Attempts at Measuring Membrane Potential in *S. cerevisiae* by the Electrochromic Probe di-4-ANEPPS

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Attempt was made to measure the membrane potential in yeast cells by the electrochromic probe di-4-ANEPPS (dibutylaminonaphthylethylene pyridinium propyl sulfonate) which has previously been used for measuring action potentials in neurons [1, 2]. This probe is believed to provide fluorescent response to changes in transmembrane electric field in nanoseconds by changing its fluorescence intensity due to an underlying wavelength shift of emission maximum. The requirements for successful measurement are (*1*) defined dependence of the fluorescence response on change in membrane potential, (*2*) low probe toxicity at the concentrations used, (*3*) reproducible incorporation of the probe solely into the outer layer of the membrane lipid bilayer (incorporation into the inner layer would give rise to two probe pools whose respective responses to membrane potential changes would be mutually opposite, hampering the measurement), (*4*) absence of any penetration of the probe into the cell.

The fluorescence of the electrochromic probe was measured in suspensions of intact cells, protoplasts and phosphatidylserine/phosphatidylcholine (20/80) liposomes. Tentative adjustment of membrane potential was done by incubating the samples in 3.5–150 mmol/L KCl, the overall molarity being adjusted in each case to 150 mmol/L by choline chloride. The effect of nonuniform staining of individual cells on the excitation spectrum of the probe was eliminated by measuring the ratio of fluorescence intensities at excitation wavelengths of 450 and 530 nm [3, 4].

The measurements showed that (*1*) the probe responds to membrane potential change by an electrochromic shift; (*2*) the cell wall hampers the penetration of the probe to the plasma membrane of yeast cells; (*3*) the actual equilibration of the probe in cell suspension should take 10–15 min but in fact the staining intensity keeps on rising even at longer intervals; (*4*) this is due to the fact that the probe is not incorporated solely into the plasma membrane but spreads gradually into the cells and liposomes, which causes persistent variations in fluorescence response to membrane potential change. This penetration brings about a fluorescence change mimicking a decrease in membrane potential, *i.e.* membrane depolarization. The probe is therefore suitable for monitoring membrane potential in yeast only over short periods of time (up to 30 min). Longer monitoring will require either a modified staining protocol or derivatization of the probe molecule. As found by using the dioctyl derivative di-8-ANEPPS, extending the aliphatic chains of the di-4-ANEPPS molecule does not prevent the dye from penetrating into the cell or liposome interior and, in addition, impairs staining.

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[1] FROMHERZ P., LAMBACHER A.: *Biochim.Biophys.Acta* **1068**, 149–156 (1991).
[2] GRINVALD A., HILDESHEIM R., FARBER I.C., ANGLISTER L.: *Biophys.J.* **39**, 301–308 (1982).
[3] MASON W.T.: *Fluorescent and Luminescent Probes for Biological Activity.* Academic Press, London–New York 1993.
[4] MONTANA V., FARKAS D.L., LOEW L.M.: *Biochemistry* **28**, 4536–4539 (1989).

Monitoring of Membrane Potential Changes in *Saccharomyces cerevisiae* by diS-$C_3(3)$ Fluorescence

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The Nernstian membrane potential $\Delta\psi$ was monitored in *Saccharomyces cerevisiae* strains S6 and RXII by using the redistribution fluorescent dye 3,3′-dipropylthiacarbocyanine iodide (diS-$C_3(3)$). The $\Delta\psi$-dependent entry of diS-$C_3(3)$ into the cells was accompanied by increased fluorescence intensity and a red shift of fluorescence maximum, λ_{max}, caused by reversible binding of the dye to proteins and other cell components. The dye redistributed across the cell membrane within 5–30 min depending on experimental

conditions. To avoid problems with variations in fluorescence intensity brought about by dye binding to cuvette surfaces, the extent of the λ_{max} shift was used instead of fluorescence intensity as a measure of $\Delta\psi$, see [1] for details. This λ_{max} response depends on the thickness of the cell wall, on the cell growth phase, cell size, and the relative cell-to-dye concentration [2]. The time courses of yeast staining monitored as time-dependent shifts of λ_{max} were assessed in different situations:

