An All-or-None Response in the Release of Potassium by Yeast Cells Treated with Methylene Blue and Other Basic Redox Dyes

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ABSTRACT Basic redox dyes, such as methylene blue, induce a loss of K^+ from yeast cells. The maximal loss, rather than the rate of loss, is related to the dye concentration, the response following a normal distribution on a plot of log-dose, *versus* percentage loss of K^+. This fact taken together with the observed correlation between K^+ loss and frequency of staining (as measured by microscopic observation), indicates that the response is all-or-none for individual cells. The response is produced by all the basic redox dyes tested (9), but by none of the acidic dyes (4). However, only the oxidized form of the dye is effective. Cations protect the cells from the basic dyes in a competitive manner, the bivalent cations (especially UO_2^{++}) being more effective than monovalent cations. It is suggested that the action of the dyes involves two steps, the first a binding to ribonucleic acid in the cell membrane (with competition from cations) and the second, an oxidation of neighboring sulfhydryl groups to the disulfide form. At a threshold level, unique for each cell, a generalized membrane breakdown occurs, resulting in the release of potassium and of other cytoplasmic constituents.

Biochemical and physiological functions of cells are usually studied by measuring the combined response of a population of cells rather than by observing the behavior of individual cells. The measurement represents the average behavior of the cells within the population. In order to facilitate the interpretation of results, it is frequently assumed that the response of individual cells is graded with respect to applied conditions, with only minor deviations from

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the measured average. However, such an assumption may not always be justified. In particular, with the application of drugs or toxic agents, the response of individual cells may be of all-or-none type, so that the observed average may represent a large response in the case of some cells, and little or no response in the rest of the population.

In permeability studies, the consequences of the different behavior of individual cells within the population were analyzed mathematically some years ago (1), but no data were available at that time to test the theoretical considerations. Recently, however, a significant variation in the rate of equilibration of red blood cells with potassium ion has been observed, using K^{42} (2). After a period of equilibration the cells were centrifuged and successive layers of the packed cells were analyzed for potassium and for K^{42}. The top portion of cells showed the most rapid exchange rate, and the middle portion the slowest. Even more dramatic differences in individual response were found in experiments on the influence of certain poisons on the potassium permeability of the human red cell (3, 4). In the case of lead, for example, an all-or-none response of individual cells was observed. Different cells in the suspension were found to have different threshold values. Consequently, at a given lead concentration, the average potassium loss as measured by analyzing large numbers of cells, represented the complete loss of potassium from some cells and little or no loss from others. The leaky cells could be separated from the uninjured cells by taking advantage of differences in fragility. Recently, the action of relatively high concentrations of NaF on K^+ exchange of human red cells has been interpreted in terms of an all-or-none breakdown of the membrane in an increasing number of cells (5).

A markedly increased permeability to potassium in yeast cells can be induced by a number of agents including certain redox dyes and mercury (6). In the course of investigating the mechanism of the effect of the redox dye, methylene blue, microscopic observation revealed that numbers of the cells were stained. It seemed possible that, as in the case of lead and the red blood cells (3, 4), the effect of methylene blue on K efflux might be all-or-none with regard to individual cells, the stained cells being those in which the threshold had been exceeded. In the present study, therefore, correlations were made between the potassium loss and the frequency of staining, as a function of methylene blue concentration. Additional studies relating to the mechanism of the effect were also carried out.

Methods

Bakers' yeast (Standard Brands, Inc.) was thoroughly washed and in the washing procedure centrifugation was at low speed so that colloidal material and cellular debris were discarded. The cells were then starved for a period of 3 hours in order to reach the stable stage of endogenous metabolism (7). For experimental use the cells

were suspended in water adjusted to pH 3.0 with HCl. The dye was added and samples were collected at appropriate times, centrifuged, and the supernatant decanted for analysis. Potassium was determined with a Beckman flame photometer. The results were expressed in terms of the percentage of the total cellular potassium released, the latter parameter being determined by boiling an aliquot of the cell suspension for 3 minutes (8), followed by flame photometry. The percentage of stained cells has been estimated by microscopic observation, after resuspending in a Thoma cell, using a flat-field high dry objective (Zeiss planachromat, 40×), a Zeiss compensating eyepiece (6.3×), and a long working distance substage condenser. Photographs were made with polaroid projection film, using a red filter. The stained and unstained cells could be easily distinguished from each other on the film. A field of 400 to 600 cells was counted.

