Volume 254, number 1,2, 17–21 FEB 07508 August 1989

The Na^+-motive respiration in *Escherichia coli*

A.V. Avetisyan, P.A. Dibrov, V.P. Skulachev and M.V. Sokolov

Department of Bioenergetics, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

Received 4 July 1989

The protonophorous uncoupler carbonyl cyanide *m*-chlorophenylhydrazone was found to inhibit the growth rate of *E. coli* and maintenance of the membrane potential ($\Delta\psi$) in intact bacterial cells. Both inhibitory effects of the protonophore were strongly decreased by Na^+. A favourable action of Na^+ could be observed only when cells were grown under conditions of low $\Delta\tilde{\mu}H$, i.e. at alkaline pH (8.6) or in the presence of the photonophore in growth medium. It was abolished by addition of ΔpH-dissipating agents, i.e. the penetrating weak base diethylamine and the Na^+/H^+ antiporter monensin, to the incubation medium. In inside-out subcellular vesicles isolated from cells grown at alkaline pH, NADH oxidation was shown to be coupled to Na^+ uptake which was markedly stimulated by protonophore and inhibited by cyanide and HQNO. It is concluded that the growth of *E. coli* at alkaline pH or in the presence of protonophore gives rise to the induction of a primary respiratory Na^+ pump(s).

Na^+-motive respiratory chain; Protonophore; Uncoupler; (*E. coli*)

1. INTRODUCTION

It has been shown in several laboratories that *E. coli* can grow under conditions of very low $\Delta\tilde{\mu}H$. Such an effect was observed in wild strains growing on glucose [1,2] or in protonophore-resistant mutants [3–7]. To explain the latter observation, the 'localized' proton cycle was postulated [6]. The sodium cycle seems to be the alternative explanation [8].

The studies carried out by Tokuda and Unemoto [9,10] and by our group [8,11–14] have shown that the marine bacterium *Vibrio aliginolyticus* possesses an Na^+-motive NADH-quinone reductase which produces $\Delta\tilde{\mu}Na$ utilized to support all types of membrane-linked work, i.e. ATP synthesis, uphill import of metabolites and rotation of the flagellum.

It is usually assumed that there is no primary respiratory Na^+ pump in *E. coli*, $\Delta\tilde{\mu}Na$ being formed at the expense of $\Delta\tilde{\mu}H$ energy by means of an Na^+/H^+ antiporter (reviews [15,16]). In this study, we demonstrate this to be the case for *E. coli* grown under conditions optimal for $\Delta\tilde{\mu}H$ formation. When, however, $\Delta\tilde{\mu}H$ is low, an electrogenic Na^+-motive respiratory system is induced.

Correspondence address: V.P. Skulachev, Department of Bioenergetics, A.N. Belozersky Laboratory of Molecular Biology and Bioorganic Chemistry, Moscow State University, Moscow 119899, USSR

Abbreviations: $\Delta\tilde{\mu}H$ and $\Delta\tilde{\mu}Na$, H^+ and Na^+ electrochemical potential differences, respectively; $\Delta\psi$, electric potential difference; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DEA, diethylamine; HQNO, 2-heptyl-4-hydroxyquinoline *N*-oxide; TPP^+, tetraphenylphosphonium cation

2. MATERIALS AND METHODS

E. coli strain K-12 Doc S (lac $i^-z^+y^+a^+$,pro^-,trp^-,his^-,met^-) was kindly supplied by Professor L. Grinius. Cells were grown aerobically at 37°C in a medium containing 22 mM KH_2PO_4, 20 mM Na_2HPO_4, 10 mM NaCl, 1 mM $MgSO_4$, 50 mM glycylglycine (pH 8.6 or 7.6), 0.05% yeast extract, 60 mM sodium succinate and marker amino acids (0.08 mg/ml) (medium 1). In medium 2 all Na^+ salts were replaced by K^+ salts.

To measure growth rate, 2×10^7 cells/ml were added to medium 1 or 2 and the light scattering at 600 nm was monitored.

