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Measurement of Membrane Potential in *Bacillus subtilis*: A Comparison of Lipophilic Cations, Rubidium Ion, and a Cyanine Dye as Probes

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Summary. Two of the commonly used probes for measuring membrane potential – lipophilic cations and the cyanine dye diS-$C_3(5)$ – indicated nominally opposite results when tetraphenylarsonium ion was added as a drug to suspensions of metabolizing *Bacillus subtilis* cells. [^{3}H]-Triphenylmethylphosphonium uptake was enhanced by the addition, indicating hyperpolarization, yet fluorescence of diS-$C_3(5)$ was also enhanced, indicating depolarization. Evidence is presented that both effects are artifactual, and can occur without any change in membrane potential, as estimated by $^{86}Rb^+$ uptake in the presence of valinomycin. The fluorescence studies suggest that tetraphenylarsonium ion displaces the cyanine dye from the cell envelope, or other binding site, into the aqueous phase.

The uptake characteristics of the radiolabeled lipophilic cations were quite unusual: At low concentrations (e.g., less than 10 μM for triphenylmethylphosphonium) there was potential-dependent uptake of the label to a stable level, but subsequent addition of nonradioactive lipophilic cation caused further uptake of label to a new stable level. Labeled triphenylmethylphosphonium ion taken up to the first stable level could be displaced by 10 mM magnesium ion, whereas $^{86}Rb^+$ uptake was unperturbed. Association of the lipophilic cations with the surface of de-energized cells was concentration-dependent, but there was no evidence for cooperative binding. This phenomenon of stimulated uptake in *B. subtilis* (which was not seen in *Escherichia coli* cells or vesicles) is consistent with a two-compartment model with access to the second compartment only being possible above a critical cation concentration. We tentatively propose such a model, in which these compartments are the cell surface and the cytoplasm, respectively.

Triphenylmethylphosphonium up to 0.5 mM exhibited linear binding to de-energized cells; binding of tetraphenylphosphonium and tetraphenylarsonium was nonlinear but was not saturated at the highest concentration tested (1 mM). The usual assumption, that association of the cation with cell surfaces is saturated and so can be estimated on de-energized cells, therefore leads to undercorrected estimates of cytoplasmic uptake in *B. subtilis*, and hence to overestimates of membrane potential. We describe a more realistic procedure, in which the estimate of extent of binding is based on a mean aqueous concentration related both to the external concentration and to the much higher internal concentration that exists in energized cells. Using this procedure we estimate the membrane potential in *B. subtilis* to be 120 mV, inside-negative. The procedure is of general applicability, and should yield more accurate estimates of membrane potential in any system where there is significant potential-dependent binding.

Key words: membrane potential, *Bacillus subtilis*, cyanine dyes, lipophilic cations, rubidium, binding models

Electrical potential across cell membranes is measured most directly by microelectrodes. Such measurements have recently been achieved on *Escherichia coli* cells enlarged by means of genetic or pharmacological interference with synthesis of the cell envelope (Felle, Stetson, Long & Slayman, 1978), but implantation of electrodes is not generally applicable to cells or organelles as small as steady-

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state growing bacteria. Experimenters have therefore resorted to the use of probe molecules which indirectly estimate membrane electrical potential, either by their potential-dependent accumulation (monitored by radiolabel) or by changes in some spectral property (such as fluorescence). (For reviews *see* Rottenberg, 1979; Waggoner, 1979; Freedman & Laris, 1981.)

Calculation of membrane potential from potential-dependent accumulation of radiolabeled ionic probes [such as triphenylmethylphosphonium ($TPMP^+$)[1] or tetraphenylphosphonium (TPP^+)] is straightforward, but involves a few key assumptions: (*i*) The probe is membrane-permeant and moves freely between the intra- and extracellular compartments. (*ii*) It does not appreciably perturb the potential it is being used to measure. (*iii*) It is charged, and therefore equilibrates at an in/out concentration ratio (strictly, activity ratio) which yields a chemical potential equal and opposite to the electrical potential across the membrane (the assumption of Nernst equilibrium). Any probe fulfilling assumption (*i*) is likely to partition appreciably into the cell membrane (and possibly other superficial or cytoplasmic components); total uptake must be corrected for this "bound label" so that only uptake into the aqueous phase of the cytoplasm is employed in the Nernst equation for estimating potential.

In the case of optical probes, the relationship between signal size and membrane potential is indirect, and a calibration must be made against some other technique such as potassium diffusion potential (Hoffman & Laris, 1974; Kashket & Barker, 1977), $TPMP^+$ uptake (Miller & Koshland, 1977), or Donnan equilibrium potential (Freedman & Hoffman, 1979). The physical basis of the potential-dependent spectral changes is usually not fully understood. For example, the cationic cyanine dyes developed and studied by Cohen, Hoffman, Waggoner and colleagues (for reviews, *see* e.g., Waggoner, 1979; Freedman & Laris, 1981) exhibit fluorescence quenching when they associate with energized cells. The quenching is believed to be caused by dimer formation and binding to cell surfaces or cytoplasmic macromolecules (Sims, Waggoner, Wang & Hoffman, 1974), but only in the case of the human erythrocyte has the mechanism been explored in detail (Hladky & Rink, 1976; Tsien & Hladky, 1978; Freedman & Hoffman, 1979). In that particular system, the major binding site was found to be oxyhemoglobin.

In the context of bacterial motility, we became interested in the physiological effects of certain lipophilic cations, particularly tetraphenylarsonium ($TPAs^+$). At concentrations of the order of 10 μM, $TPAs^+$ was reported to have marked effects both on swimming speed and on motor switching probabilities in *Bacillus subtilis* (Ordal, 1976; de Jong & van der Drift, 1978), without affecting the fluorescence of the potential-sensitive cyanine dye diS-C_3(5) (de Jong & van der Drift, 1978). In view of other evidence linking protonmotive force (PMF), which is the energy source for the bacterial motor, to control of switching probabilities (Khan & Macnab, 1980*a*), we decided to examine further the effects of lipophilic cations such as $TPAs^+$. Physiological and behavioral effects have been reported separately (Zaritsky & Macnab, 1981). Here we describe anomalous behavior of lipophilic cations and cyanine dye as probes for measurement of membrane potential in *B. subtilis*, and our investigation of the basis for these anomalies; we also offer an improved general procedure for correcting for cation bound to the surface of cells or organelles. Attention is drawn to the fact that introduction of a drug that (like the probe itself) interacts with the membrane can result in a large change in signal, but that this is not necessarily indicative of a change in membrane potential.

Materials and Methods

A. Bacterial Strains and Growth Conditions

Bacillus subtilis OI 1 (*ilv C1, leu-1*; Ordal & Goldman, 1975) was used throughout. Cultures were grown aerobically, with vigorous shaking, at 25 °C in a minimal medium (pH 7.5), based on that of Sargent (1975), with glycerol (0.5 % vol/vol) as the carbon source, and supplements of methionine, tryptophan, leucine, isoleucine, and valine (50 μg ml^{-1} each).

Cells were harvested during mid-exponential growth at a cell density of 2×10^8 ml^{-1} ($OD_{650} = 0.5$) or lower, centrifuged once (1600 × g, 10 min, 25 °C) and resuspended in 10 mM potassium phosphate, pH 7.5, 0.1 mM EDTA, 0.3 mM $(NH_4)_2SO_4$, 0.14 mM $CaCl_2$, with glycerol (0.5 % vol/vol) as an energy source. This is a slightly modified version of the *Bacillus* chemotaxis medium of Ordal and Gibson (1977). For experiments requiring a K^+ concentration lower than 18 mM, sodium phosphate and potassium phosphate were mixed in the appropriate ratio. Where additions were made as ethanolic solutions, final ethanol concentration was always less than 1 %. Valinomycin was used at 10 μM/OD_{650} = 60 μg/mg protein.

[1] *Abbreviations and symbols:* C_{in}, cytoplasmic concentration; C_{out}, external concentration; C_{env}, noncytoplasmic, cell-associated formal concentration; C_{tot}, total cell-associated formal concentration; $\bar{C}$, exponential mean concentration; CCCP, carbonyl-cyanide *m*-chlorophenylhydrazone; diS-C_3(5), 3,3′-dipropyl-2,2′-thiodicarbocyanine iodide; $\Delta\psi$, membrane (electrical) potential; ΔpH, proton chemical potential; PMF, protonmotive force or proton electrochemical potential; $TPAs^+$, tetraphenylarsonium ion; TPB^-, tetraphenylboron ion; $TPMP^+$, triphenylmethylphosphonium ion; TPP^+, tetraphenylphosphonium ion.

B. Determination of Membrane Potential ($\Delta\psi$)

1) Uptake of Radioactive Cations. Cells suspended in *Bacillus* chemotaxis medium (typically 1.2 ml at an OD_{650} of 0.4) in a small (12 mm × 35 mm) vial to reduce evaporative losses, were stirred vigorously by a magnetic bar, and aerated via a hypodermic needle. Uptake of radioactive cation ([^{3}H]−TPMP$^+$, −TPP$^+$, or −TPAs$^+$, or $^{86}Rb^+$ in the presence of valinomycin) was determined by filtration assay (Schuldiner & Kaback, 1975) using Unipore polycarbonate filters (0.6 μm pore, 25 mm dia, Bio-Rad). 50 μl aliquots of the cell suspension were added to 2 ml of chemotaxis medium, filtered, and washed with a further 2 ml (total elapsed time was about 5 sec). Filters were dried and placed in scintillation fluid (Aquasol, New England Nuclear) for counting. In double-label experiments, $^{86}Rb^+$ was counted in one channel of a Beckman model LS 3133P scintillation counter, with lower and upper discriminator settings of 400 and 1,000, respectively, while [^{3}H]-TPMP$^+$ was counted in another channel using the narrow tritium Iso-set. Gain was set at 370. Under these conditions spill-over between channels was less than 1.5%.

