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The effect of pH and temperature on the lysis of yeast cells by cationic dyes and surfactants

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Conductivity measurements in yeast suspensions, to which a stepwise increasing quantity of a cytolytic reagent was added, provided evidence towards explaining possible mechanisms of cytolysis by cationic dyes and by cationic surfactants. These lytic cations are bound to the cell surface. If uptake exceeds a critical amount, the permeability barrier of part of the yeast cells in the suspension is destroyed. The "threshold" amount of lytic agent was proportional to the quantity of yeast in the suspension, and was pH- and temperature dependent in a way which suggested an interaction with anionic membrane lipids. There were similarities between the cationic dyes, Toluidine Blue and Azure A, and the cationic surfactants, cetrimide and cetylpyridinium chloride, in their cytolytic effect on yeast cells. A mechanism of membrane breakdown is discussed.

SEVERAL authors have attributed the bactericidal action of long-chain alkylammonium compounds to their protein-denaturing properties which might result in an inactivation of cellular enzymes (Valko, 1946). Others have directed attention to the cytolytic properties of cationic surfactants. Hotchkiss (1946), Salton (1951) and others have attributed antiseptic action to the membrane-disorganizing effects of these compounds. The observed influence of surfactant alkyl chain length on bactericidal and cytolytic effectiveness suggests an interaction with membrane lipids (Cella, Eggenberger, Noel, Harriman & Harwood, 1952; Ross, Kwartler & Bailey, 1953; Ross & Silverstein, 1954; Hooghwinkel, De Rooij & Dankmeijer, 1965). Surfactants can no doubt interact with enzymes and other cell proteins, but the most likely mechanism of cytolysis appears to involve an interaction with the permeability barrier of the cell (Schulman, Pethica, Few & Salton, 1955; Pethica, 1958; Newton, 1958).

The present paper discusses lysis in yeast cells (*Saccharomyces cerevisiae*) by two cationic dyes and two cationic surfactants. Extensive use was made of conductivity measurements, because a separation of cells and extracellular fluid is not required; furthermore, leakage of ions and cytolysis are registered as conductivity changes as they occur. A rapid assessment of the influence of such variables as temperature and external pH on cytolysis can be made.

Experimental

Materials. Commercial baker's yeast (fresh Koningsgist, Delft), was washed by suspension in distilled water, centrifugation and resuspension. To obtain cells in a reproducible "starved" condition, air was bubbled through the suspension overnight to exhaust endogenous substrates. After aeration the yeast was freed from debris by fourfold centrifugation and resuspension. Centrifugation periods were for only 1–2 min at 3000 rpm to keep many of the cells suspended; these were removed with the debris. The remaining cell suspension was centrifuged at 3000 rpm for 20 min. The packed cells were used to prepare a stock suspension in distilled water, containing 35·5 g yeast/100 ml ("35·5% suspension").

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Cetylpyridinium chloride and cetyltrimethylammonium bromide (cetrimide), of high purity (>95%), were supplied by F. W. Berk, London. Sodium dodecylsulphate was supplied by Unilever Research Laboratory, Vlaardingen, through the courtesy of Dr. K. van Senden. Toluidine Blue O and Azure A as chlorides, supplied by National Aniline Division, Allied Chemical Co. were used because they were relatively pure as shown by paper chromatography (Persijn, 1961) and conductimetric analysis.

Apparatus. A Philips PR 9501 conductivity meter was used. Reagents were added from a Metrohm piston burette to a magnetically stirred yeast suspension (total volume 32 ml) in a double-walled glass vessel connected with a thermostat. After each reagent addition a time-interval (1 or 2 min respectively) was allowed to elapse before the conductivity reading. By intensive stirring a local excess of reagent was avoided.

