^+ activity of *Dunaliella* has been suggested [171,172]. Yet only the purification of the proteins in an active form would prove that these are antiporter proteins. The possibility for induction of the antiporter activity [172] may assist in the purification of the proteins and, possibly, also in cloning of the antiporter genes.

A Na^+/H^+ antiporter on the plasma membrane in *Sacharomyces cerevisiae* and *Neurospora crassa* has been suggested to be responsible for Na^+ export from the cell [Fig. 1C.1 and [173–176]]. Based on a selection for increased LiCl tolerance in *Schizosaccharomyces pombe*, a new locus, *sod*2, was recently identified [177]. As in *E. coli* (subsection 2.2), Li^+ is toxic to these cells at low concentrations relative to Na^+. This eliminates the selection for osmotolerance and has yielded mutants with increased antiporter activity excreting the toxic ion and thus conferring Li^+ resistance on the cells.

As in the case in *E. coli* (subsection 2.2), it was inferred that multiple copies of the wild-type *sod*2 gene would be sufficient to confer resistance by increasing the export capacity of the ion. Transformants of *S. pombe* with a genomic gene bank scored for Li^+ resistance yielded a strain carrying a recombinant plasmid containing *sod*2 [177]. The overexpressing of *sod*2 increased Na^+ export capacity and conferred Na^+ tolerance [177].

The predicted *sod*2 product can be placed in the broad class of transporters which possess 12 hydrophobic transmembrane domains [177]. The protein shows limited similarity to NhaI, NhaA antiporters [6,177] and NhaC [34]. The disruption of *sod*2 yielded cells incapable of exporting Na^+, hypersensitive to Na^+ (unable to grow above 125 mM) and Li^+ and sensitive to an increase in pH between pH 3.5 and 7.5 even with no addition of Na^+. These results suggest the role of *sod*2 in pH homeostasis at increasing pH and in Na^+ extrusion. The increasing sensitivity of the mutants with pH is reminiscent of the Δ*nha*A *E. coli* strain [28]. However, the pH sensitivity of Δ*nha*A is exposed above pH 7.5 and is Na^+ dependent [[28] and subsections 2.2, 2.8 and 2.9].

The amplification of *sod*2 occurred in the *sod*2 mutants. Various levels of amplification could be selected, stepwise, and the degree of such amplification correlated with the level of Na^+ or Li^+ tolerance. However, Na^+ does not induce *sod*2 transcription [177].

In plant cells, sequestering Na^+ within the vacuole plays a major role in Na^+ tolerance (see below). Although vacuolar storage of various ions in yeast has been reported there is no report yet for Na^+.

In the cytoplasmic membrane of various plant cells [178–183], a primary H^+ cycle and an Na^+/H^+ antiporter dependent secondary Na^+ cycle have been implicated (Fig. 1C.1).

An assay for the assessment of Na^+/H^+ antiport at the plasma membrane of intact plant tissue has recently been described [182]. This assay measures the effect of an artificial pH gradient on net $^{22}Na^+$ uptake by ATP depleted tissues. The uptake at pH 7.0 is compared to that at pH 3.9, obtained by acidification with either H_2SO_4 or butyric acid. With H_2SO_4, not only is the acidification of the external pH obtained, but also a transmembrane pH gradient (directed inward). On the other hand, the weak butyric acid permeates the plasma membrane in its undissociated form [184] and is not expected to establish a pH gradient or at least to establish a significantly smaller one. A pH gradient should support an outward directed Na^+ transport by Na^+/H^+ antiport at the plasma membrane, and result in a decreased net $^{22}Na^+$ uptake. Since an increased proton concentration may compete with Na^+, or directly affect the transporter, it had to be assured that the decreased net Na^+ influx results from a pH-gradient dependent enhancement of efflux, and not from a low pH dependent blockage of influx. This was achieved by comparing the effects of a lowering pH with H_2SO_4 (forming ΔpH) or with butyric acid (not forming ΔpH) and by checking the effect of these acids on Cl-uptake by the same tissue samples [185,186]. Chloride symport was observed only in the presence of ΔpH. The Na^+/H^+ antiporter activity at the cytoplasmic membrane is inhibited by uncouplers and by high K^+. These properties were used in vivo to differentiate between this antiporter activity and that at the tonoplast membrane [187].

This assay was employed to screen 16 crop plant species for Na^+/H^+ antiport at the plasma membrane of root or storage tissue [187]. Evidence for Na^+/H^+ antiport at the cytoplasmic membrane was found in four species (*Beta vulgaris; Hordeum vulgare; Lycopersicum esculentum and Triticum aestivum*) and for its absence in five other species. In seven species, the evidence was not unequivocal, implying that only positive results should be taken into account. This conclusion is further justified in view of the many known antiporters which are Na^+ and pH regulated, both at the activity and/or transcription levels, suggesting that measuring the activity at a single pH or Na^+ condition may be misleading (subsections 2.7 and 2.10). Furthermore the measurement in intact cells may be complicated by the presence of the Na^+/H^+ at the tonoplast membrane (see above).

Na^+/H^+ antiporter activity has been examined in isolated plasmalemma membranes of several plant cells including an halophite such as *Atriplex nummularia* and glycophites [178,179,183]. Proton fluxes have been followed, into and out of the vesicles with the aid of a ΔpH probe (^{14}C-methylamine or quinacrine). The cation supply after a pH gradient has been set up across the vesicle (either as a result of providing ATP to the H^+-ATPase, or by imposing an artificial pH gradient) brings about dissipation of the ΔpH but not the membrane potential, implying that the antiporter is electroneutral.

The specificity to Na^+ of the antiporter appears to be low in glycophytes and possibly higher in the halophites [179]. Other properties of the antiporter have yet to be explored. The purification and reconstitution of the activity in proteoliposomes should help in these studies.

At the tonoplast membrane of plant cells, a secondary Na^+ cycle is established by an Na^+/H^+ antiport driven by an H^+/ATPase [188–190] (and possibly also by an H^+/PPase [191]) activity derived proton motive force (Fig. 1C.1). In vivo, this activity of the tonoplast Na^+/H^+ antiporter has been measured by monitoring a metabolic-dependent Na^+ uptake in the presence of high K^+. It is assumed that under the conditions of high K^+, the Na^+/H^+ antiporter at the cytoplasmic membrane is suppressed, exposing the tonoplast activity [187].

Short-term in vivo NMR spectroscopy studies of the exposure of barley roots to salt revealed an Na^+-induced upward shift in vacuolar pH, consistent with the operation of a Na^+/H^+ antiport [192].

The operation of a vacuolar Na^+/H^+ antiport has been reported in isolated tonoplast vesicles of various plants, including glycophytes and halophytes [178,179,193,194] and in isolated vacuoles of *Beta vulgaris* [189,190,195] and *Catharantus roseus* [196].

Amiloride and its analogues were shown to competitively inhibit the vacuolar Na^+/H^+ antiport from *B. vulgaris* [189,190,193]. The binding properties of a radiolabeled 5-amino substituted amiloride analogue and its covalent labeling of tonoplast proteins after photolysis revealed a high affinity binding component in the tonoplast [197–199]. The close relationship between the dissociation constant of binding of this component with the constant of inhibition of the antiport activity suggested that this high-affinity component represents a class of sites associated with the tonoplast Na^+/H^+ antiport.

