

# Constitutive and Induced Trehalose Transport Mechanisms in Spores of the Fungus *Myrothecium verrucaria*

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## ABSTRACT

Trehalose is absorbed by two distinct systems—one constitutive, the other induced by turanose and to a lesser extent by nigerose but not by trehalose. The constitutive system is apparently mediated by a surface trehalase; the induced system has the characteristics of a permease. The specificity of the induced system is apparently limited to the  $\alpha$  glucosyl-glucose or glucosyl-fructose linkage, because absorption of kojibiose, nigerose, maltose, isomaltose, turanose, sucrose, and melezitose, in addition to that of trehalose, was increased. Absorption of  $\beta$ -linked or of galactose-containing disaccharides was not increased. The constitutive and induced trehalose-absorbing systems differ in their activity, specificity, lability to acid treatment, effects of substrate concentration, and pH optima. Both systems require oxygen, and no marked differential effects of inhibitors were observed. The activity of the induced system is proportional to log turanose concentration (from about 1 to 300  $\mu\text{g/ml}$ ), and is an approximate linear function of time of exposure (from about 1 to 50 min). Accumulation of trehalose occurred against a concentration gradient in both systems but particularly in the induced. No leakage was observed. The activity of the induced system declined slowly upon removal of the inducer. Accumulated trehalose is metabolized after activation by azide as are the endogenous trehalose reserves. The accumulated trehalose appears to enter the endogenous trehalose pool found in these spores, although some data suggest it may be more accessible. Respiratory data indicate that absorbed trehalose is available for metabolism while in transit from the external membrane to the internal pool.

It was shown previously that trehalose is absorbed and respired by *Myrothecium verrucaria* spores with essentially no lag. Treatment of spores with acid (0.1 N HCl) destroyed their ability to absorb trehalose without impairing glucose absorption or viability. Since the acid treatment also destroyed the trehalase activity, which was inferred to be at the spore surface, it was proposed that the trehalase might be involved in trehalose transport (14). Experiments to test this hypothesis showed that certain sugars, particularly turanose, greatly increased the rate of trehalose absorption. Further study of this phenomenon indicated that trehalose can be absorbed by two systems—one constitutive, the other induced by exposure to turanose or nigerose.

This report presents results of studies concerned with the mechanisms of trehalose transport into *M. verrucaria* spores and with the accumulation of trehalose by these spores. The implications of these data regarding the utilization of the endoge-

nous trehalose reserves of the spores (14) are also discussed.

## MATERIALS AND METHODS

Spores of *M. verrucaria* QM 460 were harvested from agar cultures grown at 29 C with filter paper as carbon source and were washed before use, as described previously (11). Since sporulation is complete in about 5 days, spore age is equal to culture age minus 5 days. To obtain dry weights, spores were filtered on sintered-glass funnels and were dried at 100 C.

The rate of respiration was determined by standard Warburg techniques at 30 C. All enzyme assays and metabolic studies were done at 30 C on a reciprocal shaker, unless noted to the contrary.

Total carbohydrate (soluble) was determined by use of the orcinol reagent (17), and reducing sugar, by the dinitrosalicylic acid method (19).

For acid treatment of spores, aqueous suspensions were combined with equal volumes of 0.2 N HCl. After incubation at 30 C for appropriate times, the samples were brought to approximately pH 5.8 with buffer or appropriate smaller volumes of 2 N NaOH.

Trehalose, the major carbohydrate component of the spores, was determined by heating suspensions in a boiling-water bath for about 30 min and analyzing the extract by use of orcinol reagent (see above). Extracts were checked by paper chromatography to confirm the identity of trehalose and to ascertain whether new carbohydrates had appeared (14).

Turanose treatment was effected, unless stated to the contrary, by incubating suspensions on a reciprocal shaker at 30 C with 1 mg/ml of turanose in 0.05 M phosphate buffer at pH 5.5. After 30 min, the spores were washed by centrifugation and were used directly.

Trehalase activity was determined by measuring reducing-sugar formation during incubation at 30 C in 0.05 M sodium citrate buffer at pH 3.5 containing 5 mg of trehalose per ml and a few drops of toluene.

Isomaltose was provided by F. W. Parrish; kojibiose, by K. Aso; nigerose, laminaribiose, and sophorose, by E. T. Reese.

In a few experiments, spores from atypical cultures were used. These are designated as being from "mycelial" cultures, since they tended to have a slight mycelial overgrowth over the normal, black, sporulating surface. Spores from these cultures showed only quantitative differences from the normal.

