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Factors Affecting the Rate of Heat-induced Spore Germination in *Dictyostelium discoideum*[1]

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Washed spores of *Dictyostelium discoideum*, strains NC-4H, NC-4D, and V-12, germinated rapidly after being heat shocked at or near 45.0 C for 30 min. Cultures of the slime molds were grown in association with *Escherichia coli* B/r as the host bacterium; spores taken from plates of synthetic medium had a higher final germination value than spores from complex medium containing peptone and yeast extract. Young spores germinated more rapidly than older spores. Optimal germination occurred between *p*H 6.0 and 7.0, and, of the buffers tested, potassium phosphate allowed the most rapid germination. After heat shocking, spores were diluted into fresh oxygenated buffer to provide enough oxygen for completion of germination. Germination occurred most rapidly between incubation temperatures of 22 and 25 C.

Washed dormant spores of *Dictyostelium discoideum* Raper can be induced to germinate by plating them on agar media containing peptones or amino acids (4; Cotter, M.S. Thesis, Univ. of Wisconsin, Madison, 1965; Cotter, Bacteriol. Proc., p. 17, 1966). Mild heat shock also stimulates germination of *D. discoideum* spores (4; Cotter, M.S. Thesis).

This communication reports the results of further investigations that define more precisely optimal conditions for obtaining relatively high germination rates with mild heating.

MATERIALS AND METHODS

Production of spores. Many students of the cellular slime molds have observed that media low in nutrients, although not optimal for the bacterial host, are required by many slime molds for optimal growth and fruiting.

The first medium used in this study was diluted from its original concentration so that it contained the following components: 0.2% peptone (Difco), 0.2% glucose, 0.2% yeast extract (Difco), 2% agar (Difco), and 10 mM potassium phosphate buffer at *p*H 6.5. This agar medium was called 0.2% PGY by Cotter and Raper (4).

The exact reason(s) why rich media often repress growth and normal fruiting of the slime molds is not known. It cannot be assumed that the cellular slime molds use only the bacterial cells and not some of the soluble components of the medium as nutrients for growth and development. However, it is known that certain products of bacterial growth, if in excess, render media unsuitable for slime mold growth. Thus, a second medium, originally devised by Adams (1) for bacteriophage study, was tried for simultaneous growth of *Escherichia coli* B/r and *D. discoideum*. This balanced medium, which will be termed synthetic medium throughout the text, was used to achieve rich bacterial growth without the production of a high background of inhibitory products or unused nutrients. The composition of the synthetic medium follows: NH_4Cl, 1.0 g; $MgSO_4$, 0.13 g; KH_2PO_4, 3.0 g; Na_2HPO_4, 6.0 g; glucose, 4.0 g; and distilled water, 1,000 ml. Difco agar at 1.5% was added when a solid substrate was desired, in which case the glucose was added just before the plates were poured. The *p*H of the medium was 6.5 before and after growth. The medium was used in liquid form for the growth of *E. coli* B/r alone; for the production of vegetative myxamoebae the bacteria and slime mold were grown together in shaken flasks.

Spores of *D. discoideum* used in the germination studies were obtained from stock cultures prepared as follows: 1.0-ml samples of a mixed suspension of host bacteria from liquid synthetic medium and 10^4 nonshocked spores were spread onto plates of synthetic medium which were then incubated in the light at 25 C. The large number of spores used as inoculum produced an even growth of vegetative myxamoebae, and, subsequently, an even distribution of fruiting structures occurred over the entire surface of the plate. Migration of the pseudoplasmodia was minimized because of the relatively high ionic strength of the medium. Synchronous fruiting was necessary so that all the newly produced spores would be of the same age. The usual incubation time required for fruiting was 3.5 days. The stock cultures were stored at 25 C until the spores were to be harvested.

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Preparation of the spores for heat shocking. The sorocarps were allowed to age for at least 1 day at 25 C before the spores were harvested. All operations were performed at room temperature because early work had shown that preparation of the spores at low temperature caused suboptimal germination rates. A similar slowdown in germination rate for peptone-activated spores has also been noted by other investigators (3).

