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DEVELOPMENT OF TREHALASE AND INVERTASE ACTIVITY IN *NEUROSPORA*[1]

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ABSTRACT

HILL, E. P. (University of Michigan, Ann Arbor), AND A. S. SUSSMAN. Development of trehalase and invertase activity in *Neurospora*. J. Bacteriol. **88**:1556–1566. 1964.—The levels of trehalase and invertase found during the development of *Neurospora* have been studied. Invertase activity is highest in the mycelium after growth has been completed, whereas the most trehalase activity is found in ungerminated conidia. Both enzymes show the least activity in the ascospore. Although the specific activity of trehalase varies no more than 3-fold during the spore stages, there is a 60-fold change in the mycelium. Similar but less pronounced variations in the specific activity of invertase in the mycelium occur. The lowest ratios of invertase to trehalase activity in the soluble fraction are found in conidia and ascospores, except in dormant ascospores where the ratio approaches that of older mycelium. Similar results are obtained for the enzymes in the wall fraction, except for dormant and newly activated ascospores. Moreover, the walls of young mycelium appear to have relatively more trehalase than is found at all other times. The activities of both enzymes vary about 20-fold in the wall fraction, but invertase activity fluctuates more widely than that of trehalase. Invertase activity always exceeds that of trehalase, and the cytoplasmic fraction contains more activity than that of the wall. These results are shown to contradict the hypothesis that trehalase and invertase activities are coordinately controlled. Finally, the role of trehalase in the activation of ascospores is considered in the light of these results.

Under natural circumstances, ascospores of *Neurospora* are dormant and probably remain viable in this condition for years. Activation, or the disruption of dormancy, may be accomplished by application of a heat shock (Goddard, 1935), or by treatment with furfural and related heterocyclics (Emerson, 1948; Sussman, 1953), or with aliphatic alcohols, aldehydes, and esters (Sussman, Lowry, and Tyrrell, 1959).

Upon activation and subsequent incubation, a 20- to 30-fold increase in respiratory activity occurs which is accompanied by the protrusion of the germ tube as the first visible evidence of germination (Goddard, 1935). That there are qualitative changes in the metabolism of germinating spores, as well as quantitative ones, has been borne out by the studies of Lingappa and Sussman (1959) and Sussman and Lingappa (1959), who found that the large amounts of trehalose stored in dormant cells are utilized only during germination. By contrast, lipids provide the energy for the dormant stage. Thus, ascospores of *Neurospora* are a relatively simple developing system in which there is a transition from a resting stage of low metabolic capacity to a vegetative organism differing in its morphology and respiring at a much greater rate.

The discovery that the dissimilation of trehalose is correlated with the breaking of dormancy and the activation of metabolism in ascospores suggested that the metabolic block which restrains their development is associated with their inability to utilize trehalose. Consequently, studies of the enzyme trehalase were begun and have led to its crystallization from mycelial extracts (Hill and Sussman, 1963). It was the purpose of the present investigation to determine trehalase activity at each stage in the development of *Neurospora*, including several ages of mycelium and ungerminated and germinated conidia and ascospores. Evidence has been obtained that more than one form of trehalase and invertase exist in *Neurospora* (Hill and Sussman, 1963; Eilers et al., 1964). Consequently, it seemed possible that if the activity of only one form of trehalase was limiting development, measurements of total activity might be misleading. Therefore, cells were partially frac-

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tionated in case the different forms of the enzyme were localized in different parts of the cell. To test the hypothesis that invertase and trehalase may be coordinately repressed (Metzenberg, 1962), the activities of the two enzymes were compared during development.

MATERIALS AND METHODS

Production and use of conidia. Conidia of strain 89601-A, an inositol-less mutant of *N. crassa*, were inoculated into 125-ml Erlenmeyer flasks containing 30 ml of liquid "complete" medium (Horowitz, 1947). The flasks were maintained in stationary cultures for 2 weeks at 25 C, after which the conidia were harvested by aspiration into a distilled-water trap and filtered three times through glass wool to remove mycelial fragments and large clumps of conidia. They were concentrated by centrifugation, washed with sodium phosphate buffer (0.05 M, pH 5.6, hereafter referred to as standard buffer), and stored overnight at 4 C. A 1-g (wet weight) amount of conidia was introduced into each of three 250-ml Erlenmeyer flasks containing 100 ml of sterile "minimal" medium consisting of 2% (v/v) Vogel's (*unpublished data*) salt solution, containing 2% sucrose (w/v) and 50 μg of inositol per ml. The conidia were allowed to germinate at 25 C on a centrifugal shaker and were harvested at intervals over a Büchner funnel with filter paper and washed with 500 ml of distilled water followed by 200 ml of standard buffer.

Production and use of ascospores. Ascospores of *N. tetrasperma* were grown and harvested according to the method of Goddard (1935). They were suspended in 0.8% ethylenediaminetetraacetic acid (EDTA), pH 7.0, and washed in distilled water, over a Büchner funnel with filter paper. They were air-dried and stored at 4 C until used. The spores were activated by shaking 25-ml Erlenmeyer flasks containing ascospores in 5.0 ml of standard buffer in a water bath (60 C) for 11 min. Activated spores were incubated during germination on a reciprocal shaker at 25 C.

