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Effect of metal ions on the lysis of yeast cells by cationic dyes and surfactants

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Uranyl ions and thorium ions are effective in preventing cytolysis by Toluidine Blue and Azure A because they interfere with dye uptake by the cells. Neither uranyl nor thorium ions prevent the uptake of cetrimide or cetylpyridinium chloride. Cetylpyridinium chloride is capable of releasing previously bound uranyl ions from the cell surface. Anionic groups belonging to lipid constituents of the cell membrane may be involved in the uptake of uranyl and thorium ions as well as in the binding of dye and surfactants cations respectively. Possible cytolytic mechanisms involving polar interactions at the cell surface with lytic cations are discussed.

THE pronounced cytolytic effect of cationic surfactants of the quaternary alkylammonium type, as well as the effects of cationic dyes belonging to the thiazine group (Armstrong, 1957, 1958, 1963; Passow, Rothstein & Loewenstein, 1959), may be connected with the presence of anionic groups at the surface of microbial cells which are capable of binding these substances. With yeast cells, a rôle of polar interactions is suggested by the contrasting behaviour of a cationic surfactant, such as cetylpyridinium chloride, and of an anionic surfactant such as sodium dodecylsulphate (Riemersma, 1966). In yeast suspensions having a pH between 3·5 and 6·0, the cationic surfactants cetrimide and cetylpyridinium chloride were found to have strong cytolytic effects. Extensive cytolysis appeared when a critical quantity of the surfactant uptake by the cells was exceeded. An anionic surfactant, sodium dodecylsulphate, was cytolytically effective only below pH 3·2; uptake from the medium was not complete, as with cationic substances.

Metal ions capable of interaction with anionic membrane constituents might be expected to influence these cytolytic phenomena. Some observations have been reported of a protective action by uranyl salts, which appear to inhibit lysis of yeast cells by cationic dyes and surfactants (Armstrong, 1958; Passow & others, 1959). The present paper deals with the protective effects of uranyl and thorium ions.

Experimental

Fresh commercial baker's yeast (Koningsgist, Delft) was washed, aerated and freed from cell debris (Riemersma, 1966). A 35·5% stock suspension was prepared (35·5 g fresh yeast per 100 ml). Conductivity measurements were carried out with a Philips PR 9501 conductivity meter. A double-walled glass vessel was used, connected with a thermostat (21°), and the cell constant of the conductivity cell was 0·30. Cytolytic reagents were added in small quantities (usually 0·05 ml portions) from a 5 ml piston burette (Metrohm) with adequate stirring to prevent local excess of reagent. Conductivities were read after a waiting period of 2 min (in some instances after 1 min).

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During conductivity experiments the concentration of the extracellular medium was analysed by withdrawing a small volume of suspension, usually 0·5 ml, by pipette at regular time intervals from the starting volume of 160 ml. This was filtered rapidly over a Millipore filter (HA, pore size 0·45 μ) to separate the medium from the cells. The filtrate was analysed colorimetrically at the absorption maximum of various lytic dyes. Surfactant analysis was made following in principle Auerbach's (1943) Bromophenol Blue method, as modified by Van Steveninck & Maas (1965). Metal ions were determined colorimetrically; uranyl ions were converted into a coloured thiocyanate complex having an absorption maximum at 365 mμ (Tucker, 1957). For thorium ions thoron was used as the reagent, the complex formed having an absorption maximum at 540 mμ (Sandell, 1959).

Results

EFFECTS OF METAL IONS IN CYTOLYSIS BY CATIONIC DYES

The conductivity changes in a yeast suspension to which a Toluidine Blue solution was added with and without uranyl ions are given in Fig. 1 (see the legend for experimental conditions). Up to a certain quantity of dye only minor conductivity changes were found. Curve A, obtained in the absence of uranyl ions, shows the characteristic conductivity changes

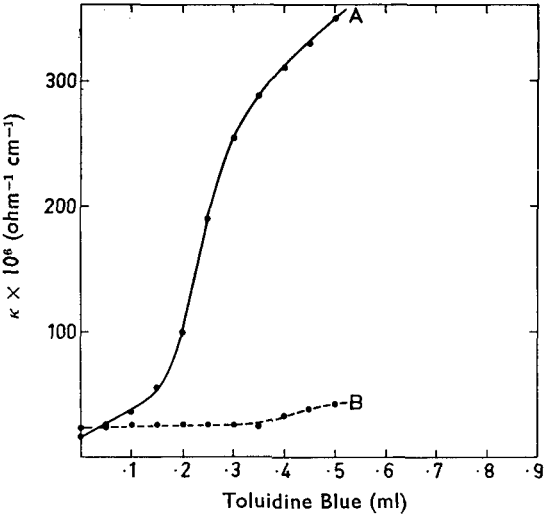

FIG. 1. Influence of uranyl ions on cytolysis caused by Toluidine Blue (21°). A. 5 ml 35·5% yeast + 27 ml water. B. 5 ml 35·5% yeast + 26 ml water + 1 ml 0·01 M uranyl nitrate.

