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Chapter 3

ESTIMATIONS AND ACTUAL MEASUREMENTS OF THE PLASMA MEMBRANE ELECTRIC POTENTIAL DIFFERENCE IN YEAST

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ABSTRACT

Estimation of the plasma membrane potential (PMP) in yeast by the fluorescence changes of various indicators have been studied by several groups for many years; with most variable results. The most used of these

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indicators have been $DiSC_3(3)$ and $DiSC_3(5)$. This contribution explores different dyes studied, methods and incubation media, as well as the different parameters analyzed. Particularly with $DiSC_3(3)$, from rather high to very low values of PMP have been calculated. Recently, we reported that fluorescence changes and accumulation of acridine yellow can be used to estimate and obtain actual values of the PMP in yeast. The experiments were performed with an old dye from a flask labeled "acridine yellow" from a commercial source. However, NMR and mass spectrometry revealed that it was thioflavin T. With the pure dye, the experiments were repeated. Also the accumulation of the dye was measured to obtain real values of PMP, mainly based in permeabilizing the cells with chitosan in the absence or presence of an adequate concentration of KCl that allowed to correct the raw data obtained. Hence, more accurate values were obtained. Moreover, results of comparing this dye with others used so far, point to thioflavin T as the best one to follow by fluorescence and measure by its accumulation the plasma membrane potential in yeast.

Keywords: yeast, plasma membrane potential, fluorescence, *S. cerevisiae,* thioflavin T

INTRODUCTION

The mechanism of K^+ transport in yeast was proposed as driven by the plasma membrane electric potential difference (PMP) generated by a H^+-ATPase (Peña et al. 1972; Peña A. 1975). This mechanism was also found in *Neurospora crassa* (Slayman, Long, and Lu 1973), later in many other yeasts and fungi (Goffeau and Slayman 1981), and also in plants (Sze 1983). Studies were also performed to measure the relationship between the changes produced by the ATPase on the external and internal pH when K^+ was added or the pH of the medium was increased (Peña A. 1975). The mechanism proposes that the H^+-ATPase produces a ΔpH, that has been measured (Peña et al. 1972), but also a plasma membrane potential difference (PMP), measured originally by the accumulation of tetraphenylphosphonium (Borst-Pauwels 1981; Vacata et al. 1981; De la Peña et al. 1982). Since then, other studies have been performed using the accumulation or the fluorescence changes of different dyes (De la Peña et

al. 1982; Peña et al. 2010; Plášek et al. 2012; Plášek and Gášková 2014), but most of those results appear to be inaccurate.

More recently (Calahorra et al. 2017a; Peña et al. 2017), the use of acridine yellow to estimate by fluorescence the PMP changes in yeast, as well as the actual values by its accumulation, were reported. As mentioned, the dye was really thioflavin T (Calahorra et al. 2019). Thioflavin T was then purchased pure, and with small variations, also similar results were obtained.

In general, the mentioned reports of the actual PMP values obtained from the accumulation of the dyes or other molecules can be taken as inaccurate mostly because none of them considered that the concentration reached inside was not only due to the influence of the PMP, but also to their internal binding because of its hydrophobic and cationic nature. Previously (Calahorra et al. 2017a), we tried to define a numeric value for the yeast PMP by using supposedly acridine yellow. In those studies it was particularly difficult to obtain satisfactory values after the addition of K^+ because only the internal binding of the dye because of its hydrophobicity was considered, but not accurately that due to its cationic characteristics. Using the pure thioflavin T (Figure 1), previous results were confirmed, and using a larger concentration, as well as different conditions, more accurate and reliable results were obtained.

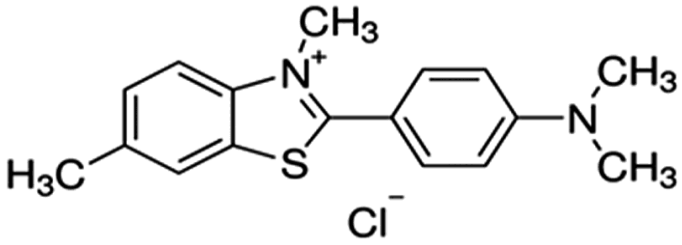

Figure 1. Chemical structure of thioflavin T.

From these results our proposal is now corrected, confirming that thioflavin T can be used to qualitatively follow and to obtain actual values and changes of the PMP in *S. cerevisiae*. Since those values could be obtained with a dye which among its properties is characterized by a lower

hydrophobicity than others, experiments performed with other dyes are also presented and analyzed.

GENERAL PROCEDURES

As in any other procedure, the particular experimental conditions are central to the results obtained. Because of this, different factors involved are described.