Photolabeling studies using the radiolabeled

amiloride analogue indicated the possible association of a 170 kDa polypeptide with the tonoplast antiporter [197–199]. A polyclonal antibody against the 170 kDa polypeptide immunoreacted with a 170 kDa polypeptide of the tonoplast membrane, while no reactivity was detected with purified plasma membrane. Most importantly, the antibody almost completely inhibited the tonoplast antiport activity [199]. Although these results suggest the association of the 170 kDa polypeptide with the vacuolar Na^+/H^+ antiport, purification and functional reconstitution in proteoliposomes are yet to be accomplished.

Sugar beet cell suspension, challenged with high level of NaCl and amiloride, showed an increased synthesis of a 170 kDa polypeptide that correlated with an increased Na^+/H^+ antiporter activity in the membranes. These results suggested the addition of more antiporter molecules to the tonoplast and/or an increase in the turnover rate of the Na^+/H^+ exchange [199].

A possible role of the cytoplasmic membrane Na^+/H^+ antiporters in pH regulation of the cytosol has been suggested [[168] and see above]. The transport of Na^+ from the cytosol, via the tonoplast antiporter, and its accumulation in the vacuole of halophytes and salt-tolerant glycophytes is an important mechanism for averting the damaging effects of Na^+ on key biochemical processes in the cytosol. In certain plants the operation of the Na^+/H^+ antiporter at the cytoplasmic membrane excretes Na^+ from the cells, and thus act synergistically with the tonoplast antiporter in maintaining the cytoplasmic Na^+ concentrations [179,199,200].

Accordingly, although the Na^+/H^+ antiporter activity of the cytoplasmic membrane is constitutive in *Atriplex*, it appears to be modified and/or induced by growing the plant cells in the presence of 400 mM NaCl [175]. Similarly as discussed above (and [199]) the presence of amiloride, an inhibitor of the tonoplast Na^+/H^+ antiporter, or an increase in the NaCl concentration in the growth medium resulted in an increase in the tonoplast Na^+/H^+ antiporter activity. The study of the Na^+/H^+ antiporters of both the cytoplasmic and tonoplast membranes is of paramount importance to the understanding of both pH homeostasis and the mechanism of salt tolerance in plants.

6. Na^+/H^+ antiporters in animals

6.1. Cloning of the plasma membrane Na^+/H^+ antiporter genes of higher animals

The plasma membrane antiporter of higher animal eukaryotes is involved in intracellular pH regulation, in regulation of intracellular volume and in net transepithelial H^+ and Na^+ secretion. The exchanger is rapidly activated in response to a variety of signals and its involvement in an array of processes in the cell cycle has been documented and extensively reviewed [81,82,201–204]. We will therefore limit our discussion to selected topics of the antiporter (Fig. 1C.2).

The use of a powerful and elegant combination of genetic techniques allowed the isolation of a c-DNA that codes for a human exchanger, Nhe1, [83]. The first step included the selection of an exchanger deficient mouse fibroblast cell line. This was based on the loading of the cells with Li^+ and exposure to an acidic pH in a medium free of Na^+ and Li^+. The wild-type exchanger containing cells rapidly died due to the exchanger-catalysed H^+ uptake and the cytoplasm acidification, thereby enriching the exchanger-deficient cells resistant to the treatment [73,205]. In addition, the isolation of mutants overexpressing the exchanger was made possible by acid-loading cells and allowing them to recover under conditions in which the exchanger activity is slowed down. Only cells overexpressing the exchanger survive this treatment [206].

The transfection of the exchanger-deficient mouse cell line with human genomic DNA and selection of cells overexpressing the human exchanger, allowed for the isolation of a genomic probe that was used for c-DNA cloning of Nhe1 [64,207]. The nucleotide sequence predicts a protein of 815 amino acids with 12 putative transmembrane helices. As in the case of many other secondary transporters from higher eukaryotes, the exchanger has two clear domains, a 500-amino-acid membrane bound NH_2-terminus and hydrophylic cytoplasmic C-terminal domain of 315 residues.

This cDNA facilitated the isolation at lower stringency, of several isoforms referred as Nhe 2, 3, 4 and β-Nhe [208–211], all of which exhibit 45–70% identity with Nhe-1 and possess a similar hydropathy profile. Yet another member of this family was fortuitously identified in *Caenorhabditis elegans* [212]. The highest degree of conservation in these forms is observed in the putative fourth and sixth transmembrane helices.

Tissue distribution studies reveal that the Nhe-1 mRNA is present at varying levels, in all tissues examined, whereas Nhe3 is expressed mostly in kidney and intestine [[209] and see also [210]]. Nhe4 is most abundant in stomach, followed by intermediate levels in colon and small intestine and less amounts in kidney, brain, uterus and skeletal muscle cells [210]. Consistent with the occurrence of the neutral NaCl absorptive process, Nhe3 message is absent from duodenum and descending colon cells. It is therefore suggested that Nhe3 might be the apical Na^+/H^+ exchanger involved in neutral NaCl absorption [209].

A classical feature of the Na^+/H^+ exchanger of the plasma membrane of animal cells is the sensitivity to

amiloride and related pyrazine derivatives [211]. Amiloride is a competitive inhibitor ($K_i = 3$ μM in a sodium-free medium) with respect to external Na^+ in many cell types and alkylation of its 5-amino group yields the most potent derivatives [213] [Ethylisopropylamiloride, (EIPA)]. As described above, there are multiple isoforms of Na^+/H^+ exchangers. One of these isoforms (Nhe2) expressed in kidney, intestine and adrenal gland cells is at least 300-fold less sensitive to EIPA [214]. Another isoform from hipocampal neurons shows practically no sensitivity to either amiloride or its analogues [215]. In addition, amiloride resistant mutants of Nhe1 have been isolated and sequenced [206]. Analysis of these findings, suggests that a specific sequence in the putative fourth transmembrane helix is responsible for amiloride recognition. Since amiloride is a competitive inhibitor of sodium it is suggested that the domain identified may be part of the sodium binding site [216].

It is worthwhile mentioning, however, that there are several seemingly unrelated antiporters from plants, algae, bacteria and insects which are inhibited by amiloride. Their sequences are still unavailable. It will be interesting to see whether the 'amiloride consensus sequence' is present in these proteins as well, despite the fact that some of them show clearly distinct properties (see below). It seems, therefore, that sensitivity to amiloride and its derivatives, although a hallmark of the Nhe type of protein, is shared by proteins which differ in stoichiometry, K_m for Na^+ and ion specificity. Moreover, antiporters which exchange ions other than Na^+ are also inhibited by amiloride [217,218].

6.2. Stoichiometry, pH sensor and regulation

It is generally agreed that the stoichiometry of the major antiporters characterized thus far in higher mammals is one to one. This conclusion is based on actual flux measurements and also on the fact that the membrane potential has no effect on the exchange activity [74,201].

