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REVERSIBLE DEACTIVATION OF NEUROSPORA ASCOSPORES BY LOW TEMPERATURE [1]

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ABSTRACT

SUN, CLARE Y., and ALFRED S. SUSSMAN. (U. Michigan, Ann Arbor.) Reversible deactivation of Neurospora ascospores by low temperature. Amer. Jour. Bot. 47(7): 589–593. Illus. 1960.—Heat-activated ascospores of *Neurospora tetrasperma* are reversibly deactivated after incubation at 4°C. for 36–48 hr. Two cycles of deactivation and reactivation are possible although the percentage germination decreases in the last cycle. By contrast, spores held at 20°C., or in glycerol at 4°C., will remain activated for much longer periods of time. If an incubation period at 20°C. greater than 30 min. is interposed before the activated spores are placed at 4°C., germination occurs despite the cold-treatment. Furfural-activated ascospores, when held at 4°C., are deactivated but can be reactivated only by heat, pointing up a difference between ascospores activated by these different means. Although a fraction of the stimulus afforded by heat-sensitization to chemical activators is preserved for 2 days at —20°C., it is dissipated completely after a short time at 4°C. These data are discussed on the basis of the suggestion that the reversible production of a substance initiates a series of steps which lead to germination. Thus, the temperature minimum of the forward reaction is greater than 4°C. whereas the back reaction proceeds at this temperature.

IN THE model proposed by Goddard (1939) to explain the activation of *Neurospora* ascospores by heat, it was assumed that a substance, S, is converted to a product, P, according to the following reaction:

$$S \rightleftharpoons P$$

The increase in respiration attendant upon activation depends directly upon the concentration of P, but its maintenance, after induction, does not require the continued presence of P. Thus, heat activation at 50–60°C. is required to shift the equilibrium of the above reaction to the right, whereas at lower temperatures it is in the other direction and P is converted back to S. Evidence for the reversibility of the activation process was provided by Goddard (1939) and by Goddard and Smith (1938) who showed that activated spores were deactivated by incubation under anaerobic conditions or in certain respiratory poisons.

If the above reaction is accepted as a working hypothesis, then certain experimental procedures are indicated. Among these, the isolation of substance P, broadly defined to include metabolites, enzymes or coenzymes may be possible. Although such a substance has not yet been isolated, recent work suggests that its function may be connected with trehalose metabolism (Lingappa, B. T. and Sussman, 1959; Sussman and Lingappa, 1959). Another approach suggested by the hypothesis is based upon the possibility that the temperature minima of the forward and back reactions differ,

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as well as the temperature coefficients. Accordingly, incubation at a suitable temperature might result in the conversion of product P to S if the minimum temperature of the forward reaction is higher than that of the back reaction. In this event reversible deactivation is to be expected and the following experiments were designed to test this possibility.

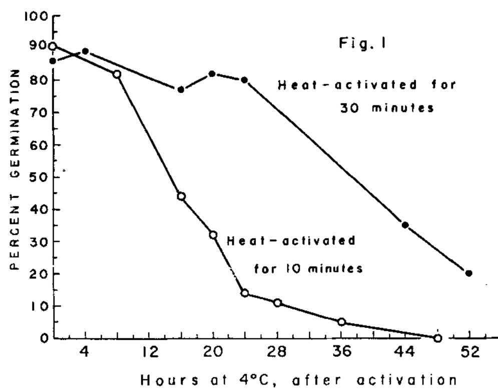
MATERIALS AND METHODS.—Ascospores of *Neurospora tetrasperma* were harvested, stored and prepared for use by the methods described previously (Sussman, 1954a). In all experiments, unless otherwise noted, ascospores that had been stored for about a year were used. The concentration of spores usually used gave a reading of 90 in the Klett colorimeter with a blue filter (Klett # 42) and was equivalent to 0.5 mg. (dry weight) per ml.

