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Effect of Ethidium Bromide on Ca^{2+} Uptake by Yeast

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Summary. Ethidium bromide and other cationic dyes have been found to inhibit monovalent cation uptake. This dye also produces in a K^+-free medium an efflux of K^+ which could be of the electrogenic type.

The study of the effects of the same cationic dyes on Ca^{2+} uptake showed a large stimulation of the uptake rate of the divalent cation of more than tenfold.

The analysis of the effects of one of the cationic dyes on Ca^{2+} uptake indicated that the efflux of K^+ is of the electrogenic type and can drive the uptake of the divalent cation.

Kinetic data on Ca^{2+} uptake indicate that, both under "normal" or under stimulated conditions, the divalent cation is taken up by the same transport system.

The addition of ethidium bromide, besides, can stimulate the uptake of Mn^{2+} and ^{14}C-glycine and could be a good weapon to magnify and study some of the characteristics of ion transport systems in yeast.

In previous studies [11, 13] it was found that different cationic dyes and amphiphilic molecules are capable of modifying monovalent cation transport in yeast cells. One of the interesting effects of some of the dyes studied, among which ethidium bromide was comprised, consisted in an efflux of K^+ produced by their addition in the presence of a substrate. In these same studies, other interactions of the dyestuffs with the system for transport of monovalent cations were studied, and it was considered interesting to analyze the effect of these substances on the uptake of divalent cations for several reasons: A transport system for divalent cations has been described in yeast [2, 6, 7, 15] which is different from that for monovalent cations. It is important to know how specific the effects of dyes can be on one or more cation transport system. The addition of cationic dyes to yeast suspensions in the presence of a substrate produces an efflux of K^+; it has to be investigated if this is a consequence, as described by Elferink and Booij [4] of the disruption of the cell, or it can generate or modify an electrical potential

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within the cell. It is also important to know if this efflux of K^+ could be used for the transport of other materials into the cell. Reports have appeared, according to which aminoacids, for instance, can be transported by using H^+ gradients of the cell [1, 16]. Recently, a report has appeared on the effects of K^+ efflux on ion transport in yeast [5]. This paper reports the results of the analysis of these possibilities of action of dyes by using ethidium bromide in yeast cells.

Materials and Methods

Commercial Baker's yeast was prepared as described previously [12]. $^{45}Ca^{2+}$ binding to yeast was measured by incubating the cells for short intervals, filtering afterwards with Millipore type filters, and washing twice with water. The cells were then resuspended in water, plated, and counted in a gas flow counter.

Calcium transport was measured by the uptake of $^{45}Ca^{2+}$, usually at short intervals. In these experiments, cells were added to the incubation mixture, previously equilibrated to the temperature of the water bath. After 2 min, in which the metabolism of the cells had started, the isotope at the desired concentration was added. Unless otherwise specified, after 2 min, an aliquot of the incubation mixture was filtered through a membrane filter of 0.45 μm, pore size. The cells were washed under vacuum once with water, twice with 5 mM $CaCl_2$, and twice more with water. Afterwards, the cells were resuspended in water, and an aliquot was taken to plate and counted with a gas flow counter. The uptake of $^{54}Mn^{2+}$ and ^{14}C-glycine was followed in much the same way, but the washing after filtration was carried out with the respective nonradioactive material, and after the resuspension of the cells, ^{54}Mn taken up was determined with a liquid scintillation counter.

K^+ movements were followed with a cationic electrode (Beckman, 39047) and a suitable recording system.

Fluorescence changes were followed in 3.0 ml capacity quartz cells (1.0×1.0 cm) in a Farrand, Mark II spectrofluorometer.

Each experiment was repeated at least three times. Typical data are presented in all the cases described.