We define *apparent*[2] *membrane potential*, abbreviated from now on as *apparent potential*, as $(RT/ZF)\ln(C_{in}/C_{out})$, where C_{out} is the external concentration of a given cationic probe, and C_{in} is its *apparent cytoplasmic concentration based on uptake data and a given set of assumptions*. Unless otherwise indicated, and exclusively prior to Section *H* of Results, apparent potential was calculated as follows: Cell-associated counts were divided by specific activity and by the cytoplasmic volume of the aliquot (3.2 μl ml^{-1} OD_{650}^{-1}; based on measurements in Khan and Macnab, 1980*a*) to yield a cell-associated (cytoplasm plus envelope) formal concentration $C_{tot} = C_{in} + C_{env}$. To estimate C_{env}, the same procedure was carried out on another aliquot of cells de-energized by addition of 100 μM CCCP, for which it was assumed that the cytoplasmic and external concentrations were the same, and hence that $C_{env} = C_{tot} - C_{out}$. Note that since ΔpH is negligible in *B. subtilis* at pH 7.5 (Khan & Macnab, 1980*b*; Shioi, Matsuura & Imae, 1980) collapse of PMF is equivalent to collapse of $\Delta\psi$. C_{env} for de-energized cells was subtracted from C_{tot} for energized cells to yield C_{in} for energized cells. Apparent potential was then given by $(RT/ZF)\ln(C_{in}/C_{out})$, equal to $59\log(C_{in}/C_{out})$ mV at 25 °C. Uptake ratio in de-energized cells was defined as C_{env}/C_{out}.

All cell-associated concentrations were expressed formally, as moles per liter of cytoplasm, regardless of whether the material was believed to be bound or free.

2) DiS-C_3(5) Fluorescence. Measurements were made on an Aminco DW-2 spectrophotometer, run in split-beam fluorescence mode, with the excitation monochromator set at 647 nm (slit width 10 nm), and a high-pass filter (RG 665, Schott, transmission below 650 nm less than 1%) inserted in the emission path, between the cuvettes and the photomultiplier tube (Hamamatsu R446; extended red-sensitivity). This procedure is similar to that used by Freedman and Hoffman (1979).

Cells suspended in *Bacillus* chemotaxis medium (2.5 ml) at an OD_{650} of 0.1 were placed in the sample cuvette (10 mm × 10 mm path), and diS-C_3(5) in ethanol was added to the desired concentration (0.1 to 1 μM). Lipophilic cations, valinomycin, or gramicidin were also added as ethanolic solutions. K^+ concentration was varied by addition of concentrated aqueous solutions of KCl. The reference cuvette contained a 0.1 μM ethanolic solution of diS-C_3(5); both cuvettes were stirred magnetically at all times, and the temperature of the cuvette compartment was controlled at 25 °C.

C. Chemicals

Chemicals were obtained from the sources listed: Carbonyl-cyanide *m*-chlorophenylhydrazone (CCCP), gramicidin, sodium tetraphenylboron (TPB$^-$), and valinomycin – Sigma; tetraphenylarsonium chloride – Strem; tetraphenylphosphonium chloride and triphenylmethylphosphonium bromide – Pfaltz and Bauer; the cyanine dye diS-C_3(5) – Dr. A.S. Waggoner, Amherst College; [^{3}H]-tetraphenylphosphonium bromide, 2.5 Ci mmol^{-1}, and [^{3}H]-tetraphenylarsonium chloride, 15 mCi mmol^{-1} – Dr. H.R. Kaback, Roche Institute of Molecular Biology; ^{86}rubidium chloride, 146 mCi mmol^{-1} – New England Nuclear; [^{3}H]-triphenylmethylphosphonium bromide – nonradioactive material *ex* Pfaltz and Bauer, tritiated by catalytic exchange to 6 Ci mmol^{-1} by New England Nuclear.

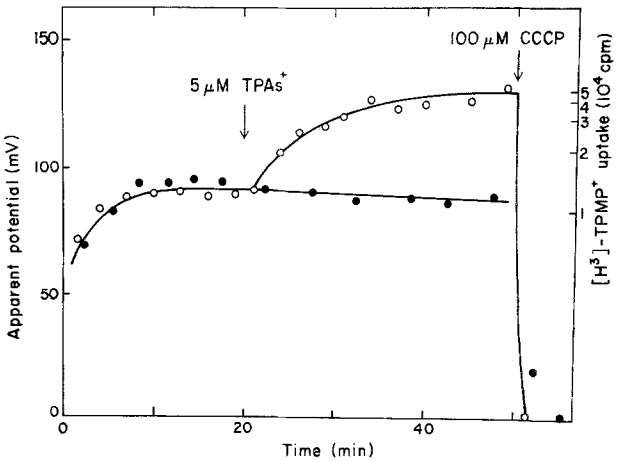

Fig. 1. Effect of TPAs$^+$ on apparent membrane potential of *B. subtilis*, as measured by [^{3}H]-TPMP$^+$ uptake at an external concentration of 5 μM. At the point indicated, 5 μM TPAs$^+$ was added to the sample (○), but not to the control (●). Finally, CCCP (100 μM) was added to both

Results

A. Effect of TPAs$^+$ on Apparent Potential

1) Measurement by [^{3}H]-TPMP$^+$ Uptake. [^{3}H]-TPMP$^+$ (5 μM) was added to two identical samples of vigorously metabolizing *B. subtilis* cells; equilibration of label was complete by about 10 min (Fig. 1). At 20 min, 5 μM TPAs$^+$ was added to one sample only. Over the next 10 min, TPMP$^+$ uptake in that sample was stimulated to almost four times the control level, i.e. the apparent potential had increased from 90 to 125 mV.[2] We have repeated such experiments many times; there was some day-to-day variation (±10 mV) in the absolute values of the apparent potential, but the stimulation of uptake was always observed. The phenomenon was insensitive to TPMP$^+$ concentration in the range 2–10 μM.

To test whether washing losses might be responsible for the low retention in samples prior to TPAs$^+$ addition, we altered the filtration procedure from the usual double wash to a single wash (elapsed time *ca.* 3 sec). This resulted in a 10% increase

[2] Electrical potentials are in all cases inside-negative. For simplicity, we omit the negative sign throughout this paper.

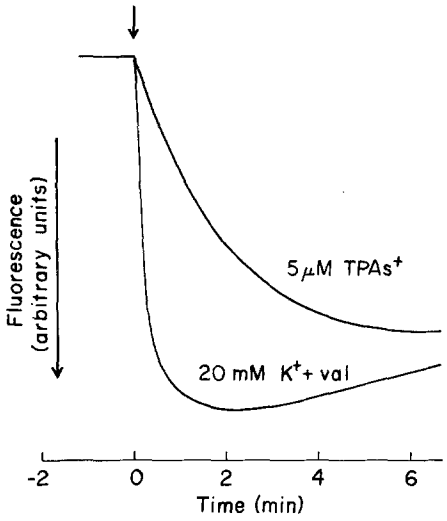

Fig. 2. Enhancement by $TPAs^+$ of the fluorescence of diS-$C_3(5)$ (0.2 μM) in *B.subtilis* suspensions. Fluorescence enhancement (here and in Fig. 4) is indicated by a downward deflection of the spectral trace. Fluorescence enhancement upon partial depolarization (by addition of valinomycin at 20 mM K^+) is shown for comparison

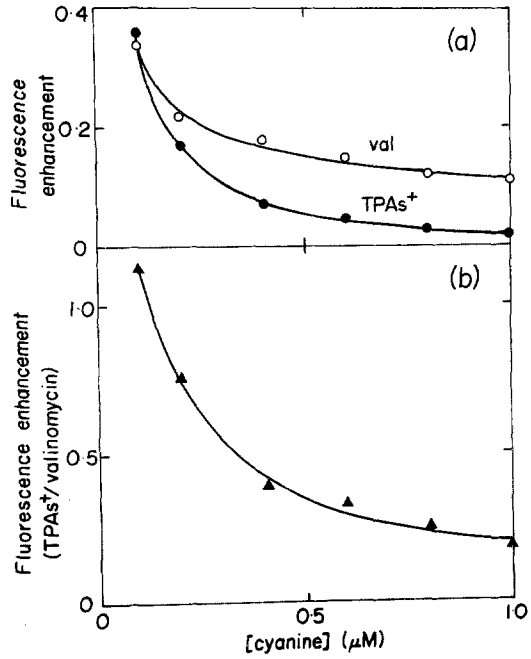

Fig. 3. Enhancement of diS-$C_3(5)$ fluorescence in *B. subtilis* suspensions as a function of dye concentration. (*a*) Enhancement by 5 μM $TPAs^+$ (•), or by valinomycin at 20 mM K^+ (○), expressed as a fraction of maximum enhancement, achieved by valinomycin at 200 mM K^+. (*b*) Enhancement by 5 μM $TPAs^+$ as a fraction of that by valinomycin at 20 mM K^+

in retention of label; the corresponding increase in apparent potential is negligible. Washing three times resulted in a slight decrease (<10%) in retention.

Analogous experiments on *E. coli* cells permeabilized with EDTA failed to show any stimulation of [^{3}H]-$TPMP^+$ uptake by $TPAs^+$ [*data not shown;* this result, and a similar one with *E. coli* vesicles, have been confirmed independently by H.R. Kaback *(personal communication)*].

2) Measurement by diS-$C_3(5)$ Fluorescence. DiS-$C_3(5)$ was mixed with vigorously metabolizing *B. subtilis* cells, and fluorescence allowed to reach a stable level. Upon addition of 5 μM $TPAs^+$, the fluorescence level was enhanced (Fig. 2; downward deflection corresponds to fluorescence enhancement, i.e. apparent depolarization). Based on the extent of enhancement by 1 μM valinomycin at various K^+ concentrations (e.g. the enhancement at 20 mM K^+ is shown in Fig. 2), the $TPAs^+$-induced enhancement would be interpreted as a depolarization of about 50 mV. $TPMP^+$ (at the concentration used in uptake experiments) gave only a slight enhancement of diS-$C_3(5)$ fluorescence, and diS-$C_3(5)$ (at the concentration used in fluorescence experiments) gave only a slight stimulation of $TPMP^+$ uptake; the slight interference that was observed further exaggerated the difference between the results obtained by the two techniques.

Thus, the situation existed where two techniques for potential measurement gave qualitatively opposite indications regarding the effect of $TPAs^+$ on membrane potential.

