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Gene-Enzyme Relationships in *Neurospora* Invertase[1]

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A spontaneous, single-gene mutation responsible for a total lack of invertase activity in *Neurospora crassa* is described. The mutation is believed to lie in the structural gene for invertase, since an immunologically cross-reacting protein is made by the mutant strain. In addition, there was no evidence for a defect in regulation of invertase activity or synthesis by the following criteria. (i) The invertaseless condition was recessive in heterokaryons; (ii) no invertase inhibitor was found in mutant extracts by mixing experiments; and (iii) none of the several sugars able to induce activity in wild-type strains was able to induce activity in the mutant strain. It was also discovered that most of the wild-type enzyme (55 to 75%) cannot be washed free from the rapidly sedimenting cell debris. This finding provided additional support for the hypothesis that *Neurospora* invertase is located within or about the cell wall.

While developing a system for the analysis of circadian rhythms in *Neurospora crassa* (15), we discovered a mutant strain lacking invertase activity (β-D-fructofuranoside fructohydrolase, EC 3.2.1.32). The strain is thus unable to utilize sucrose as a carbon source.

Gene-enzyme studies were conducted on this mutant because invertase may have an indirect role in *Neurospora* rhythmicity (15). Study was further warranted by the work of Metzenberg, Trevithick, and Marzluf, who have investigated the coordinate regulation of invertase and trehalase in *Neurospora* (8), the localization of invertase (9), the subunit structure of invertase (11), and the significance of the localization and subunit structure with respect to secretion of the enzyme (17) and uptake of sugars (7).

In this paper, we describe the discovery and and genetics of the invertaseless strain. We also present experiments designed to investigate: (i) the nutritional and enzymatic defects of the mutant strain; (ii) the nature of the enzymatic defect; and (iii) the localization of invertase.

MATERIALS AND METHODS

Strains. Most of the strains utilized were described in the accompanying paper (15). Three additional strains were used for making heterokaryons: F6A (*ad*-4), F16A (*ad*-4), and B368-4-3a (*arg*-10). Stock maintenance, preservation, designations, and gene symbols are as described before (15).

[1] Taken in part from a thesis submitted by M. L. S. to Stanford University in partial fulfillment of the requirements for the Ph.D. degree.

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Heterokaryons. Heterokaryons were made according to standard techniques (14), and the nuclear ratios were determined by plating conidia on minimal and supplemented media as described by Prout et al. (13). The equation of Atwood and Mukai (3) was used to calculate the nuclear ratio from the proportions of conidial types found.

Growth and extraction. Dry-weight growth tests were conducted according to standard techniques (14).

In studying the nature of the invertase defect, the following methods were employed. Florence flasks half-filled with appropriate media were inoculated (10^4 to 10^5 conidia/ml), incubated on a reciprocal shaker, and harvested by filtration. The mycelial pads were washed with cold water, pressed dry, and lyophilized. The lyophilized mycelium was ground in a Wiley Mill, and the powder passing a 60-mesh screen was collected. Crude extracts were made by suspending the ground powder (50 mg/ml) in buffer at *p*H 5 (sodium acetate, 0.05 M), shaking for 60 min, and centrifuging for 20 min at 25,000 $\times$ *g*. The cell debris was discarded, and the extract was dialyzed against several changes of buffer at *p*H 5 to remove endogenous glucose. All extraction steps were carried out at 4 C.

For the detection of bound invertase, 10-liter carboys were half-filled with medium (Vogel's salts plus 1.5% fructose) and were inoculated with 10^6 conidia/ml. The cultures were incubated for 3 to 4 days at 25 C with filtered, compressed air serving to agitate the medium. The mycelium was harvested by filtration, washed with cold water, and wrung dry in cheesecloth. Crude breis of this mycelium were prepared in the following five ways. (i) For glass homogenization, wrung mycelium was suspended in buffer at *p*H 5 (100 mg/ml), ground in an Omnimixer (Sorvall Inc.) for 2 to 3 min, and then homogenized

