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Acetate-nonutilizing Mutants of *Neurospora crassa*

I. Mutant Isolation, Complementation Studies, and Linkage Relationships

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Sixty mutants of *Neurospora crassa* unable to grow on acetate as sole source of carbon, but able to utilize sucrose, were isolated. On the basis of complementation tests, they were divided into seven groups, each group representing a different gene. Six of the genes have been mapped; no two are closely linked. These loci have been designated *acu-1* to *acu-7*. Mutations at four of these loci result in poor germination of ascospores.

Many microorganisms, when growing on acetate as the sole source of carbon, use the glyoxylate cycle as well as the Krebs cycle to maintain a supply of energy and precursors for the biosynthesis of cellular components. When sucrose or glycolytic intermediates are present, however, the glyoxylate cycle is not essential (6). The two enzymes of the glyoxylate cycle that are not also part of the Krebs cycle, namely, isocitrate lyase and malate synthase, as well as the acetate-activating enzyme acetyl-coenzyme A (CoA) synthetase, are usually regulated so that they are present in induced (derepressed) amounts on acetate but in low (repressed) amounts in the presence of sucrose or glycolytic intermediates (6). Thus, these enzymes are suitable for a study of enzyme regulation. Such a study has been made in the bacterium *Escherichia coli* (5), but no similar investigation, combining the genetic with the biochemical approach, has been reported for a fungus. As a first step it seemed desirable to isolate mutants that were deficient in functions required for growth on acetate but not on sucrose. This paper reports the isolation of such mutants, their division into functional groups, and their linkage relationships to other mutants of *Neurospora crassa*. (The accompanying paper describes the effects of these mutations on the activities of glyoxylate shunt and Krebs cycle enzymes.)

MATERIALS AND METHODS

Strains. The mutations were induced in the ST 74*A* wild-type strain and in an inositol-requiring strain *inos* (R233-6-8)*A* derived by backcrossing *inos* (R233) six times to ST 74*A*. The single mutant strains *ad-7* (Y175 M236)*a*, *asp* (C 123)*a*, *arom-3* (C 163)*a*, and the triple mutant *leu-2* (37501) *pan-1* (5531) *mat* (B57) *a* were kindly supplied by the Fungal Genetics Stock Centre, Dartmouth College, Hanover, N.H. The *arg-5* (27947)*a*, *arg-1* (46004)*a*, *arg-10* (B 317)*a*, and *pyr-3* (KS-48)*a* strains were from the collection maintained in this laboratory, and the double mutant *cys-1* (84605) *ylo* (Y30539y)*a* and "alcoy" strains were supplied by Noreen Murray. "Alcoy" is a stock carrying three reciprocal translocations involving six of the seven linkage groups, with a visible marker at or near each interchange point (8). Its full description is T(1;II) *al-1* (4637 T); T(IV;V) 2355 *cot* (C 102); T(III;VI) 1, *ylo* (Y30539y)*a*.

Media. Vogel's minimal medium (15) was supplemented with 1.5% sucrose ("sucrose medium") or with acetate stock solution (1 M sodium acetate adjusted to *p*H 5.8 with 1 M acetic acid) to a final concentration of 40 mM acetate ("acetate medium") Solid medium contained 1.5% agar. Amino acid supplements were added at a final concentration of 25 mg/100 ml of medium and inositol at 0.2 mg/100 ml of medium, unless stated otherwise. To induce colonial growth on solid medium, Vogel's minimal medium was supplemented with 0.2% sucrose and 0.9% sorbose (13; "sorbose medium"). Crosses were made on the medium described by Westergaard and Mitchell (17) supplemented as appropriate.

Ultraviolet light (UV) treatment. A 5-ml amount of 5- to 10-day-old conidia (10^7 conidia/ml of sterile water) was exposed in a petri dish for 225 sec to 1,300 ergs per cm^2 per sec from a low-pressure mercury lamp at a distance of 16 cm. This killed approximately 90% of the conidia.