Membrane potential in intact *E. coli* cells was measured using a TPP^+-sensitive electrode kindly supplied by Dr S.A. Novgorodov. At mid-logarithmic phase, cells were sedimented and washed twice with a medium containing 7 mM KCl,

Published by Elsevier Science Publishers B.V. (Biomedical Division)
00145793/89/$3.50

150 mM NaCl, 5 mM $MgSO_4$, 25 mM Tris-H_2SO_4 (pH 8.6 or 7.6) (medium 3) or a similar medium containing 150 mM KCl instead of NaCl (medium 4). The final sediment was suspended in medium 3 or 4 to obtain 0.3–0.5 mg protein/ml. $\Delta\psi$ was calculated according to Kamo et al. [17]. Intracellular volume was assumed to be equal to 1.7 μl/mg protein [18]. Passive TPP^+ binding was determined in the presence of 10 mM CCCP and 5 mM KCN.

Protein concentration was measured according to the Lowry method using Merck serum albumin as standard.

To obtain subcellular vesicles, cells were sedimented at 7500 × *g* for 10 min and washed with 150 mM NaCl, 5 mM $MgSO_4$, 10 mM Tricine (pH 8.2). The sediment was suspended in 0.2 M sucrose, 5 mM $MgSO_4$, 10 mM Tricine (pH 8.2) and incubated for 45 min at 30°C. After incubation, cells were sedimented again, suspended in 0.1 M K_2SO_4, 30 mM $MgSO_4$, 10 mM Tricine (pH 8.2), lysozyme (0.3 mg/ml) and incubated for 30 min at 37°C. The spheroplasts obtained were centrifuged at 9800 × *g* for 10 min and the sediment was suspended in 0.1 M K_2SO_4, 5 mM Na_2SO_4, 5 mM $MgSO_4$, 10 mM Tricine (pH 8.2), serum albumin (1 mg/ml), dithiothreitol (0.62 mg/ml). The suspension was passed once through a French press (1000 lb/inch^2) and then centrifuged at 14 000 × *g* for 15 min. The supernatant was centrifuged at 50 000 × *g* for 60 min. The sediment from subcellular vesicles was stored at liquid nitrogen temperature in 20% glycerol, 0.1 M K_2SO_4, 5 mM $MgSO_4$ and 10 mM Tricine (pH 8.2). Gel filtration with centrifugation and flame photometry techniques were used to measure Na^+ transport into subcellular vesicles, as described [13].

3. RESULTS

Growth of *E. coli* on media 1 and 2 containing succinate as the main energy source was found to occur only under aerobic conditions (not shown). Therefore, one may assume that oxidative phosphorylation was involved in the cellular energetics.

In fig.1A,B, one can see the effect of the protonophorous uncoupler CCCP upon growth of *E. coli* in the presence of high (about 170 mM) and low (about 2 mM) Na^+ concentrations, respectively. It is shown that in high Na^+ medium, much greater CCCP concentrations are required to arrest growth. This effect was observed at pH 7.6 (fig.1) as well as at pH 8.6 (not shown).

In fig.2 the effect of CCCP upon membrane potential in *E. coli* cells is demonstrated. According to fig.2A, cells grown at pH 8.6 and incubated in K^+ medium failed to maintain any $\Delta\psi$ in the presence of 7×10^{-6} M CCCP. At the same time, in Na^+ medium this concentration of CCCP decreased $\Delta\psi$ only slightly. A rather high $\Delta\psi$ was observed even with 2×10^{-5} M CCCP. Half-maximal effect was noted at an Na^+ concentration

