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Uptake and Effects of Several Cationic Dyes on Yeast

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Summary. Several cationic dyes were found to behave as inhibitors of K^+ uptake in yeast. When added at high concentrations or in a K^+-free medium, dyes can also produce an efflux of K^+. The dyes are taken up by the cells in a process that, in different degrees, for several cations requires glucose and is inhibited to a higher degree by K^+ than by Na^+.

The inhibition of cation uptake is of the competitive type with EB and close to this type with other dyes. Ca^{2+} inhibits the uptake and effects of dyes and in some cases also seems to change the inhibition kinetics on Rb^+ uptake closer to a pure competitive type.

According to preliminary experiments, the efflux of K^+ seems to be of the electrogenic type, and not due to the disruption of the cells. The data indicate that, independently of the existence of other types of interaction (which do exist), dyes seem to interact with the system for monovalent cation uptake of yeast in different degrees of specificity and energy requirement. This interaction can be followed by fluorescence or metachromatic changes or reduction of the dyes as observed in the dual wavelength spectrophotometer and can be inhibited specifically by K^+, but not by Na^+.

Cationic dyes have shown several kinds of effects on living cells of all types. Special attention has been focussed on studies with cationic dyes (the so-called basic dyes) for which, among others, actions have been described on intracellular enzymes [8, 10], on oxidative phosphorylation mechanisms [11, 13, 18, 28], and on the genetic machinery of the cell [25–27] as a consequence of the interaction of these molecules with different molecules or structures within the cell. In yeast cells in particular, studies on the effects of dyes and other cationic molecules have revealed this same kind of interactions [2, 6, 18]. Armstrong [2] had already pointed out that dyes, in order to interact with other components of the cell, had to make contact first with the surface and possibly required to be transported into the yeast cell. With ethidium bromide, it seems to be clear that a mechanism exists for its translocation into

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the cell [20], apparently through the natural transport system for monovalent cations. Chaix and Roncoli [4] reported that K^+, but not Na^+, could decrease the rate of methylene blue reduction by yeast; this could be due to a lower rate of uptake of the dye by the cell in the presence of K^+, but not of Na^+. These antecedents generated the hypothesis that many cationic dyes or molecules might show an interaction with the system for monovalent cation transport in yeast, in spite of the fact that recent data by Elferink and Booij indicated that some triphenylmethane dyes seem to act by inducing the destruction of the cell membrane in yeast [7]. The work presented here consisted in studying the relationship of several cationic dyes to K^+ transport, as well as the effects that several factors could have on the uptake of the dyes themselves, including those of K^+, as compared with Na^+.

Materials and Methods

Yeast cells obtained commercially (La Azteca, S.A.) were prepared as described elsewhere [19] and used within the same day of preparation by keeping them in ice under continuous bubbling of air.

Potassium and H^+ movements were followed with a cationic or a combination electrode and the uptake of the dyes by centrifugation, according to techniques described before [20]. After incubation, the concentrations of the different dyes used were measured by determining the absorbance of the supernatants obtained by centrifugation of the cells in the microfuge. The absorbance was measured for each dye at its absorption maximum and the values were compared with those of calibration curves prepared with known concentrations of the respective dye. Measurement of the uptake of $^{86}Rb^+$ was also described previously [20]. In experiments with dyes, these were added simultaneously to $^{86}Rb^+$, to avoid their effects previous to the addition of the monovalent cation.

To measure the displacement of $^{45}Ca^{2+}$ by dyes, the cells were first incubated for 3 min with 10 μM $^{45}CaCl_2$ in the presence of a buffer and without a substrate. After this incubation the cells were filtered through a Millipore filter (0.45 μm) and washed once with water. The cells were then suspended in water and kept in ice. The preparation obtained had a considerable amount of radioactivity bound, of which at most 10% could be removed by 6 washings with water, but more than 95% could be eliminated by washing with cold $CaCl_2$. These cells were incubated in the presence of the dyes and then separated by filtration and washed with water. After resuspending and plating the cells, the remaining radioactivity was measured in a gas flow counter.

Absorbance changes of the dyes at two wavelengths were followed in an Aminco DW2a spectrophotometer.

The material absorbing at 260 nm was measured essentially as described by Elferink and Booij [7], except that the supernatant obtained after centrifuging the cells was washed twice with approximately 3 vol dichloromethane and once with the same amount of petroleum ether, with the addition of 0.1 vol 20% NaOH.

Respiration was measured with a Clark electrode and a suitable recording system.

Results

Figure 1 shows the effects of the cationic dyes studied on both K^+ uptake and H^+ production by yeast. It is shown that, with all of them, marked effects can be produced on K^+ uptake without modifying the proton production, except for the case of nile blue at 40 μM, which produces not only an inhibition of K^+ uptake, but also an actual efflux of the monovalent cation of the cells. The results obtained with brilliant green and with methyl green are not presented, but none of them altered either K^+ or H^+ movements if compared with the controls.

The experiments presented in Fig. 1 were carried out by measuring the movements of K^+ when the cation was added to the incubation mixture at a concentration of 2.0 mM. When the experiments were carried out in the absence of K^+ added to the medium, i.e., by following the movements of the K^+ of the yeast cells, the results shown in Figs. 2

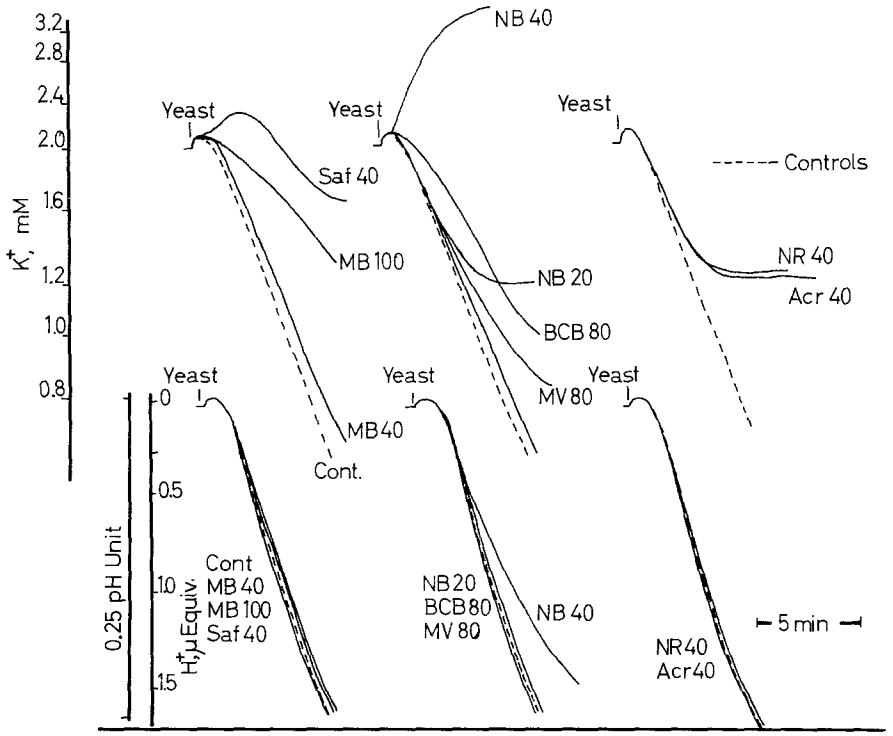


Fig. 1. Effects of several cationic dyes on the uptake of K^+ and the extrusion of H^+ by yeast. The cells (250 mg, wet wt), were added to a medium containing the indicated concentrations of the dyes and the following composition: 20 mM maleate-TEA buffer, pH 6.0; 50 mM glucose, and 2.0 mM KCl, in a final volume of 5.0 ml. Temperature was 30 °C. Figures beside the tracings indicate the concentrations of the dyes used

