# RESEARCH PAPERS

## STUDIES ON THE KINETICS OF FUNGICIDAL ACTION

### PART I. THE EFFECT OF CONCENTRATION AND TIME ON THE VIABILITY OF *Penicillium notatum* SPORES IN SOLUTIONS OF PHENOL

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Received March 17, 1961

About 98 per cent of *Penicillium notatum* spores produced germ tubes in liquid nutrient media within 12 hr. Viability was unaffected by storage in distilled water for 20 days at 4°. The sporicidal activity of phenol was measured by determining the percentage of germinated spores before and after contact with phenol solution. Spores were separated on membrane filters from fungicide by rapid filtration, and incubated in Horowitz fluid medium. The rate of death in solutions containing 0·5 to 1·25 per cent phenol followed a first order reaction. The values of the concentration exponent for 50 and 100 per cent mortalities were 12 and 10·5 respectively.

VARIOUS methods have been used for evaluating the activity of fungicides (Berry and Perkin, 1946; Cochrane, 1958; Gerrard, Harkiss and Bullock, 1960; Horsfall, 1956; Reddish, 1957). Those based on the measurement of turbidity, oxygen uptake or of dry mycelial weight are of limited use (Cochrane, 1958) and appear to be suitable for the determination of extinction times only. Complete correlation of the percentage of germinating spores and the number of colonies obtained by a roll-tube method has not always been realised (Brown and Bullock, 1960). The slide-germination method described by the American Phytopathological Society (1943) for evaluating fungicides determines fungistatic and not fungicidal activity.

The method now described gives quantitative and reproducible results and seems capable of use with a wide range of fungi and fungicides. It facilitates rapid separation of spores and fungicide after reaction, permitting the determination of both the number of viable cells and the amount of fungicide taken up by the spores.

## EXPERIMENTAL

### *Preparation of Spore Suspensions*

*Penicillium notatum* strain 15378 from the Commonwealth Mycological Institute was used. Stock cultures were prepared by incubating slopes of Oxoid Sabouraud glucose agar inoculated with a small quantity of mycelium for 4 weeks at 28°. The slopes were then stored at 4°.

Agar slopes for the preparation of spore suspensions were made from 30 ml. of Sabouraud agar in 8 oz. emulsion bottles. These were inoculated with a loopful of spores from a stock culture and incubated for 10 days at 28°. 10 ml. of water was pipetted on to the surface of the slopes

which were then lightly scraped with a platinum loop to dislodge the spores. The resulting suspension was strained through muslin, centrifuged at 2,000 r.p.m. for 2 min. and the supernatant fluid containing mycelial debris rejected. The spores were suspended in 5 ml. of water. The suspension was transferred to a 30 ml. McCartney bottle containing ten glass beads, five of diameter 8 mm. and five of diameter 4·5 mm., and shaken with a Microid shaker at a moderate speed for 10 min. to break up the spore clumps. The resulting suspension was centrifuged, the supernantant fluid removed and the spores dispersed in 10 ml. of water.

TABLE I

RATE OF GERMINATION OF *P. notatum* SPORES IN WATER, WORT, SABOURAUD, AND HOROWITZ FLUID MEDIA AND ON WORT, SABOURAUD, AND HOROWITZ AGAR

| Incubation time (hr.) | Per cent germination | | | | | | |
|---|---|---|---|---|---|---|---|
| | Water | Wort | | Sabouraud | | Horowitz | |
| | | F | A | F | A | F | A |
| 6 | 1 | 0 | 1 | 1 | 1 | 1 | 1 |
| 7 | 2 | 7 | 2 | 6 | 6 | 9 | 9 |
| 8 | 0 | 21 | 14 | 42 | 20 | 42 | 42 |
| 9 | 3 | 63 | 28 | 58 | 47 | 86 | 65 |
| 10 | 7 | 94 | 52 | 96 | 81 | 97 | 92 |
| 11 | 13 | 99 | 84 | 99 | 96 | 98 | 94 |
| 12 | 20 | 98 | 92 | 99 | 97 | 98 | 97 |
| 24 | 78 | Not countable | | | | | |

F = Fluid medium
A = Agar medium

This was repeated once more. A portion of the suspension was suitably diluted and the spore concentration determined by counting in a haemocytometer cell. The bulk suspension was then diluted with water to give $10^8$ spores per ml. Sterile apparatus and sterile distilled water were used throughout.

*Preparation of Phenol Solutions*

A stock solution containing 4 per cent w/v of Phenol B.P. in distilled water was prepared and stored at 4°. Dilutions were prepared as required using volumetric flasks and burettes.

