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Review

The role of sodium ion transport in *Escherichia coli* energetics

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I. Introduction

Escherichia coli is considered to be classical example of a bacterium using a proton cycle, but not a sodium cycle, for energy conversion. Nevertheless, the *E. coli* cell maintains the nonequilibrium transmembrane Na^+ distribution ($[Na^+]_{out} > [Na^+]_{in}$) in a wide range of $[Na^+]_{out}$ concentrations [27]. As shown in numerous studies, an inwardly directed Na^+ gradient is supported by the $\Delta\tilde{\mu}_H$-driven Na^+/H^+ antiport [57,83–84,148]. Recently vital progress has been made in the study of *E. coli* Na^+–H^+ exchange genetics: the *ant* gene which, if cloned in multicopy plasmid, stimulates Na^+/H^+ antiport activity has been cloned and sequenced [50,71]. The authors suggest that the *ant* product is the Na^+/H^+

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antiporter or, at least, one of its subunits. Unfortunately, efforts to isolate and characterize the corresponding protein have failed. It is still an open question whether the Na^+/H^+ antiporter of *E. coli* is electroneutral [141,149] or electrogenic, i.e., exchanging more than one proton per sodium ion [10,14,118]. Most likely, the apparent stoichiometry of exchange depends on pH of the medium [118].

The electrochemical sodium gradient ($\Delta\tilde{\mu}_{Na}$) formed on the *E. coli* membrane can be used by the cell for driving the uphill transport of melibiose [138], glutamate [59], proline [128], serine [53] and, possibly, succinate [113]. Since the Na^+/H^+ antiporter is reversible, $\Delta\tilde{\mu}_{Na}$ may be converted to ΔpH, which allows maintenance of the protonmotive force at a sufficiently high level for some time in case of a deficiency of energy substrates [23,118,124]. In addition, the transmembrane sodium ion circulation seems to play the major role in the intracellular pH homeostasis when bacteria grow in an alkaline environment [64,108]. As to the mechanical work of flagellar rotation, $\Delta\tilde{\mu}_H$ serves as an energy source for this in *E. coli* [78], in contrast to a number of halo- and alkalotolerant microorganisms, whose motility is directly energized by $\Delta\tilde{\mu}_{Na}$ [30,34,62,63]. $\Delta\tilde{\mu}_H$ was also found to be utilized by the reversible F_0F_1-type ATPase for the ATP synthesis in *E. coli* (see reviews in Refs. 55, 56, 124).

So, *E. coli* membrane energetics are of dual character: $\Delta\tilde{\mu}_H$ produced by primary proton pumps is utilized for the formation of nonequilibrium transmembrane sodium ion distribution; Na^+ is involved in the osmotic work, pH regulation, and $\Delta\tilde{\mu}_H$-buffering. In the past few years a number of laboratories have been obtaining evidence on the possibility of *E. coli* growth in the presence of uncoupling concentrations of protonophores [65–68,80,106,120]. In this context, a problem arises of oxidative phosphorylation at low levels of the protonmotive force. The same situation is obtained when *E. coli* grows at alkaline pH. Since $\Delta\psi$ and ΔpH are oppositely directed in this case, the protonmotive force decreases significantly as a result. The energetic dualism mentioned above seems to be of more profound nature, a factor which we ought to take into consideration in tackling this problem. Indeed, when fermentable substrates are absent, $\Delta\tilde{\mu}_H$ lowering to some critical value induced by the presence of an uncoupler or by high pH_{out} may serve as a signal for the respiring cell to switch its energetics from the proton to the sodium cycle. Below, we shall discuss possible mechanisms of such an event. The possibility of the substitution of the sodiummotive force for the protonmotive force to maintain such a vital energy-dependent function as oxidative phosphorylation would essentially increase the adaptive ability of the *E. coli* cell. Evidently, such profound reorganization of metabolism must be controlled by a high-level regulatory system. Significantly, certain data on the dependence of *E. coli* growth at high pH on the cAMP exchange system have been reported recently [2]. The possibility of the existence of an inducible Na^+ cycle in *E. coli* is an intriguing one that deserves further exploration.

II. Na^+/H^+ antiport in *Escherichia coli*

II-A. Energetics of Na^+ export from intact cells

The cation/proton (including Na^+/H^+) exchange was predicted by Mitchell [97]. The bacterium *Streptococcus faecalis* was the first procaryotic object for which the Na^+/H^+ exchange was experimentally demonstrated, in 1972 [57]. 2 years later, West and Mitchell reported that, at pH 7.1, the addition of O_2 pulse to an anaerobic *E. coli* cells suspension induced transitory acidification of the incubation medium [149]. The recurrent phase of this acidification was accelerated in the presence of Na^+; the anaerobic addition of Na^+ per se resulted in the H^+ efflux from cells. The latter effect was insensitive to the penetrating SCN^- anion; this observation indicated the electroneutral character of Na^+–H^+ exchange [149]. Tsuchiya and Takeda [142] were the first who in 1979 (using an Na^+ electrode in a hardly buffered medium) registered directly the Na^+ extrusion from *E. coli* cells induced by addition of O_2 to a suspension of anaerobic cells. N_2 bubbling caused a slow decrease in the Na^+ concentration in the medium. An anaerobic addition of glucose caused a similar, though much weaker, effect; in this case, Na^+ efflux was prevented by iodacetate, the glycolysis inhibitor. The protonophorous uncoupler, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), taken at a concentration of 20 μM, arrested the Na^+ extrusion from cells caused by aeration and the addition of glucose. Thus, the activation of respiration or glycolysis generating $\Delta\tilde{\mu}_H$ on the membrane could energize Na^+–H^+ exchange. It is remarkable that the activity of the Na^+-extruding system could be increased by a cultivation of cells in the presence of 1 mM melibiose when the Na^+, melibiose symporter was induced [142]. Apparently, the Na^+/H^+ antiport was electrogenic, because all measurements were performed at pH 8.0, when $\Delta\tilde{\mu}_H$ exists solely in the form of $\Delta\psi$ [107,125].

Later, Borbolla and Rosen [18], using substrate-depleted, Na^+-loaded cells of the ATPase mutant AN120 (*unc* A401), demonstrated an ATP-independent Na^+ extrusion induced by the addition of glucose or reduced phenazine methosulfate (PMS). The process was prevented by the protonophore FCCP and cyanide. Again, a sufficiently high pH of the incubation medium implied an electrophoretic exchange; the ratio $2H^+ : Na^+$ was proposed as the simplest stoichiometry [18].

Macnab and co-workers adopted a new approach to the direct measuring of intracellular Na^+ in respiring or

glycolizing *E. coli* cells in the process of energization [26]. This approach involved $^{23}Na^+$ NMR spectroscopy in the presence of dysprosium tripolyphosphate, a non-permeable paramagnetic shift reagent. $^{23}Na^+$ spectra thus obtained include two resonance peaks, an unshifted peak and a shifted one corresponding to Na^+_{in} and Na^+_{out}, respectively. Simultaneously, pH_{in} was monitored by ^{31}P-NMR. At neutral pH_{out}, the O_2 passing through the anaerobic cell suspension induced a rapid Na^+ efflux from the cytoplasm against its electrochemical gradient. Simultaneously, ΔpH (alkali inside) was rising. CCCP addition led to a collapse of both the sodium ion and the proton gradients. Similar results were obtained under glycolytic conditions. Thus, a precise correlation was established between $\Delta\tilde{\mu}_H$ generation and sodium gradient formation on the membrane. At an extracellular Na^+ concentration of 95 mM, endogenously respiring *E. coli* maintained its $[Na^+]_{in}$ at a low steady-state level (about 4 mM). Apparently, Na^+ was extruded from the cytoplasm by a secondary proton-driven Na^+/H^+ antiport, because the Na^+ efflux was very sensitive to the uncoupler action.