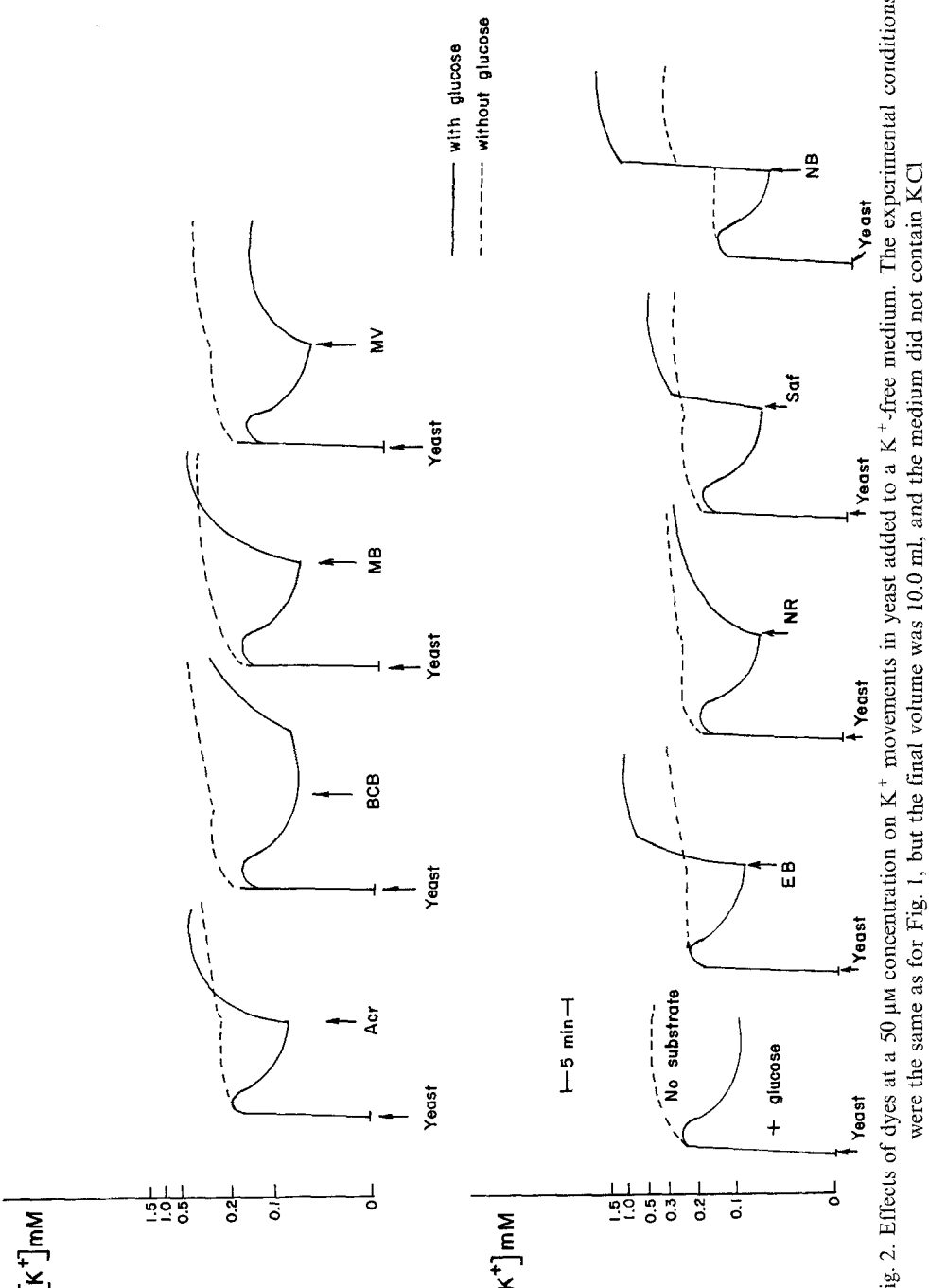


Fig. 2. Effects of dyes at a 50 μM concentration on K^+ movements in yeast added to a K^+-free medium. The experimental conditions were the same as for Fig. 1, but the final volume was 10.0 ml, and the medium did not contain KCl

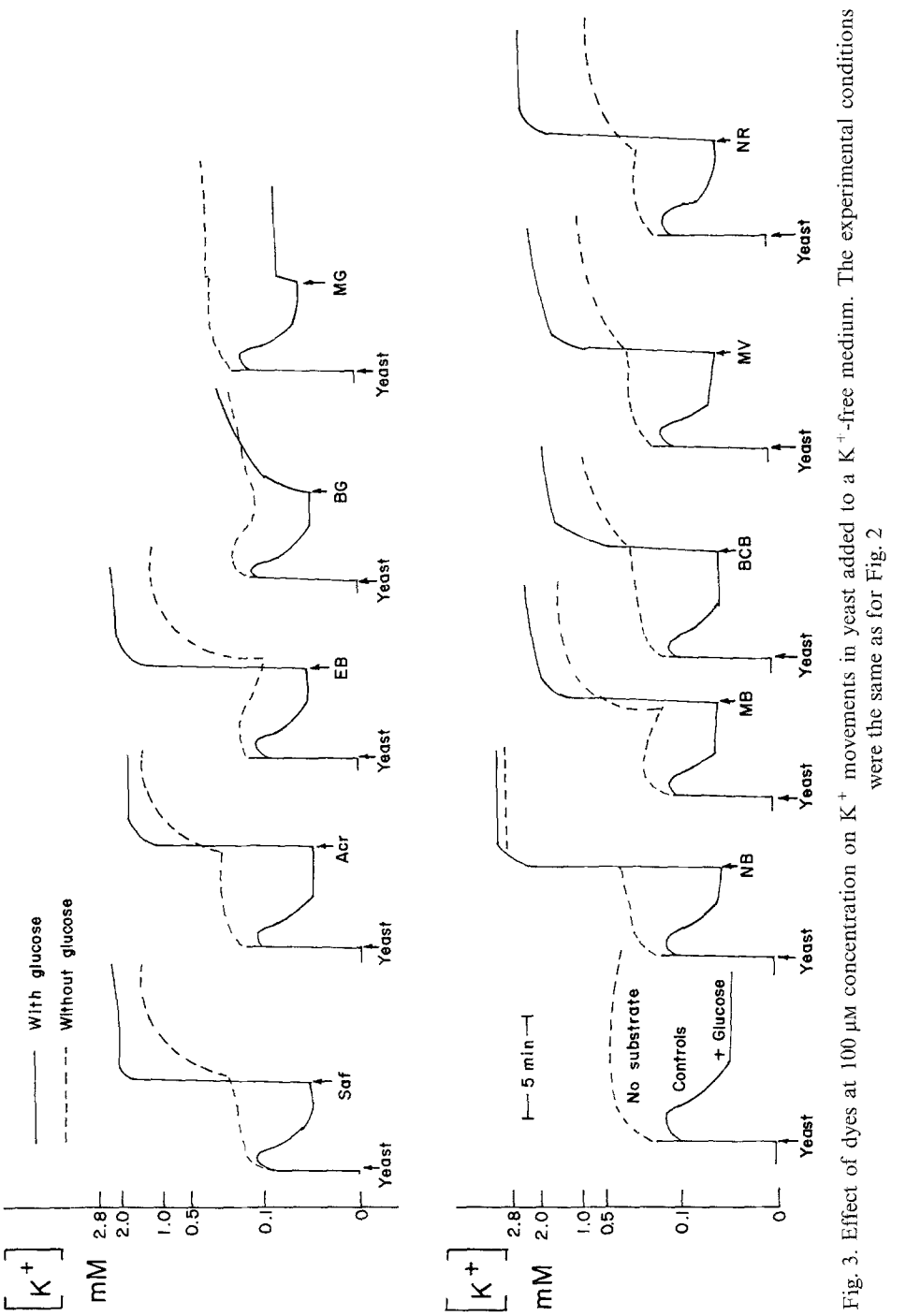


Fig. 3. Effect of dyes at 100 μM concentration on K^+ movements in yeast added to a K^+-free medium. The experimental conditions were the same as for Fig. 2

Table 1. Effect of cationic dyes (80 μM) on the release of material absorbing light at 260 nm, by yeast

	Absorbance of washed supernatant	% of value for the PCA extract
Control	0.237 ± 0.030	2.05
EB	0.754 ± 0.24	6.50
Saf	1.330 ± 0.15	11.53
NB	1.45 ± 0.25	12.5
MB	0.68 ± 0.06	5.8
MV	0.54 ± 0.06	4.6
BCB	0.85 ± 0.05	7.4
NR	1.00 ± 0.37	8.7
Acr	1.27 ± 0.21	11.03
BG	0.89 ± 0.89	7.7
MG	0.65 ± 0.38	5.6
Perchloric acid extract	11.53 ± 1.60	100.0

Cells were incubated for 8 min in the following medium: 10 mM succinate-TEA buffer, pH 6.0; 100 mM glucose; yeast cells, 125 mg, wet wt; final volume, 5.0 ml. After incubation the cells were centrifuged for 5 min in a clinical centrifuge, and the supernatant was decanted. The measurement of absorbance was performed as described under *Methods*.

and 3 were obtained. For these experiments, two concentrations of the dyes were employed, 50 and 100 μM. At the low concentration (Fig. 2), in the absence of substrate, except for nile blue which produced an efflux of K^+, none of the dyes produced a significant effect on the K^+ movements. When added in the presence of glucose, however, all the dyes, except for brilliant green and methyl green (not shown) produced a rapid efflux of K^+ from the cells. When the dyes were added at a higher concentration (100 μM) (Fig. 3), the phenomenon was exaggerated, and an efflux of K^+ was produced even in the absence of glucose, but, nonetheless, in all cases the efflux was more pronounced and rapid in the presence of glucose than when no substrate was added. The weakest action was shown again by methyl green and brilliant green, which produced a slight K^+ efflux, but only when glucose was present.

