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Interaction of Ethidium Bromide with the Transport System for Monovalent Cations in Yeast

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Summary. Ethidium was found to be taken up by yeast cells in a process that, at certain concentrations has the main following characteristics: a) a substrate is required; b) it presents cooperative kinetics, with n, according to the Hill equation ≈ 3; c) ethidium can be concentrated more than 100-fold; d) the uptake is inhibited by Ca^{2+}; e) the uptake of the dye is inhibited by monovalent cations with a selectivity pattern similar to that observed in their transport by yeast; f) ethidium inhibits the uptake of K^+, and, at concentrations up to about 250 μM produces a competitive inhibition on the uptake of Rb^+; and g) ethidium produces the same effects as K^+ on respiration and the extrusion of H^+. It is concluded that ethidium is taken up by yeast cells in a selective way by the same transport system normally employed for monovalent cation uptake.

In the course of some experiments designed to study the effects of some cationic dyes on K^+ transport in yeast, it was found that ethidium bromide, as well as other cationic dyes were able to inhibit the uptake of K^+. In view of this, and the fact that this dye has been so extensively studied in relation to its fluorescent characteristics, it was considered important to determine the kind of interaction of the molecule with the monovalent cation transport mechanism in yeast cells. This paper presents the results of such studies.

Materials and Methods

Saccharomyces cerevisiae cells from a pure strain kindly donated by La Azteca, S.A., were prepared as described previously (Peña, 1975).

The methods for the measurement of K^+ uptake, pH recording, oxygen consumption and ^{86}Rb uptake were also described previously (Peña, 1975).

Fluorescence changes of ethidium were followed in a Farrand, Mark I spectrofluorometer at 530 → 590 nm. Narrow band filters of 530 and 590 nm were placed additionally between the excitation monochromator and the sample, and between this and the analyzer monochromator, respectively.

The uptake of ethidium bromide was followed in two ways; in the first, the cells were added to the incubation mixture in centrifuge tubes previously equilibrated to the temperature

of a water bath (30 °C); after the incubation, the tubes were cooled in an ice water bath for 2 min, and then centrifuged at 3,000 rpm for 3 min. After centrifuging, the supernatants were decanted to measure the ethidium concentration. In the second method, after mixing the cells with the medium at 30 °C, aliquots were withdrawn at fixed intervals, rapidly placed in tubes of a Beckman Microfuge and centrifuged for 10 sec. The supernatant was then separated with a Pasteur pipette to measure its ethidium concentration. The ethidium concentration of the supernatants obtained was determined by measuring the fluorescence of adequate dilutions in a 20 mM maleate-triethanolamine buffer, pH 6.0. The wavelengths employed were 330 nm, excitation, and 600 nm, emission. The results were compared each time with a standard curve of ethidium bromide from 4 to 40 μM at the same pH.

Results

Ethidium bromide inhibits K^+ uptake in yeast at rather low concentrations; concentrations of 100 μM are enough to produce a clear inhibition (Fig. 1). As reported for alkyl guanidines (Peña, 1973), the dye does not produce at this concentration practically any effect on the expulsion of H^+. At higher concentrations, the addition of the dye produces an actual exit of K^+, and a strong inhibition of the H^+ expulsion from the cells.

The fact that ethidium produces an inhibition of the transport of K^+ can be due in a first analysis to the possible binding of the molecule to the surface of the cell, in a similar way to that described for ANS by Fortes and Hoffman (1974). The first efforts to measure binding to the cells by fluorescent techniques gave negative results at the concentrations of the dye usually employed in these studies (10 to 40 μM). An experiment was carried out using higher concentrations of ethidium, and measuring its disappearance from the medium by centrifugation in the microfuge, to follow the time course of the phenomenon; the results are shown in Fig. 2. In the presence of glucose as substrate, the initial entrance of ethidium is very fast, and the rate seems to be related to the concentration of the dye employed. The results showed also that although in the absence of a substrate, some uptake exists, much higher concentrations have to be used in order to observe a significant uptake of the dye by the cells. By assuming an internal water content of approximately 50 percent of the wet weight for the cells, as measured before (Peña *et al.*, 1967), internal concentrations of ethidium between 20 and 30 mM can be calculated. Calculating the final ratio between this and the external concentration, values higher than 100 can be observed in the presence of substrate. Without substrate, at 1.333 mM ethidium, internal concentrations around 15 mM can be calculated, and concentration ratios close to 20 have been estimated. This is of course by assuming