Production and use of mycelium. After inoculation, flasks containing medium prepared as described above for the germination of conidia were incubated in stationary culture at 25 C. At intervals, enough flasks were harvested to give 2.5 g (wet weight) of mycelium. The mycelium was harvested on a Büchner funnel and washed with about 200 ml of distilled water followed by 50 ml of buffer.

Preparation of extracts. Cell-free extracts of conidia, ascospores, and mycelium were prepared according to the protocol presented in Scheme 1.

The cell-wall fractions were washed three times by centrifugation in standard buffer. These wall preparations cannot be considered to be entirely free of cytoplasmic contaminants although, as will be seen, large differences are found between the enzyme activity of the two fractions. No trehalase activity, as detected by "Tes-tape" (Eli Lilly & Co., Indianapolis, Ind.), was observed in the supernatant fluid after the walls were washed, although invertase activity was still present. The wall material was suspended in standard buffer and assayed in the same manner as the soluble fraction.

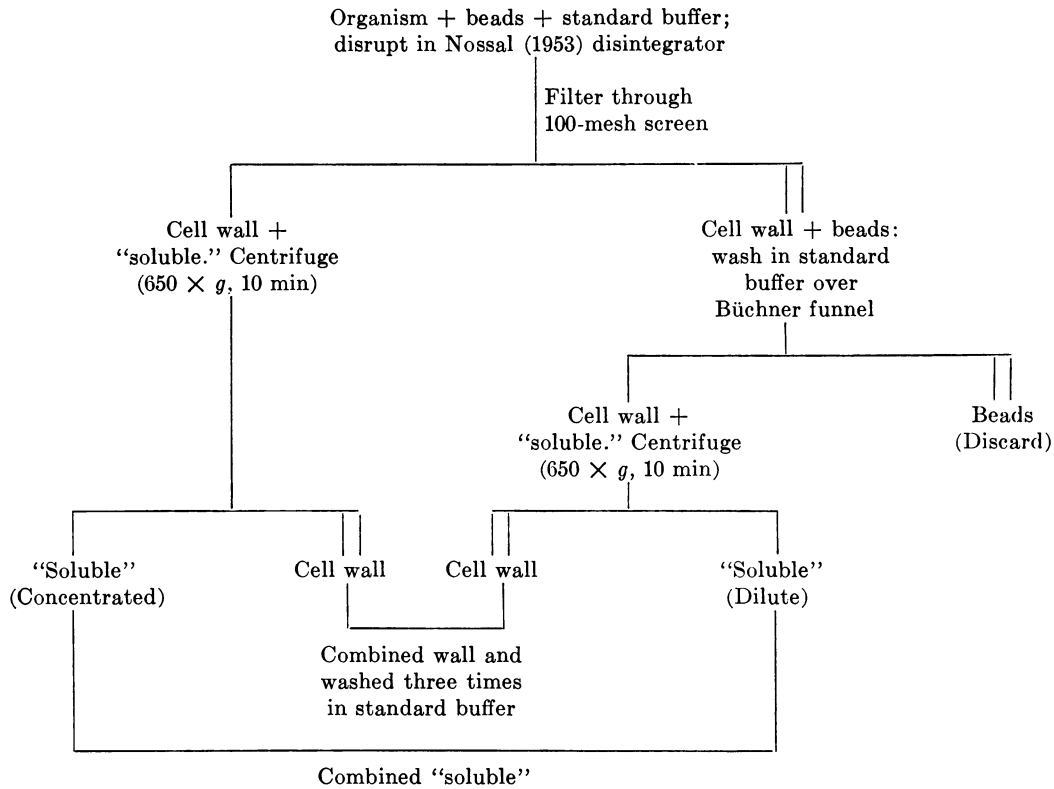
Assay procedure. The protein content of the soluble fraction of conidial and mycelial extracts was determined according to the method of Lowry et al. (1951) with bovine serum albumin as the standard. Nitrogen in the cell-wall fraction of conidia and mycelium, and all fractions of ascospores, was determined according to a modification of the method of Koch and McMeekin (1924), with $(NH_4)_2SO_4$ as the standard. The soluble fraction of the ascospores was assayed by this latter method because of its turbidity.

Trehalase activity was measured as previously described (Hill and Sussman, 1963) unless otherwise indicated. Invertase activity was assayed in the following manner: 0.1 ml of extract or cell-wall suspension was introduced into tubes containing 9.9 ml of 0.05 g/ml sucrose dissolved in standard buffer. The tubes were incubated for 30 min at 37 C, and the reaction was stopped by boiling for 5 min. Trehalase activity of the ascospore fractions was measured in a similar manner. The reducing sugar produced as a result of enzyme activity was measured as glucose by the method of Somogyi (1952) and Nelson (1944). All assays and controls were run in duplicate. Specific activities were calculated as micrograms of glucose produced per milligram of protein.

