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MEMBRANE POTENTIAL IN A POTASSIUM TRANSPORT-NEGATIVE MUTANT OF *ESCHERICHIA COLI* K-12

THE DISTRIBUTION OF RUBIDIUM IN THE PRESENCE OF VALINOMYCIN INDICATES A HIGHER POTENTIAL THAN THAT OF THE TETRAPHENYLPHOSPHONIUM CATION

EVERT P. BAKKER

Universität Osnabrück, Fachbereich Biologie/Chemie, Fachgebiet Mikrobiologie, Postfach 4469, D-4500 Osnabrück (F.R.G.)

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The membrane potential across the cytoplasmic membrane of EDTA-treated cells of a K^+ transport-negative mutant of *Escherichia coli* K-12 was estimated from the equilibrium distribution of different lipid-soluble cations. With glucose as a substrate and at low K^+_{out}, the membrane potential calculated from the distribution ratio of $^{86}Rb^+$ in the presence of valinomycin ($\Delta\psi_{Rb^+}$) was considerably higher than that indicated by the [^{3}H]tetraphenylphosphonium cation ($\Delta\psi_{TPP^+}$). The lipid-soluble anion phenyldicarbaundecaborane (PCB^-) increased $\Delta\psi_{TPP^+}$ close to $\Delta\psi_{Rb^+}$. To investigate whether these results were due to different binding of the cations to cellular components, residual Rb^+ and TPP^+ uptake was measured in cells permeabilized with 5% *n*-butanol (by volume). In those cells the distribution ratios of Rb^+, K^+ and Na^+ approached a value of 4, indicating that the uptake of all three ions was driven by a residual negative surface potential or transmembrane Donnan potential (internally negative). The distribution ratio of TPP^+ was 3–4-times higher than that of other cations and up to 10 mM TPP^+_{out} was almost independent of the added TPP^+ concentration. This extra uptake presumably represents binding of TPP^+ to the cellular membranes. Thus, at pH 7.5, $\Delta\psi_{Rb^+}$ was about 180–200 mV, whereas after correction for binding $\Delta\psi_{TPP^+}$ was 110–150 and 150–170 mV in the absence and presence of PCB^-, respectively. It is proposed that TPP^+ indicates too low a potential, because by its strong binding it decreases the negative surface potential of the cytoplasmic membrane, and thereby inhibits its own further uptake. This is taken to mean that TPP^+ distribution can be used as a qualitative probe only for the bacterial membrane potential.

Introduction

According to Mitchell's chemiosmotic hypothesis, the transmembrane electrochemical gradient of H^+ ($\Delta\tilde{\mu}_{H^+}$) plays a central role in energy transduction and transport processes in mitochondria, chloroplasts and bacteria [1–4]. Therefore, much interest attaches to the exact determination of $\Delta\tilde{\mu}_{H^+}$ and its components, Δ pH and $\Delta\psi$. Whereas independent methods indicate almost equal values for ΔpH in *Escherichia coli* [5–7], the value of the

Abbreviations: Aces, *N*-(2-acetamido)-2-aminoethanesulfonic acid; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethane sulfonic acid; Taps,3-tris(hydroxymethyl)methylaminopropanesulfonic acid; TPP^+, tetraphenylphosphonium cation; $TPMP^+$, triphenylmethylphosphonium cation; PCB^-, phenyldicarbaundecaborane anion; $\Delta\psi$, membrane potential (out minus in); $\Delta\psi_{cation}$, apparent membrane potential indicated by cation; ΔpH, transmembrane pH gradient (pH_{out} minus pH_{in}); $\Delta\tilde{\mu}_{H^+}$, transmembrane electrochemical proton gradient, or proton-motive force (out minus in; $\Delta\tilde{\mu}_{H^+} = \Delta\psi - 58\ \Delta$pH).

bacterial membrane potential varies with the method employed: The caretenoid absorption band shift [8] indicates that potentials of the order of 300–400 mV (negative inside) develop across the cytoplasmic membrane of photosynthetic bacteria [9]. By contrast, bacteria only seem to develop membrane potentials of 100–200 mV, as measured by the distribution of lipid-soluble cations (e.g., see Refs. 5 and 9–17]. However, even the different lipid-soluble cations do not always give the same results: Although few data have been published from which a direct comparison is possible, in some cases $^{86}Rb^+$ or K^+ distribution in the presence of valinomycin indicated larger potentials than did organic phosphonium cations, like $TPMP^+$ or TPP^+ [10,15,18], whereas in others the two methods gave the same results [16,19,20]. In view of this discrepency, I have made a systematic study of the membrane potential in EDTA-treated cells of *E. coli* K-12. For this purpose, I have compared $^{86}Rb^+$ uptake in the presence of valinomycin [5,21] with that of the most frequently employed phosphonium cation, TPP^+ [22,23]. To avoid interference with Rb^+ uptake through the intrinsic K^+-transport systems of the cells [24], most of the experiments were carried out with a K^+-uptake-negative strain [25]. In addition, to correct the apparent accumulation of cations for binding to cellular components, the uptake of these ions was studied in cells permeabilized by *n*-butanol [26]. My results indicate that after such corrections were made, the membrane potential indicated by Rb^+ distribution ($\Delta\psi_{Rb^+}$) was about 180–200 mV at pH 7.5, whereas that of TPP^+ was much lower. An explanation for this result may be that, due to its strong binding, TPP^+ decreased the negative surface potential of the cytoplasmic membrane, which led to an inhibition of further TPP^+ movement across this membrane. The results imply that at least in EDTA-treated cells of *E. coli* the membrane potential is underestimated when calculated from the TPP^+ distribution, and that, therefore, this distribution can serve as a qualitative measure of $\Delta\psi$ only.

