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Mechanisms of Protection of Trehalase Against Heat Inactivation in *Neurospora*

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The half-life of trehalase and invertase at 65 and 60 C was found to be much greater when intact ascospores of *Neurospora tetrasperma* were heated, as compared with extracts. By contrast, no protection was afforded these enzymes when they were heated in intact conidia and mycelium of *N. crassa* or *N. tetrasperma*. The protective effect of ascospores for trehalase was further investigated by heating ascospore extracts before and after dialysis. The removal of small molecules by dialysis lowered the heat resistance of trehalase significantly in such extracts. When the dialysate from extracts of mycelium, conidia, or ascospores was added to dialyzed enzyme extracts, that from ascospores was by far the most active. However, the same dialysates had only a small protective effect on invertase. The addition of ashed dialysates did not protect trehalase, and trehalose and glucose protected less effectively than the dialysate.

Ascospores of *Neurospora* are among the most heat-resistant fungal cells known, for not only do they need a heat shock to germinate but they survive exposure to 60 C for hours (8). By contrast, mycelium and conidia of this organism are killed by exposure to this temperature for only a few minutes. Therefore, *Neurospora* is excellent material for the study of the mechanism of heat resistance because of the opportunity it affords to compare resistant and sensitive stages of the same organism.

The experiments reported herein were designed to explore the effect of high temperatures upon two hydrolytic enzymes, trehalase and invertase, and the extent to which they are protected against heat inactivation within the ascospores, conidia, and mycelium.

MATERIALS AND METHODS

Production of conidia and mycelium. Conidial suspensions of *N. crassa* and *N. tetrasperma* were inoculated in 125-ml Erlenmeyer flasks containing 30 ml of "minimal" medium. Minimal medium consisted of 2% Vogel's (*personal communication*) salt solution, 2% sucrose, and 2% agar (50 mg of *i*-inositol per liter was added when needed for the inositol-less strain, 89601-A). The flasks were placed in an incubator at 37 C for 1 day and then under fluorescent lights in a constant-temperature room at 20 C. Conidia were harvested in phosphate buffer (0.05 M, *p*H 5.6), hereafter called "normal buffer," and were passed through glass wool to remove mycelial fragments. They were then concentrated by centrifugation at 600 × *g* and washed several times with normal buffer until no mycelial fragments were seen under a microscope. Mycelial mats were grown in liquid "minimal" medium on a reciprocal shaker at 25 C. After 5 days of growth, the mycelium was harvested and washed with distilled water and normal buffer, and pressed with a paper towel to dry.

Preparation of enzyme extracts. Strains 89601-A, an inositol-less mutant of *N. crassa*, and 394.4 of *N. tetrasperma* were used. The mycelium was suspended in normal buffer and ground with a Ten Broeck homogenizer with a Teflon pestle. Conidia were harvested either from 3-day-old cultures of *N. crassa* or from 5-day-old cultures of *N. tetrasperma*. Grinding was accomplished with alumina powder (Alcoa) with a mortar and pestle. Ascospores (1-year-old) of *N. tetrasperma* from a cross of strains 394.4 and 394.5 were produced by the methods described previously (5, 12). Grinding was accomplished in normal buffer with a mechanically driven syringe (Eilers and Fisher, *personal communication*). After grinding, all the extracts were centrifuged at 4 C at 10,000 × *g* for 10 min and the supernatant fluids were collected.

Dialysis and concentration procedures. Extracts were concentrated by vacuum dialysis performed in Visking cellulose tubing. Materials collected outside the tubing are referred to hereafter as the dialysate. Dialysates were condensed by the use of a rotary evaporator at 65 C.

Ashing procedure. Concentrated dialysates or buffer was heated in a crucible over a Bunsen burner for approximately 20 min.

Heat-inactivation procedure. Samples (3 to 5 ml) of enzyme extracts, or mixtures of enzyme extracts and reaction solutions, were pipetted into 25-ml Erlenmeyer flasks. The flasks were then immersed in a constant-temperature water bath and, at appropriate intervals, were withdrawn and immediately cooled in an ice bath.

Assays. The protein content of the enzyme extracts was measured as described by Lowry et al. (9).

