

Effect of Intracellular Carbohydrates on Heat Resistance of *Dictyostelium discoideum* Spores

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The effect of intracellular trehalose and glycogen on the survival of spores of *Dictyostelium discoideum* ATCC 25697 after exposure to supraoptimal temperatures was examined. Cells metabolically perturbed by incubation in glucose and inorganic phosphate have intracellular trehalose and glycogen concentrations fivefold and twofold higher, respectively, than those of the controls. These cells were more resistant to the lethal effects of wet heat (45° to 55°C) than were control cells. The presence of 40 mM trehalose in the buffer during heat stress increased the survival of nonperturbed cells to approximately the level of the perturbed cells. No protection was observed when cells were heated in the presence of exogenous glycogen. Glucose or disaccharides other than trehalose, when present during heat stress, had no effect on heat resistance. Nonperturbed cells preincubated in 40 mM trehalose and washed before heat stress were more resistant to killing than were controls. Cells perturbed with inorganic phosphate, which has been shown to increase trehalose concentrations but decrease glycogen concentrations, were also more resistant to the lethal effects of wet heat than were controls. The data suggest that trehalose has an effect on the wet-heat resistance of *D. discoideum*. Some possible mechanisms are suggested.

The cellular slime mold *Dictyostelium discoideum* differentiates when starved and produces constitutively dormant spores. There are two major storage carbohydrates in the spores, glycogen and trehalose. Glycogen is also a major polysaccharide in vegetative cells. The glycogen content of the cells decreases during fruiting body formation and sorocarp maturation (9, 14). Trehalose is detectable at a low level in vegetative cells but accumulates in spores (2). It is not used during dormancy but, upon germination, is rapidly degraded due to the activity of the enzyme trehalase (3).

Wright et al. (16) have found that the levels of trehalose and glycogen in *D. discoideum* can be experimentally manipulated after differentiation has occurred. Metabolic perturbation of the spores can result in a fivefold increase in trehalose and a twofold increase in glycogen over levels in nonperturbed cells. The ability to alter the intracellular concentrations of these storage carbohydrates has provided a means to examine the role of these carbohydrates in dormancy and aging. This paper reports that spores with increased intracellular concentrations of storage carbohydrates had increased resistance to wet heat. Evidence is presented indicating that, of the two saccharides, trehalose is more likely than glycogen to be responsible for the increased

heat resistance. Some possible mechanisms of heat protection by trehalose are proposed.

MATERIALS AND METHODS

Organism and culture conditions. *D. discoideum* (NC-4) ATCC 24697 was grown on nutrient agar (1% peptone, 0.1% yeast extract, 1% glucose, 1.5% agar) with *Escherichia coli* as the bacterial associate, according to methods described previously (7). After the bacterial food source had been depleted, myxamoebae of *Dictyostelium* were harvested and spread onto sterile sheets of 1.5% agar (Difco Laboratories, technical grade). The cells were then incubated at 23°C for 24 h, by which time young sorocarps had been formed. Sorocarps from half of a sheet of agar were suspended in 50 mM 2-[*N*-morpholino]ethanesulfonic acid (MES) buffer (pH 6.5; Sigma Chemical Co.). The remaining sorocarps were suspended in the same buffer containing 50 mM each D-glucose and KH₂PO₄ (J. T. Baker Co.). After the cells were filtered through cheesecloth to remove the bulk of stalk cells, the spore suspensions were incubated for 90 min at 23°C in a water bath shaker (New Brunswick Scientific Co.). The cultures were then centrifuged at low speed (3,000 rpm, 5 min; Ivan Sorvall, Inc.), washed twice with MES buffer, and resuspended in approximately 5 ml of the same buffer. The number of spores, per milliliter was determined visually by using a Levy-Hausser counting chamber and a light microscope (American Optical Corp.). The spores incubated in glucose and phosphate and in buffer only will be referred to as

perturbed and nonperturbed spores, respectively.

Heat stress. Both perturbed and nonperturbed spores were subjected to heat stress by diluting spores, to approximately 10^6 /ml, into 10 ml of MES buffer preheated to the desired temperature in a constant-temperature water bath (P. M. Tamson, Zoetermeer, Holland). Samples were removed at time zero and at 2- to 5-min intervals, serially diluted in MES buffer, and spread, with *E. coli*, onto nutrient agar in standard petri dishes. The cultures were incubated at 23°C until visible plaques were formed (usually 3 days). The number of plaque-forming units per milliliter was determined, and the data were normalized as percent plaque-forming units per milliliter at time zero.

Comparisons of total spores counts (using a Levy-Hausser counting chamber) with plaque-forming units at time zero showed an initial viability of at least 85%.