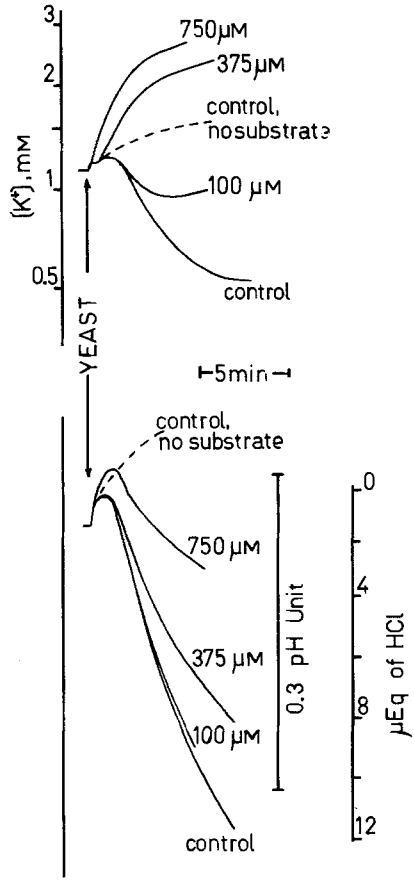

Fig. 1. Effect of different ethidium bromide concentrations on K^+ and H^+ movements in yeast. Incubation conditions: 10 mM maleate-triethanolamine buffer, pH 6.0; 50 mM glucose; 1 mM KCl; yeast, 250 mg, wet weight. Final volume, 10.0 ml. The experiments were carried out in a water jacketed vessel at 30 °C. After equilibrating the temperature, each experiment was started by the addition of yeast. A control without substrate is included for comparison

that the internal ethidium is not bound to the cell structures, but freely distributed in the cell water. In Fig. 3 the results of the uptake measured at 3 min in a similar experiment are presented. The relationship between the concentration and the uptake is sigmoidal. At low concentrations of ethidium there is a more pronounced dependence on a substrate for the uptake.

A sigmoidal relationship between concentration and uptake is an indication of a possible cooperative effect in the uptake of the dye. Fig. 3 also shows the data presented in the form of a Hill plot, in order to analyze more accurately this behavior. The value obtained for n in the

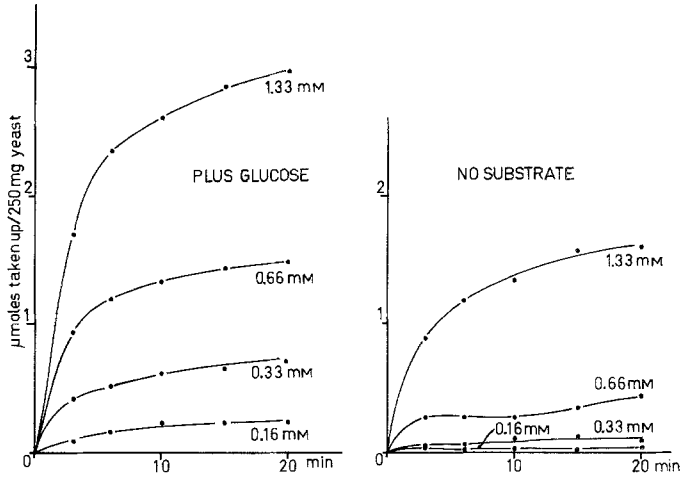

Fig. 2. Time course of ethidium uptake with and without glucose as substrate, at different concentrations of the dye. Incubation conditions: 20 mM maleate-triethanolamine buffer, pH 6.0; 33 mM glucose; yeast cells, 500 mg, wet weight. Final volume, 6.0 ml. Temperature, 30 °C. The incubation was started by the addition of yeast to the complete incubation mixture. At fixed times, aliquots were taken and rapidly centrifuged in the microfuge. Ethidium concentration was measured in the supernatants as described in Materials and Methods. Figures next to the curves indicate the ethidium concentration in each case. To compare with other experiments, results are expressed as µmoles taken up by 250 mg of yeast

