J. Membrane Biol. 81, 127–138 (1984)

The Journal of
Membrane Biology

Membrane Potential and Surface Potential in Mitochondria: Uptake and Binding of Lipophilic Cations

Hagai Rottenberg
Department of Pathology, Hahnemann University School of Medicine, Philadelphia, Pa. 19102

Summary. The uptake and binding of the lipophilic cations ethidium$^+$, tetraphenylphosphonium$^+$ (TPP$^+$), triphenylmethylphosphonium$^+$ (TPMP$^+$), and tetraphenylarsonium$^+$ (TPA$^+$) in rat liver mitochondria and submitochondrial particles were investigated. The effects of membrane potential, surface potentials and cation concentration on the uptake and binding were elucidated. The accumulation of these cations by mitochondria is described by an uptake and binding to the matrix face of the inner membrane in addition to the binding to the cytosolic face of the inner membrane. The apparent partition coefficients between the external medium and the cytosolic surface of the inner membrane (K'_o) and the internal matrix volume and matrix face of the inner membrane (K'_i) were determined and were utilized to estimate the membrane potential $\Delta\psi$ from the cation accumulation factor R_c according to the relation $\Delta\psi = RT/ZF \ln [(R_c V_o - K'_o)/(V_i + K'_i)]$ where V_o and V_i are the volume of the external medium and the mitochondrial matrix, respectively, and R_c is the ratio of the cation content of the mitochondria and the medium. The values of $\Delta\psi$ estimated from this equation are in remarkably good agreement with those estimated from the distribution of ^{86}Rb in the presence of valinomycin. The results are discussed in relation to studies in which the membrane potential in mitochondria and bacterial cells was estimated from the distribution of lipophilic cations.

Key Words mitochondria · membrane potential · surface potential · lipophilic cations · proton electrochemical potential

Introduction

Over a decade ago, Liberman et al. [12] showed that energized mitochondria accumulate lipophilic cations but not anions, while submitochondrial particles accumulate lipophilic anions but not cations. These results are compatible with the chemiosmotic hypothesis according to which mitochondria generate a negative membrane potential while submitochondrial particles, with inverted polarity, generate a positive potential [17]. Similar data were obtained with a large number of cells, organelles, vesicle preparations, liposomes and reconstituted systems [*cf.* 3–6, 9, 11, 26, 29, 30, 34, 35]. The basic assumption used in interpreting these experiments is that the membrane is permeable to lipophilic ions and that the uptake is a result of the electrochemical equilibration of these ions across the membrane. A similar uptake of inorganic cations was observed when the membrane was made permeable to cations by the addition of ionophores [*cf.* 6, 19, 22, 26]. These latter observations provided the basis for the estimation of $\Delta\psi$ in many systems [*cf.* 22, 23]. The use of radiolabeled lipophilic cations for the estimation of $\Delta\psi$ in bacterial vesicles was introduced by Schuldiner and Kaback [26]. In this system similar values of $\Delta\psi$ were obtained from the use of ^{86}Rb (+ valinomycin) and ^{3}H-TPMP$^+$.[1] In *E. coli* cells similar values were obtained by microelectrodes and TPP$^+$ [5]. However, in mitochondria and other systems it was noted that the values calculated from different cations are not identical [1, 10, 27, 33]. In several bacterial species similar discrepancies were also noted [2, 11, 34]. It is apparent that because of excess binding to membrane and other cell components the values of $\Delta\psi$ calculated from lipophilic ion distribution are often overestimated. Nevertheless, because of their convenience the use of these probes for quantitative estimation of $\Delta\psi$ is widespread. While it is recognized by most investigators that accurate estimation of $\Delta\psi$ from the distribution of lipophilic cations requires correction for binding contributions, no acceptable procedure has yet been proposed. In mitochondria, different correction procedures have been used for TPMP$^+$, without sufficient justification [10, 27, 33, 36]. More careful consideration was given to binding correction in some bacterial systems [11, 34]. But, no consideration was given, in any of these studies, to the

[1] Abbreviations used: TPP$^+$-tetraphenylphosphonium; TPMP$^+$-triphenylmethylphosphonium; TPA$^+$-tetraphenylarsonium; $\Delta\tilde{\mu}_H$-proton electrochemical potential.

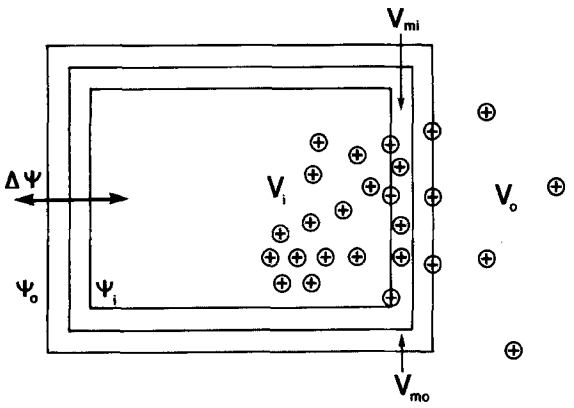

Fig. 1. Four compartment model for the analysis of ion binding and uptake in mitochondria

effect of surface potential on the binding. It is also apparent that it is not possible to generalize these correction procedures to other membrane systems and other probes.

Cafiso and Hubbell [3] have studied the binding of spin-labeled phosphonium ions in liposomes in relation to the transmembrane potential. Here, we describe mitochondrial binding of lipophilic cations by a model which is essentially identical to the model employed by Cafiso and Hubbell [3]. We consider the equilibrium distribution of lipophilic ions between four compartments (Fig. 1). The extravesicular solvent volume (V_o), the internal solvent volume (V_i), the volume of the external membrane surface (V_{mo})[2] and the volume of the internal membrane surface (V_{mi}). The mitochondrion contains two membrane systems—the inner and outer membranes. We are concerned with the distribution across the inner membrane. Of course, the outer membrane may contribute also to the binding. However, since there is no considerable membrane potential across the outer membrane, we will treat this membrane binding as part of the external surface of the inner membrane (V_{mo}). In fact, the surface area of the inner membrane is large compared to the outer membrane and it appears, with one exception, that the outer membrane, and other mitochondrial components, do not contribute significantly to ion binding (*see* Results). In the paper by Cafiso and Hubbell [3] the observable components were the total amount of bound probe on both sides of the membrane $N_{mo} + N_{mi}$, and the total amount of free probe, internal and external, $N_i + N_o$. Consequently, equations were derived to express these in terms of the partition coefficients, volumes and membrane potentials.

In studies of the binding and accumulation of radiolabeled lipophilic cations the observable parameters are the amount of external free ion N_o and the sum of the externally bound ions, the internally bound ions and internal free ions, i.e. $N_{mo} + N_{mi} + N_i$. We shall henceforce proceed to derive relations between both the membrane potential and surface potential and the above observable parameters.

