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Correlation of Enzymatic Activity and Thermal Resistance with Hydration State in Ungerminated *Neurospora* Conidia

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Ungerminated *Neurospora crassa* conidia were incubated at 0, 50, and 100% relative humidity, giving rise to conidia in dry, quasi-dry, and wet hydration states, respectively. Metabolic activity was detected by monitoring levels of reduced glutathione (GSH), oxidized glutathione (GSSG), and the soluble-amino acid pools as a function of incubation time. Wet conidia (~65% water content) exhibited significant metabolic activity as evidenced by: (i) reduction of GSSG to GSH, (ii) degradation of GSH, and (iii) changes in the pool sizes of certain amino acids. GSSG accumulated slowly in dry conidia (<5% water content) and more rapidly in quasi-dry conidia (~13% water content), indicating that enzymatic reduction of GSSG is inactive in these states. Longevity and thermal resistance were high for dry conidia and low for wet conidia, but were not influenced by variation in GSSG content. The water content of conidia exhibited a hysteresis effect in that at a given relative humidity previously dried conidia attained a lower water content than freshly harvested conidia.

In earlier studies freshly harvested *Neurospora crassa* conidia were found to have elevated oxidized glutathione (GSSG) and protein-glutathione disulfide (PSSG) levels compared with mycelia (11). These levels increased further when conidia were aged under normal atmospheric conditions but decreased sharply during the first minutes of germination. Because the total glutathione (reduced glutathione [GSH] + GSSG + PSSG) level showed only minor changes, the observed fluctuations in glutathione thiol-disulfide status result from interconversion of thiol and disulfide forms. The term glutathione thiol-disulfide status is taken here to mean the distribution of glutathione among its thiol and various disulfide forms, including PSSG, and has essentially the same meaning as the GSH-GSSG status defined and studied extensively by the Kosowers and their co-workers (21-23).

The initial objective of the present study was to elaborate the answers to two questions associated with the changes in glutathione thiol-disulfide status found in *Neurospora* conidia. First, what factors are involved in the control of GSSG levels during aging of conidia? Second, are the increased disulfide levels in aged conidia associated with increased thermal resistance? Our studies revealed that the conidial water content, determined by the relative humidity during storage, was a key element in the answers to these questions. The hydration state of conidia then became a central aspect of the study.

The water content of fungal spores spans a wide range, 8 to 88% of fresh weight, depending upon species and upon conditions of growth and harvest (17), but detailed studies of the effect of relative humidity upon the water content and properties of fungal spores have not been reported. Such studies have been carried out with a wide array of other biological materials including, for example, bacterial spores (15, 20, 25, 28). *Artemia* cysts (7, 8), seeds (19) and other agricultural products (16), processed foods (1, 2, 5, 18), and purified biopolymers, primarily proteins (6, 9, 13, 24). Such materials invariably exhibit an S-shaped plot of water sorption as a function of relative humidity or water activity. The desorption curve usually lies at higher water content than the adsorption curve, a phenomenon referred to as the hysteresis effect (16, 24). Between 0 and 10% relative humidity, most biological materials exhibit a sharp increase to as much as 5% water content. A more gradual increase is observed between 10% and about 80% relative humidity to a value for water content usually in the range 10 to 25% (6, 19, 20). This water is considered to be "bound" at charged or polar sites with varying degrees of tightness (9, 24). Above 80% relative humidity there is a sharp increase in the water content to a value usually in excess of 35% at 100% relative humidity. This is considered to represent "bulk" water accumulating in the regions between hydrated molecules (9, 24).

We describe these three general hydration

states as dry (water absent or tightly bound at <10% relative humidity), quasi-dry (bound water only), and wet (bound plus bulk water present), recognizing that a range of conditions may exist within each category. We have examined conidia conditioned under 0, 50, and 100% relative humidities to explore the glutathione thiol-disulfide status and thermal stability in dry, quasi-dry, and wet hydration states. The results demonstrate that hydration state is an important factor in determining enzymatic activity and thermal stability in *Neurospora* conidia.

MATERIALS AND METHODS

Reagents and enzymes. All inorganic chemicals were of reagent or higher grade. Enzymes, coenzymes, and other biochemicals were obtained from Calbiochem unless otherwise indicated.