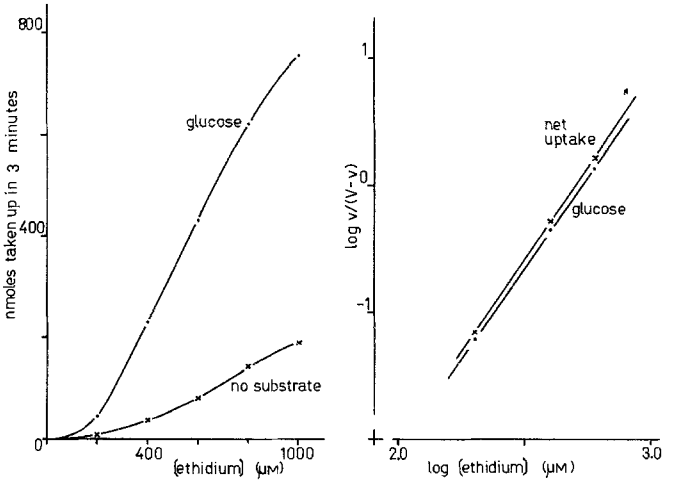

Fig. 3. Normal and Hill plots of the relationship between ethidium concentration and uptake with and without glucose. On the left side, the results of the uptake of ethidium with (●) and without (×) glucose are presented. On the right side, results are presented as the Hill plots of the same results, in the presence of glucose (●) and the results in the presence of glucose minus the values without substrate (×). Incubation: 20 mM maleate-triethanolamine buffer, pH 6.0; 50 mM glucose; yeast cells, 150 mg; ethidium concentration, as indicated. Final volume, 2.0 ml. Temperature, 30 °C; time 3 min. The uptake of ethidium was measured according to the second method described in Materials and Methods

experiment presented is 2.85 but values oscillate between 2.10 and 3.33 in seven experiments, with a mean of 2.90. It is important to point out, besides, that the subtraction of the values found in the absence of a substrate does not change the value of the slope in the Hill plot.

Since ethidium is an amphiphilic molecule of cationic nature, and results had been reported on the interference of cations with the effects of this kind of molecules in yeast (Armstrong, 1961), the effect of several concentrations of KCl on the uptake of the dye also at different concentrations was explored. KCl produced an increasing inhibition of the uptake of ethidium at all concentrations tested (Fig. 4). An experiment was carried out in which the effect of K^+ was determined on the *n* parameter of the Hill equation. The results are presented in Fig. 5; the values for the uptake of ethidium in the absence of glucose have been subtracted from those obtained with the substrate. Although the addition of 3.33 mM KCl to the incubation mixture produces a good inhibition of the uptake of ethidium, it does not produce any change in the number of sites of the system involved in its uptake.

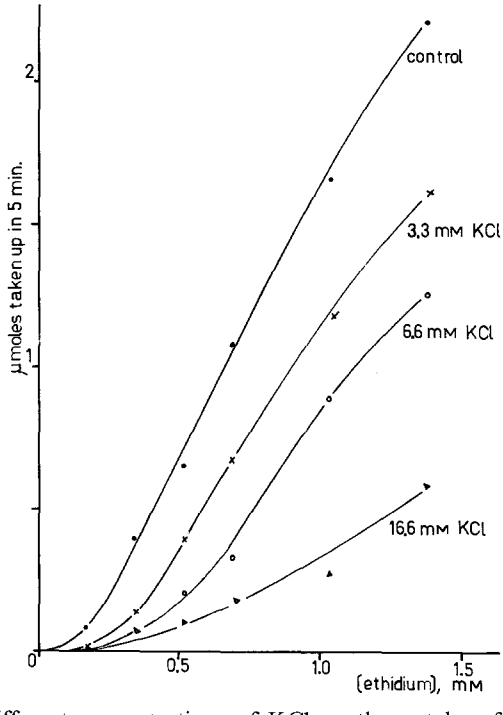

Fig. 4. Effect of different concentrations of KCl on the uptake of ethidium at different concentrations. Incubation conditions were the same as for Fig. 3 except that KCl was included as indicated, ethidium uptake was measured according to the first method described in Materials and Methods, and the incubation time was 5 min

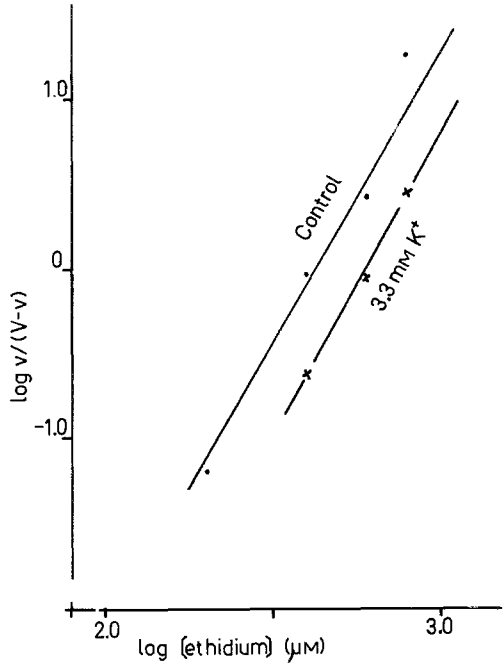

Fig. 5. Hill plot of the effect of 3.3 mM KCl on the uptake of ethidium by yeast. The incubation conditions and experimental procedure were the same as for Fig. 3, except that KCl was included as indicated. The uptake values presented are the figures resulting after subtracting the values observed in the absence of substrate from those found with glucose as substrate

Armstrong (1961), also reported a reversion of the effects of cationic amphiphilic molecules on yeast by the presence of divalent cations at relatively low concentrations. In order to explore this possibility for the case of ethidium, the effect of three concentrations of Ca^{2+} was measured on the uptake of ethidium at several concentrations (Fig. 6). The cation at low concentrations of ethidium produced a marked inhibition of the uptake. However, unexpectedly, at high concentrations of ethidium, the presence of Ca^{2+} not only did not produce the inhibition of the uptake, but resulted in a substantial enhancement of the uptake of ethidium.

