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Studies on the Basic Physiological Conditions Affecting the Spore Germination of an Obligatorily Tonophilic Fungus

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I. Introduction

Since 1940, a number of papers[1-6] have been published on the fungi characterized by the ability to grow on dry materials, such as lenses, prisms and others. In view of their habitat, two species are called "glass-moulds",[1-4] which were identified by Ohtsuki as *Aspergillus vitricolae* and *Eurotium tonophilum*. Recently, highly tonophilic fungi, *Eurotium halophilicum*[7] and *Eurotium heterocaryoticum*[8], were also isolated by C. M. Christensen et al. from dried cereals. At the same time, the present author found a fungus showing a strong resemblance to *E. halophilicum*, but being distinguished by the absence of conidial stage. This has been identified as *Eurotium halophilicum* forma *A*.[9,10] The mycelial development of these fungi was never found on usual media, but on hypertonic media containing high concentrations of osmoactive substances. This physiological property has been called "obligate tonophily" by Ohtsuki[6] and others.[9,10]

Among ordinary fungi there are others which are described as "facultatively tonophilic fungi". They are capable of growing either on usual media or on hypertonic media.[11-13] In 1911, organisms[14] belonging to *Aspergillus glaucus* group[13] or *Aspergillus restrictus* group[13] were isolated from "dried bonito" (Katsuobushi) by Hanzawa. These fungi are distinguished from the glass-moulds by their luxuriant growth on usual media.

In recent years, a number of investigations[7,11,15-20] have been published on the effects of atmospheric humidity on spore germination.

Spores of tonophilic fungi do not germinate in air saturated with water vapour i. e., in air of 100% relative humidity (RH), but can germinate in the range of 95% to 60% RH. On the contrary, the spores of ordinary fungi never germinate at relative humidity less than 80%, and an optimal humidity for their growth is 100% RH. Now, it should be quite important to establish our fundamental knowledge on spore germinations of the tonophilic fungi.

The experiments described here include the observations on morphological changes of ascospores of *Eurotium halophilicum* forma *A* during germination, and also on the effects of environmental factors on spore germination. Furthermore, experiments were carried out on the effects of salt solutions and osmotic pressure upon the viability of spores. In the following, experiments and result will be described.

II. Materials and Methods

1. Materials

Eurotium halophilicum forma *A* was used throughout, since this organism produces ascospores only and spores when germinated may be easily distinguished from the dormant ones by their characteristic appearance. For comparison, ordinary fungi such as *Aspergillus oryzae*,[19] *Aspergillus niger*[23] and *Aspergillus fischeri*[13,21] were employed in parallel.

2. Criteria of spore germination

Ascospores of *E. halophilicum* f. *A* are shown in Fig. 1. Dormant spores are lenticular, having equatorial furrows which are shallow but prominent and bordered by low ridges. Walls of ascopores are conspicuously rough, especially when they are not mounted in water (Fig. 1, A and B). Dry ascospores are $(5.5\text{–}6.0)\times 4.5\,\mu$ in diameter. When spores are suspended in water, 0.2% solution of Tween 80, or other liquid media, they swell, and the diameter increases up to $6.0\times(6.0\text{–}7.0)\mu$. Yet, walls of the spores were not separated (C). Swelling (D and E) gives rise to spore-sprouting (F). In contrast to the conidiospores,[22,23] the germinating ascospores are extremely striking not only for their size but also for their morphological changes during the course of germination. Even if contamination occurs, it may be easily recognized. The germination of spores could be divided into two phases, swelling phase and sprouting phase, as described previously in the case of *A. niger*.[19,23] Further, the swelling phase may be separated into two stages, i. e., early stage (C) and later stage (D and E) of swelling.

3. Collection of spores and preparation of spore suspension

Malt-extract agar (usual medium), malt-extract agar supplemented with 15% of sodium chloride (hypertonic medium) and modified Harrold's medium (hypertonic medium) were used for slant cultures in order to harvest spores. Inocula of *E. halophilicum* f. *A* were obtained either from old cultures on usual medium or on these hypertonic media. Mature perithecia were suspended in water or 0.2% solution of Tween 80 using a Shimazu Ultra-sonic Vibrator (Type ST-200).[24]

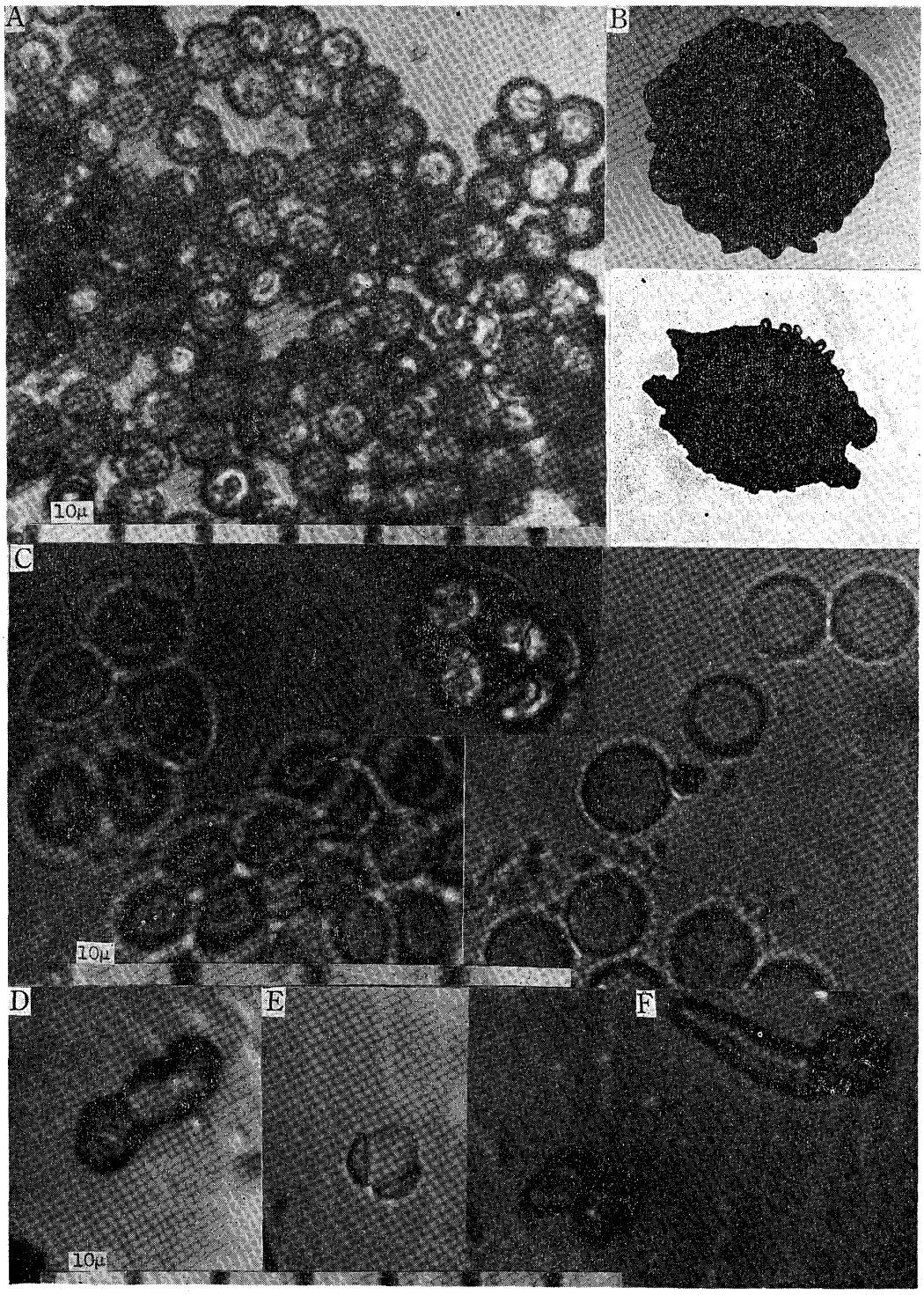

Fig. 1. Morphological changes in the course of aerial spore germination of *Eurotium halophilicum* forma *A*. A: Dormant ascospores not mounted in water, some showing side views. B: Ascospores, face and side views magnified by electron microscope (×8,000). C: Ascospores and ascus in early stage of swelling phase, mounted in water, side and face views of ascospores, and ascus (center). D, E and F (left): Ascospores in later stage of swelling phase. F (right): Ascospore in sprouting phase.

By this treatment ascospores could be dispersed homogeneously. Then the spore suspension was filtered twice with glass-filters No. 3 and No. 4 successively. By this procedure, fragments of hyphae and perithecial walls were removed, and the intact spores were collected on the glass-filter No. 4. 3–5 mg of ascospores were resuspended in water and diluted to the required concentration (ca. 8×10^7 spores per 10 ml). Concentration of the spore suspension was determined by the use of a Hitachi Electrophotometer (Type EPO-B), or by counting spore numbers under the microscope using a blood cell counting chamber (Thoma Haemacytometer). Stock suspensions when kept in a refrigerator at 0°–5°, were fully usable within a month. All procedures were made asceptically.

4. Measurement of germination

The rate of germination was shown as the percentage of germinated spores to the total of inoculated spores. The term "germinated spore" was defined as sprouted spores. The size and number of spores were measured microscopically by the use of an ocular micrometer. At least several sites in a field were examined and two or three inoculated slide-glasses were examined for a germination test. All tests were carried out under the conditions of 28° and 90% RH.

5. Equipment used for spore germination at a constant relative humidity

The following two kinds of equipment were prepared for the conditioning of constant relative humidity.

(1) Small glass-tube: Small glass-tubes were used as described by Ohtsuki[6] and others[,12] (Fig. 2-A). Spores were inoculated on the medium coating the under surface of half-sized slide-glass, and this was laid on the top of small glass-tube, containing a half volume of

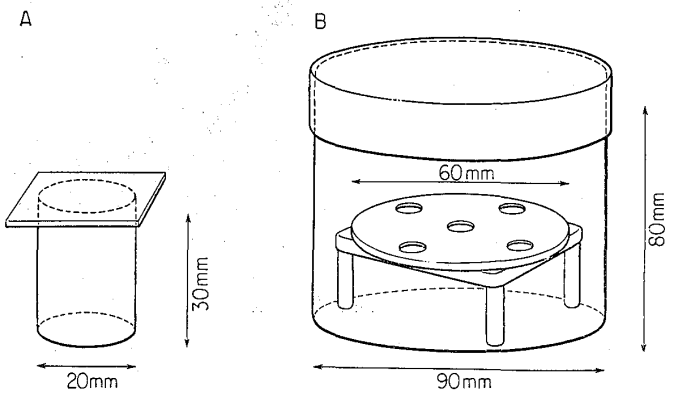

Fig. 2. Equipments used for testing of aerial spore germination at desired constant level of relative humidity. A: Small glass-tube. B: Cylindrical glass-jar.

aqueous solution of sulfuric acid[25] to maintain the inside air at a desired level of relative humidity. The tubes were incubated at 28° and subjected to frequent microscopic observation. In winter, however, this procedure was not always satisfactory, since drops of water sometimes condensed on the under surface of the slide-glass and disturbed microscopic observations.

(2) Cylindrical glass-jar: A cylindrical glass-jar (Fig. 2-B) containing a circular glass-plate for holding inoculated slide-glasses was used as a humid chamber. The jars were set to contain aqueous sulfuric acid at the bottom in order to maintain the relative humidity at a desired level. Lid of each jar was sealed with purified vaseline. This equipment was suitable for preserving inocula of spore suspensions and especially to test three or four inoculated slide-glasses at a time. Moreover, it was advantageous in that the moisture of the air inside was relatively free from condensation.

(3) Adjustment of the atmospheric humidity: Sulfuric acid solutions were used for this purpose. The relationships between the concentration of sulfuric acid and the corresponding relative humidity of the atmosphere are listed in Table 1-A. The values of the relative humidity depending on varied temperatures were obtained from the

Table 1. Concentrations of sulfuric acid solutions and corresponding relative humidities.

A. At 20°.

H_2SO_4 (%)	Relative humidity (%)
0	100
8.88	95
15.75	90
25.90	80
32.70	70
37.00	60

B. Values of RH depending on temperatures.

RH % / Temp. / H_2SO_4 (%)	Relative humidity (%)	
	20°	30°
0	100.	100.
8.88	94.6	94.5
15.75	89.4	89.5
25.90	80.3	80.1
32.7	70.1	70.0
37.69	61.7	61.4

table of Landolt-Börnstein[26] (Table 1-B). According to the tables, only a slight differences in the values of relative humidity at the different temperatures: that is, not exceeding 1% RH between 20° and 30°. Therefore, this may be negligible in further experiments. Relative humidities applied to the experiments were as follows: RH 100, 95, 90, 80, 70 and 60%.

