JOURNAL OF BACTERIOLOGY, Aug. 1973, p. 592-599

Vol. 115, No. 2
Printed in U.S.A.

Localization of Trehalase in the Ascospores of *Neurospora*: Relation to Ascospore Dormancy and Germination

LANNY I. HECKER[1] AND ALFRED S. SUSSMAN

Department of Botany, The University of Michigan, Ann Arbor, Michigan 48104

Received for publication 6 November 1972

An association of trehalase with the innermost wall (endosporium) of ascospores of *Neurospora* is suggested, because this enzyme could be lyophilized in the presence of various wall components and heated in this dried state at 65 C without loss of activity. Ground ascospore walls, purified mycelial walls, a wall fraction consisting of protein, glucan and polygalactosamine, or bovine serum albumin stabilize trehalase under these conditions. No other substances tested protected as well as the above materials. Immunofluorescent labeling of trehalase shows that it is localized in the endosporium. Therefore, it is most probable that in dormant ascospores of *Neurospora*, trehalase, and its substrate, trehalose, are physically separated. Trehalose is located in the cytoplasm, whereas trehalase resides within the protein and carbohydrate matrix of the innermost major cell wall layer of the ascospore. The association with the cell wall protects the enzyme against the heating which is necessary to activate germination. Activation, whether by heat or chemical treatment (furfural), probably involves an increase in the permeability of the ascospore plasma membrane allowing trehalose to diffuse to the vicinity of its hydrolase, thereby providing the energy and intermediates for germination.

The nonreducing disaccharide trehalose is an important storage form of carbohydrate in the ascospores of *Neurospora*. This sugar accounts for up to 14% of the ascospore's dry weight and is probably located within the cytoplasm (1, 13). Although trehalase (EC 3.2.1.28., α,α'-glucoside 1-glucohydrolase) also is present in dormant ascospores (10) and exogenous glucose can be metabolized by these spores (1), the enzyme does not hydrolyze trehalose while the spores are dormant (1, 13). Instead, lipids are metabolized until the spores are activated by a heat shock of 60 C or furfural treatment (27), whereupon the trehalose reserves are rapidly utilized as lipids continue to be metabolized at about the same rate (13).

Thus, a better understanding of dormancy in ascospores will require an explanation of how trehalose catabolism is activated during germination and how trehalase can survive the high activation temperatures. This paper reports on attempts to investigate these problems through the localization of trehalase in ascospores employing immunofluorescent labeling and other techniques.

This investigation was taken from a dissertation submitted to the University of Michigan in partial fulfillment of the requirement for the Ph.D. degree.

[1] Present address: Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee 37830.

MATERIALS AND METHODS

Trehalase. The enzyme used in this study was obtained from the mycelium of *N. crassa* strain 89601A and is from the same enzyme preparation described and used previously (9a).

Lyophilization experiments. A 5-μliter sample of trehalase was added to 0.5 ml of 0.0005 M acetate buffer, pH 5.4, or deionized water. This solution was gently mixed with the substance to be tested in test tubes made of flint glass (16 to 25 mm). The suspension was then frozen at −20 C and lyophilized. The dried mixture was then rehydrated with 5 ml of 0.0005 M buffer and the resultant suspension was thoroughly mixed and assayed for trehalase activity, or the dried suspension was heated at 65 C for 30 min and then resuspended and assayed. Thus, trehalase was tested for its ability to survive lyophilization in the presence of other materials and to withstand high temperatures in the dried state.

Trehalase was dried in the presence of the following substances: (i) polystyrene, polypropylene, pyrex, and flint-glass test tubes, without the addition of any other materials; (ii) whole ascospores (external spore surface exposed) (9a); (iii) walls of ground ascospores (internal and external wall surfaces exposed; obtained from ascospores used in previous experiments; (iv) purified cell walls of mycelium from strain 89601A. Purified mycelial walls were prepared and fractionated according to Mahadevan and Tatum (15) and their fractions listed below were used in these experiments: (v) fraction I consisting of a glucan, protein, and a galactosamine polymer; (vi) fraction III, a laminarin-like polymer; (vii) fraction IV, chitin. Other polymers used were: (viii) chitin obtained from crab shells (Sigma Chemical Co.); (ix) cellulose (Sigma Chemical Co.); (x) cellulose acetate. Several purified proteins were tested including: (xi) bovine serum albumin (crystallized and lyophilized; Sigma Chemical Co.); (xii) lysozyme (3× crystallized; Calbiochem); (xiii) cytochrome-*c* (from horse heart, free of ammonium sulfate and sodium chloride; Sigma Chemical Co.); (xiv) ribonuclease (from bovine pancreas, 5× crystallized, salt and protease free; Sigma Chemical Co.).