B. Quantitative Studies of $TPAs^+$-Enhanced Dye Fluorescence

As described in the previous section, 5 μM $TPAs^+$ caused a considerable enhancement of diS-$C_3(5)$ fluorescence, whereas de Jong and van der Drift (1978) in prior studies (in connection with *B. subtilis* motility) had reported negligible enhancement at $TPAs^+$ concentrations below 100 μM. Using a cell density (OD_{650}) of 0.1 and diS-$C_3(5)$ concentration of 0.1 μM, we found that 100 μM $TPAs^+$ caused 80% of maximum fluorescence enhancement (achieved by 1 μM gramicidin or 200 mM K^+ plus 1 μM valinomycin). De Jong and van der Drift had used the same cell density as we, but a much higher dye concentration (1 μM), and we have established that this is the principal cause of the discrepancy. Using dye concentrations from 0.1 to 1 μM, we found that the enhancement decreased from 36 to 2% of the maximum available upon potential collapse with 200 mM K^+ plus 1 μM valinomycin (Fig. 3*a*). Fluores-

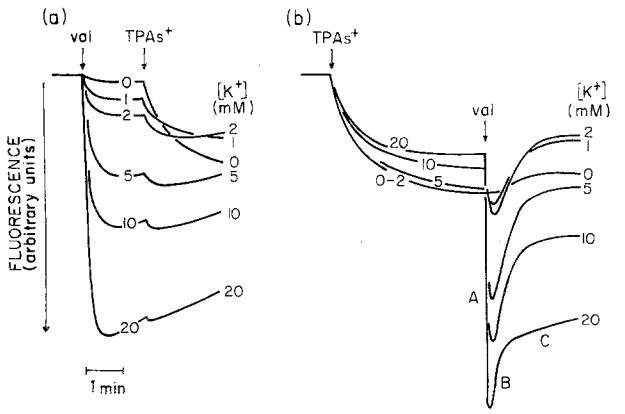

Fig. 4. Fluorescence enhancement of diS-$C_3(5)$ (0.1 μM) in *B. subtilis* suspensions, upon sequential addition of (*a*) valinomycin then 5 μM $TPAs^+$ or (*b*) 5 μM $TPAs^+$ then valinomycin, at various K^+ concentrations. The response to valinomycin in (*b*) can be resolved into a fast enhancement of fluorescence (phase *A*), a moderately fast requenching (phase *B*) and a further slow requenching (phase *C*)

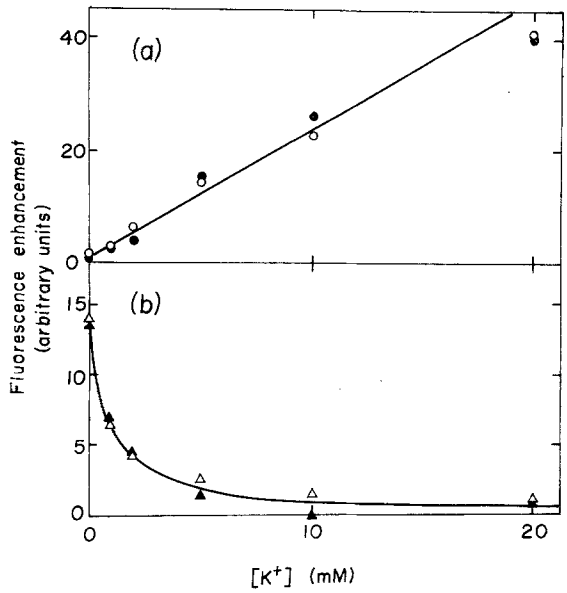

Fig. 5. Interpretation of the fast fluorescence enhancement (phase *A* in Fig. 4*b*) and moderately fast requenching (phase *B* in Fig. 4*b*) induced by valinomycin after prior addition of 5 μM $TPAs^+$, as a function of K^+ concentration. (*a*) Phase *A* (●), from Fig. 4*b*, compared to valinomycin-induced change in the absence of $TPAs^+$ (○), from Fig. 4*a*, (data prior to $TPAs^+$ addition). (*b*) Differential fluorescence increase (▲), namely, $TPAs^+$-induced signal minus phase *B* (both from Fig. 4*b*), compared to $TPAs^+$-induced change (△), after prior addition of valinomycin (from Fig. 4*a*)

cence enhancement deriving from a condition known to cause partial depolarization, namely addition of valinomycin to cells in 20 mM K^+ buffer, showed a much smaller dependency on dye concentration than did enhancement deriving from $TPAs^+$ (threefold *vs.* 18-fold over the dye concentration range studied; Fig. 3*a*). Thus the fluorescence enhancement induced by $TPAs^+$ could be interpreted as an apparent depolarization of anywhere from less than 20% to more than 100% of the depolarization caused by 20 mM K^+ plus valinomycin, depending on the dye concentration used (Fig. 3*b*). Furthermore, the magnitude of the $TPAs^+$-induced fluorescence enhancement showed a much greater day-to-day variability than did valinomycin-induced enhancement. Both features led us to believe that the $TPAs^+$-induced dye fluorescence enhancement was an artifact.

This belief was further strengthened by experiments in which, at various K^+ concentrations, valinomycin and $TPAs^+$ were added sequentially (Fig. 4*a*). If valinomycin was added to medium of very low K^+ concentration (<1 mM), so that the fluorescence enhancement was very small, subsequent addition of $TPAs^+$ still caused fluorescence enhancement, in spite of the fact that one would have expected the membrane potential to be effectively clamped by the K^+ diffusion potential. At slightly higher K^+ concentrations, valinomycin addition resulted in a severe reduction of the subsequent $TPAs^+$-induced enhancement, so that the combined signal at 2 mM K^+ was in fact smaller than when K^+ was omitted from the medium. By 20 mM K^+, the $TPAs^+$-induced signal had virtually disappeared.

When $TPAs^+$ was added first (Fig. 4*b*) there was a slight dependence of signal size on K^+ concentration. Subsequent valinomycin addition yielded a distinct triphasic signal change: A rapid increase in fluorescence (phase *A*, complete in less than 20 sec), followed by a fairly rapid decrease (phase *B*, less than 50 sec), and finally a much slower decrease (phase *C*) that was observed even when valinomycin was added in the absence of $TPAs^+$.[3] A similar triphasic response was observed when cells were subjected to $TPAs^+$ and gramicidin sequentially. We attribute phase *A* to enhancement, by valinomycin, of fluorescence from the stable level reached after $TPAs^+$ addition; the amplitude of this component from Fig. 4*b* agrees (Fig. 5*a*), throughout the K^+ range used, with the amplitude of the signal from Fig. 4*a* obtained upon simple valinomycin addition in the absence of $TPAs^+$. Phase *B* appears to be a decrease in the $TPAs^+$-induced signal as a result of the decrease in potential caused by valinomycin addition. A plot against K^+ concentration (Fig. 5*b*) of the difference between the $TPAs^+$-induced signal in the absence of valinomycin and the phase *B* com-

[3] We have not further investigated this final phase, which is most pronounced when extreme depolarization occurs.

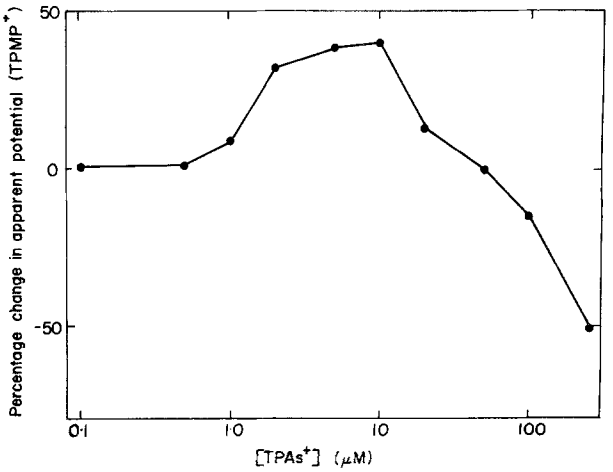

Fig. 6. Percentage change in the apparent potential of *B. subtilis* (measured by uptake of [^{3}H]-TPMP$^+$ at 4 μM external concentration, upon addition of TPAs$^+$ at different concentrations

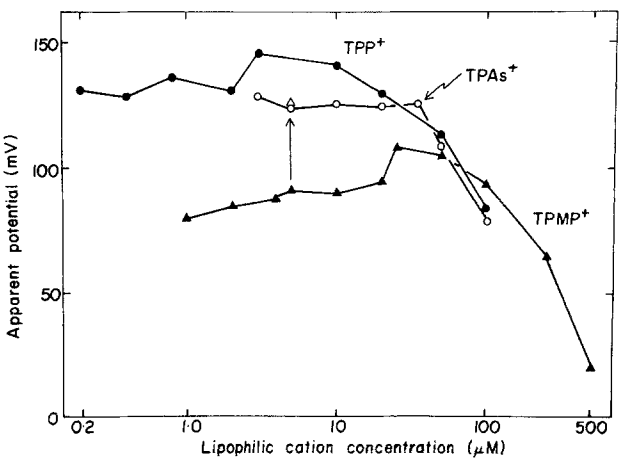

Fig. 7. Apparent potential of *B. subtilis*, measured by uptake of the tritiated cations TPP$^+$ (●), TPAs$^+$ (○), and TPMP$^+$ (▲), as a function of external cation concentration. The point (△) at 5 μM TPMP$^+$ is the apparent potential measured in the presence of 5 μM nonradioactive TPAs$^+$

ponent upon valinomycin addition (both from Fig. 4*b*), agrees well with a similar plot of the second-stage signal from Fig. 4*a*, obtained upon addition of TPAs$^+$ to cells already exposed to valinomycin.

We therefore conclude that TPAs$^+$-induced fluorescence enhancement in *B. subtilis* does not indicate depolarization, although it is a sensitive function of membrane potential. A modest reduction in membrane potential, or the use of high dye/cell ratios, greatly reduces the effect.

C. Effect of TPAs$^+$ Concentration on Apparent Potential Measured by TPMP$^+$

The uptake of [^{3}H]-TPMP$^+$ was examined over a concentration range of TPAs$^+$ from 0.1 to 250 μM (Fig. 6). Below 1 μM TPAs$^+$, TPMP$^+$ uptake was unaffected. Between 2 and 10 μM TPAs$^+$, TPMP$^+$ uptake was stimulated three- to fivefold (yielding a 30–40% increase in apparent potential, to *ca.* 120 mV), while at higher TPAs$^+$ concentrations the apparent potential declined, reaching 50% of the control value at 250 μM TPAs$^+$. TPP$^+$ at 5 μM also caused a several-fold increase in TPMP$^+$ uptake, as did the lipophilic anion tetraphenylboron (TPB$^-$) at 2 μM (*data not shown*). Unlike TPAs$^+$ or TPP$^+$, TPB$^-$ also enhanced the retention of TPMP$^+$ by cells de-energized by CCCP, although by a smaller factor (*ca.* twofold) than in energized cells. The stimulated uptake by TPB$^-$ occurred very rapidly (complete in less than 2 min), whereas TPAs$^+$-stimulated uptake of TPMP$^+$ proceeded at the characteristic rate of TPMP$^+$ alone (complete in *ca.* 10 min).

D. Effect of Cation Probe Concentration on Apparent Potential

In the experiments just described [and also in the physiological study reported separately (Zaritsky & Macnab, 1981)] TPAs$^+$ was regarded as the "drug", and TPMP$^+$ as the "probe". The chemical similarity between the two compounds makes such a distinction rather artificial, and so we next examined the effect of cation concentration upon its own uptake. Ideally, the relationship should be linear, yielding an apparent potential that is independent of cation concentration, unless the latter is high enough to change the state of the cation (e.g. by dimer formation), or to interfere with the cells' energy-producing processes.

