J. Membrane Biol. 73, 131–136 (1983)

The Journal of
Membrane Biology

Interaction of Ethidium Bromide with Yeast Cells Investigated by Electron Probe X-Ray Microanalysis

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Summary. K^+ efflux provoked by ethidium proceeds partially as an all-or-none effect by which the diffusion barrier for K^+ is disrupted and partially from still intact cells, presumably by exchange against ethidium. This is shown by the application of an electron probe microanalysis X-ray technique by which the K^+ content of a number of individual cells is analyzed.

Key Words electron probe X-ray microanalysis · *Saccharomyces cerevisiae* · ethidium · bromophenol blue · cationic dye · cytolysis

Introduction

It has been established that a variety of cationic dyes and cationic surfactants are able to induce an almost complete loss of K^+ from yeast cells [3, 6, 8, 9, 11, 13–15]. Two different mechanisms have been proposed for the dye-induced K^+ loss.

Peña et al. [11] attributed the K^+ loss induced by cationic dyes to a specific interaction of the dyes with the transport system employed for monovalent cation uptake. It is supposed that due to that interaction the dyes provoke a K^+ efflux, which efflux is proposed to be of the electrogenic type and to give rise to a hyperpolarization of the cell membrane. The stimulation of Ca^{2+} uptake in yeast cells by several cationic dyes is considered to support that notion [9].

Other authors [3, 6, 8, 14, 15], however, put forward the hypothesis that the K^+ loss induced by cationic dyes or surfactants is an all-or-none response of individual cells. Each cell has according to this view a certain threshold concentration, which if exceeded, results in the destruction of the permeability barrier for K^+ and as a consequence, in an almost complete loss of K^+ from the affected cells. This hypothesis is supported by the fact that a correlation exists between the amount of K^+ liberated and the amount of UV absorbing substances leaking from the cells if a cationic dye or surfactant is added to the yeast suspension [3]. A similar correlation is found between the amount of K^+ liberated and the number of cells which are stained with a cationic dye or which are no longer viable [6, 8, 15]. No direct experimental proof, however, has been presented, that in fact part of the cells have lost all their K^+ and that the remainder of the cells still has the normal K^+ content, as is supposed to be the case according to the all-or-none hypothesis.

We have now examined by means of the electron probe X-ray microanalysis technique [5] the distribution of K^+ over a number of cells, which are treated with different amounts of the cationic dye ethidium. This dye is chosen, since the dye has almost no side-effects on the yeast cells [11].

Materials and Methods

Yeast cells of *Saccharomyces cerevisiae* strain Delft II were starved under aeration for 20 hr. After starvation the cells were washed three times with distilled water, centrifuged and suspended in 20 mM maleate-triethanolamine buffer, pH 6.0 at a cell density of 5% (wt/vol). After a preincubation period of 15 min with or without 3% (wt/vol) glucose, ethidium bromide was added to the suspension. The suspension was kept anaerobic by bubbling N_2 through the suspension at 25 °C. At appropriate times, 10-ml samples were taken, centrifuged and the supernatant was analyzed for K^+ and ethidium. Potassium was estimated by flame photometry. Maximum loss of K^+ was determined after boiling an aliquot of the cell suspension before the addition of ethidium. The ethidium concentrations were determined by measuring the absorbance of the dye at 482 nm with a Beckman 25 spectrophotometer.

Cells of another pure strain of *Saccharomyces cerevisiae*, denoted by us as Azteca strain, were kindly donated by Dr. A. Peña (Mexico) and prepared as described elsewhere [12]. With this yeast the effect of ethidium was studied only in the presence of glucose and at 30 °C.

For vital staining of the cells a slightly modified procedure of Borst-Pauwels and Dobbelmann [2] was followed. A small aliquot of the suspension was diluted in 180 mM NaCl, the cells centrifuged and resuspended in 0.5 mM bromophenol blue in 45 mM Tris adjusted to pH 4.5 with succinic acid. The percentages of stained cells were calculated from the number of

countings (≥ 100) of both stained and unstained cells under a Zeiss photomicroscope III (Carl Zeiss, W. Germany).

