PROBES OF MEMBRANE POTENTIAL IN *ESCHERICHIA COLI* CELLS

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1. Introduction

Membrane potential ($\Delta\psi$) of cells which are too small to allow the use of microelectrodes must be indirectly determined. In bacterial cells, labelled permeant cations which distribute across the cell membrane in response to a potential difference (negative inside) are currently used [1]. At thermodynamic equilibrium, the membrane potential is related to the concentration of labelled monovalent ion C^+ inside and outside the cell by the Nernst equation:

$$\Delta\psi = (RT/F) \ln [C^+]_{in}/[C^+]_{out}$$

Here, we compare the results obtained over a large range of $\Delta\psi$ with three different probes (Ph_3MeP^+, Ph_4P^+ and Rb^+ in the presence of valinomycin). $\Delta\psi$ has been varied either by changing the ratio of external to internal potassium concentrations in the presence of valinomycin, or by allowing high concentrations of Ph_3MeP^+ to accumulate within the cell (uncoupling effect). Moreover, it has been shown [2,3] that the use of cyanine dyes allows one to monitor continuously $\Delta\psi$. Indeed, the accumulation of these dyes into the cell is $\Delta\psi$-dependent and leads to a quenching of their fluorescence. Making use of the results obtained with the labelled probes, we present here a calibration of the fluorescence quenching in terms of $\Delta\psi$, for 2 *E. coli* strains.

Abbreviations: Ph_3MeP^+, triphenylmethyl phosphonium ion; Ph_4P^+, tetraphenyl phosphonium ion; Rb^+, rubidium ion; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; Mes, 2-morpholinoethanesulfonic acid

2. Materials and methods

2.1. *Growth of bacteria and EDTA-treatment*

Escherichia coli ML 308 225 cells were grown in minimal medium A containing 1% dipotassium succinate as the sole carbon source, and harvested in mid-log phase as in [3]. EDTA-treated cells were prepared according to [4] and were suspended at 10 mg/ml in 5 mM Tris–5 mM Mes–HCl buffer (pH 7.3) containing 150 mM choline chloride. *E. coli* F was grown in NT broth and treated with EDTA as in [5]. They were resuspended in 10 mM Tris–HCl, 100 mM NaCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$ (pH 7.4) at 10 mg/ml. Valinomycin (10 μM, final conc.) was added to both EDTA-treated cells which were kept on ice until use.

2.2. *Chemicals*

[^{14}C]Ph_3MeP^+ (49.6 Ci/mol), [^{14}C]Ph_4P^+ (87 Ci/mol), ^3H_2O (1 Ci/ml) were from CEA Saclay (France). (*hydroxy*[^{14}C]*methyl*)Inulin (15.6 Ci/mol) and $^{86}Rb^+$ (760 Ci/mol) were from Amersham. The cyanine dye di I C_1 [5] (3,3′-dimethylindodicarbocyanine iodide) was a gift of Dr A. S. Waggoner.

2.3. *Measurement of intracellular volume*

The internal volume was measured according to [1]. Cells at 1 mg/ml were incubated at room temperature with a mixture of (*hydroxy*[^{14}C]*methyl*)inulin (5 μM final conc.) and ^3H_2O (5 μCi/ml). They were centrifuged through silicon oil as in [6]. The value obtained for both *E. coli* F and ML 308 225 was 2.3 μl/mg bacterial dry wt.

2.4. *Membrane potential measurements*

The use of Rb^+ requires a valinomycin treatment. To ensure that the experiments are similar when the other probes are used, the cells were also treated with

valinomycin when using Ph_3MeP^+ or Ph_4P^+. Cells at 1 mg/ml were incubated with the radioactively-labelled compounds (Ph_3MeP^+, 25 μM; Ph_4P^+, 14 μM; Rb^+, 0.5 μM; final conc.) as in fig.1,2. The amount of accumulated probe was determined either by centrifugation [6] or by filtration on EH Millipore filters (0.45 μm). Each accumulation has been corrected by subtracting blanks obtained in identical conditions except that the cells were pretreated with 50 μM CCCP. The membrane potential was calculated using the Nernst equation.

2.5. *Fluorescence measurements*

Standard conditions were as follows: the dye (0.17 μM for *E. coli* F and 0.08 μM for *E. coli* ML 308 225) was added to a cuvette containing the same buffer as that used for filtration and centrifugation experiments. Of the EDTA–valinomycin-treated cells (20 μg/ml final conc.) 5 μl were added. Fluorescence was measured at room temperature as in [3,5].

