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Regeneration of Invertase in *Neurospora crassa*

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In *Neurospora*, invertase is predominately an extracellular enzyme, and acid phosphatase is partially external in location. Both extracellular invertase and acid phosphatase were rapidly and quantitatively inactivated by acid treatment (pH 1.3). When such acid-treated cells were incubated with a suitable carbon source, a substantial regeneration of invertase activity occurred, but no restoration of acid phosphatase could be detected. The regeneration of invertase does not occur by renaturation of the inactivated enzyme, nor by secretion of a preexisting intracellular pool of invertase, but instead requires de novo enzyme synthesis. Invertase synthesis was partially repressed by glucose and mannose and was completely inhibited by 2-deoxyglucose. Acetate was found to inhibit invertase regeneration and the transport and incorporation of uracil and leucine. Several potential inhibitors of transcription, including alpha-amanitin, 5-fluorouracil, actinomycin D, and three derivatives of rifamycin, were ineffective in preventing invertase regeneration and in inhibiting the synthesis of ribonucleic acid. Conidia appeared to be very poorly permeable to these compounds.

The enzyme invertase of *Neurospora crassa* is primarily localized outside of the membrane of the cell. Metzenberg (12) showed that the enzyme of whole cells is accessible to substrate and that it is also inactivated by brief acid treatment, which does not impair conidial viability nor affect intracellular enzymes such as alkaline phosphatase. Chang and Trevithick (4) found that about 8% of the conidial invertase is actually entrapped within the cell wall, whereas most of the invertase is presumed to be free within the periplasmic space or associated with the outside of the plasma membrane.

When conidia are briefly treated at pH 1.3, the extracellular invertase is rapidly and completely inactivated. When such invertaseless conidia are incubated under certain conditions, however, a regeneration of extracellular enzyme occurs long before germination takes place (8). This reappearance of extracellular invertase could result from enzyme renaturation or the secretion of preexisting intracellular enzyme, or it might require de novo enzyme synthesis. In the last case, invertase biosynthesis could proceed by translation of a stable messenger ribonucleic acid (RNA); on the other hand, enzyme regeneration might require both transcription and translation. These possibilities are examined in this paper. Acid phosphatase, an enzyme that is also partially extracellular in location, was also inactivated by the same acid treatment. However, no regeneration of acid phosphatase was observed under conditions where substantial invertase regeneration occurred.

MATERIALS AND METHODS

Neurospora strains. The wild-type strain 74-OR23-1A was provided by the Fungal Genetics Stock Center, Humboldt State College, Arcata, Calif. Conidia were obtained from petri plates containing Fries minimal salts plus sucrose (1.5%) solidified with agar (1.5%). After growth for 6 to 7 days at 25 C, the conidia were harvested, suspended in water, and filtered through glass wool. The concentration of conidia was determined by measuring the turbidity of suspensions at 420 nm.

Inactivation of conidial invertase. Conidia suspended in distilled water at 4 C were mixed with an equal volume of cold 0.1 N hydrochloric acid and kept for 10 min in an ice bucket, when sufficient sodium acetate or sodium succinate was added to bring the pH to 5. Fructose was usually added to a final concentration of 10 mM. For invertase regneration, the treated conidia were then incubated at 37 C for various time intervals, after which cycloheximide was added.

Enzyme assays. Invertase assays were conducted as described by Metzenberg (11). To assay alkaline phosphatase, samples of conidia were incubated at 37 C in 0.05 M tris(hydroxymethyl)aminomethane-hydrochloride buffer, pH 8.0, containing 5 mM *p*-nitrophenol phosphate and 1 mM $MgSO_4$ for 15 min. The reaction was stopped by addition of 0.5 ml of 2.0

N NaOH, the conidia were sedimented by centrifugation, and the amount of free *p*-nitrophenol released was determined by measuring the absorbance at 405 nm. Acid phosphatase was assayed in a similar manner but with 30 min of incubation at pH 5.0 in succinate buffer. The activity of each enzyme was reported as the net change in absorbency observed at the appropriate wavelength under the conditions described.