Materials and Methods

Bacterial strains and growth conditions

Most of the experiments were carried out with cells of the *E. coli* K-12-derived strain TK 2242 (*F⁻, trkD1, trkA405, kdp42, nagA, lacZ, rha, thi*) [27]. The cells were grown on the minimal salt medium K 115 with glucose as a carbon and energy source [28]. Under those conditions, the synthesis was repressed of the (already mutated) Kdp K^+-uptake system, and the cells contained the very sluggish TrkF K^+-uptake system only [25]. Some of the experiments were carried out with the K^+-transport-positive strain TK 1001 (*F⁻, trkD1, kdpABC5, lacZ, rha, thi, gal*), that contained the high-rate K^+-transport system TrkA, which is constitutive [25,28]. Cells of this strain were grown on medium K 5 in the presence of glucose [25]. All of the cells were harvested in the late exponential phase of growth.

EDTA and valinomycin treatment

The outer membrane of the cells was disrupted by treatment with 1 mM EDTA [5,29,30]. For this purpose, the harvested cells were washed once with 120 mM Tris-HCl, pH 8.0, resuspended at 10 mg dry weight of cells/ml of the same medium, and incubated at 37°C. After addition of 1 mM EDTA the cells were incubated for 10 min more, during which the suspension was occasionally shaken by hand. If the cells were treated with valinomycin, this ionophore was added to the suspension 4 min after the addition of EDTA. After the 10 min incubation period, the cells were harvested by centrifugation, washed twice with 200 mM Na-Hepes buffer, pH 7.5, resuspended at 10–20 mg/ml of the same medium, and shaken at 20°C at 220 rpm in a gyratory shaker bath. After a 100-fold dilution of the suspension with distilled water, more than 90% of the EDTA-treated cells were sensitive to lysozyme treatment. The residual K^+ content of the cells was about 50 nmol K^+/mg dry weight for cells not treated with valinomycin, and about 80 nmol K^+/mg dry weight for cells treated with 10 nmol valinomycin/mg dry weight of cells. The EDTA-treated cells were used for the experiments within 4 h after their isolation.

Cation-distribution studies

The EDTA-treated cells were diluted to 1 mg dry weight/ml of the 200 mM Na-Hepes buffer (pH 7.5) in the presence of 10 mM glucose, and shaken at 220 rpm at 20°C. At zero time, $^{86}Rb^+$

was added at 100–200 μM (about 20 nCi/ml). If [^{3}H]TPP$^+$ uptake was measured simultaneously, [^{3}H]TPP$^+$ was added at a concentration of 10 μM (20–50 nCi/ml) at zero time as well; if present, PCB$^-$ was added shortly before zero time. The uptake of the labelled compounds by the cells was determined as a function of time, by separating the cells from the medium by centrifugation through silicone oil in a Beckman B Minifuge [13]. Previous experiments have shown that under the experimental conditions employed here, the rate of O_2 uptake by the cells is so slow that anaerobiosis during the incubation and subsequent centrifugation is unlikely to occur [30,31]. The whole pellet and part of the supernatant of each fraction were treated with 1 ml of 0.2 M NaOH and 4 ml Quickszint 212 as described previously [30]. Radioactivity was determined in Packard B 460 liquid-scintillation counter at a setting suitable for simultaneous ^{3}H$^+$ and ^{86}Rb$^+$ counting. A steady state of cation uptake was reached within 30–40 min after the beginning of the experiment. Data are presented as the steady state-distribution ratio, $[\text{cation}]_{in}/[\text{cation}]_{out}$, or as the apparent membrane potential, $\Delta\psi_{\text{cation}} = 58 \log([\text{cation}]_{in}/[\text{cation}]_{out})$. For those calculations the intracellular water space of the cells was taken to be 1.45 μl/mg dry weight of cells [30]. Unless stated otherwise, neither $\Delta\psi_{TPP^+}$ nor $\Delta\psi_{Rb^+}$, as presented, were corrected for binding of these cations to cellular components.

Uptake studies in n-butanol-treated cells

EDTA-treated cells were permeabilized with 5% (v/v) *n*-butanol [26] and uptake of [^{3}H]TPP$^+$ and ^{86}Rb$^+$ by these cells was measured under the conditions specified above, except that glucose was absent. The gradients of K^+ and Na^+ across the cytoplasmic membrane of the cells were determined by flame photometry, as described in Ref. 30. The pH gradient (ΔpH) was either measured by [^{14}C]benzoic acid distribution [13] or by [^{14}C]ethanolamine distribution [32] in the presence of ^3H_2O [33]. For experimental details, see Ref. 30.

Reagents and radioisotopes. The buffers Mes, Aces, Hepes and Taps were from Sigma Chemie GmbH, Taufkirchen, F.R.G. Valinomycin was either from Sigma or a gift from Professor Zähner, University of Tübingen, F.R.G. The two preparations gave identical results. TPP$^+$Br$^-$ was a gift from Dr. Michael Eisenbach, The Weizmann Institute of Science, Rehovot, Israel. It gave results identical to TPP$^+$Cl$^-$ from Fluka, Neu-Ulm F.R.G. FCCP was a gift from Dr. P.G. Heytler, Dupont de Nemours, Wilmington, DE., U.S.A. PCB$^-$ was from Dr. F.M. Harold, National Jewish Hospital and Research Center, Denver, CO, U.S.A. The radioisotopes ^{86}Rb$^+$ and ^3H_2O were from Amersham-Buchler, Brunswick, F.R.G. [^{14}C]Benzoic acid was from New England Nuclear, Boston, U.S.A., and [^{3}H]TPP$^+$Br$^-$ was from the Nuclear Research Center, Negev, Israel. Thin-layer chromatography on aluminium oxide or silica gel plates (Merck, Darmstadt, F.R.G.) with isopropanol/H_2O (7:3, v/v) as a solvent showed that the [^{3}H]TPP$^+$ used was at least 95% pure at the time of the experiments. All other reagents were of the highest purity available commercially.

