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SODIUM-DEPENDENT SUCCINATE UPTAKE IN PURPLE BACTERIUM *ECTOTHIORHODOSPIRA SHAPOSHNIKOVII*

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Summary

Succinate, malate and fumarate uptake in purple sulfur bacterium *Ectothiorhodospira shaposhnikovii*, strain 1 K MSU, obligatorily depends on the presence of Na^+. Other monovalent cations such as K^+, Li^+, NH_4^+ could not replace Na^+. Experiments with energy-depleted cells have shown that succinate uptake against its concentration gradient can be energized by artificially imposed sodium gradients (ΔpNa).

An artificial membrane potential (inside negative) inhibited ΔpNa-driven succinate uptake at pH 7.0 but stimulated it at pH 9.0.

The results confirm the suggestion that succinate uptake in *E. shaposhnikovii* is carried out in symport with Na^+.

Introduction

According to Mitchell's chemiosmotic theory [1—3] electrochemical potential gradients of cations play a major role in energization of uphill transport processes in living cells. Most possible cosubstrates for symport are H^+ and Na^+ as they are actively extruded by cells by appropriate mechanisms thus creating inwardly directed ΔpH and ΔpNa. There are many examples of solutes symport with protons in bacteria (for review see Ref. 4). It was shown, for instance, that succinate, malate and fumarate transport in *Escherichia coli* is carried out in symport with protons down ΔpH [5].

In eukaryotic cells sodium gradients formed by ($Na^+ + K^+$)-ATPase are the driving force for accumulation of different substrates via symport systems

Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.

Na^+/substrate [6,7]. Lately the role of Na^+ in bacterial transport has been studied extensively by many investigators. Frank and Hopkins [8] demonstrated that the presence of Na^+ is required for glutamate uptake in *E. coli*. B. Halpern et al. [9] reported that the presence of K^+ and Na^+ was required for active transport of glutamate in *E. coli* K-12. Stevenson [10] found that glutamate uptake by *Halobacterium salinarium* was stimulated by the addition of NaCl. Recent studies of membrane vesicles of *Halobacterium halobium* have shown that Na^+ gradients are involved in amino acid transport via symport [11—13]. Electrochemical gradient of Na^+ is also the driving force for glutamate accumulation in energy-depleted cells and membrane vesicles of *E. coli* B [14,15]. Stock and Roseman [16] demonstrated that transport of methyl-β-D-thiogalactoside via the melibiose transport system in *Salmonella typhimurium* is stimulated by Na^+ or Li^+. These studies have been extended recently by Tokuda and Kaback [17] and by van Thienen et al. [18] who have concluded that methyl-β-D-thiogalactoside transport in *S. typhimurium* is driven by an electrochemical Na^+ gradient. A similar relationship of Na^+ and Li^+ for methyl-β-D-thiogalactoside transport in *E. coli* was demonstrated by Tsuchiya et al. [19] and Lopilato et al. [20]. As Kitada and Horikoshi reported [21], the uptake of α-aminoisobutyric acid in alkalophilic species of *Bacillus* is stimulated by the addition of Na^+.

So far transport processes in phototrophic bacteria containing bacteriochlorophyll have been investigated very little. Perhaps the only exception is non-sulfur purple bacterium *Rhodopseudomonas sphaeroides* in which the presence of the common transport system for C_4-dicarboxylic acids: fumarate, succinate and malate with very low values of K_m for these substrates was demonstrated [22]. In earlier studies it was shown that the presence of Na^+ in culture medium was required to stimulate the growth rate of fresh water strain *R. sphaeroides* [23,24]. Recently preparations of membrane vesicles obtained from *R. sphaeroides* have been shown to be capable of producing alanine uptake as a result of a process sensitive to uncouplers [25]. Rinehart and Hubbard [26] reported that purple sulfur bacterium *Ectothiorhodospira halophila* requires high NaCl concentrations for maximum proline and glutamate uptake. However, the autors reject the possibility of functioning of Na^+ gradient.

The in vivo creation of Na^+ gradient in prokaryotes is a most intriguing process because ($Na^+ + K^+$)-ATPase is found only in membranes of eukaryotes. Over the past few years there appeared some papers revealing the existence of Na^+/H^+ antiport system in many bacterial species [17,27—32]. Na^+ gradient could be established via such antiport by coupling to a proton-motive force and could serve as ΔpH buffer under conditions unfavourable for $\Delta\bar{\mu}H^+$ generator [33].