Results

In a K^+-free medium with glucose as substrate, the incubation of yeast in the presence of several cationic dyes produces the efflux of K^+ from the cells. If the effect of the same dyes is measured on the binding of $^{45}Ca^{2+}$, it is found that, in the presence of glucose, the dyes increase the amount of label present in the cells. If after incubations the cells are filtered and washed with 5 mM CaCl, this treatment washes away the $^{45}Ca^{2+}$ bound to the outside of the cells, and it is shown that the dyes have produced the entrance of the divalent cation into the cell. The case of ethidium bromide at a concentration of 100 μM

Table 1. Effects of EB on $^{45}Ca^{2+}$ binding by yeast in the presence or absence of a substrate

Addition	cpm bound to yeast after incubation	
	Yeast washed with water	Yeast washed with $CaCl_2$
None	6192	320
Glucose	7192	1338
EB	6368	1778
Glucose+EB	8760	6448

After loading with $^{45}Ca^{2+}$ in the absence of substrate, filtering, and washing with water, the cells were incubated for 3 min in the following incubation mixture: 20 mM maleate-triethanolamine (TEA) buffer, pH 6.0; 100 mM glucose or 100 μM EB where indicated, and 100 mg of yeast cells. Final volume, 2.0 ml. After incubation, an aliquot of the cells was filtered and washed either with water four times, or once with water, twice with 5 mM $CaCl_2$, and twice more with water. The cells were then resuspended in water, plated, and counted in a gas flow counter

is presented in Table 1. The addition of the dye in the presence of a substrate, and even in its absence, produces a large increase in the amount of the divalent cation that was present before on the surface of the cell and now seems to be inside, in a nonexchangeable form.

The results of the experiment of Table 2 were obtained by measuring the actual uptake of $^{45}Ca^{2+}$ into the cells in the presence of ten different cationic dyes. As reported by other authors [15], the uptake of Ca^{2+} is rather slow in the control experiments. When the dyes are present, on the other hand, there is a large increase of up to 10 times in the amount of the divalent cation taken up in the first 2 min of incubation with some of them. The effects were clear and concentration-dependent with acriflavin, methylene blue, ethidium bromide, safranine and neutral red. Both brilliant green and methyl green produced only a slight effect. In view of the fact that ethidium bromide (EB) showed a clear stimulation on Ca^{2+} transport and this dye has been rather extensively studied [11, 13], showing special properties, the following experiments were carried out with this dye.

The results of a time curve in which the effect of EB on Ca^{2+} uptake was measured are presented in Figure 1. The effect of the dye was measured in the presence or absence of a substrate. 100 μM EB, even in the absence of a substrate, produced an increase of the uptake of Ca^{2+}. In the presence of glucose, the addition of the dye produced an increase of the uptake of Ca^{2+} that was more pronounced in the initial rate.

Table 2. Effect of several cationic dyes at two different concentrations on $^{45}Ca^{2+}$ uptake by yeast

		nmoles taken up in 2 min $(100\ mg)^{-1}$	
		50 μM	100 μM
Controls	4.40 3.96		
Methylene blue		5.95	22.3
Nile blue		40.99	40.0
Brilliant cresyl blue		7.54	19.6
Methyl violet		8.46	14.6
Acriflavin		29.20	52.6
Ethidium bromide		33.00	47.5
Safranine		26.60	47.5
Neutral red		30.50	43.5
Brilliant green		11.10	12.1
Methyl green		5.71	4.1

Incubation mixture: 20 mM maleate-TEA buffer, pH 6.0; 100 mM glucose; Yeast cells, 100 mg, wet wt. Final volume, 2.0 ml; temperature, 30°. The incubation mixture minus the yeast cells was equilibrated at the temperature of the water bath. Yeast cells were then added, and 2 min later, 50 μM $^{45}Ca^{2+}$ and the indicated final concentration of each dye were also added. After 2 min more in the presence of the radioactive cation, an aliquot of the mixture was taken, filtered through a Millipore filter, and washed once with water, twice with 5 mM $CaCl_2$, and twice more with water. The cells were then resuspended in water, and an aliquot was plated and counted. Results are the means of two experiments; the values of each experiment for the controls are given

After approximately 4 min, the rate of uptake was practically the same with or without the addition of the dye.

Figure 2 presents the data obtained when, measuring the uptake of Ca^{2+} at short intervals (2 min), the effect of varying the concentration of EB was studied. The effect was apparent at the two concentrations of Ca^{2+} utilized, but quantitatively more evident in the presence of glucose with 60 μM Ca^{2+}. In the presence of glucose, at both Ca^{2+} concentrations, the maximal effect was reached at 100 μM EB.