In the experiments concerned with the protective action of salts, the salts were added to the yeast suspension 2 minutes before the dye.

RESULTS

1. Potassium Loss as an All-or-None Response

The potassium content of yeast cells is very high, of the order of 0.15 to 0.20 M/kg. of cells. Yet the cells suspended in distilled water lose potassium only very slowly unless the cell membrane is damaged (9). With high concentra-

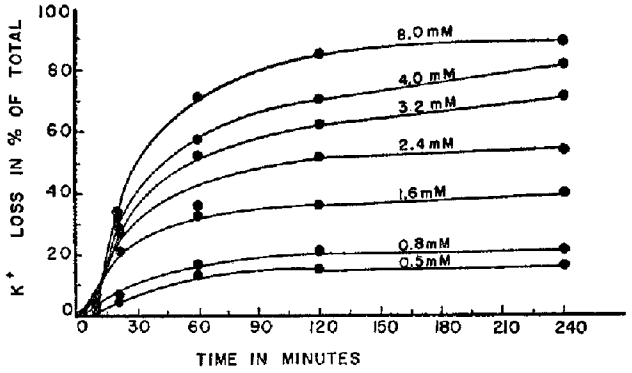

FIGURE 1. The leakage of K^+ induced by different concentrations of methylene blue. The yeast concentration was 5 mg./ml. In the control (no dye) the K^+ loss was less than 5 per cent.

tions of methylene blue (10 mM or higher) essentially all of the cellular K leaks out into the medium. With lower concentrations of the dye, the effects are intermediate, the time-course of K^+ loss being represented by a family of curves shown in Fig. 1. At any particular concentration of methylene blue, the K loss progresses rapidly for about an hour, but thereafter slows down. After 2 hours little or no further loss occurs, even though only a small fraction

of the cellular potassium may have diffused out of the cells. Primarily, then, higher concentrations of the dye are associated with a higher plateau, or maximal K^+ loss, rather than with a higher rate of loss. The log of methylene blue concentration plotted against the maximal K^+ loss is a symmetrical S-shaped curve (Fig. 2) which plots as a straight line on a log-probability graph (Fig. 3).

Several reasonable interpretations of the curves of Fig. 1 can be made. However, in view of the log-probability response shown in Fig. 3, the following concept is preferred. Each cell has a threshold tolerance for methylene blue, which if exceeded, results in a complete loss of K^+, and which if not exceeded results in little or no loss of K^+. The higher plateaus associated with higher concentrations would thereby represent an increased proportion of the

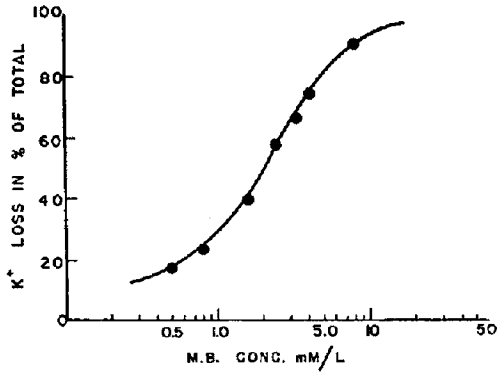

FIGURE 2. The relation of methylene blue concentration and maximal K^+ loss. The data represent averages of three experiments of the type shown in Fig. 1. The maximal loss was taken as that at 2 hours.

cells in which the thresholds had been exceeded. The dose-response curves (Figs. 2 and 3) according to this hypothesis represent the variation in threshold value within the population.

In looking for a means of testing the above interpretation, some of the treated cells were examined under the microscope. A fraction of the cells was found to be stained deep blue, whereas the remaining cells were unstained. A comparison of the percentage loss of potassium with the per cent of stained cells at different times indicated a high degree of corelation. A series of experiments was carried out using a variety of dye concentrations. After maximal K^+ loss had occurred (after 180 minutes) the K^+ loss and per cent of stained cells were determined. An excellent 1 to 1 correlation was found over the whole range (Fig. 4), indicating that the stained cells were also the leaky cells—those in which the threshold for K^+ loss had been exceeded.

