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Appearance of the Na^+-motive terminal oxidase in *Bacillus FTU* grown under three different conditions lowering the $\Delta\bar{\mu}_{H^+}$ level

Anna L. Semeykina and Vladimir P. Skulachev

Department of Bioenergetics, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, USSR

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The terminal oxidases and coupled Na^+ transport have been studied in intact cells and inside-out subcellular vesicles of alkalo- and halotolerant *Bacillus FTU* grown under different conditions. Cells grown at pH 7.5 are shown to possess a system of respiration-dependent Na^+ transport which is (i) inhibited by protonophorous uncoupler and (ii) activated by the $\Delta\Psi$-discharging agent valinomycin, suggesting that the Na^+ transport is due to cooperation of the H^+-motive oxidase and Na^+/H^+ antiporter. On the other hand, growth under conditions lowering the $\Delta\bar{\mu}_{H^+}$ level, namely (i) pH 8.6, (ii) pH 7.5 in the presence of protonophore, and (iii) pH 7.5 in the presence of low cyanide concentrations results in appearance of terminal oxidase-supported Na^+ transport which is stimulated by protonophores (the Na^+-motive oxidase). In all three cases, the appearance of ascorbate (+ TMPD) oxidation resistant to low and sensitive to high cyanide concentrations was found to occur. It is concluded that not only alkaline pH but also other conditions which lower $\Delta\bar{\mu}_{H^+}$ can cause substitution of Na^+ for H^+ as a coupling ion.

Low $\Delta\bar{\mu}_{H^+}$; Na^+-oxidase; Protometer; *Bacillus FTU*

1. INTRODUCTION

As was shown by our group [1–3], alkalo- and halotolerant *Bacillus FTU* possesses two respiratory chains, namely H^+-motive and Na^+-motive. It was also found, that while the former can be revealed in cell, grown at both neutral and alkaline pH, the latter is present only in the case of alkaline pH [4]. These data are in agreement with the concept [5] that adaptation to low $\Delta\bar{\mu}_{H^+}$ conditions ($pH_{in} < pH_{out}$) in bacteria growing in high [Na^+]media causes substitution of Na^+ for H^+ as a coupling ion. However, it was not excluded that such substitution is a specific consequence of the growth at alkaline pH rather than of a $\Delta\bar{\mu}_{H^+}$ decrease. In order to distinguish between these two possibilities, we now investigated *Bacillus FTU* cells grown at neutral pH but in the presence of agents lowering $\Delta\bar{\mu}_{H^+}$; i.e. (i) the protonophorous uncoupler pentachlorophenol or (ii) a low concentration of cyanide, which specifically inhibits the Na^+-motive terminal oxidase of *Bacillus FTU*. As will be shown below, in both cases the appearance of Na^+-motive terminal oxidase in *Bacillus FTU* takes place. This points to the crucial role of decrease in the $\Delta\bar{\mu}_{H^+}$ level, rather than in external [H^+], in switching from H^+ to Na^+ energetics.

Abbreviations: $\Delta\bar{\mu}_{H^+}$, transmembrane difference in electrochemical H^+ potential; $\Delta\Psi$, transmembrane electric potential difference; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; TMPD, *N,N,N',N'*-tetramethyl *p*-phenylenediamine.

Correspondence address: V.P. Skulachev, Department of Bioenergetics, A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119899, USSR

2. MATERIALS AND METHODS

Bacillus FTU, isolated in our laboratory [1,2], was used. The alkaline growth medium contained 0.5 M NaCl, 10 mM KCl, 15 mM $(NH_4)_2SO_4$, 2 mM KH_2PO_4, 5 mM $MgSO_4$, 1×10^{-5} M $FeSO_4$, 0.1 mM EDTA, 50 mM Tris-HCl, pH 8.6, and 60 mM sodium succinate as the only energy and carbon source. For growth at neutral pH, 30 mM HEPES-NaOH, pH 7.5, substituted for Tris-HCl. In all cases, cells were grown at 37°C under aerobic conditions. When indicated, growth medium was supplemented with 3 μM pentachlorophenol or 20 μM potassium cyanide. In the latter case, cells were grown for not longer than 12 h. In a separate experiment it was shown that the cyanide concentration left in the growth medium during this time decreased the rate of growth of *Bacillus FTU* cells 4–5-fold. The rate of growth of *Bacillus FTU* was monitored using light scattering at 600 nm.