3. Results and discussion

3.1. *Parallel determinations of Δψ with Rb^+, Ph_3MeP^+ and Ph_4P^+*

The membrane potential was determined in 2 *E. coli* strains: ML 308 225, routinely used for lactose transport studies; and *E. coli* F, recently used for investigation of phage infection energetics [5]. The membrane potential was gradually decreased by incubating the EDTA–valinomycin-treated cells either with increasing amounts of external potassium (fig.1) or with increasing amounts of unlabelled Ph_3MeP^+ (fig.2). The radioactive labelled compounds $^{86}Rb^+$, [^{14}C]Ph_3MeP^+ or [^{14}C]Ph_4P^+ were then added in trace amounts to the cells and their accumulation at equilibrium was determined either by centrifugation on silicon oil [6] or by filtration. For Rb^+ the latter technique yields Δψ values which are 10–20 mV lower than the former one (see below, fig.4a). This may be a consequence of some efflux taking place

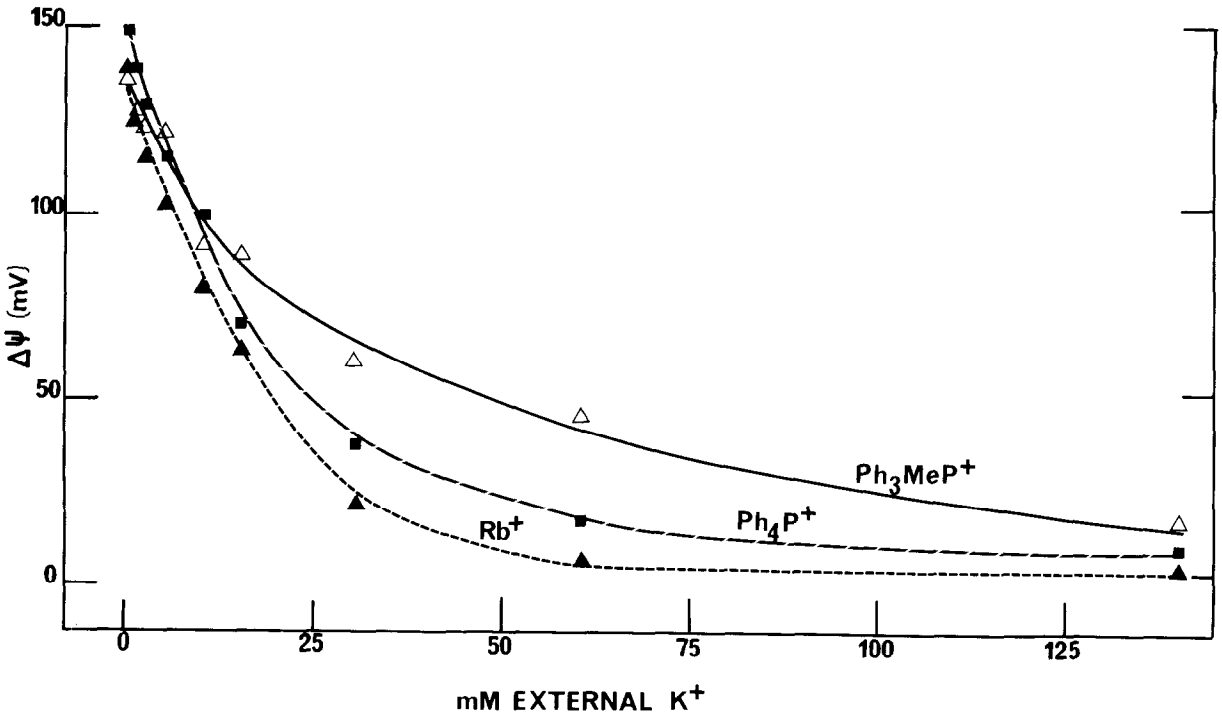

Fig.1. Variations of Δψ as a function of external [K^+]. EDTA- and valinomycin-treated *E. coli* ML 308 225 cells were incubated at 1 mg/ml in 5 mM Tris/5 mM Mes/HCl (pH 7.3) containing 150 mM Cl^-, with different ratios of K^+/choline to give final [K^+] 1, 2, 5, 10, 15, 30, 60 and 150 mM. Ph_4P^+ (14 μM, final conc.) or Ph_3MeP^+ (25 μM, final conc.) or Rb^+ (0.5 μM, final conc.) were added to the cells. After 20 min incubation at room temperature, cells were filtered: (■) Ph_4P^+; (△) Ph_3MeP^+; (▲) Rb^+.