The amount of externally bound ion is related to the external concentration, N_o/V_o, by the following expression:

$$N_{mo} = K_{mo}\alpha_o N_o V_{mo}/V_o \tag{1}$$

where K_{mo} is the ion partition coefficient for the external surface of the membrane and α_o is a function of the external surface potential ψ_o:

$$\alpha_o = e^{-ZF\psi_o/RT}. \tag{2}$$

The amount of internal free ion is related to the external concentration by the following relationship:

$$N_i = \beta N_o V_i/V_o \tag{3}$$

where β is a function of the transmembrane potential $\Delta\psi$,

$$\beta = e^{ZF\Delta\psi/RT}. \tag{4}$$

The amount of internally bound ion is related to the internal concentration, N_i/V_i, by the following expression:

$$N_{mi} = K_{mi}\alpha_i N_i V_{mi}/V_i \tag{5}$$

where K_{mi} is the internal surface partition coefficient and α_i is a function of the internal surface potential ψ_i equivalent to Eq. (2). The observable parameters are the amount of externally free ion N_o and the sum of externally bound, internally bound and internally free ion, $N_{mo} + N_{mi} + N_i$, which can be expressed as an accumulation fraction R_c, where

$$R_c = (N_{mi} + N_i + N_{mo})/N_o. \tag{6}$$

[2] The membrane volume occupied by the phospholipids on the matrix and cytosolic surface of the inner membrane is approximately half the volume of the total phospholipids of the inner membrane. Quite likely the lipophylic cations occupy only a fraction of this volume. While this volume fraction is unknown it is proportional to the total membrane volume and therefore to the membrane protein content which is routinely measured. In the applications that follow the apparent surface membrane concentration is expressed in units of nmol/mg protein. The units of the apparent partition coefficients K', (nmol/mg protein)/(nmol/μl), are therefore expressed in μl/mg protein.

From Eqs. (1), (3) and (5) we get

$$R_c = K_{mi}\alpha_i\beta V_{mi}/V_o + \beta V_i/V_o + K_{mo}\alpha_o V_{mo}/V_o \quad (7)$$

which after rearrangement and solving for β gives

$$\beta = (R_c V_o - K_{mo}\alpha_o V_{mo})/(V_i + K_{mi}\alpha_i V_{mi}). \quad (8)$$

We solve Eq. (8) to express the transmembrane potential $\Delta\psi$ as a function of the accumulation fraction R_c; thus from Eqs. (4) and (8) we get

$$\Delta\psi = (RT/ZF)\ln[(R_c V_o - K_{mo}\alpha_o V_{mo})/(V_i + K_{mi}\alpha_i V_{mi})]. \quad (9)$$

Equation (9) allows us to calculate $\Delta\psi$ from the measured accumulation factor R_c if we know the compartment volumes V_i, V_o, V_{mo}, V_{mi}, the internal and external partition coefficients K_{mo} and K_{mi} and the surface potentials ψ_i and ψ_o on each side of the membrane. For ions which do not bind extensively to the membrane such as Rb^+ the partition coefficients K_{mo} and K_{mi} are vanishingly small[3] and Eq. (9) is reduced to the Nernst equation

$$\Delta\psi = (RT/ZF)\ln(R_c V_o/V_i) = (RT/ZF)\ln(C_{in}/C_{out}). \quad (10)$$

In this study we have attempted to evaluate the binding parameters for several lipophilic cations in rat liver mitochondria so as to provide a physically justified correction procedure for the determination of $\Delta\psi$ with these ions.

Materials and Methods

Ethidium bromide was purchase from Aldrich; valinomycin, ADP, CCCP, $TPMP^+$, TPP^+ and TPA^+ were obtained from Sigma; ^{86}Rb, [^{14}C]-$TPMP^+$, [^{14}C]-TPP^+, and [^{3}H]H_2O were products of Amersham and [^{3}H]-TPA^+ was a gift from Dr. H.R. Kaback, Roche Institute of Molecular Biology, Nutley, N.J.

Rat liver mitochondria were isolated as described previously [24]. Submitochondrial particles were prepared from liver mitochondria as described previously [21]. The protein was determined by the biuret cyanide method [31] using bovine serum albumin as the standard. For the incubation of the mitochondria, in most cases, the incubation medium contained (in mM) 150 sucrose, 10 Tris, 100 NaCl, and 5 $MgCl_2$ at pH 7.4.

The membrane potential, $\Delta\psi$ and ΔpH were determined as follows [23]: Three parallel samples were prepared, each containing ^3H_2O (50 μCi/ml). Sample A, for determination of the sucrose-impermeable space (matrix space), contained [^{14}C]-sucrose (1.7 μCi/ml), sample B, for determination of ΔpH, contained [^{14}C]-DMO (1.7 μCi/ml) and sample C, for determination of $\Delta\psi$, contained $^{86}Rb^+$ (0.13 μCi/ml, 2 to 5 μM Rb^+). Mitochondria (2 to 3 mg protein), 5 μM rotenone and 0.1 μM valinomycin were added to samples. A final volume of the reaction mixture was 1.5 ml. After incubating for 1 min, the reaction was started by adding 10 mM succinate or 4 mM ATP and was stopped 3 min later by centrifuging at 12,000 × g for 3 min in a microcentrifuge Eppendorf, model 5412. A sample from the supernatant (50 μl) and the pellet are dissolved in 0.5 ml of 14% perchloric acid. After incubating overnight, the precipitate was pelleted and a sample of 0.35 ml from the extracts was mixed with 5 ml of scintillation liquid (Packard, Insta-gel) and counted (Packard, model B460C). Membrane potential and ΔpH were calculated as described [23].

The binding of ethidium$^+$, $TPMP^+$, TPP^+ and TPA^+ to mitochondria and submitochondrial particles in the nonenergized state were determined as follows: Mitochondria (2 mg protein/ml) and submitochondrial particles (1 mg protein/ml) were incubated at 25°C for 3 min in 1.5 ml of a medium containing 150 mM sucrose, 10 mM Tris-Cl (pH 7.4), 100 mM KCl, 5 mM $MgCl_2$, 5 μM rotenone, 1 μM CCCP, 0.1 μM valinomycin, and various amounts of lipophilic cations. The mitochondrial pellet was obtained by centrifugation at 12,000 × g for 3 min. The submitochondrial particles pellet was obtained by centrifugation at 144,000 × g for 30 min (at 2°C). The pellets of the mitochondria and submitochondrial particles were dissolved in 1.5 and 0.5 ml of 1% sodium dodecyl sulfate, respectively, and the amount of ethidium in the pellet was determined from the absorbance at 510 nm and in the supernatant fluid from the absorbance at 480 nm. The amounts of radiolabeled lipophilic cations were determined by liquid scintillation counting as described above except that the pellet was dissolved in 1% SDS.

Results

To obtain estimates of the binding parameters K_{mo} and K_{mi}, we determined the binding of the lipophilic cations ethidium$^+$, TPP^+, TPA^+ and $TPMP^+$ to mitochondria as a function of the cation concentrations. In this experiment the standard medium contained antimycin A, CCCP and valinomycin to abolish the energy-dependent membrane potential. The high concentration of K^+ and Mg^{2+} salts abolish the Donnan potential and the membrane surface potential [21]. Figure 2*A* shows the concentration dependence of the binding of these cations to rat liver mitochondria. The binding is characterized by a low affinity and a high capacity and can be approximated, except in the case of ethidium$^+$, by a partition coefficient. Ethidium$^+$ binds most strongly; TPP^+ and TPA^+ bind moderately and $TPMP^+$ binds weakly. To test whether binding to components other than the mitochondrial inner

[3] When ^{86}Rb binding to de-energized mitochondria and submitochondrial particles was determined as a function of Rb^+ concentration (10 μM to 10 mM), there was no significant difference between the Rb^+ concentration in the pellet and supernatant. This indicates that at high ionic strength (where the surface potential is screened) the binding of Rb^+ to the mitochondrial membrane is negligible. This is compatible with the general low affinity of the alkaline cations to biological membranes. In energized mitochondria ^{86}Rb concentration ratio was not affected by the Rb^+ concentration up to 100 μM but was diminished in higher concentrations.

