MOULD SPORE SUSPENSIONS AND POWDERS FOR USE IN FUNGICIDAL KINETIC STUDIES

PART I. PRELIMINARY EXPERIMENTS WITH *Rhizopus nigricans* AND *Penicillium digitatum*

BY M. R. W. BROWN* AND KENNETH BULLOCK

From the Department of Pharmacy of the University of Manchester

Received May 17th, 1960

Reproducible colony counts of spore suspensions of *Rhizopus nigricans* and *Penicillium digitatum* have been obtained on a malt medium containing 0·02 per cent rose bengal. Spores of these organisms suspended in different strengths of Ringer's solution and stored at 1–4° lost viability after a lag of up to 2 days. A spray-dried peptone powder containing evenly distributed *R. nigricans* spores has been prepared without loss of viability of the spores.

INVESTIGATIONS previously made in this department related the effects of storage, moisture, heat, disinfectants and ionising radiations to the viability, as judged by roll-tube colony counts, of *Bacillus subtilis* spores in aqueous suspensions, powders and oils[1–4]. The object of this work was to ascertain how far these investigations could be extended to similar preparations of mould spores.

Many methods of evaluating antifungal agents by their action on mould spores have been described[5]. With one exception[6], these methods have not enabled the results to be statistically analysed and used in kinetic studies comparable with those employed in the study of bactericides.

EXPERIMENTAL METHODS

Choice of Test Organisms

Rhizopus nigricans and *Penicillium digitatum*, both previously recommended as test organisms[7,8], were chosen because the spores of these mould separate readily into single spores, are water-wettable, grow freely on common types of medium and are large enough for direct microscopic examination. *R. nigricans* was used as the main test organism and *P. digitatum* to check selected results.

Counting Method

The techniques of standardising apparatus, making roll-tube colony counts and assessment of errors were those previously described[9,10]. The mean coefficient of variation was 4·4 per cent for *R. nigricans* and 2·2 per cent for *P. digitatum*. The goodness of fit of the values of χ^2, obtained from 100 quintuplicate counts on suspensions of *R. nigricans* spores is recorded in Table I.

Preparation of Suspensions of Spores

Malt extract was chosen as the basic medium[11,12] because the growth of both test organisms is favoured by abundant carbohydrate and slight acidity. Water used in the media was glass-distilled.

* Present address: Department of Pharmaceutics, School of Pharmacy, University of London.

Stock cultures of the test organisms were grown on a medium which contained 3 per cent Malt Extract in water, solidified with 1·5 per cent agar (medium A) and incubated at 25° for 1 week after which they were stored at 3° for up to 3 months.

Preliminary experiments showed that growth of *R. nigricans* in a liquid malt medium stimulated abundant sporulation and consequently spore

TABLE I

GOODNESS OF FIT OF VALUES OF χ^2 OBTAINED FROM COUNTS OF SUSPENSIONS OF *R. nigricans* SPORES USING SETS OF FIVE ROLL-TUBES

Value of χ^2	Expected frequency (m)	Observed frequency	Difference (x)	$\frac{\chi^2}{m}$
Under 1	9·02	11	1·98	0·43
Between 1 and 2	17·40	23	5·60	1·89
Between 2 and 3	17·79	19	1·21	0·08
Between 3 and 4	15·18	14	1·18	0·09
Between 4 and 5	11·87	10	1·87	0·30
Between 5 and 6	8·82	7	1·82	0·38
Between 6 and 7	6·33	6	0·33	1·72
Between 7 and 9	7·48	5	2·48	0·83
Over 9	6·11	5	1·11	0·21

$\chi^2 = 5{\cdot}88$ N = 8 P = 0·5–0·7

TABLE II

DISTRIBUTION OF SPORE GROUPS IN SPORE SUSPENSIONS OF THE TEST ORGANISMS

Organism	Suspension	Approximate concentration of suspension in millions per ml.	Number of slides examined	Percentage of single spores
Rhizopus nigricans	A	10	27	92
	B	16	10	92
	K	10	17	90
	Dilution of K	3	25	97
	Q	14	10	93
	Dilution of Q	6	10	95
	Further dilution of Q	3	13	97
Penicillium digitatum	M	6	5	94
	N	2	5	93
	P	7	5	94

In suspensions of the test organisms containing less than 3 million spores per ml. none of the clumps contained more than 2 spores.

suspensions of this organism were prepared by growing on a medium, containing 3 per cent Malt Extract in water, for 14 days at 25°. The resultant mycelial felts, bearing sporangia, were removed from the surface of the medium and shaken with water to remove the spores. *P. digitatum* was grown on slopes of medium A for 14 days at 25° when the spores were washed off. With both test organisms the crude spore suspensions were strained through gauze and run from a burette so that the last portion

which contained floating debris could be discarded. The spores were collected by centrifuging and resuspended in water.