Preparation of cells for microprobe analysis was carried out as follows: exactly 5 min after the addition of ethidium a sample of 10 ml was centrifuged, the cells were washed three times with ice-cold distilled water and resuspended in distilled water. During the washings no appreciable extra loss of cell K^+ occurred, as could be shown by analysis of the supernatants by flame photometry. The cell suspension was sprayed through a glass capillary tube (internal diam. about 0.5 mm) onto pure polished carbon plates, air-dried on the support and stored under vacuum at room temperature. The specimen was coated with a carbon layer to improve conductivity and, to prevent rehydration, was kept under vacuum until analysis, according to Kuypers and Roomans [5].

Yeast cells with altered K^+ contents were prepared by incubating the starved yeast cells (2% wt/vol) in 45 mM Tris adjusted to pH 4.5, provided with 3% (wt/vol) glucose and either 100 mM KCl or 600 mM NaCl, at 25 °C under anaerobic conditions. The incubation in the Na^+-containing medium for 12 hr was repeated three times after washing the cells twice with distilled water by centrifugation. This repetition was necessary in order to replace the greater part of cell K^+ by Na^+. After appropriate incubation periods, samples of 10 ml were centrifuged and the cells were washed three times with ice-cold distilled water and resuspended in distilled water. A small aliquot of the resulting suspension was sprayed onto carbon plates, as described above. The remainder of the suspension was centrifuged immediately after the spraying was completed. The pellet was dried, weighed and destructed with nitric acid according to Kotz et al. [4]. In the resulting solution K^+ was determined by flame photometry and the total phosphorous content was determined with a Technicon Autoanalyzer II C (Technicon Ireland, Ltd., Dublin) according to Meuffels [7].

X-ray data were obtained at room temperature in a Camebax scanning electron microscope model MB76 (Cameca, France), equipped with a Cameca inclined wavelength-dispersive (WD) spectrometer and a Microtrace energy-dispersive (ED) spectrometer, and processed with a Tracor Northern TN2000 data acquisition system (Tracor Europa, Bilthoven, The Netherlands).

Carbon supports containing a large number of well-separated cells were analyzed. P and K were determined with the ED spectrometer and Ca by the detection of its K_α X-rays by means of a WD spectrometer using a PET crystal. To enable simultaneous electron-probe analysis of these elements rather high beam currents (70 nA at 15 kV) resulting in a spot size of 0.35 μm, were used. In order to reduce the large dead time of the ED spectrometer under these conditions, an aperture, specially designed for this spectrometer, was used [16]. When no Ca was measured a beam current of 5 nA at 15 kV was used.

Results

Figure 1 shows that the amount of K^+ liberated from nonmetabolizing cells increases with increasing concentrations of ethidium. At concentrations below 1 mM no detectable K^+ loss is found. In the presence of glucose, the rate of K^+ loss increases considerably resulting at the higher ethidium concentrations in a far greater K^+ loss than is found in the absence of added glucose. At the lower ethidium concentrations, however, part of the K^+ liberated within the first few minutes is again reaccumulated by the cells. Reentry of K^+ is not found in the absence of glucose.

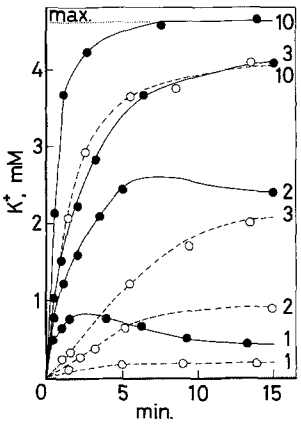

Fig. 1. The time course of K^+ appearance in the medium after addition of various amounts of ethidium and the effect of glucose upon the K^+ efflux. The final concentration of ethidium added is indicated in the Figures and is expressed in mM. Full drawn lines (●) refer to experiments carried out in the presence of 3% (wt/vol) glucose and the dotted lines (○) to experiments without added glucose. The maximum K^+ (denoted *max.* in the Fig.) refers to K^+ released in the medium after boiling the cells

