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Purification and Properties of Trehalase(s) from *Neurospora*[1,2]

E. P. HILL AND A. S. SUSSMAN

From the Department of Botany, University of Michigan, Ann Arbor, Michigan

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Trehalase has been obtained in crystalline form from the mycelium of *Neurospora crassa*. During elution from the first pass through a DEAE-cellulose column, two peaks with trehalase activity were obtained. When the first of these was rechromatographed on DEAE-cellulose, two more major peaks were found. The enzymes of these fractions were compared and found to be similar in substrate specificity, response to inhibitors, pH optima, and Michaelis constants. However, small differences in the rate of inactivation of these enzymes at 50° were detected.

INTRODUCTION

The disaccharide trehalose has been found in organisms as diverse as microbes, vertebrates, invertebrates, ferns, and phanerogams (23). In several instances, its accumulation and utilization are associated with resistant stages like diapause in insects (32), brine-shrimp eggs (5), and fungus spores (2, 15, 18, 27).

As early as 1904, Bourquelot and Herissey (4) were able to demonstrate that the disappearance of trehalose in fungi coincided with the appearance of trehalase, an enzyme whose activity had first been demonstrated in 1893 (3). Since that time this enzyme has been found in bacteria (1, 11), other fungi (10, 20, 22, 30), insects (12–14, 16, 24, 33), oysters (7), fish (31), hog intestinal mucosa (8), and higher plants (6, 10, 29). Despite their ubiquity, however, the physiological role of the sugar and enzyme is not known.

One suggestion is that their role in the case of ascospores is the provision of energy for germination, whereas lipid serves this purpose in dormant spores (26). Inasmuch as trehalose accumulates to the extent of 15% in such dormant spores, the treatment which permits germination to occur must trigger a means through which the sugar is utilized. Such a means may be the enzyme trehalase. Therefore, the properties of trehalase from *Neurospora* were determined in these experiments, as a prelude to the study of the regulatory role postulated above for this enzyme.

METHODS

Protein was measured as described by Lowry *et al.* (19) using bovine serum albumin as a standard.

Trehalase activity was measured, unless otherwise stated, in the following manner: 1 ml. sodium phosphate buffer 0.05 *M*, pH 5.6, hereafter referred to as the "standard buffer," 1.0 ml. trehalose dihydrate in standard buffer containing 5 mg./ml., and 0.1 ml. enzyme in a total volume of 2.1 ml. of standard buffer. These reactants were incubated for 30 min. at 37°, and the reaction was stopped by boiling for 5 min. Reducing sugar was measured as glucose by the method of Somogyi (25). Optical densities of all assays were measured in a Klett-Summerson colorimeter with a No. 54 filter. A minimum of triplicate samples was used in all experiments.

In experiments where the substrate specificity was determined, the glucose released was measured by the glucose oxidase method (17), modified as described below. One milliliter of substrate, in standard buffer, and adjusted to give glucose equivalents identical to trehalose, and 0.1 ml. enzyme were made up to a total volume of 2.1 ml. in standard buffer and incubated at 37° for 30 min. The reaction was stopped by boiling for 5 min. Glucose oxidase and chromogen provided