Organisms and culture conditions. Conidia of *N. crassa bd* strain (31) were used, unless otherwise specified, and were prepared, dry harvested, and germinated as described previously (11). Conidia of *N. crassa bd* NADase$^-$ strain (29) were employed in some experiments. These were grown on a vitamin-supplemented complete medium (30), omitting malt extract and adding biotin at a final concentration of 0.5 μg/liter. Large-scale preparations of these conidia were obtained by using 500 ml of 1.5% agar media autoclaved in a glass baking dish (20 by 34 cm) covered by a layer of cotton supported on three glass rods and taped to the sides of the dish. After inoculation with 1 to 2 ml of conidial suspension, dishes were incubated at 30°C for 36 to 48 h and the maintained at room temperature. After 8 to 9 days the conidia and mycelia were scraped from the surface of the agar and stored in a desiccator over $CaSO_4$ for 3 to 5 days. Conidia were isolated from mycelial debris by filtration through a very fine filter support screen (no. XX 50 02501, Millipore Corp.) and were stored at −20°C until used. Conidial viability as measured by germ tube formation was determined as previously described on samples of 150 to 250 conidia (11). Viability based upon colony formation was determined by plating 100 or more conidia on each of five 1.5% agar petri dishes prepared with Vogel 8% sorbose–0.8% glucose minimal medium (38). Freshly harvested conidia (65 ± 10% colony formation) were used to define 100% viability. Comparisons showed that viability based upon germ tube formation was essentially identical to that based upon colony formation.

Aging at controlled relative humidity. Conidia (50 to 300 mg, wet weight) were weighed into tared 15-ml scintillation vials and stored uncovered at the desired relative humidity. A desiccator containing anhydrous $CaSO_4$ was used for 0% relative humidity. Higher relative humidities were obtained by using mixtures of water and sulfuric acid (39) in wide-mouth jars containing polyethylene platforms to support the vials. The sealed humidity chambers were kept under laboratory temperature (20 ± 3°C) and lighting conditions. Water content was established from the loss in weight of a sample heated at 100 ± 5°C for 18 h or longer. Weights were determined on a Mettler H51AR balance and the repeatability was ±0.1 mg or better.

Thermal resistance. Approximately 1 mg of each conidial sample was placed in open Pyrex tubes (10 by 75 mm), and these were immediately immersed in a sand bath maintained at 100°C in an oven. Tubes were removed at intervals and cooled in water. Samples of the conidia were then germinated to test viability. An unheated sample served as control in each case to establish that the pretreatment did not result in lowered viability.

Extraction and assay methods. Conidia were extracted in hot 80% ethanol, with *N*-ethylmaleimide present when disulfides were to be assayed (11). GSH and GSSG were assayed as previously described (11), with a reliability of ±10% of the reported values, and amino acid analyses were performed according to published procedures (32).

RESULTS

Aging of dry conidia. *N. crassa* conidia were found to contain 65 ± 5% water by weight immediately after dry harvesting. When stored at 0% relative humidity, the water content dropped to about 30% after 4 h and to about 5% after 1 day. Small samples lost water more rapidly than large ones. After 3 days, less than 2% weight loss was observed when the desiccated conidia were heated at 100°C for extended periods. The extent to which this later loss represents tightly bound water, thermal dehydration, or volatiles other than water is not clear.

The effects of storage at 0% relative humidity upon the viability and glutathione content of conidia are summarized in Table 1. At room temperature there is a slow increase in the GSSG content of the conidia, accompanied by a decline in viability. In one experiment most of the glutathione was oxidized during an initial storage at 50% relative humidity (see below), after which the sample was transferred to 0% relative humidity. The decline in viability with time was no

TABLE 1. *Effect of 0% relative humidity upon the glutathione content and viability of conidia*

Treatment[a]	GSH (μmol/g)	GSSG (μmol/g)	Viability (%)[b] (colony formation)
Freshly harvested	20	0.2	(100)
79 days 0% RH[c]			90
112 days 0% RH			70
306 days 0% RH	14	2.4	35
393 days 0% RH			30
454 days 0% RH	16	1.3	15
62 days 50% RH plus 274 days 0% RH	0.5	8	50
6 days 0% RH plus 491 days 0% RH at −20°C	21	0.2	100

[a] At room temperature except as noted.
[b] All values ±10%.
[c] RH, Relative humidity.

greater in this sample than in those in which only a small fraction of the glutathione was oxidized. This indicated that the ratio of GSH to GSSG is not a factor in determining the viability of conidia stored at 0% relative humidity. When conidia were stored for 16 months in the dark at 0% relative humidity and −20°C, no loss in viability could be detected.