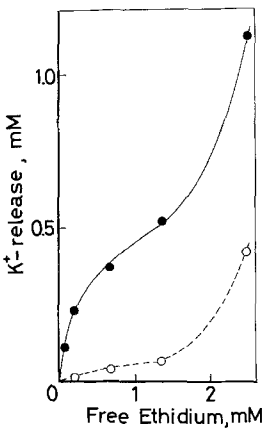

Fig. 2. The dependence of the ethidium-induced K^+ release after a 30 sec incubation upon the concentration of free ethidium, and the effect of glucose. Data of Fig. 1 are used

The amount of K^+ liberated within 30 sec after the addition of ethidium increases at relatively low ethidium concentrations less than proportionally with the free ethidium concentration in the medium (*see* Fig. 2). At concentrations of free ethidium exceeding 1 mM, however, the rate of K^+ efflux is increased more than proportionally with the ethidium concentration. This is found both in the presence and in the absence of glucose. This indicates that two processes are involved in the efflux of K^+, a process characterized by saturation kinet-

ics and a process characterized by a sigmoidal relation between the efflux rate and the ethidium concentration. The latter may point to the occurrence of an all-or-none response of the yeast cells to ethidium. This is further confirmed in Fig. 3, in which it is shown that the loss of K^+ is accompanied by an increasing number of cells which are stainable with the dye bromophenol blue. This dye only penetrates into leaky cells and not into intact cells [2]. The relation between the amount of K^+ liberated and the number of stained cells, however, is nonlinear. In fact, the amount of K^+ released from the cells exceeds the amount expected from the number of cells that become freely accessible to bromophenol blue. Therefore part of the K^+ should be released from cells which are still intact.

The indication that besides an all-or-none effect also a graded loss of K^+ from still intact cells is induced by ethidium, is now further examined by the X-ray microanalysis technique.

We first carried out a calibration of this measuring technique and determined its sensitivity. For that purpose the K^+ content of the cells was either increased by metabolic K^+/H^+ exchange or decreased by metabolic Na^+/K^+ exchange. In this way cells were obtained with mean K^+ contents ranging from 185 to 720 mmol/kg dry weight of yeast; their phosphorous (P) content was not significantly affected by the cation exchange processes and was 420 ± 21 mmol/kg dry weight of yeast. X-ray microanalysis of these cells showed that the cation exchanges applied resulted in great changes in the mean intensities of the K peak and in all cases examined a single cell K population was present. The mean intensity of the K peak of these cells showed a greater standard deviation than the corresponding K/P ratio (for each case 50 cells were analyzed). For that reason, and because the P content of the cells did not vary significantly, we used the mean K/P ratio as a measure for the K^+ content of the cells. Figure 4 shows that this K/P ratio was linearly related to the K^+ content of the cells, determined by flame photometry. The mean standard error of the mean of the K/P ratios was 0.04. In Fig. 4 also data obtained with K^+-depleted cells are included. K^+ depletion of starved cells was caused by a 5-min incubation of the cells in the presence of 1 mM cetyltrimethylammonium-bromide (CTAB) in water. Also in this case a single cell K population was present. CTAB, however, caused a significant (30%) loss of cellular phosphorous. The K/P ratio has been corrected for this P loss.

Table 1 shows that in the presence of ethidium the mean intensity of the P peak is not significantly affected. The mean intensity of the K peak, however, is greatly reduced by the dye. Since the P content of the Delft II strain is not affected by ethidium, we have used also in this series of experi-

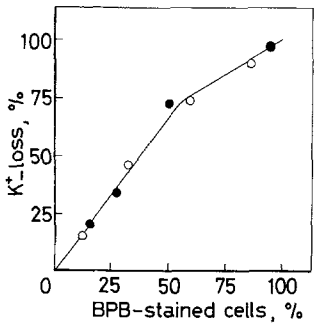

Fig. 3. Relationship between the percentage K^+ loss in the presence of ethidium and the percentage of cells stained by bromophenol blue (BPB). (●) in the presence of 3% (wt/vol) glucose, (○) without added glucose. 100% K^+ loss is determined by boiling the cells. The values of K^+ loss and the number of cells stained by BPB are corrected for the values found in the absence of added ethidium

