Measurement of Mitochondrial Membrane Potential Using Fluorescent Rhodamine Derivatives

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ABSTRACT We investigated the use of rhodamine 123 (R123), tetramethylrhodamine methyl ester (TMRM), and tetramethylrhodamine ethyl ester (TMRE) as fluorescent probes to monitor the membrane potential of mitochondria. These indicator dyes are lipophilic cations accumulated by mitochondria in proportion to $\Delta\Psi$. Upon accumulation, all three dyes exhibit a red shift in both their absorption and fluorescence emission spectra. The fluorescence intensity is quenched when the dyes are accumulated by mitochondria. These properties have been used to develop a method to dynamically monitor $\Delta\Psi$ of isolated rat heart mitochondria using a ratio fluorescence approach. All three dyes bound to the inner and outer aspects of the inner mitochondrial membrane and, as a result, were accumulated by mitochondria in a greater quantity than predicted by the Nernst equation. Binding to mitochondria was temperature-dependent and the degree of binding was in the order of TMRE $>$ R123 $>$ TMRM. The internal and external partition coefficients for binding were determined to correct for binding in the calculation of $\Delta\Psi$. All three dyes suppressed mitochondrial respiratory control to some extent. Inhibition of respiration was greatest with TMRE, followed by R123 and TMRM. When used at low concentrations, TMRM did not suppress respiration. The use of these dyes and ratio fluorescence techniques affords a simple method for measurement of $\Delta\Psi$ of isolated mitochondria. We also applied this approach to the isolated perfused heart to determine whether $\Delta\Psi$ could be monitored in an intact tissue. Wavelength scanning of the surface fluorescence of the heart under various conditions after accumulation of TMRM indicated that the mitochondrial matrix-induced wavelength shift of TMRM also occurs in the heart cytosol, eliminating the use of this approach in the intact heart.

INTRODUCTION

Numerous types of indicator molecular probes and methods have been used to estimate the electrical potential across the inner mitochondrial membrane ($\Delta\Psi$). Many of these probes can be classified as lipophilic cations or "redistribution dyes." These compounds accumulate in the mitochondrial matrix because of their charge and solubility in both the inner mitochondrial membrane and matrix space. Safranine (Akerman and Wikstrom, 1976; Nicholls, 1978), tetraphenylphosphonium (TPP) (LaNoue et al., 1986; Rottenberg, 1984; Kamo et al., 1979; Demura et al., 1985) and rhodamine 123 (Emanus et al., 1986) are members of this class. Several review articles have summarized the use of these and other indicators (Chen, 1988; Smith, 1990). In many cases, the distribution of the free dye across the inner membrane has been shown to follow the Nernst equation.

For these probes to be useful in the estimation of $\Delta\Psi$, several criteria must be met. They should be innocuous (e.g., not cause loss of mitochondrial function and/or depletion of $\Delta\Psi$) and the compound must be easily detected to estimate the distribution across the inner membrane. Many of these probes, however, exhibit significant binding to mitochondria, which complicates their use for accurate quantitation of $\Delta\Psi$. This binding causes apparent deviations from Nernstian behavior, and leads to an enhanced mitochondrial accumulation (LaNoue et al., 1986; Rottenberg, 1984). Binding can be separated into two components that are $\Delta\Psi$-dependent and $\Delta\Psi$-independent.

R123 has been used in numerous investigations to estimate $\Delta\Psi$, stain mitochondria, and monitor movement of mitochondria in cells (Emanus et al., 1986; Millot et al., 1994; Salmeen et al., 1985). R123 has been shown to inhibit the mitochondrial F_0F_1-ATPase (Modica-Napolitano and Aprille, 1987), although Emanus et al. (1986) reported that the inhibition of mitochondrial respiration was not significant with R123 concentrations below 1 μM. More recently, TMRM (tetramethylrhodamine methyl ester) and TMRE (tetramethylrhodamine ethyl ester), two fluorescent derivatives of R123, have been used to quantitate $\Delta\Psi$ by fluorescence imaging (Loew et al., 1993; Ehrenberg et al., 1988). These studies indicated that these fluorescent dyes exhibit low binding to mitochondria.

In this report we describe the use of the fluorescent dyes R123, TMRM, and TMRE to determine $\Delta\Psi$ of isolated mitochondria. All three dyes displayed a red shift in their excitation and emission fluorescence spectra upon $\Delta\Psi$-driven mitochondrial uptake. The extent of the wavelength shift was used to determine the distribution of the dye across the mitochondrial membrane and to quantitate $\Delta\Psi$ using a ratiometric approach. All three dyes exhibited significant binding to mitochondria, which occurred to a greater extent than displayed by tetraphenylphosphonium. The binding coefficients of R123, TMRM, and TMRE were determined

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to correct the observed accumulation of dye by mitochondria. These coefficients were used to establish a method to quantitate $\Delta\Psi$ using ratio fluorescence measurements.

To determine whether this method could be used in the intact heart the isolated perfused rat heart was loaded with TMRM. Maneuvers expected to collapse $\Delta\Psi$ in the heart, however, did not cause a spectral shift in the surface fluorescence of TMRM. It is proposed that the spectral shift caused by mitochondrial accumulation also occurs in rat heart cytosol, prohibiting the use of this ratio technique in determining the cytosolic-to-mitochondrial gradient of TMRM in intact heart.

METHODS

Mitochondrial isolation and incubation

Mitochondria from rat heart were isolated as described previously (Scaduto, 1994). Incubations were conducted at either 28 or 37°C in a medium composed of 135 mM KCl, 20 mM MOPS, 5 mM K_2HPO_4, and 5 mM $MgCl_2$ at pH 7.00. Substrates were added as indicated in the figure legends. Incubations also contained either R123, TMRM, or TMRE at concentrations indicated in the figure legends. In some experiments, ^{3}H-tetraphenylphosphonium (TPP, 200 μCi/μmol, 0.05 μM) was added along with the fluorescent dye. In experiments designed to set the membrane potential with potassium and valinomycin, mitochondria were incubated at either 28 or 37°C in media composed of 200 mM sucrose, 20 mM mannitol, 20 mM Hepes, and 1 mM EDTA at pH 7.00. Concentrations of potassium were added as described in the figure legends.

