

Compartmentation of Metabolic Systems in the Regulation of Dormancy in Fungus Spores

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MANDELS, G. R. 1981. Compartmentation of metabolic systems in the regulation of dormancy in fungus spores. *Experimental Mycology* 5, 278-291. Dormancy in fungus spores can be due to a variety of causes relating to structural, physiological, or biochemical functions. Based on data reported here and earlier, compartmentation of endogenous reserves or of enzymes is proposed as the mechanism controlling dormancy in spores of *Myrothecium verrucaria* and *Trichoderma reesei*. Spores of both organisms contain a pool(s) of reserves composed of trehalose, amino acids, as well as unidentified compounds. Addition of hot water extracts of these reserves to spores results in rapid increases in respiratory activity and germination. This observation coupled with other data showing the stimulation of endogenous respiration by heat, freezing, or azide demonstrates that dormancy is due primarily, if not entirely, to sequestering or compartmentation of the endogenous reserves and not of the enzymatic systems involved in utilization of metabolites for germination. Presumably these reserves are contained within the vacuoles. Data on the interactions of treatments that stimulate endogenous respiration and on the effects of metabolism of exogenous substrates indicate that the transport pathways within the cell, from the pools or from the plasma membrane, to the loci of initial metabolism are not identical and that the total regulatory system is composed of a number of separable processes. It appears probable that examination of the spores of other fungi will show that compartmentation of reserves is not of uncommon occurrence and is not an unusual cause of dormancy.

INDEX DESCRIPTORS: compartmentation; dormancy; endogenous; fungus; reserves; spores; stimulation; *Myrothecium verrucaria*; *Trichoderma reesei*.

Dormancy in spores can be due to a variety of causes involving permeability, endogenous reserves, activity of metabolic systems, or endogenous inhibitors. This mechanistic diversity is associated with tremendous variation in spore structure at cellular and subcellular levels as well as in physiology and biochemistry. Compartmentation of endogenous reserves, however, has not been recognized for its potential involvement.

Previous studies (Mandels *et al.*, 1965; Mandels and Vitols, 1967; Mandels and Maguire, 1972) with *Myrothecium verrucaria* spores led to the postulate that dormancy in this species results from the physical separation of the endogenous trehalose reserves from metabolic enzymes and that dormancy was maintained by compartmentation of endogenous reserves.

Compartmentation of intermediary metabolites—amino acids, organic acids, and carbohydrates—within cells is of widespread occurrence and is being increasingly recognized as a regulatory mechanism. Although compartmentation is involved in many systems of metabolic regulation (Stebbing, 1974; Oaks and Bidwell, 1970), application to interpretations of mechanisms of dormancy has been confined largely to situations involving insolubility of reserves. Thus, dormancy in some spores has been ascribed to the unavailability of lipid reserves (Reisener, 1976). Segregation of water-soluble metabolites from an active enzyme system is less obvious and the involvement of this phenomenon in regulating dormancy does not appear to have been fully realized or exploited. In bacteria, reserves are usually polysaccharides, lipids,

polyphosphate, etc., and not sugars (Dawes and Ribbons, 1964). Presumably the lack of ultrastructural differentiation prevents or minimizes the relative significance of compartmentation as a regulatory mechanism in procaryotes.

Dormancy in ascospores of *Neurospora* has been attributed to spatial separation of trehalose reserves from trehalase (Budd *et al.*, 1966; Hill and Sussman, 1964). Subsequently, Hecker and Sussman (1973) reported evidence indicating that, in dormant ascospores, trehalase is associated with the innermost ascospore wall, being separated from trehalose by the cell membrane. They propose that activation effects permeability changes in the membrane or disassociation of trehalase from the membrane allowing admixture of enzyme and substrate, but the mechanism is probably not so simple. For example, glucose uptake increases about 100-fold (from 0.175 to 19 $\mu\text{g}/\text{mg} \times \text{hours}$) upon activation (Budd *et al.*, 1966). This implies some change in the glucose metabolizing system and/or in the transport of glucose to its metabolic site within the cell.

This report presents additional data on *M. verrucaria* as well as results of experiments with *Trichoderma reesei* (QM6a) relating to the concept of compartmentation as a mechanism of dormancy in fungus spores. In particular, it has been found that hot water extracts of the spores contain a wide variety of metabolites which can be utilized rapidly and with no lag as germinants when added to resting spores, thus substantiating the concept of compartmentation of substrates rather than enzymatic systems as a cause of dormancy (Mandels and Maguire, 1972).

MATERIALS AND METHODS

Organisms. Conidia of *M. verrucaria* (Alb. and Schw.) Ditm. ex. Fr. QM460 and *T. reesei* (Simmons, 1977) QM6a, a wild-type strain formerly known as *T. viride*, were used. Spores of *M. verrucaria* were

harvested from agar culture having cellulose as a carbon source (Mandels and Maguire, 1972) and after washing were suspended in 50 mM KH_2PO_4 - K_2HPO_4 buffer at pH 5.5. For *T. reesei*, the carbon source was maltose and spores were suspended in the *Trichoderma* mineral salts solution (Sternberg and Mandels, 1979), unless specified to the contrary. Spore age is actually the age of the culture from which spores were obtained. For *M. verrucaria* true age is ca. 4 days less than indicated age; for *T. reesei* true age is ca. 6 days less than indicated age.

Spore extracts. Washed suspensions of spores in water at 5–10 mg/ml were autoclaved 5 min, filtered on a fine sintered glass Buchner funnel, or centrifuged and decanted through a filter. In either case, the spore residues were washed once with water, and the filtrates combined, concentrated on a rotary evaporator, and then freeze-dried. Extracts of autolyzed spores were prepared as above, but 300 ml of spore suspension was incubated at ca. 28°C with 10 ml of toluene with a magnetic stirrer for 20 h prior to extraction. The autolyzed spores were not separated from the medium prior to extraction, the extract thus being a combined room temperature autolysate and hot water extract.

