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Conversion of *Escherichia coli* Cell-Produced Metabolic Energy into Electric Form

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Abstract

The formation of membrane potential in energized *E. coli* cells has been investigated by means of ionic penetrants. The fluxes of anions and cations in opposite directions have been observed: anions moved out and cations moved into the cells. The energy-linked uptake of cations was stoichiometrically coupled with the outflow of H^+ ions from the cells. The value of a membrane potential in the energized cells calculated from a distribution of permanent cations was in the range of −140 mV (inside minus). The uptake of penetrating cations by deenergized cells has been observed following the non-enzymatic generation of a membrane potential. The influx of synthetic and natural (lactose) penetrants collapsed the non-enzymatic membrane potential. The effect of lactose was sensitive to N-ethyl maleimide. These results are in favour of the conception that in the energized *E. coli* cells an energy-linked H^+-pump generates a membrane potential which is a driving force for the transport of synthetic and some natural penetrants.

Introduction

Conversion of bacterial cell-produced metabolic energy into electrochemical gradient of H^+ ions (proton motive force) has been postulated

Abbreviations: CCCP (carbonylcyanide p-trichloromethoxyphenylhydrazone), NEM (N-ethyl maleimide), PCB^- (phenyl dicarbaundecaborane anion), $TPMP^+$ (triphenyl methylphosphonium cation), DDA^+ (N,N-dibenzyl N,N-dimethyl ammonium cation), DCCD (N,N-dicyclohexylcarbodiimide).

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by Mitchell (for review see [1-3]). According to this conception, the respiratory chain and ATPase act as two reversible electrogenic H^+-pumps. Mitchell and co-workers [4, 5] presented evidence indicating that respiration of aerobic bacteria is coupled with the electrogenic extrusion of H^+ from the cells. Recently a method for detection of membrane potential in so small objects as a mitochondria has been developed [6, 7]. It has been concluded [8] that synthetic ions penetrating artificial phospholipid membranes can be transferred across the biomembranes in response to the membrane potential generation without being bound to a translocase. Membrane potential-driven transport of ionic penetrants was observed in intact mitochondria, mitochondrial, bacterial, chloroplast particles and bacterial chromatophores (see [9, 10, 11]). This approach had been criticized by Lombardi *et al.* [12] on several grounds. These authors pointed out that under some conditions no uptake of the cationic penetrants by vesicles of *E. coli* membrane had been observed. Controversially, recent studies have shown that the vesicles [13] and intact cells of *E. coli* [14] accumulate cationic penetrants in the energy-dependent way.

It is the objective of this paper to examine the conversion of metabolic energy produced by *E. coli* cells into electric form. It has been shown that a membrane potential, negative inside, is being generated by electrogenic energy-linked H^+-pump. In the deenergized cells treated with protonophores–uncouplers of oxidative phosphorylation the non-enzymatic membrane potential can be generated by the artificially imposed pH gradient. Study of the effect of lactose on the non-enzymatic membrane potential revealed the electrogenic nature of permeation of this compound.

Materials and Methods

Reagents

DCCD, CCCP, valinomycin, PCB^- and NEM was a gift from Dr. V. P. Skulachev of Moscow State University (Moscow, U.S.S.R.), $TPMP^+$ was obtained from Chemapol (Czechoslovakya). Commercial preparates of D-lactose and Tris were recrystalyzed before use. Other reagents were analytical grade. Valinomycin and CCCP were dissolved in distilled ethanol.

Organism

The bacterial strain used in this work was *E. coli* W 945 (pro^-, lac^-, B^-, str-r) kindly provided by Dr L. M. Goldfarb of Institute of General Genetics (Moscow, U.S.S.R.) and *E. coli* ML 308-225 (i^-, z^-, y^+, a^+) kindly provided by Dr H. R. Kaback of the Roche Institute of Molecular Biology (Nutley, U.S.A.).

Culture of Bacteria

Stock cultures were maintained on solid medium, containing casein hydrolysate, yeast extract, agar and glucose (0.4 g of glucose per 100 ml). The liquid culture medium used for *E. coli* W 945 was that of Fisher *et al.* [15] and contained (in 1 litre): 4 g glucose, 7 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$, 0.1 g $MgSO_4 \cdot 7H_2O$, 5 mg $MnCl_2 \cdot 4H_2O$, 5 mg $Ca(NO_3)_2$, 2 mg $FeSo_4 \cdot 7H_2O$, 2 mg thiamine and 160 mg L-proline. The pH of medium, equal to 7.0, was controlled by H^+-sensitive electrode. The liquid culture medium used for *E. coli* ML 308-225 was that of Davis and Mingioli [16] and contained (in 1 litre): 7 g K_2HPO_4, 1 g $(NH_4)_2SO_4$, 0.5 g sodium citrate$\cdot 2H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$ and 2 g of succinic acid. In some experiments, mentioned below, instead of succinic acid 2 g of glucose was added. A small inoculum from the agar slope was added to the liquid medium. Strain W 945 was grown at 37° for 9-10 h under aerated conditions and harvested by centrifugation at 4° for 10 min at 10,000 g. Strain ML 308-225 was grown at 37° for 9 h under aerated conditions and diluted 1 : 8 with fresh medium. The stationary phase of growth corresponding to an extinction at 700 nm of about 1.7 was reached in 2.5 h before harvesting. In medium containing glucose as carbon source the cells were grown without dilution for 9 h at 37°. Cells were harvested as described above.

Preparation of washed suspensions of cells

For the preparation of suspensions of cells a Tris-EDTA treatment, exactly as described by West and Mitchell [5], was employed.

Preparation of sonicated particles

E. coli ML 308-225 cells were grown on succinate as described above. Harvested cells were washed twice with 0.01 M Tris-HCl (pH 8.0) and suspended (1 g wet wt/80 ml) in 0.03 M Tris-HCl (pH 8.0) and 20% sucrose at room temperature. The solution of 0.25 M EDTA (pH 6.6) and dry lisozyme were added to a final concentration of 0.01 M and 0.5 mg/ml, respectively. After incubation for 30 min at room temperature spheroplasts formed were pelleted at 8000 × g for 10 min. The sediment was suspended in minimal volume of 0.03 M Tris-HCl (pH 8.0) and 20% sucrose and diluted by 0.05 M Tris-H_2SO_4 (pH 7.8) to a final concentration of 1 g wet wt/25 ml and sonicated 2 × 1 min with a MSE sonifier (maximal output). After centrifugation at 10,000 × g for 15 min solid $MgSO_4$ was added to a final concentration of 5 mM and incubation was continued at 4° for 10 min. To obtain particles, suspension was centrifuged at 80,000 × g for 1 h at 4°. The pellet contained particles

which were suspended in 0.05 M Tris-H_2SO_4 and 5 mM $MgSO_4$ to a final concentration of 50-90 mg of protein/ml. The particles could be stored overnight at $-20°$ without significant loss of energy-linked transport of PCB^-.