RESULTS

Increased heat resistance of perturbed spores. The ability of perturbed and nonperturbed spores from 24-h sorocarps to withstand wet-heat stress at various temperatures was examined. The intracellular concentrations of trehalose and glycogen are given in Table 1. Metabolic perturbation by incubation in the presence of exogenous glucose and inorganic phosphate increased the concentration of trehalose 4.5-fold and that of glycogen 1.5-fold. Figure 1 shows the results from a typical heat stress experiment, in which the cells were heated at 50°C. The nonperturbed cells died more rapidly than the perturbed cells. A "90% killing time" was obtained from these experiments by determining, from the steepest parts of the curves, the times required to obtain a 90% decrease in plaque-forming units (Table 2). At 45 to 55°C, the 90% killing time was longer for the perturbed cells than for the nonperturbed cells. At 40°C there was no killing over 20 min, whereas at 60°C complete killing of both perturbed and nonperturbed cells occurred in less than 5 min.

Exogenous carbohydrate present during

TABLE 1. Intracellular metabolite concentrations in young sorocarps of *D. discoideum*^a

Incubation for 90 min in:	Concn ^b (mM) of metabolite:	
	Glycogen	Trehalose
0.05 M MES, pH 6.5	22 ± 1.8	2.3 ± 0.42
0.05 M glucose and P _i in 0.05 M MES	34 ± 2.8	10.3 ± 1.4
0.05 M P _i in 0.05 M MES	19 ± 0.9	3.1 ± 0.42

^a Data from Wright et al. (16).

^b Concentration is expressed in terms of cell volume and is normalized to the initial dry weight per cell volume relationship at aggregation (15). Data are given as mean values of three to six experiments ± standard error.

heat stress. To try and mimic or enhance the increased stability of perturbed cells, either glycogen or trehalose was added to the prewarmed (50°C) buffer. The presence of 40 mM trehalose increased the survival of the nonperturbed cells to approximately the level of the perturbed cells and provided additional protection to the perturbed cells when compared to perturbed cells heated in buffer alone (Fig. 2). No protection was observed when cells were heated in the presence of exogenous glycogen (15 mg/ml; Fig.

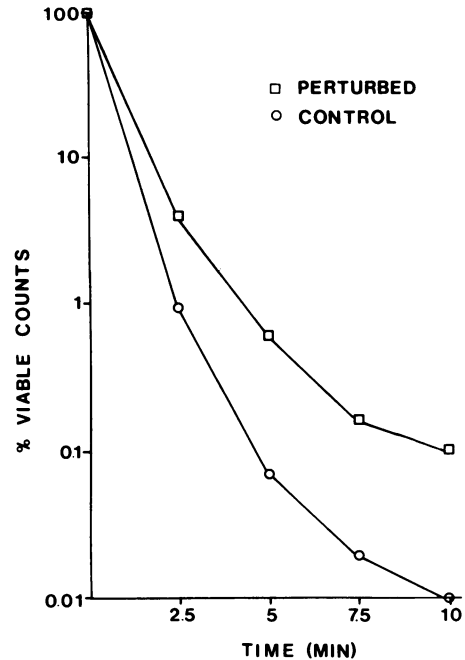


FIG. 1. Susceptibility of perturbed and nonperturbed (control) spores to heat stress of 50°C. Perturbation was done as described in the text. This and subsequent figures show data from a single representative experiment. Each experiment was repeated at least three times.

TABLE 2. 90% killing times for spores of *D. discoideum* exposed to wet heat

Temp (°C)	Mean 90% killing time ^a (min)		<i>S</i> _D ^b	<i>P</i>
	Control spores	Perturbed spores		
45	8.50	14.20	1.47	0.05
50	1.92	2.59	0.27	0.05
55	1.38	2.0	0.29	0.10

^a 90% killing time was determined as described in Results. Values given are means of four to six experiments.

^b Standard error of the mean difference.

3). The addition of disaccharides other than trehalose, including maltose, sucrose, and melibiose at 40 mM, provided no increase in heat resistance. The presence of 50 mM each glucose and inorganic phosphate, the metabolites used for perturbation, also had no positive effect on heat resistance.

It was not necessary for exogenous trehalose to be present during heat stress for it to have a protective effect (Fig. 4). When nonperturbed cells were incubated for 30 min in the presence of 40 mM trehalose, washed, and then stressed at 50°C, they were more resistant to killing than those preincubated in MES buffer alone.