Since K^+ produced a large diminution of the uptake of ethidium, it was considered important to determine how specific was the effect in comparison with the other monovalent cations. Fig. 7 shows the results obtained; to make the phenomenon less dependent on charge effects of the cations, the experiment was carried out in the presence of 0.1 mM $CaCl_2$. At 10 mM concentration, lithium and sodium ions showed some inhibition of the uptake of ethidium; however, this inhibition was low

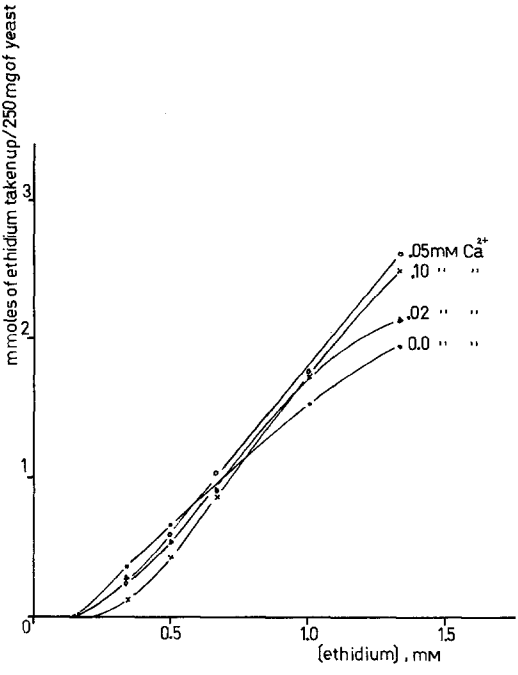

Fig. 6. Effect of $CaCl_2$ at different concentrations on the uptake of ethidium by yeast. The incubation conditions were the same as for Fig. 4, except that $CaCl_2$ was included as indicated. The figures next to the tracings indicate the $CaCl_2$ concentration (mM)

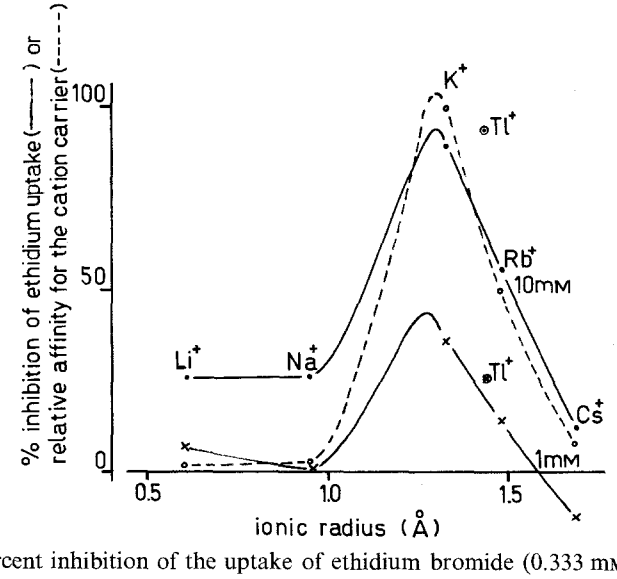

Fig. 7. Percent inhibition of the uptake of ethidium bromide (0.333 mM) in relation to the ionic radius of the different monovalent cations. Incubation conditions were the same as for Fig. 4, except that two concentrations of each monovalent cation were employed (10 and 1 mM), and all the tubes contained 0.1 mM $CaCl_2$. The dotted line represents the relative affinity of the monovalent cation carrier of yeast for the different monovalent cations, as reported by Armstrong and Rothstein (1964). Thallium salt was the sulfate

if compared to that produced by the presence of K^+ or Tl^+; Rb^+ showed an intermediate degree of inhibition, and finally Cs^+ practically did not produce any change in the amount of ethidium taken up. At a 1 mM concentration, Na^+ and Li^+ had no effect; K^+ and Tl^+ had an important inhibitory effect, Rb^+ a lower one, and Cs^+, at this concentration even produced an increase in the uptake of ethidium; this latter result, besides, was consistent in three similar experiments performed. To get an idea of the selectivity of this inhibition, the graph includes the data published by Armstrong and Rothstein (1964) on the relative selectivity of the monovalent cation transport system for the different monovalent cations, considering a value of 100 for K^+. Except for a deviation of Na^+ and Li^+, the data of the inhibition of ethidium uptake by the different cations at 10 mM concentration are very similar to those of the relative affinity of the cation transport system.