The spores were removed from the sori by holding a glass slide endwise at the level of the sori and rotating the petri dish. During this operation, we proceeded with care to avoid picking up stalks. This procedure was more difficult for the diploid variety of *D. discoideum*, NC-4D, than for the two haploid varieties (A. T. Weber, M.S. Thesis, Univ. of Wisconsin, Madison, 1967). The spores were washed from the slides into a beaker of distilled water; the resulting spore suspension was then passed through cheesecloth, which allowed passage of single spores but retained most of the spore clumps and any stalk material present. It was then placed in a 15-ml centrifuge tube which was touched to a Vortex Junior mixer to break up any remaining spore clumps and to wash the spores. This suspension was then centrifuged at 1,000 × *g* for 5 min to sediment the spores and to remove any water-soluble inhibitor(s) (2, 3, 7). The supernatant fraction was saved for studies of possible inhibitors, and the spores were suspended in 15 ml of 10 mM potassium phosphate buffer, *p*H 6.5. The spores were then washed twice by centrifugation in the above buffer and finally were suspended in 5 ml of the same buffer for heat shocking. The top and sides of the centrifuge tube above the liquid level were wiped with tissue to remove any spores that might not undergo heat shocking. The spore suspension was then shocked, generally at 45.0 C for 30 min in a Precision Freas model 161 water bath in which the temperature was controlled to ±0.02 C.

Methods of incubation after heat shocking. The shocked spores were then usually pipetted into 10 mM potassium phosphate buffer, *p*H 6.5, contained in beakers, in flasks, or in the 10 × 1 cm tubes used in the Bausch & Lomb colorimeter (Spectronic-20). This dilution procedure cooled the spores and also resulted in a final concentration of from 10^6 to 10^7 spores per ml, as measured by a Bright-line hemacytometer. Higher spore concentrations did not germinate rapidly because of oxygen depletion (Cotter, Ph.D. Thesis, Univ. of Wisconsin, Madison, 1967). The beakers, flasks, or tubes contained small magnetic stirring bars which were spun just fast enough to prevent the spores from settling. The object of the stirring was to provide some gaseous exchange into and out of the medium without causing destruction of the newly emerged myxamoebae. This gentle stirring did not prevent clumping of the myxamoebae which emerged from the swollen spores.

Flasks on rotary shakers could also be used, but these were not generally employed since the shaker had to be stopped for frequent sampling.

The spore suspensions were usually incubated at temperatures between 22 and 25 C for periods up to 5 hr.

In certain experiments, 0.5 ml of spore suspension was evenly plated onto buffered agar (10 mM potassium phosphate, *p*H 6.5; 2% Difco purified agar). Incubation of the plates was at 24 C.

Scoring germination. The various stages of germination (4) were determined by removing 1 ml of the spore suspension from the incubation vessel. The sample was pipetted into a small tube which was stirred with a Vortex Junior mixer for 30 sec to break up clumps of spores and myxamoebae; these clumps usually formed after 3 hr of incubation when the temperature was between 22 and 25 C. Then, 0.05 ml of this dispersed spore suspension was removed from the tube, placed on a glass slide, and examined under a glass cover slip. An AO Spencer microscope equipped with a 43 × objective and 15 × eyepieces was used to count at least 200 objects. Counting took less than 3 min. The objects were divided into three groups: morphologically dormant spores, swollen spores, and emerged myxamoebae (Fig. 1).

In the plate experiments, the germinating spores were examined on the buffered agar at a magnification of 645 × and then were divided into only two groups, myxamoebae and spores.

A spore was considered to have germinated completely (emergence) only when a viable myxamoeba was counted. Empty spore cases were not counted because they do not necessarily represent living myxamoebae. Myxamoeba emergence in these experiments was thus equivalent to outgrowth in a bacterial spore or to germ tube formation in a fungal spore.

RESULTS AND DISCUSSION

Morphogenetic capability of emergent myxamoebae. The myxamoebae produced during these germination studies were viable and capable of aggregating when they were placed on buffered agar, despite their smaller dimensions and an absence of prefeeding. The times required for aggregation were identical to those required for vegetative myxamoebae grown in liquid synthetic medium with *E. coli* B/r. Aggregation centers were formed about 2.5 hr after the myxamoebae were deposited onto buffered agar, and sorocarps of normal pattern were formed within 18 hr. A majority of the spores from such sorocarps were able to germinate after heat shocking.