RESULTS

Conidia. Conidia were germinated in "minimal" medium, and samples were analyzed for

SCHEME 1. *Protocol of enzyme extraction from Neurospora mycelium, ascospores, and conidia.*



enzyme activity at various times after inoculation (Fig. 1–4). Total trehalase activity in the soluble fraction decreased precipitously during the first 6 hr of incubation and more slowly through 14 hr. On the other hand, the amount of activity in the cell wall rose during the first 6 hr, followed by a drop thereafter. The figures for dormant conidia probably were strongly affected by harvest and storage conditions, so the absolute values vary considerably. Trends were similar from one experiment to another. The specific activity of trehalase in the wall fraction reflects the trend shown in total activity, except there was an increase in the former between 6 and 10 hr. However, the trend of the specific activity of the soluble fraction was very different from that of the total activity because there was a slow early decline, followed by a more rapid one after 10 hr. As was true at all stages in the development of *Neurospora*, there was much more activity in the soluble fraction than in the wall.

Total invertase activity follows a pattern similar to that of trehalase, for there was a decrease in the activity in the soluble fraction throughout the course of germination, but the percentage decrease was more than twice as great for trehalase. Moreover, invertase activity in the cell wall peaked after 6 hr and declined thereafter, very much like trehalase. It is worth noting that the increase in total trehalase activity in the wall was about 250% compared with slightly over 50% in the case of invertase. Whereas there was about a 50% drop in the specific activity of the soluble fraction of trehalase during the 14-hr period studied, that of invertase remained almost constant or rose slightly. Although the specific activity of invertase in the wall fraction increased less than that of trehalase, the trends were similar. The ratio of invertase to trehalase activity followed opposing trends in the soluble and wall fractions during the germination of conidia. Thus, there was proportionately more invertase than trehalase activity in the wall than in the soluble fraction of