Digestion of fraction I with Pronase. Fraction I was digested with Pronase B-grade (45,000 U per g; obtained from Calbiochem). A 200-mg sample of fraction I was suspended in 40 ml of buffer and digested for 30 min with 1 mg of Pronase at 40 C. This procedure was repeated four times. The buffers used were as follows: (i) 0.1 M sodium borate-0.05 M hydrochloride containing 5 mM calcium chloride, pH 7.5 (first two digestions); (ii) 0.1 M sodium borate-0.05 M hydrochloride containing 10 mM calcium chloride, pH 7.5; (iii) 0.05 M sodium Veronal hydrochloride containing 10 mM calcium chloride and 1 mM cobaltous chloride, pH 8.0 (22).

After each digestion the suspension was centrifuged; the residue was resuspended in buffer for the next digestion, and the supernatant fluid was analyzed for amino acids. After the final digestion the water-insoluble residue was pelleted, washed twice with deionized water, resuspended in deionized water, and dialyzed for 24 h in deionized water (three changes). After dialysis, this digested fraction (water-insoluble residue) was frozen and lyophilized.

The trichloroacetic acid-soluble portion of each of the above supernatant fractions was analyzed for amino acids by the ninhydrin method with leucine as a standard (25). Fraction I and the water-insoluble residue left after Pronase digestion were analyzed for sugar content by the anthrone method (26). Analysis of protein composition was carried out by the ninhydrin method after hydrolysis of the above fractions in 6 N HCl for 24 h at 110 C (15). Hexosamines were determined by the methods of Dische and Borenfreund (5) after hydrolysis for 3 h at 110 C in 3 N HCl (15).

Immunofluorescent labeling of ascospores. Ascospores were fixed in 3% glutaraldehyde in 0.05 M phosphate buffer, pH 5.6, for 2 h at 0 C, washed three times in deionized water, and the final pellet was mixed with warm 0.5% agar (14). This preparation was then dehydrated in the following series: 2-methoxyethanol, ethanol, *n*-propanol, and *n*-butanol (24 h in each with two changes). The spores were then placed in the monomer mixture of glycol methacrylate (24 h, two changes) and transferred to fresh monomer mixture, placed at 37 C for 48 h, and then for 4 to 5 h at 60 C (7, 8).

The resulting blocks were sectioned on a Sorvall Porter-Blum MT-2 ultramicrotome with glass knives. The sections were floated on drops of deionized water on glass microscope slides and left overnight to dry onto the slides.

These sections could then be labeled sequentially with rabbit anti-trehalase antibodies and fluorescein-labeled goat anti-rabbit antibodies (Miles Laboratories Inc., Kankakee, Ill.) using the sandwich technique of Nairn (20).

Experiments were performed by using the following protocol: experimental: UT + RAT + FGAR; control: (i) UT; (ii) UT + FGAR; (iii) UT + NIS + FGAR; (iv) UT + NAT + FGAR. (UT, untreated sections; NIS, nonimmunized rabbit serum; RAT, rabbit anti-trehalase antibodies; FGAR, fluorescein-labeled goat antirabbit globulin antibodies; NAT, anti-trehalase antibodies neutralized by a slight excess of purified mycelial trehalase.)

The labeled sections were covered with 50% glycerol solution and observed with a Carl Zeiss photomicroscope using excitation filter II and barrier filters 50 and 44 (4).

RESULTS

Lyophilization experiments. Trehalase was mixed with a suspension of ground ascospore walls in 0.5 ml of 0.0005 M acetate buffer, pH 5.4, and heated at 65 C for 30 min. The half-life of trehalase in this suspension was 31 min, which suggests little protective ability for walls under these conditions (9a).

It is likely that if trehalase is associated with ascospore walls it is not in solution. Therefore, residual trehalase activity was measured after the enzyme was lyophilized with various materials. Then the ability of these lyophilized preparations to survive heating at 65 C also was tested. As Katchalski et al. (12) point out, proteins are known to adsorb nonspecifically onto either charged or neutral surfaces. However, desorption occurs quite easily and in many cases, this may lead to denaturation. The point to be noted here is that denaturation or inactivation does not occur in the case of trehalase when the "proper" substances are used (Fig. 1).

