*SPORE GERMINATION IN DICTYOSTELIUM DISCOIDEUM**

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Synchronous germination is essential for the study of enzymes involved in spore germination. The present work describes some properties of the spores of *Dictyostelium discoideum*, and methods that have been developed to induce rapid and synchronous germination of these on simple media.

The first quantitative study of spore germination in the Acrasieae was conducted by Russell and Bonner.[1] Germination in *Dictyostelium mucoroides* was found to reach 98 per cent after 4 hr at 22°C in the dark on a medium containing: 1 per cent glucose, 1 per cent Bacto peptone, 0.096 per cent $Na_2HPO_4 \cdot 12H_2O$, 0.145 per cent KH_2PO_4, and 2 per cent Bacto agar. However, spores germinated at a high level only when maximum moisture was present and when the concentration of spores was less than 33 per mm^2 of agar surface. Germination was very poor on nutrient-free washed agar.

Materials and Methods.—Dictyostelium discoideum, strain NC-4, was grown together with *Escherichia coli*, strain B/r, on fresh plates of a medium containing: 0.2% Bacto peptone, 0.2% glucose, 0.2% Bacto yeast extract, 2% Bacto agar, and 10 mM potassium phosphate buffer at pH 6.5. The spore size was found to be more uniform on this than on several other media tested, the average being 8×3 μ. The number of spores inoculated per plate was 10^4. This number when suspended in 1-ml of a dilute bacterial suspension, produced uniform growth and sorocarp formation on the above agar medium.[2]

Spores were harvested by removing sori from stock cultures of known age by drawing an inoculating loop or a glass slide across the mature fructification at the level of the spore masses or sori. The spores were suspended in 1 ml of 10 mM potassium phosphate buffer at pH 6.5 and washed by shaking with a Vortex Junior mixer for 1 min. The spores were then centrifuged at 400 *g* for 5 min. The supernate was discarded and the spores were again suspended and washed in 1 ml of buffer. A second centrifugation was performed, after which the spores were resuspended in 5 ml of fresh buffer. The above procedure was necessary because spores that remained in clumps frequently did not germinate when plated. If the spores were to be germinated on an agar surface, this last suspension was counted with a Bright-line hemocytometer. The concentration of spores was adjusted so that the buffered suspension contained 2×10^4 spores/ml. A 0.5-ml aliquot of spore suspension was spread on each of the test agar plates so that the concentration of spores was 2.6/mm^2 of agar surface. A band of agar 0.5-cm wide at the edge of the plastic culture plates did not receive spores.

If the spores were to be germinated in a flask containing a magnetic stirring bar, the concentration of spores was determined after the spores were placed in the flask. The final concentration of spores in flasks was from 1×10^4 to 1×10^5 per ml. Higher concentrations resulted in inhibition of germination in some media. The stirring bars were rotated at 120 rpm. Incubation on agar plates or in flasks was at 25°C.

Spores to be heat-shocked were prepared in a similar way. These were heat-shocked in 15-ml centrifuge tubes for germination tests in flasks, or shocked in 10 by 1-cm tubes for germination tests on agar plates. The former tubes contained 5 ml of spore suspension while the latter contained 0.5 ml. Heat shocking was done in a Labline water bath which varied 0.25°C above or below the set mark. After heat shocking, the spores were quickly cooled to room temperature before being dispensed onto plates or into flasks.

All experiments were performed in the light since light versus dark experiments failed to show any difference in final amounts of germination.[3]

Plates were removed from the lighted 25°C incubator at various times and assayed for percentage germination in the following way. Air was blown across the agar to dry the surface and

to cause the myxamoebae to round up. The agar surface was then scanned in parallel strips with an AO Spencer microscope set at 200× until 100 objects had been counted. This represented 1% of the spore population on the plate and also approximately 1% of the total agar surface. A spore was considered to have germinated when an emerged myxamoeba was counted. Myxamoeba emergence in these plate experiments was thus equivalent to outgrowth in bacterial spores, or to germ tube formation in fungal spores.