## RESULTS

*Characteristics of the constitutive trehalose absorbing system.* Previous studies (14) showed that absorption of trehalose or of glucose by spore suspensions occurred with essentially no lag, and that glucose was absorbed about four times as fast as trehalose. These data also showed the mechanisms of absorption to be different, since acid treatment suppressed trehalose absorption without impairing glucose absorption. Additional experiments characterizing the trehalose absorbing system and comparing it with the glucose system are reported below.

The pH characteristics for glucose and trehalose absorption were radically different (Fig. 1). Trehalose absorption was optimal at around pH 4.5, whereas the optimum for glucose absorption was above pH 7.5. Furthermore, trehalose absorption was much more dependent upon pH than was glucose.

The effects of substrate concentration also differed radically for the two sugars. Glucose absorption was markedly influenced by concentration, whereas the rate of trehalose absorption was essentially independent of concentration over the range studied (Fig. 2).

A variety of inhibitors were tested to determine any differential effects upon absorption. No marked differences in inhibition were found for azide, fluoride, *p*-chloromercuribenzoate, 2,4-dinitrophenol, dinitrofluorobenzene, uranyl nitrate, or iodoacetate at concentrations of about  $5 \times 10^{-3}$  M. On the other hand, EDTA (ethylenediaminetetraacetic acid) at 0.1 or 0.01 M in phos-

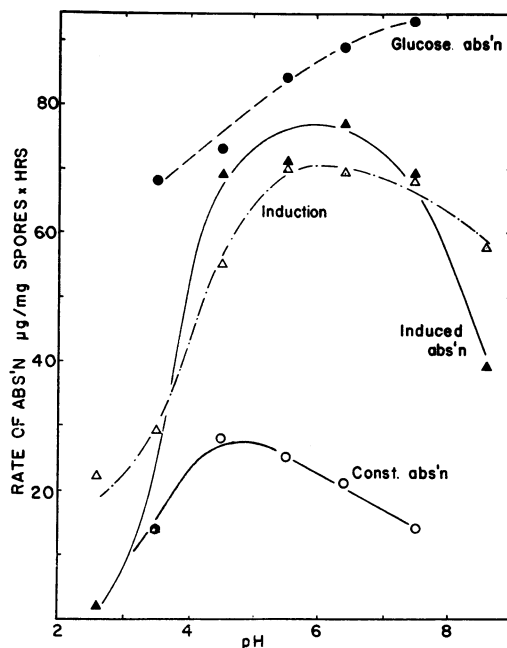


FIG. 1. Effect of pH on absorption of trehalose (○) and glucose (●) by untreated spores, and on induction (△) and activity (▲) of the induced trehalose transport system. Effect on induction was determined by incubating with turanose at 1 mg/ml for 15 min at different pH levels, washing by centrifugation, and measuring induced activity at pH 5.5. Effect on induced activity was determined by incubating with turanose at 1 mg/ml at pH 5.5 for 15 min, washing by centrifugation, and measuring trehalose absorption at different pH levels. Buffer was 0.05 M phosphate except at pH 2.6, where 0.05 M potassium hydrogen phthalate was used, and pH 3.5, where 0.05 M potassium hydrogen phthalate or sodium citrate was used.

phate buffer (pH 5.6) stimulated glucose absorption slightly (24 and 8%, respectively), whereas trehalose absorption was inhibited about 70% at both concentrations. The stimulatory effect on glucose absorption was reversed by washing the spores after 15 min of incubation with EDTA prior to adding the sugar, whereas the inhibitory effect on trehalose absorption was not. Addition of  $\text{CaCl}_2$  at 100  $\mu\text{g}/\text{ml}$  after removing the EDTA reversed the inhibition almost completely from 49 to 8%.

Attempts to determine interference in trehalose absorption by other sugars showed no significant suppressive effects, but marked stimulation was found when turanose was present (Table 1). The inductive effects of turanose are discussed below.