Mitochondrial oxygen consumption was determined at 28°C in a water-jacketed vessel fitted with a Clark electrode and a stirring apparatus. The respiratory control ratio (state 3/state 4 respiratory rate ratio) of all preparations was determined and preparations with control ratios <6 were not used. Isolated mitochondria were kept at 4°C and used within 4 h after isolation.

Isolated perfused rat hearts

Isolated rat hearts were perfused at a constant aortic pressure of 60 mmHg using the Langendorff procedure as described previously (Scott et al., 1994). Surface fluorescence of the heart was measured using the excitation source from a SPEX Fluoromax fluorometer and a detection system designed in this laboratory. Excitation light was transmitted from the Fluoromax to illuminate the left ventricle of the perfused heart using a liquid light guide. The emitted fluorescence was collected using a silica fiberoptic bundle and was directed to a photomultiplier tube operating in the photon count mode (Hamamatsu R2560). An interference filter (610 nm center, 15 nm bandpass) was placed in front of the photomultiplier to select the emission wavelength. Details of this instrument have been described previously (Scott et al., 1994).

Reagents, chemicals, and fluorescence measurements

TMRM and TMRE were found to bind significantly to polystyrene, but not to glass, acrylic, or polypropylene. All experiments in this work were performed using fluorometer cuvettes constructed of either glass or acrylic and pipette tips constructed of polypropylene. The fluorescence of these indicators was also found to be temperature-dependent. Relative to the intensity measured at 22°C, the observed fluorescence emission of TMRM at 590 nm decreased by 12.5 and 37.1% when excited at 550 nm and measured at 28 and 37°C, respectively. For this reason, all assays of TMRM, TMRE, and R123 were conducted at the same temperature used for incubation of mitochondria.

TMRM, TMRE, and R123 were dissolved in methanol and used directly. The methanol concentrations in all incubations of mitochondria were kept to $<0.5\%$ (v/v). TMRM and TMRE were obtained from Molecular Probes (Eugene, OR). R123 and all other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Fluorescent measurements of mitochondria and extracts were made using a SPEX Fluoromax fluorometer using a 90° excitation and emission optical path and a water-jacketed cuvette holder. The excitation and emission slits were 0.5 mm, to yield a bandpass of $\sim$2.1 nm. The activity of ^{3}H-TPP in samples was determined using a Beckman LS6000 scintillation counter in the auto DPM mode.

Estimation of binding partition coefficients

As discussed in Results, TMRM, TMRE, and R123 were found to bind to mitochondria, causing an apparent deviation of the $\Delta\Psi$-dependent accumulation of these probes from that predicted by the Nernst equation. Rottenberg (1984) and LaNoue et al. (1986) described the binding of TPP to mitochondria using a model in which the degree of binding to the outer and inner aspects of the inner membrane can be expressed using two partition coefficients. In the terminology of LaNoue et al. (1986), these first-order coefficients for binding to the outer and inner components of the inner mitochondrial membrane are K_o and K_i, respectively. In application of this model, the total accumulation of TMRM by mitochondria is the sum of four components; dye bound to the inside, dye bound to the outside, dye free outside the mitochondria and in the intermembrane space, and dye free in the matrix. This summation is expressed as follows.

$$[\mathrm{TMRM}]_t = K_i(\mathrm{TMRM})_m + K_o(\mathrm{TMRM})_o + V_{im}(\mathrm{TMRM})_o + V_m(\mathrm{TMRM})_m \quad (1)$$

Where $[\mathrm{TMRM}]_t$ is the total mitochondrial accumulation (in units of nmol/mg), K_i and K_o are the partition coefficients (in units of μl/mg), V_{im} and V_m are the volumes of the intermembrane and matrix spaces (in units of μl/mg), respectively, and $(\mathrm{TMRM})_o$ and $(\mathrm{TMRM})_m$ are the concentrations of free TMRM outside (i.e., in the media) and inside the matrix (in units of nmol/μl). As will be shown, the last two terms of this summation can be ignored since the volume of these spaces is insignificant relative to K_i and K_o. V_{im} and V_m have been determined in numerous studies and are generally found to be within the range of 1 to 2 μl/mg. Upon simplification,

$$[\mathrm{TMRM}]_t = K_i(\mathrm{TMRM})_m + K_o(\mathrm{TMRM})_o. \quad (2)$$

To determine the value of K_i and K_o for TMRM, mitochondria were incubated under conditions in which 1) the membrane potential was collapsed, and 2) when it was set to a fixed value using a potassium diffusion potential in the presence of valinomycin. In the absence of a membrane potential,

$$(\mathrm{TMRM})_o = (\mathrm{TMRM})_m. \quad (3)$$

Upon substitution, the total accumulation under this condition is a function of the media concentration and the sum of the two partition coefficients.

$$[\mathrm{TMRM}]_t = (K_i + K_o)(\mathrm{TMRM})_o \quad (4)$$

To determine the total binding ($K_i + K_o$), mitochondria were incubated in a KCl-based media in the presence of valinomycin and CCCP, to collapse $\Delta\Psi$, and with a varying initial concentration of TMRM. A plot of $[\mathrm{TMRM}]_t$ vs. $(\mathrm{TMRM})_o$ at equilibrium yields a slope having the value of ($K_i + K_o$) (not shown). In these experiments, TMRM was incubated with deenergized mitochondria (3 mg/ml) for 1 min at 28°C before centrifugation. Equilibrium was attained within this time frame since similar data were attained after a 5-min incubation (not shown). This line of experi-

mentation was repeated with incubations conducted at 37°C and with TMRE and R123.