Aging of quasi-dry conidia. Freshly dry-harvested conidia stored at 50% relative humidity lost water for 25 h and then stabilized at 13 ± 2% water content after 48 h. The GSSG and PSSG levels increased steadily with time, and the GSH content decreased correspondingly, until after 2 months essentially all of the glutathione had become oxidized (Fig. 1). The viability decreased slightly after 1 month. In replicate experiments the rate of GSSG formation varied somewhat but was generally within a factor of 2 of that shown in Fig. 1. The glutathione content of different samples of conidia varied from 10 to 30 μmol/g which contributed to the variation in oxidation rate. Comparison of the data of Fig. 1 with those of Table 1 indicates that oxidation of glutathione occurs 30 to 100 times faster at 50% than at 0% relative humidity.

Germination of dry and quasi-dry conidia. The effect of germination upon the thiol-disulfide status of conidia aged under controlled humidity is presented in Table 2. For conidia aged 5 days at 0% relative humidity, about 20% of the GSSG is retained by the conidia and about 20% appears in the germination medium. The remaining 60% must have been reduced to GSH. With conidia aged at 50% relative humidity, the bulk of the GSSG was reduced to GSH and retained by the conidia. Only 5% of the GSSG remained associated with the conidia and another 5% appeared in the medium. The amount of glutathione released into the medium was essentially the same in the two experiments, but the fraction present as GSSG was higher in the conidia having higher GSSG content. These observations exclude the possibility that most of the GSSG found in conidia is associated with the surface or periplasmic space and is merely washed out during germination.

Aging of wet conidia. When freshly har-

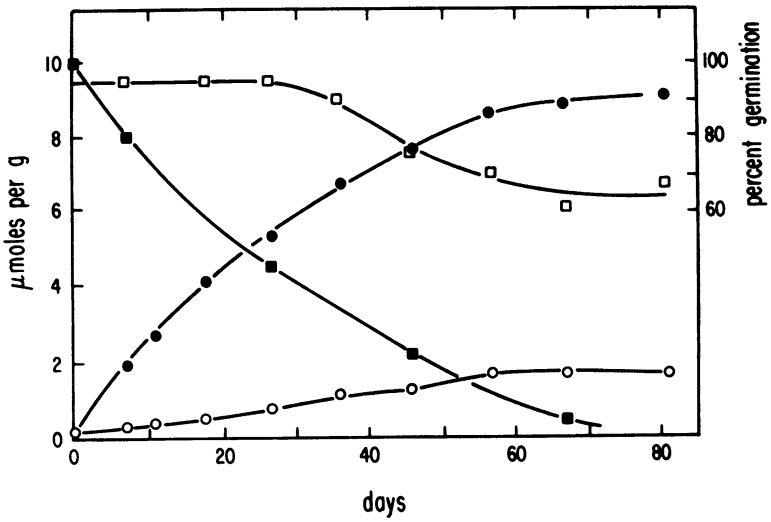

FIG. 1. *Germination and glutathione content of conidia stored at 50% relative humidity. Percent germination (□), 1/2 GSH (■); GSSG (●); and PSSG (○).*

TABLE 2. *Effect of germination upon the glutathione content of conidia*

Treatment	Time germinated (min)	Conidia[a]			Medium[a]		Total glutathione[a]
		GSH	GSSG	PSSG	GSH	GSSG	
5 days 0% RH[b]	0	17	0.5	0.3			18
	10	16	0.1	0.2	1.1	0.1	18
37 days 50% RH	0	3.8	6.5	1.4			18
	10	18	0.3	0.4	0.7	0.3	20

[a] Values shown are in micromoles per gram.
[b] RH, Relative humidity.

vested conidia were stored at 100% relative humidity, the total sample weight increased several percent during the first 9 days and then declined, approaching the original level around 18 days. The fraction of the total weight remaining after oven drying declined from 36 to 29% after 18 days, indicating an increase in the amount of water and other volatile components. A decline of ~25% in the ratio of residual dry weight after ethanol extraction to total wet weight occurred between 5 and 12 days.