The data on the inhibition of ethidium uptake by monovalent cations could be taken as an indication in the sense that this organic cation is taken up by the cell using the same transport system employed for the cations. Rb^+, according to the studies of Armstrong and Rothstein (1964) seems to be taken up by the same system as K^+. It was then thought that if Rb^+ and ethidium penetrate by the same system, the kinetics of the inhibition of the Rb^+ uptake by ethidium should be of the competitive type. Experiments were performed in which the effect of several concentrations of ethidium was determined on the initial rate of uptake of ^{86}Rb, also at different concentrations. The data are presented in Fig. 8 in the form of the double reciprocal plot. The expected results were obtained only at a concentration of ethidium of 250 μM. At this concentration, ethidium shows a very clear competitive type of inhibition on Rb^+ uptake. The K_i calculated in this experiment was 347 M, in another, the value obtained was 315 μM. At concentrations of 0.5 and 1.0 mM of ethidium the inhibition shows a complicated pattern which, in any case, does not conform to the expected classical competitive type.

It has been extensively demonstrated that the monovalent cation transport implies an exchange of the cation for H^+ (Conway & O'Malley, 1946; Rothstein & Enns, 1946; Conway & Brady, 1950). The presence of monovalent cations in the incubation medium at pH 6.0 stimulates the efflux of H^+ observed after the addition of yeast to a medium containing a substrate; Fig. 9 shows this phenomenon. It is seen that the addition of ethidium at different concentrations also produced the stimulation of the H^+ efflux. By using different concentrations, it could be seen that ethidium always produces the stimulation of the proton efflux; however,

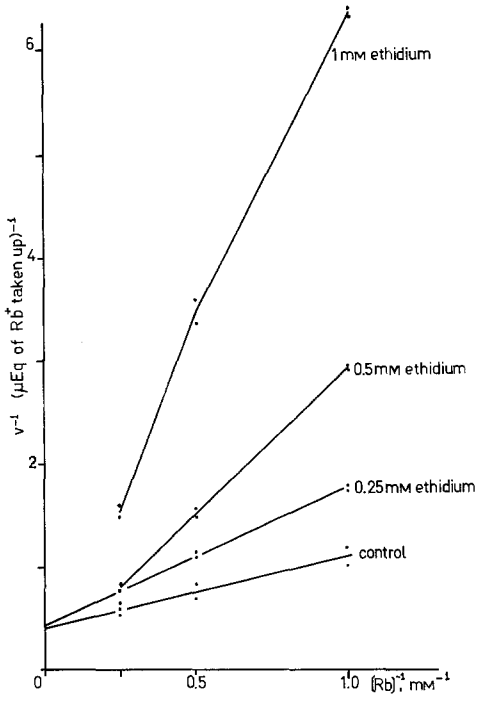

Fig. 8. Effect of different concentrations of ethidium on the uptake of ^{86}Rb by yeast; double reciprocal plot. Incubation conditons: 40 mM maleate-triethanolamine buffer, pH 6.0; 100 mM glucose; yeast, 100 mg, wet weight. Final volume, 2.0 ml. Temperature, 30 °C. The experiment was performed as described in Materials and Methods

at the higher concentrations, after the initial stimulation of the proton expulsion, a clear inhibition takes place. This inhibition could be observed also in the experiments presented in Fig. 1.

K^+ transport is an energy requiring process, and when K^+ is added to the incubation medium of yeast, metabolism, mainly glycolysis and respiration, are accelerated to provide for the energy expended during the process (Peña, Cinco, Gómez & Tuena, 1969). It was tested if ethidium could mimic K^+ also in this respect. The addition of ethidium to yeast cells with ethanol as substrate produces a stimulation of respiration of about the same extent as the alkali cation. However, at the higher concentrations of ethidium, as for H^+ efflux, there is an inhibition of respiration after the initial stimulation (results not presented).

