MOULD SPORE SUSPENSIONS AND POWDERS FOR USE IN FUNGICIDAL KINETIC STUDIES

PART II. PREPARATIONS USING *Penicillium spinulosum*

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Spore suspensions of *Penicillium spinulosum* have been prepared containing over 99·0 per cent single spores in an even distribution. About 80 per cent of the spores were able both to produce germ tubes and to form colonies in a roll-tube. A peptone powder containing an even distribution of spores has been prepared. The viable count of the powder did not show a significant fall during 5 months storage. The suspension has been successfully used to study the sporicidal and fungistatic activities of chlorocresol solutions.

BROWN and Bullock[1] found that 80 to 90 per cent of *Rhisopus nigricans* and *Penicillium digitatum* spores put out germination tubes in the custom-moist slide germination test, yet roll-tube counts of suspensions of these spores were approximately half the corresponding total (haemocytometer) counts. This failure to produce colonies in a roll-tube might be a general characteristic of mould spores or it might be peculiar to the two species used by Brown and Bullock[1]. Bain[2] working in the same laboratory and using Brown's[3] counting technique found some evidence that the same difficulty might not arise with the spores of *P. spinulosum*. The present work was undertaken to investigate more fully the suitability of *P. spinulosum* spores for use in this type of work and, if possible, to use a suspension of these spores to evaluate the antifungal activity of Chlorocresol B.P.

EXPERIMENTAL AND RESULTS

Much of the work has been duplicated by two of the authors H.N.G.[4] and A.V.H.[5]. Where conditions, techniques or results differed, those of the former will be denoted by (G) and those of the latter by (H).

Penicillium spinulosum strain 42237 of the Commonwealth Mycological Institute was used. Stock cultures were incubated on slopes containing 3 per cent Liquid Malt Extract, and 2 per cent agar, at 25° for 10 weeks (G) or 21 days (H) and subsequently stored at 4°. Spore suspensions were prepared in two ways.

(G) Preliminary experiments showed that mature spores, present on 10 day old but more abundant on 21 day old cultures, are readily detached from the conidiophores and from one another. Suspensions were therefore prepared by inoculating of the same medium from the stock culture and incubating for 21 days at 25°. The mature spores were washed off the surface with distilled water and wetted by shaking vigorously in a test-tube. After passing through a sintered glass filter SG 3 the resulting suspension consisted of 99·4 per cent single spores and none of the clumps contained more than 2 spores.

(H) To minimise the effect of a possible mutant, malt agar slopes were heavily inoculated with a mixture of spores and hypae and incubated for 17 days at 25° after which the surface of the culture was flooded with sterile water. The spore suspension was withdrawn and "atomised" by

TABLE I

THE RESULT OF STATISTICAL ANALYSES ESTABLISHING THE SUITABILITY OF THE MEDIUM USED AND ASSESSING THE ERRORS INVOLVED IN ROLL-TUBE VIABLE COUNTS OF SUSPENSIONS OF THE SPORES OF *Pencillium spinulosum*

	(G) P	(H) P
Reproducibility of medium *t* test, 3 pairs of 4 batches	0·2–0·1 0·5–0·4 0·8–0·7	0·8–0·9 0·7–0·8 0·6–0·7
Sensitivity of the medium obtained from the goodness of fit of χ^2 from replicate viable counts	20 counts N = 19 χ^2 = 10·94 P = 0·95–0·90	18 counts N = 17 χ^2 = 13·6 P = 0·5–0·7
Overall errors of diluting and pipetting mean coefficient of variation	20 quintuplicate counts 4·83 per cent	10 quintuplicate counts 1·87 per cent
Normal sampling variance. Goodness of fit of χ^2	100 quintuplicate counts N = 8 χ^2 = 6·163 P = 0·5–0·7	60 quintuplicate counts N = 8 χ^2 = 7·97 P = 0·3–0·5

passing 3 times through a hand spray. The resultant suspension contained 85 per cent of single spores and 15 per cent of clumps of two spores.