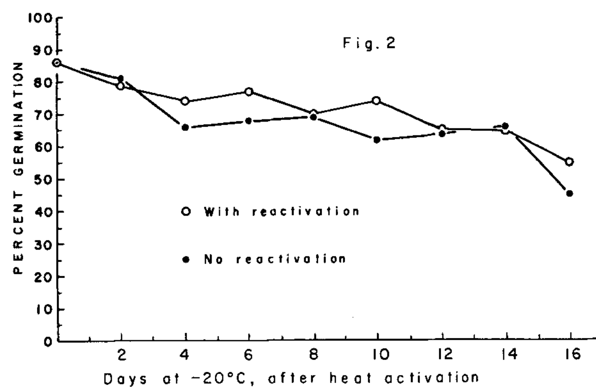
The breaking of dormancy usually was accomplished by placing ascospores suspended in water in a constant temperature bath at 60°C. for 10 min. In certain experiments the spores were activated by suspension in furfural at a final concentration of 1×10^{-3} M. When chemical activation was performed the spores were first exposed to 45°C. in a water bath for 40 min. in order to induce the recovery of sensitivity to furfural (Sussman, 1954b).

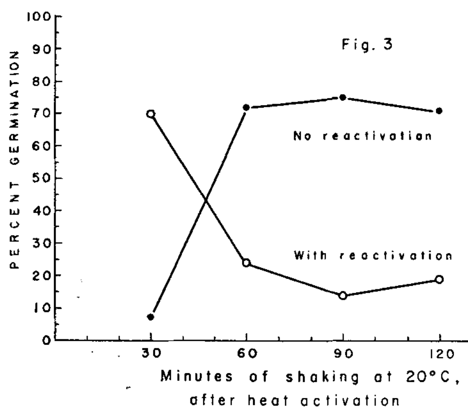
Incubation of the activated ascospores was usually performed at 27°C. for 3–4 hr. on a shaking machine with a reciprocal movement. For treatment at low temperatures, the spores were stored in the main compartment of a refrigerator (4°C.), or in the freezer compartment which was maintained at -15°C. to -20°C.

Germination counts were made on duplicate samples and at least 2 separate counts of 600 spores were averaged for each determination.

RESULTS.—*Effect of storage at low temperature upon heat-activated ascospores.*—Previous work has disclosed that the minimum temperature for the germination of ascospores of *Neurospora* is about 6°C. (Lingappa, Y., and Sussman, 1959). Consequently, attempts were made to interfere with the activation mechanism by exposure of the spores to temperatures below this minimum. Ascospores were heat-activated for 10 min. and duplicate 2-ml. aliquots were removed and incubated at 27°C. The remainder of the spores was placed at 4°C. At periodic intervals, thereafter, duplicate samples were removed, incubated at 27°C. as before, and







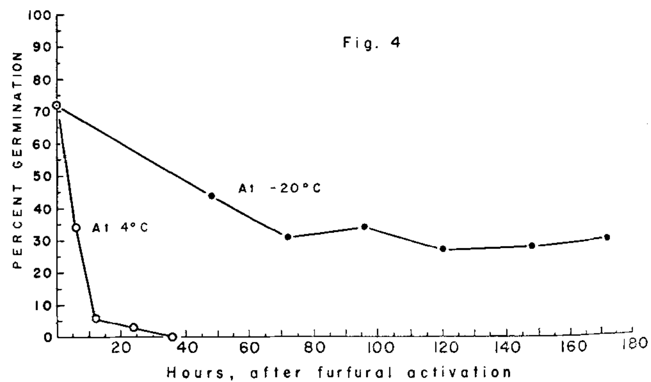


Fig. 1–4.—Fig. 1. Effect of storage at 4°C. upon the germination of ascospores activated for different lengths of time at 60°C.—Fig. 2. Germinability of heat-activated ascospores after storage for various times at —20°C. Reactivation was accomplished by exposure of spores to 60°C. for 10 min.—Fig. 3. Effect of storage at 4°C. upon germination of heat-activated ascospores incubated at 20°C. for various lengths of time after activation. Reactivation was accomplished as above. —Fig. 4. Germinability of ascospores activated by 1×10^{-3} M furfural after storage at 4° and —20°C.

the percentage germination after 4 hr. determined. The results in fig. 1 disclose that only 14% of the spores remain activated after storage at 4°C. for 24 hr. and that none germinate after 48 hr.

