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# Heat Activation of *Phycomyces blakesleeanus* Spores: Thermodynamics and Effect of Alcohols, Furfural, and High Pressure

JOHAN M. THEVELEIN,[1]* JOZEF A. VAN ASSCHE,[1] ALBERT R. CARLIER,[1] AND KAREL HEREMANS[2]

*Laboratorium voor Plantenbiochemie, Katholieke Universiteit te Leuven, Vaartstraat 24, B-3000 Leuven,[1] and Laboratorium voor Chemische en Biologische Dynamica, Katholieke Universiteit te Leuven, Celestijnenlaan 200 D, B-3031 Heverlee,[2] Belgium*

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The thermodynamic parameters for the heat activation of the sporangiospores of *Phycomyces blakesleeanus* were determined. For the apparent activation enthalpy ($\Delta H^{\neq}$) a value of 1,151 kJ/mol was found, whereas a value of 3,644 J/°K·mol was calculated for the apparent activation entropy ($\Delta S^{\neq}$). *n*-Alcohols (from methanol to octanol), phenethyl alcohol, and furfural lowered the activation temperature of *P. blakesleeanus* spores. The heat resistance of the spores was lowered concomitantly. The effect of the alcohols was a linear function of the concentration in the range that could be applied. When the log of the concentration needed to produce an equal shift of the activation temperature was plotted for each alochol against the log of the octanol/water partition coefficient, a straight line was obtained. The free energy of adsorption of the *n*-alcohols to their active sites was calculated to be −2,487 J/mol of $CH_2$ groups. Although still inconclusive, this points toward an involvement of protein in the activation process. The effect of phenethyl alcohol was similar to the effect of *n*-alcohols, but furfural produced a greater shift than would be expected from the value of its partition coefficient. When the heat activation of the spores was performed under high pressure, the activation temperature was raised by 2 to 4°K/1,000 atm. However, with pressures higher than 1,000 atm ($1.013 \times 10^5$ kPa) the activation temperature was lowered until the pressure became lethal (more than 2,500 atm). It is known that membrane phase transition temperatures are shifted upward by about 20°K/1,000 atm and that protein conformational changes are shifted upward by 2 to 6°K/1,000 atm. Consequently, heat activation of fungal spores seems to be triggered by a protein conformational change and not by a membrane phase transition. Activation volumes of −54.1 $cm^3$/mol at 38°C and −79.3 $cm^2$/mol at 40°C were found for the lowering effect of high pressure on the heat activation temperature.

No definite explanation is available concerning the primary site of action for heat or other stimuli which cause the breaking of dormancy in fungal (41) or bacterial spores (14). The way in which dormancy is broken in these spores has been studied intensively, since it is considered to be a model system in the study of cell development (41).

Spores can be activated by a variety of different treatments; some treatments, such as heat, seem to have rather a general action, whereas others, such as L-alanine in the case of *Bacillus* spore activation (22), seem to be more specific. Certain compounds are very effective in activating spores of one species, whereas simultaneous activation with a second treatment is required for optimal or even any activation in other species. Furfural is a potent activator of *Neurospora* ascospores (11), whereas simultaneous application of heat is required to induce optimal germination of the basidiospores of *Coprinus radiatus* (30).

It has been shown previously that alcohols and other organic solvents are capable of activating the spores of certain fungi (41). However, no detailed analysis has been made concerning the effect of several alcohols in relation to their chain length, and hence to their nonaqueous/aqueous partition coefficient. This has been accomplished in the present paper.

In addition, we have also calculated the free energy of adsorption of the alcohols to their

active sites in order to identify the chemical nature of the sites responsible for spore activation. Several workers have reported indirect evidence of membrane involvement during the heat activation process (1), but the experimental data so far obtained do not allow one to distinguish between a primary involvement of phospholipids and a primary involvement of proteins. Sussman (41) suggested that lipids were involved in the activation of *Neurospora* ascospores. On the other hand, several indirect arguments led Cotter to the conclusion that partial denaturation of a mitochondrial inner membrane protein was the primary event in *Dictyostelium discoideum* spore activation (9). A universal mechanism, however, for the activation of fungal spores seems more probable in view of the striking similarities between the activation behavior and subsequent physiological processes in several fungi.

The free energy data obtained with the alcohol experiments alone are not sufficient to determine unequivocally the chemical nature of the target sites of heat activation. Since it is known that the effects of pressure on membrane phase transitions and on protein conformational changes are quite different, the activation of *Phycomyces blakesleeanus* spores was carried out under high pressures.