Type of buffering compound and its effect upon germination. Spore germination in *D. discoideum* depends, to some extent, upon the solution in which the spores are suspended during and after heat shocking (Table 1). Of course, germination does not occur at all under these conditions unless the spores are shocked (4). Oxygenated distilled water permitted high germination but allowed clumping of the spores. This nonspecific clumping, i.e., in the absence of myxamoebae, made it difficult to determine the amount of germination, since agitation of the culture with a Vortex Jr. mixer failed to separate the clumps into single spores.

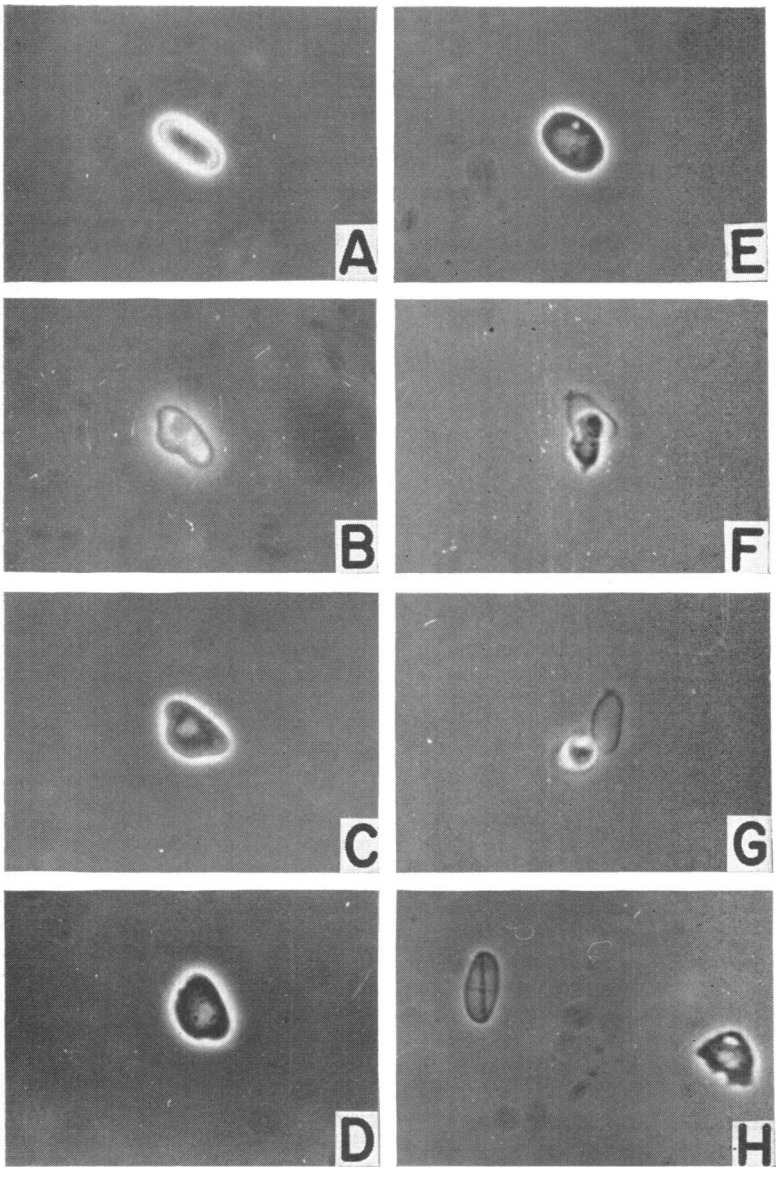

FIG. 1. *Sequence of changes observed during spore germination in Dictyostelium discoideum, strain NC-4H. (A) Dormant spore, 8 μ long; (B–E) early, intermediate, and late swelling; (F) emergence of the myxamoeba; (G–H) postemergence. In (H) the myxamoeba has moved away from the empty spore case. ×1,000. Phase microscopy.*

When less than 10 mM potassium phosphate buffer was used, clumping again occurred, sometimes as soon as incubation began. A buffer solution containing 10 mM potassium phosphate, *p*H 6.5, produced high germination without nonspecific spore clumping. Higher concentrations of phosphate (50 mM) seemed to inhibit the germination process (Cotter, M.S. Thesis, Univ. of Wisconsin, Madison, 1965).