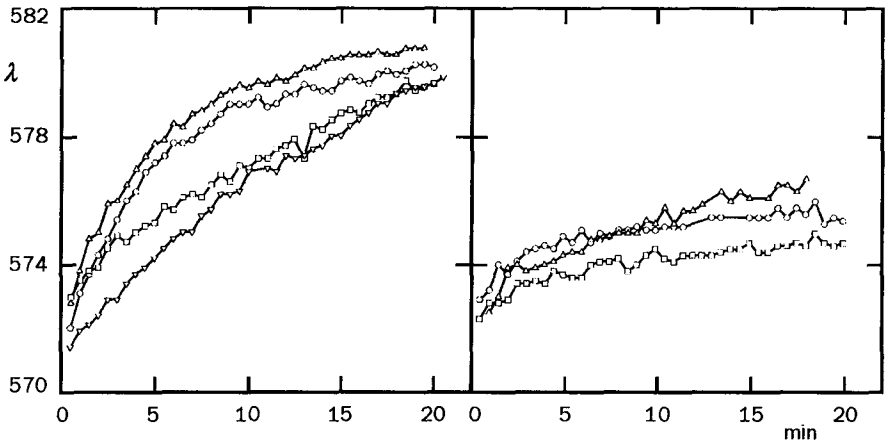

Fig. 1. Time course of diS-C_3(3) staining of yeast cell suspensions kept in air (*triangles*), bubbled with oxygen (*circles*), bubbled with N_2 (*squares*), or growing in YEPG medium containing 4 % (*W/V*) glucose (*inverted triangles*). *Left*: Wild-type *S. cerevisiae* RXII cells in the stationary growth phase were harvested by centrifugation, washed twice in distilled water and suspended in 10 mmol/L citrate-phosphate buffer (pH = 4.6) to a final concentration of 5/nL. Dye concentration – 0.1 μmol/L, measured at 25 °C, λ – fluorescence maximum (nm); time, min. *Right*: Respiration-deficient *S. cerevisiae* RXII *rho*$^-$ mutants; for details *see left*.

Aerobiosis, anaerobiosis, glucose addition: The staining process was found to be faster in cells kept in air or bubbled with oxygen than in cells bubbled with nitrogen. When the cells were cultivated at an elevated concentration of glucose (*e.g.* 4 % *W/V* instead of the standard 2 % *W/V*), the staining pattern resembled that found in N_2-flushed yeast suspensions (Fig. 1 *left*). Aerobic cells thus differ from anaerobic or glucose-repressed cells in the speed of diS-C_3(3) uptake in that the staining of aerobic cells, whether kept in air or supplied with oxygen, is about twice as fast as that found in either anaerobic or glucose-repressed cells. This lower ability of repressed (or anaerobic) cells to generate membrane potential is apparently associated with a lower contribution of the mitochondrial membrane potential to the overall $\Delta\psi$. The role of mitochondrial membrane potential contribution was documented in a theoretical simulation of intact versus mitochondria-deprived cells (*unpublished data*) and was further supported by the finding that in respiration-deficient (*rho*$^-$) mutants (Fig. 1 *right*), in which the mitochondria are not functional, the level of staining, and thus of membrane potential generation, was substantially lower than in intact cells.

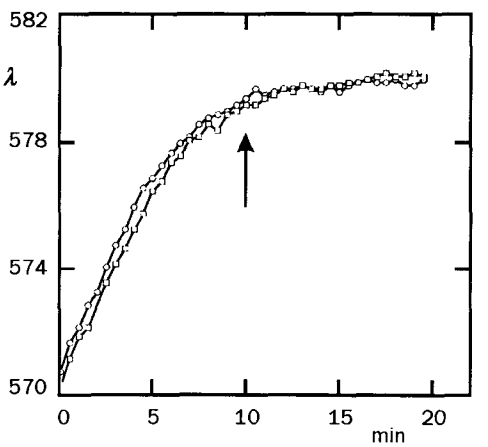

Fig. 2. Effect of hydrogen peroxide addition on the yeast staining curve. The *arrow* indicates the time when 100 mmol/L H_2O_2 was added to the cells (*circles*); *squares* – control without H_2O_2 addition. Stationary-phase *S. cerevisiae* S6 cells were treated as described in Fig. 1.

Cell depolarization by external K^+ and by uncouplers: The addition of valinomycin (1 μmol/L) slightly facilitated membrane depolarization at increased extracellular K^+ [3], while the addition of the protonophore carbonylcyanide-3-chlorophenylhydrazone (CCCP) (10 μmol/L) caused a fast membrane depolarization, *i.e.* a blue shift in λ_{max}. The depolarization was usually not complete, part of the dye remaining bound in the cells.

Oxidative stress by hydrogen peroxide: Addition of 100 mmol/L H_2O_2 to the cell suspension had no measurable effect on the λ_{max}, *i.e.* on the $\Delta\psi$ (Fig. 2). However, when the cells were first supplied with $FeSO_4$ (50 μmol/L), which strongly facilitates the generation of OH• radicals in the presence of H_2O_2 [4],

the H_2O_2 addition caused an instant red shift of λ_{max} (Fig. 3 *above*) accompanied by a simultaneous drop in fluorescence intensity (Fig. 3 *below*). These changes, however, do not reflect cell hyperpolarization but are due to an interaction of OH• radicals with extracellular free probe molecules, as found by measuring the fluorescence in buffer without cells (Fig. 4). In contrast to the free probe molecules, the dye present inside the cells (both bound and free) is protected against the radicals. Subsequent addition of CCCP