Under physiological conditions the antiporter catalyzes net uptake of Na^+ coupled to efflux of cellular H^+. However, in vivo, the system never seems to reach equilibrium and the Na^+ gradient directed inwards (5–15 fold) is never balanced by an H^+ gradient directed outwards. The reason the antiporter cannot reach equilibrium seems to be the basis of the mechanism of pH regulation: the protein is kinetically blocked above certain pH values and the dependence on the concentration of internal H^+ is quite steep, with a Hill constant of more than 2. Aronson et al. [219] observed that acid-loading of membrane vesicles from brush border, stimulated rather than inhibited Na^+ efflux, an effect contrary to that expected from competition. They proposed the existence of internal 'H^+ modifier' sites at which the operation of the exchanger is modulated. In other words, the protein is only poised to function when the intracellular pH is below a certain homeostatic value. Once the physiological set-point is reached, the activity is switched off so that the pH does not increase further; upon acidification of the cytoplasm, the activity rapidly increases to allow for efficient extrusion of H^+. As described above, NhaA, an *E. coli* antiporter, displays a similar sensor except that the 'switch' has the opposite polarity: alkaline rather than acid pH activates this antiporter as expected from its role in recycling of protons back to the cell [6–8,11,63].

A wide variety of external signals including growth factors, hormones, neurotransmitters, lectins, phorbolesters and sperm, shift the set point of the Nhe antiporters to more alkaline values and thereby allow for a higher activity and eventually increase of intracellular pH [74,202,220]. It has been proposed that this stimulation is associated with activation of protein kinase activity and phosphorylation of serine residues on the protein has been demonstrated directly [64,221,222].

Removal of the cytoplasmic hydrophylic C-terminus domain preserved the Na^+/H^+ exchange activity while abolishing the exchanger activation in response to external signals [223]. It was suggested that one of the eight serine residues in this domain is phosphorylated, but a mutation of each of the residues to alanine did not abolish growth factor activation [224]. It is still possible, however, that phosphorylation at multiple serine residues is required for mitogen activation.

Although phosphorylation plays a key role in mitogen-induced activation, a phosphorylation independent mechanism has been demonstrated upon activation during cell volume regulation. The effect of hypersomolarity on the exchanger is also manifested as an alkaline shift in the pH_i dependence of the modifier site. However, this change seems to be induced by a different process [224].

6.3. Na^+/H^+ antiporters in invertebrates

Shortly after fertilization of sea urchin eggs, a prodigious amount of acid is released from the cells via an amiloride sensitive electroneutral Na^+/H^+ antiporter which alkalizes the cytoplasm. This phenomenon, first described in the early 1930s, was intensively studied for many years and lucidly reviewed by Epel [225]. A large variety of mechanisms were proposed to explain the activation of the antiporter upon fertilization, among them phosphorylation, exocytotic insertion and others. A role of the pH changes in posfertilization events has been also postulated. Following these studies, it was found that the sperm of sea urchins also had an Na^+/H^+ antiporter responsible for raising and main-

taining a high pH_i associated with a motility increase. However, unlike the egg, the sperm antiporter is not amiloride sensitive and is sensitive to changes in membrane potentials.

In epithelia of several invertebrates an electrogenic Na^+/H^+ antiporter activity has been described which exhibits a transport stoichiometry of $2Na^+/H^+$ [226–229]. It has been suggested that these proteins exhibit two external cationic binding sites, as indicated by the fact that amiloride inhibition displays in every case two different K_is. There is no indication of an intracellular proton activator site as described for NhaA and the Nhe family. In crustaceans, the contents of the stomach lumen, receiving secretions from the hepatopancreatic epithelium, have been measured at pH 4.0–5.0 during digestion, and the suggestion was proposed that a major physiological function of this antiporter may be to provide the acidity necessary to aid in food breakdown [226,228].

7. Na^+/H^+ antiporters in subcellular organelles

The existence of Na^+/H^+ antiporters in mitochondria was postulated in 1961 by Mitchell [1], and experimentally demonstrated in 1969 by Mitchell and Moyle [3]. Later studies demonstrated that mitochondria contains two distinct Na^+/H^+ antiporters that can be distinguished by their cation selectivity and inhibitor sensitivity: one, coined the K^+/H^+ antiporter, transports all alkali cations and is sensitive to DCCD and quinone. It was purified in a functional state and identified as an 82 kDa polypeptide. Mg^{2+} depletion and/or acid pH inhibit this activity [230]. The second antiporter is specific for Na^+ ($K_m = 30$ mM) and Li^+ ($K_i = 0.5$ mM) and was reconstituted and partially purified [231]. Both activities are electroneutral and resistant to amiloride.

Although in plants, the existence of Na^+/H^+ antiporters in the vacuole membrane is well-documented (see Section 5), in animal cells, reports on activities in subcellular organelles other than mitochondria are very scattered. Chromaffin granules, the secretory vesicles from bovine adrenal medulla, have a Na^+/H^+ antiport activity [232]. This is an electroneutral antiporter with a K_m for Na^+ of 20–30 mM. Amiloride is a competitive inhibitor but with a decreased affinity (K_i 0.26 mM). The more potent EIPA does not inhibit.

An amiloride insensitive antiporter was also described in renal endocytic membranes [233]. In several studies there are reports of lack of activity in phagosomal membranes of neutrophils [82], in a microsomal fraction [234] or in another preparation of renal endosomes [235]. It is possible that the meager information available is due, on one hand, to the difficulty in preparing sufficient amounts of pure membrane preparations and on the other hand, to a different pharmacology of the subcellular activities.

8. Concluding remarks

The universality of the Na^+/H^+ antiporters in the cytoplasmic membranes of cells and organellar membranes throughout the prokaryotic and eukaryotic kingdoms is apparent. This wide occurence is irrespective of the mode of energization of the membranes; the antiporters are engaged in maintaining a secondary cycle of either Na^+ or H^+; depending on the conditions, both cycles can even alternate in a single cell.

The paradigm set by the major advances achieved in the human (*nhe*) and *E. coli* (*nha*A and *nha*B) antiporters emphasizes the importance of combined approaches, molecular biology and biochemistry, in resolving the structure and function relationship of the Na^+/H^+ antiporters, their regulation and their major