Respirometric methods. Warburg manometers or Gilson differential respirometers were used at 30°C with 1 ml of spores in the vessels, 0.5 ml substrates or other additives in the sidearms, and 0.2 ml 10% KOH in the center wells. Respiratory rates are expressed as $Q_{\text{O}_2} = \mu\text{l O}_2 \times \text{mg dry wt}^{-1} \times \text{h}^{-1}$ and represent initial (0–60 min), maximum, average, or final as indicated.

Amino acids. A Phoenix K 8000 C amino acid analyzer equipped with an Infotronics CRS 110 A system was used for *M. verrucaria* extracts and a Beckman analyzer with an Autolab system for *T. reesei* extracts.

Carbohydrates. Glucose absorption was measured by determining changes in the

supernatant after centrifuging, by the dinitrosalicylic acid method (Sumner and Somers, 1944). Carbohydrate composition of *T. reesei* extracts was determined by high-pressure liquid chromatography (hplc)¹ (Palmer, 1975).

Other methods. Where actually determined, germination is the protrusion of a germ tube as observed microscopically. While no data on percentage or rate of germination are cited, all comments noting occurrence of germination are made from microscopic observations. Cell volumes were determined by centrifuging in protein tubes (Mandels and Darby, 1953) after incubation on a reciprocal shaker at 29°C for time indicated. Dry weight was determined by filtration through sintered glass crucibles of fine porosity. Heat treatments were for the time and temperature indicated, the suspensions being shaken during treatment. For freezing treatment, spore suspensions in glass containers were frozen for 30 min or longer in a dry ice-Cellosolve mixture, and then thawed by placing in 30°C water bath. Electron microscopy was with spores fixed with 2% KMnO₄.

RESULTS

Reproducibility of results. Data in the figures and table are from representative experiments, all of which have been repeated at least once with quantitatively consistent values.

Stimulation of respiration (M. verrucaria). Previous studies with *Myrothecium* spores have documented in considerable detail the pronounced stimulation of endogenous respiration by a variety of different stimuli—freezing, heat treatment, sonication, poisons (merthiolate), inhibitors (azide, dinitrophenol, etc.) (Mandels, 1963; Mandels and Maguire, 1972). Stimulation to actually germinate (i.e., emergence of a germ tube) was rarely observed and then

¹ Abbreviation used: hplc, high-pressure liquid chromatography.

only at a very low level. Thus, these stimuli are not activators of germination as is heat treatment for *Neurospora* ascospores or bacterial spores.

Interpretation of the mechanism of action was not clear. Subsequent experiments on the effects of multiple stimuli and in particular on the effects of spore extracts which contain a variety of water-soluble metabolites, however, provide additional facts and observations which are presented below and must be considered in any interpretation.

Interactions of stimuli (M. verrucaria). The types of effects produced on a living cell by azide, heat, or freezing would be expected to differ greatly. Consequently, the responses to multiple stimuli were examined. The effects of heat and freezing (the sequence is immaterial) applied sequentially to similar spores showed the effects to be at least additive and in some cases synergistic (Fig. 1). Thus, heat and freezing must have different effects on the cell. While this is not surprising, the inference is that multiple mechanisms are con-

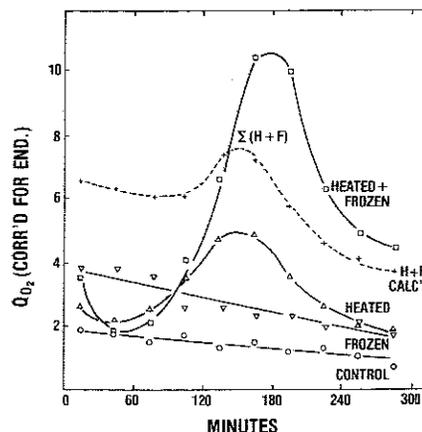


FIG. 1. *M. verrucaria*: Changes in rate of endogenous respiration of heated, frozen, or heated and frozen spores. Values corrected for rate of untreated spores. Spores 6 days old, 13.6 mg/vessel; heat 25 min at 50°C. Untreated (O—O); heated (Δ—Δ); frozen (∇—∇); heated and frozen—experimental (□—□); summation of effects of heat and freezing—calculated (+---+).

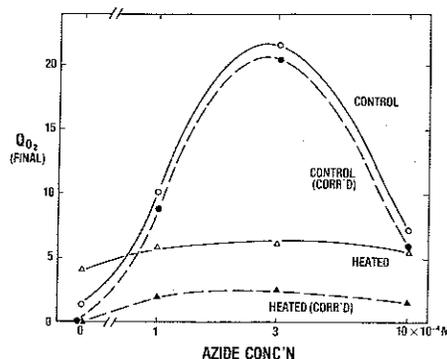


FIG. 2. *M. verrucaria*: Effect of azide concentration on endogenous respiration of heated (25 min at 50°C) spores. Spores 12 days old, 6.9 mg/vessel. Unheated spores (○—○); unheated spores corrected for respiration with no azide (●—●); heated spores (△—△); heated spores corrected for activity with no azide (▲—▲). Q_{O_2} values are those at termination, 210–270 min.

trolling endogenous metabolism and that heat and freezing affect different phases.