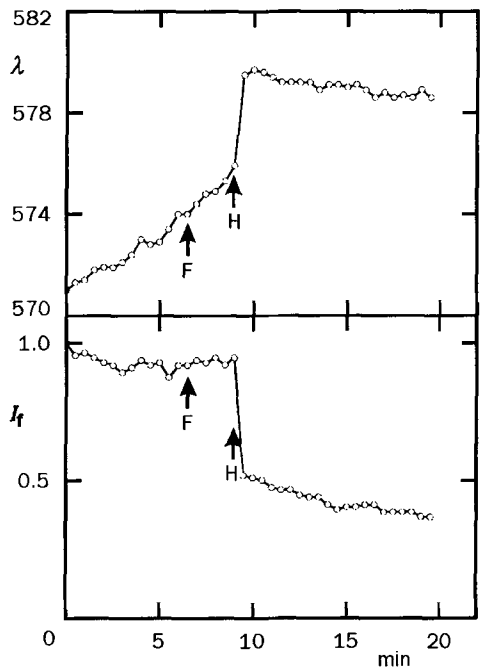

Fig. 3. Time course of change in the position of fluorescence maximum (*top*) and in fluorescence intensity (*bottom*) of diS-C_3(3) in a stained suspension of *S. cerevisiae* S6 after addition of $FeSO_4$ and H_2O_2. *Arrows* indicate additions of 50 μmol/L $FeSO_4$ (F) and 100 μmol/L H_2O_2 (H). Experimental conditions as in Fig. 1.

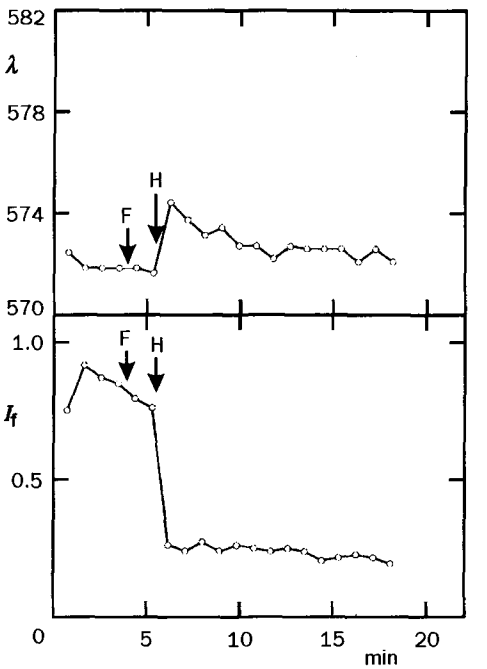

Fig. 4. Time course of change in the position of fluorescence maximum (*top*) and in fluorescence intensity (*bottom*) of diS-C_3(3) in a CP buffer (pH = 4.6) after addition of $FeSO_4$ and H_2O_2. The final concentration of the dye was 0.1 μmol/L (measured at 25 °C). Arrows indicate the times when 50 μmol/L $FeSO_4$ (F) and 100 mmol/L H_2O_2 (H) were added.

depolarizing the cells releases these undamaged probe molecules into the medium and the increased concentration of (now free) intact extracellular dye causes a blue shift in λ_{max} (Fig. 5).

The spectral shift of diS-C_3(3) fluorescence maximum (λ_{max}) may thus be successfully used for measuring $\Delta\psi$ transients in yeast cells even under extreme conditions, when part of the dye loses its fluorescence due to an interaction with radicals.

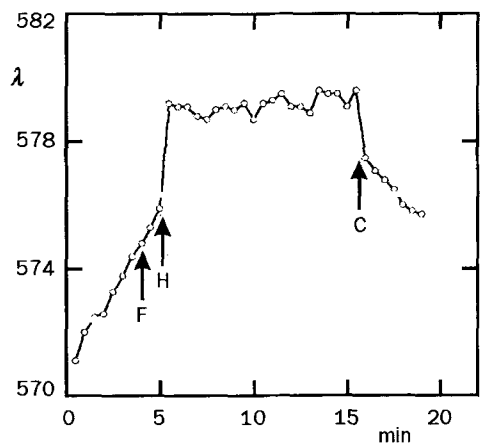

Fig. 5. Effect of adding the protonophore CCCP on the staining (λ_{max} change) of *S. cerevisiae* S6 suspension previously exposed to $FeSO_4$ and H_2O_2. Arrows indicate the times when 50 μmol/L $FeSO_4$ (F), 100 mmol/L H_2O_2 (H) and 10 μmol/L CCCP (C) were added. Experimental conditions as in Fig. 1.

