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The use of the Coulter Counter to detect the inactivation of preservatives by a non-ionic surface-active agent

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Bacterial and mould spores undergo a germination swelling process which can be accurately measured using a Coulter Counter. The efficacy of preservatives can be assessed by their action in suppressing germination swelling of spores. The antagonism between polysorbate 80 and several preservatives has been demonstrated by measuring swelling of spores in the presence of preservatives and preservative-surfactant mixtures.

AN important factor in the preservation of pharmaceutical and cosmetic two phase systems is the antagonism between non-ionic agents and many preservatives (Wedderburn, 1958, 1964). Physicochemical methods of measuring the interaction between preservatives and non-ionic surfactants have been reviewed (Mitchell & Brown, 1966). These methods include solubility and partition studies, equilibrium dialysis, pH measurements and potentiometric titration. An alternative procedure is to measure the antimicrobial efficacy of preservatives in various combinations with surfactants or in final formulations (Bolle & Mirimanoff, 1950; Barr & Tice, 1957). Although such tests are time-consuming they have the advantage that they use viable micro-organisms and the preservative is assessed under conditions of use. In a modified test of this type Judis (1962) examined the protection given by polysorbate 80 to *Eschericha coli* against chloroxylenol using the release of radioactive material from ^{14}C-labelled cells as an index of cell damage.

The swelling of mould spores during a period of some 5 hr before germ tube formation can be measured rapidly and accurately either by microscopic or by electronic methods. The use of these techniques to evaluate antifungal agents by their effect in suppressing spore swelling has been described by Barnes & Parker (1966). Bacterial spores undergo a similar swelling process which is prevented by antimicrobial agents (Gould, 1964). Measurements of spore swelling should provide a method of rapidly assessing inhibition of preservative action.

Experimental

ORGANISMS

Trichoderma species, IMI 110150. Spore suspensions were prepared from 21 day cultures by the method of Gerrard, Harkiss & Bullock (1960). *Bacillus subtilis* NCTC 3610. Spore suspensions were prepared as described by Gerrard, Parker & Porter (1961).

SIZE ANALYSIS OF DORMANT SPORES

Trichoderma. A suspension of spores in normal saline was submitted to size analysis in a Coulter Counter Model B (with Model J plotter) using a 50 μ orifice tube.

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Instrument settings: Gain trim = 48, Amplification Setting (AS) = 1·0 and Aperture Current (ACS) = 0·5. The average spore volume was 14·8 μ^3. *B. subtilis* spores were analysed in the same manner using a 30 μ orifice tube. Instrument settings: Gain trim = 100, AS = 0·25 and ACS = 0·707. The average spore volume was 0·63 μ^3.

PRESERVATIVE SOLUTIONS

Solutions containing (% w/v) (a) methyl hydroxybenzoate (0·04) and propyl hydroxybenzoate (0·02), (b) chlorocresol (0·34), (c) chlorocresol (0·1), (d) phenylmercuric nitrate (0·004), (e) cetrimide (0·04) and (f) Nipastat (0·1) were prepared in sterile water. All chemicals were of B.P. quality. Nipastat is a mixture of methyl, ethyl, propyl and butyl esters of *p*-hydroxybenzoic acid (Nipa Laboratories Ltd.).

SIZE ANALYSIS OF MOULD SPORES IN PRESERVATIVE SYSTEMS

Malt broth, 4% malt extract B.P.C. in glass distilled water pH5 (10 ml) was mixed with preservative solution (a) (10 ml), inoculated with a suspension (1 ml) of *Trichoderma* spores (approximately 60×10^6) and incubated at 25° for 5 hr. Samples (5 ml) taken from the preservative system and diluted to 50 ml with filtered normal saline were submitted to size analysis as described for dormant *Trichoderma* spores.

A control of malt broth (10 ml) and sterile water (10 ml) similarly inoculated and incubated was also submitted to size analysis. Similar experiments were conducted using the preservative solutions (b)–(f). The results are given in Table 1.

TABLE 1. THE EFFECTS OF PRESERVATIVES, ALONE AND WITH 2% POLYSORBATE 80, ON THE SIZE INCREASE DURING GERMINATION OF *Trichoderma* AND *B. subtilis* SPORES

Preservative	Percentage of swelling of untreated *Trichoderma* spores		Percentage of germination swelling of untreated *B. subtilis* spores	
	Without polysorbate 80	With polysorbate 80	Without polysorbate 80	With polysorbate 80
Nil	100·0	100·0	100·0	100·0
PMN 0·002%	0·0	0·0	0·0	0·0
Chlorocresol 0·17%	0·0	0·0	0·0	0·0
Chlorocresol 0·05%	8·4	75·0	38·4	84·7
Parabens 0·03%	13·4	100·0	12·2	63·0
Nipastat 0·05%	0·0	60·0	13·5	21·0
Cetrimide 0·02%	0·0 (spore disintegration)	66·7	13·5	43·1

SIZE ANALYSIS OF MOULD SPORES IN PRESERVATIVE-POLYSORBATE-80 SYSTEMS

Samples of malt broth (10 ml) each containing 4% w/v polysorbate (Tween) 80 (Honeywill-Atlas Ltd.) were mixed separately with preservative solutions (a)–(f) respectively (10 ml) inoculated with *Trichoderma* spores, incubated at 25° for 5 hr and the suspension submitted to size analysis as described. Malt broth (10 ml) containing 4% w/v polysorbate

80 diluted with sterile water (10 ml) was inoculated, incubated and sampled as a control (Table 1).