Uracil transport and incorporation. Samples of conidia or mycelia were incubated with 0.1 ml of uracil-2-^{14}C (2 μCi/ml) in a total volume of 1 ml at 37 C with shaking for 0, 30, and 60 min, except that 10-min uptake periods were used in some cases. The amount of uracil transported was determined by collecting the conidia on glass fiber filters and washing them well with water containing 0.1 mg of nonradioactive uracil per ml. The amount of uracil which had been incorporated into macromolecules was determined by adding 2 ml of 95% ethyl alcohol to similar incubation mixtures, which were then heated at 80 C for 30 min; 3 ml of cold 10% trichloroacetic acid was then added. After standing at 4 C for 30 min, the precipitates were collected on prewashed membrane filters (1.2-μm pore size; Millipore Corp., Bedford, Mass.) and washed well with cold 5% trichloroacetic acid containing 0.1 mg of nonradioactive uracil per ml. The filters were placed in vials and counted with 10 ml of dioxane-based scintillation fluid in a Beckman LS-120 scintillation counter.

Leucine transport and incorporation. Samples of conidia or mycelia were incubated with 0.1 mM ^{14}C-leucine (2 μCi/ml) for 0, 30, and 60 min at 37 C in a total volume of 1 ml. Leucine uptake was measured by collecting the conidia on glass fiber filters and washing them with a solution of nonradioactive leucine. The amount of leucine incorporated into protein was determined by stopping identical samples with 4 ml of 12.5% trichloroacetic acid. The samples were heated for 30 min at 80 C, when the precipitate was collected on a membrane filter and washed four times with 5-ml portions of cold 5% trichloroacetic acid.

Chemicals. Uracil-2-^{14}C (7.2 μCi/μmol) and leucine-*U*-^{14}C were purchased from New England Nuclear Corp., Boston, Mass. Three derivatives of rifamycin were kindly provided by Luigi Silvestri of Gruppo Lepetit, Milan, Italy. The structures of the rifamycin derivatives are given in reference (10). Actinomycin D was a gift from Merck, Sharp, and Dohme Research Laboratories, Rahway, N.J., and anisomycin was provided by Pfizer Co., Inc., Brooklyn, N.Y. T. Wieland, of the Max Planck Institute, Heidelberg, Germany, kindly provided a sample of alpha-amanitin. All other compounds were of reagent grade and purchased from common commercial sources.

RESULTS

Invertase regeneration. When conidia whose extracellular invertase had been quantitatively inactivated by acid treatment were subsequently incubated at 37 C under the proper conditions, considerable regeneration of the enzyme occurred. Regeneration required the presence of a carbon source (Table 1). Fructose, galactose, sucrose, and trehalose allowed much enzyme regeneration, while glucose and mannose permitted a decreased level of regeneration, but still an amount substantially above that observed in the absence of any supplement. On the other hand, 2-deoxyglucose completely inhibited the regeneration of invertase. Fructose was routinely used to obtain an optimal amount of enzyme regeneration. The regenerated extracellular enzyme was found to be firmly associated with the conidia; all of the activity remained with the cells after centrifugation or when they were collected on membrane filters. The enzyme is presumed to be trapped between the cell membrane and the cell wall.

Although invertase regeneration proceeded well in succinate buffer at pH 5, it completely failed to occur in acetate buffer under otherwise identical conditions. Acetate not only inhibited invertase regeneration but also prevented the uptake and incorporation of both uracil and leucine (Table 2). The acetate inhibition was not readily reversible because cells which had been incubated in acetate for 60 min and then washed free of the inhibitor were still unable to

TABLE 1. *Effect of different carbon sources on invertase regeneration*[a]

Carbon source	Invertase regeneration (enzyme activity)	Fructose level (%)
None	0.183	12
Galactose	1.693	111
Sucrose	1.789	118
Trehalose	1.751	115
Fructose	1.521	100
Glucose	0.696	46
Mannose	0.462	30
2-Deoxyglucose	0.034	2
None + cycloheximide	0.004	1
Fructose + cycloheximide	0.005	1

[a] Acid-treated conidia were neutralized with succinate and incubated for 60 min with the indicated sugars, each at a final concentration of 20 mM. Enzyme regeneration was stopped by the addition of cycloheximide, and the conidia were collected on 1.2-μm membrane filters and washed well to remove the sugars. The cells were then suspended in succinate buffer containing cycloheximide (0.1 mM) and assayed for invertase. Control experiments showed that no interference in the invertase assay resulted from accumulation of sugars during the prior incubation period.