2. *Properties of the Interactions between Dyes and the Cell Membrane*

Yeast cells can be protected against the deleterious effects of methylene blue by salts. The cation rather than the anion is the effective agent. Thus NaCl, $NaNO_3$, and $\frac{1}{2}$ (Na_2SO_4) are equally effective, whereas large differences are observed between different cations when the anion is chloride. Of the cations tested, the most effective is UO_2^{++}, followed by Ca^{++} and Mg^{++}. The least effective are the monovalent cations such as Na and K^+ (Table I).

The relative affinity of the dye for the cation-binding sites of the cell can be calculated from the protective action of various concentrations of salts. For

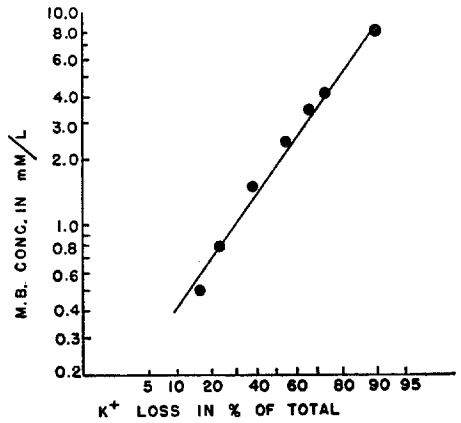

FIGURE 3. K^+ loss *versus* methylene blue concentration on a log-probability plot. The data are taken from Fig. 2.

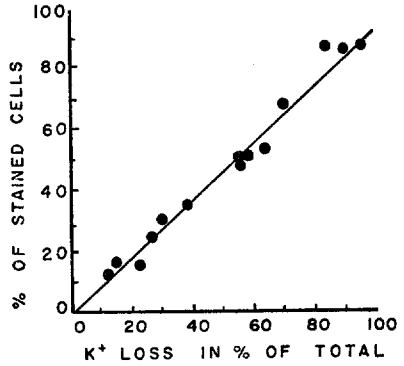

FIGURE 4. The relationship between maximal K^+ loss and the staining of yeast cells. The yeast concentration was 8 mg./ml. The methylene blue concentration varied from 0.3 to 10 mM. The maximal K^+ loss was taken as that found after 2 hours. The frequency of staining was also measured after 2 hours.

example, about 50 per cent protection from 1 mM dye is afforded by 50 mM Na^+, or by 0.5 mM Mg^{++}, or by <0.01 mM of UO_2^{++}. Thus the dye forms a less stable complex than the bivalent cations but a more stable complex than the monovalent cations. The protective effect is the same when measured in terms of K^+ loss or frequency of staining.

The relatively low affinity of the dye for the binding sites is also indicated by the fact that the action on membrane permeability is relatively independent of cell concentration. An eightfold reduction in cell concentration (from 32 mg./ml. to 4 mg./ml.), at a fixed dye concentration results in a small change in the per cent of stained cells (46 per cent as compared with 56 per cent).

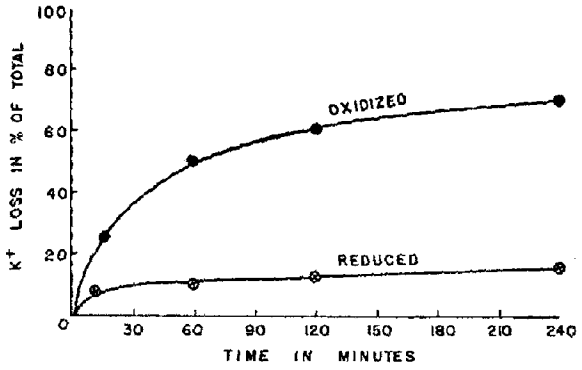

FIGURE 5. A comparison of leucomethylene blue and methylene blue. The leucomethylene blue was prepared by bubbling H_2 in the presence of catalytic platinum black. The experiment was carried out in an atmosphere of N_2. The yeast concentration was 8 mg./ml. and the dye concentration, 3 mM.