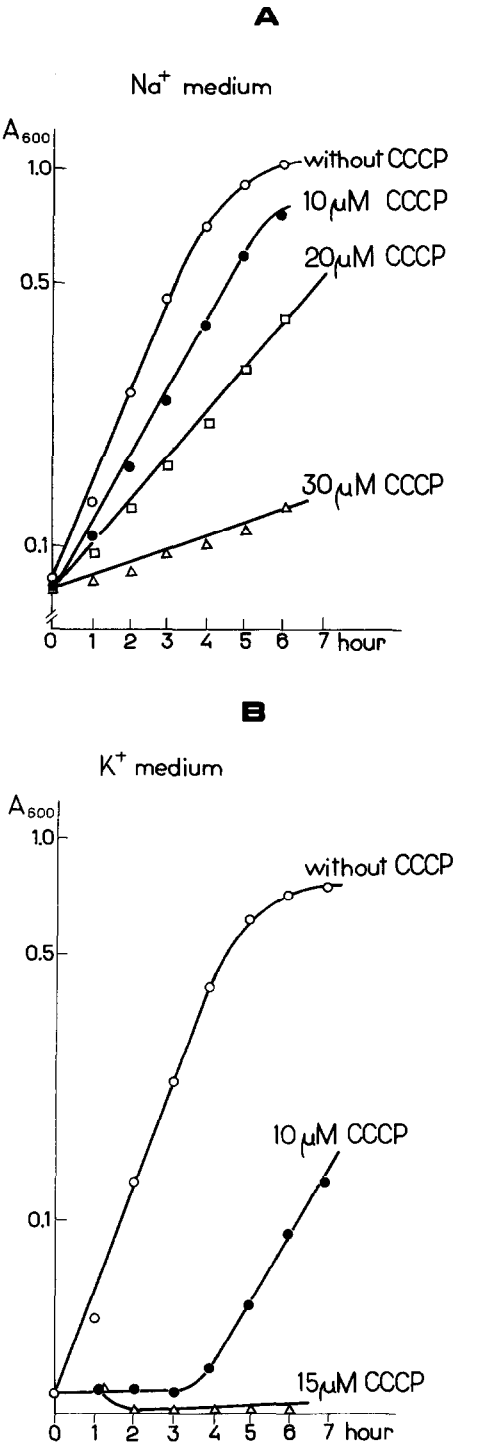
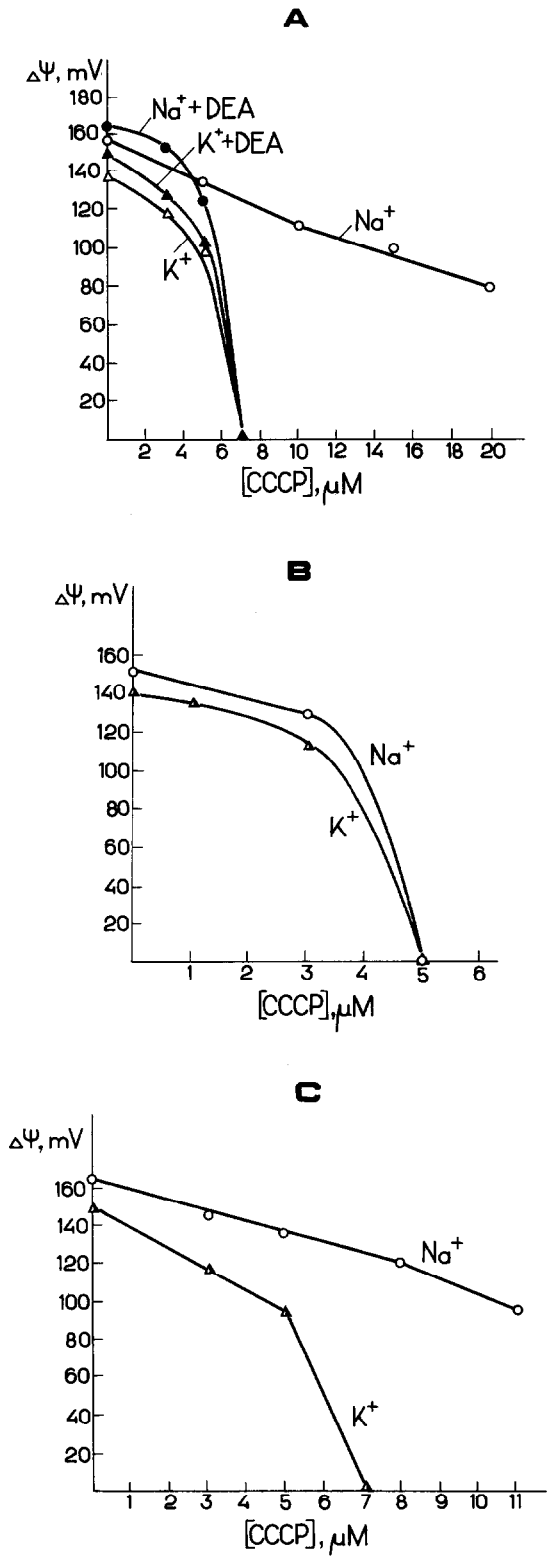


Fig.1. Na^+ decreases CCCP inhibition of *E. coli* growth rate; pH 7.6. (A) Na^+ medium (medium 1); (B) K^+ medium (medium 2).



