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Small lipid-soluble cations are not membrane voltage probes for *Neurospora* or *Saccharomyces*

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Abstract

Small lipid-soluble cations, such as tetraphenylphosphonium (TPP^+) and tetraphenylarsonium (TPA^+) are frequently used as probes of membrane voltage ($\Delta\Psi$, or V_m) for small animal cells, organelles, and vesicles. Because much controversy has accompanied corresponding measurements on 'walled' eukaryotic cells (plants, fungi), we studied their transport and relation to V_m in the large-celled fungus *Neurospora crassa* – where V_m can readily be determined with microelectrodes – as well as in the most commonly used model eukaryotic cell, the yeast *Saccharomyces cerevisiae*. We found no reasonable conditions under which the distribution of TPP^+ or TPA^+, between the cytoplasm (i) and extracellular solution (o), can serve to estimate V_m, even roughly, in either of these organisms. When applied at probe concentrations (i.e., $\leq 100\ \mu M$, which did not depolarize the cells nor deplete ATP), TPP^+ stabilized at ratios (i/o) below 30 in both organisms. That would imply apparent V_m values positive to -90 mV, in the face of directly measured V_m values (in *Neurospora*) negative to -180 mV. When applied at moderate or high concentrations (1–30 mM), TPP^+ and TPA^+ induced several phases of depolarization and changes of membrane resistance (R_m), as well as depletion of cytoplasmic energy stores. Only the first phase depolarization, occurring within the perfusion-turnover time and accompanied by a nearly proportionate decline of R_m, could have resulted from TPP^+ or TPA^+ currents per se. And the implied currents were small. Repeated testing, furthermore, greatly reduced the depolarizing effects of these lipid-soluble ions, implicating an active cellular response to decrease membrane permeability.

Key words: Membrane voltage; Lipid-soluble ion; TPP^+; Voltage probe; Yeast; *Neurospora*

1. Introduction

Over the past 15 years, synthetic lipid-soluble ions (LSI's), including several families of fluorescent dyes, have become widely used as physiological probes and labels: to evaluate, e.g., protein conformational shifts [1], intracellular ion concentrations and ion movements [2,3], changes of membrane surface charge [4,5], and transmembrane differences of electric potential [6–9]. As is true with all indirect techniques, major problems in using LSI probes have been how to calibrate them and to guarantee that their signalling is unique. Such problems are intrinsically most difficult when absolute information, rather than relative information, is needed; and use of lipid-soluble ions to measure membrane voltages in intact biological systems has proven particularly treacherous [6,10–12], as well as particularly important.

Three classes of extrinsic LSI probes have thus far been used to estimate biological membrane voltages: i) those, like di-5-ASP, which respond very rapidly (10^{-7}–10^{-10} s) to voltage steps by changing optical

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Abbreviations: DCE, dichloroethane; di-5-ASP, dipentylaminostyrylmethylpyridinium; DMG, 2,2-dimethylglutaric acid; DOC, deoxycorticosterone; DOP, dioctyl phthalate; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; LSI, lipid-soluble ion; Mes, 2-(*N*-morpholino)ethanesulfonic acid; PCB, phenyldicarbaundecaborane; PVC, polyvinylchloride; THF, tetrahydrofuran; TPA^+, tetraphenylarsonium ion; TPB^-, tetraphenylborate; $TPMP^+$, triphenylmethylphosphonium ion; TPP^+, tetraphenylphosphonium ion; Tris, tris(hydroxymethyl)aminomethane.

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properties and are presumed to display true electrochromicity [13,14]; ii) those, like merocyanine 540 or some oxonols, which respond less rapidly (10^{-3}–10^{-7} s), probably by reorienting in the membrane [9,15]; and iii) those, like quaternary phosphonium or arsonium ions or the carbocyanine dyes, which respond slowly (10 to 10^{-3} s) and have been shown to redistribute in the cytoplasm and extracellular solution surrounding the membrane [5,16,17]. LSI probes in the latter class, especially, have been widely adopted, both because of their technical ease in use and because of appropriate slowness in many important membrane processes.

The best reports on LSI probes for membrane voltage have involved careful calibration of the measured distribution [18,19] or optical signal [7,20] against other indicators, especially K^+ or Rb^+ distribution in the presence of the specific ionophore valinomycin [9,16,21,22], or – in particularly favorable cases – intracellular microelectrodes [23–25]. It has thereby been possible to determine with considerable confidence both absolute membrane voltages and changes of membrane voltage in neural tissue [24,26], in brain synaptosomes [27,28], in hepatocytes [29], in mammalian and avian erythrocytes [30–33], in cultured fibroblasts [34], in human neutrophils and lymphocytes [35,36], in Ehrlich ascites tumor cells [37–39], in protoplasts and Gram-negative species of bacteria [25,40–43], in a few samples of plant/fungal protoplasts and vacuoles [44–47], and in a variety of vesicle and organelle preparations [44,48–55].

Reports have also been registered of the use of LSI distribution probes to determine membrane voltages in intact plant and fungal cells [56–64], but those have been surrounded with controversy and evidence that in walled species, equilibrium and steady-state distributions of LSI probes are complicated and only indirectly related to membrane voltage [10,11,65–70]. After following these arguments for several years, we decided to try to use the small lipid-soluble cations tetraphenylphosphonium (TPP^+), tetraphenylarsonium (TPA^+), and triphenylmethylphosphonium ($TPMP^+$) to measure membrane voltages in the yeast *Saccharomyces* and to calibrate/verify the technique specifically on the mycelial fungus *Neurospora* – where direct measurement of membrane voltages with microelectrodes has long been possible, as well as on liposomes.

In conducting these experiments, we were unable to find any conditions in which the above-mentioned ions, used in tracer-probe concentrations (1–100 μM), distributed to equilibrium with membrane voltage in *Neurospora* or yeast; in almost all cases the probes stabilized at very low apparent concentrations in the cells, so that allowance for subcellular compartmentation would increase, rather than mitigate, the discrepancy between observed and predicted (equilibrium) distributions.

2. Materials and methods

2.1. Growth and handling of cells

Yeast strain NCYC 239 of *Saccharomyces cerevisiae* was maintained in primary stock cultures at 4°C on mineral-supplemented YPD-nutrient agar (YPD = 1% yeast extract, 2% bactopeptone, and 1.5% agar (Difco Laboratories, Detroit, MI)), containing 15 mM $(NH_4)_2SO_4$ and 2% glucose as nitrogen and carbon sources [71]. Secondary stock cultures of 100 ml liquid YPD were grown to stationary phase in shaking cultures (30°C, 24 h, reciprocating shaker at 180 cpm) to O.D. 30 (about $6 \cdot 10^8$ cells/ml). These could be stored for 2–3 weeks at 4°C, and 10-ml aliquots were removed as needed, to start 1-liter production cultures for experiments. The latter were grown to mid log-phase, harvested, washed, stored, and reactivated as previously described [72].

Neurospora crassa strain RL21a was maintained in stock cultures on N-minimal medium [73], plus 2% sucrose and 3% agar. For electrical experiments, conidia were inoculated by needle onto scratched cellophane atop minimal agar plus 2% sucrose, in 9-cm Petri dishes, and grown overnight at 25°C [74,75]. For use, centimeter squares of mycelium attached to cellophane were cut from the plate, transferred to a recording chamber on the stage of a compound microscope, and suffused with flux buffer (compositions in Table 1).

For flux experiments, *Neurospora* conidia were suspended in sterile distilled water, harvested by Millipore filtration (type RA filters; Millipore, Bedford, MA), rinsed, resuspended in distilled water, and inoculated into 160-ml suspensions of minimal medium plus 2% sucrose. These were incubated on a reciprocating shaker at 25°C for 9–13 h (mid log-phase), harvested on cheesecloth, rinsed, and resuspended in the test buffers at densities near 1 mg (dry weight) per ml. Flux experiments were carried out both on normal (high-K^+) cells and on Na^+-loaded cells, prepared by preincubation of rinsed cells (2 h, 25°C) in K^+-free, Na^+-rich buffer at pH 8.2, prior to resuspension in test buffer.