Measurement of $TPMP^+$, K^+, H^+, PCB^- and oxygen concentration in extracellular media

Concentration of $TPMP^+$ was determined in centrifugates by means of ultraviolet absorbtion spectra [11]. Samples (5 ml) of the cell suspension were taken from aerobic experimental vessel, layered over precooled 0.88 M sucrose (3 ml) in the narrow (12 mm diameter) centrifuge tubes, chilled at 2° and pelleted in the refrigeratory centrifuge at 10,000 x g for 10 min. Then samples (3 ml) were carefully taken from the upper layer and optical density at 266.5 nm was measured. Optical density values of samples in the absence of $TPMP^+$ not exceeded of 0.05. The concentrations of $TPMP^+$ in the samples were calculated from the optical density values using extinction coefficient equal to 3100 litres/mole cm, as described by Harold and Papineau [11]. To calculate the concentration of $TPMP^+$ in experiments with NEM, an absorbtion caused by NEM was subtracted from the optical density values at 266.5 nm. The concentration of $TPMP^+$ in the cellular water space was calculated from the amount of $TPMP^+$ removed from the medium. From the results of three or four parallel measurements the standard error was calculated.

The effective quantities of H^+ and K^+ in the incubation mixture were measured simultaneously by means of the glass ion-selective electrodes connected with the LPU-01 pH-meters and sensitive strip-chart recorders. The commercial silver chloride reference electrode connected with medium by 0.1 M choline chloride junction was routinally used. K^+-selective electrode was greatly insensitive to the alkalization of incubation medium by the addition of Tris-base in the absence of *E. coli*. The anaerobiosis was achieved by bubbling of oxygen-free nitrogen in the incubation medium and reagents.

The concentration of PCB^- in the incubation medium was measured using phospholipid film, as described earlier [6, 7].

The respiration of cells was measured polarographically by means of a stationary platinum electrode.

Results and Discussion

Permeant cation movements during non-enzymatic membrane potential (negative inside) generation

Figure 1 shows the time-course of the effective quantities of H^+ and K^+ ions in the anaerobic suspension of cells preincubated with DCCD,

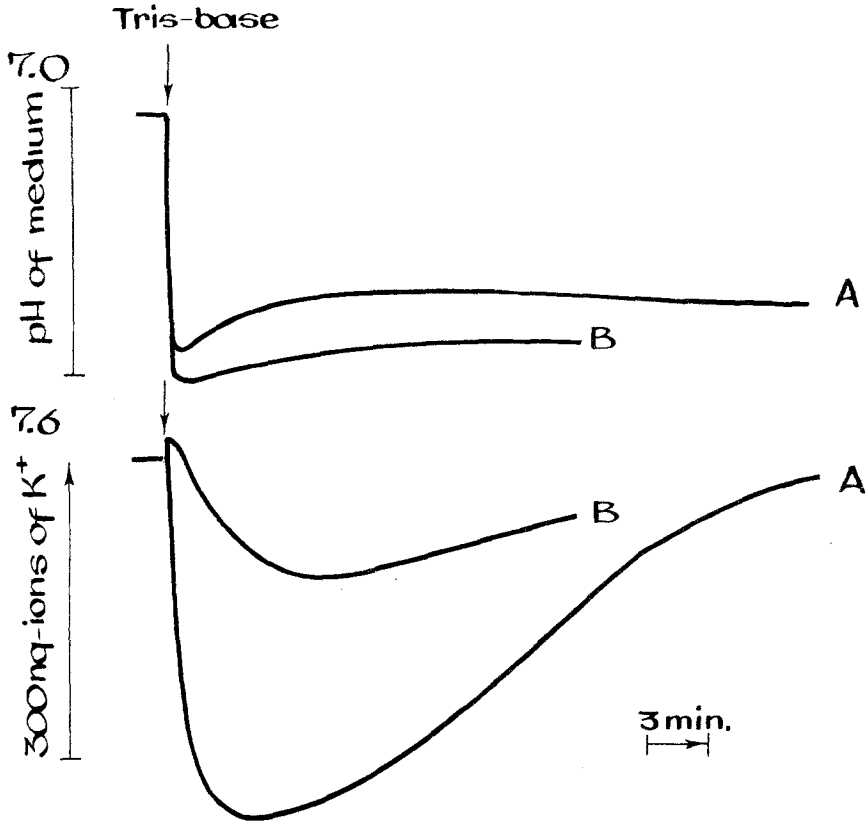

Figure 1. The K^+ uptake by deenergized *E. coli* cells in response to the non-enzymatic membrane potential in the presence (A) and absence (B) of valinomycin. The incubation medium (initial pH 7.2), containing 0.25 M sucrose, 3 mM glycil-glycine, 1.10^{-4} M DCCD and 4.10^{-6} M CCCP, was rendered anaerobic by flushing with oxygen-free nitrogen In Expt. A the incubation medium was supplemented with 2 μg/ml valinomycin. The suspension of *E. coli* ML 308-225 cells grown on glucose was added to a final concentration of 1.4 mg of dry weight per ml. After anaerobic incubation for 12 min, 50 μl of anaerobic 0.1 M Tris-base solution was injected as indicated by the arrows.

inhibitor of ATPase, and uncoupler CCCP in the presence (Expt. A) and absence (Expt. B) of valinomycin. It is seen that in the presence of valinomycin the effective quantities of H^+ and K^+ leave the anaerobic medium as a result of the alkaline Tris-base addition. The rapid alkalization of medium is followed by an exponential return towards the original pH due to an exit of H^+ ions from bacterial cells. The exit of H^+ ions coincides with the uptake of K^+ ions. After this, the pH of medium

stabilizes and the exponential return of K^+ concentration proceeds up to the original value that existed in medium before Tris-base pulse.

Experiment B, Fig. 1, demonstrates that the extent of K^+ uptake and the rate of K^+ movement is greatly diminished in the absence of valinomycin. The rate of H^+ exit from cells to alkaline incubation medium is slow in spite of the fact that the membrane is made permeable for H^+ by protonophore CCCP. The exponential nature of return of pH response is no longer observable.

In the case of experiments, such as that of Fig. 1, the final pH value depended on the quantity of Tris-base added and on the concentration of cells in the medium. No correlation between final pH and pK values was observed. Therefore, the different kinetics of K^+ uptake and medium alkalization, the absence of correlation between the final pH and pK levels and the dependence of K^+ uptake on the presence of valinomycin argue against the possibility that the Tris-base-induced K^+ accumulation is caused by direct H^+/K^+ exchange or by the appearance of dissociable groups in cell membrane with high affinity to K^+. Experiments depicted in Fig. 1 indicate that deenergized *E. coli* accumulate K^+ ions in response to the transmembrane pH gradient generation by Tris-base pulse. Since, as it is widely accepted [17-20], H^+ ions in the presence of uncoupler are considerably more mobile in the lipid membrane phase of cells than OH^- ions it seems possible to conclude that Tris-base pulse initiates diffusion of H^+ ions from more acidic intracellular volume to the alkaline external medium. Therefore, a transmembrane diffusion potential of H^+ ions, inside negative, establishes. This electrical back pressure diminishes the rate of H^+ efflux from cells. According to this point of view, the effective transmembrane diffusion of H^+ down the concentration gradient is achieved only in the presence of mobile counter-ions. Data of Fig. 1 strongly support this conception. It could be seen that the rate of H^+ exit greatly depends on the valinomycin-mediated K^+ uptake. Thus, it can be concluded that under these conditions the non-enzymatic membrane potential, with sign "minus" inside, generated by H^+ diffusion down the concentration gradient drives the K^+ uptake by deenergized *E. coli* cells.

Cells treated with $3\text{-}1.10^{-6}$ M CCCP and 1.10^{-4} M DCCD were routinally used for demonstration of Tris-base pulse-induced K^+ movements under anaerobic conditions. CCCP in concentration of 1.10^{-5} M or DCCD in concentration of 1.10^{-3} M caused the significant (80%) inhibition of pK responses. The effect of high concentrations of uncoupler or DCCD seems to be related with damage of cell membrane.