Since one of the evident effects of EB in yeast consists in the production of a K^+ efflux that requires the presence of a substrate, the effects of EB in this parameter at several concentrations of the dye were studied, with the results presented in Figure 3. EB produced an increase of the efflux of K^+ which is maximal at the 50 μM concentration. There is a difference in the concentration at which the maximal effect is reached in the K^+ efflux (50 μM), and the Ca^{2+} uptake (100 μM); however, the differences are not very large.

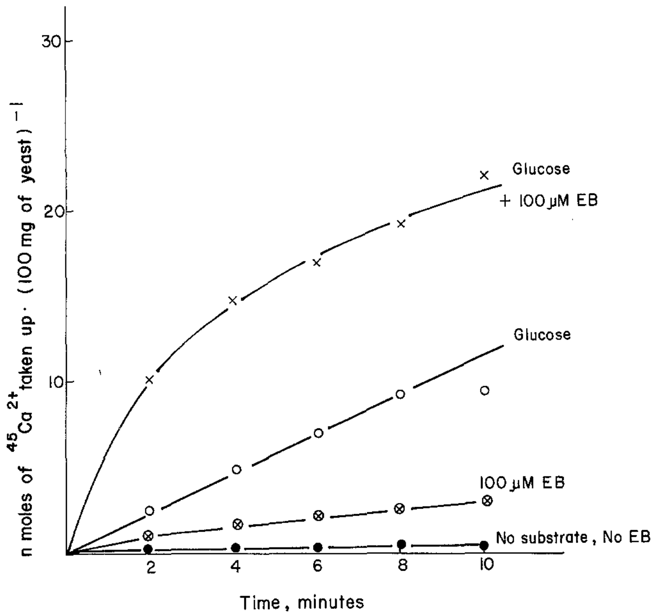

Fig. 1. Time course of $^{45}Ca^{2+}$ uptake by yeast, and the effect of EB in the presence or absence of a substrate. Experimental conditions were as for Table 2, but the aliquots to measure Ca^{2+} uptake were taken at the indicated times

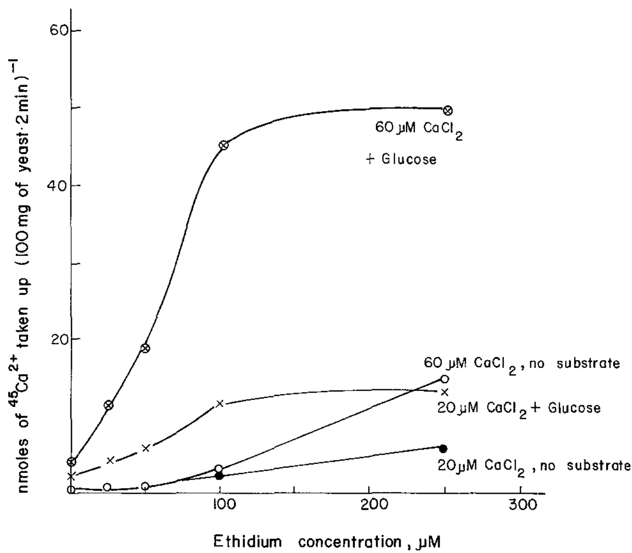

Fig. 2. Effects of EB at different concentrations on the initial (2 min) uptake of Ca^{2+} by yeast in the presence or absence of a substrate. Experimental conditions were as for Table 2

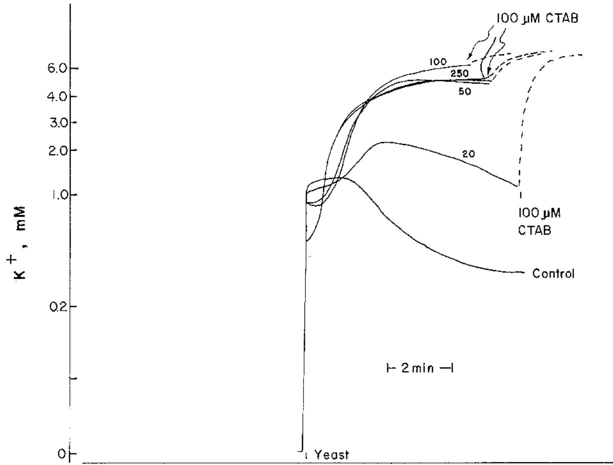

Fig. 3. Effect of several ethidium bromide concentrations on the K^+ movements of yeast incubated in a K^+-free medium. Incubation conditions were as for Table 2, but always in the presence of glucose. The final volume was 5.0 ml, and all reagents and additions were adjusted accordingly. The K^+ movements were followed with a cationic electrode