Strain and Growth Conditions

Most reports are referred to experiments performed with *Saccharomyces cerevisiae*, usually grown in YPD and collected in the exponential phase. In our experiments, this yeast from a commercial strain (La Azteca, México) obtained form an isolated single colony has always been used. Cultures are started by placing a loophole of cells in 500 mL of liquid YPD grown for 24 h at constant room temperature (30°C) in an orbital shaker at 250 rpm. In our experiments, in order to be able to see the effects of a substrate (glucose) to energize the cells, they are starved by collecting them by centrifugation, suspending them in 250 mL of water and incubating in the same shaker for 24 h. After this incubation the cells are collected by centrifugation, washed once with water and suspended in water at a ratio of 0.5 g (wet weight)·mL^{-1}.

Dyes Used

The dyes more frequently used have been $DiSC_3(3)$ (3,3'-Dipropyl thiacarbocyanine iodide), $DiSC_3(5)$ (3,3′-Dipropylthiadicarbocyanine iodide), $DiOC_6(3)$ (3,3'-Dihexyloxacarbocyanine iodide) (Sigma), but also acridine yellow (Sigma), acridine orange, safranine O, neutral red, rhodamine G and thioflavin T (Biotium) have been tested under the conditions described for each group of results.

METHODS

The procedures used have been: a) following the fluorescence changes of the dyes; b) analyzing their distribution within the cell, and c) measuring the accumulation of the dyes by the cells, but also using some correction factors due to the fact that they are not only accumulated due to the PMP, but they also bind to the internal components of the cells.

Membrane Potential Estimations by Fluorescence

As done by many groups, membrane potential changes have been estimated by measuring the fluorescence changes of the different dyes at their maximum excitation-emission wavelengths. Some authors (Plášek et al. 2012; Plášek and Gášková 2014) have actually used the changes of the absorption maxima to estimate the values, but also using complex incubation media, and have reported rather low values. We have used a more simple approach, by following the fluorescence intensity changes under different conditions and after the addition of agents known to affect both the distribution and accumulation of the dyes inside the cells, mainly in the mitochondria, cytoplasm and vacuole. We believe that the incubation medium has to be as simple as possible, and free of components that may affect the PMP. It is 10 mM MES-TEA buffer, pH 6.0; 20 mM glucose, and 10 μM $BaCl_2$ to avoid the binding to the surface of the cells; final volume, 2.0 mL. Besides, changes have been estimated by adding 5 μL 3% H_2O_2, 10μM CCCP (Carbonylcyanide-*m*-phenylhydrazine), or 10 mM KCl. As mentioned, fluorescence is followed at the corresponding maxima of excitation and emission wavelengths, using an SLM Aminco spectrofluorometer with stirring and temperature regulation at 30°C. The composition of the incubation medium is an important factor in the collection of the spectral changes (Calahorra et al. 2017b; Peña et al. 2010).

Microscopic Fluorescence Images

Fluorescence images were obtained by microscopy, providing more information about the localization of the dyes under different conditions. Microscope fluorescence images have been obtained, both with $DiSC_3(3)$ (Peña et al. 2010) and with pure thioflavin T obtained from Biotium. This combined approach allows to define what is supposed to produce the fluorescence changes.

Accumulation of Dyes

To obtain the actual PMP values, the accumulation of thioflavin T or other dyes during 10 min was measured under different conditions. In all cases, the essential medium was the same already mentioned, and the indicated concentrations of the dyes, in a final volume, 3.0 mL. Where indicated, 10 μM CCCP and, or 100 μg chitosan, low molecular weight, without or with 200 mM KCl was added. The use of an adequate incubation medium, particularly the buffer has been discussed before (Peña et al. 2010).

After equilibrating the tubes to 30°C in a water bath, 150 mg of cells were added, a) with glucose; b) with glucose plus 10 μM CCCP; c) with 10 mM KCl, and the cells were centrifuged after 10 min. In order to correct the accumulation values for the total binding of the dye to the internal components of the cells, the amount of dye remaining inside was measured as described in (b), but adding 100 μg of chitosan, which allows (by permeabilizing the cells) to quantify that total dye bound inside due to its cationic and hydrophobic nature. To obtain the value of the binding because of the cationic nature of the dye, cells were also incubated as described in (b), but in the presence of 100 μg of chitosan and 200 mM KCl, assuming that in this way the bound dye can be displaced from probable anionic sites; the remaining dye after KCl addition was taken as that bound only to hydrophobic sites. Chitosan used was low molecular weight (Sigma), dissolved in water to a concentration of 10 $mg.mL^{-1}$ by the addition of HCl to a pH around 4.8.

In all cases, using a DW2a SLM Aminco-Olis spectrophotometer, the absorbance values at their maximum absorption lambda of adequate dilutions were recorded and concentration was calculated by comparing with the absorbance of a standard curve of each.

