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The influence of pH upon the antifungal activity of phenol and benzoic acid

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The toxicity of phenol and benzoic acid to spores of *Aspergillus niger* is due to the unionised molecules.

WITH antimicrobial agents which are more active in the unionised form, e.g. organic acids (Cruess & Richert, 1929; Rahn & Conn, 1944), alteration in the pH of a reaction mixture will cause changes in the percentage ionisation and hence will influence the activity of these toxic agents. This effect is reported for the action of phenol and benzoic acid on *Aspergillus niger* spores.

Experimental

Buffered test solutions. (i) Phenol 2% in 0·05 M boric acid and 0·05 M potassium chloride solution adjusted to the required pH with 0·2 N sodium hydroxide. (ii) Benzoic acid 0·19% in 0·05 M potassium hydrogen phthalate solution adjusted to the required pH with 0·2 N sodium hydroxide or 0·2 N hydrochloric acid.

The pH values of the test solutions and of the reaction mixtures were determined with a "Cambridge" pH meter calibrated with standard buffers. Addition of the spore suspension to the test solutions caused a pH change of less than 0·1 unit and no further change occurred during the reaction period. The methods of preparation of spore suspensions and of evaluation of the fungicidal activity of the test solutions were as described by Winsley (1964) and are similar to those of Chauhan & Walters (1962). The reaction temperatures were 35 $\pm$ 0·1° with phenol and 25 $\pm$ 0·1° with benzoic acid.

Fungistatic activity. To appropriate quantities of concentrated buffered Oxoid Sabouraud liquid medium were added solutions of phenol 1% or benzoic acid 0·2%, water to 9 ml, and 1 ml of a suspension containing 5×10^7 *A. niger* spores. A series of concentrations was made for each pH and replicated ten times. The tubes were incubated at 36° for 5 days. Growth did not occur after this time in any tube previously negative. Controls without the test substances were made.

Results

Previous experiments showed that unmodified Sabouraud liquid medium of pH about 5·4, incubated at 36°, provided optimal recovery conditions for both damaged and undamaged spores. Approximately 99% of the latter were viable.

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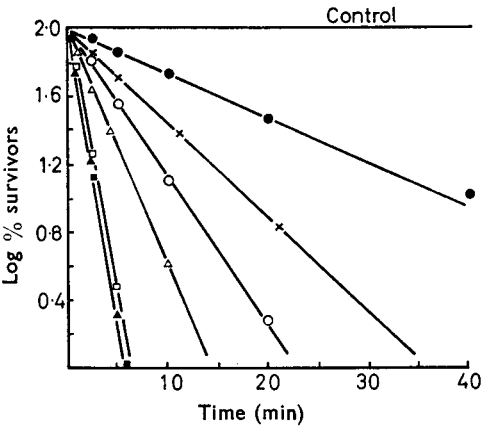

FIG. 1. Log survivor-time curves for the viability of *A. niger* spores in 1% phenol solutions at pH 5·3–9·5. Reaction temperature 35°. Control: buffer solution without phenol. □ = 5·3 (99·99). ▲ = 6·95 (99·5). ■ = 8·1 (98·7). △ = 9·1 (86·3). ○ = 9·2 (83·4). × = 9·3 (79·9). ● = 9·5 (71·5). Figures in brackets are percentages of phenol unionised.

Linear log survivor-time curves were obtained for the viability of *A. niger* spores in 1% phenol solutions buffered at pH 5·3 to 9·5 (Fig. 1). Fig. 2 shows the relationship between the pH and the percentage of undissociated phenol with time for 50 and 99% mortalities. Fig. 5 shows the same relationship for benzoic acid.

The calculated regressions of log percentage undissociated phenol and benzoic acid on log time to cause 50% and 99% mortalities are linear (Figs 3 and 6 respectively).