The use of tris(hydroxymethyl)aminomethane (Tris) adjusted to *p*H 7.0 with HCl did not allow as high germination as phosphate buffer. In addition, nonspecific clumping occurred in the Tris-chloride buffer.

Potassium citrate (*p*H 6.5) and sodium pyrophosphate (*p*H 7.0) did not allow emergence of myxamoebae from swollen spores until after 5 hr of incubation. The slow release of myxamoebae

TABLE 1. *Spore germination in liquid culture as a function of the suspending medium*[a]

Suspending medium	Percentage of swollen spores	Percentage of emerged myxamoebae
Distilled water	8.0	90.0
Potassium phosphate, 5 mM	10.0	88.0
Potassium phosphate, 10 mM	8.0	90.0
Tris-chloride, 10 mM	40.0	58.0
Potassium citrate, 10 mM	96.0	0.0
Sodium pyrophosphate, 10 mM	80.0	0.0

[a] The spores were placed in the above media and shocked at 45.0 C for 30 min. Subsequent incubation was at 25 C, and percentage of germination was determined 5 hr after dilution.

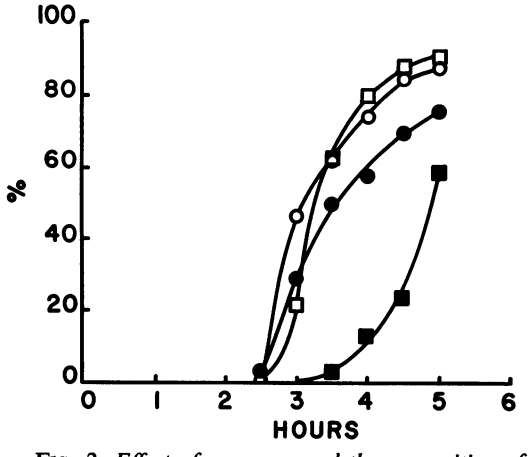

FIG. 2. *Effect of spore age and the composition of the growth medium on the rate (%) of emergence of myxamoebae from swollen spores. Symbols: ○, 1-day-old spores produced on synthetic medium agar; ●, 4-day-old spores produced on synthetic medium agar; □, 1-day-old spores produced on 0.2% PGY agar; and ■, 4-day-old spores produced on 0.2% PGY agar. Incubation temperature, 25 C.*

was not a result of *p*H change, although these compounds had little buffering capacity in the *p*H range used.

Effect of spore age and growth medium on the rate of spore germination. A dramatic effect was produced when the spores for germination tests were obtained from two contrasting growth media. Young spores (1 day old) from the two different media had similar germination rates after they were heat shocked at 45 C for 30 min. However, 4-day-old spores showed a difference in the rate of germination, depending upon the medium on which they were produced (Fig. 2). Figure 3 presents a summary of experiments with spores produced on 0.2% PGY agar and compares them to those spores produced on synthetic medium. The final amount of spore swelling, as well as the final amount of myxamoebae emergence, is greater for spores produced on synthetic medium. Cycloheximide was used to allow the normal swelling rate while preventing myxamoeba emergence (4).

Beyond this point, most experiments were conducted with spores produced on synthetic medium that had not aged for more than 3 days. Accumulated evidence had shown that these conditions of age and substrate provided spores that were optimal for rapid germination.

For the production of myxamoebae, liquid synthetic medium was superior to 0.2% PGY medium. With the latter medium, considerable numbers of the host bacteria still remained, as a rule, along with the vegetative myxamoebae at the end of their growth period. However, with synthetic medium, very few bacteria were observed in the culture at the stationary phase of myxamoeba growth. No other liquid nutrient medium investigated in this laboratory produced similar results.