*Induction of a trehalose transport system.* The increased rate of trehalose absorption induced by turanose was not due to an increased rate of

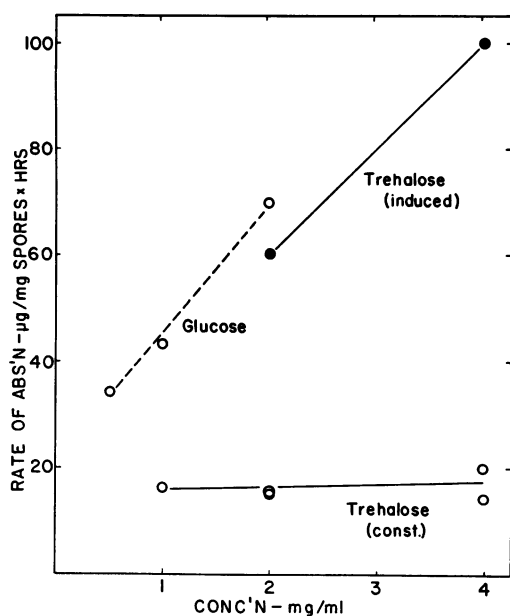


FIG. 2. Effect of glucose or trehalose concentration on rate of absorption by control (○) and turanose-treated (●) spores.

metabolism, as determined by measuring respiration, nor was there any effect of turanose treatment on trehalase activity.

The effect of exposure to turanose was not reversed by washing the spores, although there was a gradual reversal over a period of several hours (Fig. 3). The magnitude of the turanose effect was a function of both turanose concentration and duration of exposure. The response was essentially proportional to log of turanose concentration from 1 to 300  $\mu\text{g/ml}$  ( $2.9 \times 10^{-6}$  to  $870 \times 10^{-6}$  M). By extrapolation, a significant response should be detectable at concentrations approaching 0.1  $\mu\text{g/ml}$  (Fig. 4). Determination of the effect of duration of exposure showed a more or less linear response up to 50 min (Fig. 5).

To determine whether induction would occur in the absence of oxygen, special precautions were required to exclude oxygen while the spores were being washed free from turanose. This was accomplished by a technique which permits spores to be washed by centrifugation under anaerobic conditions (12). No induction occurred when oxygen was excluded during both incubation with turanose and the two washing cycles

TABLE 1. Specificity of induction of the trehalose transport system and of the activity of the turanose-induced system

Sugar	Configuration <sup>a</sup>		Relative specificity as inducer <sup>b</sup>	Relative specificity of induced system <sup>c</sup>	Absorption by control spores
Trehalose.....	$\alpha$ 1-1	G-G	(1)	5	+
Kojibiose.....	$\alpha$ 1-2	G-G	(1)	$\infty$	0
Nigerose.....	$\alpha$ 1-3	G-G	2	4	+
Maltose.....	$\alpha$ 1-4	G-G	(1)	$\infty$	0
Isomaltose.....	$\alpha$ 1-6	G-G	(1)	$\infty$	0
$\beta\beta$ Trehalose.....	$\beta$ 1-1	G-G	(1)	1	+
Sophorose.....	$\beta$ 1-2	G-G	(1)	1	+
Laminaribiose.....	$\beta$ 1-3	G-G	(1)	1	+
Cellobiose.....	$\beta$ 1-4	G-G	1	1	+
Gentiobiose.....	$\beta$ 1-6	G-G	1	1	+
Sucrose.....	$\alpha$ 1-2	G-F		2	+
Turanose.....	$\alpha$ 1-3	G-F	5	3	0 $\rightarrow$ +
Melibiose.....	$\alpha$ 1-6	Gal-G	1	0	0
Lactose.....	$\beta$ 1-4	Gal-G	(1)	0	0
Melezitose.....	$\alpha$ 1-3, $\beta$ 2-1	G-F-G	1	$\infty$	0 $\rightarrow$ +
Raffinose.....	$\alpha$ 1-6, $\alpha$ 1-2	Gal-G-F		0	+

<sup>a</sup> G-G, glucosyl-glucose; G-F, glucosyl-fructose; Gal-G, galactosyl-glucose; G-F-G, glucosyl-fructose-glucose; Gal-G-F, galactosyl-glucose-fructose.

<sup>b</sup> Rate of absorption of trehalose after exposure of spores to the sugar indicated relative to rate by untreated spores. Sugars at 1 mg/ml in 0.05 M phosphate buffer at pH 5.5. Incubation with inducer for 30 min except for figures in parentheses, in which case substrate and inducer were added together.

<sup>c</sup> Rate of absorption of indicated sugar after turanose treatment relative to rate with untreated spores.