To determine the individual partition coefficients K_i and K_o, mitochondria were incubated in the presence of a known membrane potential. Under these conditions, $(TMRM)_m$ is considerably greater than $(TMRM)_o$ at equilibrium, and the accumulation of TMRM is almost entirely due to the first term of the above summation (Eq. 1). Under these conditions, the above two-term summation (Eq. 2) can be arranged to the following equation.

$$[TMRM]_t = (K_o + ZK_i)(TMRM)_o \quad (5)$$

where

$$Z = (TMRM)_m/(TMRM)_o \quad (6)$$

This relationship indicates that upon energizing (i.e., when $Z > 1$), the accumulation of TMRM is augmented by $\Delta\Psi$-driven accumulation of the dye. Since Z has the same value for any monovalent cation at equilibrium, the value of Z was set to a know quantity by incubation of mitochondria in media containing either 0.5 or 2.0 mM potassium in the presence of valinomycin. Under these conditions,

$$Z = (K^+)_m/(K^+)_o = (TMRM)_m/(TMRM)_o. \quad (7)$$

Fig. 1 illustrates the results of typical experiments with mitochondria incubated with varying initial concentrations of TMRM at 28°C and at 37°C. The value for $(K^+)_m$ was taken to be 120 mM (Akerman and Wikstrom, 1976; Sakanoue et al., 1997; Rossi and Azzone, 1969; Reers et al., 1991). Similar incubations were conducted using TMRE and R123. With knowledge of Z, $(Z K_i + K_o)$, as in Fig. 1, and $(K_i + K_o)$, determined under deenergized conditions, both K_i and K_o were calculated.

RESULTS

Properties of TMRM, TMRE, and R123

Fig. 2 illustrates the fluorescence spectra of TMRM when incubated in the presence of rat heart mitochondria and after addition of the uncoupler CCCP. Accumulation of TMRM by mitochondria causes considerable fluorescence quenching and a red shift in both the excitation and emission spectrum. The spectra obtained following CCCP addition were identical to those of the supernatant following removal of the deenergized mitochondria by centrifugation (not shown), indicating that CCCP caused complete release of the dye from mitochondria. Spectra obtained from deenergized mitochondria were the same as spectra of TMRM in solution in the absence of mitochondria. When the difference in the excitation spectrum between the coupled and uncoupled state was determined (Fig. 2), 546 and 573 nm appeared as the wavelengths of maximal difference. The spectra obtained using TMRM, as well as the response to uncoupling, were indistinguishable from those obtained using TMRE (not shown).

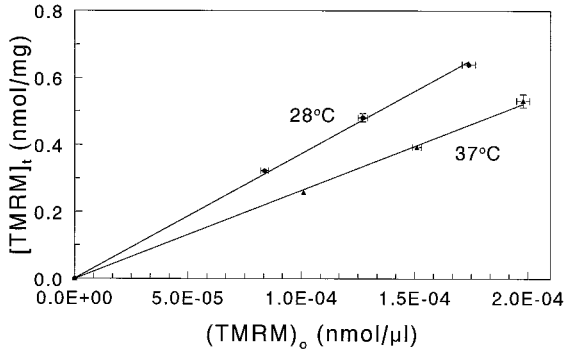

FIGURE 1 Binding of TMRM by energized mitochondria. Mitochondria (0.25 mg/ml) were incubated in sucrose-based media containing 0.5 mM potassium, as described in Methods, and either 0.167, 0.25, or 0.333 μM TMRM at 28°C (*top line, circles*) and at 37°C (*bottom line, triangles*). Valinomycin (20 nM) and rotenone (1 μg/mg) were also added to set a potassium diffusion potential. Reactions were started by the addition of mitochondria. After 30 s, mitochondria were removed by centrifugation and the residual amount of TMRM in the media was calculated by fluorescence assay of the supernatant. The data illustrate the amount of TMRM associated with the mitochondria as a function of the residual amount of TMRM in the media at equilibrium. Shown are the mean ± SE from four incubations at each concentration of TMRM.

The spectral properties of R123 and the effect of CCCP were also examined. The spectra of R123 were strikingly similar to the spectra of TMRM and TMRE, except that the wavelengths of maximal excitation and emission were blue-shifted (not shown). In coupled mitochondria, the excitation and emission wavelengths of peak intensities of R123 were 510 and 534 nm, respectively. Upon deenergizing with either CCCP or dinitrophenol, these wavelengths shifted to 500 and 526 nm. The maximum difference in the excitation spectra between the coupled and uncoupled state with rhodamine 123 occurred at 497 and 520 nm. These spectra obtained with rhodamine 123 were essentially identical to those obtained previously by Emanus et al. (1986).

Fig. 3 illustrates the change in the fluorescence intensities at 546 and 573 nm excitation, as well as the 573/546 excitation fluorescence ratio, as a function of time as mitochondria are progressively deenergized by increasing concentration of dinitrophenol in the presence of TMRM. These data illustrate that the intensity at both wavelengths responds to DNP and that gradual uncoupling causes an increased proportion of the dye to be released by mitochondria. These changes are in accordance to those expected as a result of changes in $\Delta\Psi$ and the associated wavelength shift as TMRM is released by mitochondria.

We reasoned that since mitochondrial accumulation of TMRM (and of TMRE and R123) is dependent on $\Delta\Psi$, it should be possible to utilize this wavelength shift to estimate $\Delta\Psi$. Note that the magnitude of the change in the ratio (~2.1-fold) is much greater than the change in the intensity measured at each of the individual wavelengths (~50% at 546 nm). Thus, the use of a signal ratio yields a measurement proportional to $\Delta\Psi$ that has a wider dynamic range than the signal obtained using a single wavelength. Moreover, the use of a ratio would make the method less sensitive to fluctuations in dye concentration and the fluorometer excitation energy.

Estimation of binding partition coefficients

Tetraphenylphosphonium (TPP) and related compounds have been used extensively for estimation of $\Delta\Psi$. Measure-