II-B. $Na^+ : H^+$ stoichiometry

Sub-bacterial vesicles (right-side-out or everted) represent a very useful model system for studying ionic exchange processes. The standard approach includes the testing of an Na^+-induced H^+ flux across the vesicular membrane by measuring the fluorescence change of acridine dyes; occasionally this method is supplemented with the flow dialysis of $^{22}Na^+$ for direct registration of an Na^+ transport [10,11,14,19,110,118]. This experimental technique was used for study of the Na^+–H^+ exchange stoichiometry. In 1978, Schuldiner and Fishkes, investigating energy-dependent, FCCP-sensitive $^{22}Na^+$ export from the *E. coli* right-side-out subcellular vesicles, reported that at acid (< 6.6) pH_{out} this process is ΔpH-driven, while at higher pH the Na^+ transport becomes $\Delta\psi$-driven [118]. The authors suggested that the Na^+/H^+ antiporter stoichiometry is pH-dependent and changes from an electroneutral process in an acidic medium to electrogenic one at more alkaline pH values. Beck and Rosen [14], using everted vesicles, showed that the penetrating anion SCN^-, discharging $\Delta\psi$ on the membrane, strongly reduced the rate of Na^+/H^+ exchange at pH values between 7.0 and 8.7 (in these experiments the ΔpH was estimated from the energy-dependent quenching of quinacrine fluorescence, and the uptake of $^{22}Na^+$ by vesicles was measured). The data obtained by Beck and Rosen indicate that the antiport was electrogenic ($H^+ : Na^+ > 1$) at pH_{out} above 7.0, too. Later, Leblanc and co-workers investigated in detail the downhill sodium efflux from the $^{22}Na^+$-loaded right-side-out vesicles [10,11]. It was found that (i) respiration-linked $\Delta\tilde{\mu}_H$ as well as artificially imposed $\Delta\psi$ (interior negative) and/or ΔpH (interior alkaline) strongly stimulated the downhill Na^+ efflux rate (it should be pointed out in this context that this acceleration cannot be accounted for by an increase in passive Na^+ leaks, if we take into consideration both the $\Delta\psi$ and ΔpH directions); (ii) in the absence of $\Delta\tilde{\mu}_H$, the Na^+ efflux rate along its chemical gradient (Δp*Na* was about 90 mV) is low and insensitive to protonophore FCCP; (iii) $\Delta\psi$ itself enhances the Na^+ efflux at pH_{out} between 6.6 and 7.5 but not at pH_{out} 5.5. On the other hand, when a ΔpH higher than some threshold ΔpH value (about 70 mV) was simultaneously imposed, $\Delta\psi$ was proved effective to accelerate the Na^+ efflux rate at pH_{out} 5.5 [10]. The authors concluded from the data obtained that (a) the downhill Na^+ efflux from *E. coli* membrane vesicles is mediated by the Na^+/H^+ antiport; (b) the Na^+/H^+ antiport is electrogenic in the pH range from 5.5 to 7.5; (c) in an acidic medium (pH 5.5) the process of Na^+–H^+ exchange may be inhibited by acidification of the vesicles' interior; (d) the Na^+–H^+ exchange reaction is rate-limited by the Na^+ extrusion coupled H^+ uptake [10]. In the next paper [11], these authors demonstrated that the high concentration of protons in the vesicles' interior inhibits the Na^+–H^+ exchange by decreasing the affinity of the antiporter for internal Na^+ ions. As pH_{in} decreases from 7.7 to 6.8, the apparent K_m of antiporter for Na^+ increases from 3.5 to 40 mM; according to the authors' estimation, at pH_{in} 5.5, the K_m value may reach 100 mM. It was concluded that protons compete with sodium ions for the internal Na^+ binding site of the carrier [11]. At the same time, kinetical analysis data obtained in this work demonstrated that imposed $\Delta\psi$ (interior negative) increases the maximal rate of Na^+–H^+ exchange. Thus, the apparent inefficiency of $\Delta\psi$ in an acidic medium [10] may be explained. Indeed, at pH 5.5 the Na^+ concentration within vesicles (which was equal to 10 mM in these experiments) is much lower than the antiporter K_m value for Na^+. Observing that $\Delta\psi$ of favourable direction (but not ΔpH) affects the charge translocation across the membrane, the authors suggest that the exchange stoichiometry is higher than unity in the pH range between 5.5 and 7.5; but they do not exclude the alternative possibility, namely, that $\Delta\psi$ increases the number of active antiporter molecules [11]. However, the latter explanation is rather formal because it is difficult to visualize the actual mechanism, while the electrogenic character of exchange (more than one H^+ per Na^+) should certainly result in the influence of $\Delta\psi$ on the transmembrane current of Na^+.

In their fundamental work, Macnab and co-authors [27], studying the relationship between Δp*Na* and the protonmotive force steady-state levels in endogenously respiring *E. coli* cells, investigated $\Delta\tilde{\mu}_{Na}$ as a function of $\Delta\tilde{\mu}_H$ at different pH levels of the medium. The authors assumed that the Na^+/H^+ antiport with nH^+

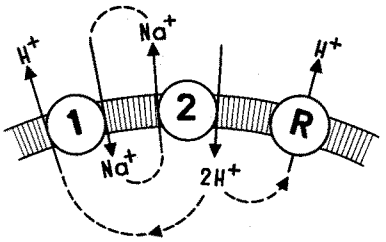

Fig. 1. Hypothetical futile cycle for Na^+ (from Ref. 27, with changes). R = respiratory chain. See the text for further comments.

per Na^+ stoichiometry is the only way to produce a Na^+ gradient on the membrane, and determined an apparent stoichiometry, n_{app}, by the equality $\Delta\tilde{\mu}_{Na} = n_{app}\Delta\tilde{\mu}_H$. It was calculated from experimental data that at $pH_{out} < 7.2$ for *E. coli* MRE600 used in this study, $n_{app} = 1.13$ and thus indicated a 'slightly electrogenic' antiport. A pH_{out} increase above 7.2 caused the n_{app} value to increase to 1.26, i.e., the electrogenic character of the exchange was intensified. To explain the *nonintegral* n_{app} values obtained, the authors suggested an original scheme that involved a simultaneous operation of 1 : 1 Na^+/H^+ and 1 : 2 Na^+/H^+ antiporters working in opposite directions. The combined action of the two exchangers with an *integral* stoichiometry results in a futile cycle for Na^+, with a net inward H^+ flux that is balanced by H^+ extrusion via respiratory $\Delta\tilde{\mu}_H$ generators (see Fig. 1). At steady-state conditions, the actual nonintegral n_{app} value will be determined by the relative Na^+ conductances of system 1 and system 2. The pH-dependent increase in n_{app} within the framework of this model may be explained by an increase in the conductance of the electrogenic antiporter relative to the electroneutral one [27]. In the simplest case, the same exchanger would be able to operate with different stoichiometries [27]; however, it is not clear how the switching from one operating mode to the other one should occur. The authors indicate that the scheme involving a Na^+ uniporter instead of an electroneutral Na^+/H^+ antiporter is likewise compatible with their experimental data. The Na^+ uniporter allows Na^+ ions to return into the cell without any H^+ exchange. Such a variant is considered as an unlikely one. On the other hand, systems symporting substrates with Na^+ ions apparently may be considered as a leakage current of the same kind. That is why a comprehensive analysis of the in vivo situation should involve the existence of such systems, especially since NMR measurements are carried out in an extremely dense cell suspension that damages a number of cells and causes some Na^+-symportable substrates to be released into the incubation medium. It should be also emphasized here that the extremely small changes in the n_{app} value were assumed by the authors as a basis of certain theoretical considerations. Therefore, even nonsignificant errors in determination of the levels of the proton or soDium electrochemical gradients would be crucial. Accurate quantitative measurmeents of $\Delta\psi$ as well as ΔpH or Δp*Na* is, in general, a highly complicated experimental task: conventional approaches (e.g., use of penetrating ions and weak acids/bases) actually give corresponding values to within 10 mV. One may hope that the NMR technique used by the authors is a more reliable method; however, additional confirmation of the data obtained would be advisable.

Unfortunately, all experiments in the study [27] were performed at pH_{out} below 7.6. It might be of some interest to monitor the situation at higher pH_{out} when the noticeable ΔpH of the opposite direction ($[H^+]_{in} > [H^+]_{out}$) is present. (It was found by Macnab and co-workers [27] that at pH_{out} 7.2–7.6 the correlation between ΔpH and Δp*Na* is disturbed: ΔpH continues to decrease and drops to zero at pH_{out} 7.5, while Δp*Na* tends to increase.) In any event, for all the conditions examined, the $\Delta\tilde{\mu}_{Na}$ proved higher than $\Delta\tilde{\mu}_H$; in other words, *E. coli* prefers to store a 'membrane-linked' energy in the form of the sodium, rather than the protonmotive force.

It should be mentioned that nearly in all the studies concerning the Na^+–H^+ exchange in *E. coli* cell cultures were grown on standard media at optimized (about neutral) pH levels, whereas important information may be obtained if cells are cultivated at pH above 8.0 for subsequent measurement.

II-C. Specificity

An investigation of Na^+–H^+ exchange in subbacterial vesicles has allowed a number of independent Na^+-transporting systems involved in this process to be established. Rosen's laboratory has carried out a wide program of studies on transmembrane cation/proton exchange in *E. coli*; as a result, two systems of this kind have been found [14]. However, there is only one system possessing equal affinity for Na^+ and Li^+ (NHA system) that functions in vivo *; it not only generates Δp*Na* on the membrane but removes toxic Li^+ from the cytosol as well, whereas the KHA system, possessing a significantly higher affinity for K^+ than Na^+ under actual conditions (high $[K^+]$ in the cytoplasm), operates as K^+/H^+ antiporter [14]. Later, a specific inhibitor for the *E. coli* Na^+/H^+ antiport was identified [99]. It was amiloride that was found to inhibit the exchange competitively, with K_i 40 μM. Earlier, amiloride was used as a eucaryotic Na^+–H^+ exchange inhibitor. At the same time, the K^+/H^+ antiport in *E. coli* proved to be insensitive to amiloride if its concentration was below 1

* A new paper by Padan et al. (J. Biol. Chem. 264, (1989) 20297–20302) shows two *nha* genes, encoding two systems differing in ion specificity and pH sensitivity.

mM. It is noteworthy that, by the recently obtained data [131], 0.5 mM amiloride completely arrests the motility of alkalophilic *Bacillus* which possesses a $\Delta\tilde{\mu}_{Na}$-driven flagellar motor. On the other hand, it should be taken into account that, according to Leblanc et al. (Na^+/K^+ exchange in bacteria and organelles, in Na^+/H^+ exchange (1988) (Grinstein, S., ed.), pp. 103–117, CRC Press, Cleveland, OH), amiloride at 0.5 mM or above can act as an uncoupling agent. Therefore, the additional investigation of the specificity of the amiloride action seems to be necessary.

