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The measurement of transmembrane electrical potential with lipophilic cations

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Abstract

The binding of lipophilic cation probes of membrane potential to cells was re-examined. Even concentrations of probe molecules as low as 100 nM were found to reduce $\Delta\Psi$ and thus many commonly used techniques for $\Delta\Psi$ determination are inappropriate. Binding was found to be a linear function of probe concentration and independent of pH. The proportionality constant for binding has been equated to an 'apparent binding volume' for $[^3H]TPP^+$ with units of $\mu l/mg$ dry weight of cells. This 'apparent binding volume' is thermodynamically equivalent to the volume of cell membrane multiplied by the partition coefficient of $[^3H]TPP^+$ for cell membrane and was equivalent to 9.10 ± 0.33 $\mu l/mg$ dry weight in *Enterococcus faecalis*. It was concluded that the most accurate method for $\Delta\Psi$ determination was to use nanomolar concentrations of lipophilic cations and appropriate correction for energy dependent binding.

Keywords: Lipophilic cation probe; Tetraphenylphosphonium ion; Membrane potential; (*E. faecalis*)

1. Introduction

The proton electrochemical potential gradient (Δp) across a membrane barrier consists of a transmembrane electrical potential ($\Delta\Psi$) and a transmembrane proton gradient (ΔpH) as described in the equation,

$$\Delta p = \Delta\Psi - Z \cdot \Delta pH \quad (1)$$

where $Z = 2.3\ RT/F$ and R is the gas constant, T is temperature and F is the Faraday constant. The constant Z is used to convert pH units to millivolts. Δp must normally be determined by measuring $\Delta\Psi$ and ΔpH separately and this is commonly achieved by monitoring the distribution of radiolabeled probes across the cell membrane and then calculating the intracellular and extracellular concentrations with the known cell volumes.

However, as recently as 1990 Bakker [1] concluded: "In drawing the balance one has to conclude that after almost twenty years of determinations of bacterial membrane potentials and cytoplasmic pH values, one is still faced with very crude methods that often give inaccurate and sometimes even unreliable results. In particular the exact value of the membrane potential of neutrophilic bacteria is uncertain."

These methods have recently been reviewed by Bakker [1], Kashket [2] and Rottenberg [3]. The results presented in this report indicate that even concentrations of probe molecules as low as 100 nM could reduce $\Delta\Psi$. This means that many commonly used techniques for $\Delta\Psi$ determination are inappropriate. In addition, a model of concentration dependent binding of lipophilic cations to cell membranes will be presented.

The equilibrium distribution of radiolabeled lipophilic cations is the only method with the sensitivity to utilise nanomolar concentrations of probe. ^{86}Rubidium has been used to measure $\Delta\Psi$, however, it has been shown [2] that in bacterial cells exhibiting active K^+ uptake, even with apparently adequate amounts of valinomycin, the K^+ concentration gradient was no longer maintained in thermodynamic equilibrium with $\Delta\Psi$ by the ionophore. Thus, for *Enterococcus faecalis* (previously *Streptococcus faecalis*, [4]), which is known to accumulate up to 500 mM K^+ by active transport [5], $^{86}Rb^+$ distribution may not accurately reflect $\Delta\Psi$. Thus, it is important that the probe cations be lipophilic so that the rate of independent electrogenic transport of the probe is much faster than active transport processes. Organic ions with diffuse charge distribution such as tritiated tetraphenylphosphonium ion ($[^3H]TPP^+$) have found widespread use since they were first introduced

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by Schuldiner and Kaback in 1975 [6]. The major disadvantage associated with this technique is that due to their lipophilic nature a considerable fraction of the ions associated with cells may be bound to membranes and it is therefore necessary to correct for the amount of bound ions before $\Delta\Psi$ can be calculated.

To deal with this problem it is necessary to construct an appropriate model of probe binding. The simplest model, though probably not the correct one, is to assume that binding is saturable and that a constant amount of $[^3H]TPP^+$ binds to the cells [7]. The amount of $[^3H]TPP^+$ taken up by deenergised cells is then subtracted from that of energised cells. With the known cytoplasmic volume of the cells the corrected $[^3H]TPP^+$ uptake data are then converted into $\Delta\Psi$. Indeed, this model is still in common use today [8]. However, as has been reported for deenergised cells of *Escherichia coli* and *Bacillus alcalophilus* [9,10] and confirmed for *E. faecalis* in the present study, $[^3H]TPP^+$ binding is proportional to the $[^3H]TPP^+$ concentration. The concentration of intracellular $[^3H]TPP^+$ and therefore the amount of $[^3H]TPP^+$ binding is dependent on $\Delta\Psi$. Models for this energy-dependent binding have been presented in the literature and will be described below.