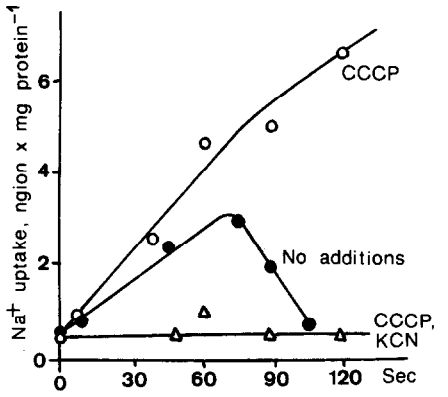


Fig.3. CCCP-stimulated Na^+ uptake by *E. coli* inside-out subcellular vesicles oxidizing NADH. Incubation mixture, 50 mM K_2SO_4, 5 mM Na_2SO_4, 30 mM $MgSO_4$, 25 mM potassium acetate, 0.1 M Tricine-KOH (pH 7.75). At zero time, 5 mM NADH was added. Other additions: 1×10^{-5} M CCCP, 10 mM KCN.

of about 3 mM (not shown). Addition of the penetrating weak base, DEA, sensitized $\Delta\psi$ to the action of CCCP in Na^+ medium. Without CCCP, DEA slightly increased $\Delta\psi$. The CCCP sensitivity of $\Delta\psi$ was unaffected by DEA in low Na^+ medium. DEA could be replaced by the Na^+/H^+ antiporter monensin (not shown).

All the above effects were observed for a pH value of the incubation medium of 8.6 (fig.2A) as well as pH 7.6 (not shown). However, the pH of the growth medium proved to be critical. The Na^+ effect upon $\Delta\psi$ was observed in bacteria grown at pH 8.6 (fig.2A) but not at pH 7.6 (fig.2B). The presence of CCCP in the growth medium resulted in the appearance of the Na^+ effect even when the pH of this medium was 7.6 (fig.2C).

In inside-out subcellular vesicles of *E. coli* grown at pH 8.6, it was shown that NADH oxidation was coupled to Na^+ uptake. Na^+ transport was strongly stimulated with CCCP and abolished by cyanide (fig.3) and HQNO (not shown).

Fig.2. Effect of Na^+ upon CCCP inhibition of membrane potential in intact *E. coli* cells. Na^+, Na^+ medium 3; K^+, K^+ medium 4. Where indicated, media were supplemented with 50 mM DEA. (A,B) Cells grown in medium 1 at pH 8.6 and 7.6, respectively; (C) medium 1 (pH 7.6) supplemented with 1.5×10^{-5} M CCCP.

4. DISCUSSION

All the above data can be readily explained by assuming that primary respiration-linked Na^+ pump(s) are induced in *E. coli* grown under conditions of low $\Delta\tilde{\mu}H$, i.e. at alkaline pH or in the presence of protonophore. Such pump(s) could be responsible for energy conservation in CCCP-containing growth medium (fig.1), formation of a CCCP-resistant $\Delta\psi$ in intact cells (fig.2A,C) and CCCP-stimulated Na^+ uptake coupled to NADH oxidation by inside-out subcellular vesicles (fig.3).