Thus, when trehalase was lyophilized in either deionized water or 0.0005 M acetate buffer in polystyrene, polypropylene, pyrex or flint-glass test tubes it survived to a considerably extent (Fig. 1). (One hundred percent activity is the activity of trehalase in 0.05 M buffer and, since all the trehalase suspensions in

these experiments were assayed in 0.0005 M buffer, the maximal trehalase activity should be between 110 and 115%; 9a). Furthermore, most of the activity that survived lyophilization also was stable to heating at 65 C except for enzyme lyophilized in flint-glass test tubes (Fig. 1). Thus, all further experiments were done in flint glass because, if the substance mixed with trehalase did not protect, the enzyme would be inactivated by the flint glass during lyophilization and subsequent heating. If trehalase was dried in any of the above containers while suspended in 0.05 M actate buffer, no more than 10 to 30% of its activity remained after lyophilization, even without heating.

If trehalase and ground ascospore walls are freeze-dried, the enzyme activity is completely stabilized during lyophilization and during the heat treatment (Fig. 1). Ground spore walls have their internal wall layers exposed so as a control, whole ascospores with only their outer wall layer exposed were freeze-dried with trehalase. In the latter case only about half of the trehalase activity remained after lyophilization; almost all of it was stable to heating. These

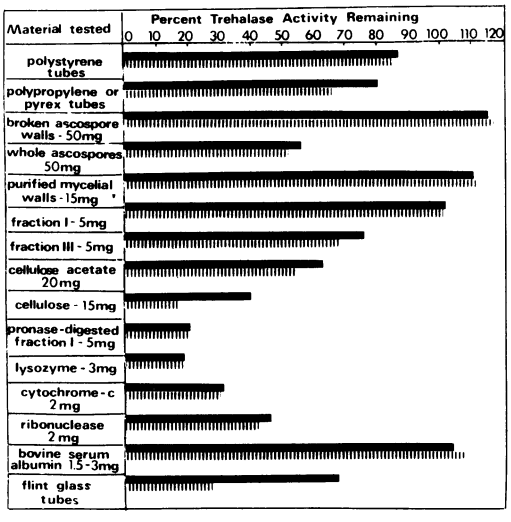

FIG. 1. *Effect of lyophilization and subsequent heating at 65 C on mixtures of trehalase and various substances. The solid lines represent the percentage of trehalase activity remaining after lyophilization. The striped lines represent the percentage of trehalase activity after the freeze-dried mixture has been heated at 65 C for 30 min. One hundred percent activity is the activity of trehalase in 0.05 M acetate buffer, pH 5.6. All assays were done in 0.0005 M acetate buffer, pH 5.4, and compared to 100% activity. Chitin obtained from crab shells or Neurospora cell walls (15) completely abolished trehalase activity during lyophilization, so the results of its use are not given in this figure.*

effects are not due to differences in the amount of surface area, because the same results were obtained over a wide range of amounts of whole and ground spores.

Examination of the work of Lowry and Sussman (14) suggests that the innermost wall of the ascospore, the one which becomes continuous with the germ bud, probably is equivalent to the mycelial wall. Therefore, cell walls were obtained from the mycelium of *Neurospora crassa* strain 89601A and were processed according to the methods of Mahadevan and Tatum (15). These cell walls protected trehalase fully during lyophilization and the heating step that followed. The association of trehalase with the cell wall achieved during freeze-drying is not a very strong one, because the enzyme may be separated from the walls by resuspension in aqueous solution and is totally recovered in the supernatant fluid after centrifugation.

Purified cell walls were fractionated (15) and fraction I, which consists of peptides, glucan, and polygalactosamine, was the most effective one in stabilizing trehalase during lyophilization, although not quite to the extent of unfractionated cell walls. Fraction II, which consists of a hydrolysate of wall polysaccharides, was not tested. Fraction III, which is a laminarin-like polymer, is not very effective in protecting trehalase from loss of activity during lyophilization, but the surviving activity is quite stable to heating. Chitin obtained both from crab shells and the cell walls of *N. crassa* (fraction IV) completely obliterates trehalase activity during freeze-drying.

The water-insoluble residue left after Pronase treatment of fraction I inactivates trehalase during lyophilization. This residue consists of 43% of the original dry weight of fraction I, but it has approximately the same proportions of neutral sugars, amino acids, and hexosamines (fraction I: 80, 18, 1 to 2% analyzable material; Pronase-treated residue: 77, 21, 1 to 2%). The Pronase-treated fraction also was fluffier and slightly darker in color than fraction I. Of the 57% that is solubilized during Pronase treatment, only an amount equivalent to 0.5% of the original dry weight of fraction I could be detected as trichloroacetic acid-soluble amino acids by the ninhydrin method.