In the first of these Zaritsky et al. [11] assumed that the amount of $[^3H]TPP^+$ bound to the cells was proportional to the exponential mean between the free concentrations of $[^3H]TPP^+$ in the cytoplasm and the medium, although no experimental evidence was presented to support this hypothesis. The second model was published by Lolkema et al. [12] and assumed separate binding constants for binding dependent on the intracellular probe concentration, the extracellular probe concentration and for probe binding to the cytoplasmic membrane. In cells of the Gram-negative bacteria *Rhodopseudomonas sphaeroides*, these workers calculated that 47% of $[^3H]TPP^+$ binding sites were located at the inside of the cytoplasmic membrane and 53% at the outside of the cytoplasmic membrane [13].

The model of Rottenberg [14] used the description of lipophilic ion binding of Cafiso and Hubbell [15] in which binding took place in two boundary regions located just below the level of the membrane/solution interface (Fig. 1). The lipophilic ions in these boundary regions are said to be in rapid equilibrium with their respective aqueous solutions and it is equilibration between the two membrane boundary regions which is subject to the highest energy barrier to transport. This model will be adopted for the present study as the assumption of independent partition coefficients on the two sides of the cell membrane has good support from the work of Cafiso and Hubbell. Both Rottenberg and Lolkema et al. point out that the inclusion of divalent cations and a high ionic strength in the medium substantially reduce the surface potential of the membrane and hence the amount of lipophilic cation binding. We have therefore introduced 10 mM $MgSO_4$ and 150 mM KCl into the experimental buffer.

2. Materials and methods

2.1. Materials

$[^3H]$Tetraphenylphosphonium bromide ($[^3H]TPPBr$) (26 Ci/mmol), $[^{14}C]$methylamine (60 mCi/mmol) and $[^{14}C]$5,5-dimethyl-2,4-oxazolidinedione (DMO) (50 mCi/mmol) were obtained from Amersham, Amersham, UK. Valinomycin was obtained from Calbiochem and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) from Sigma. All other chemicals were reagent grade and obtained from commercial sources, except for TPPBr which was synthesised in the laboratory [16].

2.2. Preparation of intact cells

Enterococcus faecalis cells (Queensland University Culture Collection No. 011768) were grown as described previously [17] and washed twice in the appropriate ice-cold buffer before being resuspended in the same buffer to a final density of 80–100 mg bacterial dry weight/ml. All buffers contained 10 mM KH_2PO_4, 5 mM $MgSO_4$, 2 mM EDTA, 150 mM KCl and 100 mM of one of the following biological buffers (the so-called Good buffers): (a) 3-tris(hydroxymethyl)aminopropanesulfonic acid (Taps), pH 8.6; (b) 3-(*N*-morpholino)propanesulfonic acid (Mops), pH 7.6; (c) piperazine-*N,N'*-bis(2-ethanesulfonic acid) (Pipes), pH 6.8; or (d) piperazine-*N,N'*-bis(2-ethanesulfonic acid) (Pipes), pH 6.6.

Cell suspensions were kept on ice for no more than 2 h before use, at which time they were warmed to 20°C 10 min before the beginning of the experiment.

2.3. Non-specific binding of $[^3H]TPP^+$

Each assay mix contained 200 μl of bacterial cell suspension with a mixture of 120 μM CCCP and 10 μM valinomycin to deenergise the cells. After a 10 min incubation $[^3H]TPPBr$ was added to give a concentration in the range 10 nM to 1 μM and the incubation continued for a

