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Some Characteristics of Ca^{2+} Uptake by Yeast Cells

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Summary. Experiments were performed to obtain information on: (i) the specific properties of Ca^{2+} binding and transport in yeast; (ii) the relationship between both parameters; (iii) similarities to or differences from other biological systems as measured by the effects of inhibitors; and (iv) the effects of mono and divalent cations, in order to get some insight on the specificity and some characteristics of the mechanism of the transport system for divalent cations in yeast.

The results obtained gave some kinetic parameters for a high affinity system involved in the transport of Ca^{2+} in yeast. These were obtained mainly by considering actual concentrations of Ca^{2+} in the medium after substracting the amounts bound to the cell. A k_m of 1.9 μM and a V_{max} of 1.2 nmol $(100\ mg \cdot 3\ min)^{-1}$ were calculated.

The effects of some inhibitors and other cations on Ca^{2+} uptake allow one to postulate some independence between binding and transport for this divalent cation.

Of the inhibitors tested, only lanthanum seems to be a potent inhibitor of Ca^{2+} uptake in yeast.

The effects of Mg^{2+} on the uptake of Ca^{2+} agree with the existence of a single transport system for both divalent cations.

The actions of Na^+ and K^+ on the transport of Ca^{2+} offer interesting possibilities to study further some of the mechanistic properties of this transport system for divalent cations.

The first studies on the uptake of divalent cations by yeast were started in 1958 almost simultaneously by Conway et al. [3] and Rothstein et al. [17]. These studies, however, were designed only to determine the dependence of the uptake on metabolism, some relationships of the uptake of different divalent and monovalent cations, and the effect of some inhibitors. This work, besides, was focused mainly on the uptake of magnesium ions. Investigations have also been carried out in which other characteristics and applications of the properties of divalent cation transport were described [1, 4, 5, 8, 11]. Other cations have been studied as well [for a review, *see* reference 7]. Recently, a more specific and detailed study on Ca^{2+} transport was published by Roomans et al. [15], but more with the idea of defining some aspects of surface charge and membrane potential on the uptake of divalent cations. In view of this generally uncertain situation on the characteristics of the uptake of divalent cations by yeast, the present work was undertaken, with the purpose of: (i) defining the kinetic constants of the phenomenon, trying to eliminate binding as a factor causing error in the quantification of uptake; (ii) characterizing the binding characteristics of Ca^{2+}; (iii) gaining some insight about the meaning of binding in the general process of Ca^{2+} uptake; (iv) exploring the effects and possibly the mechanism of action of several substances described as inhibitors of Ca^{2+} uptake in other systems; and (v) obtaining some information about the mechanism of the uptake of divalent cations. Later on, experiments will be designed to ascertain in detail the specificity of the uptake system in order to define if there is one or several systems employed by yeast to take up the different kinds of divalent cations that the cell is capable of transporting.

Materials and Methods

Yeast cells were obtained commercially, incubated in a nutritive medium for 8 to 10 hr and starved overnight as described previously [14]. The uptake of Ca^{2+} was measured as before [13], but the incubation time with the isotope ($^{34}Ca^{2+}$) was 3 min. After incubation in the presence of $^{45}Ca^{2+}$, an aliquot was filtered through Millipore-type filters and the cells were washed with 10 mM cold

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$CaCl_2$ to remove by exchange the externally bound $^{45}Ca^{2+}$. The binding of the isotope was measured by incubating the cells in the presence of $^{45}Ca^{2+}$ for short intervals without substrate to avoid uptake, filtering an aliquot on Millipore-type filters, and washing twice with water. This washing does not remove a significant amount of the bound cation, and under these conditions the amount of $^{45}Ca^{2+}$ entering the cells is practically null. Experiments were repeated at least three times.

Results

Results have been published on the values of the kinetic parameters of divalent cation uptake by yeast. Armstrong and Rothstein [1], for instance, obtained a value of 600 μM for Ca^{2+}. Values of a similar order of magnitude can be inferred from the work by Roomans et al. [15]. Besides, Rothstein et al. had found that the uptake of Sr^{2+} and Ca^{2+} was much slower than that of Mn^{2+} and Mg^{2+} under similar conditions [17]. Under our previous experimental conditions [13], the value of the kinetic constants for Ca^{2+} uptake is similar to that of other investigations. The K_m for the uptake was around 75 μM, and the V_{max} was around 5 nmol·(100 mg·3 min)$^{-1}$; these values were obtained, however, by plotting the double reciprocal values of the velocity of uptake against concentration of added divalent cation (results not shown).