There are two possible explanations for the deleterious effect of Pronase-treated fraction I: (i) the residue left after Pronase treatment is itself harmful to trehalase; (ii) some Pronase is left on this residue which inactivated trehalase. To test these hypotheses, trehalase was mixed with a suspension of the Pronase-treated water-insoluble residue (using the same concentra-

tions as were used for lyophilization). This suspension was kept at 0 C and at room temperature prior to being assayed. The suspension held at 0 C for 1 h or at room temperature for one half hour retained 88% of its original activity, whereas enzyme held at room temperature for 1 h retained 71% of its original activity. The small amount of inactivation in the above experiment cannot account for all of the activity lost during lyophilization (during which the suspension was never above 0 C), so it is likely that the water-insoluble residue left after Pronase treatment is itself harmful to trehalase during lyophilization and perhaps in suspension as well.

Neither cellulose nor cellulose acetate was able to protect trehalase during lyophilization. Lysozyme, cytochrome *c*, and ribonuclease seemed to inactivate trehalase during lyophilization, but bovine serum albumin was able to protect it almost as well as purified mycelial walls (Fig. 1).

Thus, trehalase can be protected best during lyophilization and subsequent heating at 65 C by ground ascospore walls, purified mycelial walls, fraction I (consisting of peptides, glucan, and polygalactosamine), and bovine serum al-

Immunofluorescent localization. The localization of trehalase in ascospores was studied through the use of immunofluorescent labeling techniques. Sections of ascospores embedded in glycol methacrylate were indirectly labeled (20), with the controls as outlined in the Materials and Methods section. Throughout these experiments none of the controls exhibited fluorescence associated with walls of either dormant or germinating ascospores (Fig. 2a, 2b, 3a).

The cytoplasm of ascospores often fluoresces brightly in both control (Fig. 2b, 3a) and experimental preparations. But distinguishing between autofluorescence and labeled fluorescence usually is not too difficult for the observer. However, the long exposure times necessary to obtain adequate photomicrographs reduce the distinction between the two types of fluorescence, because autofluorescence has a greater half-life. Also, because of the thickness of the sections used (1 to 2 μm) it is possible to have cytoplasm superimposed over pieces of cell wall. Thus, autofluorescence makes it difficult to determine whether or not there is any trehalase in the cytoplasm.

Figures 2a and b are dormant ascospore controls. There is no visible fluorescence in the

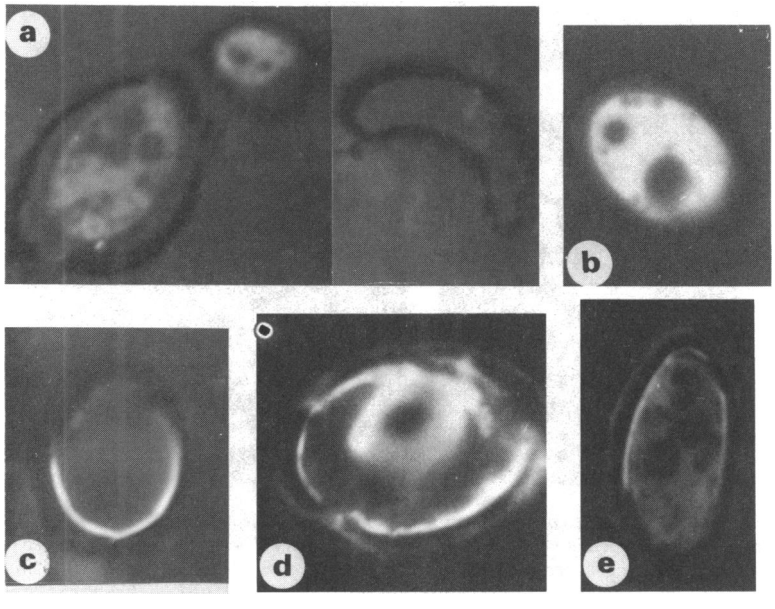

FIG. 2. *The immunofluorescent localization of trehalase in dormant ascospores. a, Dormant ascospore control showing cytoplasmic autofluorescence, but no labeled fluorescence on ascospore walls; ×1,800. b, Dormant ascospore control showing strong cytoplasmic autofluorescence; ×2,100. c, Labeled shell of dormant ascospore showing fluorescence on the innermost ascospore wall; ×2,150. d, Labeled shell of dormant ascospore showing fluorescence on the innermost ascospore wall layer. The central portion of this shell contains a tangential section of the inner wall surrounding a tangential section of one of the outer wall layers (black dot in the center); ×2,100. e, Labeled dormant ascospore with intact cytoplasm; ×1,750.*