Evidence validating the influence of membrane potential on the accumulation of permeant cations is shown in Fig. 2. Experiments A and B demonstrate the time-course of $TPMP^+$ quantities in cells. At zero time the cells were added to aerobic medium containing $TPMP^+$. At intervals indicated samples were quickly taken from suspension, chilled and

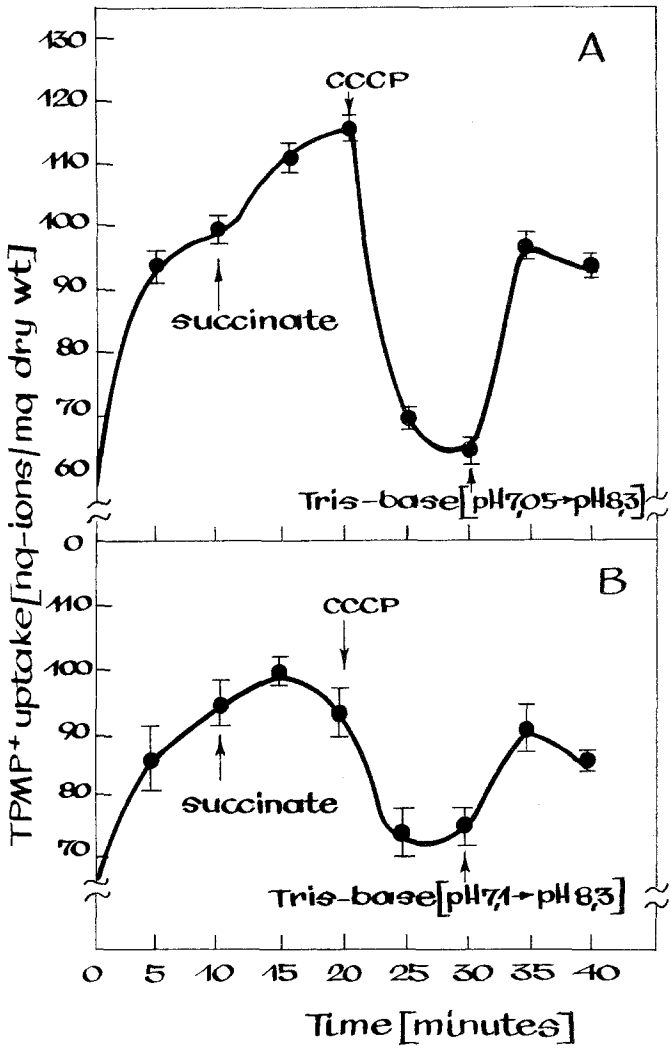

Figure 2. The $TPMP^+$ uptake by *E. coli* cells in the absence (A) and presence (B) of NEM. The aerobic incubation medium (initial pH 7.2) contained 0.25 M sucrose, 3 mM glycil-glycine, 1.10^{-6} M PCB^- and $1.83.10^{-4}$ M $TPMP^+$. In the Expt. B the incubation medium was supplemented with 5.10^{-4} M NEM. The suspension of *E. coli* ML 308-225 cells grown on succinate was added at a zero time to a final concentration of 1.2 mg dry wt/ml. Further additions were made: 4.10^{-3} M succinate, 1.10^{-6} M CCCP and solid Tris-base. The latest addition shifted the pH of medium from 7.1 to 8.3.

analyzed. It is seen (Expt. A) that cells accumulate $TPMP^+$ in the absence of added respiratory substrates. Additional $TPMP^+$ uptake is obtained after the addition of succinate to the suspension of cells. Treatment of cells with uncoupler CCCP causes the efflux of $TPMP^+$. Pulse of alkaline Tris-base leads to the extensive uptake of $TPMP^+$ by the deenergized cells. These results support the view that transmembrane pH gradient imposed by Tris-base pulse in the presence of protonophores generates the diffusion potential of H^+ ("minus" inside) which in turn, drives the uptake of cationic penetrants.

Assuming that 1 g of dry weight of cells corresponds to 2.7 ml of intercellular water [21], one can calculate that internal concentration of $TPMP^+$ accumulated in the uncoupler-sensitive way would be 18.1 mM, while its external concentration is 0.067 mM. The concentration ratio of 260 would be in equilibrium with a Nernst potential of −142 mV, negative inside. Earlier [14], the value of membrane potential in energized *E. coli* cells of the order of −140 mV was calculated from the distribution of DDA^+. These values are illustrative only because exact activity of internal $TPMP^+$ or DDA^+ used in calculations is unknown.

Early calculations [22] of the membrane potential value in *E. coli* cells from distribution of Cl^- ions gave the value of 29 mV, minus interior, but, unfortunately, no evidence excluding the electroneutral Cl^- exchange across membrane for some internal anion (s) was presented.

Experiment B of Fig. 2 shows the effect of NEM on the $TPMP^+$ uptake. At zero time the cells were added to the incubation medium supplemented with 5.10^{-4} M NEM. It is seen that cells accumulate the same quantity of $TPMP^+$ as in Expt. A. The addition of succinate induces no $TPMP^+$ uptake. Inhibition of succinate-induced $TPMP^+$ uptake seems to be related with NEM's effect on respiration. The polarographic measurements showed that at concentration of 5.10^{-4} M NEM inhibited succinate oxidation by 77%.

The cells treated with NEM retained ability to accumulate some quantity of $TPMP^+$ in the energy-linked way. It is seen that the addition of CCCP to the suspension of cells leads to the efflux of $TPMP^+$. The extra uptake of $TPMP^+$ takes place whenever a membrane potential is induced by Tris-base pulse. Results of this experiment indicate that the artificially imposed membrane potential is still observed when respiration-linked transport of $TPMP^+$ is inhibited by NEM

The next set of measurements was undertaken with a view to examine the effect of synthetic and natural penetrants on the artificially imposed membrane potential. The effect of $TPMP^+$ is depicted in Fig. 3. Experiment A shows that the addition of $TPMP^+$ lowers the value of membrane potential in cells, the fact being indicated by the efflux of K^+ that had accumulated during non-enzymatic potential generation. Experiment B shows that preincubation of cells with $TPMP^+$ practically prevents the non-enzymatic potential generation by Tris-base pulse. The