K^+ Efflux and Uptake

It was found that several of the cationic dyes affect the response of a K^+ selective electrode, so the efflux they produced was followed by incubating cells as follows. Cells (150 mg wet weight) were incubated for 10 min at 30ºC in 10 μM MES-TEA, pH 6.0, 20 μM glucose with 200 μM of each dye, in a final volume of 5 mL. After incubation, cells were centrifuged and K^+ in the supernatant was measured by flame photometry (Carl Zeiss PF5). Total K^+ in the cells was obtained by boiling an equal number of cells with no dye added, during 10 min.

To measure the uptake, after incubating in a similar medium, but with 5 mM KCl, the tubes were cooled in ice, centrifuged, and washed with 4.0 mL of ice cold water also by centrifugation. The final pellet obtained was resuspended in 5.0 mL of water and placed in boiling water for 10 min. After centrifugation, the K^+ concentration was measured in the supernatant. To calculate the internal concentration, an internal water volume of 0.45 $mL.g^{-1}$ of cell wet weight was considered (Sánchez et al. 2008).

Calculation of the Vacuolar and Cytoplasmic Volumes

In previous and recent experiments microscopic images showed that thioflavin T, under basal conditions (only with glucose), does not accumulate in the vacuole, so it was required to calculate the vacuolar and cytoplasmic volumes as described before. Shortly, from images obtained in the microscope, the mean relative radii of vacuoles and cytoplasm for 30 cells were obtained with Image J software. Knowing the internal water

volume of the cells (Sánchez et al. 2008), the values of the internal water volumes could be obtained, with the results shown in Table 1.

Table 1. Calculation of the internal volumes

Total cell water per g of cells	**0.372 mL (Sánchez et al. 2008)**
Mean radii of 30 cell images	0.821 relative value
Mean radii of their vacuole images	0.404 relative value
Apparent mean cell volume	2.318
Apparent mean vacuole volume	0.276
Vacuole/cell volume ratio	0.119
Total vacuole water per g cells	0.0442 mL
Cytoplasm volume per g of cells	0.3277 mL
Total water for 150 mg cells	0.0558 mL
Cytoplasm volume for 150 mg cells	0.0492 mL
Vacuole volume for 150 mg cells	0.00663 mL
Cytoplasm/total volume ratio	0.882

RESULTS

Fluorescence Changes

Changes obtained with pure thioflavin T are shown in Figure 2, when added to yeast cells at similar concentrations (15 µM) to those used before (Calahorra et al. 2017a).

The result was similar to that reported before with $DiSC_3(3)$ (Peña et al. 2010), and also with the supposed acridine yellow (Calahorra et al. 2017a; Calahorra et al. 2019), now thioflavin T. A low increase of fluorescence that reached a constant value after approximately 2 min, that can be interpreted as a result of the accumulation of the dye by the mitochondria, where due to the high concentration reached, its fluorescence is quenched. It was then followed by a small increase when oxygen was exhausted, due to a partial deenergization of the mitochondria, that however still can maintain its membrane potential by using the ATP synthesized by glycolysis. In fact, this

small increase can be reversed by adding hydrogen peroxide. Then a small concentration of CCCP (10 µM) added to fully deenergize the mitochondria, produced a large fluorescence increase, that was followed by a large and slow decrease of fluorescence after adding 20 mM KCl.

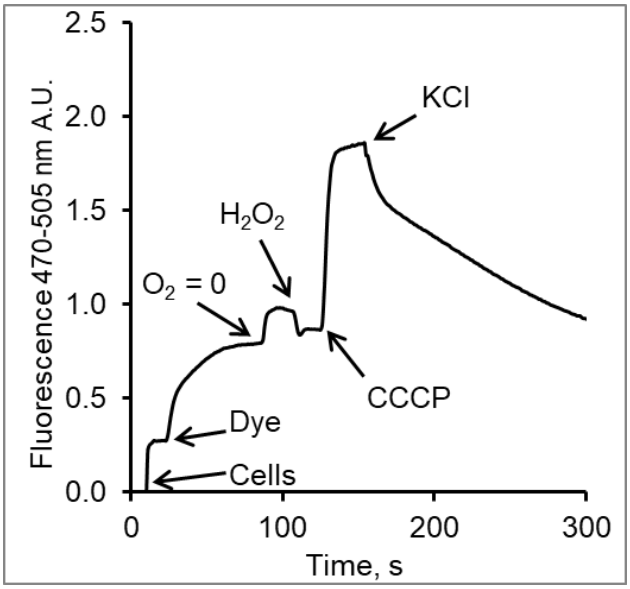

Figure 2. Fluorescence changes of thioflavin T with yeast cells. The incubation medium was 10 mM MES-TEA buffer, pH 6.0, 20 mM glucose, 10 µM $BaCl_2$, to a final volume of 2.0 mL. Tracings show the changes after the addition of 50 mg of yeast cells; 15 µM thioflavin T; the exhaustion of oxygen (O_2= 0); the addition of 5µL of 3% H_2O_2; the addition of 10 µM CCCP, and the addition of 20 mM KCl. Fluorescence changes were followed in an SLM spectrofluorometer at 470 nm excitation and 505 nm emission wavelengths respectively.