Results

^{86}Rb$^+$ uptake in the presence of valinomycin

Rhoads et al. [34] have shown that Rb$^+$ is accepted by the constitutive *E. coli* K^+-transport systems. To circumvent interference with these systems, ^{86}Rb$^+$ uptake was studied in strain TK 2242 [27], under conditions where it only contained the very sluggish TrkF K^+-uptake system (Ref. 25; see Materials and Methods). With glucose as a substrate and in the absence of valinomycin, EDTA-treated cells of this strain indeed hardly took up any ^{86}Rb$^+$ (Fig. 1). Panel A of this figure also shows that the ionophore up to concentrations of 30 nmol/mg (30 μM) was rather ineffective when added immediately before the beginning of the experiment. Therefore, I resorted to adding valinomycin during the EDTA treatment (see Materials and Methods). Thus, the cells were preincubated with the ionophore for at least 90 min before the start of the experiment. Fig. 1B shows that with this pretreatment 10 ng valinomycin/mg cells caused a rapid and, in extent, saturating uptake of ^{86}Rb$^+$ by the cells.

In Fig. 2 the effect of valinomycin on ^{86}Rb gradients in strain TK 2242 is compared with that in strain TK 1001, which rapidly transports Rb$^+$ via its TrkA system [25,34]. In the presence of

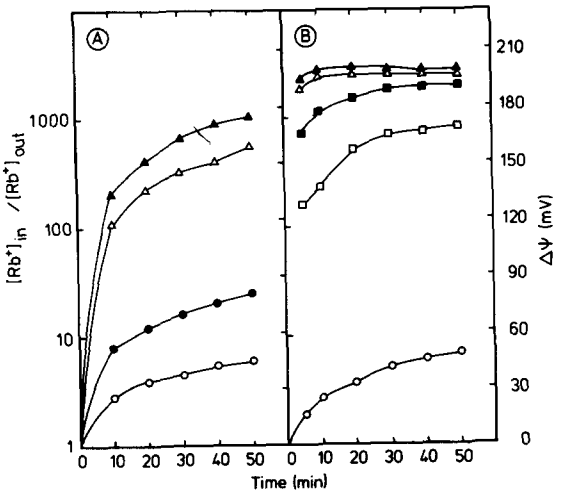

Fig. 1. Effect of preincubation with valinomycin on $^{86}Rb^+$ uptake by *E. coli* TK 2242. (A) Valinomycin was added immediately before the experiment; (B) valinomycin was added during the EDTA treatment (see Materials and Methos). For the experiment the EDTA-treated cells were resuspended at 1 mg dry weigh/ml in a medium containing 10 mM glucose, 200 μM $^{86}RbCl$, 200 mM Na-Hepes, pH 7.5, and valinomycin at the following concentrations (in nmol/mg): (○——○) 0, (●——●) 0.1, (□——□) 1, (■——■) 3, (△——△) 10, (▲——▲) 30.

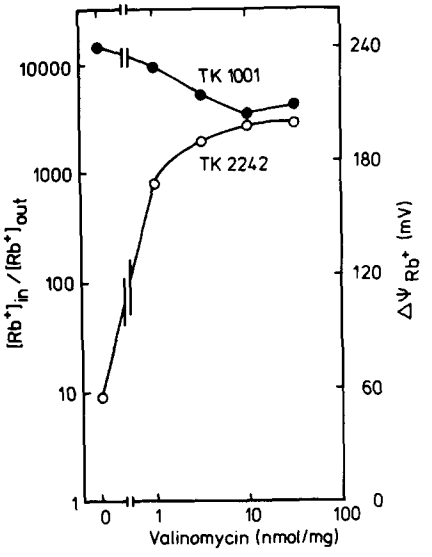

Fig. 2. Comparison of steady-state $^{86}Rb^+$ uptake by strains TK 1001 and TK 2242 as a function of the valinomycin concentration. EDTA cells were treated as described in the legend to Fig. 1B. (○——○) Strain TK 2242 (*trkA*$^-$), (●——●) strain TK 1001 (*trkA*$^+$).

saturating amounts of ionophore (10 nmol/mg or greater) the steady-state distribution of $^{86}Rb^+$ in both strains converged to the same value. This indicates that under those conditions the TrkA system did not influence $^{86}Rb^+$ distribution. Since at a valinomycin concentration of 10 nmol/mg binding of $^{86}Rb^+$ to cellular components is negligible (see below), this implies that in the presence of this amount of valinomycin the membrane potential is equal to $\Delta\psi_{Rb^+}$, both in K^+-uptake-negative and -positive mutants of *E. coli*.

Comparison with [^{3}H]TPP$^+$ distribution

The results of Figs. 1 and 2 indicated that at pH 7.5 *E. coli* developed a membrane potential of about 200 mV. Since this value was much higher than the potential of 140–160 mV calculated from [^{3}H]TPP$^+$ distribution under similar conditions [30], I carried out some experiments in which the two methods were compared directly. Fig. 3 shows a double-label experiment in which the steady-state distribution of $^{86}Rb^+$ and [^{3}H]TPP$^+$ in valinomycin-treated cells was measured as a function of the TPP$^+$ concentration. Remarkably, at low concentrations the distribution ratio of TPP$^+$ increased with TPP$^+$ concentration, and optimal TPP$^+$ accumulation was reached at about 10 μM

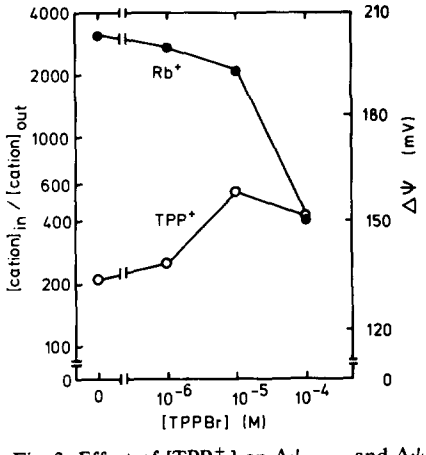

Fig. 3. Effect of [TPP$^+$] on $\Delta\psi_{TPP^+}$ and $\Delta\psi_{Rb^+}$ in *E. coli* TK 2242. Cells were treated with EDTA and 10 nmol valinomycin/mg dry weight of cells, as described in the legend to Fig. 1B. Experimental details as in Fig. 1, except that [^{3}H]TPPBr was present at the concentration indicated. (●——●) $^{86}Rb^+$ (○——○) [^{3}H]TPP$^+$.