Analysis of the extracellular fluid. At intervals during the determination of a conductivity curve a small known volume of suspension was rapidly filtered through a Millipore filter (HA, 0·45 μ pore size). Potassium in the extracellular fluid was determined by flame photometry. An estimate of extracellular nucleotide concentration was obtained by measuring the absorbance at 260 mμ. Chloride ions were titrated

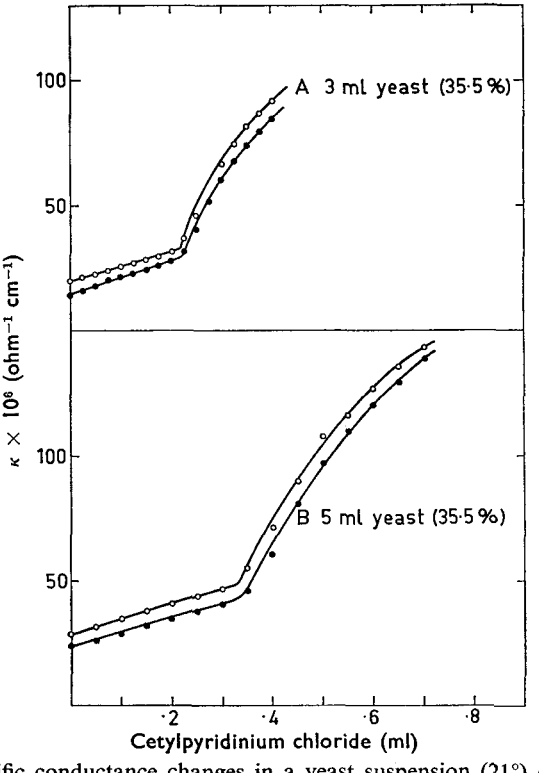

FIG. 1. Specific conductance changes in a yeast suspension (21°) during stepwise addition of 0·01M cetylpyridinium chloride. A. 3 ml 35·5% yeast + 29 ml water. B. 5 ml 35·5% yeast + 27 ml water.

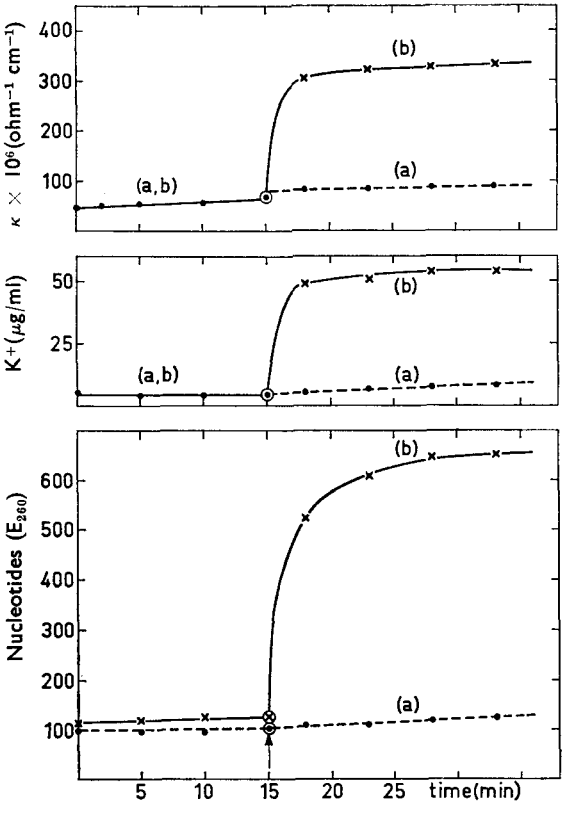

FIG. 2. Specific conductance, potassium concentration, and nucleotide concentration in the extracellular medium as a function of time. The suspension contained 50 ml 35·5% yeast + 110 ml water at 20°. At t = 15 min (*a*) 2 ml 0·01M cetrimide; (*b*) 5 ml 0·01M cetrimide were added (previously determined critical quantity: 3·4 ml 0·01M cetrimide).

conductimetrically, in an 80% ethanol medium, with 0·001M silver nitrate in 80% ethanol. Cationic surfactants were determined with the Bromophenol Blue method, as described by Auerbach (1943) and modified by Van Steveninck & Maas (1965). A similar method was used to determine dodecylsulphate, with the basic dye Azure A to form a coloured complex. This complex was extracted from an aqueous solution containing 0·01M sulphuric acid by means of 1,2-dichloroethane.