Statistical analyses of a large number of roll-tube colony counts of both types of suspension of *P. spinulosum* spores using the technique and rose bengal medium described by Brown[3] are summarised in Table I. Limits of error are similar to those previously reported using *Bacillus subtilis*, *R. nigricans* and *P. digitatum*[3,7].

TABLE II

COMPARISON OF TOTAL HAEMOCYTOMETER COUNTS AND VIABLE ROLL-TUBE COUNTS IN MILLIONS OF SPORES PER ML.

Experiment number	1	2	3	4	5	6	7	8	9	Mean
Total count	6·7	4·75	6·75	8·5	7·15	7·15	7·1	6·0	5·1	
Method A										
Viable count	5·79	3·79	5·25	5·32	5·85	6·08	5·13	5·28	4·35	
Per cent viability	86·4	79·7	77·8	62·6	81·8	85·0	72·2	88·0	85·3	79·86
Method B										
Viable count	5·87	4·18	5·64	6·98	7·01	6·88	6·10	6·26	5·24	
Per cent viability	87·6	88·1	83·6	82·1	98·0	96·2	85·9	104·3	102·7	92·0

Relation between Total Count, Viable Count and Percentage Germination

(G) Total and colony counts were made on 9 different spore suspensions containing about 7×10^6 spores per ml. Total counts were made on a Thoma slide (Table II). Preliminary experiments had shown that when a spore suspension is measured using a dry pipette, the water retained in the pipette contained a lower concentration of spores than did the original suspension. Making conventional serial dilutions effects a concentration

of spores greater than would be expected in the higher dilutions. Two methods of dilution were therefore used. This concentration effect with *P. spinulosum* spores has been investigated and will be reported elsewhere.[8]

(H) Drops of a suspension containing about 10^6 spores per ml. in sterile tap water on a microscope slide were incubated at 25° for 24 and 48 hours in a moist chamber (closed Petri dish containing some moist filter paper). 300 spores on each slide were examined for formation of a germ tube. Table III shows that approximately 80 per cent of the mature spores germinated, a figure in close agreement with that for the percentage forming colonies.

Spores on the surface of malt agar, which after 24 hours at 25° were observed microscopically to have formed germ tubes, were transferred to a

TABLE III

DETERMINATION OF THE PERCENTAGE GERMINATION OF *P. spinulosum* SPORES IN SUSPENSIONS

Age of the culture from which the spore suspension was prepared (days)	Percentage germination of spores (mean of four slides)	
	After 24 hours incubation	After 48 hours incubation
4	1	1
5	5	19
6	2	—
14	24	50
15	46	52
16	60	71
17	79	80
18	72	79
21	—	82
22	62	77
30	87	87

second plate of malt agar, covered with thin layer of the same medium and incubated again at 25°. Out of 165 spores with germ tubes, 161 produced colonies in 24–48 hours, i.e., 97·5 per cent.

Preparation of a Powder Containing an even Distribution of P. spinulosum *Spores*

Using the method and conditions described by Brown and Bullock[1] a suspension containing about 2×10^5 *P. spinulosum* spores per ml. in 10 per cent peptone solution was spray dried. About 3 per cent mortality was found on drying. This was within the experimental error of counting. All the 13 viable counts obtained during a period of 5 months, when examined statistically, were the same within the error of counting showing that a stable powder had been produced. An analysis of variance (Tables IV and V) showed that the spores were evenly distributed in the powder.