Very little is known about the Na^+–H^+ exchange molecular mechanisms. Leblanc and colleagues [32] used chemical modifiers to assess the role of various amino acids in the Na^+/H^+ antiport. The histidyl reagent diethylpyrocarbonate $(EtO)_2C_2O_3$ prevented both the active Na^+ uptake by everted sub-bacterial vesicles and the energy-dependent Na^+ efflux from Na^+-loaded right-side-out membrane vesicles. The inhibitory effect of $(EtO)_2C_2O_3$ was completely reversed by hydroxylamine treatment of vesicles. Carboxyl group modifiers as well as -SH reagents were found to be unable to modify the Na^+ transport. Apparently, histidine residues are directly involved in the Na^+–H^+ exchange reaction.

In 1986, Rosen and colleagues [104] demonstrated the ΔpH-driven $^{22}Na^+$ uptake by proteoliposomes which were prepared from *E. coli* phospholipids and an octyl glucoside extract of the inner membrane of the same bacterium. Using the methods of direct Na^+ transport measurement, the authors confirmed the earlier observation of Tsuchiya and co-authors [143] concerning Na^+/H^+ antiport in proteoliposomes obtained from the same components. Switching on the proteoliposomal respiratory chain to initiate an antiport, Tsuchiya and associates measured the ΔpH-dependent quenching of acridine fluorescence.

II-D. Genetics of Na^+/H^+ antiport

In 1980, Zilberstein and co-authors [153] isolated a mutant of *E. coli* which was unable to grow at alkaline pH, either on melibiose (symported with Na^+) or on succinate. Compared with wild-type cells, the energy-dependent Na^+ efflux from the cytoplasm of this mutant was decreased [153]; subsequently, it was shown on this mutant that its Na^+ symporters for melibiose and glutamate had a K_m for Na^+ 10-fold higher than that in the wild type [155]. To explain the pleiotropic action of this mutation the authors used a model offered for *Bacillus alcalophilus* by Guffanti and others [51]. This model proposes the existence of a common subunit for the Na^+/H^+ antiporter and Na^+-dependent transport systems; then a point mutation impairing the function of this hypothetical subunit must inhibit the Na^+–H^+ exchange as well as the symport of substrates with Na^+. However, sub-bacterial vesicles obtained from the mutant appeared to be normal with respect to Na^+/H^+ antiport [48]. Moreover, surprisingly enough, the mutation was mapped in the α-subunit of RNA polymerase [16]. It is not yet clear how this mutation may influence Na^+-coupled processes in the cell.

Another *E. coli* mutant, capable of growing on melibiose in the presence of 10 mM Li^+, was isolated in Tsuchiya's laboratory [105]. At the concentration used, Li^+ inhibiting pyruvate kinase is toxic to the wild type [145]. The mutant obtained (W3133-2S) possessed two mutations: one mutation (in *mel B*) increased the Na^+, melibiose symporter affinity for Na^+ and the other mutation (*ant*-up) strongly enhanced the Na^+/H^+ antiporter activity [105]. Apparently, the latter mutation, allowing for a rapid removal of Li^+ from the cytoplasm, enabled the cell to survive. Everted vesicles of W3133-2S exhibit elevated Na^+–H^+ exchange activity relative to the parental strain. Proceeding from the acridine fluorescence monitoring data, the authors suggested that the K_m of the Na^+/H^+ antiporter in the *ant*-up mutant should be lower than K_m in the wild type [105]. Using the mutant W3133-2S, Goldberg and co-authors [50] designed a strain which carries the *ant*-up only; the mutation was localized near 0.5 min on the *E. coli* map. The presence of the mutant gene increased the V_{max} of the Na^+/H^+ antiporter by 4-fold. On the other hand, the normal *ant* gene introduced into the cell in multiple copies stimulated antiport activity. In this study, Na^+–H^+ exchange activity was determined with the aid of acridine fluorescence; in addition, $^{22}Na^+$ uptake by everted subcellular vesicles was measured. The simplest explanation of these data, as the authors reasonably concluded, is that *ant* is the structural gene for the Na^+/H^+ antiporter which is affected by the *ant*-up mutation. Subsequent investigations showed that the *ant* gene codes for a single polypeptide which has a mass of 35 kDa according to SDS electrophoresis on polyacrylamide gel. The amino acid sequence of this protein was deduced from the cloned gene. On the basis of hydrophobicity profile analysis, ten transmembraneous segments were postulated in the most probable resultant conformation [71].

After their work of many years, in 1987 Tsuchiya's group succeeded in detecting an *E. coli* mutant with an impaired Na^+/H^+ antiporter [64]. The criterion of the search was the inability of bacteria to grow on serine as a sole carbon source. As shown in the same laboratory, the constitutive serine permease of *E. coli* co-transports Na^+, whereas induction of the H^+,serine symporter occurs only in the presence of leucine or methionine [53]. Three possibilities may account for the unability of cells to grow on serine: (i) the Na^+/H^+ antiporter is defective; (ii) the Na^+,serine symporter is defective; (iii) cells are unable to metabolize serine. The authors used an ingenious strategy for isolating mutants for the first

type. They tested the ability of clones that do not grow on serine to accumulate Na^+ in response to the addition of serine. If the system removing Na^+ from the cytoplasm is damaged, the cells must be more active in accumulating Na^+ in response to the addition of the substrate symported with Na^+; according to the authors, the wild-type cells treated with amiloride exhibited the same behaviour in the control experiment. As a result, the strain HIT-1, which carries a single point mutation (the frequency of reversion 10^{-8}), was isolated. According to the quenching fluorescence data, the HIT-1 everted membrane vesicles possessed a normal K^+/H^+ antiporter, but were unable to change ΔpH on the membrane in response to the addition of Na^+ or Li^+

III. Na^+ exchange and regulation of intracellular pH

III-A. The phenomenology of pH homeostasis

A sudden shift in external pH results in an immediate corresponding transient perturbation of *E. coli* pH_{in} [79,109,126,154,156]. After a pH jump, the cytoplasmic pH returns to its initial level within a few minutes [107]. The addition of a membrane-permeant weak acid (or base) to the cells at constant pH_{out} produces the same effect [79]. These perturbations of cytoplasmic pH are accompanied by a transient rise in $\Delta\psi$; yet the respiration rate is not affected [109]. Thus, *E. coli* cells possess a very effective system which maintains pH_{in} at an acceptable value.

Macnab and his collaborators [125] used ^{31}P nuclear magnetic resonance of methylphosphonate and phosphate to measure pH homeostasis in *E. coli*. A simple linear relation between pH_{in} and pH_{out} was established:

$$pH_{in} = 7.6 + 0.1(pH_{out} - 7.6) \quad (1)$$

According to the Eqn. 1, ΔpH of the appropriate direction (alkaline-inside) existing on the cell membrane in an acidic medium turns into zero at pH_{out} 7.6 and changes its sign under more alkaline conditions; at pH_{out} 8.5, inverted ΔpH is about 53 mV. Internal pH increases from 7.4 at pH_{out} 5.5 to 7.8 at pH_{out} 9.0. It should be mentioned that by the data of MacNab et al., the precise steady-state pH_{in} value at constant external pH was dependent on the strain used as well as on the osmolarity of the incubation medium; it was found that the rise in osmolarity correlates with the increase in pH_{in} [27]. The actual value of pH_{in} might be dependent on the mode of cell energization, the composition of the incubation mixture, and the method of pH registration. These factors might explain some discrepancies of the pH_{in} values determined experimentally by several groups [16,73,107,108,125]. However, the elegant study referred to in Ref. 125 apparently provides the best insight into the quantitative aspects of pH homeostasis.

III-B. The role of Na^+

Cation–proton exchangers seem to be the natural candidates to the role of molecular devices providing the pH homeostasis [16,84,108,124]. Indeed, at low external pH, the K^+/nH^+ antiporter ($n > 1$) mediating the K^+ uptake by the cell coupled to the extrusion of protons could convert $\Delta\psi$ to ΔpH. On the other hand, in alkaline medium an electrogenic Na^+/H^+ antiporter operating in the opposite direction allows protons to return to the cell in exchange for Na^+, and this results in the acidification of the intracellular volume. Finally, at neutral pH the return of protons removed from the cytoplasm by proton pumps may occur via electroneutral Na^+–H^+ exchange. In principle, pH homeostasis in an acid medium may also be supported by the cooperative action of K^+ uniporter and $\Delta\tilde{\mu}_H$ generators [124]; recently, Konings and co-authors confirmed this possibility experimentally for *Rhodobacter sphaeroides* [1]; in an alkaline environment the '$\Delta\tilde{\mu}_{Na}$-generator plus H^+ uniporter' pair would play an analogous role [124].