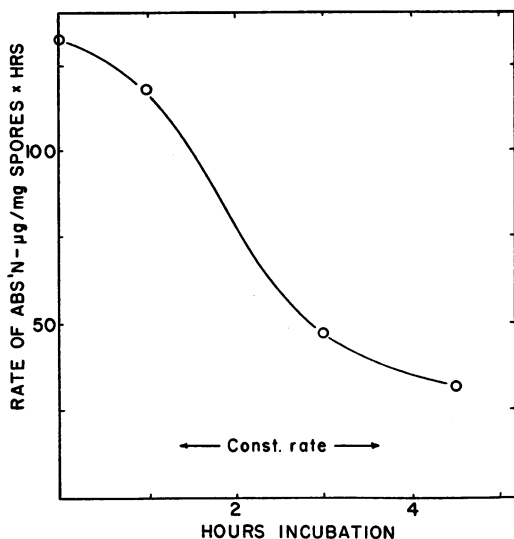


FIG. 3. Reversal of turanose-induced trehalose transport system. Spores were treated with turanose (1 mg/ml) for 30 min, washed, and incubated in water at 30 C for various times. Trehalose was then added to measure rate of absorption.

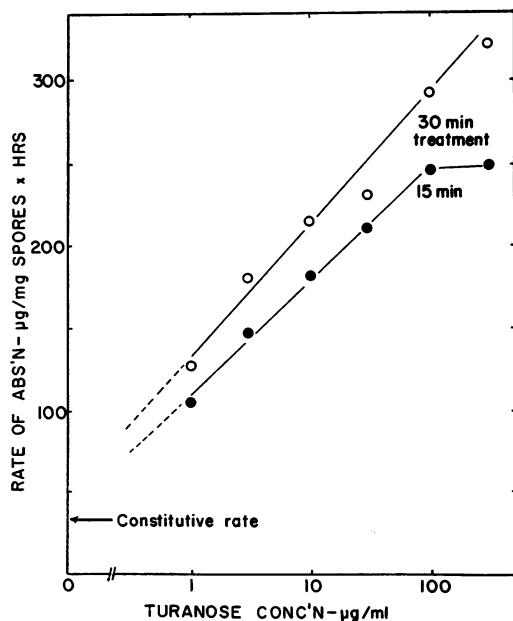


FIG. 4. Effect of turanose concentration on induction of trehalose transport system. These data were obtained by use of spores from "mycelial" culture. Symbols: 15-min turanose treatment, ●; 30-min turanose treatment, ○.

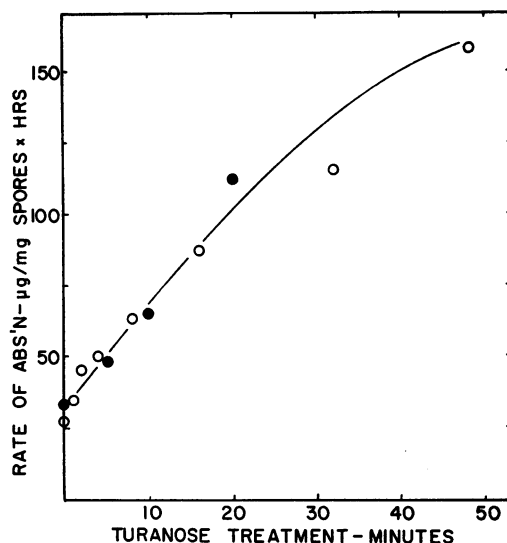


FIG. 5. Effect of duration of turanose treatment on induction of trehalose transport system. Spores were from cultures either 14 (○) or 19 (●) days old.

essential to remove traces of turanose prior to admitting air.

The temperature characteristics of induction were not determined, although it was established that none occurred at 4 C. The effect of pH on induction was quite similar to that on absorption by the induced system (Fig. 1).

*Specificity of inducers and of the activity of the transport system.* Several experiments to determine the ability of other sugars to induce a trehalose transport system showed only nigerose to have a significant effect (Table 1). It is noteworthy that trehalose has no activity as an inducer. Although pretreatment with maltose increased trehalose absorption, the effect is probably due to impurities (*unpublished data*). It should be noted that both turanose and nigerose are  $\alpha$  1-3 linked sugars, the former being a glucosyl-fructose, the latter a glucosyl-glucose dimer.

Experiments to determine the specificity of the induced system showed that pretreatment of spores with turanose increased the rate of absorption of kojibiose, nigerose, maltose, isomaltose, turanose, sucrose, and melezitose, in addition to trehalose (Table 1). Thus, the transport system appears to be specific for  $\alpha$ -linked glucosyl-glucose or glucosyl-fructose oligosaccharides. Melibiose (galactosyl  $\alpha$ -1,6-glucose) is not absorbed by either the constitutive or the induced system.