Spore suspensions of the test organisms were examined microscopically to determine the percentage of single spores and the results are recorded in Table II. Sixteen fields, each containing approximately from 5 to 12 spores depending upon the concentration of the suspension used were examined on each slide.

Discrepancy Between Total Count and Viable Count

During this work it was observed that the colony counts for both test organisms were only about half the total count obtained by calculating the mean of three counts using Thoma chambers.

Investigation of the Inhibitory Effect of Rose Bengal on the Growth of the Test Organisms

The excessive spreading of fungal colonies was a serious difficulty and prevented an accurate colony count. To reduce their size it has been recommended[6] that the medium should be more concentrated than that

TABLE III

MAXIMUM PERCENTAGE GERMINATION OF *Rhizopus nigricans* SPORES

Experiment	Number of plates examined	Number of fields per plate	Maximum percentage germination	
			Standard medium	Rose bengal medium
A	3	10	89	90
B	3	10	90	94
C	3	10	93	93

used for normal culture. This procedure alone was found unsatisfactory and experiments were made to investigate the effects of rose bengal on the growth of the test organisms since this dye in a concentration of 1 in15,000 has been recommended[13] as an anti-spreading agent.

Microscopic determination of the maximum percentage germination. Petri dishes containing medium A as a standard, and dishes containing medium A with 1 in 15,000 rose bengal added were streaked with a suitable dilution of a spore suspension of *R. nigricans* and incubated at 25°. The spores were examined microscopically and the maximum number of spores which germinated is recorded as a percentage in Table III. Each field viewed contained about 20 spores.

Effect of rose bengal on the colony count. A roll-tube medium which contained 6 per cent Malt Extract solidified with 2·5 per cent agar was used as a standard and was compared with a medium containing the same constituents with 1 in 15,000 rose bengal. Five roll-tubes of each medium were inoculated with 1 ml. quantities from the same dilution of a spore suspension of *R. nigricans*. The dilutions were adjusted to give counts of not more than about 50 colonies per roll-tube to prevent overlap of the colonies that would otherwise occur on the standard medium. The results were compared by means of the *t* test and are seen in Table IV.

Experiments with *R. nigricans* have indicated that concentrations of rose bengal of up to 1 in 3,000 in the standard medium do not reduce the colony counts when compared with those obtained by using 1 in 15,000 rose bengal[10]. But as the concentration of rose bengal increased it became

TABLE IV

RESULTS OF COMPARISONS OF COLONY COUNTS OF THE TEST ORGANISMS OBTAINED ON MEDIA CONTAINING DIFFERENT CONCENTRATIONS OF ROSE BENGAL

Media compared by *t* test	Organism	Experiment (replicates)	*t*	P
(*a*) 6 per cent Malt Extract	*Rhizopus nigricans*	A	0·8974	0·4–0·5
(*b*) 6 per cent Malt Extract and 1 in 15,000 rose bengal		B	0·6822	0·5–0·6
		C	0·0623	0·9
a) 6 per cent Malt Extract and 1 in 15,000 rose bengal	*Rhizopus nigricans*	D	0·3556	0·7–0·8
(*b*) 6 per cent Malt Extract and 1 in 5,000 rose bengal		E	0·2192	0·8–0·9
		F	0·8082	0·4–0·5
(*a*) 6 per cent Malt Extract	*Penicillium digitatum*	M	0·4150	0·6–0·7
(*b*) 6 per cent Malt Extract and 1 in 5,000 rose bengal		N	0·7960	0·4–0·5
		O	0·8781	0·4–0·5

easier to count large numbers of colonies. Consequently, the experiment was repeated using the standard medium containing 1 in 5,000 and 1 in 15,000 rose bengal and the colony counts obtained were compared (Table IV).

Colony counts of *P. digitatum* spores on the standard medium with 1 in 5,000 rose bengal added were compared with those obtained without the dye (Table IV).