To obtain the cells with depleted pools of endogenous respiratory substrates and ATP, the previously described procedure was used [2].

Ultrasonic inside-out subcellular vesicles were prepared as was described previously [2].

The Na^+ content in the subcellular vesicles was assayed by means of the gel-filtration and centrifugation procedure of Penefsky [6]. Respiratory chain dependent Na^+ uptake was initiated by adding TMPD. To arrest the reaction, 0.04 ml of the incubation mixture was centrifuged in a gel-filtration column (D = 10 mm) containing Sephadex G-50 coarse pre-equilibrated with 0.1 M Tris-H_2SO_4 and 10 mM $MgSO_4$, pH 8.2. The eluate was diluted 10-fold with bidistilled water and [Na^+] was measured with a PFM flame photometer. The above procedure was found to decrease extravesicular [Na^+] by a factor of 5×10^3 [3].

Oxygen consumption was monitored with a standard Clark-type oxygen electrode.

The protein concentration was measured by the Lowry method.

3. RESULTS

Fig. 1A and B show the effect of protonophorous uncoupler, CCCP, on Na^+ uptake by inside-out *Bacillus FTU* vesicles, energized by oxidation of ascorbate and TMPD. One can see that the vesicles from *Bacillus FTU* grown at pH 8.6 respond to the TMPD addition to the ascorbate-containing medium by Na^+ uptake which is strongly stimulated by CCCP.

As was shown elsewhere [3,4], such an effect is due to cooperation of (i) the electrogenic Na^+-motive terminal oxidase and (ii) protonophore-mediated electrophoretic H^+ efflux, which discharges the oxidase-produced $\Delta\Psi$ and allows large-scale Na^+ uptake to occur.

Quite different relationships were revealed when vesicles from *Bacillus FTU* grown at pH 7.5 were studied (Fig. 1B). Here the CCCP addition completely abolished the Na^+ uptake while valinomycin increased the Na^+ uptake rate. According to our previous study [4], the Na^+ uptake under these conditione is a result of cooperation of three systems, i.e. (1) H^+-motive oxidase, (2) valinomycin-mediated electrophoretic K^+ efflux converting the oxidase-produced $\Delta\Psi$ to ΔpH and (3) Na^+/H^+ antiporter.

Cyanide titration of respiration of the intact *Bacillus FTU* cells (Fig. 1C) showed that the *Bacillus FTU* cells, when grown at pH 7.5, possess only one type of oxidase which is sensitive to low cyanide concentrations. Under