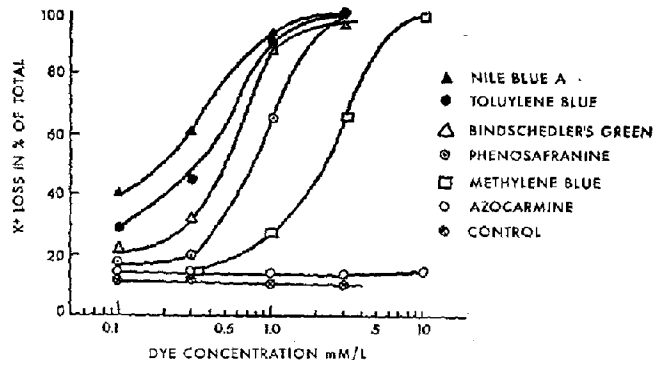

FIGURE 6. The K^+ leakage induced by various basic and acidic redox dyes. The yeast concentration was 8 mg./ml. The K^+ leakage was measured after 2 hours.

Only the oxidized form of the methylene blue is effective. Hence leuco-methylene blue, produced by exposure to H_2 in the presence of catalytic platinum black, had only a small effect (Fig. 5).

A number of other redox dyes of varying chemical structure and potential were tested. Although they were not equally effective, all the basic dyes

TABLE I

THE PROTECTIVE ACTION OF CATIONS AGAINST THE EFFECTS OF METHYLENE BLUE

The methylene blue concentration was 3 mM, yeast 9 mg./ml., and time of exposure, 30 minutes.

Protective agent	Concentration	Frequency of stained cells
	M/liter	*per cent*
None	—	60
NaCl	0.01	58
	0.03	51
	0.10	25
	0.30	11
KCl	0.03	48
	0.1	28
Triethylamine	0.1	56
	0.3	55
$MgCl_2$	0.001	40
	0.003	24
$CaCl_2$	0.0005	20
	0.001	13
	0.003	6
UO_2Cl_2	0.00001	5
	0.00002	2

tested, regardless of chemical structure or potential, induced a leakiness toward K^+ and staining of cells. In contrast, none of the acid dyes had such an effect. The action of representative dyes is shown in Fig. 6.

DISCUSSION

In studies with populations of cells, the behavior of individual cells can be observed only in special cases, as for example, colony formation in micro-organisms, hemolysis in red blood cells, or staining of cells. In most cases,

especially when chemical parameters are measured, only the "average" behavior of the population can be measured. For convenience, the assumption is usually made that the contributions of the individual cells to the observed over-all effect are almost alike. On this basis, the increasing effect associated with increasing concentrations of a chemical agent is usually interpreted in terms of the reaction of the chemical agent with increasing numbers of receptors in the cells, resulting in a graded response. Relationships of this kind can often be fitted by the Michaelis-Menten equation, or some other mass-law derivation. On the other hand, in situations such as described in this paper, in which cells behave in an all-or-none fashion, the response to dosage represents something quite different. It represents the variation of threshold values of individual cells within the population.

Why do cells show a threshold response to some agents and a graded response to others? No unequivocal answer can be given. However, certain suggestions can be made, based on the nature of the interaction between the cell and the agent, and on the nature of the observed response.

The response measured in the present paper is the loss of total cellular potassium by affected cells. Normally, yeast cells are relatively impermeable to K^+, and are very impermeable to anions (9). Consequently the small amounts of K^+ that leak out are exchanged for H^+. In the present case, the K^+ leakage is not K^+-H^+ exchange, but a leakage of K^+ together with cellular anions, especially phosphates. Furthermore, the cell has become permeable to the dye molecules themselves, with resultant staining of the cytoplasm. Thus the response constitutes a general breakdown of the permeability barrier, rather than a specific change in the permeability to one substance.[1]

The nature of the chemical reaction which induces the breakdown of the membrane can be inferred from a number of observations. Firstly, a binding of the redox dye to a negative site in the cell surface must occur. For this reason, acidic dyes, regardless of redox potential, have no effect whereas basic dyes of either high or low potential, all produce leakage. For the same reason, cations added to the medium competitively protect the cell by preventing the binding of the dye. The binding of the dye is followed by a second irreversible reaction, the disruption of the membrane.