In the case of spores placed in flasks, the process of germination was broken down into three phases: activation, swelling, and emergence. A 0.1-ml sample of spore suspension was removed from a flask and sandwiched between a cover glass and a glass slide. The microscope was set at 450× and 100 objects were counted in each determination. The objects were placed into three groups: dormant spores, swollen spores, and emerged myxamoebae.

The basic medium used in these studies consisted of 10 mM potassium phosphate buffer pH 6.5 solidified with 2% Difco purified agar. All other media were made using this buffered agar with additions, unless stated otherwise. All media requiring adjustment of pH were made and then adjusted to pH 6.5 with KOH, after which the agar was added.

Excellent germination was obtained with a tryptic digest of casein, "N-Z-Case Amine." Amino acid composition of this peptone was supplied by the Sheffield Chemical Company[4] and served as a basis for medium A. Medium A contained the basic buffer agar plus the following amino acids in grams per 100 ml of medium. Filtration of the amino acid solution was necessary before addition of the agar.

Glycine	0.0180	L-Aspartic	0.0590
L-Alanine	0.0330	L-Glutamic	0.2200
L-Valine	0.0550	L-Proline	0.1060
L-Leucine	0.1030	L-Histidine	0.0305
L-Isoleucine	0.0450	L-Tryptophan	0.0140
L-Serine	0.0670	L-Phenylalanine	0.0460
L-Threonine	0.0370	L-Tyrosine	0.0350
L-Lysine	0.0650	L-Cystine	0.0051
L-Arginine	0.0360	L-Methionine	0.0235

A series of media numbered I, II, and III were then prepared using amino acids in 10 mM quantities in the usual buffer agar.

Medium I, pH 6.5 without adjustment		Medium II, adjusted to pH 6.5 with KOH		Medium III, filtration necessary, pH 6.5 without adjustment	
Glycine	10 mM	L-Lysine	10 mM	L-Tryptophan	10 mM
L-Alanine	10 mM	L-Arginine	10 mM	L-Phenylalanine	10 mM
L-Valine	10 mM	L-Aspartic	10 mM	L-Tyrosine	saturated at 20°C
L-Leucine	10 mM	L-Glutamic	10 mM		
L-Isoleucine	10 mM	L-Proline	10 mM	L-Cystine	saturated at 20°C
L-Serine	10 mM	L-Histidine	10 mM		
L-Threonine	10 mM			L-Methionine	10 mM

A medium containing vitamins (vitamin medium) was prepared by adding the following to the buffer agar base: 10 μg pyridoxine hydrochloride/ml, 0.05 μg biotin/ml, 10 μg thiamine hydrochloride/ml, 40 μg nicotinamide/ml, and 10 μg riboflavin/ml.

Media containing buffer agar base plus 10 mM L-tryptophan, 10 mM L-phenylalanine, and 10 mM L-methionine were designated 10 mM L-try, phe, met. Other media will be more easily explained in connection with the experiments where these were employed.

All agar media used in this study were liqufied by steaming for 30 min in an Arnold steam sterilizer, and cooled to 45°C before plating. Each plastic Petri dish received 20 ml of media.

Results.—All experiments showed that increasing the spore concentration decreased the final amounts of germination except when these were placed on 1 per cent peptone agar plates. No difference in spore germination capacity was found in spores between 1 and 10 days of age. Figure 1 shows progressive stages in the process of germination as it occurs in liquid culture and on agar. Figure 2 shows