The reproducibility of the medium containing 1 in 5,000 rose bengal and 6 per cent Malt Extract was tested by inoculating 5 roll-tubes from

TABLE V

RESULTS OF COMPARISONS OF COLONY COUNTS OBTAINED ON SUCCESSIVE BATCHES OF MEDIA

Batches compared by *t* test	*t*	P
1 and 2	0·4999	0·6–0·7
2 and 3	0·6163	0·5–0·6
3 and 4	1·0236	0·3–0·4

successive batches with 1 ml. taken from the same dilution of a suspension of *R. nigricans* spores. The counts obtained from successive pairs of batches were compared by means of the *t* test (Table V).

Viability of spore suspensions after storage. To test for any loss of viability of the spores on storage, suspensions of the test organisms were stored at 3° and colony counts were made after the time intervals shown in Figure 1. In this experiment the spores were suspended in water and in dilutions of Ringer's solution.

Spray drying of suspensions of spores. The apparatus and techniques of the spray drying process have been previously described[14]. A suspension of *R. nigricans* spores was added to a 10 per cent solution of peptone in water, previously cooled to about 3°, so as to give a colony count of about 20,000 per ml. This suspension was cooled by an ice-water jacket

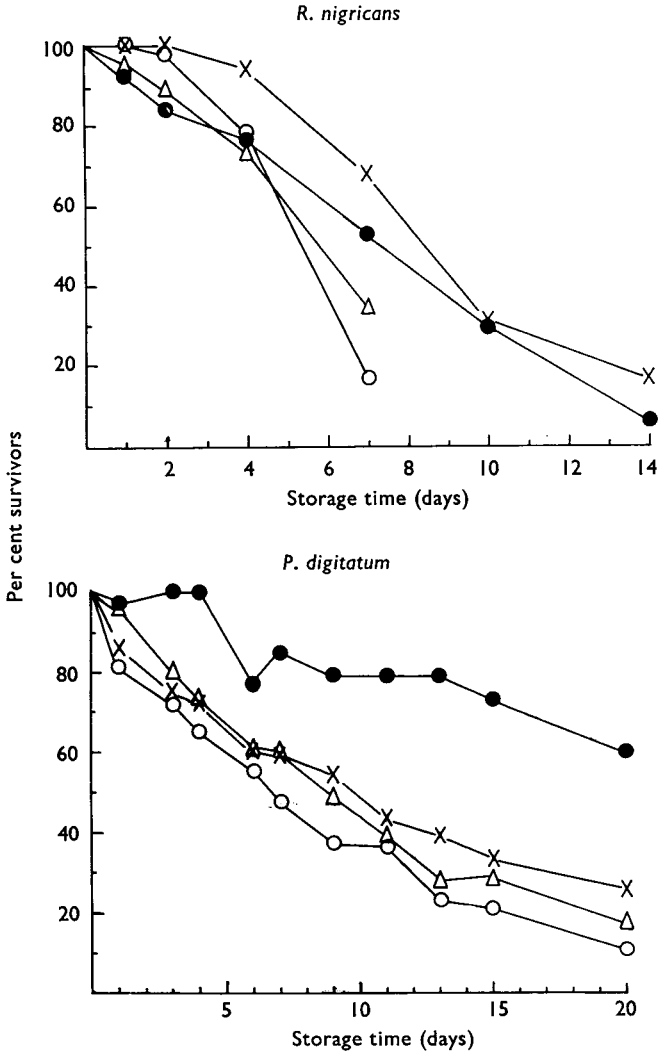

FIG. 1. Effect of storage at 3° upon the colony count of spores of the test organisms suspended in water and in different strengths of Ringer's solution.

● Water.
○ Ringer's solution.
△ ½ strength Ringer's solution.
× ¼ strength Ringer's solution.

and fed to the spray drier with a main inlet temperature of 80°. The spray dried powder was mixed in a revolving jar for 24 hours. Evenness of distribution of the spores was confirmed by an analysis of variance of the viable counts of 8 random samples of the powder each made in quintuplicate. See Tables VI and VII. Colony counts of the reconstituted

TABLE VI

QUINTUPLICATE PLATINGS OF 8 SAMPLES OF THE SAME SPRAY-DRIED POWDER CONTAINING *R. nigricans* SPORES

Sample	1	2	3	4	5	6	7	8
Weight of powder (g.)	0·0680	0·0675	0·0501	0·0664	0·0528	0·0679	0·0589	0·0712
Volume of diluent (ml.)	6·8	6·8	5·0	6·6	5·3	6·8	5·9	7·1
Roll-tube counts	194	171	210	193	184	200	198	190
	189	180	180	177	183	183	200	185
	210	198	183	172	174	189	190	196
	187	202	210	211	188	195	204	192
	194	207	200	201	182	176	197	200
Total	974	958	983	954	911	943	989	963

