Biochimica et Biophysica Acta, 681 (1982) 85–94
Elsevier Biomedical Press

BBA 41097

THE EFFECT OF 'PROBE BINDING' ON THE QUANTITATIVE DETERMINATION OF THE PROTON-MOTIVE FORCE IN BACTERIA

JUKE S. LOLKEMA, KLAAS J. HELLINGWERF AND WIL N. KONINGS *

Department of Microbiology, State University of Groningen, Kerklaan 30, 9751 NN Haren (The Netherlands)

(Received December 1st, 1981)

Key words: Nonsaturable probe binding; Proton-motive force quantitation; Bacterial bioenergetics; Tetraphenylphosphonium

The electrical potential across the cytoplasmic membrane of bacteria can be calculated from the distribution of the lipophilic cation tetraphenylphosphonium between the bulk phases of the medium and the cytoplasm. In order to determine the bulk phase concentrations, information about the binding of the probe to the cellular components is required. In de-energized cells of *Rhodopseudomonas sphaeroides* the binding appears to be proportional to the free probe concentration. The bulk phase concentrations can only be determined when knowledge is available about the distribution of the binding of the probe over the different cellular components. In this report, models for binding are presented which are based on the assumption that the binding is an energy-independent process. These models allow a proper calculation of the electrical potential when the binding of the probe to the different cellular components is known.

I. Introduction

The chemiosmotic theory, as proposed by Mitchell [1], states that biological energy converters like bacteria, mitochondria and chloroplasts are able to transform chemical, redox or light energy into the electrochemical energy of a proton-motive force ($\Delta\tilde{\mu}_{H^+}$). In bacteria this conversion is catalyzed by primary H^+ pumps, localized in their cytoplasmic membrane. Various processes are energetically linked to the free energy of the proton-motive force. For instance, solute transport into bacterial cells is catalyzed by secondary transport systems, which couple the transport of the metabolite across the cytoplasmic membrane to the simultaneous movement of H^+ [2].

Recent investigations on quantitative aspects of the chemiosmotic theory have indicated a role of 'localized' chemiosmotic phenomena [3–6] in bioenergetics. These experiments, as well as determinations of H^+ : solute stoichiometries in secondary transport [7,8], depend heavily on the quantitative reliability of current methods to determine the magnitude of the proton-motive force. Two components contribute to this electrochemical H^+ gradient: the concentration gradient of the

* To whom correspondence should be addressed.

Abbreviations: TPP^+, tetraphenylphosphonium ion; $TPMP^+$, triphenylmethylphosphonium ion; TPB^-, tetraphenylborate ion; Mes, 2-(*N*-morpholino)ethanesulphonic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*-2-ethanesulphonic acid.

Glossary: $\Delta\tilde{\mu}_{H^+}$, proton-motive force; $\Delta\psi$, electrical transmembrane potential difference; ΔpH, transmembrane H^+ concentration gradient; C_o, probe concentration in the medium before adding the cells; C_i intracellular free probe concentration; C_i^b, intracellular bound probe concentration; C_{cm}, cytoplasmic membrane probe concentration; C_{om}, outer membrane probe concentration; C_e, extracellular probe concentration; V, volume of the system; x, fractional internal volume; y, fractional cytoplasmic membrane volume; z, fractional outer membrane volume; f_{cm}, ratio of fractional cytoplasmic membrane and intracellular volume; f_{om}, ratio of fractional outer membrane and intracellular volume; K_i, binding constant for binding to intracellular components; K_{cm}, cytoplasmic membrane partition coefficient; K_{om}, outer membrane partition coefficient; k, first-order proportionality constant between flux and concentration; $N_{b(f)}$, number of bound (free) probe molecule.

0005-2728/82/0000–0000/$02.75 © 1982 Elsevier Biomedical Press

ion (ΔpH) and the difference in electrical potential between the two aqueous phases ($\Delta\psi$). In the formula:

$$\Delta\bar{\mu}_{H^+} = \Delta\psi - Z\,\Delta pH \quad (1)$$

$Z = 2.3RT/F$, and R, T and F have their usual meaning *.

At present, no reliable method is available for a direct determination of the total proton-motive force. Therefore, quantitation via separate measurements of ΔpH and $\Delta\psi$ is required. Distribution measurements of membrane-permeant indicator molecules or ions (the so-called 'probes') have been found to be most reliable [9–11]. Several techniques can be used to measure the transmembrane distribution of these probes, such as filtration assay [12], silicon-oil centrifugation [13], potential measurements across black lipid membranes [14] and flow dialysis [15,16]. Each technique has its own advantages and disadvantages, and a specific application area, and these have been extensively discussed (e.g., see Ref. 17).

Less attention has been paid to the properties of the probes used to measure ΔpH and $\Delta\psi$. It is generally assumed that these probes show an ideal behaviour. For measurements of ΔpH, with an inside alkaline polarity, and benzoic acid as a probe, this assumption turned out to be correct. We demonstrated that the ΔpH values, measured with two completely independent methods, ^{31}P-NMR and the distribution of benzoic acid, are essentially the same [18]. Measurements of $\Delta\psi$ appear to be more problematic. Frequently, it has been observed that binding of the indicator probe to, e.g., bacteria occurs (see Section V). However, proper corrections for this binding have not been made mainly because it is generally (and erroneously [8]) assumed that binding of these $\Delta\psi$ probes to membranes and/or proteins is already saturated at the applied probe concentrations (between 1 and 100 μM). In this paper, the demands for application of a probe for $\Delta\psi$ measurements will be discussed with special emphasis on the most commonly used $\Delta\psi$ probe in bacteria, TPP^+. A model for a quantitatively correct calculation of $\Delta\psi$ will be presented which can be used if probe binding occurs.