That the length of the heat-activation treatment influences the rate at which the percentage germination decreases during storage at 4°C. also may be seen in fig. 1. Thus, despite the fact that there is very little difference in the number of spores which germinate after activation periods of 10 and 30 min., the "decay" of germinability at 4°C. is much slower in the latter case. In fact, after 52 hr. 20% of the spores still germinated.

In order to determine whether activated spores stored at low temperatures lose their viability, or are simply deactivated, they were exposed to 60°C. for 10 min., after removal from 4°C., and incubated at 27°C. It is clear from the data in table 1 that the latter is the case and that storage at 4°C. results in the gradual deactivation of ascospores.

The number of cycles of activation and deactivation of which the ascospores are capable was studied by storing activated cells for 144 hr. at 4°C. At 48-hr. intervals during this time, samples were withdrawn, reactivated, incubated at 27°C. and the percentage of germination determined. Parallel samples were incubated at 27°C. without being heat-activated again and served as a check upon the level of germination after cold-storage. As the data in table 2 reveal, the limit is only 2 cycles under the conditions of these experiments and, even after the second cycle, there is about a 30% decrease in germinability.

Some of the experiments at 4°C. reported above were repeated at –20°C. Thus, activated ascospores were stored at this temperature for various periods of time after which they were incubated at 27°C. and the percentage germination determined. Figure 2 shows that even after 16 days (384 hr.) at this low temperature, if the spores are placed at 27°C., about 50% will germinate with no additional heat treatment. Furthermore, these data also reveal that another heat activation has only a small effect upon activated spores stored at –20°C.

That there are at least 2 phases in the development of activated ascospores, recognizable through differences in their physiological characteristics, has been suggested by Sussman et al. (1956) and Lingappa, Y., and Sussman (1959). Therefore, a period of shaking at 20°C. was interposed between the treatment at 4°C. and the final incubation at 27°C. during which germination occurs. In these experiments, the spores were maintained at 4°C. for 48 hr. and the length of the first exposure to 20°C. varied as indicated in fig. 3. Ascospores incubated at 20°C. for up to 30 min. before transfer to 4°C. were reversibly deactivated in accordance with the results obtained previously. On the other hand, when such spores are incubated for longer than 30 min. at 20°C., they maintain their state of activation even after storage at 4°C. for 48 hr. The provision of another heat-treatment to such spores results in a considerable decrease in their germinability.

Thus far, all the evidence supports the notion that a reversible chemical reaction, like that discussed in the introduction, occurs as a result of activation. In order to subject this model to a further test, ascospores were immersed in 100% glycerol, a treatment which results in the loss of most of the cell's water. Observation of the spores after such treatment reveals that almost all of the spores have collapsed such that they are crescent-shaped when observed laterally. This means of drying the cells was used because it had been observed that complete viability is retained during this treatment (Lowry and Sussman, unpublished observations). Accordingly, activated ascospores were immersed in 100% glycerol and stored at 4°C. for various

TABLE 1. *Effect of reactivation upon ascospores incubated at 4°C. after their first activation treatment. Spores were removed from 4°C., reactivated by suspension in water at 60°C. for 10 min., and then incubated at 27°C.*

Time of storage at 4°C. (hr.)	Percentage germination	
	control spores	reactivated spores
36	5	85
60	0	60

TABLE 2. *Cycles of activation and deactivation of ascospores of N. tetrasperma due to treatment at 4°C. Ascospores were activated and stored at 4°C. for 48-hr. periods after which they were reactivated and aliquots were incubated at 27°C. or returned to 4°C.*

Hrs. after first activation	Number of cycles	Percentage germination	
		not reactivated	reactivated
0	Control	86	–
48	1	0	87
96	2	0	63
144	3	0	0