## MATERIALS AND METHODS

**Spores.** Strain 1$^+$ of *P. blakesleeanus* (from the collection of W. Halbsguth) was grown and the sporangiospores were harvested as previously described (43). The spores used were not older than 14 days. Usually more than 90% of the spores germinated after a heat activation of 3 min at 50°C.

**Activation.** Activation was done in test tubes for 3 min in 0.1 M potassium phosphate buffer, pH 6.5 (controls). The same buffer was used for activation with alcohols and with furfural. The spores were activated at a concentration of 3 mg/ml in a water bath at the indicated temperatures. After a 3-min heat treatment the spores were chilled in an ice bath, collected by centrifugation, and washed twice with distilled water.

**Activation under pressure.** In a first series of experiments with pressures of up to 420 atm (42,560 kPa), the spores were activated for 3 min at a concentration of 1 mg/ml in the column of a high-pressure liquid chromatograph (Waters Associates). Potassium phosphate buffer (0.1 M, pH 6.5) was used with or without the addition of *n*-alcohols. The high-pressure liquid chromatograph column with the spores was cooled by immersion in an ice bath, and then the pressure was applied with a high-pressure liquid chromatograph pump. This took only a few seconds. Any possible effect due to a rise in temperature caused by the application of pressure could be completely excluded in this way. The column was heated in a water bath and cooled for 30 s in an ice bath after the activation treatment before the pressure was released. The pH shift brought about by the pressure application was too small to affect the activation temperature.

In a second series of experiments with pressures up to 2,500 atm, 3 mg of spores was activated for 4 min in a specially constructed metal cylinder (volume, 0.5 ml). The cylinder with the spores was cooled in an ice bath, and then the pressure was applied with an oil pump in a few seconds. The cooling and pressure release after activation (in 0.1 M potassium phosphate buffer, pH 6.5) were achieved as described above.

**Germination percentages.** Germination percentages were determined as previously described after 8 h of germination in a minimal nutrient medium at a concentration of 3 mg/ml (43). For larger numbers of samples, a model TA Coulter Counter (Coulter Electronics, Hertfordshire, England) was used to determine the germination percentages. The apparatus was able to make a particle size distribution of a particle mixture based upon volume. A total of 10,000 spores were counted in an electrolyte solution of 1% NaCl. Upon activation, the germinating spores shifted from a size class with a diameter between 10.08 and 12.70 μm to a higher size class with a diameter between 16.00 and 20.16 μm. The spore fraction remaining in the small-size class was inversely related to the germination percentage.

**Partition coefficients.** The partition coefficient of furfural in an octanol-water system was determined with the diphenylamine test of Ashwell (2). The other partition coefficient data were taken from Leo et al. (25).

**Free energy of adsorption of the *n*-alcohols to their active sites.** The standard free energy of adsorption of the *n*-alcohols per methylene group was calculated by the method of Schneider (37):

$$\Delta G_t^0 = 2.303RT \log \frac{\text{mol fraction}_{i+1}}{\text{mol fraction}_i}$$

where $i$ and $i + 1$ are two successive homologs, $R$ is the gas constant (8.314 J/mol per °K), and $T$ is an absolute temperature (e.g., 313°K) at which all of the *n*-alcohols in a certain concentration (see Fig. 4) bring about the same germination percentage (arbitrarily taken at 50°C). At this temperature all of the *n*-alcohols (in the concentrations shown in Fig. 4) have shifted the activation temperature by 3.2°K.

## RESULTS

**Thermodynamics of heat activation.** Spores of *P. blakesleeanus* were heated for 3 min at different temperatures and subsequently introduced into the germination medium. When the germination percentages were determined after 8 h, a typical sigmoidal activation curve was obtained (Fig. 1). Such curves allow a calculation of the thermodynamic parameters of fungal spore heat activation, as first carried out by Cotter (8). He assumed that there was a two-state equilibrium between an activated form and an inactive form of a protein, which were responsible for the activated state and the dor-