II. Demands on $\Delta\psi$ probes in distribution methods

In distribution measurements the magnitude of $\Delta\psi$ is calculated from the Nernst equation:

$$\Delta\psi = Z \log[A]_{in}/[A]_{out} \quad (2)$$

in which [A] represents the activity of the indicator probe in either one of the two aqueous bulk phases. Usually, the activity of the probe is taken to be equal to its concentration (see below). An ideal $\Delta\psi$ probe should have the following properties.

(i) It should pass rapidly energy-transducing membranes in the ionized form, either passively or facilitated, but in the latter case only in response to $\Delta\psi$. Preferentially, the resistance of the membrane to probe permeation should be very low. Ideally, the free energy of probe ions in the hydrophobic interior of biological membranes should be equal to or lower than the free energy of the probe in the bulk aqueous phase. However, this requirement will often be in conflict with the next property.

(ii) It should not bind to the membrane or to intracellular or extracellular constituents. All interactions which lead to an apparent accumulation into the cells under de-energized conditions will be considered as binding. According to this definition, binding includes association of the probe molecules with cellular constituents, partitioning into the cellular membrane(s) and the effects due to differences in the activity coefficient of the probe in the aqueous bulk phases.

(iii) It should be detectable at very low concentrations. As $\Delta\psi$ probes are membrane-permeant ions they may contribute to an interconversion of $\Delta\psi$ into ΔpH [15,19,20]. The $\Delta\psi$ probe should be used at that concentration at which the apparent $\Delta\psi$ is maximal [21].

(iv) It should be biologically inert. The probe should not be metabolized, nor should it be toxic for growth of the cells in the concentration range used.

Requirement ii is the main subject of this report

* Electrochemical potential ($\bar{\mu}_{H^+}$) is defined as $\bar{\mu}_{H^+} = \mu_{H^+} + F\psi$, where μ_{H^+} is the chemical potential. In this equation the electrochemical potential $\Delta\tilde{\mu}_{H^+} = \Delta\bar{\mu}_{H^+}/F$ and is expressed in mV.

because binding of $\Delta\psi$ probes to biological structures is a quite common property (see Section V).

III. Results

IIIA. Binding of TPP^+ to intact cells of Rhodopseudomonas sphaeroides

Binding of TPP^+ to intact cells of *R. sphaeroides* is proportional to the free probe concentrations up to concentrations as high as 100 μM (Fig. 1). In a binding experiment the internal and external free probe concentrations are equal (de-energized cells). However, under energized conditions the probe will accumulate in the cells, making the internal concentration different from the external one. It is necessary, therefore, to know whether the internal concentration, the external concentration or both determine the amount of binding before $\Delta\psi$ can be calculated. We will now investigate sequentially the effect of probe binding on the $\Delta\psi$ calculation for these three possibilities in the case where the distribution is followed by changes in the external probe concentration.

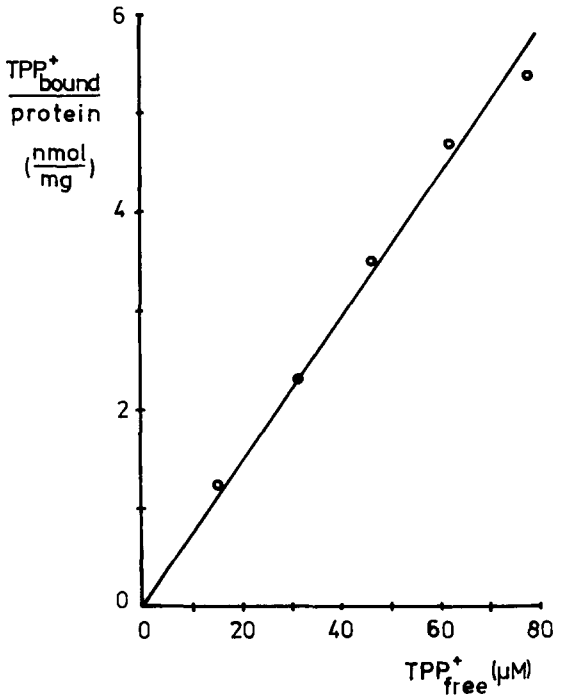

Fig. 1. Concentration-dependent binding of TPP^+ to cells of *Rps. sphaeroides*. Cells were grown as described in Ref. 16, harvested and washed twice in a buffer containing 100 mM KCl, 20 mM Mes, 20 mM Hepes, 2 mM EDTA, 5 mM Mg^{2+}, 15 μg/ml chloramphenicol, pH 7.8. Cells were de-energized with 1% toluene during an incubation for 1 h at 37°C. The change in TPP^+ concentration upon the addition of the cells was measured with a TPP^+-sensitive electrode constructed according to Shinbo et al. [50]. The protein concentration was 2.3 mg/ml. Using a conversion factor of 3 μl internal volume per mg protein (Ten Brink, B., unpublished observation), a value for $K_i + f_{cm}K_{cm} + f_{om}K_{om}$ of 25 can be calculated (see text).