Sporicidal Effects of Chlorocresol

A suspension containing about 10^5 spores in 10 ml. was mixed (time of running in 20 sec.) with 10 ml. of chlorocresol solution of twice the concentration to be investigated. In these preliminary experiments this reaction mixture was kept at room temperature. At the desired intervals

1 ml. quantities of reaction mixture were diluted (20 sec.) by addition to 99 ml. of sterile water. Six separate 1 ml. quantities of the well mixed dilution were transferred to roll-tubes and counted after 48 hours incubation at 24°. To check that this dilution was sufficient to quench the sporicidal activity of the chlorocresol, colony counts of *P. spinulosum* in

TABLE IV

REPLICATE VIABLE COUNTS OF 10 RANDOM SAMPLES OF SPRAY-DRIED PEPTONE POWDER CONTAINING *P. spinulosum* SPORES

Sample No.	Count per g. × 10^{-4}	Sample No.	Count per g. × 10^{-4}
1	132	6	136
2	134	7	102
3	113	8	97
4	123	9	107
5	118	10	137

roll-tubes containing a concentration of 0·001 per cent of chlorocresol in the medium were made and no reduction in numbers were observed. A control experiment with sterile water in place of chlorocresol solution was always run to ascertain the correct figure for the initial count. Timing was from the last drop of chlorocresol added to the spores to the diluting out of the reaction mixture. It was estimated that, because of the delivery time from the burette and pipette, some spores might have been exposed

TABLE V

THE ANALYSIS OF VARIANCE OF THE VIABLE COUNTS OF 10 SAMPLES OF SPRAY-DRIED POWDER

Source of variations	Sum of squares	N	Mean squares
Difference between samples ..	2,037·8	9	226·4
Difference between individuals	7,080·4	20	354·0
		29	

Variance ratio = 1·563
P = 0·2

to a varying concentration of chlorocresol for up to 28 seconds longer than others. This mixing-time factor obviously had the greatest effect in the experiment using 0·1 per cent chlorocresol, where 90 per cent of the spores failed to produce colonies after 2 minutes exposure. In this experiment the reaction mixture was kept in a 10 ml. burette so that 1 ml. quantities could be run off at 5 second intervals. Figure 1 shows the time against log per cent survivor curves using 0·05, 0·066 and 0·1 per cent chlorocresol solutions.

Fungistatic Experiments with Chlorocresol

In these experiments it was necessary to have accurately known and increasing concentration of chlorocresol in the roll-tube medium. Four ml. quantities of a medium containing 7·5 per cent malt extract, 0·025 per cent rose bengal and 3·75 per cent agar were delivered from a heated

container by means of a Matburn pressure operated ampoule filling apparatus into the roll-tubes. The mean delivery weight was 4·1190 g. (coefficient of variation 0·07 per cent. After autoclaving the plugged tubes the mean weight was 4·1177 (coefficient of variation 0·75 per cent.). In the experiments 1 ml. of chlorocresol solution 6 times the concentration

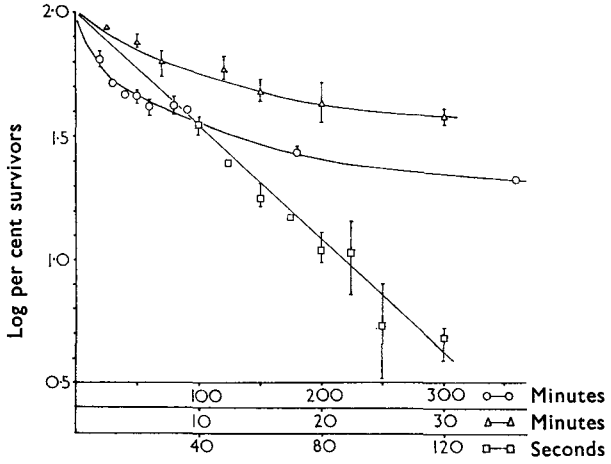

FIG. 1. The sporicidal effects of chlorocresol on spores of *P. spinulosum* in aqueous suspension at room temperature. Three experiments were made with each strength of chlorocresol. Experimental points denote the average values; vertical lines indicate variation. ○—○ = 0·05 per cent, △—△ = 0·066 per cent, □—□ = 0·1 per cent chlorocresol.