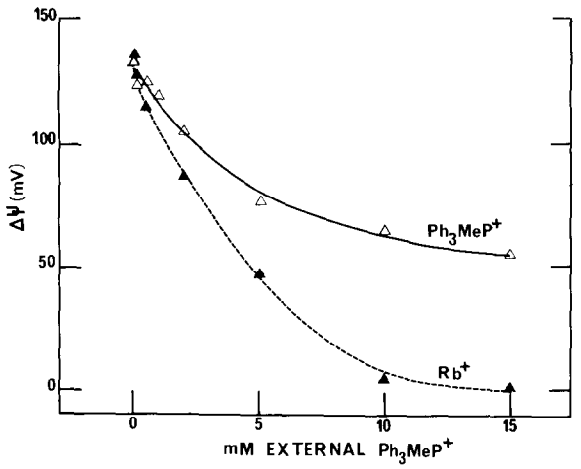

Fig.2. Variations of $\Delta\psi$ as a function of external $[Ph_3MeP^+]$. Experimental conditions were identical to those of fig.1 except that KCl and choline chloride concentrations were kept at 1 mM and 150 mM, respectively. External labelled Ph_3MeP^+ was varied from 0–15 mM.

during washing and filtration. We could not use the centrifugation technique to follow Ph_4P^+ or Ph_3MeP^+ accumulation. Indeed, the affinity of these cations for silicon oil is not negligible (1/100 as compared to $1/10^6$ for Rb^+). Fig.1,2 show the data obtained with the filtration technique for *E. coli* ML 308 225. Similar results were found for *E. coli* F (not shown). $\Delta\psi$ was calculated using the internal cell volume of 2.3 μl/mg dry wt. Recently a value of 1 μl/mg dry wt has been reported for *E. coli* ML 308 225, using sucrose to discriminate between periplasmic and cytoplasmic volumes [4]. Using this last value would only shift upwards all the $\Delta\psi$ data we report here by some 20 mV. The data in fig.1,2 show that at >100 mV the 3 radioactive probes give similar $\Delta\psi$ values. As $\Delta\psi$ is decreased to <100 mV there is an increasing dispersion of the values obtained with the 3 probes. This is particularly significant when Ph_3MeP^+ is used to depolarize the cell membrane. For high external Ph_3MeP^+ concentrations, the accumulation of $[^{14}C]$-Ph_3MeP^+ still indicates $\Delta\psi$ = 60 mV, whereas the accumulation of $^{86}Rb^+$ indicates $\Delta\psi \sim 0$ mV (fig.2). This suggests a possible binding of the lipophilic probes to the membrane. Thus we have measured the accumulation of $[^{14}C]Ph_3MeP^+$ in de-energized (CCCP-treated) cells, in the presence of increasing external concentrations of Ph_3MeP^+. Under these conditions there is no energy-dependent accumulation, and the amount of Ph_3MeP^+ accumulated within the cells should correspond to calculated internal concentration equal to the external one. Fig.3 clearly shows that this is not the case, indicating a concentration-dependent binding of Ph_3MeP^+ to the membrane. This binding cannot be determined experimentally for $\Delta\psi$ values different from 0 mV. Nevertheless, as the internal concentration of the probe is raised, due to $\Delta\psi$-dependent accumulation, the binding is probably larger than in CCCP-treated cells. Therefore, the blanks obtained by treating the cells with CCCP are underestimated and thus, the $\Delta\psi$ values shown in fig.1,2 are overestimated. This could explain the discrepancy observed at low $\Delta\psi$ between Ph_3MeP^+ and Rb^+. As $\Delta\psi$ increases, we observe that the difference between Rb^+ and Ph_3MeP^+ decreases suggesting that