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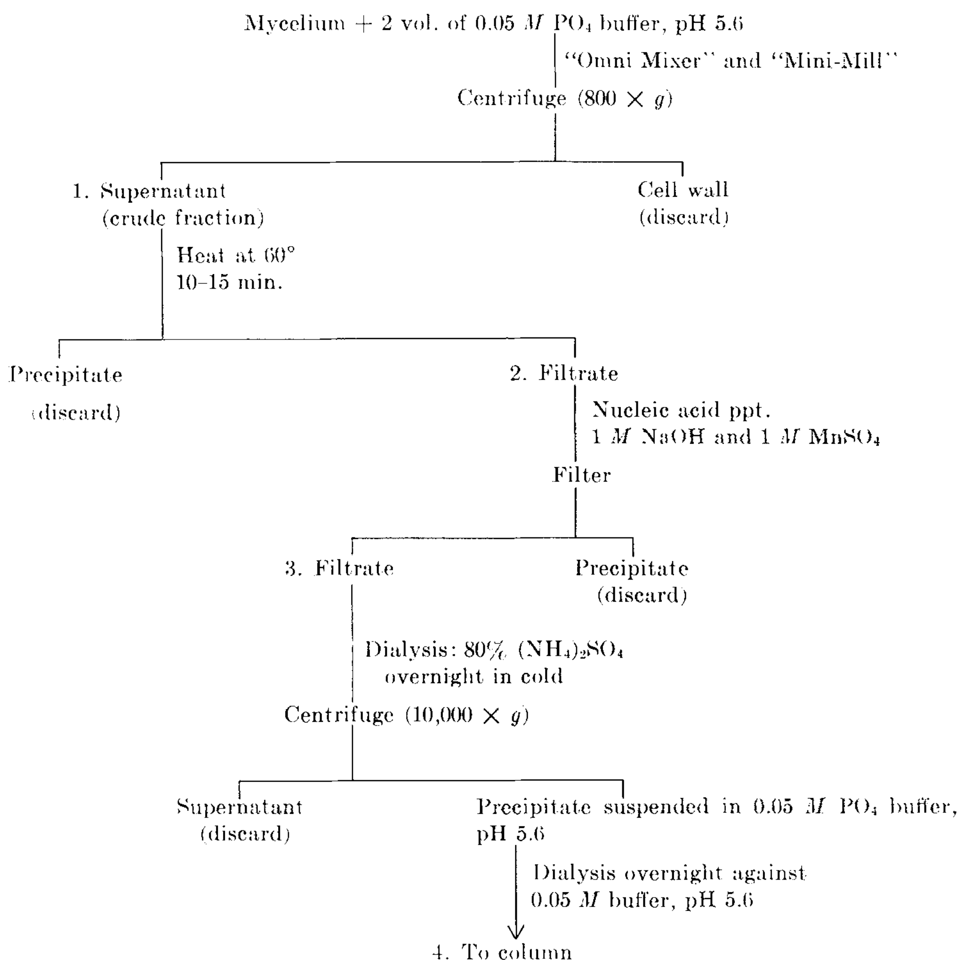


FIG. 1. Protocol of extraction of trehalases from *Neurospora* mycelium. Conditions of assay described in text.

with the "Glucostat reagent" (purchased from Worthington Biochemical Corp., Freehold, N. J.) were added, and the entire reaction mixture was incubated for 30 min., unless otherwise indicated, at 37°. The reaction was stopped by adding 0.1 ml. of 4 N HCl per tube. The color formed was read at 400 mμ in a Beckman DU spectophotometer.

The glucose oxidase and chromogen were prepared in the following manner: The enzyme and chromogenic agent were 22 and 44%, respectively, of the concentrations suggested by the company. These changes were introduced to reduce the high background color which was obtained when the amounts recommended by the supplier were used. All standards, controls, and reaction mixtures were assayed at these concentrations.

One unit of activity is defined as that amount of enzyme that will produce 1 μg. of reducing sugar, measured as glucose, in 30 min. at 37° in standard buffer.

PREPARATION OF THE ENZYME EXTRACT FROM *NEUROSPORA*

A conidial suspension of an "inositol-less" strain of *Neurospora crassa*, designated as 89601-A, was introduced into each of three 9-l. carboys filled with 6-l. of sterile medium. The medium consisted of 50 mg./l. inositol, 2% Vogel's (28) salt solution, and 2% sucrose. The carboys were sparged with compressed air during a growth period of 5 days at 25°. The mycelium was harvested by filtering through coarse muslin, washed with twice its volume of distilled water, pressed as dry as possible with paper towels, and immediately frozen. Total yield of mycelium per liter of medium was *ca.* 40 g. (wet weight). After 26 days in the frozen condition, the mycelium was thawed and the enzyme was extracted according to the protocol in Fig. 1.

The mycelium was macerated by grinding in

twice its volume of standard buffer for 4–40-sec. periods, with a 5-min. cooling interval, in an "Omni-Mixer" whose cup was immersed in ice. The resulting brei was further homogenized in a Gifford-Wood "Mini-Mill." This was accomplished by placing 50 ml. brei with 50 ml. glass beads (Minnesota Mining and Manufacturing Co. Super-Brite pavement markers) in the "Mini-Mill" cup and grinding for 3 min. The rotor was spaced 2.5 mm. from the stator, and the rheostat was set at 100. The cup was always immersed in ice during this operation.