The objectives of the present study were to investigate the effects of extracellular pH and Na^+ on the uptake of succinate by the purple sulfur bacterium *Ectothiorhodospira shaposhnikovii* which uses C_4-dicarboxylic acids as electron donors in the process of photosynthesis and as carbon sources. The obtained results provide direct evidence that cells of *E. shaposhnikovii* accumulate succinate down electrochemical sodium gradient $\Delta\bar{\mu}Na^+$, suggesting a Na^+/succinate symport.

Methods

The cultures of *E. shaposhnikovii*, strain 1 K, from the collection of Microbiology department of Moscow State University were grown in Larsen medium [34] plus 0.5% $NaHCO_3$, 0.1% NaCl and 0.2% sodium succinate. The medium was adjusted to pH 8.0 with H_3PO_4. Glass flasks were completely filled with the medium to create anaerobic conditions. The cultures were incubated at 30°C for 40—42 h under two 60-W incandescence lamps. Cells were harvested by centrifugation at 4°C for 15 min at 8000 × *g* and washed twice with the experimental medium.

In the experiments with succinate uptake energized by artificial ΔpNa and $\Delta\psi$, cells were energy depleted by 10 μM CCCP in the argon atmosphere for 1 h in the darkness. All experiments with energy-depleted cells were performed in glass thermostated vessels wrapped up in light-proof paper. To create anaerobic conditions argon bubbling was conducted through the reaction medium prior to the start of the uptake experiments and during the experiments as well. The reaction medium in the vessel was continuously stirred by a magnetic stirrer.

To create ΔpNa energy-depleted cells suspended in 25 mM Tris-HCl at a density of 8 mg protein/ml were saturated with 500 mM KCl and then diluted 40-fold into 25 mM Tris-HCl of the same pH containing 500 mM NaCl, 10 μM CCCP and 6.7 μM [^{14}C]succinate. The value of ΔpNa in some experiments was varied by preliminary equilibration of cells in media containing NaCl concentrations less than dilution medium. In these cases the concentration of KCl was varied so that the final salts concentration should be 500 mM.

An artificial membrane potential (inside negative) in energy-depleted cells was created through the imposition of the K^+ or H^+ diffusion potentials. To impose K^+ diffusion potential energy-depleted cells were suspended at a density of 8 mg protein/ml in 25 mM Tris-HCl (pH 7.0 or 9.0) containing 490 mM KCl and 20 μM valinomycin. After 1 h exposure in the given medium the cells were diluted 40-fold in 25 mM Tris-HCl of the same pH containing 10 μM CCCP, 20 μM valinomycin and 6.7 μM [^{14}C]succinate. In this case valinomycin-mediated electrogenic K^+ efflux created temporal membrane potential (inside negative).

For the assays in which $\Delta\psi$ was formed by setting up a proton diffusion potential, energy-depleted cells were suspended in 25 mM Tris-HCl (pH 7.0) at a density of 8 mg protein/ml. In an hour the cells were diluted 40-fold into 25 mM Tris-HCl (pH 9.0) containing 10 μM CCCP and 6.7 μM [^{14}C]succinate. Membrane potential (inside negative) was formed during CCCP-mediated electrogenic H^+ efflux.

To measure the light-induced succinate uptake the washed cells were suspended in 25 mM Tris-HCl at the required pH and supplements (see legends to the figures) up to a density of 200 μg protein/ml. After incubation for 1 h in the appropriate medium the cells were transferred into the glass thermostated vessels. Cell suspension in the vessel was illuminated by a 60 W incandescence lamp ($0.032\ J \cdot cm^{-2} \cdot s^{-1}$) and was bubbled with argon. The reaction was initiated by the addition of [^{14}C]succinate to give a final concentration of 6.7 μM.

In all experiments the samples of the cell suspension (1 ml) were withdrawn at the appropriate periods of time after the start of the uptake and were filtered through membrane filters (pore size 0.4 μm). Then the filters were washed once with 5 ml of reaction medium, dried and counted for radioactivity in Mark-II (Nuclear-Chicago) liquid scintillation spectrometer.

Protein was measured as described by Lowry et al. [35] using bovine serum albumin as a standard.