Results

Conductivity measurements were made in yeast suspensions to which successive quantities of 0·01M cetrimide were added, as specified in the legend of Fig. 1. With increasing surfactant additions, a phase of relatively minor conductivity changes was followed by a more rapid increase. By taking samples of suspension and analysing the extracellular fluid after Millipore filtration, the break of the conductivity curve

was found to occur at a "critical" surfactant addition beyond which there was rapid release of cellular potassium ions and of nucleotides.

Additions below the critical quantity of surfactant caused a negligible immediate release of intracellular constituents and a slightly increased continuous leakage of potassium ions and of nucleotides.

Fig. 2 shows conductivity changes as a function of time, after one addition of cetrimide in an amount below the cytolytic threshold (*a*) and above this threshold (*b*). The yeast suspension (*a*) showed negligible ion losses from the cells, mostly due to an increased rate of steady leakage. In (*b*) there was a sizeable and relatively rapid ion loss from the cells which approached a plateau. Cetylpyridinium chloride behaved similarly.

From samples of extracellular medium after Millipore filtration it could be shown that there was no equilibrium surfactant concentration

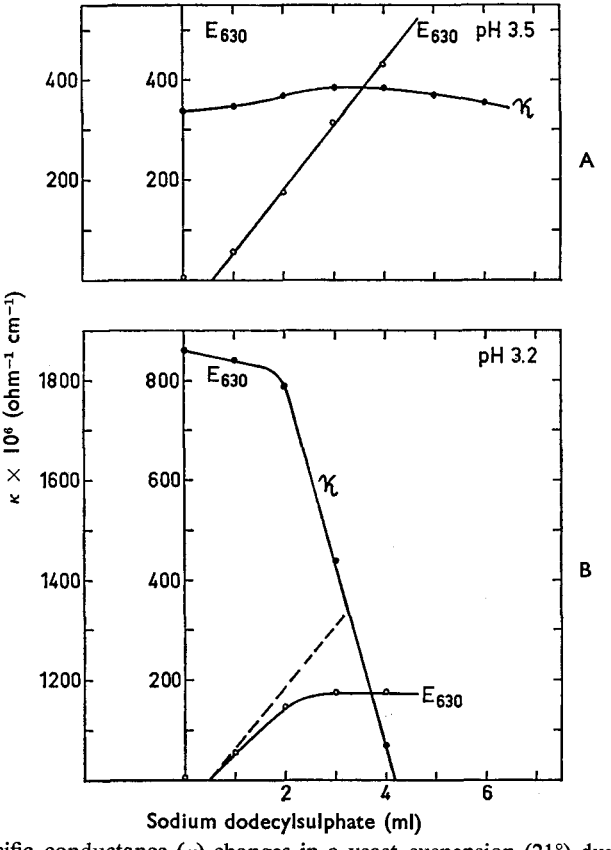

FIG. 3. Specific conductance (κ) changes in a yeast suspension (21°) during stepwise addition of 0·05M sodium dodecylsulphate. The lines for E_{630} indicate the rise in dodecylsulphate concentration in the extracellular medium (determined as a complex with Azure A having an absorption maximum at 630 mμ), as a function of total sodium dodecylsulphate added. A. pH 3·5, 28 ml 35·5% yeast + 100 ml water. B. pH 3·2, 28 ml 35·5% yeast + 100 ml water. Case B, only, shows surfactant uptake and cytolysis.