*Choice of Recovery Medium*

The suitability of the growth medium was assessed by slide-germination and surface-plating methods.

20 ml. quantities of Oxoid Wort and Sabouraud agar and Horowitz (1947) agar were poured into Petri dishes. Two one drop volumes of a spore suspension of *P. notatum* containing $10^8$ spores per ml. were placed on each plate with a standard dropping pipette. Immediately afterwards, one drop volumes of water and double-strength Oxoid Wort broth, Oxoid Sabouraud fluid medium and Horowitz (1947) fluid medium

were placed on separate microscope slides, with standard dropping pipettes. The procedure was duplicated. One drop of the *P. notatum* spore suspension was added to each slide and mixed with the medium; a square of side about $\frac{3}{4}$ in. was previously outlined on each slide by smearing with Canada balsam to prevent the liquid spreading. The plates and slides were incubated at 28°, the latter in humid chambers prepared by placing glass tubes as supports within Petri dishes containing absorbent cotton wool moistened with water.

The slides and plates were examined microscopically at 1 hr. intervals and the percentage of germinated spores determined by a differential count of 100 spores. The mean of two experiments (Table I) shows that the rate of germination was most rapid in Horowitz fluid medium.

TABLE II

EFFECT OF STORAGE OF *P. notatum* SPORE SUSPENSION AT 4° ON THE RATE OF GERMINATION IN WATER AND HOROWITZ FLUID MEDIUM

| Incubation time (hr.) | Per cent germination | | | | | |
|---|---|---|---|---|---|---|
| | Water | | | Horowitz medium | | |
| | Storage period (days) | | | | | |
| | 0 | 10 | 20 | 0 | 10 | 20 |
| 6 | 1 | 2 | 2 | 1 | 1 | 2 |
| 7 | 2 | 1 | 1 | 9 | 5 | 8 |
| 8 | 0 | 2 | 3 | 42 | 42 | 50 |
| 9 | 3 | 0 | 5 | 86 | 84 | 75 |
| 10 | 7 | 7 | 8 | 97 | 95 | 90 |
| 11 | 13 | 5 | 8 | 98 | 96 | 98 |
| 12 | 20 | 14 | 17 | 98 | 97 | 99 |
| 24 | 78 | 76 | 70 | Not countable | | |

To determine the effect of storage upon the viability and rate of germination of an aqueous spore suspension, counts were made as described above on a fresh spore suspension using water and Horowitz fluid medium, and after it had been stored for 10 and 20 days at 4°. The results are shown in Table II.

*Effect of Phenol on Viability and Rate of Germination*

60 ml. quantities of a suspension containing $10^8$ spores per ml. and a solution of phenol of twice the required strength were mixed after equilibrating at 25°. 20 ml. amounts of the resulting suspension were immediately distributed into each of six test tubes. 10 ml. of water was mixed with 10 ml. of the parent spore suspension to provide a control aqueous suspension containing no phenol.

Five min. after mixing, one of the reaction suspensions was filtered under reduced pressure through a membrane filter (Oxoid) supported in a Seitz filter unit. The filtrate was stored at 4° for subsequent determination of phenol concentration. $2 \times 10$ ml. quantities of water were

immediately passed through the filter to remove the remaining traces of phenol solution.

The filter was removed from the Seitz unit and the spores washed off into a test tube with 2 × 5 ml. quantities of water. A drop of the resulting suspension was added to a drop of double-strength Horowitz fluid medium on each of two microscope slides and incubated at 28°. Differential spore counts were made after 6 hr. and subsequently at 1 hr.

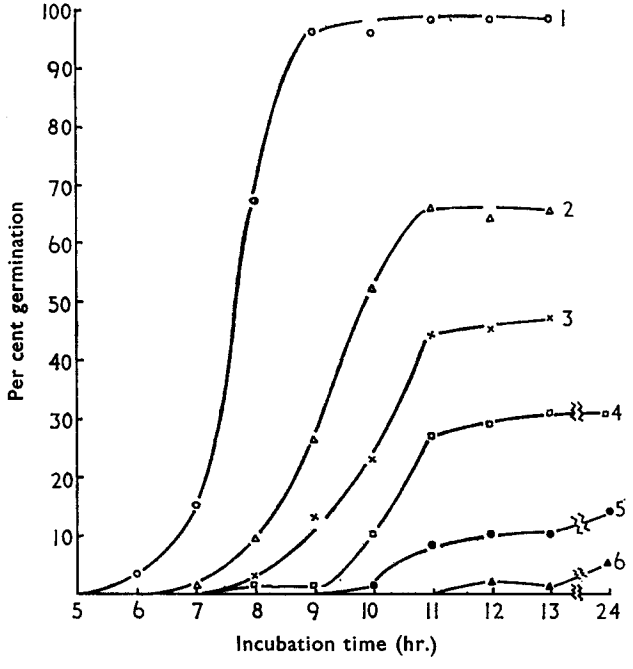


FIG. 1. The effect of time of contact on the viability and rate of germination of *P. notatum* spores treated with 1 per cent phenol.
1. Control (spores in distilled water).
2–6. 5, 10, 20, 40 and 60 min. contact with phenol respectively.

