

**SPORE GERMINATION PROMOTER OF
Dictyostelium discoideum EXCRETED
BY *Aerobacter aerogenes***

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SUMMARY

The nutrient medium in which *Aerobacter aerogenes* was grown, contains a spore germination promoter (SGP) for the cellular slime mould *Dictyostelium discoideum*. SGP can cause synchronous spore germination in a short time, and triggers the germination process in just a few minutes. Germination-promoting capacity of SGP decreases as it comes in contact with increasing number of spores. When spores activated by SGP are stored at 4 °C, they gradually return to the dormant state. SGP is comparatively heat-stable, but is unstable at pH above 10 or under 3.

INTRODUCTION

When spores of the cellular slime mould, *Dictyostelium discoideum*, are cultured on nutrient agar with bacteria they germinate, feed on the bacteria by phagocytosis and proliferate by division. On exhaustion of the bacterial supply there is an interphase of several hours after which the cells aggregate to form pseudoplasmodia. Subsequently, each pseudoplasmodium produces a fruiting body composed of spores and stalk cells (Bonner, 1967; Olive, 1975). The aggregation is induced by a response of the surrounding cells to c-AMP that is secreted periodically by some cells. The c-AMP pulse is relayed by the surrounding cells; and response to this signal results in the formation of a multicellular aggregate. Such periodic behaviour continues until the formation of the fruiting body is completed (Robertson *et al.* 1971; Alcantara & Monk, 1974).

The spores of *D. discoideum* germinate under suitable conditions of temperature, moisture, air and nutrients. The spores become able to germinate when fruiting is complete (Cotter & Raper, 1966, 1968*a, b*). However, the spores contain a germination inhibitor (SGI) and, thus, the germination process must be triggered. Most of the spores do not germinate even when they are cultured on plain agar at optimum temperature after washing off the SGI in water. If the density of the spore population is high, spores do not germinate readily even when they are cultured with bacteria on nutrient agar, unless the SGI is washed away (Cohen & Ceccarini, 1967; Ceccarini & Cohen, 1967; Tanaka, Yanagisawa, Hashimoto & Yamaguchi, 1974; Tanaka, Hashimoto, Yanagisawa, Abe & Uchiyama, 1975).

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Synchronous germination of spores can be induced by subjecting them to heat shock (Cotter & Raper, 1968 *a*) or γ -ray irradiation (Hashimoto & Yanagisawa, 1970; Khoury, Deering, Levin & Altman, 1970; Hashimoto, 1971). It is believed that heat or γ -rays act on the mitochondria in the spore (Cotter, 1973). But initiation of germination by a heat shock of, for example, 45 °C for 30 min, or by γ -rays would be rare in natural circumstances. In the work on the isolation of SGI and other studies, the authors normally allowed spores to germinate in the presence of *Aerobacter aerogenes*. Bacon & Sussman (1973) who have used heat shocks in their studies of germination, have reported that the SGI is *N,N'*-dimethylguanosine. This substance, however, does not exhibit any inhibitory effect on spore germination when spores are incubated with *A. aerogenes*. We are trying to identify a substance that inhibits germination even in the presence of *A. aerogenes*.

On further investigation of the germination process of *D. discoideum* and of the mechanism by which the spores detect the presence of *A. aerogenes*, as described later, we found that the spores respond to a specific substance that is secreted by growing *A. aerogenes*.

MATERIALS AND METHODS

Stocks of *D. discoideum*, NC-4, were maintained on a lawn of *A. aerogenes* on nutrient agar which contained 5.00 g glucose, 0.50 g yeast extract, 5.00 g proteose peptone, 2.25 g KH_2PO_4 , 1.50 g $\text{K}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.50 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 20.00 g of agar in 1000 ml of distilled water.

Spores used in the germination experiment were obtained by incubating the spores on nutrient agar with *A. aerogenes* for 5–8 days at 22 °C. Except for special cases, SGI on the spores was not washed off.

A. aerogenes was cultured on A-medium, consisting of the nutrient medium above without the agar. The culture was then centrifuged and filtered through a Millipore filter (pore size, 0.45 μm) to remove all traces of bacteria. Since spores at a density of $1 \times 10^6/\text{ml}$ showed the same percentage germination in both the unfiltered culture medium and the filtrate, the filtrate was used as the crude spore germination promoter (SGP).