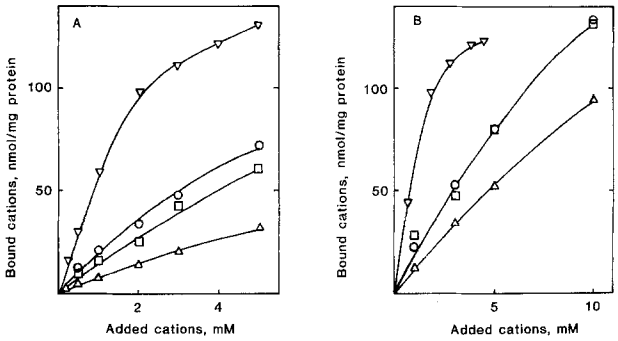

Fig. 2. Binding of lipophilic cations to nonenergized rat liver mitochondria and submitochondrial particles. Membranes were incubated with either ethidium$^+$ (▽), ^{3}H-TPP$^+$ (○), ^{3}H-TPA$^+$ (□), or ^{3}H-TPMP$^+$ (△) at the indicated concentrations. Conditions and experimental protocol as described in Materials and Methods. (*A*) Rat liver mitochondria. (*B*) Submitochondrial particles

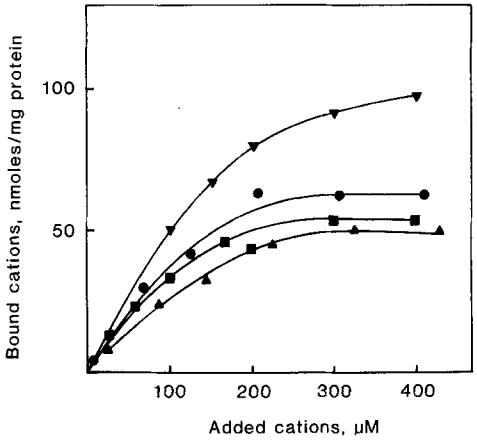

Fig. 3. Binding of lipophilic cations to energized mitochondria. Condition and experimental protocol as in Fig. 2*A* except that 10 mM Na-succinate was added to the incubation medium 5 min prior to centrifugation. Ethidium$^+$ (▼), TPP$^+$ (●), TPA$^+$ (■), TPMP$^+$ (▲)

membrane contribute to over-all binding in mitochondria we prepared inner membrane vesicles (submitochondrial particles). Figure 2*B* shows the binding of these cations to submitochondrial particles. The pattern of the binding is the same as in mitochondria. The amount of bound cation, nmol/mg protein, is approximately twice as high for TPMP$^+$, TPP$^+$ and TPA$^+$ but almost the same for ethidium$^+$. Since submitochondrial particles contain twice the amount of inner membranes (per mg protein) compared to mitochondria (as judged from the content of cyt *a*) and bind twice as much TPMP$^+$, TPA$^+$ and TPP$^+$ as mitochondria it is concluded that the binding of these ions can be accounted for mostly by the binding to the inner membrane. On the same basis ethidium binding appears to be equally distributed between the inner membrane and other mitochondrial components (possibly DNA) which are lost in the preparation of the inner membrane vesicles. The binding shown in Figs. 2*A* and 2*B* is contributed by both surfaces of the membrane so that no distinction between K_{mi} and K_{mo} can be made on the basis of these data.

Figure 3 shows the concentration dependence of the binding and uptake of the lipophilic cations in coupled mitochondria energized by succinate oxidation. The generation of a transmembrane potential results in a great increase in the apparent affinity of lipophilic cations but the binding appears to saturate at relatively low concentrations. This pattern can be explained, qualitatively, by the model discussed above. The formation of a negative membrane potential induces cation accumulation in the matrix and increases by several orders of magnitude the internal concentration. This high matrix concentration leads to extensive binding to the internal surface of the inner membrane which contributes greatly to the accumulation despite the low external concentration. Because the internal concentration is so high the internal binding is orders of magnitude greater than the external binding which results in increased apparent affinity. The saturation of binding appears because at higher cation concentration the membrane potential is collapsed, so that the internal concentration does not increase with external concentration and the accumulation appears to be saturated. Notice also that, since the binding is mostly to the inner face of the membranes it appears that the capacity is actually reduced. To see these aspects of the binding and accumulation more clearly, we analyze the data of Figs. 2 and 3 in a formal binding analysis (ignoring, for the time being, the fact that a certain fraction of the uptake is contributed by the internal free ion). Figure 4*A* shows the results of Fig. 2*A*, the binding of lipophilic cations to nonenergized mitochondria plotted as a double-reciprocal plot, 1/bound *vs.* 1/free. Here as in all subsequent calculations, we have subtracted from the total cation content the amount of bound cation to give the correct external free concentration. If the binding could be described by a simple partition coefficient all the lines would extrapolate to the origin, while the slope would indicate the value of K.

This approximation is fairly satisfactory but the lines in fact cross the Y-axis indicating that the binding would be saturated at very high concentration. This is reasonable since the volume of the membrane surface is limited. In fact binding of 250 nmol/mg protein means that there would be as many mol-