Purification of Trehalase on Columns of DEAE-Cellulose

Diethylaminoethylcellulose (DEAE-cellulose) (purchased from California Corporation for Biochemical Research, Los Angeles 63, Calif.) was prepared for use as follows: 100 g. of the dry powder was allowed to sink into 4 l. of 0.5 *N* NaOH. After settling, the DEAE-cellulose was washed with 10 l. of demineralized water in 1-l. aliquots. Each time the "fines" were siphoned off. The DEAE-cellulose slurry was then washed with 4 l. of standard buffer before being placed into a column. The column was formed by allowing enough slurry to settle by gravity until a bed 2.5 cm. × 30 cm. was made. The column was further washed with 2 l. of standard buffer before the concentrated enzyme was applied. The enzyme was applied to the column and washed with 1 l. of standard buffer. A gradient elution system was established by placing 1 l. of standard buffer in the mixer and 1 l. of standard buffer supplemented with 1 *M* NaCl in the reservoir. The column was adjusted to deliver 1 drop/sec.

The enzyme from fractions 8 and 11 was diluted with standard buffer so as not to produce over 800 μg. glucose/ml. enzyme. The enzyme thus diluted was used in inhibitor, H-ion concentration and heat-stability assays.

Heat Stability

Enzyme aliquots, 3.5 ml., were placed in test tubes immersed in a water bath at the appropriate temperature. At the designated time intervals 0.1-ml. aliquots were withdrawn, placed in test tubes, plunged into an ice bath, and assayed for glucose.

Inhibitor Studies

The enzyme in 0.1-ml. aliquots was placed in the presence of inhibitors at various concentrations for 30 min. at 37°. After incubation in the presence of the inhibitor, trehalose was added and the reaction mixture was incubated for an additional 30 min. at 37° and assayed for glucose.

H-Ion Concentration

Sodium phosphate buffer, 0.05 *M*, was adjusted to various H-ion concentrations with a model G Beckman pH meter. The substrate was dissolved in demineralized water. Incubation of the reactions mixture and assay for glucose was performed as previously described. Although the buffer capacity of phosphate is low in parts of the range pH 3.5–5.5, there was no change in the pH during the experiment.

RESULTS

The elution pattern of trehalase, shown in Fig. 2, from a crude extract applied to a DEAE-cellulose column suggested the presence of two different proteins that show trehalase activity. Trehalase from the first peak, between volumes 500 and 800 ml., was reprecipitated in 80% $(NH_4)_2SO_4$, dialyzed against standard buffer, and reapplied to the column. The elution pattern from the second application to a DEAE-cellulose column indicated that fractions 7 and 11 came off at approximately the same place. On the other hand, fraction 8 was eluted much sooner than was fraction 12, suggesting a difference in the proteins of these two samples.

Another difference in behavior is shown in the heat stabilities (Figs. 3*a* and 3*b*), although the differences are not very pronounced. At 50° fraction 8 (Fig. 3*a*) lost activity slightly whereas fraction 11 (Fig. 3*b*) increased in activity. At the other temperatures, however, the patterns are quite similar. The half-life of both enzymes is similar, that for fraction 8 being 4 min. at 70° and that for fraction 11 being 3 min. at 70°.

A summary of the purification achieved in the steps outlined in Fig. 1 is provided in Table I. The greatest purification of trehalase attained on DEAE-cellulose was approximately 250-fold as shown in Table I. In further attempts to purify the enzyme (fraction 11) by ethanol fractionation, it was found that it crystallized, upon the addition of enough absolute ethanol to make a 40% solution, when allowed to stand in the deep freeze overnight. The crystals were collected by centrifugation or filtration over a Büchner funnel. Upon a series of three recrystallizations, activity was found to remain in the crystals.