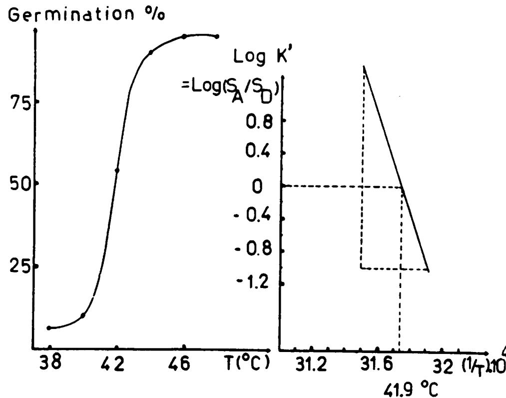


FIG. 1. *Sigmoidal activation curve of the spores of P. blakesleeanus heated for 3 min at the indicated temperatures. From the slope of the straight line in the plot of log K′ against 1/T, $\Delta H^{\#}$ can be calculated. $S_A$, Fraction of activated spores; $S_D$, fraction of dormant spores.*

mant state of the spores, respectively. This can be expressed by an equilibrium constant, $K'$, which is the ratio of the fraction of activated spores to the fraction of dormant spores. When log $K'$ is plotted against $1/T$ (Fig. 1), $\Delta H^0$ can be calculated from the following equation: slope = $-\Delta H^0/2.303R$. At 41.9°C (Fig. 1), when 50% of the spores germinate, log $K' = 0$. Hence, $\Delta S^0$ can be calculated from the following equations: $\Delta G^0 = -2.303RT \log K' = \Delta H^0 - (T \times \Delta S^0)$. Cotter analyzed heat activation in a purely thermodynamic way without taking into account the kinetic aspects. Indeed, activation at suboptimal temperatures does not result in an equilibrium state since longer heating at these temperatures also raises the germination percentage until, after prolonged periods, the maximum percentage is obtained. Hence, the entropy and enthalpy values have a dual origin; there is a thermodynamic and a kinetic contribution, and therefore we refer to them as activation parameters.

We calculated a value of 1,151 kJ/mol for the apparent activation enthalpy ($\Delta H^{\#}$) and a value of 3,644 J/°K·mol for the apparent activation entropy ($\Delta S^{\#}$). Cotter reported a $\Delta S^{\#}$ of 7,113 J/°K·mol for *D. discoideum* spores and a $\Delta S^{\#}$ of 6,276 J/°K·mol for the spores of *Neurospora tetrasperma* (8). He concluded that the high entropy changes of fungal spore heat activation pointed to a conformational change of the polar part of a membrane protein.

**Effect of *n*-alcohols on heat activation.** When spores were heated at different temperatures in the presence of *n*-alcohols, the sigmoidal activation curve shifted toward lower values. Figure 2 shows a typical example, the lowering effect of *n*-butanol on the activation temperature. The magnitude of the shift produced by the alcohol varied linearly with the concentration in the range that could be applied (Fig. 3). There was indeed an upper limit for the higher alcohol concentrations since they caused a lethal effect which resulted in lower germination percentages at the optimum activation temperature. Besides, the addition of *n*-alcohols caused a decrease in the heat resistance of the spores, even at low concentrations (Fig. 2).

The same results were obtained with the other *n*-alcohols, but the concentration required to produce the same effect with each alcohol was reduced with increasing chain length. A straight line was obtained when the log of the concentration of each alcohol needed to produce an equal shift was plotted against the log of the octanol/water partition coefficient (Fig. 4). Such a correlation is usually taken as an indication of a

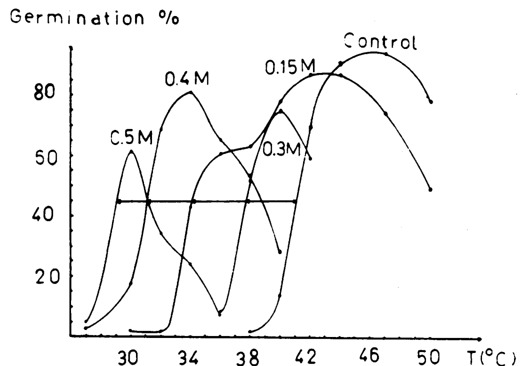


FIG. 2. *Germination percentage as a function of temperature with different concentrations of 1-butanol.*

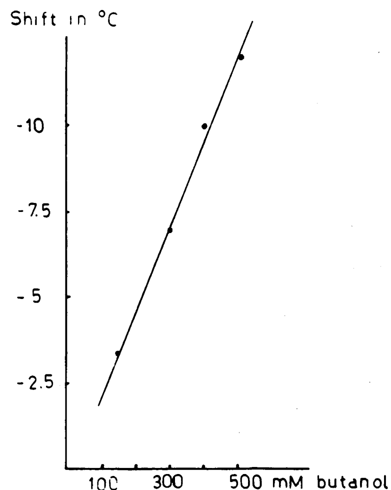


FIG. 3. *Shift of the activation temperature as a function of the concentration of 1-butanol.*