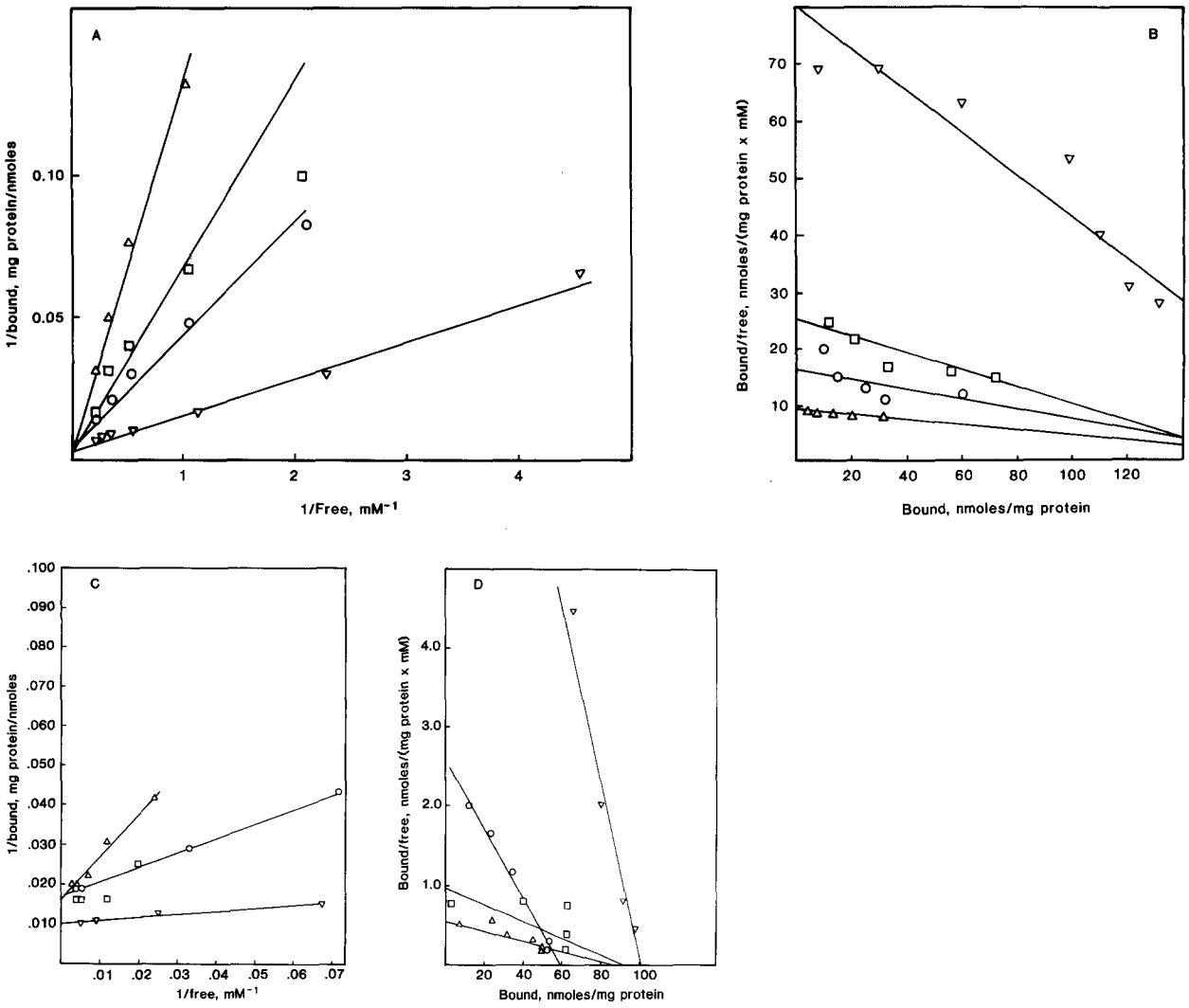

Fig. 4. Analysis of apparent binding parameters in energized and nonenergized mitochondria. The data are taken from Figs. 2*A* and 3. Figure 4*A* shows a double reciprocal plot 1/bound *vs.* 1/free for nonenergized mitochondria. Figure 4*B* shows Scatchard plots (bound/free *vs.* bound) of the same data. Figure 4*C* shows reciprocal plots for energized mitochondria and Fig. 4*D* shows Scatchard plots of the same data. Symbols indicate: Ethidium$^+$ (▽), TPP$^+$ (○), TPA$^+$ (□), and TPMP$^+$ (△)

ecules of lipophilic cations in the membrane as phospholipid molecules, which no doubt would lead to membrane disintegration[4]. Figure 4*B* shows the same data plotted as a Scatchard plot. The extrapolated capacity is very high, over 250 nmol/mg protein. Figure 4*C* shows the data of Fig. 3, the binding of lipophilic cations to energized mitochondria, in a double-reciprocal plot. The saturation and the highly increased affinity appear more clearly in this plot. The Scatchard plot (Fig. 4*D*) shows that the capacity is greatly decreased when compared to nonenergized mitochondria. This formal treatment does not take into account the effect of $\Delta\psi$ on the uptake nor the effect of the uptake on $\Delta\psi$ and internal binding. It is therefore of limited use for the description of the binding to energized mitochondria. However, the binding parameters for nonenergized mitochondria (Fig. 4*A*) can be used for the description of uptake if we know $\Delta\psi$.

To study the effect of surface charge on the binding of TPP$^+$ we have incubated the mitochondria in a salt-free medium with increasing concentrations of $MgCl_2$. Figure 5*A* shows that in nonenergized mitochondria the binding is reduced to a small fraction as the Mg^{2+} concentration is increased. KCl and NaCl similarly affect the binding

[4] The phospholipid content of rat liver mitochondria is approximately 200 nmol/mg protein.

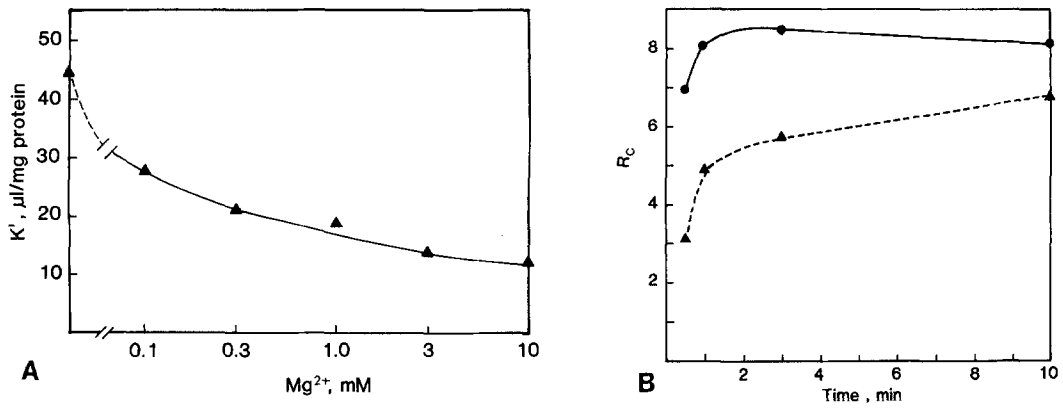

Fig. 5. Effect of surface potential on binding and uptake of ^{3}H-TPP$^+$ in rat liver mitochondria. Figure 5*A* shows the effect of $MgCl_2$ on the external binding of TPP$^+$ in nonenergized mitochondria. Mitochondria (2 mg protein/ml) are incubated in 0.25 M sucrose, 5 mM NaHEPES (pH 7.5) and 5 µM rotenone. In Fig. 5*B*, the time course of the uptake in the presence of 5 mM Na_2 succinate ± 10 mM $MgCl_2$ is shown. No Mg^{2+} (▲); +10 mM Mg^{2+} (●)

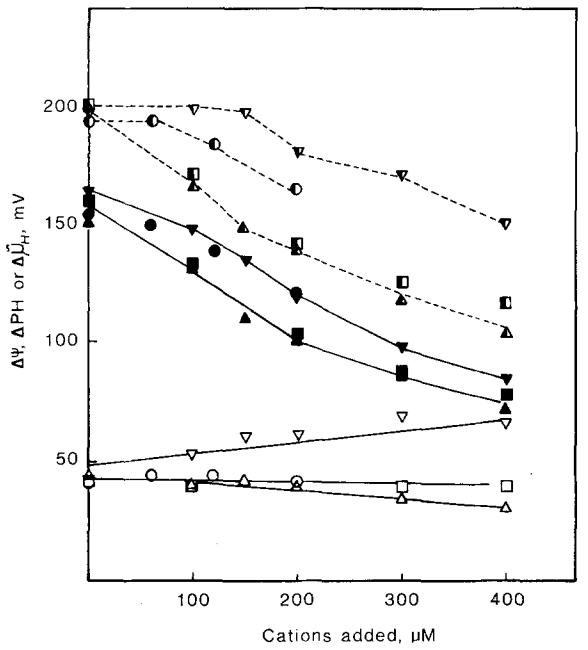

Fig. 6. Effect of lipophilic cation on $\Delta\tilde{\mu}_H$. Conditions and experimental protocol as described in Materials and Methods. The symbols indicate: Ethidium$^+$ (▽, ▼, ▼), TPP$^+$ (△, ▲, ▲), TPA$^+$ (□, ■, ◧) and TPMP$^+$ (○, ●, ◐). Open symbols ΔpH; closed symbols $\Delta\psi$; half-closed symbols $\Delta\tilde{\mu}_H$