(see also Refs. 14 and 16). However, this concentration of TPP^+ already decreased $\Delta\psi_{Rb^+}$ by about 10 mV (Fig. 3). Conversely, the presence of valinomycin plus 200 μM Rb^+ also slightly decreased $\Delta\psi_{TPP^+}$ (not shown). Both effects may be due to a small interconversion of $\Delta\psi$ into ΔpH caused by electrogenic uptake of the two cations by the cells [30].

The uptake of both Rb^+ and TPP^+ by the cells was rapidly reversed by the addition of 10 μM FCCP (not shown) to levels comparable with that in cells preincubated with this protonophore (see below). This indicates that neither of the two cations bound irreversibly to cellular components.

Fig. 4 shows a comparison between the distributions of Rb^+ and TPP^+ in metabolizing cells that were depolarized by increasing concentrations of K^+_{out} [5,35]. As expected, the distribution of Rb^+ closely followed that of K^+. By contrast, the distribution of TPP^+ varied much less with that of K^+. Assuming that K^+ and Rb^+ distributions measure $\Delta\psi$ correctly (see above), these results indicate that TPP^+ distribution underestimates $\Delta\psi$

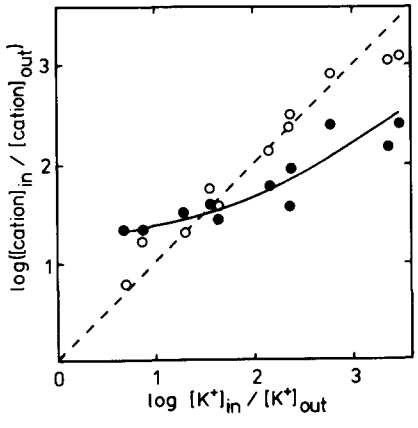

Fig. 4. The gradients of $^{86}Rb^+$ and [^{3}H]TPP^+ as a function of that of K^+ in *E. coli* TK 2242. Experimental details as in Fig. 3, except that [TPPBr] was 10 μM and that different concentrations of K^+ were added, ranging from 0.1 to 100 mM. One set of incubations received [^{3}H]TPPBr and ^{86}RbCl, and was used to determine the gradient of these cations across the membrane. A parallel set of incubations received only nonradioactive TPPBr and RbCl and was used for the determination of the K^+ gradient. The data shown are the combined results with three different preparations of cells. (●——●) $[TPP^+]_{in}/[TPP^+]_{out}$ as a function of $[K^+]_{in}/[K^+]_{out}$, (○——○) $[Rb^+]_{in}/[Rb^+]_{out}$ as a function of $[K^+]_{in}/[K^+]_{out}$.

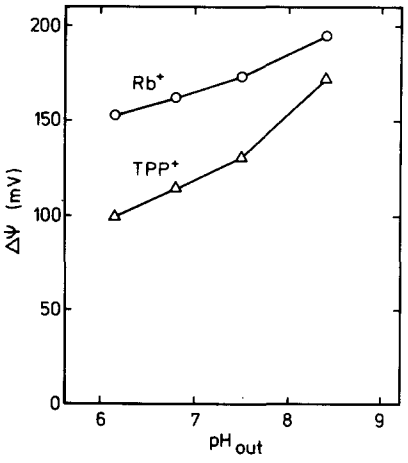

Fig. 5. pH dependence of $\Delta\psi_{Rb^+}$ and $\Delta\psi_{TPP^+}$ in *E. coli* TK 2243. EDTA- and valinomycin-treated cells were washed and resuspended at 1 mg/ml in either one of the following buffers: 200 mM Na-Mes, pH 6.1; 200 mM Na-Aces, pH 6.7; 200 mM Na-Hepes, pH 7.5; 200 mM-Taps, pH 8.4. To all incubations glucose (10 mM), [^{3}H]TPPBr (10-μM) and ^{86}RbCl (200 μM) were added. (○——○) $\Delta\psi_{Rb^+}$ (△——△) $\Delta\psi_{TPP^+}$.

at high potentials, indicates about the right value at $\Delta\psi = 80$–100 mV, and overestimates $\Delta\psi$ below this value.

Fig. 5 shows that between pH 6.1 and 8.4 $\Delta\psi_{Rb^+}$ was always much larger than $\Delta\psi_{TPP^+}$. The pH

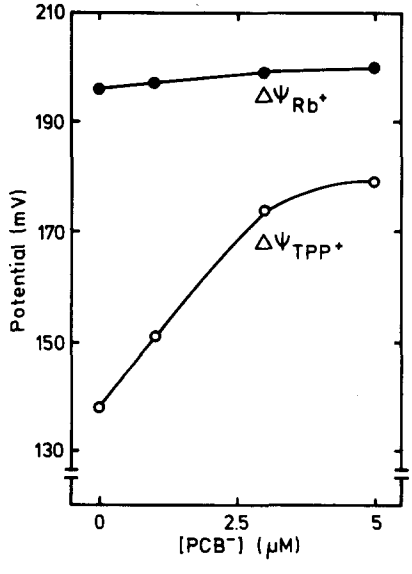

Fig. 6. Effect of PCB^- on $\Delta\psi_{TPP^+}$ and $\Delta\psi_{Rb^+}$ in *E. coli* TK 2242. Cells were treated at pH 7.5 as described in the legend to Fig. 5. (●——●) $\Delta\psi_{Rb^+}$, (○——○) $\Delta\psi_{TPP^+}$.

profiles obtained for the two cations support the notion that the membrane potential in *E. coli* increases with pH [5,30,36–38]. Note, however, that this dependence may not be as steep as assumed, since $\Delta\psi_{Rb^+}$ was much less pH dependent than $\Delta\psi_{TPP^+}$ (Fig. 5, see also Ref. 39).