In the germination experiments, 1 ml of medium was taken in a test tube and the spore density was adjusted to about $1.0 \times 10^6/\text{ml}$. The preparation was cultured on a reciprocal shaker (stroke 5 cm, 120 cycles/min) at 22 °C. Specimens were sampled several times while being cultivated to measure the density of spores and cells by a haemocytometer. Division of the germinated cells is disregarded because the incubation period is short and no bacteria are present.

RESULTS

The medium in which *A. aerogenes* had been grown overnight was filtered as described, then diluted with distilled water to obtain concentrations between 0.2 and 100%. These solutions were further diluted with equal volumes of fresh A-medium. The effects of these solutions on spore germination were then assayed as described above. The medium was sampled over a period of several hours and the percentage germination calculated (Fig. 1). Dormant and swelling figures were counted as spores, while amoeboid figures were regarded as cells. Most of the spores capable of germination germinated within 2–2.5 h and very few germinated thereafter. In the control

experiment, without filtrate, the observed percentage germination was 10–15% even after 24 h. In the following experiments, except for special cases, germination was measured at 2.5 h. These experiments establish the presence of a spore germination promoter (SGP) in the medium in which *A. aerogenes* has been growing.

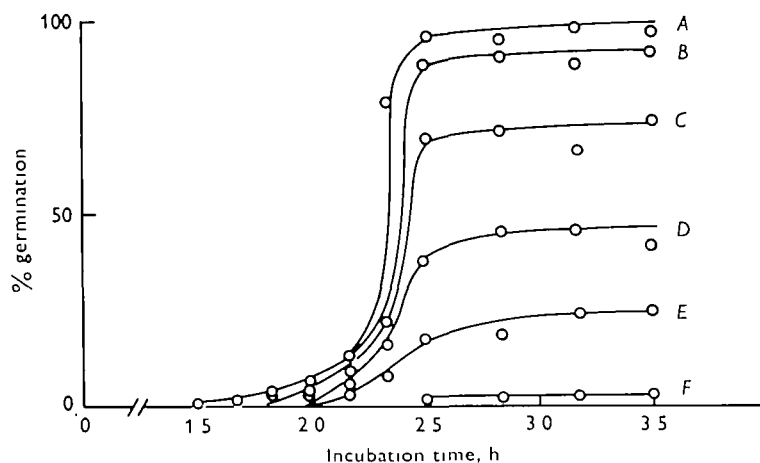


Fig. 1. Percentage germination against various SGPS concentrations: A, B, C, D, E, F, 50, 10, 5, 2, 1, and 0% respectively.

Effects of culture condition of A. aerogenes on SGP

Solution containing SGP (SGPS) was prepared by adding 10 ml of overnight-cultured *A. aerogenes* to 1000 ml of A-medium and incubating it at 22 or 30 °C. The culture medium was sampled at fixed time intervals and freed from bacteria. Culture medium containing 0.1–50% SGPS was prepared, incubated with spores, and the amount of germination checked after 2.5 h (Fig. 2A, B).

When incubated at 22 °C, *A. aerogenes* multiplied for 5 days and then continued in its stationary phase. At 30 °C, multiplication was maintained for only 3 days before levelling off. The concentration of SGP also increased until the density of the bacteria ceased to increase. The cell density of *A. aerogenes* at saturation was 2×10^8 /ml at 22 °C and 5×10^8 /ml at 30 °C. Similarly, at 22 °C, 10% SGPS gives 90% germination and at 30 °C, 5% SGPS gives 90% germination. Hence it can be concluded that the concentration of SGP is closely correlated with the density of *A. aerogenes*, or perhaps with the total number of bacterial divisions. At 30 °C the concentration of SGP tended to decrease after 30 days incubation. At 22 °C a decrease was observed only after a much longer period.

Effects of exposure time to SGP

SGPS cultured for 3 days at 30 °C which gave 90% germination at the 5% concentration, as shown on Fig. 2A, was used in the following experiments. The SGP was removed at different times by centrifugation and resuspension of the spores in a

solution of 50% A-medium and 50% distilled water and the percentage germination was determined.