N-methyl-N'-nitrosoguanidine (NG) treatment. A similar conidial suspension was shaken at 30 C for 30 min wth 100 μg of NG per ml. This killed approximately 85% of the conidia. The conidia were then

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centrifuged, and the pellet was suspended in sterile water.

Filtration enrichment. The method used was essentially that described by Woodward, De Zeeuw, and Srb (18). The mutagen-treated ST 74*A* conidia were shaken at 30 C in 50 ml of acetate medium for 130 hr, and the suspension was filtered through cheesecloth whenever visible growth appeared. The whole suspension was then mixed with 100 ml of sorbose medium containing 0.5% agar and poured onto a solid layer of sorbose medium containing 1.5% agar, previously set in 12 × 12 inch (30.5 × 30.5 cm) plates. These were constructed from 12 × 12 inch sheets of plate glass, which formed the top and bottom, and 0.5 × 0.5 inch (1.3 × 1.3 cm) nylon strips which were fixed to the bottom with silicone grease to form the sides. Survivors were isolated after incubation for 36 hr at 30 C.

Mutant enrichment by inositol-less death. A modification of the method described by Lester and Gross (7) was used. The mutagen-treated inositol-requiring conidia were shaken for 6 hr at 30 C in 7 ml of acetate medium containing 25 μg of inositol per ml. After being harvested and washed three times by centrifugation and resuspension in sterile water, the conidia were shaken in 5 ml of acetate medium without inositol for 120 hr. They were then plated in sorbose medium containing inositol, as described for filtration enrichment.

Growth tests. All survivors of the mutant enrichment procedures were tested for their ability to grow in 2 ml of liquid acetate medium containing inositol. Presence or absence of growth was scored after 2 days of incubation at 30 C. Samples of those strains which showed no growth were tested in a more quantitative manner. Approximately 2 × 10^6 conidia of each isolate, and of ST 74*A* and *inos* (R233-6-8)*A* as controls, were inoculated into duplicate 100-ml flasks containing 25 ml of sucrose plus inositol medium, and into similar duplicate flasks containing 25 ml of acetate plus inositol medium. The cultures were incubated without agitation at 25 C for 70 hr, harvested, dried in an oven at 60 C for 24 hr, and weighed.

Complementation tests. Samples (2 ml) of molten agar-sucrose medium containing inositol were set in 10 × 1 cm tubes (not sloped) arranged in a standard matrix. Drops (0.05 ml) of dense conidial suspension of the mutants to be tested were added in the appropriate pairwise combinations to the tubes, which were then incubated at 25 C until many new conidia had formed on the mycelial growth. These new conidia (of which some from each culture were presumed to be heterokaryotic) were then tested for their ability to grow in 2-ml lots of acetate medium containing inositol. The tests were scored for growth after incubation for 48 hr at 30 C. Tubes containing two drops of the same suspension served as controls.

Crossing procedure. The method used was as described by Catcheside (1).

Cross analysis, method 1. Ascospores suspended in sterile water were heat-shocked at 59 C for 35 min and spread onto selective media on which only certain progeny classes could grow. After incubation for 16 to 20 hr at 25 C, the ascospores were examined at 40× magnification and classified as growing, germinated but not growing, or nongerminated.

Cross analysis, method 2. Heat-shocked ascospores were plated on a medium on which all progeny should grow and were incubated at 25 C for 6 to 7 hr. A random sample of germinated ascospores was picked off, transferred to slopes of a similar medium, and incubated further until conidia were abundant. Conidia from each isolate were then tested for their ability to grow in selective liquid media.

Analysis of "alcoy" crosses. The method used was that detailed by Perkins (8).

Mating-type tests. These tests were made and scored as described by Smith (10).

RESULTS

Mutant isolation. Sixty acetate-nonutilizing mutants were obtained from a series of isolation experiments. The proportion of such mutants among the survivors ranged from 15 to 40% after inositol-less death and from 1.3 to 3.7% after filtration enrichment. Two to three times as many mutants were recovered in experiments where NG was used as the mutagen instead of UV. Each of these mutants was given an isolation number preceded by the letters JI.