in a 10-ml glass homogenizer. (ii) For sonic treatment, wrung mycelium was suspended in buffer at *p*H 5 (100 mg/ml), ground in an Omnimixer for 2 to 3 min, and then sonically treated for 2 to 15 min at 7 amp in a Branson Sonifier (model S 75). (iii) For acetone-ground material, wrung mycelium was washed with cold acetone (−20 C), air-dried, ground in cold acetone for 3 to 4 minutes in an Omnimixer, collected by filtration, washed with warm acetone (20 to 25 C), and air-dried. The acetone powder was then suspended in buffer at *p*H 5 (50 mg/ml). (iv) For lyophilization, wrung mycelium was lyophilized, ground, and suspended as described in the preceding paragraph. (v) For dry ice-ground material, the directions of Metzenberg (10) were followed exactly. Fractionation of the breis into extracts, washes, and pellets was done by centrifugation at 4,000 × *g* for 20 min. Breis and pellets were allowed to incubate in buffer for 1 hour with shaking before centrifugation. All steps were conducted at 0 to 4 C.

Assays. Invertase activity in crude extracts from shake cultures was assayed by a method modified from Metzenberg (8). The assays were conducted by (i) adding 25 μmoles of sucrose in 0.1 ml of water to 0.4 ml of suitably diluted extract or purified invertase in buffer at *p*H 5, (ii) incubating for 15 min at 37 C, (iii) stopping the reaction by boiling for 5 min, and (iv) measuring the glucose produced by adding commerical Glucostat reagent (Worthington Biochemical Co.) directly to the incubation tube. Assays for invertase activity in the brei from carboy cultures were conducted as previously described (15). Trehalase activity was determined according to the method of Hill and Sussman (5). Specific activity units are as defined by the respective authors. Protein determinations were done by the method of Lowry et al. (6) with bovine serum albumin as the protein standard.

Immunology. Antibody preparations (anti-invertase) were prepared by Antibodies Inc. from 10 mg of lyophilized invertase suspended in Freund's adjuvant and injected into three rabbits. Invertase was purified from the STA 4 strain of *N. crassa* by Metzenberg's procedure (10). The invertase (50-fold purified; specific activity, 10,000) gave one predominant band on disc electrophoresis, and a single symmetrical peak in the model E ultracentrifuge and in glycerol density gradients (Braymer, Sargent, and Woodward, *unpublished data*). Sera from rabbits injected with *Neurospora* adenylosuccinase and β-D-galactosidase (with their respective preinjection sera) were used as controls. The antisera were diluted into 0.01 M phosphate buffer, *p*H 8.0, when required.

Immunodiffusion experiments were conducted by double diffusion in agar plates (4) made from 0.01 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 8.0) and 1.5% agar (Difco); incubation was at room temperature.

Solubilization experiments. Washed cellular debris ("walls") from the suspension of ground, lyophilized mycelium was suspended either in borate buffer (0.2 M, *p*H 8.5) or in 1% Triton X-100 (Rohm and Haas) and was incubated on ice for 1 hr. Separation into supernatant fractions and pellets was as described above for the fractionation of mycelial breis.

RESULTS

Origin of the inv gene. A strain (*pan* 724-2a) characterized by sparse growth on minimal medium supplemented with pantothenate and by thin, periodic conidia formation in growth-tube cultures was discovered by T. H. Pittenger (*unpublished data*) among the progeny from a cross between *pan*-2(B3)A and *nic*-1, *al*-2a (both of the St. Lawrence background). Dry-weight growth tests (*see below*) established that this strain was unable to utilize sucrose and suggested that the strain lacked invertase activity. The strain was therefore crossed to STA 4 wild type to characterize the presumed invertaseless condition genetically. Six asci dissected showed a 1:1 segregation of the invertaseless condition (locus designated invertase, *inv*). The invertaseless condition also showed a 1:1 segregation in random isolates from six different crosses (343:357; *inv*:*inv*$^+$), providing additional evidence that this defect is attributable to a single gene. The gene is located on the right arm of linkage group V, 3.3 map units distal to *pab*-2 (15).

The *inv* gene presumably arose through spontaneous mutation in one of the two strains crossed by Pittenger and existed in a population of predominantly *inv*$^+$ nuclei. No mutagenic agents were used in the recent history of either Pittenger strain.