As shown in Fig. 3, when the spores were kept in contact with SGP for 2 min, they showed the same percentage germination as when kept in SGP throughout.

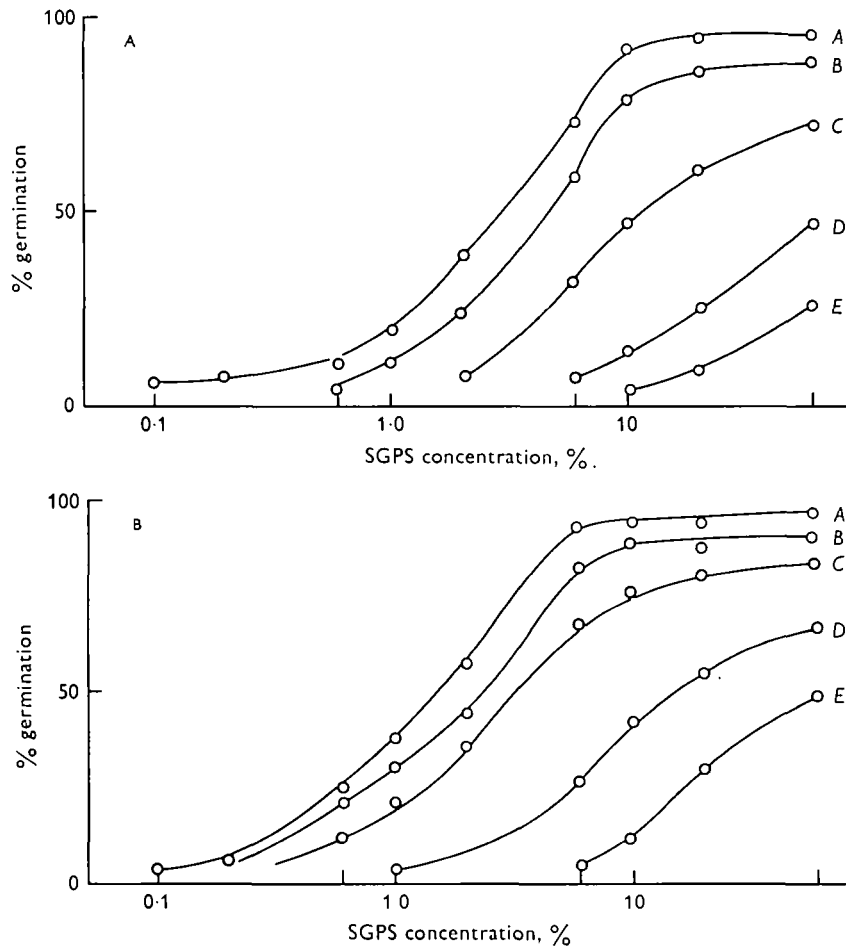


Fig. 2A, B. Accumulation of SGP in A-medium and the resultant percent germination at 2.5 h incubation for various *A. aerogenes* incubation times at 22 °C (A) and 30 °C (B). In both graphs, A, B, C, D, E, represent 5, 3, and 1 days and 12, and 6 h respectively.

This suggests that SGP acts as a trigger of the germination process and that contact with the spores is not necessary for the subsequent steps of germination. The curve of Fig. 3 was obtained at room temperature but a similar result was obtained when the experiment was conducted in an ice-water bath.

When the spores were kept in contact with SGP for a longer period, a significant fall in the percentage germination was observed at 1–2 h contact (Fig. 4A). The percentage germination dropped to 40% after 1.5 h contact. But the percentage germination re-

covered to 90% when the incubation was continued for another 1 h, and the results at 3.5 h are indicated by the dotted line on Fig. 4A. A further study was made of spores activated by SGP for 1.5 h. The spores were centrifuged to remove SGP, and incubated in the shaker bath for 2 h to investigate the percentage of germination. As indicated in Fig. 4B, the germination of centrifuged spores (*A*) was delayed for approximately 1 h compared with spores incubated with SGP (*B*).