Microscopic Images

Consistent with these results, the images in Figure 3 show: a) with glucose alone, a rather low fluorescence is located in the mitochondria, in agreement with the idea that these organelles highly concentrate the dye to a degree that results in quenching of fluorescence, which also happens with $DiSC_3(3)$ (Peña et al. 2010); b) after the addition of 10 µM CCCP, there is a

large increase of fluorescence, no longer concentrated in the mitochondria, consistent with the large fluorescence observed after the addition of the uncoupler in the spectrofluorometer tracings; c) the further addition of 20 mM KCl produced a large decrease of the observed fluorescence.

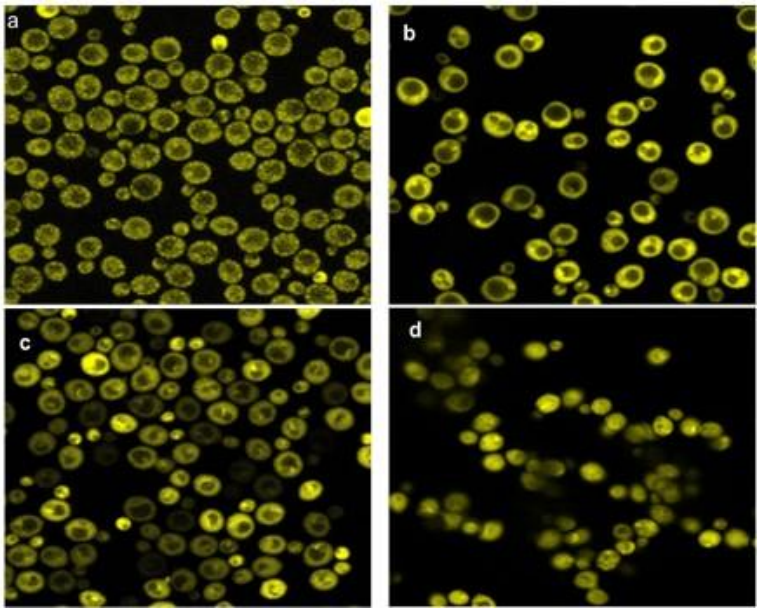

Figure 3. Fluorescence images of cells observed by microscopy under different conditions. Images were obtained as described under methods using the same medium described for Figure 2, containing 10 mM MES-TEA buffer, pH 6.0, 10 µM $BaCl_2$, 20 mM glucose, 50 mg of cells, wet weight, followed by the dye (15 µM). a: image obtained approximately 5 min after adding the cells in the buffered medium with $BaCl_2$ and glucose. b: image from the same cells approximately 5 min after adding 10 µM CCCP. c: image obtained 5 min after the addition of 20 mM KCl and d: image obtained 5 min after the addition of 100 µg chitosan and 200 mM KCl.

After the addition of chitosan (100 µg), also a decrease, but not the disappearance of fluorescence was observed, indicating that still a significant amount of the dye remains bound inside the cell, due to its hydrophobic and cationic nature. The combined addition of chitosan and 200 mM KCl produced a further decrease of fluorescence (data not shown), but not its disappearance, indicating that under these conditions, although this K^+ concentration displaces the dye bound due to its cationic nature, part of

it still remains because of its hydrophobic character. In the absence of chitosan, the dye did not enter the vacuole, but in its presence, it distributed uniformly inside the cell d). Results are perfectly consistent with those presented before (Calahorra et al. 2017a), although a lower concentration was used.

Other Dyes

Also other dyes have been used to follow the fluorescence changes; most of those tested show similar changes to those described for thioflavin T. Tracings are shown in Figure 4.