Nutritional deficiency. To diagnose the biochemical deficiency of strain *pan* 724-2a, dry-weight growth tests were performed. The extensive stimulation of growth by various mixtures of nutrients (top part, Table 1) suggested a carbon source involvement, and this involvement was verified by use of individual sugars as carbon sources (bottom part, Table 1). The data showed that sucrose is not utilized as a carbon source, but that its hydrolysis products, glucose and fructose, are readily used. This result could be explained most easily by assuming that this strain lacks the enzyme invertase.

Absence of invertase activity in inv strains. Invertase activity measurements were made on the crude extracts of several strains (Table 2). Less than 0.1% of the STA 4 level of activity (not significantly above background) was found in strains unable to utilize sucrose. Trehalase activity was also determined, because Metzenberg (8) had suggested that invertase and trehalase are coordinately regulated. Normal to high levels of trehalase were found in the *inv* strains. The simplest interpretation of these data is that the *inv* mutation represents a defect in the invertase structural gene.

Nature of the invertaseless condition. Three approaches provided no evidence for a regulatory

TABLE 1. *Dry-weight growth tests on strain pan 724-2a*

Supplement[a]	Weight[b]
	mg
None	1.1
Casamino Acids (Difco), 0.5%	28.5
Yeast extract (Difco), 0.5%	35.1
Casamino Acids, 0.5%, plus yeast extract, 0.5%	41.8
Glucose, 1.5%	127.4
Fructose, 1.5%	111.0
Sucrose, 1.5%	3.5

[a] Basic medium contained Vogel's salts and 4 μg of calcium pantothenate per ml. Cultures were grown for 72 hr at 34 C.

[b] Average of three mycelial mats.

TABLE 2. *Invertase and trehalase levels in crude extracts derived from the parents and isolates of ascus E[a]*

Strain[b]	Specific activity (units/mg of protein)	
	Invertase	Trehalase
STA 4, *inv*$^+$	268	
pan 724-2a, *inv*	0.1	
E1A, *inv*	0.1	138
E3A, *inv*	0.1	462
E5a, *inv*$^+$	208	106
E7a, *inv*$^+$	325	232

[a] Cultures were grown for 96 hr at 25 C on Vogel's salts, 4 μg of pantothenate per ml, and 1.5% maltose. Galactose (0.6%) was added at 72 hr.

[b] The strains were judged *inv*$^+$ or *inv* by their ability or inability, respectively, to utilize sucrose as a carbon source.

role of the *inv* gene. First, none of the common carbohydrates which serve as a carbon source for the *inv* strains (xylose, fructose, maltose, glucose, mannose, or glycerol) could induce these strains to produce invertase (Table 3), although they stimulated invertase production in wild-type strains. If there is a defect in sensitivity to inducers, the defect pertains to all inducers.

Second, no evidence for a soluble invertase inhibitor was found in crude extracts of *inv* strains, as demonstrated by assays of mixed, crude extracts from wild-type and *inv* strains (Table 4).

Finally, the invertaseless condition was recessive in heterokaryons. Forced heterokaryons grew at a normal rate on sucrose, and the invertase level in crude extracts of such heterokaryons was roughly proportional to the percentage of wild-type nuclei (Table 5). The observed invertase levels were slightly higher than predicted from the nuclear ratios in the inoculating conidia; this effect was presumably due to a small change in the nuclear ratio or to more complete derepression of the invertase locus in *inv*$^+$ nuclei.

A protein immunologically related to wild-type invertase, i.e., a cross-reacting material (CRM; 16), was found in crude extracts of *inv* strains. Such evidence provided more direct proof that the *inv* gene directs the synthesis of a protein whose conformation is nearly normal even though it is enzymatically inactive.

The result of diffusing anti-invertase serum against purified invertase is shown in Fig. 1. The precipitin line formed (line A) is believed to represent Metzenberg's heavy invertase (11), because the purified invertase preparations that yield this line sediment as a single peak in the analytical ultracentrifuge with an $S_{20,w}$ of 10.7

TABLE 3. *Invertase activity in crude extracts of strain E3 grown on various carbon sources[a]*

Carbon source[b]	Weight	Invertase activity (units/mg of protein)
	mg	
Galactose	<5	
Raffinose	<5	
Xylose	800	0.02
Trehalose	870	0.00
Fructose	910	0.00
Maltose	810	0.01
Sucrose	<5	
Glucose	850	0.01
Mannose	980	0.00
Glycerol	740	0.20

[a] Cultures were grown for 96 hr at 25 C on Vogel's salts and a carbon source (1.5%).