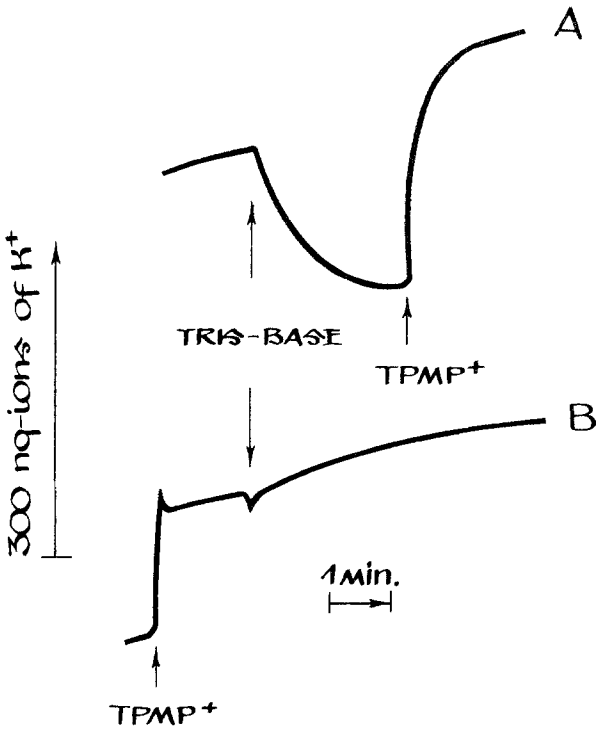

Figure 3. The effect of $TPMP^+$ on the non-enzymatic membrane potential. The anaerobic incubation medium was as in Fig. 1A except that 2.10^{-5} M CCCP was present. The suspension of *E. coli* ML 308-225 cells grown on succinate was added to a final concentration of 1.2 mg dry wt/ml. After anaerobic incubation for 8 min 1 mM Tris-base and 7.10^{-4} M $TPMP^+$ were added. The addition of Tris-base shifted the pH of the medium from 7.1 to 7.8.

effect of $TPMP^+$ on membrane potential reveals the electrogenic nature of movement of this cation across the cell membrane.

Qualitatively the same results were obtained in experiments with lactose (Fig. 4). The addition of lactose to medium lowered the non-enzymatic membrane potential (Expt. A). Preincubation of cells with lactose induced a decay of artificially-imposed potential (Expt. B). Experiment C shows that treatment of cells with NEM prior to injection of the Tris-base practically abolishes the effect of lactose on the membrane potential. It is seen that the addition of $TPMP^+$ to the incubation medium initiates the efflux of K^+. This fact correlates with data of Fig. 2 indicating that artificially-imposed membrane potential drives the uptake of $TPMP^+$ in the presence of NEM. With regard to the effect of lactose on the membrane potential, it seems possible to

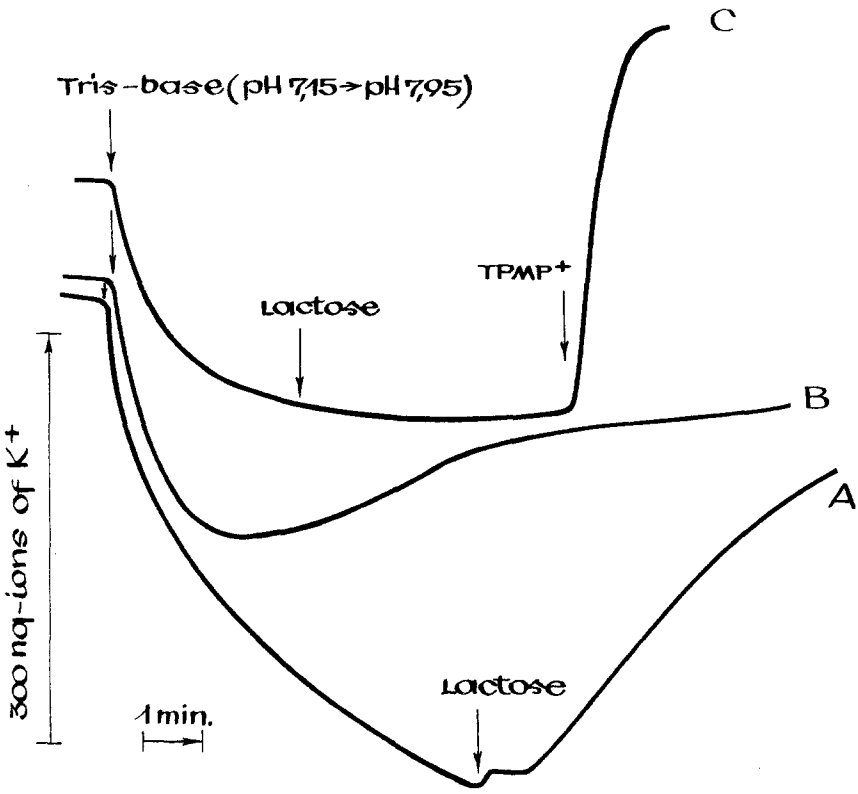

Figure 4. The effect of lactose on the non-enzymatic membrane potential. The anaerobic incubation medium was as in Fig. 3 except that 3.10^{-6} M CCCP was present and DCCD was omitted. In Expt. B the incubation medium was supplemented with 4 mM lactose and in Expt. C—with 4.10^{-5} M NEM. The suspension of *E. coli* ML 308-225 cells grown on succinate was added to a final concentration of 0.92 mg dry wt/ml. After the anaerobic incubation for 8 min 1 mM Tris-base, 4 mM lactose and 7.10^{-4} M $TPMP^+$ were added. The addition of Tris-base shifted the pH of medium from 7.1 to 7.9.

conclude that the entry of lactose down its concentration gradient via the specific NEM-sensitive porter is of electrogenic nature. Earlier the evidence of electrogenic character of lactose movement in *E. coli* cells was presented by West and Mitchell [5].

Transport of penetrating anions in the intact cells and sonicated membrane particles

The energy-linked transport of penetrating anion PCB^- was employed to detect the membrane potential in mitochondria, bacteria and

reconstituted energy-conserving proteoliposomes [23-25]. Suggestions that the PCB^- anion, as an excellent penetrant for artificial phospholipid membranes, should penetrate the phospholipid part of biological membranes in response for membrane potential generation was confirmed by recent observation [26].

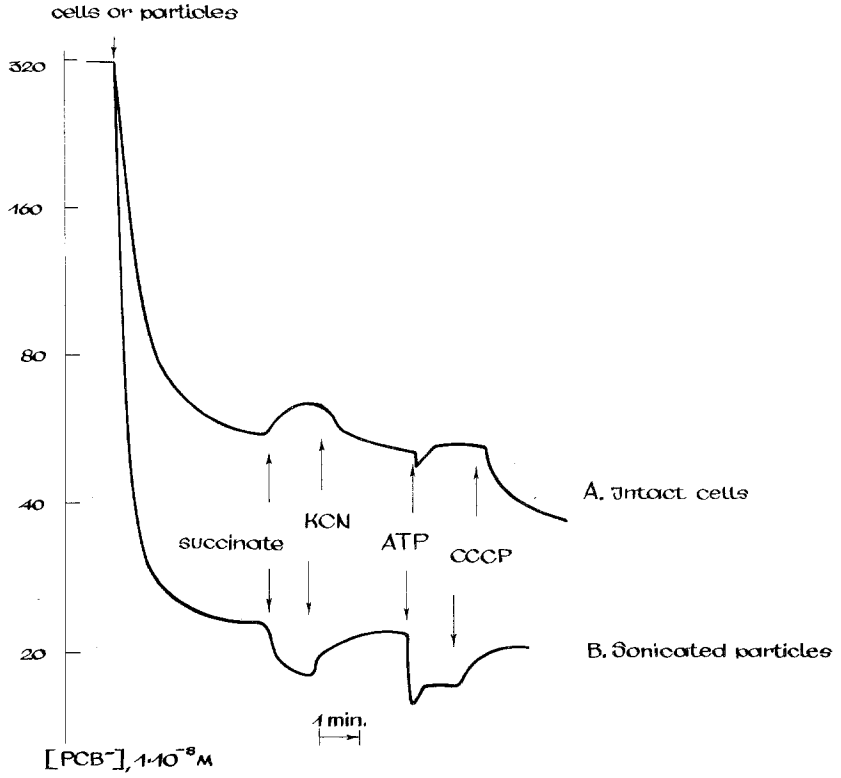

Figure 5. The effect of intact cells and sonicated membrane particles on the concentration of PCB^- in the medium. The aerobic incubation medium (initial pH 7.4) contained 0.05 M Tris-H_2SO_4, 5.10^{-3} M $MgSO_4$ and $3.2.10^{-6}$ M PCB^- The suspension of *E. coli* ML 308-225 cells grown on succinate (A) or sonicated particles of these cells (B) was added to the final concentration of 2.6 mg dry wt/ml and 1.8 mg of protein/ml, respectively. Further additions consisted of: 8.10^{-3} M succinate, 0.01 M KCN, 2.10^{-3} M ATP and 2.10^{-6} M CCCP.