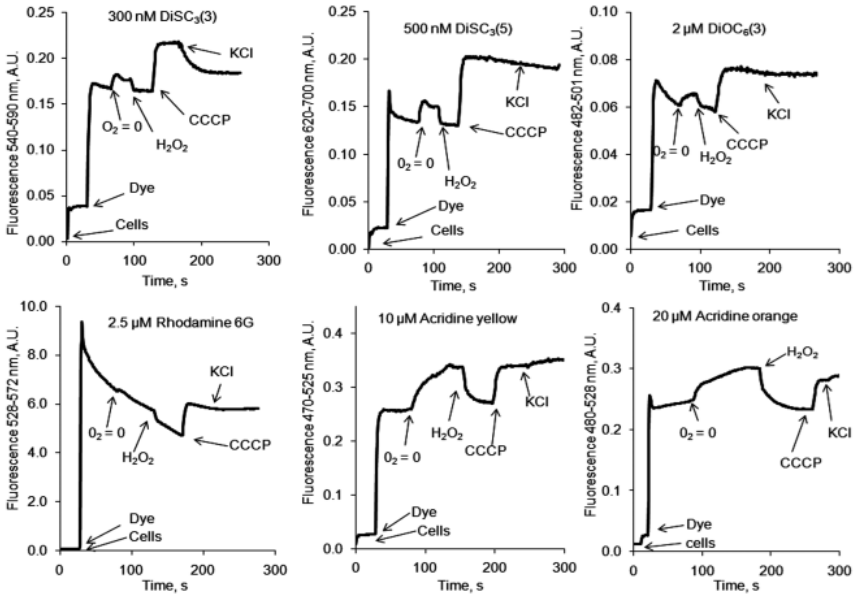

Figure 4. Fluorescence changes of several dyes upon their interaction with yeast cells. The incubation conditions were the same used in Figure 2. Fluorescence was followed at the maximal excitation-emission wavelengths of the respective dyes. The concentration used is indicated in each case, obtained as the best by following the changes with that concentration producing the clearest changes. Similar changes were observed in the other figures, except for the changes produced after the addition of KCl.

In all cases, most changes were similar to those observed with thioflavin T, however, probably the clearest difference was that the response to the addition of KCl was not observed, except in the case of $DiSC_3(3)$. Another interesting finding was that with rhodamine 6G, all concentrations tested showed a decrease in its fluorescence, probably because this dye requires higher concentrations to observe the changes that concentrate it in the cell, with the consequent quenching of fluorescence.

PMP VALUES OBTAINED BY THE ACCUMULATION OF DYES

Corrections

Previous PMP values (in millivolts) obtained by measuring the accumulation of $DiSC_3(3)$ at two concentrations in the presence of 10 μM CCCP (Peña et al. 2010) were around -168 mV and -140 mV when 5 mM KCl was added

The measurement of the dye accumulation at concentrations similar to those of the fluorescence experiments produces uncertain results because practically all the dye is taken, as shown by the already described images of the cells. In new experiments, using thioflavin T, a larger concentration was used, with more reliable results. Since the microscope images (Figure 3) and also previous accumulation experiments, clearly showed that mitochondria concentrate the dye, this was avoided by the addition of 10 μM CCCP. In the first experiment performed as described, but using 300 μM thioflavin T, apparent values for the PMP of -220 mV with glucose were obtained, which decreased to -184.7 mV by the addition of CCCP, and to -146.8 mV after the further addition of 10 mM KCl. It is so clear that values with glucose alone are overestimated because a large part of the dye accumulates in the mitochondria, but also that even in the presence of CCCP, the amount of dye inside the cells is not only due to the PMP difference, but also to its binding to the internal components of the cells.

To correct the values obtained in the presence of glucose and CCCP, adding chitosan, that permeabilizes the cells (Peña et al. 2013), a large amount of the dye that remains bound to their internal components can be estimated, and this is then subtracted from the total accumulated in the absence of chitosan.

In previous work (Calahorra et al. 2017a), in cells permeabilized with chitosan, the amount of dye bound because of its cationic nature was estimated by attempting to displace it by the addition of 10 mM KCl, but this concentration is not enough to displace all the dye bound to anionic sites. In intact cells, after the addition of 10 mM KCl, the internal K^+ concentration reaches around 200 mM; for that reason, in the new experiments, after the addition of chitosan, where the cells are already permeabilized, that concentration of KCl (200 mM) was added to achieve maximal displacement of the dye.

In the Presence of Glucose

To correct the values obtained for the apparent PMP with glucose plus, the cytoplasmic bound dye in the cells was measured after the addition of 100 μg of chitosan, and this concentration was then subtracted from the total accumulated in the cytoplasm of intact cells in the presence of glucose (plus CCCP) to calculate what was considered the actual PMP value. The corrected internal concentration divided by the external one obtained for glucose plus CCCP gave an internal/external concentration ratio whose negative log is -2.67, which multiplied by 60 results in a corrected value of -173.0 mV.

Glucose + KCl

The corrected PMP values obtained when chitosan and 200 mM KCl were added were obtained as follows: The external concentration obtained after chitosan +200 mM KCl allowed estimating the amount of dye remaining within the cell due to its hydrophobicity, and by the difference from the total amount in the absence of KCl, that bound because of its cationic nature. The corrected internal/external concentration ratio in the presence of glucose plus KCl in intact cells was obtained, and from its

negative log, a PMP value of -117.8 mV was calculated. Only values in the presence of CCCP are presented because in its absence they are influenced by the large accumulation by the mitochondria.