[b] Order from top to bottom represents decreasing ability to induce (8).

TABLE 4. *Mixing of crude extracts from inv*$^+$ *and inv strains[a]*

Extracts	Invertase activity (units/ml)	
	Predicted	Observed
STA 4, *inv*$^+$		5.07
E3, *inv*		0.00
patch, *inv*$^+$		0.59
STA 4:E3, 1:1	2.53	2.41
STA 4:E3, 1:5	0.67	0.70
STA 4:patch, 1:1	2.83	2.74

[a] Cultures were grown for 96 hr at 32 C on Vogel's salts and 1.5% maltose. Galactose (0.6%) was added at 72 hr.

TABLE 5. *Invertase level in heterokaryons containing inv⁺ and inv nuclei*[a]

Strain	Per cent of nuclei		Invertase level (units/mg of protein)	
	inv	*inv⁺*	Predicted	Observed
Heterokaryon				
E1 (*inv*, *pan*)/ F16 (*ad*)	85.1	14.9	35.7	54.6
E1 (*inv*, *pan*)/ F6 (*ad*)	74.7	25.3	10.4	17.5
R4 (*inv*, *pan*)/B368 (*arg*)	98.4	1.6	1.7	2.9
Homokaryon				
E1	100	0		0.1
R4	100	0		0.7
F16	0	100		239
F6	0	100		39.4
B368	0	100		58.8

[a] Cultures were grown for 96 hr at 34 C on Vogel's salts and 1.5% fructose (E1/F16 was grown on 1.5% sucrose). Appropriate supplements were added for growth of the homokaryons.

(Braymer, Sargent, and Woodward, *unpublished data*). In addition, a bright green region, indicating invertase activity, forms over this line when a liquid invertase assay solution (Braymer, Sargent, and Woodward, *unpublished data*) is added to the surface of the plate. The results of diffusing anti-invertase serum against crude extracts of various strains are also shown in Fig. 1. Strain STA 4 and several other wild-type strains with high levels of invertase activity yielded two invertase-specific precipitin lines: an A line, homologous with the purified invertase line (heavy invertase), and a B line found in all of the extracts examined. Extracts of timex and many other strains, including patch and clock, yielded the B line but not the A line.

Since timex has no A antigen as judged by double-diffusion in agar, the identity of the protein responsible for the B line is critical in deciding whether timex has an invertase CRM. The B protein is believed to be the light form of invertase reported by Metzenberg (11), because (i) their mobilities in disc electrophoresis are similar, (ii) the control sera yielded no B line when diffused against crude extracts, (iii) purified invertase removed all of the B antibodies in absorption experiments, and (iv) the B protein was produced in one experiment by the treatment of heavy invertase with 8 M urea (Sargent, Ph.D. Thesis, Stanford University, Palo Alto, Calif.). The treatment of heavy invertase with urea, base, salt, or heat leads to a variety of immunological forms of invertase which have not yet been completely characterized.

Although the four lines of evidence given above suggest that the B protein is indeed light invertase, direct confirmation by enzymatic activity was not obtained. The liquid invertase assay solution did not produce a green halo, indicative of invertase activity, around the B line as it did around the A line. A plausible explanation for this is based on the observation that the anti-invertase serum contained neutralizing antibodies (causing a loss of enzymatic activity) in addition to precipitating antibodies. Since the precipitated heavy invertase does retain enzymatic activity, it is conceivable that the neutralizing antibodies react selectively with the light form of invertase (Sargent, Ph.D. Thesis, Stanford University, Palo Alto, Calif.).