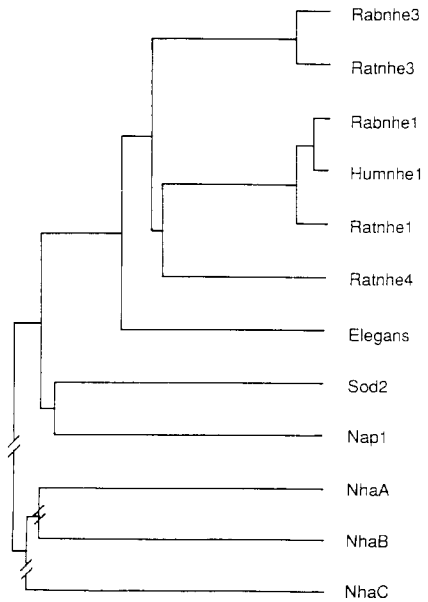

Fig. 2. Evolutionary relationship between Na^+/H^+ antiporters. The dendogram was constructed using the LINE UP algorithm with the following sequences: Rabnhe1 and 3: Nhe1 and Nhe3 antiporters from rabbit (Accession No. X61504 and M87007); Humnhe1: Nhe1 from man (Accession No. JO3163); Ratnhe1, 3 and 4: Nhe1, 3 and 4 from rat (Accession No. M85299, M85300, M85301); Elegans: putative antiporter from *C. elegans* (Accession No. M23064); Sod2: from *S. pombe* (Accession No. S99129); Nap1: from *Enterococcus hirae* (Accession No. 587401); NhaA and NhaB: from *Escherichia coli* (Accession No. JO3879, M83655); NhaC: from *B. firmus* (Accession No. M73530). As seen in the figure, the various Nhe antiporters from rabbit, rat and man are clearly related to one another and the one from *C. elegans* is in the same family. Although showing a much lower degree of homology the proteins from *E. hirae* and *S. pombe* display weak but possibly significant relationship to the Nhe family. NhaA, B and C show only remote homologies, which do not suggest their belonging to the same family, even though they are included in the plot.

roles in homeostasis of pH, Na^+ and volume of cells. These studies showed that, although a single polypeptide comprises an antiporter, there are many different polypeptides with Na^+/H^+ exchange activity. Only in the higher eukaryotes (from *C. elegans* to human) there is a distinct homology between the various antiporters (Fig. 2), which is reflected in their similar properties and function. Using *nha*A as a DNA probe to screen DNA of many bacteria by Southern hybridization, it has been shown that *nha*A is widely spread among *Enterobacteriaceae* [33] but no hybridization was found with other groups of bacteria. *nha*B shows similar spread pattern as *nha*A (Kotler, Y., Schuldiner, S. and Padan, E., unpublished results). However, strikingly, there is very little homology between NhaA ad NhaB proteins and the other putative prokaryotic antiporters or the eukaryotic Nhe family (Fig. 2). Does this mean that the residues essential for activity are very few and can be fulfilled by various different combinations? Cloning of additional antiporter genes and identification of the residues involved in the active site of the Na^+/H^+ antiporters are instrumental to answer this qestion.

The multiplicity of antiporters in one cell implicates that the understanding of their integrative regulation is a key to the understanding of their role in cell physiology. Both NhaA and Nhe are involved in an intricate signal transduction. Furthermore as predicted from the central role of the antiporters in pH homeostasis the activity of many is dramatically regulated by pH. This regulation is adjusted to the pH range through which the Na^+/H^+ activity is required; Nhe1 progressively alkalinizes the cytoplasm below pH 7; NhaA progressively acidifies it with increasing pH above pH 7.6.

Histidine at position 226 has been shown to participate in the pH sensor of NhaA. Further study of this pH sensor and that of other antiporters with different pH profiles is most intriguing.

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References

[1] Mitchell, P. (1961) Nature 191, 144–146.
[2] Mitchell, P. and Moyle, J. (1967) Biochem. J. 105, 1147–1162.
[3] Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 9, 149–155.
[4] Harold, F.M. and Papineau, D. (1972) J. Membr. Biol. 8, 45–62.
[5] Murer, H., Hopfer, U. and Kinne, R. (1976) Biochem. J. 154: 597–604.
[6] Padan, E. and Schuldiner, S. (1992) in Alkali Cation Transport Systems in Prokaryotes (Bakker, E.P., ed.), pp. 3–24, CRC Press, Boca Raton.
[7] Schuldiner, S. and Padan, E. (1992) in Alkali Cation Transport Systems in Prokaryotes (Bakker, E.P., ed.), pp. 25–51, CRC Press, Boca Raton.
[8] Schuldiner, S. and Padan, E. (1993) Int. Rev. Cytol. 137C, 229–266.
[9] Nakamura, T., Hsu, C. and Rosen, B.P. (1986) J. Biol. Chem. 261, 678–683.
[10] Tsuchiya, T., Misawa, A., Miyaka, Y., Yamasaki, K., and Niiya, S. (1982) FEBS Lett. 142, 231–234.
[11] Taglicht, D., Padan, E. and Schuldiner, S. (1991) J. Biol. Chem. 266, 11289–11294.
[12] Macnab, R.M. and Castle, A.M. (1987) Biophys. J. 52, 637–647.
[13] Zilberstein, D., Padan, E. and Schuldiner, S. (1980) FEBS Lett. 116, 177–180.
[14] Ishikawa, T., Hama, H., Tsuda, M. and Tsuchiya, T. (1987) J. Biol. Chem. 262, 7443–7446.
[15] McMorrow, I., Shuman, H.A., Sze, D., Wilson, D.M. and Wilson, T.H. (1989) Biochim. Biophys. Acta 981, 21–26.
[16] Rowland, G.C., Giffard, P.M. and Booth, I.R. (1984) FEBS Lett. 173, 295–300.
[17] Giffard, P.M., Rowland, G.C., Kroll, R.G., Stewart, L.M.D., Bakker, E.P. and Booth, I.R. (1985) J. Bacteriol. 164, 904–910.
[18] Thomas, M.S. and Glass, R.E. (1991) Mol. Microbiol. 5, 2719–2725.
[19] Pinner, E., Kotler, Y., Padan, E., and Schuldiner, S. (1993) J. Biol. Chem. 268, 1729–1734.
[20] Ohyama, T., Imaizumi, R., Igarashi, K. and Kobayashi, H. (1992) J. Bacteriol. 174, 7743–7749.
[21] Niiya, S., Yamasaki, K., Wilson, T.H. and Tsuchiya, T. (1982) J. Biol. Chem. 257, 8902–8906.
[22] Umeda, K., Shiota, S., Futai, M. and Tsuchiya, T. (1984) J. Bacteriol. 160, 812–814.
[23] Yazyu, H., Shiota, S., Futai, M. and Tsuchiya, T. (1985) J. Bacteriol. 162, 933–937.
[24] Goldberg, B.G., Arbel, T., Chen, J., Karpel, R., Mackie, G.A., Schuldiner, S. and Padan, E. (1987) Proc. Natl. Acad. Sci. USA 84, 2615–2619.
[25] Karpel, R., Olami, Y., Taglicht, D., Schuldiner, S. and Padan, E. (1988) J. Biol. Chem. 263, 10408–10414.
[26] Mackie, G.A. (1980) J. Biol. Chem. 255, 8928–8935.
[27] Mackie, G.A. (1986) Nucleic Acids Res. 14, 6965–6981.
[28] Padan, E., Maisler, N., Taglicht, D., Karpel, R. and Schuldiner, S. (1989) J. Biol. Chem. 264, 20297–20302.
[29] Karpel, R. (1990)Ph.D. Thesis, Hebrew University, Jerusalem.
[30] Pinner, E., Padan, E. and Schuldiner, S. (1992) J. Biol. Chem. 267, 11064–11068.
[31] Brey, R.N., Beck, J.C. and Rosen, B.P. (1978) Biochem. Biophys. Res. Commun. 83, 1588–1594.
[32] Plack, R.H., Jr. and Rosen, B.P. (1980) J. Biol. Chem. 255, 3824–3825.
[33] Pinner, E., Carmel, O., Bercovier, H., Sela, S., Padan, E. and Schuldiner, S. (1992) Arch. Microbiol. 157, 323–328.
[34] Ivey, D.M., Guffanti, A.A., Bossewitch, J.S., Padan, E. and Krulwich, T.A. (1991). J. Biol. Chem. 266, 23483–23489.
[35] Ivey, D.M., Guffanti, A.A., Zemsky, J., Pinner, E., Karpel, R., Padan, E., Schuldiner, S. and Krulwich, T.A. (1993) J. Biol. Chem. 268, 11296–11303.
[36] Tabor, S. and Richardson, C.C. (1985) Proc. Natl. Acad. Sci. USA 82, 1074–1078.