Table 2. Values of the PMP (in mV) of yeast cells under different conditions. Raw and corrected values. n=5

Condition	Raw values	Corrected values
Glucose-CCCP	-184.7 ± 3.6	-173.0 ± 9.6
Glucose-CCCP-KCl	-146.8 ± 3.7	-117.8 ± 3.5

The Use of Other Dyes

One of the main differences of thioflavin T and other dyes is that its hydrophobicity, measured as the distribution coefficient between water and dichloromethane is as follows:

Table 3. Distribution coefficients of several dyes between dichloromethane and buffer

Dye	Distribution Coefficient
Thioflavin T	9.4
$DiSC_3(3)$	26.6
$DiSC_3(5)$	893.4
$DiOC_6(3)$	164.3
Safranin O	0.36
Neutral Red	3.37
Rhodamine 6 G	2.16
Acridine yellow	12.5
Acridine orange	0.94

Hydrophobicity of dyes measured as their distribution coefficients between 2 mL of 10 mM MES-TEA, pH 6, and 2 mL of dichloromethane. The dyes, 120 μM, were added and vigorously stirred in a vortex mixer. Then the mixture was centrifuged for 5 min at 3000 RPM. The absorbance of the water layer was measured at the maximal lambda value of each dye and its concentration calculated by comparison with a standard curve.

Clearly, thioflavin T has a low distribution coefficient between dichloromethane and water, indicating that it is among the least hydrophobic of the dyes tested, and values of the PMP could be obtained from its accumulation. $DiSC_3(3)$, with the lowest hydrophobicity of other similar dyes and higher than those of thioflavin T. That dye, as shown in Table 4 was tested, and gave results not very different from those with thioflavin T. Results were then compared with other dyes at 200 μM: rhodamine 6G, safranin O, neutral red, real acridine yellow, and acridine orange.

Table 4. Raw and corrected values of the PMP of yeast cells with different dyes

	Raw values	**Corrected values**
Thioflavin T 333 μM (n = 5)		
Glucose-CCCP	-184.7 ± 3.6	-173.0 ± 9.6
Glucose-CCCP-KCl	-146.8 ± 3.7	-117.8 ± 3.5
$DiSC_3(3)$ 167 μM (n = 3)		
Glucose-CCCP	-210.4 ±3.9	-181.9 ± 5.9
Glucose -CCCP-KCl	-202.0 ±4.9	-166.5 ± 7.8
Rhodamine 6G 200 μM (n = 3)		
Glucose - CCCP	-194.9 ± 9.8	-173.3 ± 9.5
Glucose -CCCP-KCl	-147.1 ± 17.4	-155.2 ± 16.7
Safranin 200 μM (n = 3)		
Glucose-CCCP	-169.1 ± 1.8	-146.1 ± 2.1
Glucose-CCCP-KCl	-150.0 ± 3.2	-163.0 ± 2.9
Neutral red 200 μM (n = 3)		
Glucose-CCCP	-173.3 ± 25.3	-149.7 ± 36.2
Glucose -CCCP -KCl	-138.2 ± 31.4	-154.7 ± 31.6
Acridine yellow 300 μM (n = 4)		
Glucose -CCCP	-209.8± 8.5	-195.4 ± 9.6
Glucose CCCP KCl	-155.4± 12.0	-141.9 ± 9.7

Values were obtained as described in the text for thioflavin T at the indicated concentrations. Means of 3 to 5 experiments, each ± standard deviation.

Most of them gave rather similar results, except for safranin O and neutral red, with values that after correction by the addition of KCl, in the presence of chitosan, for some reason we cannot explain, were higher than

in its absence. Results with acridine orange are not presented because it gave similar results under all the different conditions.

Collateral Effects of High Concentrations of Thioflavin T on the Acidification Capacity, K^+ Transport, and Respiration

Particularly in the case of thioflavin T, concentrations used for the accumulation experiments (333 μM), higher than those used before, require observing their effects on three important physiological parameters: the capacity of the cells to acidify the medium, to take up potassium, and respiration. In the case of thioflavin T, it did not inhibit acidification of the medium, but on the contrary, increased it for about two minutes, and then returned to a rate almost similar to that of the control. One of the main *S. cerevisiae* functions, proton pumping, on which many other transport systems depend, was not inhibited, but also increased, probably because of the energy required for the uptake of the dye at these concentrations. Regarding K^+ uptake, tracings could not be obtained because the K^+ selective electrode responds to the addition of the dye. However, the effect of all the dyes tested was also estimated on two parameters: a) the uptake of K^+, adding 5 mM KCl to the incubation media, followed by measuring its accumulation in the presence of 200 μM of each dye, and b) the efflux of K^+ produced under the same conditions, but in the absence of externally added KCl. Results are shown in Figure 5.