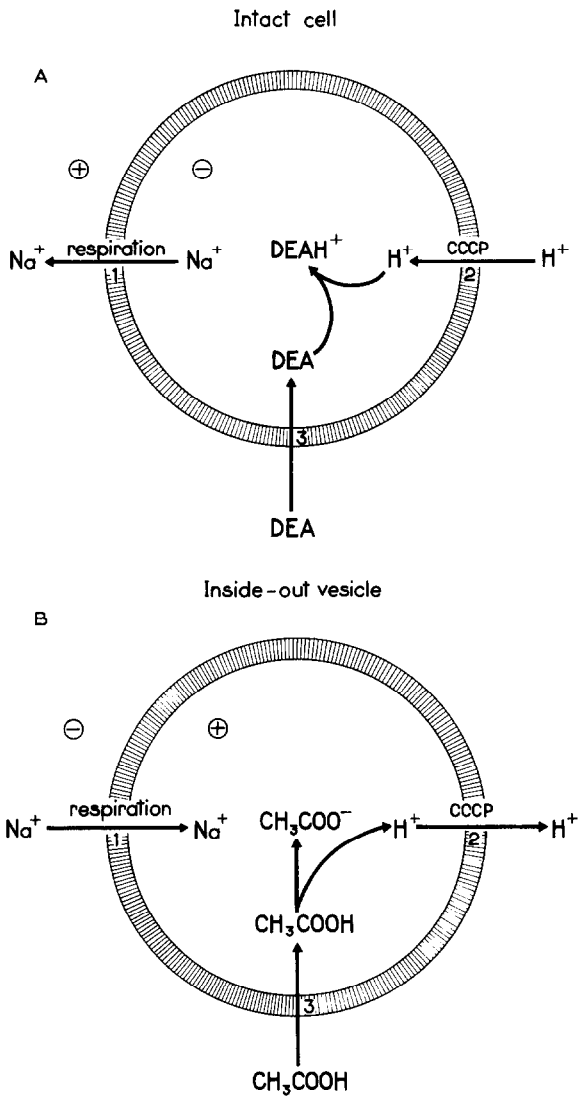


Fig.4. Interplay of primary respiratory Na^+ pump(s), CCCP and penetrating weak base or acid in *E. coli* grown under low $\Delta\tilde{\mu}H$ conditions.

The very fact that CCCP strongly stimulates Na^+ transport in the vesicles excludes any explanations of this effect by cooperation of the H^+-motive respiratory chain and Na^+/H^+ antiporter. At the same time, this stimulation could be predicted, suggesting that it is an electrogenic Na^+ pump that transports the Na^+. The formation of $\Delta\psi$ accompanying such transport prevents the large-scale Na^+ uptake by inside-out vesicles. Discharge of $\Delta\psi$ by CCCP-mediated H^+ efflux can abolish this limitation. To prevent ΔpH formation due to cooperation of the Na^+ pump and CCCP-mediated H^+ flux, acetate or DEA were added to subcellular vesicles or intact cells, respectively. These relationships are illustrated by fig.4.

It should be emphasized that, according to the data obtained, Na^+-motive respiration appears to be absent from cells grown at pH 7.6 without CCCP, i.e. under conditions of high $\Delta\tilde{\mu}H$. This indicates that the *E. coli* Na^+ cycle enzymes are induced only when $\Delta\tilde{\mu}H$ is lowered.

The discovery of the primary Na^+ pump in *E. coli* clearly shows that the occurrence of this type of bioenergetic mechanisms is not confined to such peculiar forms of life as alkalotolerant or some anaerobic bacteria.

REFERENCES

[1] Kinoshita, N., Unemoto, T. and Kobayashi, H. (1984) J. Bacteriol. 160, 1074–1077.
[2] Onoda, T. and Oshima, A. (1988) J. Gen. Microbiol. 134, 3071–3077.
[3] Ito, M. and Ohnishi, Y. (1981) FEBS Lett. 136, 225–230.
[4] Ito, M., Ohnishi, Y., Itoh, S. and Nishimura, M. (1983) J. Bacteriol. 153, 310–315.
[5] Jones, M.R., Quirk, P.G., Campbell, I.D. and Beechey, R.B. (1986) Biochem. Soc. Trans. 13, 888–889.
[6] Jones, M.R. and Beechey, R.B. (1987) J. Gen. Microbiol. 133, 2579–2766.
[7] Sedgwick, E., Hou, C. and Bragg, P.D. (1984) Biochim. Biophys. Acta 767, 479–492.
[8] Skulachev, V.P. (1988) Membrane Bioenergetics, Springer, Berlin.
[9] Tokuda, H. and Unemoto, T. (1982) J. Biol. Chem. 257, 10007–10014.
[10] Tokuda, H., Sugasawa, M. and Unemoto, T. (1982) J. Biol. Chem. 257, 788–794.
[11] Chernyak, B.V., Dibrov, P.A., Glagolev, A.N., Sherman, M.Yu. and Skulachev, V.P. (1983) FEBS Lett. 164, 38–42.

[12] Dibrov, P.A., Lazarova, R.L., Skulachev, V.P. and Verkhovskaya, M.L. (1986) Biochim. Biophys. Acta 850, 458–465.
[13] Dibrov, P.A., Skulachev, V.P., Sokolov, M.V. and Verkhovskaya, M.L. (1988) FEBS Lett. 233, 355–358.
[14] Dibrov, P.A., Kostyrko, V.A., Lazarova, R.L., Skulachev, V.P. and Smirnova, I.A. (1986) Biochim. Biophys. Acta 850, 449–457.
[15] Krulwich, T.A. (1983) Biochim. Biophys. Acta 726, 245–264.
[16] Dimroth, P. (1987) Microbiol. Rev. 54, 320–340.
[17] Kamo, N., Muratsugy, M., Hogoh, R. and Kobatake, J. (1979) J. Membrane Biol. 49, 105–121.
[18] Ahmed, S. and Booth, I.R. (1983) Biochem. J. 212, 105–112.