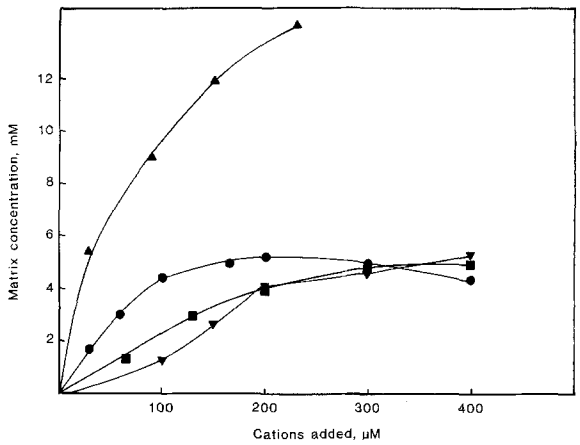

Fig. 7. Calculated matrix concentration of lipophilic cation in energized mitochondria. The matrix concentration is calculated from the data of Figs. 3 and 6. The data of Fig. 3 are used only for calculation of the external free concentration (total added cation-bound)/(volume). The determined values of $\Delta\psi$ from Fig. 4 are used to calculate the matrix concentration assuming electrochemical equilibrium $\Delta\psi = 59 \log (C_{in}/C_{out})$. Symbols as in Fig. 3

at the concentration range 10 to 200 mM (*not shown*). This effect is most probably due to the reduction and screening of the external surface charge [21]. In energized mitochondria, the external surface charge greatly affects the rate of equilibration of TPP$^+$. Without Mg^{2+} (or high concentration of monovalent salts) equilibrium is not obtained even after 10 min of incubation, while with Mg^{2+} equilibrium is established within 1 min (Fig. 5*B*). In a separate experiment mitochondria were incubated for 20 min with continuous supply of oxygen. After 20 min there was no significant difference between the binding in the presence and absence of Mg^{2+}. Thus, at equilibrium, the surface potential does not appear to affect the binding of TPP$^+$. This difference in the effect of surface potential on TPP$^+$ binding in energized and nonenergized mitochondria is compatible with Eq. (7) [*see also* Eq. (11), below]. In energized mitochondria most of the accumulation is ac-

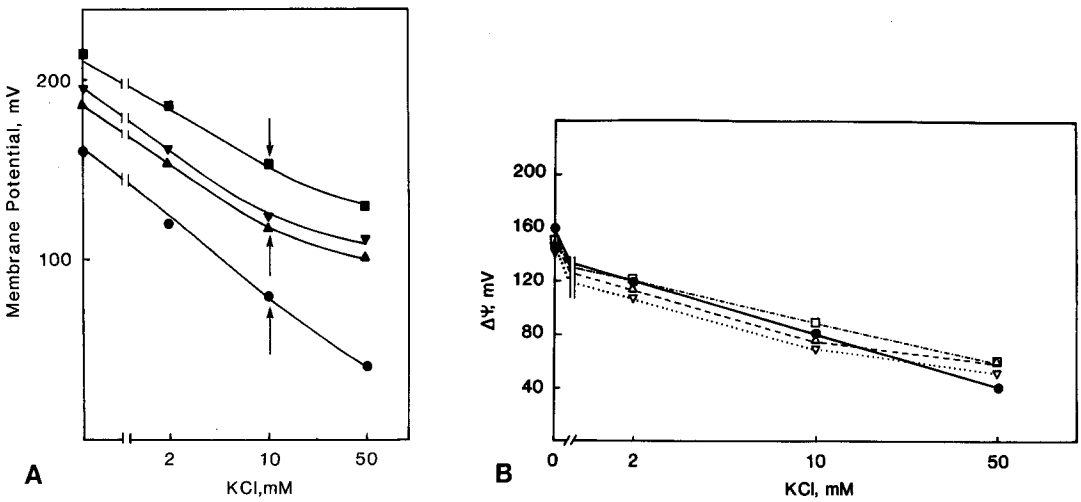

Fig. 8. Effect of KCl on the estimated membrane potential from the distribution of ^{86}Rb, ^{14}C-$TPMP^+$, ^3H-TPA^+ and ^{14}C-TPP^+. Conditions and protocol as described in Materials and Methods except that ^{14}C-methanol was used instead of ^3H_2O for volume determination in the ^3H-TPA^+ experiment. The lipophilic ion concentrations were 5 μM (TPP^+ and TPA^+) or 24 μM ($TPMP^+$). The pellet was dissolved in 1% SDS. The symbols indicate: TPP^+ (■, □); TPA^+ (▼, ▽); $TPMP^+$ (▲, △), and Rb^+ (●). In *A* the potential was calculated from the distribution of each ion without corrections for binding (Eq. 10). In *B* the potential was estimated from the lipophilic ion distribution using Eq. (11) and binding parameter of Table 1.

counted for by increased internal concentration and internal binding. When R_c is large, $K_{mi}\alpha_i\beta V_{mi} + \beta V_i \gg K_{mo}\alpha_o V_{mo}$ since the external binding is negligible compared to uptake and internal binding. To elucidate the relationship between the apparent binding parameters and transmembrane potential we have determined the relationship between $\Delta\psi$ (measured by the distribution of ^{86}Rb in the presence of valinomycin), ΔpH and the concentration of the lipophilic cations. Figure 6 shows the results of these experiments. All the lipophilic cations collapse the membrane potential as their concentrations are increased. Their effectiveness in reducing the potential is approximately the same, regardless of their affinity. This effect is probably due to the electrogenic transport of these cations across the mitochondrial membrane and the resulting short circuit of the membrane potential. However, only ethidium shows the expected parallel increase in ΔpH. It is possible that lipophilic cations increase anion permeability thereby stimulating anion efflux and H^+-coupled anion uptake which limit the magnitude of ΔpH. The decreased membrane potential with increased concentration of lipophilic cations leads to the apparent saturation of binding of these cations in energized mitochondria. This is demonstrated in Fig. 7 in which the calculated internal concentration of the lipophilic cations is plotted as a function of the external concentration. The internal concentration is calculated from the external free concentration and the measured $\Delta\psi$ assuming that the cations are at electrochemical equilibrium (data from Fig. 6). It is observed that after reaching a value of several mM the internal concentration does not increase with further increase of external concentration, because of the reduction in $\Delta\psi$. In this Figure we estimated the matrix-free cation concentrations from the measured ^{86}Rb distribution but not from the extent of total cation uptake. This is in contrast to the numerous studies in which the energy-dependent binding of the lipophilic cations is measured, assumed to be free in the matrix, and used to calculated $\Delta\psi$. To find the extent to which these procedures overestimate the potential, we show in Fig. 8*A* an experiment in which the membrane potential, measured from the distribution of ^{86}Rb, is compared with the potential calculated from the distribution of TPP^+, TPA^+ and $TPMP^+$. Ethidium would indicate even higher potentials. However, because the accurate measurement of free ethidium is more difficult in our assay, we have not included these data in the Figure. Valinomycin is included in all the systems, and the potential is reduced by increasing concentrations of KCl. It is observed that the uptake of all lipophilic cations, when used for calculation of $\Delta\psi$, overestimates the potential, when compared to ^{86}Rb. The overestimation increased from $TPMP^+$ to TPA^+ and TPP^+, and gets exceedingly high in the case of ethidium$^+$ (*not shown*). Hence, the overestimation appears to be a reflection of the amount of internally bound lipophilic cations. We have observed similar differences in calculated $\Delta\psi$ from

Table 1. Effect of lipophilic cation on $\Delta\psi$[a]

Cation	Cation added (nmol)	Cation bound (nmol/2 mg p)	R_c	K' (μl/mg p)	$\Delta\psi$ calculated (mv)	$\Delta\psi$ measured (mv)
$TPMP^+$	100	48	0.92	3.7	130	135
TPA^+	100	68	2.1	7.2	139	137
TPP^+	70	60	6.0	11.1	157	150
Ethidium$^+$	200	160	4.0	31.2	123	118

[a] $\Delta\psi$ was measured by ^{86}Rb distribution (Fig. 6) or calculated from the cation uptake (Fig. 3) utilizing Eq. (11). The apparent internal and external partition coefficients were assumed to be equal and were estimated from Fig. 4*A* as described in the text.