In view of the report of Elferink and Booij [7], who found that triphenylmethane dyes, among them brilliant green and methyl green, can produce the disruption of yeast cells, the cells were incubated with the dyes and then were centrifuged. The dye was washed from the supernatant and the material remaining that could absorb light at 260 nm measured, as a test of the disruption of the cells. As shown in Table 1, some of the dyes produce an increased appearance of absorbance at

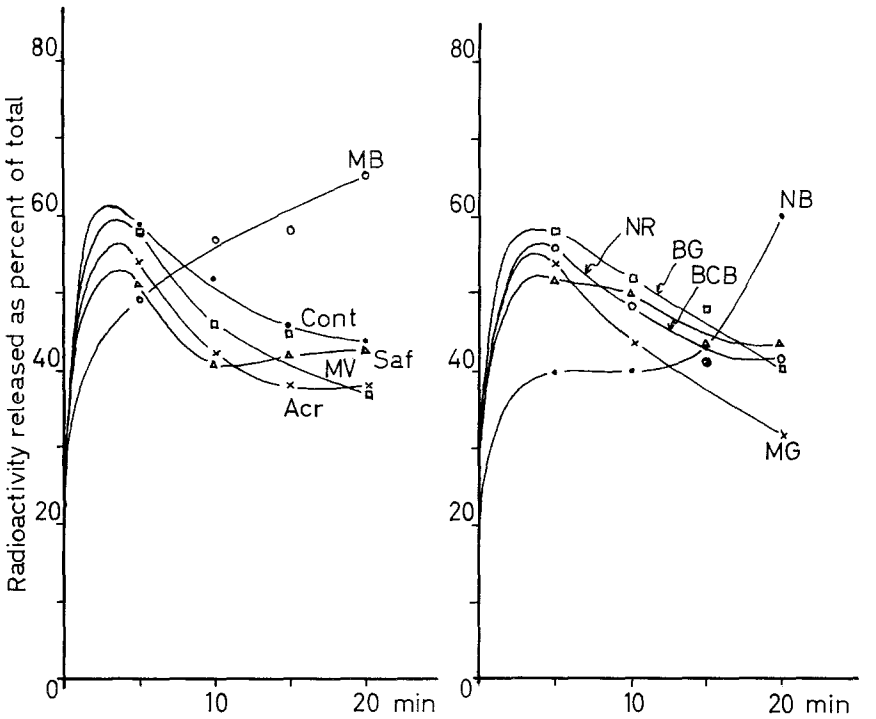


Fig. 4. Effect of cationic dyes on the efflux of $^{45}Ca^{2+}$ in yeast cells previously loaded with the isotope. Cells were loaded previously by incubating them for 30 min with 100 μM $^{45}CaCl_2$, 20 mM MES-TEA buffer, pH 6.0; 400 mM glucose, and 1.5 g yeast, in a final volume of 25 ml, and filtering and washing with 100 μM cold $CaCl_2$. After resuspending in water, the cells were incubated in the following mixture: 20 mM maleate-triethanolamine, pH 6.0; 50 mM glucose; 100 mg yeast cells; final volume, 2.0 ml; temperature 25°. At the indicated times, aliquots were removed and centrifuged for 10 sec in the microfuge. An aliquot of the supernatant was plated and counted. The data are the means of four experiments carried out on different days

260 nm in the medium of incubation. However, the highest values obtained were of only about 12% of that obtained by the complete disruption of the cells with perchloric acid. Further test of the cell integrity was provided by the experiment presented in Fig. 4; yeast cells were loaded with $^{45}Ca^{2+}$ by incubation with the isotopes in the presence of glucose for 30 min. The cells were filtered and washed with 100 μM $CaCl_2$ and resuspended in water. Afterwards, the cells were incubated in the absence or in the presence of the dyes, and the efflux of radioactivity was measured by counting the supernatant obtained after centrifuging the cells in the microfuge at different times. After an important initial efflux of the divalent cation, the cells again took up the radioactivity, essentially in a similar way, except for those incubated in the presence

Table 2. Effects of several cationic dyes on the $^{86}Rb^+$ uptake by yeast

	No. of experiments	% inhibition of uptake	
		0.8 mM Rb^+	4.0 mM Rb^+
Control	22	–	–
Safranine	3	76±6	43 ±12
Acr	4	73±5	49 ±3
MB	3	33±15	4.6±20
NR	3	65±13	40 ±5
BCB	3	64±4	16 ±9
MV	3	48±11	11 ±12
NB	3	86±2	63 ±10

Incubation medium: 20 mM maleate-TEA buffer, pH 6.0; 100 mM glucose. After equilibrating the temperature in a water bath at 30 °C, 100 mg of yeast cells, wet wt, were added. After exactly 2 min, $^{86}Rb^+$ was added at a concentration of 4.0, 2.0, 1.33 or 0.8 mM, followed immediately by the dye at a final concentration of 100 μM. The final volume was 2.0 ml. After 2 min an aliquot was taken and filtered through a Millipore type filter, 0.45 μm pore size, washing once with water, twice with 5 mM RbCl, and twice more with water. The cells were then resuspended in water, and an aliquot was plated and counted. One control was always run with each dye. The values for the Lineweaver-Burk equation were obtained by the least squares method.

of MB[1] or NB, in which the pattern of movement of Ca^{2+} was different from the rest of the cases.

Given the fact that dyes can produce an inhibition of the uptake of K^+, and also an efflux of the monovalent cation present within the cells, it was important to determine the effects of the dyes on the initial rates of uptake of $^{86}Rb^+$, to ascertain their action on the *influx* of monovalent cations. To avoid the effect of K^+ extruded from the cells by the addition of the dye in the absence of external cations, both dyes and $^{86}Rb^+$ were added simultaneously in each case; besides, different concentrations of Rb^+ were employed, in order to get more information about the inhibition produced by each dye.

The results obtained are presented in Table 2; there is an actual inhibition of the uptake of the cation that can be measured on a percentual basis. By measuring this inhibition at the highest and the lowest Rb^+ concentrations employed, it can be seen that at high concentrations of the alkali cation, the inhibition decreases. Besides, there are changes

[1] *Abbreviations:* CTAB, cetyltrimethylammonium bromide; TEA, triethanolamine; FCCP, *p*-trifluoromethoxycarbonylcyanide-phenylhydrazone; EB, ethidium bromide; Saf, safranine; NB, nile blue; MB, methylene blue; MV, methyl violet; BCB, brilliant cresyl blue; NR, neutral red; BG, brilliant green; MG, Methyl green; Acr, acriflavin.