Changes in the glutathione thiol-disulfide state and viability during storage at 100% relative humidity are shown in Fig. 2. During the first few days little change occurred in the GSH, GSSG, or PSSG levels, and viability remained high. After 5 days, however, GSSG and PSSG levels increased, and the GSH content fell. At the same time viability began a steady decline, until after 18 days only 2% of the conidia were capable of germ tube formation.

Some components of the amino acid pool showed marked changes during storage at 100% relative humidity, as did the total glutathione pool (Fig. 3). The decline in total glutathione after 10 days was comparable to the increase in the glycine pool, suggesting that the glutathione was degraded to its component amino acids. The glutamine pool decayed exponentially from 5 days onward, whereas the asparagine pool declined only after 10 days. Glutamic acid and aspartic acid levels increased between 5 and 12 days, then declined after 15 days. Although undetected before 12 days, γ-aminobutyric acid appeared in the amino acid pool between 12 and 19 days at a level of 12 to 15 μmol/g.

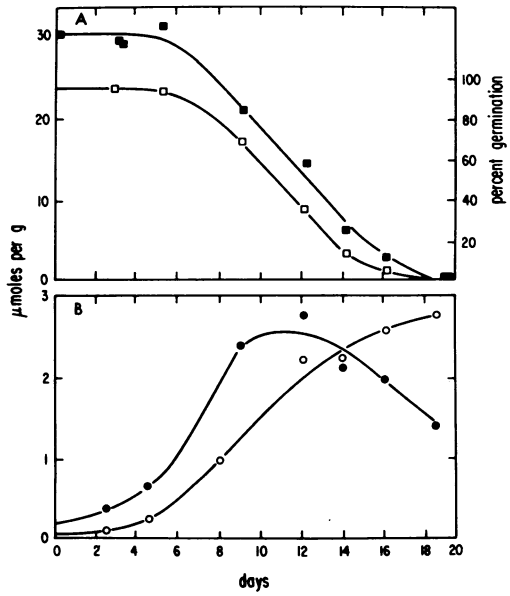

FIG. 2. *Germination and glutathione content of conidia stored at 100% relative humidity. (A) Percent germination* (□); *GSH* (■). *(B) GSSG* (●); *PSSG* (○).

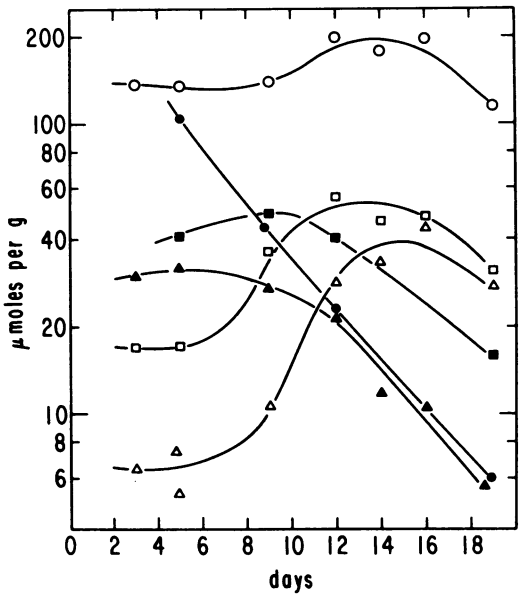

FIG. 3. *Amino acid and total glutathione (GSH + half-GSSG + PSSG) of conidia stored at 100% relative humidity. Glutamic acid* (○); *glutamine* (●); *aspartic acid* (□); *asparagine* (■); *total glutathione* (▲); *and glycine* (△).