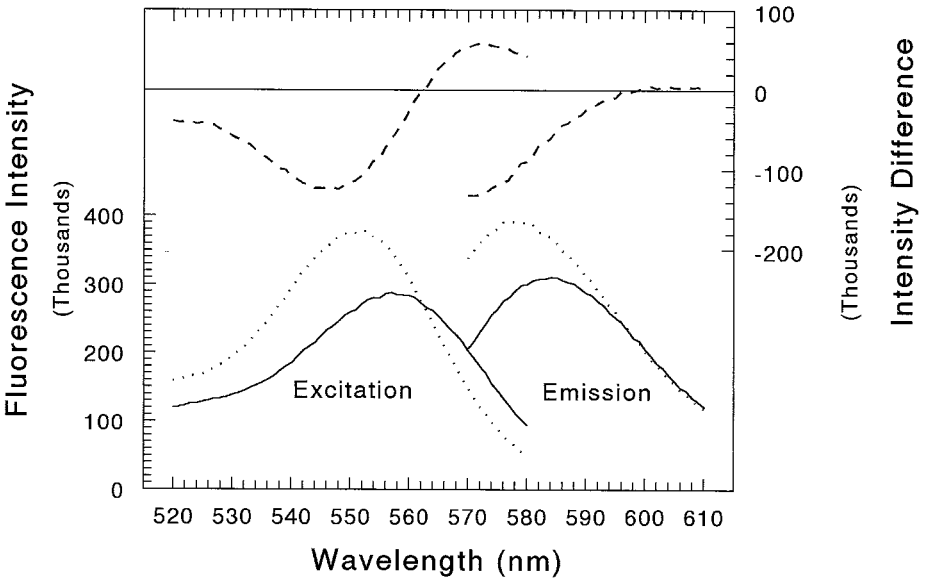

FIGURE 2 Fluorescence spectra of TMRM-loaded mitochondria. Mitochondria (0.5 mg/ml) were incubated with TMRM (0.5 μM) in media containing 10 mM glutamate and 5 mM malate as substrates. The excitation spectra were scanned using 590 nm emission and the emission spectra were scanned using 560 nm excitation (*solid lines*). Two minutes after the initial scan of energized mitochondria, CCCP (0.5 μM) was added and the scans were repeated (*dotted lines*). The top traces illustrate the difference spectra between the control and CCCP-treated mitochondria (i.e., energized control–CCCP) (*dashed lines*). The maximum differences in the excitation spectra occurred at 546 and 573 nm. All excitation scans were corrected for wavelength-dependent changes in excitation energy using a reference photometer.

ment of $\Delta\Psi$ based on the uncorrected distribution of TPP, however, overestimates $\Delta\Psi$ due to binding of TPP to mitochondrial membranes (LaNoue et al., 1986; Rottenberg, 1984; Demura et al., 1985). The accumulation of free TPP in the mitochondrial matrix is in accordance with the Nernst equation. $\Delta\Psi$ can be estimated from the observed total accumulation of TPP by mitochondria if this binding is taken into account in the quantitation of distribution of free TPP across the inner mitochondrial membrane (LaNoue et al., 1986).

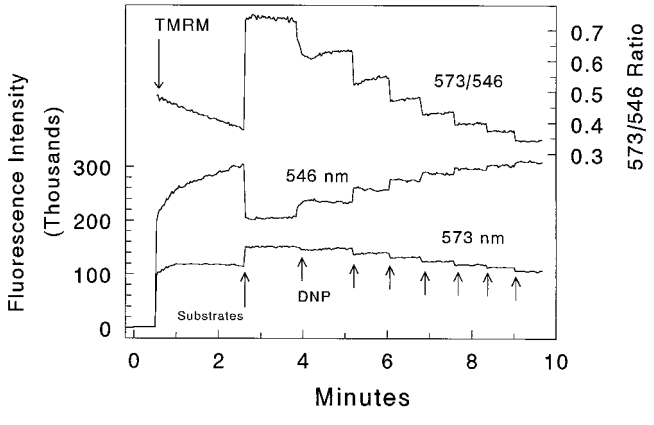

FIGURE 3 Response of TMRM fluorescence to mitochondrial deenergizing by dinitrophenol. Rat heart mitochondria (0.5 mg/ml) were incubated at 28°C. The lower two traces indicate the fluorescence intensity measured at excitation wavelengths of 546 and 573 nm using an emission detection wavelength of 590 nm. The top trace indicates the ratio between these signals. The first arrow illustrates the addition of TMRM (0.2 μM) to the suspension. The second arrow indicates the addition of the substrates 10 mM glutamate and 5 mM malate. Subsequent arrows indicate the addition of dinitrophenol (DNP). Dinitrophenol additions yielded final concentrations of 2.5, 5, 7.5, 10, 12.5, 17.5, and 22.5 μM. Note that before the addition of substrates, the 573/546 ratio steadily declined, indicative of mitochondrial deenergizing and a redistribution of TMRM from mitochondria to the media. The accumulation of TMRM afforded by the addition of substrates was progressively decreased by the subsequent addition of dinitrophenol.

To directly compare the properties of TMRM with those of TPP, rat heart mitochondria were incubated in the presence of both TMRM and ^{3}H-TPP during titration of $\Delta\Psi$ with DNP. Low concentrations of DNP stimulate electron transport and decrease $\Delta\Psi$ and NADH (Scaduto, 1994) to levels that remain fairly stable for periods of several minutes. After a 2-min incubation with DNP, mitochondria were removed from the media by centrifugation (15,000 $\times$ *g*) and the residual TPP and TMRM in the supernatant were determined by scintillation counting and fluorescence assay measurements, respectively.

As shown in Fig. 3, low concentrations of DNP generated discrete distributions of TMRM across the mitochondrial membrane as indicated by the stable fluorescence intensities and intensity ratio at each concentration of DNP. With knowledge of the initial amount of TMRM and ^{3}H-TPP added to the media before addition of mitochondria, the accumulation of these two lipophilic cations could be directly compared under conditions in which the $\Delta\Psi$ was lowered to several steady-state levels using DNP. Fig. 4 illustrates the relative accumulation of ^{3}H-TPP by mitochondria plotted as a function of the relative accumulation of TMRM. The slope of this line indicates that mitochondrial accumulation, and hence the degree of mitochondrial binding, of TMRM is ~3-fold greater than the accumulation of TPP. The fact that this relation is linear illustrates that binding of TMRM and the effect of $\Delta\Psi$ can be evaluated using an approach used previously for evaluation of TPP binding. That is, the extent of binding can be predicted based on the concentration of free dye in the matrix and the appropriate binding coefficients.