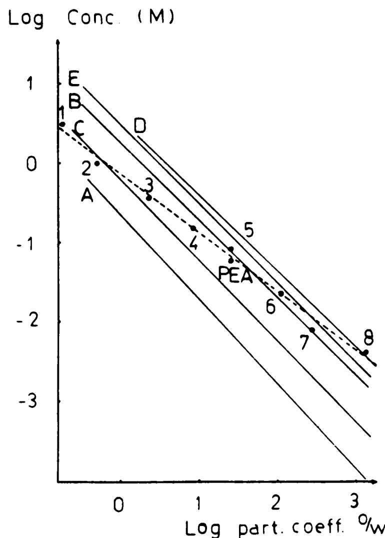


FIG. 4. *Log of the concentration needed to produce the same shift (3.2°K) in activation temperature with each alcohol (1 through 8, alcohols from methanol to 1-octanol; PEA, phenethyl alcohol). Comparison with other systems: line A, inhibition of the tadpole reflex (29); line B, resistance decrease in synthetic lipid membranes (17); line C, inhibition of erythrocyte hemolysis (38); line D, change in the order parameter of a spin label incorporated in lipid bilayers (33); line E, decrease of the activity of the ($Na^+$ + $K^+$)-adenosine triphosphatase and of the order parameter of the phospholipid annulus surrounding the enzyme (16). part. coeff. o/w, Octanol/water, partition coefficient.*

hydrophobic action site. The phenomenon is well known, particularly from the study of (alcohol) anesthetics (39); the equipotent concentrations of the members of a homologous series such as the alcohols differ by a factor of about 3 in their effect on physiological processes. In our system the factor had a value of 2.6, which is near the lower limit of the values obtained in other systems (37).

In Fig. 4 the linear relation obtained for the effect of *n*-alcohols on heat activation is compared with the same relation in some other systems. These include (i) the inhibition of the tadpole reflex (Fig. 4, line A) (29), (ii) the decrease in resistance in synthetic lipid membranes (line B) (17), (iii) the inhibition of erythrocyte hemolysis (line C) (38), and (iv) the change in the order parameter of a spin label incorporated into lipid bilayers (line D) (33). (Order parameters are directly related to the mobility of the spin label in the membrane.) Systems (ii) and (iv) clearly show that alcohols have a profound effect on the structural organization of phospholipids. In the other systems, however, the nature of the action site is not exactly known and there is still controversy concerning whether phospholipids or proteins are involved (12, 24, 35). Finally, we compared our results with those of Grisham and Barnett (16), who studied the effect of alcohols both on the activity of sheep kidney ($Na^+$ + $K^+$)-adenosine triphosphatase and on the order parameter of a spin label incorporated in the phospholipid annulus of the enzyme (Fig. 4, line E). From these studies it was concluded that the denaturation proceeded by causing changes in the association of the phospholipids with the protein.

**Free energy of adsorption of the *n*-alcohols to their active sites.** In certain cases, the standard free energy of adsorption of alcohols may indicate whether they act on lipids or on proteins (see below). We calculated, by the method of Schneider (37), a standard free energy of adsorption of −2,487 J/mol of $CH_2$ groups. In other biological systems the values range from −2,665 J/mol of $CH_2$ groups for the anesthetic effect of alcohols on *Paramecium* mobility to −4,519 J/mol of $CH_2$ groups for the reversible suppression of spontaneous movement in tadpoles (37).

**Effect of phenethyl alcohol and furfural on heat activation.** Phenethyl alcohol and furfural are known as potent activators of *Neurospora* ascospores (11, 26). Neither had any activation effect at room temperature on *P. blakesleeanus* spores. However, when they were applied simultaneously with heat treatment, an effect similar to that of *n*-alcohols was found. The activation temperature shifted toward lower values for both phenethyl alcohol (data not shown) and furfural (Fig. 5). At higher concentrations the optimal germination percentages became lower, as observed with the *n*-alcohols. As Fig. 4 shows, the relationship between effec-

