STUDIES WITH PURPLE ADENINE MUTANTS IN NEUROSPORA CRASSA. IV. LACK OF COMPLEMENTATION BETWEEN DIFFERENT ad-3A MUTANTS IN HETEROKARYONS AND PSEUDOWILD TYPES

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IN Neurospora a useful method of screening for alleles in a series of mutants with identical biochemical requirements is the test for complementation in heterokaryons. Mutants that complement in such tests and enable the heterokaryon to grow on minimal medium are considered nonallelic and are assumed to result from mutation at different genetic loci; whereas mutants that do not complement are considered allelic and are assumed to result from mutation at the same locus. The simplicity of this test has been somewhat complicated by the demonstration of allelic complementation at a number of loci (MITCHELL and MITCHELL 1956; FINCHAM and PATEMAN 1957; GILES, PARTRIDGE and NELSON 1957; CASE and GILES 1958a,b; CATCHESIDE and OVERTON 1958; LACY and BONNER 1958; LACY 1959; WOODWARD, PARTRIDGE, and GILES 1958). The identity of such complementing allelic mutants is not completely obscured by this interaction; however, such complementation is usually quite different from that obtained with nonallelic mutants. The latter grow at wild-type rate, whereas the former generally grow more slowly. The identity of complementing allelic mutants is also apparent in intercrosses since prototrophs are recovered at very low frequencies (10^{-3} or lower) or not at all, and the origin of such prototrophs shows no strict correlation with an exchange of markers in adjacent regions. In the analysis of *ad-3* mutants (DE SERRES 1956) quite different results were obtained. Whereas at other loci only a small proportion of the mutants show allelic complementation (see CATCHESIDE and OVERTON 1958), all the *ad-3* mutants studied were capable of apparent allelic complementation and all the heterokaryons grew at wild-type rate. Moreover, the origin of prototrophs in crosses between members of the two complementing groups of mutants showed a definite correlation with marker exchange. Such evidence was interpreted as indicating that these two groups of *ad-3* mutants were not identical, and that in this region purple adenine mutants resulted from mutation of two separate but closely linked loci (*ad-3A* and *ad-3B*) rather than one. In more comprehensive analyses of the mutants at each locus, no further evidence for complementation was found in any pairwise combination within each series.

Recently the accumulation of many new *ad-3* mutants has made it possible to investigate the phenomenon of complementation in this region in more detail.

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The data presented here are from a series of experiments to test for complementation among 13 different *ad-3A* mutants. Tests were made in forced and unforced heterokaryons and in pseudowild types (PWT's).

Strains

The *ad-3A* mutants A1-A10 and *ad-3B* mutant B17 were derived from the St. Lawrence wild-type strain 74A and are isogenic. Mutants A4-A10 were supplied by DR. MARY E. CASE, Yale University. The strains of *ad-3A* mutants 38701, 38709, and 68306 (BEADLE and TATUM 1945) were selected F_2 progeny from backcrosses to 74A. All 13 *ad-3A* mutants were classified as alleles on the basis of preliminary heterokaryon tests with *ad-3A* and *ad-3B* tester strains and their behavior in crosses with various *ad-3B* mutants (to be presented in detail in a subsequent paper). The *hist-2 ad-3A*(2) *nic-2* strain has been described previously (DE SERRES 1956).

The symbols used to refer to series of nonallelic mutant strains with the same biochemical requirement (i.e., *ad-1*, *ad-2*, *ad-3*, *ad-4*, etc.—adenine-requiring) are those proposed by BARRATT, NEWMEYER, PERKINS, and GARNJOBST (1954). The modified symbols *ad-3A* and *ad-3B* have been used to indicate further complexity within an apparently allelic series (DE SERRES 1956). Individual mutant designations which denote the position of a given mutant in the *ad-3* region used interchangeably in this paper are as follows: *ad-3A*(1) or A1; *ad-3A*(38701) or 38701; *ad-3B*(2) or B2, etc. The symbols used to designate mutant strains with other biochemical requirements are as follows: *hist*, histidine-requiring; *nic*, niacin-requiring; *pan*, pantothenate-requiring; *inos*, inositol-requiring; *pyr*, pyrimidine-requiring; and *am*, α-amino nitrogen-requiring.