Additional experiments exposing heated or frozen spores to azide substantiate the above conclusion. Heated spores have no or very little response to azide (Fig. 2). Frozen spores, however, display a pro-

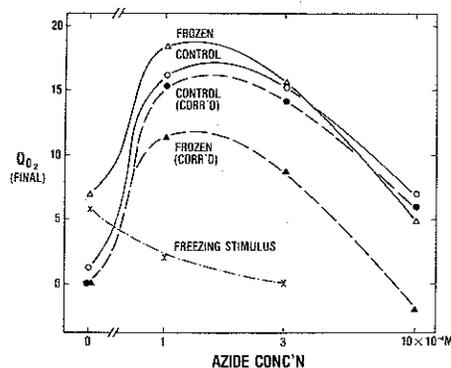


FIG. 3. *M. verrucaria*: Effect of azide concentration on endogenous respiration of frozen spores. Spores 20 days old, 7.3 mg/vessel. Untreated spores (○—○); untreated spores corrected for respiration with no azide (●—●); frozen spores (△—△); frozen spores corrected for respiration with no azide (▲—▲); effect of azide on freezing stimulation: respiration of frozen spores corrected for respiration of untreated spores with azide (x—x). Q_{O_2} values are those at termination, 150–210 min.

nounced effect (Fig. 3), i.e., if azide is added to spores which had been frozen, the stimulatory response is similar to that observed in control spores. Conversely, however, the stimulatory effect of freezing is not additive to that of azide. While the meaning is not clear, the evidence implies involvement or activation of the same or related mechanism by azide and by freezing although quantitatively the azide effect is much greater than the freezing effect.

Differences in the mechanisms of activation (or stimulation) are also indicated by effects of spore age on the degree of stimulation. The effect is greatest on the response to azide, being roughly inversely proportional to spore age; response to freezing is relatively unaffected by spore age; with heated spores the stimulation is less with older spores (as with azide), although limited data indicate a nonlinear relation (Mandels and Maguire, 1972).

Response of "activated" spores to exogenous substrates (M. verrucaria). Despite the presence of about 20% trehalose reserves, endogenous respiration is very low. Addition of exogenous trehalose, glucose, or various other sugars increases respiration with essentially no lag (Mandels *et al.*, 1965; Mandels and Vitols, 1967). Thus, trehalose can be transported directly into the cells and metabolized immediately. This indicates that metabolic enzymes are available and in an active form in resting *M. verrucaria* spores.

The interaction of stimuli such as heat, freezing, and azide with exogenous substrates is pertinent to an examination of dormancy. While exogenous respiration on glucose is significantly faster in the presence of azide (the effect being a function of azide concentration) (Fig. 4), or in previously frozen cells, heated cells respire glucose more slowly than controls (Figs. 4 and 5).

In all these analyses of data on effects of exogenous substrates, it is assumed that endogenous respiration continues at the

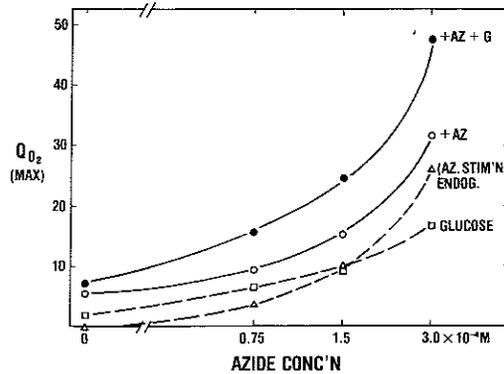


FIG. 4. *M. verrucaria*: Effect of azide concentration on rate of exogenous (glucose) respiration. Spores 21 days old, 4.4 mg/vessel. Endogenous (○—○); exogenous (+1 mg glucose/ml) (●—●); azide stimulation of endogenous respiration (△---△); glucose respiration, i.e., endogenous respiration plus azide subtracted from exogenous respiration plus azide (□---□). Respiration data are maximum rates.

same rate. While this may not be strictly true, endogenous levels are so low that even complete repression would not materially change any conclusions.

Whereas the stimulatory effect of azide is

enhanced in the presence of exogenous sugars, the rapid respiration on sucrose and yeast extract is suppressed at all levels tested (unpublished). This is ascribed to inhibition of some step in the complex of reactions involved in the germination process which, in the case of *M. verrucaria*, requires a complex of metabolites. Increases in cell volume (growth) are similarly inhibited. Germination is not observed in the presence of azide despite the great stimulatory effect on respiration, presumably because only the initial stage(s) in breaking dormancy is affected by azide. The subsequent more complex, synthetic reactions are suppressed.

Spore extracts (M. verrucaria). The quantity and diversity of water-soluble components in extracts of autoclaved spores (Mandels and Maguire, 1972) suggest that they should have a marked effect on spore metabolism. This is readily demonstrated by effects on spore swelling (germination) and even greater effects on respiration (Figs. 6 and 7). Spore extracts

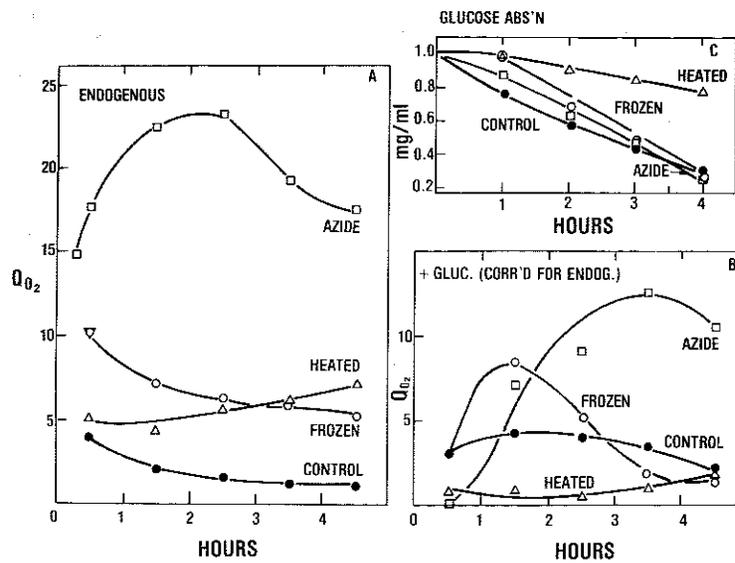


FIG. 5. *M. verrucaria*: Effects of azide, freezing, and heat on respiration rate and glucose absorption. Spores 20 days old, 8 mg/vessel. (A) Endogenous respiration; (B) exogenous respiration on glucose corrected for endogenous; (C) glucose absorption. Spores + azide— 2×10^{-4} M for A, B; 1.5×10^{-4} M for C (□); heated spores—25 min; 50°C (△); frozen spores (○); control spores (●). Glucose at 1 mg/ml where added.