IV. The model

IVA. Binding dependent on the internal free probe concentration

In this case, binding consists of binding to macromolecules such as ribosomes or nucleic acids or a lower activity of the probe in the intracellular aqueous compartment compared to the external medium. For the time being, we will take the bound concentration (C_i^b) to be proportional to the free concentration of the probe inside the cells (C_i), although at probe concentrations higher than 100 μM some saturation can be observed (see Section V). With a proportionality constant K_i we obtain:

$$C_i^b = K_i C_i \tag{3}$$

We will call C_e the external probe concentration after adding the cells and C_o the probe concentration before addition of the cells but corrected for the dilution caused by adding the cells. C_o and C_e are the measurable parameters. From the Law of Mass Action it follows that (see also Fig. 2A):

$$C_o = (1-x)C_e + x(C_i + C_i^b)$$

in which x is the fractional internal volume of the cells [20]. Together with Eqn. 3 this can be rearranged to:

$$C_o = (1-x)C_e + x(1+K_i)C_i$$

or:

$$\frac{C_o}{C_e} = 1 - x + x(1+K_i)\frac{C_i}{C_e} \tag{4}$$

Insertion of this expression in Eqn. 2 shows that $\Delta\psi$ can now be calculated correctly from C_o and C_e

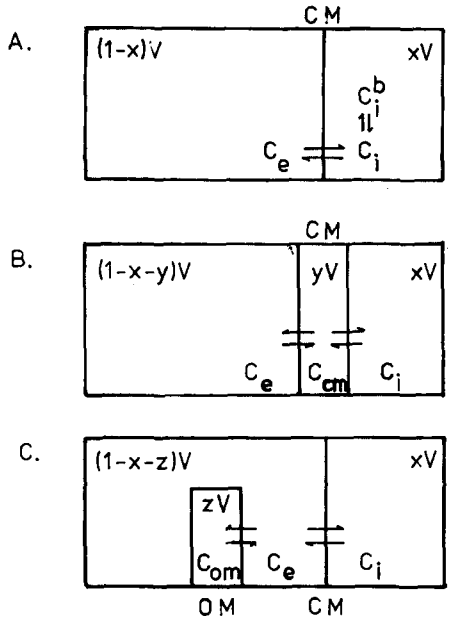

Fig. 2. Schematic diagram of the different models for binding of TPP^+ to bacteria: (A) binding to intracellular components, (B) partitioning in the cytoplasmic membrane and (C) partitioning in the outer membrane (or binding to extracellular components). V, total volume of the system; x, y and z, volume fraction of the intracellular compartment, the cytoplasmic membrane and the outer membrane, respectively. C_i, C_i^b, C_e, C_{cm} and C_{om} represent the concentration of TPP^+ in the intracellular aqueous bulk phase, bound to intracellular constituents, in the extracellular aqueous bulk phase, bound to intracellular constituents, in the extracellular aqueous phase, partitioned in the cytoplasmic membrane and partitioned in the outer membrane, respectively. The arrows indicate the various ways of probe transfer in the system.

if x is known:

$$\Delta\psi = Z \log\left\{\frac{\frac{C_o}{C_e} + x - 1}{x(1+K_i)}\right\}$$

This equation demonstrates that binding of the probe to intracellular components leads to an apparent increase in the fractional internal volume of the cells by a factor $1 + K_i$. The value of K_i can easily be calculated from a binding experiment (with $C_i = C_e$) and Eqn. 4. In that case:

$$\frac{C_o}{C_e} = 1 + xK_i \qquad (5)$$

If binding is due to adsorption to cytoplasmic components, K_i has the meaning of an apparent binding constant; when, on the other hand, the underlying mechanism is loss of activity of the probe in the intracellular aqueous phase, K_i is the reciprocal of the activity coefficient. The experiment depicted in Fig. 1 yields a K_i of 25, which would mean that, if all binding occurs to intracellular constituents, only 4% of the intracellular probe is free in solution.

IVB. Binding dependent on both the internal and external probe concentrations

A second possibility is that probe binding occurs at the cytoplasmic membrane, at both its intracellular and extracellular side. Apart from saturation and asymmetry effects, this is mathematically indistinguishable from a partitioning of the probe between the aqueous bulk phases and the hydrophobic membrane phase. If we ignore the fact that the surface of the inner half of the membrane is somewhat smaller than that of the outer half, then the unidirectional flux (J) through a membrane/water interface will be $J = k_{i,o}C$, assuming this is a simple first-order process (cf. Fig. 2B). Under steady-state conditions, the net flux into the membrane, being the sum of the four unidirectional fluxes through the membrane/water interfaces, equals zero:

$$J_{net} = -2k_oC_{cm} + k_iC_e + k_iC_i = 0$$

where k_o and k_i are the first-order rate constants of probe transfer through the membrane/water interfaces and C_{cm} the probe concentration in the membrane, for which it follows:

$$C_{cm} = K_{cm}/2(C_e + C_i) \qquad (6)$$

in which $K_{cm} = \frac{k_i}{k_o} \cdot K_{cm}$ is the partition coefficient for the probe between the aqueous and membrane phase. The Law of Mass Action can be used to derive:

$$C_o = (1-x-y)C_e + yC_{cm} + xC_i$$

where y is the fractional volume of the membrane.