Some dyes produced a small increase of the K^+ efflux, and mainly acridine yellow and acridine orange clearly increased the efflux of the monovalent cation. This corresponded in a reverse way with the uptake of K^+.

It was also found that thioflavin T did not inhibit respiration, but stimulated it, and CCCP still further stimulated it (not shown). This means that at these concentrations (100 μM to 300 μM) the presence of the dye results in an increase of both basal and maximal (uncoupled) mitochondrial respiratory capacity.

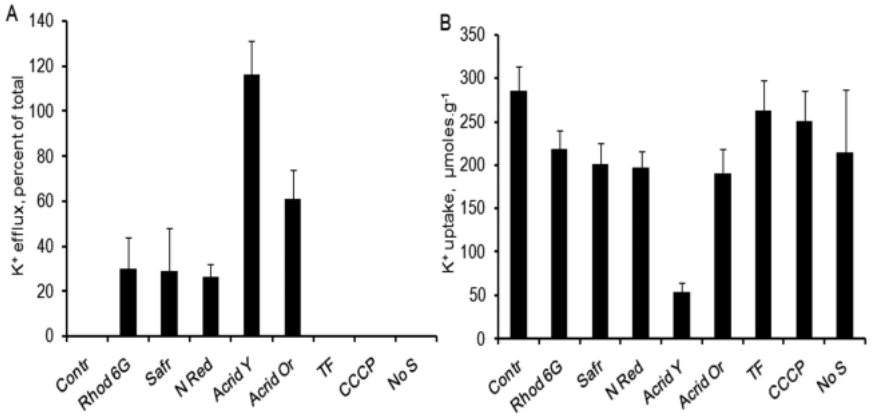

Figure 5. Effects of different dyes at a 200 µM concentration on K^+ uptake and efflux. (A) Efflux in a medium without KCl, and (B) Uptake in the presence of 5 mM K^+, as described in Methods. Contr = Control, Rhod 6G = Rhodamine 6G, Safr = Safranine, N Red = Nile red, Acrid Y = Acridine yellow, Acrid Or = Acridine orange, TF = Thioflavine, No S = no substrate added. Values are means ± standard deviation of 3 to 4 different experiments.

Particular Comments on Dyes

Although it would appear that any fluorescent or colored dye might be used to actually measure the PMP of yeast cells with the adequate corrections described, there are some pertinent observations on several of them: $DiSC_3(3)$, $DiSC_3(5)$ and $DiOC_6(3)$. Although the first is the least hydrophobic of those tested, in experiments performed to correct the values for its displacement by KCl it was found that after the treatment with chitosan, a large part of it remains bound to the cells, and only a very small amount could be displaced by the high concentrations of KCl. This should be a valuable argument to eliminate it, as well as $DiSC_3(5)$ and $DiOC_6(3)$, which are even more hydrophobic as useful for the quantitative measurement of the PMP.

Safranin O and neutral red. Because of the unexplainable behavior, with the corrected values higher in the presence of KCl than in its absence, they

are not to be adequate for the purpose of measuring the real values of the PMP in yeast.

Rhodamine 6G. This dye appeared to be also adequate, with similar values to those for thioflavin T, except for the fact that fluorescence intensity did not decrease upon the addition of KCl.

Acridine yellow. Although values obtained are more or less close to those with thioflavin T, the corrected figures for the PMP in the absence of KCl appear to be rather high.

Thioflavin T. This is a cationic dye with a low hydrophobicity, and what appears to be a balance between these two properties, that may be proposed to measure the actual values of the PMP in yeast, given the correction factors used.

CONCLUSION

It would appear that in principle, any more or less hydrophobic cationic dye could be used to in fact measure the real PMP difference in yeast; results point to thioflavin T as the best dye for that purpose. It should be made clear that the dye used in the previously reported experiments (Calahorra et al. 2017a, b; Peña et al. 2017) was not acridine yellow, but thioflavin T (Calahorra et al. 2019).

The use of thioflavin T allowed the best approximation to the estimation by fluorescence and measurement by its accumulation, of the PMP in *S. cerevisiae*. Results also show that neither the fluorescence changes nor the simple accumulation values under different conditions can be taken as such without considering the uptake of the dye by the mitochondria and its binding due to both its hydrophobic and cationic nature. Already the fluorescence changes observed indicate that a large proportion of the dye was accumulated by the mitochondria, and a low concentration of CCCP (10 to 20 μM) is enough to deenergize the mitochondria without an effect on the plasma membrane of yeast (Peña 1975). This fortunate situation allows eliminating the participation of the mitochondria. This conception was supported by the observation of the fluorescence images that clearly showed

that with glucose alone, although the cells captured a large proportion of the dye, it was located largely in the mitochondria, and fluorescence was faint because the high accumulation resulted in quenching. Also from the microscope images it could be concluded that after permeabilizing the cells with chitosan (not shown) an important portion of the dye remained inside imputable to its hydrophobic and cationic characteristics.