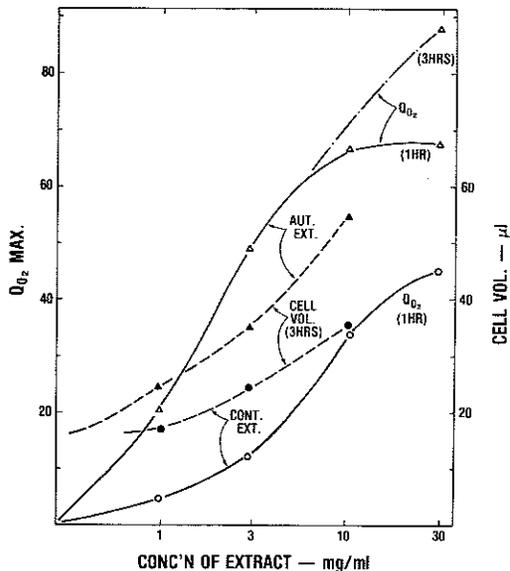


FIG. 6. *M. verrucaria*: Effect of concentration of extracts from untreated (O) or autolyzed (Δ) spores on maximum rate of respiration (—) and on spore swelling (---). Respiration data: spores 18 days old, 7.8 mg/vessel (5.2 mg/ml); respiration rates are at 60 min, except for high value for 30 mg/ml of extract from autolyzed spores which is value at 3 h. Cell volume data at 3 h. See Materials and Methods for preparation of extracts.

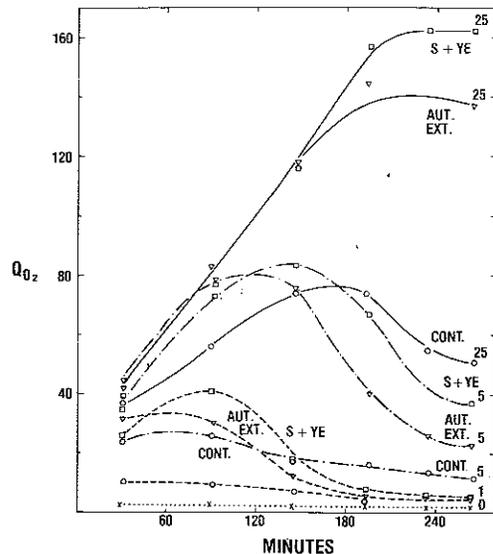


FIG. 7. *M. verrucaria*: Comparison of effect of different concentrations of extracts from control (O) or autolyzed (∇) spores with an artificial medium (\square) (sucrose-yeast extract) and no substrate (X) on course of respiration rates. 3.66 mg spores/vessel; substrate concentrations as mg/ml final; sucrose and yeast extract at equal concentrations totaling level indicated. —, 25 mg/ml; ---, 5 mg/ml; ····, 1 mg/ml; - · - · = 0 mg/ml. See Materials and Methods for preparation of extracts.

have a much greater effect (ca. 2 \times) on older spores, whereas azide has a greater effect on young spores (Mandels and Maguire, 1972).

Earlier publications on the physiology of *M. verrucaria* have reported the spores to contain (on dry weight basis) about 3% fat, 9.5% total nitrogen, and 33% total carbohydrate. Water-soluble carbohydrates included 18.6% trehalose, 2% mannitol, and 0.3% glucose (Mandels *et al.*, 1965). Subsequently, it was shown that water-soluble extracts contained about 23 free amino acids comprising about 8.7% of the dry weight (Mandels and Maguire, 1972).

Aqueous extracts from autolyzed spores (spores incubated with toluene for ca. 20 hours prior to extraction) are much more effective in promoting respiration than extracts from intact spores (Fig. 6). This increase presumably results from the action

of freed autolytic enzymes since inactivation (heat) prior to toluene treatment negates the change. The increase may also be due to increases in the total amount of amino acids in the extract (ca. 1.8 \times), but changes in composition are considered more important.

Respiratory activity (T. reesei). Despite the presence of considerable endogenous reserves of trehalose and a variety of amino acids (Table 1), *T. reesei* conidia do not germinate in distilled water, buffer, or nutrient salts and show a very low endogenous respiratory activity (Q_{O_2} = ca. 1.5). Addition of glucose or other respirable sugars to spores suspended in PO_4 buffer results in an immediate increase in rate of respiration to an essentially constant level (Fig. 8). In nutrient salts medium, however, the rate increases linearly. Measurements in media lacking individual essential ele-

TABLE 1
Composition of *Trichoderma reesei* Spores
and Spore Extracts

Component	Percentage dry weight	
	Spores	Spore extract
Water soluble	30.0	100
Lipid (CHCl ₃ -CH ₃ OH solution)	15.0	—
Glucose	2.5	6
Trehalose	13.0	44
Unidentified carbohydrate	4.5	10
Amino acids ^a	3.5	8
Unknown	—	32

^a Identified amino acids (major components include: alanine, aspartic acid, cystathionine, glutamic acid, histidine, 1-methyl histidine, serine, threonine).

ments show that (NH₄)⁺ is the critical ion promoting this effect in the salts medium. (NH₄)⁺ cannot be replaced by NO₃⁻ or urea.