Heterokaryon analyses

Heterokaryon tests with genetically unmarked strains: Initially, tests for complementation among the 13 *ad-3A* alleles were made with genetically unmarked strains as described previously (DE SERRES 1956). Heavy conidial suspensions ($1–5 \times 10^8$ conidia/ml) from each mutant were combined in all possible combinations, two at a time, on petri plates containing minimal agar. Combinations with *ad-3B* mutant B17 were included to ensure that all 13 *ad-3A* strains were of the appropriate genotype with reference to genes controlling heterokaryon formation and maintenance (GARNJOBST 1955). Within 24 hours at 27° C all *ad-3A* + *ad-3B* combinations formed heterokaryons with wild-type linear growth rate, which showed that all strains were fully compatible. All *ad-3A* combinations, however, were negative even after 5–7 days' incubation. Beyond this time in control experiments in which inoculum size and other aspects of the experimental procedure were varied, wild-type growth was observed with certain *ad-3A* combinations. At times both the heterokaryon and one of the homokaryotic controls grew, thus implicating reversion rather than allelic complementation. But, when no growth of either homokaryon was apparent, it was necessary to test for the possibility of reversion and the presence of adenine-independent nuclei. In such cases the tests were always positive, clearly indicating that such delayed growth

was caused by reversion of one of the homokaryons rather than allelic complementation. In a number of replicate experiments genetically unmarked strains of these 13 mutants were used, and there was no evidence of allelic complementation in any pairwise combination.

Since the mutants used in these tests were genetically unmarked and the presence of two different *ad-3A* mutant nuclei in the same cytoplasm cannot be readily proved, the possibility was considered that such negative evidence might be inconclusive. In theory, negative tests could also result from (1) some type of protoplasmic incompatibility (GARNJOBST and WILSON 1956); (2) disproportionate nuclear ratios attributable to inadequate nuclear mixing (PITTENGER and ATWOOD 1956); or (3) insufficient interaction between presumably defective gene products to produce functionally active enzyme (GILES, PARTRIDGE and NELSON 1957; FINCHAM and PATEMAN 1957; WOODWARD, PARTRIDGE and GILES 1958). To test these alternative interpretations of the negative results, a more comprehensive series of experiments was planned using forced heterokaryons between genetically marked strains. Such marked strains permit identification of different *ad-3* mutants in the same cytoplasm as well as precise control of nuclear ratios.

Selection and tests with genetically marked strains: Each of the 14 *ad-3* strains and wild-type strain 74A was crossed to a double mutant strain (319-OR5-8a) *inos* (inositol) *pan-2* (pantothenate) to obtain mating type A *inos* and A *pan-2* (referred to hereafter as *inos* and *pan*) derivatives of each mutant containing the same factors controlling heterokaryon formation and maintenance. From the cross of 74A, *inos* and *pan* strains were set up as standards and *ad-3 inos* and *ad-3 pan* derivatives of each mutant were tested with these two standard strains in forced heterokaryons on minimal medium in growth tubes to ensure that all derivatives were fully compatible. Linear growth rates of such forced heterokaryons were compared with the heterokaryon *inos* + *pan* and isogenic wild type strains of both mating types (74A and 74-OR8-1a). The data in Table 1 indicate that all such heterokaryons grow at wild type rate and that the *inos* and *pan* derivatives of each mutant have the same heterokaryon factors. The tests also show the complete dominance of the *ad-3* wild type alleles over each of the 14 *ad-3* mutant alleles used in the present experiments in heterokaryons.

Tests for complementation between genetically marked strains on minimal medium: To test for complementation in *ad-3A* + *ad-3A'* combinations, conidial suspensions of equivalent concentration of each of the 15 *pan* derivatives and the 15 *inos* derivatives were mixed two at a time on minimal medium supplemented with 0.1 mg of adenine/liter to establish forced heterokaryons in each of the 225 combinations. Conidia from each heterokaryon were tested in liquid minimal medium and in adenine-supplemented medium. Growth on the adenine-supplemented medium confirmed the presence of heterokaryotic macroconidia in each heterokaryon. Growth on minimal medium indicated complementation between the *ad-3* combinations. Since the average nuclear number per conidium is reduced by growing strains of Neurospora on minimal medium (HUEBSCHMAN 1952), the nuclear ratio of genetically different *ad-3A* mutant nuclei in heterokaryotic conidia should be approximately equal. None of the forced heterokaryons between

