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CORRELATION OF THE METABOLIC EFFECTS OF BENZALKONIUM CHLORIDE WITH ITS MEMBRANE EFFECTS IN YEAST*

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Abstract—To non-growing suspensions of baker's yeast at pH 5·5 were added varying amounts of the cationic surfactant, benzalkonium chloride. The percentage of cells stained by Nile Blue dye in the presence of calcium chloride, the percentage of potassium lost from the cells, the percentage inhibitions of rates of anaerobic glucose utilization and CO_2 production, and the decarboxylation of exogenous pyruvate (as percentage of maximal rate of decarboxylation) were found to be equal for any given surfactant concentration in the range of concentrations used. Results were interpreted as an all-or-none phenomenon for individual cells in suspension, i.e. as the concentration of surfactant was raised, greater numbers of cells simultaneously released all or nearly all of their potassium, ceased fermenting, and commenced to decarboxylate pyruvate. The results are discussed.

INTRODUCTION

CATIONIC surface-active agents produce changes in both membrane permeability and metabolism. Hotchkiss[1] reported the loss of phosphorus- and nitrogen-containing compounds from staphylococci treated with cationic surfactants. Armstrong[2] showed a correlation between the cytolytic effects of cationic surfactants and their inhibitory effects on acid production and CO_2 production in yeast.

Poisons other than surfactants are known to increase the permeability of the red blood cell[3, 4] or of the yeast cell[5] in an all-or-none manner. Thus, each cell in suspension shows no membrane response until the concentration of agent becomes critical for that cell; at the critical concentration the membrane permeability to potassium suddenly increases with a resultant complete loss of this ion from the cell. The cells in a suspension would behave as a population, with greater numbers of cells losing their potassium as the concentration of agent was increased.

The aforementioned findings suggested that both the membrane response and certain metabolic responses of yeast to the cationic surfactant, benzalkonium chloride,† might be of an all-or-none nature. Results reported here lead to the conclusions that the effects of benzalkonium chloride on the membrane and on certain metabolic measurements are indeed all-or-none in nature. (In this paper the effects of the surfactant on the membrane were measured in terms of K^+ loss, but it is not implied that K^+ loss is the only result of alteration in the membrane produced by this agent.)

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† Benzalkonium chloride, a mixture of cationic quaternary ammonium derivatives of varying alkyl chain lengths was supplied by Mr. Irwin Shupe of Winthrop Laboratories.

METHODS

All experiments were performed at pH 5·5 on non-growing suspensions of commercial baker's yeast (standard brands). The yeast had been washed four times and aerated for 4 hr prior to experiment. Manometric measurements of oxygen consumption or CO_2 production were made by the usual Warburg techniques.

Concentration of potassium ion in supernates from the suspensions was determined by flame photometry with lithium as internal standard. Total cellular potassium was estimated by boiling an aliquot of the yeast suspension for 2–3 min[6] and measuring the potassium in the supernate.

The dye, Nile Blue, was used to determine whether or not surfactant affected the membrane in an all-or-none manner. It was found that as surfactant concentration was raised, greater numbers of cells were stained. In any particular count there appeared only two types of cells—cells deeply stained and cells apparently unstained. The cells were protected from the effects of the dye by previous addition of calcium chloride to give a final calcium concentration of 0·005 M. The concentration of dye in the suspensions was 0·037 mM. At this dye concentration 97–100 per cent of the cells were stained when calcium chloride was not present. In the presence of calcium, however, the staining was reduced to 0–3 per cent. However, cells which have undergone membrane changes stain even in the presence of calcium. The effects of benzalkonium chloride on potassium loss and staining could thus be determined by adding surfactant to a yeast suspension, allowing the suspension to stand for 30 min with occasional shaking, withdrawing aliquots for potassium analysis, and then adding $CaCl_2$ and dye to other aliquots. After 45 min the aliquots containing the dye were then placed in a hemocytometer and the percentage of stained cells was determined.

A commercial glucose oxidase (Glucostat-Worthington Biochemical Corp.) preparation was utilized for glucose analysis. Alcohol was determined by use of a commercial alcohol dehydrogenase preparation (Determatube C-alc-Worthington Biochemical Corp.)