In previous studies on the uptake of Ca^{2+}, data do not exist on the value of the kinetic characteristics of this phenomenon. It was interesting, then, to determine both the values of K_m and V_{max} for the process and at the same time to ascertain the effects of EB on these parameters. Besides, in the studies carried out on the uptake of divalent cations in yeast, it has been found that uptake of the cations is very low in starved yeast, and that it is increased severalfold by a preincubation of the cells with glucose, K^+, and inorganic phosphate [6, 15]. The experiment of Figure 4 was then designed to also determine the effects of preincubation and EB on the uptake of Ca^{2+} by yeast from the kinetic point of view. The results showed that (i) the preincubation of the cells with glucose, K^+, and inorganic phosphate, contrary to the reported results of other investigators with other divalent cations [6, 15], did not produce an increase of the Ca^{2+} uptake by yeast; (ii) the stimulation produced by EB on the uptake of the divalent cation was smaller in the case of the preincubated cells than in those not preincubated, and (iii) the changes produced on the uptake of Ca^{2+} are more evident in the V_{max} than in the K_m of the transport system for the cation.

Previously [13], it had been reported that Ca^{2+} prevents the fluorescence changes that the addition of a substrate induces in yeast with EB. Since these fluorescence changes seem to be related to the penetration of the dye [13], it seemed interesting to investigate if the addition of

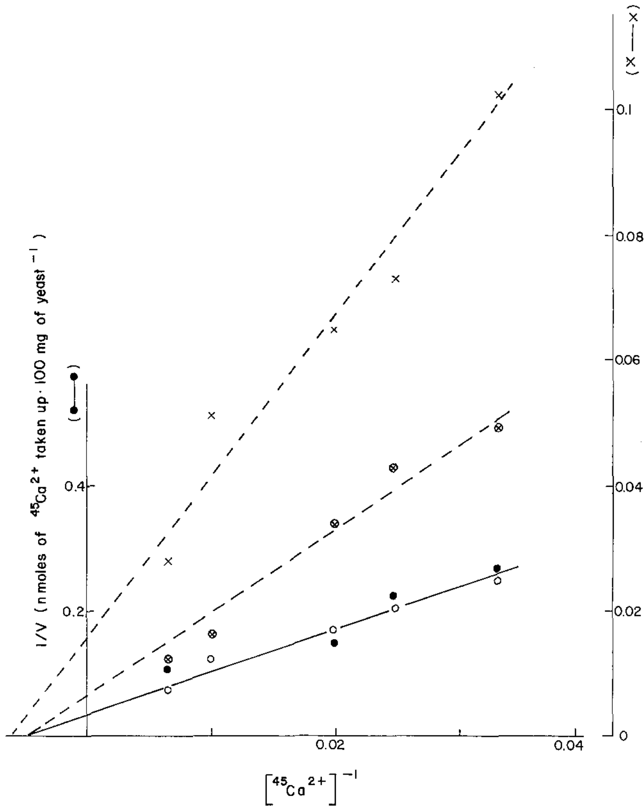

Fig. 4. Effects of EB (100 μM) on the uptake of Ca^{2+} by yeast, at different concentrations of the divalent cation. Incubation conditions were as for Table 2, but several concentrations of Ca^{2+} and also two kinds of yeast were used. One lot of yeast was preincubated for 40 min in a mixture containing 40 mM KH_2PO_4 buffer, pH 6.0, 0.4 M glucose, and 3 g of yeast, wet wt in a volume of 10 ml, at 30°C. After the incubation the cells were filtered with a Millipore filter (0.45 μm), washed with water, and resuspended in water, to be used as for the control experiment. Symbols: o, fresh, control yeasts; ⊗, fresh, EB treated cells; ●, preincubated controls; ×, preincubated cells plus EB