The experiments depicted in Fig. 5 demonstrate the energy-linked transport of PCB^- in *E. coli* cells and sonicated membrane particles. It is seen that the addition of cells or particles to the medium containing PCB^- results in a decrease in the effective quantity of PCB^-, the effect being due to high distribution coefficient for PCB^- in the lipid-water

system [6]. Addition of oxidizable substrate succinate to the medium after the termination of PCB^- absorbtion induces an efflux of PCB^- from cells (Expt. A). When sonicated particles were used instead of intact cells, the additional PCB^- uptake had been obtained (Expt. B). Cessation of respiration by a respiratory inhibitor cyanide induces an uptake of PCB^- by cells (Expt. A) and an efflux of PCB^- urom particles (Expt. B). It is seen that particles take up PCB^- again in response to the ATP addition while addition of ATP to the suspension of cells has a little non-specific effect on PCB^- concentration. Deenergization of particles with CCCP (Expt. B) induces an efflux of PCB^- that had accumulated during the energized state. Expt. A shows that the treatment of cells with uncoupler leads to the uptake of anions. The PCB^- uoncentration in medium stabilizes at a level lower than that observed before succinate addition. Pretreatment of cells or particles with CCCP abolished the energy-linked PCB^- responses to succinate and ATP addition (not shown).

According to polarographic measurements the suspension of cells retained the ability to oxidize the endogenous respiratory substrates. This fact indicates that prior to the addition of exogenous substrates there exists an energized state in cells. Thus, the extra PCB^-uptake after the addition of uncoupler seems to be affected by deep deenergization of cells.

It was shown earlier [23] that energization induces an efflux of PCB^- urom intact mitochondria and an uptake of this anion by the "inside out" sonicated mitochondrial particles. Recently van Thienen and Postma [27] presented evidence indicating the "inside out" membrane orientation in sonicated *E. coli* particles. Results of Fig. 5 demonstrate that sonicated particles, but not intact cells, use ATP as energy source for PCB^- transport. In other experiments (not shown) the effectiveness of NADH to support PCB^- transport in particles, but not in cells, has been observed. All these facts correlate with "inside out" orientation of membrane in particles, since only the ATPase or NADH dehydrogenase oriented to the outside can react with non-penetrating substrates, such as ATP and NADH. Therefore, it can be concluded that the penetrating anion response depends on the membrane orientation. Experimental facts testify to the energy-linked formation of membrane potential with sign "minus" inside cells and "plus" inside sonicated particles.

pH and pK responses of cell suspension coupled with accumulation of catonic penetrants

According to chemiosmotic conception, fluxes of ionic penetrants, lowering the back pressure of the membrane potential, would stimulate the electrogenic energy-linked H^+-pump. The next set of experiments was undertaken to examine such possibility.

The time-course of the effective quantities of H^+ entering the aerobic incubation medium with *E. coli* cells in response to $TPMP^+$ addition is shown in Fig. 6. Experiment A was carried out in the absence of PCB^- and Expt. B—in the presence of this anion. It is seen that H^+ ions enter (or OH^- ions leave) the medium as a result of $TPMP^+$ addition. Treatment of cells with protonophorous uncoupler results in an influx of

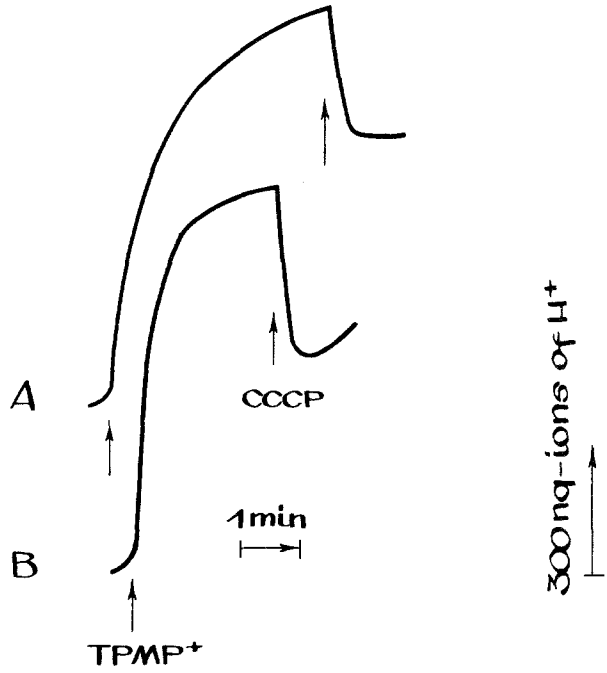

Figure 6. The effect of energy-linked $TPMP^+$ uptake on the pH of aerobic cell suspension in the absence (A) and presence (B) of PCB^-. The aerobic incubation medium (initial pH 7.2) contained 0.25 M sucrose and 3 mM glycil-glycine. In Expt. B the incubation medium was supplemented with 1.10^{-6} M PCB^-. The suspension of *E. coli* ML 308-225 cells grown on succinate was added to a final concentration of 1.4 mg dry wt/ml. Afterwards aerobic incubation for 5 min 7.10^{-4} M $TPMP^+$ and 1.10^{-6} M CCCP were added.

H^+ in both experiments. The rate of H^+ translation after the addition of $TPMP^+$ depends on the presence of PCB^- in medium (compare Figs. 6A and 6B). Earlier it has been shown [14] that the uncoupler-sensitive pH response to DDA^+ addition was practically abolished in the absence of PCB^-. This fact correlates quite well with lower permeability of DDA^+ through phospholipid membranes in the absence of PCB^- [7].