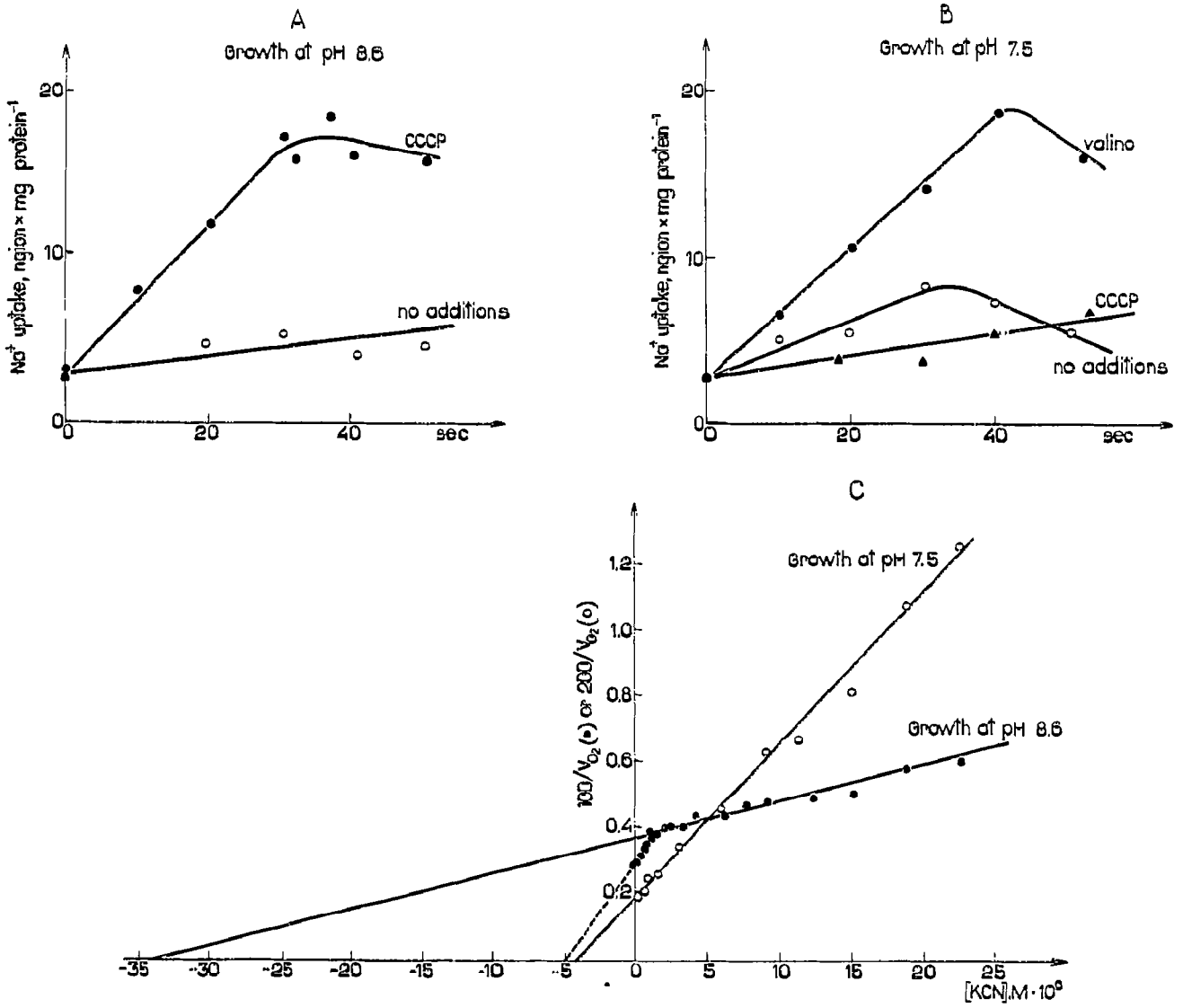

Fig. 1. Effect of pH of the growth medium on the terminal oxidase-supported Na^+ uptake inside-out subcellular vesicles of *Bacillus FTU* (A,B) and upon cyanide sensitivity of oxidase(s) in *Bacillus FTU* cells (C). Incubation mixture contained 0.1 M K_2SO_4, 2.5 mM NA_2SO_4, 10 mm $MgSO_4$, 50 mM tricine-KOH, pH 8.2, 50 mM diethyl ammonium acetate, 15 mM Tris-ascorbate, vesicles, 3.3 (A) and 4.7 (B) mg protein per ml or cells, 0.4 mg protein per ml (C). In A and B the mixture was supplemented with 4×10^{-6} M triphenyltin. At zero time, 0.5 mM TMPD was added to initiate electron transfer. Other additions: 1×10^{-5} M CCCP (A, B and C) and 1×10^{-5} M valinomycin (B). In C the cells were depleted in their pools of endogenous respiratory substrates (see Methods). Initial rates of respiration without cyanide were 340 (growth at pH 8.6) and 170 (growth at pH 7.5) ng-atom O per min per mg protein.