The fact that Na^+ circulation is needed for the regulation of a cytosolic pH is now well established for some alkalophilic *Bacillus* strains which grow at pH_{out} from 9.0 to 11.0 [81,85–86]. In the case of neutrophilic *E. coli*, some data were also obtained indicating that the Na^+/H^+ antiport is involved in pH homeostasis. Thus, a simple point mutant HIT-1 with defective Na^+/H^+ antiporter, which was isolated in Tsuchiya's group, was unable to grow at pH > 8.5 [64]. Interestingly, a rise in external pH from 7.0 to 7.5 resulted in the 2-fold decrease of the growth rate. Such a significant effect cannot be explained by alkalinization of the cytoplasm up to a nonphysiological value, because in *E. coli* the ΔpH is about zero (and, thus, $\Delta\psi$ is comparatively high) at $pH_{out} = 7.5$ [125]. The HIT-1 membrane had a normal K^+/H^+ antiporter [64], which argues against the earlier supposition about the main role of K^+–H^+ exchange in maintaining the pH_{in} in *E. coli* at high medium pH [20]. Moreover, according to the data obtained by Macnab and co-workers [125], in the 'alkali-sensitive', defective in K^+/H^+ antiport mutant KHA-1 obtained by Plack and Rosen [111], the pH homeostasis was not affected. The activation of the Na^+/H^+ antiporter via an increase in the cytosolic pH, discovered in Leblanc's group (see above), may be yet another indication that Na^+ is involved in pH_{in} maintenance.

On the other hand, the *E. coli* growth rate was claimed to be independent of Na^+ addition to the medium [14,17,125]. However, it is important to stress that Na^+ is usually present in growth media at appreciable (to a few hundred micromolar) concentrations as a contaminant even if not added deliberately. Therefore the Na^+-transporting device with a sufficiently high affinity for Na^+ may operate in such cases as well. Wilson and co-workers [93], using plastic tubes and a

medium with a very low Na^+ concentration for cell growth, reported that Na^+ is absolutely necessary for *E. coli* growth at pH 8.5. The minimal possible concentration of Na^+ in the growth medium was found to be 100 μM for a normal cell and 50 μM for the *ant*-up mutant; amiloride inhibited the growth at pH 8.5 in the presence of the added Na^+. The authors concluded that the Na^+/H^+ antiport is essential for the growth of *E. coli* in alkaline environment [93].

Thus, the transmembrane Na^+ circulation clearly plays a central role in the pH homeostasis of the *E. coli* cytosol at high pH.

IV. Na^+-coupled import of substrates

IV-A. H^+,solute and Na^+,solute symports in E. coli

According to the chemiosmotic theory, $\Delta\tilde{\mu}_H$ produced by proton pumps can be used directly for the import of solutes via H^+, substrate cotransporters [98]. H^+-symports ensure the accumulation of phosphate [117], glycine, histidine, lysine, phenylalanine [69], alanine [31], DL-lactate [58], pyruvate [91] and β-galactosids [146–148] in *E. coli* cells. In the latter case, the purified permease (*lac y* gene product) was incorporated into liposomes in which lactose accumulation, driven by an artificially imposed ΔpH (inside alkaline) and/or membrane potential (inside negative) occurred [44,70]; proteoliposomes, containing both the lactose carrier and the *o* cytochrome oxidase from *E. coli,* exhibited H^+,lactose symport driven by the $\Delta\tilde{\mu}_H$-forming ubiquinole oxidation [92].

The transportation of a few substrates in *E. coli* cells is, however, Na^+-dependent (see Refs. 37, 87, 124). Apparently, the Na^+-dependence of transport per se does not mean Na^+ symport. A standard approach for clarifying the role of Na^+ is to study the kinetic parameters (K_m and V_{max}) of the transport as a function of Na^+ concentration. If the kinetic characteristics are not affected by sodium ions, then Na^+ clearly does not serve as a coupling ion. In principle, the Na^+ uptake by cells in response to the addition of a substrate or its non-metabolized analogue (as well as the uptake of substrate driven by an artificially imposed transmembrane sodium gradient) could be considered as direct evidence of Na^+-symport. However, attempts to prove this are limited by the rapid formation and dissipation of Na^+ gradient (especially if the cell membrane possesses an Na^+/H^+ antiporter). The Δp*Na* developed may be too small for measurement in the case of electrogenic transport (see Ref. 87). These problems are compounded by the fact that the same substrate can be transported via different systems using distinct coupling ions. If one of the alternative carriers is inducible, it is possible to separate them by cultivating cells in an appropriate medium. Furthermore, the energy source for one and the same transport system can be complex: H^+ or Na^+ may be used as coupling cations depending on concrete conditions, and when the 'mixed' Na^+,H^+ symport occurs, both ions will cross the membrane simultaneously with the substrate.

The above-mentioned circumstances make it difficult to interpret experimental data. On the other hand, such a variety of relationships between Na^+ and H^+ in the transport of solutes in the same bacterium may be useful for elucidating the coupling molecular mechanisms and their evolution.

IV-B. Mixed Na^+,H^+,glutamate symport

As early as 1969 it was established that Na^+ stimulates the glutamate transport in *E. coli* by increasing the permease affinity for the substrate [45]. Later, in various laboratories it was demonstrated that an artificially imposed ΔpNa can drive the glutamate accumulation in intact cells or in right-side-out membrane vesicles [59,90,137]. In 1982 Tsuchiya and co-authors, using octyl glucoside, extracted and incorporated into liposomes membrane proteins from the *E. coli* strain W3133-2S with elevated Na^+/H^+ antiport activity [143]. The obtained proteoliposomes were able to (i) oxidize either NADH or the reduced TMPD in a cyanide-sensitive fashion; (ii) hydrolyze ATP with the coupled formation of ΔpH on the membrane, this process being inhibited by DCCD; (iii) perform Na^+–H^+ exchange; (iv) accumulate glutamate in response to artificially imposed $\Delta\psi$ and Δp*Na*. Glutamate transport was completely arrested by 2 μM of the Na^+-ionophore, dianemycin.

Later, Anraku and co-authors [46], using right-side-out membrane vesicles from *E. coli*, demonstrated that protons as well as Na^+ ions specifically stimulate glutamate binding to the carrier. Vesicles obtained from a mutant with a defective Na^+-dependent glutamate transport were unable to bind the substrate. The protonophorous uncouplers CCCP and SF6847, as well as the Na^+–H^+ exchanger monensin, the K^+–H^+ exchanger nigericin or a combination of the protonophore with monensin failed to arrest the substrate binding to the carrier in wild-type vesicles, which indicates this stage to be energy-independent. Varying the H^+ and Na^+ concentrations in the medium, the authors determined a number of characteristics of the process. While the maximum number of binding sites remained constant, an apparent dissociation constant was found to be a linear combination of the reciprocals of the Na^+ and H^+ concentraitons. To explain these results, a model for the binding and transport of glutamate was proposed [46,47]. According to this model: (i) Na^+ and H^+ bind to the unloaded glutamate carrier via random-order binding on the external surface of the membrane; (ii) then glutamate binds to the Na^+/H^+/carrier complex;

(iii) the positively charged $Na^+/H^+/Glu^-$/carrier complex is translocated across the membrane; (iv) Glu^- and both the cations dissociate on the internal surface, and the carrier returns to its initial state. In this way, both the proton and the sodium act as *syn*-coupling ions of the glutamate transport, and $\Delta\psi$, ΔpH, $\Delta p Na$ or any combination of them can serve as a driving force.

This proposition was confirmed in the next series of experiments carried out in the same group, when the glutamate accumulation driven by artificially imposed ionic gradients was being studied [47]. It was shown that the simultaneous imposition of ΔpH (interior alkaline) and $\Delta p Na$ ($[Na^+]_{out} > [Na^+]_{in}$) leads to a several hundred-fold increase in the glutamate concentration inside the membrane vesicles. $\Delta p Na$ or ΔpH, taken separately, were less effective. Since the valinomycin-induced K^+ potential (interior negative) had driven the transport, the process was apparently electrogenic. While the K_m of the glutamate transport was strongly dependent on the Na^+ and H^+ concentrations in the medium, $\Delta\psi$ of an appropriate direction increased V_{max} (from 10 to 30 nmol/min per mg protein) without influencing the K_m value. Li^+ ions did not substitute for Na^+ in the glutamate transport [47].