With Eqn. 4 this results in:

$$\frac{C_o}{C_e} = 1 + x\left(\frac{C_i}{C_e} - 1\right) + y\left\{\frac{K_{cm}}{2}\left(\frac{C_i}{C_e} + 1\right) - 1\right\} \quad (7)$$

Since there will only be a significant effect of the partitioning phenomenon on the calculation of $\Delta\psi$ if the term containing y is of the same order of magnitude as that containing x and $y \ll x$, we make the assumption that $K_{cm} \gg 1$. Furthermore, y is expressed in x, since for a specific bacterium, grown under well defined conditions, there is a constant factor between x and y, $f_{cm} = y/x$, which means that f_{cm} can be regarded as the fractional volume of the cytoplasmic membrane in the bacterium. This simplifies Eqn. 7 to

$$\frac{C_o}{C_e} = 1 - x\left(1 - \frac{f_{cm}K_{cm}}{2}\right) + x\frac{C_i}{C_e}\left(1 + \frac{f_{cm}K_{cm}}{2}\right) \quad (8)$$

Together with the Nernst equation (Eqn. 2) this results in:

$$\Delta\psi = Z \log\left\{\frac{\frac{C_o}{C_e} - 1 + x\left(1 - \frac{1}{2}f_{cm}K_{cm}\right)}{x\left(1 + \frac{1}{2}f_{cm}K_{cm}\right)}\right\}$$

The term $f_{cm}K_{cm}$ can be determined from a binding experiment, using Eqn. 8:

$$\frac{C_o}{C_e} = 1 + xf_{cm}K_{cm} \quad (9)$$

IVC. Binding dependent on the extracellular free probe concentration

The binding of the probe may as well be due to partitioning in the outer membrane of gram-negative bacteria or to adsorption to bacterial cell walls. Again there is no phenomenological difference between these two possibilities (cf. subsection IVB) and we will derive the equations for a correct $\Delta\psi$ calculation, for the case of partitioning of the probe in the outer membrane (Fig. 2C). As we assume that the probe external to the cytoplasmic membrane is homogeneously distributed in the aqueous bulk phase we can define the partition coefficient of the probe in the outer membrane (K_{om}) as $K_{om} = C_{om}/C_e$ in which C_{om} is the probe concentration in the outer membrane. Similar to subsection IVA and IVB, the Law of Mass Action gives:

$$C_o = (1 - x - z)C_e + zC_{om} + xC_i$$

in which z is the fractional volume of the outer membrane. Using $f_{om} = z/x$ and $K_{om} \gg 1$ it follows that:

$$\frac{C_o}{C_e} = 1 - x(1 - f_{om}K_{om}) + x\frac{C_i}{C_e} \quad (10)$$

This equation can be used to correct the calculation of $\Delta\psi$ for binding of the probe to sites external to the cytoplasmic membrane in the form of:

$$\Delta\psi = Z \log\left\{\frac{\frac{C_o}{C_e} - 1 + x(1 - f_{om}K_{om})}{x}\right\}$$

In a binding experiment, assuming that all binding is due to partitioning in the outer membrane:

$$\frac{C_o}{C_e} = 1 + xf_{om}K_{om} \quad (11)$$

IVD. Simultaneous binding of the probe to several cellular components

Comparison of Eqns. 5, 9 and 11 shows that it cannot be concluded from a binding experiment what the relative contributions of the three possibilities (subsections IVA–IVC) are to the probe binding. If they occur simultaneously, a binding experiment will yield the arithmetical sum of the three contributions:

$$\frac{C_o}{C_e} = 1 + x(K_i + f_{cm}K_{cm} + f_{om}K_{om})$$

Therefore, for a quantitatively reliable $\Delta\psi$ measurement, the contribution of the various fractions to probe binding has to be assayed separately for each bacterium studied, e.g., through binding experiments on relevant subcellular fractions. When these separate contributions have been quantitated, $\Delta\psi$ can be calculated according to the formula:

$$\Delta\psi = Z \log\left\{\frac{\frac{C_o}{C_e} - 1 + x\left(1 - \frac{1}{2}f_{cm}K_{cm} - f_{om}K_{om}\right)}{x\left(1 + K_i + \frac{1}{2}f_{cm}K_{cm}\right)}\right\}$$

V. Discussion

VA. Magnitude of the corrections due to probe binding

It is instructive to see how binding of the probe to cellular components affects the calculation of $\Delta\psi$. In experimental practice, two cases can be distinguished: one can either measure the probe accumulation by filtration or by silicon-oil centrifugation and interpret uptake of probe by binding under de-energized conditions as an electrical potential or one can correct for this probe binding [4,22]. The latter procedure is very similar to flow-dialysis experiments in which a proper reference level is determined [20]; otherwise probe binding in flow-dialysis results in the same error as that with filtration or silicon-oil centrifugation. In Fig. 3A and B the error which is caused by binding of the probe in the calculation of $\Delta\psi$ is presented for the two experimental procedures. In both parts of this figure, the calculation is based on the amount of binding of TPP^+ to intact cells of *Rps. sphaeroides*, as measured in the experiment described in Fig. 1. As would be expected the largest error is made if binding of the probe depends on intracellular components exclusively (case a); the smallest error is made in case c, if binding occurs to extracellular components like the outer membrane. In all these cases the magnitude of the correction also depends on the cell density as this parameter contributes to the changes in both external and internal concentrations of the probe. Lowering the cell density will increase the correction in cases a and b and decrease it in case c. For the calculations in Fig. 3, a cell density was chosen that is routinely used in measurements with an ion-selective electrode. The magnitude of the corrections is generally 70–80 mV, for cases a and b, except for very low potentials (Fig. 3B). In case c, a significant error is made only when this binding is considered as a resting potential (Fig. 3A). At this moment, the exact contribution of the intracellular contents, cytoplasmic membrane and