In experiments where pyruvate was used as substrate, acetaldehyde was determined by a modified Barker–Summerson[7] method for lactate. Apparently acetaldehyde, and not lactate, was being produced in these experiments, since zinc lactate standards required an initial 5-min heating period to convert the lactate to acetaldehyde. Barker and Summerson[7] also found the 5-min heating period to be necessary for conversion. The supernates from the pyruvate experiments, however, yielded acetaldehyde-like material without the initial 5 min of heating. An independent check for acetaldehyde was made by means of a modification of the method of Friedemann, *et al.*[8] Material in the supernates was volatilized at room temperature and reduced pressure, trapped in sodium bisulfite, and titrated by the method of Friedemann *et al.*[8] The apparent levels of acetaldehyde were quantitatively similar to, though more variable than, those obtained by the modified Barker–Summerson method just described.

Effects of benzalkonium chloride on the cell membrane as measured by net potassium loss

In Fig. 1 is shown a plot of potassium ion concentration in the medium as a function of time for various surfactant concentrations. Since no K^+ was added to the suspensions, any K^+ found in the suspending medium had to come from the cells. With the exception of the suspension containing the highest concentration of surfactant, most of the K^+ that was going to be lost from the cells had been lost by the time the first

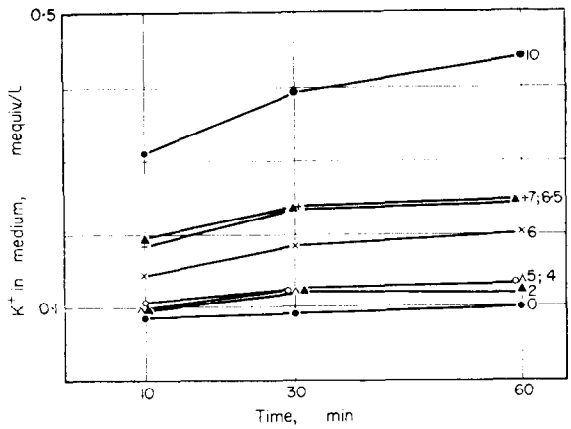

FIG. 1. Leakage of potassium vs. time, for different concentrations of benzalkonium chloride. Yeast concentration was 5 mg/ml wet weight. Suspensions were buffered at pH 5·5 with 0·03 M tris-succinate tartarate made by adding solid tris-(hydroxymethyl) aminomethane to the acids until appropriate pH was reached. Numbered curves represent mg of surfactant/g yeast. One experiment.

samples (10 min) were taken. In the time interval 30–60 min the further loss of K^+ was slight. In Fig. 2 the data of Fig. 1 are replotted to show net K^+ loss vs. surfactant concentration. It can be seen that K^+ loss increased slightly until a surfactant concentration of 0·004–0·005 (mg surfactant/mg yeast) was reached. Above this concentration the net K^+ loss rose sharply until it amounted to about 90–100 per cent of the total cellular K^+ at 0·010 surfactant concentration. Although in Figs. 1 and 2 the results for

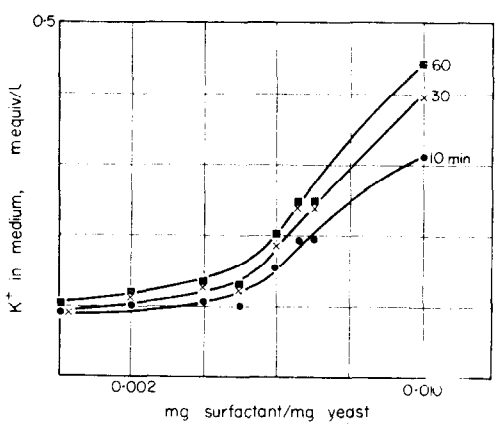

FIG. 2. Potassium leakage as a function of benzalkonium chloride concentration (replotted from Fig. 1).

only one experiment are shown, the K^+ loss in ten experiments was similar to that shown in the figures and was independent of the presence of, or the type of, substrate.