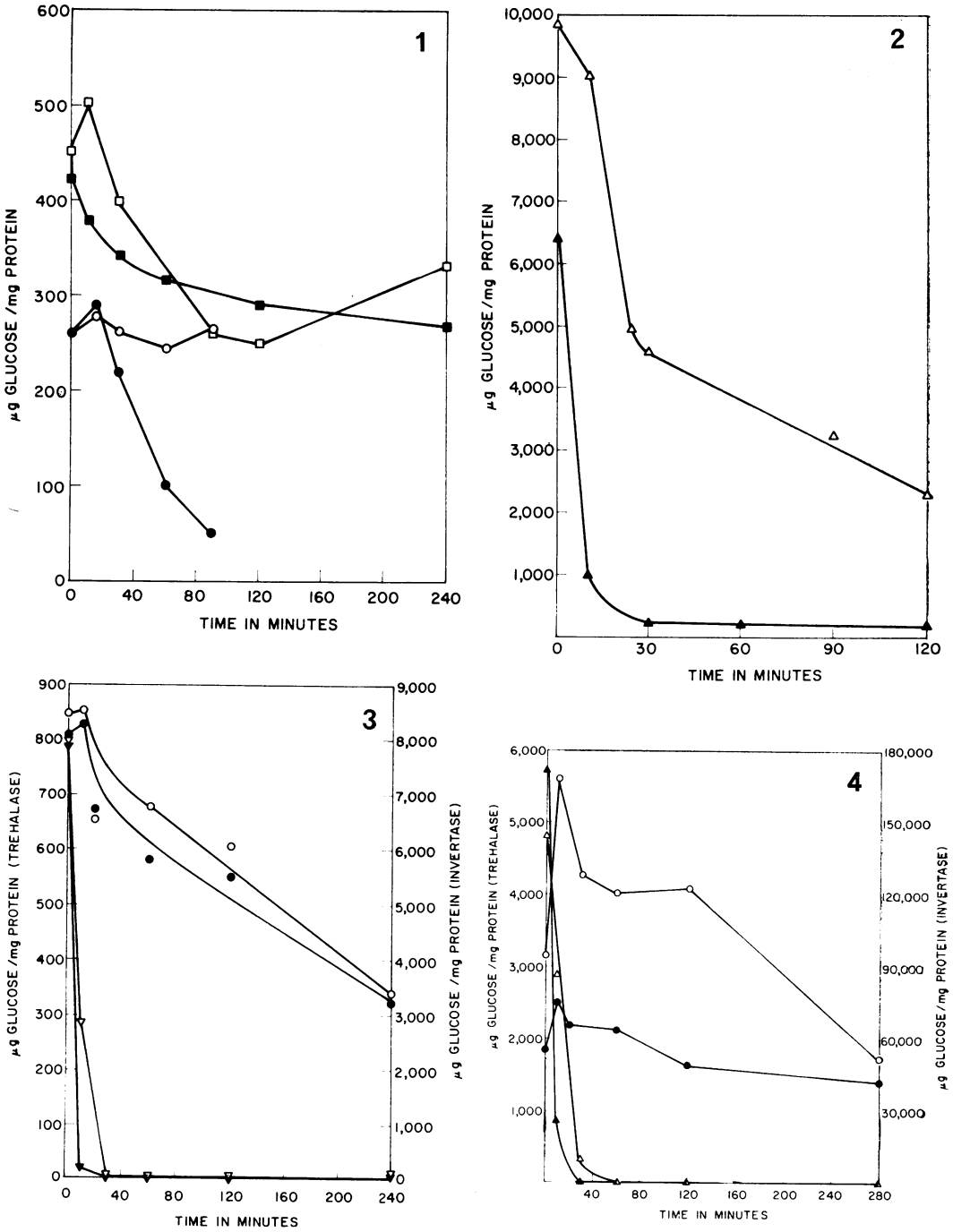

FIG. 1. *Trehalase activity after heating ascospores and extracts of Neurospora tetrasperma at 60 and 65 C. Symbols: □, intact ascospores at 60 C; ■, extracts at 60 C; ○, intact ascospores at 65 C; ●, extracts at 65 C.*

FIG. 2. *Invertase activity after heating ascospores and extracts of Neurospora tetrasperma at 60 C. Symbols: △, intact ascospores; ▲, extracts.*

FIG. 3. *Trehalase and invertase activity after heating conidia of Neurospora crassa and extracts thereof at 60 C. Symbols: ○, trehalase activity of extracts from heated intact conidia; ●, trehalase activity of heated conidial extracts; ▽, invertase activity of extracts from heated intact conidia; ▼, invertase activity of heated conidial extracts.*

FIG. 4. *Trehalase and invertase activity after heating mycelium of Neurospora crassa and extracts thereof at 60 C. Symbols: ○, trehalase activity of extracts from heated mycelium; ●, trehalase activity of heated mycelial extracts; △, invertase activity of extracts from heated mycelium; ▲, invertase activity of heated mycelial extracts.*

Trehalase and invertase activities were determined by the methods of Hill and Sussman (4, 5).