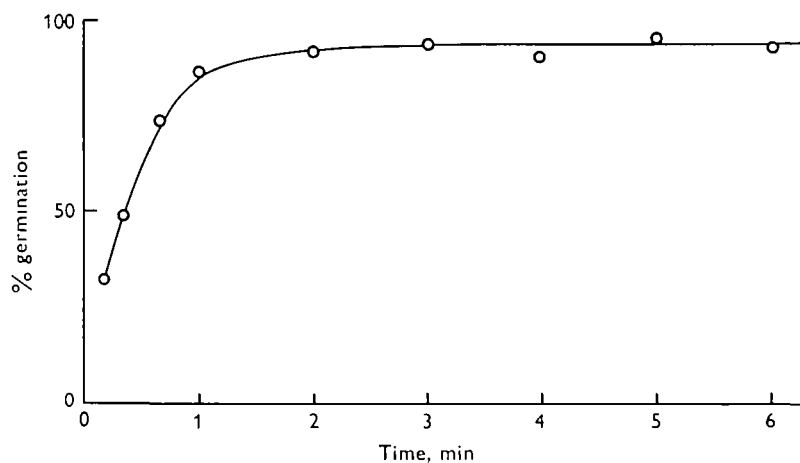


Fig. 3. Spore contact time with SGP vs. percentage germination at 2.5 h incubation.

The time at which the percentage germination started to decrease corresponded to the time when the spores started to swell. Since there is expansion of cytoplasm and separation of organelles within the spores when they are swollen, the centrifugation applied to remove SGP probably affected the organelles causing the germination delay. After 2 h incubation the spores are not affected by centrifugation because germination has begun.

Absorption of SGP

A medium was made with 5% of the SGPS prepared as in the previous experiments, 50% A-medium and 45% distilled water. Spores which had been washed free of SGI were added to the medium to a density of about 1.0×10^8 /ml and allowed to stand at room temperature for 5 min. One millilitre of this suspension was transferred to a test tube and incubated in a shaker at 22 °C for 2.5 h. The rest of the spore suspension was filtered through an 0.8- μ m pore size Millipore filter to remove the spores. Again, SGI-free spores were suspended in this filtrate at a concentration of about 1.0×10^8 /ml, allowed to stand for 5 min for contact with SGP, and a 1-ml portion was transferred to a test tube, incubated for 2.5 h and germination determined. The above procedure was repeated. The germination-promoting capacity of the SGPS declined with every successive step, although it was left in contact with the spores, as indicated in Fig. 5A. If the horizontal axis is logarithmically scaled for total spore numbers suspended in SGPS, and the vertical axis is linearly scaled for percentage germination a linear relationship is observed down to a germination of 10% (Fig. 5B).

In this experiment, SGI-free spores were used in order to eliminate the possibility of SGI accumulation. In other words, it was confirmed that the promoting capacity of SGPS decreases as it comes in contact with increasing numbers of spores.