Because the response of individual cells could not be ascertained from the data of Figs. 1 and 2, the staining technique with Nile Blue in the presence of calcium was used to show individual cell response to surfactant. Calcium chloride and Nile Blue dye were added to suspensions previously subjected to the action of surfactant (see

Methods). It was assumed that the cells in the population absorbed dye as the result of an increased permeability brought about by surfactant. Results for net K^+ loss (as percentage of total cellular K^+) and percentage staining are shown in Fig. 3. It is evident that the percentage K^+ loss and percentage cells stained could be represented

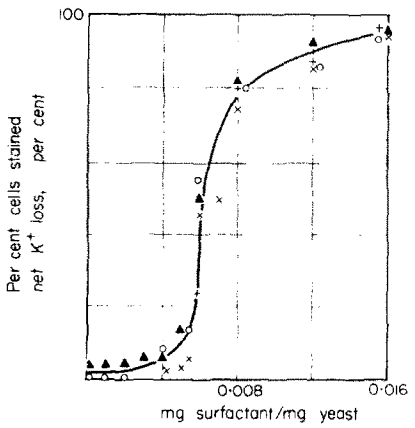

FIG. 3. Simultaneous staining and potassium loss for different concentrations of benzalkonium chloride. Total potassium loss was corrected for the control potassium loss. Yeast concentration and buffer same as in Fig. 1. See text for procedure. Two experiments. Symbols: + and ×: % staining. ▲ and 0: % net K^+ loss.

by the curve shown. In Fig. 4 the data of Fig. 3 and of two other experiments are plotted to show the variation of the experimental points from the line representing equal percentage K^+ loss and percentage staining. From Fig. 4 it can be seen that the percentage K^+ loss and percentage staining were approximately equal for each surfactant concentration. These data are best explained as representing an all-or-none phenomenon.

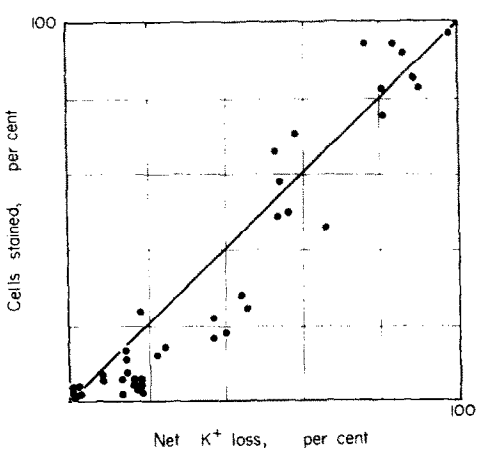

FIG. 4. Relationship of staining to potassium loss due to surfactant. Conditions same as in Fig. 3. Benzalkonium chloride concentration: 0–16 mg/g yeast (wet weight). Values corrected for control potassium loss. Four experiments.

Effects of benzalkonium chloride on metabolism

Decarboxylation of pyruvate. In confirmation of the findings of Barron *et al.*[9] it was found in the present study that intact yeast fails to metabolize pyruvate under anaerobic conditions at pH 5·5. At this pH the negatively charged pyruvate ion cannot penetrate the cell membrane.[9, 10] Because of the ability of benzalkonium ion to affect membrane permeability, it was decided to study the effects of surfactant on the metabolism of exogenous pyruvate. In Fig. 5 it is seen that decarboxylation of pyruvate by

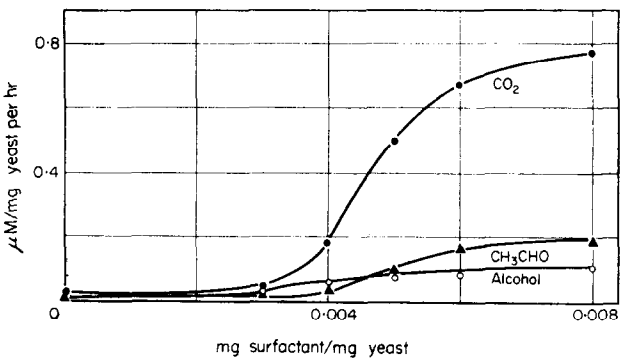

FIG. 5. Anaerobic decarboxylation of pyruvate vs. benzalkonium chloride concentration. Yeast, 10 mg/ml wet weight. Sodium pyruvate concentration was 0·05 M. Buffered as in Fig. 1. Nitrogen atmosphere. Average of two experiments.