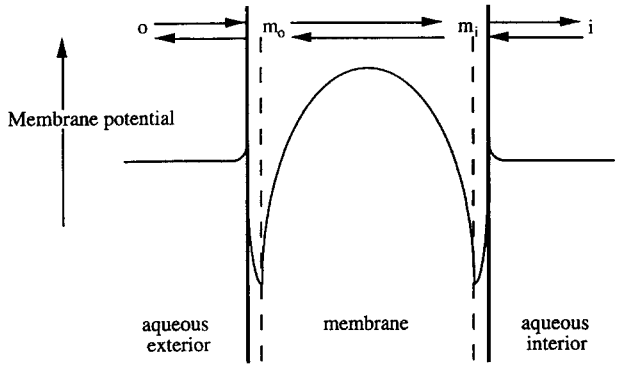

Fig. 1. Potential energy profile for a hydrophobic ion across a cell membrane. There are free energy minima for these ions at 'boundary' regions (m_o and m_i) lying below the membrane surface interface (from Cafiso and Hubbell [15]).

further 30 min. The reaction mixtures were prepared in 400 μl Eppendorf microfuge tubes and at the end of the second incubation the mixtures were centrifuged for 2 min in a Hettich microliter centrifuge at 12000 × *g*. The supernatant was checked for optical clarity and an 80 μl aliquot withdrawn with a Hamilton glass syringe and incubated for 24 h at 37°C with 0.5 ml of 5% (w/v) SDS. The microfuge tube was then cut at the top of the pellet and any remaining drop of supernatant removed by holding the edge of a tissue to the pellet surface. The pellets were resuspended with a glass syringe in 0.5 ml of 5% SDS and incubated under the same conditions as the supernatant. The pellet and supernatant incubations were then centrifuged and a 100 μl aliquot of the clear supernatant was removed from each and added to 4 ml of ACS scintillation fluid (Amersham) and counted on a Packard Tri-Carb 2000 series liquid scintillation counter. The radioactivity was recorded as dpm using an appropriate quench curve generated with chloroform.

In some experiments cells were deenergised by incubation with 1% toluene for 1 h at 37°C. Processing of the assay mixtures was the same as that outlined above.

2.4. The effect of [^{3}H]TPP$^+$ concentration on apparent $\Delta\Psi$

A cell suspension was prepared at pH 8.6 in the 100 mM Taps buffer described above. 800 μl of bacterial suspension and enough distilled and deionized water to bring the final volume to 1.0 ml were incubated with a range of [^{3}H]TPPBr concentrations from 10 nM to 1 μM for 2 min. At time zero 10 mM D-glucose was added. ^{1}H-NMR spectroscopy showed that 5 min after glucose addition lactate concentration reached a maximum level indicating that glycolysis was complete and that thermodynamic equilibrium could be assumed (results not presented). Duplicate 200 μl aliquots were withdrawn at 10 min and 20 min and rapidly centrifuged and processed as described for the determination of [^{3}H]TPP$^+$ binding.

2.5. Δp determination

$\Delta\Psi$ and ΔpH were determined simultaneously in separate parallel experiments [18]. A 20 nM concentration of [^{3}H]TPPBr was used as the $\Delta\Psi$ probe and 6.7 μM [^{14}C]DMO as the ΔpH probe. In a third experiment at pH 8.6, 4.7 μM [^{14}C]methylamine was also used as a ΔpH probe. The external pH was recorded continuously throughout the experiment with a combination pH electrode. All assays were processed as outlined above.

3. Results

3.1. [^{3}H]TPP$^+$ binding

When the membrane is totally deenergised (zero $\Delta\Psi$) the free [^{3}H-TPP$^+$]$_i$ will be equal to the free [^{3}H-TPP$^+$]$_o$. Lolkema et al. [12] have shown that the addition of CCCP and valinomycin completely deenergise the membranes of typical Gram-positive bacteria. Under these conditions the concentration of [^{3}H]TPP$^+$ bound per mg dry weight of cells, [^{3}H-TPP^+_B], could be calculated from the total dpm in the pellet after correction for free [^{3}H]TPP$^+$ in the cytoplasm and the extracellular matrix of the pellet. The amount of free [^{3}H]TPP$^+$ in these compartments can be calculated from the [^{3}H]TPP$^+$ in the supernatant and the known volumes of the compartments [19]. This can be expressed in the following equation,

$$[^3\mathrm{H}]\mathrm{TPP}^+_\mathrm{B} = \frac{\left(^3\text{H-TPP}_\mathrm{P} - (V_\mathrm{i}/\mathrm{mg} + V_\mathrm{m}/\mathrm{mg}) \times W \times {}^3\text{H-TPP}_\mathrm{S}/\mu\mathrm{l}\right)}{\mathrm{dpm/nmol}\ ^3\text{H-TPP}^+ \times W} \quad (2)$$

where V_i/mg and V_m/mg are the volumes of intracellular and matrix water per mg dry weight of cells, and W is the mg dry weight in the 200 μl aliquot. ^{3}H-TPP$_P$ and ^{3}H-TPP$_S$ are the dpm in the pellet and supernatant, respectively, and the units of [^{3}H]TPP^+_B are nmol/mg dry weight.