For TPP$^+$ (Fig. 7), the apparent potential (*ca.* 130 mV) was independent of concentration below about 2 μM. In the range 2–10 μM, distinctly higher values (*ca.* 145 mV) were obtained. Above 10 μM TPP$^+$, the apparent potential progressively declined. It might appear that the variation throughout the entire concentration range 0.2 to 10 μM is within experimental error, but this is not so. First, it must be realized that an increase of 15 mV represents an 80% increase in uptake. More importantly, it was obvious in two-step experiments that uptake was stimulated at higher concentrations: For example, the data points at 0.2 and 3 μM are from such an experiment; after measurement of uptake at 0.2 μM TPP$^+$ (yielding an apparent potential of 131 mV), nonradioactive TPP$^+$ was added to raise the chemical concentration to 3 μM, whereupon a large stimulation of uptake of label occurred (yielding an apparent potential of 146 mV).

In the case of $TPAs^+$ (Fig. 7), we were limited to concentrations of 3 μM and greater by the low specific activity of the material available. The apparent potential was approximately constant (at 125 mV) up to 35 μM $TPAs^+$ and declined at higher concentrations.

$TPMP^+$ at 10 μM or less gave an apparent potential of about 90 mV (Fig. 7); at around 20 μM there was an abrupt rise in apparent potential (to about 110 mV), and from 50 μM up the apparent potential declined. Again, as with TPP^+, the stimulation of uptake of [^{3}H]-$TPMP^+$ was directly evident in two-stage experiments upon addition of nonradioactive $TPMP^+$.

Equilibration of TPP^+ and $TPAs^+$ was much faster than that of $TPMP^+$ (time constants of about 0.6, 2 and 4 min, respectively).

E. Effect of $TPAs^+$ on Apparent Potential Measured by $^{86}Rb^+$ and [^{3}H]-$TPMP^+$ in the Presence of Valinomycin

In an attempt to determine whether lipophilic cations were causing hyperpolarization, we simultaneously measured the effect of $TPAs^+$ on [^{3}H]-$TPMP^+$ and $^{86}Rb^+$ uptake in the presence of valinomycin.

[^{3}H]-$TPMP^+$ (4 μM) and $^{86}Rb^+$ (8 μM) were added to cells in 5 mM K^+ (Fig. 8). Upon addition of valinomycin, $^{86}Rb^+$ equilibrated rapidly and some [^{3}H]-$TPMP^+$ was released (indicating that 5 mM K^+ was above the equilibrium concentration for the pre-existing potential). At this stage, the apparent potential indicated by [^{3}H]-$TPMP^+$ (60 mV) was much lower than that indicated by $^{86}Rb^+$ (100 mV). When 5 μM nonradioactive $TPAs^+$ was added subsequently, the two radiolabels behaved in a strikingly different manner. [^{3}H]-$TPMP^+$ uptake was greatly stimulated (as noted previously in experiments where valinomycin was absent), yielding an apparent potential of 134 mV, whereas the apparent potential indicated by $^{86}Rb^+$ remained unchanged at 100 mV. Similar results were obtained when 30 μM nonradioactive $TPMP^+$ was added instead of 5 μM $TPAs^+$.

At higher $TPAs^+$ concentrations (>20 μM), [^{3}H]-$TPMP^+$ indicated an initial rise in apparent potential followed by a gradual decline, whereas $^{86}Rb^+$ indicated only the gradual decline (*data not shown*).

The results of these double-label experiments argue strongly that $TPAs^+$-stimulated uptake of $TPMP^+$ is *not* a consequence of hyperpolarization.

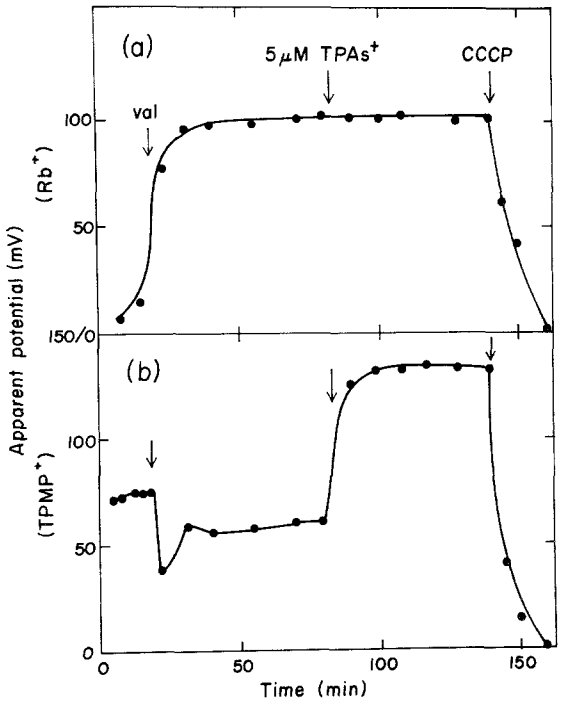

Fig. 8. Effect of 5 μM $TPAs^+$ on the apparent potential of *B. subtilis*, measured by uptake of (*a*) $^{86}Rb^+$ and (*b*) [^{3}H]-$TPMP^+$ in a double-label experiment in the presence of valinomycin at [K^+] = 5 mM

F. Effect of Mg^{2+} on Apparent Potential

Divalent cations are known to bind to the gram-positive cell surface (Hughes, Stow, Hancock & Baddiley, 1971; Beveridge & Murray, 1976; Doyle, Matthews & Streips, 1980). We wished to see whether they would affect uptake of lipophilic cations. After equilibration of 5 μM [^{3}H]-$TPMP^+$, 10 mM $MgCl_2$ was added; this caused release of about 80% of the potential-dependent uptake of label, and hence a drop in apparent potential from 90 to 55 mV. Subsequent addition of 5 μM $TPAs^+$ produced the usual stimulation of uptake, yielding an apparent potential of 105 mV. At a concentration of 1 mM, Mg^{2+} had only a slight effect on $TPMP^+$ uptake (*data not shown*). In a double-label experiment at 2 mM K^+ in the presence of valinomycin (Fig. 9), [^{3}H]-$TPMP^+$ was still released (the apparent potential dropping from 75 to 30 mV) upon addition of 10 mM Mg^{2+}, and taken up upon addition of 5 μM $TPAs^+$, whereas the apparent potential of $^{86}Rb^+$ remained constant at 125 mV throughout.

G. Binding of Lipophilic Cations to De-energized Cells

Concluding that lipophilic cations do not cause hyperpolarization, we attempted to determine the

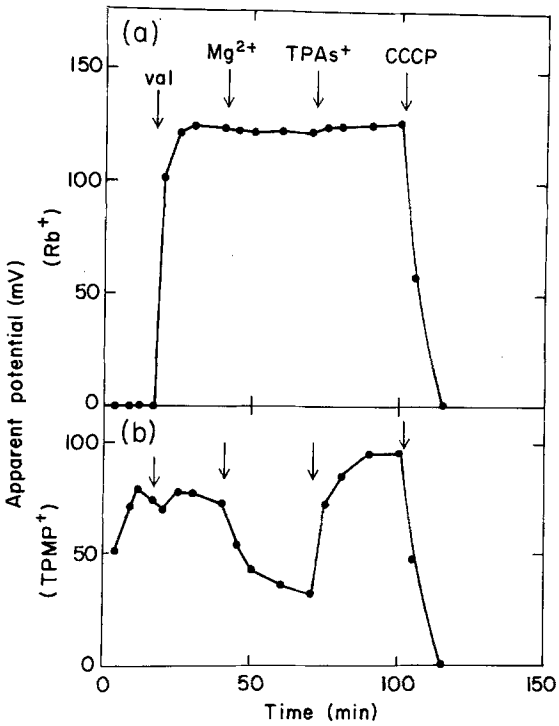

Fig. 9. Effect of successive addition of 10 mM Mg^{2+} and 5 μM $TPAs^+$ on the apparent potential of (*a*) $^{86}Rb^+$ and (*b*) [^{3}H]-$TPMP^+$ in a double-label experiment in the presence of valinomycin at $[K^+]=2$ mM

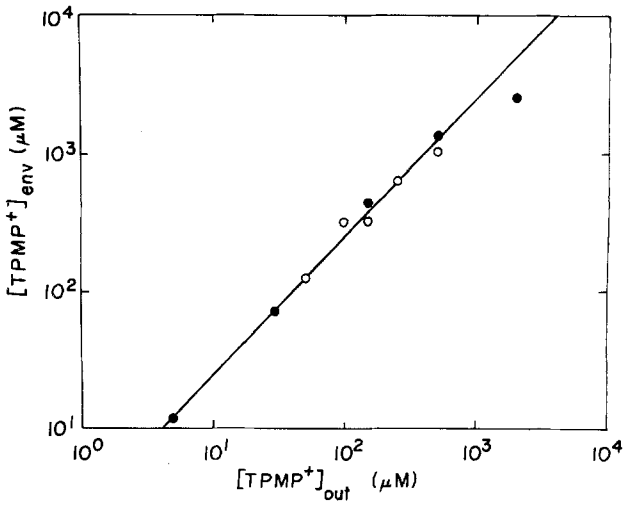

Fig. 10. Uptake of $TPMP^+$ by de-energized *B. subtilis* cells. Cells were either exposed to [^{3}H]-$TPMP^+$ at a low concentration (5 μM), CCCP (50 μM) added, and then successive additions of nonradioactive $TPMP^+$ made (●), or de-energized by CCCP at the $TPMP^+$ concentration indicated (○). $[TPMP^+]_{env}$ was calculated from total formal concentration minus cytoplasmic concentration (assumed equal to external concentration). A linear regression of the double logarithmic plot gave $y=0.38+1.01x$ (correlation coefficient of 0.98), i.e. $[TPMP^+]_{env}=2.40\times[TPMP^+]_{out}^{1.01}$. The datum point at 2 mM $TPMP^+$ was excluded from the linear regression

cause of the stimulated uptake of $TPMP^+$. Since a stable level had been reached prior to $TPAs^+$ addition (Fig. 1), the stimulation had to be explained in thermodynamic rather than kinetic terms, i.e. as a shift from one equilibrium state to a new one, either by increasing the $TPMP^+$ partition coefficient into an already available compartment, or by making a new compartment available. The description in terms of equilibria rather than steady states is based on the reasonable assumption that there is no active transport system involved.