For studies of cell aggregation, also, the presence of few bacteria after growth of the

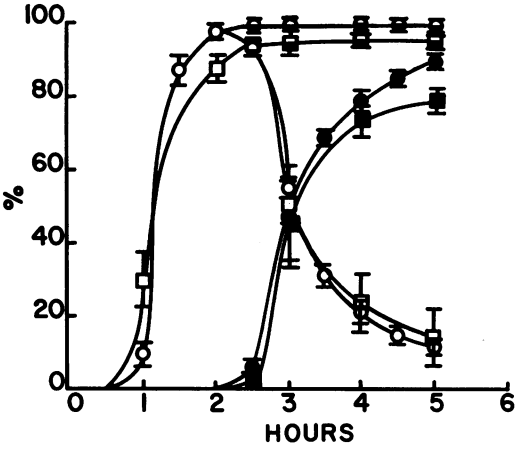

FIG. 3. *Germination of 1- to 3-day-old spores from synthetic medium agar compared to spores from 0.2% PGY agar. The incubation temperature after heat shocking was 25 C. The mean and standard deviation above and below this mean for 10 experiments are plotted. Symbols: spores from synthetic medium are represented by circles, while spores from 0.2% PGY medium are represented by squares. Without cycloheximide; open symbols, percentage of the spore populations which is swollen; closed symbols, percentage of the spore populations present as myxamoebae. With 100 μg of cycloheximide per ml: half-closed symbols, percentage of the spore populations which is swollen.*

myxamoebae is a distinct advantage, since only one low-speed washing is needed to separate most of the residual bacteria from the myxamoebae.

Influence of pH on spore germination. Several experiments demonstrated that the rate, as well as the final amount of germination, was the same when dormant spores were heat shocked and incubated at *p*H 6.0, 6.5, and 7.0. When *p*H below 6.0 or above 7.0 was used for heat shocking and incubation, the rate and final amount of germination were reduced. Thus, the *p*H range, which was optimal for spore germination, was similar to that for growth (6).

Activation occurred at a low *p*H, although spore swelling and myxamoeba emergence were slowed down. The results of an experiment in which spores were heat shocked at *p*H 4.0 are presented in Fig. 4. Incubation was at 25 C. The initial reaction was *p*H 4.0, and at various time intervals the *p*H was adjusted to 6.5 with KOH. Although a small amount of swelling occurred at *p*H 4.0, rapid swelling occurred only after *p*H adjustment to 6.5.

Optimal temperature for heat shocking. It has been shown that temperatures of 40.0 and 50.0 C for 15 min did not result in germination when plated spores were examined after 7 hr of incubation at 25 C (4). A recheck of heat shocking at 50.0 C for 30 min showed that spores not only were activated but also were damaged at this temperature. Swelling of the spores and some emergence of myxamoebae from the swollen spores occurred, but this action took place much later than usual (Table 2). A temperature of 55.0 C for 30 min caused death of the spores, since they never germinated after plating and incubation at 25 C.

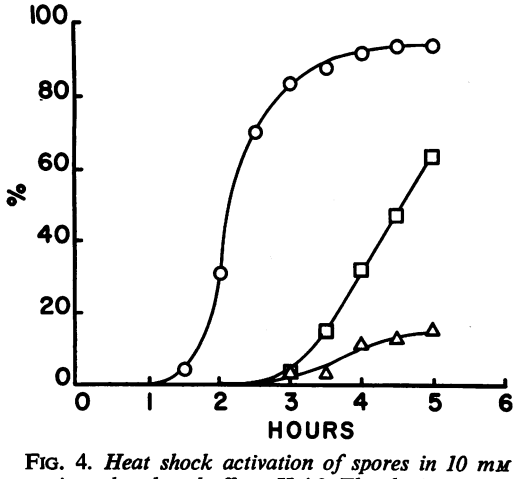

FIG. 4. *Heat shock activation of spores in 10 mM potassium phosphate buffer, pH 4.0. The abscissa refers to the time after shocking. Symbols: ○, percentage of swollen spores plus free myxamoebae when the pH was adjusted to 6.5 directly after heat shocking; □, percentage of swollen spores when the pH was adjusted to 6.5 after 3 hr of incubation at pH 4.0; △, percentage of swollen spores when the pH was maintained at 4.0.*

TABLE 2. *Comparison of the effects of different heat shock temperatures upon spore germination in Dictyostelium discoideum*[a]

Time after plating	Heat-shock temp	No. of determinations	Mean percentage of emergence of myxamoebae
hr	*C*		
10	25[b]	1	0.0
10	45.0	2	99.0
10	50.0	3	0.0
10	55.0	3	0.0
24	25[b]	1	0.5
24	45.0	2	99.0
24	50.0	3	17.0
24	55.0	3	0.0

[a] The spores were produced on synthetic medium and allowed to age for 2 days before harvesting. After heat shocking at the indicated temperatures for 30 min, the spores were plated on buffer agar and incubated at 24.0 C.