6. Methods of inoculation

(1) Inoculation with a platinum-loop: This usual method was used mainely for the observations of morphological changes.

(2) Inoculation with separated ascospores: A cover-glass was placed over perithecia on a slide-glass and moved about so that the perithecia and asci were crushed and the ascospores were set free. The separated ascospores were transferred to the desired media by tapping the cover-glass slightly.

(3) Inoculation with spore suspension: 0.02–0.05 ml of spore suspension was used as an inoculum. When washed spores were needed, this method was applied using cylindrical glass-jars. The pre-treatments of agar to be used was made as follows: 0.1–0.5 ml of agar medium was smeared on half-sized slide-glasses, this was kept in a desiccator over calcium chloride for four days, and finally in a cylin-

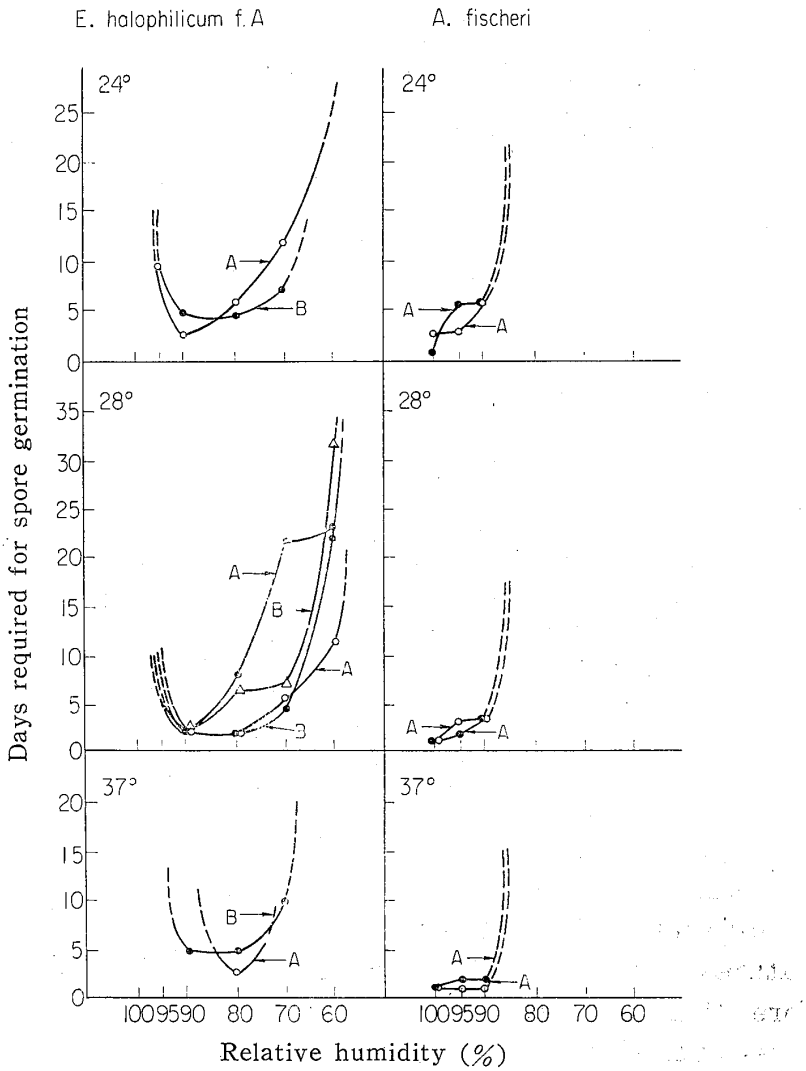

Fig. 3. Effects of inoculation procedure on germination. ○—○: Germination on dried medium after 4-day adjustment of water content at desired level of relative humidity. ●—●: Germination on dried medium. △—△: Germination on agar medium without drying. A. Inoculation with a platinum-loop of spores. B. Inoculation with a drop of spore suspension.

drical glass-jar for succeeding four days.

In order to know the effects of inoculation procedures, on which the water content of media must depend, some germination experiments were carried out. The results are shown in Fig. 3. In these experiments, *A. fischeri* was used as the control. For the ordinary fungus, the inoculation with a platinum-loop on dried medium with or without the adjustment of moisture content were almost the same as regards the effects on germination. The germination was more intensely affected by temperature rather than by the inoculation process. In the case of the tonophilic fungus, the inoculation with a platinum-loop (method 1 and 2) on dried media with adjustments of moisture content were suitable for germination experiments. The inoculation with spore suspension (method 3) on dried medium was also applicable. Therefore, both procedures were employed in this investigation.

III. Experiments and Results

A. Morphological changes of spores in the course of germination

1. Swelling phase

Observations were made in a cylindrical glass-jar at 28° and 90% RH. Ascospores were inoculated on dried malt-extract agar media with and without adjustment of water content prior to inoculation.

As shown in Fig. 1-C, spores began to swell in a day or two. The rough surface of the spore wall became smoother. This process occurs at an early stage of the swelling phase. Spores which were suspended in various solutions including distilled water, 0.2% solution of Tween 80, Czapek's solution with and without carbon and nitrogen sources, 3% malt-extract solution and 15% solution of sodium chloride, swelled slightly ; the swelling being arrested by exposure to lower temperature. In a later stage of the swelling phase, the spore wall split into two parts at the site of the furrow (Fig. 1-D, E and left F) after two or three days of culture. The two halves of the spore wall seemed to be connected by a hinge-like structure (Fig. 1-F). The size of the spores at this stage markedly increased to 10-12 μ, namely about 1.5-2.0 times as large as dormant spores (Table 2).

2. Sprouting phase

In two- or three-day cultures, germ tubes sprouted out, which were about 5 μ in diameter and larger than those of mycelial cells developed later. If malt-extract agar were replaced either by 3% agar or by Czapek's agar without carbon and nitrogen sources, the germ tubes ceased to develop and soon degenerated. In the case of the tonophilic fungus, 50% of the spores commenced to germinate on malt-

Table 2. Morphological changes of spores in the course of germination.

Dormant spores	Swollen spores		Sprouted spores
μ in diam.	(in early swelling stage) μ in diam.	(in later swelling stage) μ in diam.	μ in diam. of germ tube
4.6×(5.3-5.8) Harrold's agar*	5.8×7.0 Distilled water**	10-11 Dried malt-extract agar*	5.0 Dried malt-extract agar*
〃 〃	(5.0-5.8)×(6.5-7.0) Tween 80 solution	10-11 〃	5.0 〃
4.6×(5.0-5.8) Czapek's agar	(5.8-6.5)×(6.5-7.0) Cz-C, N solution	10-12 〃	5.0 〃
〃 〃	6.4×7.0 15% NaCl solution	10-11 〃	5.0 〃
4.6×(5.3-5.8) Czapek's agar	5.8×7.0 Czapek's solution	10-11 〃	5.0 〃
〃 〃	6.0×7.2 Malt-extract liquid medium	12 〃	5.0 〃

*: Culture medium. **: Suspension medium.

extract agar after 2-day incubation and complete germination was occured in 3 days (98-100%), while the germination rate of the conidiospores of *A. niger*[22,23] and of *A. oryzae*[13] were 90% and 97% after 9 hours and 6 hours of incubation, respectively, on complete media.

B. Effects of environmental factors on sphore germination

1. Ultra-sonic vibration

Spores collected from a slant culture were transferred to 5 ml of Tween 80 solution and treated with a Shimazu Vibrator. By this means, perithecia and asci were disrupted, leaving homogeneous suspensions of ascospores. To examine the effect of this method on germination, pro-

Table 3. Germination capacity of spores vibrated with ultra-sonic waves.

No.	Conditions of vibration		Germination rate %		Days required for 50% germination
	Position of test-tube, mm	Time in min.	2 days	3 days	
1	above 3-5	10	85.0	99.3	1.3
2	above 3-5	20	79.0	98.5	1.3
3	above 3-5	30	88.0	100.0	1.3
4	in contact with oil surface	10	78.0	99.0	1.3
5	below 3-5	5	22.0	90.0	2.5
6	below 3-5	10	79.4	97.0	1.3

visional tests were made at varied distances and varied vibration times as shown in Table 3. Test-tubes containing spore suspensions were suspended loosely with copper wire (1 mm in diam.). To avoid an excessive rise in temperature during the vibration, the time of vibration should be less than 30 minutes. Thereafter, the test-tubes were immediately cooled with tap water and stored in a refrigerator. Germination tests were made on spore suspensions obtained as described on page 27.

The results are shown in Table 3 and Fig. 4. The vibration conditions of Test No. 3 were found to be the most suitable for germination; yet, for the sake of time-saving, the conditions of Test No. 1 were employed for further experiments.

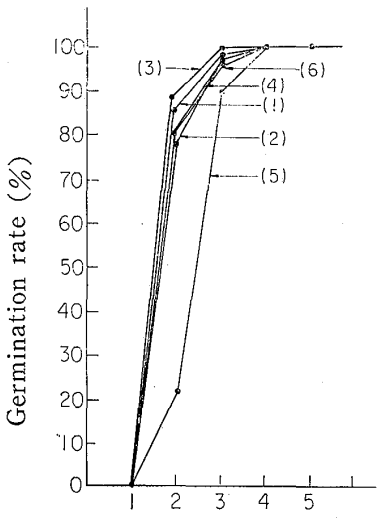

Fig. 4. Influence of ultrasonic vibration on germination. Figures in parenthesis show the test numbers listed in Table 3.

2. Suspension medium

(1) *E*ffects of suspension medium on germination[19,23]: Ascospores of the tonophilic fungus were suspended in various media including distilled water, 0.2% solution of Tween 80 and Czapek's salt solution lacking both carbon and nitrogen sources (Cz-C, N), using the vibration technique. Germination tests were made as described above.

The results obtained are shown in Fig. 5-A (I) and B.

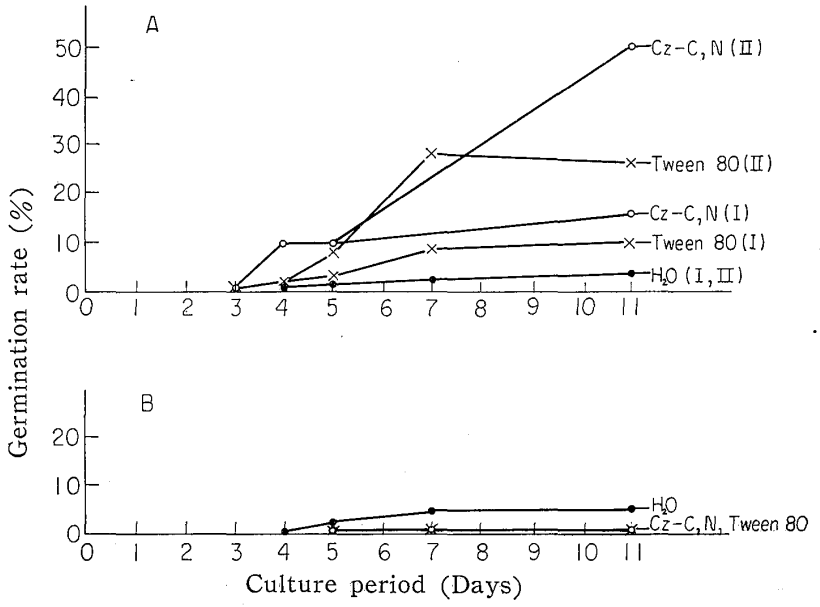

Fig. 5. Effects on germination of media used to suspend spores.
I: Spores not pre-incubated. II: Spores pre-incubated.

(2) Effects of pre-incubation: The spore suspensions were incubated for 20 days at 28°, and then 0.02 ml of each suspension was used as an inoculum. Germination tests are shown in Fig. 5-A (II). The germination rates of pre-incubated spores are listed in Table 4. From the experiments (1) and (2), it may be seen the germination rates of spores suspended in water with and without pre-incubation were found to be the lowest. Germination was slightly promoted by Tween 80 solution, and more markedly by Cz-C, N.

As described above, the germination rate is affected to an appreciable degree by the condition of preliminary suspension as well as the kind of suspension media.

Table 4. Effects of pre-incubation on germination.

Days of cultures	Increments of germination after 20-day pre-incubation (%)		
	Distilled water	Tween 80 solution	Cz-N solution
3	0	0	0
4	0	0	0
5	0	5	0
7	0	18	11
11	0	18	35
15	0	17	30

3. Temperature

(1) Effects of pre-incubation at various temperatures on germination[27,23]: (a) Ascospores of the fungus were suspended in 15% sodium chloride solution and pre-incubated for one day at the following temperatures: 0°, 24°, 28°, 37° and 63°. Subsequently, 0.05 ml of each suspension was inoculated into 5 ml of liquid malt-extract medium, to which sodium chloride was added up to 15%. Sets of such cultures were incubated at 24°, 28° and 37°, respectively. Control experiments were done on usual malt-extract medium without extra 15% sodium chloride.