Alternating quasi-dry and wet hydration states. The foregoing results suggested that dehydration inactivates the metabolic processes necessary to maintain glutathione in the reduced state. Further evidence of this was obtained by exposing conidia alternately to 50 and 100% relative humidities. The GSSG level elevated by aging at 50% relative humidity could be returned to a level typical of freshly harvested conidia simply by storing the conidia at 100% relative humidity for approximately 20 h (Fig. 4). Moreover, this oxidation-reduction cycle could be repeated many times. Summing over all of the cycles shown in Fig. 4 revealed that the equivalent of over 18 μmol of GSH per g was oxidized to GSSG and subsequently reduced back to GSH. No significant change in total glutathione was detected during this process. A decline in viability similar to that observed for continuous aging at 50% (Fig. 1) was also detected in this experiment.

Rehydration of dry conidia. When conidia were first dehydrated at 0% relative humidity then stored at 50 to 95% relative humidities, there was a rapid uptake of water during the

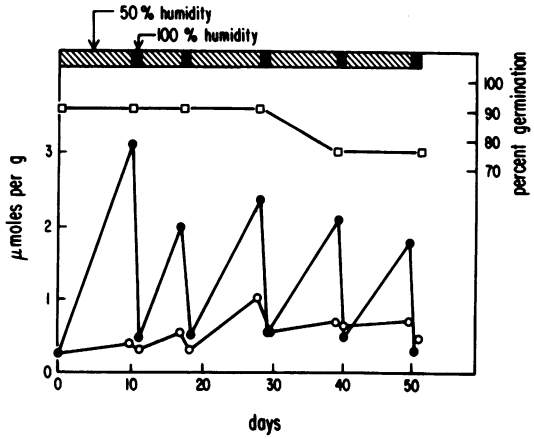

FIG. 4. *Germination and glutathione content of conidia stored alternately at 50% relative humidity and 100% relative humidity. Percent germination* (□); *GSSG* (●); *and PSSG* (○).

first 24 h, after which further change was very slow or negligible. At 100% relative humidity a slow increase followed the initial sharp increase. However, even after 8 days the water contents were substantially below the values obtained by dehydration of freshly harvested conidia. Thus, conidia exhibit the hysteresis effect observed with other biological systems. Figure 5 shows the water and GSSG contents for desiccated conidia stored at various relative humidities for 8 days. Both the water content (5 to 10%) and the rate of GSSG accumulation (~0.08 μmol/g per day) at 50% relative humidity were significantly lower than that of freshly harvested conidia stored at 50% relative humidity (~13% water content and ~0.2 μmol of GSSG per g per day). For purposes of later comparison, Fig. 5 also includes data obtained by Skujins and McLaren (36) for the water uptake of dry crystalline urease and for enzymic activity ($^{14}CO_2$ release) of a lyophilized mixture of urease and [^{14}C]urea, both measured as a function of relative humidity.

Thermal resistance in dry and wet conidia. Conidia were pretreated under various conditions of relative humidity, incubated at 100°C for 5- and 60-min intervals, and germinated. The results (Table 3) demonstrate that dehydration, with little or no change in GSSG content, gives rise to a marked increase in thermal resistance. Aging at 50% relative humidity, with the accompanying 10-fold increase in GSSG content, gave no appreciable enhancement in the thermal resistance of dry conidia. Conidia aged at 50% relative humidity and then placed at 100% relative humidity (with reversion of water content to a high level and GSSG content to a low level) behaved like freshly harvested conidia in exhibiting no thermal resistance at 100°C.

DISCUSSION

The major features of dry, quasi-dry, and wet conidia as identified in these studies and elaborated below are summarized in Table 4. Dry and quasi-dry conidia contain ≤15% water. Based upon results with other complex systems containing carbohydrates and proteins (5), it is expected that diffusion rates will be greatly reduced at this low water content. The decline in diffusion coefficient with decreasing water content is a function of molecular weight, being more pronounced for larger molecules, so that enzymes and large coenzymes are expected to be immobilized in dry and quasi-dry conidia. Small nonpolar molecules such as oxygen, however, retain some mobility even in anhydrous solids (37). Although enzymatic reduction of GSSG becomes diffusion limited, oxidation of GSH in quasi-dry conidia, and more slowly in dry conidia, can reasonably involve molecular oxygen. Such oxidation may contribute to the very low oxygen uptake in anhydrobiotic dormant systems so that such uptake should not be taken a priori as evidence of metabolic activity involving enzymatic respiratory processes.