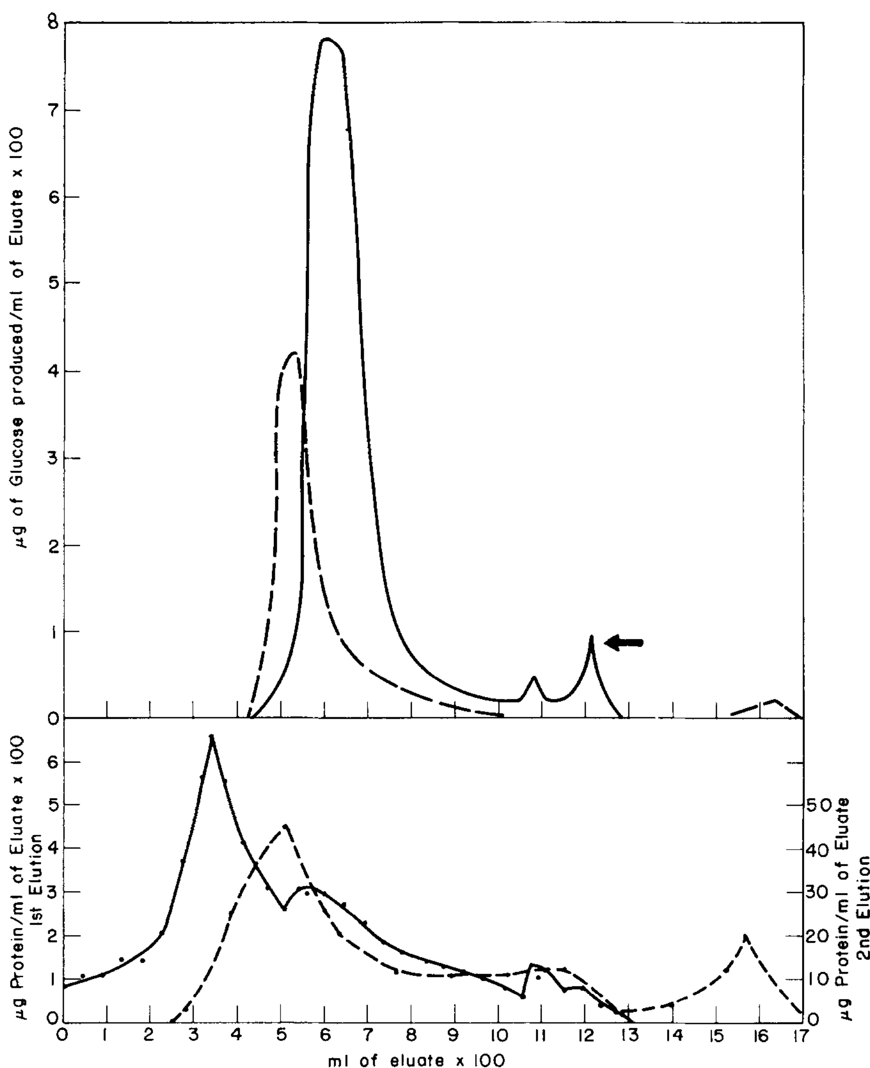
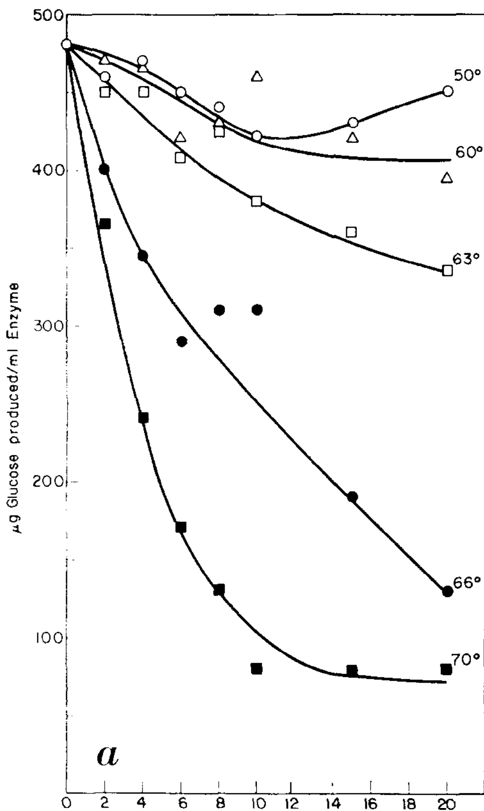