[37] Poulsen, L.K., Larsen, N.W., Molin, S. and Andersson, P. (1989) Mol. Microbiol. 3, 1463–1472.
[38] Rahav-Manor, O., Carmel, O., Karpel, R., Taglicht, D., Glaser, G., Schuldiner, S. and Padan, E. (1992) J. Biol. Chem. 267, 10433–10438.
[39] Thelen, P., Tsuchiya, T. and Goldberg, E.B. (1991) J. Bacteriol. 173, 6553–6557.
[40] Devereux, J., Haeberli, P. and Smithies, O. (1984) Nucleic Acids Res. 12, 387–395.
[41] Padan, E., Arbel, T., Rimon, A., Bar-Shira, A. and Cohen, A. (1983) J. Biol. Chem. 258, 5666–5673.
[42] Borbolla, M.G. and Rosen, B.P. (1984) Arch. Biochem. Biophys. 22, 98–103.
[43] Bassilana, M., Damiano, E. and Leblanc, G. (1984) Biochemistry 23, 5288–5294.
[44] Bassilana, M., Damiano, E. and Leblanc, G. (1984) Biochemistry 23, 1015–1022.
[45] Schuldiner, S. and Fishkes, H. (1978) Biochemistry 17, 706–710.
[46] Castle, A.M., Macnab, R.M. and Schulman, R.G. (1986) J. Biol. Chem. 261, 3288–3294.
[47] Castle, A.M., Macnab, R.M. and Schulman, R.G. (1986) J. Biol. Chem. 261, 7797–7806.
[48] Taglicht, D., Padan, E. and Schuldiner, S. (1993) J. Biol. Chem. 268, 5382–5387.
[49] West, I.C. and Mitchell, P. (1974) Biochem. J. 144, 87–90.
[50] Pan, J.W. and Macnab, R.M. (1990) J. Biol. Chem. 265, 9247–9250.
[51] Beck, J.C. and Rosen, B.P. (1979) Arch. Biochem. Biophys. 194, 208–214.
[52] Mochizuki-Oda, N. and Oosawa, F. (1985) J. Bacteriol. 163, 395–397.
[53] Leblanc, G., Bassilana, M. and Damiano, E. (1988) in Na^+/H^+ exchange (Grinstein, S., ed.), pp. 103–117, CRC Press.
[54] Taglicht, D. (1992) Ph.D. Thesis, Hebrew University of Jerusalem.
[55] Padan, E., Zilberstein, D. and Rottenberg, H. (1976) Eur. J. Biochem. 63, 533–541.
[56] Zilberstein, D., Schuldiner, S. and Padan, E. (1979) Biochemistry 18, 669–673.
[57] Cabantchik, Z.I. (1990) in Blood Cell Biochemistry, 1. Erythroid Cells (Harris, J.R., ed.), pp. 359–364, Plenum Press, New York and London.
[58] Padan, E., Patel, L. and Kaback, H.R. (1979) Proc. Natl. Acad. Sci. USA 76, 6221–6225.
[59] Padan, E., Sarkar, H.K., Viitanen, P.V., Poonian, M.S. and Kaback, H.R. (1985). Proc. Natl. Acad. Sci. USA 82, 6765–6768.
[60] Kaback, H.R. (1988) Annu. Rev. Physiol. 50, 243–256.
[61] Okamura, M.Y. and Feher, G. (1992) Annu. Rev. Biochem. 61, 861–896.
[62] Damiano, E., Bassilana, M. and Leblanc, G. (1985) Eur. J. Biochem. 148, 183–188.
[63] Gerchman, Y., Olami, Y., Rimon, A., Taglicht, D., Schuldiner, S. and Padan, E. (1993) Proc. Natl. Acad. Sci. USA 90, 1212–1216.
[64] Sardet, C., Counillon, L., Franchi, A. and Pouyssegur, J. (1990) Science 247, 723–726.
[65] Karpel, R., Alon, T., Glaser, G., Schuldiner, S. and Padan, E. (1991) J. Biol. Chem. 266, 21753–21759.
[66] Silver, S. and Walderhaug, M. (1992) Microbiol. Rev. 56, 195–228.
[67] Padan, E., Zilberstein, D. and Schuldiner, S. (1981) Biochim. Biophys. Acta 650, 151–166.
[68] Booth, I.R. (1985) Microbiol. Rev. 49, 395–378.
[69] Padan, E. and Schuldiner, S. (1986) Meth. Enzymol. 125, 337–352.
[70] Slonczewski, J.L., Macnab, R.M., Alger, J.R. and Castel, A. (1982) J. Bacteriol. 152, 384–399.
[71] Zilberstein, D., Agmon, V., Schuldiner, S. and Padan, E. (1984) J. Bacteriol. 158, 246–252.