From these arguments, it was obvious that by permeabilizing the cells with chitosan, the total amount of bound dye within the cells could be measured. But not only this, also in the cells permeabilized with chitosan, adding a large enough concentration of KCl (200 mM), the dye bound to anionic sites could be displaced, remaining only that bound to hydrophobic molecules; then, by difference the cationic binding could be obtained. In this way the concentration values obtained with glucose plus CCCP could be corrected by subtracting from the total accumulated. In the case of the cells incubated with 10 mM KCl the situation is the reverse: the uptake of K^+ results in an internal dye concentration decrease not only owed to the decrease of the PMP by the entrance of positive charges, but also because it results in an accumulation of around or more than 200 mM of the cation, which displaces the dye from its binding to anionic sites. By the procedure described, the amount and concentration of the dye displaced by K^+ when in intact cells 10 mM KCl was added could be obtained; then this concentration of the dye could be added to that found in intact cells incubated in the presence of KCl (with CCCP). These correction factors can be obtained with enough approximation and used to calculate the real PMP values. It is important to remark that all values of the PMP require the presence of 10 to 20 μM CCCP to eliminate the additional and very large accumulation of the dye by the mitochondria.

The results obtained are higher than those reported by other authors (Vacata et al. 1981); (De la Peña et al. 1982), and definitely much higher than those proposed by (Plášek et al. 2012) and (Plášek and Gášková 2014). The two latter reports also have a problem, they assume that the PMP of yeast is collapsed by a low concentration (10 μM) of either CCCP or FCCP, which as mentioned is not enough to increase the H^+ conductivity of the plasma membrane of yeast. This also can be confirmed by the experiments

shown in Figure 5, in which it is clear that CCCP at the concentration used (10 μM) did not affect the uptake, neither increased the K^+ efflux, that would be expected if the uncoupler at that concentration affected the PMP.

With the corrected values, values were lower in the presence of KCl (Table 2), but the decrease was perhaps not so large as expected from the fluorescence changes. This can be explained because although in the presence of K^+, a large decrease of PMP would be expected, former studies (Peña et al. 1969; Peña 1975) showed that the addition of the cation immediately results in an increase of the H^+ pumping by the H^+-ATPase of the plasma membrane. Then this expenditure of ATP produces an increase of ADP which is responsible for the acceleration of glycolysis (and respiration), and by that the ATP levels are recovered. It is so that the H^+-ATPase activity, supported by an increased rate of glycolysis can maintain the PMP values not much lower than those present in the absence of K^+. In the mentioned studies it was found that in fact, glycolysis is accelerated in the presence of potassium, but also that the acceleration persists after K^+ has been taken up by the cells, probably because energy is necessary not only to capture the cation; after this happens, a new equilibrium is established between its uptake and efflux, due to the high concentrations reached inside. In fact, Rothstein and Bruce (1958) described that after K^+ has been taken up by yeast a steady state is established in which an efflux-influx equilibrium takes place, that not surprisingly, is accompanied by the partial recovery of the PMP at the expense of an accelerated glycolysis. It is so that the H^+-ATPase activity, supported by an increased rate of glycolysis can maintain the PMP values not much lower than those present in the absence of K^+.

Using a similar procedure; i.e., with the correction factors, $DiSC_3(3)$, the least hydrophobic of the cyanines, produced results not far different from those with thioflavin T, even though it binds inside with a large affinity because of its hydrophobicity. On the other hand, using 200 μM safranin O, rhodamine 6G and neutral red, for some reason, values obtained in the presence of CCCP were lower than those obtained with thioflavin T. Also, we cannot explain why values after adding KCl were higher than in its absence.

Another dye used before is ethidium bromide, but it has a problem, it appears to be transported by the same carrier as K^+ (Peña and Ramírez 1975). It seems that even small variations in structure make some dyes unsuitable, even for the qualitative estimation of the PMP.

All previous results reported regarding the quantitative value of the PMP in yeast cells are probably wrong because they did not consider the internal binding of the agents used under different conditions.

In summary, the studies performed allow to propose the use of thioflavin T to estimate by fluorescence, and measure by its corrected accumulation, the actual PMP values of yeast cells.

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CONFLICT OF INTEREST

The authors have declared that no conflict of interest exists.

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