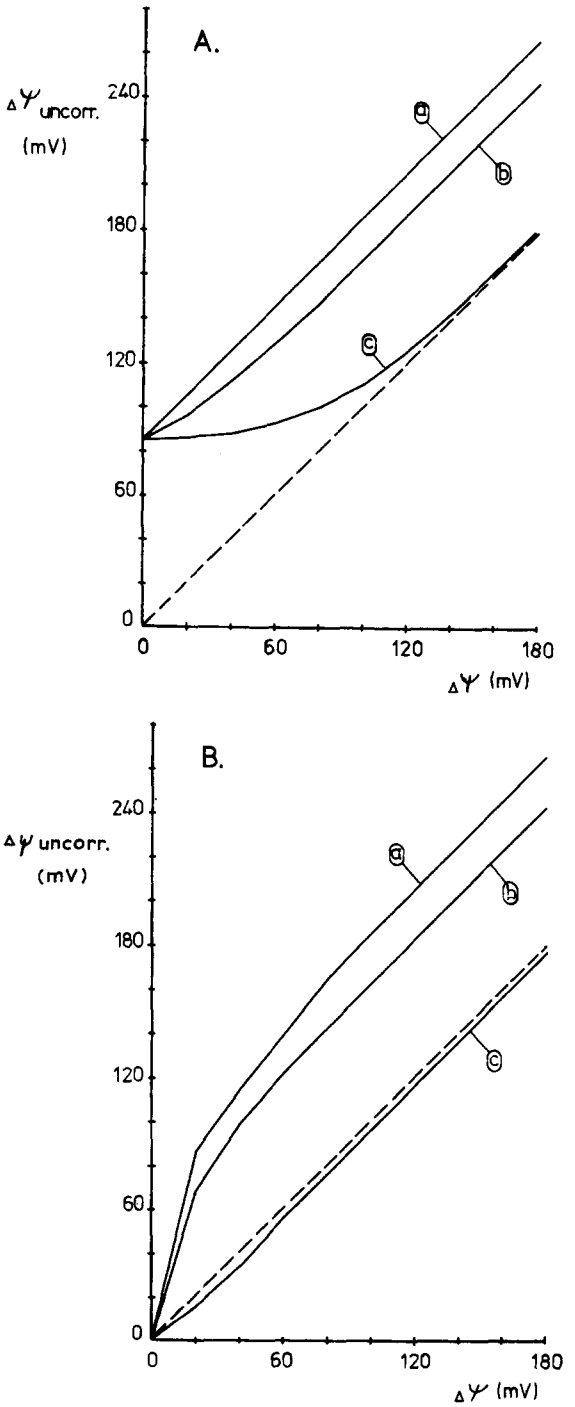

Fig. 3. The uncorrected $\Delta\psi$ (calculated by ignoring probe binding) as a function of the quantitatively correct $\Delta\psi$. The calculation is based on the binding described in Fig. 1 and an internal volume fraction (x) of 0.005 (corresponding to a protein concentration of 1.7 mg/ml). a, b and c refer to the models for probe binding presented in Fig. 2. The dashed line gives the $\Delta\psi$, properly corrected for probe binding. In A it is assumed that the binding of probe (under de-energized conditions) is due to a resting potential and in B this binding is subtracted from the accumulation of the probe.

outer membrane to probe binding it not exactly known and the true error in $\Delta\psi$, which is made when one ignores the binding of TPP^+ to *Rps. sphaeroides* cells, lies somewhere in between cases a and c.

The effects of probe binding has been neglected so far, mainly for the reason that probe binding is assumed to be saturated at low probe concentrations. If that were the case for the binding of TPP^+ to *Rps. sphaeroides*, the error in the calculation of $\Delta\psi$ would only be 6 mV at a $\Delta\psi$ of 120 mV and 0.7 mV at 180 mV.

VB. Saturation in the binding of TPP^+

At high concentrations of the probe, saturation in the binding occurs (see also Ref. 8). To ensure that the assumption of proportionality between bound and free probe still holds under conditions of a specific experiment, it is important to know the changes in probe concentrations in the aqueous bulk phases on the two sides of the membrane (C_i and C_e). Fig. 4. shows the internal probe concentration (C_i, relative to the starting concentration) as a function of the $\Delta\psi$ for cases a and b. It turns out that due to probe binding, the internal concentration increases much slower with $\Delta\psi$ than would be the case when no binding occurs. This is even more pronounced in case a when the binding is determined completely by the internal free probe concentration. It should be kept in mind that in this experiment a drastic decrease in the external probe concentration must occur in order to reach the equilibrium distribution ($C_o/C_e = 130$ at 180 mV in case a). Without binding of the probe, C_i/C_o increases up to a value of 67 and 167 at $\Delta\psi$ values of 120 and 180 mV, respectively. In the case of probe binding to extracellular components, no problems with saturation of the probe binding occur, since the starting concentration of the probe can always be chosen so that changes in the external probe concentration during energization fall within a linear part of the binding curve. The conclusion from Fig. 4 then must be that with a reasonable choice of the starting concentration of the probe ($C_o \approx 1$ μM) with a $\Delta\psi$ of up to 180 mV, the assumption of a non-saturable binding is correct, since in the most unfavourable case (b) under these conditions the free internal probe concentration only increases to 15 μM. This certainly is