In view of the failure of resting spores to utilize endogenous trehalose, it is signifi-

cant that exogenous trehalose is readily metabolized with no lag, the kinetics being similar to those for glucose.

Stimulation of endogenous respiration (T. reesei). As with *M. verrucaria* spores, the low endogenous respiration is stimulated very significantly by a variety of methods—lyophilizing, freezing (Fig. 9), heat treatment (Fig. 10), or by azide or dinitrophenol (Fig. 11). It is noteworthy that the effects of all these stimuli, except 50°C heat treatment, are inhibitory when an exogenous substrate—glucose is provided (data for azide and glucose not shown; DNP and glucose not tested). The positive effects are thus on the metabolic system effecting the utilization of endogenous reserves whereas the metabolism of exogenous substrates is suppressed as might be expected.

Effect of spore extracts on metabolism (T. reesei). The low-molecular-weight, water-soluble metabolites that can be ex-

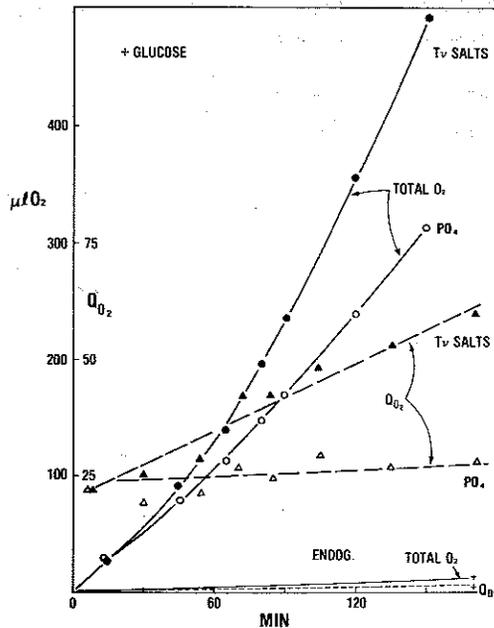


FIG. 8. *T. reesei*: Kinetics of endogenous or exogenous (glucose at 5 mg/vessel) respiration of spores in nutrient salts or 0.06 M phosphate buffer at pH 5.7. Spores 31 days old, 4.44 mg/vessel. Total O₂ uptake (○); Q₀₂ (Δ); nutrient salts (○, Δ); buffer (○, Δ); endogenous respiration (+---+).

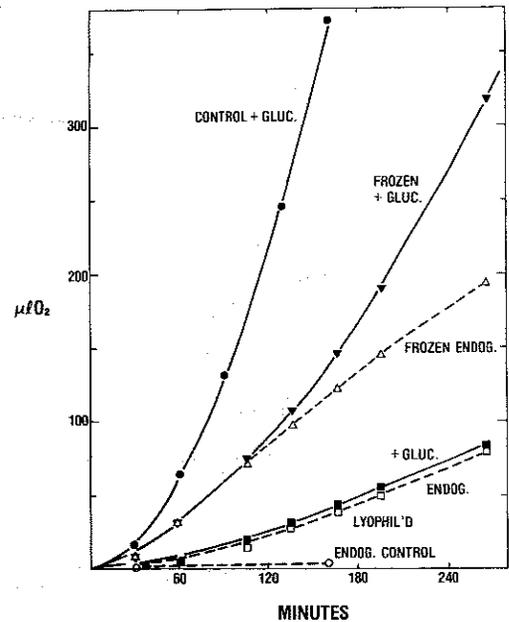


FIG. 9. *T. reesei*: Effects of freezing and lyophilization on endogenous or exogenous (glucose—10 mg/vessel) respiration. Spores 23 days old, 4.1 mg/vessel; nutrient salts. Control spores (○); frozen spores (Δ); lyophilized spores (□); solid symbols, + glucose; empty symbols, endogenous.

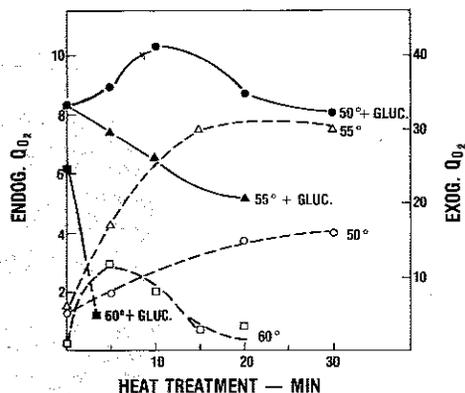


FIG. 10. *T. reesei*: Effects of time and temperature of heat exposure on rates of endogenous (open symbols, dashed lines; left-hand ordinate) and exogenous (glucose—15 mg/vessel) (closed symbols, solid lines; right-hand ordinate) respiration. (Note: Data from several experiments—age and concentration of spores differ, but not significantly.) 50°C (○ or ●); 55°C (△ or ▲); 60°C (□ or ■).

tracted from *T. reesei* spores by simple hot water treatment (Table 1) are comparable to those from *M. verrucaria* and the effects of the extracts on spore metabolism are similar, the response being rapid and extensive (Fig. 12). While the initial rate is not a func-

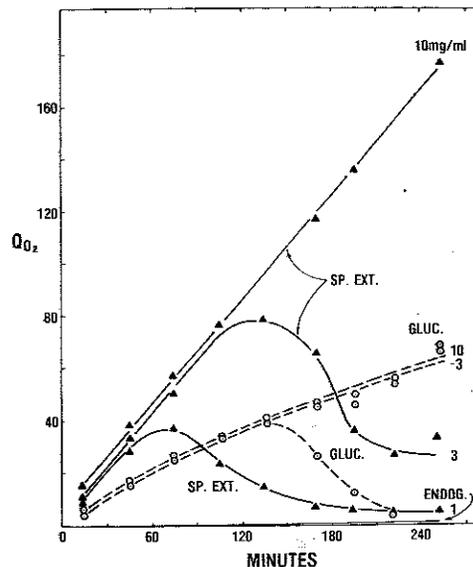


FIG. 12. *T. reesei*: Effect of spore extract (▲) or glucose (○) concentration at 0, 1, 3, 10 mg/ml as indicated on rate of respiration. 3.14 mg spores/vessel; nutrient salts medium; concentrations indicated are final in mg/ml. SP. EXT. = spore extract; G Gluc. = glucose.

tion of concentration, the magnitude of response is.