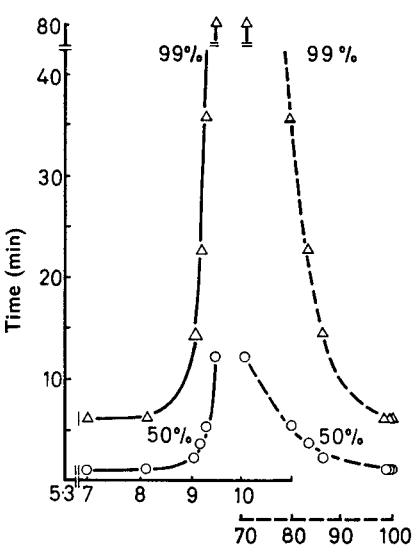

FIG. 2. Relationship between time for 50 and 99% mortality of *A. niger* spores in 1% phenol solution and pH (solid lines) and percentage of unionised phenol molecules (broken lines). Temperature 35°.

The times for 50% and 99% mortalities in a 1% solution of phenol containing only undissociated molecules were calculated to be 0·87 and 5·1 min, respectively. For benzoic acid the corresponding times are 15·9 and 32·8 min. With benzoic acid, as the reaction pH approached and exceeded the pK_a value (4·2), there was a decrease in mortality for a

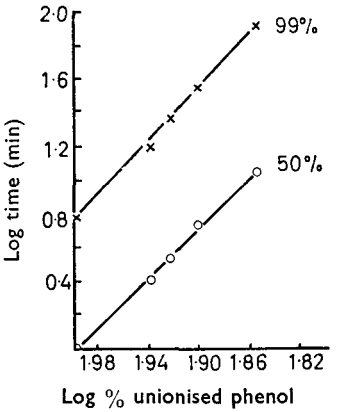

FIG. 3. Relationship between log percentage unionised phenol and log time for 50 and 99% mortality of *A. niger* spores in 1% phenol solution at 35°.

given contact time; curvilinear log survivor-time curves were obtained (Fig. 4). At least 98% of the spores were viable after exposure to buffer solutions alone (pH 2·2, 5·3 and 9·5) for 60 min (Figs 2 and 4).

The minimum inhibitory concentrations of phenol and benzoic acid at different pH values are summarised in Table 1.

TABLE 1. EFFECT OF pH ON THE FUNGISTATIC CONCENTRATION OF PHENOL AND BENZOIC ACID ON *A. niger* SPORES

pH (reaction mixture)	Percentage molecules unionised	Min. inhibitory conc. (%)	Conc. of unionised molecules in m.i.c. (%)
Phenol 5·4	> 99·99	0·066	—
7·0	99·87	0·068	—
9·0	88·1	No growth in control	
Benzoic acid 2·2	99·01	0·017	0·0168
4·0	61·32	0·032	0·0184
4·6	28·47	0·065	0·0185
4·9	16·63	0·11	0·0182
5·1	11·19	0·15	0·0168

Discussion

The shapes of the log survivor-time curves obtained respectively with solutions of phenol and benzoic acid of constant concentration but of different pH values (Figs 1 and 4) were identical to those obtained by

changing the concentration of these agents in unbuffered solutions in water (Winsley, 1964). Increasing the pH value of the reaction mixtures resulted in an increase in the amount of ionised phenol and benzoic acid present*; this was accompanied by a decrease in the death-rate. Apart from change of pH, all other reaction conditions were constant, therefore the change in death-rate with pH must have been due to change in the concentration of the effective form of the fungicide. The slopes of the regressions in Figs 3 and 6 may be considered as concentration coefficients for unionised molecules. To cause 50 and 99% mortalities these are 7·7 and 8·2 for phenol and 3·8 and 4·1 for benzoic acid.