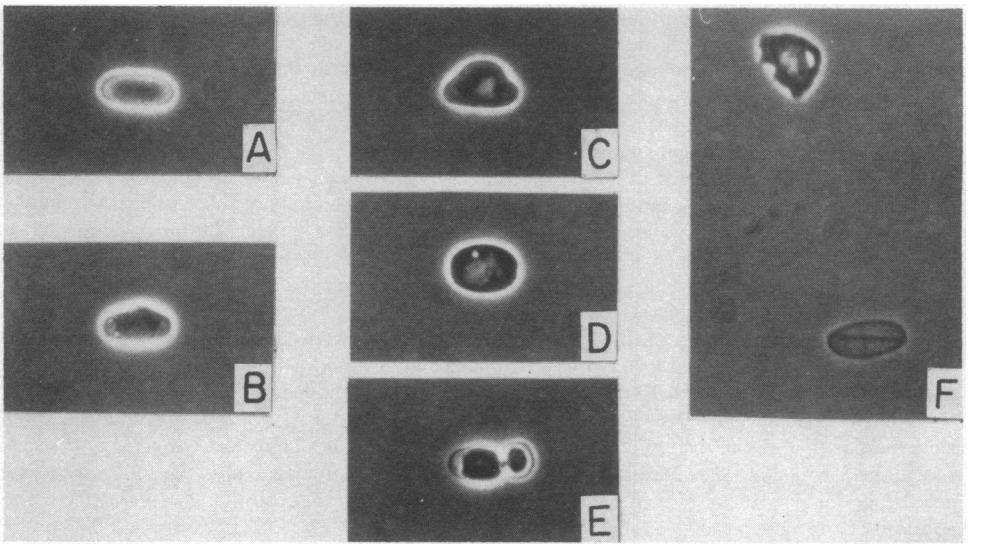

FIG. 1.—Sequence of changes observed during spore germination. ×800. Phase microscopy. (*A*) Dormant spore, 8 μ long; (*B–D*) early, intermediate, and late swelling, respectively; (*E*) emergence of the myxamoeba; (*F*) postemergence. The myxamoeba has moved away from the empty spore case.

that a medium similar to that of Russell and Bonner[1] produced germination kinetics in *D. discoideum* that resemble those of *D. mucoroides* as reported by these authors. It is seen that glucose and phosphate are not needed for germination. Figure 3 presents the kinetics of the swelling and emergence stages of *D. discoideum* spores in liquid culture. Table 1 and Figure 4 demonstrate that other peptone mixtures such as 1 per cent N-Z-Case Amine permit high germination in a syn-

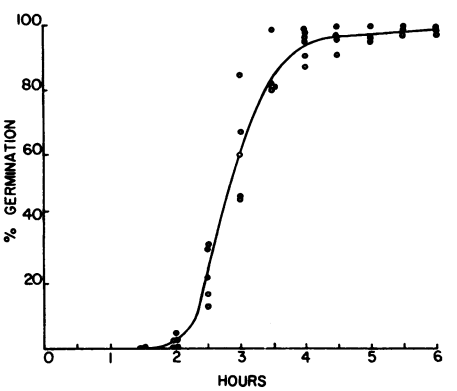

FIG. 2.—The kinetics of spore germination on agar media with and without glucose and phosphate. Closed circles represent germination on media containing 1% peptone, 1% glucose, and 10 mM phosphate buffer (modified Bonner's agar). Open circles represent germination on agar media containing 1% peptone without phosphate or glucose. Three experiments were performed on each medium with spores of different ages.

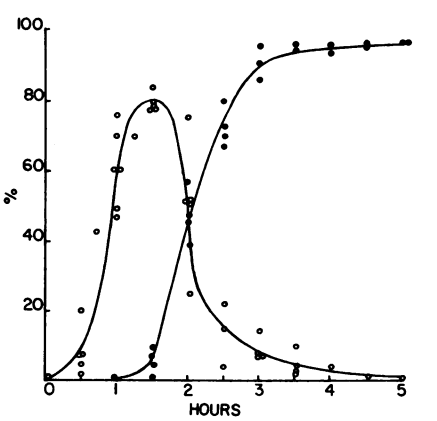

FIG. 3.—The germination kinetics of spores incubated in 1% peptone buffer solution. A total of seven experiments is presented. Germination is broken down into two stages: open circles represent spores during the swollen state, and closed circles represent myxamoebae following their emergence.