^{86}Rb and TPP^+, using the flow dialysis method [20] (Robertson & Rottenberg, *unpublished*). Therefore, these differences are not an artifact of the centrifugation method. At high potential $TPMP^+$ indicate a potential 28 mV higher than $^{86}Rb^+$ and TPP^+ 55 mV higher. At low potential $TPMP^+$ overestimates by 60 mV and TPP^+ by 84 mV. In order to estimate the membrane potential directly from the accumulation of the lipophilic cations we must therefore use Eq. (9) with the proper binding parameters. Although, the parameters estimated from Fig. 2 do not distinguish between the external and internal binding constants K_{mo} and K_{mi}, we can assume as a first approximation that $K_{mo} = K_{mi}$. Since we have used high salt concentrations in these experiments the surface potentials ψ_i and ψ_o are exceedingly small and hence $\alpha_i = \alpha_o = 1$. The surface membrane volumes V_{mo} and V_{mi}, are related to the membrane content, usually measured as mg mitochondrial protein. Therefore, we express the membrane concentration as nmol/mg protein and the partition coefficients in these units: (nmol/(mg protein)/(nmol/μl) which is equivalent to μl/mg p(protein). The terms $K_{mo}\alpha_o V_{mo}$ and $K_{mi}\alpha_i V_{mi}$ are considered as an apparent external partition coefficient K'_o and internal partition coefficient K'_i, respectively, and Eq. (9) can be written as

$$\Delta\psi = (RT/ZF)\ \ln[(R_c V_o - K'_o)/(V_i + K'_i)]. \qquad (11)$$

Assuming that $K_{mo} = K_{mi}$ and $V_{mo} = V_{mi}$, then $K'_i = K'_o$. The partition coefficients estimated from Fig. 4*A* are equal to $K'_i + K'_o$ since both surfaces contributed to the binding. Assuming that $K'_i = K'_o$ we get the following binding parameters: $TPMP^+$ 3.7 μl/mg p, TPA^+ 7.2 μl/mg p, TPP^+ 11.1 μl/mg p and ethidium$^+$ 31.2 μl/mg p (Table 1). To check the validity of Eq. (11) we use the accumulation factors derived from Fig. 3. At 100 μM added $TPMP^+$ the binding is 48 nmol/2 mg p, hence the free external concentration is 52 μM and $R_c = 0.92$. Since $V_o = 1000$ μl and $V_i = 2$ μl, $\Delta\psi = 59 \log [(0.92 \times 1000 - 3.7)/(2 + 3.7)] = 130$ mV. This value can be compared with the value of 135 mV obtained from ^{86}Rb distribution in the presence of 100 μM $TPMP^+$ (Fig. 6). It is observed that the correction for externally bound $TPMP^+$ (in the numerator) is very small but that the correction for internally bound $TPMP^+$ (in the denominator) is very large as expected from the difference in internal and external concentrations. Table 1 shows the comparison of the calculated and measured values of $\Delta\psi$ in the presence of $TPMP^+$, TPP^+, TPA^+ and ethidium$^+$. All the accumulation factors are calculated from the data of Fig. 3 and the measured $\Delta\psi$ are taken from Fig. 6. Considering all the approximations and the fact that the uptake of the cations and the determination of $\Delta\psi$ were done in separate experiments, the agreement between calculated values and determined value is striking. We can apply the same correction method to the measurements of Fig. 8*A*. Figure 8*B* shows the result of this correction procedure. The accumulation ratios that were used to calculate $\Delta\psi$ in Fig. 8*A* were corrected using Eq. (11) with the apparent binding constant listed in Table 1. It is observed that the correction is fairly good although most of the values are somewhat underestimated at high potential and overestimated at low potential. One possible reason for this discrepancy is that the assumption $K'_i = K'_o$ is not valid. We have therefore used a second approach to estimate the internal binding constants. In Fig. 7 we have calculated the internal concentration of the cations expected from the measured ^{86}Rb distribution. These internal concentrations can be related to the amount of internal binding. We corrected the total binding for the amount of internally free cation and external binding and plotted the internal binding *vs.* the internal concentration from which we get the internal binding partition coefficient. These data are shown in Fig. 9. It is observed that the internal binding coefficients are adequately described by partition coefficients except for the TPA^+ data which are scattered. This is compatible with our explanation that the apparent saturation of binding in energized mitochondria is due to the collapse of $\Delta\psi$. The partition values obtained from Fig. 9 are shown in Table 2. These values differ considerably from those cal-

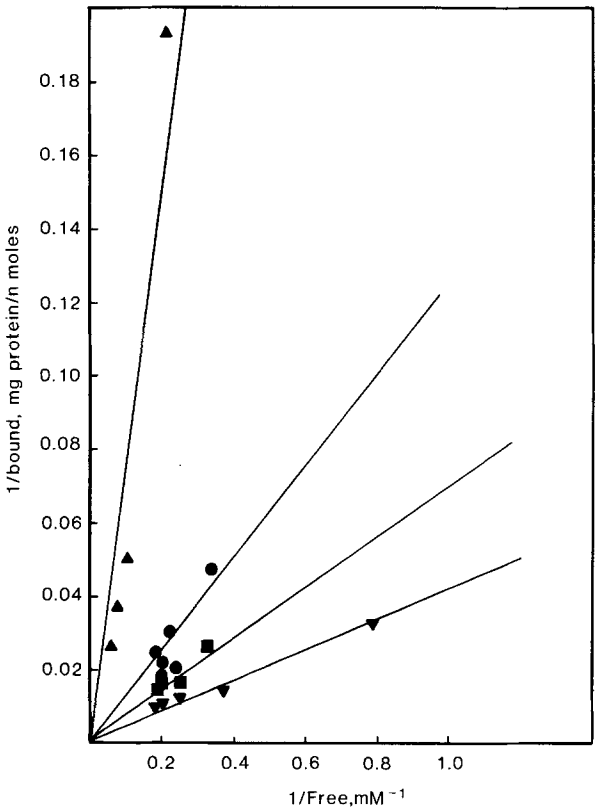

Fig. 9. Binding parameters of matrix cations binding in energized mitochondria. The results are calculated from the experiments of Figs. 3 and 6. The internal concentration is calculated as in Fig. 7. The internal bound cation is estimated from the amount of total binding after substruction of internal free (N_i) and external bound ion (N_{mo}). The figure shows reciprocal plots of the internal bound (N_{mi}) against the matrix concentrations. The data are best fit by a partition coefficient. Symbols as in Fig. 3

Table 2. Estimated internal and external partition coefficients[a]

Cation	K_i (μl/mg p)	K_o (μl/mg p)
$TPMP^+$	2.4	5.0
TPP^+	7.9	14.3
TPA^+	14.3	—
$Ethidium^+$	23.3	39.1

[a] The internal partition coefficients were estimated from Fig. 9. The external partition coefficient was estimated from Fig. 4*A* and the internal partition coefficients as described in the text.