One of the important facts already described in the literature [16] is the ability of yeast cells to bind cations with similar affinities for different alkali cations; for example, values for Mn^{2+} of 40 to 80 μM for the dissociation constants, as well as a high number of sites with high affinity for the cations, have been described. If the number of binding sites is high, it is possible that, in the presence of significant amounts of yeast, the binding can produce a decrease in the concentration of free cation in the medium available for the transport systems of the cell. The Scatchard plot [18] of Fig. 1 shows that, in fact, our yeast cell preparation has the ability to bind Ca^{2+} ions with high affinity. To calculate the high affinity constants, the four points of the graph obtained at the lowest concentrations were disregarded and the next four points were used. From the line obtained with these points, n was obtained by extrapolation and K_d from its slope. The low affinity constants were not calculated, since the curve seems to continue bending as higher Ca^{2+} concentrations are added. The dissociation constant of this binding has a value of 14 μM, and the number of high affinity sites is 1.22 nmol/mg of yeast. By counting the number of cells and with adequate calculations we find that there are 69 million of these double sites per cell; at a 125 μM Ca^{2+} concentration, 90% of these sites are occupied. By this same procedure it is also possible to calculate the actual concentration of free Ca^{2+} in the medium and thus to correct the values of the kinetic constants in the transport experiments. As shown in Fig. 2, in the form of a Hofstee plot [10], if the concentrations of Ca^{2+} in the medium are corrected for the amounts bound by the cells in each experimental point, different values are obtained, especially for K_m. The curve is of the same type as that reported by Roomans et al. [15], but the velocity

Abbreviations: MES, 2(N-morpholino) ethane sulfonic acid; TEA, Treithanolamine.

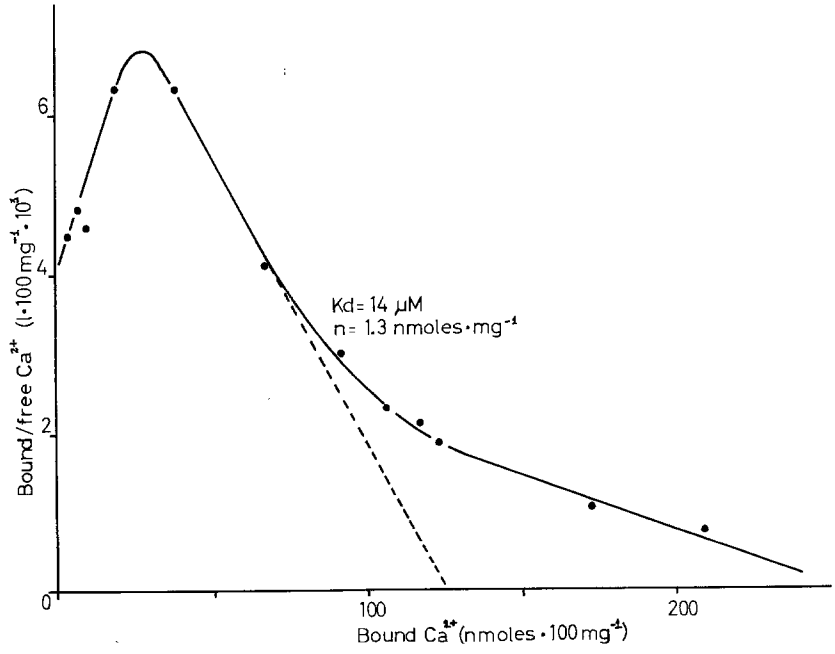

Fig. 1. Scatchard [18] plot of Ca^{2+} binding by yeast. Experimental conditions: 100 mg of yeast were incubated briefly (1 min) at 30° in the following medium: 10 mM MES-TEA buffer, pH 6.0; from 2.5 to 250 μM $^{45}CaCl_2$; final volume, 2.0 ml; an aliquot was taken, filtered through a Millipore-type filter and washed twice with water. The cells were then suspended in water, and an aliquot was plated and counted in an end-window gas-flow counter. The dotted line was used to calculate the "high affinity" constants