Effect of PCB^-

Grinius et al. [22] have shown that addition of catalytic amounts of lipid-soluble anions increases the permeability of black-lipid membranes for phosphonium ions. Subsequently, Harold and coworkers showed in their pioneering studies on $\Delta\psi$ in bacteria that TPB^- or PCB^- increased the extent of uptake of lipid-soluble cations by both bacteria [10] and bacterial membrane vesicles [40]. Therefore, I tested the effect of PCB^- on $\Delta\psi_{TPP^+}$ and $\Delta\psi_{Rb^+}$. Fig. 6 shows that PCB^- greatly enhanced $\Delta\psi_{TPP^+}$, but hardly affected $\Delta\psi_{Rb^+}$. Thus, at 10 μM TPP^+, 5 μM PCB^- decreased the difference between $\Delta\psi_{TPP^+}$ and $\Delta\psi_{Rb^+}$ from 55 to 20 mV (Fig. 6). At 30 μM TPP^+, 10 μM PCP^- even decreased this difference from 30 to 8 mV (not shown). I will come back to this point in the Discussion.

Uptake of TPP^+ and Rb^+ by permeabilized cells

To assess the binding of TPP^+ and Rb^+ to cellular components, I measured the uptake of these cations in cells that were depolarized by different methods (Table I). Residual cation uptake was lowest after addition of either 5% (v/v) *n*-butanol or 100 mM K^+ to the valinomycin-treated cells. In the presence of 0.5% (v/v) toluene [41] uptake of TPP^+ and Rb^+ was slightly higher, but FCCP [16,19,20,36], added at either 3 or 10 μM, induced uptake of the two cations 3-times as high as that in *n*-butanol-treated cells. Under those conditions, FCCP did not depolarize the cells completely, since FCCP plus *n*-butanol treatment caused the same low level of cation uptake as did *n*-butanol alone (not shown).

Table I also shows that the cells took up a larger fraction of the TPP^+ added than they did for Rb^+; Table II shows that the apparent accumulation of Rb^+ by the *n*-butanol-treated cells was similar to that of Na^+ and K^+, whereas that of H^+ was somewhat lower. Since the amounts of these cations present in the system differed by orders of magnitude, it seems likely that uptake of these cations by the *n*-butanol-treated cells was driven by a residual electrical potential. This may either be a transmembrane Donnan potential, created by the excess polyanions in the cytosol, or a surface potential, created by negatively charged groups on the membrane surface, or a combination of both.

Presumably, the enhanced uptake of TPP^+ by the butanol-treated cells in comparison to that of Rb^+ (Tables I and II) represents mainly binding to cellular components. Fig. 7 shows that this binding was almost independent of the added TPP^+ concentration, up to 10 mM TPP^+ added to the system, indicating that the cells have at least 150

TABLE I

COMPARISON OF DIFFERENT METHODS TO DEPOLARIZE *E. COLI* CELLS COMPLETELY

EDTA- and valinomycin-treated cells of strain TK 2242 were incubated as described in Materials and Methods, except that glucose was omitted. [^{3}H]TPPBr was present at 10 μM and ^{86}RbCl at 200 μM.

Addition	TPP^+_{bound} [a] (pmol/mg)	$[TPP^+]_{in}/[TPP^+]_{out}$ [b]	Rb^+_{bound} [a] (pmol/mg)	$[Rb^+]_{in}/[Rb^+]_{out}$ [b]
5% (v/v) *n*-butanol	170	12	600	4.0
0.5% (v/v) toluene	280	19	550	3.8
3 μM FCCP	550	38	1400	10
10 μM FCCP	540	37	1200	8
100 mM KCl	230	16	430	3

[a] Assuming that all cations taken up were bound to cellular components.

[b] Assuming that all cations taken up accumulated in the cytosol.

TABLE II

CATION DISTRIBUTION IN *n*-BUTANOL-TREATED CELLS

The cells were treated with EDTA and valinomycin as described in Materials and Methods and were resuspended at 1 mg/ml in 20 mM Na^+-Hepes, pH 7.5, 10 μM TPPBr and the different radiochemicals (see Materials and Methods). n.d., not determined.

KCl added	$\frac{[TPP^+]_{in}}{[TPP^+]_{out}}$	$\frac{[Rb^+]_{in}}{[Rb^+]_{out}}$	$\frac{[K^+]_{in}}{[K^+]_{out}}$	$\frac{[Na^+]_{in}}{[Na^+]_{out}}$	$\frac{[H^+]_{in}}{[H^+]_{out}}$ [a]	$\frac{[H^+]_{in}}{[H^+]_{out}}$ [b]
–	15.5	5.0	n.d.	4.3	3.4	1.3
10 mM	14.5	4.5	4.1	4.1	3.1	1.2

[a] As determined by [^{14}C]ethanolamine distribution.

[b] As determined by [^{14}C]benzoic acid distribution.

nmol of TPP^+-binding sites/mg dry weight of cells. Presumably, those binding sites are located at the interface of the membranes present in the system. Results not shown indicated that this binding of TPP^+ was only slightly pH dependent, in that it increased by about 15% from pH 6.1 to pH 8.4. Similar results on TPP^+-binding patterns obtained with somewhat different methods were recently reported both for EDTA-treated cells of *E. coli* [41] and for *Bacillus subtilis* cells [16] (see Discussion).

I also tested the effect of PCB^- on TPP^+ uptake by the *n*-butanol-treated cells. At 10 μM TPP^+, the presence of 5 μM PCB^- raised the ratio $TPP^+_{bound}/TPP^+_{free}$ from about 0.018 to 0.023. Under those conditions, PCB^- did not affect residual $^{86}Rb^+$ uptake by the cells (cf. Fig. 6).