[1] VEČEŘ J., HEŘMAN P., HOLOUBEK A.: Diffusion membrane potential in liposomes: setting by ionic fluxes, absolute calibration and monitoring of fast changes by spectral shifts of diS-C_3(3) fluorescence maximum. *Biochim.Biophys.Acta*, submitted for publication.

[2] DENKSTEINOVÁ B., GÁŠKOVÁ D., HEŘMAN P., VEČEŘ J., SIGLER K., PLÁŠEK J., MALÍNSKÝ J., p. 151 in *Proc. 2nd Conf. on Fluorescence*, Prague: *Fluorescence Microscopy and Fluorescent Probes* (J. Slavík, Ed.). Plenum Press, New York and London 1996.
[3] GÁŠKOVÁ D., DENKSTEINOVÁ B., HEŘMAN P., VEČEŘ J., SIGLER K., BENADA O., PLÁŠEK J.: Fluorescent probing of membrane potential in yeast: the role of cell wall in assays with diS-C_3(3), in preparation.
[4] HALLIWELL B., GUTTERIDGE J.M.C.: *Free Radicals in Biology and Medicine*, 2nd Edn. Clarendon Press, Oxford 1993.

Vicilin Storage Proteins Inhibit Yeast Growth and Glucose Stimulated Acidification of the Medium by Cells

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Vicilins (V) are seed storage proteins of the 7S globulin class, which are present in seeds of leguminous and other plants [1]. These proteins are characterized as oligomers of 150–170 kDa formed by three similar subunits of about 40–70 kDa with no disulfide linkages [2]. V can bind to chitin or to N-acetyl glucosamine containing poly- and oligomers [3]. The aim of the work was to learn whether vicilin can inhibit yeast growth and whether its target is the plasma membrane (PM). We found that V inhibited the growth of *Candida albicans* and *Saccharomyces cerevisiae* in a dose-dependent manner. The degree of inhibition depended on the plant species used for isolation of V and on yeast used as a target. For example, in the case of *S. cerevisiae* it was more than 90 % for vicilins from *Vigna unguiculata* (cv. *pitiuba*) and about 65 % for V from *V. radiata*. To testify whether V interacted with the yeast plasma membrane we monitored their effect on the glucose-stimulated acidification of the incubation medium. It was found that V inhibited the glucose-stimulated acidification of the medium also in a dose dependent manner. The mean value of inhibition of the acidification by V of *V. unguiculata* (cv. *pitiuba*) in the case of *S. cerevisiae* was 60 %, while nystatin showed a 95 % inhibition. A nearly full effect of V occurred after a 2-min preincubation with cells before supplying glucose and was not increased after a 10-min preincubation. The inhibition did not depend on the incubation medium used (water, Tris·HCl buffer or Tris·HCl buffer and glucitol). The test with Tripan Blue did not show any increase in permeability of the PM to molecules of its size after the V treatment, while it showed a permeabilization of about 85 % of cells after the nystatin treatment which occurred simultaneously with a 95 % inhibition of medium acidification. We supposed that possibly V can permeabilize plasmalemma for ions and, as a result, inhibit yeast growth. We found, however, that the cell inorganic phosphate content was not decreased by the V treatment, while it was decreased more than 90 % after incubation with nystatin. To test the possibility of permeabilization of plasmalemma by V only for H^+ ions, we added V to control cells after 30 min of acidification; V neither stopped acidification nor initiated a retrograde H^+ transport. Once again nystatin exhibited a different behavior: it initiated a retrograde H^+ transport when it was added to control cells or to the V-treated cells. In the last case, however, the retrograde H^+ transport was smaller. One can assume that V modifies the H^+ permeability of the plasmalemma neither at pH 5 (when added after medium acidification) nor at pH 6.5. However, we cannot rule out the possibility of an increase of H^+ permeability of the plasmalemma by V at pH 6.5. An interesting preliminary finding which can help us in understanding the mechanism of the V effect is the formation of spores after a few days of incubation of V with yeast. Washing out of V from the spores with water and a following incubation of the spores in a new medium without V restored normal yeast growth. This indicates a reversibility of the yeast growth inhibition by vicilins. We suppose that the binding of vicilins to yeast cells, probably through chitin-like components of the plasmalemma, results in perturbation of the membrane, inhibition of H^+ pumping, limited nutrition of cells and finally to spore formation.

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[1] DERBYSHIRE E., WRIGHT D.J., BOULTER D.: *Phytochemistry* **15**, 3–24 (1976).
[2] CASEY R., DOMONEY C., ELLIS N.: *Oxford Surveys of Plant Molecular Biology*, Vol. 3, pp. 1–95. Oxford University Press, Oxford 1986.
[3] SALES M.P., GOMES V.M., FERNANDES K.V.S., XAVIER FILHO J.: *Braz.J.Med.Biol.* **29**, 319–326 (1996).