As shown in Table 5, mycelial growth was absent at 37°, when the spores were pre-incubated at any temperature between 0° and 63°. However, all the pre-incubated spores except those pre-incubated at 63° showed mycelial growth at 28°. Thus, it follows that pre-incubation temperatures lower than 37° are not effective for germination, whereas the temperatures higher than 63° show a lethal effect.

(b) Similar experiments was carried out using suspension media and culture media which both lacked sodium chloride. In these experiments, spores were suspended in 0.2% Tween 80 solution and pre-incubated overnight at 0°, 24°, 28°, 37° and 60°, respectively. After incubation, these suspended spores were examined for germination at

Table 5. Growth influenced by pre-incubation at various temperatures.

Temp.*	0°				24°				28°				37°				63°
Medium**	+NaCl			−	+NaCl			−	+NaCl			−	+NaCl			−	+NaCl
Temp.*** / Days	24°	28°	37°	28°	24°	28°	37°	28°	24°	28°	37°	28°	24°	28°	37°	28°	28°
6	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
7	−	−	−	−	−	−	−	±	−	−	−	±	−	−	−	±	−
10	−	+	−	±	−	+	−	±	−	+	−	±	−	+	−	±	−
12	−	++	−	±	−	++	−	±	−	++	−	±	−	++	−	±	−
18	−	+++	−	±	−	+++	−	±	−	+++	−	±	−	+++	−	±	−
22	−	+++	−	±	+	+++	−	±	+	+++	−	±	+	+++	−	±	−
35	+	+++	−	±	++	+++	−	±	++	+++	−	±	+++	+++	−	±	−
47	+	+++	−	±	++	+++	−	±	++	+++	−	±	+++	+++	−	±	−
50	+	+++	−	±	++	+++	−	±	++	+++	−	±	+++	+++	−	±	−

*: Temperatures of pre-incubation. **: Medium with and without 15% NaCl are represented by (+NaCl) and (−), respectively. ***: Temperatures of cultivation. Growth rates: −, no growth. ±, slight growth around inside wall of test-tube. +, slight growth on the surface of liquid medium. ++, formation of a thin layer on liquid surface. +++, formation of aerial mycelia. +++, favorable growth. +++, luxuriant growth. ±, Growth on malt-extract agar slant containing no NaCl.

Table 6. Effect of pre-incubation at various temperatures on germination.

Temp.* / RH% / Days	0°					24°					28°					37°					60°				
	95	90	80	70	60	95	90	80	70	60	95	90	80	70	60	95	90	80	70	60	95	90	80	70	60
2	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
5	−	+++	−	−	−	−	++	++	−	−	−	++	++	−	−	−	+++	−	−	−	−	++	−	−	−
7	−	+++	+++	−	−	−	+++	+++	−	−	−	+++	+++	−	−	−	+++	+++	−	−	−	+++	−	−	−
11	−	+++	+++	+++	−	−	+++	+++	+++	−	−	+++	+++	+++	−	−	+++	+++	+++	−	−	+++	+++	+++	−
16	−	+++	+++	+++	−	−	+++	+++	+++	−	−	+++	+++	+++	−	−	+++	+++	+++	−	−	+++	+++	+++	−
63	−	+++	+++	+++	−	−	+++	+++	+++	−	−	+++	+++	+++	−	−	+++	+++	+++	−	−	+++	+++	+++	−

*: Temperatures of pre-incubation. Degrees of germination: −, none. ++, 10–20%. ++, 20–50%. +++, 50–80%. +++, more than 80%.

relative humidity ranging from 100% to 60%. The results are summarized in Table 6. Germination of the fungus spores was scarecely affected by any temperature of pre-incubation; the spores pre-incubated at 37° germinated almost completely within the range of 90–70% RH. Even the spores pre-incubated at 60° were able to germinate at 28°.

(2) Heat-resistance of spores: Heating in a temperature ranging

Table 7. Conditions of heat-treatment.

Temperatures °C	Heating time	
	Minutes	Hours
50	10 20 30 / 60	4.5
60	10 20 30 / 60	3 / 5 14
80	5 10 20 / 40 60	3
100	1 2 3 5 10 20	

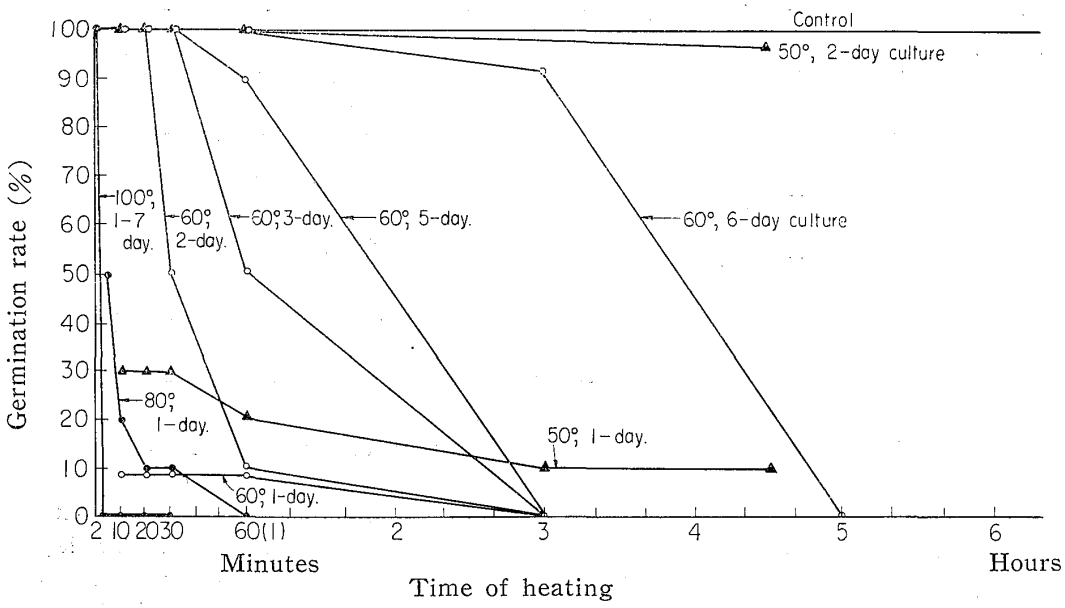

Fig. 6. Heat-resistance of ascospores of *Eurotium halophilicum* forma *A*.

over 50°–100° for varied lengths of time was tested. Germination was tested with each 0.02 ml of aqueous suspension of the heated spores on the dried malt-extract agar. The times and temperatures of heating are listed in Table 7, and the results are shown in Fig. 6. Germination of spores heated at 50° was evidently delayed and suppressed at the beginning, but after 2 days it reached to the maximum level. Heating at 60° for 3 hours brought about a suppression of the initial germination, but this recovered with time. In the case of heating at 80° for 5 minutes, germination did not occur even in 4-day culture, while on the 5th day, 50% germination was observed. Heating at 100° for 2 minutes have rise to a lethal effect.

Above results show that the spores are more resistant against heating than the mycelial cells,[30] and that the accelerating effect of heating[23] is hardly perceptible.

4. Temperature and humidity requirements for germination

As seen in Fig. 3 and Table 6, the temperature and humidity requirements for spore germination of the tonophilic fungus showed a

marked difference from those of ordinary fungi. In order to compare these characteristic behaviors in two groups of fungi. the following experiments were carried out. *A. niger* and *A. oryzae* were used in addition to *A. fischeri* and *E. halophilicum* f. *A.* Spores of these fungi were inoculated on dried malt-extract agar and on chemically clean glass, and subjected to the desired level of relative humidity within the range of 100% to 60% RH, and at various temperatures such as 0°, 15°–20° (room temperature), 24°, 28° and 37°, respectively. The results are shown in Fig. 7.

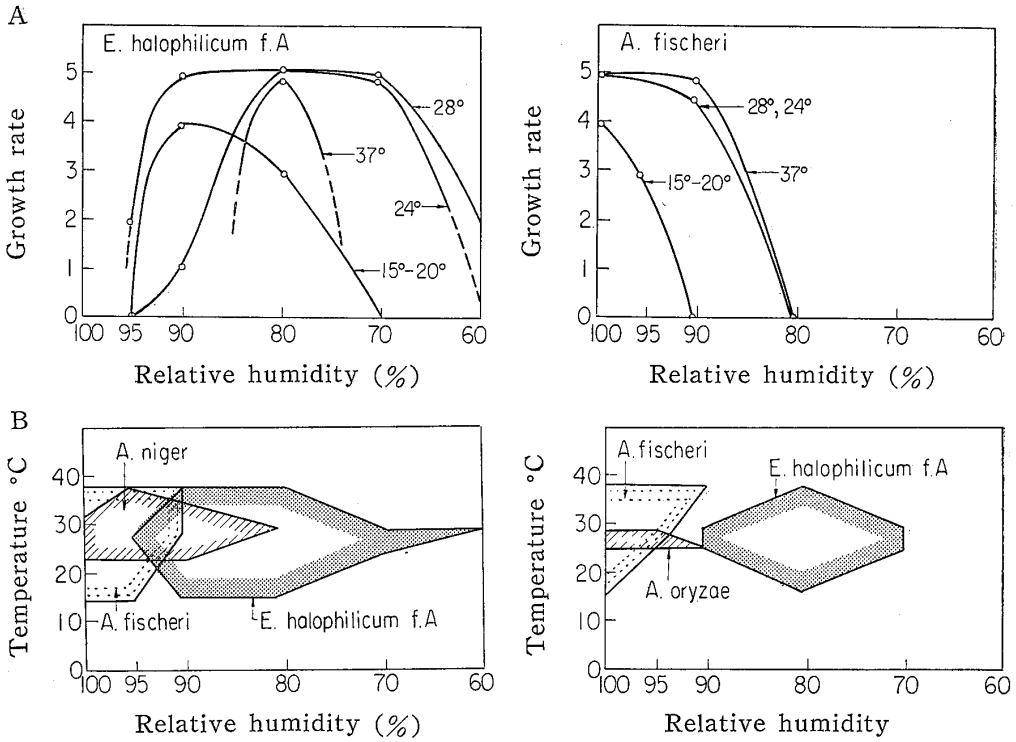

Fig. 7. Spore germination and mycelial growth under conditions of various temperatures and relative humidities. A. Germination and subsequent mycelial growth on dried malt-extract agar medium. B. Range of temperature and relative humidity required for germination of various fungi. The left and right parts show the rates of germination on dried malt-extract agar and on chemically clean slide-glass, respectively. Growth rate: 0, no growth. 1, slight. 2, fair. 3, ordinary. 4, favorable. 5, luxuriant.

In A (Fig. 7), germination rate and subsequent growth of each fungus are shown. In B, the relation between temperature and relative humidity required for each fungus is shown. These figures indicate that spore germination of the tonophilic fungus is never seen at 100% RH, although this relative humidity is optimal for ordinary fungi at any temperature examined so far. The graphs in B, clearly represent this characteristic and more distinctly in the cultures grown on chemically clean glass.

5. Choice of germination medium

In order to choose the media suitable for germination, twelve kinds of media[9,13] listed in Table 8 were examined. Cultures were made as described above (p. 27). The results are given in Table 9. The germi-

Table 8. Composition of culture media used.

No. 1. Czapek's medium: water 1L, $NaNO_3$ 3 g, K_2HPO_4 1 g, $MgSO_4 \cdot 7H_2O$ 5 g, KCl 5 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, sucrose 30 g, agar 30 g.
No. 2. Modified Czapek's agar medium: sucrose 400 g replacing sucrose 30 g of Medium No. 1.
No. 3. Malt-extract agar medium (Blakeslee, 1915): water 1L, malt-extract 20 g, peptone 1 g, glucose 20 g, agar 30 g.
No. 4. Modified Moyer's medium (sporulation medium for *Aspergillus flavus*): glucose 165g, Bacto-peptone 1g, $MgSO_4 \cdot 7H_2O$ 0.05g, KH_2PO_4 0.06g, KNO_3 0.5g, Fe (as tartarate) 0.04g, agar 30g (original Moyer's medium has 25g agar): distilled water to make 1L.
No. 5. Malt-extract agar medium: malt-extract (3%) 1L, agar 30g.
No. 6. Modified Harrold's medium: malt-extract (2%) 1L, sucrose 400g, yeast-extract 5g, agar 30g.
No. 7. Bouillon-peptone agar: water 1L, beef-extract 3g, Bacto-peptone 5g, agar 30g.
No. 8. Sucrose agar: sucrose 1%, agar 3%.
No. 9. Glucose agar: glucose 1%, agar 3%.
No. 10. Fructose agar: fructose 1%, agar 3%.
No. 11. Mannose agar: mannose 1%, agar 3%.
No. 12. Simple agar medium: agar (3%) only.
(No. 13.) Chemically clean slide-glass.