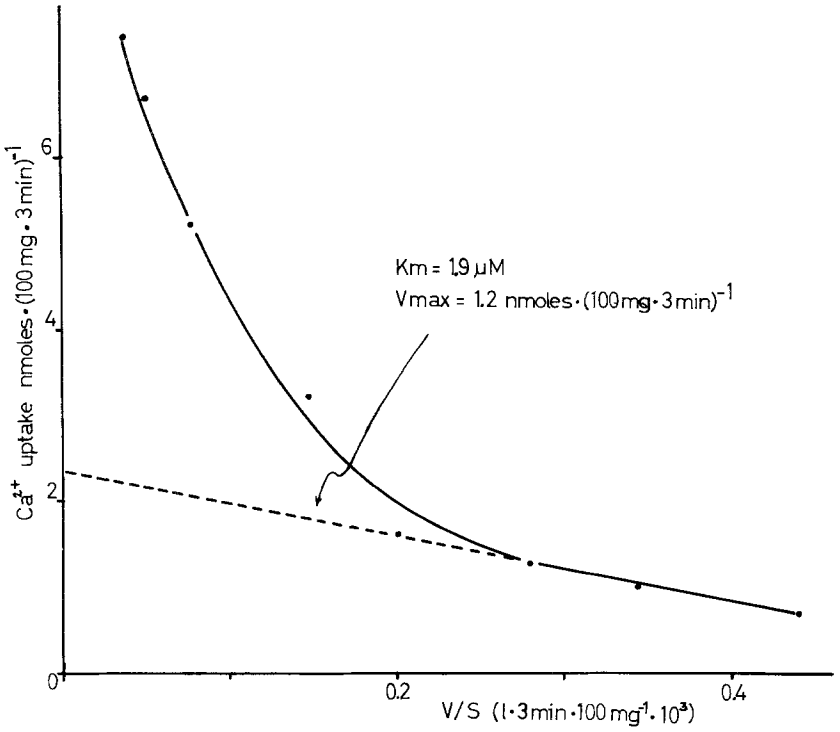

Fig. 2. Hofstee [10] plot of the uptake of Ca^{2+} at different concentrations. Incubation conditions: 10 mM MES-TEA buffer, pH 6.0; 50 mM glucose; yeast cells, 100 mg; final volume, 2.0 ml; temperature, 30°. The cells were added to the incubation mixture previously equilibrated to the bath temperature; after 3 min the indicated concentrations of $^{45}CaCl_2$ were added; exactly 3 min later an aliquot was taken, filtered, and washed twice with 10 mM $CaCl_2$. The cells were then resuspended in water and an aliquot was plated and counted. As in Fig. 1, the kinetic constants were obtained from the dotted line

values are lower. There is a high affinity uptake, and a bending of the line at higher concentrations of the cation, as reported by the aforementioned authors. Taking into account the decrease of the actual concentration of the cation by the binding to the cells, with the four points at the lowest concentration, the K_m for the high affinity transport of the cation decreases to 1.9 μM, with a V_{max}, of approximately 1.2 nmol·(100 mg of yeast·3 min)$^{-1}$.

One of the important facts to study in transport systems is the possibility of inhibiting them with several kinds of agents. In the case of Ca^{2+} transport, several inhibitors have been reported; Ruthenium red was described as a potent inhibitor of Ca^{2+} uptake in mitochondria [12]; lanthanum has been used also [2], and Rothstein and Hayes described uranyl as an inhibitor of cation binding in yeast [16]. In view of these antecedents, it was considered interesting to test the inhibitors; in order to have more information about them, their effects were determined both on binding and transport of Ca^{2+}.

Ruthenium red was used from 0.25 to 25 μM. As shown in Fig. 3, this inhibitor produces a slight inhibition of Ca^{2+} binding, depending on the concentrations. One effect of the dye on the uptake of Ca^{2+}, however, was rather unexpected, since at very low concentrations, it produced a consistent stimulation of the uptake.

Another of the inhibitors studied was uranyl (Fig. 4). Again, as with ruthenium red, low concentrations of this cation produced a stimulation of the uptake and an inhibition at higher values; the latter, at 100 μM, is higher than 80%. The effect on binding was similar to that of ruthenium red, but higher concentrations of uranyl were used. Inhibition at 100 μM does not exceed 50%; it is progressively higher as the concentration of the inhibitor is increased.

Figure 5 shows the effects observed for La^{3+} also on both binding and uptake of Ca^{2+} under experimental conditions similar to those used for the other inhibitors. With this inhibitor, both parameters were decreased with increasing concentrations. Besides, with La^{3+}, the inhibition of the uptake was obtained at much lower concentrations than that of the binding. Approximate calculations of the half maximal inhibitory concentrations were obtained by double reciprocal plots of percent inhibition and La^{3+} concentration and gave values of around 150 and 4 μM for binding and uptake, respectively.