Liposomes were prepared from acetone-washed asolectin by modification of the procedure described by Kagawa and Racker [76]. Approx. 1240 mg of commercial asolectin (Avanti Polar Lipids, Birmingham, AL) was rinsed on a small Buchner funnel with $3 \cdot 10$ ml of reagent grade acetone (J.T. Baker, Phillipsburg, NJ), transferred to a round-bottom flask, dried under a stream of nitrogen, and rehydrated by sonication to clarity (about 8 min in a bath sonicator; Model B12, Branson Equipment, Shelton, CT) into an oxygen-free solution containing 20 mM potassium-Mes (pH 6.5, usually, or 5.0) and supplemented with 150-, 300-, or 350-mM KCl (see Fig. 3). Vesicles were sedimented at 49000 rpm ($230000 \times g$) for 2 h in a Beckman L2-65B

ultracentrifuge with an SW50.1 rotor. The pellet was resuspended in fresh buffer at a density of ~300 mg/ml, and 0.25-ml samples were diluted into 14.75 ml of test buffer for the transport assays.

2.2. Solutions

Compositions of the growth media are given above. Variations on several different 'standard' buffer solutions were used for flux and electrical measurements, and the compositions of the standard solutions are listed in Table 1. The buffers Mes, Hepes, and Tris were obtained as reagent grade chemicals from Calbiochem-Behring (LaJolla, CA), and DMG and citric acid were obtained from Sigma (St. Louis, MO).

In electrophysiological experiments (Figs. 6,7,8, 10,11), 1 mM NH_4Cl was added to the standard buffers to suppress electrical transients which can arise from NH_4^+ depletion [77]. Other variations from the standards included omission of Ca^{2+} or glucose, addition of KCl (1 to 300 mM), or changed pH. The particular variations are noted in each figure legend, along with the time and concentration for each addition of LSI.

The three main lipid-soluble ions used in these experiments, TPA^+, TPP^+, and $TPMP^+$, were chosen because their extensive and well-documented use with bacteria and bacterial protoplasts [55,78–80] has made them very attractive for prospective studies of membrane voltage in plants and fungi [56,58,63]. All were obtained as chloride salts from ICN Pharmaceuticals (Plainfield, NY). The three LSI salts were dissolved directly into flux buffer (maximal practical concentration for all three, about 60 mM). For experiments using ion-specific electrodes, cells were added to flux buffer already containing the LSI, in order to allow pre-equilibration of the recording electrode; but for electrophysiological experiments, control voltages and resistances were determined in LSI-free buffer, prior to perfusion of the chamber with the test concentration.

Table 1
Compositions of standard buffer solutions

Name species	Buffer ions concn. (mM)	pH	KOH (mM)	NaOH (mM)	$CaCl_2$ (mM)	Glucose (% w/v)
K·Hepes	Hepes 30	8.2	25	0	1	1
Na·Hepes	Hepes 30	8.2	0	25	1	1
K·Mes	Mes 20	6.5	14	0	0	0
Na·Mes	Mes 20	6.5	0	14	0	0
K·DMG	DMG 20	5.8	25	0	1	1
Na·DMG	DMG 20	5.8	0	25	1	1
Tris·CIT	citrate 15	4.5	20 mM Tris		0	0

DMG, 2,2-dimethylglutaric acid; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

2.3. Ion electrodes and recording apparatus

Uptake of TPP^+ from external concentrations at or below 100 μM was monitored by means of ion-specific electrodes fabricated from LSI-doped PVC membranes [81–83]. Separate 10 mM solutions (50 ml) of TPP-chloride and sodium tetraphenylborate (NaTPB; Pfaltz and Bauer, Stamford, CT) were prepared and mixed 1:1 to yield a white precipitate of TPP · TPB salt. An equal volume of dichloroethane (DCE; 100 ml) was added, and the mixture stirred vigorously overnight at room temperature. The organic phase was then drawn off through a separatory funnel and dryed on a rotary evaporator. The residue was redissolved in one-half volume (50 ml) of tetrahydrofuran (TFH). In parallel manipulations, dioctyl phthalate (DOP, a plasticizer; Eastman Kodak, Rochester, NY) and PVC were dissolved successively in TFH in the ratio 0.4 g/1.5 ml/10 ml. The resulting solution was mixed gently with one-quarter volume (3 ml) of the above LSI salt in THF, and the uniform mixture was poured into a large glass Petri dish (16-cm), whence the solvent was allowed to evaporate (overnight, room temperature and pressure, under a loose dust-cover). The resulting membrane, about 0.2 mm thick, was stripped with a razor blade, punched with a thin-walled glass tube, and glued to the end of a piece of 5 mm PVC tubing which had been moistened with THF. After several hours of drying, the half-closed cylinder was fitted onto a suitable glass tube, and the whole structure was filled with 1 M KCl + 10 mM TPPCl. Individual electrodes constructed in this way could be stored for several weeks with the tip immersed in the same solution. PVC, DCE, and THF were obtained as reagent grade chemicals from Aldrich Chemical (Milwaukee, WI).

These electrodes were connected, via a salt bridge (5 M NaCl), to an Ag-AgCl half-cell, and that and a calomel reference electrode were connected to an electrometer amplifier (Model 610B, Keithley, Cleveland, OH) circuited as diagrammed in Fig. 1. The PVC-membrane electrode was calibrated against flux buffer containing 1, 3, 10, 30, 100, 300, and 1000 μM TPP^+, and gave a linear response of 58 to 60 mV/log unit increase of $[TPP^+]_o$, over the entire range. Parallel measurements of solution pH (liposome experiments) were made with a separate pH- and reference-electrode combination (Model 4094L, A.H. Thomas, Swedesboro, NJ), arranged as in Fig. 1, with both strip-chart and digital readout. All measurements were carried out in a thermostatted vessel, at 30°C for liposomes and yeast, and at 25°C for *Neurospora*; the cell suspension (15 ml) was bubbled with air through a small glass frit and stirred with a magnetic flea. Most electrodes showed a small offset of voltage (about 20 mV) from the equilibrium voltage for TPP^+ (E_{TPP}), and experiments were therefore conducted by first

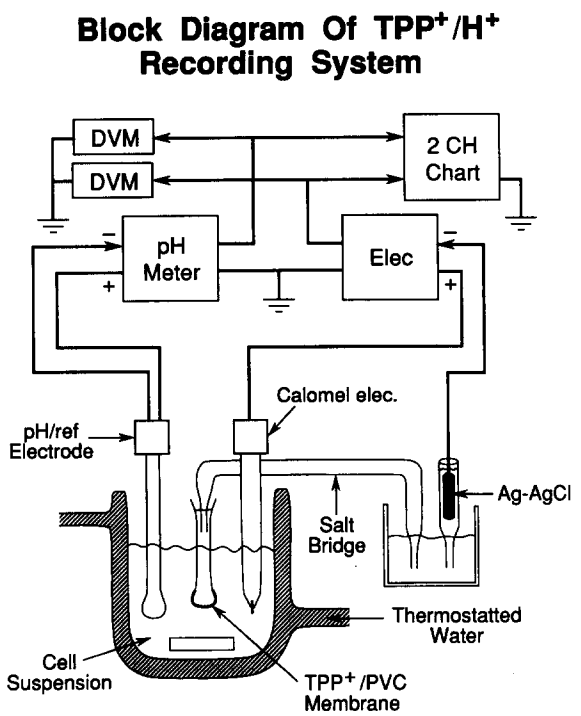

Fig. 1. Block diagram of TPP^+ recording system. DVM, digital volt meter; 2 CH, 2-channel chart recorder; Elec., high impedance electrometer (Model 610B, Keithley, Cleveland, OH). Working volume of chamber, 15 ml; temperature, 30°C for liposomes and yeast, 25°C for *Neurospora*. During use with cells of yeast or *Neurospora*, suspension was bubbled with a fine air stream.

equilibrating the electrode with flux buffer containing the desired TPP^+ concentration, then adding cells or liposomes. Depletion of solution TPP^+ by the transporting membranes was then calculated from the change in electrode reading (see, e.g., Fig. 2).