In all the assays the temperature was set at 35°C. The details of methods used are explained in legends to the figures.

Chemicals. Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), valinomycin and Tris were obtained from Sigma, U.S.A. Tetra-*n*-butylammonium hydroxide was purchased from Chemapol, Czechoslovakia. [2,3-^{14}C]Succinate (1.5 Ci/mol) was purchased from Sojuzizotop, U.S.S.R.

All other chemicals were reagent grade and obtained from commercial sources.

Results

Preliminary experiments have shown that light-induced uptake of succinate, malate and fumarate in *E. shaposhnikovii* at substrate concentrations below 100 μM strongly depends on the presence of Na^+ in reaction mixture. Na^+ cannot be replaced by other monovalent cations such as K^+, Li^+, NH_4^+ or divalent cations such as Mg^{2+}, Mn^{2+}, Ca^{2+}. The uptake of succinate at maximum rate needs relatively high (more than 50 mM) NaCl concentrations (Fig. 1).

The results of the experiments with energy-depleted cells confirm the suggestion that inwardly directed sodium gradient can serve as the immediate driving force for succinate accumulation against its concentration gradient (Fig. 2). In this case the initial rate and accumulation level of succinate uptake increase with the increase of ΔpNa value. The Na^+ gradient 500 : 1 ensures a 25-fold increase of intracellular succinate concentration as compared with the external medium. In these calculations the following ratio was used: 5 μl of intracellular volume corresponds to 1 mg of cellular protein [22]. Dissipation of artificial ΔpNa is accompanied by the efflux of the accumulated succinate out of the cells down its concentration gradient.

Experiments with energy-depleted cells also have shown (Fig. 3) that at pH 7.0 succinate accumulation is driven only by ΔpNa. Imposition of membrane potential (inside negative) prevents succinate accumulation at pH 7.0 even in the presence of ΔpNa. On the contrary, the highest level of succinate accumulation at pH 9.0 was observed at simultaneous imposition of ΔpNa and $\Delta\psi$. In all cases no uptake was observed in the absence of ΔpNa.

To examine more closely the requirements for $\Delta\psi$ and ΔpNa each was artificially imposed individually or in combination. In this series of experiments, $\Delta\psi$ (inside negative) was created by a proton diffusion potential. As shown in Fig. 4 simultaneous application of a proton diffusion potential and a sodium gradient produced a large transient uptake of succinate. The individual components ($\Delta\psi$ and ΔpNa) alone were less effective but still capable of producing uptake. Outwardly directed sodium gradient did not produce succinate uptake against its concentration gradient even in the presence of $\Delta\psi$.