TABLE 1

Linear growth rate of forced heterokaryons of genetically marked ad-3 *mutants on minimal medium as compared with various wild-type strains*

Forced heterokaryons	Mean growth rate (cm/hr, 28°–32°C)	Forced heterokaryons	Mean growth rate (cm/hr, 28°–32°C)
A1 I + P	0.47	38701 I + P	0.48
A1 P + I	0.47	38701 P + I	0.47
A2 I + P	0.48	38709 I + P	0.48
A2 P + I	0.47	38709 P + I	0.48
A3 I + P	0.48	68306 I + P	0.48
A3 P + I	0.48	68306 P + I	0.49
A4 I + P	0.48		
A4 P + I	0.48	Controls	Mean growth rate (cm/hr, 28°–32°C)
A5 I + P	0.47	B17 I + P	0.49
A5 P + I	0.48	B17 P + I	0.46
A6 I + P	0.48	I + P	0.47
A6 P + I	0.48	74A	0.48
A7 I + P	0.50	74a	0.48
A7 P + I	0.49		
A8 I + P	0.48		
A8 P + I	0.48		
A9 I + P	0.48		
A9 P + I	0.49		
A10 I + P	0.47		
A10 P + I	0.48		

different *ad-3A* mutants grew on minimal medium, but conidia from all heterokaryons grew on the adenine-supplemented medium. The *ad-3A inos* + *ad-3B pan*, *ad-3A pan* + *ad-3B inos*, *ad-3 inos* + *pan*, and *ad-3 pan* + *inos* combinations also grew on minimal medium. In tests where there is no question of the presence of genetically different *ad-3A* nuclei in the same cytoplasm in almost equal nuclear ratios, no evidence for *ad-3A* complementation was found in any pairwise combination tested.

Tests for complementation on adenine-supplemented medium: The experiments of WOODWARD, PARTRIDGE, and GILES (1958) showed a very precise correlation of the time required for complementation to occur and of the level of complementation with the distance separating a given pair of *ad-4* alleles on a complementation map. Heterokaryons between mutants in adjacent cistrons require more time for the initiation of the complementation reaction and grow more slowly than heterokaryons between mutants separated by a number of cistrons. WOODWARD suggested that complementation results from a random exchange of defective gene products at either the RNA or polypeptide level in the cytoplasm

to yield enzymically active protein. He observed that the time required for growth of slow-growing heterokaryons to commence could be shortened within certain limits by increasing conidial concentration in the inoculum. Since similar variation in conidial concentration (up to about 5×10^8 conidia/ml) was without effect on forced or unforced heterokaryons of the 13 *ad-3A* mutants in the present study, tests of another type were developed to see if such negative results were attributable not to the complete lack of formation of functional enzyme but to formation at a low rate. If the rate of formation barely exceeds the degradation rate, it might permit, at a maximum, only very slow growth. With the use of suboptimal supplementation, the rate of linear growth of *ad-3* heterokaryons can be controlled precisely. The formation of functional enzyme when different *ad-3A* mutant nuclei are present in a common cytoplasm should result in a more rapid linear growth rate than with either mutant type alone. If the growth of heterokaryons containing complementing *ad-3A* mutants is similar to the growth of partial *ad-3B* revertants on minimal medium (DE SERRES 1958), we might expect a long lag period of very slow growth followed by a progressive increase in linear growth rate until wild-type growth rate is finally attained.

The rates of linear growth obtained with *ad-3A* mutants with suboptimal levels of supplementation are given in Figure 1. The range of the growth rates of forced heterokaryons of each strain (e.g., A1 *inos* + A1 *pan*) are given for each level tested. The variation in linear growth rates obtained with different mutants in such tests is the same as that obtained with the same mutant in replicate experiments. With these levels of supplementation, the mycelium also shows a deep purple pigmentation. As the supplement is increased to 80 or 160 mg of adenine/liter, the amount of pigmentation decreases and the mycelium becomes phenotypically wild type in color. If complementation between *ad-3A* mutants is equivalent to complementation between *ad-3A* and *ad-3B* mutants, then in *ad-3A* heterokaryons we would expect a marked increase in growth rate and a decrease in the intensity of pigmentation of the mycelium.