Finally, I examined whether residual TPP^+ uptake by the *n*-butanol-treated cells was completely due to binding or also in part driven by the residual electrical potential postulated above. For this purpose, an experiment was carried out in

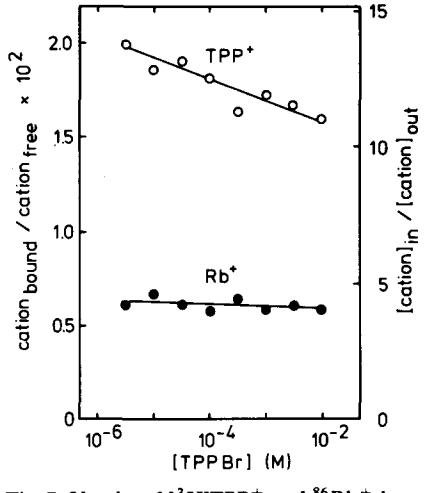

Fig. 7. Uptake of [^{3}H]TPP^+ and $^{86}Rb^+$ by permeabilized cells of *E. coli* TK 2242. Cells were treated with EDTA and with 10 nmol valinomycin/mg as described in Materials and Methods. 5% (v/v) *n*-Butanol was added 5 min prior to the addition of radioactivity. [^{86}RbCl] was 200 μM and [[^{3}H]TPPBr] as indicated on the abscissa. (●——●) $^{86}Rb^+$, (○——○) [^{3}H]TPP^+. For the scale of the left-hand ordinate it is assumed that all cations taken up are bound to the cells; for that of the right-hand ordinate it is assumed that all cations taken up are free in the cytosol.

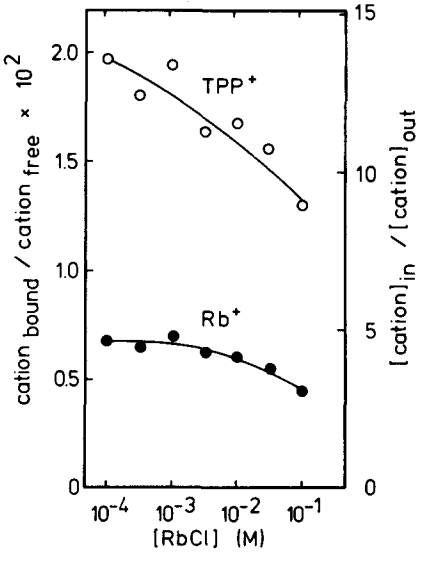

Fig. 8. Influence of [RbCl] on the uptake of [^{3}H]TPP^+ and $^{86}Rb^+$ by permeabilized cells. Details and symbols as in Fig. 7, except that [^{3}H]TPP^+ Br was present at 10 μM and [^{86}RbCl] was as indicated on the abscissa.

which residual cation uptake was measured as a function of the RbCl concentration (Fig. 8). The $^{86}Rb^+$ distribution decreased with increasing Rb^+ concentrations, indicating that the residual potential also decreased. High Rb^+ concentrations lowered the TPP^+ distribution by about the same proportion as that of $^{86}Rb^+$ (Fig. 8). This may indicate that TPP^+ uptake by permeabilized cells was in part driven by a residual surface or Donnan potential (see Discussion).

Discussion

Since intact bacteria are in general too small to tolerate microelectrodes, indirect methods have to be employed to estimate $\Delta\psi$ in these systems. For quantitative purposes, the best method is to calculate $\Delta\psi$ from the distribution of lipid-soluble cations [33], and two types of such cations are frequently used: first, K^+ or Rb^+ in the presence of valinomycin [5,21], and second, organic phosphonium ions like $TPMP^+$ and TPP^+, first introduced by Skulachev and co-workers [22].

The experiments presented here show that at least in the presence of valinomycin, at membrane potentials exceeding 100 mV, $\Delta\psi_{Rb^+}$ significantly exceed $\Delta\psi_{TPP^+}$. For instance, at pH 7.5 and low $[K^+_{out}]$, $\Delta\psi_{Rb^+}$ was 180–200 mV, whereas $\Delta\psi_{TPP^+}$ ranged between 130 and 160 mV (Figs. 1–3 and 6). I interpret these results to mean that the use of TPP^+ led to an underestimation of the membrane potential, and that $^{86}Rb^+$ distribution indicated $\Delta\psi$ correctly. This contention is based on the following considerations: (i) in the presence of saturating amounts of valinomycin, the gradient of $^{86}Rb^+$ was equal in the K^+-transport-positive and -negative mutant (Fig. 2). This implies that under those conditions the native K^+-transport systems were effectively short-circuited by the electrogenic diffusion pathway provided by valinomycin and that, consequently, the membrane potential was reduced to an Rb^+ (or K^+) diffusion potential (Figs. 2 and 4). (ii) $^{86}Rb^+$ showed negligible background binding (Tables I and II, Figs. 7 and 8). Even if the residual uptake in the permeabilized cells were due to binding rather than to a Donnan and/or surface potential (see Results), calculations as in Ref. 16 showed that this would reduce $\Delta\psi_{Rb^+}$ by maximally 10 mV only. (iii) the uptake of $^{86}Rb^+$ was rapidly reversed by FCCP, indicating that the high level of uptake of this cation was not due to irreversible binding to cellular components.

One is left, then, with the conclusion that TPP^+ indicates too low a potential. I have excluded some trivial explanations for this result, such as contamination with chemically degraded TPP^+, anaerobiosis, or incomplete EDTA treatment (see Materials and Methods). Instead, a possible explanation could be that due to its strong, and energy-dependent binding (see below), TPP^+ decreases the negative surface potential of the cytoplasmic membrane, and thereby effectively inhibits its further equilibration with the membrane potential. It should be stressed that this effect would be kinetic rather than thermodynamic, and can only become important for ions that are not very permeant anyway. Studies with artificial membranes show that the latter is indeed the situation for TPP^+ [22]. A similar effect of surface potential on the apparent permeability towards a permeant anion has been reported in bacteriorhodopsin-containing proteoliposomes. In this system, the permeant anion SCN^- has been shown to monitor $\Delta\psi$ only in vesicles carrying a positive surface charge; In negatively charged proteoliposomes no SCN^- uptake was observed, despite the fact that also these vesicles were expected to develop a membrane potential (positive inside) [42].