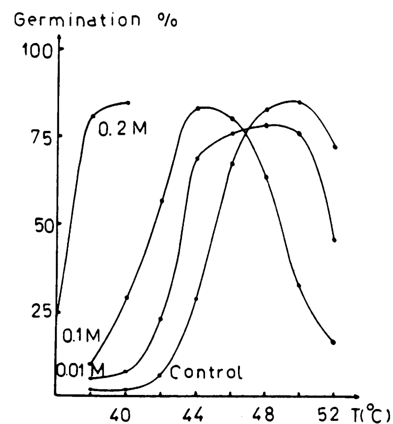


FIG. 5. *Lowering effect of furfural on the heat activation temperature of the spores of P. blakesleeanus.*

tiveness and partition coefficient of phenethyl alcohol fits in the straight line obtained with the *n*-alcohols. However, furfural produces a greater shift (4°K at 0.1 M) than would be expected from the value of its partition coefficient (log = 0.13). It is not clear which other property of the furfural molecule causes this deviation.

**Activation under high pressure.** It is known that high pressure has very different effects on membrane phase transitions and on protein conformational changes; the midpoint of the transition temperature is raised by about 20°K/1,000 atm for the former and approximately 2 to 6°K/1,000 atm for the latter (5, 19).

The application of pressure during the activation of *Phycomyces* spores produced two kinds of effects. At low pressures (less than 500 atm) the activation temperature was raised by about 2 to 4°K/1,000 atm (Fig. 6), but at higher pressures the effect was reversed and a lowering of the activation temperature by about 5°K/1,000 atm occurred in the pressure range of 1,000 to 2,000 atm (Fig. 7). A plot of the shift in activation temperature as a function of pressure is shown in Fig. 8. The dual effect of pressure on the spores clearly indicates a protein involvement: low pressures counteract, whereas high pressures enhance the thermal denaturation of proteins (42). Moreover, the upward shift of the activation temperature with low pressures amounts to only 2 to 4°K/1,000 atm, which is clearly different from the shift of 20°K/1,000 atm for membrane phase transitions.

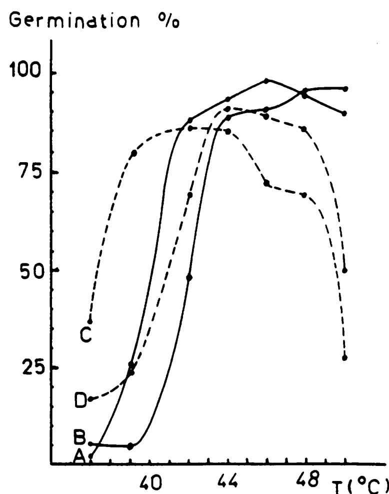


FIG. 6. *Effect of 420 atm of pressure on the activation temperature with and without 150 mM 1-butanol. Curve A, Control; Curve B, control + 420 atm; Curve C, 150 mM 1-butanol; Curve D, 150 mM 1-butanol + 420 atm.*

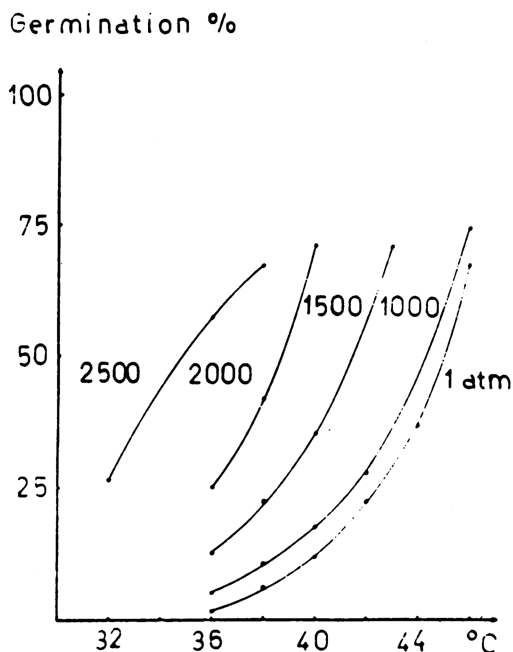


FIG. 7. *Lowering effect of high pressures (1,000 to 2,500 atm) on the activation temperature.*