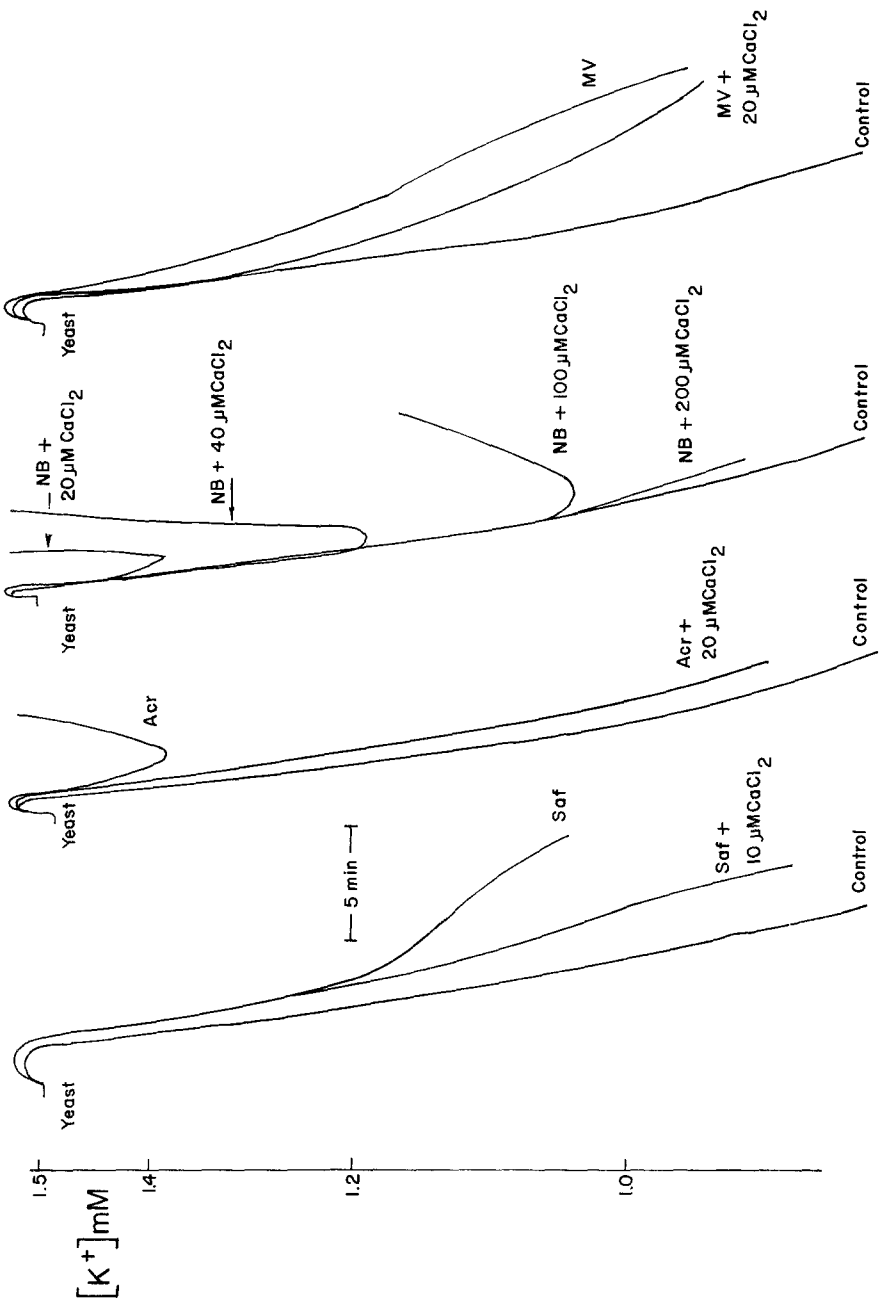


Fig. 5. Reversion by Ca^{2+} of the effects of some dyes on the uptake of K^+ by yeast. The experimental conditions were the same as for Fig. 1, but the indicated concentrations of $CaCl_2$ were included

in the kinetic parameters that in general are more significant for the K_m than for the V_{max} (*see also* Fig. 5); in other words, by these criteria, the inhibition resembles the competitive type, without being purely so, which is somewhat different than what was reported before for ethidium bro-

Table 3. Uptake of different dyes by yeast. Substrate requirement and effects of K^+ and Na^+

	Control no substrate	nmol dye taken up in 3 min by 125 mg yeast				
		Glucose	No substrate +KCl	Glucose +KCl	No substrate +NaCl	Glucose +NaCl
EB	19±14	69±27[a]	27±4	25±9[e]	32±16	51±21
Saf	108±15	144±23[b]	80±10	90±18[d]	87±17	108±23
Acr	147±11	175±12[a]	112±7	130±12[d]	122±10	143±26
NB	187±8	184±23	193±3	188±4	193±2	190±2
MV	190±3	190±7	180±11	186±5	191±2	191±3
MB	130±27	164±12[b]	100±13	91±32[d]	103±29	133±5[e]
BCB	149±11	148±16	141±6	125±31	140±10	142±11
NR	186±5	183±7	174±21	168±7	180±	177±7

Incubation conditions: 10 mM maleate TEA buffer, pH 6.0, 50 mM glucose (where indicated); 5 mM KCl or NaCl (where indicated); dyes 40 μM; yeast, 125 mg wet wt; final volume, 5.0 ml; time; 3 min. Dye uptake was measured as described under *Methods*. Data are given as the mean values of 5 experiments with the SD of each value.

[a] P 0.01 and [b] P 0.05 with respect to controls without substrate.

[c] P 0.01 with respect to controls with glucose.

[d] P 0.01, and [e] P 0.02 with respect to controls with glucose.

Statistic significance was calculated by means of the student t test.

mide [20]. Data with methyl green and brilliant green, which do not appreciably inhibit the uptake of K^+, are not presented.

If dyes produce an effect on K^+ uptake by yeast, it is important to define if they are taken up by the cells and, if so, to investigate some characteristics of their uptake. The parameters studied were the requirement for glucose and the effects of the monovalent cations K^+ and Na^+ on the uptake of the dyes; Table 3 shows the results of experiments in which this exploration was made. From the point of view of the substrate requirement, it can be seen that, unlike ethidium bromide which shows a significant requirement for glucose, other dyes are taken up in more than 90% of the amount present without any differences in the presence or absence of glucose. There is, on the other hand, an intermediate group, represented essentially by safranine, acriflavine, and methylene blue, for which the uptake is increased by 12 to 33% in the presence of glucose.

As to the effects of K^+ or Na^+ on the uptake of the dyes, there are also three groups of dyes; the first is again represented by ethidium bromide; its uptake is inhibited 64% by K^+ and only 26% by Na^+ at a 5 mM concentration. The second group shows a smaller inhibition

of uptake by monovalent cations, with a less selective inhibition if K^+ and Na^+ are compared; it includes safranine, acriflavin, and methylene blue. To only a minor extent, brilliant cresyl blue also shows this selective inhibition by K^+. With the rest of the dyes, the inhibition by Na^+ or K^+ was either very low or also nonselective between cations.

Up to this point, there seemed to be some correlation between the effects of some of the dyes and their penetration. The effect of ethidium has been dealt with somewhere else [20], but some of the other inhibited Rb^+ uptake with kinetic characteristics close to the competitive type showed a partial requirement of glucose to be taken up by the cells, and K^+, to a greater extent than Na^+, inhibited its uptake.

It is possible that, as postulated for ethidium bromide [20], these dyes might interact with the K^+ carrier, but presented some other kind of interaction and/or penetration besides. They could interact with the membrane of the cell in nonspecific sites, different from those of K^+ transport, by means of the negative charge that the cell surface has been shown to have [9]. Divalent cations, which can bind to this surface [24] and block the effects of some cationic detergents on yeast [3], might also block the action of dyes by forming a positive layer on the surface of the cell. The possibility was tested with only four of the dyes: safranine, acriflavine, nile blue, and methyl violet. As shown in Fig. 5, Ca^{2+} is capable of reverting the inhibition of K^+ uptake produced by the dyes. With nile blue, perhaps the most active of the dyes tested, higher concentrations of Ca^{2+} were required to produce full reversion of the inhibition produced on K^+ uptake.

Another way of testing the characteristics of the interaction of the dyes with the cells was by determining the inhibition kinetics produced by dyes in the presence and absence of low concentrations of Ca^{2+} that do not produce any effect on the uptake of Rb^+ *per se*. As shown in Fig. 6 for acriflavin, safranine, and methylene blue, the inhibition of Rb^+ uptake produced by the dyes, that in the absence of Ca^{2+} is of a noncompetitive type, diminishes and tends to acquire this form when the divalent ion is present in the incubation mixture. Although results are not shown, at the low concentrations tested for Ca^{2+} (60 μM) and in agreement with the data of Theuvenet and Borst-Pauwels [24], no effect was found for this cation on the uptake of Rb^+ in the control experiments.