Growth tests. Many of the mutants were tested quantitatively for their ability to grow on acetate and sucrose media, both supplemented with inositol. Some of the results are given in Table 1. Since mutants from all complementation groups (*see* Fig. 1) were tested, the results show that a

TABLE 1. *Growth responses of acetate-nonutilizing mutants on Vogel's minimal medium containing inositol with sucrose or acetate as carbon source*

Strain (JI isolation no.)	Complementation group (*acu* no.)	Growth on sucrose (dry wt)[a]	Growth on acetate (dry wt)[a]
		mg	*mg*
ST74*A*		75.2	18.1
inos-6-8*A*		76.6	14.3
22	3	72.6	0[b]
38		58.5	0
48[c]	1	61.0	0
14	2	62.3	0
17	4	73.2	Trace[d]
18	5	63.2	0
31	6	58.0	0
49[c]		70.0	0
45[c]	7	71.6	

[a] The dry weights are averages from duplicate flasks.

[b] Zero denotes no visible growth beyond germination of conidia.

[c] These strains still contain the *inos* mutation as they were isolated by inositol-less death.

[d] Trace denotes too little to harvest and dry (<1 mg).

mutation in any one of these seven cistrons is capable of preventing growth on acetate medium without substantially affecting growth on sucrose.

Complementation tests. On the basis of the growth tests on heterokaryotic conidia, the mutants tested were arranged into seven groups (Fig. 1). Each of these groups was considered to consist of mutants of a single cistron. The corresponding loci have been termed *acu* loci, since the genes corresponding to these loci enable acetate to be utilized for growth. The loci are distinguished from one another by the use of a number following the *acu* description (e.g., *acu-1*); the number assigned to each group is shown in Fig. 1. It is important that these mutants are not confused with those mutants of *N. crassa* that *require* acetate for growth, which are termed *ac* mutants (16). *Acu-6* is the only one of the *acu* loci within which allelic complementation was observed. Nine of the 17 mutants at this locus showed complementation; the mutants can be divided into three classes and represented by a linear complementation map in the now conventional way (2).

Mapping. Representatives from each complementation group were crossed to the reciprocal