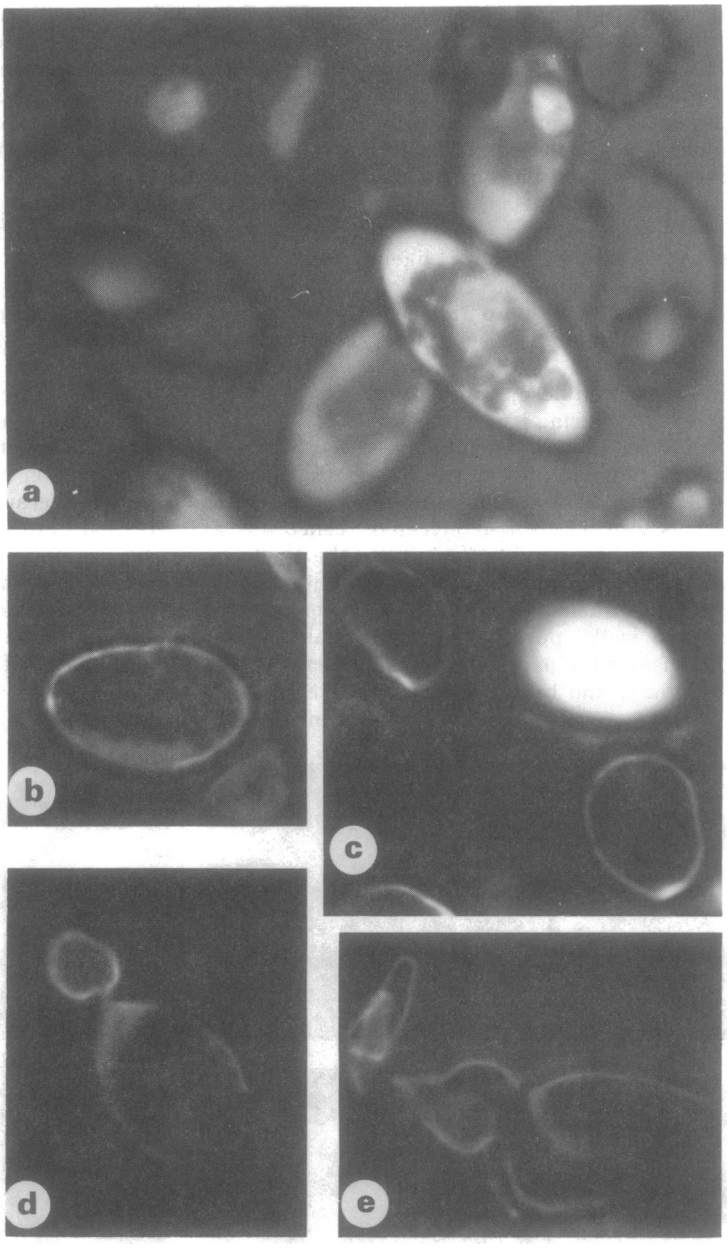

FIG. 3. *The immunofluorescent localization of trehalase in germinating ascospores. a, Germinating (3 h) ascospore control. Autofluorescence is seen in the central group of three ascospores and the two germ buds in the upper left corner. None of the spore walls show any fluorescence; ×1,850. b, Germinating (3 h) ascospore showing labeled fluorescence in the innermost ascospore wall; ×1,900. c, Labeled shells of germinating (3 h) ascospores. The central spore shows a brightly labeled tangential section of the innermost spore wall. All other labeled spores are shown in cross-section; ×1,850. d, Germinating (4 h) ascospore showing labeled fluorescence on the wall of the germ bud as well as the innermost wall of the ascospore; ×1,700. e, Labeled germinating (4h) ascospore showing separation of cell walls and cytoplasmic contents of the ascospore, the germ bud and the protruding hypha. A connection can be seen between the innermost spore wall (on the left hand side of the spore) and the wall of the germ bud; ×2,000.*

cell walls, but the cytoplasm shows some autofluorescence. Frequently, because of the difficulty in fixation of ascospores, the cytoplasmic contents will be lost, leaving the three major wall layers as a shell (14). The fluorescent stain can very easily be seen on the inner wall layer of these shells (Fig. 2c, d). Dormant spores usually will not stain if the spore cytoplasm remains intact, but exceptions to this rule can sometimes be observed (Fig. 2e). The fact that dormant ascospores with intact cytoplasm usually are not labeled and germinating spores in the same condition often are, may indicate some change in the permeability of the ascospore plasma membrane during activation. Alternatively, there may be a change in the association between trehalase and the spore wall, making the enzyme more accessible to external agents.