By incubating the cells with $^{45}Ca^{2+}$ it should be possible to "label" the negative sites of the surface and afterwards to test the ability of the dyes to displace $^{45}Ca^{2+}$, as described in Table 4. The addition of

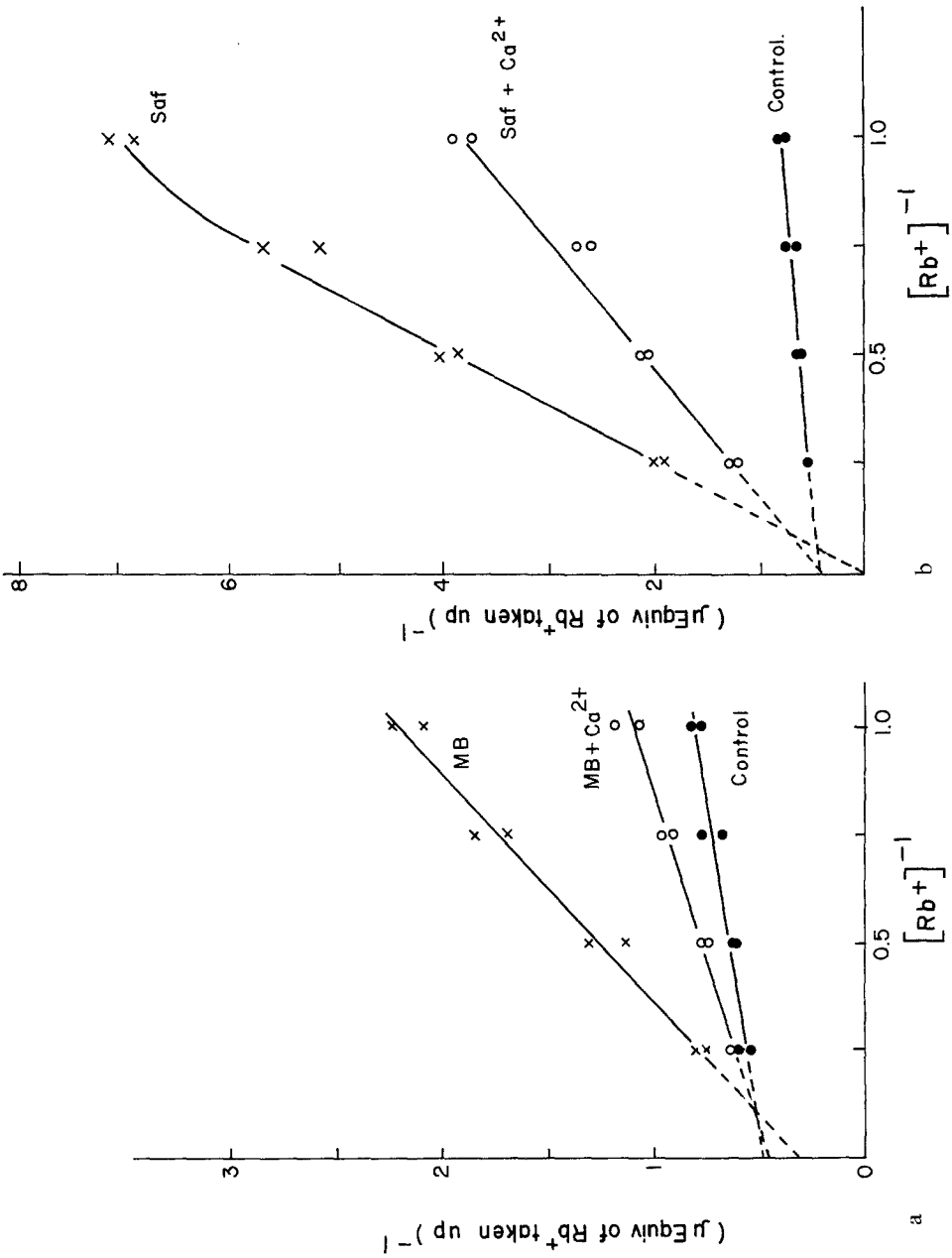
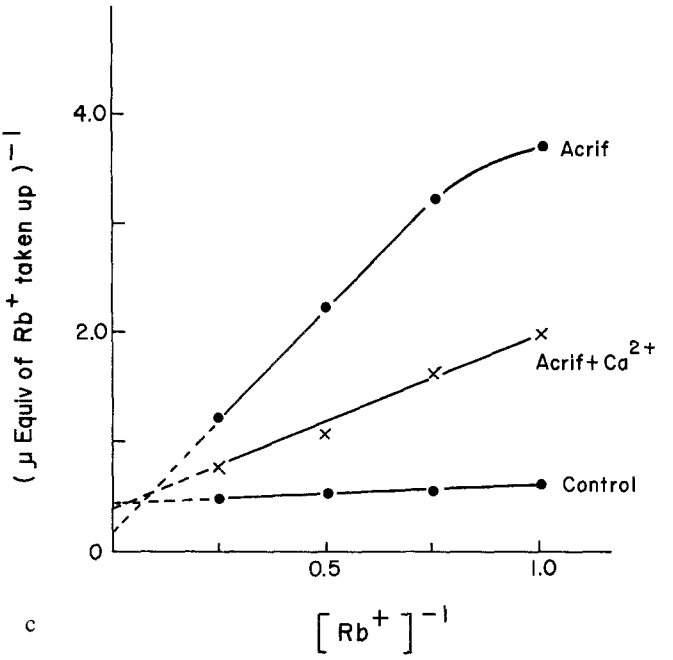


Fig. 6. Changes produced by Ca^{2+} on the inhibition kinetics of $^{86}Rb^{+}$ uptake. Experimental conditions were the same as for Table 2, but where indicated, 60 µM $CaCl_2$ was included in the incubation mixture. The dye concentration was 150 µM

the dyes, if they interact with the negative surface of the cells, should displace at least part of the radioactivity bound. Since the uptake of the dyes, their effect on K^+ movements and possibly their interaction with the membrane are favored by glucose; the experiment was performed both in the presence and in the absence of glucose. In the absence



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of glucose most of the dyes showed an almost negligible effect of displacement of $^{45}Ca^{2+}$; only nile blue and methyl green produced a noticeable lowering of the radioactivity upon incubation. In the presence of glucose, surprisingly, some of the dyes not only do not decrease the radioactivity present in the cells, but actually increase it. This is the case of MB, MV, Acr, EB and Saf. With some of the dyes, the effect has been further studied and it seems to involve the actual penetration of the divalent cation into the cell, since a significant part of the radioactivity is no longer exchangeable by cold $CaCl_2$ [17].

Under the assumption that Ca^{2+} might decrease the interaction of the dyes with nonspecific sites of the membrane, it could be expected that the uptake of dyes occurring in the presence of the divalent cation took place in a larger proportion through more specific sites and, therefore, were more sensitive to the competitive presence of monovalent cations, particularly K^+. The data of Table 5 show the results of the experiment that was carried out with four dyes. Although 40 μM $CaCl_2$ produced a decrease of the dye uptake, the addition of K^+ under these conditions did not produce a larger inhibition of the uptake than when added in the absence of Ca^{2+}.

Safranine has been studied from the point of view of its interaction with membrane systems of mitochondria [5] or liposomes [1]. When

Table 4. Effect of cationic dyes on $^{45}Ca^{2+}$ binding to yeast cells in the presence or absence of glucose

	Cpm remaining in yeast (100 mg) after incubation with or without the dyes, filtering, and washing	
	No substrate	Plus glucose
Controls	3018	3697
	3018	3713
MB	2971	3961
NB	2798	3594
BCB	2915	3512
MV	3360	3941
Acr	2850	4151
EB	3066	4410
Saf	3103	4197
NR	3078	3707
BG	2972	3450
MG	2708	2994

3 g yeast were incubated for 3 min at 30° with 10 μM $^{45}Ca^{2+}$ (293,000 cpm). After filtering and washing twice with water; the cells (100 mg) were resuspended in water and incubated for 2 min in the following mixture: 20 mM maleate-TEA buffer, pH 6.0; 100 mM glucose where indicated, and 50 μM of the respective dye in a final volume of 2.0 ml. After 2 min at 30°, an aliquot of the mixture was filtered and washed 3 times with water. The cells were then resuspended in water, plated, and counted.