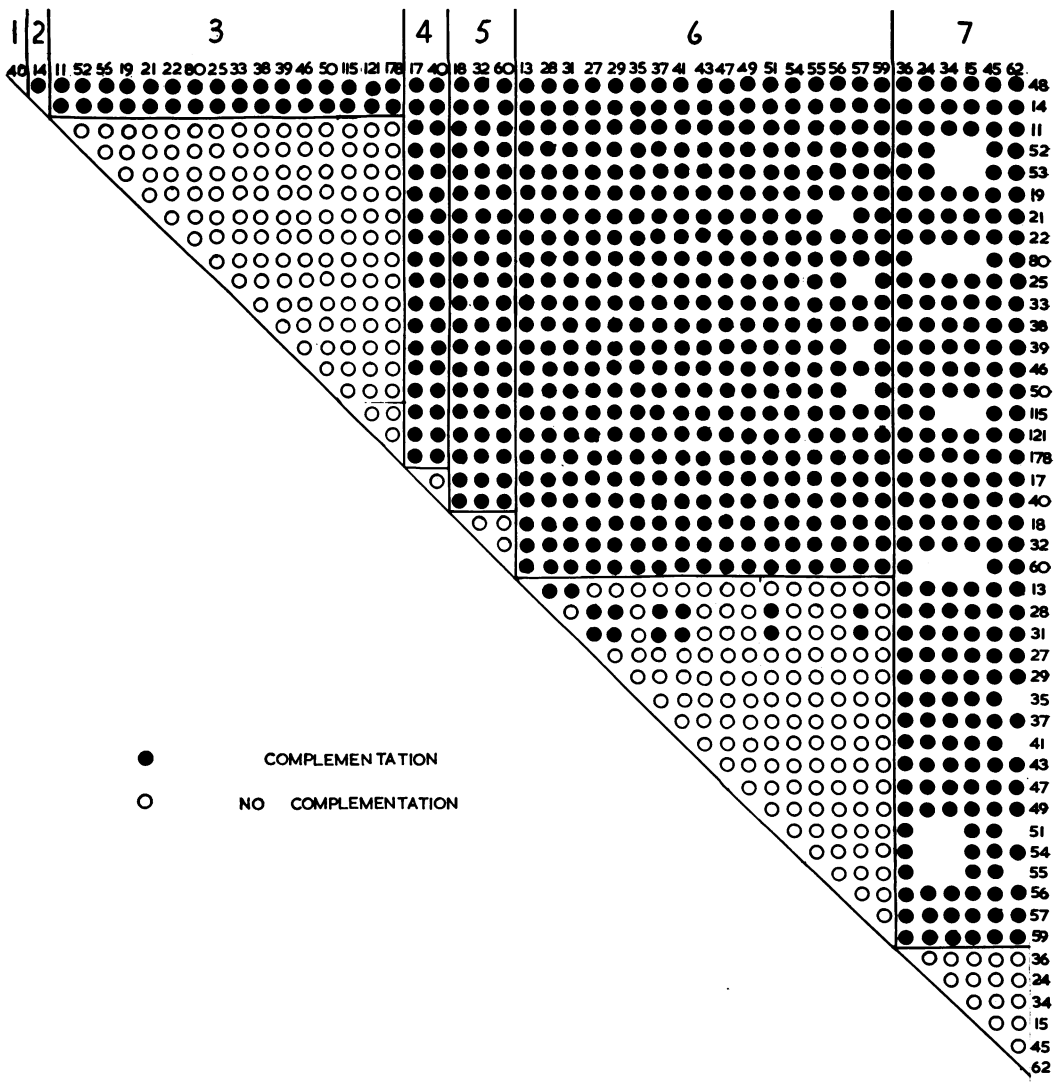

FIG. 1. *Results of complementation tests. The mutants have been arranged into seven groups on the basis of their ability to complement other mutants. Each mutant is shown by its JI isolation number. The acu number assigned to each group is shown at the top of the matrix.*

translocation stock "alcoy." All loci, except *acu-7*, showed linkage to one of the "alcoy" markers. These linkage relationships were subsequently confirmed by the analysis of crosses between each *acu* mutant and genetic markers in the two linkage groups marked by the relevant mutation in "alcoy." The results of these crosses are shown in Tables 2 and 3. Since *acu-7* (JI 36) showed no linkage to any "alcoy" marker, the locus presumably was either in linkage group VII or at the extreme left end of group I, III, IV, or V (8). The results of crosses of *acu-7* (JI 36) with a marker in group VII and with markers within 50 map units of the ends of groups I and IV are also shown in Tables 2 and 3.

The results in Table 2 show that *acu-3* (JI 11),

TABLE 2. *Analysis by method 2 of progeny from crosses between acu mutants and known genetic markers*