Table 9. Spore germination affected by the kinds of culture media.

Medium No. / Days	Spore germination												
	1	2	3	4	5	6	7	8	9	10	11	12	13
1	−	−	−	−	−	−	−	−	−	−	−	−	−
2	−	−	+～−	−	+～−	−	−	−	−	−	−	−	−
3	++	+++	+++	+	+++	+++	−	+	+～−	−	++	−	−
5	+++	+++	+++	+++	+++	+++	+～−	+	+～−	±	++	−	−
6	+++	+++	+++	+++	+++	+++	++	+++	+～−	±	++	+～−	−
10	+++	+++	+++	+++	+++	+++	++	+++	+～−	±	++	++	+～−

Germination rate: −, no germination. ±, swollen but not yet sprouted spores. +, less than 10%. ++, less than 30%. +++, less than 50%. +++, less than 80%. +++, more than 80%.

nation rates of cultures grown on Czapek's medium containing both nitrogen and carbon sources (No. 1) and Czapek's medium containing much sucrose (No. 2), or natural media such as malt-extract agar and yeast-extract agar (Nos. 3–6), were greater than those cultures on the media containing either organic nitrogen source only (No. 7) or carbon source only (Nos. 8–11). On chemically clean glass (No. 13) and dried

agar medium (No. 12), germination rates did not exceed 1% and 20%, respectively, even after prolonged cultivation.

From these results, it may be assumed that for complete germination spores require nutrients such as carbon source together with nitrogen source.

6. Effects of pH on germination

(1) pH of suspension medium; Washed spores were suspended in Cz-C, N. The pH was adjusted to 1.8, 4.2, 5.2, 6.0, 7.2, 8.2 and 10.6, respectively. Germination tests were made with these spore suspensions and again with the same suspensions which were preliminarily incubated at 28° for two weeks. Dried malt-extract agar was used under the conditions of 28° and 90% RH. The results are represented

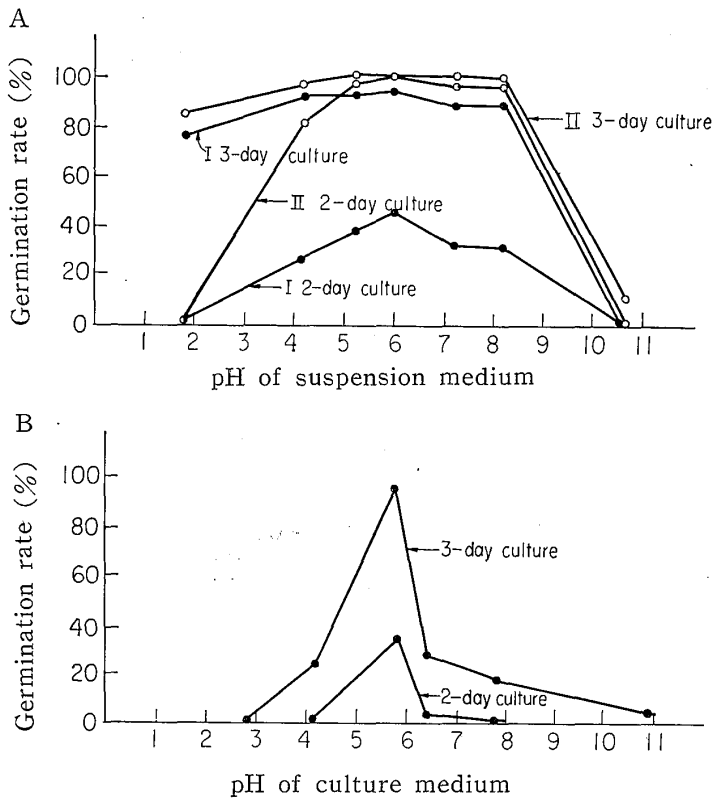

Fig. 8. Effect of pH on spore germination. A. Effects of pH of suspension medium on germination. I: Germination obtained with spores which were cultivated immediately after suspending. II: Germination of spores pre-incubated at 28° for two weeks. B. Effects of pH of culture medium on germination.

in Fig. 8. A in Fig. 8 shows that the germination rate was quite low at both extremes of pH range, namely at pH 1.8 and 10.6, but was gradually increased later. The optimal pH of the suspension medium measured as about 6.0.

(2) pH of culture medium: In this experiment, spores suspended

in water were inoculated on dried malt-extract media, pH of which were adjusted to 2.8, 4.2, 5.8, 6.4, 7.8 and 11.2, respectively. As shown in Fig. 8–B, the effects of pH under these conditions were more conspicuous than in Experiment (1). The optimal pH of the culture medium was 5.8.

From these data, it may be assumed that spores in the swelling phase are strongly affected by chemical composition of the medium rather than by pH of the suspension medium, and that pH of the culture medium affects germination rate more markedly than that of the suspension medium.

7. Nutritional requirements for germination

(1) Sugar requirement: From the data in Table 4 and 9, and also in Fig. 5, it is presumed that the nutrients may promote the germination of spores. 0.1 ml of spore suspension (in water) was transferred into 5 ml of single sugar solution (20 mg/ml) in a test-tube, whereby glucose, mannose, galactose, fructose, maltose, lactose and sucrose were used as sugar source. Spores suspended were inoculated on both dried simple agar and dried malt-extract agar, and cultured at 28° and 90% RH. Control experiments were made with aqueous suspension in the same way. Here, the following three types of experiments were carried out. In Experiment A, germination test were made immediately after suspension. In Experiment B, the suspended spores were pre-incubated at 28° for 3 weeks and then tested for germination. In Experiment C, the pre-incubated spores were washed twice, with 20 ml of distilled water, and re-suspended in 5 ml of distilled water per test-tube.

The results obtained with 4–6 day cultures are shown in Fig. 9. In Experiment A, germination was promoted by glucose, maltose, mannose, and galactose, in this sequence. The germination rates in these sugars were almost equal to those in cultures on dried malt-extract agar, while the effects of fructose and sucrose were unexpectedly low. In Experiment B, germination rates on malt-extract agar were relatively low after three days. On a simple agar medium, the germination tendency was rather striking: The greatest rate of germination was found to be about 40% in the case of maltose in 6-day cultures. Therefore, it seems that the pre-incubation tends to reduce the resting time of spores. If the period of pre-incubation is too long, the spores may consume their reserve material necessary for sprouting. This may be the reason why the spores do not germinate rapidly on simple agar medium. But still, these spores possess a germination capacity, since a luxuriant germination occurs when they are transferred to malt-extract agar. Experiment C has shown that spores never germinate on simple agar medium even in 15-day cultures. This is be-

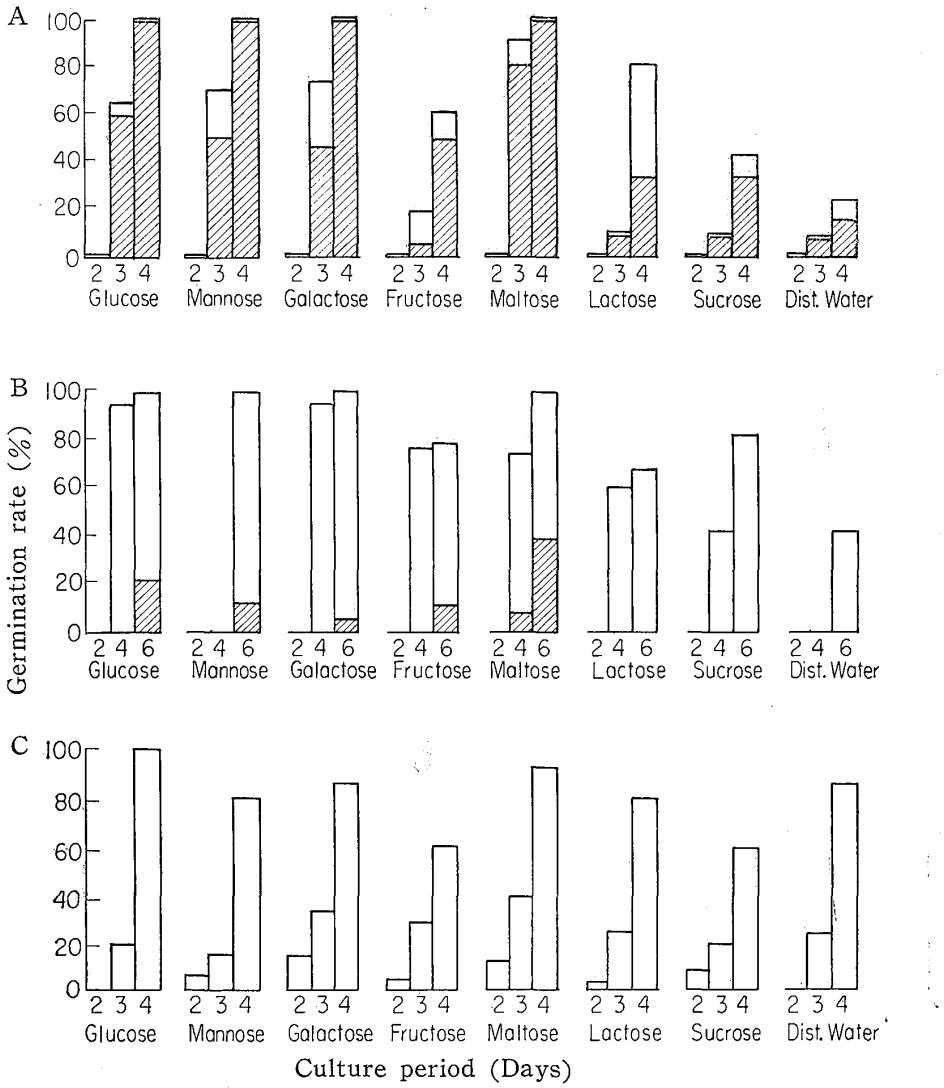

Fig. 9. Effects of sugars on spore gemination. A: Germination of spores suspended in various sugar solutions. B: Germination of suspended spores incubated for three weeks at 28° prior to inoculation. C: Germination of spores washed after 3-week pre-incubation. ▨: Germination on dried simple agar medium. □: Germination on dried malt-extract agar medium.

cause of the lack of sugar source. Similar tendency may be seen in the case of germination on malt-extract agar, but in lesser degree. By prolonged pre-incubation and washing, spores would consume by themselves all the reserves which are indispensable for energy source.

(2) Amino acid and vitamin requirements[23,31,32]: Each substance was dissolved in Cz-C, N, in concentrations of 10, 50, 100, 200 and 400μg per ml. Washed spores were suspended in each solution and 0.02 ml of spore suspension were inoculated on dried simple agar medium and cultured at 28° and 90% RH. Germination rates were measured at regular intervals for about two weeks. Experiments were processed in the same way as described in the preceding section. In Experiment

A, germination tests were made immediately after suspension of spores. Tests were made with thirteen amino acids: L-tryptophan, DL-serine, L-alanine, L-proline, L-arginine hydrochloride, L-leucine, L-asparagine, L-glutamic acid, L-methionine, L-cystine, glycine, tyrosine and L-lysine: and also with nine vitamins: niacinamide (B_3), biotin (H), pyridoxine hydrochloride (B_6), rivoflavin (B_2), cyanocobalamin (B_{12}), thiamine hydrochloride (B_1), p-aminobenzoic acid, calcium pantothenate (B_5) and L-ascorbic acid (C). Typical data obtained from the experiments show that the rate of germination was only slightly enhanced as compared with that of the control experiments made on simple agar medium, with an only exception of p-aminobenzoic acid, which had a suppressive effect for germination (Fig. 10).

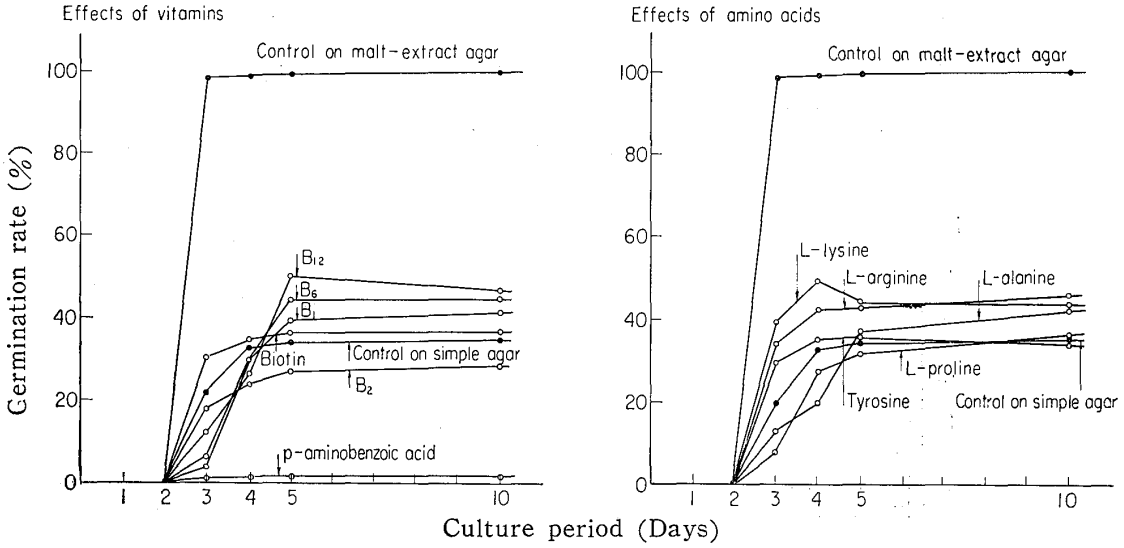

Fig. 10. Effects of various nutrients on germination (A). ○—○: Single nutrient added. ●—●: No nutrient added.