The possibility of complementation between different *ad-3A* mutants was tested by comparing the growth rates of four forced heterokaryons, two heterozygous for a given pair of *ad-3A* mutants and the other two homozygous (e.g., A1 *inos* + A3 *pan* and A3 *inos* + A1 *pan* compared with A3 *inos* + A3 *pan* and A1 *inos* + A1 *pan*). All possible pairwise combinations of the 13 *ad-3A* mutants were tested in duplicate at ~ 30°C. Most of the combinations were tested with supplementation of both 5 and 10 mg of adenine/liter. Representative results of tests made with mutants A1, A2, and A3 over a wider range of supplementation are given in Table 2.

No evidence of complementation was found between any pairwise combination of the 13 *ad-3A* alleles, that would be indicated by either a change in the growth rate expected with a given level of supplementation or by a change in the level of pigmentation of the mycelium. There was no greater variation in the growth rate with a given level of adenine supplementation with forced heterokaryons heterozygous in the *ad-3A* region than with forced heterokaryons homozygous for a given *ad-3A* mutant.

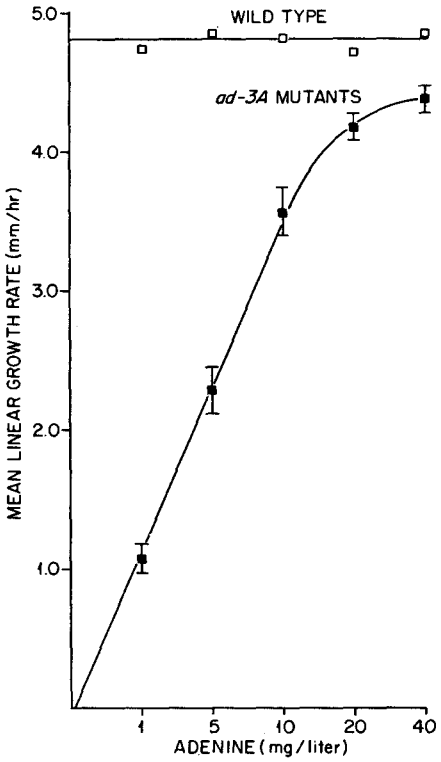


FIGURE 1.—Average linear growth rates of forced heterokaryons homozygous for different *ad-3A* mutants or homozygous for the wild-type allele on minimal medium supplemented with various levels of adenine at 28°–32°C. Vertical lines are 95 percent confidence limits of the mean.

TABLE 2

Linear growth rates of forced heterokaryons heterozygous and homozygous for* ad-3A *mutants A1, A2, and A3 on differentially supplemented media*

Forced heterokaryons	Growth rate with various levels of adenine (mg/liter)				
	1	5	10	20	40
inos + *pan*	0.39	0.39	0.44	0.45	0.45
A1 *inos* + A1 *pan*	0.17	0.24	0.38	0.42	0.42
A2 *inos* + A2 *pan*	0.18	0.27	0.40	0.43	0.45
A3 *inos* + A3 *pan*	0.16	0.26	0.37	0.43	0.41
A1 *inos* + A2 *pan*	0.15	0.28	0.40	0.43	0.42
A2 *inos* + A1 *pan*	0.15	0.26	0.40	0.44	0.44
A1 *inos* + A3 *pan*	0.15	0.23	0.40	0.41	0.42
A3 *inos* + A1 *pan*	0.16	0.25	0.40	0.40	0.42
A2 *inos* + A3 *pan*	0.13	0.19	0.35	0.43	0.46
A3 *inos* + A2 *pan*	0.15	0.26	0.35	0.42	0.44

* Rate: centimeters per hour during a 96-hr period at 28°–32°C.