Figure 7 demonstrates the time-course of pK responses on the addition of $TPMP^+$. The pK traces have not been corrected for the

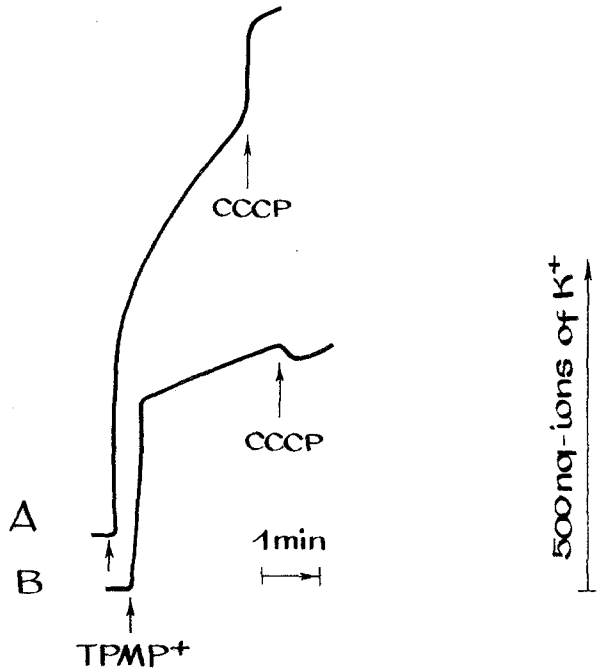

Figure 7. The effect of energy-linked $TPMP^+$ uptake on the pK of aerobic cell suspension in the presence (A) and absence (B) of valinomycin. The aerobic incubation medium was as in Fig. 6. In Expt. A the incubation medium was supplemented with 2 μg/ml valinomycin. The suspension of *E. coli* W 945 cells was added to a final concentration of 1 mg dry wt/ml. Afterwards aerobic incubation for 5 min 7.10^{-4} M $TPMP^+$ and 1.10^{-6} M CCCP were added.

significant, but low, reactivity of the K^+ ion-selective electrode to $TPMP^+$ ions. In Expt. A aerobic suspension of cells preincubated with valinomycin was used. It is seen that the addition of $TPMP^+$ results in a fast efflux of K^+ ions from cells. The addition of CCCP stimulates the effective entry of K^+ ions to the medium.

Experiment B, Fig. 7, was carried out in the absence of valinomycin added. The pK trace shows non-specific response of K^+ ion-selective electrode on the addition of $TPMP^+$. It is seen that in the absence of valinomycin the uptake of $TPMP^+$ by cells causes only a slow exit of K^+ ions. The addition of CCCP has a small effect on the pK of medium. The step change in pK corresponds only to the dillution effect.

The experiments depicted in Figs 6 and 7 demonstrate the crucial difference of pH and pK responses on $TPMP^+$ addition. The reversion of pH response by uncoupler indicates that H^+, but not K^+, ions are transported against their own concentration gradient.

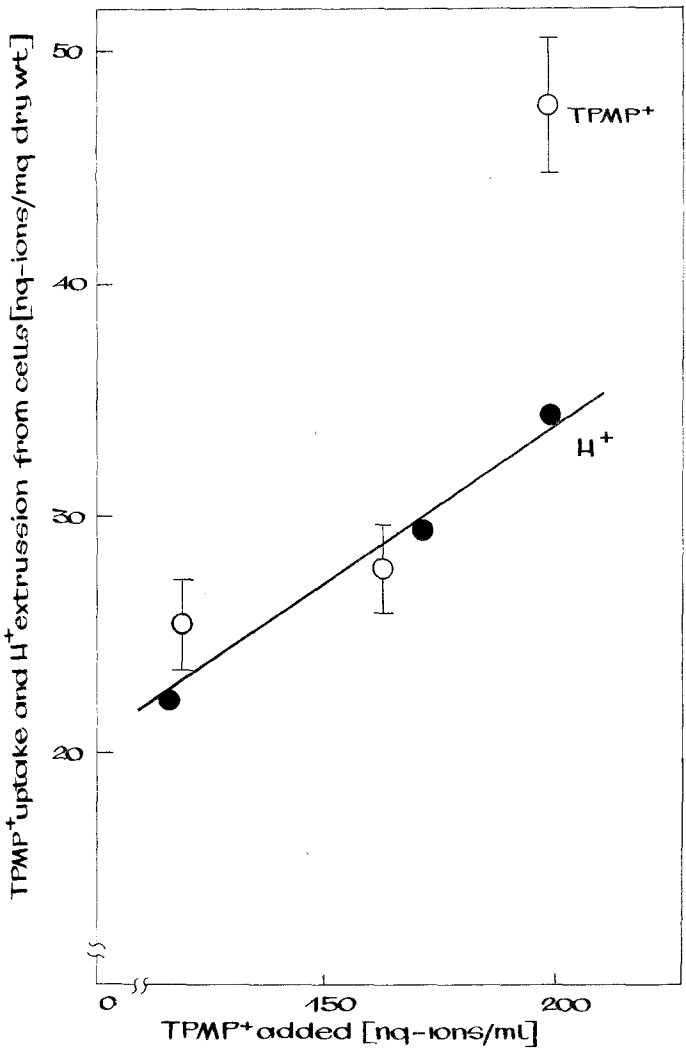

Figure 8. Correlation between the energy-linked uptake of $TPMP^+$ and H^+ extrusion from cells. The aerobic incubation medium was as in Fig. 6. The suspension of *E. coli* W 945 cells was added to a final concentration of 1.1 mg dry wt/ml. After aerobic incubation for 10 min the solutions of $TPMP^+$ were added to the final concentrations shown in the figure. The parallel measurements of pH responses on the $TPMP^+$ addition were obtained from cells from the same stock suspension. The quantities of $TPMP^+$ accumulated and H^+ extruded on the energy-linked way were calculated after the addition of CCCP to a final concentration of 1.10^{-6} M.

Model of electrogenetic H^+-pump predicts rigid stoichiometry between ionic penetrant accumulated and H^+ ion extruded under conditions when side reactions, such as electroneutral H^+/cation antiport, are minimal. The correlation between the quantity of $TPMP^+$ accumulated and H^+ extruded in the energy-linked way is depicted in Fig. 8. It was not possible to measure both $TPMP^+$ and H^+ movements in the same suspension simultaneously because the withdrawal of portions of the suspension for $TPMP^+$ determinations disturbed the pH measuring system. Parallel sets of measurements were therefore done on cells from the same stock suspension. Figure 8 demonstrates that the effective quantities of H^+ entering and $TPMP^+$ leaving the incubation medium in the energy-linked way depend on the amount of $TPMP^+$ added. It is seen that at low concentrations of $TPMP^+$ the stoichoimetric ratio of effective $H^+/TPMP^+$ translocation is close to 1.

Figure 9 demonstrates the time course of the effective quantities of H^+ entering the aerobic medium as a result of energy-linked accumulation of K^+ ions. It is seen (Fig. 9A) that the valinomycin-induced inflow

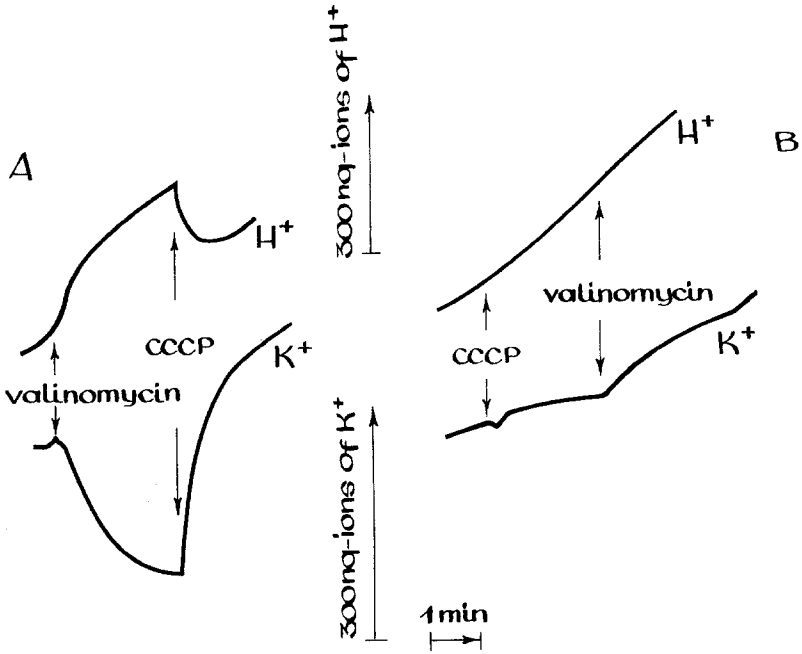

Figure 9. The effect of valinomycin on the pH and pK of aerobic cell suspension. The incubation medium as in Fig. 6 was supplemented with 7.10^{-3} M KCl. The suspension of *E. coli* W 945 cells was added to a final concentration of 1.1 mg dry wt/ml. After aerobic incubation for 5 min 2.76 μg/ml valinomycin and 1.10^{-6} M CCCP were added.