E. faecalis cells were deenergised with CCCP and valinomycin, and the amount of [^{3}H]TPP$^+$ bound to the cells per mg dry weight was calculated with Eq. (2). The result at four different pH values (6.6, 6.8, 7.6 and 8.6) is plotted against free [^{3}H]TPP$^+$ concentration in Fig. 2 with a log-log scale. While this provides the best graphical representation of the data the slope was obtained from a linear depiction. The slope was a straight line with a value of 9.10 ± 0.33(S.D.) fmol/mg dry weight per μM, which is equivalent to 9.10 ± 0.33 μl/mg dry weight. The slope represents the apparent binding volume of [^{3}H]TPP$^+$ and can be used in calculations of $\Delta\Psi$ to correct for probe binding. Deenergisation of the cells with 1% toluene for 1 h at 37°C gave similar results. Thus, [^{3}H]TPP$^+$ binding in *E. faecalis* cell suspensions has been shown to be linear and independent of pH.

In order to calculate $\Delta\Psi$ it was first necessary to determine the cytoplasmic [^{3}H]TPP$^+$ concentration. The assumption was made that half of the [^{3}H]TPP$^+$ binding volume was in equilibrium with the extracellular compartment and the other half with the cytosol as suggested by the results of Lolkema et al. [13]. [^{3}H-TPP$^+$]$_i$ could then be calculated from the following equation,

$$[^3\text{H-TPP}^+]_\mathrm{i} = \frac{^3\text{H-TPP}_\mathrm{P} - \left\{(V_\mathrm{m}/\mathrm{mg} + B/2) \times W \times {}^3\text{H-TPP}_\mathrm{S}/\mu\mathrm{l}\right\}}{(V_\mathrm{i}/\mathrm{mg} + B/2) \times W} \quad (3)$$

where B was the effective [^{3}H]TPP$^+$ binding volume in μl/mg dry weight. $\Delta\Psi$ could then be determined from the Nernst Equation.