the control suspensions was negligible. Above 0·004 surfactant concentration decarboxylation occurred at increasing rates. The concomitant appearances of acetaldehyde and alcohol and the failure to detect lactate indicated that the carboxylase enzyme was involved rather than the dismutation reaction occurring anaerobically in intact yeast below pH 4.[9]

In the aerobic experiments of Fig. 6 the same type of decarboxylation curve as seen in Fig. 5 was obtained. Below 0·004 surfactant, pyruvate was metabolized at the theoretical R.Q. of 1·2. The aerobic decarboxylation curve was obtained by assuming a

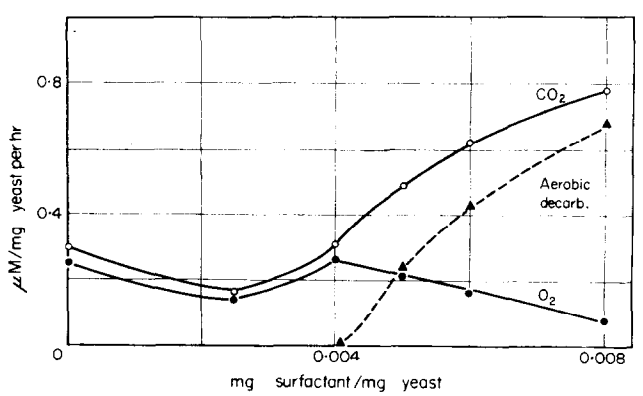

FIG. 6. Aerobic decarboxylation of pyruvate vs. benzalkonium chloride concentration. Conditions similar to those of Fig. 5, except air replaced nitrogen in flasks. Average of two experiments.

continued 1·2 value for R.Q. at the higher surfactant concentrations and subtracting 1·2 times the oxygen consumption from total CO_2 production at the corresponding surfactant concentration. In both the anaerobic and the aerobic experiments it was found that decarboxylation of pyruvate occurred in or on the cell, since the supernatant showed no ability to decarboxylate.

The correlation of the membrane effects with the metabolic effects in surfactant treated cells

In Fig. 7 the percentage inhibition of fermentation is plotted against percentage net K^+ loss. It can be seen that the anaerobic metabolism as measured by glucose utilization or CO_2 production showed results similar to the results of percentage staining vs. percentage K^+ loss obtained in Fig. 4. In addition, in Fig. 7 it is seen that the decarboxylation of pyruvate expressed as the percentage of the maximum decarboxylation rate was equal to the percentage K^+ loss for each surfactant concentration used.

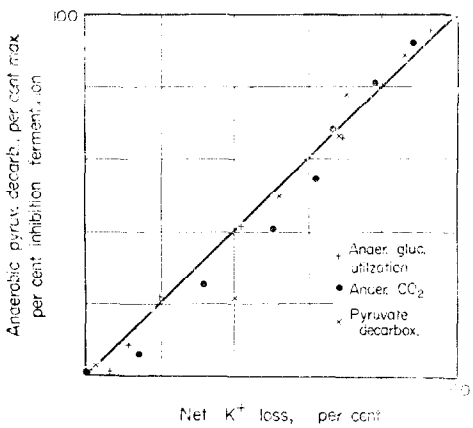

FIG. 7. Relationship of potassium loss to fermentation and anaerobic decarboxylation of pyruvate. Glucose concentration was 0·1 M for anaerobic CO_2, 0·03 M for anaerobic glucose utilization. Yeast, 5 mg/ml (wet weight), with glucose substrate, 10 mg/ml (wet weight), with pyruvate. All suspensions buffered as given in Fig. 1. Potassium loss corrected for control potassium loss. Benzalkonium chloride concentration: 0–16 mg/g yeast. Pyruvate and anaerobic CO_2 determinations by single experiments. Anaerobic glucose utilization, two experiments.

DISCUSSION

It is evident from the foregoing experiments that there is a high correlation between K^+ loss on the one hand and fermentation or decarboxylation of pyruvate on the other hand. As surfactant concentration is increased, greater numbers of cells simultaneously release all or nearly all of their K^+, cease to ferment glucose, and commence to decarboxylate pyruvate.