*Metabolism of absorbed trehalose.* Spores of

*M. verrucaria* contain relatively large amounts of trehalose as endogenous reserves—about 20% of the dry weight (14). It was significant, therefore, to determine whether trehalose was absorbed against a concentration gradient and whether it was accumulated within spores or was metabolized as it was absorbed.

Paper chromatography of hot-water extracts of spores before and after absorbing trehalose showed no qualitative changes in carbohydrate composition. Quantitative tests, however, showed significant increases in trehalose (Fig. 6) and slight increases in mannitol. No reducing sugars were found.

Calculation of the actual trehalose concentration within spores is not possible, but, if it is assumed that both endogenous and absorbed trehalose are uniformly dissolved within the spores (1) and that the centrifuged spore volume represents the actual spore volume (2), it can be shown that trehalose is absorbed against a concentration gradient by the constitutive as well as by the induced transport systems. Using the

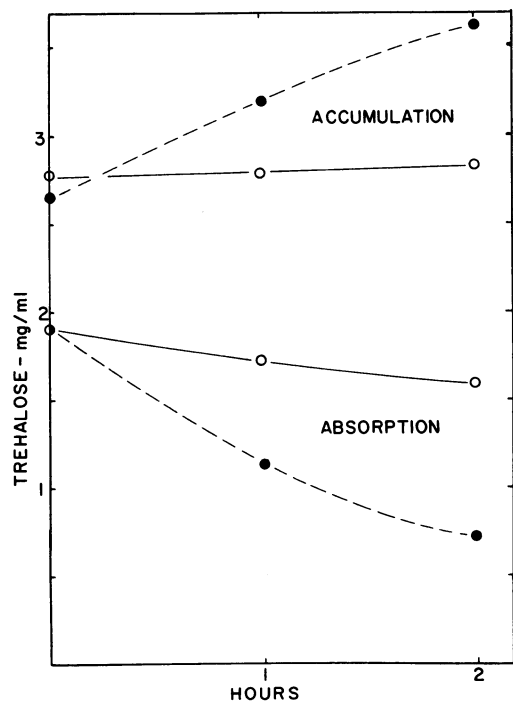


FIG. 6. Absorption and accumulation of trehalose by untreated (○) and turanose-treated (●) spores. Spores from 9-day-old culture at 14.8 mg/ml; absorption shown by decrease in trehalose content of supernatant fluid; accumulation expressed as trehalose content of extract of spores in 1 ml of suspension.

data of Fig. 6 and previous data (13) showing the centrifuged spore volume to be 3.1  $\mu$ liters/mg (dry weight), the trehalose concentration within turanose-treated spores increased from 169 to 230 mM, the initial concentration in the medium being 5.6 mM. The final concentration in control spores was 180 mM.

Endogenous respiration of turanose-treated spores was not significantly different from that of control spores. Respiration of treated spores suspended in trehalose solution, however, was appreciably greater, although not enough to account for the increased rate of absorption (Fig. 7). Experiments to determine whether turanose-treated spores, which had accumulated trehalose, respired more rapidly than "unfortified" spores showed only a slight difference. The inability to oxidize accumulated trehalose was also evidenced by the marked decline in respiratory rate after about 2 hr, by which time all exogenous trehalose had been absorbed (Fig. 7). It is noteworthy, however, that the respiratory rate did not decline until about 30 min after absorption was complete.

To determine whether accumulated trehalose could be respired after activation (14), turanose-treated spores were allowed to accumulate trehalose

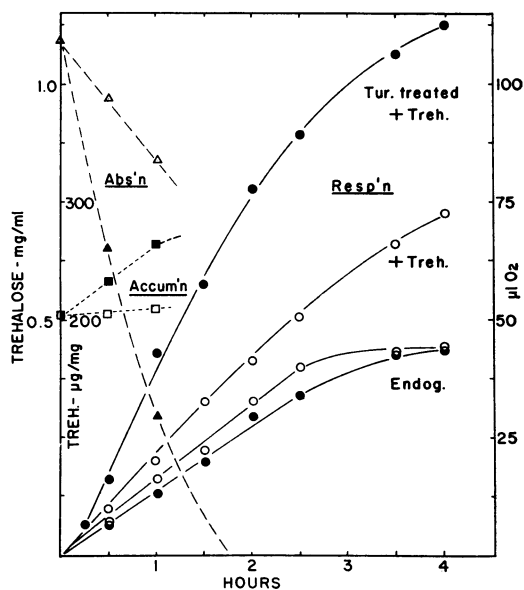


FIG. 7. Trehalose absorption ( $\Delta$ ), accumulation ( $\square$ ), and respiration ( $\circ$ ) by control (open symbols) and turanose-treated (solid symbols) spores. Spores from 14-day-old culture were suspended in 0.5 M phosphate buffer, pH 5.5, and were used in the following amounts: 6.9 mg/ml in absorption and accumulation study, and 3.45 mg per vessel in respirometers.