Ca^{2+} could prevent or reverse the fluorescence changes of the dye added, under the same experimental conditions under which the uptake of the divalent cation was measured. The results of Figure 5 show that the addition of Ca^{2+} two minutes after the addition of yeast to the incubation mixture produced only a slight decrease of the fluorescence curve observed in the absence of the divalent cation. A similar experiment was performed to determine the effects of Ca^{2+} on the K^+ efflux under similar experimental conditions, since it has been said that divalent cations are taken up into yeast cells in an exchange for K^+ [2, 6]. As shown in Figure 6, upon the addition of Ca^{2+}, there was only a slight change

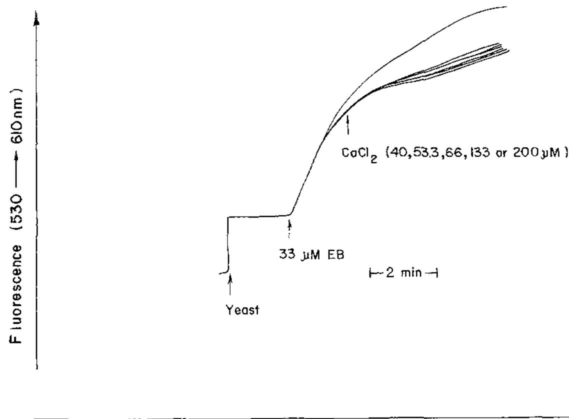

Fig. 5. Fluorescence changes of EB added to yeast in the presence of glucose and the effect of the addition of several concentrations of $CaCl_2$. The incubation conditions were as for other experiments. The final volume was 3.0 ml, but the amounts of reagents were adjusted accordingly to maintain the same concentrations; EB and $CaCl_2$ were added where indicated, at the indicated concentrations

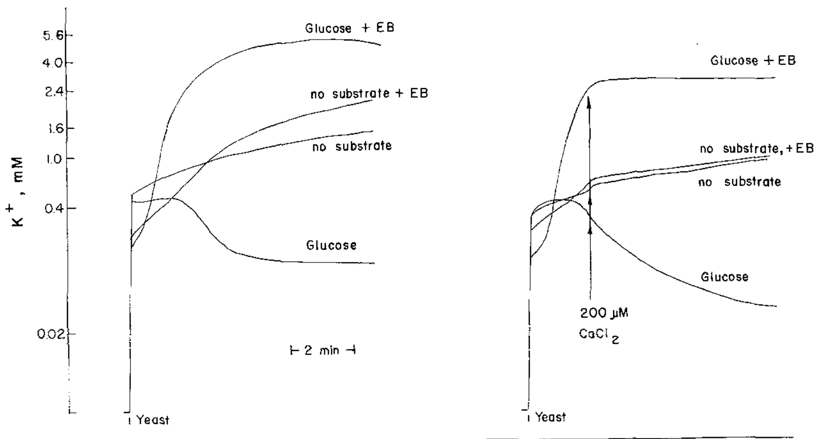

Fig. 6. Effect of $CaCl_2$ on the K^+ efflux by EB under different conditions. Experimental conditions were as for Figure 5. EB concentration was 100 μM, and $CaCl_2$, 200 μM

of the K^+ efflux produced by EB in the sense of a decrease of its rate.