[1] Other reported effects of basic dyes on cells may well be manifestations of the generalized membrane defect. These include the well known antiseptic and bacteriostatic action of basic dyes such as gentian violet, brilliant green, acriflavine, basic fuchsin, cresyl violet, etc., and the inhibition of metabolism (10–12) by methylene blue, crystal violet, methyl green, fuchsin, methyl violet, malachite green, acridines, and brilliant green. The effects on metabolism are similar to those on the membrane in the following respects: (1) only basic dyes are effective, (2) protection is afforded by salts, and (3) the effect once established is irreversible.

Other actions of acid and basic dyes, such as those reported by Conway (8) on potassium transport are related to redox potential and would seem to represent a specific effect on the transport mechanism, rather than a generalized breakdown of the membrane. The action of methylene blue on Na^+ transport (13) may be mediated by a similar mechanism.

The binding of certain basic dyes by yeast cells has been studied directly (14, 15).[2] The binding curve for trypaflavine and for methylene blue follows a typical absorption isotherm. All cations tested compete for the binding sites, with the order of effectiveness as follows: Al^{+++}, Fe^{+++}, Mn^{++}, Zn^{++}, Ca^{++}, Co^{++}, Mg^{++}, Sr^{++}, K^{+}, Na^{+} (15). Related observations have been made with bacteria (19) and spores of *Neurospora* (20).

The nature of the negative sites responsible for cation binding has been investigated in some detail in yeast (21, 22). The binding sites are of two chemical species, phosphoryl and carboxyl, both located on the cell surface. The phosphoryl groups are able to form a chelate system with the cations and are therefore particularly effective binding sites. UO_2^{++} forms an exceptionally stable complex with the phosphoryl sites. Consequently, UO_2^{++} is a particularly effective protective agent against the redox dyes (Table I). The nature of the substance which contributes the phosphoryl groups can be inferred from the following observations: (1) Massart *et al.* (14) suggest that the binding sites are nucleoproteins based on the similar affinities for basic dyes. (2) The old observations that basic dyes are especially effective agents against Gram-positive bacteria also support the concept that nucleic acids are involved, for the Gram-staining materials seem to be ribonucleic acid at least in part (23). (3) Ribonuclease has been found to reduce the binding of Sr^{++} and Ca^{++} by plant cells and the staining of the cell surface of sea urchin eggs by toluidine blue (24). (4) Finally, treatment of yeast cells with ribonuclease reduces the binding of methylene blue by a considerable amount (15). It seems rather convincing, therefore, that the cation-binding sites of the cell membrane are in part the phosphoryl groups of ribonucleic acid.

The nature of the site involved in membrane breakdown is not so clear. The dyes that are effective are listed in a footnote.[3] They range in redox potential from $+0.24$ to -0.29. Although a number of different chemical structures are represented, in all the compounds the positively charged group is adjacent to a ring in which the resonant structure occurs. Only the oxidized

[2] Some of the dyes, particularly methylene blue, have been used to stain "dead" yeast cells (16, 17), and recently the uptake of the dye by such cells has been quantitatively measured (18). In this case, however, the membrane of the cell had already been damaged. The binding (and staining) therefore represents not only the interaction of the dyes with membrane sites, but also with many cytoplasmic constituents. In this regard, methylene blue must be used with caution as a staining agent for dead cells, for the dye itself in sufficient concentrations can "kill" cells. Protection is afforded by salts, but the staining reaction is reduced competitively by salts.

[3] All basic dyes tested in this and in a previous study (6) produced K^{+} leakage. These include: Bindschedler's green, toluylene blue, phenosafranine, safranine O, methylene blue, Nile blue A, neutral red, thionine, and brilliant cresyl blue.

No acid dyes tested produced K^{+} leakage. These include: 2,6-dibromoquin, 1-chloromide; benzenone-3-chlorophenol; 2,6-dichlorophenol indophenol, and azocarmine G.

Dyes were purchased commercially from National Aniline, Matheson Coleman and Bell, or Allied Chemical and Dye Corporation.

form is effective, suggesting that the membrane site is an oxidizable entity. Study with heavy metals (25) throws some light on the situation. Hg^{++} produces an effect on the membrane which is similar to that of the redox dyes in every respect. In fact the actions of Hg^{++} and of methylene blue are additive, suggesting that the two agents act on the same sites. No protection against the mercury effect was found with cations, but sulfhydryl groups were very effective in this respect. One membrane ligand which might be acted upon by both the oxidized forms of the redox dyes and by Hg^{++} is the sulfhydryl group, with the formation of –S–S– or –S–Hg–S– linkages. If –SH groups are involved, they must be sterically or electrostatically protected from redox substances in the medium, such as the acid dyes, or the basic dyes in the presence of protecting cations. However, when the basic dye is bound to the phosphoryl groups of the membrane, the adjacent sulfhydryl groups become accessible. The proximity of the phosphoryl groups and sulfhydryl groups suggests that the membrane entity involved is ribonucleoprotein.