TABLE 2. *Invertase regeneration and leucine and uracil transport in acetate and in succinate*[a]

Determination	Activity observed	
	In acetate	In succinate
Invertase regeneration ...	0.020	1.485
Leucine transport	627	33,850
Leucine incorporation ...	40	27,145
Uracil transport	487	27,961
Uracil incorporation	37	3,541

[a] Acid-treated conidia were neutralized with either sodium acetate (0.13 M) or sodium succinate (0.06 M) to pH 5. Identical samples were utilized for invertase regeneration, leucine transport and incorporation, and uracil transport and incorporation for 60 min as described in Materials and Methods. Invertase regeneration is expressed as enzyme activity; all other values are net counts per minute determined in each assay.

regenerate the enzyme after resuspension in succinate buffer.

Inhibition by cycloheximide. The regeneration of invertase was completely inhibited by the addition of 0.1 mM cycloheximide. Anisomycin, another inhibitor of protein biosynthesis in eukaryotes, also completely inhibited invertase regeneration. When regeneration was allowed to proceed for various intervals, the addition of cycloheximide immediately prevented further increases in the amount of enzyme. These results imply that protein biosynthesis is required for invertase regeneration (5). The question immediately arises, however, as to whether it is invertase per se that is synthesized during the regeneration process, or whether the secretion or a preexisting intracellular pool of invertase requires the synthesis of another protein involved somehow in the secretion step.

Intracellular invertase. Conidia which had been acid-treated to destroy external invertase and untreated, normal conidia were examined for their content of intracellular enzyme by incubation with the polyene antibiotic tyrocidine. This antibiotic is known to cause the formation of roughly spherical holes, approximately 12.5 nm in diameter, in steroid-containing membranes (3). These membrane lesions permit ready permeation by low-molecular-weight substrates, allowing direct assay of intracellular enzymes in whole cells.

The constitutive alkaline phosphatase of conidia is almost exclusively localized within the permeability barrier; only about 3% of the total enzyme activity, which is near the limit of sensitivity of the assay, could be detected with untreated conidia (Table 3). Treatment of the conidia with tyrocidine or with 50% dimethylsulfoxide revealed the presence of a large complement of cryptic alkaline phosphatase, although tyrocidine was by far the most efficient in this regard and was, therefore, utilized for subsequent studies with invertase.

Normal conidia have only approximately 10% of their total invertase in an intracellular location; the remainder is apparently all outside the permeability barrier (Table 4). When normal conidia were simply incubated for 60 min, a substantial increase (60 to 70%) in both the intracellular and extracellular enzyme was observed. This increase in the amount of invertase was prevented by the presence of cycloheximide, although no turnover of the original activity occurred.

When acid-treated conidia were examined, it was found that the external invertase was completely absent and some of the intracellular enzyme was missing. Acid-treated conidia have a very limited quantity of intracellular invertase, which cannot nearly account for the restoration of enzyme that occurs during regeneration (Table 4). Indeed, during the regeneration process an increase in both the internal and external enzyme levels occurred. Therefore, it is

TABLE 3. *Exposure of cryptic alkaline phosphatase of conidia*[a]

Treatment	Alkaline phosphatase activity
Expt 1	
DMSO	
0%	0.043
10%	0.116
20%	0.119
30%	0.240
40%	0.556
50%	0.740
60%	0.471
Expt 2	
Control	0.050
Tyrocidine (50 μg/ml)	1.710
Tyrocidine (100 μg/ml)	1.695

[a] In the first experiment, samples (1 ml) of a conidial suspension (optical density at 420 nm = 4.0) were incubated with dimethylsulfoxide (DMSO) at the indicated final concentrations in a total volume of 2 ml for 30 min at 30 C. The cells were washed with water twice, resuspended in 1 ml of water, and assayed for alkaline phosphatase. In the second experiment, 1 ml of a similar conidial suspension was incubated with tyrocidine at the final concentrations shown for 30 min at 37 C, when assays for alkaline phosphatase were conducted.

clear that regeneration does not occur by secretion of preexisting intracellular invertase, but that it represents de novo synthesis of the enzyme.

Acid phosphatase. The major fraction of acid phosphatase is internal, although a significant fraction, approximately 25%, of this enzyme is extracellular in location (Table 5). After conidia were incubated for 60 min in the presence or absence of cycloheximide, no change was observed in the amount of extracellular enzyme, indicating that neither new synthesis nor turnover was taking place during this period. A slight decrease (14%) of intracellular acid phosphatase was observed during this same period, suggesting that a limited turnover of the internal form might take place. When conidia were subjected to the usual acid

TABLE 4. *Distribution and regeneration of invertase in normal and acid-treated conidia*[a]