Analysis of ad-3A *crosses*

Another method of detecting interallelic complementation is by formation of PWT's (PITTENGER 1954) in allelic crosses. Inclusion of different allelic mutants in the same nucleus by nondisjunction of homologous chromosomes and the eventual breakdown of the disomic into a heterokaryon has been a particularly useful and sensitive test for complementation in the analysis of *pyr-3* mutants (MITCHELL and MITCHELL 1956) and the *am* locus (PATEMAN and FINCHAM 1958). CASE (personal communication) found in the analysis of *pan-2* mutants that the PWT test is a more sensitive test for complementation than the heterokaryon test, especially in detecting complementation between mutants very closely linked on a complementation map. This is especially significant with the *pan-2* system, since all of the mutants were induced in wild-type strain 74A or essentially isogenic derivatives, and it does not seem that the difference in sensitivity can be attributed to small differences in genetic background that might affect heterokaryon formation and maintenance.

Crosses with a marked strain of mutant ad-3A*(2):* Various difficulties in the analysis of interallelic crosses of *ad-3A* or *ad-3B* mutants have been discussed previously (DE SERRES 1956). In brief, whereas *ad-3A* × *ad-3B* crosses are fully fertile, interallelic crosses produce very few perithecia and ascospores. In addition, about 90 percent of the ascospores are sterile and even with a number of crosses of the same type, the large populations of viable ascospores necessary for an adequate analysis of such crosses can be obtained only with considerable difficulty.

Because of these crossing problems, the analysis was focused on crosses of the 13 *ad-3A* mutants to a marked strain of mutant A2 (a *hist-2 ad-3A*(2) *nic-2*). The presence of closely linked adjacent markers (*hist-2* about two units to the left and *nic-2* about three units to the right) permits identification of individual components of any PWT's formed as well as an ordering of individual *ad-3A* mutants, with reference to the markers and mutant A2, if any wild-type progeny are recovered. All crosses were made on liquid synthetic cross medium (WESTERGAARD and MITCHELL 1947) supplemented with 1.0 mg of adenine, 0.1 mg of histidine, and 0.01 mg of niacin per milliliter and two percent sucrose in test tubes with filter paper partitions.

All crosses were analyzed by the overlayering technique of NEWMEYER (1954). It was not possible to include all mutants in this analysis, however, since crosses involving mutants A3 and A9 were sterile, no mature perithecia were formed with those of A3, and there were numerous perithecia but no ascospores in those of A9. Also too few ascospores were formed in the crosses of A6 and A7 and selfings of all mutants for plating analysis. Analysis of the selfing of A2 was made possible only by pooling the contents of a very large number of cross tubes.

Since the majority of ascospores in such crosses are not ejected from the perithecia, they were collected in the following manner: The entire contents of the cross tubes were macerated with a spatula in distilled water, and the macerate was filtered through a series of stainless steel sieves or several layers of cheese-

cloth to remove the coarse debris. Ascospores were then freed from conidia and the remaining fine debris by repeated centrifugation, decanting, and washing. Such a method vastly increased the quantity of ascospores available from a given cross with little or no change in viability.

Aliquots of ascospores of known concentration suspended in a viscous 0.15 percent agar solution were plated both (1) in minimal medium supplemented with histidine, adenine, and niacin to estimate the total viable population and (2) in minimal medium supplemented with histidine and niacin to recover adenine-independent progeny. All adenine-independent progeny were subcultured, tested on differentially supplemented media to determine genotype with reference to the adjacent markers, and then crossed to the appropriate mating type of the marked strain *hist-2 ad-3A*(2) *ad-3B*(35203) *nic-2* (GILES, DE SERRES, and BARBOUR 1957) to distinguish between true and pseudowild-type progeny. Pseudowild types are readily distinguished in the analysis of such crosses since they yield 100 percent adenine-requiring progeny, whereas true wild types give only 50 percent such progeny. In addition, a sample of the adenine-independent progeny from crosses of true wild types was tested to determine if their genotypes were the same as the adenine-independent parental type. Such tests detect pseudowild type progeny resulting from nondisjunction of adenine-independent recombinants and a parental chromosome.