intervals up to 13 hr. and again, where indicated, at 24 hr. The remaining reaction mixtures were treated similarly after 10, 20, 40, 60 and 80 min. respectively, and the control suspension as soon as possible after mixing to obtain the initial number of viable spores.

The experiment was repeated using different concentrations of phenol.

## RESULTS

### *Effect of Medium on the Germination of* P. notatum *Spores*

Germination began in each nutrient medium and in water within 7 hr. but the rate of germination in the following 5 hr. differed markedly and was more rapid in the nutrient liquid media than on the surface of the corresponding solid media; it was most rapid in Horowitz fluid medium

## TABLE III

EFFECT OF CONCENTRATION OF PHENOL ON THE VIABILITY AND RATE OF GERMINATION OF *P. notatum* SPORES

| Phenol concentration (per cent) | Contact time (min.) | Per cent germination — Incubation time* (hr.) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 24 |
| 0·5 | 0 | 3 | 22 | 73 | 96 | 96 | 97 | 97 | 97 | — |
| | 5 | 2 | 14 | 59 | 86 | 96 | 96 | 96 | 96 | — |
| | 10 | 1 | 8 | 44 | 84 | 88 | 90 | 90 | 91 | — |
| | 20 | 0 | 6 | 37 | 83 | 93 | 90 | 90 | 89 | — |
| | 40 | 0 | 7 | 27 | 76 | 86 | 93 | 92 | 91 | — |
| | 60 | 0 | 2 | 19 | 64 | 77 | 87 | 88 | 87 | — |
| | 80 | 0 | 0 | 15 | 62 | 80 | 88 | 87 | 86 | — |
| 0·75 | 0 | 4 | 26 | 69 | 96 | 95 | 95 | 97 | 97 | — |
| | 5 | 1 | 9 | 48 | 86 | 90 | 92 | 93 | 89 | — |
| | 10 | 0 | 7 | 45 | 82 | 80 | 86 | 89 | 85 | — |
| | 20 | 0 | 6 | 34 | 76 | 79 | 81 | 86 | 84 | — |
| | 40 | 0 | 4 | 36 | 71 | 72 | 80 | 80 | 83 | — |
| | 60 | 0 | 2 | 9 | 58 | 65 | 68 | 69 | 68 | — |
| | 80 | 0 | 1 | 8 | 37 | 60 | 65 | 65 | 68 | — |
| 0·875 | 0 | 3 | 22 | 73 | 96 | 96 | 97 | 97 | 97 | — |
| | 5 | 0 | 6 | 40 | 77 | 80 | 78 | 80 | 80 | — |
| | 10 | 0 | 2 | 26 | 67 | 70 | 71 | 78 | 78 | — |
| | 20 | 0 | 0 | 17 | 41 | 70 | 70 | 73 | 73 | — |
| | 40 | 0 | 0 | 2 | 25 | 43 | 54 (65) | 67 | 66 | — |
| | 60 | 0 | 0 | 0 | 1 | 11 | 26 (41) | 50 | 52 | — |
| | 80 | 0 | 0 | 0 | 0 | 0 | 2 (20) | 32 | 28 | — |
| 1 | 0 | 3 | 15 | 67 | 96 | 96 | 98 | 98 | 97 | — |
| | 5 | 0 | 1 | 9 | 26 | 52 | 66 | 64 | 65 | — |
| | 10 | 0 | 0 | 3 | 13 | 23 | 36 (44) | 45 | 47 | — |
| | 20 | 0 | 0 | 1 | 1 | 10 | 15 (27) | 21 (29) | 31 | 31 |
| | 40 | 0 | 0 | 0 | 0 | 1 | 3 (8) | 10 | 10 | 14 |
| | 60 | 0 | 0 | 0 | 0 | 0 | 0 | 3 | 2 | 5 |
| | 80 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 1·125 | 0 | 3 | 28 | 80 | 94 | 93 | 95 | 96 | 96 | — |
| | 5 | 0 | 0 | 0 | 2 | 4 | 3 (12) | 21 (28) | 20 (28) | 24 |
| | 10 | 0 | 0 | 0 | 0 | 0 | (3) | 4 (9) | 7 | 10 |
| | 20 | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| | 40 | Nil | | | | | | | | |
| 1·25 | 0 | 3 | 28 | 80 | 93 | 95 | 95 | 96 | 96 | — |
| | 5 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 0 | 2 |
| | 10 | Nil | | | | | | | | |