TABLE 1

COMPARISON OF SPORE GERMINATION ON MEDIA ENRICHED WITH PEPTONES OR GLUCOSE AND ON BUFFER AGAR ALONE

Time of assay (hr)	Agar medium used	No. of determinations	Mean per cent germination	Standard deviation
7	1% N-Z-Case Amine	10	98.3	4.3
7	1% Bacto peptone	61	98.4	—
8	1% Bacto peptone	8	98.6	0.8
7	0.05% Bacto-peptone	4	66.8	5.0
7	1% glucose	5	0.0	—
14	1% glucose	2	1.0	0.0
7	Buffer	61	0.08	0.002

TABLE 2

GERMINATION OF SPORES ON COMPONENT PARTS OF PEPTONES

ime of assay (hr)	Agar medium used	No. of determinations	Mean per cent germination	Standard deviation
7	Medium A	5	67.6	1.2
7	Vitamin medium	5	0.4	0.4
7	1 gm Ashed peptone	5	2.3	0.1

chronous manner. Table 1 also indicates that the peptone fraction of Russell and Bonner's medium[1] was responsible for the germination reported. Because 1 per cent Bacto peptone in buffer agar always yielded high levels of germination, and buffer agar without peptone supported very low levels, the two media were used as controls in all experiments. Difco purified agar was used in all experiments since Bacto agar produced variable results.

When the component parts of peptones were tested (exclusive of peptides), it was found that the amino acid fraction was mainly responsible for germination (Table 2). It was next demonstrated that only one group of the amino acids in an arbitrary mixture of these promoted spore germination (Table 3). The data show that some inhibition occurred when all three groups of amino acids were present together in 10 mM concentration.

Experiments with a medium containing buffer agar and 10 mM L-tryptophan, 10 mM L-phenylalanine, and 10 mM L-methionine (10 mM L-try, phe, met) revealed that most of the potential to induce germination among the five amino acids in medium III resided in these three (Table 4). Also, it should be noted that a racemic mixture of tryptophan, phenylalanine, and methionine produced the same amount of germination as the L enantiomers. Table 4 reveals, however, that the D enantiomers of these three amino acids produced less germination than the L enantiomers. Attempts to increase the final amount of germination on media containing tryptophan, phenylalanine, and methionine by increasing the concentration of all three amino acids failed, as shown in Table 4. Use of only two of the three amino acids showed a further loss in the final amount of germination (Table 5). No single amino acid in 10

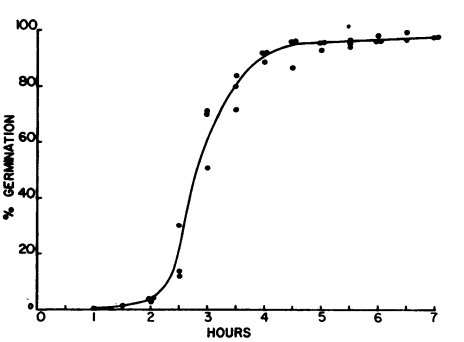

FIG. 4.—Germination kinetics of spores on buffer agar containing 1% N-Z-Case Amine. Results of three experiments with spores of different ages are shown.

TABLE 3

SPORE GERMINATION ON DIFFERENT MIXTURES OF AMINO ACIDS

Time of assay (hr)	Agar medium used	No. of determinations	Mean per cent germination	Standard deviation
7	Medium (I, II, III)	5	47.2	1.5
7	Medium I	10	0.1	—
24	Medium I	5	2.0	0.2
7	Medium II	10	0.1	—
24	Medium II	10	0.6	0.3
7	Medium I, II	10	0.0	—
7	Medium III	12	67.7	2.2
24	Medium III	5	92.8	1.6

TABLE 4

SPORE GERMINATION ON MIXTURES OF TRYPTOPHAN, PHENYLALANINE, AND METHIONINE

Time of assay (hr)	Agar medium used	No. of determinations	Mean per cent germination	Standard deviation
7	100 mM L	3	0.0	—
7	50 mM L	3	0.7	0.2
7	10 mM L	25	55.8	2.2
24	10 mM L	5	84.8	1.1
34	10 mM L	1	90.0	—
7	5 mM L	3	51.3	2.0
7	10 mM DL	5	54.4	3.7
34	10 mM DL	1	86.0	—
7	10 mM D	5	28.0	4.1
24	10 mM D	5	57.6	1.9

TABLE 5

SPORE GERMINATION ON MIXTURES OF TWO AMINO ACIDS

Time of assay (hr)	Agar medium used	No. of determinations	Mean per cent germination	Standard deviation
7	10 mM L-try, phe	2	7.5	0.5
7	10 mM L-try, met	2	20.0	2.0
7	10 mM L-phe, met	2	0.5	0.5

mM concentration supported more than 5 per cent germination after 7 hr of incubation at 25°C.[3]

Chelating agents such as EDTA, citrate, and glycylglycine failed to induce germination when incorporated into the buffer agar medium.