halose and then were placed in respirometers containing azide in the side arms. Although the respiratory rate of "fortified" spores was not significantly different from the controls, as noted above, the azide stimulation was about twice as great (Fig. 8). The high endogenous  $Q_{O_2}$  of 35 in fortified spores is noteworthy. In a similar experiment, a  $Q_{O_2}$  of 60 was attained after 5 hr and remained at this level for the duration of the experiment (7 hr), by which time considerably more oxygen was absorbed than could account for the complete oxidation of the original endogenous trehalose.

*Characteristics of the induced transport system in comparison with the constitutive system.* Data from a variety of experiments show the characteristics of the induced system to be distinctly different from those of the constitutive. The induced was much more resistant to acid treatment (Fig. 9) and had a different pH optimum (Fig. 1) than the constitutive system, i.e., 4.5 versus 6.5. The effects of trehalose concentration were also markedly different. Absorption of trehalose by control spores was not affected by concentration, whereas absorption by turanose-treated spores was influenced markedly (Fig. 2).

Attempts to find differential effects of inhibitors were unsuccessful.

Spore age had a marked effect on the rate of

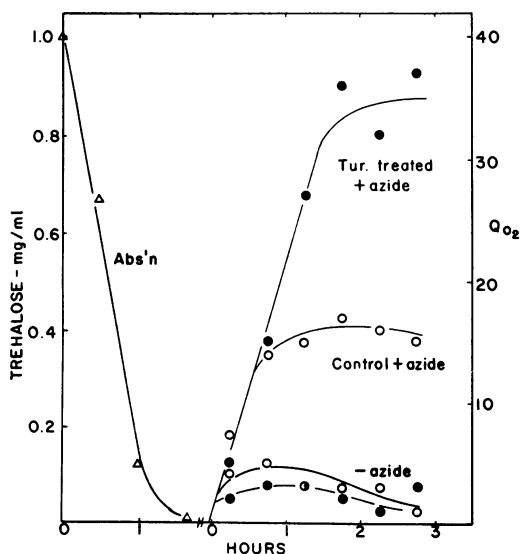


FIG. 8. Effect of azide ( $10^{-3} M$ ) on rate of respiration of untreated and of turanose-treated, trehalose-fortified spores. Spores from 22-day-old "mycelial" culture were suspended in  $0.05 M$  phosphate buffer, pH 5.5; turanose-treated spores (5.5 mg/ml) were incubated with trehalose before being placed in respirometers.

trehalose absorption (Fig. 10). Although this was more obvious in turanose-treated spores, the limited data available indicate the same effect with control spores. The relative rates of absorption by the induced and constitutive systems thus appear to be about the same. Data obtained with spores from the atypical "mycelial" cultures showed that trehalose was absorbed by these spores at about the same rate as by normal spores, but showed significantly increased response to turanose treatment (Fig. 10).

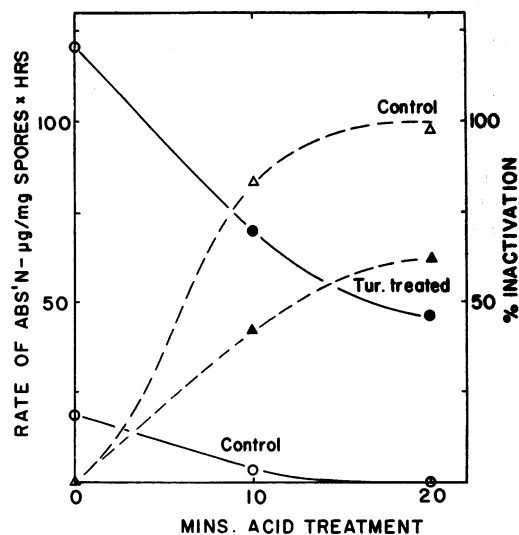


FIG. 9. Effect of acid treatment on absorption of trehalose by control and turanose-treated spores. Spores (8.3 mg/ml) were from 25-day-old culture; acid treatment was in  $0.1 N$  HCl; absorption of trehalose was determined at 1 mg/ml in  $0.05 M$  phosphate buffer, pH 5.5; data are also expressed as per cent activation ( $\Delta$ ).