FIG. 2. Upper curves: gradient elution pattern of *Neurospora* trehalase(s) from a DEAE-cellulose column. The solid line represents the elution pattern of trehalase from a partially purified extract after one passage through the column. The two peaks are numbered fractions 7 and 8 (Table I). The eluates were collected in 9.2-ml. aliquots. No activity was detected until *ca.* 435 ml. irrigant had passed through the column. The dotted line represents the elution pattern of trehalase activity after passage of the enzyme from the first peak between 505 and 785 ml., after it had been reconcentrated, dialyzed, and reapplied to the same column. The two major peaks are numbered fractions 11 and 12 (Table I). The column had previously been cleansed of protein with 0.5 *N* NaOH and reequilibrated with standard buffer. The 2nd elution was collected in 25.5-ml. aliquots. See text for an explanation of the gradient system. Arrow indicates peak from which fraction 8 was derived. Lower curves: gradient elution pattern of protein recovered from *Neurospora* extracts on DEAE-cellulose. The solid line describes the elution pattern of the 1st application and the dotted line corresponds to that of the 2nd application.

Michaelis constants (K_s) were derived as a means of comparing the trehalases from the several fractions. A comparison between trehalases recovered from two different extractions is given in Table II. No differences are obvious.

The substrate upon which fractions 8 and 11 is active includes only trehalose (Table



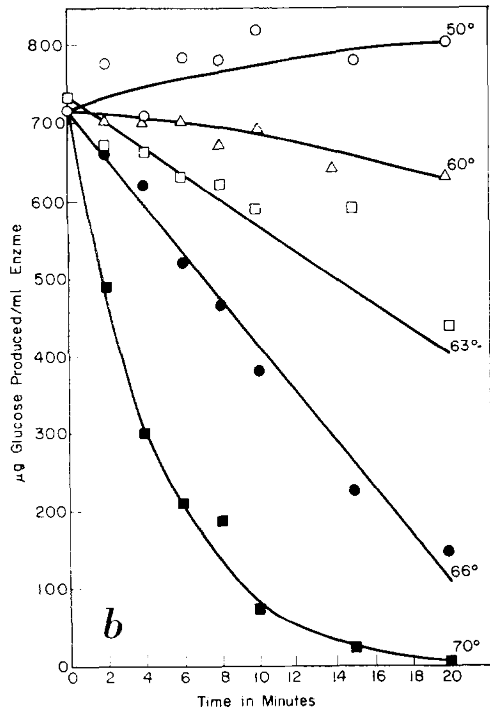


FIG. 3

FIG. 3. Heat stability of *Neurospora* trehalase *a*. Enzyme from fraction 8. *b*. Enzyme from fraction 11. Conditions of assay are described under *Methods*.

III). Fraction 12 showed more activity on sucrose, melibiose, and cellobiose than did the other two fractions. However, it should be noted that the specific activity of fraction 12 was much lower than those of the other fractions (Table II) so that contaminant proteins could have been responsible for the cross specificity. Moreover, glucose oxidase has traces of maltase, trehalase, and invertase, as indicated by high values for the respective sugar controls, so that, under the conditions of the assay, no appreciable utilization of sugars except trehalose is demonstrated with purified trehalase from fractions 8 and 11.

The following inhibitors were without appreciable effect on the trehalase activities of either fraction 8 or fraction 11: NaN_3, Na arsenate, $CuSO_4 \cdot 5H_2O$, gluconic δ-lactone, urea, $NiCl_2 \cdot 6H_2O$, aniline, NaF, and iodoacetate. All inhibitors were used at concentrations which ranged from 1×10^{-5} to 1×10^{-2} *M* except $CuSO_4 \cdot 5H_2O$ which was used at 1×10^{-5} to 1×10^{-3} *M*.

The pH optima shown in Fig. 4 are similar for both enzymes. The maximum rate at which glucose formed was found to be at pH 5.5 with a sharp decline in rate toward more alkaline conditions.