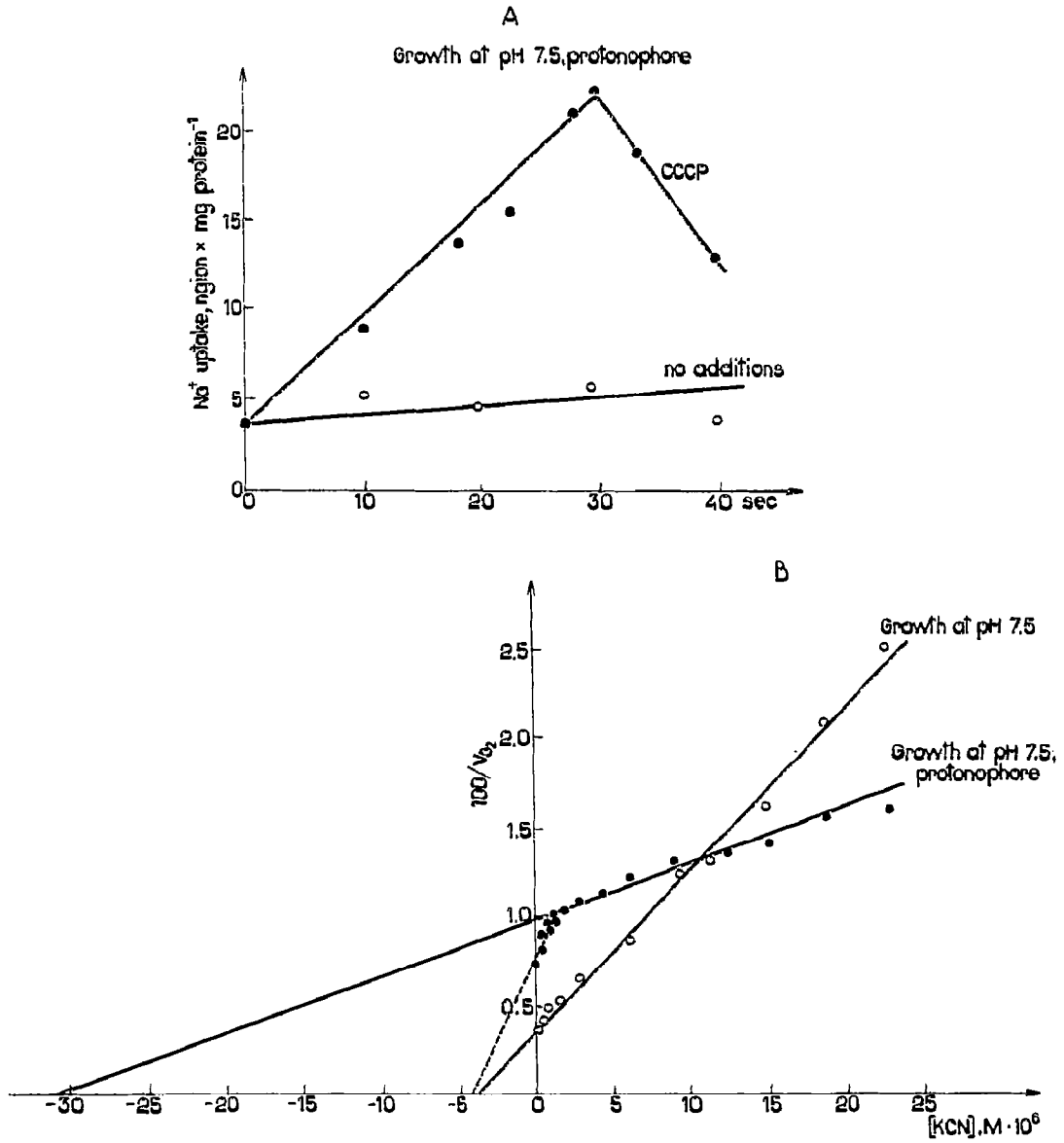

Fig.2. Appearance of activity of the Na^+-motive terminal oxidase in *Bacillus FTU* grown at pH 7.5 in the presence of the protonophore pentachlorophenol. (A) Na^+ uptake by the *Bacillus FTU* vesicles; (B) cyanide sensitivity of oxidase(s) in the *Bacillus FTU* cells. Vesicles, 3.1 mg protein per ml (A); cells, 0.3 mg protein per ml (B). Other conditions as in Fig. 1. Initial rates of respiration without cyanide were 170 (growth without protonophore) and 130 (growth with 3×10^{-6} M pentachlorophenol) ng-atom O per min per mg protein.

alkaline growth conditions, there appears one more terminal oxidase activity, resistant to low and sensitive to high cyanide concentration. As was shown earlier [3,4], oxygen consumptions sensitive to low and high cyanide concentrations are due to activities of H^+- and Na^+-motive terminal oxidases, respectively.