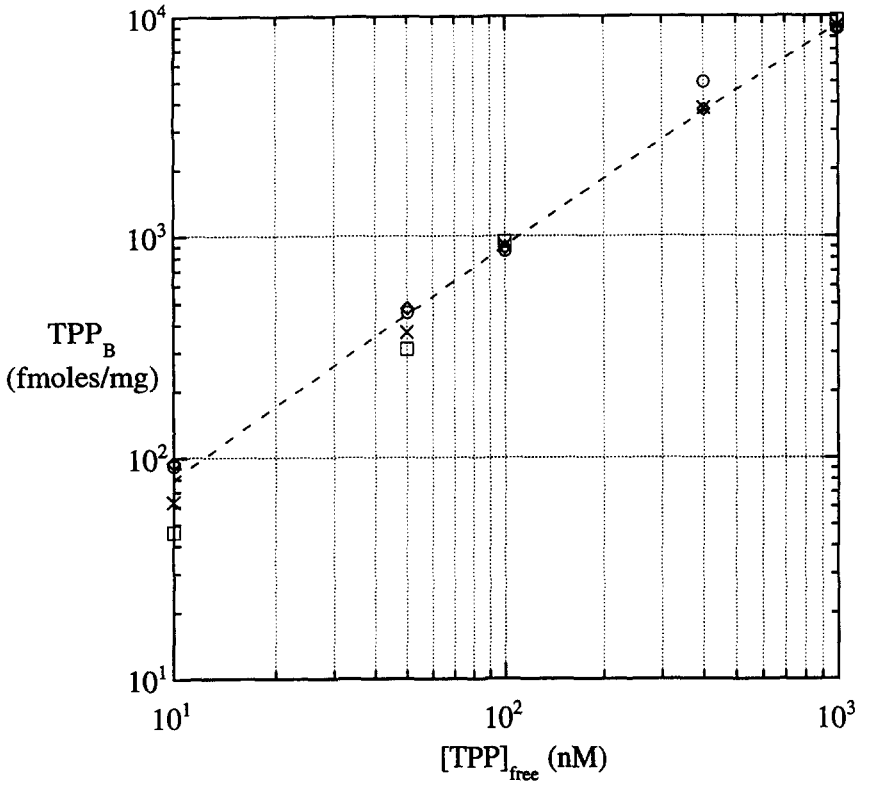

Fig. 2. Concentration-dependent binding of [^{3}H]TPP$^+$ to cells of *E. faecalis*. Cells were resuspended at pH 8.6 (circles), pH 7.6 (squares), pH 6.8 (diamonds), or pH 6.6 (crosses) and [^{3}H]TPP$^+$ binding determined as described in Section 2.

Extraction of [^{3}H]TPP$^+$ from the pellet was complete in 24 h as shown by the failure of a second extraction of the cell pellet with 5% SDS to increase the [^{3}H]TPP$^+$ concentration above that expected from a simple dilution of the pellet. In some experiments [^{3}H]TPP$^+$ binding in the range 1 to 100 μM was also examined and was found to be linear.

3.2. Effect of [^{3}H-TPP$^+$] on apparent $\Delta\Psi$

Fig. 3 shows a plot of $\Delta\Psi$ against [^{3}H]TPP$^+$ concentration for *E. faecalis* cells at pH 8.6 energised with 10 mM D-glucose. $\Delta\Psi$ was reasonably constant in the range 10 to 100 nM [^{3}H]TPP$^+$ but was observed to decrease at higher [^{3}H]TPP$^+$ concentrations. For this reason a concentration of 20 nM [^{3}H]TPPBr was chosen for $\Delta\Psi$ determinations.

3.3. Estimation of Δp

Preliminary experiments indicated that 5 min after *E. faecalis* cells were fed with glucose glycolysis was essentially complete, and that both $\Delta\Psi$ and ΔpH changed only slowly in the next 30 min indicating that thermodynamic equilibrium had been reached. For this reason measurements of $\Delta\Psi$ and ΔpH were made at 10 and 20 min after feeding with glucose in the present study. The results at four different pH values are shown in Fig. 4a and 4b.

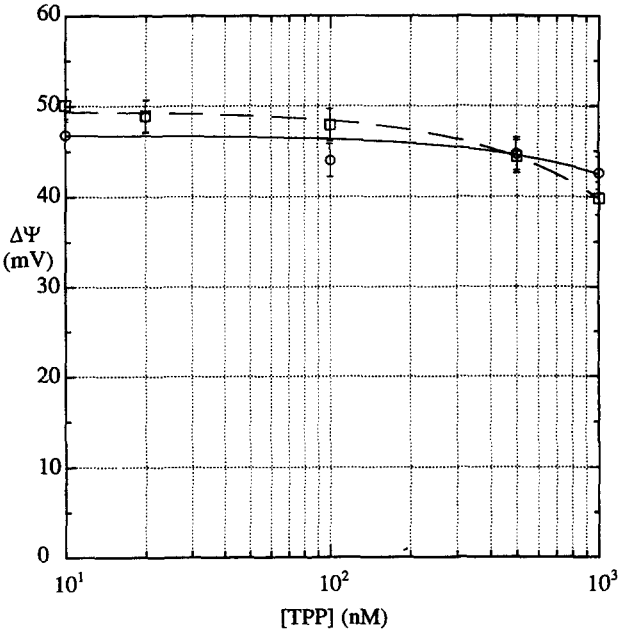

Fig. 3. Effect of $[^3H\text{-}TPP]_{free}$ on the apparent membrane potential. Aliquots were taken for $\Delta\Psi$ determination 10 min (circles) and 20 min (squares) after the addition of 10 mM D-glucose to thick cell suspensions of *E. faecalis* at pH 8.6 that had been preincubated with [^{3}H]TPPBr as described in Section 2.