Cross	Linkage group of marker	Progeny genotypes	No. observed	Conclusions [% recombination between *acu* locus and marker(s)]
(1) *acu-3*(JI 11)*A* × *pyr-3* (KS-48)*a*	IVR	*acu-3* +[a] + *pyr-3* + + *ace-3 pyr-3*	1 37 39 0	*acu-3* and *pyr-3* not linked (51%)[b]
(2) *acu-3*(JI 11)*A* × *inos*-(R233)*a*	VR	*acu-3* + + *inos* + + *acu-3 inos*	1 73 18 1	*acu-3* linked to *inos* (20%)[b]
(3) *acu-7*(JI 36)*A* × *arg-10*(B317)*a*	VIIR	*acu-7* + + *arg-10* + + *ace-7 arg-10*	2 33 36 0	*acu-7* and *arg-10* not linked (51%)[b]
(4) *acu-1*(JI 48)*inos*(R233-6-8)*A* × *pyr-3 a*	IVR(*pyr-3*) and *VR*(*inos*)	+ + *pyr-3* *acu-1 inos* + + *inos* + *acu-1* + *pyr-3* + *inos pyr-3* *acu-1* + + + + + *acu-1 inos pyr-3*	12 10 5 4 10 9 15 18	*acu-1* linked to *inos* (34%) but not to *pyr-3* (51%)
(5) *acu-1*(JI 48)*inos*(R233-6-8)*A* × *asp a*	VR	+ *asp* + + *inos asp* + *inos* + + + +	26[c] 7 5 1	*acu-1-inos* (31%) *acu-1-asp* (15%) *asp-inos* (21%) Probable order: *acu-1 asp inos*
(6) *acu-2*(JI 14)*A* × *leu-2 pan-1 mat a*	IVR	+ *pan-1 leu-2 mat* + *pan-1 leu-2* + + *pan-1* + *mat* + *pan-1* + + + + *leu-2 mat* + + + + + + + *mat* + + *leu-2* +	62[d] 14 7 1 0 1 1 3	*acu-2-pan-1* (6%) *acu-2-leu-2* (11%) *acu-2-mat* (21%) *pan-1-mat* (20%) *leu-2-mat* (28%) *leu-2-pan-1* (12%) Linkage in probable order: *leu-2 acu-2 pan-1 mat*
(7) *acu-4*(JI 17)*A* × *arg-1 a*	I	+ *arg-1 a* + + *a* + + *A* + *arg-1 A*	18[c] 1 6 4	*acu-4-arg-1* (24%) *acu-4*-mating type (35%) *arg-1*-mating type (17%) Linkage in probable order: *acu-4 arg-1 mat*

[a] Plus denotes the wild-type allele.
[b] Recombination values based only on *acu*$^+$ progeny since *acu* progeny were nonviable.
[c] Only *acu*$^+$ progeny scored.
[d] No *acu-2* progeny were isolated from this cross.

TABLE 3. *Analysis by method 1 of progeny from crosses between acu mutants and known genetic markers*

Cross	Linkage group of marker	Supplement to acetate + inositol medium	Progeny scored			Germination (%)	*acu*⁺ progeny among germinated spores (%)	Estimated no. of *acu*⁺ spores germinated	Estimated % recombination (prototrophs/estimated total *acu*⁺)
			Non-germinated	Germinated but not growing	Growing				
acu-3(JI 11)*A* × *ad-7a*	VR	Adenine	356	74	144	39	66		
		—	353	242	9	41		166	5.4
acu-3(JI 11)*A* × *asp a*	VR	Asparagine	552	72	176	31	71		
		—	1,317	530	25	30		394	6.3
acu-5(JI 18)*A* × *arg-5 a*	IIR	Arginine	41	357	325	94	50[a]		
		—	64	933	30	94		481	6.2
acu-5(JI 18)*A* × *arom-3 a*		—	12	920	35	99	50[b]	477	7.4
acu-6(JI 49)*inosA* × *cys-1 ylo a*	VII	Tyrosine + methionine	231	30	204	50	87		
		—	632	674	12	52		598	2.0
acu-2(JI 14)*A* × *pyr-3 a*	IVR	Uridine	520	3	180	26	98		
		—	616	178	15	24		189	7.9
acu-7(JI 36)*A* × *arg-1 a*	IL	Arginine	271	179	583	74	76.5		
		—	350	486	292	69		595	49
acu-7(JI 36)*A* × *pyr-3 a*	IVR	Uridine	290	130	292	59	69		
		—	894	266	181	53		308	58.8

[a] Assumed 50% since 357 and 325 are not significantly different and *acu* spores are not expected to germinate better than *acu*⁺.