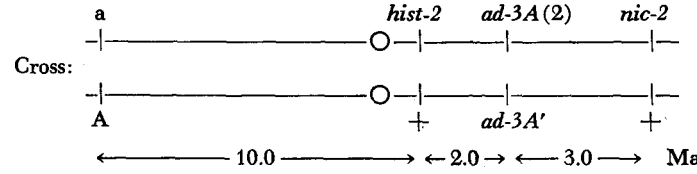
Since *ad-3A* + *ad-3B* combinations show complete complementation, estimates of the frequency of pseudowild type progeny expected in these crosses were obtained from crosses of various *ad-3B* mutants to the same *hist-2 ad-3A*(2) *nic-2* strain (stock culture 74A-YU192-1a) used in the present analysis. Although the analysis of such *ad-3A* × *ad-3B* crosses (DE SERRES unpublished) has shown that crosses of an occasional mutant may have a markedly higher frequency of pseudowild-type progeny than other essentially isogenic allelic mutants, the average expected frequency of such progeny for *ad-3B* mutants induced in a 74A background is one per 16,339 viable progeny and for *ad-3B* mutants of different origin one per 5,256.

The numbers of adenine-independent progeny of both types recovered from individual crosses are given in Table 3. No adenine-independent progeny were recovered from the selfing of mutant A2, but there were a number of adenine-independent progeny in all the other crosses. The frequency of true wild-type progeny show that all mutants are closely linked to A2. However, the marked lack of asymmetry in the numbers of true wild type progeny of crossover genotype (H++ or ++N) makes it impossible to order the mutants with reference to A2. The pattern of marker segregation among the true wild-type progeny in these crosses is quite similar to that obtained by CASE and GILES (1958b) with very closely linked mutants in group B5 of the *pan-2* locus that give a similar frequency of *pan* phototrophs in intercrosses.

Whereas adenine-independent progeny were recovered from all the intercrosses, pseudowild types were found among the progeny of only two crosses. However, neither of the pseudowild type isolates resulted from interallelic complementation between different *ad-3A* mutants. One from the cross A8 × A2,

TABLE 3

Frequency and genotypes of adenine-independent isolates obtained from crosses of ad-3A *mutants with a marked strain of* ad-3A *mutant A2*



Strain	Total viable ascospores × 10^4*	Adenine-independent progeny							
		Pseudowild types		True wild types					
		Expected	Found	No.	Frequency	Genotype			
						H++	++N	H+N	+++
A1	19.4	11.9	0	68	0.035	13	17	12	26
A2	4.8	. . .		0	. . . .				
A4	68.1	41.7	0	104	0.015	17	18	34	35
A5	84.4	51.7	1†	35	0.004	2	5	12	16
A8	107.4	65.7	1†	109	0.010	11	13	32	53
A10	4.7	2.9	0	16	0.034	1	1	7	7
38701	5.2	9.8	0	18	0.035	4	2	4	8
38709	3.0	5.7	0	9	0.029	3	3	3	0
68306	6.9	13.0	0	17	0.025	4	6	4	3

* Pooled data from several experiments on the same cross.
† See text for discussion.

which tested originally as + + + with reference to markers, was actually a disomic resulting from nondisjunction of a niacin-requiring recombinant with an A8 parental chromosome. The other pseudowild type, also + + +, was recovered from the cross A5 × A2. In this case, nondisjunction involved a nonrequiring recombinant (+ + +) and an A5 parental chromosome. The + + + chromosome carried a new morphological mutation, presumably of spontaneous origin, which greatly restricted the ultimate size of colonies formed by conidia homokaryotic for the mutation. The recovery of these rare PWT's between recombinant and parental chromosomes among the adenine-independent progeny from these crosses indicates that PWT's involving different *ad-3A* mutants with mutant A2 must have also occurred at the higher frequencies expected. Thus the failure to recover PWT progeny involving different *ad-3A* mutants may be interpreted as a failure to obtain interallelic complementation in any of the combinations tested in this manner.