Apparent membrane voltages (V_m) of the cells or vesicles were calculated from steady-state TPP^+-depletion curves via the formula

$$V_m = \frac{RT}{F} \ln \left[\frac{(C_{o0} - C_{o\infty}) v_o + C_{i0} v_i}{C_{o\infty} v_i} \right] \quad (1)$$

in which C_{o0} and $C_{o\infty}$ are the starting and final extracellular TPP^+ concentrations, C_{i0} is the starting intracellular concentration, v_o is the extracellular volume, and v_i is intracellular water (intraparticle water). To obtain v_i for cells, 10 ml of reserve suspension was put through a Millipore filter, and the resulting cell mat was rinsed three times with 10 ml distilled water, removed from the filter, dried overnight at 90°C, and weighed. Since the ratio of intracellular water to dry weight (ICW/DW) for *Neurospora* is 2.54 [84], and for *Saccharomyces* is 2.3–2.6 [85,86], the average value in the present experiments was taken to be 2.5. Thus v_i for each cell sample was calculated as 2.5 × the total pellet dry weight. To obtain v_i for liposomes, the stock suspension was mixed with ^3H_2O and [^{14}C]sorbitol, and the apparent internal space was calculated from the difference of accessible volumes for the two isotopes. The resultant ratio, liposome dry weight: contained H_2O volume, was 0.67.

2.4. Chemical measurements

ATP was measured by a modification of the method previously described [87]. Cells were harvested in the usual manner for flux experiments, but the cell pellets were dashed immediately (before rinsing) into 5 ml of 50% ethanol/water at 80°C then capped and extracted for 30 min. Thereafter, the samples were chilled on ice, and frozen at −70°C for storage. For analysis, they were thawed in a 25°C-waterbath and filtered through polycarbonate filters. Pellets were removed, dried overnight at 90°C, and weighed. Filtrates were stored on ice until assayed (in duplicate) by injection of 0.1 ml into a standard mixture of firefly luciferase.

2.5. Electrophysiological techniques

Basic procedures for measuring membrane voltage were described previously [88]. Large, mature hyphae (15–20 mm diameter) from the cellophane cultures were selected for recording. Conventional borosilicate capillaries (1 mm o.d.; Hilgenberg Glas, Malsfeld, Germany) containing an internal fiber were used to pull electrodes with tips of < 0.5 μm diameter. These were filled by injection of 1 M KCl through the butt, mounted on an agar/salt bridge (1 M KCl) and an Ag-AgCl half-cell, and led to the input of a capacity-compensated electrometer amplifier (input impedance = $3 \cdot 10^{12}$ ohm; Model 750, WP Instruments, Sarasota, FL). For estimation of membrane resistance (R_m, see Fig. 8), two or three microelectrodes were inserted into single *Neurospora* hyphae, one to inject square pulses of current, one nearby to monitor membrane voltage and the current-induced changes of voltage (ΔV), and in some experiments another 100–200 mm away to monitor the decrement of current signal. *Absolute* values of membrane resistance were calculated by means of core-conductor theory previously developed for *Neurospora* hyphae [89], and *relative* membrane resistance, with changing conditions, was estimated by squaring the ratio of ΔV values for each pair of conditions, as expected for a one-dimensional linear cable: $R_{m2}/R_{m1} = (\Delta V_2/\Delta V_1)^2$.

For some experiments (see Figs. 6 and 7), separate LSI sensor electrodes were constructed to monitor ionic changes in the immediate extracellular space. These sensors, which were ordinary microcapillary pipettes filled with 1 M potassium citrate, had large tip voltages (−25 to −100 mV) and were sensitive to the ionic strength of the bathing medium. They responded smoothly and rapidly to additions of 5 mM LSI (or more) to the usual 25–30 mM salt in the flux buffers.

For mechanical stability of *Neurospora* studied with penetrating microelectrodes, extracellular free $[Ca^{2+}]$ must be maintained at least at 0.1 mM, and that concentration or higher (usually 1 mM), was present in the flux buffers for electrophysiological experiments. All such experiments were carried out at the ambient temperature, 21–23°C.

2.6. Computations

Data analysis via flux and regression equations (Figs. 3,4) was carried out by the generalized least-squares algorithm of Marquardt [90]. Display graphics for Figs. 3–5 and 8–10 were created via a program written by Dr. Bliss Forbush, III (Yale Department of Cellular and Molecular Physiology). Cellular ion concentrations are stated throughout as 'mM', meaning mmol/kg cell water.

3. Results

3.1. Distribution of TPP^+ in liposomes

The first step in demonstrating the feasibility of using LSI distributions to measure membrane voltages generally is to establish a reference condition under which they clearly do work. We decided to simplify this task by adopting a purely chemical system, asolectin liposomes which had been selectively permeabilized for H^+ or K^+ ions.

Typical records from two experiments with TPP^+ are shown in Fig. 2, for liposomal membranes 'clamped' by a pH difference in the presence of the protonophore FCCP. The TPP^+-sensitive electrode (upper trace in each pair) registered movement of that ion in synchrony with imposed changes of pH (lower traces), as well as in response to membrane-active agents (e.g., DOC). Fig. 3B summarizes results from all liposome trials of this type, with apparent intravesicle TPP^+ concentration ($[TPP^+]_i$) calculated via Eq. (1). Fig. 3A gives corresponding results for liposomes 'clamped' by K^+ differences in the presence of the specific ionophore valinomycin. For both sets of experiments, the data were easily described by a straight line with a Nernst slope, 1:1 between the log (concentration) scales, or (−)60 mV/l.u. on the voltage scale, at 30°C.

The major conclusion from these experiments is that, for liposomal membranes, LSI's do seem to equilibrate with membrane voltages set by $[H^+]$ or $[K^+]$ differences, but only over a range of about 2.5 log units (300-fold; 150 mV). Calibration tests showed this limitation to be intrinsic to the liposomes, not to performance of the TPP^+ electrodes. The same point is also clear from the traces of Fig. 2, since maximal changes

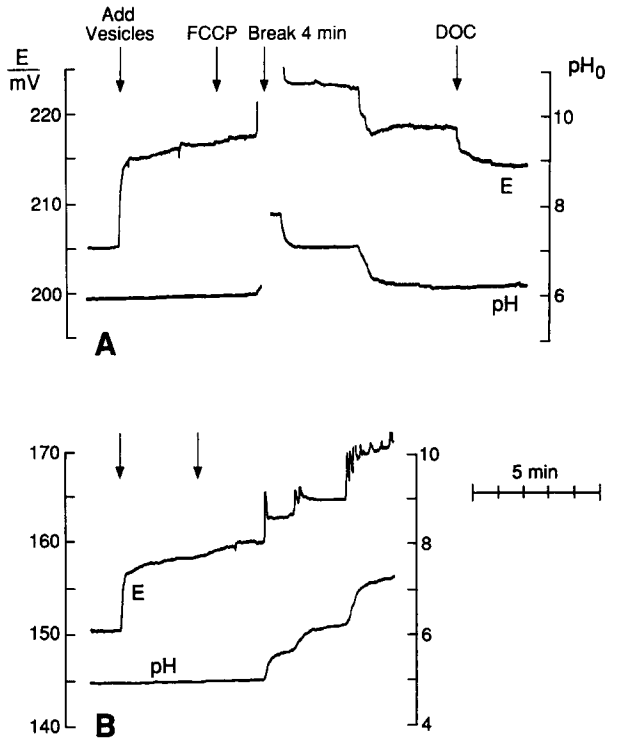

Fig. 2. Uptake and release of TPP^+ by liposomes, in response to changes of extracellular pH. (A) Asolectin vesicles prepared in K·Mes (pH 6.0) + 350 mM KCl, then diluted into the same buffer + 10 μM TPP^+ for recording. The uncoupling agent FCCP (10 μM in 0.1% ethanol, final concentrations) was injected at the second arrow, and 1 mM DOC (a weak ATPase inhibitor [91]) was added at the fourth arrow. External pH was changed by injecting KOH or HCl, where indicated by sudden shifts in the lower trace. (B) Vesicles prepared similarly to the above, but diluted into K·Mes (pH 5.0) + 350 mM KCl + 100 μM TPP^+. FCCP added at the second arrow, and KOH at the three successive jumps of pH_o. Upper trace (each panel): responses of the TPP^+ electrode, with increasing recorded voltage (negative) indicating removal of TPP^+ from free solution. Apparent intraliposomal TPP^+ concentration calculated by assuming all shifts to be strictly between the vesicles and free solution. The TPP^+-distribution ratio and apparent liposomal membrane voltage were calculated from these records, via text Eq. (1).

of ~15 mV – less than 50% depletion – were involved. Scatter of the data points in Fig. 3, however, probably originated from the electrodes, not the liposomes, since individual electrodes tended to be noisy (see, e.g., end of upper record, Fig. 2B).