A plausible example of the first kind would be positively cooperative binding of lipophilic cation at the cell surface. We therefore measured the extent of binding of cation to cells de-energized by 50 μM CCCP,[4] as a function of cation concentration. Since we were anticipating the possibility that binding in energized cells might be determined in part by the cytoplasmic concentration, we included measurements up to the millimolar range. Binding of $TPMP^+$ was linear over the range 5–500 μM (Fig. 10), with an uptake ratio $[TPMP^+]_{env}/[TPMP^+]_{out}$ of 2.4;[5] a slight degree of saturation was evident by 2 mM. In an experiment where [^{3}H]-$TPMP^+$ was added at low concentration (5 μM) to de-energized cells, successive additions of nonradioactive $TPMP^+$ at 30, 150 and 500 μM caused no further uptake of label, while addition of 2 mM caused a 30% displacement. $TPAs^+$ at 500 μM likewise failed to enhance uptake of 150 μM $TPMP^+$ in de-energized cells. Similar extents of binding were obtained when potential collapse was achieved by 400 mM K^+ plus valinomycin, rather than CCCP.

TPP^+ and $TPAs^+$ gave decreasing uptake ratios as external concentration was increased. For TPP^+, uptake ratios at 10 and 100 μM were 8.0 and 5.2, respectively; for $TPAs^+$, 4.6 and 3.3, respectively; however, even at 1 mM, binding was not saturated. The affinity of the cations for the cell surface was thus found to be $TPP^+ > TPAs^+ > TPMP^+$. None showed positive cooperativity of binding that could have explained stimulation of uptake in energized cells.

[4] With 100 μM CCCP (the concentration used as the final stage of experiments with energized cells) a precipitate formed at the higher cation concentrations studied. 50 μM CCCP sufficed to cause complete de-energization of cells as judged by motility loss, and $TPMP^+$ release.

[5] This is calculated on the assumption that the label has equilibrated in the cytoplasm; if label does not enter the cytoplasm, the uptake ratio should be increased by unity, to 3.4.

H. Analysis of Cation Uptake Data using a Two-Compartment Model

The stimulation of $TPMP^+$ uptake by $TPAs^+$ (Fig. 1; Fig. 8*b*) in the absence of a potential change (Fig. 8*a*) and in the absence of cooperative binding (Fig. 10) led us to explore a two-compartment model for lipophilic cation uptake according to which one compartment, *A*, is accessible to lipophilic cations at all concentrations whereas the other compartment, *B*, only becomes accessible at higher concentrations, both levels of uptake being potential-dependent (Fig. 1; *cf.* data before and after CCCP addition). We postulate that compartment *A* is some aspect of the cell envelope and that compartment *B* is the cytoplasm (*see* Discussion).

Using this model, we first analyzed the data from two-stage experiments in which the initial cation concentration was low enough that only the first level of uptake was achieved. Then, for example, the uptake of $TPMP^+$ (at 5 μM) into compartment *B* was the incremental uptake upon addition of 5 μM $TPAs^+$; this was approximately three times the uptake into compartment *A* (Fig. 1), and led to an apparent potential of 120 mV. Similarly, in a two-stage experiment using [^{3}H]-TPP^+ as label, a shift in the chemical concentration from 0.2 to 3 μM by addition of nonradioactive TPP^+ (data points from Fig. 7) gave an incremental uptake of label corresponding to an apparent potential of 120 mV.

Next the question arose of how to analyze data from single-stage experiments. If the cation concentration was low enough that only compartment *A* was occupied (<1 μM for TPP^+; <3 μM for $TPAs^+$; <10 μM for $TPMP^+$), analysis by the Nernst equation would not be legitimate because, although uptake into-compartment *A* was potential-dependent, the compartment was of undefined volume and composition.

If the cation concentration in a single-stage experiment was above the critical concentration for uptake into compartment *B*, the problem reduced to one of correcting for the uptake into compartment *A*. Since uptake into compartment *A* was potential-dependent (Fig. 1; Fig. 8*b*), a correction based on de-energized cells at the external concentration of the energized cell experiment would be an underestimate, and hence the potential would be overestimated. We describe this as correction according to the *external model* (Fig. 11*a*); it was the method used for Figs. 1 and 6–9.

The potential-dependent uptake into compartment *A* presumably is a reflection of increasing chemical potential in the region where electrical potential is decreasing. An extreme model would be one in which the entire compartment *A* was at the same electrical potential as the cytoplasm, in which case the cation concentration in compartment *A* would be related solely to the cytoplasmic concentration (the *internal model*; Fig. 11*b*). An intermediate case would have the concentration in compartment *A* related to both internal and external concentration; for simplicity, we have assumed that the drop in electrical potential and the compensating rise in chemical potential occurs linearly across compartment *A* (the *exponential mean model;* Fig. 11*c*) and hence that the mean concentration in compartment *A* is determined by the partition coefficient and by the exponential mean of the external

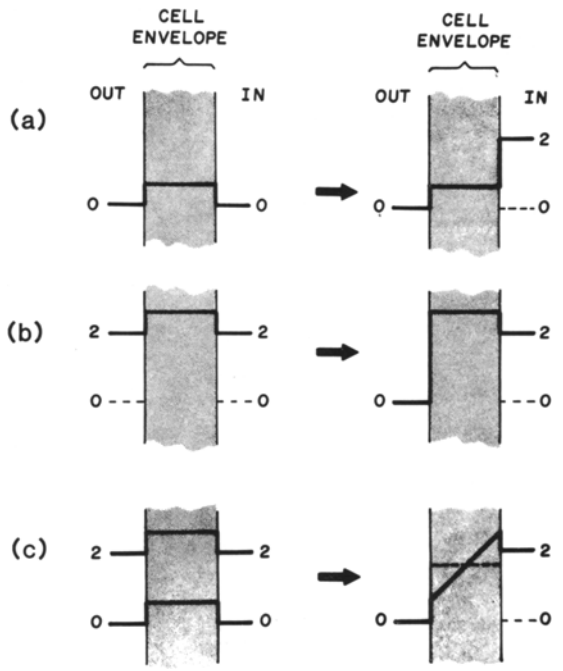

Fig. 11. Models for association of lipophilic cations with a cell envelope. Illustrations on the left refer to information obtained from de-energized cells, which is then used to infer the extent of association in energized cells (illustrated on the right). Numbers refer to the logarithmic concentration of the cation in the external medium (*OUT*) and the cytoplasm (*IN*). For illustrative purposes, energized cells have been assumed to maintain a 100-fold concentration gradient. The stepwise changes in concentration at the aqueous interfaces indicate partition into the cell envelope. (*a*) *External model:* Association assumed independent of potential, i.e. related to external concentration only. (*b*) *Internal model:* Association assumed potential-dependent, and related to cytoplasmic concentration only. (*c*) *Exponential mean model:* Association assumed potential-dependent, and related to the exponential mean of the external and cytoplasmic concentrations, $(C_{in} - C_{out})/\ln(C_{in}/C_{out})$

and internal concentrations, i.e. by $\bar{C}=(C_{in}-C_{out})/\ln(C_{in}/C_{out})$.[6]

In applying a correction for uptake into compartment A, using any model where the uptake was dependent on the cytoplasmic concentration, the difficulty arose that the latter was not known until the potential had been calculated. We therefore adopted an iterative procedure that insisted on internal agreement, i.e. a test value $C_{in(test)}$ for the cytoplasmic concentration had to generate a correction C_{env} to the total uptake C_{tot} such that the calculated cytoplasmic concentration $C_{in(calc)}$ was equal to $C_{in(test)}$.

$$C_{in(test)} \xrightarrow[\text{binding model}]{C_{out}} C_{env} \xrightarrow{C_{tot}} C_{in(calc)}$$

A specific illustration follows, assuming the exponential mean model: In a particular experiment at 3 μM TPP$^+$ (C_{out}), C_{tot} for energized cells was 913 μM. A test value $C_{in(test)}$ of 350 μM, corresponding to a potential of $59\log 350/3=122$ mV, yielded $\bar{C}=(350-3)/\ln(350/3)=73$ μM, which in turn (using the calibration curve of TPP$^+$ binding to de-energized cells) indicated $C_{env}=460$ μM. $C_{in(calc)}$ for the energized cells therefore was $913-460=453$ μM, which is higher than the original test value of 350 μM, yielding a ratio $C_{in(test)}/C_{in(calc)}$ of 0.77. The test value was therefore adjusted upwards, and the calculation repeated. Typically, five iterations were needed before a ratio of 1.0 ± 0.05 was reached. (Note that a 5% error in concentration yields a much smaller error in potential.) In the present example, a satisfactory solution was $C_{in(test)}=410$ μM, which gave $\bar{C}=83$ μM, $C_{env}=505$ μM, $C_{in(calc)}=408$ μM, $C_{in(test)}/C_{in(calc)}=1.00$, and $\Delta\psi=126$ mV.

When the procedure was carried out assuming the internal model, lower estimates of potential were of course obtained. In the above example, a cytoplasmic concentration of 157 μM was necessary to produce a self-consistent calculation, resulting in a potential estimate of 101 mV.

The data of Fig. 7 were re-analyzed (Fig. 12) using the self-consistent correction procedure, the exponential mean model, and the appropriate calibration

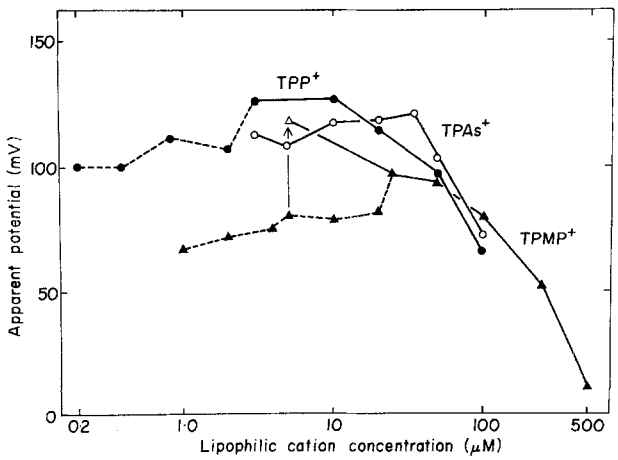

Fig. 12. Apparent potential of *B. subtilis* measured by TPP$^+$ (●), TPAs$^+$ (○), and TPMP$^+$ (▲), as a function of cation concentration. The primary data are the same as in Fig. 7, but have been corrected according to the exponential mean model (*see* Results, Section *H*). TPMP$^+$ data below 25 μM, and TPP$^+$ below 3 μM have been linked by dashed lines because the correction procedure is invalid if access to the cytoplasm has not occurred. The point (△) for 5 μM TPMP$^+$ was measured in the presence of 5 μM TPAs$^+$; the procedure is valid for this point