Figure 3a is a control slide for ascospores that have been activated and allowed to germinate for 3 h at 25 C. The bright cytoplasmic autofluorescence in the central spore is conspicuous. No labeling can be seen on the cell walls of ascospores with or without cytoplasmic contents. There are two germ buds in the upper left-hand corner which show cytoplasmic autofluorescence, but there is no label on their walls.

During germination the cytoplasmic contents of the ascospore become increasingly vacuolated and less dense as can be seen by comparing Fig. 3a and b to 2a, b, and e. Figure 3b shows a labeled germinating ascospore with its cytoplasm intact and a brightly fluorescing ring around the inner spore wall. Once again, fluorescence is particularly strong in ascospore shells, whose cytoplasmic contents have been washed away. Figure 3c shows a tangential section through the inner spore wall flanked by the cross sections of labeled spore shells.

Figure 3e suggests localization in the wall rather than in the membrane. Thus, in this figure, the wall and cytoplasm have clearly separated showing that the immunofluorescent label is in the cell wall. Further evidence that trehalase is localized in the inner spore wall is given by the fact that fluorescent label also appears in the walls of the germ buds (Fig. 3d). These walls are continuous with the endosporium or inner spore wall (14). In Fig. 3e the bottom half of the ascospore wall is shown to be continuous with the wall of the germ bud.

DISCUSSION

Localization of trehalase. Our previous work suggests that trehalase probably does not exist in the cytoplasm of ascospores because of the adverse effects of pH and ionic strength of the cytoplasm on trehalase activity and heat stability. Also, cytoplasmic localization would not account for the fact that trehalase does not hydrolyze trehalose while spores are dormant (9a). That trehalase is associated with cell walls in all stages of the life cycle of *Neurospora* has been shown, but these results were obtained from relatively crude preparations after grinding mycelia, conidia and ascospores, and most of the trehalase activity recovered was in the "soluble fraction". More recently (29) it has been demonstrated that much of the trehalase from conidia of *N. crassa* resides within the cell wall. Because high levels of both trehalase and trehalose exist in ascospores as well as conidia (9), it was suggested that trehalase may be associated in some way with the ascospore walls (9a).

Both lyophilization and immunofluorescent experiments in this paper indicate that trehalase is associated with the innermost ascospore wall (endosporium). Thus, purified mycelial walls, which are continuous with the endosporium, are just as effective as ground ascospore walls in stabilizing trehalase during lyophilization and subsequent heating at 65 C. If purified mycelial walls are fractionated (15), only fraction I, consisting of peptides, glucan, and polygalactosamine, is nearly as effective as the walls themselves in stabilizing trehalase during lyophilization. In as much as fraction I is solubilized from purified cell walls by 16 h in NaOH (15), the slight drop in this fraction's ability to protect trehalase, as compared to purified walls, is to be expected. Fraction III, which is a laminarin-like polymer (15), does not effectively stabilize trehalase during lyophilization, and fraction IV (chitin) completely obliterates trehalase activity during lyophilization.

Pronase-digestion of fraction I releases both protein and carbohydrates because the water-insoluble residue and the starting material are similar in composition. Very few free amino acids were released by this treatment, so Pronase treatment must have resulted in the release of glycoproteins or peptides and carbohydrates, or both. This interpretation agrees with the results obtained by others who have used either Pronase or a combination of it and other hydrolytic enzymes (2, 11, 18). Because the last two Pronase treatments of fraction I were buffered to permit the action of exopeptidases (21), and it has been observed that intact hyphal walls of *N. crassa* are best digested by alternating sequences of laminarinase and Pronase (11),

it is probable that Pronase treatments leave a residue of glucan and polygalactosamine. This is consistent with the lyophilization experiments which show that glucans (fraction III, cellulose, cellulose acetate) do not effectively protect trehalase and that N-acetylglucosamine causes a loss of trehalase activity. Therefore, the Pronase-labile component that is effective in stabilizing trehalase probably is a protein. This view is further supported by the finding that bovine serum albumin is effective in stabilizing trehalase during lyophilization.

Trehalase is probably adsorbed by or entrapped within the purified walls or other protective substances during lyophilization, because of the ease with which it can be removed from this association in aqueous solution. This agrees with the conclusions of Chang and Trevithick (3) who find that both trehalase and invertase are only physically confined in the walls of *Neurospora* mycelium and can be released by any of several procedures which can disrupt cell wall integrity. Furthermore, these authors cite a private communication by Wrathal which states that invertase may be associated with proteins of low molecular weight in cell walls.