Table 5. Effect of K^+ on dye uptake by yeast in the presence or absence of 40 μM $CaCl_2$

	nmol dye taken up in 3 min by 125 mg yeast			
	Without $CaCl_2$		Plus 40 μM $CaCl_2$	
	Control	5 mM KCl	Control	5 mM KCl
Acr	144±7	102±4(30)	116±2	88±13(25)
Saf	113±4	62±5(46)	79±8	61±17(73)
BCB	131±17	123±9(7)	135±2	116±7(6)
MB	122±14	69±11(44)	88±12	59±20(33)

Experimental conditions: 20 mM maleate-TEA buffer, pH 6.0; 50 mM glucose, plus the indicated concentrations of KCl or $CaCl_2$. After equilibrating the temperature in a water bath at 30°, 125 mg yeast were added. Two min later, the dyes at a final concentration of 50 μM were added; after 3 min an aliquot was taken and centrifuged in a microfuge for 10 sec. The dye remaining in the supernatant was measured by the absorbance at the appropriate wavelength. Final volume was 5.0 ml. Data are the means of 4 experiments, ±SD. Figures in parentheses are the percent inhibition produced by K^+ in relation to the respective control.

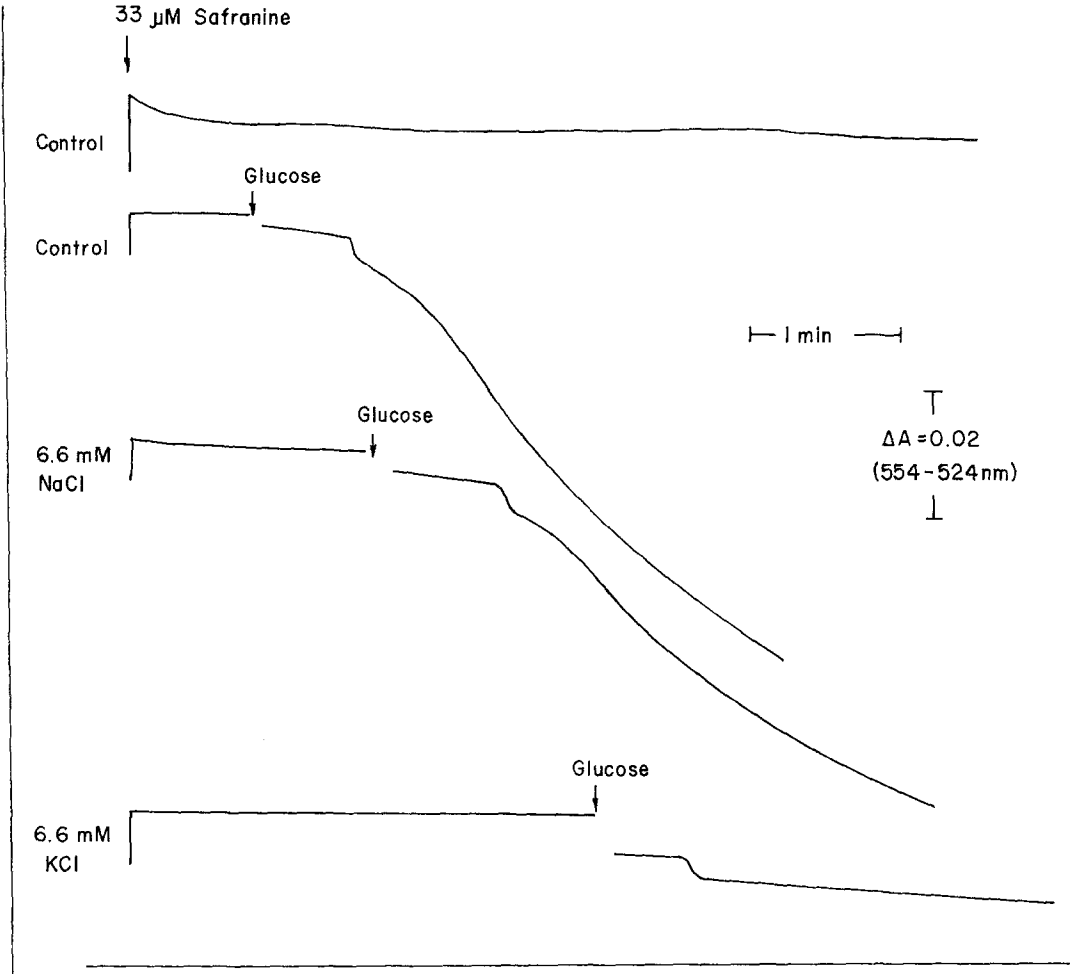

Fig. 7. Effects of substrate, K^+ and Na^+ on the absorbance changes (554–524 nm) of safranine produced by yeast. Incubation mixture: 20 mM maleate TEA buffer, pH 6.0; yeast, 150 mg, wet wt; final volume, 3.0 ml. The experiment was carried out at room temperature. Safranine was added at a 33 μM concentration and glucose at 16.6 mM

it interacts, apparently by being transported into the matrix space, by virtue of the stacking of its molecules, its absorbance spectrum changes, and this can be followed in a dual wavelength spectrophotometer. With the same instrument, the reduction of methylene blue can be followed. In this latter case, it can be assumed that its penetration into the cell is a prerequisite to its reduction, so that the change can be taken as an index of the uptake of the dye. Acriflavine, on the other hand, shows a change in its spectrum when placed in an organic solvent of low dielectric constant, like dioxane or butanol, and this change can also

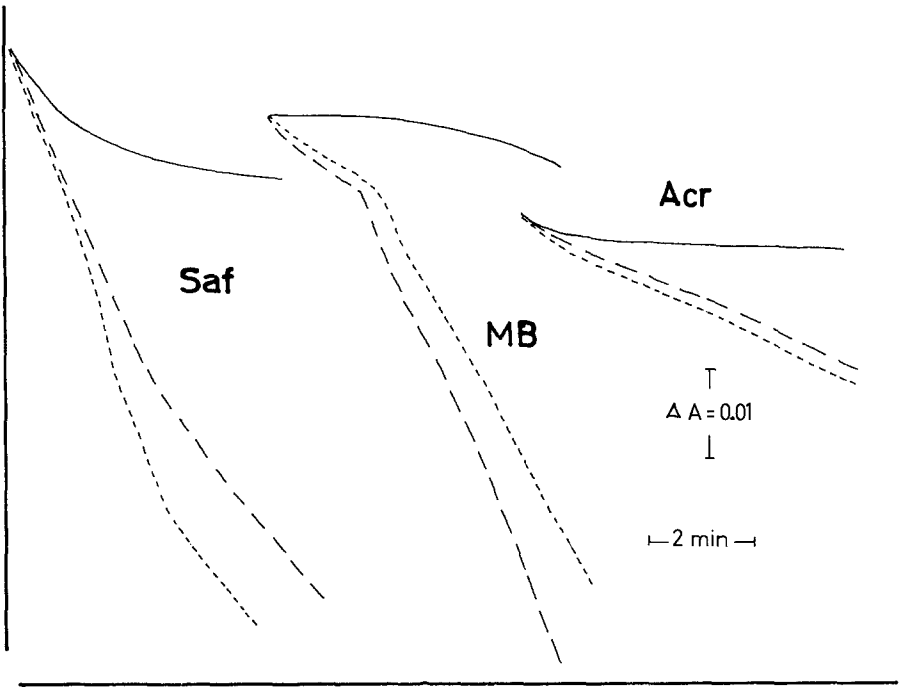

Fig. 8. Effects of Na^+ and K^+ on the absorbance changes of safranine (554–524 nm), acriflavine (475–465 nm), and methylene blue (640–650 nm) during their interaction with yeast cells. The incubation medium was the same as for Fig. 6, but the buffer concentration was 13.3 mM and the yeast amount 100 mg, wet wt. The concentrations of the dyes were 33.3 μM. Key: ——, control; -----, 6.6 mM NaCl; —, 6.6 mM KCl