RESULTS

Effect of heating in vivo and in vitro. Ascospores (25 mg/ml) of *N. tetrasperma* were suspended in distilled water and exposed to 60 and 65 C for varying periods of time, after which extracts (1 ml of extract equivalent to 25 mg ascospores) were prepared and trehalase activity was determined (Fig. 1). An extract of another lot of ascospores was exposed for the same time intervals to these temperatures and trehalase activity also was determined (Fig. 1). The data reveal that, whereas the activity of trehalase in the intact ascospores that were heated to 65 C did not change very much, the activity in extracts diminished rapidly after even 15 min of heating. In the first 15 min, the extracts actually showed increased activity. At 60 C, the trehalase activity of intact spores also was enhanced after 10 min of heating, but, thereafter, diminished, rising again after 240 min. Extracts held at this temperature lost only about one-third of their activity after 4 hr. Therefore, considerable protection was afforded trehalase by intact spores at 65 C, but the effect was less obvious at 60 C.

Parallel experiments with invertase at 60 C (Fig. 2) revealed that this enzyme also was protected when heated in ascospores, as compared with extracts. The notably greater heat resistance of trehalase in these extracts also is apparent from these data.

The same types of experiments were repeated at 60 C with intact conidia (25 mg/ml) and extracts (1 ml of extract equivalent to 25 mg of conidia) of *N. crassa* (Fig. 3). However, in this case there was little, if any, protection afforded the enzymes by the intact conidia. A slight increase in trehalase activity was observed at 10 min both when the enzyme was heated in extracts and in whole cells. Parallel results were obtained when conidia and extracts thereof from *N. tetrasperma* were used.

When these experiments were performed on the enzymes of the mycelium of *N. crassa* (Fig. 4), trehalase activity was almost doubled after 10 min of heating at 60 C in the intact mycelium (70 mg/ml), and a smaller increase was observed in extracts (1 ml of extract equivalent to 72 mg of mycelium) as well. Moreover, there was little protection given trehalase by the intact cells, for, if anything, there was a faster loss of activity after 120 min when the mycelium itself was heated.

As for invertase activity, there was some protection by the mycelium but much less than was observed in the case of ascospores, and no activation has ever been observed in repeated experiments.

Mechanism of protection by intact cells. The protective effect of ascospores upon trehalase activity was investigated by heating extracts (1 ml of extract equivalent to 25 mg of ascospores) of these cells before and after dialysis. As Fig. 5 reveals, the removal of small molecules by dialysis before heating markedly lowers the heat resistance of trehalase in ascospore extracts. That dialysis of extracts (1 ml of extract equivalent to 23 mg of conidia) of conidia of *N. crassa* under the same conditions does not have the same effect may be seen in Fig. 6. The results were similar when conidia of *N. tetrasperma* were used.

These experiments suggested the presence in ascospores of a dialyzable substance (or substances) which protects trehalase against thermal inactivation, so its isolation was attempted. The material which was removed by the dialysis of extracts from 3 g of ascospores was prepared as described in Materials and Methods, and an amount equivalent to 0.5 g of spores was added back to enzyme extracts which had been thoroughly dialyzed. Such reinforced extracts, and controls lacking the agent, were heated to 65 C for various times and their trehalase activity was determined. The results, which are presented in Fig. 7, disclose that considerable protection is afforded dialyzed trehalase extracts from ascospores by concentration of the materials which are removed by dialysis. Moreover, the activity of the heated extracts was increased about 25% above