DISCUSSION AND CONCLUSIONS

The properties of the trehalases from *Neurospora* resemble those of the trehalases from other sources in several respects. Thus, the Michaelis constants of the enzyme from yeast (6), seeds of *Plantago* (6), June bugs (6), silkworms (24), wax moths (16), and blowflies (13) range from 1.3×10^{-4} *M* to 9×10^{-4} *M*; the values obtained for *Neurospora* are intermediate. However, the Michaelis constant of the trehalase from pig intestinal mucosa is 3×10^{-3} *M* and deviates markedly from these values (9). In general, where purified trehalases have been used, trehalose is the only substrate that is utilized (9, 13, 16, 24). Although the enzyme from June bugs was partially purified (6), it retained activity on maltose and sucrose but further work

TABLE I

SUMMARY OF STEPS IN THE PURIFICATION OF TREHALASE FROM *NEUROSPORA*

Conditions of assay are described in text. Steps are numbered to coincide with those numbered in Figs. 1 and 2.

Fraction	Total activity	Total protein	Recovery activity	Specific activity	Purification factor[a]
	units	*mg.*	%		
1. Crude	4,083,700	8.984×10^3	(100)	454	—
2. After heat treatment	3,654,000	4.891×10^3	89.4	747	1.6
3. After nucleic acid treatment	3,536,000	3.648×10^3	86.5	969	2.1
4. After buffer dialysis	2,236,600	637	54.7	3,510	7.7
5. 1st application to column	2,110,000	601	54.7	3,510	7.7
6. Recovered from 1st column elution	1,462,890	133	35.8	10,974	24.1
7. Peak 1: at the crest of the peak	At vol. 598 ml. 71,760	2.708	—	26,499	58.3
8. Peak 2: at the crest of the peak	At vol. 1210.0 ml. 990	0.669	—	14,798	32.6
9. Applied to 2nd column. Act. eluted in Peak 1.	705,600	17.940	—	39,331	86.6
10. Peak 1, 2nd elution	At vol. 436–842 ml. 665,833	10.574	16.5	62,023	136.6
11. Peak 1, 2nd elution. At the crest of the peak	At vol. 535.0 ml. 107,100	0.928	—	115,409	254.2
12. Peak 2, 2nd elution. At the crest of the peak	At vol. 1640.0 ml. 5,100	0.515	—	10,873	23.9

[a] Compared to the specific activity of the crude extract.

will be necessary before its broad specificity can be accepted.

If trehalase is involved in the germination of *Neurospora* ascospores, it might be expected to survive the high temperatures (60° for 5–30 min.) required to break the dormancy of these cells. As can be seen in Figs. 3*a* and *b*, the trehalases of the *Neurospora* mycelium survive treatment at 60° very well although their activity is rapidly destroyed at 70°. By contrast, our experiments (unpublished) have shown that invertase, which also has been isolated from *Neurospora* (22), loses activity rapidly at 60°. In fact, the heat step incorporated in the purification procedure described in Fig. 1 takes advantage of this and rids the extracts of invertase activity which otherwise far exceeds that of trehalase. Similar differences in heat resistance have permitted the separation of trehalase from invertase in hog intestinal mucosa (8), and from maltase in the wax moth (16). However, there are cases where trehalase is relatively more heat sensitive than other carbohydrases (3).

A comparison of the properties of fractions 8, 11, and 12 from the DEAE-cellulose column reveals that the Michaelis constants are very similar (Table II), as are the pH

TABLE II

MICHAELIS CONSTANTS FOR *NEUROSPORA* TREHALASES

Data calculated by least-squares analysis of nine points.

Source of enzyme	Purification factor[a]	K_m	Average
Fraction 8	32.6	3.2×10^{-4}	—
Fraction 11	254	6.3×10^{-4}	5.7×10^{-4}
		5.1×10^{-4}	
Fraction 12	23	4.7×10^{-4}	-
Pooled fraction[b]	98	4.2×10^{-4}	2.7×10^{-4}
		2.2×10^{-4}	
		1.4×10^{-4}	
		4.3×10^{-4}	
		1.7×10^{-4}	

[a] Purification factor defined as in Table I.