Sample	Total enzyme	External enzyme		Internal enzyme	
		Amt	%	Amt	%
Normal conidia					
Control	8.996	8.060	90	0.936	10
Incubated	14.235	12.610	89	1.625	11
Incubated + cycloheximide	9.529	7.996	84	1.533	16
Acid-treated conidia					
Control	0.492	0.071	14	0.421	86
Incubated	5.247	4.009	76	1.238	24
Incubated + cycloheximide	0.655	0.093	14	0.562	86

[a] Wild-type conidia were collected, and one portion was subjected to acid treatment as described in Materials and Methods. Fructose was added to normal and treated cells to a final concentration of 10 mM. In each case, samples of control cells were maintained at 4 C, while identical samples were incubated for 60 min at 37 C in the presence and absence of cycloheximide. After the incubation period, cycloheximide was added to all samples to prevent any invertase synthesis during subsequent steps. To determine the total enzyme content, cells were incubated with tyrocidine (100 μg/ml) for 30 min at 37 C before invertase was assayed. External invertase activity was assayed in identical samples of whole cells which were not treated with tyrocidine. Internal invertase activity was calculated as total enzyme minus the extracellular activity. All values given were corrected by use of blanks which were treated in the same way except that the invertase assay was terminated immediately upon addition of the substrate. In duplicate experiments, even less external invertase (0.030) was detectable in acid-treated cells.

TABLE 5. *Distribution of acid phosphatase in normal and acid-treated conidia*[a]

Sample	Total enzyme	External enzyme		Internal enzyme	
		Amt	%	Amt	%
Normal conidia					
Control	1.235	0.272	22	0.958	78
Incubated	1.077	0.280	26	0.797	74
Incubated + cycloheximide	1.074	0.271	25	0.803	75
Acid-treated conidia					
Control	0.729	0.047	6	0.682	94
Incubated	0.679	0.034	5	0.645	95
Incubated + cycloheximide	0.689	0.049	7	0.640	93

[a] Wild-type conidia were collected, and one portion was subjected to the usual acid treatment. Fructose was added to both normal and treated cells to a final concentration of 10 mM. Samples of control cells were maintained at 4 C while identical samples were incubated for 60 min at 37 C. A third series of samples was incubated for the same period in the presence of cycloheximide. Following the incubations, cycloheximide was added to all samples to prevent any enzyme synthesis during subsequent steps. Total acid phosphatase content was assayed in cells treated with tyrocidine. External enzyme was determined in untreated cells, and the internal enzyme was calculated as total enzyme minus the extracellular activity. All values were corrected by use of blanks which were treated in the same way except that the phosphatase assay was terminated immediately upon the addition of substrate. Assays were conducted as described in Materials and Methods.

treatment, the extracellular acid phosphatase was almost completely inactivated, but only a slight loss of the internal enzyme occurred. No restoration of the extracellular acid phosphatase was observed under conditions where substantial invertase regeneration was taking place.

Inhibitors of RNA synthesis. It was of interest to determine whether invertase regeneration in treated conidia depended upon the synthesis of messenger RNA or could proceed with preexisting messenger; a number of potential inhibitors of transcription were used to investigate this question. Neither invertase regeneration nor RNA synthesis was inhibited by alpha-amanitin (20 μg/ml), 5-fluorouracil (5 mM), or actinomycin D (50 μg/ml) even after preincubation for 30 min with these compounds. Nor did preincubation of conidia with 0.5% ethylenediaminetetraacetic acid (10 min at 37 C in phosphate buffer) render either RNA synthesis or invertase regeneration sensitive to inhibition by actinomycin D. These results strongly suggest a very poor penetration of actinomycin D through either the cell membrane or the nuclear membrane, as noted by others (15, 18, 20, 22, 23), because effective local concentrations even lower than 5 μg/ml would be expected to cause almost complete inhibition of transcription.

Rifamycin is a potent inhibitor of RNA synthesis in bacteria but is not effective in eukaryotes. However, certain rifamycin derivatives have been found to inhibit RNA synthesis in higher organisms (10). Accordingly, three rifamycin derivatives, rifamycin AF-013, rifamycin AF-05, and rifamycin AF-ABDP-cis, were tested to see whether they would inhibit transcription in *Neurospora*. None of these compounds significantly inhibited the incorporation of uracil by conidia. Furthermore, conidia were found to germinate and grow at 25 or 37 C in medium containing each of these inhibitors (100 μg/ml); therefore, it is concluded that they are ineffective as inhibitors of transcription in whole cells of *Neurospora*. The failure of these agents to inhibit RNA synthesis in conidia is probably a permeability problem (19), because they are each quite effective in inhibiting all or certain of the three RNA polymerase species of yeast (2) and animals cells (10) when tested in vitro.