DISCUSSION

Dormancy, a phenomenon characterized by low, resting endogenous metabolic activity, represents the response of an organism to some environmental situation, and can result from morphological, physiological, and/or biochemical diversity as well as from a variety of extracellular factors. Extensive examination of these conditions has been given elsewhere (see Mandels and Norton, 1948; Sussman and Douthit, 1973, for example) and need not be repeated. Obviously, there are quantitative as well as qualitative considerations so that it is appropriate to consider the degree of dormancy. This is particularly pertinent when comparing a laboratory situation with that of the natural environment of the organism. Thus, in laboratory experiments with *M. verrucaria*, 100% germination oc-

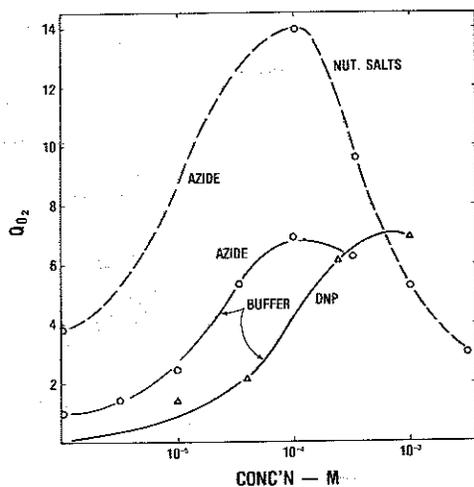


FIG. 11. *T. reesei*: Effect of azide (○) or dinitrophenol (DNP; △) concentration on rate of endogenous respiration. Spores 35 days old, 2.1 mg/vessel, nutrient salts (---). Spores 37 days old, 5.7 mg/vessel, 0.033 M phosphate buffer, pH 5.5 (—). Q_{O_2} = average value 0–300 min.

curs rapidly in a complex medium, such as sucrose and yeast extract, whereas under conditions approximating a soil environment, the normal habitat, germination is very slow and sparse, yet adequate and probably advantageous for survival. At least an occasional spore will germinate in the complete absence of any exogenous nutrients. Thus, even within one type of spore of one organism, dormancy is a variable phenomenon, the requirements for individual spores following some normal distribution curve.

Before attempting to develop an interpretation of dormancy in *M. verrucaria* spores, the relevant data presented in this report and elsewhere are summarized here:

(1) *M. verrucaria* spores possess a constitutive dormancy mechanism, i.e., they do not germinate when placed in water or nutrient salt solution and have a low endogenous Q_{O_2} (ca. 3) under these conditions (Mandels and Norton, 1948; Sussman and Douthit, 1973).

(2) Endogenous reserves of trehalose and free amino acids are present amounting, respectively, to ca. 20 and 2% of the dry weight.

(3) Metabolic utilization of endogenous reserves can be activated by a variety of treatments—exposure to azide or DNP being particularly effective, but sublethal heat or freezing are also stimulatory. While the effects of short-term exposure to azide are not reversible, continued exposure for 8–20 hours can result in irreversible stimulation.

(4) Exogenous trehalose, as well as other sugars, are respired with essentially no lag, but rapid germination does not occur.

(5) Sucrose and yeast extract promote both rapid germination and rapid lag-free respiratory increases (Mandels *et al.*, 1956).

(6) Hot water extracts of spores containing the water-soluble endogenous reserves are rapidly respired with no lag and cause rapid germination.

(7) Extractives from spores autolyzed

under toluene are much more effective than extracts from enzyme-inactivated or control spores. This is presumably due primarily to depolymerization of reserves and/or to transformation of complexed substrates and not to quantity of extractives which increases only slightly (ca. 1.3 \times). Since extracts of azide-treated spores do not have a greater effect than extracts of control spores (not published), it appears that metabolites do not accumulate and that the stimulatory effects of azide may not be due to hydrolytic or other autolytic changes in the endogenous reserves.

(8) The effects of spore extracts are due to their complex composition and not solely to trehalose.

(9) A multiplicity of substrates is required for rapid germination of *M. verrucaria* spores. Attempts to formulate a simple, synthetic medium composed of only one or few each of amino acids, nitrogen bases, carbohydrates, and accessory growth factors show slight increases in germination rates with each increase in complexity of the medium. Only when a complex such as yeast extract and carbohydrate is used, does rapid germination occur (ca. 100% in 3 hours). Yeast extract presumably does not act as a catalyst or activator, since relatively high concentrations are required, but rather as a complex of substrates. Germination does occur in experimental culture on simple substrates (i.e., glucose, etc.) and can be observed even in the complete absence of any exogenous substrates.

(10) Sparse germination, ca. 1%, occurs only occasionally during treatment with azide or DNP at concentrations which promote the greatest stimulation of respiration and percentage germination is low (Mandels and Norton, 1948). While a variety of treatments activate respiration, it is noteworthy that none stimulates germination.