[b] Control.

Experiments with temperatures closer to 45.0 C showed that the optimal temperature range for heat shocking was very narrow. Some quantitative data that illustrate this point, i.e., for shocking temperatures very near 45.0 C, are given in Table 3. On the average, temperatures slightly below 45.0 C resulted in rapid spore swelling and rapid emergence of myxamoebae from the swollen spores, but not all the spores in the population were activated; thus, the final percentage of germination was reduced. The remaining spores did not swell. Temperatures slightly above 45.0 C caused a lower rate of swelling of the spores, and emergence of the myxamoebae was slower also. However, high levels of germination were obtained with extended incubation (24 hr), and the small number of ungerminated spores had swollen. The data also show that emergence is usually 2 to 3% higher on plates when compared to rate studies such as those conducted in liquid (Fig. 6).

Optimal temperature for incubation after shocking. In these experiments, the heat-shocking temperature was again 45.0 C for 30 min; but the incubation temperature was varied, and the percentage of germination was compared to that obtained at 25.0 C. The controls for these experiments are shown in Fig. 3 as the curve for germination of spores from synthetic medium at 25.0 C. Thus, an incubation temperature of 25.0 C resulted in a population containing 90%

myxamoebae 5 hr after heat shocking. The effect of incubation at 22.0 C was compared with that at 28.0 C (Fig. 5). The data suggest that high incubation temperatures favor rapid swelling of the heat-activated spores, but these temperatures cause a decrease in the rate of myxamoebae emergence from these swollen spores. At 22.0 C, after 5 hr of incubation, 95.5% of the spores had germinated to release myxamoebae.

A compromise incubation temperature of 23.5 C produced rapid and synchronous germination after heat shocking at 45.0 C for 30 min (Fig. 6). The kinetics of spore swelling resembled those at an incubation temperature of 25.0 C. The kinetics of myxamoeba emergence, however, more closely

TABLE 3. *Comparative effects upon germination of heat shocking spores of Dictyostelium discoideum at temperatures near 45.0 C*[a]

Time after plating	Spores heat shocked at	No. of determinations	Age of spores	Mean percentage of emergence of myxamoebae
hr	*C*		*days*	
4	44.5	3	2	88.8
4	45.0	3	2	75.2
4	45.5	3	2	58.8
4	46.0	1	2	21.0
5	44.0	1	1	91.0
5	44.5	1	1	94.0
5	45.0	1	1	99.0
7	44.0	2	1	90.7
7	44.5	2	1	94.0
7	45.0	2	1	98.8
24	44.0	3	2	94.3
24	44.5	3	2	99.1
24	45.0	3	2	99.5
24	45.5	2	2	99.8
24	46.0	2	2	98.0

[a] The spores were shocked for 30 min at the indicated temperatures, plated on buffer agar, and incubated at 24 C.

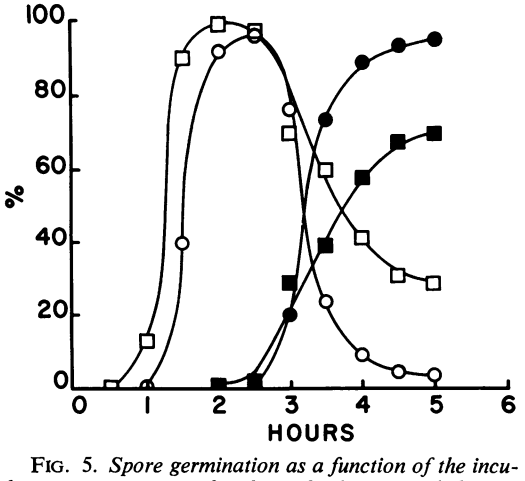

FIG. 5. *Spore germination as a function of the incubation temperature after heat shocking. Symbols:* ○, *percentage of swollen spores at 22.0 C;* ●, *percentage of myxamoebae at 22.0 C;* □, *percentage of swollen spores at 28.0 C;* ■, *percentage of myxamoebae at 28.0 C. For comparison of spore germination at 25 C see Fig. 3.*