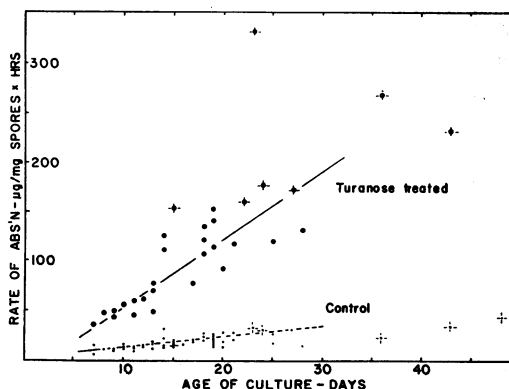


FIG. 10. Effect of spore age on rate of absorption of trehalose by constitutive and induced systems. Spore age is approximately the culture age minus 5 days; points with crosses are for spores from mycelial cultures.

## DISCUSSION

Numerous studies on the absorption of organic molecules, particularly carbohydrates and amino acids, by microorganisms have led to the postulation of a variety of mechanisms involving both active and passive transport phenomenon (2-4, 8-10, 20). The probable multiplicity of mechanisms as well as unknown structural details of membranes at molecular levels have so far prevented definitive treatment.

Most studies with microorganisms have been with bacteria, mainly *Escherichia coli*, although yeasts have also received attention. No studies concerned with carbohydrate transport mechanisms in fungus spores have come to our attention.

The studies reported have shown that trehalose absorption by *M. verrucaria* spores can occur by two distinct systems: one constitutive, and the other induced by turanose or other  $\alpha$  1,3 glucosyl-glucose or glucosyl-fructose disaccharides. The two systems differ with respect to their acid lability, pH optima, substrate concentration dependence, specificity, and activity. On the other hand, both require aerobic conditions and do not function at low temperatures. No differences were found with respect to inhibitors. Accumulation of trehalose against a concentration gradient occurs with both systems, although greater accumulation was observed with the induced.

It is possible that the constitutive system involves mediation by the surface trehalase, as proposed earlier (14). If so, hydrolysis of trehalose at the osmotic barrier must be followed by immediate transport of the resulting glucose into the cells, since glucose cannot be detected in the medium unless metabolism is blocked or the cells are killed. The apparent coupling of trehalose hydrolysis with glucose transport would appear to differentiate this system from that proposed for absorption of sucrose by yeast (6).

A variety of data indicate the participation of trehalase in trehalose absorption: trehalase activity is approximately equivalent to the rate of trehalose uptake; both trehalase activity and trehalose uptake are essentially independent of substrate concentration over the range studied; sensitivity to acid treatment is essentially the same; EDTA inhibition is reversed by  $\text{Ca}^{+2}$  in both. Since glucose is absorbed about three times as fast as trehalose, the rate-limiting step in trehalose absorption by the constitutive system must be trehalase activity and not glucose uptake.

Oxygen is not required for trehalase activity. In fact, glucose appears in the medium if metabolism is blocked by suspending cells in trehalose

solution in the absence of oxygen. Since absorption of trehalose by noninduced spores does not occur under these conditions, oxygen must be associated directly or indirectly with the transport of the hydrolytically produced glucose into the cell.

The mechanism for glucose transport is clearly different from the constitutive trehalose system. This is shown by the difference in pH characteristics, effects of substrate concentration, and resistance to acid treatment of the glucose system (14) and also to the qualitatively different effects of EDTA.

If hydrolysis of trehalose to glucose is prerequisite to its utilization by noninduced spores, then some intracellular mechanism must convert part of the absorbed glucose into trehalose.

The specificity of the constitutive trehalose absorbing system is presumably dependent upon the specificity of the surface trehalase, which is unknown. Nigerose is the only additional  $\alpha$ -linked glucosyl-glucose disaccharide absorbed by noninduced spores. It is assumed, however, that nigerose is absorbed via an induced system, since the rate of absorption increases during incubation. Furthermore, nigerose treatment induces formation of the trehalose absorbing system. Absorption of  $\beta$ -linked glucosyl-glucose disaccharides probably occurs by some other constitutive system(s).