If the efflux of K^+ plays an important role in uptake of Ca^{2+}, as suggested by other authors [6, 7], it is important to investigate if the external concentrations of the monovalent cation have an influence on the rate of uptake of Ca^{2+}. As Figure 7 shows, and in agreement with

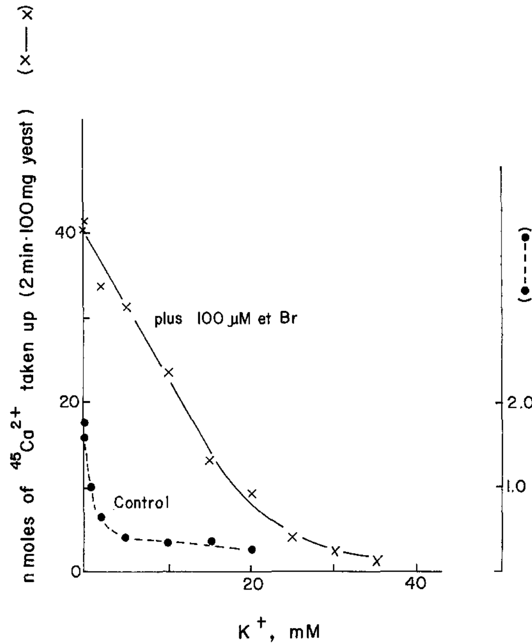

Fig. 7. Inhibition of Ca^{2+} uptake by different concentrations of K^+ in the presence or absence of EB (100 µM). Experimental conditions were as for Table 1, but the indicated concentrations of KCl were included

other investigations, in the absence of EB, concentrations of K^+ lower than 5 mM are able to considerably inhibit the uptake of Ca^{2+}. When, however, the same experiment was carried out in the presence of EB, concentrations of K^+ up to 20 mM produced a linear decrease in the uptake of Ca^{2+}, and low concentrations of K^+ showed only a slight effect on the uptake of Ca^{2+}.

According to several of the experiments carried out up to this point, a correlation seemed to exist between the initial rate of uptake of Ca^{2+} and the efflux of K^+ produced by EB. Figure 8 and Table 2 show the correlation between the rate of Ca^{2+} taken up by the cells and the efflux of K^+ observed under different conditions of incubation. As previously reported, *P*-trifluoromethoxy-carbonylcyanide phenylhydrazone (FCCP) produced a decrease in the amount of K^+ taken up by yeast [10]. Also in agreement with previous studies [11], according to which the efflux of K^+ produced by EB and other dyes is linked to the energy state of the cell, FCCP, as an uncoupler, diminishes the amount of K^+ leaving the cells under the influence of the dye (Fig. 8). In concordance with this, the amount of Ca^{2+} taken up by the cells keeps parallel

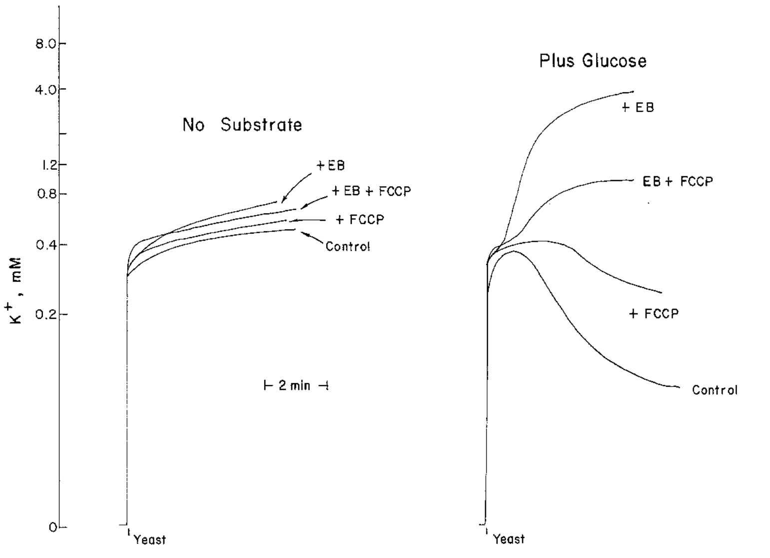

Fig. 8. Effects of FCCP on the movements of K^+ in yeast incubated in a K^+ free medium under different conditions. The experimental conditions were essentially similar to those of Figure 6. Additions were as indicated. EB concentration was 100 μM and FCCP was 60 μM

with the amount of K^+ that at the time of addition of the divalent cation (2 min) has left the cell. The results in the absence of substrate are more difficult to analyze; however, also in this case, FCCP produces a slight decrease in the amount of K^+ that leaves the cell in response to EB, and the uncoupler also reverses the stimulation of Ca^{2+} uptake produced by EB. The uptake of the divalent cation observed in the absence of a substrate without EB in the incubation medium is too low to allow a careful analysis (Table 3).