In Experiment B, Czapek's salt solution plus 3% sucrose (Cz-N) was used as basal suspension medium. To this medium, 10–400 μg/ml of various amino acids and vitamins were added. Spores were suspended in these solutions and the germination rate was measured. Control experiments were made on both dried malt-extract agar and dried simple agar using spore suspension in Cz-N. Some of the representative results are depicted in Fig. 11.

As seen from the figures, germination rate depends upon the kind of substance but not upon its concentration. The germination curves may be classified into four groups as follows. Group I: Substances of this group showed an inhibitory effect on germination. Namely, the curves being lower as compared with the control on simple agar medium. L-tryptophan, DL-serine, p-aminobenzoic acid belong to this group. Concentrations exerted little influence. Group II: Substances

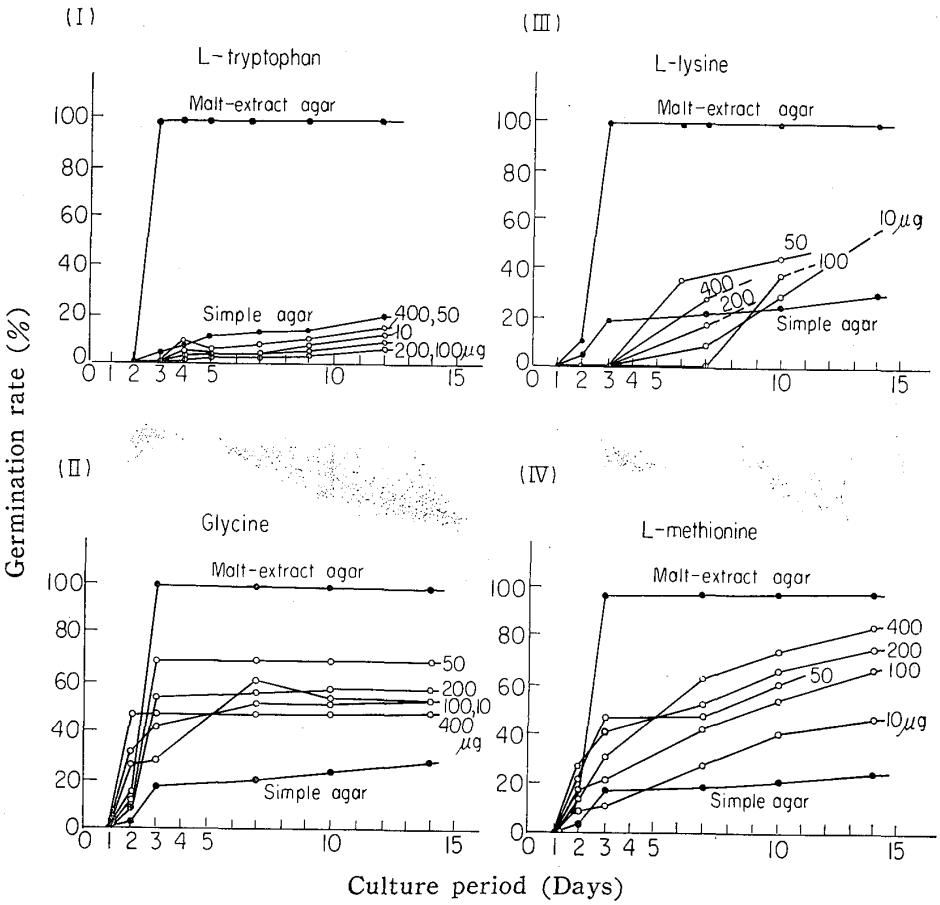

Fig. 11. Effects of individual amino acids and vitamins on germination (B).
○—○: Single nutrient added. ●—●: No nutrient added.

of this group showed a promotive effect. Germination was initiated earlier than that in the controls, and the highest rate of germination exceeded the rate that was found on simple agar. This group includes L-asparagine, L-alanine, L-glutamic acid, L-arginine, L-cystine, glycine, and vitamins B_1, B_2, B_3, B_5, B_6 and B_{12}. Group III: While the substances of this group showed promoting effects, germination was initiated later than that in the control experiments. It was increased gradually and varied with concentration of added substance. However, no definite relationship was observed between concentration and germination rate. This group includes L-lysine, L-proline, and L-leucine. Group IV: The substances of this group showed a promoting effect. Germination was initiated earlier than the controls, while an optimal germination appeared later than in the control experiments on dried malt-extract agar. The shape of germination curves were dissimilar. The highest rate of germination was nearly equal to that of the group II. This group includes L-methionine, tyrosine and vitamin C.

In Experiment C, spore suspensions such as prepared in preceding experiment (B) were pre-incubated at 28° for three weeks. The results

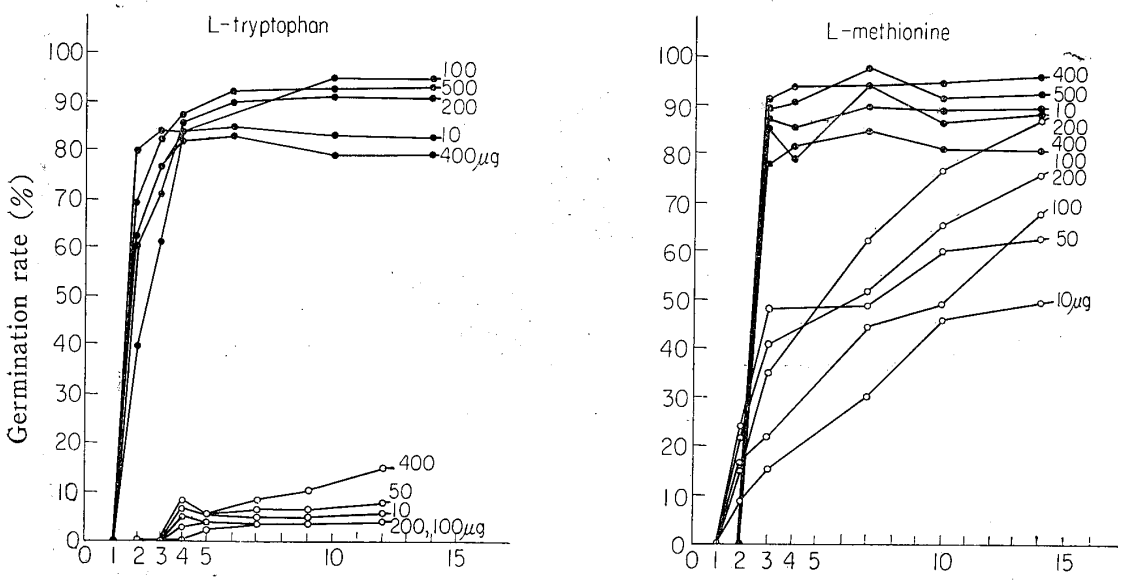

Fig. 12. Effects of individual amino acids and vitamins on germination (C).
○—○: Data from Experiment B. ●—●: Pre-incubated spore suspensions.

of the subsequent germination tests are shown in Fig. 12. The promoting effects on germination were quite remarkable, even in the case of L-tryptophan. The lags in germination curves were appreciably shortened as compared with those of the control experiments and the highest percentage of germination was measured at 80–95%. In all, the values were 8–9 times as high as those obtained in Experiment B. In the case of L-methionine, the germination curves were quite similar in entire range of concentration and the highest germination rate appeared in 3–4 day culture.

C. Effects of salts on germination

1. Survival test of spores exposed to concentrated salt solutions

The effects of fifteen salts[9,29,33] (Table 10) on spore germination were examined. Spores were cultured at 28° for 45 days on malt-extract agar slants, which were added with salts in a series of concentrations ranging from 0 to 55%.

As shown in Table 10, mycelial growth was completely inhibited by LiCl, $LiNO_3$ and NH_4NO_3. No growth could be found on media containing K_2SO_4, but when the same slants were rinsed with water, fungal development was found on them. Such states were recorded by the symbol ± in the table. In the presence of much salt, agar became difficult to coagulate on autoclaving at 15 lb for 15 minutes; this is indicated by ( ). Less coagulability caused by autoclaving is often accompanied by the change in colour and acidity of the medium,

Table 10. Effects of salts at various concentrations.

Basic medium / Salt % / Salts	Malt-extract agar											
	0	5	10	15	20	25	30	35	40	45	50	53
NaCl	−	−	⫼	⫼	⫼	−	−	−	−	−	−	−
NaBr	−	−	−	⫽	⫽	⫽	+	−	−	(−	−	−)
$NaNO_3$	−	−	−	⫼	⫼	⫼	⫼	⫽	⫽	±	+	+
Na_2SO_4	−	−	−	−	−	(−	−	−	−	+	+	−)
$MgCl_2$	−	−	−	⫽	⫼	⫽	⫽	(−	−	−	−	−)
$CaCl_2$	−	−	−	⫽	−	(−	−	−	−	−	−	−)
KCl	−	−	+	⫽	⫼	⫼	⫼	⫼	−	−	−	−
KBr	−	−	−	−	+	⫽	⫽	+	+	+	−	−
KNO_3	−	−	−	−	+	⫽	⫽	+	+	+	+	+
$(NH_4)_2SO_4$	−	−	−	⫼	(⫼	⫼	⫼	⫽	⫽	−	−	−)
NH_4Cl	−	−	⫼	⫽	(−	−	−	−	−	−	−	−)
K_2SO_4	−	−	−	±	±	±	±	±	±	±	±	±
LiCl	−	−	−	−	−	(−	−	−	−	−	−	−)
$LiNO_3$	−	−	−	(−	−	−	−	−	−	−	−	−)
NH_4NO_3	−	−	−	(−	−	−	−	−	−	−	−	−)

( ): Agar liquefied by autoclaving. −, no growth. ±, no growth or only germinated spores. +, slight growth. ⫽, fair growth. ⫼, ordinary growth. ⫼, favorable growth. ⫼, luxuriant growth.

whereas the reduction of pH was not lower than 4.0, which was still tolerable for the fungus. Spores harvested from salt-containing slants were transferred to malt extract liquid medium, which was added with sodium chloride up to 15% and cultured at 28° for about a month. As shown in Table 11, almost all of the spores were brought into germination and formation of mycelia after a week. However, spores from the slants containing $LiNO_3$ (5–30% and 50–55%) did not germinate. These results reveal that the salt-resistance of spore cells is higher than that of mycelial cells.

2. Effects of salt concentration on germination[27,28,33–38]

The results of experiments with salt solutions are shown in Table 12 and Fig. 13. Spores were suspended in fifteen salt solutions, and 0.02 ml portions of the suspensions were employed as inocula.

(1) Spore suspension in concentrated salt solution: Germination tests were made immediately after preparation of spore suspensions.

Table 11. Survival tests on spores*.

Salts \ Salt %	Growth on liquid malt-extract medium containing 15% NaCl											
	0	5	10	15	20	25	30	35	40	45	50	55
NaCl	+	+	/	/	/	+	+	+	+	+	+	+
NaBr	+	+	+	/	/	/	/	+	+	+	+	+
$NaNO_3$	+	+	+	/	/	/	/	/	/	+	+	+
Na_2SO_4	+	+	+	+	+	+	+	+	+	/	/	/
$MgCl_2$	+	+	+	/	/	/	/	+	+	+	+	+
$CaCl_2$	+	+	+	/	+	+	+	+	+	+	+	+
KCl	+	+	/	/	/	/	/	/	+	+	+	+
KBr	+	+	+	+	/	/	/	/	/	/	+	+
KNO_3	+	+	+	/	/	/	/	/	/	/	/	/
$(NH_4)_2SO_4$	+	+	+	/	/	/	/	/	/	+	+	+
NH_4Cl	+	+	/	/	+	+	+	+	+	+	+	+
K_2SO_4	+	+	+	+	+	+	+	+	+	+	+	+
LiCl	+	+	+	+	+	+	+	+	+	+	+	+
$LiNO_3$	+	−	−	−	−	−	−	+	+	+	−	−
NH_4NO_3	+	+	+	+	+	+	+	+	+	+	+	+

*: Spores which failed to germinate on media containing extremely low or high concentrations of salts. /: Ranges at which spores germinated and grew. −: No growth. +: Growth.