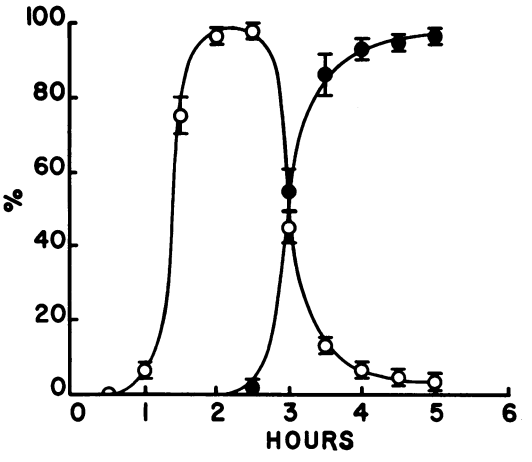

FIG. 6. *Spore germination as a function of the incubation temperature, 23.5 C. The mean and one standard deviation above and below the mean for six experiments are presented. Symbols:* ○, *percentage of the spore population which is swollen;* ●, *percentage of the population present as myxamoebae.*

TABLE 4. *Presence of bacteria during the germination of heat-shocked spores of Dictyostelium discoideum*[a]

Time after shocking	Percentage of myxamoebae present	Bacteria/ml	Bacteria/ml consumed by the myxamoebae (approximate)
hr			
0	0.0	2.4×10^2	0
1	0.0	2.6×10^2	0
2	0.0	2.3×10^2	0
3	37.5	2.3×10^2	0
4	75.0	1.6×10^2	1.0×10^2
5	89.0	0.8×10^2	1.8×10^2

[a] Two-day-old spores were heat shocked at 45.0 C for 30 min and then diluted with buffer to a final spore concentration of 3.9×10^6/ml; 20 ml of this suspension was incubated at 25.0 C. Viable bacteria were determined by plating 1 ml of suspension in nutrient agar and incubating at 35 C; this procedure would rapidly kill the myxamoebae. The triplicate plates were read after 2-day incubations, and the results were averaged.

resembled those obtained at 22.0 C. After 5 hr of incubation at 23.5 C, 96.5% of the heat-activated spores had germinated to myxamoebae.

Germination in various strains of D. discoideum. Every strain of *D. discoideum* tested responded to the heat shocking process. Strains NC-4H, NC-4D, V-12, and AC-4 from Raper's collection germinated rapidly after heat shocking. Strain B (from Maurice Sussman) and ACR-12 (also from Raper's collection) responded to the heat shocking, but these strains germinated more slowly than the preceding four strains. This topic will be covered more fully in a later paper in this series.

Bacterial contamination during spore germination. Bacterial contamination should be minimal for physiological studies during spore germination. This was not a problem in the spore suspensions employed in this study (Table 4). Only one bacterium was present for every 1.6×10^4 spores at time-zero. After 5 hr of incubation, about 180 bacteria could have been engulfed by the myxamoebae that emerged from the 89% of the spore population which germinated in this experiment. Assuming a Poisson distribution, each myxamoeba that ingested any bacteria engulfed only one. In this case, then, there were 1.9×10^4 myxamoebae which did not ingest any bacteria to every myxamoeba which did consume one bacterium. From the above data, we would hardly expect that the consumed bacteria would change the enzymatic pattern of the entire myxamoebae population enough to be detectable.

Emergent myxamoebae from washed spores should constitute ideal material for investigating macromolecular syntheses. For example, the problem of contaminating bacterial ribonucleic acid (RNA) which can mask slime mold RNA, as reported by Inselburg and Sussman (5) for myxamoebae produced by vegetative growth, would not arise.

ACKNOWLEDGMENTS

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