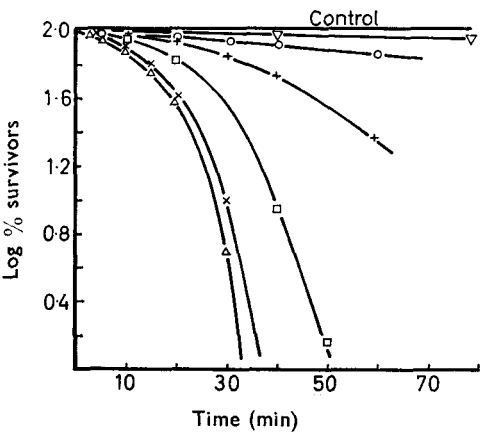

FIG. 4. Log survivor-time curves for the viability of *A. niger* spores in 0·18% benzoic acid solutions at pH 2·3–5·3. Reaction temperature 25°. Control: buffer solution without benzoic acid. △ = 2·3 (98·76). × = 2·9 (95·2). □ = 3·25 (89·9). + = 3·7 (76·0). ○ = 4·05 (58·5). ▽ = 5·3 (7·4). Figures in parentheses are percentages of unionised benzoic acid.

When the toxic agent is incorporated in the recovery medium, the pH of the medium affects not only the degree of ionisation of the agent, but also the growth of the fungus.

In general, the range of pH of the medium over which growth and germination of fungi occur is pH 3 to 8 with an optimum of 4·5 to 6·5 (Cochrane, 1958). The effect of ionisation on the fungistatic concentration of phenol could not be assessed since growth did not occur in the control at pH 9·0. With benzoic acid, whereas the concentration required to inhibit growth increased with pH, the corresponding concentration of unionised molecules remained almost constant from pH 2·2 to 5·1 (Table 1). Rahn & Conn (1944) likewise found that the inhibitory concentration of benzoic acid against *Saccharomyces ellipsoideus* increased with pH, varying from 0·042% at pH 3·5 to 1·27% at pH 5·8, whereas the corresponding percentages of unionised molecules were 0·035% and 0·028%. In contrast, Evans & Dunbar (1964) found that

* Ionisation % $= \frac{100}{1 + \text{antilog } (pK_a - pH)}$ (Albert, 1965)

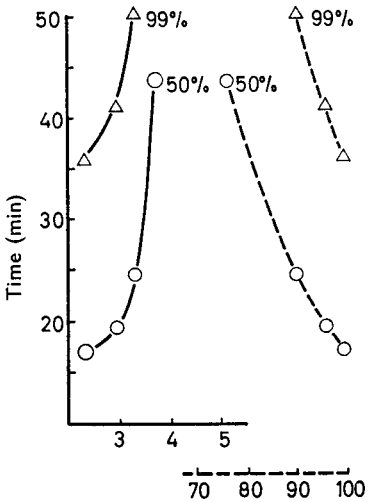

FIG. 5. Relationship between time for 50 and 99% mortality of *A. niger* spores in 0·18% benzoic acid solution and pH (solid lines) and percentage of unionised benzoic acid molecules (broken lines). Temperature 25°.

a decreasing amount of unionised molecules inhibited the growth of *A. niger* as the pH was raised above the pK_a, presumably because the benzoate anions possessed some of the activity of the intact molecules; 0·02% of undissociated acid was inhibitory at pH 4·0 but at pH 5·0, 0·006% was sufficient.

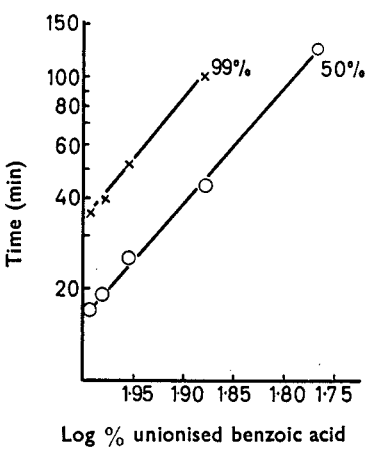

FIG. 6. Relationship between log percentage unionised benzoic acid and log time for 50 and 99% mortality of *A. niger* spores in 0·18% benzoic acid solution at 25°.

The results reported here indicate that unionised molecules are the predominantly active form; penetration to or beyond the cell membrane is more easily achieved by neutral molecules (Davson & Danielli, 1952; Albert, 1963, 1965).

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