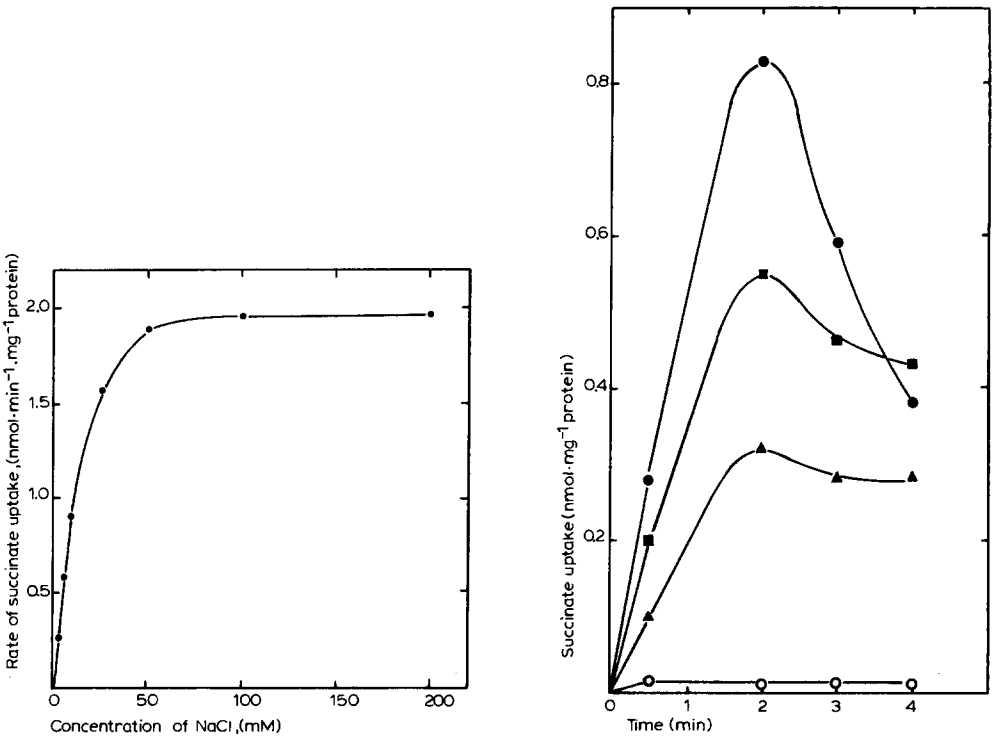

Fig. 1. Light-induced succinate uptake in *E. shaposhnikovii* as a function of NaCl concentration at pH 7.5.

Fig. 2. Succinate uptake energized by sodium gradients at pH 7.5. Cells were energy depleted by 10 μM CCCP as described under Methods. ●, 1 mM NaCl and 499 mM KCl intracellular; 500 mM NaCl extracellular, creating a ΔpNa of 500 : 1. ■, 10 mM NaCl and 490 mM KCl intracellular; 500 mM NaCl extracellular, creating a ΔpNa of 50 : 1. ▲, 50 mM NaCl and 450 mM KCl intracellular; 500 mM NaCl extracellular, creating a ΔpNa of 10 : 1. ○, 500 mM NaCl intra- and extracellular (control).

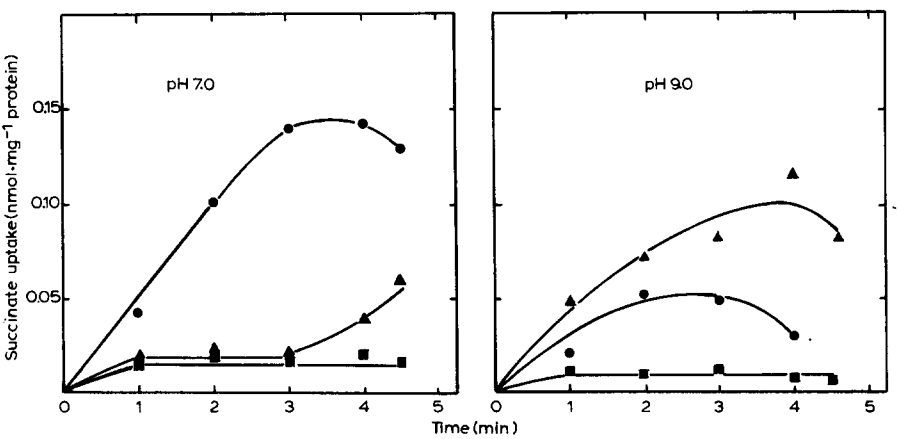

Fig. 3. Role of membrane potential in succinate transport energized by artificial sodium gradient ΔpNa at different pH values. Cells were energy depleted by 10 μM CCCP and $\Delta\psi$ (inside negative) was imposed as a K^+ diffusion potential as described under Methods. ●, 10 mM NaCl and 490 mM KCl intracellular; 500 mM NaCl extracellular, creating ΔpNa. ▲, 10 mM NaCl, 490 mM KCl and 20 μM valinomycin intracellular; 500 mM NaCl and 20 μM valinomycin extracellular, creating ΔpNa and $\Delta\psi$. ■, 500 mM NaCl intra- and extracellular (control).