These binding constants represent the binding due to $\Delta\Psi$-independent and $\Delta\Psi$-dependent events, respectively. Table 1 illustrates these partition coefficients for TMRM,

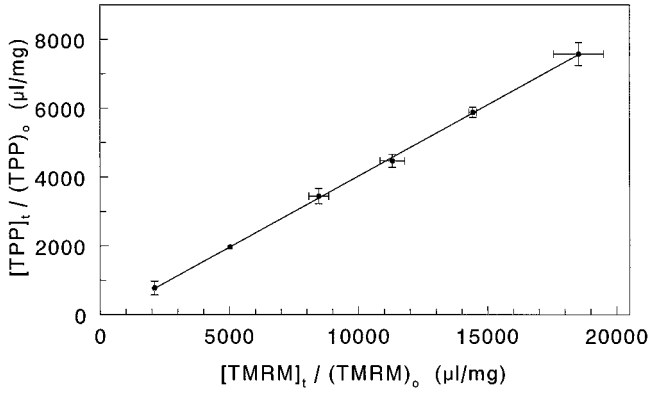

FIGURE 4 Relative accumulation of TPP and TMRM by energized mitochondria. Mitochondria (0.5 mg/ml) were incubated with ^{3}H-TPP (0.05 μM) and with TMRM (0.5 μM) in media containing 10 mM glutamate and 5 mM malate as substrates. Also added was either 0, 1.25, 2.5, 5, 10, or 20 μM dinitrophenol to lower the membrane potential. Each point represents incubations conducted with a different concentration of DNP from 0 to 20 μM (points from *right* to *left*). After 30 s, the mitochondria were removed from the incubation media by centrifugation. The remaining concentration of ^{3}H-TPP and TMRM in the media was calculated from scintillation counting (^{3}H-TPP) or fluorescence assay (TMRM) of the supernatant after the removal of mitochondria. Plotted is the amount of dye associated with the mitochondria, relative to the amount in media (for ^{3}H-TPP vs. TMRM). Shown are the mean ± SE from four incubations at each concentration of dinitrophenol. The slope of this line is 0.4, indicating a higher degree of TMRM uptake relative to the uptake of ^{3}H-TPP.

TMRE, and R123 at 28 and at 37°C. K_o and K_i were determined as described in Methods. The data of Fig. 1 illustrate the temperature sensitivity of mitochondrial binding, which is reflected in the constants shown in Table 1. As expected from the data of Fig. 4, the value of the coefficients for TMRM are greater than the values determined previously for TPP (LaNoue et al., 1986).

Interference with coupled respiration

As the initial concentration of TMRM is increased, the accumulation of TMRM by energized mitochondria departs from that expected (see Discussion). Because of this effect, and the fact that R123 is known to suppresses mitochondrial respiration (Modica-Napolitano and Aprille, 1987), we investigated the effects of TMRM, TMRE, and R123 on coupled respiration. Fig. 5 illustrates the influence of TMRM, TMRE, and R123 on state 3 respiration rates of mitochondria when incubated at 0.5 mg protein/ml. All dyes decreased rates of respiration as the concentrations of the indicators were increased. Respiration was most affected by the presence of TMRE, whereas TMRM did not suppress respiration until the concentration of indicator was >0.25 μM. Rates of state 4 respiration were not affected by any of the indicators in the concentration range tested, nor were rates of uncoupled respiration (not shown). It is interesting that the order of the degree of inhibition by these probes (TMRE > R123 > TMRM) is same as the order of the magnitude of their binding to mitochondria (Table 1). This suggests that the enhanced accumulation of these indicators by $\Delta\Psi$-dependent binding may lead to their toxicity.

Changing the protein concentration in the incubation altered the sensitivity of mitochondria to inhibition of respiration. This arises because the amount of indicator accumulated by mitochondria at a given level of $\Delta\Psi$ is dependent upon both the initial amount of indicator employed and the mitochondrial protein concentration (see Discussion). In other experiments, incubations were conducted at two mitochondrial protein concentrations and with lower concentrations of TMRM. As expected, incubations containing a lower concentration of mitochondria displayed greater sensitivity to TMRM (not shown). We observed that even at protein concentrations as low as 0.25 mg/ml, TMRM could be used at concentrations up to 0.5 μM without causing an inhibitory effect on state 3 respiration.

TABLE 1 Binding partition coefficients for TMRM, TMRE, and R123

Indicator	Temp. (°C)	K_o	K_i
TMRM	28	88	33
	37	95	25
TMRE	28	129	60
	37	170	28
R123	28	109	37
	37	120	26

The binding partition coefficients were determined as described in the text. Values represent the coefficients calculated using the slope parameters obtained from duplicate or triplicate experiments as described in the text and in the legend to Fig. 1. In all instances, these slope parameters differed by <5% from the average of replicate experiments.

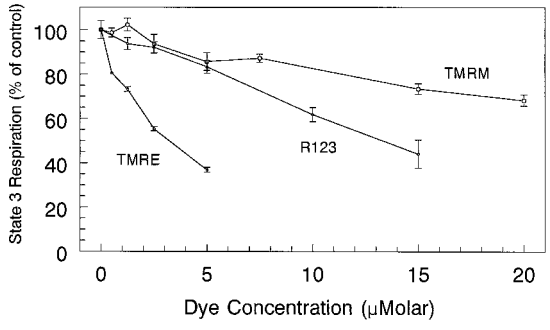

FIGURE 5 Effect of TMRM, TMRE, and R123 on mitochondrial respiration. Mitochondria (0.5 mg/ml) were incubated in media containing 20 mM glutamate, 10 mM malate, and varying concentrations of either TMRM, TMRE, or R123 at 28°C. Oxygen consumption was monitored as described in Methods. State 3 respiration was initiated by addition of 1 mM ADP. Each point represents the mean ± SE from three incubations. Each dye was tested using a different preparation of mitochondria. For comparison, the data were normalized to 100% using the rate obtained in each preparation in the absence of added dye. The initial 100% rate for the three traces were 381 ± 15, 432 ± 1374 ± 2 natoms oxygen/min per mg for TMRM, TMRE, and R123, respectively.

Procedure for calibration of the fluorescence ratio

With knowledge of the partition coefficients (Table 1), the excitation intensity ratio signal can be calibrated to determine $\Delta\Psi$. Media containing 0.33 μM TMRM was used. Before the addition of mitochondria, the fluorescence intensity at one excitation wavelength was determined. This measurement was used as an indication of the total dye concentration using a standard curve made by serial dilution of TMRM in incubation media. Mitochondria and DNP were then added in the presence of substrates to attain discrete levels of $\Delta\Psi$. After a steady state had been reached, the fluorescence of the suspension at both excitation wavelengths was measured and the contents of the cuvette were immediately centrifuged to pellet the mitochondria. The fluorescence of the supernatant was then determined to calculate $(TMRM)_o$ from a standard curve. From the initial fluorescence (before addition of mitochondria) and the fluorescence of the supernatant, the amount of dye taken up by mitochondria was calculated by subtraction. This value is converted to a per mg basis to obtain the term $[TMRM]_t$. The concentration of free TMRM in the matrix, $(TMRM)_m$, is then calculated from Eq. 2 above. $\Delta\Psi$ was then estimated from $(TMRM)_m$ and $(TMRM)_o$ using the Nernst equation in the usual manner. A plot of the ratio fluorescence measured in the suspension of mitochondria versus $\Delta\Psi$ yields a standard calibration curve.