Another interesting question emerging from the results obtained was if the conditions produced by EB, whichever they may be, can be translated by the cell into an increased uptake of other materials. The effect of EB was, accordingly, studied under the same experimental conditions, on the uptake of the cells of one aminoacid, glycine, and another divalent cation, Mn^{2+}. The results presented in Table 4 show that EB stimulates the uptake of these materials both in the presence and in the absence of a substrate. Although these are only preliminary data, it is interesting that the uptake of these two substances was increased only about twofold. In some of the results presented, the uptake of Ca^{2+} was increased more than fifteen times in comparison with the controls in the absence of EB.

Table 3. Effects of EB (100 μM) and FCCP (60 μM) on $^{45}Ca^{2+}$ uptake by yeast

	nmoles of $^{45}Ca^{++}$ taken up · (2 min × 100 mg of yeast)$^{-1}$	
	No substrate	Plus glucose
Control	0.13	1.46
FCCP	0.19	2.83
EB	2.73	35.7
FCCP+EB	0.12	6.30

Experimental conditions were as for Table 2, but $^{45}CaCl_2$ concentration was 50 μM, EB was 100 μM and FCCP, 60 μM.

Table 4. Effects of EB on the uptake of $^{54}Mn^{2+}$ and ^{14}C-glycine by yeast

Additions	nmoles taken up by 100 mg of yeast in 4 min		
	$^{54}Mn^{2+}$	^{14}C-Glycine	
		800 μM	400 μM
No substrate	2.83	0.56	
	2.75	0.46	
+Glucose	8.73	3.46	2.90
	8.52	4.06	2.98
No substrate, +EB	3.81	0.74	
	3.31	0.91	
+Glucose	21.5	9.16	5.43
	22.2	9.96	5.38

Experimental conditions were as for $^{45}Ca^{2+}$ uptake experiments (Table 2), with the following variations: $^{54}MnCl_2$ concentration was 50 μM, and ^{14}C-glycine was 800 or 400 μM. The incubation time after the additions of the labeled substances was 4 min. For more details consult *Materials and Methods.*

Discussion

Ethidium bromide has been shown to inhibit K^+ uptake by yeast [11, 13]. However, it has been shown in the experiments reported here to strongly stimulate the uptake of Ca^{2+}. In agreement with the results of other authors [6, 7], these results invalidate the postulation of Conway and Gaffney [2] in the sense of the existence of a common cationic carrier in yeast. On the other hand, the results support the hypothesis of the interaction of EB with the K^+ transport system of the cell in an energy requiring process that, when K^+ is absent from the outside

of the cell, produces a large efflux of the monovalent cation [13]. The data are against an effect due to the disruption of the cell structure, as proposed by Elferink and Booij [4]. It would be difficult to expect an increase in the uptake of anything in broken cells. The results are further reinforced by the stimulatory effect found also on the uptake of Mn^{2+} and glycine. The data are more in agreement with the production by EB of an electrogenic K^+ efflux from the cell. The negative potential increased within the cell by the efflux of K^+ can then be utilized to drive the entrance of other cations or substances. The main postulate would be that EB produces a change of the K^+ carrier that is translated into an absence of coupling to the electrochemical potential of the cell. Under such conditions, the dye would provoke the efflux of K^+ according to its concentration gradient. The efflux of K^+ then would produce an increase of the negative potential within the cell. It should be taken into account that EB does not greatly affect the H^+ pumping activity of the yeast cell [13].