3.2. TPP^+ distribution into cells

Experiments similar to the above were carried out with suspensions of *Saccharomyces* and *Neurospora*, with the averaged results shown in Fig. 4. As with the liposomes, internal concentrations of TPP^+ stabilized within 1–2 min for both organisms. More than 90% of TPP^+ uptake occurred via a single-exponential process, described by Eq. (2):

$$C_i = C_{i\infty}(1 - e^{-\alpha t}) \quad (2)$$

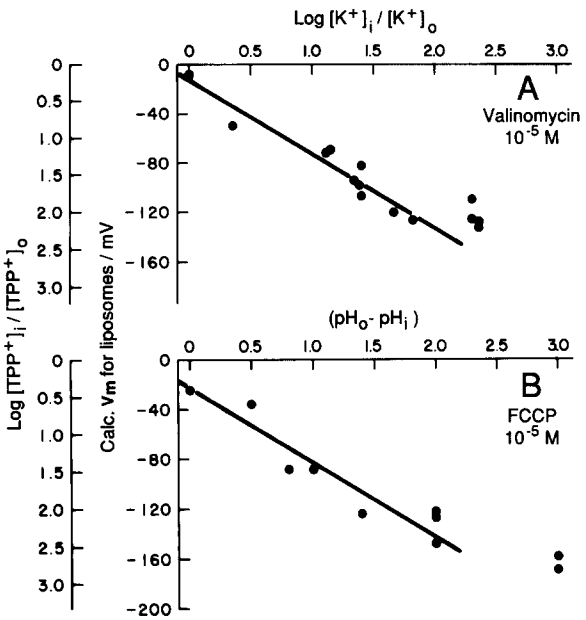

Fig. 3. Stable TPP^+ distributions in liposomes, compared with the transmembrane ratio of potassium (A) or protons (B). Summary of five experiments like that in Fig. 2, using 10 μM valinomycin to create K^+ permeability, or 100 μM FCCP to create H^+ permeability. The lines, drawn constrained to the theoretical slope of 60 mV/log unit, do not differ significantly from the unconstrained regression lines, having slopes of 59.0 mV/l.u. (A) and 56.0 mV/l.u. (B). Points beyond the line segments drawn were excluded from the fits. Apparent membrane voltages (inner ordinate scales) calculated via text Eq. (1). Thus, TPP^+ distribution is a reasonable estimator of membrane voltage in liposomes, up to about (−)150 mV, but not beyond that limit. The small offsets of both intercepts from the origin (10–20 mV negative) suggest a roughly 2-fold underestimate of liposomal volume.

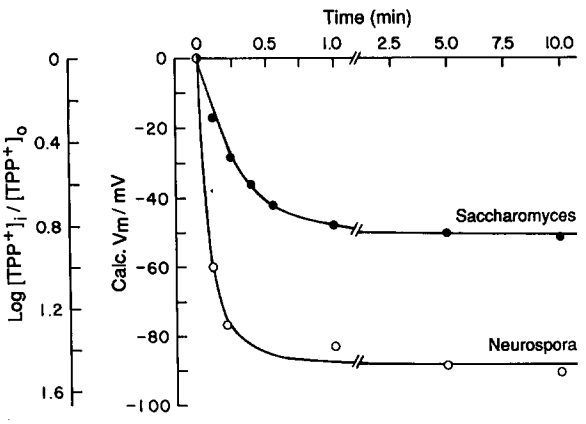

Fig. 4. Uptake of TPP^+ by yeast and *Neurospora*, as a function of time. Experiments similar to those of Fig. 2, but with cell suspensions instead of liposome suspensions. More than 90% of transported TPP^+ was taken up as a single exponential component, and each curve has been drawn from a least-squares fit of $[TPP^+]_i$ (vs. time) to text Eq. (2). Rate constants: for *Saccharomyces*, 2.93/min; for *Neurospora*, 5.14/min (half-times of 14 s and 8 s, respectively). Flux buffers: Tris·CIT + 10 μM TPP^+ for *Saccharomyces*; K·Hepes + 100 μM TPP^+ for *Neurospora*. Apparent membrane voltages calculated via text Eq. (1), with $v_o / v_i \sim 170$ and 400, respectively, for the two species.

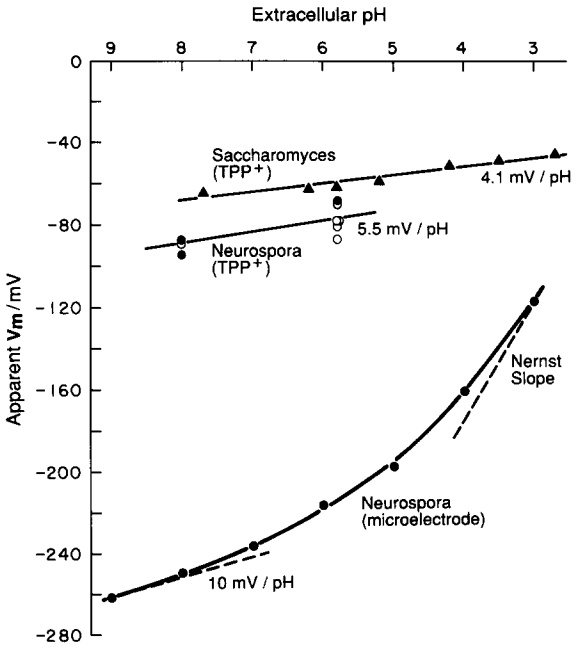

Fig. 5. TPP^+-distribution estimates of membrane voltage in yeast and *Neurospora* (upper two plots), compared with electrode measurements on *Neurospora* (bottom curve). TPP^+ estimates from five experiments similar to those of Fig. 4. Lines drawn by least-squares to the data plotted. Electrode measurements from previously published experiments [74]. Clearly, steady-state TPP^+ distribution does not approach equilibrium with the directly measured membrane voltage at any tested pH_o; furthermore, TPP^+ distribution is practically insensitive to pH_o, while measured membrane voltage is very sensitive, at least for *Neurospora* below pH 6. Solutions as follows: for yeast, K·DMG (pH 2.7–7.8) + 10 μM TPP^+; for *Neurospora*, K·DMG or K·Hepes, plus 10 μM TPP^+, with (●) or without (○) Ca^{2+}.

but both rate and extent of uptake were greater for *Neurospora* ($\alpha = 5.14$/min; $C_{i\infty} = 2.7$ mM with $[TPP^+]_o \approx 90$ μM) than for *Saccharomyces* ($\alpha = 2.93$/min; $C_{i\infty} = 66$ μM with $[TPP^+]_o \approx 9.5$ μM), corresponding to i/o ratios of about 29 and 7, respectively, for the two species. Furthermore, as is demonstrated in Fig. 5, (upper two plots) the distribution ratio – now described as apparent membrane voltage – proved only slightly sensitive to extracellular pH (4–6 mV/l.u.) for both species. Comparison of the *Neurospora* data with direct electrical measurements of membrane voltage (Fig. 5, lower plot) showed the TPP^+-distribution results to be essentially unrelated to the actual membrane voltage, both in absolute magnitude and in pH-sensitivity.

The disparity was largest at high pH, with the electrically measured membrane voltage being more than 150 mV negative to that calculated from TPP^+ distribution. That fact underscores another point: if the limitation on distribution measurements observed with liposomes (linearity up to −150 mV, vesicle interior negative) applies also to cells, then the normal membrane voltages of *Neurospora* are simply out of range

for the distribution technique. But the *actual* limitation with cells of *Neurospora* is even more severe, since membrane voltages negative to −150 mV were observed at all pH_o values above 4, but the diffusion voltages for TPP^+ (E_{TPP}) were more than 50 mV positive to −150 mV at all pH_o values.

While no unequivocal direct measurements of resting membrane voltage in this species of yeast are yet available, the overall resemblance of the TPP^+ measurements to those in *Neurospora*, in both magnitude and lack of pH dependence, clearly argues that the LSI measurements on *Saccharomyces* are just as specious as those on *Neurospora*.