IV-C. $Na^+(Li^+)$,proline symport

For a long time, the transport of proline, a typical neutral amino acid, in *E. coli* cells was considered a classical example of the protonic symport. This point of view was based on a series of observations concerning the ability of $\Delta\psi$ of the 'right' sign to energize the proline uptake against its concentration gradient in the absence of added Na^+ [15,43,60,61,88,112,135]. In Anraku's laboratory it was shown that the binding of proline to the carrier is energy-independent and has a sharp maximum at pH 5.3 [4]; Mogi and Anraku obtained some evidence for a 2 : 1 stoichiometry [100–102]. At the same time, it was observed that preincubation of sub-bacterial vesicles with Na^+ inhibits the proline transport [61,88,103]. Kayama and Kawasaki reported that 10 mM Li^+ (but not Na^+) stimulated the proline uptake by intact *E. coli* cells [76]; the addition of proline to the suspension was not accompanied by transmembrane proton movement, which indicated the possibility of Li^+,proline symport [77]. A direct piece of evidence for Li^+,proline symport was obtained by Tsuchiya and co-authors [144], who used an Li^+-selective electrode to register the Li^+ uptake by *E. coli* cells in response to the anaerobic addition of proline. The Li^+ accumulation was suppressed by a competitive inhibitor of the proline transport. The transport was absent in cells grown on 20 mM glucose [144]. The latter observation agrees with the data concerning the inducible character of the proline transport system in *E. coli* [103,127]. Then Stewart and Booth [128] demonstrated that the simultaneous addition of Na^+ and labelled proline to the cell suspension leads to amino acid uptake; if cells were preincubated in an Na^+-containing medium, the Na^+ accumulation in the cytoplasm resulted in the inhibition of the proline transport. The addition of Na^+ to the cells increased the V_{max} of the proline transport without affecting the K_m. The authors therefore concluded that an Na^+,proline symport takes place [128]. Chen et al. [28] confirmed the data of Stewart and Booth as well as the above-mentioned results of Tsuchiya's group. It was shown that in the absence of a protonmotive force, the *E. coli* cells with the induced system of transport and utilization of proline can accumulate proline by Na^+- or Li^+-, but not by H^+-symport. The RM2 strain, which lacks the *put P* gene coding for proline porter I, was unable to accumulate proline by $Na^+(Li^+)$-symport; the pattern of the radioactive proline uptake by the cells of a strain lacking the proline porter II was the same as that obtained in the case of the wild-type strain [28]. Thus, proline porter I apparently mediated the Na^+,proline symport. Harmaline, known as a potent inhibitor of the sodium-dependent transport in animal cell membrane [25,121], was found to be a competitive inhibitor of the Na^+,proline cotransport in *E. coli* [28]. According to the data of Chen et al., the proline carrier (i) has a low K_m for Na^+ (37 μM). That is why the Na^+-coupled proline co-transport was not recognized previously: the use of glassware and inorganic reactants without special purification to remove sodium contaminations gave a 50 to 100 μM Na^+ background in the media [28].

Recently the proline carrier I (*put P* gene product) was incorporated into liposomes from *E. coli* phospholipids [29]. The proteoliposomes thus obtained accumulated proline against its concentration gradient in response to the simultaneous imposition of $\Delta p Na$ ($[Na^+]_{out} > [Na^+]_{in}$) and the $\Delta\psi$ formed by valinomycin addition to the K^+-loaded proteoliposomes (inside negative), while the artificially imposed $\Delta\tilde{\mu}_H$ had no effect. The authors have estimated the Na^+ : proline stoichiometry as 1 [29].

Thus, it seems to be firmly established that the *put P* gene product mediates the Na^+ symport of proline. In contrast to the glutamate transport, Li^+ may substitute for Na^+ in the proline symport. In vivo, Na^+ apparently serves as a coupling ion. It should be mentioned that, parallel to the system described above, there exists a proline porter II (*pro P* gene product) in *E. coli*. As was recently shown on intact cells and on sub-bacterial vesicles, the latter porter, which may be rapidly activated ($\tau_{1/2}$ about 1 min) by hyperosmotic stress, is energized by $\Delta\tilde{\mu}_H$ [96].

The nucleotide sequence of the *put P* gene has been determined, and the corresponding primary structure was predicted [104a]. In a recent paper by Yamato et al. [150a], a detailed model of the secondary structure of

the proline carrier is suggested. According to this model, 12 transmembrane helices connected by hydrophilic loops form a functional symporter molecule. Furthermore, basing on the analysis of *put P* mutants, the authors suggested that a cation-binding site of the carrier is formed by the five contacting amino acid residues (Glu-22, Cys-141, Arg-257, Cys-281 and Cys-344) located in different segments (150a).

IV-D. Na^+,serine(threonine) symport

A recently described system of serine(threonine) import may serve as another example of the Na^+-coupled transport of amino acid in *E. coli* [53]. As was originally assumed, the *E. coli* cell symports serine with H^+ [31]. Three distinct serine transport systems are now known in this bacterium; one of them transfers threonine, too [88,134]. Tsuchiya's group studied the energetics of this system in detail [53]. It was established that: (i) the serine transport is stimulated by Na^+, but not by K^+, Li^+, NH_4^+ or choline; (ii) the addition of serine or threonine to the cell suspension causes an Na^+ uptake from the medium and a H^+ extrusion (in the presence of Na^+); (iii) an artificially imposed $\Delta\tilde{\mu}_{Na}$ (inside negative, $[Na^+]_{in} < [Na^+]_{out}$) on the membrane of energy-depleted cells strongly stimulates the serine uptake; (iv) this system has a K_m for Na^+ equal to 21 μM; (v) the Na^+,serine(threonine) carrier is suppressed in cells grown in a mixture of amino acids; (vi) Na^+ increases the V_{max} of transport without affecting the K_m.

It is remarkable that this porter has a highly specific requirement for Na^+ as a coupling ion. In *E. coli* at the present time this is the sole example of a substrate symport exclusively with Na^+. As in the case of the Na^+,proline symport, a half-maximum concentration of Na^+ for serine transport was found to be too low to recognize the Na^+-dependence of transport without the use of plastic vessels and thorough control of $[Na^+]$ in the experimental solutions [53]. The authors point out that a high concentration of Na^+ inhibits the serine [53] and the proline [88] transport; since a similar situation (according to the data of Lombardi and Kaback [88]) takes place in the transport of glycine, aspartic acid, phenylalanine and cysteine, they may be cotransported with Na^+ as well [53].

IV-E. Melibiose uphill transport: three coupling ions

For the first time, the Na^+-symport of thiomethylgalactoside (TMG), mediated by the melibiose carrier was shown in the *Salmonella typhimurium* intact cells by Stock and Roseman [130], who demonstrated that the addition of TMG to the cells causes Na^+ uptake and, vice versa, Na^+ stimulates the TMG uptake. Subsequently, the $Na^+(Li^+)$ symport of TMG was demonstrated in subcellular vesicles from this organism [136]. In *E. coli*, melibiose can enter the cell via the lactose transport system (*lac Y* gene product) or via the melibiose carrier itself (coded for by the *mel B* gene), which is induced if the cell grows in the presence of melibiose. Namely, the latter transport system operates as an Na^+ symporter [89,138,140].

Using a *lac Y* mutant, Tsuchiya and co-authors showed that Na^+ and Li^+ had the same stimulatory effect on the uptake of radioactive TMG by cells [110]; an apparent K_m for TMG was 0.21 mM at 5 mM Na^+. K^+, Rb^+, Cs^+, NH_4^+ and choline were found to be ineffective in stimulating the sugar uptake. The proton conductor CCCP, at a concentration of 16 μM, completely inhibited the TMG transport irrespective of the presence of Na^+, but in the presence of a protonophore an artificial proton diffusion potential (outside positive) as well as ΔpLi or $\Delta\tilde{\mu}_{Li}$ of an appropriate direction caused a reversible uptake of TMG by the cells [89]. Tsuchiya and his colleagues, using Na^+ instead of Li^+, obtained analogous data [138].

Subsequently, kinetic parameters of the Na^+ stimulation of the melibiose transport were determined; the K_m for Na^+ was equal to 0.3 mM [105]. Leblanc and co-authors [33], studying the galactosides binding to the carrier, showed that Na^+ increased the affinity of the carrier for the substrate; H^+ played the role of a competitive inhibitor of this activation. Within the framework of the model suggested by these authors, the substrate may bind to the porter only after Na^+ (or H^+) association with the protein [33]. Dissociation of the complex on the other side of the membrane occurs in a reverse (mirror-like) way – first the sugar molecule is released, and then Na^+; the latter step (Na^+ release on the inner surface of the membrane) was postulated to be a rate-limiting reaction [13].

As a matter of fact, not only $Na^+(Li^+)$ but also H^+ may be used as a coupling ion in the melibiose co-transport [105,140]; for example, it is possible to observe an H^+ uptake from the medium by the cells in response to the addition of melibiose [105]. The $\Delta\psi$ of the 'correct' sign, imposed on the membrane of subbacterial vesicles, changes the K_m (but not the V_{max}) if H-symport occurs, but in the case of Na^+-symport $\Delta\psi$ increases V_{max} without affecting the K_m [12]. Yet TMG cannot be symported with H^+; in this case, Na^+ or Li^+ serve as coupling ions [89,138,140].