The previous results seemed to indicate that some separation exists between binding and transport of Ca^{2+} in yeast; some inhibitors produce a slight block of binding without inhibiting or even producing a stimulation of transport; La^{3+} inhibits both. Another way to test this consists in assessing the effects of monovalent cations on both parameters. This was also important for several reasons; Conway and Beary postulated that divalent and monovalent cations are transported by the same carrier system in yeast [3]. This was postulated because the authors

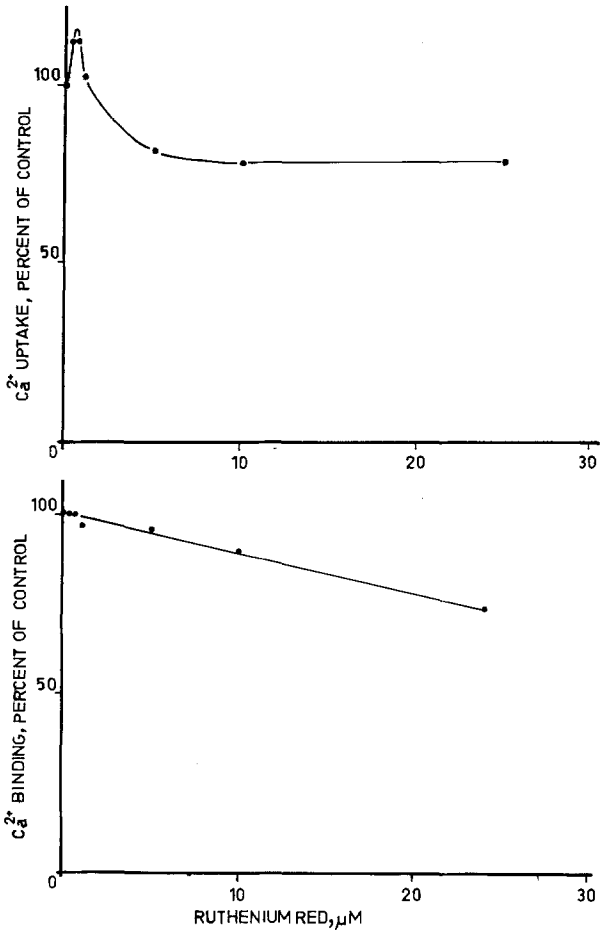

Fig. 3. Effects of ruthenium red at different concentrations on binding and uptake of Ca^{2+}. Experimental conditions were as described for Figs. 1 and 2, but different concentrations of ruthenium red were included in the incubation mixture; the concentration of Ca^{2+} was 75 μM

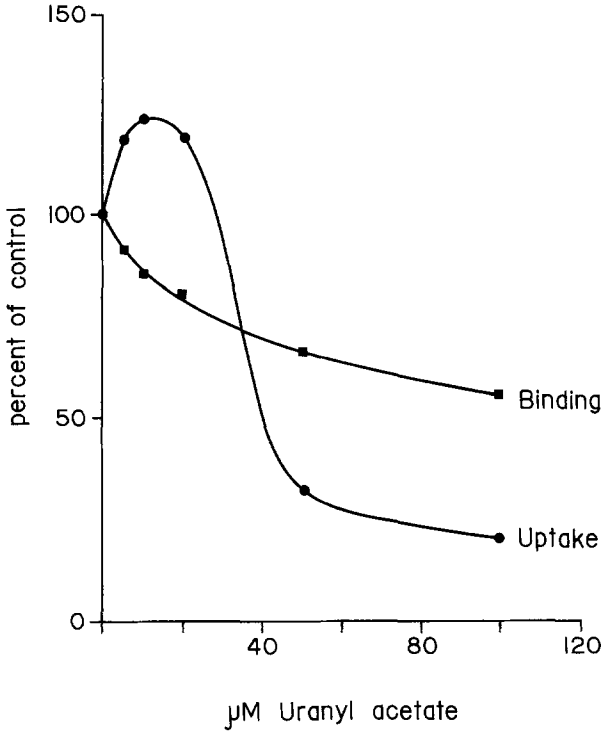

Fig. 4. Effects of uranyl acetate at several concentrations on both binding and uptake of Ca^{2+}. Experimental conditions were as described for Figs. 1 and 2, but with the inclusion of several concentrations of uranyl acetate. The Ca^{2+} concentration was in all cases 75 μM