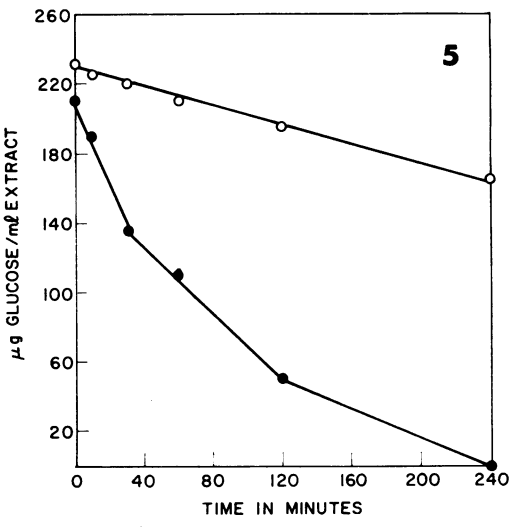

FIG. 5. *Trehalase activity of extracts of ascospores of Neurospora tetrasperma dialyzed before and after exposure to 65 C. Symbols: ●, dialyzed before; ○, dialyzed after.*

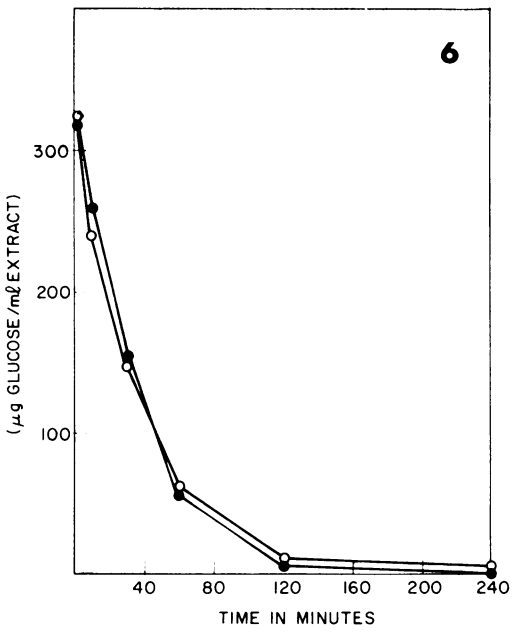

FIG. 6. *Trehalase activity of conidial extracts of Neurospora crassa dialyzed before and after exposure to 65 C. Symbols: ●, dialyzed before; ○, dialyzed after.*

that of the controls by the addition of the materials.

When these dialyzable substances were incinerated at approximately 800 C, their protective action was strongly reduced (Table 1). In fact, the half-life of trehalase in the control preparation (approximately 30 min) was greater than that of extracts to which the ashed diffusate was added. Buffer, at the same concentration as that in the ashed dialysate, also was incinerated, and added to dialyzed extracts, but was without significant effect (half-life, approximately 30 min).

The possibility that trehalase is protected by its substrate in extracts from *Neurospora* has been ruled out by prior treatment of dialysates with purified trehalase. Following this treatment, the dialysate was mixed with an equal volume of trehalase from ascospores and heated for 30 min at 65 C, but the protective effect was not eliminated. To test whether the product of trehalase action protects the enzyme against heat denaturation, glucose in amounts up to 0.1 M was incubated at 65 C with trehalase for 30 min. The sugar was then removed by dialysis overnight at 4 C, and the activity was determined. The protection afforded by glucose was slight as compared with that of the dialysate.

It was also of interest to learn whether dialysates from conidial and mycelial extracts protected trehalase against heat inactivation.

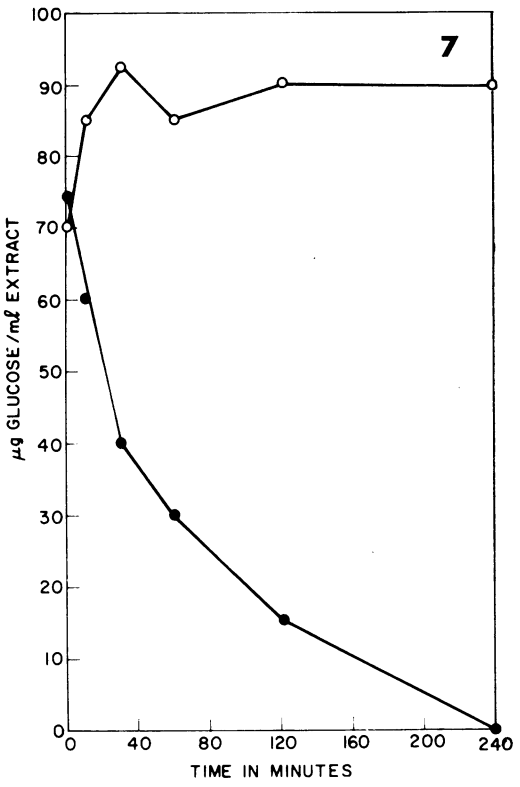

FIG. 7. *Trehalase activity of dialyzed extracts of ascospores of Neurospora tetrasperma after exposure to 65 C in the presence and absence of dialysate. Symbols: ○, with dialysate; ●, without dialysate.*