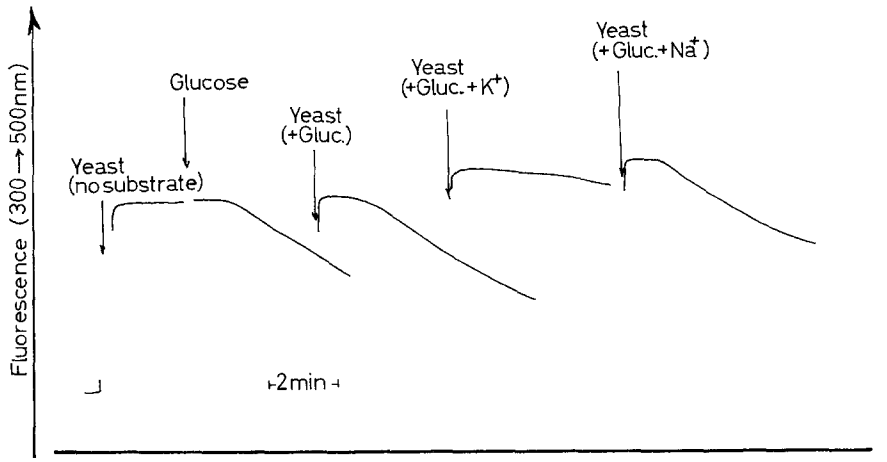

Fig. 9. Effects of substrate, Na^+ and K^+ on the fluorescence of acriflavine upon its interaction with yeast. The experimental conditions were the same as in Fig. 7, but the yeast concentration was 50 mg, wet wt, and fluorescence at 300 → 500 nm was registered

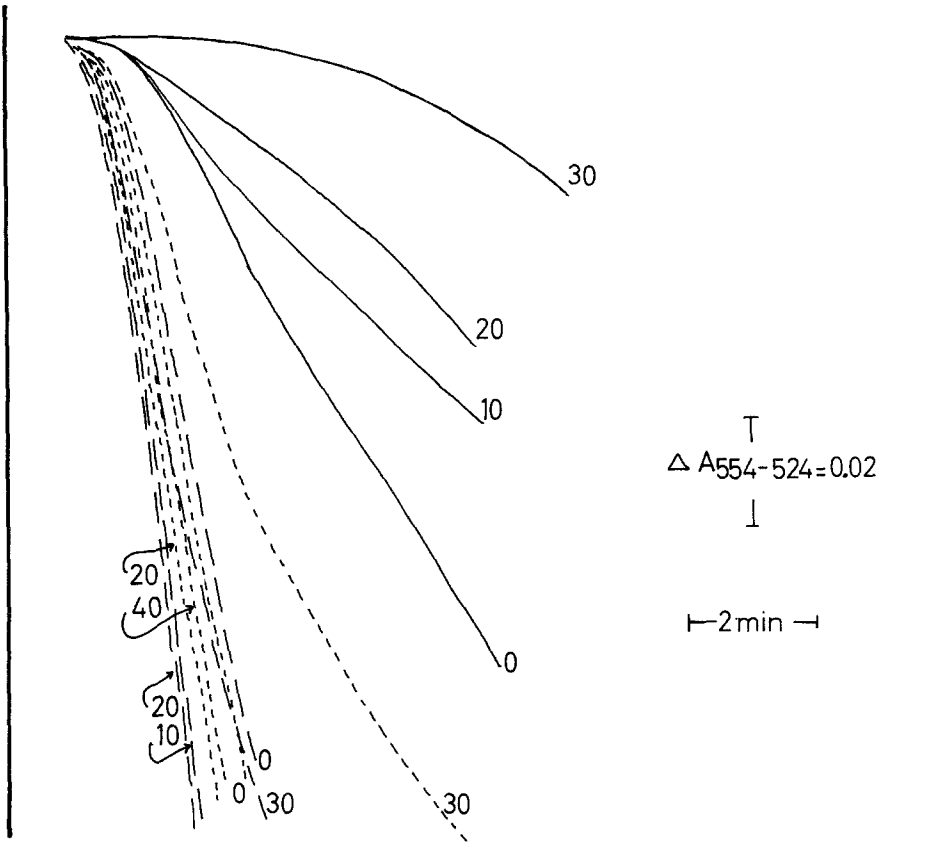

Fig. 10. Effect of different concentrations of Ca^{2+} and Na^+ and K^+ on the metachromatic changes of safranine produced by its interaction with yeast. The experimental conditions were the same as for Fig. 7, except that the indicated concentrations (μM) of $CaCl_2$ were included. The key to the figure is the same as for Fig. 7

be followed as an indication of its interaction with the membrane(s) of the cell. With this in mind, the color changes of the three dyes were followed upon their interactions with the yeast cells. The idea was to investigate the energy (substrate) requirements of the interactions, as well as the effects that both K^+ or Na^+ could have, using the color changes as an index.

The experiment of Fig. 7 shows the detected changes with safranine; the color change requires a substrate, and, besides, it can be inhibited by K^+ but not by Na^+ at 6.6 mM concentration. The complete results are not presented, but similar tracings were obtained by using methylene blue or acriflavine at the adequate wavelengths, under similar conditions. In Fig. 8 it can be seen that with the three dyes the changes observed

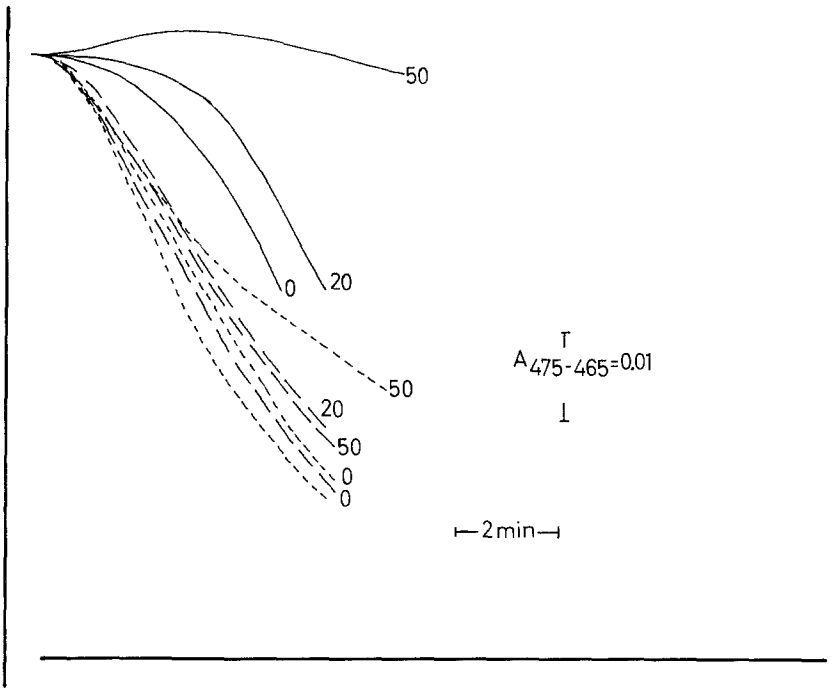

Fig. 11. Effect of different concentrations of Ca^{2+} on the inhibition of the metachromatic changes of acriflavine produced by either Na^+ or K^+ upon its interaction with yeast. The experimental conditions were as those of Fig. 9

in the presence of a substrate can be prevented by the addition of K^+, but not by the addition of an equal concentration of Na^+.

In the case of acriflavin, in Fig. 9, the results of one experiment are presented, in which the interaction of the dye was monitored by recording the fluorescence changes of the dye at $300 \rightarrow 500$ nm. Also in this case, the changes observed require a substrate, and are reverted by K^+, but not by Na^+.

The metachromatic changes observed with the aforementioned dyes can be prevented specifically by K^+ and not by Na^+, depending on the dye concentration used; as the concentration is increased, both the energy requirement and the specificity of the reversion by K^+ tend to disappear. Besides, the phenomenon is not absolutely constant, and with some yeast preparations there is not a clear reversion of the color changes by K^+. In these cases, however, it was found that, at least with safranine and acriflavine, the addition of Ca^{2+} can produce the recovery of the cells to the state in which the metachromatic changes can be inhibited by K^+ and not by Na^+ (Figs. 10 and 11).

Discussion

The K^+ transport system of yeast seems to be sensitive to an already large variety of cationic molecules. Guanidine, and particularly alkylguanidines, can inhibit the uptake of monovalent cations by yeast [15] and the same seems to be true for ethidium bromide [20]. The data presented in this paper show the ability of most of the molecules tested to interact with this same transport system, though in different degrees of effectiveness and specificity. As tools in the study of transport mechanisms, dyes offer the advantage of being easy to detect and to measure their concentration changes and uptake by the cells. A good example of this is given by the experiments of Figs. 7 to 10.