TABLE VII

ANALYSIS OF VARIANCE OF QUINTUPLICATE PLATINGS OF 8 SAMPLES OF THE SAME SPRAY-DRIED POWDER

Source of variation	Sum of squares	N	Mean square	Variance ratio	P
Difference between samples	860·4	7	122·9	1·02	0·2
Difference between individuals	3866·0	32	120·8	—	—
Total	4726·4	39	—	—	—

TABLE VIII

COLONY COUNTS OF *R. nigricans* SPORES IN A SPRAY-DRIED PEPTONE POWDER AFTER DIFFERENT PERIODS OF STORAGE

Period of storage (days)	0	12	24	35	53
Count (mean count of 5 roll tubes)	173	196	198	166	173

suspension showed no loss of viability of the spores as a result of the spray drying process. Colony counts of the powder were made after the time intervals as shown in Table VIII.

DISCUSSION

It has been shown (Table II) that suspensions of the test organisms can be prepared containing about 97 per cent of single spores for *R. nigricans* and about 94 per cent for *P. digitatum.*

Figure 1 shows that spores of the test organisms rapidly lost viability when suspended in water and in dilutions of Ringer's solution. The suspensions were examined microscopically for the production of germ tubes and none were observed. However, germination might have advanced to a stage which preceded germ tube production but at which

viability was rapidly lost due to lack of nutrients. Consequently it was decided to use freshly prepared spore suspensions of the test organisms. *R. nigricans* spores were suspended in one-quarter strength Ringer's solution and *P. digitatum* in water since under these conditions there was a lag of about 2 days before viability decreased.

The mean coefficients of variation for both test organisms are regarded as satisfactory and may be compared with 3·79 per cent obtained by Berry and Michaels[15] who have designated this figure as the standard error of the counting method since it represents the sum of the variances due to manipulative technique, sampling and counting errors, and errors due to variability of medium. The goodness of fit of values of χ^2 shown in Table I is satisfactory and indicates that the counting technique is capable of giving reproducible results.

Table III shows that both the standard medium and the rose bengal medium allow about 90 per cent germination of suspensions of *R. nigricans* spores.

The presence of 1 in 5,000 rose bengal in the roll-tube medium did not lower the colony count of spore suspensions of either test organism when compared with that obtained on the standard malt medium (Table IV), but it facilitated the counting of the colonies. On the basis of these results the roll-tube medium chosen for subsequent work was Malt Extract 6 per cent, rose bengal 1 in 5,000, agar 2·5 per cent, water to 100 per cent. Table V shows that different batches of this medium offer similar facilities for the growth of colonies.

There is an apparent conflict between the results obtained in this work with rose bengal and those obtained by Smith and Dawson[13]. They found that a concentration of rose bengal of 1 in 10,000 reduced the colony count of soil fungi while a medium containing smaller amounts of rose bengal did not do so. However, these workers did not use a known suspension of single spores of one species but used a soil suspension as a source of mixed organisms. It is possible that the rose bengal increased the lag phase of growth by an amount which differed for various fungal species so that fast growing fungi may have produced colonies which inhibited the growth of more slowly developing fungi. Bain[16] has since confirmed our findings with rose bengal and using a similar technique to that we have described has obtained reproducible colony counts of spore suspensions of *Penicillium spinulosum* and *Aspergillus niger*.

Table VIII shows that colony counts of *R. nigricans* spores in a spray-dried peptone powder did not decrease significantly during 7 weeks. It is intended that similar preparations of free flowing, spray-dried powders containing evenly distributed mould spores be used to study the viability of moulds in systems of low moisture content.

Experimental results have indicated that whilst about 90 per cent of *R. nigricans* spores are capable of developing germ tubes on the surface of agar plates (Table III), only about 50 per cent are capable of producing colonies in roll-tubes, and similar results have been obtained with *P. digitatum*[10]. For suspensions of bacterial spores it has been found possible[17,18] to reduce the discrepancy between the total and colony

counts of some organisms by heat and chemical stimulation of the spores. As yet such treatment has not been attempted. However, the cause of this discrepancy should be investigated before these test organisms are used in fungicidal experiments designed to correlate uptake of fungicide and rate of kill.

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