TABLE 1. *Effect of ashing of dialyzable materials from ascospores of Neurospora tetrasperma upon their capacity to protect trehalase from such spores against thermal inactivation at 65 C*

Time of heating	Trehalase activity[a] in extracts containing			
	Control[b]	Ashed dialysate	Ashed[c] buffer	Dialysate
min				
0	260	200	140	260
10	220	100	120	—
30	130	20	60	—
60	90	10	10	200
120	20	6	2	—
240	4	0	0	—

[a] Activity = micrograms of glucose per milliliter of extract.

[b] Dialyzed enzyme suspended in 0.05 M phosphate buffer.

[c] An 80-ml amount of 0.05 M phosphate buffer was dried and incinerated over a Bunsen burner for approximately 20 min.

Therefore, 2-ml amounts of such dialysates from *N. tetrasperma* (2 ml of conidial dialysate equivalent to 1.76 g; 2 ml of mycelial dialysate equivalent to 6.6 g) were added to equal volumes of enzyme from all stages and heated at 65 C for 60 min. Trehalase activity was determined after the preparation was dialyzed for 24 hr against normal buffer. The data in Table 2 indicate that the protective materials occur in all stages of the life cycle of *Neurospora* but that the greatest amount is present in ascospores. Almost exactly similar results have been obtained with dialysates from conidia and mycelium of *N. crassa*.

That invertase as well as trehalase is protected considerably when heated within intact ascospores is revealed in Fig. 2. Therefore, the effect upon heated invertase of dialysates which protect trehalase was studied. Equal volumes of dialyzed enzyme extracts from mycelium of *N. tetrasperma* and of a dialysate from ascospores were heated at 50 C for 20 min. Then the mixture was dialyzed against normal buffer for 24 hr at 4 C and invertase activity was determined. The results reveal that, although there was little protective effect of the dialysate upon invertase, this material markedly increased the heat stability of trehalase.

TABLE 2. *Effect of dialyzable materials from mycelium, conidia, and ascospores (Neurospora tetrasperma) upon the heat resistance of trehalase at 65 C for 30 min*

Source of enzyme	Addenda[a]	Trehalase activity		Relative protective activity[c]
		Amt of glucose/ml extract	Amt of glucose/g[b]	
		μg	μg	
Conidia	Normal buffer	260	—	—
	Conidial dialysate	510	156	12
	Mycelial dialysate	520	52	4
	Ascospore dialysate	680	1,272	100
	Unheated control	360	—	—
Mycelium	Normal buffer	210	—	—
	Conidial dialysate	460	151	15
	Mycelial dialysate	450	50	5
	Ascospore dialysate	540	1,000	100
	Unheated control	410	—	—
Ascospores	Normal buffer	140	—	—
	Conidial dialysate	270	81	13
	Mycelial dialysate	280	28	4
	Ascospore dialysate	340	606	100
	Unheated control	310	—	—

[a] Two-ml amounts of dialyzed enzyme extracts were added to 2 ml of dialysate. Two ml of conidial dialysate equivalent to 3.2 g of conidia, 2 ml of mycelial dialysate equivalent to 10 g of mycelium, and 2 ml of ascospore dialysate equivalent to 0.66 g of ascospores.

[b] Computed on basis of activity per unit (wet weight) of starting materials of each dialysate. The control activities with buffer alone were subtracted.

[c] Computed using activity (micrograms of glucose per gram) of ascospores as 100%.

DISCUSSION

At least five theories to account for the survival of thermophiles can be proposed, including the following: (i) innate resistance of the organism's enzymes or nucleic acids, or both; (ii) rapid resynthesis of enzymes or nucleic acids ["dynamic" theory of thermophilism (1)]; (iii) protection of enzymes and nucleic acids by intracellular substances; (iv) resistance conferred upon enzymes or nucleic acids, or both, by association with a structural component(s) of the cell; (v) high activation energy of enzymes in thermophiles, or low entropy of activation (11). It is probably to be expected that a combination of these factors will be found to explain thermal resistance, and, in fact, Bubela and Holdsworth (3) have presented data which implicate at least three of the above in the case of enzymes of *Bacillus stearothermophilus*. To these must be added the innate heat stability of the flagellar and cytoplasmic proteins of this organism, a characteristic which has been emphasized by several groups (2, 6, 7, 10).