In the next series of experiments, we studied *Bacillus FTU* cells grown at neutral pH but under conditions which, like high pH, must results in de $\Delta\bar{\mu}_{H^+}$ decrease, i.e. in the presence of (i) protonophore pentachlorophenol (Fig. 2), or (ii) low concentration of cyanide (Fig. 3). In both cases, ascorbate (+ TMPD)-supported Na^+ uptake by inside-out vesicles appears to protonophore-stimulated (Figs. 2A and 3A). The cyanide titration of respiration in *Bacillus FTU* cells showed the appearance of a second terminal oxidase activity, sensitive to high cyanide concentrations, an effect similar to that induced by high pH (Figs. 2B and 3B).

4. DISCUSSION

The above data indicate that substitution of Na^+ for H^+ as a coupling ion is not only the bioenergetic mech-

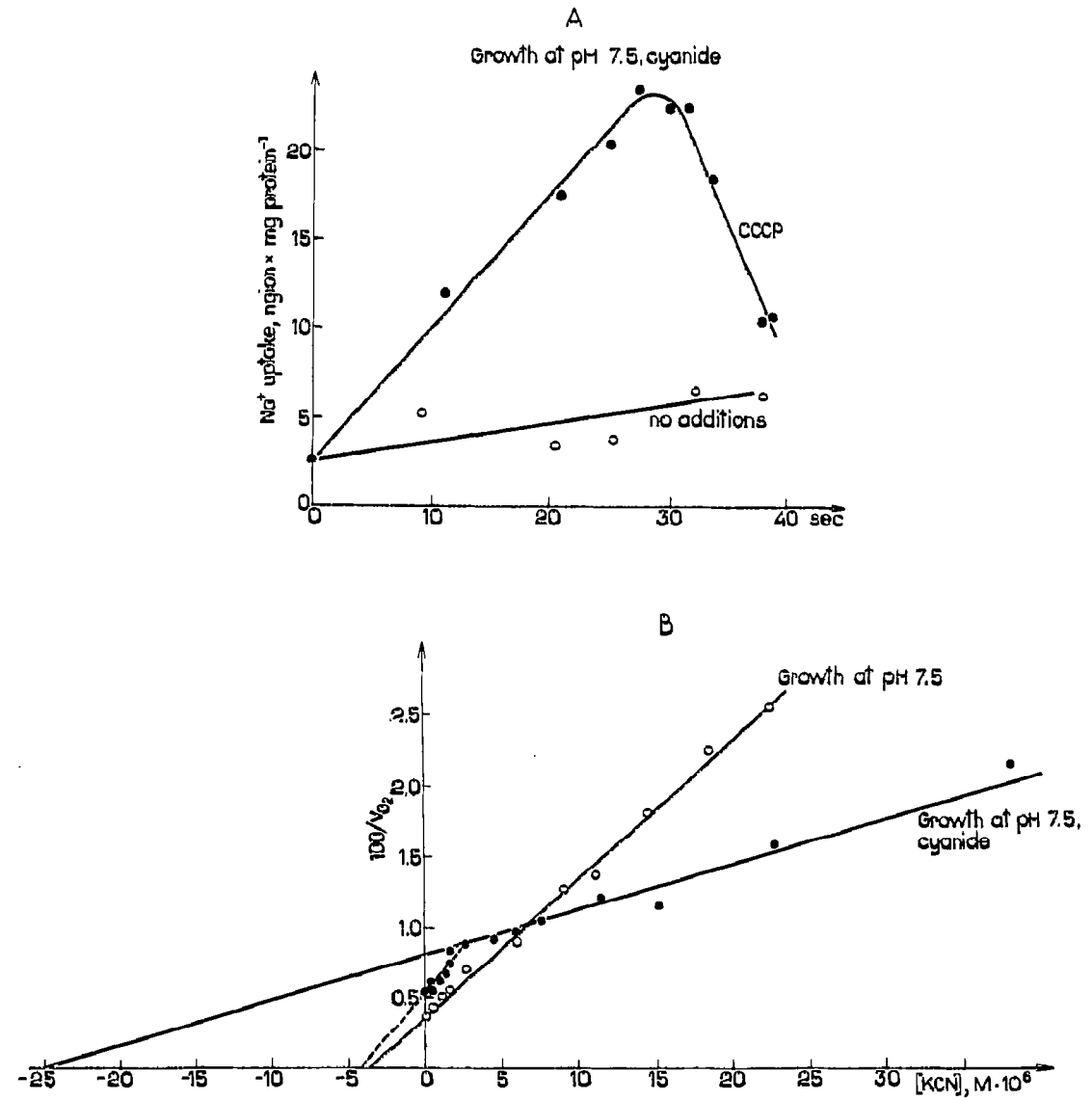

Fig. 3. Appearance of Na^+-motive terminal oxidase activity in *Bacillus FTU* grown at pH 7.5 in the presence of 2×10^{-5} M cyanide. (A) Na^+ uptake by *Bacillus FTU* vesicles; (B) cyanide sensitivity of the oxidase(s) in the *Bacillus FTU* cells. Vesicles, 3.8 mg protein per ml (A); cells, 0.4 mg protein per ml (B). Other conditions as in Fig. 1. Initial rates of respiration without cyanide in het incubation mixture were 170 (growth at pH 7.5 and 190 (growth in the presence of 2×10^{-5} M cyanide) ng-atom O per min per mg protein.

anism of bacterial adaptation to alkaline medium, but also a way to survive at neutral pH under conditions where $\Delta\bar{\mu}_{H^+}$ is low. Within the framework of this concept, high pH is a particular case of an adaptive mechanism of more general importance. It should be pointed out that data supporting this hypothesis have been recently obtained for *Escherichia coli* [4]. It was shown that bacteria grown at low $\Delta\bar{\mu}_{H^+}$, i.e. at alkaline pH or in the presence of a protonophore, possess primary Na^+-pumps in the respiratory chain, i.e. Na^+-motive NADH-quinone reductase and terminal oxidase.