Two additional points must also be emphasized. First, intracellular sequestration of the lipophilic probe into a non-diffusional compartment, often invoked to account for discrepancies, is irrelevant in this case, because it should give averaged intracellular concentrations which are too high, not too low; and second, metabolic damage to the cells – which might be imagined to result from TPP^+ treatment – cannot account for the low distribution ratios of the LSI, since no short-term effects of 10–100 μM TPP^+ were observed on electrically measured V_m, oxygen consumption, or ATP levels (data not shown).

3.3. Depolarization at higher LSI concentrations

The very fact that TPP^+ or TPA^+ at concentrations below 1 mM had no immediately measurable effect on membrane electrical parameters raised the question of whether their entry might be entirely non-ionic. We therefore used higher concentrations (1–30 mM) of several LSI's to investigate this question. Effects on membrane voltage are illustrated in Figs. 6 and 7. At concentrations of 20–30 mM, approaching the limit of aqueous solubility for TPP^+ or TPA^+, depolarization was rapid but multiphasic. And only the initial component of response, amounting to 35–80 mV depolarization, *could* have reflected current actually transported by lipid-soluble ions, as can be seen by comparing record d of Fig. 6 and record a of Fig. 7 with the others. The sensor trace below these two records shows that fluid exchange in the recording chamber was complete (in the immediate vicinity of the cell under study) within 15 s, which coincided with the duration of initial depolarization in all the other records.

Moderate concentrations of LSI's, in the range of 1–2 mM, produced effects similar to those of the higher concentrations, but acted about 3-fold more slowly (Fig. 7, records b,c). In addition to the multiphasic time-course of depolarization, records with 1–2 mM TPP^+ and TPA^+ show two features which are strong indications of metabolic involvement, during washout of the LSI's: a tendency toward oscillation of the membrane voltage [92,93]; and a delay, whereby removal of extracellular TPP^+ immediately following the 15-s entry period (up arrow in Fig. 7 record b) apparently did not alter the time-course of continuing depolarization for at least 1–2 min, as if primary action were from an intracellular reservoir.

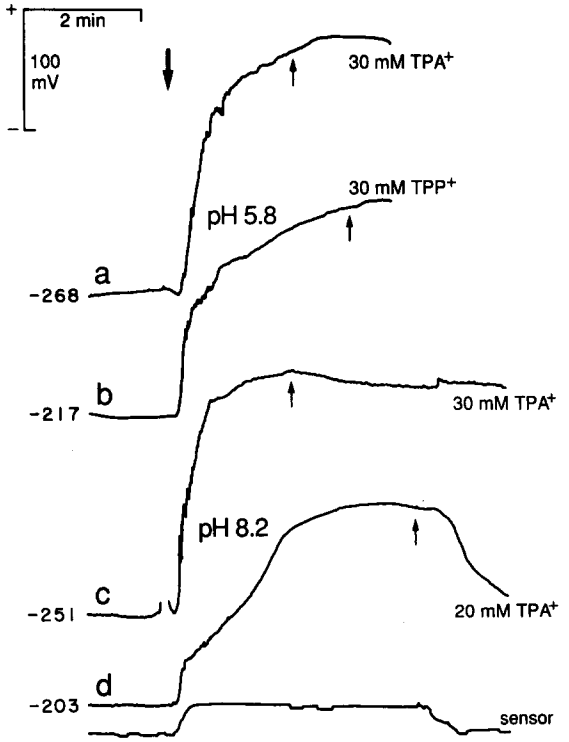

Fig. 6. Multiphasic depolarization of *Neurospora* by high LSI concentrations. TPA^+ or TPP^+ injected into the recording chamber at down arrow (zero time). Washout begun at up arrows. An initial rapid component of depolarization coincided with the rise of LSI concentration (record d, lower trace), but at least two slower components followed. Even for *fresh* preparations, the individual waveforms were quite variable. Conspicuously slow second components, as in record d, *always* followed prior insults, such as anoxia or mechanical stress (see also Fig. 11). Records a, b: recording buffer was Na·DMG+NH_4^+; c, d: Na·Hepes+NH_4^+.

3.4. Membrane resistance measurements and the first phase of depolarization

A more precise view of charge flow associated with LSI uptake can be obtained by examining the behavior of membrane resistance. With an explicit circuit model for the cell membrane, the resistance change corresponding to a particular voltage change, upon LSI entry, can be calculated. An appropriate equivalent circuit for normal *Neurospora* hyphae is shown in Fig. 8A (inset) – in which I_p represents a current source due to the electrogenic proton pump (functioning, at −200 mV, far from its reversal potential, which is beyond −400 mV [89]); R_d and E_d represent the diffusional resistance and equilibrium voltage; R_L and E_L represent the corresponding properties for LSI^+

entry; and closure of the switch (S) represents initiation of the experiment by addition of extracellular TPP^+ or TPA^+. R_L can then be calculated from the observed depolarization (ΔV_m) as

$$\frac{R_L}{R_d} = \frac{E_d - E_L + R_d I_p - \Delta V_m}{\Delta V_m} \quad (3a)$$

whence the fractional change (reduction) in overall membrane resistance (ΔR_m) should be simply

$$\Delta R_m = \frac{R_d}{R_d + R_L} \quad (3b)$$

In this formulation, the absolute value of R_d does not matter. Rough figures for the voltage terms in Eq. (3a) are $E_d = -40$ mV and $R_d I_p = -160$ mV [88,89]. $E_L$would initially be undefined, but uptake at > 100 mM/min [94], with 20 mM outside, would bring E_L to ~ 60 mV within 1 s and to ~ 0 mV in 15 s, making $+30$ mV a reasonable average for the initial phase of depolarization. Since the initial depolarizaton during rapid perfusion of the recording chamber with 20–30 mM TPP^+ or TPA^+ is 35–80 mV over 15 s (see Figs. 6 and 7), ΔR_m should be 15–35%.

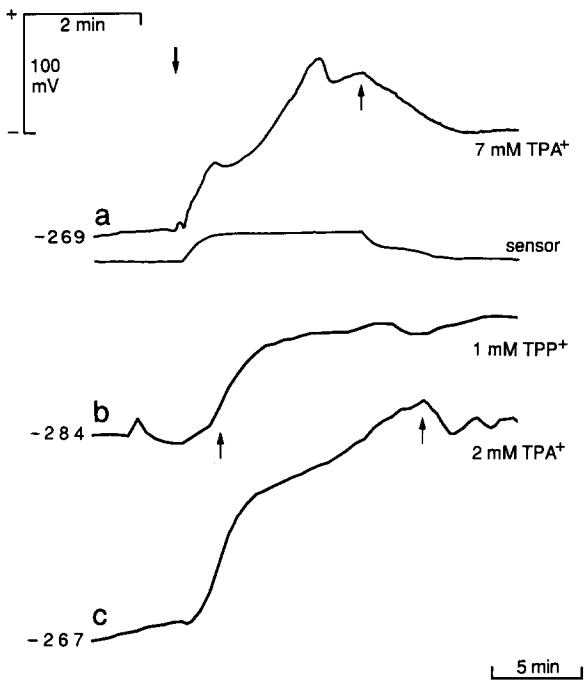

Fig. 7. Initial effects of moderate LSI concentrations (7–1 mM) upon recorded membrane voltage in *Neurospora*. TPP^+ or TPA^+ admitted to the chamber at down arrow, and washout begun at up arrows. Again, depolarization was multiphasic in all cases, and no convincing differences in the response wave form could be attributed to the particular LSI. Note that even with early washout (record b), considerable depolarization was sustained, consistent with secondary effects of intracellular accumulation. For records b and c, original traces were digitized and replotted by computer, in order to compress the time scale; separate time scale for these two curves is at lower right. Solutions: a, Na·Hepes *without* Ca^{2+}; b, c, Na·Hepes.