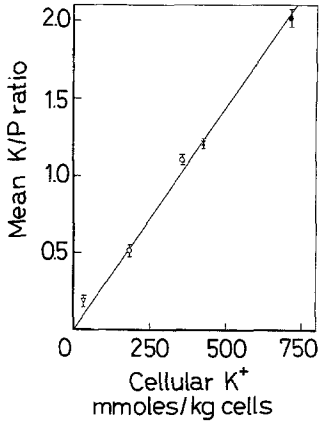

Fig. 4. Relationship between the mean K/P ratio of single cells determined by X-ray microanalysis and the K^+ content determined by flame photometry. (●) K^+-loaded cells, (○) Na^+-loaded cells, (×) starved control cells, (▽) K^+-depleted cells. The cellular K^+ content is expressed in mmol/kg dry weight of yeast. The bars denote the standard error of the mean ($n = 50$) (*see also* text)

Table 1. Mean intensities of the peaks of K and P at various ethidium concentrations

EB (mM)	K (±SE)	P (±SE)	*n*
0	4979±129	3049±118	152
0.75	4329±132	3019± 66	200
1	4323±165	3135± 71	200
3	730±102	3169±136	127

EB means ethidium concentration. The peak intensities are expressed in X-ray counts min^{-1}. SE means standard error of the mean. *n* the number of cells examined. The yeast used is *S. cerevisiae* Delft II.

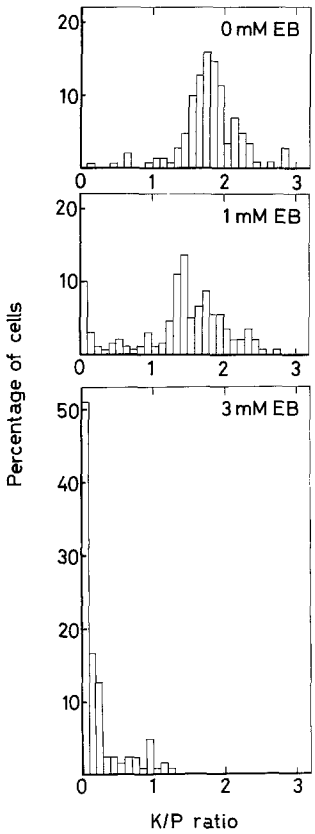

Fig. 5. X-ray microanalysis of the K distribution over the cells of the Delft II strain and the effect of ethidium upon this distribution. The K/P ratio is taken as a measure for the K content of the cells. Same experiment as in Table 1

ments the intensity of the P peak as an internal standard for the cell mass excited by the electron beam and expressed the K^+ contents of the cells in relative values, namely the ratios of the K peak and the P peak. Figure 5 shows the distribution pattern of the K/P ratios both in the absence and in the presence of ethidium. In the absence of ethidium two populations of cells are found, a small one with a very low K/P ratio consisting of 2% of the total number of cells examined and a large population with a mean K/P ratio of 1.78. The population of K^+-poor cells has a K/P ratio <0.5. This population likely consists of completely leaky cells, since also 2% of the cells appeared to be stainable with bromophenol blue. We will denote yeast cells with a K/P ratio <0.5 as broken cells, and cells with a K/P ratio >0.5 as intact cells. The population of broken cells greatly increased on raising the ethidium concentration. The mean K/P ratio of the intact cells also decreased significantly, *see* Table 2 ($P<0.0001$ for both the difference between K/P of the blank and K/P found with either 0.75 or 1 mM ethidium and the difference between K/P found at either 0.75 or 1 mM ethidium and at 3 mM ethidium). Table 2 shows that the amount of K retained in the broken cells is small and comprises less than 5% of the K being present in the originally intact cells. In addition it is seen that especially at low ethidium concentrations a relatively great part of the K^+ liberated is lost from cells which are considered to be still intact, and which have lost only part of their K^+. The total amount of K^+ loss calculated compares rather well with the amount of K^+ released to the medium.