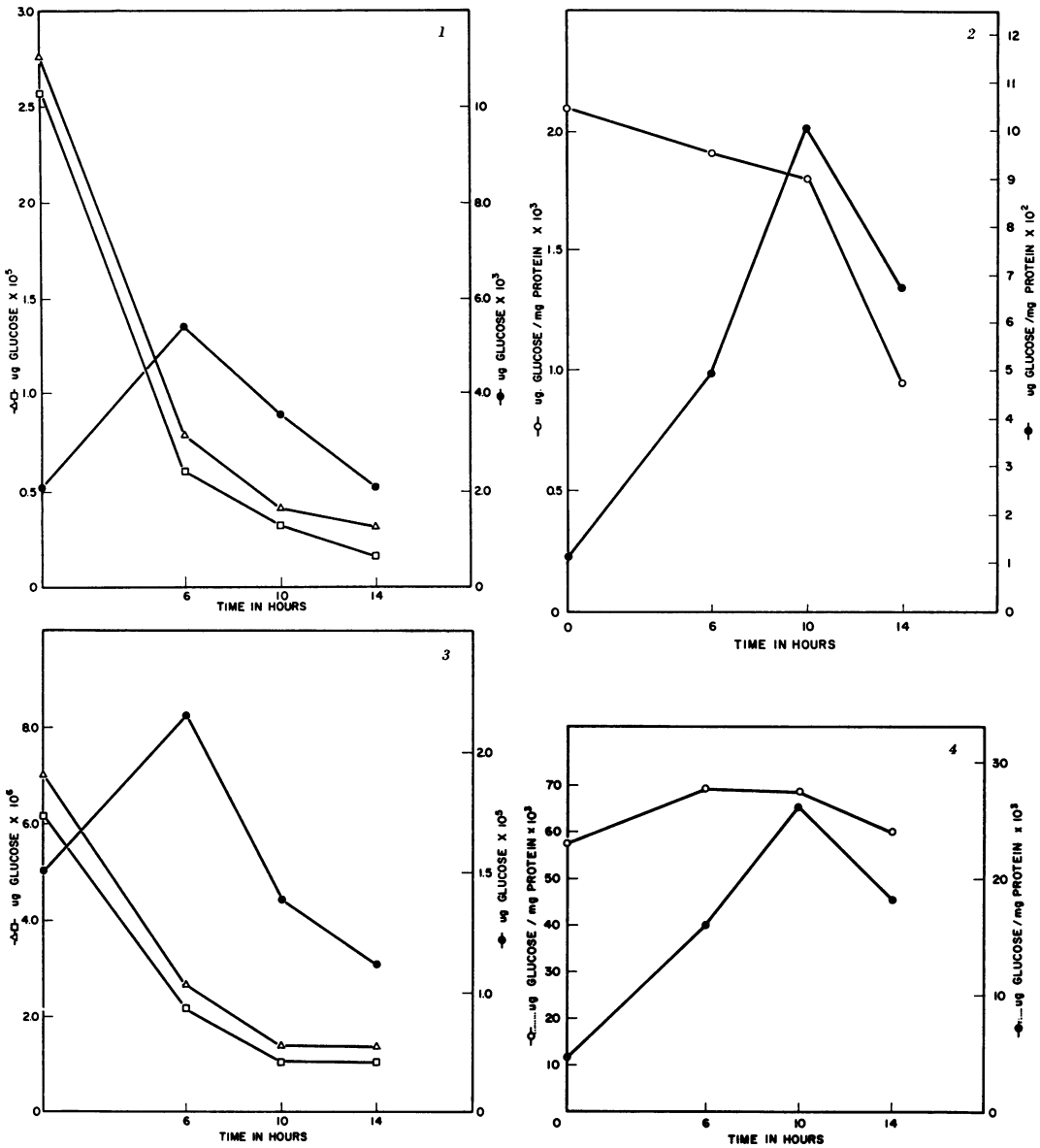


FIG. 1–4. *Activity of trehalase and invertase in conidia of Neurospora crassa. (1) Total activity of trehalase per gram (wet weight) of organism. (2) Specific activity of trehalase. (3) Total activity of invertase per gram (wet weight) of organism. (4) Specific activity of invertase. The total recoverable activity* (△) *was obtained by adding the amount of enzyme activity found in the combined soluble fraction* (□) *to that in the combined cell-wall fraction* (●) *after the two had been separated by centrifugation, but before the walls were given the final three washes. Specific activity of soluble concentrated fraction,* ○; *specific activity of cell-wall fraction,* ● *(after the walls were given the final three washes). It should be noted that the activity in the final three washes of the wall fraction is not included in these graphs so that the total activity is not the sum of the activities in the wall and soluble fractions. However, very little activity is found in these washes so that the difference is small.*

TABLE 1. *Protein nitrogen in extracts of conidia and mycelium from Neurospora crassa and ascospores from N. tetrasperma**

Stage	Time	Fraction	
		Soluble	Cell wall
Conidia	Hr of incubation		
	0	123.6	17.9
	6	32.8	11.0
	10	17.5	3.4
	14	18.8	3.5
Mycelium	Days of incubation		
	3	37.5	15.1
	6	37.0	6.8
	9	38.0	8.8
	13	41.0	10.7
	16	62.0	14.7
	20	30.6	12.6
	27	10.8	22.6
Ascospores	Min after activation		
	Dormant	19.7	28.8
	0	18.5	27.1
	30†	26.2	35.1
	60	21.3	37.9
	120	37.4	28.6
	180	39.2	21.6

* Figures are the average of two different experiments. Results are expressed as milligrams per gram (wet weight).

† Only one sample was taken at this time.

dormant conidia. However, the opposite was found 14 hr after inoculation.

Conidia as they were harvested had relatively little trehalase or invertase activity in the wall. However, as germination proceeded through 14 hr, an increasing proportion of these activities appeared in the wall. The relatively low ratios of specific activities throughout probably reflect the small amount of nitrogen present in the walls as compared with the soluble extracts. Data on the variations in the protein nitrogen content of the two fractions (Table 1) indicate that the protein nitrogen decreases throughout the course of the germination of conidia.

Mycelium. Total and specific activities of trehalase during the growth of *Neurospora* over a 27-day period are plotted in Fig. 5 and 6; growth for 18 days is shown in Fig. 9. At 3 days, which was as early as the determinations were made, there was very little trehalase activity in either the soluble or cell-wall fractions. Enzyme was synthesized gradually in both fractions until the ninth day, after which rapid synthesis ensued. After 20 days, activity in the soluble fraction diminished, whereas that in the cell wall continued to increase. These relationships were paralleled for the most part in the data on specific activity, except that the latter increased less rapidly than the total activity between 13 and 16 days after inoculation. Moreover, the specific activity of the soluble fraction continued to rise after 20 days, instead of falling, as in the case of total activity.

Invertase activities during growth are provided in Fig. 7 and 8. Total soluble invertase activity was much higher than trehalase activity from the start of mycelial growth, and its synthesis was rapid throughout the third to sixteenth days after inoculation, except for the period between 6 and 9 days. It is significant, perhaps, that this period of diminished synthesis of invertase coincided with the beginning of conidiation. The peak of total invertase activity in the soluble fraction was reached at 16 days, in contrast to trehalase for which the peak was reached at 20 days. However, the specific activities in the soluble fraction were much more parallel. As for the total activity in the cell wall, invertase was synthesized rapidly from the start, leveled off at 13 days, and increased again after 20 days. Specific activity in the wall increased rapidly from the start of growth but leveled off after 13 days. Compared with the specific activity of invertase in the wall, that of trehalase began later but peaked later as well, increasing even beyond 20 days.

In 3-day-old mycelium, the wall had a much lower ratio of invertase to trehalase activity than did the soluble fraction. Therefore, the trend toward higher ratios noted in the soluble fraction of germinating conidia seems to continue beyond 14 hr and the opposite appears to be true for the ratio of activities in the wall, at least until 3 days after inoculation. The walls acquired invertase faster than trehalase from 3 to 9 days so that at 9 days the ratio of activities in the two fractions was not very different. After 13 days, the ratio decreased in both fractions, as would be expected from the data in Fig. 5–8.