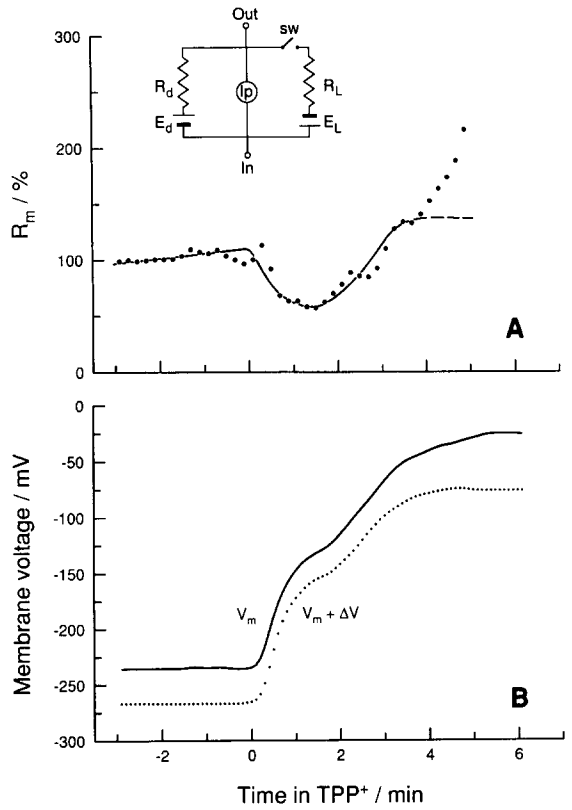

Fig. 8. Measurement of resistance change during initial TPP^+-induced depolarization. (A, inset) Equivalent circuit model for the *Neurospora* membrane. R_d, R_L: Resistances of normal ion diffusion regime, and LSI regime, respectively. E_d, E_L: equilibrium voltages for the two diffusion regimes (value changes continuously for E_L). I_p: current driven by the plasma membrane's electrogenic proton pump. (A, main) Time-plot of calculated membrane resistance (R_m), relative to control. Basic experiment as in Figs. 6c,d, but using two intracellular electrodes: one to measure voltage and the other to inject square pulses of current (5 s, $3 \cdot 10^{-9}$ A). (B) Voltage trace (V_m, upper curve) and superimposed voltage responses (ΔV). (The first (rapid) phase of depolarization is usually slower in multiple-electrode experiments than in single-electrode or ion-flux experiments because of mechanical sensitivity of the preparation.) Again, three phases of depolarization are evident in the V_m trace. *Relative* membrane resistance, R_m, was calculated as $100 \cdot \Delta V^2/(\Delta V_{t=0})^2$, to accommodate spread of current in the hyphal filament of *Neurospora* [89,95]. K·Hepes buffer (pH 8.2) at ambient temperature; 20 mM TPP^+. (The sudden large increase of R_m at 3.5 min was an artifact due to closing of hyphal crosswalls.) Membrane resistance dropped $\sim 35\%$ during the initial rapid phase of depolarization, stabilized during the slower second phase, then progressively increased in the third phase.

Data from a typical sequence of resistance measurements, during the onset of TPP^+ uptake, are shown in the main panels of Fig. 8. The simple measurement of membrane potential (V_m) is shown by the solid trace in panel B, on which are superimposed the steady-state voltage displacements (ΔV) in response to fixed square pulses of current, $3 \cdot 10^{-9}$ A. 20 mM TPP^+ was injected into the perfusion stream at 0-time, and relative membrane resistance, calculated (from ΔV^2) as described in Materials and methods, is plotted as the

dotted curve in panel A. It can be seen that membrane resistance fell 35% during the first phase of depolarization. Subsequent depolarization, at least out to 3.5 min (see legend to Fig. 8) was accompanied by a slow *increase* of membrane resistance. The voltage parameters of this experiment require recalculation of the expected resistance change, with $R_d I_p = -195$ mV and $\Delta V_m = 100$ mV during initial TPP^+ uptake, from which Eqs. (3) yield $R_L/R_d = 1.65$, and the expected value of $\Delta R_m = 38\%$.

The observed coincident decline of membrane voltage and resistance indicates that at least a fraction of the LSI influx occurs *as ions*, but the magnitude of ionic flux, per se, depends upon the absolute value of R_d. That ranges from ~15 to ~3 kΩ cm^2 (Slayman, C.L., Gradmann, D. and Blatt, M.R., unpublished experiments; Ref. [89]), so the range of currents implied by the experiment of Fig. 8 is 7–30 μA/cm^2. Those numbers correspond to 5–22 mM/min flux on the basis of cell volume, compared with total observed LSI influxes under comparable conditions of 55–60 mM/min (Figs. 5 and 9 of Ref. [94]), i.e., 3–10-fold larger.

3.5. ATP depletion and the second phase of depolarization

Under the general conditions of these experiments, 20 mM TPP^+ caused a rapid decline of cytoplasmic ATP concentration to about 25% of the control value, followed by a small rebound and subsequent further decline, as is shown in Fig. 9. This sort of quasi-oscillation, with lengthening period, has been reported previously during prolonged use of respiratory blocking agents or mitochondrial uncoupling agents [77], again pointing to metabolic effects (mitochondrial uncoupling?) of the higher LSI concentrations. Quantitatively, the resolvable initial time-course of ATP decline, which lasts for 2–3 min, is readily superimposed (Fig. 9, inset) on the time-course of membrane voltage, averaged for all experiments like those in Fig. 6, records a–c. This makes it likely that the second phase of depolarization, also lasting out to 2–3 min, occurs simply *because* of the withdrawal of ATP, according to the known Michaelian behavior of the rheogenic plasma-membrane ATPase ($K_{m(ATP)} \approx 1.5$ mM [98]). Thus, with ATP falling from ~2 mM at 15 s (end of the first-phase depolarization) to 0.7 mM (minimum at 2–3 min), pump velocity would diminish from ~60% of its maximum to about ~30%, yielding an average depolarization of about 80 mV, which is indeed near the average observed for the second phase of depolarization (Figs. 6 and 9).

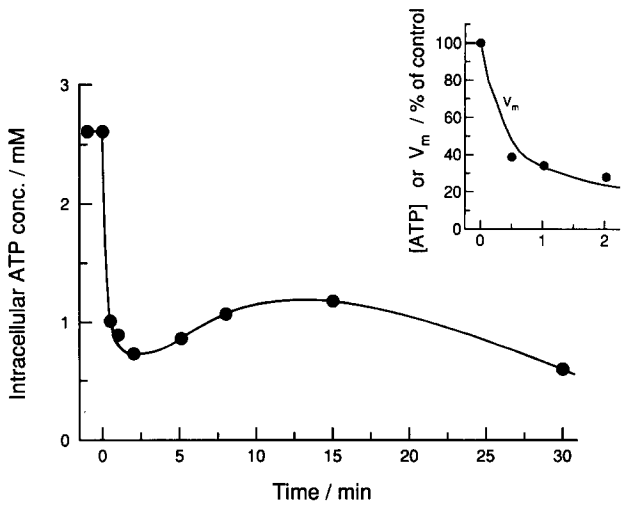

Fig. 9. Response of ATP concentration in *Neurospora* to 20 mM TPP^+. Cells incubated in K·Hepes. Oscillations of ATP and other metabolites in response to metabolic down-shifts or up-shifts are well known in microbial systems [96,97], and time curves like this for ATP have been seen in specific response to submaximal respiratory inhibition [77]. (Inset) Comparison of initial [ATP] points with the time-course of membrane voltage, averaged for curves a,b, and c in Fig. 6.

3.6. Comparison of uptake levels at high $[LSI^+]_o$ with V_m

By means of a series compartmental model [94] it is possible to compute sequential LSI concentrations in both a free cytosolic compartment and an internal storage compartment, from total TPP^+-influx data. Then from the calculated cytosolic concentrations and the known extracellular concentrations of TPP^+, the diffusion potentials (E_{TPP}) can be calculated via the Nernst equation. A comparison of such results with voltage traces from comparable electrophysiological experiments is shown in Fig. 10. The salient feature in comparison is that cytoplasmic [TPP^+] did seem – eventually – to approach equilibrium with the V_m; but essential caveats are that it happened only when the cells were strongly depolarized, whether $[TPP^+]_o$ was high (20 mM) or only moderate (2 mM), and that the *rate* of approach was independent of the actual membrane voltage.