Wall-bound invertase. Although there were no previous indications that crude extracts of *Neurospora* do not contain all of the invertase synthesized by this organism, the matter was investigated because it is conceivable that the *inv* mutation affects the localization and not the structure of the enzyme, and because of several reports of wall-bound invertase in yeast (12) and higher plants (1, 18). The data in Table 6 demonstrate that with a variety of extraction techniques most of the activity found in a crude brei remains with the "wall" fraction (pellet of 4,000 × *g*, 20-min centrifugation) and that only 13.0 to 38.5% of the activity is found in the crude extract. Repeated washings of the walls yielded very little additional invertase, suggesting that the enzyme is tightly bound to or trapped within the cell wall. Neither borate buffer nor the detergent Triton X-100 was able to solubilize the activity (Table 7). The *inv* strains had no activity in the

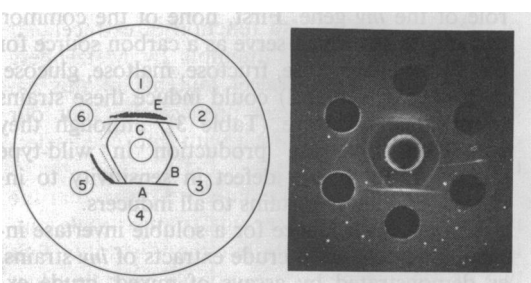

FIG. 1. *Precipitin lines found when anti-invertase was diffused against Neurospora crude extracts and invertase preparations. The center well contained the serum. The wells contained urea-treated invertase (1); crude extracts of timex (2) and patch (3); purified invertase (4); and crude extracts of STA 4 (5) and clock (6). Dotted lines represent nonspecific (non-invertase) precipitin lines.*

TABLE 6. *Extraction of wild-type invertase by various procedures*[a]

Fraction	Invertase units				
	Glass homogenized	Sonic treated for 15 min	Acetone powder	Lyophilized and ground	Frozen and blended
Brei (*p*H 5.0, 0.05 M NaOAc)	100%	100%	100%	100%	100%
Crude extract	22.6	38.5	13.0	24.7	24.5
First wash	4.0	3.4	8.4	17.7	7.3
Second wash	0.6	0.6	1.6	3.8	1.4
"Walls"[b]	67.9	72.2	54.8	54.7	73.4
Lost/gained[c]	−4.9	+14.7	−22.2	+0.9	+6.6

[a] STA-4 wild type was grown for 96 hr at 25 C on Vogel's salts and 1.5% fructose.
[b] Obtained by centrifugation at 4,000 × *g* for 20 min.
[c] The difference between the total units in the brei and the sum of the units in the extract, washes, and "walls."

TABLE 7. *Treatment of cell walls with borate buffer and Triton X-100*[a]

Fraction	Invertase units	
	Borate buffer	Triton X-100
Suspended walls	100%	100%
Supernatant fluid from		
First treatment	10.5	17.0
Second treatment	2.4	
Wall after treatment	58.5	59.2
Lost/gained	−28.6	−23.8

[a] Brei from STA 4 wild type which was grown for 96 hr at 25 C on Vogel's salts and 1.5% fructose.

mycelial brei, providing strong evidence that the defect is not one of enzyme localization.

DISCUSSION

The discovery of a spontaneous invertase mutant in *Neurospora* should be helpful for the study of the biochemistry and function of extracellular enzymes (7). Previous attempts by Metzenberg (8) and ourselves to select mutants of this enzyme directly have been unsuccessful, but our efforts are being continued with improved selection techniques.

Although the data presented do prove that the invertaseless condition results from a single mutation, they do not rule out the possibility that two or more genes are responsible for the stucture of invertase by contributing different polypeptide chains. The presence in vivo of heavy and light forms of invertase that can be interconverted (11) and the ability to produce a variety of immunologically unrelated forms of invertase on treatment with urea, base, salt, and heat suggest that invertase is a complex of several polypeptide chains. Colvin and Braymer (*personal communication*) do in fact have preliminary evidence that a minimum of two different polypeptide chains are found in invertase. The presence of a CRM in the *inv* strain implies that a deletion covering the structural gene(s) for invertase is not the genetic lesion responsible for the invertaseless condition. The defect could be a point mutation or a deletion of one structural gene if more than one gene is responsible for producing the enzyme.

The data presented here on invertase localization extend the interpretation of Metzenberg (9). His results demonstrated that invertase is located between the cell membrane and a barrier to proteolytic enzyme attack on invertase; thus, he proposed that the enzyme is in free solution between the cell membrane and cell wall; i.e. "intramural" (17). As Trevithick and Metzenberg have pointed out (17), their results do not show whether all of the *Neurospora* invertase is in free solution in the intramural space.