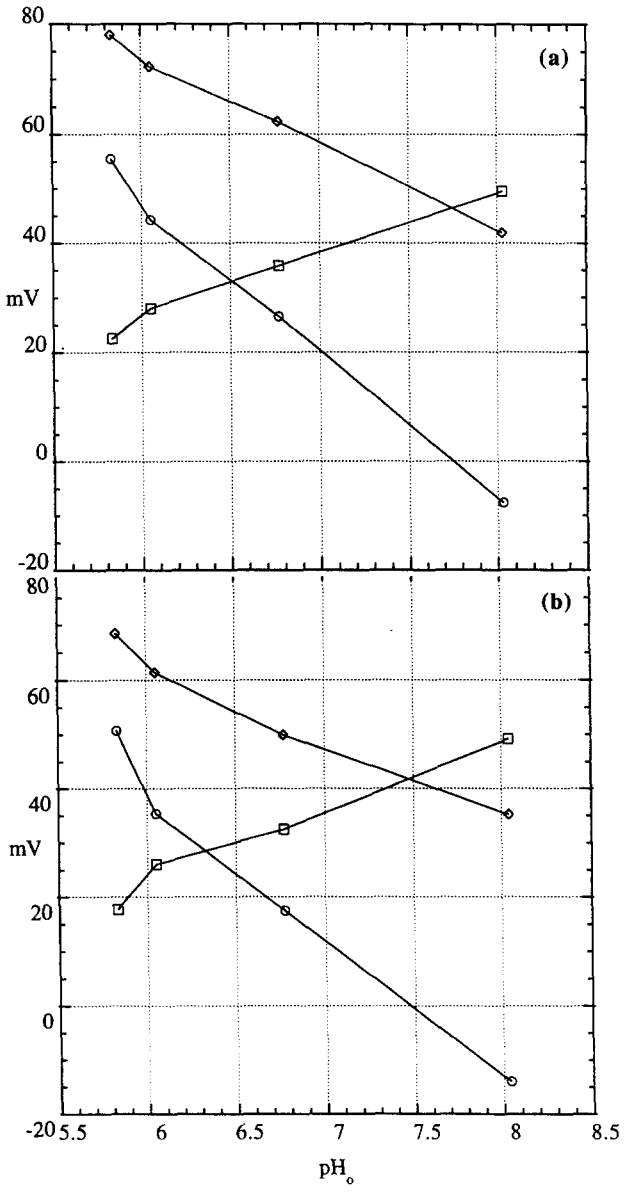

Fig. 4. $\Delta\Psi$, ΔpH and Δp as functions of extracellular pH. At time zero 10 mM glucose was added to thick cell suspensions of *E. faecalis* suspended in the buffers described in Section 2. Aliquots were taken at 10 min (a) and at 20 min (b) and $\Delta\Psi$ (squares) and ΔpH (circles) were determined. Δp (diamonds) was calculated from these results.

Upon addition of 10 mM D-glucose an immediate acidification of the external medium took place. At each pH_o $\Delta\Psi$ and ΔpH varied only slightly between 10 and 20 min after feeding and both were found to be functions of the pH_o. As the pH_o was raised $\Delta\Psi$ increased and ΔpH decreased. Thus, the effect of pH_o on Δp was much less than on either $\Delta\Psi$ or ΔpH, although $\Delta\Psi$ did not compensate fully for the change in ΔpH and as a result Δp was observed to decrease as pH_o increased. Above pH 7.6 pH_o is greater than pH_i, reversing the sense of the normal pH gradient. The ΔpH measurements at pH_o 8.1 were made with both the weak acid DMO and the weak base methylamine. The same ΔpH of -0.2 pH units was obtained with each probe.

4. Discussion

In contrast to many other reports [7,20], Zaritsky et al. [11] and Lolkema et al. [12] showed that lipophilic cation binding to cells was proportional to the free probe concentration in their cell systems. In this study binding of [^{3}H]TPP$^+$ to *E. faecalis* cells was also found to be directly proportional to [^{3}H]TPP$^+$ concentration, however, in contrast to the results of Lolkema et al. [12], binding was pH independent. The assumption that [^{3}H]TPP$^+$ binding was saturating in previous studies of *E. faecalis* would have led to an overestimation of $\Delta\Psi$ [21].