There were great differences during development in the ratios of invertase specific activity in the soluble fraction to that in the wall. For example, there was a difference of over 40-fold

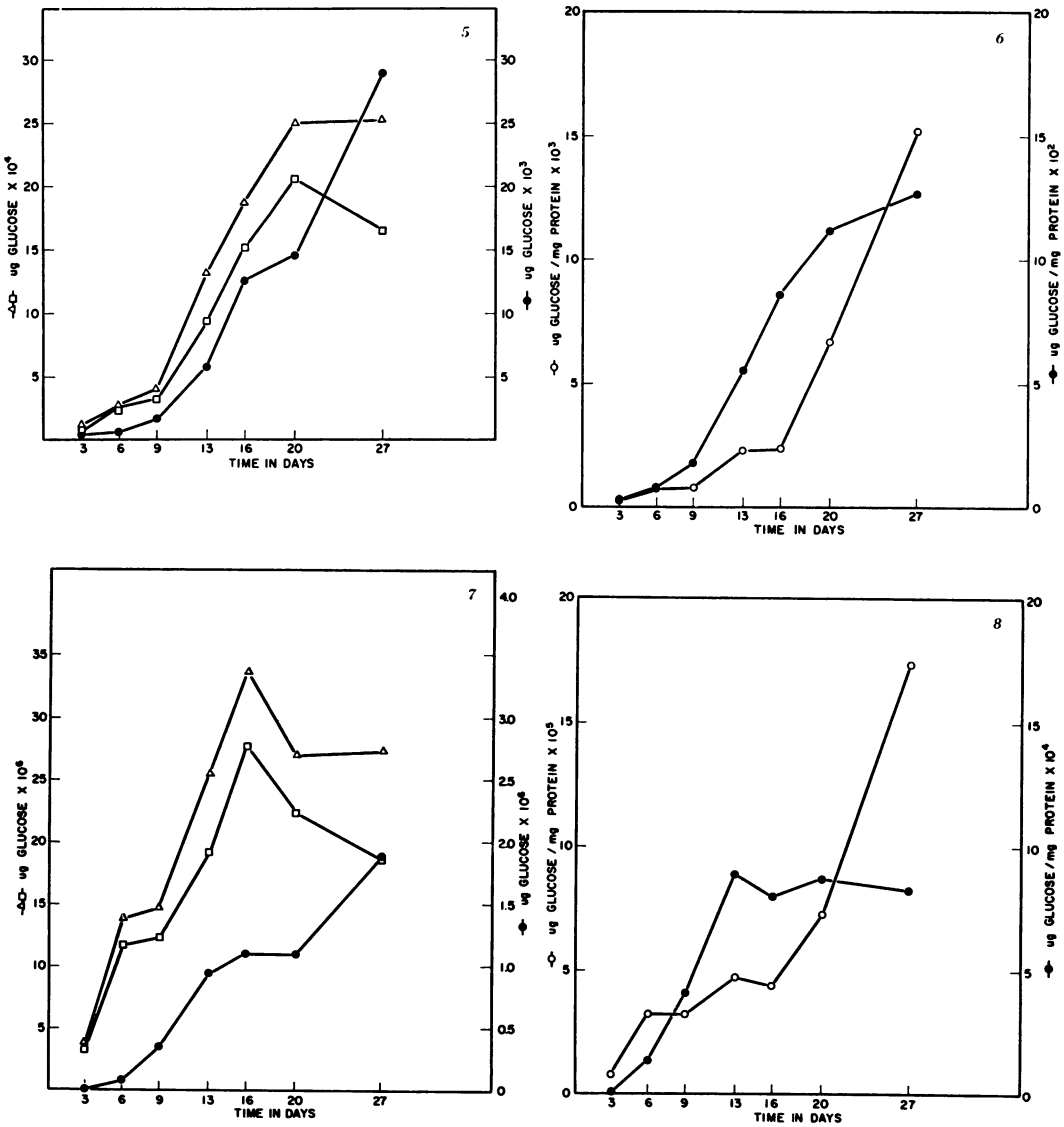


FIG. 5–8. *Activity of trehalase and invertase in mycelium of Neurospora crassa. (5) Total activity of trehalase per gram (wet weight) of organism. (6) Specific activity of trehalase. (7) Total activity of invertase per gram of organism. (8) Specific activity of invertase. Symbols as in Fig. 1–4.*

between 3- and 13-day-old mycelium in this respect, whereas the ratios of trehalase specific activities were much more constant. Table 1 reveals that the protein nitrogen reached a maximum at 16 days but diminished at 27 days. By contrast, that in the wall increased at 27 days, probably reflecting the amount of wall material relative to protoplasm in old mycelium.