Control experiments were made with spores suspended in water. The results are shown in Fig. 13-(1). Three salts, LiCl, $LiNO_3$ and NH_4NO_3 suppressed severely the germination, being in agreement with the results shown in Table 10. Germination was also suppressed intensely but not completely by $CaCl_2$ and NH_4Cl. Other salts showed a similar effect to a lesser extent. The effect of K_2SO_4 was the least of all, and was comparable to that of KNO_3.

(2) Germination of spores pre-incubated in concentrated salt solution: Spores in concentrated salt solutions were pre-incubated at 28° for three weeks. Results are shown in Fig. 13-(2). In general, germination was suppressed by salts, the spores being damaged most severely by LiCl, $LiNO_3$, NH_4NO_3 and NH_4Cl. KBr, NaBr and $MgCl_2$ also caused the suppression to a lesser extent, whereas KNO_3, $(NH_4)_2SO_4$, KCl and $CaCl_2$ showed more or less promoting effects on germination as compared with the preceding experiment (1).

(3) Germination of spores which were washed after pre-incuba-

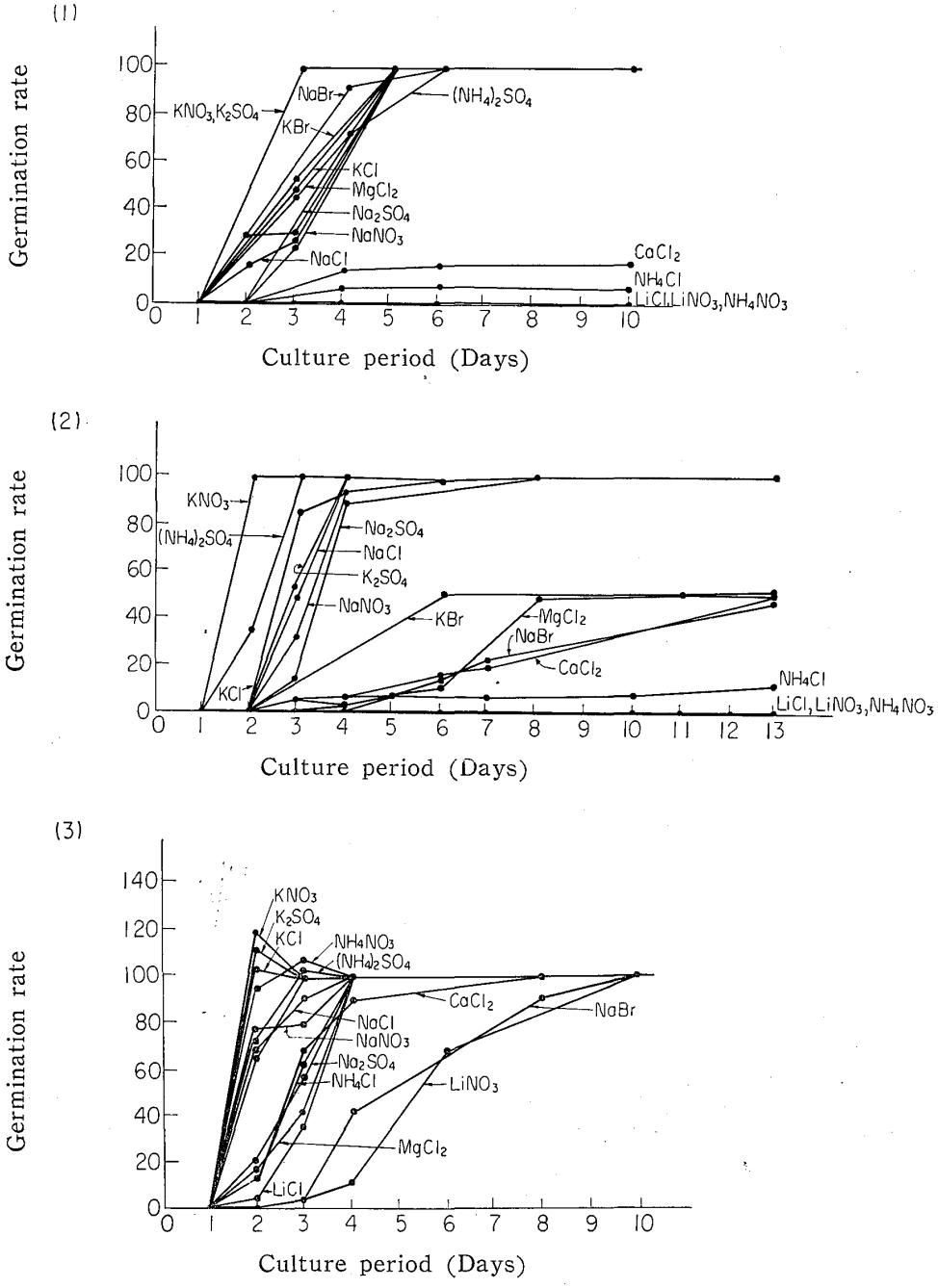

Fig. 13. Effects of highly concentrated salt-solutions on germination. (1): Germination of spores suspended in concentrated salt-solutions· (2): Germination of spores pre-incubated in concentrated salt-solutions. (3): Germination of spores washed after pre-incubation.

tion: The spores pre-incubated with salt solutions were washed twice with distilled water and tested for germination capacity. The results are shown in Fig. 13-(3). Removal of the salts resulted in a decrease in suppressing effects. Under these conditions, potassium salts (KCl, KNO_3 and K_2SO_4) and ammonium salts (NH_4NO_3 and $(NH_4)_2SO_4$) seem to promote a germination capacity.

Table 12. Salt concentrations of suspension solutions.

Salt % / Salts	Concentrations of salts which permitted fungal growth											
	0	5	10	15	20	25	30	35	40	45	50	55
NaCl			+	+	+		30					
NaBr				+	+	+	+		40			
$NaNo_3$				+	+	+	+	+	+		50	
Na_2SO_4							30			+	+	+
$MgCl_2$				+	+	+	+	35				
$CaCl_2$				+						45		
KCl			+	+	+	+	+	+			50	
KBr					+	+	+	+	+	+	50	
KNO_3				+	+	+	30 +	+	+	+	+	+
$(NH_4)_2SO_4$				+	+	+	+	+	40 −			
NH_4Cl			+	+	30							
K_2SO_4				15 ±	±	±	±	±	±	±	±	±
LiCl										45		
$LiNO_3$									40			
NH_4NO_3											50	

+ : Range at which fungal growth was attained. ± : Range at which fungal growth was uncertain. Numerical values denote the percentage of salt-solutions used for spore suspension.

3. Transfer of spores pre-incubated in concentrated salt solutions to dilute ones[17,36-43]

Five salts were chosen according to their suppressive effects on spore germination. NaCl, $MgCl_2$, $CaCl_2$, LiCl and NH_4NO_3 were added to the aqueous spore suspensions up to the concentrations of 5.13 M (30%), 3.67 M (35%), 4.05 M (45%), 5.3 M (25%) and 3.125 M (25%), respectively. The original spore suspension in each salt was diluted with sterilized distilled water. The transmittance of the spore suspension was measured by an electrophotometer, and the number of spores in each suspension was estimated by a Thoma Haemacytometer. Germination tests were made on the spore suspensions by incubation at 28° for 7-9 hours giving the results shown in Fig. 14. The transmittance of the suspension and both number and germination capacity of the pre-incubated spores were then examined again.

As shown in Fig. 14, the transmittance of the salt (NaCl) solutions decreased rapidly until two hours after dilution, and then became con-

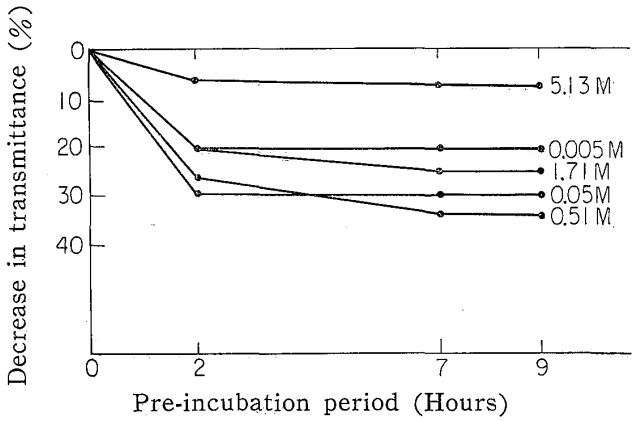

Fig. 14. Changes in transmittance of spore suspension caused by reduced concentration of NaCl.

Table 13. Decrease in number of spores in NaCl solutions.

Concentration of NaCl in suspension Mol	Number of spores*		
	Incubation time Hours		Rate of decrease
	0	9	%
5.13	3.66×10^7	2.62×10^7	28.7
1.71	2.19×10^7	1.12×10^7	48.8
0.51	8.896×10^6	5.31×10^6	40.2
0.05	1.136×10^7	7.52×10^6	33.8
0.005	1.84×10^6	1.76×10^6	4.3

*: Spore cells per ml.

stant at a level which was maintained for subsequent seven hours. Changes in transmittance may be explained by the changes in cell wall, probably because of a decrease in osmotic pressure following dilution.

As listed in Table 13, the number of spores decreased in the dilute salt solutions after 9-hour incubation. Such a decrement seems to be dependent on the degree of dilution: The decrease being minimum (4.3%) at the lowest concentration.

The results of experiments shown in Fig. 15 account for the relationship between dilution effect and spore germination. Here, germination of spores in original salt solution (NaCl 5.13 M) was remarkably reduced by the 9-hour pre-incubation (from 50% to 30% after 2 days and 80% to 50% after 3 days). Higher salt concentrations exhibit a suppressive effect on germination. On the other hand, a suppressive effect of dilution appeared in the cultures after 2–4 days or later. The germination rate of spores that was measured on 2-day culture in most dilute salt solution (0.005 M) was higher than that of the spores pre-incubated for 9 hours in the same salt solution. Accordingly, it fol-

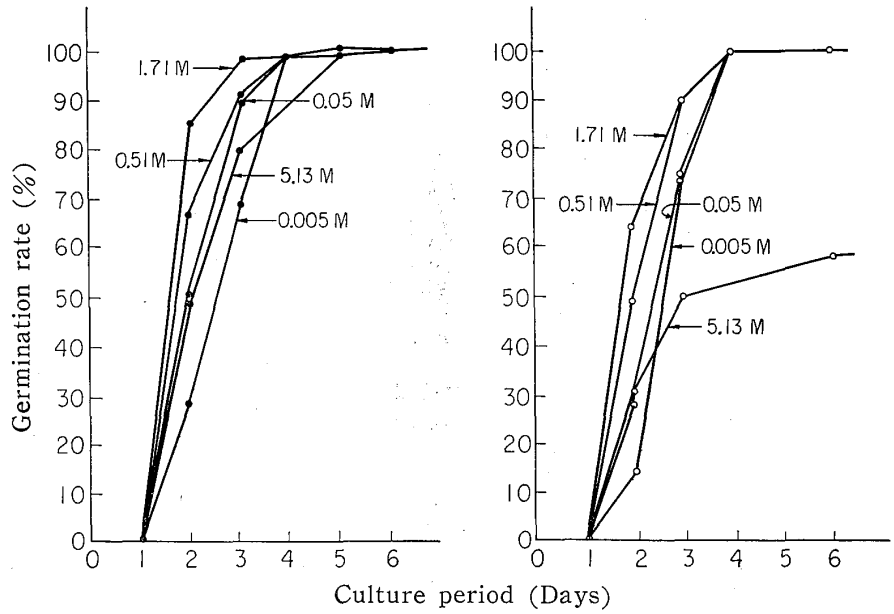

Fig. 15. Different rates of germination in various concentrations of NaCl.

lows that the germination capacity of the suspended spores was restored by dilution of the salt solution. Fig. 16 shows this fact more clearly.

Results obtained from similar experiments carried out with solutions of $MgCl_2$, $CaCl_2$, LiCl and NH_4NO_3 are shown in Figs. 17, 18, 19 and 20, respectively. Spores were pre-incubated in salt solutions for 7 hours. The transmittance of spore suspensions were measured on dilute salt solutions after 7-hour pre-incubation. In all diluted solutions spore number decreased during this pre-incubation period.

In the case of $MgCl_2$, the germination rate was lowest in the original solution (3.67 M) (Fig. 17). Germination increased after dilution without pre-incubation, the optimal concentration being 0.31 M. In the most dilute salt solution (0.061 M), the germination rate was lower than that in 0.31 M. In testing the effect of pre-incubation, it was found that the germination rate in pre-incubated spore suspensions was accelerated higher in proportion to the degree of dilution.