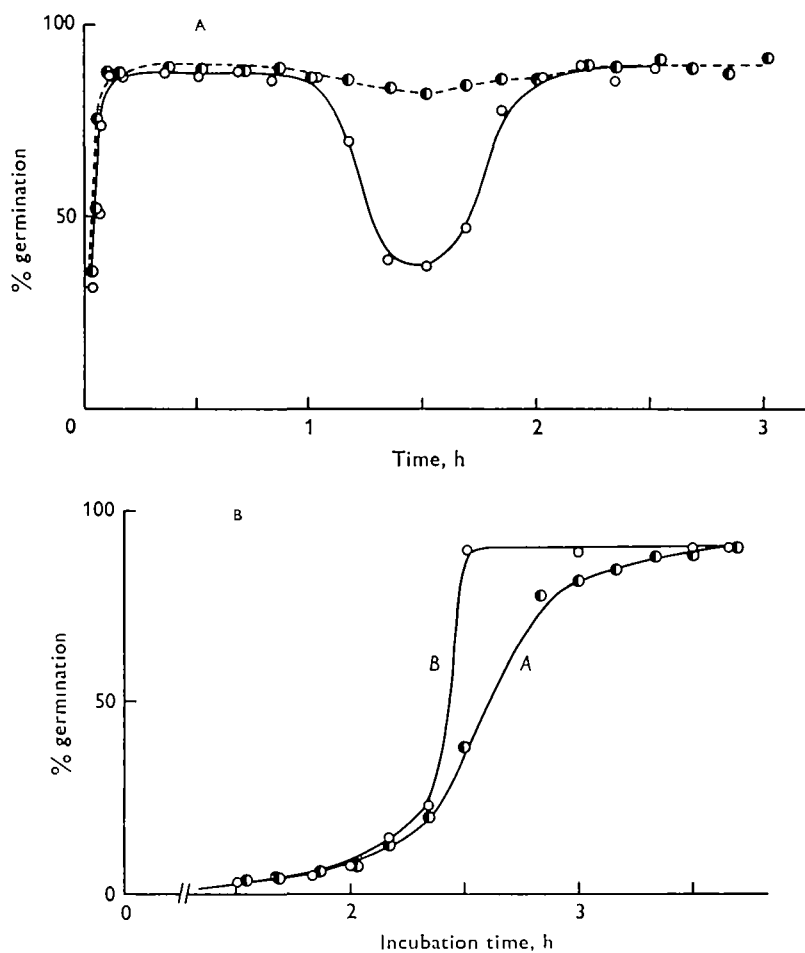


Fig. 4A, Spore contact time with SCP vs. percentage germination. ○, 2.5 h incubation, ●, 3.5 h incubation. B, Germination comparison of SGP contact time. A, 1.5 h contact, B, kept in contact.

Stability of activated spores at 4 °C

Spores were activated by immersing them in a medium containing 5% SGPS for 5 min and immediately freed from SGP by centrifugation (200 g, 3 min). The spores were then suspended in distilled water and maintained at 4 °C. An aliquot of the activated spore suspension was taken out every hour, diluted to 1×10^6 /ml with 50% of A-medium and 50% of distilled water and incubated at 22 °C for 2.5 h. As shown in Fig. 6, the percentage germination declined with time.

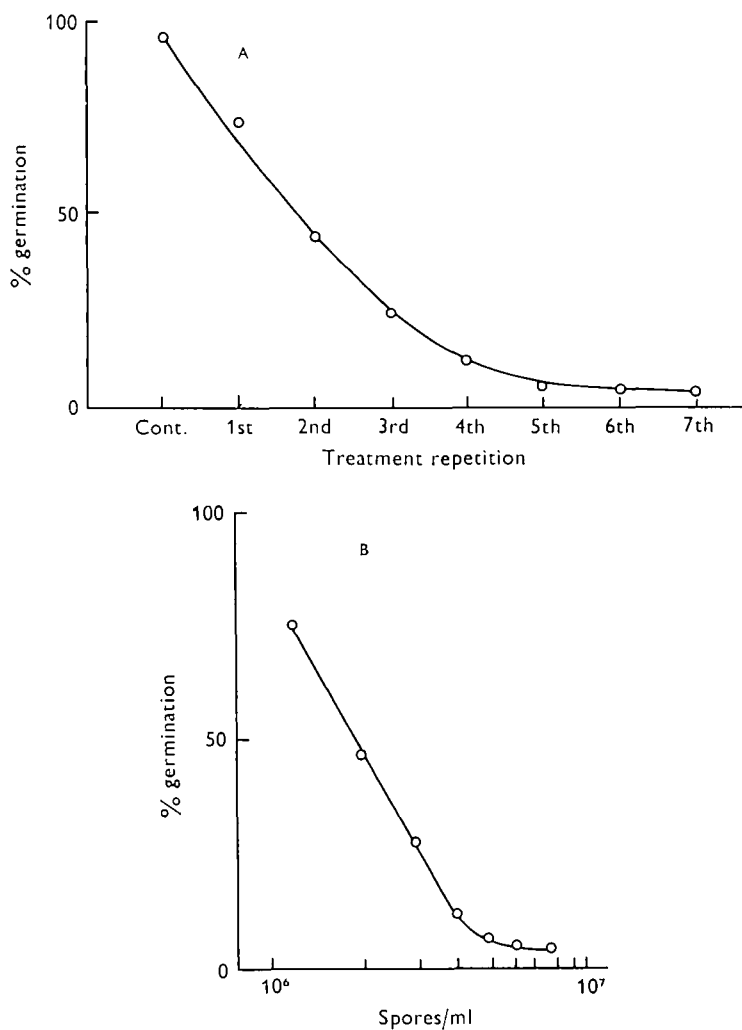


Fig. 5. A, Absorption of SGP; % germination at 2.5 h incubation is shown against the number of treatments. B, Absorption of SGP to total spore numbers. % germination is shown against spores/ml.