found out that K^+ inhibits the uptake of divalent cations; however, Rothstein [17] (quoted in reference 8) found that preincubation with K^+ in the medium stimulated Mn^{2+} transport; since then, it is a common practice in measuring the uptake of divalent cations to stimulate it by preincubating the cells with phosphate, K^+ and glucose [7, 8, 15, 17]; it has also been postulated that phosphate is involved in the formation of the carrier for divalent cations [7, 11]. These facts raise the importance of studying the effects of K^+ on both the binding and the uptake of calcium ions and, besides, of comparing its effects with those of other monovalent cations, sodium, for instance, on the basis that they should have similar effects on binding, but not necessarily so on the uptake of Ca^{2+}. Figures 6 and 7 show the results obtained; with Na^+, an inhibition of binding was observed, but the uptake was little affected; it is possible, as shown in Fig. 6, to calculate the concentration of this monovalent cation that is required to produce half maximal inhibition of the binding of Ca^{2+}; the value is 13 mM; the maximal inhibition that can be obtained is 96%; data are only approximate because deviations are observed from linearity on the double reciprocal plots. At low concentrations of Na^+, a small inhibition of transport was observed, but above 10 mM, a definite and constant tendency to stimulate was observed as the concentration was increased (Fig. 6).

K^+ (Fig. 7) also produced a progressive inhibition of Ca^{2+} binding as its concentration was increased. The double reciprocal plots also gave some deviation from linearity at higher concentrations, but from the linear part of the line, the highest attainable inhibition of binding was close to 90%, and the calculated concentration of K^+ required to obtain half maximal inhibition was around 8 mM. A very important difference, however, was observed on the effects on Ca^{2+} uptake, in comparison with Na^+. With K^+, a marked inhibition was also observed for the uptake. Both

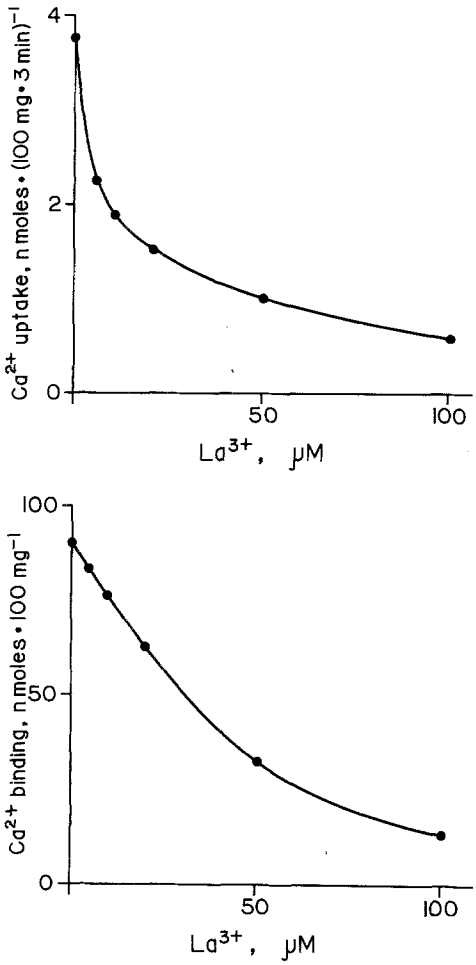

Fig. 5. Effects of $LaCl_3$ on the binding and uptake of Ca^{2+} by yeast. Experimental conditions were as for previous figures, but varying concentrations of $LaCl_3$ were included in the incubation medium