For wet conidia the situation is substantially different. Appreciable bulk water is present

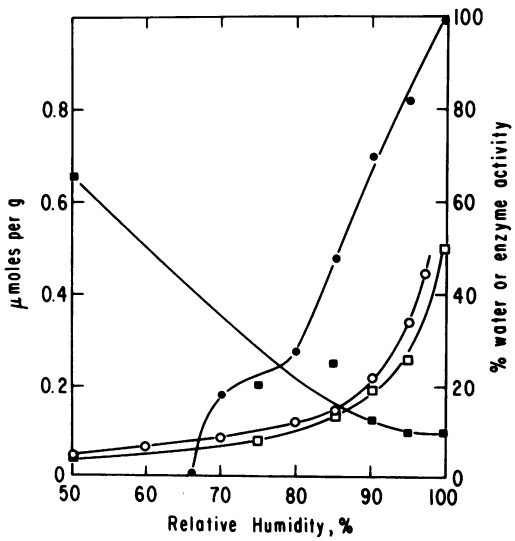

FIG. 5. *Effect of storage for 8 days at different relative humidities upon the water content* (□, *right scale*) *and GSSG content* (■, *left scale*) *for N. crassa bd NADase⁻ strain conidia. Variation in water content of crystalline urease* (○, *right scale*) *and relative enzyme activity of a mixture of [^{14}C]urea and urease* (●, *right scale*) *with relative humidity as reported by Skujins and McLaren (36).*

TABLE 3. *Effect of aging upon the water content, glutathione content, and thermal stability of conidia*

Treatment	% Water (wt/wt)	GSSG (μmol/g)	PSSG (μmol/g)	Germination[a] after heating at 100°C for:	
				5 min	60 min
Freshly harvested	65	0.23	0.13	<10	<10
1 day 0% RH[b]	4	0.27	0.22	70	70
4 days 50% RH	13			80	
1 day 0% RH plus 6 days 50% RH plus 1 day 0% RH	7	2.7	0.44	75	60
6 days 50% RH plus 1 day 0% RH	6	2.5	0.34	90	60
1 day 0% RH plus 6 days 50% RH plus 1 day 100% RH	42	0.22	0.16	<10	<10

[a] Germ tube formation. All values ±10%. Unheated controls ≥90%.
[b] RH, Relative humidity.

TABLE 4. *Summary of major differences among dry, quasi-dry, and wet conidia*

Hydration state	Water content (%)	Relative humidity (%)	Longevity	Thermal resistance	Rate of GSSG accumulation (μmol/g per day)	State of water	Inferred degree of molecular immobilization	Enzyme activity
Dry	<5	0	Months	High	~0.005	Absent or tightly bound	Nearly complete	Negligible
Quasi-dry	5-15	50	Weeks	High	~0.2	Bound	Extensive	Very limited
Wet	40-70	100	Days	Low	<0.02[a]	Bound plus bulk	Little or none	Substantial

[a] During the first several days.

within the conidium, and molecular diffusion coefficients can be expected to approach values representative of dilute solutions (5). Enzymatic activity in wet conidia would then not be greatly restricted by limitations on enzyme, substrate, and cofactor mobility. The observation that GSSG was converted to GSH in aged conidia upon storage at 100% relative humidity (Fig. 3) indicates that glutathione reductase is active in wet conidia. Such reduction utilizes reduced nicotinamide adenine dinucleotide phosphate, and possibly reduced nicotinamide adenine dinucleotide (40). Enzymatic regeneration of reduced pyridine nucleotides appears to occur in wet conidia because ≥9 μmol of GSSG per g can be reduced and fresh conidia contain a total of only ~4 μmol of pyridine nucleotides per g, less than half of which is in the reduced state (33).