As mentioned before, with low concentrations of ethidium, up to 20 μM, as usually employed in fluorescence studies, no enhancement of fluorescence was observed with yeast, whether in the presence or absence of a substrate. By using higher concentrations, however, and with the require-

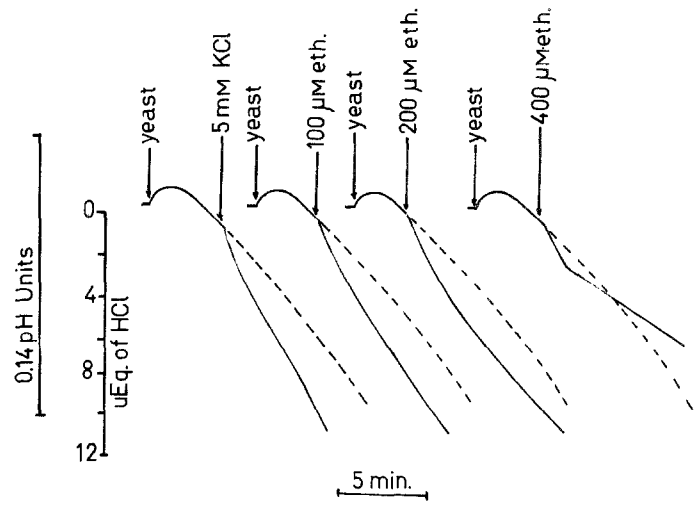

Fig. 9. Effect of 5 mM KCl, or different concentrations of ethidium on the H^+ extrusion by yeast. Incubation conditions: 20 mM maleate-triethanolamine buffer, pH 6.0; 50 mM glucose; yeast cells, 250 mg, wet weight. Final volume, 10.0 ml. Temperature, 30 °C. The H^+ scale was constructed by the addition of HCl to the incubation mixture minus the yeast

ment of a substrate, a clear enhancement of fluorescence is observed (Fig. 10). In general terms, the phenomenon behaves like the uptake of the dye. It requires a substrate at low concentrations, but at the higher ones employed (500 μM) a slight enhancement of fluorescence occurs in the absence of a substrate. As with the uptake of ethidium, the fluorescence enhancement is inhibited by the presence of K^+ at rather low concentrations.

Results on the further study of the inhibition of the fluorescence enhancement of ethidium by monovalent cations are presented in Fig. 11. In the upper tracings the inhibition produced by increasing concentrations of Na^+, K^+ and Ca^{2+} is shown. As with the uptake, Ca^{2+} is the most effective cation, producing an effect at concentrations as low as 50 μM; K^+ is also very effective, and Na^+ inhibits the fluorescence enhancement only at rather high concentrations. The lower tracings of Fig. 11 show the selectivity pattern of the inhibition of this fluorescence enhancement for the several monovalent alkali cations. Again, the selectivity is approximately the same observed for the inhibition of the uptake of the dye. The experiments on uptake, however, are difficult to compare strictly with those of fluorescence measurements. At the high concentrations of

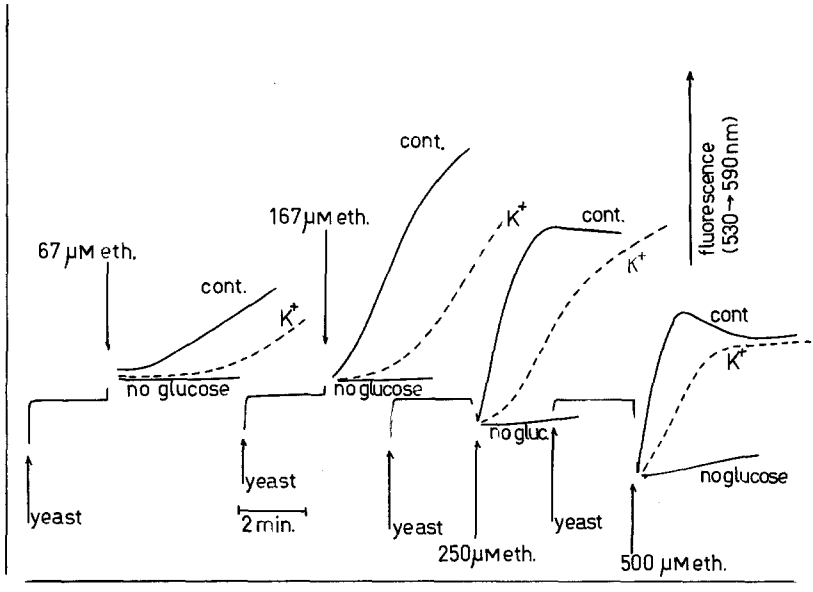

Fig. 10. Ethidium fluorescence at different concentrations of the dye, and the effect of substrate (glucose), and KCl. Incubation conditions: 20 mM maleate-triethanolamine buffer, pH 6.0; 33 mM glucose; 1 mM KCl (where indicated); yeast cells, 75 mg; final volume, 3.0 ml