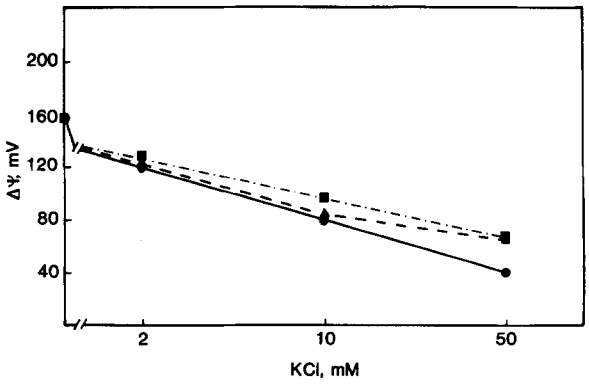

Fig. 10. Membrane potentials as estimated from the distribution of lipophilic cations after correction for internal and external binding. Results are from Fig. 8*A*. The membrane potential was calculated from Eq. (11) with the parameters listed in Table 2. Rb^+ (●), $TPMP^+$ (▲), TPP^+ (■)

culated from the experiment of Fig. 4*A*. In Fig. 10 we show the results of the corrections using the newly estimated K_i. To estimate K_o we used again the partition coefficients obtained in Fig. 4*A*, since $K_p = K_o' + K_i'$, then $K_o' = K_p - K_i'$. The estimated K_i' and K_o' from this procedure are shown in Table 2. We have omitted TPA^+ from this treatment, since such a procedure would yield no external binding which is extremely unlikely. Except for TPA^+, the corrected external partition coefficients appear to be twice as large as the internal coefficients. This difference may be due to the anisotropic nature of the mitochondrial membrane or to the contribution to binding from the outer membrane. As shown in Fig. 10 the corrections using the partition coefficients of Table 2 are very good particularly at high potential. At very low values of membrane potential, the potentials estimated by lipophilic cations appear to be overestimated. We believe that this is caused by the uncertainty of the internal volume. In high KCl (in the presence of valinomycin), mitochondria swell considerably, up to 3 μl/mg protein [25], and the value of V_i becomes an important parameter in the calculation of $\Delta\psi$. Inspection of Eq. (11) shows that at higher values of K_i' the calculated $\Delta\psi$ is less sensitive to an error in V_i. Thus, it is possible that, when the value of V_i is in question, the membrane potential estimated from lipophilic cation distribution may be more accurate than ^{86}Rb provided the values of K_i' are reliable.

Discussion

Binding and Uptake of Lipophilic Cation in Mitochondria

The suggestion by Liberman et al. [12] that the uptake of lipophilic cations in energized mitochondria

is driven by the mitochondrial membrane potential in a process of relaxation to equilibrium is fully supported by the binding analysis and the comparison with ^{86}Rb distribution. However, because of the extensive membrane binding, much of the uptake is accounted for by internal membrane binding which greatly increases as the matrix concentrations of the cations are elevated. Indeed, as the comparison between the binding to energized and nonenergized mitochondria shows, the extent of binding in both cases is correlated with the apparent partition coefficients of the cations. As expected, the electrogenic uptake leads to a collapse of $\Delta\psi$ at high cation concentrations. It is interesting that, even though the apparent binding affinity varies greatly, the effect on the potential is quite similar. This suggests that the collapse of $\Delta\psi$ is not due to internal binding. In general, the reduction of $\Delta\psi$ should depend on the rate of transport, which could be similar for the cations tested. Since the external surface concentration is higher for the more lipophilic cations, equal rate of transport would suggest that another factor limits the mobility in the membrane. It is possible that in mitochondria the transport of lipophilic cations depends on charge-pair formation which determine the rate of transport. In this respect it should be noted that in liposomes the transport of lipophilic cations is very slow unless lipophilic anions are added to the membrane [29]. The negative surface charge also exerts a considerable effect on the rate of transport. The surface potential increases the binding of the cations to the external surface but apparently the surface charge inhibits the transport. This latter effect may be due to a larger difference in the electrostatic potential between the positive hydrophobic membrane core and the negative polar interface in the absence of Mg^{2+} [21]. This potential difference in the membrane core is rate limiting for translocation across the membrane. Lipophilic cations also inhibit oxidative phosphorylation [7, 8]. This inhibition is not due to the collapse of $\Delta\psi$ and appears to result from the binding of the cations to the internal surface, rather than their translocation across the membrane (*unpublished data*).

Calculation of $\Delta\psi$ from Lipophilic Cation Uptake in Mitochondria

$TPMP^+$ has been used extensively for the determination of $\Delta\psi$ in mitochondria (*cf.* 1, 9, 10, 15, 18, 27, 32, 33, 36]; TPP^+ has also been used recently for that purpose [13, 14, 16, 28]. Similar studies were conducted both with TPP^+ and $TPMP^+$ in many bacterial systems. However, the problem of the binding correction in bacterial cells has been considered in only a few recent studies [11, 34]. In some studies of $TPMP^+$ distribution in mitochondria [9, 15, 32], no correction for binding was made for the calculation of $\Delta\psi$. This may lead to overestimation of 25 to 60 mV. In numerous studies for Azzone's laboratory [*cf.* 1, 18, 36], a correction was based on the binding of $TPMP^+$ to nonenergized mitochondria. In our model, this is equivalent to correction for the external binding only. At high potentials (>140 mV) this correction is negligibly small (<1 mV) and does not correct for the 25 to 30 mV overestimation observed under this condition due to internal binding. The corrections becomes increasingly larger at lower potentials (of course, it is adequate at $\Delta\psi = 0$), but under most conditions of interest it does not provide a substantially reduced value. More adequate corrections were based on the comparison of K^+ (in the presence of valinomycin) and $TPMP^+$ uptake [10, 27, 33]. In these experiments, which are similar to our Fig. 8*A*, a correlation is established between the two sets of measurements of $\Delta\psi$. Holian and Wilson [10] found a linear correlation with a slope of 0.85 and an intercept of 50 mV. This means that at low potential the value obtained from $TPMP^+$ overestimates the potential by ~50 mV and at high potential the overestimation is ~25 mV which is in good agreement with our Fig. 8*A*. This calibration curve was used directly to correct the potential estimated from $TPMP^+$. Holian and Wilson present a large number of measurements where the corrected values are compared with values estimated from K^+ distribution. In experiments which are similar to their calibration experiments (i.e. valinomycin and various external K^+) the agreement is fairly good. However, under some conditions, persistent discrepancies up to 80 mV were noted. More recently, Wilson and Forman [33] have presented another calibration curve, covering only the high potential range (>75 mV) in which the intercept was 28 mV and the slope close to 1. They also reported that in a similar calibration with TPP^+ about 77% of the TPP^+ appears to be bound. Scott and Nicholls [27] used similar procedures (i.e. assuming that a constant fraction (60%) of the accumulated $TPMP^+$ is bound). Similar corrections were made in studies which utilized TPP^+ for the measurement of $\Delta\psi$ [13, 28]. In these correction procedures no account is taken of external binding which can be substantial at low potentials, in particular at low salt concentrations (Fig. 5). These procedures also do not take into consideration the effects of changes in internal volume, which may greatly change the proportion between bound and internal free ions.

Two recent studies of lipophilic cations uptake

in bacterial cells [11, 34] consider, among other models, a model which is essentially identical to the one described here. No comparison was made in these studies with ^{86}Rb or K^+ uptake but it appears that, in these cells, both internal and external binding contribute to the total accumulation of lipophilic cations.

In summary we suggest that, if necessary, lipophilic cations could be used to estimate $\Delta\psi$ in mitochondria provided that necessary precautions are taken and adequate corrections are applied. Equation (11) can be used to estimate the potential from the TPP^+ and $TPMP^+$ distribution using the apparent binding constants in Table 2 and the measured accumulation factors and internal volumes. The internal binding parameter K_i' appears to be dependent on the internal pH (*not shown*) and therefore requires separate determination in those experiments in which the internal pH is greatly modulated. The external binding parameter, while less important under most conditions is greatly affected by the external surface potential (Fig. 5). It should be newly determined under conditions of high pH and low salt concentrations which maximize the external surface potentials [21]. It should also be noted that at high surface potentials the uptake is slow and it must be established that the cations have reached equilibrium. With $TPMP^+$, because the value of K_i' is similar to the internal volume, an accurate estimation of the internal volume is necessary for reliable estimates of $\Delta\psi$. With TPP^+, the value of V_i has relatively small effect on the calculated potential and may not require accurate measurement with each experiment. This may prove an advantage over $TPMP^+$ or even Rb^+ in a situation where the accurate measurement of the volume is not possible. Finally, because lipophilic cations affect several membrane processes and collapse the potential they must be used in low concentrations, preferably below 5 nmol/mg protein.