[b] Assumed.

acu-7 (JI 36), and *acu-2* (JI 14) progeny from sexual crosses are almost completely nonviable. The poor viability of thes and also *acu-6* (JI 49) progeny is also evident in Table 3, since in each case progeny growing on supplemented acetate plus inositol medium greatly outnumbered those which had germinated but were not growing. Even those presumed *acu-2*, *acu-3*, *acu-6*, and *acu-7* ascospores which did germinate (*see* Table 3) frequently failed to grow further after transfer to sucrose medium, which explains the almost total lack of *acu* progeny in the data of Table 2. Similar crosses with other alleles at these loci have shown similar viabilities, so the phenomenon is locus-specific rather than allele-specific. Recombination frequencies were calculated from the data of Tables 2 and 3. Where the *acu* progeny tended to be nonviable, recombination frequencies were calculated on the basis of the acetate-utilizing progeny only. It was assumed in making the calculations from the data of Table 3 that the frequency of *acu* spores among those germinated was the same on both supplemented and unsupplemented acetate medium. This frequency was calculated from the results for supplemented medium, and, together with the data for unsupplemented medium, was used to calculate the proportion of acetate-utilizing recombinants among the germinated acetate-utilizing progeny.

The observed linkage relationships enabled six of the seven *acu* loci to be located on a linkage group. The *acu-7* (JI 36) locus showed no linkage to *arg-10* in group VII or to markers in groups I and IV. These results, and the results of the cross with "alcoy," suggest that *acu-7* is probably at the extreme left end of group III or V.

DISCUSSION

The data of Table 1 confirm that the mutants selected as unable to grow on acetate grow at a more or less normal rate on sucrose. Such mutants would be expected to have deficiencies in the central area of metabolism concerned with

energy production and the synthesis of precursors for many cellular components. The biochemical analyses in the accompanying paper confirm this.

Although conidia of all *acu* mutants germinate and grow on sucrose medium, ascospores bearing a mutation at the *acu-2*, *acu-3*, *acu-6*, or *acu-7* locus usually either fail to germinate on sucrose or acetate medium or, if they produce a germ tube, often fail to grow further. Attempts to improve germination and growth of these ascospores by supplementing with Krebs cycle intermediates and related compounds have proved unsuccessful (*unpublished data*). Mutations at the loci *acu-1*, *acu-4*, and *acu-5* do not appear to affect germination.

Lipid and trehalose have been shown to be the major sources of carbon and energy for ascospore germination (12). Correlation of the germination characteristics and biochemical deficiencies in *acu*-mutants also suggests strongly that lipid utilization, involving acetyl-CoA as an intermediate, is a key process in ascospore germination. Consistent with this is the fact that mutants of the *acu-5* group, which are defective in acetyl-CoA synthesis from free acetate (*see* following paper), have normal ascospore germination; utilization of free acetate is not expected to be involved in utilization of lipid reserves. If trehalose were utilized throughout germination along with lipid, then at no time would the spore be utilizing acetate as sole source of carbon and the *acu* mutations would presumably be of no crucial consequence. However, trehalose breakdown appears to occur chiefly during the first 2 hr after spore activation (12), whereas lipid breakdown continues until germination is complete. The importance of lipid in the later stages is also suggested by the observation that even those mutant ascospores which germinate often fail to reach the stage when growth can continue by using the carbon supplied in the external medium. A further observation pertinent to this discussion on ascospore germination is that *suc* mutants (11), which cannot grow on sucrose without a Krebs cycle dicarboxylic acid supplement, germinate well. These mutants can utilize acetate as well as wild type (*personal observations*).

The study of conidiation by Turian and Matikian (14) has shown that this morphological change is accompanied by an increase in isocitrate lyase activity and aerobic respiration. Mutants at all the *acu* loci conidiate well on sucrose, indicating that the deficiencies in the *acu* mutants are not critical for conidia formation under conditions where they do not limit growth.