DISCUSSION

Chang and Trevithick (4) observed that mid-log-phase mycelia have a 10-fold greater specific activity of invertase than do conidia. In this paper it was demonstrated that, when conidia are simply incubated with a carbon source, approximately a 60% increase in invertase activity occurs within 1 h. Since the increase in invertase was prevented by cycloheximide, it apparently represented de novo enzyme synthesis. This synthesis had to utilize endogenous substrates and demonstrates that conidia were already in a highly active metabolic state long before germination occurred. The transport systems for phenylalanine (19), thymidine, and deoxyadenosine (14) of conidia were also found to increase dramatically long before germination, showing approximately a 100% increase in 1 h.

The use of tyrocidine and similar related polyene antibiotics to reveal intracellular enzymes in *Neurospora* is a valuable technique. Scott and Metzenberg (16) showed that tyrocidine was efficient in exposing a cryptic compartment of aryl sulfatase in conidia. In the present study, it was observed that pretreatment of cells with dimethylsulfoxide, which makes alpha-glucosidase available for assay in yeast (1), partially exposed intracellular alkaline phosphatase, but not nearly as thoroughly as did tyrocidine. Further evidence that tyrocidine acts at the membrane level was provided by the observation that it completely prevented both sulfate accumulation and the retention of preexisting pools of intracellular sulfate in both conidia and mycelia (Marzluf, unpublished data).

The present findings have shown that invertase, acid phosphatase, and the constitutive alkaline phosphatase differ markedly in their cellular distributions, displaying approximately a 90, 25, and 2% extracellular localization, respectively. Acid phosphate, however, has been shown to consist of two distinct forms (21), and it would be interesting to know their individual distributions within the cell.

Acid treatment was shown to destroy external invertase and acid phosphatase almost completely and also to cause loss of some of the intracellular activity of each of these enzymes. When such treated conidia were incubated in the presence of a suitable carbon source, considerable invertase was regenerated, but no restoration of acid phosphatase activity was found. The regeneration of extracellular invertase was clearly not due to a reassociation of enzyme subunits or other type of renaturation of the inactivated enzyme, because protein synthesis was required for this process. Nor did the regeneration of extracellular invertase occur by the secretion of preexisting intracellular enzyme, but rather involved considerable de novo synthesis of invertase. Our results showed that

cycloheximide not only inhibited synthesis of additional invertase but also prevented any secretion of the intracellular enzyme still possessed by the treated conidia. This result suggests that the processes of synthesis and secretion may be coupled in some manner, or that the synthesis of some accessory protein may be required for invertase secretion.

Acetate was found to inhibit invertase regeneration and the transport of uracil, leucine, and glucose completely (8). This result suggests that it has a general cellular effect, perhaps exerted at the membrane level. It has been suggested that acetate mimics the effect of dinitrophenol in *Bacillus subtilis*, perhaps by uncoupling oxidative phosphorylation (17), a mechanism that possibly could also explain its various effects in *Neurospora*. The complete inhibition of invertase regeneration by 2-deoxyglucose is interesting, particularly in view of the suggestion (6, 7) that this sugar analogue may prevent the synthesis of the carbohydrate moiety of mannan proteins in yeast protoplasts. In such protoplasts, 2-deoxyglucose prevented the appearance of invertase and acid phosphatase, which are both mannan proteins, but not the appearance of alkaline phosphatase, which is not. *Neurospora* invertase is also a glycoprotein and was found to contain about 11% mannose and 3% glucosamine (9, 13). It has been suggested that the carbohydrate moiety of glycosylated proteins may determine the extracellular fate of the protein (24).

It has been difficult to obtain a clear answer concerning whether or not invertase regeneration requires transcription, presumably to produce a messenger RNA which encodes invertase. The various potential inhibitors of RNA synthesis tested were found to be unsatisfactory for studies with *Neurospora* conidia, apparently because of their poor permeability. Because mannose and glucose depress invertase regeneration and because the synthesis of invertase is also known to be controlled by repression involving these same sugars (10), this can be viewed as indirect evidence that invertase regeneration requires transcription. However, the repression of invertase synthesis exerted by glucose and mannose is only presumed to act at the level of transcription and conceivably could be involved in translational control.