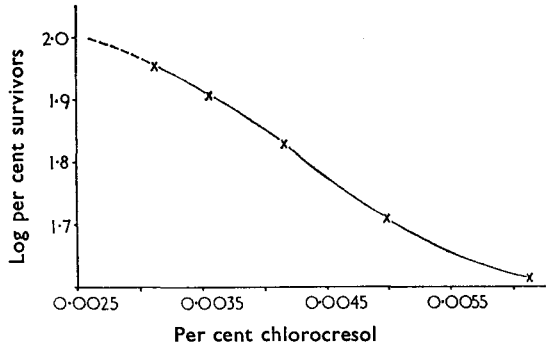

FIG. 2. Fungistatic effects of chlorocresol on the spores of *P. spinulosum* at 25°.

which it was desired to test and 1 ml. of a spore suspension having a viable count of 70 per ml. was added to each tube. In the critical strengths the chlorocresol not only prevented some of the spores from producing colonies, but also reduced the rate of colony growth. The control tubes containing no chlorocresol attained their maximum growth after 48 hours at 24°. All tubes containing chlorocresol could be counted after five days incubation. The count in those tubes containing chlorocresol 1 in

16,000 and 1 in 20,000 continued to increase up to 8 days incubation, whereas those containing chlorocresol 1 in 24,000 and 1 in 28,000 gave the same count as recorded after 5 days incubation. Tubes containing chlorocresol 1 : 32,000 showed confluent growth after 8 days incubation. In these experiments therefore the counts were recorded after 48 hours for the controls, after 5 days with 1 in 32,000 and after 8 days with all the other strengths of chlorocresol. The results are shown in Figure 2.

Discussion

From the work described and from the earlier work of Berry and Perkins[6] and Brown and Bullock[1] it appears that aqueous suspensions of the spores of different species of moulds can be prepared which are suitable for studying the kinetics of fungicidal action. As a test organism *P. spinulosum* offers certain advantages. The spores are of a medium size but large enough for microscopic observation of germ tubes. On rose bengal medium they form discrete compact easily counted colonies. As shown in Tables II and III there is a close agreement between the percentage of the spores forming colonies in a roll-tube and the percentage putting out a germ tube in a moist chamber. Further, as Table II shows, the viable count is about 80 per cent of the total count which is better than the 50 per cent found by Brown and Bullock for *R. nigricans* and *P. digitatum.*

The best way of obtaining a spore suspension containing the highest proportion, over 99 per cent, of single spores is method G described above using a 21 day old culture and filtration through a sintered glass filter SG 3.

Table I confirms that colony counts of mould spore suspensions can be replicated with errors comparable to those found with colony counts of *B. subtilis* spore suspensions. It should be pointed out that favourable results of statistical analyses of viable counts can be obtained with spore suspensions which are not ideal since they were obtained by (*a*) Brown when only 50 per cent of the total spores were forming colonies and (*b*) by Brown and Harkiss above with suspensions containing different proportions of single and grouped (double) spores, as well as by Gerrard above with suspensions containing over 99 per cent single spores.

It has been found possible to prepare peptone powders stable on storage containing an even distribution of *P. spinulosum* spores. These are being used to investigate the effects of heat and gaseous fungicides on contaminated powders.

In experiments using 3 strengths of chlorocresol reasonably close agreement has been obtained between three replicate experiments with different spore suspensions. Except in the instance of 0·1 per cent chlorocresol where the experiment was completed in 2½ minutes, the time log per cent survivor graphs appear not to be straight lines. The reason for the deviations from linearity needs investigation.

References

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After Mr. Brown presented Part I and Mr. Gerrard presented Part II there was a DISCUSSION. The following points were made.

The rose bengal medium had been successfully used for counting other organisms; a fungistatic effect had not been observed with high concentrations of the dye but the dye might enhance the fungistatic action of added substances. The toxic effects of Ringer's solution as a diluent were confirmed. Fungistatic concentrations should be clearly defined, as the concentration inhibiting the growth of the organism may not be the same as the concentration inhibiting germination of the spores.