These experiments (Fig. 1 and 2) reveal that trehalase from *Neurospora* ascospores is innately more heat-resistant than invertase from the same cells, and the same is true for these enzymes from vegetative stages (5). Consequently, a diversity of enzymes probably exists in ascospores, in terms of their innate resistance to heat. Unless further work shows that only those enzymes that are required by ascospores during the time when they must survive exposure to high temperatures are innately heat-resistant, then it must be concluded that other means must exist in ascospores to protect their cellular machinery.

The protection afforded trehalase and invertase when heated in ascospores of *N. tetrasperma* suggests that the arrangement of the enzymes in the spore, protective substances, or both, account for some of the heat resistance of these spores. That protective substances contribute toward enhancing the durability of trehalase is established by the dialysis experiments reported herein. In contrast, lower activity of such substances has been demonstrated in similar experiments with invertase, so that other protective mechanisms must be invoked to explain the survival of this enzyme after activation and the protective effect of ascospores.

The fact that conidia and mycelium of *Neurospora* do not protect trehalase and invertase against heat denaturation, and that these stages

do not survive very long at temperatures above 40 C (8), argues that the protective effect observed with ascospores is of selective advantage. This conclusion is fortified by the much lower concentration of protective agents in the dialysates from conidia and mycelium, as compared with that in dialysates from ascospores.

The protective substance(s) from *Neurospora* has not been identified, but ashing destroys the activity of the dialysates (Table 1). Although minerals are not conclusively ruled out as the active protective principle, the fact that conidial and mycelial extracts are similar in mineral composition to those from ascospores, yet protect very little, decreases the likelihood that minerals are involved. Furthermore, it does not appear as though trehalose, the substrate of trehalase, or glucose, the product, is the protective agent of this enzyme. Analysis of the dialysates is being continued to identify the active material.

A summary of the data on the heat resistance of trehalase and invertase from *Neurospora* appears in Table 3. Although the half-lives at 65 C of trehalase in undialyzed extracts of ascospores and conidia are widely divergent (240 and 53 min, respectively), those of dialyzed ones are much more similar (60 and 43 min, respectively). Although these data are too meager to permit the conclusion that the enzymes from the two types of spores are identical, experiments in progress may answer this question. As in the case of many other enzymes, the heat resistance of purified preparations is much lower than that of crude extracts. The wide divergence in the half-lives of undialyzed trehalase extracts noted above is not mirrored by the data for invertase in Table 3. This further reflects the small degree of protection afforded this enzyme by dialyzable materials in extracts.

TABLE 3. *Summary of half-lives of trehalase and invertase at 60 and 65 C in extracts and intact cells of Neurospora*

Cell type	Conditions of heating	Half-life (min)		
		Invertase, 60 C	Trehalase	
			60 C	65 C
Ascospores (*N. tetrasperma*)	In cells	22	>240	>90
Ascospores	In undialyzed extracts	3–4	>240	>240
Ascospores	In dialyzed extracts	—	—	60
Conidia (*N. crassa*)	In cells	8	195	—
Conidia	In undialyzed extracts	5	190	53
Conidia	In dialyzed extracts	—	—	43
Mycelium (*N. crassa*)	In cells	14	>280[a]	—
Mycelium	In extracts	6–7	>280	—
Mycelium[b]	In purified extracts (peak 1)	—	>20	8 (66 C)
Mycelium[b]	In purified extracts (peak 2)	—	>20	13 (66 C)

[a] This value was obtained by using the activity at the start as the basis of the calculation. If the highest activity was used (Fig. 4), the half-life would be about 200 min.

[b] Data from Hill and Sussman (6) on enzymes purified 254-fold (peak 1) and 24-fold (peak 2).

The increased trehalase activity after heating, which was noted in some of these experiments (Fig. 3 and 4), may be of significance with regard to the activation of dormant ascospores. Thus, whereas dormant cells do not metabolize trehalose, activated ones do, so that it is possible that increased trehalase activity may explain the greater respiratory rate that is observed after the breaking of dormancy (13). However, heat stimulation of trehalase is not unique to ascospores; it has been observed in other stages as well (5), so its relevance to the dormant condition is still uncertain. It appears from the data in Fig. 7 that dialysates not only protect trehalase against heat denaturation but increase its activity after heating (activate) as well. Whether the material which regulates trehalase activity is the same as that which protects remains to be learned.

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