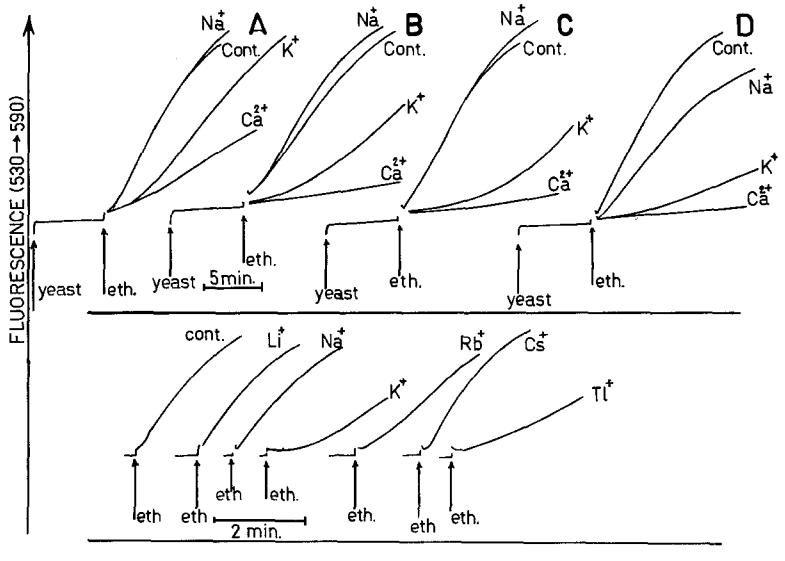

Fig. 11. Ethidium fluorescence at 0.166 mM concentration, and the effects of different concentrations of cations (upper tracings), or 1 mM monovalent cation concentration (lower tracings). The incubation conditions were the same as for Fig. 10, except that cations were added as indicated. In all cases, yeast cells were added 2 min before ethidium. In the upper tracings, the concentrations of Na^+ and K^+ were 0.5, 1.0, 2.0 and 5.0 mM, and for Ca^{2+} 0.05, 0.1, 0.2 and 0.5 mM for tracings *A*, *B*, *C* and *D*, respectively

yeast and ethidium bromide employed there are quenching problems which make it impossible to derive quantitative data from the fluorescence measurements.

Discussion

The experiments presented seem to indicate that ethidium is taken up by yeast cells in a way very similar to that of the monovalent cations. The most important fact, however, seems to be that ethidium not only penetrates by a mechanism similar to that of the monovalent cations, but seems to utilize the same transport system. The facts in favor of this idea are the following: (1) Especially at low concentrations, the uptake of ethidium requires a substrate. (2) As for monovalent cations, the transport results in the accumulation of the dye, not necessarily in a free state, since the fluorescence experiments indicate that part, at least, of the ethidium taken up is bound to the cell structures. (3) It has been found that monovalent cation uptake takes place in a process which results in an exchange for H^+ (Conway & O'Malley, 1946; Rothstein & Enns, 1946; Conway & Brady, 1950), and it has been postulated, besides, that this exchange occurs because there is a H^+ pump involved in the uptake of the cations (Peña, Cinco, Gómez & Tuena, 1972); the addition of ethidium to yeast cells in the presence of a substrate accelerates the extrusion of H^+ as K^+ does. (4) The requirement of a substrate for cation uptake seems to be due to the endergonic character of the process and as a consequence of this, the presence of a cation in the medium accelerates the energy conserving pathways of the cell (Peña *et al.*, 1969, 1972); in agreement with this, the addition of ethidium to the incubation medium, again, as K^+ does, accelerates the respiratory rate of yeast. Ethidium on the other hand, seems to be able to penetrate in the absence of a substrate by virtue of its concentration gradient, and perhaps by its affinity for the internal structures of the cell. (5) Monovalent cations inhibit the uptake of ethidium, and the selectivity of this inhibition is similar to that reported by Armstrong and Rothstein (1964) for the uptake of monovalent cations in yeast; except for Li^+ and Na^+, there is a good correspondence between the selectivity in the inhibition of ethidium uptake, and that of cation transport. (6) At low concentrations, ethidium behaves as a competitive inhibitor of Rb^+ uptake which may mean that both cations are taken up by the same system. Even at higher concentrations of ethidium, although the kinetics are abnormal, there is a clear diminution of the inhibition produced by the dye as the Rb^+ con-

centration increases. The kinetic data on the entrance of ethidium result in a sigmoid curve, which in the Hill plots gives n close to 3; Borst Pauwels, Wolters and Henricks (1971) have also reported sigmoid kinetics for the transport of Rb^+ in yeast, provided that low concentration ranges are used; on the other hand, Borst Pauwels *et al.* (1971) and Borst Pauwels (1973), as well as Armstrong and Rothstein (1967) have postulated the existence of a carrier system for K^+ with an active site which transports the cation, and a modifier site which is also sensitive to cations. We have no explanation for the value of 3 obtained for n with ethidium. Perhaps this organic cation has one additional site to penetrate. (7) The results on the inhibition of ethidium uptake by K^+ at different concentrations of the dye presented in the form of a Hill plot also show that the inhibition does not produce a change in the value of n; this datum would be more in agreement with a possible competition between both cations for the same sites of transport.