A definitive description of the induced transport system is not possible. Presumably, it involves an enzyme whose formation is induced only under aerobic conditions at physiological temperatures by certain carbohydrates having a common stereochemical configuration and whose activity is a function of time of incubation with, and concentration of, the inducer. Although no evidence of inducer uptake was found, the experimental method would not have detected trace quantities.

The induction thus appears similar to typical enzyme induction phenomena (7, 15, 16). Attempts to determine whether protein synthesis occurred during induction by use of inhibitors such as chloramphenicol or actinomycin were ambiguous. Since no exogenous nitrogen was required for induction, it is assumed that if protein synthesis occurs it is at the expense of the endogenous amino acid pool (14).

The characteristics of the induced system agree with its proposed enzymatic nature. It has marked stereochemical specificity, its activity is proportional to substrate concentration (within the limits observed), it has a characteristic pH activity function, and it apparently accumulates trehalose against a gradient. The transport mecha-

nism thus meets many of the criteria employed in defining a permease (4, 8).

Uniform dispersal was assumed in calculating the internal trehalose concentration. It seems likely, however, that the initial endogenous trehalose reserves, as well as the accumulated trehalose, are not dispersed uniformly through the cell but are localized in a structure(s), rendering them inaccessible to metabolic utilization without suitable activation. Such structures may be comparable to the storage bodies shown by electron microscopy in *Botrytis cinerea* conidia (1).

Previous data regarding the endogenous reserves of these spores (14) are pertinent to further consideration of the problems of accumulation and incorporation of exogenous trehalose into an endogenous pool.

Trehalose is the major endogenous reserve, amounting to about 20% of the dry weight. Although it is not released from intact spores, it is readily leached by any lethal treatment. Although metabolism of endogenous trehalose occurs at appreciable rates only after activation by some treatments, such as azide or heat, exogenous trehalose is respired with essentially no lag after its addition to the medium. The mechanism of trehalose metabolism is not known, but is apparently not mediated by trehalase which is at the spore surface. The inability of spores to utilize their endogenous trehalose reserves is ascribed most probably to physical separation from the metabolic system, although other mechanisms are conceivable. The higher respiratory rate of turanose-treated spores in solutions containing trehalose is maintained only while exogenous trehalose is available. After depletion of the medium, the rate declines, approaching the endogenous level. The accumulated trehalose is in a metabolically inactive situation comparable to that of the endogenous trehalose, but can be metabolized after activation of the spores. These data imply that an intracellular translocation system, possibly simple diffusion, is interposed between the cell membrane and the trehalose pool, and that trehalose is available as a respiratory substrate while in transit. Presumably, the rate of transport into the cell is greater than into a metabolically inactive pool, and a separate transport mechanism at the membrane confining the endogenous pool transports trehalose against a concentration gradient. Presumably, the trehalose is in a free, osmotically active form as shown for  $\beta$ -galactosides in *Escherichia coli* (18).

These hypotheses, relative to the absorption, accumulation, and metabolism of trehalose, are shown diagrammatically in Fig. 11.

It is questionable whether accumulated tre-

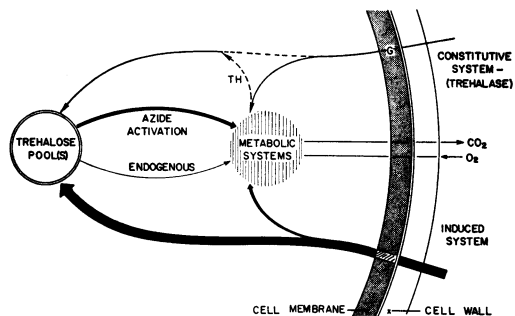


FIG. 11. Diagrammatic representation of trehalose absorption and accumulation by constitutive and induced transport systems in *Myrothecium verrucaria* spores. Dashed lines for constitutive trehalase system indicate uncertainty regarding trehalase participation. TH, trehalase; G, glucose; width of lines indicates relative rates.

halose enters the endogenous trehalose pool or whether it becomes metabolically isolated in some other structure. The response of fortified, as compared with control, spores to azide indicates that accumulated trehalose is more accessible to respiratory metabolism. This could be a concentration effect, but it seems unlikely, since the fortified spores do not contain sufficient additional substrate to account for a doubling of the respiratory rate. The possibility of two pools of differing exchangeability for accumulation of amino acids in microorganisms has been discussed elsewhere (2).

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