which occurred beyond a critical dye quantity. In the presence of uranyl ions the large conductivity increase normally occurring beyond the critical dye quantity was eliminated (curve B, 3×10^{-4}M uranyl). Subsequent experiments showed that a concentration as low as 10^{-4}M uranyl

was effective in shifting the cytolytic threshold far to the right with Azure A as well as with Toluidine Blue.

Thorium had similar effects. Calcium ions had a pronounced effect in shifting the break of the conductivity plot to the right, but only in a concentration of 10^{-3}M. Sodium and potassium had to be present in a 10^{-1}M concentration to give a corresponding effect.

Analysis of the extracellular medium at several points of the conductivity curve, following Millipore filtration of 0·5 ml samples taken from the suspension, showed that uranyl and thorium ions (3×10^{-4}M) prevented the uptake of Azure A by the cells. Normally the whole quantity of added dye is bound by the cells. With uranyl or thorium ions present the dye was completely recovered from the medium.

It proved possible to remove previously bound Azure A by adding a uranyl solution, in the following way. A small volume of yeast suspension to which a sub-critical quantity of dye had been added was filtered through a Millipore filter. Subsequently a few ml of 0·001M uranyl nitrate solution was added to the packed cells on the filter. Filtration of this liquid gave a dark blue filtrate containing the total dye quantity originally bound by the cells.

EFFECTS OF METAL IONS IN CYTOLYSIS BY SURFACTANT

Uranyl ions inhibited yeast cytolysis by cetrimide (see Fig. 2). The critical quantity of cetrimide at which extensive cytolysis began was shifted to the right. Thorium ions had a similar effect, postponing cytolysis to even higher cetrimide quantities. Uranyl and thorium ions exerted a similar protective action with cetylpyridinium chloride.

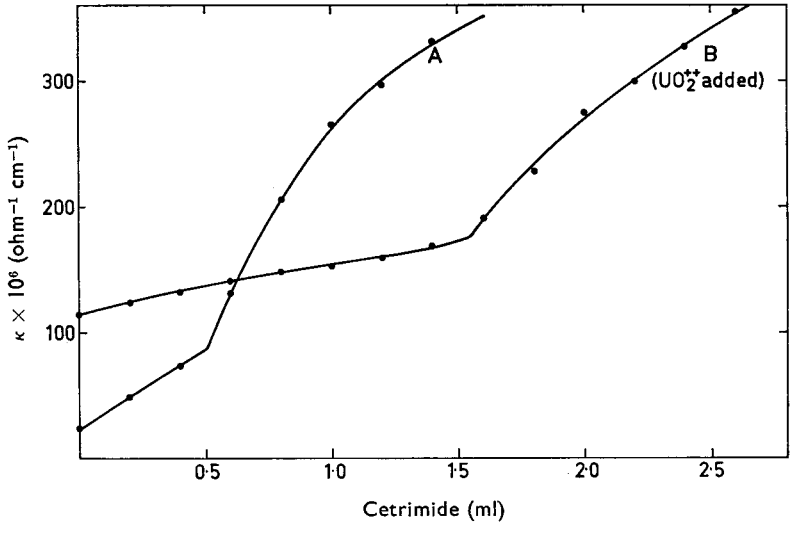

FIG. 2. Influence of uranyl ions on cytolysis by cetrimide (21°). A. 7 ml 35·5% yeast + 25 ml water. B. 7 ml yeast + 24 ml water + 1 ml 0·01 M uranyl nitrate.

Other divalent ions were less effective in shifting the cytolytic surfactant quantity to higher values. Calcium ions had little protective effect, in contrast to what was observed with the two dyes. Sodium and potassium ions also had a negligible influence on the conductivity curves, even in high concentrations.