The analysis of the transport of ethidium at different concentrations could also show that this entrance of ethidium, possibly through the same system as the other monovalent cations meets the characteristics indicated in the last paragraph at the low concentrations, as shown by the following data: it is only at low concentrations that ethidium does not produce an inhibition of the expulsion of H^+; it seems that, if the transport system for monovalent cations involves a proton pump and a carrier for the cations, it is only at low concentrations of ethidium that the interaction takes place only with the cation carrier; at higher ones, an interaction with the H^+ pump seems to exist also. It is only at low concentrations that there is an almost absolute requirement of a substrate for the entrance of the cation. Also at low concentrations only ethidium shows a clear competitive inhibition of the entrance of Rb^+; at higher concentrations the effect is more difficult to analyze. Finally, the same situation exists in relation to the effect on respiration; although at all concentrations tested, ethidium produced a stimulation of respiration, at higher ones, after the initial stimulation an inhibition was produced. It seems that ethidium at higher concentrations can penetrate the cells by other, unspecific pathways. Besides, at the higher concentrations it can be accumulated to such an extent that it can affect other functions of the cell besides K^+ transport.

There is another point which deserves mention; that is the effect of Ca^{2+} on the uptake of ethidium. The divalent cation at low ethidium concentrations produces an inhibition of the uptake of the dye; in experiments not presented here it was also found that the effect of Ca^{2+}

is larger than that of K^+. This effect is relatively easy to explain in the light of present knowledge. It has been found, for instance, by Armstrong (1961) that cations, especially La^{3+} and divalent ones, produce a diminution of the interaction of amphiphilic cationic molecules with yeast. Fortes and Hoffman (1974) found that negative amphiphilic molecules when bound inhibit the movement of anions through the erythrocyte membrane. Similar effects of local anesthetics on ion translocation have been reported by Papa, Guerrieri, Simone and Lorusso (1972) with submitochondrial particles. In all these cases a charge competition seems to be established on the membrane surface, and ions bound to the membrane inhibit the translocation of other ions with the same charge. This might also be the case in the inhibition of the uptake of ethidium by Ca^{2+}. The stimulation produced by the divalent cation on the transport of ethidium at the higher concentrations of the dye is more difficult to explain; however, since the transport of ethidium through the membrane produces its accumulation, when high concentrations of the dye are employed, due to the concentration reached within the cell, some leakage might occur. In the presence of Ca^{2+}, and at high dye concentrations, it is possible that a smaller inhibition took place on the entrance than on the leakage of ethidium and this could give rise to a higher net accumulation.

Ethidium, from the studies performed, offers as its main particular feature what seems to be a relatively high specificity for the natural transport system of the yeast cell for the monovalent cations. The uptake of ethidium by this system in what seems to be also a specific way, may be at the same time an indication of some unspecificity of this system. In previous work on this line, it has been shown that alkyl guanidines are also capable of inhibiting K^+ transport in yeast without affecting the H^+ extrusion, and the inhibition, on kinetic grounds, is of the competitive type (Peña, 1973).

It would not be difficult to imagine that an organic cation like ethidium could inhibit the uptake of monovalent cations in yeast. Some studies (Papa *et al.*, 1972; Fortes & Hoffman, 1974) demonstrate that amphiphilic molecules can inhibit the transport of ions of the same sign, and vice versa, ions seem to be able to block the interaction of amphiphilic molecules with membranes (Armstrong, 1961); this seems to be due to changes in the charge of the membrane surface (Rothstein, 1968). What is new and difficult to explain about the data presented here is the apparent selectivity of ethidium for the monovalent cation transport system, especially if one compares the characteristics of ethidium with those of K^+. At the moment it is difficult to think of any kind of explanation related

to the possible interaction of the dye with the transport system. However, the data of Hille (1971) and of Peña (1973) also point out to the possible interaction of other molecules with what has been thought of as a very specific system in biology.

It is important to point out that ethidium apparently inhibits K^+ transport by substitution; the dye is transported into the cell instead of K^+, and by this mechanism is capable of reproducing many of the effects observed with the transport of K^+, as already mentioned in the first part of this Discussion.

Finally, in the experiment of Fig. 7 a stimulation of ethidium uptake was observed in the presence of 1 mM Cs^+. This finding could bear some relationship to the stimulation of monovalent cation transport already reported by Borst Pauwels *et al.* (1971) and Borst Pauwels, Schnetkamp and Van Well (1973).

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