Table 2. Mean K/P ratios, number of cells present in the population of broken cells and the population of intact cells and percentage K^+ loss

	EB (mM)	$(\bar{K}/P)_b$	n_b (%)	R_b (%)	$(\bar{K}/P)_i$	n_i (%)	R_i (%)	R_t (%)	R_m (%)
D	0	0.233	2.0	–	1.783	98	–	–	–
	0.75	0.180	12	9.0	1.612	88	8.4	17	18
	1	0.113	16	13.1	1.603	84	8.5	22	26
	3	0.107	85	78	1.182	15	5.1	83	82
A	0	0.218	2.7	–	0.667	97	–	–	–
	0.37	0.060	3.9	1.1	0.654	96	1.9	3	8
	0.75	0.038	44	38	0.558	56	9.2	47	39

The indices b, i and t refer to the broken cells, intact cells and broken and intact cells together, respectively. The index o refers to the control without ethidium. EB is the concentration of ethidium added, n is the number of cells expressed in percents of the total number of cells examined. $\bar{K}/P$ is the mean K/P ratio of the cell population concerned. R is the K lost from the cells taking the amount of K in the intact cells of the control as 100%. $R_t = R_b + R_i$ is the total amount of K lost expressed in percentage of the control value. R_m is the percentage of K released to the medium, being determined by means of flame-photometry in the supernatants of yeast cells after 5 min incubation with ethidium. D and A refer to the Delft II, and Azteca strain, respectively. The $\bar{K}/P$ values of the Azteca strain are corrected for losses of P during incubation with ethidium. R_b and R_i are calculated as follows:

$$R_b = 100(n_b - n_{b,o})\{(\bar{K}/P)_{i,o} - (\bar{K}/P)_b\}/(\bar{K}/P)_{i,o}$$

and

$$R_i = 100 n_i\{(\bar{K}/P)_{i,o} - (\bar{K}/P)_i\}/(\bar{K}/P)_{i,o}.$$

The cells are incubated for 5 min in the presence of 3% (wt/vol) glucose before analysis by means of the X-ray microprobe technique. The values for the Delft II strain are obtained from the data presented in Fig. 4. In addition data found at 0.75 mM ethidium are included. The incubation temperature was 25 and 30 °C for the Delft II and the Azteca strain, respectively.

We have carried out similar experiments with the Azteca strain. With this yeast also two populations were found, one with a low K^+ content and one with a relatively high K^+ content. With this yeast the mean P content decreased with increasing concentrations of ethidium. Therefore we corrected our K/P ratios for the decrease in phosphorous content. The results are given in Table 2. Also with this yeast part of the K^+ released arose from

Table 3. Ca/P and K/P ratios in yeast cells suspended in a Ca^{2+}-containing medium. Accessibility of the populations of cells with a low K/P and a high K/P ratio for Ca^{2+}

	Ca (mM)	EB (mM)	$(\bar{K}/P)_b$	$(\bar{Ca}/P)_b$ ($\pm$SD)	n_b	$(\bar{K}/P)_i$	$(\bar{Ca}/P)_i$ ($\pm$SD)	n_i
A	50	0	0.04	1.38±0.41	50	–	–	–
N	0.5	0	–	–	–	1.69	0.17±0.10	26
N	50	0	–	–	–	1.73	0.23±0.09	24
N	0.5	3	0.22	1.09±0.42	10	1.28	0.33±0.17	24
N	50	3	0.18	1.52±0.63	3	1.26	0.18±0.08	26

A: acetone powders; N: normal yeast, strain Delft II. The cells (5% wt/vol) are incubated without glucose for 5 min in the presence of ethidium or in the absence of the dye. Then the cells are washed three times with ice-cold distilled water and are incubated in either 0.5 or 50 mM $CaCl_2$ at 0 °C. *n* is the number of cells examined. EB is the ethidium concentration added. SD means standard deviation. *See also* subscript to Table 2.

still intact cells. Apparently this yeast is much more sensitive for ethidium than the Delft II strain. At 0.75 mM ethidium 44% of the cells are permeabilized instead of 12% as found with the Delft II strain. The values of total K^+ loss calculated from the analysis of the individual cells agrees less with the values for K^+ released to the medium determined by flame photometry than is found with the Delft II strain. This may be attributed to differences in the relative loss of phosphorous from the individual cells.