Figures within brackets indicate germinated spores and spores swollen before germination.
* Spores washed free from phenol solution and incubated in Horowitz fluid medium.

(Table I). The slide-germination method using this medium was therefore selected for subsequent experiments.

The percentage germination after 12 hr. incubation was approximately 98 per cent in all the nutrient media except Wort agar in which it was 92 per cent. In water, however, it was only 20 per cent. After 24 hr. incubation the growth of the germ tubes in the nutrient media had produced a dense mycelial mass in which the parent spores were barely visible, whereas in water the germ tubes, now produced by 78 per cent of the spores, were still in a very early stage of growth.

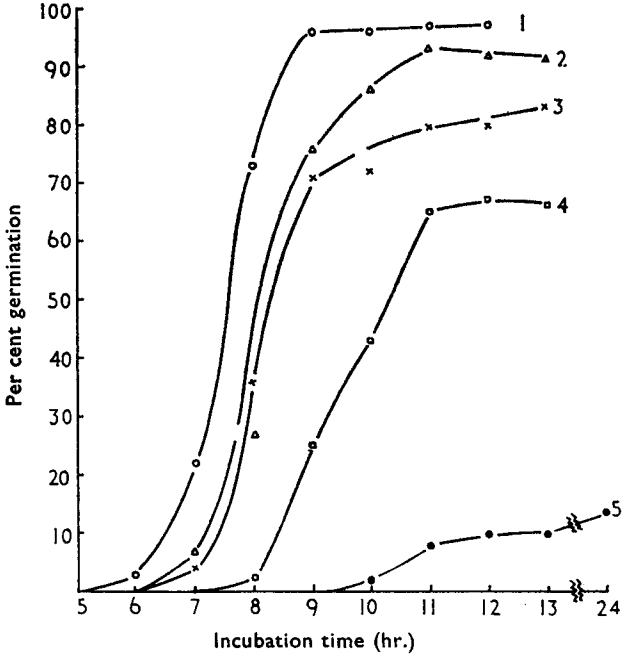


FIG. 2. The effect of concentration of phenol on the viability and rate of germination of *P. notatum* spores. Time of contact = 40 min.
1. Control (spores in distilled water).
2–5. 0·5, 0·75, 0·875 and 1 per cent phenol respectively.

*Effect of Storage at* 4°

Table II shows that the viability and the rate of germination of a freshly prepared suspension of spores in distilled water was unaffected by storage for 20 days at 4°.

*Effect of Phenol*

In the weaker concentrations with short contact times the effect of phenol was mainly to increase the duration of the lag phase before germination began; the percentage of spores killed was small (Table III) The duration of the lag phase and the number of spores killed increased with the phenol concentration and the time of contact. This is shown in Figs. 1 and 2, which are drawn from data in Table III.

## Discussion

The method employed for preparing the aqueous spore suspensions was satisfactory as the spores were uniformly distributed and at least 96 per cent were found to be viable on all occasions after incubation in

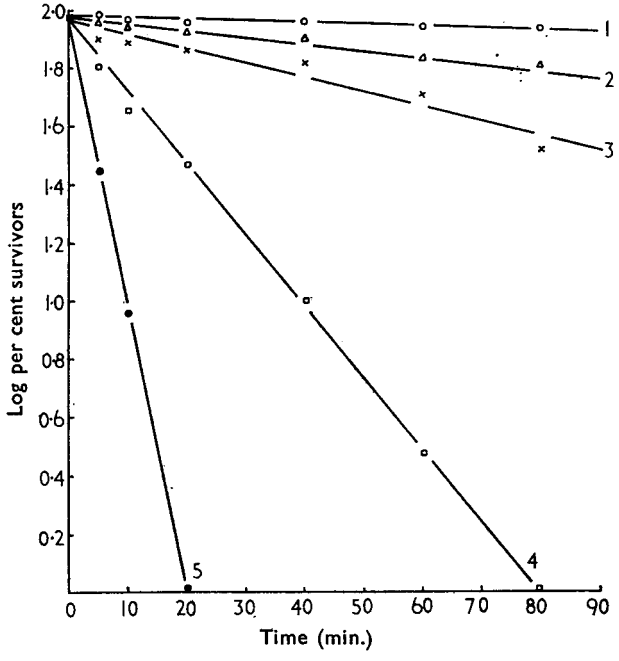


Fig. 3. Log per cent survivor-time curves for *P. notatum* spores in solutions of phenol.
1–5. 0·5, 0·75, 0·875, 1 and 1·125 per cent phenol respectively.