TABLE 3. *Effect of dehydration in glycerol upon the deactivation of ascospores of N. tetrasperma at 4°C. Activation was accomplished at 60°C. for 10 min. and the spores were immediately immersed in glycerol and stored at 4°C.*

Hr. storage at 4°C.	Percentage germination
0	82
12	76
24	74
36	75
48	70
60	69
72	57
84	54
96	60
120	55

lengths of time. Thereafter, the glycerol was removed by centrifugation in 3 changes of water and the spores were incubated in water at 27°C. and the percentage of germination determined. In direct contrast to activated ascospores that have been stored at 4°C. in water (fig. 1), those dried in glycerol before such treatment are deactivated to only a small extent after 48 hr. and fully 55% germinate even at the end of 5 days (table 3).

Effect of storage at low temperatures on furfural-activated ascospores.—Emerson (1954) has drawn attention to certain differences between activation by chemical means and by heat. With this in mind, ascospores (1 mg. per ml.) were heat-sensitized at 45°C. for 40 min. and, after being cooled, were mixed with an equal volume of 2×10^{-3} M furfural. The spores were exposed to the furfural for 10 min. and then were washed in several changes of dist. water by centrifugation. They were stored at 4°C., or at –20°C., for varying lengths of time, iucubated at 27°C and the percentage germination determined. As can be seen in fig. 4, the general effects of cold temperatures upon heat-activated ascospores were observed for chemically activated ones as well. However, the decline in germinability is more precipitous in the case of furfural-activated spores for only 6% still germinate after only 12 hr. at 4°C. Also, in the case of spores held at –20°C. germinability decreases at a more rapid rate but fully 30% germinate after 7 days at this temperature.

Reactivation of furfural-activated ascospores, held at 4°C. for 5 days, was attempted by the several means listed in table 4 but germination was elicited only by heating at 60°C.

Ascospores maintain their peak in responsiveness to furfural during a very short time-interval shortly after they are released (Emerson, 1954). Consequently, except during that short time, heat-sensitization is a necessity before germination can be induced maximally by chemical means (Sussman, 1954b; Sussman et al., 1959). Thus, it was of interest to determine whether the enhanced responsiveness to chemical activation of heat-sensitized ascospores could be maintained by incubation at low temperatures. Accordingly, ascospores (1 mg. per ml.) were held at 45°C. for 40 min. and immediately transferred to 4° or –20°C. Aliquots were removed at intervals, brought to room temperature, exposed to 1×10^{-3} M furfural (final concentration) for 10 min. and washed free of the chemical by centrifugation as described before. After incubation at 27°C., germination was counted and the results outlined in table 5. Although some of the enhanced responsiveness induced by heat-sensitization is retained for 2 days at –20°C., it is lost completely after only a day at 4°C. Moreover, even at –20°C., the stimulus induced by heat-sensitization is dissipated after 3 days.

Discussion.—The reversible deactivation of ascospores of *N. tetrasperma* during incubation at 4°C. can be interpreted to mean that, at this temperature, the back reaction proceeds faster than the forward one. On the other hand, when activated spores are held at –20°C. (fig. 2), or at 4°C. dried in glycerol (table 3), neither reaction proceeds and deactivation does not occur.

As in the case of furfural-activated ascospores that have been deactivated by incubation under anaerobic conditions, those in which the reversal has been effected by low temperature are not reactivated by reincubation in furfural (Emerson, 1954; table 4). That the cells have not been killed by this treatment is clear from the fact that incubation at 60°C. will reactivate such spores.

In the present experiments only 2 cycles of activation and deactivation were possible, that is, ascospores were activated 3 times and deactivated twice. However, the percentage germination after the last activation has decreased about ⅓. These experiments are in agreement with those of Goddard (1939) who mentions in a footnote to his table 2 that one lot of spores germinated after the same number of cycles of activation and deactivation by anaerobic incubation. A difference that arises concerns the continued high percentage of germination, even after activation 3 times.