Ascospores. Trehalase activity in extracts of ascospores was followed for 3 hr after activation (Fig. 10 and 11). At 2 hr after activation, there was an increase of about 400% in total trehalase activity in the soluble fraction. During the third hour after activation, such activity decreased. Trehalase activity in the wall increased only slightly over the 3-hr germination period, and, except in the third hour, the proportion of the total activity contributed by the cell wall decreased. The specific activity of the soluble and wall fractions paralleled the total activity

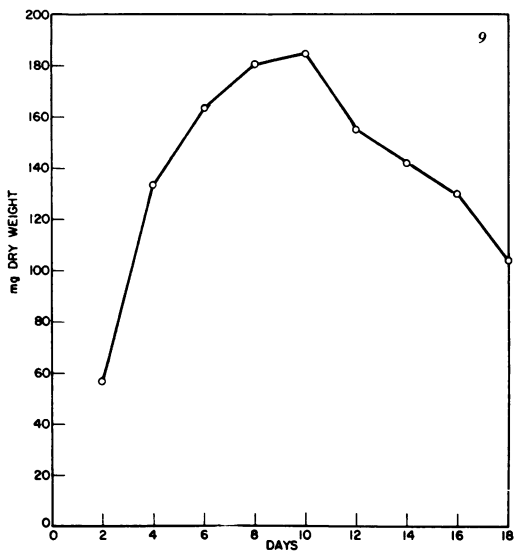


FIG. 9. *Growth of strain 89601-A of Neurospora crassa. Each point represents the average dry weight from four flasks.*

fairly closely as to the general trends, except immediately after activation when the specific activity of the soluble fraction increased more steeply than the total activity. The diminished specific activity in this fraction after 30 min also differed from the curve for total activity at this time.

In marked contrast to the results with trehalase, invertase activity, both total and specific, fell during the first hour after activation (Fig. 12 and 13). This decrease was most marked for the enzyme in the wall, whose activity was reduced almost 75% at the end of 1 hr. Thereafter, invertase in the soluble fraction increased until at the end of 2 hr there was about 50% more total activity. The contrast in the curves for specific activity of trehalase and invertase was even greater than for total activity, because there was a decrease in the latter enzyme throughout the germination period. In the case of trehalase, there was an increase in the specific activity in both the wall and soluble fractions, except in the third hour when the activity in the latter was sharply diminished. It is worth noting that the proportion of invertase in the wall was greater in the ascospore than at any other stage.

DISCUSSION

The hypothesis that the restraint to the development of dormant ascospores lies in their inability to utilize trehalase involves several alternative mechanisms. (i) A key enzyme is synthesized *de novo* when dormancy is broken as through the formation of an inducer, or the breakdown of a repressor. (ii) An inhibitor of this enzyme is destroyed when spores are activated. (iii) A precursor is converted into the enzyme by activation, in a manner analogous to the trypsinogen-trypsin transformation. (iv) The enzyme and its substrate are separated spatially in the cell, and activation brings them together. (v) A series of interlocking enzyme reactions are shifted from one steady-state level to another, as suggested by Delbrück (1949). Of course, these possibilities may not be mutually exclusive. For example, both enzyme activity (mechanisms i–iii) and contiguity of substrate and enzyme (mechanism iv) could be affected when dormancy is broken.

As shown in Fig. 10 and 11, trehalase activity can be extracted from dormant ascospores. Although a 4-fold rise in trehalase is demonstrable over the germination period, it is not proportional to the 20- to 30-fold rise in respiration which occurs during this time. Therefore, it might be concluded that mechanisms i and ii are invalid, because they would require that there be no activity before dormant spores are activated. Whereas *de novo* synthesis of trehalase seems to be ruled out by these data, the dismissal of mechanism ii is not yet justified, because the techniques used to extract the enzyme may have dissipated an inhibitor. Consequently, further experiments are necessary before this hypothesis can be ruled out completely. Furthermore, it is still possible that other enzymes besides trehalase are concerned with the dissimilation of trehalose in vivo and with the activation process. However, no such enzymes have yet been found, but it must be admitted that this is only negative evidence and cannot be conclusive. The heat resistance of *Neurospora* trehalase (Hill and Sussman, 1963), especially as compared with that of another carbohydrase (invertase) with which it coexists in ascospores, suggests that this enzyme's properties are of the kind that are necessary for it to be able to function during the activation treatment. Moreover, the different behavior of trehalase and invertase after activation (Fig. 14), especially when compared with their roughly parallel trends at all other stages, is evidence for a special role of trehalase during the breaking of dormancy. In this connection, the data on specific