DISCUSSION

The present experiments with 13 *ad-3A* mutants, although they provide no evidence for interallelic complementation at this locus, do give some answers to the question of the significance of the negative results obtained in heterokaryon tests with genetically unmarked *ad-3* strains. Since essentially all the mutants used in the analysis of the *ad-3* region were induced in the same wild-type strain,

they have the same genes controlling heterokaryon formation and maintenance and should be fully compatible (GARNJOBST and WILSON 1956). In none of the present experiments with forced heterokaryons of *ad-3A* mutants nor in previous experiments with various *ad-3A* + *ad-3B* combinations (DE SERRES 1956) has there been any indication that *ad-3* mutants do not form fully compatible heterokaryons. Thus we have the reassurance that, when conidial mixtures of different genetically unmarked *ad-3* mutants are made on minimal medium, a negative reaction indicates that the mutants are not capable of complementation.

Studies of groups of allelic mutants of diverse origin have shown that interallelic complementation is widespread in Neurospora (see CATCHESIDE and OVERTON 1958). There is no substantial body of data as yet on complementation analyses on primary (produced in the original wild-type strain) mutants of spontaneous origin, but five out of seven secondary (produced in a wild-type revertant) *ad-4* mutants of spontaneous origin are capable of complementation (WOODWARD, PARTRIDGE, and GILES 1958); whereas only 30 percent of the ultraviolet-induced *arg-1* mutants (CATCHESIDE and OVERTON 1958) and 21 percent of the X-ray-induced primary *ad-4* mutants were capable of complementation. Of the *ad-3A* mutants used in the present experiments, A2 and A5 were of spontaneous origin, A1, A3, A4, and A6-A10 were derived from X-irradiated conidia, and mutants 38701, 38709, and 68306 were induced by ultraviolet light (BEADLE and TATUM 1945). Thus it seems highly unlikely that the failure to obtain complementation can be attributed to the origin of the mutants.

Since various *ad-3A* intercrosses with mutant A2 yielded adenine-independent progeny, there seems little doubt that the *ad-3A* region consists of an undetermined number of mutable sites. Failure to obtain interallelic complementation does not necessarily mean that alteration of any one or combination of these sites produces mutants with identical defects. If complementation occurs by dissociation and reassociation of polypeptide chains of enzyme molecules with nonidentical defects to produce nondefective, functionally active enzyme, then the present analysis shows only that the 13 *ad-3A* mutants have some defect in common. Some of these mutants may be capable of complementation but only with *ad-3A* mutants of a type not included in the present analysis.

The most probable explanation for the failure to obtain allelic complementation among the present group of *ad-3A* mutants is the somewhat limited sample size. Although it is possible that complementation cannot occur among *ad-3A* mutants, this point has certainly not been proved in the present analysis. Mutations that occur at points within the *ad-3A* region that might permit complementation could be relatively rare events in themselves or even show a mutagen specificity. We hope to get more conclusive evidence of complementation in the *ad-3* region by increasing the sample size to a couple of hundred *ad-3A* and *ad-3B* mutants and using mutants of different mutagenic origin. This approach has been made feasible by the development of a forward-mutation technique (DE SERRES and KØLMARK 1958) that permits ready identification and recovery of *ad-3* mutants in treated populations of conidia.

SUMMARY

(1) Heterokaryon tests on a series of 13 genetically unmarked *ad-3A* strains of independent origin showed no evidence for interallelic complementation in any pairwise combination.

(2) The possibility that such negative results might be inconclusive was investigated with forced heterokaryon tests on genetically marked strains.

(3) No evidence for interallelic complementation in any pairwise combination of these mutants was found in forced heterokaryons grown on unsupplemented medium or medium supplemented with suboptimal levels of adenine.

(4) The recovery of true wild type progeny from crosses of different *ad-3A* mutants with a marked strain of mutant A2 suggest that the *ad-3A* region consists of an undetermined number of mutable sites, but the lack of asymmetry in marker segregation among the adenine-independent progeny did not permit an ordering of the mutants with reference to mutant A2 and the adjacent markers *hist-2* and *nic-2*.

(5) The failure to recover pseudowild type progeny involving both *ad-3A* parental chromosomes from the fertile intercrosses provides further evidence for the absence of interallelic complementation in these combinations.

(6) The present experiments firmly substantiate the original interpretation of the negative heterokaryon tests with genetically unmarked but isogenic *ad-3* mutants: that, under the experimental conditions used, such results are significant and provide evidence for the absence of complementation.

(7) The present data are consistent with the *ad-3A* locus being a one-cistron locus with an undetermined number of recombinable, mutable sites.

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