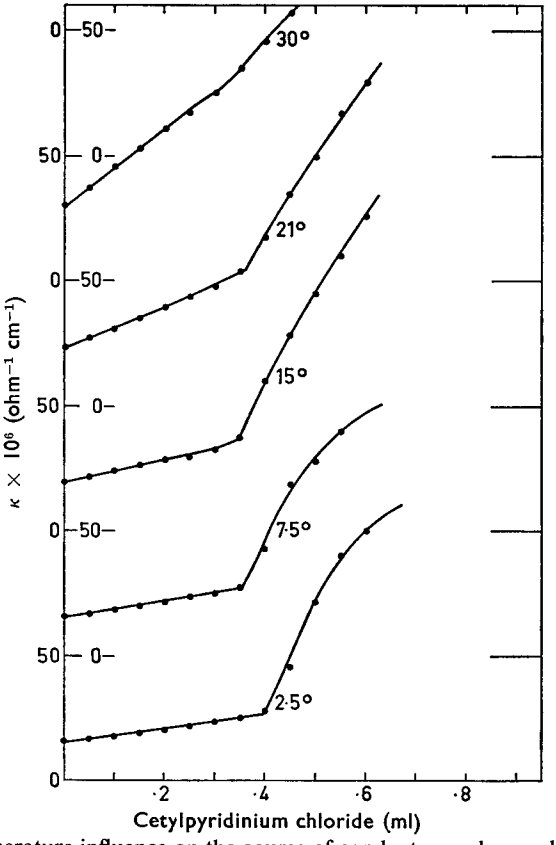

FIG. 4. Temperature influence on the course of conductance change during stepwise addition of 0·01M cetylpyridinium chloride to a yeast suspension (5 ml 35% yeast + 27 ml water; temperatures in °C).

in the medium; all the added surfactant ions were bound by the cells. Determinations of chloride and bromide ions in the medium showed that these ions were not taken up by the cells during surfactant addition.

In experiments with varying quantities of yeast in the same volume of suspension, the critical cytolytic surfactant quantity increased linearly with the quantity of yeast (Fig. 1). The slope of this line gives an estimate of the quantity of surfactant taken up before cytolysis began. This value is 1·8 μmole cetylpyridinium chloride per g fresh yeast, 1·9 μmole/g for tetradecylpyridiniumchloride, and 1·9 μmole/g for cetrimide.

The same sequence of conductivity changes was observed with Toluidine Blue and Azure A as with the cationic surfactants, but the break in the conductivity curve corresponded to a higher uptake of reagent. Again the whole of the added lytic substance was bound by the cells. Conductivity changes ran parallel with increased release of intracellular potassium to the medium.

The threshold cytolytic quantity of dye showed a linear dependence on the yeast quantity; 5·7 μmole Azure A/g yeast and 5·6 μmole Toluidine

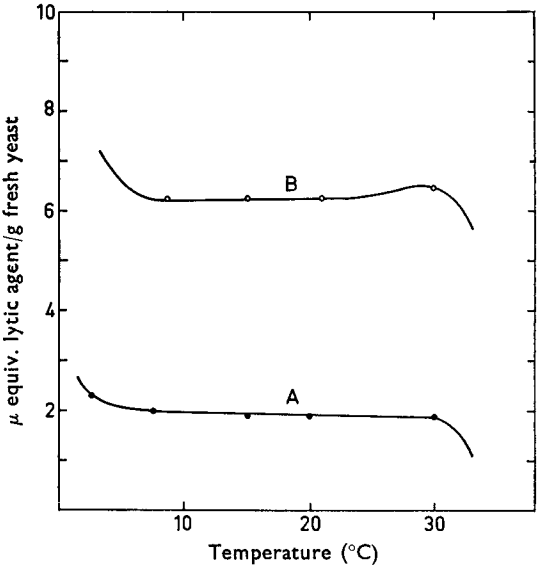

FIG. 5. Cytolytic threshold quantity as a function of temperature, for cetylpyridinium chloride and Azure A, on the basis of specific conductance curves. A. 0·01M cetylpyridinium chloride, added to a suspension containing 5 ml 35·5% yeast + 27 ml water. B. 0·05M Azure A added to a suspension of 7 ml 35·5% yeast + 25 ml water.

Blue/g yeast respectively were required to induce rapid cytolysis. Analysis of the extracellular medium showed that no free dye cations remained in solution, hence the total dye quantity added was bound by the cells.