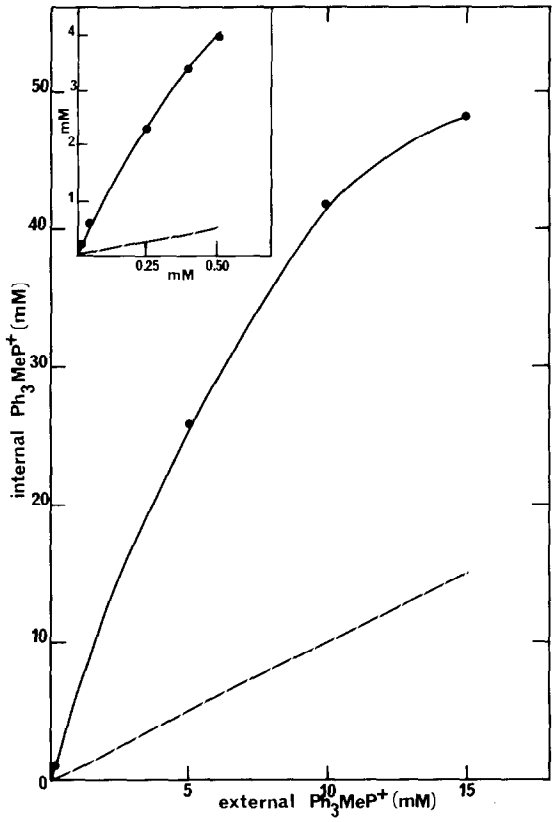

Fig.3. Ph_3MeP^+ binding to CCCP-treated cells. Experimental conditions were identical to those of fig.2 except that the cells were pretreated with 50 μM CCCP. $[Ph_3MeP^+]_{in}$ is plotted as a function of external $[Ph_3MeP^+]$: (——) experimental data, $[Ph_3MeP^+]_{in}$ is calculated assuming that all Ph_3MeP^+ accumulated is free in solution; (– – –) theoretical curve, $[Ph_3MeP^+]_{in} = [Ph_3MeP^+]_{out}$. The difference between the 2 curves reflects the concentration-dependent binding of Ph_3MeP^+.

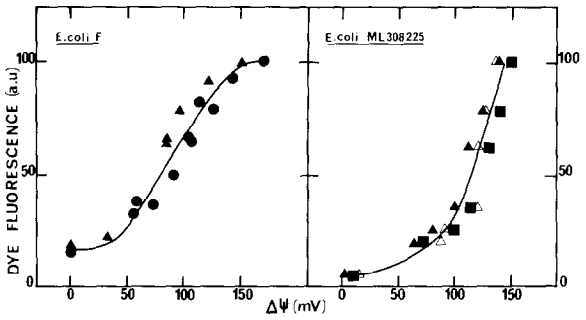

Fig.4. Calibration of the fluorescence quenching in terms of $\Delta\psi$. The dye fluorescence is expressed in arbitrary units (a.u.). (a) *E. coli* F cells. $\Delta\psi$ values are determined from the accumulation of Rb^+ using the filtration technique (▲) and the centrifugation technique (●). (b) *E. coli* ML 308 225 cells. $\Delta\psi$ values are determined from the accumulation of Rb^+ (▲); Ph_3MeP^+ (△) and Ph_4P^+ (■) using the filtration technique.

the non-specific binding of Ph_3MeP^+ may also be $\Delta\psi$-dependent.

3.2. *Calibration of the fluorescence quenching in terms of* $\Delta\psi$

E. coli F and ML 308 225 cells were incubated in increasing amounts of potassium or Ph_3MeP^+ as above. Fluorescence quenching was measured on aliquots of samples used to measure the different $\Delta\psi$ values and plotted against these different $\Delta\psi$ values obtained either for *E. coli* F (fig.4a) or *E. coli* ML 308 225 (fig.4b). In both cases, the calibration curves display a linear part from 40–130 mV for *E. coli* F and from 90–140 mV for *E. coli* ML 308 225. However, at low $\Delta\psi$ the curves are non-linear. This is also true in the case of *E. coli* F at >130 mV. This excludes the use of the dye in those regions. The reasons for non-linear response of cyanine dyes to $\Delta\psi$ in other systems, have been discussed on theoretical grounds [7]. The obvious differences in the calibration curves for the 2 *E. coli* strains incubated in different media, stress the fact that an individual calibration is needed for each particular system.

4. Conclusions

Rb^+, Ph_3MeP^+ and recently Ph_4P^+ have been widely used in various organelles, cells and vesicles in order to determine the transmembrane potential difference. In *E. coli*, as long as the cells were studied under fully energized state, there were no significant discrepancies between the values reported by several authors using different techniques (centrifugation, filtration or flow dialysis) and different probes [8–11]. The comparative study presented here confirms that at 100–150 mV the $\Delta\psi$ values are roughly identical with Ph_3MeP^+, Ph_4P^+ and Rb^+ using filtration or centrifugation techniques. However, the understanding of various mechanisms which depend upon $\Delta\psi$ requires to vary and determine $\Delta\psi$ in a large range (of $\Delta\psi$), including the low values. We show here that as $\Delta\psi$ decreases to <100 mV, there is an increasing dispersion of the $\Delta\psi$-values obtained with the 3 probes. Under these conditions, the choice of the probe becomes crucial. Ph_3MeP^+, in view of its non-specific binding should be excluded for determinations of low $\Delta\psi$-values unless corrections are performed. Ph_4P^+ and Rb^+ yield similar $\Delta\psi$-values; however Ph_4P^+ avoids the valinomycin treatment which may be inconvenient under some experimental conditions. Finally, regarding the cyanine dye, calibration allows its use for quantitative studies in *E. coli* cells over a fairly large range of $\Delta\psi$. This is of particular interest to monitor continuously transient $\Delta\psi$ variations [5].

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