[b] Pooled fraction was obtained by mixing all fractions showing trehalase activity after two passes through the DEAE-cellulose column. This material was prepared during a different run than that outlined in Table I.

optima (Fig. 4), substrate specificities (Table III), and response to inhibitors. On the other hand, fraction 11 appears to behave differently than fraction 8 in response to incubation at 50° in that its activity is increased over a period of 20 min. at this temperature whereas that of fraction 8 is decreased. The significance of this difference is not clear in view of the similarities in half-life of the two fractions when exposed to higher temperatures. The possibility must be considered that these two peaks are artifacts caused by passage through the DEAE-column inasmuch as they were recovered after the rechromatography of fraction 7.

Two forms of trehalase have been reported to exist in pupae of the silkworm, *Bombyx mori*, on the basis of differences in elutability from DEAE-cellulose columns (24). As in the case of the enzymes from *Neurospora*, the silkworm trehalases were similar with respect to substrate specificity, Michaelis constants, and pH optima. These situations are reminiscent of the isozymes of lactic dehydrogenase from the mouse in which no differences could be found except for a small one in charge and one in the isoelectric point (21). As was pointed out in the work on lactic dehydrogenases, at least five kinds of molecular changes can account for the occurrence of different but

TABLE III

SUBSTRATE SPECIFICITY OF PURIFIED *NEUROSPORA* TREHALASES

Conditions of assay are described under *Methods*. All experiments were run in triplicate except for the maltose samples of fractions 11 and 12.

Substrate	Fraction number: 8[a]			11			12		
	Optical density		Glucose produced/0.1 ml. enzyme[b]	Optical density		Glucose produced/0.1 ml. enzyme[c]	Optical density		Glucose produced/0.2 ml. enzyme[d]
	Enzyme + sub.	Sub. control	*mg.*	Enzyme + sub.	Sub. control	*mg.*	Enzyme + sub.	Sub. control	*mg.*
Trehalose	.319	.203	23.1	.578[a] × 4	.288[a]	240.0	.425[a]	.288[a]	27.5
Sucrose	.023	.019	0.8	.075	.078	0	.164	.078	5.8
Lactose	.023	.016	1.4	.054	.056	0	.065	.056	1.8
Melibiose	.062	.059	0.6	.181	.193	0	.207	.193	3.6
Raffinose	.002	.001	0	.053	.047	1.2	.058	.047	2.2
Maltose	.372	.373	0	.415[a]	.425[a]	0	.366[a]	.425[a]	0
Cellobiose	.129	.159	0	.245	.240	0.1	.263	.240	4.6

[a] Incubated in the presence of glucose oxidase for 10 min.

[b] 3.671 μg. protein/0.1 ml. enzyme assayed.

[c] 3.155 μg. protein/0.1 ml. enzyme assayed.

[d] 4.040 μg. protein/0.2 ml. enzyme assayed.

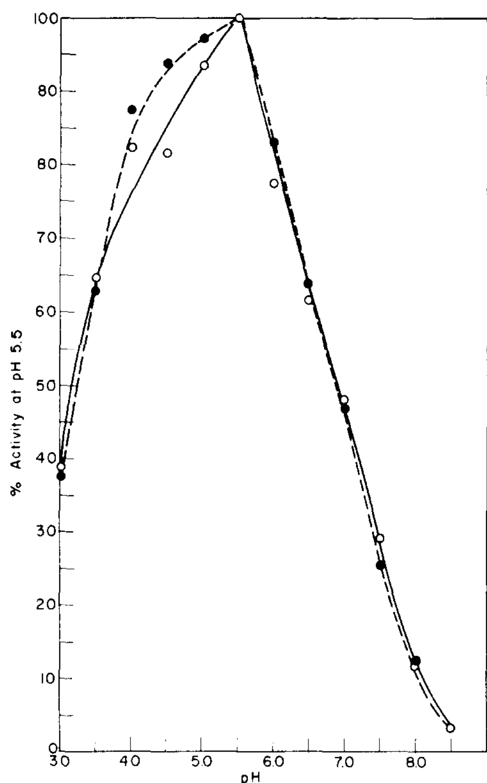


FIG. 4. Effect of pH upon trehalase eluted in fraction 8 (dotted line) and fraction 11 (solid line). Conditions of assay are described in text.

closely related isozymes. Studies of the several fractions from *Neurospora* which show trehalase activity are being continued in order to determine the significance of the differences between them in terms of the types of molecular changes mentioned above.

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