The calibration curve in Fig. 6 was obtained using 0.33-μM TMRM and 0.25 mg/ml mitochondria. This curve can then be used to estimate $\Delta\Psi$ under other conditions, provided that the concentration of mitochondria and TMRM used in the standardization procedure is maintained. This latter point deserves emphasis since the concentration of mitochondrial protein significantly alters the degree of light scattering by the sample, and it will affect the fluorescence signal strength. As discussed below, it also affects the relative distribution of the dye across the inner membrane.

As an alternative method, calibration of the ratio fluorescence signal can be accomplished without determination of the binding coefficients. In this case, mitochondria must be incubated with the fluorescent indicator under conditions in which $\Delta\Psi$ is determined by other means. The $\Delta\Psi$, as measured by another method, is then plotted as a function of the fluorescence ratio to obtain a calibration curve. It should be emphasized, however, that the calibration of the fluorescence ratio using any method is sensitive to several variables, including the mitochondrial protein concentration and temperature.

Fig. 7 illustrates the measurement of $\Delta\Psi$ in isolated mitochondria using this method. Shown is the effect on $\Delta\Psi$ caused by the stimulation of respiration with a limiting amount of ADP.

TMRM surface fluorescence in the perfused heart

Rat hearts were perfused and loaded with TMRM to determine whether spectral changes in TMRM could be observed in the intact heart upon alterations in $\Delta\Psi$. Hearts were perfused and loaded with TMRM by recirculation of 20 ml buffer containing 0.5 μM TMRM for 5 min. This procedure was sufficient to increase the fluorescence of the heart ~70-fold above background levels. TMRM loaded rapidly and was well retained upon switching to a control buffer not

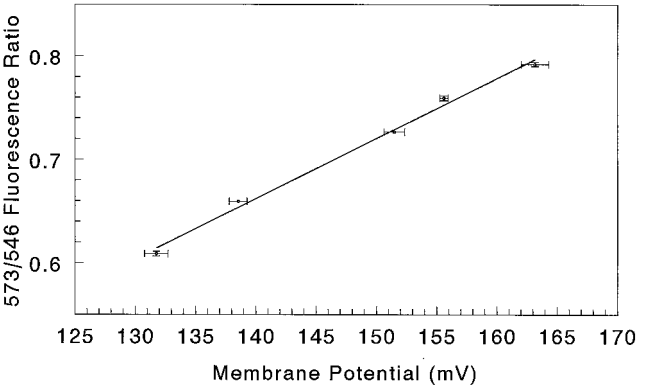

FIGURE 6 Calibration of the mitochondrial TMRM fluorescence ratio for estimation of $\Delta\Psi$. Mitochondria (0.25 mg/ml) were incubated in the presence of 0.33 μM TMRM. The fluorescence of the media containing TMRM was determined before addition of mitochondria. A low concentration of DNP was added to each incubation, as described in the legend to Fig. 4. After 30 s the mitochondria were removed from the incubation media by centrifugation. The concentration of TMRM remaining in the media was calculated from a fluorescence assay of TMRM in the supernatant. The initial total amount of TMRM in the cuvette and the amount remaining in the media were used to calculate the amount of TMRM associated with mitochondria (i.e., $[TMRM]_t$). $\Delta\Psi$ was calculated using the binding constants at 28°C (Table 1) and the equation described in Results (Eq. 2). Plotted is the observed fluorescence ratio as a function of $\Delta\Psi$. Shown are the mean ± SE from four incubations at each concentration of dinitrophenol. In all subsequent experiments, this standard curve can be used to monitor $\Delta\Psi$ provided that the same protein and dye concentrations are used. In this example, $\Delta\Psi$ can be calculated from the fluorescence ratio using the equation of $\Delta\Psi = 171.9$ (573/546 ratio) + 26.2.

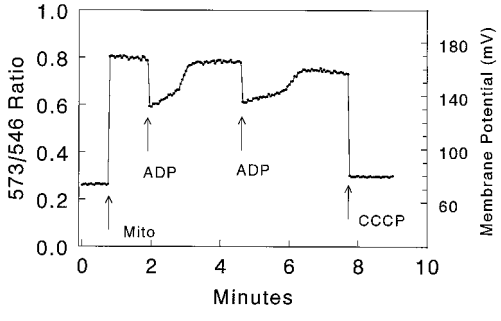

FIGURE 7 Monitoring $\Delta\Psi$ with TMRM during ADP-stimulated respiration. Mitochondria (0.25 mg/ml) were incubated with 10 mM glutamate, 5 mM malate, and 0.33 μM TMRM. Fluorescence at 546 and 573 nm excitation was monitored using an emission wavelength of 590 nm. The experiment was initiated by the addition of mitochondria to the cuvette containing TMRM and substrates. At the indicated times, ADP (0.17 mM, final conc.) and CCCP were added (0.5 μM). $\Delta\Psi$ was calculated from the observed 573/546 fluorescence ratio as described in Results and in the legend to Fig. 6. Note that addition of mitochondria immediately caused a wavelength shift due to mitochondrial accumulation and that ADP addition caused a transient decrease in $\Delta\Psi$ due to stimulated phosphorylation.

containing TMRM. Following loading, leakage of TMRM from the heart was <5%/h.