Characteristics of SGP

Crude SGPS was concentrated under reduced pressure in a rotary evaporator at 45 °C and was dialysed against distilled water. Then, both dialysed samples and dialysate were fractionated into 100, 80, and 60% ethanol-soluble and ethanol-insoluble materials. SGP activity was detected in the 80% and 60% ethanol-soluble fractions of the sample dialysed and in the 80% ethanol-soluble fraction of dialysate. However, there were cases when SGP was not detectable in the dialysed solution. The presence of an enzyme in the crude SGPS that acts on SGP is suggested by the above experiment. This 'enzyme' can be inactivated by 5-min heat treatment at 95 °C.

When heat treatment of the crude and fractionated SGP at 100 °C was prolonged

for 1 h the SGP activity dropped to one-fifth of that of non-treated SGP. When the SGP were heat treated (95 °C for 5 min) after adjustment of pH with phosphate buffer, they were inactivated completely when the pH was more than 10. On the other hand, the activity was reduced to one-fifth after heat treatment when pH was lower than 3.

SGP was not soluble in a chloroform-methanol (1:1) solution. The fact suggests that the molecule is a non-lipid substance.

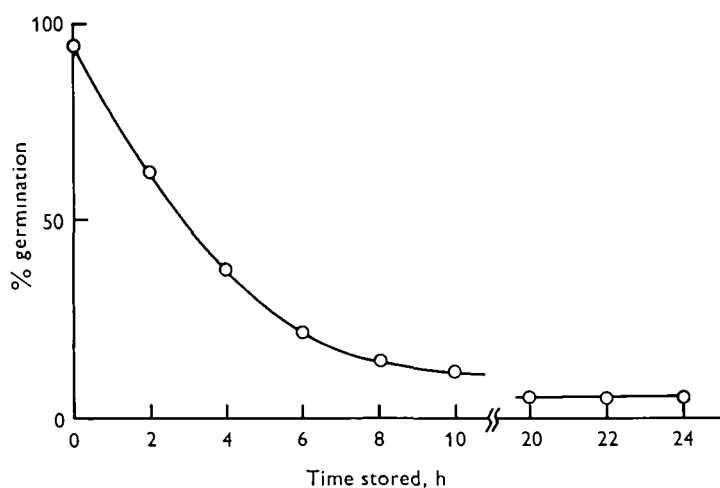


Fig. 6. Percentage germination at 2.5 h vs. time stored at 4 °C is shown.

A paper-chromatographic study was carried out with various combinations of solvents; however, an appropriate solvent could not be found. The result was that either the solute did not migrate at all or, if it migrated, the band became broadened with the constituent moving to the very end. Ninhydrin reaction of the crude SGP on the paper-chromatograph was negative.

The treatment of SGP with several different kinds of celluloses and amberlites showed that its activity was lost with anion-absorbing IRA-100, GE and DEAE, maintained with cation-absorbing IR-120-B, PCM and lowered with MB-3 which absorbs both ions. The results demonstrate that SGP is positively charged.

SGP was not inactivated when treated with the following enzymes for 30 min at 37 °C and 5 min at 95 °C: pepsin 0.4 mg/ml, pH 3.0; trypsin 0.33 mg/ml, pH 8.0; RNase 0.5 mg/ml, pH 5.0; pronase 0.33 mg/ml, pH 8.0; DNase 0.16 mg/ml, pH 8.0.