[72] Kashket, E.R. (1983) FEBS Lett. 154, 343–346.
[73] Pouyssegur, J., Sardet, C., Franchi, A., L'Allemain, G. and Paris, S. (1984) Proc. Natl. Acad. Sci. USA 81, 4833–4837.
[74] Grinstein, S., Rotin, D. and Mason, M.J. (1989) Biochim. Biophys. Acta 988, 73–97.
[75] Krulwich, T.A. (1986) J. Membr. Biol. 89, 113–125.
[76] Slonczewski, J.L., Rosen, B.P., Alger, S.R. and Macnab, R.M. (1981) Proc. Natl. Acad. Sci. USA 78, 6271–6275.
[77] Bingham, R.J., Hall, K.S. and Slonczewski, J.L. (1990) J. Bacteriol. 172, 2184–2186.
[78] Olson, E.R. (1993) Mol. Microbiol. 8, 5–14.
[79] Schuldiner, S., Agmon, V., Brandsma, J., Cohen, A., Friedman, E. and Padan, E. (1986) J. Bacteriol. 168, 936–939.
[80] Padan, E. and Schuldiner, S. (1987) J. Membrane Biol. 95, 189–198.
[81] Grinstein, S. (ed.) (1988) in Na^+/H^+ Exchange CRC Press, Boca Raton, Fl.
[82] Grinstein, S. and Furuya, W. (1988) Am. J. Physiol. 23, C272-C285.
[83] Sardet, C., Franchi, A. and Pouyssegur, J. (1989) Cell 56, 271–280.
[84] Slonczewski, J.L. (1992). pH-regulated genes in enteric bacteria. ASM News 58, 140–144.
[85] Kobayashi, H., Murakami, H. and Unemoto, T. (1982) J. Biol. Chem 257, 13246–13252.
[86] Kobayashi, H. (1985) J. Biol. Chem. 260, 72–76.
[87] Kakinuma, Y. (1987) J. Bacteriol. 169, 4403–4405.
[88] Kashket, E.R. (1981) J. Bacteriol. 146, 377–384.
[89] Krulwich, T.A., Guffanti, A.A., Bornstein, R.F. and Hoffstein, T. (1982) J. Biol. Chem. 257, 1885–1889.
[90] Krulwich, T.A., Federbush, J.G. and Guffanti, A.A. (1985) J. Biol. Chem. 260, 4055–4058.
[91] McLaggan, D., Selwyn, M.Y. and Dawson, A.P. (1984) FEBS Lett. 165, 254–258.
[92] Sugiyama, S.H., Matsukura, H. and Imae, Y. (1985) FEBS Lett. 182, 265–268.
[93] Bakker, E.P. and Mangerich, W.E. (1981) J. Bacteriol. 147, 820–826.
[94] Walderhaug, M.O., Dosch, D.C. and Epstein, W. (1987) in Ion Transport in Prokaryotes (Rosen, B.P. and Silver, S., eds.), p. 85, Academic Press, San Diego.
[95] Rosen, B.P. (1986) Annu. Rev. Microbiol. 40, 263–286.
[96] Mugikura, S., Nishikawa, M., Igarashi, K. and Kobayashi, H. (1990) J. Biochem. 108, 86–91.
[97] Kinoshita, N., Unemoto, T. and Kobayashi, H. (1984) J. Bacteriol. 160, 1074–1077.
[98] Gold, L. and Stromo, G. (1987) American Society for Microbiology, Washington, D.C., pp. 1302–1307.
[99] Henikoff, S., Haughn, G.W., Calvo, J.M. and Wallace, J.C. (1988) Proc. Natl. Acad. Sci. USA 85, 6602–6606.
[100] Christman, M.F., Storz, G. and Ames, B.N. (1989) Proc. Natl. Acad. Sci. USA 86, 3484–3488.
[101] Storz, G., Tartaglia, L.A., Farr, S.B. and Ames, B.N. (1990) Trends Genet. 6, 363–368.
[102] Shewchuk, L.M., Verdine, G.L. and Walsh, C.T. (1989) Biochemistry 28, 2331–2339.
[103] Helmann, J.D., Wang, L., Mahler, I. and Walsh, C.T. (1989) J. Bacteriol. 171, 222–229.
[104] Storz, G., Tartaglia, L.A. and Ames, B.N. (1990) Science 248, 189–194.
[105] Straus, D.B., Waller, W.A. and Gross, C.A. (1987) Nature 329, 348–351.
[106] Lanyi, J.K. (1979) Biochim. Biophys. Acta 559, 377–398.
[107] Krulwich, T.A. and Ivey, D.M. (1990) in Bacterial Energetics (Krulwich, T.A., ed.), pp. 417–447. Academic Press