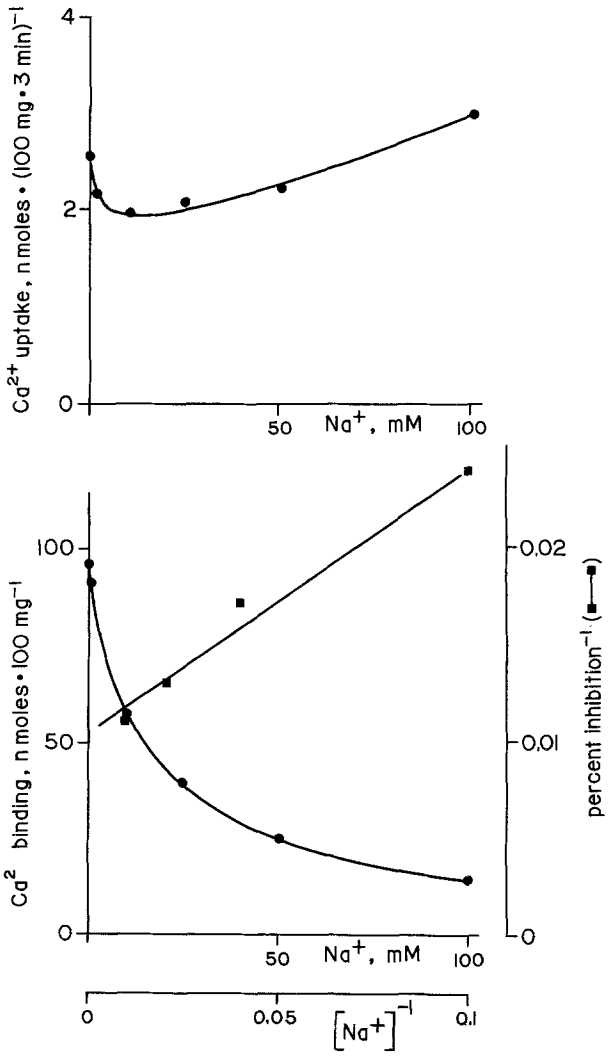

Fig. 6. Effects of Na^+ on the binding and uptake of Ca^{2+} by yeast. Experimental conditions were as for Figs. 1 and 2, but several concentrations of NaCl were included in the incubation medium, with a constant Ca^{2+} concentration of 75 μM. For the binding data, also the double reciprocal plot of the percent inhibition *VS.* the Na^+ concentration is presented (× --- ×)

at high (100 mM) and at low (1 mM) concentrations, a clear deviation from linearity was observed on the double reciprocal plots; from the linear part of this curve, however, a maximal inhibition of 62% and a K_i for K^+ of around 8.0 mM could be calculated.

The last point to be tested in this exploration of the general properties of the Ca^{2+} uptake by yeast was the effect of another divalent cation, both on uptake and binding. This was essential, since according to the literature it should be expected that Mg^{2+} inhibited both the uptake and the binding of Ca^{2+}. In agreement with this (Fig. 8), Mg^{2+} inhibited both parameters. However, the characteristics of the effects on both phenomena were different from the kinetical point of view. The double reciprocal plots of the effect of Mg^{2+} on Ca^{2+} uptake show, for two experiments combined on the same graph, two components; the one with higher affinity shows a maximal inhibition of 65% with an inhibition constant of 3 μM. The "low affinity" component shows a maximal inhibition of 84% and an inhibition constant of 17.5 μM. The effect on binding, as shown on the double reciprocal plot, shows a simple relationship between inhibition and the concentration of Mg^{2+}. Maximal inhibition was close to 100%, and K_i was 66 μM, a value significantly higher than that of the K_m of Ca^{2+}.

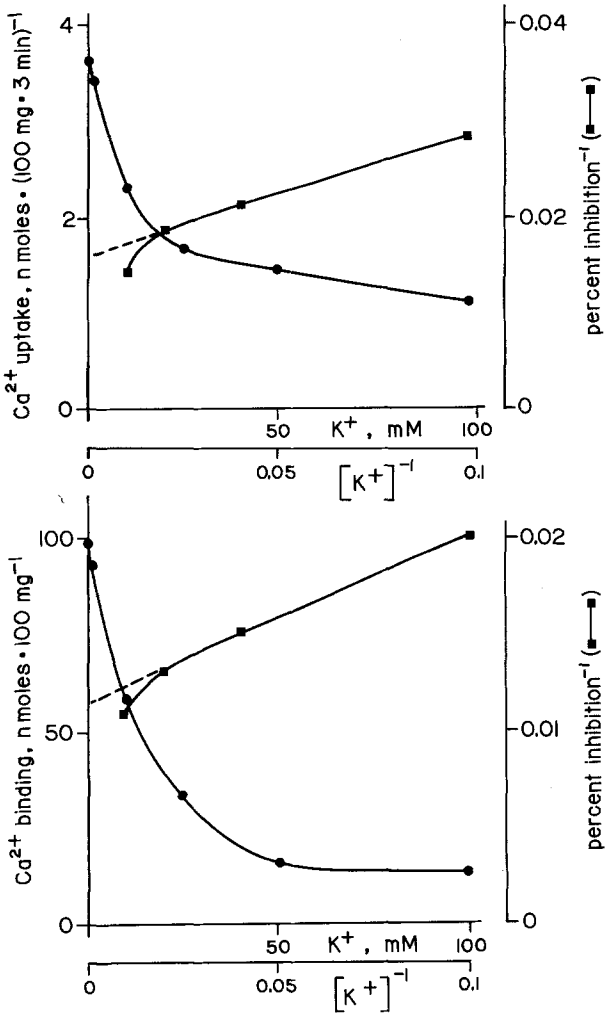

Fig. 7. Effects of K^+ on binding and transport of Ca^{2+} by yeast. Experimental conditions were as for Figs. 1 and 2, but several concentrations of KCl were included in the incubation medium, which contained a constant concentration of Ca^{2+} of 75 μM. The double reciprocal plots of percent inhibition against K^+ concentration are also presented (× --- ×)