In experiments with right-side-out membrane vesicles from the *lac Y* mutant, the stimulation of the TMG transport by Na^+ and Li^+ ions was demonstrated; in this study the transport was supported by the oxidation of reduced PMS [89]. It is noteworthy that the Li^+ ions, stimulating the TMG transport, inhibit the transport of the other substrate – melibiose [133,139]. Thus, a coupling ion varies depending on the substrate transported: melibiose is transported exclusively with Na^+ or H^+ (and Li^+ inhibits this transport); TMG is transported

with Na^+ or Li^+, but not with H^+; and methyl α-galactoside may be transported across the membrane with any of the three cations [105,139,140]. Later, Tsuchiya and his collaborators succeeded in isolating mutants which were unable to symport melibiose with H^+, whereas Na^+ and Li^+ were effective as coupling ions [105]; a second independent mutation leads to enhanced $Na^+(Li^+)/H^+$ exchange activity in both mutants. In keeping with the concept of evolution from H^+ to Na^+ bioenergetics [124,150], the authors [105] considered the *E. coli* melibiose carrier as an example of a 'transitional stage': although this porter is still able to utilize the 'ancient' coupling ion – the proton, a single mutation may cause it to lose such an ability; thus, the existence of the mutants described is the logical consequence of an evolutionary approach [105]. A few aspects of the interrelationships between H^+ and Na^+ acting as coupling ions will be discussed below.

The structural gene of the melibiose carrier in the wild type (*mel B*) was cloned and sequenced as reported in [54,151]. Then, having analyzed the sequence of *mel B* genes from the mutants mentioned above, Tsuchiya and colleagues found that a single amino acid replacement (of proline 122 with serine) in the hydrophobic domain of the protein results in the change of cationic specificity of the carrier [152].

The basic data concerning the Na^+ involvement in the metabolite import in *E. coli* are summarized in Table I. Apart from the substrates considered above, it also included succinate which is symported with Na^+ by the cell of the sulphuric purple bacterium [72]. Although *E. coli* is believed to import succinate with protons [52,91], there is some evidence obtained from the studies on sub-bacterial vesicles [113] arguing in favour of a possible Na^+ participation on this process.

V. Transport of Na^+, K^+ and the level of the protonmotive force

V-A. Na^+ and K^+ gradients as $\Delta\tilde{\mu}_H$ buffer

In 1978, Skulachev formulated a hypothesis [122,123] previously supported by experimental data in *Halobacterium halobium* and *Escherichia coli* [106a,118]. According to the hypothesis, $\Delta\psi$, the primary product of operation of the bacterial protonic generators, can be converted to the ΔpK and ΔpH of the opposite direction by means of an electrophoretic K^+ influx. The ΔpH formed serves as a driving force for an electroneutral Na^+/H^+ antiport; as a result, ΔpNa is produced. Thus, both constituents of the protonmotive force are buffered by gradients of the respective ions: K^+ (for the membrane potential) and Na^+ (for the pH difference). In this case, a sudden stop of all the protonic pumps would not lead to an immediate dissipation of $\Delta\tilde{\mu}_H$: for some time the lowering of $\Delta\psi$ will be compensated by the K^+ efflux from the cell down the potassium gradient, and in turn, ΔpH will be stabilized by the accumulation of the pre-extruded Na^+ in the cytoplasm in exchange for H^+. The same result may be obtained if the electrogenic ($Na^+ : H^+ < 1$) Na^+/H^+ antiport substitutes for an electroneutral one and/or the K^+ uniport is replaced by the K^+,H^+ symport [38,122].

The hypothesis was experimentally confirmed for a wide range procaryotic objects, including *E. coli* [5,21–23,106a,118]. In particular, it was established that: (i) the Na^+ and K^+ gradients may stabilize the $\Delta\tilde{\mu}_H$ level, the intracellular ATP concentration and the motility rate under the conditions of a shortage in external energy supply (thus, the *E. coli* motility may be supported in this way for as along as 10–15 min); (ii) all

TABLE I

Na^+-dependent transport systems of E. coli

Substrate	Coupling ion	$[Na^+]$ [a] (μM)	Na^+/ substrate ratio	Effect of Na^+ on kinetic parameters of transport	R [b]	S [c]	Ref.
Glutamate	H^+,Na^+		1:1	K_m decrease	+	–	45, 46, 59, 90, 137
Proline	$Na^+(Li^+)$	37	1:1	V_{max} increase	+	+	28, 29, 96, 104a, 128, 150a
Serine (threonine)	Na^+	21		V_{max} increase	–	–	53, 88, 134
Melibiose	Na^+, H^+						33, 54, 89
TMG	Li^+ (Na^+)						140, 151
Methyl α-galactoside	$Na^+(Li^+,H^+)$	300		K_m decrease	–	+	
Succinate	$H^+(Na^+?)$				–	–	113

[a] Na^+ concentration causing half-maximal effect.
[b] Reconstitution into proteoliposomes.
[c] Nucleotide sequence.

energy-dependent functions supported by the Na^+ and K^+ gradients have a characteristic three-phase dynamics, including the stabilization on an intermediate level. A computer modelling of the Na^+/K^+ gradient as a $\Delta\tilde{\mu}_H$ buffer was in excellent accord with the experimental data [38,39].

The described elegant self-regulating system is certainly $\Delta\tilde{\mu}_H$-dependent. It can retard the $\Delta\tilde{\mu}_H$ dissipation only after the H^+-pumps are switched off.

As to the molecular mechanisms of the Na^+/K^+ buffer, the data concerning the Na^+/H^+ antiport are summarized in the previous sections of this review. The electrophoretic K^+ uptake by *E. coli* cells is described in the literature [115]; it runs via the TrkA system, which uses ATP as an allosteric activator [8,129]. Probably, TrkA symports potassium ions with protons [7] using $\Delta\psi$ as well as ΔpH to drive the process. In addition, *E. coli* cells can build up the K^+ gradient on the membrane by means of the K^+-ATPase (kdp system). This enzyme, which has an extremely high affinity for K^+ ($K_m = 2$ μM), is induced only in cells growing in a medium with a low (about a few hundred micromolar) K^+ concentrations [40] when the electrophoresis of K^+ ions across the membrane, down $\Delta\psi$, cannot maintain a sufficiently high K^+ content in the cytoplasm.

V-B. The growth of E. coli cells in the absence of the protonmotive force

The fact of the existence of a special mechanism for $\Delta\tilde{\mu}_H$ buffering points, in particular, to the importance of the protonmotive force for procaryotic cells. Nevertheless, $\Delta\tilde{\mu}_H$, as was found in a number of independent studies, is not necessary for the bacterial survival, growth and reproduction. The ability of *E. coli* to grow in the presence of protonophores has received particular attention [66–68,80,106,120]. Cells of the wild type, placed into a medium supplemented with a high amount of glucose [80,106], as well as a number of specially isolated mutants demonstrate such an ability [65–68,120]. If, growing on glucose, the cells can produce ATP via glycolytic phosphorylation, and the absence of $\Delta\tilde{\mu}_H$ on the membrane has no adverse effect on the viability. However, the mutants isolated by Jones and Beechey [68] exhibited an oxidative phosphorylation in the presence of protonophores, whereas the parent strain DocS is inhibited by the ionophores. The coupling membrane of DocS shows enhanced accessibility for lipophilic ionophores; it is essential that the above-mentioned mutants retain the increased permeability of the cellular envelope. To explain the oxidative phosphorylation on the membrane short-circuited with respect to protons of protonophore-resistant mutants, the authors used a variant of the 'local coupling' scheme [42]. However, an alternative possibility may be a substitution of a coupling ion (Na^+ instead of H^+). A similar type of coupling (the 'sodium cycle' [123a]) has been shown in the marine alkalotolerant bacterium *Vibrio alginolyticus* possessing an Na^+-ATP systhase driven by the primary Na^+-motive respiratory chain [35,36]. The sodium cycle supports the energetics of *V. alginolyticus* (which has a pH optimum for growth at 8.6) in an alkaline medium when $\Delta\psi$ and ΔpH are oppositely directed. *E. coli* can grow at slightly acid and neutral, as well as moderate alkaline (to 8.7–8.8) pH. In the latter case, the opposition of two components of the protonmotive force confronts one with the same problem as that in the presence of the protonophore (Fig. 2). It may be proposed that, in the absence of fermentable substrates, cultivation in an alkaline or a protonophore-containing medium causes a lowering of $\Delta\tilde{\mu}_H$ on the *E. coli* membrane to a specific critical level that serves as a signal for 'switching' the cellular energetics from the H^+ to the Na^+ cycle. Such a proposition does not contradict the numerous data summarized in the previous sections of this review, because, as mentioned above, to study Na^+–H^+ exchange, *E. coli* is usually cultivated in standard media optimized for growth (e.g., see Ref. 75) concerning the effects of K^+ and Na^+ on the $\Delta\tilde{\mu}_H$ of respiring *E. coli* at alkaline pH). There are a few predictions deduced from the proposed hypothesis. Firstly, Na^+ should be necessary for respiring *E. coli* survival when $\Delta\tilde{\mu}_H$ is low; secondly, the membrane of cells grown under these conditions should contain respiratory sodium-motive pump(s), providing a sufficient