Heat resistance in phosphate-perturbed cells. Incubation of spores in the presence of 50 mM KH_2PO_4 (in MES buffer, pH 6.5) resulted in a 15% decrease in the intracellular glycogen concentration, whereas the trehalose concentration increased 1.5-fold (Table 1). If heat resistance were conferred by the glycogen, no increase in resistance would be expected in phosphate-perturbed cells compared to the control. How-

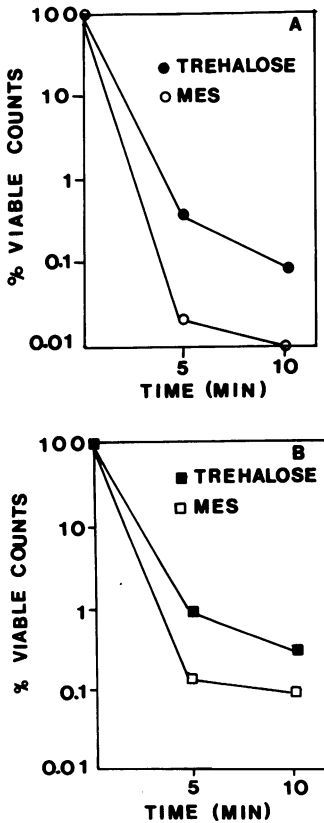


FIG. 2. Susceptibility of nonperturbed (A) and perturbed (B) spores subjected to heat stress (50°C) while in the presence (●, ■) and absence (○, □) of 40 mM trehalose.

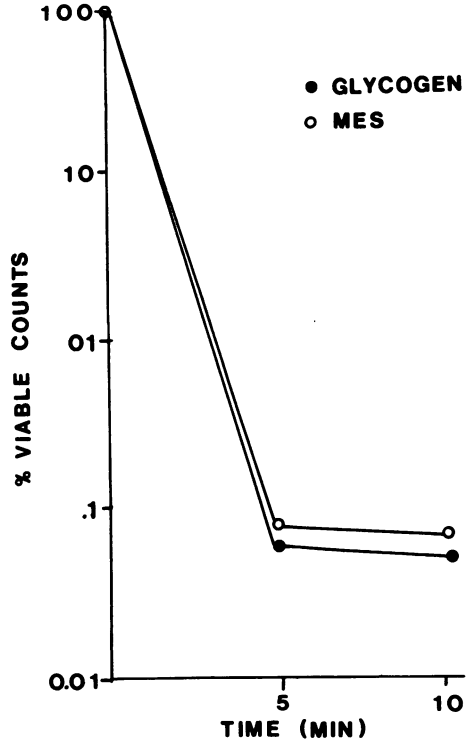


FIG. 3. Susceptibility of nonperturbed spores subjected to heat stress (50°C) while in the presence (●) or absence (○) of 15 mg of glycogen per ml.

ever, the phosphate-perturbed cells were more resistant to killing than the control (Fig. 5), suggesting that trehalose affected heat resistance.

DISCUSSION

Our data showed that intracellular storage carbohydrates increased the resistance of *D. discoideum* spores to killing by wet heat. They also suggested that trehalose was the carbohydrate primarily responsible for heat resistance.

Trehalose, which is often found in fungal spores, is thought to serve primarily as an energy source upon germination. In most fungi which store trehalose, it is not metabolized until germination is activated (1, 5, 10-12). Although the suggestion has been made that trehalose may also be involved in maintenance of spore dormancy by enhancing the ability of spores to withstand environmental stress (13), there have been no data to substantiate that idea. Our data demonstrated, for the first time, that trehalose does have a physiological function—enhancement of heat resistance—in addition to serving as an energy reserve.

The figures presented in this paper have all

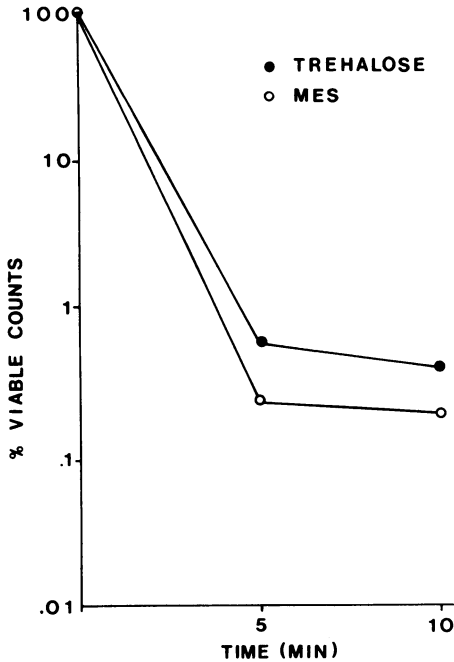


FIG. 4. Heat stress (50°C) susceptibility of nonperturbed spores preincubated for 30 min either in 40 mM trehalose in MES (●) or in MES alone (○). The spores were washed and resuspended in MES buffer before heat stress.