Activation of dormant ascospores. Ascospores of *Neurospora* can be activated by heat shock or low concentrations of chemicals such as furfural, other furans, and heterocyclics. High concentrations of organic solvents such as methanol, ethanol, acetone, ethyl ether, and benzene also can activate ascospores and are probably nonspecific in their action (28). Sussman (27) states that aged ascospores that cannot respond to furfural will germinate if heated at 45 C (a temperature unable to stimulate germination) in the presence of this chemical. Thus, heat and furfural seem to work synergistically.

As suggested above, trehalase is associated with the innermost ascospore wall so that it could be separated from trehalose by the cell membrane. The action of heat or chemical activators could be through a change in the permeability of the ascospore membrane, dissociation of trehalase from its association with the endosporium, or both, allowing trehalose and its hydrolase to become contiguous.

Support for this hypothesis can be found in the data of Eilers and Sussman (6) which indicate that labeled furfural binds mainly to ascospore cell walls and an "intermediate fraction" which probably consists of membranous material and very small wall fragments. More furfural is adsorbed by homogenized walls than by an equivalent amount of whole spores (6), which may be indicative of increased surface area due to grinding, as the authors postulate, or that the inner walls of ascospores are the actual site of binding. Trehalase may be released from mycelial walls of *N. crassa* by a 60-C heat treatment (the activation temperature for ascospores) (Krenitzky and Sussman, unpublished data).

Work on other organisms that supports these conclusions includes that of Maheshwari and Sussman (16) who have postulated that reversible changes in membrane structure and permeability are caused in rust urediospores by cycles of extreme cold and subsequent warming (40 C). In addition, Mattingly and Best (19) find that there is a nonenzymatic lysis of the cell walls of the psychrophile *Bacillus psychrophilus* at elevated temperatures (37 and 45 C) which occurs with the solubilization of 50% of the cell wall's dry weight. Mandels et al. (17) have also found that treatment of the spores of *Myrothecium verrucaria* with either toluene or heat (60 C for 20 min) results in the release of the internal stores of trehalose. Similarly, Souza and Panek (24) used toluene to disrupt the barrier between trehalose and trehalase in yeast, thereby allowing hydrolysis of the sugar at other than the usual time in the cell cycle (initial "lag" phase of a growth cycle). Consequently, it seems reasonable to assume that activation of ascospores involves a change in the membrane that separates trehalose and trehalase, possibly coupled with the loosening of trehalase from its association with the cell wall.

Because trehalase appears to remain on the cell wall at least for the first 4 h after activation of the ascospore (Fig. 3d and e), the change in the membrane caused by activation probably allows trehalose to pass through the membrane and to be hydrolyzed. This agrees with the observation that as germination proceeds, the contents of the cytoplasm of ascospores appear to become less dense. The glucose produced by trehalose hydrolysis could re-enter the cytoplasm either by a glucose transport system (22, 23) or by diffusion and provide the ascospore with the intermediates and energy for germination.

The reader is referred to a review by Sussman (27) for a discussion of other hypotheses on the role of trehalase and trehalose during ascospore germination.

ACKNOWLEDGMENTS

This investigation was supported by National Science Foundation Grant # GB6811X.

We thank Edward Allen for sectioning ascospores, Robert Lowry and David Sternberg for technical advice, and Rowland Davis for his helpful discussions throughout these

studies. We thank Helen Gay for the use of her fluorescent microscope and Louis Martonyi for his assistance in photography.

LITERATURE CITED

1. Budd, K., A. S. Sussman, and F. I. Eilers. 1966. Glucose-C^{14} metabolism of dormant and activated ascospores of *Neurospora*. J. Bacteriol. **91**:551-561.
2. Buck, K., and M. A. Obaidah. 1971. The composition of the cell wall of *Fusicoccum amygdali*. Characterization of 2 cell wall polysaccharides from *F. amygdali*. Biochem. J. **125**:461-472.
3. Chang, P. L. Y., and J. R. Trevithick. 1972. Release of wall-bound invertase and trehalase in *Neurospora crassa* by hydrolytic enzymes. J. Gen. Microbiol. **70**:13-22.
4. Chang, P. L. Y., and J. R. Trevithick. 1970. Biochemical and histochemical localization of invertase in *Neurospora crassa* during conidial germination and hyphal growth. J. Bacteriol. **102**:423-429.
5. Disché, A., and G. Borenfreund. 1950. A spectrophotometric method for the microdetermination of hexosamines. J. Biol. Chem. **184**:517-522.
6. Eilers, F. I., and A. S. Sussman. 1970. Furfural uptake by *Neurospora* ascospores. Planta **94**:265-272.
7. Feder, N., and T. P. O'Brien. 1968. Plant microtechnique: some principles and new methods. Amer. J. Bot. **55**:123-142.
8. Fulcher, R. G., and A. A. Holland. 1971. Fluorescent antibody staining of 1-2 μM sections of hyphae of *Ophiobolus graminis* Sacc. embedded in glycol methacrylate. Arch. Mikrobiol. **75**:281-284.
9. Hanks, D. L., and A. S. Sussman. 1969a. The relationship between growth, conidiation and trehalase activity in *Neurospora crassa*. Amer. J. Bot. **56**:1152-1159.