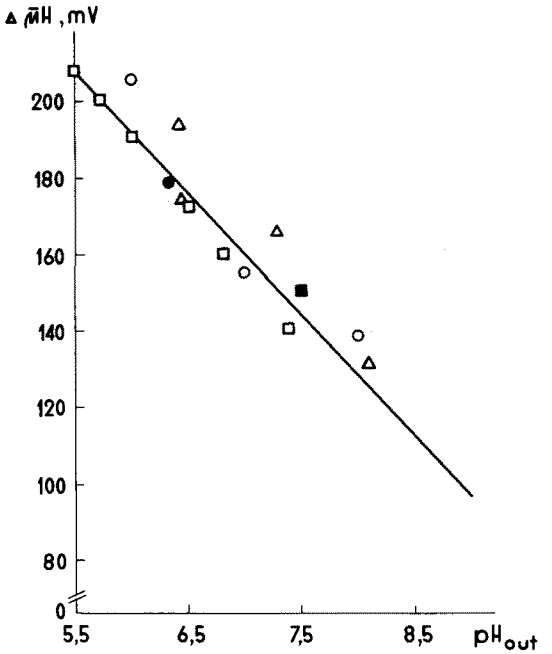

Fig. 2. Effect of external pH on the $\Delta\tilde{\mu}_H$ level in *E. coli* cells. The theoretical straight line is drawn in accordance with calculations of Slonchewski et al. [125]. Symbols indicate the experimentally measured $\Delta\tilde{\mu}_H$ values; data are taken from Ref. 108 (○), 41 (□), 74 (△), 82 (●), and 9 (■).

$\Delta\tilde{\mu}_H$ level for phosphorylation. Accordingly, the membrane potential generated by means of the sodium pump(s) should be resistant to the uncoupling concentrations of protonophore and (within a specified range) dependent on the Na^+ concentration in the medium.

Recently, using the *E. coli* strain DocS, we tried to verify these suggestions [6]. It was found that the cells, growing on succinate at pH 7.6 and 8.6, show spontaneous resistance to high (15–20 μM) concentrations of the uncoupler CCCP, if the medium contains 150 mM Na^+. Lowering of the external sodium concentration to 2 mM renders the cells sensitive to CCCP. Then the respiring cells grown at a low $\Delta\tilde{\mu}_H$ (external pH 8.6 or presence of 15 μM CCCP at pH_{out} 7.6) were able to maintain a relatively high $\Delta\psi$ in the Na^+ medium at pH 8.6 in the presence of CCCP; thus, the addition of 10 μM CCCP to an experimental mixture containing 150 mM Na^+ caused a decrease in $\Delta\psi$ from -160 to -110 mV only. At the same time, K^+ substitution for Na^+ in the medium led to complete abolition of $\Delta\psi$ by 7 μM CCCP (Fig. 3a). The addition of a small amount of Na^+ to the K^+ medium prevented the uncoupler-mediated $\Delta\psi$ collapse; the half-maximum Na^+ concentration was about 2 mM (Fig. 3b). Apparently, $\Delta\psi$ generation in the protonophore-containing medium was accompanied by reverse ΔpH formation; the simultaneous addition of the uncoupler and diethylamine, a penetrating weak base, abolished the membrane potential, whereas diethylamine did not affect $\Delta\psi$ without CCCP (Fig. 3a). Cyanide, at respiration-arresting concentrations, as well as a combination of CCCP and monensin, inhibited the uncoupler-resistant $\Delta\psi$.

The effect of $\Delta\psi$ protection by external Na^+ persisted at external pH 7.6 if the cells were grown at reduced $\Delta\tilde{\mu}_H$. Cultivated under normal conditions (pH 7.6 without uncoupler), the cells were unable to maintain a protonophore-resistant $\Delta\psi$ at both pH 7.6 and pH 8.6, even if sodium ions were present in the experimental mixture. Finally, inside-out subcellular vesicles were obtained from *E. coli* DocS grown at reduced $\Delta\tilde{\mu}_H$ (pH_{out} 8.6). These vesicles exhibited Na^+ uptake supported by NADH oxidation; CCCP was found to stimulate the Na^+ transport [6]. A detailed study on the respiration-linked Na^+ transport under these conditions is now in progress.

The data obtained appear to agree with the idea of an inducible Na^+ cycle in *E. coli*. Besides, this hypothesis could explain the protonmotive force-independent oxidative phosphorylation phenomenon described by Jones and Beechey if the Na^+ cycle becomes constitutive in the mutants isolated. It is noteworthy that above-mentioned mutants can normally accumulate proline in the presence of a protonophore [68]. The authors assumed that, under their experimental conditions, proline was symported with protons; however, it should be stressed that the proline import in *E. coli* can be energized by $\Delta\tilde{\mu}_{Na}$.

Our results might seem to be at variance with the recent study of Wilson et al. [93] concerning the strong requirement of *E. coli* for Na^+/H^+ antiport to grow at alkaline pH. Why in this case was the Na^+ cycle not induced? The point is that for growth experiments, Wilson et al. placed the cells in a medium supplemented with 0.2% glucose, and the standard Medium 63 (pH about 7.0) was used to cultivate the cells for Na^+/H^+ antiporter activity assay [93]. Thus, both methods of cultivation are distinct from ours (no glycolysis, respiration as the only energy source, the uncoupler- or pH_{out}-induced lowering of $\Delta\tilde{\mu}_H$). Apparently, under our conditions the sodium cycle induction becomes a crucial factor for survival.

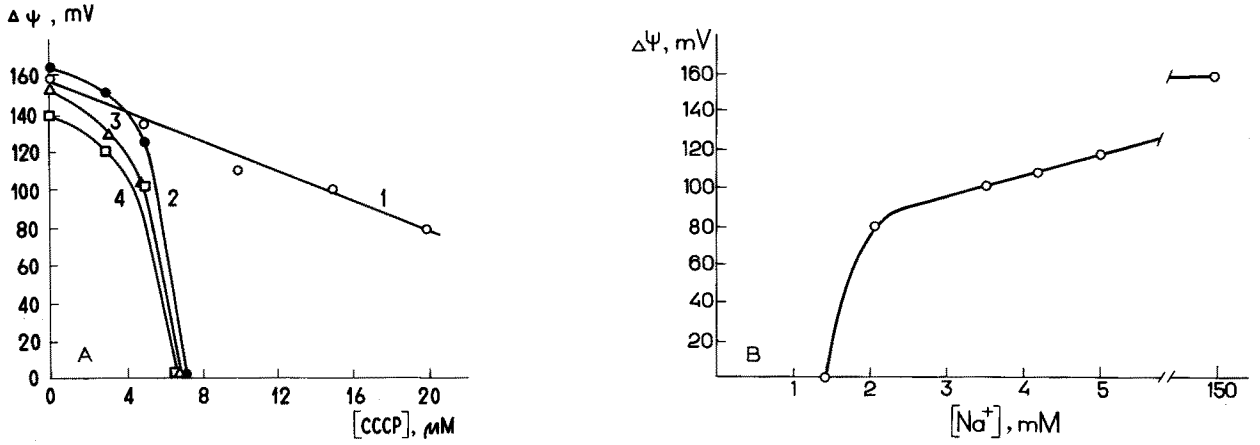

Fig. 3. Generation of the Na^+-dependent, uncoupler-resistant membrane potential by *E. coli* cells grown at lowered $\Delta\tilde{\mu}_H$ in the absence of fermentable substrates. (a) Uncoupler titration of $\Delta\psi$ in various incubation media. Curves 1, 2, the medium contains 150 mM Na^+; curves 3, 4, the medium contains less than 300 μM Na^+; curves 2, 3, 50 mM diethylamine was added to the medium. (b) Na^+ dependence of membrane potential at pH 8.6 with 7 μM protonophore CCCP in the medium. Cells were grown at pH 8.6 for all experiments.

VI. Conclusion

Today, we may form a clear view of the Na^+ exchange in *E. coli* cells grown under conditions ensuring the maintenance of a high level of protonmotive force on the membrane. In this case, $\Delta\tilde{\mu}_{Na}$ is developed by means of the Na^+/H^+ antiport, which seems to be electrogenic (more than one H^+ per Na^+); its precise stoichiometry depends on pH of the medium (the $H^+ : Na^+$ ratio increases with the increase in pH_{out}).

As to the energetics of the Na^+ transport in *E. coli* growing at low $\Delta\tilde{\mu}_H$, it still remains obscure. At least, the two following considerations strongly argue for the possibility of Na^+ cycle induction by *E. coli* under the 'energetic stress' (lowered $\Delta\tilde{\mu}_H$ in the absence of glycolysis): the existence of mutants which are able to perform oxidative phosphorylation (and to transport proline) in the absence of the protonmotive force [68], as well as the spontaneous resistance of the growth of *E. coli* DocS cells (respiring on succinate) to the uncoupler in a high-Na^+ medium and the ability of these cells (cultivated at low $\Delta\tilde{\mu}_H$) to generate a protonophore-resistant membrane potential [6]. The inducible Na^+ cycle hypothesis entails a few questions: (i) What serves as a direct signal to induce the sodium energetics? (ii) What kinds of molecular mechanism are responsible for the switching from one coupling ion to another? (iii) How do the proton and the sodium cycles interact in the same membrane? Undoubtedly, to answer these questions and to verify the initial proposition, in-depth studies with the use of subcellular systems are needed. However, some considerations may be outlined even at the present stage.