3.7. Adaptation processes

Transport experiments are normally carried out just a single time on a single batch of cells, so that the population is treated only once with the test species. Electrophysiological experiments, on the other hand, are usually conducted so that test reagents can repeatedly be added and removed. The latter kind of protocol is useful in studying long-term time dependencies, and has in the past revealed adaptation of the *Neurospora* membrane to metabolic changes by up- and down-regulation of both the proton pump and the ensemble membrane 'leaks' [77,89,99]. In the present experiments, voltage measurements made during re-

peated addition and removal of bath TPP^+ or TPA^+ showed striking reduction in the amplitude of depolarization with second and third trials. This effect is illustrated in Fig. 11, records a and c, which should be compared with Fig. 6b and Fig. 7a, respectively. In most such experiments, the control membranes were slightly depolarized (incompletely recovered), immediately before second or third trials. However, on average this could account for less than 50% of the response decrement in multiple trials of LSI's. The remainder was seen as increased (negative) membrane voltages at steady state in the sustained presence of TPP^+ or TPA^+. Even more surprisingly, transient pretreatment of the cells with unrelated metabolic inhibitors, such as cyanide or azide, also greatly attenuated the depolarizing effect of LSI's, as is demonstrated in Fig. 11, records b.

It is apparent, therefore, that fungal cells have a metabolic defensive barrier which – at least in the short term – can slow down damage due to non-physiologic lipid soluble ions. Conceivably, such a barrier could operate in naive cells at low LSI concentrations, thus accounting for the failure of TPP^+ to approach equilibrium with membrane voltage (Figs. 4 and 5). Comparison of Fig. 11 with Figs. 6 and 7, then, would suggest the barrier to be overwhelmed by high concentrations of LSI, but restored by interrupted treatment or by other transient metabolic blockades. Metabolically triggered resistance to general membrane-active agents, such as lipid-soluble ions, is an astonishing process, which has been described previously on other microorganisms (see, e.g., Ref. [100]) but has not yet been adequately explained. It is important to note, however, that such processes can easily falsify simple interpretations of LSI transport-kinetic data, as well as uncritical distribution 'measurements' of membrane voltage.

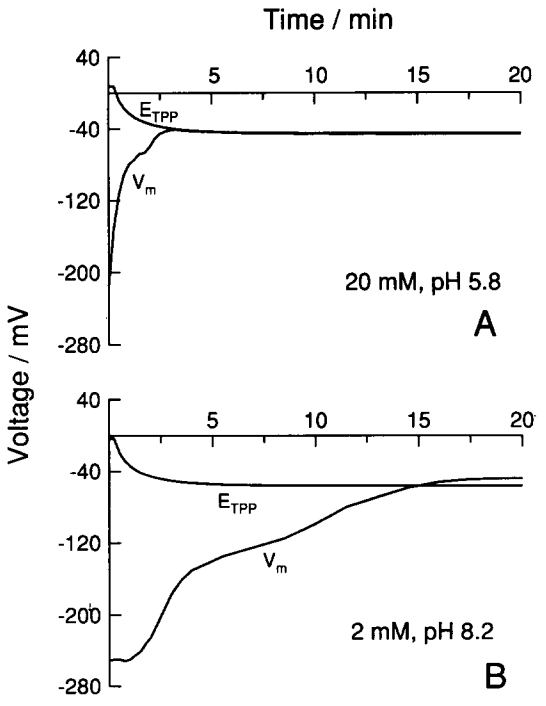

Fig. 10. Approximate equilibration of TPP^+ with membrane voltage in strongly depolarized *Neurospora*. (A) Equilibrium voltage for TPP^+ (E_{TPP}) calculated from ^{3}H-TPP^+ uptake (Slayman et al. [94], Fig. 1); approximate membrane voltage (V_m) calculated as the average for all curves similar to Fig. 6 a,b, and scaled to the average maximal value at pH 5.8 (−215 mV). (B) Equilibrium voltage for TPP^+ also calculated from ^{3}H-TPP^+ uptake; approximate V_m taken as Fig. 7c, scaled to the average maximal value at pH 8.2 (−250 mV).

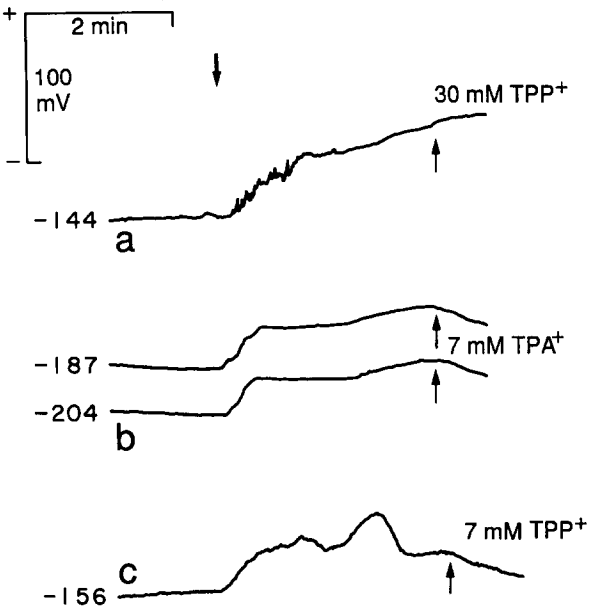

Fig. 11. Insult-induced desensitization of *Neurospora* membrane voltage (V_m) to treatment with LSI's. (a) Record made 16 min after onset of washout from Fig. 6, rec. b; V_m had recovered about 60% from the first TPP^+ treatment; solution: Na·DMG + NH_4^+. (b) Two separate cells in a single preparation, with records made 10 min after washout from respiratory blockade by 1 mM sodium azide; V_m had recovered 90% from azide effect; solution as above. (c) Record made 11 min after onset of washout from a trial like that in Fig. 7, rec. a, but with TPP^+; V_m recovered about 25% from the earlier treatment; solution: Na·Hepes + NH_4^+. Down arrow, LSI entry to chamber; up arrows, beginning of washout.

4. Discussion

4.1. Underlying assumptions

In order to use lipid-soluble ions as distribution probes for membrane voltage in biological systems, four critical assumptions are needed: (1) that transfer of the particular LSI species across the membrane under study is passive and not influenced by either primary or secondary active processes; (2) that transfer occurs with the LSI only in a known *charged* state; (3) that no significant subcompartmentation of the LSI occurs, on either side of the membrane, and (4) that

entry of the LSI does not itself alter the membrane voltage. Other ancillary assumptions may also be necessary, depending specifically upon the mode of assay for the LSI. For example, if a fluorescence assay is used, that the fluorescence calibration curve extends over a sufficient range and is independent of the measuring conditions. Of course the simple LSI's – like TPP^+, TPA^+, and $TPMP^+$ – are non-fluorescent and are assayed by more direct chemical means, either in cells or in the bathing medium. Therefore, the present results are concerned with the four critical assumptions and not with serious ancillary ones.

4.2. Intrinsic limitations of the measurements

Our choice of asolectin liposomes as a reference system to check the method of measurement was based on their structural simplicity [76], which should guarantee a close approach to assumptions (1) and (3). The finding that TPP^+ entry into liposomes follows the expected membrane voltage only up to about (−)150 mV (Fig. 3) confirms some previous reports [101,102]. The implied limitation presumably lies in localized depletion of LSI somewhere along the transit pathway, leading either to a band of nearly zero conductance, or to a very large microscopic electric field which is not reflected in the macroscopic ion concentrations. These two ideas actually converge, when LSI's are considered (for thermodynamic reasons) to concentrate at the aqueous/lipid interfaces across phospholipid bilayer membranes [103,104]. In that circumstance, enormous surface charges can build up, positive with TPP^+ and other lipid-soluble cations, and a deep well of cation exclusion results in the bilayer interior. Past workers have tried to countermand this effect by doping membranes with lipid-soluble anions, such as tetraphenylborate (TPB^-) or phenyldicarbaundecaborane (PCB^-), and have had limited empirical success [22,50,105]. However other investigators have pointed out that this maneuver easily leads to spurious binding of the probe ion and the high likelihood of transport via a neutral complex [106,107], thus violating critical assumptions (2) and (3) listed above.

The intrinsic methodological limitation implied by the results in Fig. 3 have not generally been regarded as important in studies of bacteria or animal cells, whose expected membrane voltages rarely (bacteria) or never (animal cells) exceed (−)150 mV; but that limitation is very important in the present context, since plant and fungal cells do routinely function at greater membrane voltages, in many cases much greater.