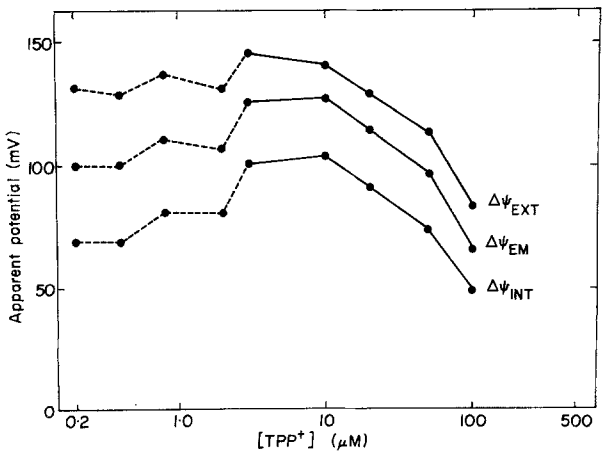

Fig. 13. Apparent potential $\Delta\psi$ of *B. subtilis* measured by TPP$^+$, as a function of TPP$^+$ concentration, corrected according to the three models described in Fig. 11. *EXT*=external model, *EM*=exponential mean model, *INT*=internal model. Dashed lines, as in Fig. 12

binding curves. Because TPP$^+$ has the highest binding affinity, potential estimates based on its uptake were lowered more (*ca.* 20 mV) than estimates based on TPAs$^+$ (*ca.* 15 mV) or TPMP$^+$ (*ca.* 6 mV). A comparison of the TPP$^+$ uptake data corrected for cell-envelope association according to the external, exponential mean and internal models is given in Fig. 13. The curves are almost parallel, but not quite, partly because TPP$^+$ binding is nonlinear, and partly because the corrections according to the three models have somewhat different potential dependencies. An analysis of the three models as a function of partition coefficient and membrane potential is given in the Appendix.

[6] A linear rise in chemical potential across compartment A implies that the equivalent aqueous concentration (strictly, activity) at a distance x from the exterior is $C=C_{out}\exp(bx)$, with $C_{in}=C_{out}\exp(bl)$, where l is the width of compartment A and $b=ZF\Delta\psi/RTl$. Then $\bar{C}=\int_0^l C\,dx \Big/ \int_0^l dx=\frac{C_{out}}{bl}[\exp(bl)-1]$. But $bl=\ln(C_{in}/C_{out})$; hence $\bar{C}=(C_{in}-C_{out})/\ln(C_{in}/C_{out})$. Note that since the rise in chemical potential across compartment A is assumed to derive from the membrane potential, the expression for mean aqueous concentration is still valid for calculation of uptake into compartment A, with C_{in} now signifying the cytoplasmic concentration that would be in Nernst equilibrium with the external concentration if equilibrated access were possible.

Discussion

Dye fluorescence and distribution of radiolabeled ions are invaluable techniques for estimating membrane potential in small cells and organelles. Yet the opposite effects exerted by $TPAs^+$ on "potential" measured by diS-$C_3(5)$ fluorescence and [^{3}H]-$TPMP^+$ uptake in *B. subtilis* demonstrate clearly that these techniques are prone to artifacts, and must be used with caution. Had we employed dye fluorescence alone, and used a low dye/cell ratio, we would have concluded that $TPAs^+$ causes *depolarization.* Had we employed $TPMP^+$ uptake alone, we would have concluded that $TPAs^+$ causes *hyperpolarization.* Because both techniques were employed, and also because de Jong and van der Drift (1978) had reported no fluorescence change under their experimental conditions, we were alerted to the fact that at least one of the techniques contained an artifact.

The developers of dye fluorescence techniques have pointed out the dangers of assuming that a change in signal upon introducing a drug necessarily represents a change in membrane potential (e.g., Waggoner, 1979; Freedman & Laris, 1981). For example, hydrophobic anions such as CCCP (in apparent contradiction to their known potential-collapsing properties) can cause fluorescence quenching, perhaps by forming neutral hydrophobic aggregates which are non-fluorescent. The artifact we have encountered seems to be of the opposite sort, a fluorescence enhancement that may result from displacement, by the lipophilic cation $TPAs^+$, of the cationic dye into the aqueous phase from a location where it was nonfluorescent. This location could be some aspect of the cell surface or, as in the case of erythrocytes (Tsien & Hladky, 1978), a binding site on a macromolecule within the cell. The effect is most pronounced at high $TPAs^+$ concentrations and low dye concentrations, further suggesting competition for a binding site. The effect is highly potential-dependent. This can give rise to an effect, puzzling on first encounter, of a combined fluorescence enhancement from $TPAs^+$ and valinomycin addition that is less at high K^+ concentration than it is at low (Fig. 4), because of a loss of $TPAs^+$-induced enhancement which exceeds the gain in valinomycin-induced enhancement. The potential dependence is likely to be simply a local $TPAs^+$ concentration dependence, the cation being at a higher activity in energized cells. The sensitivity of the $TPAs^+$-induced fluorescence enhancement to small changes in potential may be responsible for the day-to-day variability noted in the Results section.

The displacement artifact can be minimized by the use of higher dye/cell ratios, but it should be borne in mind that high ratios can cause undesirable photodynamic effects (Manson, Tedesco, Berg, Harold & van der Drift, 1977; Miller & Koshland, 1978). The problem of displacement artifacts may be less severe in larger cells, because of a lowersurface/volume ratio. Nonetheless, our results emphasize the importance of establishing, for any given system, conditions under which the dye is a reliable indicator of membrane potential.

In the case of radiolabeled cation uptake, we encountered a serious analytical problem that manifested itself in several ways. Different ions, or the same ion at different concentrations, yielded different values of apparent potential. The range was considerable, from 90 mV as measured by 5 μM $TPMP^+$ to 110 mV (25 μM $TPMP^+$) or 146 mV (3 μM TPP^+). Also, addition of another ion such as $TPAs^+$ caused an apparent hyperpolarization of some 35 mV, even in the presence of valinomycin with $^{86}Rb^+$ showing no change in uptake. We conclude that the variability in apparent potential is artifactual, i.e., that stimulated uptake does not occur because of hyperpolarization. The slow depolarization at high lipophilic cation concentration appears to be real, being indicated by release of $^{86}Rb^+$ as well as of [^{3}H]-$TPMP^+$.

Since neither *E. coli* vesicles nor EDTA-treated cells of *E. coli* displayed $TPAs^+$-stimulated $TPMP^+$ uptake, it seems likely that the phenomenon is related to the peculiar structure of the gram-positive cell surface, and particularly to the massive amount of negatively charged teichoic acid and peptidoglycan it possesses.

We postulate that there are two compartments in *B. subtilis* for potential-dependent uptake of cation, one of which (compartment *B*) is only available above a critical concentration of either a single cationic species, or of a combination of such species. The arguments in support of this model can be summarized as follows:

(i) Cationic uptake was stimulated from one stable level to another (Fig. 1).

(ii) Upon collapsing the membrane potential with CCCP, uptake was reduced to a much lower level than the first steady-state level (Figs. 1 and 8*b*).

(iii) Both auto-stimulated (e.g., $TPMP^+$ by $TPMP^+$; Fig. 7) and hetero-stimulated (e.g., $TPMP^+$ by $TPAs^+$; Figs. 1 and 6) uptake were observed.

(iv) Stimulated uptake was *not* a consequence of hyperpolarization. We consider the failure of $TPAs^+$ to stimulate $^{86}Rb^+$ uptake in the presence of valinomycin (Fig. 8*a*) conclusive in this regard.

(v) We were unable to find any evidence for cooperative binding. Binding curves as a function of cation concentration were either linear ($TPMP^+$; Fig. 10) or showed the gradual saturation that is

characteristic of low-affinity nonspecific binding (TPP^+ and $TPAs^+$).

If this two-compartment model is correct, several questions arise. Which compartment (if either) is the cytoplasm? What is the other compartment? What is the meaning of apparent potential calculated from compartment *A* uptake only? When both compartments are accessible and if one is the cytoplasm, what is the best way to correct for uptake into the other compartment? What is the barrier to entry into compartment *B*, and why is it overcome by raising the cation concentration?

The results provide circumstantial evidence to suggest that compartment *A* may be some aspect of the cell surface, and not the cytoplasm. Firstly, the apparent potential obtained with 5 μM [^{3}H]-$TPMP^+$, in the presence of valinomycin at 5 mM K^+, was 60 mV (Fig. 8*b*); this would imply that the internal K^+ concentration was only 50 mM, which is much lower than published values,[7] e.g., 400 mM (Matsuura, Shioi, Imae & Iida, 1979) or the value of 248 mM indicated in this study by Rb^+ uptake (apparent potential 100 mV, Fig. 8*a*). Secondly, in experiments below the critical concentration of $TPMP^+$ and TPP^+ the apparent potentials differed substantially, $TPMP^+$ indicating about 90 mV, TPP^+ indicating about 130 mV (Fig. 7); if compartment *A* was the cytoplasm, the Nernst equation should have given the same potential value regardless of the cation used. In fact, these apparent potentials support the hypothesis that compartment *A* is the *cell surface*, because they indicate that it has a much higher affinity for TPP^+ than for $TPMP^+$, in agreement with binding measurements on de-energized cells (Results, Section *G*). In contrast, uptakes of TPP^+ and $TPMP^+$ into compartment *B* yielded the same apparent potential, 120 mV (Results, Section *H*), supporting its identification as the cytoplasm. Thirdly, Mg^{2+} caused displacement of $TPMP^+$ from compartment *A* in the absence of any change in potential as indicated by $^{86}Rb^+$ (Fig. 9), but did not prevent $TPAs^+$ from enabling $TPMP^+$ uptake into compartment *B*; these results suggest that compartment *A* is the cell surface, with Mg^{2+} competing with $TPMP^+$ for anionic binding sites. Finally, the fact that access to compartment *B* required prior access to compartment *A* is more readily reconciled with *A* being the cell surface and *B* the cytoplasm than *vice versa*.