Experiments were performed to determine if heat shocking of spores would reduce the requirement for external activating molecules. Figure 5 summarizes experiments in which spores were heat-shocked in buffer and then plated on various media. The 1 per cent peptone and buffer agars were included primarily as controls. However, it was found that heat shocking for 15 min at 45°C increased the amount of germination on buffer agar from 0 per cent to 65 per cent. Heat shocking of spores also increases the amount of germination on dilute peptone medium and on 10 mM L-try, phe, met medium when compared to the 25°C controls with unheated spores (Fig. 5). Thus, it is clear that spores shocked at 45°C achieve a higher final amount of germination than do nonshocked spores. Experiments to determine the optimal duration of heat shock at 45°C showed that from 20 to 30 min was best, as may be seen in Figure 6. Spores shocked longer than 30 min showed some inactivation when plated on 1 per cent peptone medium and on buffer medium. Figure 7 presents the germination kinetics of spores shocked 30 min at 45°C. Spores which were heat-shocked and placed in flasks of buffer produced at least 60 per cent ger-

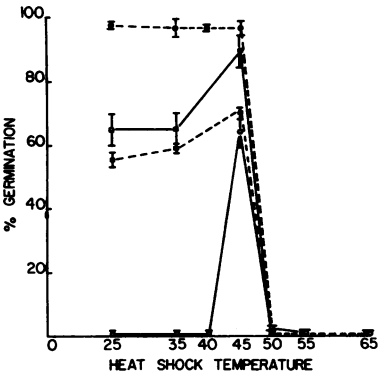

FIG. 5.—Germination of spores heat-shocked at various temperatures for 15 min. The per cent germination was measured after 7 hr incubation at 25°C. The mean and standard deviation for seven experiments is presented. Closed circles and dotted line is for 1% peptone buffer agar. Open squares and solid line is for 0.05% peptone buffer agar. Solid squares with dotted line is for 10 mM L-try, phe, met agar. Open circles with solid line is for buffer agar.

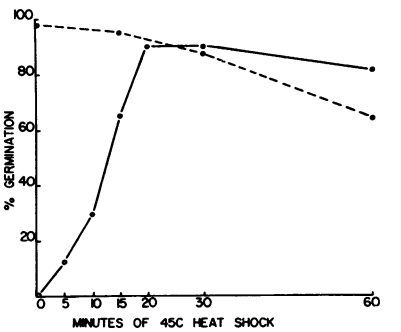

FIG. 6.—Germination of spores heat-shocked at 45°C for different time periods. The spores were plated on media and scored for germination after 7 hr incubation at 25°C. Closed circles and dotted line is for 1% peptone buffer agar. Open circles with solid line is for buffer agar. The average of three experiments is presented in each case.

mination after 7 hr of incubation at 25°C. The exact percentage was difficult to determine because of clumping of the emerged myxamoebae.

Studies with inhibitors such as Acti-dione, which prevents protein synthesis in other stages of development,[5] show inhibition of the germination process. In liquid culture using 1 per cent peptone with 250 μg Acti-dione/ml, the spores swell normally, but myxamoebae do not emerge from the spores even after the controls show more than 95 per cent emergence. The inhibition is reversible, however, since myxamoebae emerged, leaving the empty spore cases, when the swollen Acti-dione-treated spores were transferred to distilled water or phosphate buffer. Heat-shocked spores respond in a similar manner when exposed to Acti-dione. The exact mechanism of inhibition is presently under investigation.