[108] Krulwich, T.A., Guffanti, A.A. and Seto-Young, D. (1990) FEMS Microbiol. Rev. 75, 271–278.
[109] Krulwich, T.A. and Guffanti, A.A. (1992) J. Bioenerg. Biomemb. 24, 587–599.
[110] Krulwich, T.A. and Guffanti, A.A. (1989) Annu. Rev. Microbiol. 43, 435–463.
[111] Hirota, N., Kitada, M. and Imae, Y. (1981) FEBS Lett. 132, 278–280.
[112] Imae, Y. and Atsumi, T. (1989) J. Bioenerg. Biomemb. 21, 705–716.
[113] Reed, R.H. (1986) in Microbes in Extreme Environments, (Herbert, R.A. and Codd, G.A., eds.), pp. 55–81. Academic Press, London.
[114] Krulwich, T.A., Hicks, D.B., Seto-Young, D. and Guffanti, A.A. (1988) Crit. Rev. Microbiol. 16, 15–36.
[115] Hoffmann, A. and Dimroth P. (1991) Eur. J. Biochem. 201, 467–473.
[116] Kushner, D.J. and Kamekura, M. (1988) in Halophilic Bacteria (Rodriguez-Valira, F., ed.), Vol. 1, pp. 109–138, CRC Press, Boca Raton, Florida.
[117] Murakami, N. and Konishi, T. (1989) Arch. Biochem. Biophys. 271, 515–523.
[118] Konishi, T. and Murakami, N. (1990) Biochem. Biophys. Res. 170, 1339–1345.
[119] Niwano, K., Murakami, N., Okazawa, K. and Konishi, T. (1991) Biochem. International 25, 173–179.
[120] Lanyi, J.K. and MacDonald, R.E. (1976) Biochemistry 15, 4608–4614.
[121] Eisenbach, M., Cooper, B.S., Garty, H., Jhonstone, R.M., Rottenberg, H. and Kaplan, S.R. (1977) Biochim. Biophys. Acta 465, 599–613.
[122] Konishi, T. Murakami, N. and Miyazawa, Y. (1992) Biochim. Biophys. Acta 1140, 1–5.
[123] Murakami, N. and Konishi, T. (1990) Arch. Biochem. Biophys. 281, 13–20.
[124] Garcia, M.L., Guffanti, A.A. and Krulwich, T.A. (1983) J. Bacteriol. 156, 1151–1157.
[125] Krulwich, T.A., Mandel, K.G., Bornstein, R.F. and Guffanti, A.A. (1979) Biochem. Biophys. Res. Commun. 91, 58–62.
[126] Koyama, N., Ishikawa, Y. and Noshoh, Y. (1986) FEMS Microbiol. Lett. 34, 193–196.
[127] Kitada, M., Onda, K. and Horikoshi, K. (1989) J. Bacteriol. 171, 1879–1884.
[128] Krulwich, T.A. (1983) Biochim. Biophys. Acta 726, 245–264.
[129] Ivey, D.M., Guffanti, A.A. and Krulwich, T.A. (1992) in Alkali Cation Transport Systems in Prokaryotes (Bakker, E.P., ed.), pp. 101–124, CRC Press, Boca Raton.
[130] Kitada, M. and Horikoshi, K. (1992) J. Bacteriol. 174, 5936–5940.
[131] Kudo, T., Hino, M., Kitada, M. and Horikoshi, K. (1990) J. Bacteriol. 172, 7282–7283.
[132] Ivey, D.M., Guffanti, A.A., Shen, Z., Kudyan, N. and Krulwich, T.A. (1992). J. Bacteriol. 174, 4878–4884.
[133] Dimroth, P. (1992) in Alkali Cation Transport Systems in Prokaryotes (Bakker, E.P., ed.), pp. 77–100, CRC Press, Boca Raton.
[134] Dimroth, P. (1992) in Alkali Cation Transport Systems in Prokaryotes (Bakker, E.P., ed.), pp. 139–154, CRC Press, Boca Raton.
[135] Dimroth, P. (1987) Microbiol. Rev. 51, 320–340.
[136] Heefner, D.L. and Harold, F.M. (1980) J. Biol. Chem. 255, 11396–11402.
[137] Heefner, D.L. and Harold, F.M. (1982) Proc. Natl. Acad. Sci. USA 79, 2798–2802.
[138] Schonheit, P. (1992) in Alkali Cation Transport Systems in Prokaryotes (Bakker, E.P., ed.), pp. 179–202, CRC Press, Boca Raton.
[139] Muller, V. and Gottschalk, G. (1992) in Alkali Cation Transport Systems in Prokaryotes (Bakker, E.P., ed.), pp. 155–177, CRC Press, Boca Raton.
[140] Tokuda, H. (1992) in Alkali Cation Transport Systems in Procaryotes (Bakker, E.P., ed.), pp. 125–138, CRC Press, Boca Raton.
[141] Unemoto, T., Tokuda, H. and Hayashi, M. (1990) in Bacterial Energetics (Krulwich, T.A., ed.), pp. 33–54. Academic Press.
[142] Ken-Dror, S., Lanyi, J.K., Schobert, B., Silver, B. and Avi-Dor, Y. (1986) Arch. Biochem. Biophys. 244, 766–772.
[143] Skulachev, V.P. (1987) in Ion Transport in Procaryotes (Rosen, B.P. and Silver, S., eds), pp. 131–164, Academic Press, New York.
[144] Skulachev, V.P. (1988) in Membrane Bioenergetics, pp. 293–326, Springer, Berlin.
[145] Dibrov, P.A., Lazarova, R.L., Skulachev, V.P. and Verkhovskaya, M.L. (1986). Biochim. Biophys. Acta 850, 458–465.
[146] Sakai, Y., Moritani, C., Tsuda, M. and Tsuchiya, T. (1989) Biochim. Biophys. Acta 973, 450–456.
[147] Sakai-Tomita, Y., Tsuda, M. and Tsuchiya, T. (1991) Biochem. Biophys. Res. Commun. In press.
[148] Dibrov, P.A. (1991) Biochim. Biophys. Acta 1056, 209–224.
[149] Avetisyan, A.V., Bogachev, A.V., Murtasina, R.A. and Skulachev, V.P. (1992) FEBS Lett. 306, 199–202.
[150] Avetisyan, A.V., Bogachev, A.V., Murtasina, R.A. and Skulachev, V.P. (1993) FEBS Lett. 317, 267–270.
[151] Skulachev, V.P. (1984) Trends Biochem. Sci. 9, 483–485.
[152] Tokuda, H. (1989) J. Bioenerg. Biomembr. 21, 693–704.
[153] Tokuda, H. and Unemoto, T. (1985) Microbiol. Sci. 2, 65–71.
[154] Nakamura, T., Tokuda, H. and Unemoto, T. (1984) Biochim. Biophys. Acta 776, 330–336.
[155] Laubinger, W. and Dimroth, P. (1988) Biochemistry 27, 7531–7537.
[156] Dimroth, P. (1991) Bio. Essays 13, 463–468.
[157] Muller, V., Blaut, M. and Gottschalk, G. (1987) Eur. J. Biochem. 162, 461–466.
[158] Becher, B., Muller, V. and Gottschalk, G. (1992) FEMS Microb. Lett. 91, 239–244.
[159] Kobayashi, H., Suzuki, T., Konoshita, N. and Unemoto, T. (1984) J. Bacteriol. 158, 1157–1160.
[160] Shibata, C., Ehara, T., Tomura, K., Igarashi, K. and Kobayashi, H. (1992) J. Bacteriol. 174, 6117–6124.
[161] Kakinuma, Y., Igarashi, K., Konishi, K. and Yamato, I. (1991) FEBS. Lett. 292, 64–68.
[162] Kakinuma, Y. (1987) J. Bacteriol. 169, 3886–3890.
[163] Waser, M., Hess-Bienz, D., Davies, K. and Solioz, M. (1992) J. Biol. Chem. 267, 5396–5400.
[164] Reizer, J., Reizer, A. and Saier, Jr., M.H. (1992) FEMS Microbiol. Lett. 94, 161–164.
[165] Speelmans, G., Poolman, B., Abee, T. and Konings, W.N. (1993) Proc. Natl. Acad. Sci. U.S.A (In press).
[166] Sussman, M.R. and Harper, J.F. (1989) Plant Cell 1, 953–960.
[167] Frommer, W.B., Hummel, S. and Riesmeier, J.W. (1993) Proc. Natl. Acad. Sci. U.S.A (In press).
[168] Katz, A., Bental, M., Degani, H. and Avron, M. (1991) Plant Physiol. 96, 110–115.
[169] Haro, R., Garciadeblas, B. and Rodriguez-Navarro, A. (1991) FEBS Lett. 291, 189–191.
[170] Katz, A., Kaback, H.R. and Avron, M. (1986) FEBS Lett. 202, 141–144.
[171] Katz, A., Pick, U. and Avron, M. (1989) Biochim. Biophys. Acta. 983, 9–14.
[172] Katz, A., Pick, U. and Avron, M. (1992) Plant Physiol. 100, 1224–1229.
[173] Rodriguez-Navarro, A. and Asensio, J. (1977) FEBS Lett. 75, 169–172.