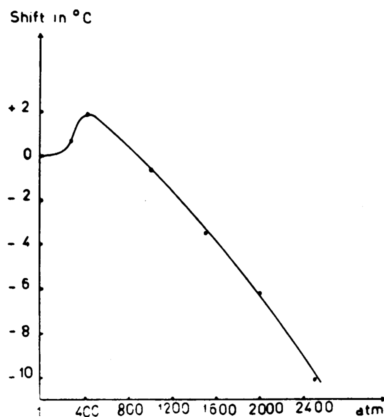


FIG. 8. *Shift of the spore activation temperature as a function of pressure.*

When the *n*-alcohols (from methanol to octanol) were added during the activation, the application of pressure did not change their effectiveness in lowering the activation temperature of the spores (Fig. 6). It was stated earlier that higher concentrations of alcohols resulted in lower optimum germination percentages. The same effect was seen with high pressures. The highest pressure that could be used without abolishing viability completely was about 2,500 atm. This is much lower than the 10,000 atm that can be tolerated by spores of many bacterial

species (4, 23). A stimulating effect of high pressure on the activation of fungal spores has not been described previously. Bacterial spores are more resistant to high pressures than to relatively low pressures. Low pressures, as opposed to high pressures, trigger the germination of bacterial spores. Since the resultant vegetative forms are much more sensitive to high pressures, the bacteria are more readily killed (6, 7, 15, 36).

Although not further investigated, we noticed also that low pressures enhanced the heat resistance of the spores at temperatures higher than those needed for activation (Fig. 6). The same effect was seen when alcohols were added during the heat treatment (Fig. 6).

**Activation volume.** The relation between germination percentage and pressure is shown in Fig. 9 for two different temperatures: 38 and 40°C. Activation volumes ($\Delta V^{\neq}$) of $-54.1$ $cm^3$/mol at 38°C and $-79.3$ $cm^3$/mol at 40°C were calculated from the following equation (5): $d \log k/dP = -\Delta V^{\neq}/2.303RT$, where $k$ is the fraction of activated spores divided by the fraction of dormant spores after a 3-min activation time, $R$ is 82.06 $cm^3 \cdot atm/°K \cdot mol$, and $P$ is pressure.

## DISCUSSION

The main purpose of this work was to discriminate between a membrane phase transition and a protein conformational change as the primary mechanism of heat activation. Several workers have proposed, on the basis of indirect evidence, a membrane involvement during the heat activation process of fungal spores (1). Hecker and Sussman (18) postulated that heat activation, as

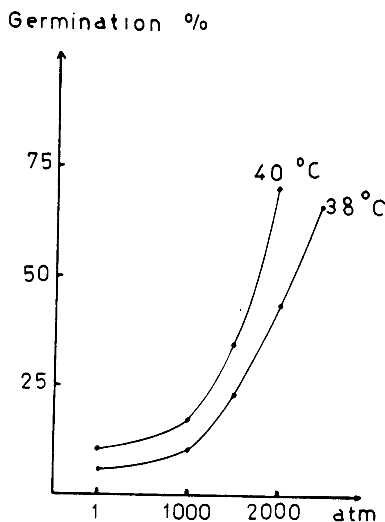


FIG. 9. *Activation of the spores as a function of pressure at 38 and 40°C.*

well as chemical activation, of *Neurospora* ascospores results in an increased permeability of the ascospore plasma membrane, thereby allowing trehalose to diffuse toward the cell wall-bound trehalase enzyme. The energy and intermediates made available in this way would allow the germination of the spore to occur. Evidence for changes in membrane particle topography upon activation has been obtained in *D. discoideum* (20) and *P. blakesleeanus* (28). These changes were already visible in *D. discoideum* spores at the end of the heat activation period, but in the spores of *P. blakesleeanus* they became evident only several hours after the heat activation. Thus, this phenomenon could be related to the germination process of the *Phycomyces* spores and not to the activation process. In the case of *D. discoideum* spores, there was no indication that the observed changes in membrane particle topography were directly related to the germination induction. These changes might be a nonspecific effect of the heat treatment.

The similar activation effects of both furfural and phenethylalcohol on the sporangiospores of *P. blakesleeanus,* on the ascospores of *N. crassa* (11) and *N. tetrasperma* (41), and on the basidiospores of *C. radiatus* (30) suggest a common mechanism of activation in the spores of these molds.

A linear relationship between the effectiveness of the members of homologous series and their nonaqueous/aqueous partition coefficient is frequently taken as an indication of a membrane localization of the active sites involved. However, it has been shown previously that *n*-alcohols can inhibit water-soluble enzymes like luciferase (21) and that they can induce conformational changes in proteins such as bovine plasma albumin and $\beta$-lactoglobulin in proportion to their anesthetic potency (3). Hence, the problem remains whether *n*-alcohols act on phospholipids or on proteins.