Although mutants that were being selected in this work were likely to have deficiencies associated with respiration, it is of interest that no cytoplasmically inherited mutants were isolated.

Insufficient three-point crosses with close outside markers were performed to locate all the *acu* loci accurately, but it is clear that no two of them are closely linked; with the exception of *acu-1* and *acu-3*, which are quite far apart on linkage group V, and the possible exception of *acu-7*, which has not been assigned to a linkage group, all the loci are on different chromosomes. This, then, is yet another example in a fungus of genes that are concerned with related metabolic functions being unlinked, in contrast to the frequent finding of close linkage of metabolically related genes in bacteria (4). This example seems of special interest in that two of the *acu* genes control enzymes which appear to be formed coordinately over a wide range of conditions (3).

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LITERATURE CITED

1. CATCHESIDE, D. G. 1964. Brief comments on heterokaryosis and crossing methods. Neurospora Newsletter **6**:17–18.
2. CATCHESIDE, D. G., AND A. OVERTON. 1958. Complementation between alleles in heterokaryons. Cold Spring Harbor Symp. Quant. Biol. **23**:137–140.
3. FLAVELL, R. B., AND J. R. S. FINCHAM. 1968. Acetate-nonutiliing mutants of *Neurospora crassa*. II. Biochemical deficiencies and the roles of certain enzymes. J. Bacteriol. **95**:1063–1068.
4. HOROWITZ, N. H. 1965. The evolution of biochemical synthesis—retrospect and prospect. p. 15–23. *In* V. Bryson and H. J. Vogel [ed.], Evolving genes and proteins. Academic Press, Inc., New York.
5. KORNBERG, H. L. 1966. The role and control of the glyoxylate cycle in *Escherichia coli*. Biochem. J. **99**:1–11.
6. KORNBERG, H. L., AND S. R. ELSDEN. 1961. The metabolism of C_2 compounds in micro-organisms. Advan. Enzymol. **23**:401–470.
7. LESTER, H. E., AND S. R. GROSS. 1959. Efficient method for selection of auxotrophic mutants of Neurospora. Science **129**:572.
8. PERKINS, D. D. 1964. Multiple interchange stocks for linkage detection. Neurospora Newsletter **6**:22.
9. SINGLETON, J. R. 1953. Chromosome morphology and the chromosome cycle in the ascus of *Neurospora crassa*. Am. J. Botany **40**:124–144.
10. SMITH, B. R. 1962. Mating type tests using a plating technique. Neurospora Newsletter **1**:14.
11. STRAUSS, B. S. 1957. Deficiency of oxaloacetate decarboxylase in *suc* mutants of Neurospora. J. Biol. Chem. **225**:535–544.

12. SUSSMAN, A. S. 1966. Dormancy and spore germination, p. 733–769. *In* G. C. Ainsworth and A. S. Sussman [ed.], The fungi, vol. 2. Academic Press, Inc., New York.
13. TATUM, E. L., R. W. BARRATT, AND V. M. CUTTER 1949. Chemical induction of colonial paramorphs in Neurospora and Syncephalostrum. Science **109**:509–511.
14. TURIAN, G., AND N. MATIKIAN. 1966. Conidiation of *Neurospora crassa*. Nature **212**:1067–1078.
15. VOGEL, H. J. 1964. Distribution of lysine pathways among fungi: evolutionary implications. Am. Naturalist **98**:435–446.
16. WEIN, J., D. C. APPLEBY, AND P. S. LEIN. 1951. Acetate formation in Neurospora studied with biochemical mutants. Arch. Biochem. Biophys. **34**:72–80.
17. WESTERGAARD, M., AND H. K. MITCHELL. 1947. Neurospora. V. A synthetic medium favouring sexual reproduction. Am. J. Botany **34**:573–577.
18. WOODWARD, V. W., J. R. DE ZEEUW, AND A. M. SRB. 1954. The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia, followed by filtration and selective plating. Proc. Natl. Acad. Sci. U.S. **40**:192–200.