The hypothesis that cytoplasmic uptake of lipophilic cations in *B. subtilis* is self-gated is a rather startling one. Are there alternative explanations for the uptake data? We have considered the following possibilities: (*i*) *Lipophilic cations cause hyperpolarization:* This explanation is not compatible with the unchanged $^{86}Rb^+$ uptake in double-label experiments. Also, it is suspect because apparent potential prior to the stimulation of uptake varied from 90 to 130 mV according to the probe used, and could be drastically reduced by addition of Mg^{2+} ion. (*ii*) *The cations always have access to the cytoplasm; partition into some other compartment is increased at high cation concentration.* Uptake data in de-energized cells do not support this.[8] If there is a change in partition, it must only occur in energized cells, i.e., it must require membrane potential *per se*, or some other metabolic process. The objection regarding variability of apparent potential in the low concentration range also applies. Further, according to this explanation one would have to accept the lowest potential estimate obtained as being the most accurate; this leads to unreasonably low values (e.g. 30 mV for 5 μM $TPMP^+$ in the presence of valinomycin and Mg^{2+}, with $^{86}Rb^+$ reporting 125 mV, Fig. 9). (*iii*) *The cations never have access to the cytoplasm; partition into the cell surface is increased at high cation concentration.* Again, the uptake data in de-energized cells do not support this. Variability of apparent potential is explained by this model (as it is by the gated uptake model). All physiological effects (inhibition of macromolecular synthesis, reduction of swimming speed, alteration of motor switching probabilities; Ordal, 1976; de Jong & van der Drift, 1978; Zaritsky & Macnab, 1981) would have to result from binding of cation to external sites. (*iv*) *There is no change in access or partition; washing losses are large at low cation concentration.* There are two reasons to doubt this explanation. First, elimination of the second wash or addition of a third wash made only a slight difference in retention; one would have to assume most of the loss was occurring in less than a few seconds, whereas uptake of $TPMP^+$ has a time constant of

[7] Chaustova, Grinius, Griniuvienė, Jasaitis, Kadziauskas & Kiaušinytė (1980) obtained a value of 50 mM for the cytoplasmic K^+ concentration, but this was after addition of valinomycin to K^+-free buffer; after equilibration, the external K^+ concentration was 0.2 mM. The membrane potential was estimated at 140 mV, some hyperpolarization being expected under these conditions.

[8] The extent of binding by de-energized cells at high external cation concentration could be appreciably greater than in energized cells where the internal concentration (if access was possible) was high but the external concentration low. It is reasonable to ask whether this difference could have obscured a cooperative binding phenomenon in the cytoplasm. At 5 μM $TPMP^+$, a typical total uptake by energized cells was 150 μM, which therefore is an upper limit to the cytoplasmic concentration under these conditions; the incremental $TPMP^+$ uptake upon $TPAs^+$ addition was around 500 μM. Total uptake of $TPMP^+$ at 150 μM by de-energized cells was 510 μM, and so a further increment of 500 μM would easily have been detected.

4 min. Second, to explain the difference in apparent potential, the loss of $TPMP^+$ would need to be much faster than that of TPP^+, yet uptake of $TPMP^+$ is almost ten times slower than that of TPP^+; one would have to invoke a different mechanism for uptake and release. The reason why washing losses from the cytoplasm would decline abruptly at high cation concentration is also obscure.

For the reasons given, none of the above explanations seems satisfactory. We therefore propose the model of gated cytoplasmic access, but acknowledge that it has not been directly demonstrated.

If we are correct in concluding that the first level of uptake is not into the cytoplasm, it is obvious that membrane potentials calculated as though it were will not give the correct quantitative result, for three fundamental reasons. First, the assumed compartment volume will be incorrect; second, the partition coefficient will not be unity as is assumed in applying the Nernst equation; third, there is no *a priori* reason to suppose that compartment *A* will be at the same potential as the cytoplasm – indeed, it is likely to be at some potential intermediate between that of the external medium and the cytoplasm. Uptake into compartment *A* is, however, potential-dependent. If it differs from potential-dependent cytoplasmic uptake by a constant factor, the apparent potential will differ from the true membrane potential by a constant amount.[9]

For *B. subtilis* with $TPMP^+$ as the probe, compartment *A* takes up approximately one-third the label of compartment *B*, and therefore potentials based on compartment *A* (Miller & Koshland, 1977; Khan & Macnab, 1980*b*) are underestimated by *ca.* 28 mV (Results, Section *H*). For TPP^+, the error is in the opposite direction; compartment *A* takes up approximately 1.7 times as much label as compartment *B*, and therefore potentials obtained at $[TPP^+] < 1\ \mu M$ are overestimated by *ca.* 14 mV. It is fortuitous that the errors in both cases are fairly small; this may explain why they have been overlooked in previous studies.

What cationic probe should be used for measurement of potential in *B. subtilis*? None seems entirely suitable. $^{86}Rb^+$ did not display any artifacts that we were aware of, but it can only be used in the presence of valinomycin, and hence at zero potassium potential and an imposed electrical potential. The lipophilic cations clearly have artifacts associated with their uptake. If the two-compartment model is valid, these ions should be used at concentrations sufficiently high to enable entry into the cytoplasm, but not so high as to cause depolarization. This compromise does not seem to be possible in the case of $TPMP^+$ (Figs. 7 and 12) unless a facilitating ion such as TPP^+, $TPAs^+$ or TPB^- is added. TPB^- greatly increases the kinetics of uptake of $TPMP^+$, but also increases its binding. [Shioi et al. (1980) obtained a value of 140 mV at pH 7.5 using $10\ \mu M$ $[^3H]$-$TPMP^+ + 2\ \mu M$ TPB^-; since the data were corrected according to the external model, we consider that this value is an overestimate.] TPP^+ or $TPAs^+$ apparently gain entry to the cytoplasm, even when used at quite low concentrations; $TPAs^+$ has a somewhat lower affinity for the cell envelope than TPP^+, which is an advantage in view of the uncertainty regarding the best procedure for applying a correction for envelope-associated material; however, $[^3H]$-$TPAs^+$ has only been manufactured at low specific activity, and there is no commercial supplier. Finally, it should be remembered that all of these lipophilic cations have major effects on the physiological state of the cells (Zaritsky & Macnab, 1981).

The peculiar properties of *B. subtilis* with respect to lipophilic cation uptake have forced us to consider critically what would be the best correction procedure in *any* system where binding is appreciable. The method applied in the past by ourselves (Khan & Macnab, 1980*a*, *b*) and others has been to regard the differential uptake between energized and de-energized cells as the potential-dependent cytoplasmic uptake [correction according to the external model (Fig. 11*a*)]. This procedure assumes that non-cytoplasmic uptake ("bound label") is potential-independent, either because it is determined solely by the external concentration, or because it is saturated; in other words it assumes that the high cytoplasmic concentration has no effect on binding. In retrospect this is an assumption that has been made rather naively, without experimental support. We find in fact that binding to de-energized *B. subtilis* cells is unsaturable, even at millimolar levels (Results, Section *G*; Fig. 10). Also, the first-stage uptake in energized cells, which we suggest may be at the cell surface, is potential-dependent (Fig. 1) and roughly linearly dependent on external concentration.

If binding is potential-dependent or depends on the cytoplasmic concentration as well as the external concentration (especially where these concentrations differ by a factor of 100, at a membrane potential of 120 mV) it is not obvious how to determine the appropriate correction. For any particular assumption regarding the relative contributions of exterior and cytoplasm to binding, however, it is possible to

[9] This assumption is precisely correct only if the partition coefficient is constant as a function of concentration (which is true for $TPMP^+$; Fig. 10) and if compartment *A* is at the cytoplasmic potential. If compartment *A* is at the mean potential, the error will show some potential-dependence (*see* Appendix).

arrive at a self-consistent estimate of the membrane potential even though the cytoplasmic concentration is not known *ab initio* (Results, Section *H*).

We have analyzed the data according to two extreme assumptions, (*i*) that only external concentration determines binding (the *external model*, Fig. 11*a*; used throughout Results, prior to Section *H*), and (*ii*) that only internal concentration determines binding (the *internal model*, Fig. 11*b*). We have also analyzed the data according to the assumption (*iii*) that the extent of binding is determined by the exponential mean of the external and cytoplasmic concentrations (the *exponential mean model*, Fig. 11*c*), this assumption being a more neutral one regarding the relative roles of the external medium and the cytoplasm.[10]

The values obtained by the three models differ considerably (spanning about 45 mV; Fig. 13). Ultimately, the only satisfactory criterion for deciding which is the most accurate is a direct measurement of the extent of binding in energized cells. Shen, Boens and Ogawa (1980) have attempted to measure TPP^+ binding in energized mitochondria, using ^{31}P NMR. The assumption was made that the peak deriving from bound TPP^+ would be too broad to be detected, and therefore that the extent of binding could be estimated from the missing intensity; however, since the result obtained was inconsistent with the extent of binding inferred indirectly from a comparison of TPP^+ and Rb^+ uptake data, Shen et al. (1980) question the validity of the line-broadening assumption when applied to low-affinity, nonspecific binding.

In the case of *B. subtilis*, the hypothesis of gated access to the cytoplasm suggests an independent measure of the extent of binding in energized cells, which we find agrees best with the exponential mean model. For example, in two-stage uptake experiments using TPP^+, a potential of 120 mV was calculated from the second-stage (compartment *B*) uptake at 3 μM (Results, Section *H*); when the total uptake at 3 μM was corrected for binding according to the external, exponential mean, and internal models, values of 146, 126 and 101 mV, respectively, were obtained (Results, Section *H*).

Similarly, the potential calculated from the differential uptake of $TPMP^+$ upon addition of 5 μM $TPAs^+$ was 120 mV. Correction of total uptake according to the three models gave 125, 117 and 97 mV, respectively. Here, because the binding affinity of $TPMP^+$ is lower, the choice of correction procedure is not as critical as it was with TPP^+. However, the extent of uptake of $TPMP^+$ into compartment *A* favors the exponential mean model. The formal concentration in compartment *A* corresponds to an effective aqueous concentration of 88 μM, which is far higher than the external concentration of 5 μM, substantially lower than the internal concentration (216 μM) calculated according to the internal model, but in quite good agreement with the self-consistent exponential mean concentration (105 μM).

Another criterion for the relative merits of various binding models is the extent to which they reconcile apparent potentials obtained with various cations of different binding affinities. Comparing Fig. 7 (based on the external model) with Fig. 12 (based on the exponential mean model), one might argue that the latter produces somewhat better agreement between the apparent potentials measured by TPP^+, $TPAs^+$, and $TPMP^+$ in the range where they have gained access to the cytoplasm, but the case is not very convincing. Part of the reason why this criterion does not discriminate more effectively is the fact that the biggest difference in binding affinity is between TPP^+ and $TPMP^+$, especially at low concentrations (say 2 μM external) before TPP^+ begins to saturate, but the comparison cannot be made, because at that concentration $TPMP^+$ has only shown the first level of uptake. Although discrimination between the models by this criterion is poor, it is worth re-emphasizing that choice of model is important, as the 45 mV range of estimates of potential in Fig. 13 clearly shows.