The present studies allowed us to find out that dyes, besides inhibiting K^+ transport and promoting its efflux under certain conditions, can be taken up by the cells in a process that, in different degrees for each one, requires the presence of a substrate, and can be inhibited by monovalent cations, more so by K^+ than by Na^+. These results pose an interesting possibility, i.e., that cationic organic molecules in general can be translocated by the natural carriers of the cells and also interact with sites that "normally" should probably be occupied by divalent cations. In much the same way that there are competitive inhibitors of enzymatic reactions, more or less selective inhibitors can be found for the transport mechanisms of cations, as postulated by Hille [12].

Although the data of Figs. 2 and 3 could indicate that the inhibition of K^+ uptake could be due to the production of an efflux of the cation, the data obtained on the effects on $^{86}Rb^+$ uptake at short incubation periods show clearly that there is an actual inhibition of the influx of cations. It has to be pointed out that the dyes and $^{86}Rb^+$ were added simultaneously to avoid the extrusion of K^+ that is produced if the dye is added in the absence of cations in the medium. The percent inhibition of Rb^+ uptake by the dyes decreases upon increasing the concentration of the monovalent cation, and this is what one would expect if some kind of competition existed between Rb^+ and the dyes.

Elferink and Booij [7] have suggested that the effect of some triphenylmethane dyes may affect yeast by disruption of the cell structure. Although this may be so at high concentrations of the dyes, at the levels used throughout the work, this action is minimal. The measurement of the material absorbing at 260 nm present in the incubation medium showed that this effect is low. The experiments represented in Fig. 1, besides, show that the cells maintain their ability to pump out protons,

a property that would be difficult to carry out by broken cells. Another proof of the integrity of the cells is the increase that most of the dyes produce in the uptake of Ca^{2+} (see Table 4, and Reference 17); it would be impossible for disrupted cells to take up Ca^{2+}. The experiments represented in Fig. 4 also indicate that yeast cells incubated in the presence of dyes maintain their ability to keep and recover Ca^{2+} also when loaded previously with this ion. Even if methylene blue and nile blue produce some efflux of Ca^{2+}, the general behavior of cells treated with dyes is similar to that of the control cells.

Passow *et al.* [14] showed that methylene blue can produce the complete destruction of some cells, while leaving part of them intact. This possibility can not be definitely ruled out; however, our data can be explained in a more simple way than by assuming the existence of two kinds of cells. If this were the case, it would be necessary to postulate that the dyes selectively break some kind of cells, but also in some way produce the stimulation of Ca^{2+} uptake or binding by the remaining cells, and that the breakage of part of them does not show any changes in the proton pumping activity.

If it is accepted that K^+ and dye uptake are related, according to the data obtained, three kinds of dyes could be considered; the first is represented by EB only and is characterized by an important requirement of glucose for the uptake and a substantial inhibition of the uptake by K^+, which is not equaled by Na^+. In previous work [20] it was found that although the dye can bind to the cells in the absence of glucose, its fluorescence at $530 \rightarrow 600$ nm is strictly dependent on the presence of glucose at low concentrations. Perhaps the most important characteristic of EB is that it produces an inhibition of $^{86}Rb^+$ transport which is of a pure competitive type [20] within low concentration ranges of the dye.

The second group is represented by those dyes that fulfill the three aforementioned criteria, but only partially, i.e., they show a relative substrate requirement for uptake; their uptake is also inhibited partially by K^+, but to a smaller extent by Na^+, and finally, the inhibition kinetics of $^{86}Rb^+$ uptake is close to but not clearly of the competitive type. To this group belong acriflavin, safranine, and methylene blue. An interesting proof of the interaction with a K^+ specific site was provided by the blocking of absorbance changes of Saf, MB, and Acr by K^+ but not by Na^+, and the strict requirement for glucose of these changes. It seems that the spectrophotometric measurement of metachromatic changes can be used to monitor more specific interactions. The

measurement of the absolute uptake of the dye by centrifugation, on the other hand, gives the sum of both specific and nonspecific interactions of the dye with the cell.

The third group is represented by those dyes that do not fulfill either one or more of the mentioned criteria. A clear limitation between this and the second group does not exist, and probably very few of the dyes belong absolutely to this third group.

The data, in a very simple way, could be visualized trying to correlate the behavior of the dyes as to their uptake with their effects on K^+ or Rb^+ uptake. It seems that dyes of the first group (EB only) can be taken up by the cells only, or almost only, through the K^+ transport system [20]. Dyes of the second group would seem to be taken up by this transport system also, but not exclusively. The uptake by other route(s) could be substrate independent and not sensitive towards K^+. Dyes of this group would be only partial and noncompetitive inhibitors of monovalent ion transport, and K^+ would only to a minor degree inhibit their transport. The localization of unspecific uptake sites would be different from that of the K^+ transport system, since Ca^{2+} at low concentrations can inhibit dye uptake, but not K^+ transport [24]. The experiment of Table 5 would disagree with this model; as mentioned before, it would be expected that, if in the presence of Ca^{2+} only or mostly nonspecific sites are blocked, there should be a more pronounced inhibition by K^+ on dye uptake percentally when the divalent cation was present. The same can be said about the data on $^{45}Ca^{2+}$ displacement by dyes of the second group. These latter data, however, could be explained in terms of the relative values of the affinity constants of Ca^{2+} and dyes for the sites involved and/or by other interaction sites without the ability to bind Ca^{2+}.

As to the third group, it was already said that perhaps very few dyes fully belong to it. Some of them show some substrate requirement; for some, uptake is inhibited by K^+ and others show a slight tendency to the competitive inhibition of Rb^+ uptake. Some of them displaced $^{45}Ca^{2+}$ from the cells when incubated in the absence of glucose.

The model imagined may be an oversimplification of the facts, and it is possible that more than one extra route for the entrance of dyes is involved besides the K^+ transport system. However, several of the dyes tested seem to be taken up, at least partially, through the K^+ transport system.

Another effect of the dyes consists in the efflux of K^+ they produce from the cells. The measurement of the amount of material absorbing

light at 260 nm and other tests lead us to conclude that the integrity of the cell is maintained. Besides, as mentioned before, the cells in the presence of EB, for instance, even at 250 μM, are able to take up $^{45}Ca^{2+}$ at an increased rate [17], which would not be possible with broken cells. Perhaps the most plausible explanation is an interaction of the dyes with the K^+ carrier which now allows the efflux of K^+; this transport system normally seems to work in such a way that K^+ is kept within the cell once it is taken up. The mechanisms of K^+ efflux are subject to influences and conditions that have been studied with certain detail by Ryan and Ryan [23] and by Rothstein [22]. The efflux of potassium ions depends very much on the presence of other ions in the medium and in the cell, and particularly on the internal pH of the yeast cell. The efflux, on the other hand, is also determined largely by the relative affinities of the monovalent cations, including H^+, for the transport system. Besides K^+ that has been taken up by yeast can be made to leave the cells by the addition of uncouplers [16, 17]. At certain dye concentrations, the efflux of K^+ they produce requires the presence of a substrate. The substrate, however, does not seem to be required for the production of the effect because it causes the uptake of the dye. Many of the dyes tested can be taken up in the absence of a substrate and still do not produce the K^+ efflux unless the substrate is added. It appears that the energy source is necessary for the interaction of the dye with the K^+ carrier. In agreement with this assumption are the results of the spectral changes of absorbance of safranine, acriflavin, and methylene blue (Figs. 7–11) which, in contrast with their uptake, require a substrate and are highly sensitive to K^+, and much less to Na^+. The dyes might represent a way to study changes of the carrier state produced by an efficient energy source in the cell. Cases like this have been described in bacteria [21]; energization changes the state of the galactose carrier. The interaction of the dyes with the carrier seems to be based on their cationic nature, since the effects cannot be reproduced with anionic dyes.

Finally, although it is difficult to decide if the uptake of the dyes represents the actual penetration of the molecules into the cell or only their binding to the surface, there are indications that the substances actually penetrate. The first is the amount taken up by the cells; although it might so happen, it is difficult for such amounts of dye to remain bound exclusively to the surface. The second proof may be more conclusive; the data are not presented, but most of the dyes used produce some inhibition of respiration except for BG and MG, which do not

inhibit. At the 100 μM concentration, the lowest inhibition of respiration was produced by neutral red, and it was of 80% as the mean value of two experiments. Although some of the dye may remain bound to the surface, it is evident that at least part of it penetrates into the cell. Finally, in the case of methylene blue, the data of Fig. 8 show that it is reduced by the cells, and, most probably, this requires that the molecules are taken inside.

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