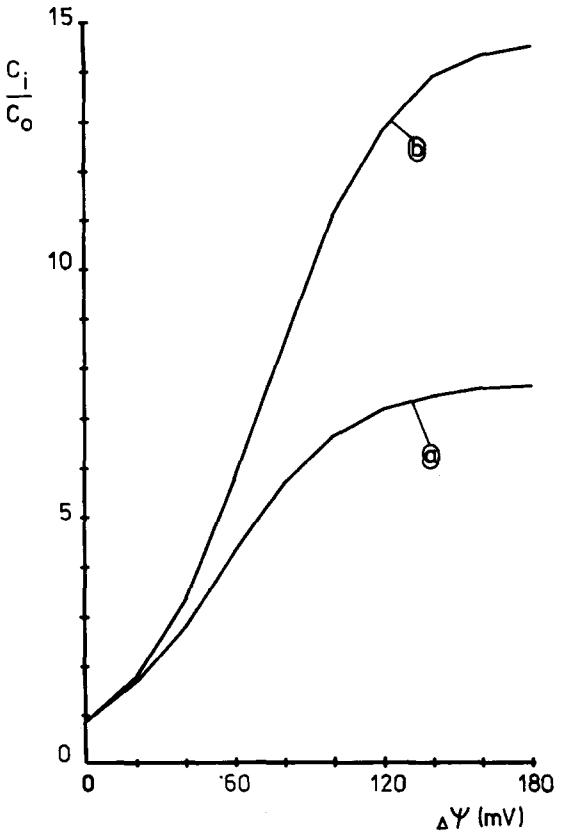

Fig. 4. The increase in internal free probe concentration as a function of $\Delta\psi$, in the case of probe binding to intracellular components (a) or partitioning in the cytoplasmic membrane (b). For details of the calculation see legend to Fig. 3.

within the linear range of probe binding in *Rps. sphaeroides* (Fig. 1) and *Escherichia coli* [8]. An important consequence of this probe binding in cases a and b is that for a certain $\Delta\psi$, much larger changes in external probe concentration occur. This increases the sensitivity of this method compared to $\Delta\psi$ measurements in which no probe binding occurs. This may be an important aspect in $\Delta\psi$ measurements in diluted suspensions of growing bacteria.

VC. Literature data on correction for probe binding

One class of probe molecules consists of large organic ions containing a delocalised charge which are therefore hydrophobic in character. For the positively charged probes dibenzyldimethylammonium, $TPMP^+$ and TPP^+ several reports have

been made on their binding to prokaryotic [4,8,23–27] and eukaryotic [28–35] cells as well as to isolated mitochondria [36,37]. The simplest correction is made by [26,27,31,35] subtracting the amount of probe bound under de-energized conditions from the total amount of accumulated probe under energized conditions. This procedure can lead to significant errors as can be seen from Fig. 3. The error in $\Delta\psi$ calculated from the distribution of these probes has been evaluated from comparison with another class of probes [23,25,36,37]: the combination of an ion with an ion-specific ionophore, predominantly the combination K^+/valinomycin. The general conclusion from these studies is that binding of the lipophilic probes occurs, though there is disagreement as to the nature and extent of the binding (see also Ref. 24). It should be noted, however, that the magnitude of the error in $\Delta\psi$ due to probe binding cannot be determined from these comparisons because the possibility cannot be excluded that $\Delta\psi$ calculations from the accumulation ratio of K^+ are also overestimations, since binding of K^+ to biological material has been reported [38–41].

More reliable are the comparisons with direct measurement of the transmembrane potential using microelectrodes. This method is only applicable for eukaryotic cells [30,34] and 'giant' bacteria [42]. In neuroblastoma [30] and giant *E. coli* cells [42], a saturated binding of the probe was reported at the concentrations used. In contrast, in yeasts, a concentration-dependent binding has been observed [34]. The latter observation has also been made with human erythrocytes [29]. In these experiments the measured probe accumulation was compared with the expected accumulation according to the Goldman equation.

Summarizing the results from the literature, probe binding is often observed but a discrepancy exists with regard to its effect on $\Delta\psi$ determinations. Possible reasons for this discrepancy and points that should be paid attention to when correcting for binding are:

(1) The probe concentration is important. The binding can be concentration dependent at low or high concentrations. It seems tempting to use saturating probe concentrations, but this is conflicting with demand iii in Section II.

(2) It is not enough to determine the binding under de-energized conditions at one probe concentration. At all probe concentrations possible under energized conditions, the value and the localization of the binding should be determined.

(3) Binding should be determined under de-energized conditions. There is no guarantee that the membrane potential is zero when simply no energy source is provided. Under these conditions, 'resting' potential from endogenous energy sources or a Donnan equilibrium can exist. The addition of toluene or uncouplers and heat treatment can have different effects upon the binding.

(4) Different methods of measuring the binding may yield different values for the binding.

(5) The accumulations of two probes should be compared under exactly the same conditions. This also means that the accumulation of one probe must be measured in the presence of the other to exclude specific effects of the probes on the $\Delta\psi$ generation or dissipation.