The parallel course of the stimulation of K^+ and of Ca^{2+} uptake are a good indication that the phenomenon described may be as proposed. After the initial stimulation of Ca^{2+} uptake produced by the efflux of K^+, a similar rate of uptake is reestablished in the cells with EB, in comparison with the control cells. Although the correlation is not perfect, there is also some correspondence between the concentration of EB and its effects on both K^+ efflux and Ca^{2+} uptake. The addition of FCCP to those cells containing EB in the presence of a substrate produced a diminution of the efflux of K^+ and also produced a decrease of the stimulatory action of the dye on Ca^{2+} uptake. In this experiment, it is difficult to explain the increase of the uptake of Ca^{2+} produced by FCCP alone in the presence of glucose, although it has been proposed that this uncoupler can behave as a cation carrier [8]. The data obtained agree with those of Foury *et al.* [5] which show a correspondence between the efflux of K^+ and the uptake of Ca^{2+}, though a discrepancy exists with respect to the effects on the uptake of aminoacids found by these authors. This latter discrepancy, however, could be due to the different kind of yeast employed. Besides, Dio-9 seems to act more like an ionophore capable of producing an exchange of K^+ for other cations.

An important test on the nature of the phenomenon is given by the kinetic analysis of the Ca^{2+} uptake under different conditions. Although large differences existed in the uptake in three different conditions, it is clear from Figure 4 that it happened mainly at the expense of a change in V_{max}, and not on the value of K_m, which might indicate

that the ion, under all conditions tested, is being transported by the same system.

Although for the natural phenomenon of divalent ion transport Fuhrman and Rothstein [7] have postulated the existence of an exchange system of K^+ for divalent cations, the experiments in which the movements of K^+ were followed showed that the addition of Ca^{2+}, if any, had the effect of stopping the efflux of K^+ that was taking place under the influence of EB. This fact, however, cannot be taken as an absolute indication that Ca^{2+} is not taken up in an exchange for K^+. The effect observed in Figure 6 might be due to the reversion of the EB effect that Ca^{2+} has been shown to produce [11] and not to the fact that the divalent ion is not transported in an exchange for K^+. The analysis of the changes produced by Ca^{2+} on the fluorescence of EB, was carried out because it has been found also [11] that EB, as well as other dyes, are concentrated within yeast cells. It might be, then, that after being taken up they are exchanged for the divalent cations. The fluorescence changes, that are roughly equivalent to the uptake of the dye [13], showed that this is not the case; the addition of Ca^{2+}, although producing a decrease of the rate of development of fluorescence of EB in relation to the control experiment, did not produce a reversion of the fluorescence increase already produced, which would be expected if the dye were being exchanged for Ca^{2+}.

The efflux of K^+ produced by the addition of EB can increase the uptake of Mn^{2+} and glycine besides Ca^{2+}. The kinetic data also seem to indicate that the route of entrance of the divalent cation in the presence or absence of EB is the same. At this point of the investigation it is difficult to decide if the full mechanism of uptake is the same as under "natural" conditions; however, the results of the inhibition produced by external K^+ on both the "normal" and the EB-stimulated Ca^{2+} uptake indicate that this might be so. When yeast cells are placed in a K^+-free medium, a small amount of K^+ leaves the cell (Fig. 8), and this might increase the negative potential created by proton pumping [10] within the cell; if the amount of K^+ efflux is small (Fig. 6), it is clear that also a small concentration of K^+ placed in the outside of the cell can be enough to block its effect. In the absence of EB, a small concentration of external K^+ is enough to produce a large blocking of Ca^{2+} uptake. In the presence of EB, it produces a large efflux of K^+ without affecting proton pumping [13], the negative potential increase (hyperpolarization) created has to be much larger, and can only be neutralized by larger concentrations of K^+ in the outside, which is what

was observed in terms of Ca^{2+} uptake rates. With EB, much larger concentrations of K^+ in the outside were required to reverse the Ca^{2+} uptake observed. Furthermore, the ratio of external K^+ to Ca^{2+} transport was practically linear within a certain concentration range, which is in agreement with an interaction with a potential mediated mechanism of uptake.

In agreement with proposals of other investigators [1, 2, 6, 16], the data presented, if interpreted according to our views, could indicate the importance of the electric component of the electrochemical potential [9] in the transport mechanism in yeast. The yeast cell invests a large amount of energy in the accumulation of K^+, which can work as an activator of enzymes or complete systems and also as an important factor in other, more complex functions of cells [14]. Our interpretation of the data points to the accumulation of K^+ as the storage of a form of energy that can be used in time for the uptake of other materials, such as divalent cations and at least one of the aminoacids, as postulated by other authors [3, 16].

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