In an earlier paper from this laboratory[11] it was shown that low concentrations (below 0·004–0·005 mg surfactant/mg yeast) of benzalkonium ion stimulate oxygen consumption at pH 5·5 and inhibit the mechanism responsible for the Pasteur effect. At such low surfactant concentrations, membrane permeability as measured by net K^+ loss (Fig. 2) is not affected appreciably. The actions of benzalkonium chloride on yeast under the given conditions can thus be divided into two concentration zones.

Zone I: The prelytic zone below 0·004–0·005 (mg surfactant/mg yeast) surfactant concentration. In this zone oxygen consumption is increased and the Pasteur mechanism is inhibited,[11] but membrane permeability and fermentation are not appreciably altered. Zone II: Above 0·004–0·005 concentration all-or-none responses occur with regard to release of K^+, inhibition of fermentation, and decarboxylation of exogenous pyruvate. In this zone oxygen consumption also declines[11] but it has not been determined as yet whether this measure of metabolism follows an all-or-none pattern. In zone II it becomes evident that fermentation of glucose and decarboxylation of pyruvate are directly and inversely correlated, respectively, with the presence of an intact cell membrane. The inhibition of fermentation in zone II probably is not the result of direct inhibition of fermentation enzymes by surfactant, because direct studies on enzyme systems show that much higher concentrations of surfactants are needed to inhibit various enzymes than are needed to inhibit cellular metabolism.[1] It is more likely that benzalkonium ion either inhibits glucose transport to the fermentation sites or that benzalkonium causes loss from the cell (with consequent dilution) of necessary coenzymes or cofactors needed in fermentation. Perhaps both of these factors are responsible for inhibition of metabolism. However, alteration of the membrane does not necessarily cause release of fermentation enzymes themselves from the cell.[12]

The finding that pyruvate is decarboxylated when membrane permeability is affected is in agreement with previous findings.[10, 12] It may be suggested that the carboxylase enzyme is located in the periphery of the cell, protected from the external medium perhaps by the cell membrane alone. In the present studies, however, it was found that surfactant did not confer on the cells an ability to metabolize anaerobically glucose-1-phosphate, glucose-6-phosphate, fructose-6-phosphate, hexose diphosphate, or 3-phosphoglycerate. Such findings suggest that penetration of the cell membrane alone is not sufficient to bring about metabolism of an intermediate. The inability of surfactant-treated yeast to metabolize the above intermediates under anaerboic conditions lends support to the contention of Rothstein *et al.*[12] that the fermentation enzymes are in a structure or matrix which is not directly affected by an increase in membrane permeability. However, it is possible that loss of other cofactors for fermentation is more critical than loss of cocarboxylase needed for decarboxylation of pyruvate. Thus, failure to ferment phosphorylated intermediates could be accounted for on this basis.

If the postulated fermentation structures in the cell do exist, there are several possible explanations for the finding that pyruvate, but not the phosphorylated intermediates tested, are metabolized anaerobically when the membrane is no longer intact. One possibility is the inactivation by surfactant of one or more of the enzymes responsible for the ultimate conversion of 3-phosphoglycerate to pyruvate. In a preliminary experiment in this laboratory the anaerobic metabolism of glucose was inhibted nearly 100 per cent by treating yeast cells with benzalkonium chloride. The control suspension and the surfactant-treated suspension were then alternately frozen and thawed to break down further the cell structure. Metabolism in the control and surfactant-treated suspensions was then rechecked. The surfactant-treated preparation after freezing and thawing exhibited over 50 per cent of the activity of the frozen and thawed control preparation. Although such an experiment does not conclusively prove that inactivation of the enzymes is not involved in the action of surfactant, it does place such an explanation in doubt. Furthermore, if the fermentation structures are well-organized

for the best metabolic usage of glucose, it seems unlikely that the interior enzymes of the fermentation chain, but not hexokinase or carboxylase at either end of the chain, should be inhibited. Two other explanations are at hand: Either carboxylase is not a part of the fermentation structure, or a further barrier exists to the penetration of the fermentation structure by the phosphorylated intermediates. Experimental evidence is not at present available to allow a choice of one over the other.

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