This hypothesis may explain the following observations: (i) the effect of PCB^- on TPP^+ uptake (Fig. 6). PCB^- is expected to prevent the decrease in surface potential by TPP^+, and thereby allow a better equilibration of TPP^+ with $\Delta\psi$. Note that PCB^- hardly increased the residual binding of TPP^+ by *n*-butanol-treated cells (see Results). This indicates that the effects of PCB^- on TPP^+ uptake (Fig. 6) were not due to binding of PCB^--TPP^+ complexes to the membrane. (ii) the difference between $\Delta\psi_{TPP^+}$ and $\Delta\psi_{Rb^+}$ increased with $\Delta\psi$ (Fig. 4). At low $\Delta\psi$ less TPP^+ will bind to the cells and consequently, TPP^+ will equilibrate more extensively.

However, this hypothesis does not explain the finding that at very low potentials $\Delta\psi_{TPP^+}$ exceeded $\Delta\psi_{Rb^+}$ (Fig. 4). Most likely, this effect is due to the stronger binding of TPP^+ to cellular material (Tables I and II, Figs. 7 and 8). It can also not account for the puzzling observation that

TPP^+ stimulated its own uptake at low concentrations (Fig. 3). A similar phenomenon has been reported for other phosphonium cations [14,16]. One must assume that, independent of the postulated effect of TPP^+ on the surface potential, the permeability of the cytoplasmic membrane increases with TPP^+ concentration.

An extra complication in the calculation of $\Delta\psi_{TPP^+}$ arises because of binding of TPP^+ to cellular membranes. This has been generally recognized, and two models have been applied to correct for binding: (i) a model in which binding saturates at low TPP^+ concentration; this implies that, independent of the total amount taken up, TPP^+ uptake has to be corrected by subtraction of a constant amount from it to obtain intracellular TPP^+ concentrations [16,19,20,36,39]. (ii) A model according to which TPP^+ binding does not saturate [43]. Since the results of Fig. 7, and those of others [16,41] indicated that the number of TPP^+-binding sites is much larger than the total amount of TPP^+ taken up by energized cells (Figs. 3–6), the latter model appears to be more appropriate. In a recent refinement of this model, Zaritsky et al. [16] have proposed that the amount of TPP^+ bound is a function of both $[TPP^+]_{out}$ and $[TPP]_{in}$ ('exponential mean model'). Thus, energy-dependent changes in binding can be taken into account: From (a) the uptake of TPP^+ by energized cells, (b) its uptake in permeabilized cells, and (c) a residual electrical Donnan or surface potential in those cells (see Results), it can then be calculated that an apparent value for $\Delta\psi_{TPP^+}$ of 160 mV (Fig. 3) has to be corrected to 147 mV and of 180 mV (in the presence of 5 μM PCB^-, Fig. 6) to 166 mV. These corrected potentials become 130 and 147 mV, respectively, if conditions c is not taken into account.

One clear effect of those binding corrections is to make the discrepency with the 'real' membrane potential (as presumably indicated by $\Delta\psi_{Rb^+}$) even larger. In any case, the underestimation of $\Delta\psi$ by TPP^+ distribution can easily amount to about 50 mV in valinomycin/EDTA-treated cells of *E. coli* at pH 7.5. This has some important consequences for quantitative aspects of energy coupling. First, with a drop in apparent potential from 200 mV ($\Delta\psi_{Rb^+}$) to 150 mV ($\Delta\psi_{TPP^+}$), the stoichiometry between $\Delta\tilde{\mu}_{H^+}$ and the phosphate potential ΔG_p with glucose as a substrate would increase from 2.6 to 3.3 (taking $\Delta pH = 0$ [6,30] and $\Delta G_p = 50$ kJ/mol (Bakker, E.P., unpublished results). Secondly, it has been reported that at high pH_{out}, $\Delta\tilde{\mu}_{H^+}$ is smaller than the lactose gradient maintained across the cytoplasmic membrane of cells or membrane vesicles of *E. coli* [41,44]. This was interpreted to mean that under those conditions two H^+ may accompany the influx of one molecule of lactose into the cell [45]. In most of these experiments $TPMP^+$ or TPP^+ was used to measure $\Delta\psi$ (Refs. 41, 44 and 46; see, however, also Ref. 36). In view of the findings reported here it would be interesting to repeat those experiments with $^{86}Rb^+$ in the presence of valinomycin as a probe for $\Delta\psi$. Very recently, Ahmed and Booth [20,46] reported on such experiments. They observed that at high $\Delta\tilde{\mu}_{H^+}$ the stoichiometry between H^+ and lactose transport was always close to 1 : 1, and that it only approached a value of 2 : 1 at low $\Delta\tilde{\mu}_{H^+}$.

Finally, I would like to make the following recommendations for the measurement of membrane potentials in bacteria: At present, the most reliable method appears to be to use $^{86}Rb^+$ distribution in the presence of valinomycin. However, this method can only be employed at low $[K^+_{out}]$, since at elevated $[K^+_{out}]$, the K^+-diffusion potential will clamp the membrane potential at a value lower than that generated by the electrogenic H^+ pumps of the cells in the absence of valinomycin. It is important to make sure that the valinomycin concentration is high enough to completely short-circuit those cellular transport systems that accept Rb^+. To this end one may have to preincubate the cells with relatively high concentrations of the ionophore (Fig. 1). At $[K^+_{out}] >$ 0.5–1 mM it may be unavoidable to employ TPP^+ distribution as a measure of $\Delta\psi$. Again it is important to establish the optimal concentration of the probe (Fig. 3). It may be advantageous to add catalytic amounts of PCB^-, since this compound increased $\Delta\psi_{TPP^+}$ considerably under conditions at which it hardly affected the binding of TPP^+ to the membrane (Fig. 6 and Results). However, even under those conditions $\Delta\psi$ may be underestimated, be it by maximally 20 mV (Fig. 6).