Actinomycin D in the dark at 250 μg/ml had no effect on the germination of spores whether activated by peptone or heat shock. Finally, the specific activity of the enzyme trehalase increases during the germination process.[6]

Discussion.—From the simple experiments described above there appear to be at least three stages in the process of spore germination in *Dicytostelium discoideum:* activation, swelling, and emergence. The activation stage may be defined as that stage from the time the spores are exposed to the activating event until the first sign of swelling. In the swelling stage the initial lateral protuberance of the spore (Fig. 1*B*) enlarges until the entire spore is swollen (Fig. 1*D*). A loss in refractility also occurs during the swelling stage. The latter part of this stage is characterized by the appearance of granules and one or more contractile vacuoles (Fig. 1*D*). The swelling stage ends with the longitudinal splitting of the spore case (Figs. 1*E–F*).[7] The emergence phase lasts but a short time during which the myxamoeba escapes from its spore case.

The process of spore germination can be triggered by amino acids and/or heat

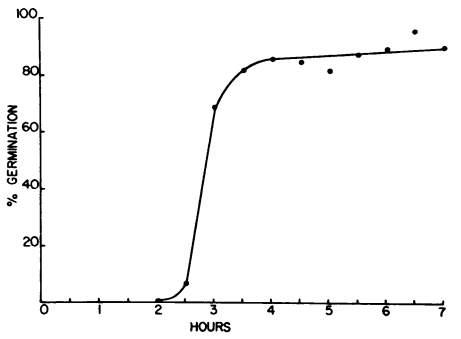

FIG. 7.—The germination kinetics of spores shocked 30 min at 45°C. The spores were plated on buffer agar.

shock. Once the swelling stage is under way, no addition of substances is required for the completion of the germination process. This was demonstrated by experiments in which peptone-activated spores in the swollen stage were transferred to buffer. The swollen spores completed the process of germination. Also, heat-activated spores germinated in buffer without the presence of added sources of carbon or nitrogen. These results support the hypothesis that some internal source of energy, possibly trehalose, is used during the germination process.[8] At any rate, the enzyme trehalase increases its specific activity during the germination process.

The fact that the enzyme trehalase and heat-shock activation both have optima of 45°C may mean that there is a connection between the two.[9, 3] However, 45°C may just be the maximum temperature that any enzyme can tolerate in the slime molds before denaturation begins to occur. It has been shown by Blaskovics and Raper[10] that myxamoebae of *D. mucoroides* are destroyed at 42°C.

Heat-shock activation could conceivably involve amino acids, if in the process of heating some spores die and release internal components into the medium. However, no experiments thus far have revealed the presence of ninhydrin-positive materials in the wash water after heat shocking.

It is probable that substances other than the few amino acids shown to induce germination in this study would produce positive results if the proper concentrations were used. This appears likely from the results which show that the concentrations of the amino acids greatly affect the percentage of germination. Spore germination resulting from the presence of the D enantiomers of tryptophan, phenylalanine, and methionine seems to infer that the process of germination is not totally steriospecific. This suggests that a racemase may be involved during amino acid activation of the spores. The presence of a racemase in the spores might explain the almost identical germination produced with 10 mM L-try, phe, met medium and 10 mM DL-try, phe, met medium. An alternate hypothesis for the germination obtained with L or DL enantiomers of amino acids is that they act as chelating agents. However, experiments with EDTA and other chelating agents failed to elicit any detectable response. As in all spore systems, much more work needs to be done before a working hypothesis can be advanced for the sequence of biochemical events that lead to spore activation and subsequent germination.

Dembitzer[11] has demonstrated that spores of *D. discoideum* contain a cellulose wall 0.1 μ thick. The breaching of this barrier may require a cellulase. Acti-dione inhibition of the synthesis of this enzyme along with other proteins in the swelling stage might explain the failure of swollen spores to enter the final stage of germination.

Earlier investigations have presented evidence for and against the hypothesis that *de novo* protein synthesis controls differentiation in the cellular slime molds.[12–14]

It is hoped that the systems for spore germination developed and described herein may aid in resolving this question.

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