Thus, spores contain adequate endogenous reserves for germination, as well as a metabolic system capable of functioning

actively with no lag if substrates or spore extracts are added exogenously. The effective concentrations are at a level which suggests that components of the extracts are acting as metabolic substrates rather than as triggers. It is concluded, therefore, that the endogenous reserves are sequestered or compartmentalized, and that metabolic systems are present in an active state. Dormancy therefore results primarily, if not entirely, from sequestering or compartmentation of the endogenous reserves, and not of the enzymatic systems involved in utilization of metabolites for germination. It is interesting and significant to note that ultracentrifugation of spores at about 226,400g for ca. 5 minutes had no effect on endogenous or exogenous (glucose) respiration. The coexistence of trehalose and trehalase in yeast cells has been ascribed to cytoplasmic membrane barriers, the substrate being bound to the particulate fractions, whereas the enzyme is in a soluble form (Souza and Panek, 1968).

The variety of treatments and the complexity of their interactions stimulating endogenous metabolism in the spores studied here indicate that the steps from a passive dormant condition to active metabolism and germination are controlled by a diversity of phenomenon. Compartmentation of reserves appears to be the primary control, but transport to different intracellular sites is also involved. Only when appropriate metabolites undergo a particular sequence of reactions does germination and growth occur.

The following scheme for the mechanisms controlling dormancy and germination is proposed, based on the observations summarized above and on concepts of cell structure and function (Mahler and Cordes, 1971). The metabolites necessary for germination and the early stages of growth and development are present within the resting cell, but are confined within two large vacuoles, which can be seen by visual microscopy, but are clearly visible in electron mi-

crographs (Fig. 13). These reserves are mostly soluble, consisting largely of trehalose and a diversity of amino acids. They are easily extracted by destroying the compartmenting membranes by hot water or toluene without rupturing the spores. Some significant fraction of the endogenous reserves, unavailable by simple hot water extraction, is converted by autolysis of the spores under toluene into available metabolites—the quantity of extractives increasing only slightly, but the activity greatly. If germination occurs in nature under less than ideal conditions, these reserves presumably become available at a slow rate for fairly extensive proliferation of mycelium until a suitable substrate is encountered.

While the basic control(s) governing dormancy can be ascribed to compartmentation, complexity of regulatory mechanisms is indicated by examining their interactions. Thus, azide added to heated spores has very little effect whereas spores which had been frozen respond comparably to controls.

The effects of these stimuli on metabolism of exogenous substrates is pertinent. Concentrations of azide which stimulate endogenous respiration will almost completely block germination and growth on sucrose and yeast extract. Furthermore, respiration on glucose is depressed in heat-activated spores, but is enhanced in spores which had been frozen or in the presence of azide.

Presumably, the transport pathways within the cell from the endogenous pool are not identical with those from the plasma membrane to the loci of initial metabolism. If the systems were similar, the response to exogenous substrates should be additive or at least not diminished in "stimulated" cells. Earlier work on the transport of trehalose into *M. verrucaria* spores (Mandels and Vitols, 1967) indicated that exogenous trehalose can accumulate against a concentration gradient within an endogenous pool and can also be respired while in