Similar experiments were carried out with the solutions of $CaCl_2$. The results (Fig. 18) differed to some extent from those of the two salts described above. At any concentration, the germination rate of the spore suspension (with or without pre-incubation) showed an inverse correlation with the salt concentration, indicating that this salt exerts a suppressive effect on germination. On the other hand, at a definite concentration, the germination rate of spores without pre-incubation was always lower than that of spores pre-incubated in the salt solutions.

In the case of LiCl (Fig. 19), the germination rate of spores sus-

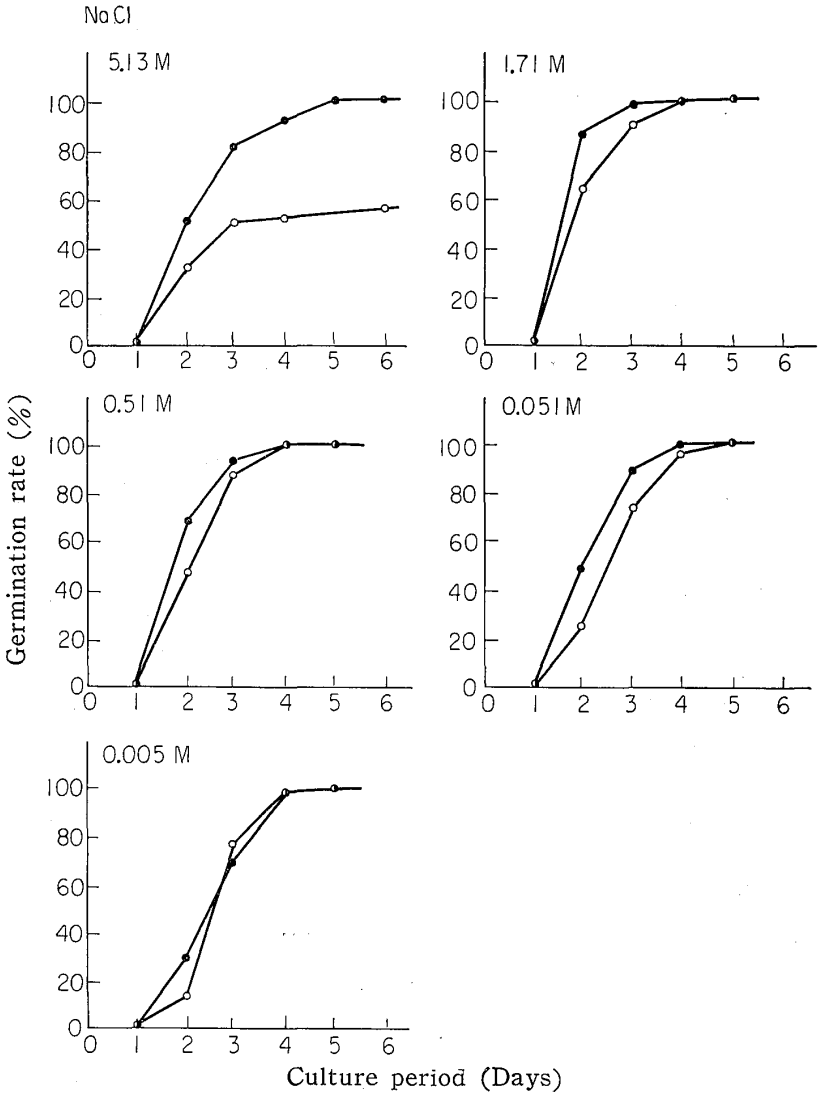

Fig. 16. Differences in germination rates related to concentrations of NaCl. ●—●: Spores suspended in dilute salt-solutions without pre-incubation. ○—○: Spores suspended in dilute salt-solutions after 9-hour pre-incubation.

pended in dilute salt solution without pre-incubation was higher than that of the spores pre-incubated in the same salt solution for 7 hours. The lower the concentration of LiCl, the higher was the rate of germination. These facts are in good agreement with the results obtained above (Table 10, 11 and Fig. 13).

In the case of NH_4NO_3 (Fig. 20), germination rates of spores suspended in the salt solutions without pre-incubation were highest at 1.25 M, lower at 0.625–0.063 M, and lowest at 3.125 M. The germination rate of spores pre-incubated in salt solutions were lowest at 1.25 M and higher at other concentrations.

For comparison, the results obtained so far, together with those regarding $LiNO_3$, are shown in Fig. 21. In this figure, differences in

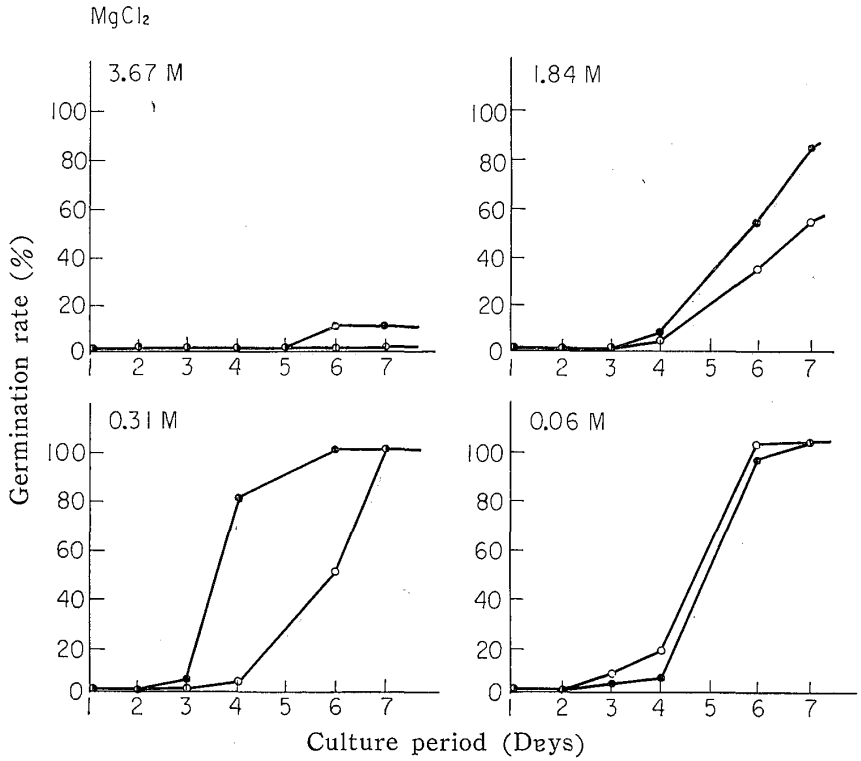

Fig. 17. Differences in germination rates related to concentrations of $MgCl_2$. ●—●: Spores suspended in dilute salt-solutions without pre-incubation. ○—○: Spores suspended in dilute salt-solutions after 7-hour preincubation.

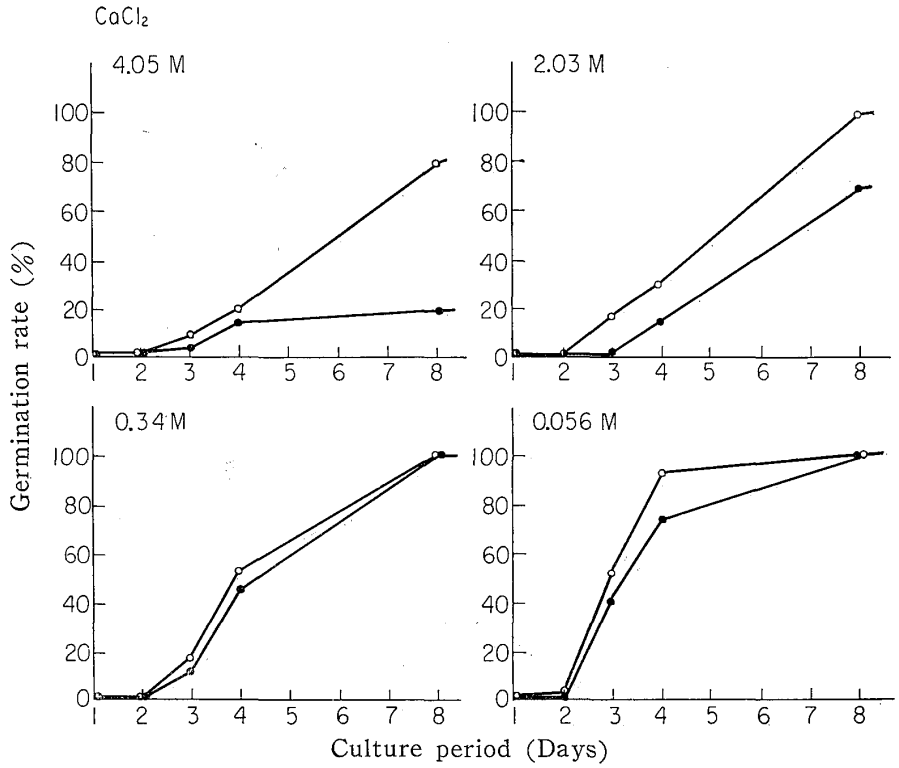

Fig. 18. Differences in germination rates related to concentrations of $CaCl_2$. ●—●: Spores suspended in dilute salt-solutions without pre-incubation. ○—○: Spores suspended in dilute salt-solutions after 7-hour pre-incubation.

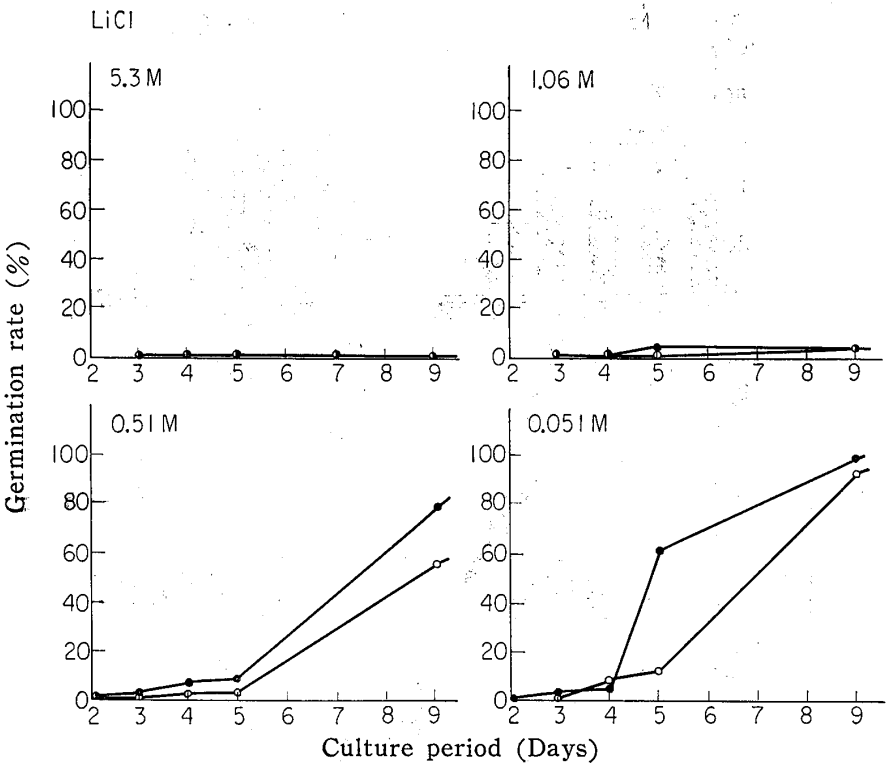

Fig. 19. Differences in germination rates related to concentrations of LiCl. ●—●: Spores suspended in dilute salt-solutions without pre-incubation. ○—○: Spores suspended in dilute salt-solutions after 7-hour pre-incubation.