It has been argued by Cotter that the temperatures at which membrane phase transitions occur in biological systems do not lie in the temperature range of heat activation of spores (8). This argument, however, seems quite insufficient since no data are available on the phase transition temperature of spore membranes. Moreover, it is possible that a phospholipid phase transition does not occur all over the membrane but is restricted to specific phospholipids around a certain membrane protein. The phase transition temperature of such a phospholipid annulus can be quite different from that of the bulk of the membrane lipids (40). Instead of a phospholipid phase transition, Cotter favored a protein conformational change as the possible

primary mechanism for the heat activation process (8). From several indirect arguments he concluded that a protein in the mitochondrial membrane was affected by heat activation (8, 41). If membranes were involved in the heat activation process or the chemical activation process or both, three possibilities would remain for the chemical nature of the action sites: (i) the nonpolar portion of the lipids, (ii) the nonpolar region of lipoprotein complexes, and (iii) the hydrophobic region of protein molecules. The $\Delta G$ for the adsorption of $CH_2$ groups to proteins ranges from −418 to −2,343 J/mol. Lower values from −2,720 to −4,602 J/mol of $CH_2$ groups have been found for proteins which undergo conformational changes (37). The $\Delta G$ values for the adsorption of $CH_2$ groups from an aqueous phase to a completely nonpolar phase range from −2,929 to −3,347 J/mol (39). The value of −2,487 J/mol for the free energy of adsorption of the alcohols per methylene group seems to indicate a protein as the target site rather than lipids.

Grisham and Barnett have shown that changes in the association of phospholipids with the ($Na^+ + K^+$)-adenosine triphosphatase from sheep kidney were responsible for the denaturation of the enzyme (16). A similar situation could be possible with the protein responsible for the activation of the spores of *P. blakesleeanus,* although at the moment no distinction can be made between a conformational change in which a protein is inactivated and one in which a protein is activated. The latter possibility is illustrated by the spore enzyme trehalase, which can be activated by the same heat treatment which activates the spores (43). Moreover, this heat activation can be shifted with alcohols toward lower temperatures in the same way that the activation of the spores can be shifted (unpublished data). Despite this striking correlation, the activation of the trehalase enzyme cannot be responsible for the activation of the spores since the enzyme can be selectively inactivated with a 0.1 M HCl treatment without altering the activation properties of the spores (44).

Since the effect of pressure on membrane phase transitions (shift of 20°K/1,000 atm) is quite different from the effect of pressure on protein conformational changes (shift of only 2 to 6°K/1,000 atm), it was possible to discriminate between the two possibilities. The results obtained were clearly consistent with those expected for a protein conformational change. Moreover, this conclusion was corroborated by the fact that at high pressures the effect was reversed, which is consistent with a pressure-stimulated protein denaturation. Some restrictions, however, must be made since our results were obtained through kinetic experiments. The activation parameters therefore have a dual origin: a large equilibrium parameter and a small kinetic parameter. We can only postulate that the contribution of the kinetic parameter is negligible and hence that the pressure dependence of 20°K/1,000 atm (obtained under equilibrium conditions) is also valid for our system. The assumption that the temperature and pressure activation parameters are largely thermodynamic in nature seems justified in view of the high values obtained.

Another possible source of restrictions is the applicability of the pressure effects on phospholipids to biological membranes. Recent reports (13, 27, 31, 34, 46) indicate that the pressure effects can also be observed in more complex systems, so that the possible involvement of lipids in the activation of the spores would have been easily detected.

In bacterial spores as well, the target site of heat activation is not yet known. However, the effect of pressure on temperature-induced germination of bacterial spores is also consistent with the concept of a protein conformational change as the primary action site (32).

From the effects of pressure and *n*-alcohols we conclude that the primary target site of the heat activation of fungal spores is a protein, which may be hydrophobic in nature. The protein involved appears to undergo a conformational change. The temperature of this transition is lowered by alcohols, a phenomenon which is well documented (10, 45). The effect of high pressure on the denaturation of proteins by alcohols has also been described by Johnson et al. (21). Finally, we can mention that direct calorimetric evidence has been obtained for changes in the protein denaturation behavior upon activation of the spores of *P. blakesleeanus* (F. Van Cauwelaert and M. Verbeke, unpublished data).

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