DISCUSSION

Although, phenethyl alcohol (PEA) has been reported as a spore germination promoter for *Neurospora* (Lingappa, Lingappa & Turian, 1970), the participation of PEA in its natural germination process is improbable. On the other hand, the SGP reported here is believed to contribute to the natural germination process of the cellular slime mould. The SGI of the cellular slime mould, which has previously been reported elsewhere (Tanaka *et al.* 1974, 1975), is not a general respiratory inhibitor but acts as a specific inhibitor of natural spore germination. It is, therefore,

meaningful to analyse the germination mechanism by purification of both SGP and SGI. It is also of interest to note that germination of cellular slime mould spores is not promoted by PEA.

In the study of chemical characteristics, it was found that SGP was undoubtedly present in the 80% ethanol-soluble fraction of the dialysate and the 60% and 80% ethanol-soluble fractions of the dialysed material. The 3 fractionated substances were similar in chemical behaviour except for their dialysis and hydrophylic properties. A possible explanation is that the SGP-containing substance decomposes and migrates to other fractions without losing any of its spore germination capacity.

As demonstrated in Fig. 6, the spores regained their dormancy when they were washed and maintained at 4 °C after the spores had been subjected to SGP of the minimum concentration which produces synchronous germination. The same spores can germinate synchronously when they come in contact with SGP again, although the period over which germination occurs is prolonged. On the other hand, when the spores are maintained at 4 °C while they are in contact with the minimum concentration of SGP for a synchronous germination, neither an incubation at 22 °C nor subsequent washing and supplying of fresh SGP, will induce any germination.

A possible explanation of these observations is the presence of multiple germination sites where SGP acts to trigger the germination circuit. When the number of triggered sites exceeds a threshold value the germination process takes place immediately, and amoebae emerge after 2.5 h in the above experimental condition, but when the triggered sites are fewer than the threshold value the germination process takes longer. If activated spores were kept under severe conditions to prevent the germination process – for example, the ambient temperature is 4 °C – the germination circuit at the sites which had been triggered was destroyed and unable to revive. But, if unactivated germination sites remain to be triggered, supply of fresh SGP can induce germination.

Further experiments are required to amplify this speculation because it has not taken the SGI factor into consideration.

Furthermore, the heat-shock-activated spores which are made dormant by maintaining them at low temperature can be reactivated by another heat-shock and it seems that heat-shock and SGP promote germination by different mechanisms.

Generally, a cellular slime mould adapts well to different circumstances. For instance, when a cellular slime mould is isolated from the soil in the field, the amoebae can grow, develop and form fruiting bodies at 12 – 30 °C. Nevertheless, when the strain is continuously subcultured at 22 °C, for a few years, both the capacity for cell growth and morphogenesis deteriorate unless the temperature is kept around 22 °C. Similarly, cellular slime moulds that have long been cultured with *A. aerogenes*, show marked deterioration of cell growth when the bacterial strain is changed to *Escherichia coli*.

Accordingly, when SGP from *A. aerogenes* is used on *D. discoideum* NC-4 which had hitherto been cultured on *E. coli*, synchronous germination is observed but it takes longer than the germination of NC-4 spores that had been cultured on *A. aerogenes*. Furthermore, the filtrate of A-medium which contained cultured *E. coli* did not induce synchronous germination of NC-4 spores which are subcultured with *A.*

aerogenes or *E. coli*. Synchronous germination becomes less obvious, that is, the time required for the individual germination process becomes widely dispersed, when the spores are incubated with *E. coli*. SGP appears to be specifically secreted by *A. aerogenes* in large quantity.

Induction of synchronous germination by highly purified SGP requires addition of more than 10% of A-medium. However, it is probable that among the components of A-medium, proteose peptone or buffer constitute the essential requirement; further evidence indicates that the ionic concentration of the medium plays an important part in germination.

Germination of spores cannot be observed when an adequate amount of *A. aerogenes* in Pad diluting fluid (KCl 1.50 g and MgCl₂ 0.50 g in 1000 ml of distilled water) is used for incubation at 22 °C. The bacteria scarcely grow in this fluid.

Nor can SGP activity be found in either the supernatant of the distilled water suspension of pregrown *A. aerogenes* on nutrient agar or in the supernatant of the nutrient agar homogenate on which *A. aerobacter* were cultured. Therefore we conclude that SGP is secreted in quantity when *A. aerogenes* is cultured in A-medium.

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