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References

1 Mitchell, P. (1966) Biol. Rev. 41, 445–502
2 Mitchell, P. (1979) Science 206, 1148–1158
3 Harold, F.M. (1977) Curr. Top. Bioenerg. 6, 83–149
4 Kell, D.B. (1979) Biochim. Biophys. Acta 549, 55–99
5 Padan, E., Zilberstein, D. and Rottenberg, H. (1976) Eur. J. Biochem. 63, 533–541
6 Navon, G., Ogawa, S., Shulman, R.G. and Yamane, T. (1977) Proc. Natl. Acad. Sci, U.S.A. 74, 888–891
7 Slonczewski, J.L., Rosen, B.P., Alger, J.R. and Macnab, R.M. (1981) Proc. Natl. Acad. Sci. U.S.A. 78, 6271–6275
8 Jackson, J.B. and Crofts, A.R. (1969) FEBS Lett. 4, 185–189
9 Jackson, J.B. and Clark, A.J. (1981) in Vectorial Reactions in Electron Transport and Ion Transport in Mitochondria and Bacteria (Palmieri, F., Quagliariello, E., Siliprandi, N. and Slater, E.C., eds.), pp. 371–379, Elsevier/North-Holland, Amsterdam
10 Harold, F.M. and Papineau, D. (1972) J. Membrane Biol. 8, 45–62
11 Schuldiner, S. and Kaback, H.R. (1975) Biochemistry 14, 5451–5461
12 Bakker, E.P., Rottenberg, H. and Caplan, S.R. (1976) Biochim. Biophys. Acta 440, 557–572
13 Kashket, E. and Barker, S.L. (1977) J. Bacteriol. 130, 1017–1023
14 McCarty, J.E.G., Ferguson, S.L. and Kell, D.B. (1981) Biochem. J. 196, 311–321
15 Guffanti, A.A., Blumenfeld, H. and Krulwich, T.A. (1981) J. Biol. Chem. 256, 8416–8421
16 Zaritsky, A., Kihara, M. and MacNab, R.M. (1981) J. Membrane Biol. 63, 215–231
17 Friedberg, I. and Kaback, H.R. (1980) J. Bacteriol. 142, 651–658
18 Bakker, E.P. and Harold, F.M. (1980) J. Biol. Chem. 255, 433–440
19 Ghazi, A., Schechter, E., Letellier, L. and Labedan, B. (1981) FEBS Lett. 125, 197–200
20 Ahmed, S. and Booth, I.R. (1981) Biochem. J. 200, 573–581
21 Mitchell, P. and Moyle, J. (1969) Eur. J. Biochem. 7, 471–484
22 Grinius, L.L., Jasaitis, A.A., Kadziauskus, Yu. P., Liberman, E.A., Skulachev, V.P., Topali, V.P., Tsofina, L.M. and Vladimorava, M.A. (1970) Biochim. Biophys. Acta 216, 1–12
23 Zilberstein, D., Schuldiner, S. and Padan, E. (1979) Biochemistry 18, 669–673
24 Epstein, W. and Laimins, L. (1980) Trends Biochem. Sci. 5, 21–23
25 Rhoads, D.B., Waters, F.B. and Epstein, W. (1976) J. Gen. Physiol. 67, 325–341
26 Kashket, E.R., Blanchard, A.G. and Metzger, W.C. (1980) J. Bacteriol. 143, 128–134
27 Epstein, W., Whitelaw, V. and Hesse, J. (1978) J. Biol. Chem. 253, 6666–6668
28 Epstein, W. and Kim, B.S. (1971) J. Bacteriol. 108, 639–644
29 West, I.C. and Mitchell, P. (1972) J. Bioenerg. 3, 445–462
30 Bakker, E.P. and Mangerich, W.E. (1981) J. Bacteriol. 147, 820–826
31 Michels, M. and Bakker, E.P. (1981) Eur. J. Biochem. 116, 513–519
32 Repaske, D.R. and Adler, J. (1981) J. Bacteriol. 145, 1196–1208
33 Rottenberg, H. (1979) Methods Enzymol. 55, 547–569
34 Rhoads, D.B., Woo, E. and Epstein, W. (1977) Biochim. Biophys. Acta 469 (1977) 45–51
35 Padan, E. and Rottenberg, H. (1973) Eur. J. Biochem. 40, 431–437
36 Booth, I.R., Mitchell, W.J. and Hamilton, W.A. (1979) Biochem. J. 182, 687–696
37 Letellier, L. and Schechter, E. (1979) Eur. J. Biochem. 102, 441–447
38 Felle, H., Porter, J.S., Slayman, C.L. and Kaback, H.R. (1980) Biochemistry 19, 3585–3589
39 Kashket, E. (1981) J. Bacteriol. 146, 377–384
40 Altendorf, K., Hirata, H. and Harold, F.M. (1975) J. Biol. Chem. 250, 1405–1412
41 Ten Brink, B., Lolkema, J.S., Hellingwerf, K.J. and Konings, W.N. (1981) FEMS Lett. 12, 237–240
42 Hellingwerf, K.J. Arents, J.C. Scholte, B.J. and Westerhoff, H.V. (1979) Biochim. Biophys. Acta 547, 561–582
43 Heinz, E., Geck, P., Pietrzyk, C., Burckhardt, G. and Pfeiffer, B. (1977) in Molecular Aspects of Membrane Transport (Oxender, D. and Fox, C.F., eds.), pp. 65–73, Alan R. Liss, Inc., New York
44 Ramos, S. and Kaback, H.R. (1977) Biochemistry 16, 4271–4275
45 Rottenberg, H. (1976) FEBS Lett. 66, 159–163
46 Ahmed, S. and Booth, I.R. (1981) Biochem. J. 200, 583–589