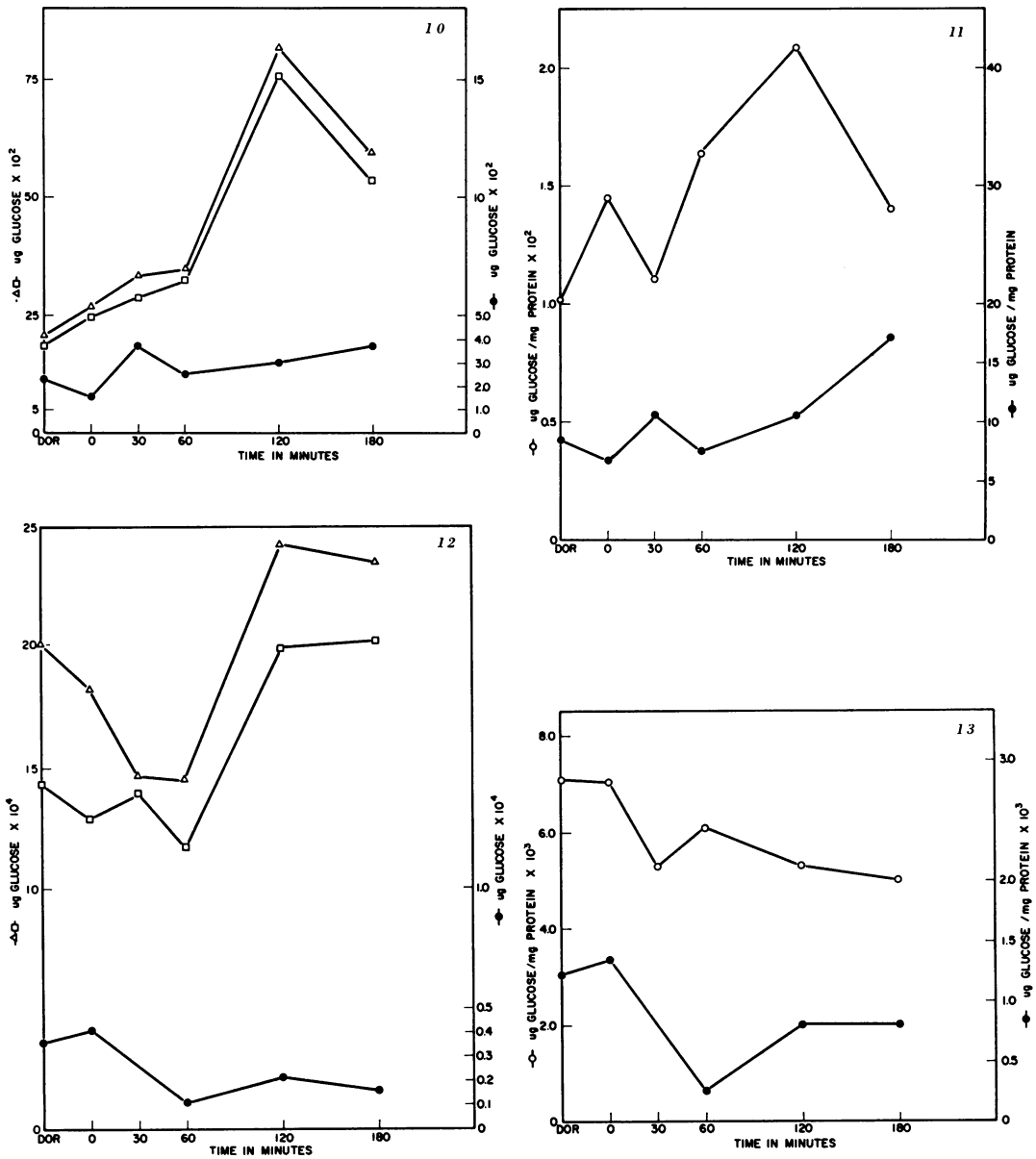


FIG. 10–13. *Activity of trehalase and invertase in ascospores of Neurospora tetrasperma. (10) Total activity of trehalase per gram of organism. (11) Specific activity of trehalase. (12) Total activity of invertase per gram of organism. (13) Specific activity of invertase. Symbols as in Fig. 1–4.*

activity provided in Fig. 11 and 13 are especially revealing, for there is an increase in trehalase activity immediately after the heat-shock has been applied, whereas that of invertase remains constant or decreases slightly. That such an increase in trehalase activity is not characteristic of all spore stages may be seen from the data in Fig. 1–4, which show that such activity actually diminishes during the germination of conidia. Although the decrease in specific activity between 0 and 30 min after activation is unexplained, the overall kinetics of the specific activities of invertase and trehalase remain very different.

Although, as indicated above, control of trehalase levels in ascospores during activation probably does not involve an effect upon the genome