Our data (Table 6) demonstrate that a certain portion (25 to 45%) of mycelial invertase is readily solubilized by a variety of techniques and therefore probably does represent an activity in free solution. The experiments also show that there is a portion (55 to 75%) of the activity that remains bound to the cell debris. This activity is presumably bound at the inner surface of the wall, bound within the wall, or mechanically trapped within the fibrillar matrix of the wall. Since lyophilization, freeze-thawing, or treatment with acetone and detergent does not markedly increase the amount of activity solubilized as compared to glass homogenization, a fourth alternative, that the "bound" activity is trapped in solution between the membrane and wall of hyphal fragments, is rendered unlikely. In summary, it appears that *Neurospora* invertase may be found in three different locations: in the medium as a secreted enzyme, in the intramural space as a free enzyme, and bound to or trapped within the cell wall.

Trevithick and Metzenberg (17) have shown that the secreted and intramural enzyme differ, at least with respect to the percentage of heavy and light forms. There is no evidence yet available regarding possible differences between the "bound" and intramural enzyme except for their localization. The inability of borate buffer to solubilize invertase as it does grape invertase (2) implies that *Neurospora* invertase may be bound differently from that found in higher plants, although both types are located within the cell wall and presumably fulfil the same hydrolytic function.

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LITERATURE CITED

1. Arnold, W. N. 1965. β-Fructofuranosidase from grape berries. Biochim. Biophys. Acta **110**:134–147.
2. Arnold, W. N. 1966. β-Fructofuranosidase from grape berries. II. Solubilization of a bound fraction. Biochim. Biophys. Acta **128**:124–129.
3. Atwood, K. C., and F. Mukai. 1955. Nuclear distribution in conidia of *Neurospora* heterocaryons. Genetics **40**:438–443.
4. Campbell, D. H., J. S. Garvey, N. E. Cremer, and D. H. Sussdorf. 1964. Methods in immunology. W. A. Benjamin, New York.
5. Hill, E. P., and A. S. Sussman. 1963. Purification and properties of trehalase(s) from *Neurospora*. Arch. Biochem. Biophys. **102**:389–396.
6. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
7. Marzluf, G. A., and R. L. Metzenberg. 1967. Studies on the functional significance of the transmembrane location of invertase in *Neurospora crassa*. Arch. Biochem. Biophys. **120**:487–496.
8. Metzenberg, R. L. 1962. A gene affecting the repression of invertase and trehalase in *Neurospora*. Arch. Biochem. Biophys. **96**:468–474.
9. Metzenberg, R. L. 1963. The localization of β-fructofuranosidase in *Neurospora*. Biochim. Biophys. Acta **77**:455–465.
10. Metzenberg, R. L. 1963. The purification and properties of invertase of *Neurospora*. Arch. Biochem. Biophys. **100**:503–511.
11. Metzenberg, R. L. 1964. Enzymically active subunits of *Neurospora* invertase. Biochim. Biophys. Acta **89**:291–302.
12. Myrbäck, K. 1957. Studies on yeast invertase. Soluble and insoluble invertase (saccharase) of Baker's yeast. Arch. Biochem. Biophys. **69**:138–148.
13. Prout, T., C. Heubschman, H. Levene, and F. J. Ryan. 1953. The proportions of nuclear types in *Neurospora* heterocaryons as determined by plating conidia. Genetics **38**: 518–529.
14. Ryan, F. J. 1950. Selected methods of *Neurospora* genetics. Methods Med. Res. **3**:51–75.
15. Sargent, M. L., and D. O. Woodward. 1969. Genetic determinants of circadian rhythmicity in *Neurospora*. J. Bacteriol. **97**:861–866.
16. Susskind, S. R., C. Yanofsky, and D. M. Bonner. 1955. Allelic strains of *Neurospora* lacking tryptophan synthetase: A preliminary immunochemical characterization. Proc. Natl. Acad. Sci. U.S. **41**:577–582.
17. Trevithick, J. R., and R. L. Metzenberg. 1966. Molecular sieving by *Neurospora* cell walls during secretion of invertase isozymes. J. Bacteriol. **92**:1010–1015.
18. Vaughan, D., and I. R. MacDonald. 1967. Development of soluble and insoluble invertase activity in washed storage tissue slices. Plant Physiol. **42**:456–458.