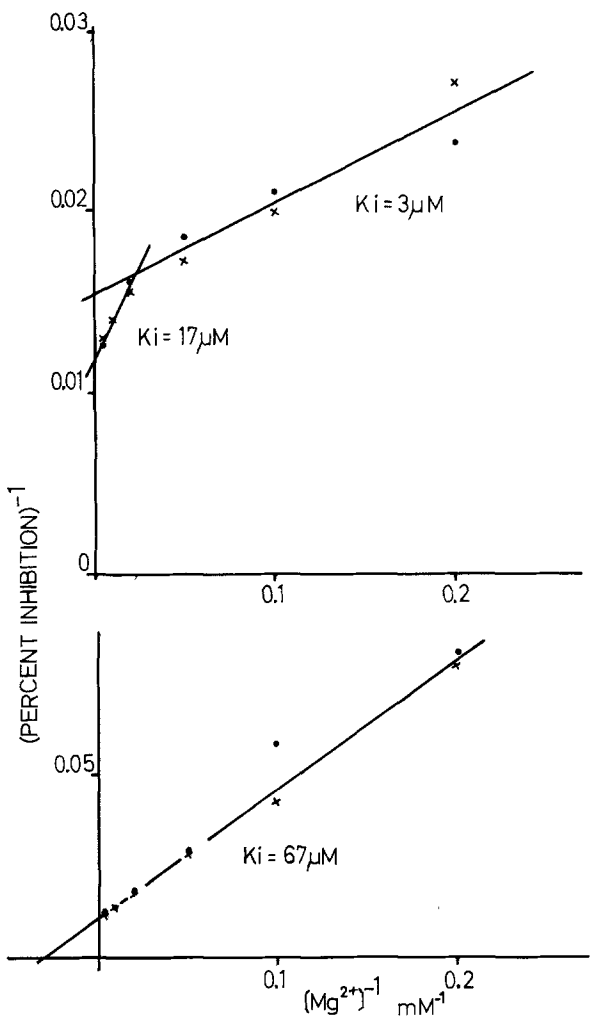

Fig. 8. Effects of Mg^{2+} on binding and transport of Ca^{2+} by yeast. Experimental conditions were as for Figs. 1 and 2, but varying concentrations of $MgCl_2$ were added to the incubation medium, with a constant 75 μM concentration of Ca^{2+}

Discussion

First, it must be said that there are methodological differences in the experiments reported here and those of other authors [1, 3, 7, 8, 11, 17]. In previous experiments with our yeast it was found that preincubation of the cells with glucose, potassium, and phosphate did not increase the rate of uptake of Ca^{2+} [13]. In view of this, we did not preincubate the cells under these conditions. Secondly, the buffer used in our experiments was MES (2 (N-morpholino) ethane sulfonic acid), which has a very low capacity to bind divalent cations [9].

We have tried to determine more precise values of the kinetic constants for the uptake of Ca^{2+} because, besides the problem of the buffer systems used in some of the previous work in other cases, the effect of the binding of the transported divalent cations was not considered [1, 3, 7, 8, 17]. Previous data from this laboratory, for instance, had both problems [13]. In fact, under those conditions a value of approximately 100 μM was found for the K_m of the transport system for Ca^{2+}. Under the conditions reported here and discounting bound Ca^{2+} from the total, the K_m is much lower and, of course, the maximal rate of uptake is not very much altered. It seems to be clear that the main reason for the difference resides in the fact that binding of the cation is so extensive that it produces a high decrease of the actual concentration of the cation in the medium. The variation of the slope of the curve has been interpreted by Roomans et al. [15] as due to the changes that Ca^{2+} itself pro-

duces on the surface of the cell. The data presented in the figure were obtained for the lowest concentrations of the divalent cation employed.