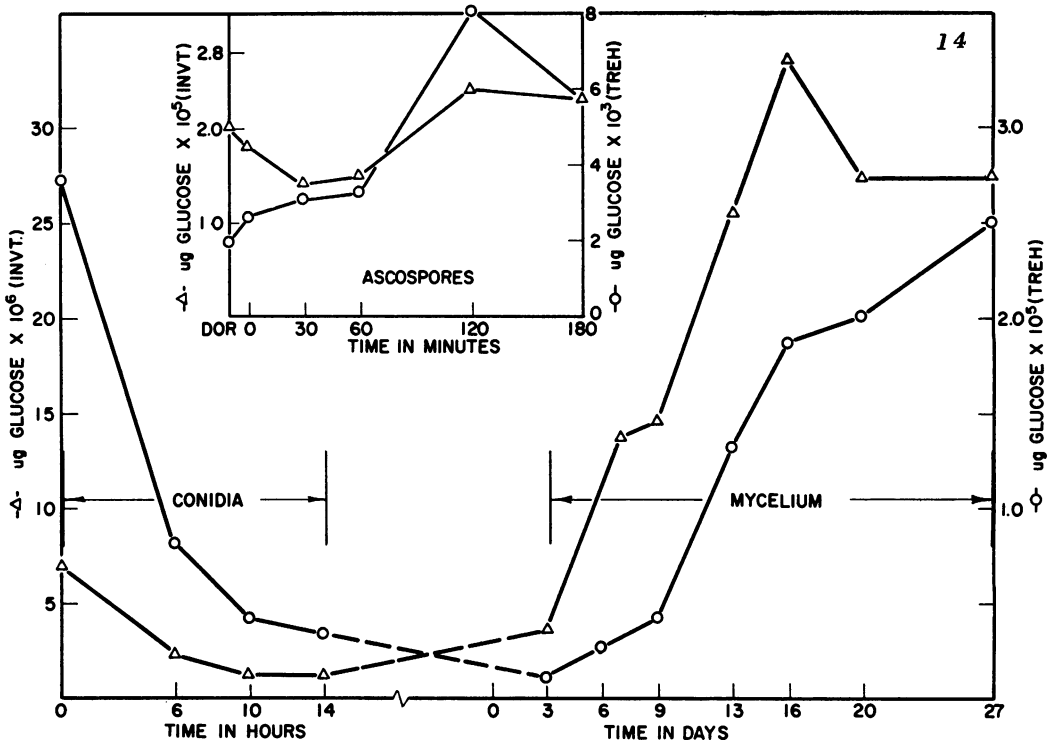


FIG. 14. *Summary of the total recoverable activity of trehalase and invertase in conidia, mycelium, and ascospores of Neurospora.*

and protein synthesis directly, it is important to know what controls exist at other times during development. Metzenberg (1962) reported that there is coordinate repression of invertase and trehalase activities in *N. crassa* for the following reasons. (i) There is a parallel effect of certain sugars upon the levels of these enzymes. (ii) A mutant which produces abnormally large amounts of both enzymes has been shown to be due to an effect at a single locus. (iii) The levels of invertase and trehalase are affected similarly during the course of growth.

However, the present work, and that reported elsewhere (Eilers et al., 1964; Metzenberg, 1964), suggest that Metzenberg's original hypothesis requires revision. First, the greatest total trehalase activity appears in dormant conidia, whereas such activity peaks in old mycelium in the case of invertase (Fig. 1, 7, and 14). Then, the greatest proportion of trehalase activity in the soluble fraction is found in dormant conidia, not in young mycelium, as in the case of invertase. Furthermore, the synthesis of trehalase in the mycelium is most rapid after 6 to 8 days of growth, and the peak in total activity is reached after 27 days; invertase is synthesized most rapidly after 3 days (Fig. 5, 7, and 14), and the peak in total activity occurs at 13 days. In physiological terms, the greatest rate of trehalase synthesis does not occur until after the log phase of growth is reached (Fig. 9 and 14), whereas that for invertase occurs while the growth rate is maximal. Therefore, significant differences may exist in the roles of these enzymes during growth. Other discrepancies between the levels of these enzymes were noted during the discussion of activation and can be found in the data on specific activities and in the ratio of activity in the soluble fraction to that in the wall. That the situation is more complex than was originally believed is suggested by the work of Hill and Sussman (1963), who found at least two different trehalases after purification of extracts from *Neurospora* mycelium. More recently, Eilers et al. (1964) and Metzenberg (1964) established the presence of two invertases in this organism. In the former case, a mutant was described which was deficient in one of these enzymes, suggesting that the two forms of the enzyme may be separately controlled.

If the premise is correct that the induction of specialized enzyme systems must precede specialization at the functional and morphological levels, then it should be possible ultimately to analyze development in *Neurospora* in terms of the causal enzymatic events. However, as Zalokar (1959) pointed out, morphological factors cannot be ruled out as causal factors in differentiation because they may produce new chemical environments. Thus, large differences in oxygen supply may be produced in parts of the mycelium such as those buried under the colony. In turn, the oxygen supply may control entire respiratory systems, as in yeast (Slonimski, 1956). Therefore, the interpretation of the fact that the peak in trehalase activity is correlated with the cessation of growth in *Neurospora* mycelium is difficult. However, it is known that several other enzymes are formed at this time, including tyrosinase and D-amino acid oxidase (Horowitz, 1953, *personal communication*) and β-galactosidase (Zalokar, 1959), so there is considerable change in the organism's enzymatic apparatus at this time.

Large enzymatic differences exist between conidia and ascospores of *Neurospora*, including the 100-fold, or greater, level of trehalase activity in the former. This difference is only about one-third as large in the case of invertase. The functional significance of these differences may lie in the fact that ascospores are resting cells in which low respiratory activity exists and in which lipids are the energy source during dormancy (Lingappa and Sussman, 1959). An interesting question raised by these data concerns the accumulation of much greater amounts of trehalase in conidia than in the mycelium upon which they are formed. In this respect, trehalase is similar to the β-glucosidases studied by Eberhart (1961) and nicotinamide adenine dinucleotidase (Zalokar and Cochrane, 1956).

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