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LITERATURE CITED

1. Adams, B. G. 1972. Method for decryptification of α-glucosidase in yeast with dimethyl sulfoxide. Anal. Biochem. **45**:137-146.
2. Adman, R., L. D. Schultz, and B. D. Hall. 1972. Transcription in yeast: separation and properties of multiple RNA polymerases. Proc. Nat. Acad. Sci. U.S.A. **69**:1702-1706.
3. Bard, M. 1972. Biochemical and genetic aspects of nystatin resistance in *Saccharomyces cerevisiae*. J. Bacteriol. **111**:649-657.
4. Chang, P. L. Y., and J. R. Trevithick. 1972. Distribution of wall-bound invertase during the asexual life-cycle of *Neurospora crassa*. J. Gen. Microbiol. **70**:23-29.
5. Horowitz, N. H., H. M. Feldman, and P. L. Pall. 1970. Derepression of tyrosinase synthesis in *Neurospora* by cycloheximide, actinomycin-D, and puromycin. J. Biol. Chem. **245**:2784-2788.
6. Kuo, S. C., and J. O. Lampen. 1972. Inhibition by 2-deoxy-D-glucose of synthesis of glycoprotein enzymes by protoplasts of *Saccharomyces*: relation to inhibition of sugar uptake and metabolism. J. Bacteriol. **111**:419-429.
7. Liras, P., and S. Gascon. 1971. Biosynthesis and secretion of yeast invertase. Eur. J. Biochem. **23**:160-165.
8. 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.
9. Meachum, Z. D., H. J. Colvin, and H. D. Braymer. 1971. Chemical and physical studies of *Neurospora crassa* invertase. Molecular weight, amino acid and carbohydrate composition, and quaternary structure. Biochemistry **10**:326-331.
10. Meilhac, M., A. Tysper, and P. Chambon. 1972. Animal DNA-dependent DNA polymerases 4. Studies on inhibition by rifamycin derivatives. Eur. J. Biochem. **28**:291-300.
11. Metzenberg, R. L. 1962. A gene affecting the repression of invertase and trehalase in *Neurospora*. Arch. Biochem. Biophys. **96**:468-474.
12. Metzenberg, R. L. 1963. The localization of β-fructofuranosidase in *Neurospora*. Biochim. Biophys. Acta **77**:455-465.
13. Metzenberg, R. L. 1963. The purification and properties of invertase of *Neurospora*. Arch. Biochem. Biophys. **100**:503-511.
14. Schiltz, J. R., and K. D. Terry. 1970. Nucleoside uptake during the germination of *Neurospora crassa* conidia. Biochim. Biophys. Acta **209**:278-288.
15. Schneider, R. P., and W. R. Wiley. 1971. Transcription and degradation of messenger ribonucleic acid for a glucose transport system in *Neurospora*. J. Biol. Chem. **246**:4784-4789.
16. Scott, W. A., and R. L. Metzenberg. 1970. Location of aryl sulfatase in conidia and young mycelia of *Neurospora crassa*. J. Bacteriol. **104**:1254-1265.
17. Sheu, C. W., W. N. Konings, and E. Freese. 1972. Effects of acetate and other short-chain fatty acids on sugar and amino acid uptake of *Bacillus subtilis*. J. Bacteriol. **111**:525-530.
18. Subramanian, K. N., and G. J. Sorger. 1972. Regulation of nitrate reductase in *Neurospora crassa*: regulation of transcription and translation. J. Bacteriol. **110**:547-553.
19. Tisdale, J. H., and A. G. DeBusk. 1970. Developmental regulation of amino acid transport in *Neurospora crassa*. J. Bacteriol. **104**:689-697.
20. Tisdale, J. H., and A. G. DeBusk. 1972. Permeability problems encountered when treating conidia of *Neurospora crassa* with RNA synthesis inhibitors. Biochem.

Biophys. Res. Comm. **48**:816–822.
21. Toh-E, A., and T. Ishikawa. 1971. Genetic control of the synthesis of repressible phosphatases in *Neurospora crassa*. Genetics **69**:339–351.
22. Totten, R. E., and H. B. Howe. 1971. Temperature-dependent actinomycin-D effect on RNA synthesis during synchronous development in *Neurospora crassa*. Biochem. Genet. **5**:521–532.
23. Turner, J. R., K. Terry, and W. H. Matchett. 1970. Temporal separation of transcription and translation in *Neurospora*. J. Bacteriol. **103**:370–374.
24. Winterburn, P. J., and C. F. Phelps. 1972. The significance of glycosylated proteins. Nature (London) **236**:147–151.