The changes in amino acid pools (Fig. 4) are also indicative of enzymatic activity in wet conidia. The appearance of γ-aminobutyric acid is interesting because it is otherwise found in *Neurospora* only during germination (32). It suggests that the pathway for degradation of glutamic acid to γ-aminobutyric acid with accompanying generation of reduced pyridine nucleotides may be functional in wet conidia in much the same manner as postulated for germinating conidia (32). Disappearance of glutathione with corresponding appearance of glycine (Fig. 4) suggests that the enzymes degrading glutathione (26) are active in wet conidia. The decrease in both total and residual dry weights during aging of wet conidia also suggests the presence of substantial degradative activity. However, not all metabolic processes are active in wet conidia. Conidia do not form germ tubes in 100% relative humidity. Further, Mirkes (27) found that ≤3% of the ribosomes are present as polysomes in freshly harvested conidia, showing that protein synthesis is inactivated.

There are reasons to believe that the decreased metabolic activity in quasi-dry conidia can result directly from the effects of dehydration on individual enzyme-catalyzed processes. The data of Skujins and McLaren (36) show that in a simple enzyme-substrate system enzymatic activity drops to zero not far below the transition from the wet to quasi-dry state (Fig. 5). Similar observations have been made in studies of the effects of water upon enzyme activities as examined in connection with the problems of food spoilage (1, 2). Several mechanisms may be important in such inactivation. Decreased water activity will decrease the rate and may even reverse the direction of reactions in which water is a reactant. Dehydration may cause conformational or other structural changes which transform the enzyme to an inactive state. In the absence of these effects, enzymatic reactions ultimately become limited by the effect of dehydration upon diffusion of substrates and co-

factors to the enzyme, as discussed above.

Dry and quasi-dry conidia exhibit substantial resistance to heating at 100°C, independent of the GSSG content, whereas wet conidia exhibit no resistance. Conidia which survive 5 min at 100°C tend to survive to 60 min, a phenomenon which probably results from the fact that dehydration competes with thermal inactivation, the relative humidity and water content not being controlled during heating. Conidia surviving 5 min have become dehydrated and are thereby stabilized to further heating. Thus, dehydration appears to be essential to thermal resistance in conidia, probably via a direct effect upon the stability of macromolecules. Dry proteins survive heating more readily than wet proteins (4), and lyophilized enzymes have been observed to withstand extended heating under vacuum at temperatures in excess of 200°C (P. Price, personal communication). The molecular basis for this is seen in the results of Altman and Benson (3) who studied the kinetics of thermal denaturation of ovalbumin and showed that the reaction rate increased as the 12th power in sorbed water. As these authors point out, to denature protein it is necessary to break hydrogen bonds in the α-helical and other structural regions of the native protein. These bonds are ordinarily replaced by hydrogen bonds to water in the crucial unfolding steps, explaining the high-order dependence upon water. In the absence of water, such replacement presumably cannot occur, and destruction of the native protein structure would then require much higher temperatures. Analogous effects might stabilize other macromolecules, including nucleic acids, in their native state.

It appears that changes in hydration state and thiol-disulfide status are closely associated and occur in a broad spectrum of systems having dormant states. The present results and conclusions parallel those of Clegg (7, 8) who has conducted a careful study of the effect of hydration upon *Artemia* cysts. Preliminary results show that GSSG in dry cysts becomes reduced upon hydration (10). Some of the effects of hydration state upon GSSG reduction and thermal resistance found in *Neurospora* conidia have also been observed with wheat embryos (R. Fahey, unpublished data), suggesting that similar phenomena occur in seeds. Bacterial spores are formed in water at near-unit water activity, but the recent work of Gould and co-workers (14, 15) suggests that changes occurring during spore formation greatly reduce the water activity in the central core and thereby establish heat resistance. According to the present arguments, enzyme inactivation as a result of immobilization would also be expected. Although sporeforming bacteria, along with most other gram-positive bacteria, appear to lack glutathione (12), conversion of coenzyme A from thiol to disulfide forms has been found by the Setlows (34, 35) to accompany spore formation in bacilli, and reduction of such disulfides was found to occur early in germination. Thus, changes in hydration state and thiol-disulfide status occur in fungal, animal, plant, and bacterial systems during transition to and from the dormant state.

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