9a. Hecker, L. I., and A. S. Sussman. 1973. Activity and heat stability of trehalase from the mycelium and ascospores of *Neurospora*. J. Bacteriol. **115**:582-591.

10. Hill, E. P., and A. S. Sussman. 1964. Development of trehalase and invertase activity in *Neurospora*. J. Bacteriol. **88**:1556-1566.
11. Hunsley, D., and J. H. Burnett. 1970. The ultrastructural architecture of the walls of some hyphal fungi. J. Gen. Microbiol. **62**:203-218.
12. Katchalski, E., I. Silman, and R. Goldman. 1971. Effect of microenvironment on the mode of action of immobilized enzymes. Adv. Enzymol. **34**:445-536.
13. Lingappa, B. T., and A. S. Sussman. 1959. Endogenous substrates of dormant, activated and germinating ascospores of *Neurospora tetrasperma*. Plant Physiol. **34**:466-473.
14. Lowry, R. J., and A. S. Sussman. 1968. Ultrastructural changes during germination of ascospores of *Neurospora tetrasperma*. J. Gen. Microbiol. **51**:403-409.
15. Mahadevan, P. R., and E. L. Tatum. 1965. Relationship of the major constituents of the *Neurospora crassa* cell wall to wild-type and colonial morphology. J. Bacteriol. **90**:1073-1081.
16. Maheshwari, R., and A. S. Sussman. 1971. The nature of cold-induced dormancy in urediospores of *Puccinia graminis tritici*. Plant Physiol. **47**:289-295.
17. Mandels, G. R., R. Vitols, and F. W. Parrish. 1969. Trehalose as an endogenous reserve in spores of the fungus *Myrothecium verrucaria*. J. Bacteriol. **90**:1589-1598.
18. Marét, R. 1972. Chimie et morphologie submicroscopique des parois cellulaires de l'Ascomycète, *Chaetomium globosum*. Archiv. Mikrobiol. **81**:68-85.
19. Mattingly, S. J., and G. K. Best. 1972. Effect of temperature on the integrity of *Bacillus psychrophilus* cell walls. J. Bacteriol. **109**:645-651.
20. Nairn, R. O. (ed.). 1964. Fluorescent protein tracing, p. 335. (2nd ed.) The Williams and Wilkens Co., Baltimore.
21. Narahashi, Y. 1970. Pronase, p. 651-664. *In* C. G. Perlmann and L. Lorand (ed.), Methods in enzymology, vol. XIX. Academic Press Inc., New York.
22. Neville, M. M., S. R. Suskind, and S. Roseman. 1971. A derepressible active transport system for glucose in *Neurospora crassa*. J. Biol. Chem. **246**:1294-1301.
23. Scarborough, G. A. 1970. Sugar transport in *Neurospora crassa*. J. Biol. Chem. **245**:1694-1698.
24. Souza, N. O., and A. D. Panek. 1968. Location of trehalase and trehalose in yeast cells. Arch. Biochim. Biophys. **125**:22-28.
25. Spies, J. R. 1957. Colorimetric procedures for amino acids, p. 468-474. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. III. Academic Press Inc., New York.
26. Spiro, R. G. 1966. Analysis of sugars found in glycoproteins, p. 4-5. *In* E. F. Neufeld and V. Ginsburg (ed.), Methods in enzymology, vol. VIII. Academic Press Inc., New York.
27. Sussman, A. S. 1969. The dormancy and germination of fungus spores: dormancy and survival. Symp. Soc. Exp. Biol. **23**:99-121.
28. Sussman, A. S., and H. O. Halvorson. 1966. Spores: their dormancy and germination, p. 354. Harper and Row, New York.
29. Sussman, A. S., S. Yu and S. Wooley. 1973. Localization of trehalase in *Neurospora*. Ind. J. Bot. (In Press).