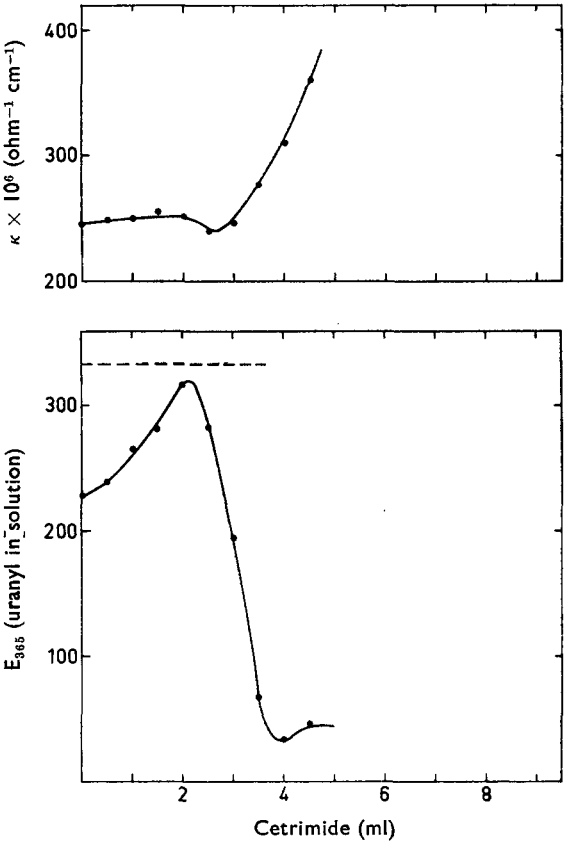

FIG. 3. Relation between uranyl concentration in the extra-cellular medium (measured as the absorbance of a thiocyanate complex E_{365}) and the specific conductivity changes of a yeast suspension during stepwise addition of 0·02M cetrimide (21°). Conditions: 28 ml 35·5% yeast + 94 ml water + 6 ml 0·01M uranyl nitrate. Uranyl determined as described in the text. Dotted line indicates the value of E_{365} if all the uranyl ions present are free in solution; removal of uranyl from the cells is incomplete.

Surfactant determinations in the extracellular medium, obtained after Millipore filtration of cell suspension samples, showed that virtually all the added surfactant cations are bound by the cells, whether or not uranyl or thorium ions are present. Apparently these metal ions do not prevent uptake of *surfactant* cations, as they do *dye* cations.

In fact rather than preventing surfactant ion uptake, uranyl was itself removed from the cell surface by successive surfactant additions. In the

experiment in Fig. 3, for instance, first a small uranyl nitrate quantity was added to a yeast cell suspension and subsequently the effect of a series of cetrimide-additions on conductivity, and on the extracellular uranyl ion concentration, was determined. Under the conditions specified, the uranyl concentration in the medium gradually rose to a value corresponding to an almost complete removal of uranyl ions from the cells, at which point lysis occurred. Lysis was followed by uranyl ion binding by released cytoplasmic material, and a corresponding drop in the uranyl concentration. Similar experiments with cell suspensions to which thorium nitrate was added showed that no comparable removal of thorium ions is effected. This ion evidently was bound more strongly at the cell surface than uranyl.

METAL ION BINDING BY YEAST CELLS

By adding increasing quantities of uranyl salt to a constant quantity of yeast, and by determining unbound uranyl after centrifugation, the existence of a definite plateau for uranyl uptake was confirmed (see also Rothstein, Frenkel & Larrabee, 1948b; Van Steveninck & Booij, 1964). Thereby an approximate value for the uptake capacity of the cell surface was obtained. To obtain a more precise figure, a constant uranyl quantity above the saturation level was added to a series of increasing yeast quantities and the quantity of bound uranyl was determined as a function of the yeast quantity. In 8 volumetric flasks (50 ml) were placed respectively, in duplicate, 1, 2, 5, and 10 ml 14·2% yeast suspension, 0·15 ml 0·15M hydrochloric acid, and 1 ml 0·01M uranyl nitrate; the volume was made up to 50 ml with water. After mixing and standing 10 min this suspension was centrifuged for 10 min at 300 rpm, 25 ml supernatant was transferred to a series of Erlenmeyer flasks, to which subsequently 10 ml ascorbic acid (2%) and 15 ml ammonium thiocyanate (50%) were added. After standing for 1 hr the absorbance was measured in 10 mm cuvettes at 365 mμ. A straight-line dependence was found between the quantity of yeast and the quantity of uranyl bound. The slope of this line gave a binding capacity of 3·5, 3·3 and 2·8 μ-equiv. UO_2/gram yeast, in three separate series.