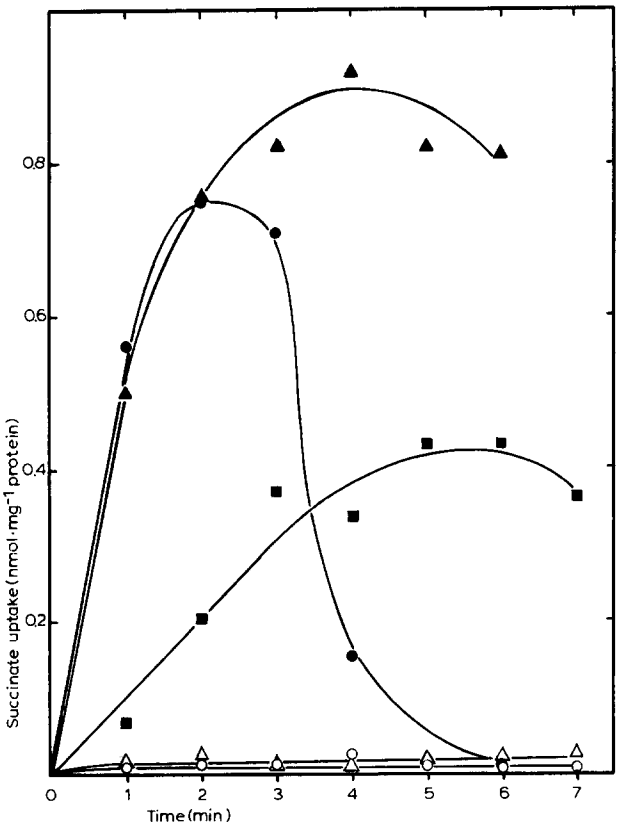

Fig. 4. Succinate uptake energized by a proton diffusion potential and/or ΔpNa. Cells were energy depleted by 10 μM CCCP and a Δψ (inside negative) was created by a shift in the pH of the external medium by two pH units in the presence of CCCP as described under Methods. ●, 500 mM NaCl, pH 7.0 intracellular; 500 mM NaCl, pH 9.0 extracellular, creating Δψ. ▲, 500 mM KCl, pH 7.0 intracellular; 500 mM NaCl, pH 9.0 extracellular, creating Δψ and ΔpNa. ■, 500 mM KCl, pH 9.0 intracellular; 500 mM NaCl, pH 9.0 extracellular, creating ΔpNa. ○, 500 mM NaCl, pH 7.0 intracellular; 500 mM KCl, pH 9.0 extracellular, creating Δψ with a reverse ΔpNa. △, 500 mM NaCl, pH 9.0 intra- and extracellular (control).

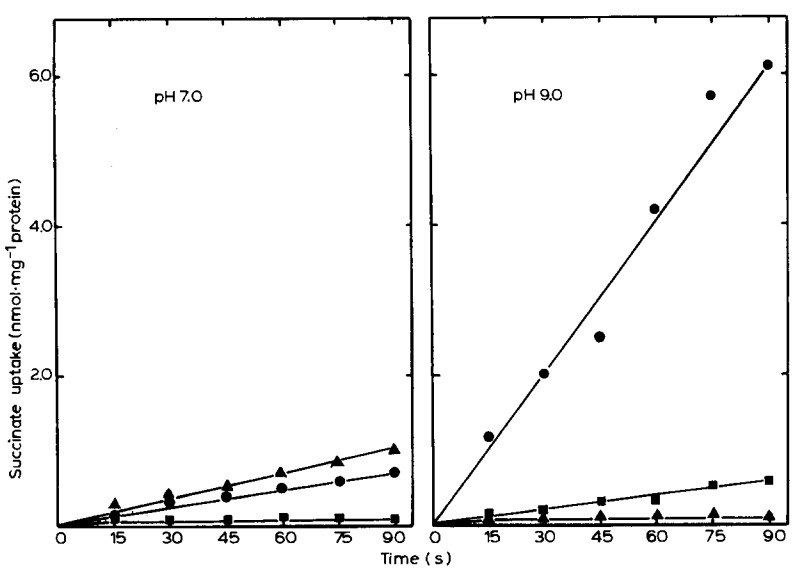

Fig. 5. Dependence of light-induced succinate uptake on added CCCP or tetra-*n*-butylammonium at different pH values. Cells were suspended in 25 mM Tris-HCl containing 50 mM NaCl. Inhibitors were added 10 min before the starting of reaction by labelled succinate. ●, control. ■, 10 μM CCCP. ▲, 2 mM tetra-*n*-butylammonium.

Light-induced succinate uptake is inhibited by CCCP, a well-known proton conductor which dissipates both ΔpH and $\Delta\psi$ produced by $\Delta\mu H^+$ generators (Fig. 5). CCCP inhibits succinate uptake both at pH 7.0 and at pH 9.0. At the same time lipid-soluble tetra-*n*-butylammonium cation inhibits light-induced succinate uptake at pH 9.0 but increases accumulation rate by 40% at pH 7.0. Such a difference in tetra-*n*-butylammonium action at different values of pH is all the more interesting because the rate of light-induced succinate uptake at pH 9.0 is much higher than that at pH 7.0 (Fig. 5).