The similar effects of dye and surfactant cations suggest that, in cytolysis, interactions with charged groups at the cell surface may be involved. Varying the cell surface charge by the external pH should according to this hypothesis have an effect on cytolysis. Conductivity experiments were undertaken with the initial pH of the cell suspension at 2·8, 3·0, 3·2, 3·5, 4·0, 5·0, 6·0, 6·5, 7·0, by the addition of 0·1N hydrochloric acid or tris (hydroxymethyl)aminomethane. Over the pH range 3·5 to 6·0 the cytolytic threshold quantity of cetylpyridinium chloride or cetrimide did not change with extracellular pH. At a pH of 3·2 or lower and at pH 6·5 and higher, small quantities of the surfactants cause rapid cytolysis.

For Toluidine Blue and Azure A, at pH 3·5 and pH 7·0 the critical quantity was shifted to zero, so that very small dye quantities were sufficient to cause cytolysis.

An anionic surfactant, sodium dodecylsulphate was similarly examined. At pH values above 3·5 no cytolysis occurred, nor were dodecylsulphate ions taken up by the cells. At a pH of 3·2, the conductivity curve showed a break corresponding to the point where rapid cytolysis occurred while there was a large uptake of dodecylsulphate ions (Fig. 3). Uptake was far from quantitative; in the medium a large equilibrium concentration of dodecylsulphate was found—in contrast to the cationic surfactants.

At 30° and higher the yeast cells become extremely sensitive to surfactants. Between 10° and 30° the threshold quantity is constant, though

continuous ion leakage from the cells occurs more rapidly with higher temperatures (Fig. 4). Below 10° an increased quantity of surfactant was needed to cause cytolysis. Similar phenomena were found with the dyes; here at 2–3° cytolysis was completely inhibited (Fig. 5).

Discussion

The two cationic surfactants and two dyes, although differing in molecular structure, exhibit striking similarities in their cytolytic effects. The cation is strongly and completely bound by the yeast cells and, at a critical quantity bound per gram cells, an extensive release of cytoplasmic constituents occurs. But with an anionic surfactant lytic effects were seen only at low pH. Neutral substances such as lower alcohols (C_1, C_2, C_3, C_4) also cause lysis in yeast cells but ion release is relatively slow and gradual (Blennemann, Janocha, Keller & Netter, 1963).

It appears that quaternary ammonium surfactants have only a slight disruptive effect on the yeast cell membrane below the critical quantity corresponding to the break in the conductivity curves. The result is a slight acceleration of the steady ion leakage which is always found in yeast cell suspensions, and which may be ascribed to membrane penetration by surfactant molecules (cf. Booij & Bungenberg de Jong, 1956). As Fig. 2 shows, beyond the break in the conductivity curve a different type of ion release occurs. At this point a large quantity of intracellular ions are released in a relatively short time. The time-course of the changes in extracellular potassium and nucleotides suggests that some cells suffer complete membrane breakdown and thus release their total ion content to the medium. For benzalkonium chloride, Sharff & Maupin (1960) found a close agreement between the curves representing potassium release and the percentage of cells stainable by Nile Blue. Potassium loss may thus be due to a complete membrane breakdown of some of the cells in the suspension (all-or-none effect), although this would have to be confirmed by further counting experiments.

To obtain complete lysis of all the cells present in a yeast suspension, 40–100 times as much surfactant as is necessary to reach the cytolytic threshold is required. This may be due to the variation of individual cells in their resistance to surfactant action. Another factor is probably even more important, namely that after passing the critical surfactant quantity the content of lysed cells competes with the membrane of the remaining intact cells for the added surfactant.

The cytoplasm of the yeast cell is probably surrounded by a bimolecular lipid membrane (Gorter & Grendel, 1926; Danielli & Harvey, 1935; see also Booij & Bungenberg de Jong, 1956; Davson, 1962) which functions as a permeability barrier. The membrane is in turn surrounded by a relatively thick cell wall which confers osmotic stability; hence the cells can be put in distilled water without lysis. The main constituents of the membrane are known to be the polysaccharides glucan and mannan. Some protein and lipid constituents are also present (Northcote & Horne, 1952; Nickerson, 1964).