The work of Halbsguth and Rudolph (1959) on sporangiospores of *Phycomyces blakesleeanus* suggests that dormancy is reversible in fungi other than *Neurospora*. Thus incubation at 25°C. in water, or under anaerobiosis, resulted in the deac-

Table 4. *Reactivation of furfural-activated ascospores stored at 4°C. for 5 days after activation. A final concentration of 1×10^{-3} M furfural was used to activate throughout*

Reactivation treatment	Percentage germination
Controls (activated in furfural before incubation at 4°C.)	72
Incubated 5 days at 4°C. and provided:	
no further treatment	0
60°C. for 10 min.	84
45°C. for 40 min.	0
45°C. for 40 min. followed by re-exposure to furfural	0
re-exposure to furfural	0

Table 5. *Effect of storage at low temperatures on enhanced responsiveness to chemical activators induced by heat-sensitization at 45°C. for 40 min. Germinability was tested by exposing spores to 1×10^{-3} M furfural for 10 min. followed by incubation at 27°C.*

Days of storage at low temperature	Percentage germination when stored at: 4°C.	–20°C.
0	50	50
1	0	15
2	0	17
3	--	0

tivation of heat-treated spores. Several cycles of activation and deactivation were possible although complete inactivation was not achieved in later cycles and, beginning with the fifth activation, such treatment reduced the percentage germination.

Further evidence for the step-wise transformation of the dormant ascospore to the vegetative (mycelial) condition is furnished by these data. Thus, fig. 3 reveals that reversion to the dormant state does not occur at 4°C. if incubation at 20°C. for longer than 30 min. is interposed between the activation treatment and exposure to low temperature. Antecedent to this change in the physiology of the activated spore is the loss of heat-resistance which is complete between 20–30 min. after activation and can be detected within minutes after activation. That a fermentative type of metabolism ensues immediately upon activation has been shown by Sussman et al. (1956). This is followed by a different type of metabolism characterized by the use of lipids and the loss of fermentative capacity (Goddard, 1939; Lingappa, B. T., and Sussman, 1959). Many of these observations are summarized in table 6 which describes the physiological markers for the stages between activation and germination.

Three methods are now known for reversing the activation of ascospores of *Neurospora* including, incubation under anaerobic conditions, in poisons like cyanide (Goddard, 1939) and exposure to 4°C. The representation of the events involved in the activation and reactivation processes presented by Goddard, and discussed in the introduction, does not imply that a unique mechanism can be formulated at this time. The fact that activation is reversible can be interpreted in several ways, which include: (1) Activation results in the production of a substance which triggers the changes which result in germination; the reaction is reversed by restriction of the synthesis of this material so that the steps which follow this initial one are not induced and the cell reverts to the dormant state.

TABLE 6. *Summary of the stages between activation and germination and the physiological markers which characterize them in Neurospora tetrasperma*

		Physiological marker:			
Condition of spores	Min. after activation	$Q^a_{O_2}$	R.Q.[a]	thermal[b] resistance	deactivability at 4°C.
Dormant	–	0.5	0.6	+	–
Activated: Stage #:					
1	0–20	0.5–4	1.2	+	+
2	30–60	4 –10	1.0	–	+
3–n	60–150	15 –30	1.0	–	–
Germinating	150	30	0.6	–	–

[a] Data taken from Lingappa, B.T., and Sussman (1959), Goddard (1939) and Holton (1958).
[b] Data taken from Lingappa, Y., and Sussman (1959).

(2) An inhibitor which maintains the cell in the dormant condition may be destroyed as a result of activation; deactivation would then be caused by the resynthesis of the inhibitor, prevention of its destruction or a combination of both. (3) Reactions involving more than one reactant at each step, or several steps which form a feedback system could also account for these observations.

At present, no decision between these alternatives is possible in the case of *Neurospora* ascospores. However, it seems likely that an important aspect of the change in the metabolism of these cells upon activation is the initiation of the use of trehalose (Lingappa, B. T., and Sussman, 1959: Sussman and Lingappa, 1959). Thus, lipids are the endogenous substrate of dormant spores whereas trehalose is utilized only after activation. How this change is initiated and how many steps are antecedent to it are still unknown.

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