(6) When TPB^- is used to accelerate the permeation of the probe through the membrane, an additional binding may be introduced [33]. The same binding may occur in the K^+/valinomycin system depending on the valinomycin concentration.

VD. The localization of the probe-binding sites

An important point made in this report is that it is necessary to know the localisation of the binding sites before $\Delta\psi$ can be calculated from the measured probe accumulation. The hydrophobic and ionic properties of the probes make binding to cytoplasmic components like proteins or nucleic acids and to the membranes very likely. In gram-negative bacteria, mitochondria and chloroplasts, both outer and inner membranes can absorb probe molecules. The permeability of the probes TPP^+ or $TPMP^+$ through membranes of different organisms varies considerably. In general, the permeability of membranes of eukaryotic cells is much lower than that of bacterial and mitochondrial membrane. Differences in the amount of probe bound to or partitioned in the membranes of different organisms can therefore occur. Preliminary binding experiments on membrane fractions of *Rps. sphaeroides* indicate that partitioning in the cytoplasmic membrane is an important fraction of the total binding to the whole cells. Re-

cently, Casadio et al. [43] reported a large adsorption of the probe TPB^- to chromatophores of *Rps. capsulata*. The existence of a potential difference over a membrane introduces a special problem which is ignored in the calculation in subsection IVB: namely, that the binding could be $\Delta\psi$ dependent. The term '$\Delta\psi$ dependent binding' can refer to three effects:

(1) The amount of probe bound to the membrane is changed when a $\Delta\psi$ is generated because the free probe concentrations at both sides of the membrane are changed. We will call this concentration-dependent binding.

(2) When light is the energy source for $\Delta\psi$ generation, the light may, independently of the existence of a $\Delta\psi$ affect the partition coefficient. We will call this light-dependent binding.

(3) The existence of a $\Delta\psi$ influences directly the partition coefficient. This is what we call $\Delta\psi$-dependent binding.

Effect 1 is determined in a binding experiment. The effect of light on the binding can be determined under conditions where a $\Delta\psi$ cannot be generated. A light-dependent binding of the probes safranine and 9-aminoacridine to bacteriorhodopsin sheets has been reported by Kell and Griffiths [44]. The real $\Delta\psi$-dependent binding is much harder to measure. It requires the measurement of the amount of probe bound under energized conditions.

Membrane partition of lipophilic ions in bimolecular lipid membranes has been described as taking place in two specific regions inside the membrane, separated by a potential energy barrier. These two 'boundary regions' are near the water/membrane interfaces [45–47]. Using this scheme Cafiso and Hubbell [48] derived an expression for the ratio of bound (N_b) to free (N_f) probe molecules as a function of a potential difference over the membrane of phospholipid vesicles. With the potentials in units of Z, the same relationship in the case of $\Delta\psi$-independent binding yields:

$$\frac{N_b}{N_f} = \tfrac{1}{2} x f_{cm} K_{cm} \frac{1 + e^{\Delta\psi}}{x + (1-x)e^{\Delta\psi}}$$

Comparison of this equation with the one derived by Cafiso and Hubbell [48] leads to the assumptions made in subsection IVB. Thus, it is necessary that the membrane should be symmetrical towards the two bulk aqueous phases. The surface difference due to the curving of the membrane of a bacterial cell ($\phi \approx 1$ μm) will be negligible. The compositional asymmetry, however, may cause differences in partition coefficient and surface potential at both sides of the membrane. The latter effect may be diminished by using media of high ionic strength. The partition coefficients, reflecting the standard free energy difference of the probe between the boundary region and the adjacent bulk aqueous phase, can differ as a result of compositional differences between the two boundary regions and the inner and outer aqueous phase. Secondly, the effective membrane volume, the partition coefficient and the surface potential should not be affected by the applied potential difference. Furthermore, it is required that the probe is adsorbed to instead of absorbed in the membrane.

After we have established the distribution in the localization of the TPP^+ binding to whole cells of *Rps. sphaeroides*, the next step will be to check the correctness of the use of the equation in subsection IVD. As mentioned above, this requires the measurement of the amount of probe bound under energized conditions.

References

1 Mitchell, P. (1961) Nature 191, 144–148
2 Konings, W.N., Hellingwerf, K.J. and Robillard, G.T. (1981) in Comprehensive Biochemistry (Bonting, S.L. and De Pont, J.J., eds.), pp. 257–283, Elsevier, Amsterdam
3 Van Dam, K., Wiechmann, A.H.C.A., Hellingwerf, K.J., Arents, J.C. and Westerhoff, H.V. (1978) in Proceedings of the 11th Meeting of the Federation of European Biochemistry Societies, Copenhagen, 1977 (Nichols, P., Møller, J.V., Jørgensen, P.T. and Moody, A.J., eds.), vol. 45, pp. 121–132, Pergamon Press, Oxford
4 Michel, H. and Oesterhelt, D. (1980) Biochemistry 19, 4615–4619
5 Kell, D,B. (1979) Biochim. Biophys. Acta 549, 55–99
6 Westerhoff, H.V., Simonetti, A.L.M. and Van Dam, K. (1981) Biochem. J., in the press
7 Zilberstein, D., Schuldiner, S. and Padan, E. (1979) Biochemistry 18, 667–673
8 Ten Brink, B., Lolkema, J.S., Hellingwerf, K.J. and Konings, W.N. (1981) FEMS Microbiol. Lett. 12, 237–240
9 Rottenberg, H. (1975) J. Bioenerg. 7, 61–74
10 Ferguson, S.J., Jones, O.T.G., Kell, D.B. and Sorgato, M.C. (1979) Biochem. J. 180, 75–85
11 Michels, P.A.M., Hellingwerf, K.J., Lolkema, J.S., Friedberg, I. and Konings, W.N. (1981) Arch. Microbiol. 130 357–361