SIZE ANALYSIS OF BACTERIAL SPORES IN PRESERVATIVE SYSTEMS

Samples of double strength nutrient broth, Oxoid granules CM 67, pH 7·5 (10 ml) were mixed separately with preservative solutions (a)–(f) respectively (10 ml), inoculated with a suspension (1 ml) of *B. subtilis* spores (approx. 80×10^6) and incubated at 32° for 4 hr. Samples (5 ml) were taken from each preservative system, diluted to 50 ml with filtered normal saline solution and submitted to analysis as described for dormant spores of *B. subtilis*.

A control consisting of equal volumes (10 ml) of double strength nutrient broth and sterile water was similarly inoculated and after incubation submitted to size analysis. The results are given in Table 1.

SIZE ANALYSIS OF BACTERIAL SPORES IN PRESERVATIVE-POLYSORBATE 80 SYSTEMS

Samples of double strength nutrient broth (10 ml) each containing 4% w/v polysorbate 80 were mixed with preservative solutions (a)–(f) respectively (10 ml), inoculated with *B. subtilis* spores and incubated at 32° for 4 hr; samples were size analysed as described. Double strength nutrient broth (10 ml) containing 4% w/v polysorbate 80 diluted with sterile water (10 ml) was inoculated, incubated and sampled as a control (Table 1).

Results and discussion

The average volume of dormant mould spores (14·8 μ^3) increases to 22·5 μ^3 within 4 hr and to 24·5 μ^3 after 5 hr incubation. The germination swelling of the bacterial spores represents an increase in volume from 0·63 μ^3 to 0·86 μ^3 increasing to 2·07 μ^3 on emergence (Fig. 1). At the concentrations in contact with the spores (viz. chlorocresol 0·05 and 0·17, combined hydroxybenzoate esters 0·03, Nipastat 0·05, phenylmercuric nitrate 0·002 and cetrimide 0·02% w/v) the preservatives all suppress the swelling of mould and bacterial spores to some extent. In the presence of chlorocresol (0·05%) and the hydroxybenzoate ester mixture (0·03%) *Trichoderma* spores show an increase in size of less than 15% of that shown by untreated spores. The other preservatives prevent swelling completely and the shape of the analysis plot for cetrimide and mould spores suggests their disintegration. Spores of *B. subtilis* have greater resistance to the preservatives and only phenylmercuric nitrate and chlorocresol at the higher concentration (0·17%) completely suppress swelling. Chlorocresol (0·05%) allowed a swelling of some 38%.

Polysorbate 80 alone (2%) in the nutrient media does not prevent swelling of the spores nor does it inhibit the effect of phenylmercuric nitrate or chlorocresol (0·17%) in suppressing spore swelling (Table 1). The surfactant interferes however with the action of chlorocresol (0·05%), hydroxybenzoate esters, Nipastat and cetrimide. With polysorbate 80 in these preservative systems both mould and bacterial spores swell.

The validity of our experimental approach is confirmed by the demonstration of mutual antagonism known to exist between polysorbate 80 and antimicrobial agents such as chlorocresol, the hydroxybenzoates and cetrimide. These are compounds known to interact with the polyoxyethylenes. The interaction between polysorbate 80 and chlorocresol is of interest in that at a concentration of 0·05% the phenolic compound is inactivated whereas at 0·17% it retains activity.

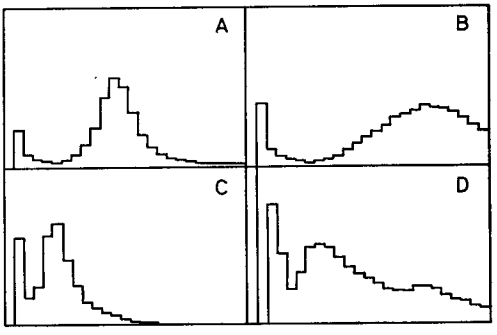

FIG. 1. The increase in size of *Trichoderma* and *B. subtilis* spores during germination, measured with a Model B Coulter Counter and Model J plotter. A. *Trichoderma* spores, peak size 14·81 μ^3. B. *Trichoderma* spores after 5 hr incubation, peak size 24·5 μ^3. C. *B. subtilis* spores, peak size 0·63 μ^3. D. *B subtilis* spores after 4 hr incubation, peaks at 0·86 μ^3 and 2·07 μ^3.

Preliminary investigation of aqueous mixtures of chlorocresol (absorption maximum 280 mμ) and polysorbate 80 (absorption maximum 234 mμ) shows that between 220 mμ and 300 mμ their absorption curves are the simple summation of the curves of their components. The precise nature of the interaction between the surfactant and chlorocresol is being studied.

It has been reported that phenylmercuric nitrate is not inactivated by polysorbate 80 (2%) (Wedderburn, 1958) and in agreement we found a 2% concentration did not interfere with the efficiency of the preservative (0·002%). As anticipated we found that the level of surfactant used (2%) inactivated the hydroxybenzoate ester mixture, the proprietary mixture (Nipastat) and the cetrimide at the concentrations used.

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