The issue of proper correction for bound label is one of general concern, wherever cationic probes are used to measure membrane potential. As an indication that problems may arise in systems other than *B. subtilis*, we may cite the observation by Shen et al. (1980) that TPP^+ uptake in energized mitochondria exceeded Rb^+ uptake (in the presence of valinomycin) by a factor of 6.5 ± 2 over a wide range of values of apparent potential.

The magnitude of the problem in any particular system will depend primarily on the binding affinity of the cation. As a rough guide, if in de-energized cells, organelles, or vesicles the uptake ratio C_{env}/C_{out} is 2 or greater, there is cause for concern (*see* Appendix). Cell surfaces possessing a large number of negatively charged groups (e.g. teichoic acids, or phospholipids with neutral head groups) are likely to fall into this category.

[10] The choice of the exponential mean, however, is arbitrary; it assumes a linear potential drop and a constant partition coefficient. This constant-field assumption is commonly made in membrane bioenergetics (e.g. in the Goldman-Hodgkin-Katz equation) but would be inappropriate if, for example, binding occurs only at the inner and outer faces of the membrane, and does so with different affinities. In that case a weighted arithmetic mean should be used (*cf.* Sims et al., 1974; Tsien & Hladky, 1978).

If, in any particular system, binding is more heavily dependent on cytoplasmic concentration than the exponential mean model dictates, the problem is even more severe. For example, if binding is almost exclusively at internal sites, even an uptake ratio C_{env}/C_{out} of 1 will result in substantial errors (up to 18 mV) if the external model for correction is used, the errors being greatest for highly energized cells (*see* Appendix).

Conclusions and Recommendations

The results presented in this paper are complex, encompassing dye fluorescence and radiolabel uptake, lipophilic cations as drugs as well as probes, anomalous uptake characteristics, binding measurements in de-energized cells, and theoretical models for correction for binding. Some of the findings may be peculiar to *B. subtilis*, others are likely to be of more general importance. Our principal conclusions and recommendations are given below:

In B. subtilis:

1. Lipophilic cations can displace cyanine dye from sites where fluorescence is quenched to sites where it is not; this results in a false indication of depolarization. Use of high dye/cell ratios minimizes the artifact.

2. Low concentrations ($<10\,\mu M$) of lipophilic cations ($TPMP^+$, TPP^+, $TPAs^+$) do not affect membrane potential significantly.

3. Higher concentrations cause depolarization; by *ca.* 100 μM, membrane potential is reduced by 50%.

4. Although no change in potential is actually occurring, the *apparent* potential indicated by lipophilic cation uptake at low concentrations is increased by further addition of lipophilic cation, and decreased by addition of Mg^{2+}.

5. We tentatively suggest that, below a critical cation concentration, potential-dependent uptake occurs into a cell-surface compartment only, and that, above that critical concentration, the cytoplasm becomes accessible for potential-dependent uptake.

6. Even at concentrations as high as 2 mM, binding of cation to de-energized cells is far from saturation. Binding affinities are in the order $TPP^+ > TPAs^+ > TPMP^+$.

7. Membrane potential calculated from cytoplasmic uptake is *ca.* 120 mV.

Generally:

8. The common assumption, that the extent of binding of cation in energized and de-energized cells is the same, is not a reasonable one to make without supporting evidence in the system under study.

9. The extent of binding in de-energized cells should be measured over a range of concentrations, from the external concentration to be used in energized cell experiments up to the cytoplasmic concentration anticipated in such experiments.

10. If estimates of the membrane potential based on correction for binding at the external and internal concentrations, respectively, are significantly different, it is desirable to establish the extent of binding in energized cells by some independent technique.

11. If no suitable technique is available, correction according to the exponential mean model is preferable to correction based on external concentration alone, or internal concentration alone.

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Appendix

Comparison of Binding Models as a Function of Potential and Binding Affinity

We examine here the difference in apparent potential when a given extent of uptake C_{tot} of a cationic probe is analyzed by various models. Apparent potential according to any model M is given by

$$\Delta\psi_M = (RT/ZF)\ln(C_{in}/C_{out}) = (RT/ZF)\ln[(C_{tot}-C_{env})/C_{out}]$$

where C_{env} is the correction for binding according to that model. Let k = the uptake ratio C_{env}/C_{out} in de-energized cells; for simplicity we assume k is a constant (i.e. binding is linear).

Then C_{env} is given by:

0	for the *zero model* (no correction),
kC_{out}	for the *external model*,
$k\bar{C} = k(C_{in}-C_{out})/\ln(C_{in}/C_{out})$	for the *exponential mean model*, and
$kC_{in} = kC_{tot}/(k+1)$	for the *internal model*.

For $C_{tot} = C_{env} + C_{in}$, we use as a matter of convenience the function

$$C_{tot} = [e^n + k(e^n - 1)/n] \times C_{out}$$

since it yields (without the need for iteration) a self-consistent value of $C_{in} = e^n C_{out}$ when the exponential mean model is applied. [Note that use of this function does not impose any restriction regarding binding, and has no effect on the relationships among potentials calculated according to the various models. C_{tot} is the independent parameter from which these potentials are calculat-

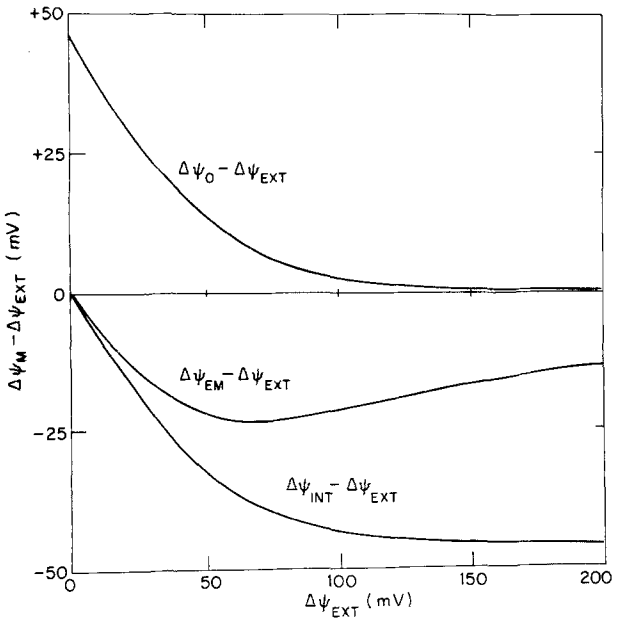

Fig. A-1. Difference between the apparent potential $\Delta\psi_M$ according to a given correction model M, and the apparent potential $\Delta\psi_{EXT}$ according to the external model, as a function of potential (represented by $\Delta\psi_{EXT}$). 0 = Zero model, EXT = external model, EM = exponential mean model, INT = internal model. Uptake ratio $k = 5$

ed; n is a dependent parameter, and is an implicit function of C_{tot}.]

Hence C_{in}/C_{out} is given by:

$e^n + k(e^n - 1)/n$ for the *zero model*,
$e^n + k(e^n - 1)/n - k$ for the *external model*,
$e^n + k(e^n - 1)/n - k(C_{in}/C_{out} - 1)/\ln(C_{in}/C_{out}) = e^n$ for the *exponential mean model*. and
$[e^n + k(e^n - 1)/n]/(k + 1)$ for the *internal model*.

Let $\Delta\psi_0$, $\Delta\psi_{EXT}$, $\Delta\psi_{EM}$, and $\Delta\psi_{INT}$ be apparent potentials according to the zero, external, exponential mean and internal models, respectively. Fig. A-1 shows $\Delta\psi_M - \Delta\psi_{EXT}$, calculated according to various models M, as a function of $\Delta\psi_{EXT}$. [Since apparent potential according to all of the models is a monotonically increasing function of C_{tot}, as presumably is true potential, the choice of one of the apparent potentials (here $\Delta\psi_{EXT}$) for the abscissa function provides a convenient representation of the behavior of the ordinate function with respect to the (unknown) true potential $\Delta\psi$]. When $\Delta\psi_{EXT}$ is zero, so also are $\Delta\psi_{EM}$ and $\Delta\psi_{INT}$, since the external and internal concentrations are the same; $\Delta\psi_0$ is $59\log(k+1)$ mV. As potential is increased, the correction according to the external model goes asymptotically to zero, and so the distinction between the zero and the external models ($\Delta\psi_0 - \Delta\psi_{EXT}$) rapidly disappears. The correction according to the exponential mean model also asymptotically approaches zero, but much more slowly, because the fractional correction on C_{tot} is $k\bar{C}/C_{tot}$, rather than just kC_{out}/C_{tot} in the external model; at high potential, these expressions reduce to $k/(n+k)$ and $[n/e^n] \times [k/(n+k)]$, respectively. As a result, the difference in apparent potential $|\Delta\psi_{EM} - \Delta\psi_{EXT}|$ passes through a maximum, and then approaches zero very gradually. $\Delta\psi_{INT}$ is always $59\log(k+1)$ mV lower than $\Delta\psi_0$; $-59\log(k+1)$ mV is also the limit of $\Delta\psi_{INT} - \Delta\psi_{EXT}$ and $\Delta\psi_{INT} - \Delta\psi_{EM}$ at high potential.

Suppose potential has been calculated according to the external model, when in fact the exponential mean model is the appropriate one for the system of interest. For $k = 5$ (the value used for Fig. A-1), the maximum error occurs at $\Delta\psi_{EXT} = 69$ mV. The "true" potential $\Delta\psi_{EM}$ is 46 mV, i.e. the error is 23 mV.

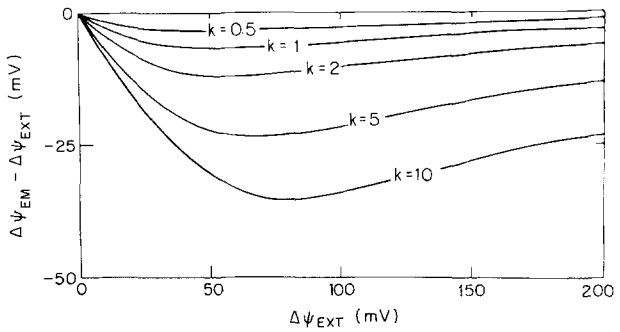

Fig. A-2. Effect of size of k upon the difference in apparent potential $\Delta\psi_{EM} - \Delta\psi_{EXT}$, as a function of $\Delta\psi_{EXT}$

Some idea of how sensitive the error is to the magnitude of k can be seen from Fig. A-2. Assuming the exponential mean model is appropriate, the maximum error in estimating potential using the external model is only about 3.5 mV for $k = 0.5$ and 6.5 mV for $k = 1$; these errors are probably too small to matter, considering the reproducibility of the experiments. However, at higher values of k, the errors are significant; at $k = 10$, the maximum error is about 35 mV.

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