12 Kaback, H.R. (1974) Methods Enzymol. 31, 698–709
13 Harris, E.J. and Van Dam, K. (1968) Biochem. J. 106, 759–766
14 Liberman, E.A. and Topali, V.P. (1968) Biochim. Biophys. Acta 163, 125–136
15 Ramos, S., Schuldiner, S. and Kaback, H.R. (1976) Proc. Natl. Acad. Sci. U.S.A. 73, 1892–1896
16 Hellingwerf, K.J. and Konings, W.N. (1980) Eur. J. Biochem. 106, 431–437
17 Rottenberg, H. (1979) Methods Enzymol. 55, 547–569
18 Nicolay, K., Lolkema, J.S., Hellingwerf, K.J., Kaptein, R. and Konings, W.N. (1981) FEBS Lett. 123, 319–323
19 Westerhoff, H.V., Scholte, B.J. and Hellingwerf, K.J. (1979) Biochim. Biophys. Acta 547, 544–560
20 Hellingwerf, K.J., Arents, J.C., Scholte, B.J. and Westerhoff, H.V. (1979) Biochim. Biophys. Acta 547, 561–582
21 Michel, H. and Oesterhelt, D. (1976) FEBS Lett. 65, 175–179
22 Kashket, E.R., Blanchard, A.G. and Metzger, W.C. (1980) J. Bacteriol. 143, 128–134
23 Sprott, G.D. and Usher, J.R. (1977) Can J. Microbiol. 23, 1683–1688
24 Kamo, N., Muratsugu, M., Hongoh, R. and Kobatake, Y. (1979) J. Membrane Biol. 49, 105–121
25 Ghazi, A., Schechter, E., Letellier, L. and Labedan, B. (1981) FEBS Lett. 125, 197–200
26 Harold, F.M. and Papineau, D. (1972) J. Membrane Biol. 8, 27–44
27 Bakker, E.P., Rottenberg, H. and Caplan, S.R. (1976) Biochim. Biophys. Acta 440, 557–572
28 Cheng, K., Haspel, H.C., Vallano, M.L., Osotimehin, B. and Sonenberg, M. (1980) J. Membrane Biol. 56, 191–210
29 Deutsch, C.J., Holian, A., Holian, S.K., Daniele, R.P. and Wilson, D.F. (1979) J. Cell. Physiol. 99, 79–94
30 Deutsch, C.J., Erecinska, M., Werrlein, R. and Silver, I.A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 2175–2179
31 Lichtshtein, D., Kaback, H.R. and Blume, A.J. (1979) Proc. Natl. Acad. Sci. U.S.A. 76, 650–654
32 Scott, I.D. and Nicholls, D.G. (1980) Biochem. J. 186, 21–33
33 Hoeberichts, J.A. and Borst-Pauwels, G.W.F.H. (1975) Biochim. Biophys. Acta 413, 248–251
34 Vacata, V., Kotyk, A. and Sigler, K. (1981) Biochim. Biophys. Acta 643, 265–268
35 Komor, E. and Tanner, W. (1976) Eur. J. Biochem. 70, 197–204
36 Azzone, G.F., Pozzan, T., Massari, S., Bragadin, M. and Pregnolato, L. (1978) Biochim. Biophys. Acta 501, 296–306
37 Shen, C., Boens, C.C. and Ogawa, S. (1980) Biochem. Biophys. Res. Commun. 93, 243–249
38 Cope, F.W. and Damadian, R. (1970) Nature 228, 76–77
39 Ling, G.N. and Cope, F.W. (1969) Science 163, 1335–1336
40 Okorokov, L.A., Lichko, L.P. and Kulaev, I.S. (1980) J. Bacteriol. 144, 661–665
41 Damadian, R. (1969) Science 165, 79–81
42 Felle, H., Porter, J.S., Slayman, C.L. and Kaback, H.R. (1980) Biochemistry 19, 3585–3590
43 Casadio, R., Venturoli, G. and Melandri, B.A. (1981) Photobiochem. Photobiophys. 2, 245–253
44 Kell, D.B. and Griffiths, A.M. (1981) Photobiochem. Photobiophys. 2, 105–110
45 Ketterer, B., Neumcke, B. and Läuger, P. (1971) J. Membrane Biol. 5, 225–245
46 Anderson, O. and Fuchs, M. (1975) Biophys. J. 15, 795–830
47 Benz, R. and Conti, F. (1981) J. Membrane Biol. 59, 91–104
48 Cafiso, D.S. and Hubbell, W.L. (1978) Biochemistry 17, 187–195
49 Cafiso, D.S. and Hubbell, W.L. (1980) Biophys. J. 30, 243–264
50 Shinbo, T., Kamo, N., Kurihara, K. and Kobatake, Y. (1978) Arch. Biochem. Biophys. 187, 414–422