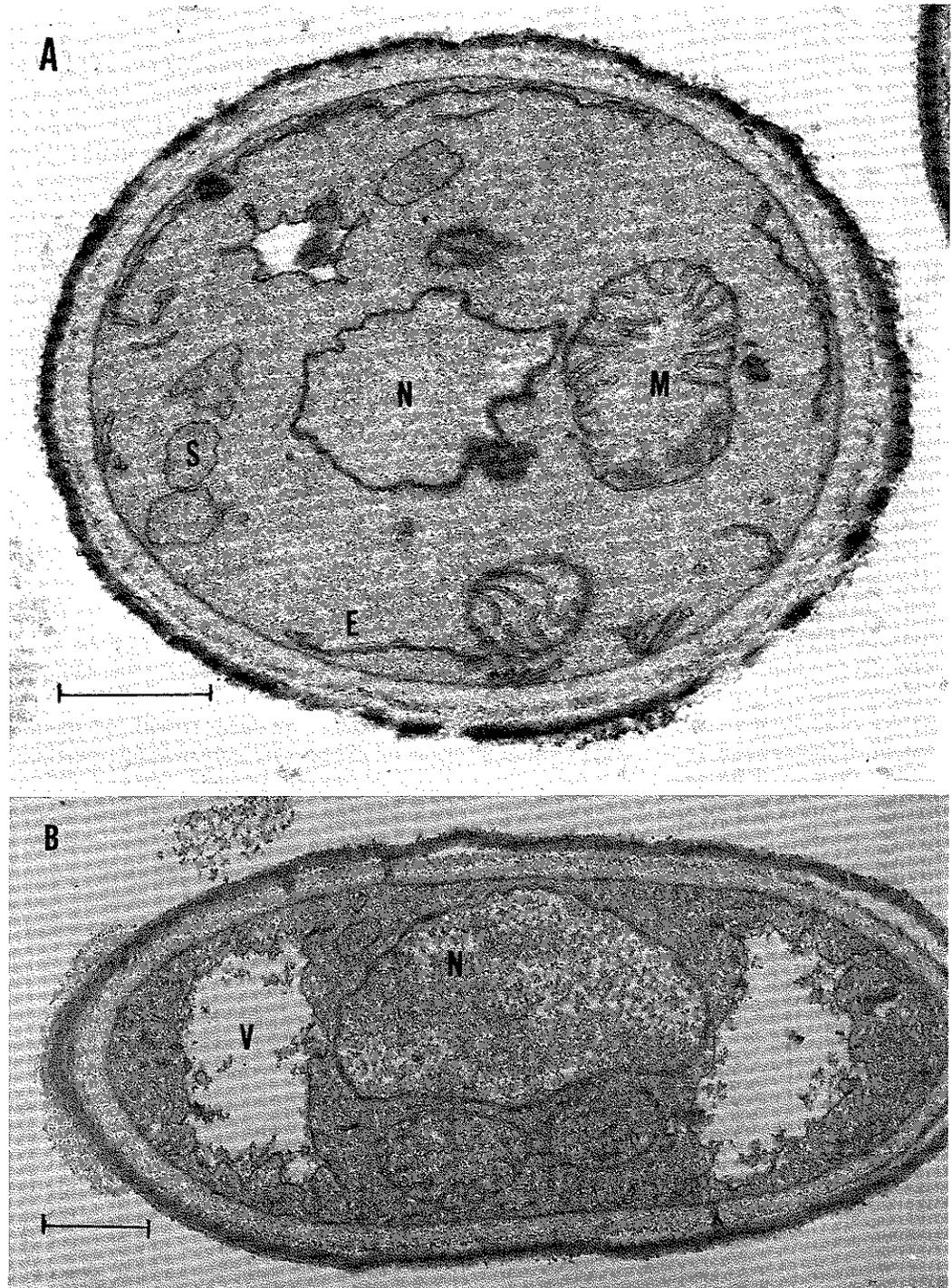


FIG. 13. Electron micrographs of *M. verrucaria* spores. (A) Cross section; (B) longitudinal section. N = nucleus; E = endoplasmic reticulum; M = mitochondria; V = vacuole; S = storage body. Bar = 0.5 μ m.

transit before entering the pool. This behavior has also been shown in *Neurospora* (Subramanian *et al.*, 1973) for the transport of exogenous arginine into a vesicular pool of endogenous, sequestered arginine. During cytoplasmic transport the arginine is available for rapid protein biosynthesis. Similarly, Goldstein *et al.* (1980) reports data with a marine fungus *Thraustochytrium aurieum* supporting the concept of physiological compartmentation, the disposition of a substance within the cell depending upon its endogenous or exogenous origin.

We can visualize that reaction of azide with some vacuolar membrane proteins results in a disruption of their permeability characteristics which cause sequestration. The reserves are then free to diffuse to metabolic mitochondrial sites. Continued growth and anabolic metabolism is prevented by the inhibitory effects of azide on biosynthetic processes occurring on the endoplasmic reticulum. Thus, respiration is stimulated, but growth is not. Furthermore, azide suppresses the exogenous respiration on sucrose and yeast extract which is much higher than the azide-stimulated endogenous respiration. Azide also suppresses germination. If spore extracts are added they are rapidly transported through the plasma membrane into the cells where they then pass (diffuse?) into the undisrupted metabolic system.

The impaired capacity of heat-activated spores to respire exogenous glucose could be due to effects of heat on transport mechanisms, into or inside the cells. The stimulatory effect of heat on endogenous respiration could be on vacuolar membranes releasing some reserves to mitochondrial respiratory sites. If this is true, then the quantity or nature of reserves released by heat is much less than that released by azide. Also, as with azide, levels of heat exposure which stimulate respiration impair spore viability (Mandels *et al.*, 1965). Freezing also disrupts some vacuolar mem-

branes releasing endogenous reserves to the respiratory cycle. While the effects of freezing are not quantitatively as great as those of azide, the effects on the cell appear more similar since exogenous respiration is not impaired as with heat.

Essentially all of the conclusions relative to *M. verrucaria* spores can be applied to *T. reesei*—the observations are similar. Other than quantitative differences only two distinctions have been observed. Perhaps most significant is the respiratory behavior of untreated spores toward exogenous substrates. With *M. verrucaria* the rate on simple sugars is essentially constant; the O₂ uptake is proportional to time and is the same in nutrient salts as in buffer. The response of *T. reesei* spores to exogenous sugars in buffer is similar to that of *M. verrucaria*, but if available nitrogen (NH₄⁺, but not NO₃⁻) is also added, a growth type of response occurs, and the rate increases linearly—not exponentially—with time. Interestingly, this linear relation has been shown for respiratory response during germination of *M. verrucaria* and also for spores of *Bacillus megaterium* (Mandels *et al.*, 1956). These data are indicative that dormancy in *T. reesei* can be partially alleviated by providing inorganic N along with a carbon source, the spores being less dependent upon complex nitrogen sources.

The second difference observed related to the effects of azide or freezing or respiration of exogenous sugars: both of these stimuli of endogenous respiration had a suppressive effect on the ability to oxidize exogenous glucose whereas with *M. verrucaria* the effects tend to be additive.

The subcellular structure of *T. viride* conidia (Rosen *et al.*, 1974) appears quite similar to that of *M. verrucaria*, except that endoplasmic reticulum is lacking in the mature spore. Whether *T. reesei* spores are different is not known.

The frequency of occurrence of intracellular localization of metabolites in pools as a dormancy regulating mechanism can only

be surmised, but it would appear to be not an uncommon phenomenon, at least in fungi.

Although essentially all data reported here relate to metabolic activity, concurrent microscopic observations for germination were always made and were negative except where noted otherwise. It is fundamentally significant that in the organisms studied here, only provision of extracted, endogenous reserves as exogenous substrates promotes the complete reactivation sequence culminating in germination. Comparison with the simple heat or furfural treatment sufficing for *Neurospora ascospore* activation (Sussman and Douthit, 1973) is challenging.

It is interesting to relate these concepts to studies with bacteria showing that, while dormant spores of *Bacillus megaterium* do not contain free amino acids, they do appear shortly after germination (Setlow, 1978). It is tempting to speculate that fungus spores do not have the great resistance to chemical and physical agents that characterizes bacterial spores, because fungal spore dormancy is based upon ultrastructural separation of metabolites, the membranes involved, as well as those not associated with dormancy, being easily damaged by heat, etc. Since integrity of membrane is a requisite for viability, such treatments kill the cells. Mechanisms regulating dormancy in bacterial spores are different.

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