Binding by itself is an important parameter to discuss. It is interesting that the yeast cell has a very high affinity for Ca^{2+}; this is probably due to the large carbohydrate content of the cell wall. Besides, the capacity of yeast to bind the cation is also impressive, particularly when the calculations are made to determine the number of sites that exist per cell, which amounts to 69 million. This means that if each site for Ca^{2+} has two negative charges there must be twice that amount of single negative sites. Another fact to consider (which will be dealt with later on) is the role that this binding may play on the process of uptake.

The curve of Fig. 1 shows at least three components. The first one, at very low concentrations of Ca^{2+}, has a negative slope due to a sigmoidal character of the curve in the direct plots of binding *vs.* concentration. A more detailed analysis of this section of the curve would be necessary before arriving at any conclusions. One plausible explanation of the changes in the slope of the rest of the curve is the change of the surface charge that the progressive binding produces on the cell surface; this effect has also been analyzed for the uptake of Ca^{2+} in the work of Roomans et al. [15].

The study of the effects of inhibitors of Ca^{2+} transport reported in other systems is important for two reasons: first, it may help to establish similarities (or differences) between transport systems; and, second, if differences are found in the effects on binding and uptake, results may help to define the role that binding has on the uptake of the divalent cation. As to the first point, it is evident that ruthenium red and uranyl are rather poor inhibitors of Ca^{2+} transport in yeast; in fact, at very low concentrations they produce a stimulation of the uptake that is difficult to explain by an increase of the actual concentration of Ca^{2+} in the medium, because the inhibitors at these concentrations hardly affect the binding of the divalent cation. Lanthanum is a good inhibitor of Ca^{2+} transport in yeast, as indicated by the low value of its K_i.

The lack of an obligatory relationship between binding and transport is demonstrated by the different effects of uranyl and ruthenium red on both parameters; besides, the inhibition constant of La^{3+} for binding is much higher than that for transport. This difference of effects on binding and transport is also observed with Na^+ and Mg^{2+}.

Although with less affinity, Na^+ can inhibit the binding of Ca^{2+} and probably, by increasing the concentration of the free divalent cation in the medium, produces an increase in its uptake at rather high concentrations (Fig. 6). The double reciprocal plots of percent inhibition of binding against concentration give for Na^+ an apparent value for K_i of around 10 mM and a maximal inhibition value of 96%, which indicates a potential ability to displace Ca^{2+} totally from its binding sites. However, the effect of low concentrations of Na^+ on the uptake is difficult to explain; there is an inhibition that might be similar to that observed with K^+ within this concentration range.

The effects of K^+ on binding are similar to those of Na^+, both quantitatively and qualitatively. In this respect, both monovalent cations seem to behave in the same way. The effects on the uptake of Ca^{2+}, on the other hand, are different; K^+ produced a large inhibition of the uptake at both low and high concentrations. The graphs of the effects of Na^+ and K^+ on the transport of Ca^{2+} by yeast seem to be similar at low concentrations. Although K^+ seems to be a better inhibitor of this phenomenon than Na^+ at the lowest concentration employed in the experiments presented in Figs. 6 and 7, Na^+ is a better inhibitor than K^+. The difference of behavior of both monovalent cations seems to be at the higher concentrations. These data are interesting because of two main facts from previous workers: first, it has been postulated that the uptake of divalent cations by yeast takes place by the exchange for internal K^+ [8], and, second, the experiments of Conway and Gaffney [5] on the preparation of a Ca^{2+} yeast showed that it is difficult to achieve the accumulation of large amounts of Ca^+ within the cells unless they are previously loaded with Na^+. Although no direct experiments have been carried out with Ca^{2+}, the differences of effects between Na^+ and K^+ suggest that there may be some important role of these cations in the uptake. The postulation of Rothstein has found some support in the fact that several substances that produce the efflux of K^+ from the cell produce a large increase in the rate of Ca^{2+} uptake [13]. The facts are also interesting with respect to the exchange that has been described in several biological systems, by means of which Ca^{2+} can be transported through biological membranes in exchange for Na^+ [6]. In this respect, experiments are in progress in which Na^+ and Ca^{2+} yeasts [6] will be used to test the characteristics of Ca^{2+} uptake.

As to the experiments presented in Fig. 8 on the effects of Mg^{2+} on the transport of Ca^{2+}, it seems that, at least for these two cations, there may be a single transport system. Further experiments will define this with a larger series of divalent cations and more profound kinetic analyses, in order to determine the justification of such an assumption. The

figure shows another interesting fact that requires further exploration: the presence of two components dependant upon the concentration of Mg^{2+}, which inhibits Ca^{2+} uptake.

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