of K^+ ions is coupled to the outflow of an equal number of H^+ ions. In other sets of measurements the stoichiometric ratio of effective H^+ and valinomycin-induced K^+ translocation varied from 0.9 to 1.3. This ratio correlates quite well with data of West and Mitchell [5]. Figure 9A also shows that the addition of CCCP to the suspension of cells treated with valinomycin leads to the exit of K^+ and to the uptake of H^+ ions. The stoichiometric ratio of the uncoupler-induced inflow of H^+ and outflow of K^+ varied from 1.5 to 5.0 in different sets of measurements.

Figure 9B shows that the addition of valinomycin to the cell suspension incubated with uncoupler induces outflow of K^+. It is seen that the outflow of K^+ from deenergized cells has a small effect on the pH of suspension. This fact indicates that under some conditions the translocation of K^+ could be compensated by movements of other ions, not H^+.

From the results of experiments presented in Figs 8 and 9 it can be concluded that in *E. coli* cells the energy-dependent inflow of cationic penetrants is stoichiometrically coupled with the outflow of an equal number of H^+ or with the influx of an equal number of OH^- ions. These results favour the supposition that in the energized state an H^+- or OH^--pump generates a negative membrane potential inside *E. coli* cells.

The next sets of measurements were taken to examine the role of ATPase in the energy-linked H^+ extrusion. Figure 10 demonstrates the time-course of effective quantities of H^+ entering the medium, as the result of $TPMP^+$ addition. In the experiments shown by Fig. 10A aerobic suspensions of cells were employed. It is seen that the addition of $TPMP^+$ causes an energy-dependent pH response in spite of the presence of ATPase inhibitor DCCD (Expt. B). Figure 10B depicts the pH responses in a medium containing the respiratory inhibitor potassium cyanide. Under these conditions the addition of $TPMP^+$ induces an energy-dependent pH response only in the absence of DCCD (Expt. A). It can be seen (Expt. B) that preincubation of cells with DCCD prior to the $TPMP^+$ mddition completely removes the energy-linked pH response. Thus, it seems possible to conclude that the functioning of the H^+-pump under conditions where the activity of the respiratory chain is blocked by inhibitors depends on the hydrolysis of ATP formed during glycolysis.

Consideration of the experiments of Fig. 10 leads to the view that the energy input from either the hydrolysis of high energy phosphates or the oxidation of respiratory substrates is utilized by the energy-linked H^+-pump for membrane potential generation. The results of Fig. 10 are in good agreement with the evidence published by Klein and Boyer [28] indicating that membranes of *E. coli* can transform both energy inputs to produce a high energy membrane state and with the recent report of West and Mitchell [29] that the H^+-translocating ATPase exists in *E. coli* cells.

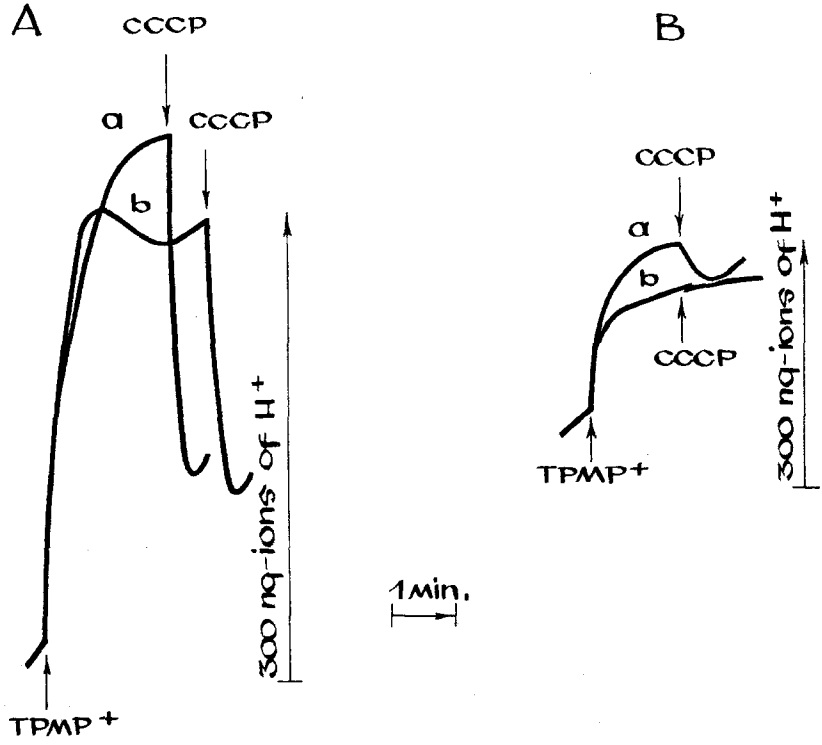

Figure 10. The effect of DCCD on the energy-linked pH responses in the absence (A) and presence (B) of KCN. The incubation medium (as in Fig. 6 supplemented with 1.10^{-6} PCB^- 10 mM K_2SO_4 (A) and 10 mM KCN (B)) contained: (a) no further additions; (b) 5.10^{-5} M DCCD. The suspension of *E. coli* ML 308-225 cells grown on succinate was added to the final concentration of 1.2 mg dry wt/ml. After incubation for 4 min 7.10^{-4} M $TPMP^+$ and 4.10^{-6} M CCCP were added.

The permeability of artificial phospholipid and biological membranes by synthetic penetrants depends on many factors [6, 7]. The experiments shown below demonstrate the effect of ionic strength on the stimulation of *E. coli* cell H^+-pump by cationic penetrants. Figure 11 shows the effect of ionic strength on the time-course of entry of effective quantities of H^+ to the medium as a result of $TPMP^+$ addition. The experiments in Fig. 11A were carried out in the absence of PCB^- and those in Fig. 11B in the presence of this anion. It is seen that in the absence of PCB^- the pH responses to $TPMP^+$ addition are removed in the medium with high ionic strength. In the presence of PCB^- (see Fig. 11B) the energy-linked pH responses are still observed under the same conditions. The quantitively analogous results were obtained in experiments where incubation medium with NaCl was used (not shown).