Finally we have examined whether in fact the cells of the Delft II strain with a K/P ratio >0.5 are still intact by determining the uptake of Ca^{2+} into the cells before and after treatment with ethidium. In order to avoid metabolically dependent Ca^{2+} uptake into the cells glucose was omitted. Also the low temperature employed may greatly prevent Ca^{2+} uptake via an energy-linked process. As shown in Table 3 the Ca/P ratio in cells with a K/P ratio <0.5 was far greater than in cells with a K/P ratio >0.5. In the latter population the Ca^{2+} uptake was not significantly increased by ethidium indicating that in the nonmetabolizing cells at least at 0 °C no appreciable ethidium-induced Ca^{2+} uptake [9] occurred.

Discussion

The results obtained with the X-ray microanalysis technique show clearly that apart from an all-or-none effect of ethidium giving rise to an almost complete loss of K^+ from the cells, a second effect is also involved consisting of a partial loss of K^+ from still intact cells. The still intact cells are not stainable with bromophenol blue and are characterized by a still relatively high K^+ content. These results also explain why the relation between the percentage K^+ loss and the number of cells which are stained with bromophenol blue is nonlinear. A closer look at the data presented in [15] also shows that with the cationic surfactant benzalkonium the K^+ loss is higher than expected from the number of stainable cells. This is, however, not true for the interaction of methylene blue with yeast [8]. The low Ca/P ratios found in cells with a relatively high K^+ content further confirms that these cells have a still intact membrane.

The K^+ loss from still intact cells is probably underestimated. As shown in Fig. 1, K^+ released during the first minutes can be reaccumulated again, provided that glucose is present. This reabsorption should be ascribed to K^+ uptake by still intact cells. Since the mean K^+ content of the intact cells found after 5-min incubation is decreased in the presence of ethidium we have to assume that initially the intact cells release part of their K^+ presumably by exchange against ethidium, which is accumulated into the yeast cells [10, 13]. Due to the increase in the K^+ concentration of the medium the uptake of ethidium in the yeast cells decreases [13]. This will give rise to a decrease in the K^+ efflux and then K^+ can be again absorbed via the metabolism-dependent monovalent cation uptake mechanism [1].

Comparison of the effects of ethidium on the two strains of yeast used shows that great differences in sensitivity for the dye may exist between two strains. Brunner et al. (*personal communication*) showed that large differences in sensitivity for ethidium also occur between mutants of the yeast *Kluyveromyces lactis*.

The observation of Peña [9] that ethidum enhances Ca^{2+} uptake into yeast supports the view that part of the cells are still intact. An important consequence of our study is, that due to the presence of broken cells, uptake of Ca^{2+} will be underestimated and that therefore the enhancement of

Ca^{2+} uptake is greater than calculated on the basis that all cells are still intact.

The complex relation between the initial rate of K^+ efflux and the concentration of free ethidium also supports the notion that two processes are involved in the interaction of ethidium with the yeast. Probably the K^+ efflux within the first 30 sec after the addition of ethidium is due to exchange of cellular K^+ and ethidium from the medium. Uptake of ethidium in nonmetabolizing cells is much lower than the uptake in metabolizing cells [13], which may explain the much lower initial rate of K^+ efflux from the nonmetabolizing cells. As stated already under results the initial efflux of K^+ found at the highest ethidium concentration may be mainly due to the all-or-none destruction of the yeast cell membrane. In accordance with this notion Peña et al. [13] showed that at low ethidium concentrations metabolic proton extrusion is not affected (indicating that the cells are still intact), whereas at high ethidium concentrations net proton pumping is inhibited as well (due to proton influx into the cells with a disrupted membrane).

We are grateful to Professor A.M. Stadhouders, Laboratory of Submicroscopic Morphology, University of Nijmegen, for his hospitality and facilities at our disposal. We thank Drs. P.G.M. Peer, Department for Mathematical and Statistical Service of the Faculty of Science, for carrying out the statistical analysis. The skilled assistance of Mr. P. Spaay in part of the experiments is gratefully acknowledged. The yeast strain Delft II was kindly supplied by Gist-Brocades at Delft. This study was supported in part by a grant from the Netherlands Organization of the Advancement of Pure Research (Z.W.O.) through the Netherlands Foundation of Biophysics.

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Received 8 November 1982; revised 1 December 1982