Having determined that [^{3}H]TPP$^+$ binding was energy dependent it was then necessary to design an experimental protocol in which an appropriate [^{3}H]TPP$^+$ concentration was used to determine $\Delta\Psi$. Hoffmann and Dimroth [10] reported that the apparent $\Delta\Psi$ was independent of [^{3}H]TPP$^+$ concentration in the range 10 nM to 50 nM but that at 1 μM [^{3}H]TPP$^+$ it substantially decreased. These results were confirmed in the present study. Furthermore, these results indicate that there is no general efflux system for [^{3}H]TPP$^+$ in these bacteria [22] as otherwise the apparent membrane potential would have increased at higher [^{3}H]TPP$^+$ concentrations as the efflux system begins to saturate and proportionally more [^{3}H]TPP$^+$ accumulates in the cells.

The vast majority of $\Delta\Psi$ studies of bacterial cells have used substantially higher concentrations of [^{3}H]TPP$^+$ on the assumption that at high concentrations of [^{3}H]TPP$^+$ binding was saturated. It is well known that high concentrations of [^{3}H]TPP$^+$ can lead to an underestimation of $\Delta\Psi$, particularly if this was a high value [9]. Bakker has speculated that this could be caused by intense binding of [^{3}H]TPP$^+$ to the cytoplasmic membrane, elicited by the high $\Delta\Psi$ and high [^{3}H-TPP$^+$], causing the surface potential on the inner face of the membrane to become less negative. This would lead to an inhibition of further [^{3}H]TPP$^+$ penetration and to an underestimation of $\Delta\Psi$. Thus, the assumption that binding was saturated led to an overestimation of $\Delta\Psi$ and the high [^{3}H]TPP$^+$ concentration usually chosen resulted in an underestimation partially compensating each other. In this study it was felt to be important to use the low concentrations of [^{3}H]TPP$^+$ that do not lead to a reduction in apparent $\Delta\Psi$. The rapid separation of cells and media by centrifugation may also be an advantage over filtration assays used by others, as pointed out by Hoffmann and Dimroth [10].

The observation that [^{3}H]TPP$^+$ concentrations above 100 nM caused a reduction in apparent $\Delta\Psi$ also had important implications for the selection of the method of determination of lipophilic cation distribution. Methods that rely on selective electrodes or EPR require concentra-

tions of at least 2 μM so one was left with the radiolabeled lipophilic cations as the only viable alternative.

The problem of energy dependent [^{3}H]TPP$^+$ binding to bacterial cells has been overcome by constructing an appropriate model. Rottenberg's model of binding in mitochondria [14] was based on the work of Cafiso and Hubbell using vesicles [15]. In these models [^{3}H]TPP$^+$ bound at both intra- and extracellular surfaces with both bound pools in rapid equilibrium with their respective aqueous solutions. It was equilibration between these two membrane boundary regions which was subject to the highest energy barrier to transport. The size of these two pools of bound [^{3}H]TPP$^+$ has been determined in the present work. The proportionality constant for binding has been equated to an 'apparent binding volume' for [^{3}H]TPP$^+$ with units of μl/mg dry weight of cells. This 'apparent binding volume' is thermodynamically equivalent to the volume of cell membrane multiplied by the partition coefficient of [^{3}H]TPP$^+$ for cell membrane. Its introduction marks a major simplification in the determination of $\Delta\Psi$ as it is then a relatively simple matter to introduce the necessary corrections into the equation to determine [^{3}H-TPP$^+$]$_i$ (Eq. (3)).

The results of Lolkema et al. where 47% of [^{3}H]TPP$^+$ binding sites were located at the inside of the cytoplasmic membrane and 53% at the outside of the cytoplasmic membrane [13] were used in the present study to allot the two pools of apparent [^{3}H]TPP$^+$ binding volumes. Lolkema et al. fractionated their cells into cell wall, outer cell membrane, inner cell membrane and cytoplasmic constituents and measured the amount of binding to each fraction. The assumption was made that binding did not change during fractionation, which may or may not be the case.

The conclusion to be drawn from this work is that the most accurate method for $\Delta\Psi$ determination was to use nanomolar concentrations of lipophilic cations and appropriate correction for energy dependent binding. The $\Delta\Psi$ values we report are substantially lower than those reported for similar cells. The experimental conditions used in this report were designed to be compatible with NMR experiments and it is likely that the use of thick cell suspensions and cells that had completed glycolysis would lead to lower values of $\Delta\Psi$. Nevertheless, the methodological issues highlighted by this study should be applicable to a wide range of experimental conditions.

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