Discussion

Two possible mechanisms for Na^+ stimulation of succinate transport were considered: (1) Na^+ is a 'cofactor' in the activation of the transport system which involves ΔpH-driven H^+/succinate symport, and (2) Na^+ gradient ΔpNa is the immediate source of energy and succinate is transported into the cell in symport with Na^+. As shown in Fig. 2 the gradient of Na^+ can be the immediate driving force for succinate uptake even in the absence of proton-motive force. These results are the direct evidence of Na^+/succinate symport. The Na^+ gradient being inwardly directed creates succinate gradient outwardly directed. An alternative explanation of the obtained results might be the following: ΔpNa via Na^+/H^+ antiport system creates ΔpH (inside alkaline) and the latter is the immediate driving force for succinate uptake. However, creation of substantial ΔpH capable of energizing succinate uptake seems impossible as the experiments were performed in the presence of 10 μM CCCP. The concentration of the uncoupler was high enough to inhibit succinate uptake even in the presence of such a strong $\Delta\mu H^+$ generator as light (Fig. 5).

To distinguish between H^+ and Na^+ as the critical cation in process of succinate transport an experiment was performed in which these two ions moved in opposite directions. A pH gradient was established (inside acid) and the outward movement of protons was facilitated by CCCP (Fig. 4). In this case the attachment of H^+ to the complex carrier-succinate (or carrier-succinate-Na^+) will not allow its transport inside because of large ΔpH (inside acid). Results show that succinate can be transported against ΔpH (inside acid), this makes us exclude completely the suggested mechanism of H^+/succinate symport with Na^+ as a 'cofactor'. In this experiment only the Na^+ would be available for inward cotransport with succinate (especially in the absence of ΔpNa). The different effect of $\Delta\psi$ on ΔpNa-driven succinate uptake at different pH values was unexpected to some extent (Fig. 3). Firstly, these results can reflect the different charge of ternary complex carrier-succinate-Na^+ (minus at pH 7.0 and plus at pH 9.0). According to this possibility $\Delta\psi$ would influence the movements of the charged complex within the membrane and hence the rate and level of succinate uptake. Secondly, these results can indicate the change of unloaded carrier charge at pH change (from plus at pH 7.0 to minus at pH 9.0). A model presented by Rottenberg [36] supposes the ternary complex between a carrier, substrate and cosubstrate to be always neutral during translocation. With respect to this model $\Delta\psi$ would be expected to influence only the return of unloaded carrier to the outer surface of the membrane. However, we cannot exclude the possibility of the ternary complex being actually charged

(positively or negatively), because the molecular structure and actual charge of binding groups for succinate and sodium are unknown.

A considerable difference in accumulation level of succinate uptake at pH 7.0 and 9.0 was rather difficult to explain when ΔpNa was the only driving force of the uptake (Fig. 3). The accumulation level of succinate down ΔpNa at pH 7.0 exceeds the level at pH 9.0 3-fold though the rate of light-induced uptake of succinate at pH 9.0 considerably exceeds the rate at pH 7.0 (Fig. 5). This discrepancy in the findings may be explained if we suggest that dissipation rate of the Na^+ gradient increases at higher pH value (9.0). Perhaps this could be a reflection of the higher numbers of Na^+ cotransported with succinate at pH 9.0 compared with pH 7.0. An increase in stoichiometry of Na^+/substrate symport at higher pH for methyl-β-D-thiogalactoside transport in *S. typhimurium* was supposed by Tokuda and Kaback [17]. The similar increase in stoichiometry for symport of neutral substrates and anions with protons in *E. coli* was shown by Ramos and Kaback [37].

The data presented in Fig. 5 indicate that $\Delta\psi$ (inside negative) is not required for succinate uptake at pH 7.0 but is necessary for the uptake at pH 9.0. The precise point of tetra-*n*-butylammonium action on the uptake (transport or creation of ΔpNa) is not possible to be detected from these results. The method of tetra-*n*-butylammonium probe within wide range of pH values can be used, nevertheless, for the approximate statement of the pH regions where the uptake depends or does not depend on $\Delta\psi$.

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