To evaluate the use of TMRM in the heart, the surface fluorescence of dye-loaded hearts was determined. The wavelength for excitation was varied from 537 to 582 in 3-nm increments while monitoring the emission at 610 nm. We found that either perfusion with no added substrate or the addition of 50 μM dinitrophenol did not alter the excitation spectrum of TMRM in the heart when compared to the spectrum obtained by perfusion with 5 mM pyruvate. These spectra were compared to the excitation spectrum of TMRM dissolved in the perfusate buffer. We found that all the spectra obtained from the heart were red-shifted to the same extent relative to the spectrum obtained from the perfusate (not shown). Perfusion with no substrate and/or with 50 μM DNP are conditions expected to decrease $\Delta\Psi$ markedly. These data illustrate that the wavelength shift of TMRM caused by mitochondrial accumulation (Fig. 2) does occur in the heart, but it is independent of dye localization between the mitochondrial matrix and the cytosol, indicating that this ratiometric approach cannot be used in the heart in situ. Perfusion of hearts with no substrate and/or with 50 μM DNP also did not cause a change in the absolute values of fluorescence measured at these wavelengths (not shown). In other experiments with 50 μM CCCP, the tissue TMRM fluorescence decreased markedly due to washout from the heart, but there was no change in the fluorescence excitation ratio.

DISCUSSION

The method used to determine $\Delta\Psi$ described in this work is based on the observation that R123, TMRM, and TMRE are accumulated within the mitochondrial matrix in accordance to the Nernst equation, and that the fluorescent properties of the dyes are altered upon mitochondrial accumulation. The major changes in the fluorescent properties of these indicators upon mitochondrial accumulation include a fluorescence quenching and a red shift in the wavelength of maximum excitation and emission energy. Because of these changes, the degree of mitochondrial dye accumulation can be determined by monitoring the fluorescence. If the extent that these indicators bind to mitochondria is known, the distribution of free indicator across the mitochondrial membrane can be estimated for measurement of $\Delta\Psi$. This method has its foundation in the quantitation of the Nernstian distribution of an indicator across the mitochondrial membrane, although the calibration procedure using a fluorescence ratio is largely empirical in design. Nonetheless, the method is simple and direct and can be easily employed using a standard fluorometer. This method alleviates the difficulty in using either electrodes or radioactive tracers to estimate the distribution of lipophilic cations, such as TPP, in the measurement of $\Delta\Psi$.

The partition coefficients for mitochondrial binding of TMRM, TMRE, and R123 have been determined in this study using procedures previously established for the nonfluorescent indicator TPP. With these binding parameters, the matrix concentration of free dye can be determined from the difference between the total dye concentration and the media concentration at equilibrium. This is used to establish an empirical calibration curve between the shift in the ratio of fluorescence excitation energy and $\Delta\Psi$. As indicated in Table 1, the binding of these dyes to mitochondria is significant. The value for K_i for TMRM at 28°C was 33, indicating that the total amount of TMRM in the matrix space would be ~33-fold higher than the amount predicted from the direct application of the Nernst equation in the absence of binding (see Eq. 1).

Although this method describes the use of a fluorescence ratio, the same calibration procedure can be employed using a single excitation and emission wavelength. In this instance, it would be preferable to use the excitation wavelength of 546 nm since it is more sensitive to changes in the distribution of TMRM than 573 nm (Figs. 2 and 3). We employed a fluorescence ratio since this would make the method inherently more sensitive than using a single excitation and emission wavelength. As shown in Fig. 3, the changes in fluorescence properties caused by decreasing $\Delta\Psi$ with DNP addition caused a 2.1-fold change in the 573/546 ratio, but only a 50% change in the absolute fluorescence measured at 546 nm. The use of a ratio signal also lowers errors arising from drift in either the excitation energy of the fluorometer or the gain of the emission detection system.

To use this method in estimation of $\Delta\Psi$, however, several factors must be taken into account. A change in the protein concentration will disturb the distribution ratio of the indicator across the mitochondrial inner membrane, and hence the observed fluorescence ratio. This effect can be illustrated by graphing an extension of the relationships describing mitochondrial accumulation that were presented in Results. Fig. 8 illustrates the fraction of the total dye that is present in the media at equilibrium after mitochondrial uptake, as a function of protein concentration and of $\Delta\Psi$. This fraction, f, is

$$f = (\mathrm{TMRM})_o/(\mathrm{TMRM})_i \quad (8)$$

where $(\mathrm{TMRM})_i$ equals the initial total concentration of TMRM in the media (in units of nmol/μl). This fraction can be calculated from the fact that the total amount of dye added initially is distributed between mitochondria and the media at equilibrium, as

$$1000\ (\mathrm{TMRM})_i = \mathrm{P}[\mathrm{TMRM}]_t + 1000\ (\mathrm{TMRM})_o \quad (9)$$

where P represents the protein concentration (in units of mg/ml). The equation for $[\mathrm{TMRM}]_t$ described above (Eq. 1) can be substituted into this equation to calculate f, the fractional amount of the dye remaining in the media at equilibrium. This relation is

$$f = 1000/[(\mathrm{P}\ K_o) + (\mathrm{P}\ Z\ K_i) + (V_m\ \mathrm{P}\ Z) + (\mathrm{P}\ V_{im}) + 1000] \quad (10)$$

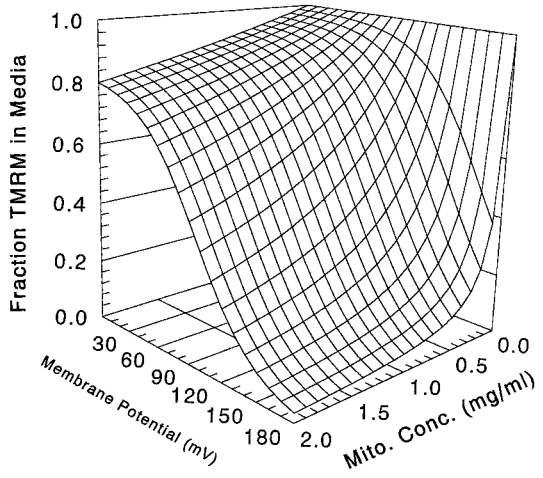

FIGURE 8 Effect of $\Delta\Psi$ and mitochondria concentration on the uptake of lipophilic cations by mitochondria. The relative uptake of a lipophilic cation was calculated based on the model in which mitochondrial binding can be estimated by the partition coefficients K_o and K_i. Shown is the theoretical uptake of TMRM at 28°C, plotted as the fraction of the total TMRM concentration remaining in the media, as a function of $\Delta\Psi$ and of the mitochondria protein concentration. The equations for these calculations are presented in the text.

where Z represents the relation shown in Eq. 6. Z is calculated from the Nernst equations as follows:

$$Z = 10^{(\Delta\Psi F/2.303\,R\,T)} \tag{11}$$

where $\Delta\Psi$ is the membrane potential in volts and F, R, and T have their usual meaning. The diagram in Fig. 8 was generated using the binding coefficients for TMRM at 28°C shown in Table 1.