Horowitz medium for 12 hr. About 85 per cent of the spores in suspensions containing $10^8$ per ml. occurred as single cells and the remainder as clumps of 2 to 5 spores.

There was no increase with longer incubation periods than 12 hr. in the number of either untreated or phenol-treated spores germinating in

TABLE IV

Concentrations of phenol required for 50 and 100 per cent death of *P. notatum* spores in 5, 10, 20, 40, 60 and 80 min.

| Contact time (min.) | 5 | 10 | 20 | 40 | 60 | 80 |
|---|---|---|---|---|---|---|
| Log LD 100 .. | 0·11 | 0·097 | 0·0512 | 0·0325 | 0·0150 | 0 |
| Log LD 50 .. | 0·03 | $\bar{1}$·9925 | $\bar{1}$·9775 | $\bar{1}$·9575 | $\bar{1}$·945 | $\bar{1}$·92 |

Horowitz fluid medium (Tables I and III). The percentage of germinated spores after 12 hr. incubation could therefore be regarded as the percentage survivors.

Fig. 3 shows a linear relationship between the log per cent survivors and time for phenol concentrations of 0·5 to 1·125 per cent which indicates that the rate of death of *P. notatum* spores in solutions of phenol follows a first order reaction.

The concentrations of phenol required for 50 and 100 per cent mortalities after 5, 10, 20, 40, 60 and 80 min. respectively, were obtained by plotting the log per cent survivors against log concentration of phenol (Table IV). The calculated regression lines for the relation log concentration : log time for these percentage mortalities are shown in Fig. 4. The slopes of these lines give values for n of 12 for 50 per cent and 10·48 for 100 per cent mortality. Calculation of n from data in Table III using the equation $C^n t$ = a constant, give values of 13·4, 11·8, 11·8 and 13·1 for 50, 70,

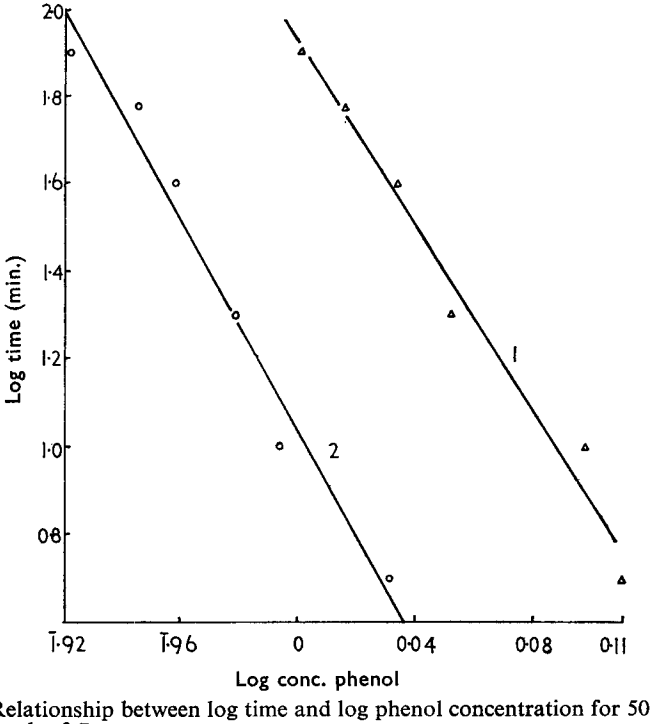


FIG. 4. Relationship between log time and log phenol concentration for 50 and 100 per cent death of *P. notatum* spores.
1. 100 per cent death. 2. 50 per cent death.

90 and 98 per cent mortalities respectively. These values are considerably greater than those of 4·4 and 5·6 obtained for phenol against *E. coli* by Withell (1942) and Jordan and Jacobs (1945).

These high values of n may be explained on the basis that a certain critical concentration of phenol must be adsorbed on to the spore surface for death to occur. Below this concentration there will be a rapid fall in the percentage of spores killed. The relation between the amount of phenol taken up by the spores and the percentage kill is under investigation.

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