4.3. Cell-imposed restrictions

The primary observation about TPP^+ (and TPA^+ or $TPMP^+$) used as a tracer probe for membrane voltage in *Saccharomyces* or *Neurospora* is that cellular uptake of the ions is monophasic. Monophasic uptake has tempted other investigators to use the *rate* of LSI entry, rather than end concentration, as a measure of membrane voltage, particularly when the approach to steady-state is slow (half-time > 1 h [58]). Such slowness has been observed with microalgae, higher plants [47], and filamentous fungi [60], and with all genera of yeast examined [57,61,65,68].

However, slow entry of LSI's into cells – especially when associated with multiple phases [61,68] – seems a clear warning that at least one of the four critical assumptions does not hold. This can be inferred from several facts of the present experiments: (a) that entry of TPP^+ into liposomes, which lack proteins and other metabolic machinery, is rapid (and monophasic; Fig. 2); (b) that changes of membrane voltage and permeability occur over intervals of 10–100 s in cells with millimolar TPP^+ (Figs. 7 and 11) or lower; (c) that other metabolic events can easily affect LSI permeability (Fig. 11; also Komor et al. [100]); and (d) that a variety of bona fide cellular transport processes occur on the longer time scale and might easily influence LSI movements.

Despite the monophasic time-course of probe (external concentration $\leq 100\ \mu M$) LSI uptake by *Saccharomyces* and *Neurospora* (Fig. 4 above; and cf. Refs. [56,70]), steady-state levels achieved in the present experiments proved surprisingly low (Fig. 4). They were also surprisingly insensitive to extracellular pH (Fig. 5), which is known to have a substantial effect on the actual membrane voltages of *Neurospora* [74] and upon TPP^+ distributions in another yeast, *Rhodotorula glutinis*. For *Neurospora* the case now is clear: steady-state distributions of TPP^+ (and TPA^+ or $TPMP^+$) bear no meaningful relationship to actual membrane voltages at any pH_o within the range tolerated by the organism. Indeed, the discrepancy at high pH_o is so large (nearly 150 mV) that $[TPP^+]_i$ would be 300-fold too dilute in the total cytoplasm, under steady-state conditions. Since the distribution curves for TPP^+ into *Saccharomyces* closely parallel those for *Neurospora* (Figs. 4 and 5), it is safest to conclude that TPP^+ distribution is unrelated to membrane voltage in both cases. The observation that treatment of *Neurospora* with *high* concentrations of LSI's does yield transmembrane distributions near equilibrium with the membrane voltage (Fig. 10) is interesting, but not very useful; in this condition the cells are are strongly depolarized (Figs. 6 and 7), and would be generally unable to carry out normal membrane processes.

The results of Figs. 4 and 11 also reveal a fifth critical assumption underlying the use of LSI probes for membrane voltage: viz., that the cells must have no adaptive mechanism to resist entry of the probes. The phenomenon of electrostatic exclusion from the membrane – a purely physical mechanism reducing appar-

ent LSI permeability – has been discussed above in connection with the liposome data (Fig. 3). But for both yeast and *Neurospora* exclusion is even more severe: in no case did measured TPP^+ distribution ratios even approach the saturating range (300-fold, 150 mV) observed for pure liposomes. Indeed, actual steady-state ratios were all below 30-fold.

The nature of this extraordinary exclusion process(es) is unknown, but of several plausible types of mechanisms, the simplest would be that the physical permeability of the membrane to LSI's declines progressively during loading, so that after a few minutes it reaches such a low value (say, $< 3\%$ of control) that further net entry cannot be resolved on the time-scale of these experiments. Such an interpretation would be consistent with the observation that pretreatment with higher concentrations of LSI's, or with specific metabolic inhibitors, sharply reduces the immediate depolarization at high concentrations of LSI's (Fig. 11).

A second type of mechanism would be an active transporter which could extrude LSI's from the cytoplasm. The plausibility of such a device derives from two quite separate observations: (a) that bacteria and cultured mammalian cells possess generalized drug-resistance systems, which may be membrane ATPases [108]; and (b) that plant and fungal cells, which have normal membrane voltages (-150 to -350 mV) well in excess of the diffusion voltages for common ions, must possess extrusion systems to deal with excess influx of Na^+, K^+, and NH_4^+ encountered under many circumstances of growth. Although active extrusion of K^+ and NH_4^+ by plants and fungi has been little studied (but see Peña and Ramirez [109]), appropriate mechanisms are required strictly on thermodynamic grounds. And since some uptake systems for ammonium and alkali metal cations in fungi function rather non-specifically, exchanging a wide range of amino cations [110–112], it is reasonable to expect extrusion systems to do likewise.

4.4. High-concentration effects

All direct electrical experiments carried out on *Neurospora* at TPP^+ (and TPA^+ or $TPMP^+$) concentrations at or below 100 μM seemed to satisfy the fourth critical assumption listed above, of not influencing the membrane voltage, at least over our usual test interval of about 10 min. Short-term toxicity, thus, is slight, probably in part because of the unexpectedly slow entry of LSI's. Although electrode-based continuous measurements of LSI uptake from the medium could not be made at concentrations ≥ 1 mM, tracer-flux measurements [94] could be made, along with the gamut of electrical measurements.

The salient result of treating *Neurospora* with (1–30) millimolar TPP^+ or TPA^+, in the presence of added (1 mM) extracellular calcium, is rapid, but clearly multiphasic, depolarization of the plasma membrane. Three components are discernable in most records: (I) an initial rapid component (half-times, $t_{0.5}$, of a few seconds under rapid-flow conditions), which coincides with solution exchange within the recording chamber (Figs. 6 and 7), and is accompanied by a nearly proportional fall of membrane resistance (Fig. 8A); (II) a slower second component ($t_{0.5} \approx 20$ s) during which membrane resistance stabilizes, but cytoplasmic ATP concentration declines roughly in parallel with the voltage (Fig. 9); and (III) a slow ($t_{0.5} \geq 1$ min) and highly variable third component which may include small oscillations of voltage and is accompanied by some recovery of both ATP level (slow transient; Fig. 9) and membrane resistance (Fig. 8A).

A plausible interpretation of these results is as follows. (I) represents partial short-circuiting of the membrane by the LSI ion-flow; as was remarked above (Fig. 8 ff) and is demonstrated later [94], TPP^+ or TPA^+ *current*, as such, is only a small fraction of the total LSI influx. (II) represents a decrease in activity of the plasma-membrane's electrogenic proton pump, consequent upon the decline of cytoplasmic ATP. Although this might seem surprising to readers familiar with other P-type ATPases (the Na^+,K^+-ATPase, for example, or the Ca^{2+}-ATPase of sarcoplasmic reticulum), which have *micro*molar K_m values for ATP [98], it works in fungi because those H^+-ATPases have *millimolar* K_m values for ATP [87,98], at least under most operant conditions. Interpretations (I) and (II) are both supported quantitatively by the data. Finally, (III) probably reflects complex metabolic events, including transport regulatory processes invoked by the metabolic downshift due to *intracellular* lipophilic ions. One such process, demonstrated in the companion paper (Figs. 10–12 [94]), is a time-dependent shut-down of the LSI-induced efflux of cytoplasmic alkali metal cations. This general interpretation is also consistent with a host of other observations on *Neurospora*, in which transport turnover is modulated by metabolic shifts [77,99,113,114].

5. Conclusions

The elementary conclusion from these experiments is that certainly with *Neurospora*, and evidently with *Saccharomyces* as well, small lipid-soluble cations cannot be used as distribution probes for membrane potential. The reasons are many, but they are mostly subsumed by the statement that such ions – or more specifically tetraphenylphosphonium (TPP^+) and its congeners – do not, within reasonable time (several tens of minutes) reach equilibrium with physiologic membrane voltages, despite the fact that high-con-

centration experiments reveal a finite electrophoretic component of LSI entry. Limiting factors are of at least three types, revealed under different conditions: intrinsic to bilayer physics (restriction to a concentration ratio [i/o] of ~300-fold, or (−)150 mV), inhibited by normal physiology (restricted to ~30-fold, or (−)85 mV), and further inhibited, with some delay, by metabolic downshifting (restriction to ~7.0-fold, or (−)50 mV). In view of the fact that normal membrane voltages for *Neurospora* can lie between −200 and −300 mV, the use of TPP^+ and its congeners as membrane voltage probes for the organism must be abandoned; and that fact makes their use as voltage probes for similar kinds of cells – i.e., other fungal cells, and plant cells – highly suspect.

6. Acknowledgements

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