(i) Under natural conditions, $\Delta\tilde{\mu}_H$ at the *E. coli* membrane may be essentially lowered when the bacterial environment is alkaline. Therefore it may be expected that the monitored parameter is pH_{out}, pH_{in}, ΔpH, or $\Delta\tilde{\mu}_H$. It is known that the *E. coli* chemotaxis system involves a special pH_{in}-sensing receptor (the cell senses acidification of the intracellular volume as a repellent); this mechanism mediates both the taxes (a) away from the acid external pH and (b) from the membrane-permeant weak acids (see Refs. 49, 79, 114). On the other hand, alkalinization of the cytoplasm induces the SOS function in *E. coli* [119]. At the same time, alkalinization (but not acidification) of the external medium without pH_{in} increase activates the heat-shock response [132]; thus, the alkaline shift of pH_{out} serves as a signal. In contrast to these cases, our data [6] concerning the Na^+ influence on both the protonophore-resistant growth and the $\Delta\psi$ generation in *E. coli* at pH_{out} 7.6, indicate that the Na^+ cycle induction occurs when $\Delta\tilde{\mu}_H$ exists in the form of the membrane potential ($\Delta pH = 0$) and the pH_{in} value is normal and constant. It seems probable that the $\Delta\tilde{\mu}_H$ (or $\Delta\psi$) decrease, which is registered by a special sensor, may initiate the sodium cycle induction. The existence of such a $\Delta\tilde{\mu}_H$-receptor (so-called 'protometer') was postualted in Ref. 49 in order to explain some aspects of the bacterial chemotaxis, in particular, the repelling action of an uncoupler upon *E. coli*.

The cyclic AMP (cAMP) exchange plays a major role in *E. coli* metabolism regulation by means of the catabolic repression system. Recently, Ahmad and Newman [2] reported that *E. coli* mutants, deficient in the adenyl cyclase (*cya*$^-$) and the catabolite activator protein (*crp*$^-$), grew more slowly when the medium was alkalinized from pH 6.0 to 7.8, whereas the growth rates of parent strains were little, if any, affected by the alkaline pH_{out}. It is noteworthy that in the *cya*-mutant, the uptake of proline and glutamate was simultaneously reduced. At the same time, the mutant cells exhibited a normal membrane-bound ATPase activity and a diminished, but pH-independent oxygen consumption rate [2]. These observations may indicate that cAMP controls the Na^+ cycle induction.

(ii) Apparently, the simplest way for a transition to Na^+ coupling is to induce a de novo synthesis of the Na^+-motive respiratory pumps and Na^+-ATP synthase, in addition to the available protonic generators. Another variant is the synthesis of a special membrane protein, operating as an 'Na^+-adaptor', which can form a complex with a proton pump and thus allow the use of Na^+

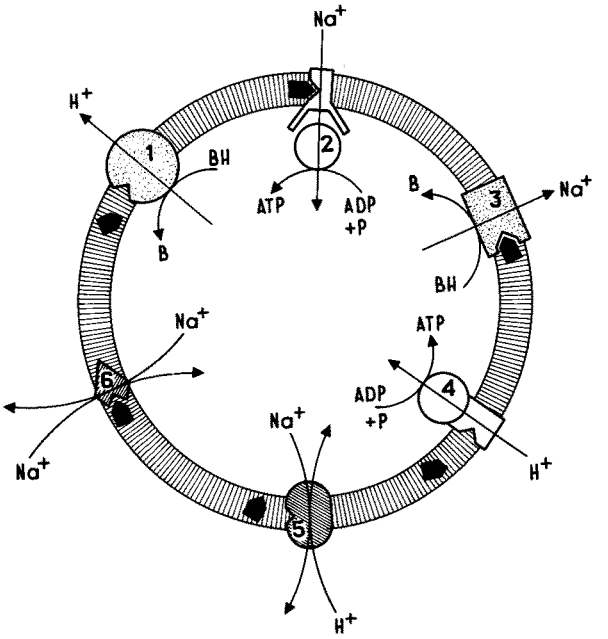

Fig. 4. Hypothetical mechanism of Na^+ cycle induction in *E. coli* by means of the 'Na^+-adaptor' (■). 1, respiratory H^+ pump; 2, ATP synthase possessing (as a result of adaptor binding) an altered specificity for cations; 3, after adaptor binding, the respiratory pump extrudes Na^+ instead of H^+; 4, H^+-ATP synthase; 5, Na^+/H^+ antiporter; 6, formation of the Na^+/H^+ antiporter-adaptor complex changes the protein conformation; thereupon only Na^+ (not H^+) may bind to the carrier.

instead of H^+ (or, maybe, parallel with the proton, if the adaptor impairs the specificity of the pump for the translocated ion). Fig. 4 illustrates this possibility. In this connection, it may be noted that the *E. coli unc*-operon, coding for the membrane H^+-ATPase, contains the *unc I* gene. The role of the *unc I* product is yet obscure; it is not essential for the synthesis, assembly or function of the enzyme and is not present in the ATPase complex [94], but a mutation in this gene decelerated the cells growth rate at pH > 8.3 [95].

The question about the operational mechanism of a hypothetical Na^+ adaptor is closely related to our concept of the physicochemical origin of ions translocation across the membrane. Apparently, a small shift in the protein conformation may be sufficient to change the carrier specificity for the translocated ions. Recently Boyer [24] called attention to this point. According to Boyer, the coordination bond formation between the ion translocated and crown-ether-like protein structures (not the reversible protonation of some protein groups, as was earlier accepted) during transmembrane ion transport plays a key role in bioenergetic coupling. Boyer pointed out that, in aqueous solutions, the proton exists in the H_3O^+ form (hydronium ion), which is a steric analog of the Na^+ ion and, therefore, may form complexes with crown ethers [24]. In the light of this hypothesis, it may be assumed that the addition of the Na^+-adaptor to any proton pump slightly changes the cation-binding cluster geometry so as to adapt it to the coupling ion replacement.

(iii) The question about the coexistence of both the proton cycle and the sodium one in the same membrane seems to be nontrivial as well. $\Delta\tilde{\mu}_{Na}$ produced by primary Na^+ pumps will be converted to $\Delta\tilde{\mu}_H$ via the Na^+/H^+ antiporter, which should result in a total uncoupling if the membrane is permeable to protons. Such an unfavourable situation could be prevented if the Na^+-adaptor could lower the conductance of the Na^+/H^+ antiporter by decreasing its affinity for H^+, thereby transforming it into an 'Na^+/Na^+ antiporter'. Other aspects relevant to the coexistence of the two energetics will be determined by a concrete set of membrane energy transductors. Thus, some transport systems are either able to use both coupling ions, or are otherwise represented by distinct independent carriers operating only with Na^+ or H^+. As to the mechanical work, the *E. coli* flagellum has a low (about 30 mV) energetic threshold and therefore can rotate at a markedly lowered protonmotive force level. For this reason, modification of the locomotive apparatus of any kind seems to be unnecessary. Unfortunately, the energy requirements for the motility of *E. coli* cells growing in alkaline media or in the presence of protonophores have not yet been studied.

Evolutionary aspects of the problem are very intriguing. There are two points of view based, in general, on comparative analysis of the transport systems energy supply. According to the first, Na^+ energetics is a relatively late evolutionary acquisition [124], added (as in the case of the animal cell plasmalemma) to the original proton cycle persisting in mitochondria, chloroplasts and in most procaryotes. An opposite viewpoint is represented by the assumption of the primacy of Na^+ cycle formation in primordial microorganisms which inhabited the salty sea water [116].

Both hypotheses provide for the existence of some 'intermediate forms', and *E. coli* may be related to them. However, there is yet a third possibility. If the chemistry of the transmembrane ion translocation via generators consists in the coordination of Na^+ (or H_3O^+) by a crown ether-like cluster of enzyme, one may presume that the ancient 'archetypical' pump, possessing a low specificity, transferred both ions with equal probability. A divergent evolution resulted in the subsequent separation of the H^+ and Na^+ pumps. Mitochondria, living in the homeostatic cytoplasm, employ the H^+ cycle; the physical mechanism of charge separation in photosynthesis was the reason why H^+ became a coupling ion for chloroplasts; a number of marine bacteria might have preferred the Na^+ energetics; finally, a large group of species (including *E. coli*) occupying habitats with fluctuating pH might have preserved the archaic 'mixed' $Na^+(H^+)$ pumps or have made $\Delta\tilde{\mu}_{Na}$-generators inducible. One may hope that further studies will help to clarify the *E. coli* strategy.

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