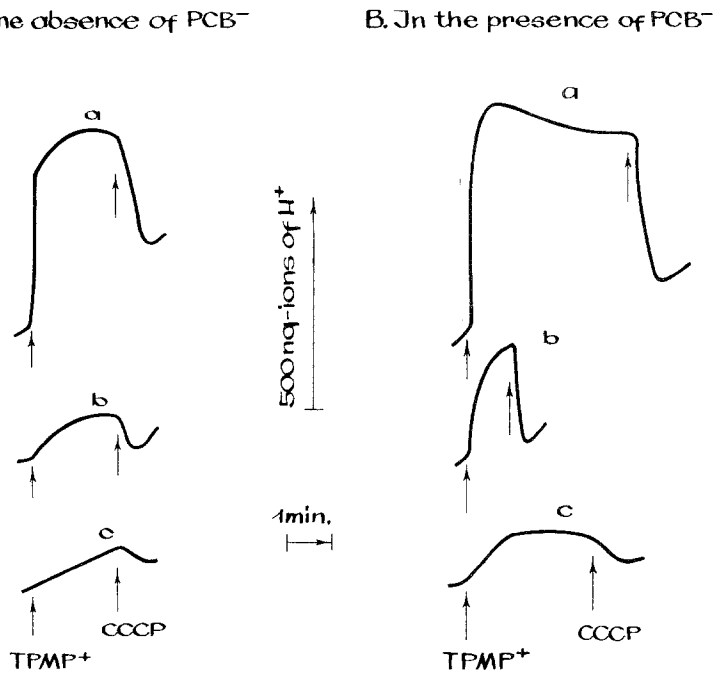

Figure 11. The effect of ionic strength on the pH responses coupled with $TPMP^+$ accumulation in the absence (A) and presence (B) of 1.10^{-3} M PCB^-. The aerobic incubation medium contained: (a) 0.25 M sucrose, 3 mM glycil-glycine (pH 7.2); (b) 0.06 M KCl, 0.13 M sucrose, 3 mM glycil-glycine (pH 7.2); (c) 0.125 M KCl, 3 mM glycil-glycine (pH 7.2). The suspension of *E. coli* W 945 cells was added to a final concentration of 1.1 mg dry wt/ml. After aerobic incubation for 5 min 7.10^{-4} M $TPMP^+$ and 1.10^{-6} M CCCP were added.

The effect of ionic strength indicates that the surface potential could regulate the permeability of *E. coli* membrane for cationic penetrants. Thus, the failure of Lombardi *et al.* [12] to demonstrate the energy-linked DDA^+ uptake by membrane vesicles of *E. coli* could be to some extent, at any rate, due to the low permeability of membrane to cationic penetrants in the absence of lipophilic anions in the medium of high ionic strength that these authors used.

Conclusion

The experiments presented above can be summarized as follows:

(1) The membrane potential can be generated in *E. coli* cells treated with uncoupler and ATPase inhibitor by artificially imposed trans-

membrane pH gradient under anaerobic conditions. The membrane potential generation is followed by the uptake of cationic penetrants.

(2) Direction of fluxes of ionic penetrants in energized cells are characteristic of the membrane potential with sign "minus" inside: anions move out and cations move into the cells. The value of membrane potential in energized cells calculated from the distribution of cationic penetrants is of the order of 140 mV.

(3) Orientation of membrane potential depends on the orientation of bacterial membrane. Intact cells and "inside out" sonicated particles are characterized by opposite orientation of membrane potential.

(4) Energy-dependent efflux of H^+ ions against its own concentration gradient is stoichiometrically coupled with the uptake of cationic penetrants. This fact indicates that an electrogenic H^+-pump generates a membrane potential in energized *E. coli* cells. Energy for H^+-pump operation could be supplied either by respiration or by ATP hydrolysis.

(5) Influx of lactose into the cells down its own concentration gradient diminishes the non-enzymatic membrane potential. This supports the view [1, 5] that transport of lactose across bacterial membrane is of electrogenic nature.

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References

1. P. Mitchell, *Symp. Soc. Gen. Microbiol.*, **20** (1970) 121.
2. F. M. Harold, *Bact. Rev.*, **36** (1972) 172.
3. P. Mitchell, *J. Bioenergetics*, 4 (1973) 63.
4. P. Scholes and P. Mitchell, *J. Bioenergetics*, 1 (1970) 309.
5. I. C. West and P. Mitchell, *J. Bioenergetics*, 3 (1972) 445.
6. E. A. Liberman and L. M. Tsofina, *Biofizika U.S.S.R.*, 14 (1969) 1017.
7. L. L. Grinius, A. A. Jasaitis, Yu. P. Kadziauskas, E. A. Liberman, V. P. Skulachev, V. P. Topali, L. M. Tsofina and M. A. Vladimirova, *Biochem. Biophys. Acta*, **216** (1970) 1.
8. E. A. Liberman and V. P. Skulachev, *Biochim. Biophys. Acta*, **216** (1970) 30.
9. V. P. Skulachev, in: *Current Topics in Bioenergetics*, D. R. Sanadi (ed.), Vol. 4, Academic Press, New York and London, 1971, p. 127.
10. H. Hirata, K. Altendorf and F. M. Harold, *J. Biol. Chem.*, **249** (1974) 2939.
11. F. M. Harold and D. Papineau, *J. Membrane Biol.*, **8** (1972) 45.
12. F. J. Lombardi, J. P. Reeves and H. R. Kaback, *J. Biol. Chem.*, **248** (1973) 3551.
13. H. Hirata, K. Altendorf and F. M. Harold, *Proc. Nat. Acad. Sci., U.S.A.*, **70** (1973) 1804.
14. B. Griniuviené, V. Chmieliauskaité and L. Grinius, *Biochem. Biophys. Res. Commun.*, **56** (1974) 206.

15. R. J. Fisher, K. W. Lam and D. R. Sanadi, *Biochem. Biophys. Res. Commun.*, 39 (1970) 1021.
16. B. D. Davis and E. S. Mingioli, *J. Bacteriol,* 60 (1950) 17.
17. P. Mitchell and J. Moyle, *Biochem. J.*, 104 (1967) 588.
18. P. Mitchell and J. Moyle, *Biochem. J.*, 105 (1967) 1147.
19. U. Hopfer, A. L. Lehninger and T. E. Thompson, *Proc. Nat. Acad. Sci., U.S.A.*, 59 (1968) 484.
20. V. P. Skulachev, A. A. Jasaitis, V. V. Navickaité, L. S. Yaguzhinsky, E. A. Liberman, V. P. Topali and L. M. Tsofina, in: *Mitochondrial Structure and Function,* L. Ernster and Z. Drahota (eds), Academic Press, New York and London, 1969, p. 275.
21. H. H. Winkler and T. H. Wilson, *J. Biol. Chem.*, 241 (1966) 2200.
22. S. G. Schultz, N. L. Wilson and W. Epstein, *J. Gen. Physiol.*, 46 (1962) 159.
23. L. E. Bakeeva, L. L. Grinius, A. A. Jasaitis, V. V. Kuliene, D. O. Levitsky, E. A. Liberman, I. I. Severina and V. P. Skulachev, *Biochim. Biophys. Acta,* 216 (1970) 13.
24. L. L. Grinius, M. D. Ilina, E. I. Mileykovskaya, V. P. Skulachev and G. V. Tikhonova, *Biochim. Biophys. Acta,* 283 (1972) 442.
25. A. A. Jasaitis, I. B. Nemeček, I. I. Severina, V. P. Skulachev and S. M. Smirnova, *Biochim. Biophys. Acta,* 275 (1972) 485.
26. A. A. Jasaitis, L. V. Chu and V. P. Skulachev, *FEBS Letters,* 31 (1973) 241.
27. G. van Thienen and P. W. Postna, *Biochim. Biophys. Acta,* 323 (1973) 429.
28. W. L. Klein and P. D. Boyer, *J. Biol. Chem.*, 247 (1972) 7257.
29. I. C. West and P. Mitchell, *FEBS Letters,* 40 (1974) 1.