[174] Rodriguez-Navarro, A. and Ortega, M.D. (1982) FEBS Lett. 138, 205–208.
[175] Rodriguez-Navarro, A., Sancho, E.D. and Pertez-Lloveres, C. (1981). Biochim. Biophys. Acta 640, 352–358.
[176] Ortega, M.D. and Rodriguez-Navarro, A. (1986) Physiol. Plant. 66, 705–711.
[177] Jia, Z.P., McCullough, N., Martel, R., Hemmingsen, S. and Young, P.G. (1992) EMBO J. 11, 1631–1640.
[178] Braun, Y.M., Hassidim, H., Lerner, R. and Reinhold, L. (1988) Plant Physiol. 87, 104–108.
[179] Hassidim, M., Braun, Y., Lerner, H.R., Reinhold, L. (1990) Plant Physiol. 94, 1795–1801.
[180] Clint, G.M. and MacRobbie, E.A.C. (1987) Planta 171, 247–253.
[181] Ratner, A. and Jacoby, B. (1976) J. Exp. Bot. 27, 843–854.
[182] Jacoby, B. and Teomi, S. (1988) Plant Sci. 54, 103–106.
[183] Cooper, S., Lerner, H.R. and Reinhold, L. (1991) Plant Physiol. 97, 1212–1220.
[184] Reid, R.J., Smith, F.A. and Whittington, J. (1989) J. Exp. Bot. 40, 883–891.
[185] Jacoby, B. and Rudich, B. (1980) Ann. Bot. 46, 493–498.
[186] Jacoby, B. and Rudich, B. (1985) Plant Cell Environ. 8, 235–238.
[187] Mennen, H., Jacoby, B. and Marschner, H. (1990) J. Plant Physiol. 137, 180–183.
[188] Sze, H. (1985) Annu. Rev. Plant Physiol. 36, 175–208.
[189] Blumwald, E. (1987) Plant Physiology 69, 231–734.
[190] Blumwald, E. Cragoe, E.J., Jr., and Poole, R.J. (1987) Plant Physiol. 85, 30–33.
[191] Rea, P.A. and Sanders, D. (1987) Physiol. Plant 71, 131–141.
[192] Fan, T.W.M., Higashi, R.M., Norlyn, J. and Epstein, E. (1989) Proc. Natl. Acad. Sci. USA 86, 9856–9860.
[193] Blumwald, E. and Poole, R.J. (1985) Plant Physiology 78, 163–167.
[194] Garbarino, J. and Dupont, F.M. (1988) Plant Physiol. 86, 231–236.
[195] Niemiets, C. and Willenbrink, J. (1985) Planta 166, 545–549.
[196] Guern, J., Malthieu, Y., Kurkdjian, A., Manigault, P., Manigault, J., Gillet, B., Beloeil, J.C. and Lallemand, J.Y. (1989) Plant Physiol. 89, 27–36.
[197] Barkla, B.J., Charuk, J.H.M., Cragoe, E.J., Jr. and Blumwald, E. (1990) Plant Physiol. 93, 924–930.
[198] Barkla, B.J. and Blumwald, E. (1991) Proc. Natl. Acad. Sci. USA 88, 11177–11181.
[199] Barkla, B.J. and Blumwald, E. (1992) in Research in Photosynthesis, Vol. IV, 219–226, Kluwer, Dordrecht.
[200] Jacoby, B. (1993) in Handbook of Plant and Crop Stress, Vol. 1 (Pessarakli, M., ed.), Marcel Dekker, New York, in press.
[201] Aronson, P.S. (1985) Annu. Rev. Physiol. 47, 545–560.
[202] Moolenaar, W.H. (1986) Annu. Rev. Physiol. 48, 363–376.
[203] Haussinger, D. (1988) in pH homeostasis – mechanisms and control, pp. 1–479, Academic Press, San Diego.
[204] Wakabayashi, S., Sardet, C., Fafournoux, P., Pouyssegur, J. (1992) Rev. Physiol. Biochem. Pharmacol. 119, 157–186.
[205] Pouyssegur, J., Franchi, A., Lagarde, A. and Sardet, C. (1988) In Na^+/H^+ Exchange, (Grinstein, S., ed.), pp. 337–347, CRC Press.
[206] Franchi, A., Cragoe, E. Jr. and Pouyssegur, J. (1986) J. Biol. Chem. 261, 14614–14620.
[207] Franchi, A., Perucca-Lostanlen, D., and Pouyssegur, J. (1986) Proc. Natl. Acad. Sci. USA 83, 9388–9392.
[208] Tse, C.M., Ma, A.I., Yang, V.W., Watson, A.J.M., Levine, S., Montrose, M.H., Potter, J., Sardet, C., Pouyssegur, J. and Donowitz, M. (1991) EMBO J. 10, 1957–1967.
[209] Tse, C.M., Brant, S.R., Walker, M.S., Pouyssegur, J. and Donowitz, M. (1992) J. Biol. Chem. 267, 9340–9346.
[210] Orlowski, J., Kandasamy, R.A. and Shull, G.E. (1992) J. Biol. Chem. 267, 9331–9339.
[211] Benos, D.J. (1988 in Na^+/H^+ exchange (Grinstein, S., ed.), pp. 121–136, CRC Press.
[212] Marra, M.A., Prasad, S.S. and Baillie, D.L. (1992) Mol. Gen. Genet., in press.
[213] Zhuang, Y., Cragoe, E.J., Shaikewitz, T., Glaser, L. and Cassel, D. (1984) Biochemistry 23, 4481–4488.
[214] Haggerty, J.G., Agarwal, N., Reilly, R.F., Adelberg, E.A. and Slayman, C.W. (1988) Proc. Natl. Acad. Sci. USA 85, 6797–6801.
[215] Raley-Susman, K.M., Cragoe, E.J., Sapolsky, R.M. and Kopito, R.R. (1991) J. Biol. Chem. 266, 2739–2745.
[216] Counillon, L., Franchi, A. and Pouyssegur, J. (1993) Proc. Natl. Acad. Sci. USA 90, 4508–4512.
[217] Wright, S.H. and Wunz, T.M. (1989) Am. J. Physiol. 256, F462–F468.
[218] Prasad, P.D., Leibach, F.H., Mahesh, V.B. and Ganapathy, V. (1992) J. Biol. Chem. 267, 23632–23639.
[219] Aronson, P.S., Nee, J. and Suhm, M.A. (1982) Nature 299, 161–163.
[220] Schuldiner, S. and Rozengurt, E. (1982) Proc. Natl. Acad. Sci. USA 79, 7778–7782.
[221] Sardet, C., Fafournoux, P. and Pouyssegur, J. (1991) J. Biol. Chem. 29, 19166–19171.
[222] Sardet, C., Wakabayashi, S., Fafournoux, P. and Pouyssegur, J. (1991) in Biological Signal Transduction (Ross, E.M. and Wirtz, K.W.A., eds.), pp. 253–269, Springer.
[223] Wakabayashi, S., Sardet, C., Fafournoux, P., Pouyssegur, J. (1992) Proc. Natl. Acad. Sci. USA 89, 2424–2428.
[224] Grinstein, S., Woodside, M., Sardet, C., Pouyssegur, J. and Rotin, D. (1992) J. Biol. Chem. 267, 23823–23828.
[225] Epel, D. (1988) in Na^+/H^+ Exchange (ed. S. Grinstein), pp. 209–223.
[226] Ahearn, G.A. and Franco, P. (1990) Am. J. Physiol. 259, F758–F767.
[227] Ahearn, G.A. and Franco, P. (1991) J. Exp. Biol. 158, 495–507.
[228] Ahearn, G.A., Franco, P. and Clay, L.P. (1990) J. Membrane Biol. 116, 215–226.
[229] Shetlar, R.E. and Towle, D.W. (1989) Am. J. Physiol. 257, R924–R931.
[230] Li, X., Hegazy, M.G., Mahdi, F., Jezek, P., Lanes, R.D. and Garlid, K.D. (1990) J. Biol. Chem. 265, 15316–15322.
[231] Garlid, K.D., Shariat-Madar, Z., Nath, S. and Jezek, P. (1991) J. Biol. Chem. 266, 6518–6523.
[232] Haigh, J.R. and Phillips, J.H. (1989) Biochem. J. 257, 499–507.
[233] Gurich, R.W. and Warnock, D.G. (1986) Am. J. Physiol. 251, F702–F709.
[234] Mircheff, A.K., Ives, H.E., Yee, V.J. and Warnock, D.G. (1984) Am. J. Physiol. 246, F853–F858.
[235] Sabolic, I., Haase, W. and Burckhardt, G. (1985) Am. J. Physiol. 248, F835–F844.