Another example of induction of Na^+-energetics by low $\Delta\bar{\mu}_{H^+}$ can be found in the investigations of Kakinuma et al. [7,8], who showed that Na^+-ATPase of *Streptococcus faecalis* can be induced by growing bacteria in the presence of the uncoupler CCCP or at high pH. The same effect was also observed in a mutant deficient in H^+-ATPase, the main $\Delta\bar{\mu}_{H^+}$ generator in these bacteria. In this context we may also mention recent observation of Quirk et al. [9] who reported an increase in cytochrome oxidase level in *Bacillus firmus* growing at high pH or, alternatively, at neutral pH in the presence of protonophore.

All these data strongly support the involvement of a $\Delta\bar{\mu}_{H^+}$-sensor ('protometer'), a hypothetical system which allows a bacterium to monitor the $\Delta\bar{\mu}_{H^+}$ level, in regulation of bacterial energetics (for details, see [4, 10-12])

REFERENCES

[1] Verkhovskaya, M.L., Semeykina, A.L. and Skulachev, V.P. (1988) Dokl. Akad. Nauk SSSR 303, 1501–1503.
[2] Semeykina, A.L., Skulachev, V.P., Verkhovskaya, M.L., Bulygina, E.S. and Chumakov K.N. (1989) Eur. J. Biochem. 183, 671–678.
[3] Kostyrko, V.A., Semeykina, A.L., Skulachev, V.P., Smirnova, I.A., Vaghina, M.L. and Verkhovskaya, M.L. (1991) Eur. J. Biochem. 198, 527–534.
[4] Avetisyan, A.V., Dibrov, P.A. Semeykina, A.L., Skulachev, V.P. and Sokolov, M.V. (1991) Biochim. Biophys. Acta (accepted).
[5] Skulachev, V.P. (1984) Trends Biochem. Sci 9, 483–485.
[6] Penefsky, H.S. (1977) J. Biol. Chem. 252, 2891–2899.
[7] Kakinuma, Y. and Harold, F.M. (1985) J. Biol. Chem. 260, 2086–2091.
[8] Kakinuma, Y. and Igarashi, K. (1989) J. Bioenerg. Biomembr. 21, 679–691.
[9] Quirk, P.Q., Guffanti, A.A., Plass, R.J., Clejan, S. and Krulwich, T.A. (1991) Biochim. Biophys. Acta 1058, 131–140.
[10] Glagolev, A.N. (1980) J. Theor. Biol. 82, 171–185.
[11] Baryshev, V.A., Glagolev, A.N. and Skulachev, V.P. (1981) Nature 292, 338–340.
[12] Skulachev, V.P. (1988) Membrane Bioenergetics, Springer-Verlag, Berlin.