I thank Dr. T. Higuti for his help in the execution and analysis of these experiments, Mr. E. Eisenberg for technical assistance and Dr. J. Hoek for critical reading of this manuscript. This study was supported by Grants GM 28173 from NIGMS and AA-3442, AA5662 from NIAAA.

References

1. Azzone, G.V., Bragadin, M., Pozzan, T., Dell, A.P. 1977. Proton electrochemical potential in steady state rat liver mitochondria. *Biochim. Biophys. Acta* **459**:96–109
2. Bakker, E.P. 1982. The distribution of rubidium in the presence of valinomycin indicates a higher potential than that of the tetraphenylphosphonium cation. *Biochim. Biophys. Acta* **681**:85–94
3. Cafiso, D.S., Hubbell, W.L. 1978. Estimation of transmembrane potentials from phase equilibria of hydrophobic paramagnetic ions. *Biochemistry* **17**:187–195
4. Cafiso, D.S., Hubbell, W.L. 1982. Transmembrane electrical currents of spin-labeled hydrophobic ions. *Biophys. J.* **39**:263–272
5. Felle, H., Porter, J.S., Slayman, C.L., Kaback, H.R. 1980. Quantitative measurements of membrane potential in *Escherichia coli*. *Biochemistry* **19**:3585–3590
6. Harold, F.M. 1972. Conservation and transformation of energy by bacterial membranes. *Bacteriol. Rev.* **36**:172–230
7. Higuti, R., Hokota, M., Arakai, N., Hattori, A., Toni, I. 1978. Sidedness of inhibition of energy transduction in oxidative phosphorylation in rat liver mitochondria by ethidium bromide. *Biochim. Biophys. Acta* **503**:211–222
8. Higuti, T., Arakaki, N., Niimi, S., Nakasima, S., Saito, R., Tani, I., Ota, F. 1980. Anisotropic inhibition of energy transduction in oxidative phosphorylation in rat liver mitochondria by tetraphenylarsonium. *J. Biol. Chem.* **255**:7631–7636
9. Hoek, J.B., Nicholls, D.G., Williamson, J.R. 1980. Determination of the mitochondrial protonmotive force in isolated hepatocytes. *J. Biol. Chem.* **255**:1458–1464
10. Holian, A., Wilson, D.F. 1980. Relationship of transmembrane pH and electrical gradients with respiration and adenosine 5′-triphosphate synthesis in mitochondria. *Biochemistry* **19**:4213–4221
11. Lalkema, J.S., Hallingworf, K.J., Koning, W.N. 1982. The effect of probe binding on the quantitative determination of the proton-motive force in bacteria. *Biochim. Biophys. Acta* **681**:85–94
12. Liberman, E.A., Topaly, V.P., Tsofina, L.M., Jasaitis, A.A., Skulachev, V.P. 1969. Mechanism of coupling of oxidative phosphorylation and the membrane potential of mitochondria. *Nature (London)* **222**:1076–1078
13. Locke, R.M., Nicholls, D.G. 1981. A re-evaluation of the role of fatty acids in the physiological regulation of the proton conductance of brown adipose tissue mitochondria. *FEBS Lett.* **135**:249–252
14. Lotscher, H.R., Winterhalter, K.H., Carafoli, E., Richter, C. 1980. The energy state of mitochondria during the transport of Ca^{2+}. *Eur. J. Biochem.* **110**:211–216
15. Meyer, A.J., Van Woerkom, G.M., Steinman, R., Williamson, J.R. 1981. Inhibition by Ca^{2+} of carbamoylphosphate synthetase. *J. Biol. Chem.* **256**:3445–3446
16. Mikes, V., Dadak, V. 1983. Berberine derivatives as cationic fluorescent probes for the investigation of the energized state of mitochondria. *Biochim. Biophys. Acta* **723**:231–239
17. Mitchel, P. 1968. Chemiosmotic Coupling and Energy Transduction. Glyn Research, Bodmin, Cornwall, U.K.
18. Pietrobon, D., Zoratti, M., Azzone, G.F., Stucki, J.W., Walz, D. 1982. Nonequilibrium thermodynamic assessment of redox-driven H^+ Pumps in mitochondria. *Eur. J. Biochem.* **127**:483–494
19. Pressman, B.C. 1965. Induced active transport of ions in mitochondria. *Proc. Natl. Acad. Sci. USA* **53**:1076–1083
20. Ramos, S., Schuldiner, S., Kaback, H.R. 1976. The electrochemical gradient of protons and its relationship to active transport in *Escherichia coli* membrane vesicles. *Proc. Natl. Acad. Sci. USA* **73**:1892–1896
21. Robertson, D.E., Rottenberg, H. 1983. Membrane potential and surface charge in mitochondria. *J. Biol. Chem.* **258**:11039–11048
22. Rottenberg, H. 1975. Measurements of transmembrane electrochemical proton gradients. *J. Bioenerg.* **7**:63–76

23. Rottenberg, H. 1979. The measurement of membrane potential and ΔpH in cell, organelles, and vesicles. *Methods Enzymol.* **LV:**547–569
24. Rottenberg, H., Robertson, D.E., Rubin, E. 1980. The effect of ethanol on the temperature dependence of respiration and ATPase activities of rat liver mitochondria. *Lab. Invest.* **42:**318–326
25. Rottenberg, H., Solomon, A.K. 1969. The osmotic nature of the ion-induced swelling of rat-liver mitochondria. *Biochim. Biophys. Acta* **193:**48–57
26. Schuldiner, S., Kaback, H.R. 1975. Membrane potential and active transport in membrane vesicles from *Escherichia coli*. *Biochemistry* **14:**5451–5416
27. Scott, I.D., Nicholls, D.G. 1980. Energy transduction in intact synaptosomes. *Biochem. J.* **186:**21–33
28. Shen, C., Boens, C.C., Ogawa, S. 1980. Steady-state measurements of the internal phosphorylation potential and the cross membrane electrochemical potential for proton in respiring mitochondria. *Biochem. Biophys. Res. Commun.* **93:**243–249
29. Skulachev, V.P. 1971. Energy transformation in the respiratory chain. *Curr. Top. Bioenerg.* **4:**127–190
30. Skulachev, V.P. 1979. Membrane potential and reconstitution. *Methods Enzymol.* **55:**586–603
31. Szarkowska, L., Klingenberg, M. 1963. On the role of ubiquinone in mitochondria. *Biochem. Z.* **338:**674–695
32. Williamson, J.R., Steinman, R., Coll, K., Rich, T.L. 1981. Energetics of citrulline synthesis by rat liver mitochondria. *J. Biol. Chem.* **256:**7287–7297
33. Wilson, D.F., Forman, N.G. 1982. Mitochondrial transmembrane pH and electrical gradients: Evaluation of their energy relationships with respiratory rate and adenosine 5′ triphosphate synthesis. *Biochemistry* **21:**1438–1444
34. Zaritzky, A., Kihara, M., MacNab, R.M. 1981. Measurement of membrane potential in *Bacillus subtilis:* A comparison of lipophilic cations, rubidium ion, and a cyanine dye as probes. *J. Membrane Biol.* **63:**215–231
35. Zilberstein, D., Schuldiner, S., Padan, E. 1979. Proton electrochemical gradient in *Escherichia coli* cells and its relation to active transport of lactose. *Biochemistry* **18:**669–673
36. Zoratti, M., Pietrobon, D., Azzone, G.V. 1982. On the relationship between rate of ATP synthesis and H^+ electrochemical gradient in rat liver mitochondria. *Eur. J. Biochem.* **126:**443–456

Received 4 August 1983; revised 3 January 1984