A similar procedure was adopted to determine the binding capacity for thorium ions. In a series of 16 volumetric flasks (50 ml) respectively, and in duplicate, were placed 1, 3, 5, 10, 15, 20, 25, 30 ml 7·1% yeast suspension, 1·0 ml hydrochloric acid 0·15M and 2 ml thorium nitrate (1000 μg thorium/ml). After adding water to 50 ml, mixing and standing for 5–10 min the suspensions were centrifuged. 5 ml amounts of supernatant were transferred to a series of volumetric flasks (50 ml), to which 2·5 ml 6M hydrochloric acid and 5 ml 0·1% thorin [2-(2-hydroxy-3,6-disulpho-1-naphthylazo)benzene arsonic acid] were added. The volume was brought to 50 ml with water and the absorbance was measured at 545 mμ. The slope of the line obtained for bound thorium as a function of the yeast quantity gave respectively 4·7, 5·4, and 5·9 μ-equiv. Th/g yeast in three separate series. The differences reflect variations among batches of yeast cells rather than errors inherent in the analytical procedure, since the difference between duplicate determinations was generally not

more than 1–2%; the same holds for the figures regarding the binding capacity for uranyl.

Rothstein & others (1948) gave for uranyl ions a binding capacity of 2·2 μ-equiv. uranyl ion/g yeast, while Van Steveninck & Booij (1964) found 4·5 to 6·2 μ-equiv./g. For thorium ions Rothstein & others gave no data, while Van Steveninck & others found an uptake of 8·5 to 10·4 μ-equiv./g yeast. Variations in the culture conditions and in pre-treatment may in part account for the divergence of these figures.

Discussion

The critical quantity of cationic dye beyond which extensive cytolysis occurs in a yeast suspension is greatly increased in suspensions containing uranyl or thorium ions (Fig. 1). Experiments with cationic surfactants as lytic substances also demonstrated a pronounced effect of uranyl or thorium ions (Fig. 2). Uranyl and other metal ions in fact interfered with the uptake of basic dyes by the yeast cells.

The uptake of cationic surfactants, on the other hand, occurred whether or not metal ions were present. Here the protective action of metal ions is less pronounced; it is restricted to only a few metal ions such as uranyl and thorium. Postponement of cytolysis to higher surfactant quantities may in these instances be the result of an increased stability of the cell membrane due to bound metal ions. The membrane can apparently take up relatively larger quantities of the cationic surfactant in the presence of uranyl or thorium than in their absence, before becoming unstable. In the case where uranyl ions are present, surfactant uptake is accompanied by a removal of uranyl from the cell surface. Cetyltrimethylammonium ions have a large enough affinity to certain anionic sites to displace uranyl ions. This affinity cannot be explained solely on the basis of Coulomb interactions between these quaternary ammonium ions and negative groups. The strong and apparently irreversible binding of surfactant cations by the cell surface suggests, apart from Coulomb interactions between ionic groups, an important contribution of van der Waals' interactions between alkyl chains. One way in which such interactions could occur is by the penetration of surfactant alkyl chains into the membrane lipid bilayer. Another, although less likely way, is an accumulation of associated surfactant cations against the cell membrane. With dye cations, given their removability from the cell surface by heavy metal ions, a less intimate combination with membrane constituents is suggested.

Studies by earlier authors have led to the hypothesis that phosphate groups at the yeast cell surface are responsible for binding thorium and uranyl ions (Booij, 1940; Rothstein & Larrabee, 1948; Rothstein & others 1948; Rothstein & Hayes, 1956; Van Steveninck & Booij, 1964). The competitive character of cationic surfactant binding and uranyl binding, as discussed in this paper, suggests that the same phosphate groups might also be involved in surfactant binding. Given the analytical data about the lipid composition of yeast cells, it is tempting to regard membrane phospholipids as directly involved in surfactant binding (Riemersma, 1966).

Phosphate groups belonging to phosphoinositides, phosphatidic acid, and other anionic lipids could combine with the "head" of the surfactant cation, while the surfactant "tail" could enter into the hydrophobic layer of the membrane. In this way the membrane bilayer would form a mixed micelle with surfactant cations, which could be transformed into globular micelles once a certain critical uptake quantity was passed. This type of mechanism is in agreement with an all-or-none type of cytolysis, and with the relatively large surfactant uptake required to reach the cytolytic threshold (of the same order as the quantity of membrane lipids). In the case of the cationic dyes, a dye accumulation in the form of a counter-micelle against the membrane must be assumed which leads to membrane breakdown once a certain critical quantity is passed.

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