The anionic groups which interact with cytolytic cations are unlikely

to be located in the extraneous cell wall. It would be hard to explain the occurrence of membrane breakdown and the instantaneous release of cellular constituents following the accumulation of lytic cations in the cell wall. A more plausible hypothesis is that the anionic groups involved are themselves part of the lipid permeability barrier of the cell. Observations on the protection afforded by certain metal ions against cytolysis by cationic surfactants also agree with this.

Although proteins may also be part of the membrane-complex in a wider sense, the relative pH-independence and temperature-independence of the critical quantities of dye and surfactant, necessary for cytolysis, argue against a major role of protein in the binding of lytic cations.

The critical quantities of the four cationic reagents may be compared with the estimated number of lipid molecules in the cell membrane (Van Steveninck & Booij, 1964). Assuming that the membrane is a lipid bilayer, the number of lipid molecules in the membrane is approximately *twice* the number of polar groups of a representative phospholipid that can be accommodated at the outward surface of the membrane. Per gram yeast approximately 10^{10} cells are present, while the cell surface is about $120\,\mu^2$ ($= 120 \times 10^{-12}$ m^2). Assuming a cross-section of lecithin, a representative phospholipid, of 40 Å^2 ($=40 \times 10^{-20}$ m^2), we arrive at 6×10^8 molecules of lecithin per cell. The number of membrane phospholipids per gram yeast is 6×10^{18} or approximately $10\,\mu$moles/g. The cross-section of 40 Å^2 represents a lower limit, found only in monolayers of saturated phospholipids. Depending on the acyl groups of the various phospholipids in the membrane a more or less "condensed" state of the lipids can be expected (Van Deenen, 1965). At a cross-section of 60 Å^2, 6·7 μmoles/g yeast would be present as membrane lipids, which is about 4 times the quantity of cationic surfactant required for cytolysis.

The composition of the yeast cell membrane is not known, but figures have been obtained for the phospholipid composition of the whole yeast cell. An analysis of the kind of yeast used in our experiments, carried out by F. A. Deierkauf in this laboratory, gave the following quantities of lipid (μmoles P) per gram yeast: phosphatidylcholine + phosphatidyl ethanolamine 51·8; phosphatidyl serine 5·2; mono-phosphoinositide 12·6; di-phosphoinositide 0·7; phosphatidic acid 1·8; unidentified 1·8; giving a total of 73·9 μmoles P.

Given the relative importance of anionic lipids in the lipid composition of the yeast cell, a role of lipid anions in the binding of lytic cations may be considered likely. Anionic phosphatides are not only a major lipid component in yeast but figure predominantly in the chemical composition of the protoplast membrane of other micro-organisms such as *Micrococcus lysodeikticus* (Gilby, Few & McQuillen, 1958; Wolfe, 1964). A negatively charged membrane surface could account for the quantitative and immediate uptake of dye or surfactant cations as observed. Membrane breakdown could be envisaged as a special case of lipid-surfactant interactions as have been studied already with isolated lipids *in vitro*.

Micelles of phosphoinositides in water, obtained by sonic dispersion, consist of concentric layers of lipid lamellae. They have been found to

be transformed into globular lipid micelles by the action of cetrimide (Thompson & Dawson, 1964; *cf.* Bangham, 1963). The same type of phase transition may be responsible for the disruption of a cell membrane, composed in part of anionic lipids, under the influence of cetrimide and related lytic substances. Such a mechanism would imply a total membrane breakdown once a critical quantity of surfactant cations has been taken up. The observations regarding a critical surfactant quantity required for beginning all-or-none cytolysis strongly suggest such a mechanism. The relative pH-independence of the critical quantity of lytic agent required for cytolysis, as well as the observed dependence of cytolytic behaviour on the temperature, are both in agreement with the idea that anionic lipids are responsible for the net charge of the membrane, and are involved in an interaction with the membrane-disrupting agents discussed.

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