To return to the question of the all-or-none response, it is apparent that the redox dyes do not act on specific functional units such as enzymes, but rather, they act by altering a chemical ligand which is an integral part of the structure of the cell membrane. With a small number of such alterations per cell, no physiological response is observed. However, as the number of disulfide links is increased, the stress on the membrane finally reaches a threshold, which if exceeded leads to a breakdown of the membrane and the loss of cellular constituents. The variation in thresholds within the population may be related to the ages of the cells within the population (unpublished observations).

REFERENCES

1. SHEPPARD, C. W., and HOUSEHOLDER, A. S., *J. Appl. Physics*, 1951, **27**, 510.
2. JOYCE, C. R. B., *Quart. J. Exp. Physiol.*, 1958, **43**, 299.
3. PASSOW, H., and TILLMAN, K., *Arch. ges. Physiol.*, 1955, **262**, 23.
4. GRIGARZIK, H., and PASSOW, H., *Arch. ges. Physiol.*, 1958, **267**, 73.
5. ECKEL, R. E., *J. Cell. and Comp. Physiol.*, 1958, **51**, 109.
6. ROTHSTEIN, A., and BRUCE, M., *J. Cell. and Comp. Physiol.*, 1958, **51**, 439.
7. STIER, T. J. B., and STANNARD, J. N., *J. Gen. Physiol.*, 1935–36, **19**, 461.
8. CONWAY, E. J., and KERNAN, R. P., *Biochem. J.*, 1955, **61**, 32.
9. ROTHSTEIN, A., Relationship of the cell surface to electrolyte metabolism in yeast, *in* Electrolytes in Biological Systems, (A. Shanes, editor), Washington, American Physiological Society, 1955.
10. MASSART, L., PEETERS, G., and WUYTS-ROBIETTE, J., *Arch. internat. pharmacod.*, 1947, **75**, 162.
11. DELEY, J., PEETERS, G., and MASSART, L., *Biochim. et Biophysica. Acta*, 1947, **1**, 393.
12. ARMSTRONG, W. McD., *Arch. Biochem. and Biophysics*, 1958, **73**, 153.
13. TAKADA, H., *J. Inst. Polytechn., Osaka City Univ., Series D*, 1956, **7**, 115.

14. MASSART, L., PEETERS, G., and VANHOUCKE, A., *Arch. internat. pharmacod.*, 1947, **75,** 210.
15. HIRAOKA, J., and TAKADA, H., *J. Inst. Polytechn., Osaka City Univ., Series D,* 1957, **8,** 79.
16. MILLS, D. R., *Food Research,* 1941, **6,** 361.
17. RAHN, O., and BARNES, M. N., *J. Gen. Physiol.*, 1932–33, **16,** 579.
18. BORZANI, W., and VAIRO, M. L. R., *J. Bact.*, 1958, **76,** 251.
19. MCCALLA, T. M., *J. Bact.*, 1940, **40,** 23.
20. SUSSMAN, A. S., and LOWRY, R. J., *J. Bact.*, 1955, **70,** 675.
21. ROTHSTEIN, A., FRENKEL, A., and LARRABEE, C., *J. Cell. and Comp. Physiol.*, 1948, **32,** 261.
22. ROTHSTEIN, A., and HAYES, A. D., *Arch. Biochem. and Biophysics,* 1956, **63,** 87.
23. STACEY, M., *in* The Nature of the Bacterial Surface, (A. A. Milne and N. W. Pirie, editors), Springfield, Illinois, Charles C. Thomas, 1949, 29.
24. LANSING, A. I., and ROSENTHAL, T. B., *J. Cell. and Comp. Physiol.*, 1952, **40,** 337.
25. PASSOW, H., and ROTHSTEIN, A., *J. Gen. Physiol.*, 1959, **43,** in press.