Note that at low concentrations of mitochondria, a greater fraction of the available dye remains in the media and is not taken up by mitochondria. Higher values of $\Delta\Psi$ are required for dye uptake as the protein concentration is lowered. This would suggest that at low concentrations of mitochondrial protein, this method would be less sensitive to changes in $\Delta\Psi$. It is reasonable to assume that the sensitivity of this method can be predicted by the slope of the fractional uptake as a function of $\Delta\Psi$ since the distribution of the indictor between the mitochondria and media leads to the observed changes in fluorescence intensities (Fig. 2). Thus, TMRM would not be well suited to discriminate between low values of $\Delta\Psi$ when the mitochondrial protein concentration is also low. This analysis also predicts that high values of $\Delta\Psi$ cannot be measured accurately using mitochondrial protein concentrations >1 mg/ml.

In determining the concentration of mitochondria to use with this method, there are additional factors that require consideration. As the mitochondrial concentration is decreased, these dyes cause a greater inhibition of respiration. This is not factored into the theoretical treatise of the indicator distribution shown Fig. 8. An additional consideration is the fact that an increase in mitochondrial protein concentration causes added light scattering and inner filtering effects in the measurement of fluorescence. These effects contribute to a lowered signal strength and an increased background signal. The sensitivity of a particular fluorometer to these effects should also be examined when using mitochondrial protein concentrations higher than ~0.5 mg/ml.

The sensitivity of the method is less dependent on the initial concentration of TMRM. The relationship between mitochondrial protein concentration, $\Delta\Psi$, and fraction uptake of TMRM shown in Fig. 8 is independent of the initial dye concentration. As the initial concentration of dye is increased, the media concentration at equilibrium is expected to change in parallel with the amount associated with mitochondria. As shown in Fig. 1, the proportion of dye taken up by mitochondria as the dye concentration is increased is a constant. This is not the case, however, when the total dye concentration reaches higher values. This linear relation breaks down at higher dye concentrations because there is a limited ability of mitochondria to accumulate these indictors. The use of TMRM concentrations higher than those used in the experiment shown in Fig. 1 would have caused the curve to bend downward. We found that at resting membrane potentials, this relation was linear only with use of initial media concentrations of TMRM below ~0.4 μM using 0.25 mg/ml mitochondria. After this point, the accumulation of TMRM was less than anticipated at a given membrane potential. This effect could be due to a functional limitation in the maximal TMRM concentration obtainable in the matrix or to a decrease in $\Delta\Psi$ due to such a limitation. This property is identical to what has been observed in the study of TPP accumulation by Rottenberg (1984). Experimentally, this linearity can be extended to higher concentrations of TMRM by increasing the mitochondrial protein concentration (not shown), in effect lowering the dye content per mg mitochondria. This observation further supports the view proposed by Rottenberg (1984) that mitochondria can accumulate a limited quantity of dye before either disruption of $\Delta\Psi$ or the dye being unable to faithfully indicate $\Delta\Psi$. As discussed by Rottenberg, this limitation is to be expected considering the high degree of $\Delta\Psi$-driven accumulation in the matrix space. In practice, the lack of an effect of the indicator on $\Delta\Psi$ should be checked by testing the linearity of the amount of dye accumulated by energized mitochondria as the initial concentration is varied, as shown in Fig. 1.

The use of TMRM and TMRE as indicators of mitochondrial membrane potential has become more popular largely because of claims that, unlike tetraphenylphosphonium, these indicators do not exhibit appreciable binding to mitochondria (Loew et al., 1993; Farkas et al., 1989). These indictors have been used to directly determine $\Delta\Psi$ in video images of cells using the observed ratio of intensities between mitochondria and cytosol. These estimates were performed without correction for fluorescence quenching or mitochondrial binding. It was assumed that these indicators did not exhibit mitochondrial binding because the mito-

chondrially associated fluorescence dissipated following addition of an uncoupler to collapse $\Delta\Psi$. In this report we found that a significant portion of the binding to mitochondria is $\Delta\Psi$-dependent, and thus cannot be assessed by collapsing $\Delta\Psi$. Although there is some binding of these indicators to the external surfaces of mitochondria (i.e., K_o), and this binding is lower with TMRM than other rhodamine-based indicators, the majority of the binding is $\Delta\Psi$-dependent. This effect can be seen in Fig. 8. The fractional uptake of the dye in the absence of a membrane potential is relatively low. In practice, we had to increase the protein concentration to 3 mg/ml in these incubations to determine the extent of $\Delta\Psi$-independent binding. Since the majority of the binding of these indicators to mitochondria is $\Delta\Psi$-dependent, the loss of mitochondrially associated dye upon collapse of $\Delta\Psi$ is not a valid criterion for assessment of mitochondrial binding.

It was unfortunate that we were did not observe spectral shifts in the surface fluorescence of TMRM-loaded rat heart upon perfusion with the uncoupler DNP. The data suggest that the spectrum of TMRM in the cytosol of rat heart is similar to the spectrum of the dye when in the mitochondrial matrix. We were unable to induce a change in the 573/546 intensity ratio in perfused hearts under any tested condition. In other experiments, we monitored the rate of TMRM uptake from the perfusion media. Likewise, this rate was unaffected by addition of DNP (data not shown).

During the course of this work, a study was reported using rhodamine 800 as a fluorescent probe of membrane potential in liver mitochondria and hepatocytes (Sakanoue et al., 1997). In similar fashion to our observations with TMRM and TMRE, rhodamine 800 was shown to undergo a red shift in the excitation and emission spectra and a quenching of fluorescence when accumulated by mitochondria. The maximal differences in the excitation spectra occurred at 730 and 685 nm.

In summary, a fluorescence ratio method is described for measurement of $\Delta\Psi$ of isolated mitochondria. The binding constants for TMRM, TMRE, and R123 with rat heart mitochondria have been determined so that $\Delta\Psi$ can be estimated by taking into account the significant binding of these indicators to mitochondria. The indicator of choice for these measurements is TMRM since it exhibits no inhibitory effect on mitochondrial respiration when used at low concentrations.

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