shown the results of representative experiments which had been repeated at least three times. This was done because there was considerable variability in the data from experiment to experiment under any one set of experimental conditions. However, the qualitative effect, i.e., the increased heat resistance of the perturbed spores, of those heated in the presence of trehalose, or of those preincubated in trehalose was almost always seen. Wright et al. (16) noted a similar problem with the values for trehalose and glycogen contents after perturbation. They stated that, although the standard errors were large, this was due to variability between experiments in both the control and experimental values, since within each experiment the same relative changes in trehalose or glycogen levels were seen. They further stated that the problem arose from the fact that "we are dealing with a population of organisms which cannot be synchronized perfectly, at a stage of differentiation when the levels of trehalose . . . are changing rapidly." We partially overcame this inherent problem by treating the values from each experiment as matched pairs and determining statistical significance by using the standard error of the mean difference (Table 2).

We are not suggesting that trehalose alone

determines spore heat resistance. Other cellular structures such as components of the wall or cytoplasmic membrane may also be involved. The data do not entirely exclude the possibility that intracellular glycogen may also contribute to heat stability. However, several lines of evidence indicate that trehalose has a prominent and possibly unique role in increasing the heat stability of the spores. Cells that had been perturbed with inorganic phosphate and had only increased trehalose levels (glycogen content actually declined, Table 1) still showed increased heat resistance compared to controls (Fig. 5). Nonperturbed cells, when provided with external trehalose, showed an increased heat resistance similar to that exhibited by perturbed cells (Fig. 2). Spores of *D. discoideum* are known to be permeable to a number of small metabolites (9, 16), and it is likely that trehalose can readily enter the cells. The fact that cells preincubated in trehalose and washed before being subjected to heat stress were more resistant to killing than were the controls (Fig. 4) indicates that exogenous trehalose was entering the cells. Since other disaccharides, when provided exogenously, had no effect on heat resistance, trehalose may have unique properties which account for its ability to increase the heat resistance of spores.

Although the data do not suggest the way in

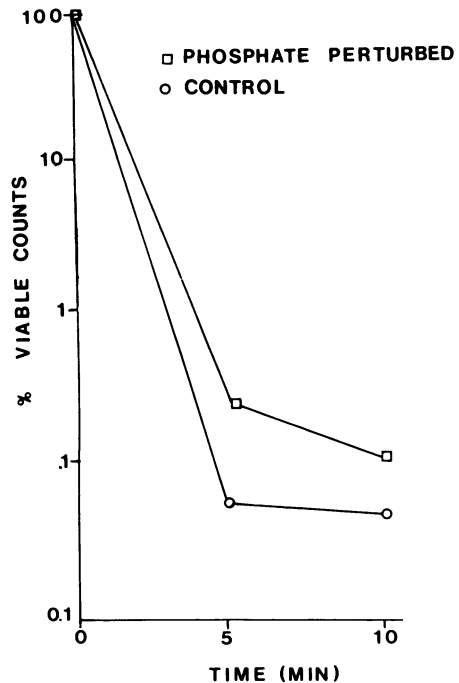


FIG. 5. Heat stress (50°C) susceptibility of spores perturbed by 90 min incubation in 50 mM KH_2PO_4 (□) or in MES buffer (○).

which trehalose might increase heat resistance, some mechanisms can be proposed. Trehalose is known to stabilize trehalose 6-phosphate synthetase (6) and trehalase (K. Killick, personal communication) *in vitro*. The extra trehalose may provide additional heat resistance by serving as a source of additional energy for repair of heat-induced damage. A third, more intriguing possibility is that trehalose might be aiding to dehydrate the cytoplasm, leading to increased heat stability. Muller and Hohl (8) obtained some indirect evidence that trehalose is localized in vesicles within the spores. If that is correct, calculations of the volume of the vesicles, based on the diameter of cross sections of the vesicles, suggest that the effective concentrations of trehalose within the vesicles would be in the molar range. Thus, storage of trehalose may serve an osmoregulatory function in *D. discoideum*. If the localization of trehalose in vesicles can be established, it might provide an interesting parallel to the concept to the osmoregulatory cortex in bacterial endospores and its role in heat resistance (4).

The data do not indicate which step or steps in plaque formation are affected heat and or trehalose. Subdivision of the process by a comparison of the percentage and rates of germination and emergence of perturbed and nonperturbed spores after heat stress would provide additional information about the physiological function of trehalose.

ACKNOWLEDGMENTS

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