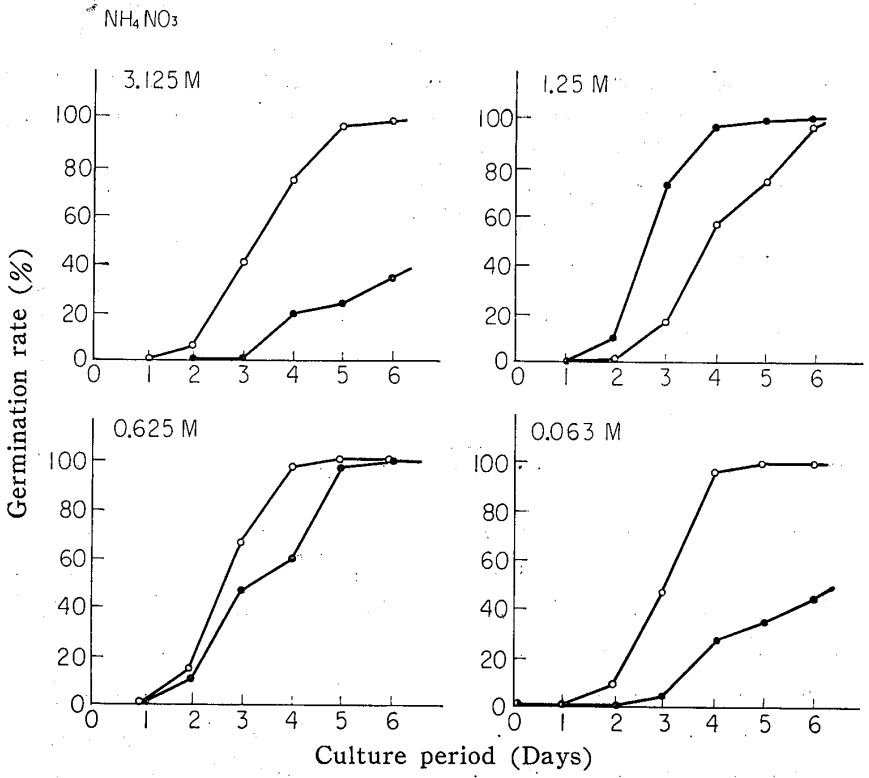

Fig. 20. Differences in germination rates related to concentrations of NH_4NO_3. ●—●: Spores in dilute salt-solutions without pre-incubation. ○—○: Spores suspended in dilute salt-solutions after 7-hour pre-incubation.

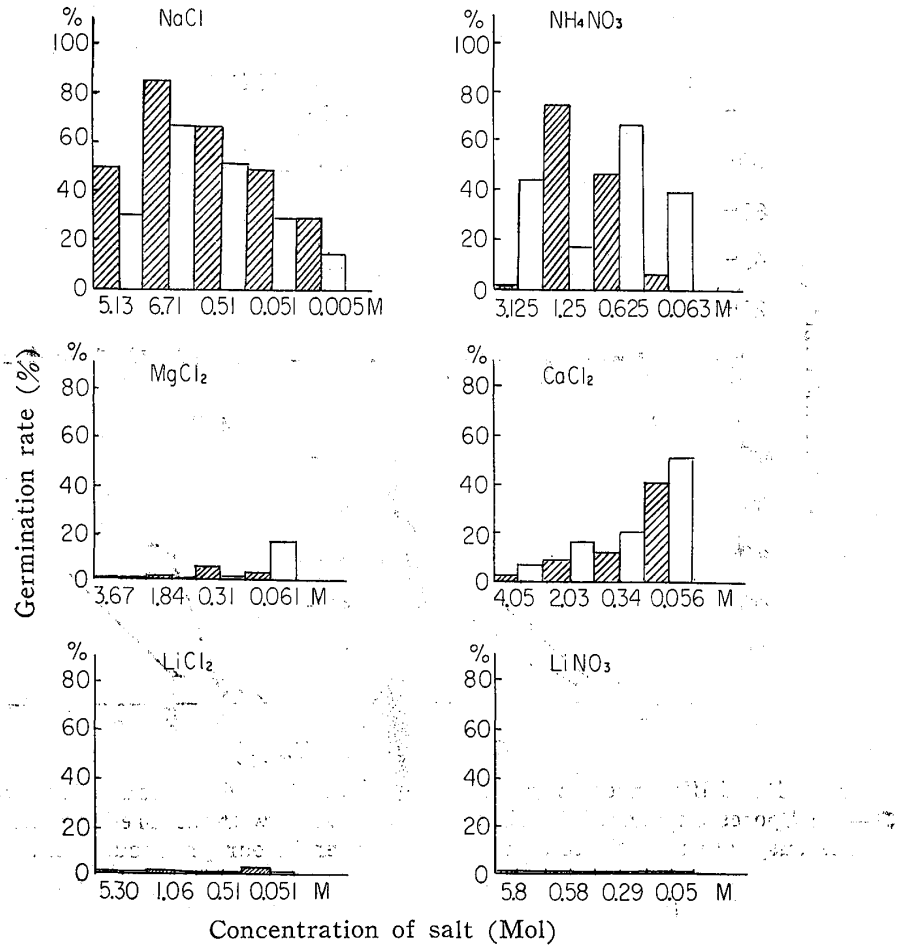

Fig. 21. Effects of dilution of salt-solution on spore germination. ▨: Germination rates of spores suspended in diluted salt-solutions without pre-incubation. □: Germination rates of spores suspended in diluted salt-solutions with pre-incubation. Except a case of NaCl (2-day cultures), data were obtained from 3-day cultures.

the effects of various salts on spore germination are shown more clearly in connection with concentration and dilution effects of the salt solution.

IV. Discussion

Heretofore, research on the problem of obligate tonophily has been directed mostly to the growth of *Aspergillus vitricolae* and *Eurotium tonophilum*[1-4,11]. In these fungi, the former forms only conidiospores, while the latter forms conidiospores and ascospores. On account of this, fluctuation has been observed in the results of experiments worked out with spores of the latter. The organism used in this study is quite suitable for the present purpose because of the fact that it lacks a conidial stage and forms only ascospores[9,10], whose characteristic shape serves to facilitate further experiments (Fig. 1).

Of all environmental factores affecting ascospore germination, atmospheric humidity seems to be the most critical one. They cannot germinate in an atmosphere saturated with water vapour (100% RH). The upper and lower limits of relative humidity, at which they can germinate, are 95% at 24°–28° and 70–60% at 28°, respectively (Figs. 3 and 7, Table 6). In marked contrast to these findings, other ordinary fungi, such as *Aspergillus niger*, *A. oryzae*, *A. flavus*, *A. fumigatus*, *Penicillium notatum*, *Curvularia* sp., *Alternaria* sp., *Cladosporium herbarum* and *Monascus araneosus*, were able to germinate best at 100% RH and a lower limit was found at 90–80% RH.[12] The same is true in the case of conidiospores of *A. niger*[15] and *A. oryzae*.[13]

Recently, attention has been paid to the viability of conidiospores of *Aspergilli* preserved at various temperatures and humidities[18]. For instance, Terui and Mochizuki[19] have studied the effects of humidity on the respiration rate of conidiospores of *A. oryzae*. Goos and Tschirsch[16] studied the effects of temperature and relative humidity on the conidiospores of *Gloeosporium musarum*. Schein and Rotem[17] studied the longevity of uredospores of *Ulomyces phaseoli* under various conditions of temperature and relative humidity. The findings described in these papers suggest certain approaches to the problem of clarifying tonophilic nature of the tonophilic fungi.

Judging from the results obtained by the present study, it is surmised that the fundamental properties of these organisms are not essentially different from those of fungi, with only exception of their moisture requirement for germination.

On the basis of the morphological change of spores occurring in their swelling and sprouting phases (Fig. 1 and Table 4), it is inferred that the early and later stages of the swelling phase may be brought about by some physiological changes comparable to the endogenous and exogenous swelling as has been seen in the germination of *A. niger*[23]. The nutritional requirements characteristic for the ascospores of *Eurotium halophilicum* forma *A* (Figs. 9–12) also support this possibility. Considering the differences found between the requirements for sugar and other substance, it seems that sugars may serve to supply energy, while other substances may be utilized for certain metabolic systems.

Here, especially noteworthy is the fact that the pre-incubated spores of the tonophilic fungus may swell but not able to sprout in nutritional solutions. On the contrary, both swelling and sprouting phases of ordinary fungi may occur in these same solutions.

As reported previously[1–6,8,10,12], high concentrations of salts are needed to support the mycelial growth of tonophilic fungi. However, it becomes evident that the germination of the tonophilic fungi was suppressed by concentrated salt solutions. This appeares distinctly in

the case of LiCl, $LiNO_3$ and NH_4NO_3, but removal of these salts makes it possible to restored the germinating capacity in various degrees (Fig. 13). These observations may suggest that spores of this fungus are halotolerant rather than halophilic, and that its tonophily is comparable with the halotolerance of halophilic bacteria[41,45] and of halophilic yeasts.[34,35] The enhancement of germination capacity by removal of salts in the tonophilic fungus may be arranged in the following order: $K^+ > Na^+ > Mg^{++} > Ca^{++} > Li^+$. This agrees with the previous record on *Zygosaccharomyces major*, strains N5 and N8[34]. On the other hand, it has been reported that high concentrations of $MgCl_2$ and $CaCl_2$ sometimes induce the swelling of germ-tubes, though it is not known whether this phenomenon is the same as that of halophiic bacteria[43,45].

According to the recent literatures, investigations of salt effects on the growth of microorganisms have been made not only with halophilic bacteria[32,33,41,45,46] and halophilic yeasts[27,23,34-36] but also with a halophilic *Chlamydomonas*[42,47]. These investigations suggest that salts may play a significant role in germination as well as in growth of the tonophilic organisms. The halophilic bacteria,[37,39,41,43] halophilic yeasts[27,23,34-36] and a halophilic *Chlamydonas*[42] were damaged when they were transferred into the salt solutions of lower concentrations. This has never been examined in detail on tonophilic fungus. As described above, ascospores of the tonophilic fungus are able to germinate after dilution of concentrated salt solutions in which they have been preincubated, although they have suffered some damage (Fig. 14 and Table 13). In this case, less concentrated salt solutions rather promote often spore germination (Fig. 13). An apparent inconsistency of these phenomena may ce explained in part by the experiments with NaCl, as shown in Fig. 16.

On the whole, NaCl shows a suppressive effect on the germination of fungal spores, but the rate of germination depends upon the degree of dilution. But, no reasonable explanation of this phenomenon has been obtained so far. Spore cells must have certain active transport system and or osmotic regulation mechanisms similar to those already suggested in a halophilic *Chlamydomonas*.[47] Such mechanisms would permit the cells to survive even after transfer to dilutie salt solutions. However, if the osmotic change in external salt solutions were too abrupt, the spore cells might burst up or at least their germination capacity might be suppressed. With other salts examined so far, results are too complicated to be explained.

For the present, the mechanism of tonophily are still obscure and has much to be investigated. But tonophilic fungi may be particularly important and interesting organism for investigating permeability of cell membranes, halophilic enzymes and active transport systems.

Recent investigations on the halophilic enzymes[43-57] in halophilic bacteria suggest approaches which might be followed in the effort to clarify the problems with respect to tonophily and halophily of tonophilic fungi.

V. Summary

Some basic physiological conditions affecting the ascospore germination of the obligatorily tonophilic fungus, *Eurotium halophilicum* forma *A* have been studied. This was facilitated by availability of a fungal strain which forms only ascospores. The results obtained are summarized as follows.

1. The process of ascospore germination in the obligatorily tonophilic fungus can be divided into two phases, swelling and sprouting. The swelling phase is divided into early and later stages. Spores in each stage can be distinguished morphologically. The early stage corresponds to the stage of exogenous swelling and the later to endogenous swelling of the conidiospores of *Aspergillus niger*.

2. The effect of environmental factors on the ascospore germination of the fungus have been studied in detail. The optimal conditions were 28°, 90% RH and pH 5.6–6.0. Dried malt-extract agar smeared on a slide-glass are found to be most favorable for aerial germination of ascospores. Spore germination occurs within the ranges of 15°–37°, 95–60% RH, and pH 1.8–10.6 on this culture medium. Heating of the spores at 50°–100° before inoculation shows a suppressive and sometimes lethal effect.

3. Germination tests on dried simple agar medium have revealed that addition of various amino acids, vitamins (10–400 μg/ml) and sugars (20 mg/ml) to the basal suspension medium (Czapek's salt solution without C and N sources) are almost indifferent for germination. However, spore germination was promoted when a substance was added to the basal suspension medium together with sucrose (30 mg per ml). And, pre-incubation of spores in the suspension medium at 28° for three weeks was most favorable for germination.

It seems probable that external substances diffuse into the spore cells in an earlier stage, and certain metabolic change may take place at a later stage within the cell, whereupon the spores may be driven into the sprouting phase.

4. The effects of fifteen kinds of salts on germination have also been examined. On the whole, the salts added to the suspension medium at higher concentrations showed suppressive effect on germination. The effect varies with the kind of salts and depends on a pre-incubation period. Among the salts tested, LiCl, $LiNO_3$ and NH_4NO_3

suppressed germination almost completely. However, the spores were able to germinate better, when salts were removed by washing.

5. Spores, whose germination was suppressed by highly concentrated salt solutions, recovered their capacity of germination after dilution of the salt solutions. In the case of NaCl, an optimal concentration (1.71 M) lies between 5.13 M and 0.005 M salt solutions. As regards LiCl and $LiNO_3$, suppressive effect decreased with an increase of dilution rate. On the other hand, decreases in spore number and a corresponding decreases in transmittance have been observed in the solutions of NaCl, $MgCl_2$ LiCl and NH_4NO_3 after pre-incubation at 28° for 7–9 hours in the concentrated solutions.

From all these results, certain parallelism exist in the effects of salts during pre-incubation.

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