STUDIES WITH PURPLE ADENINE MUTANTS IN NEUROSPORA CRASSA. I. STRUCTURAL AND FUNCTIONAL COMPLEXITY IN THE AD-3 REGION[1]

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A LARGE number of adenine-requiring strains with a purple phenotype have now been obtained for study of the problem of the reversibility of X-ray-induced mutations at the *ad-3* locus in *Neurospora crassa*. However, subsequent investigations have shown that these phenotypically similar mutants represent two distinct groups on the basis of physiological (heterokaryon) and genetic (crossing) analyses. In this paper, it will be shown that these groups represent two closely linked loci in the *ad-3* region. Studies on the mutability of X-ray-induced purple adenine mutants will be considered in a subsequent paper.

MATERIALS

New mutant strains

The 24 purple adenine mutant strains used in this investigation were obtained by the "filtration technique" of WOODWARD, DE ZEEUW, and SRB (1954) on untreated or X-irradiated macroconidia of the St. Lawrence wild type strain 74A. The isolates of these mutants used in the present investigation, with the exception of the strains with additional genetic markers, are the originals and not strains extracted from crosses to other mutant or wild type strains. The genetic background of the 24 purple adenine strains may be thus considered essentially identical.

The non-purple adenine strain, E1, was also derived from the St. Lawrence wild type strain 74A. This strain utilizes adenine but not hypoxanthine for growth (N. J. JOHNSON unpublished) and will be referred to as an adenine-specific strain.

Standard mutant strains

The other mutant strains used were: *hist-2* (histidine), C94 (HAAS, *et al.* 1952); *ad-3* (purple adenine), 35203, 38701, 38709, 45601, 68306; and *nic-2* (nicotinic acid), 43002 (BEADLE and TATUM 1945). Isolates of these mutant strains were obtained from the culture collections of PROF. N. H. GILES or DR. P. ST. LAWRENCE of Yale University.

The terminology used to refer to mutant strains is that proposed by BARRATT, NEWMEYER, PERKINS, and GARNJOBST (1954).

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METHODS

The medium used was the standard Neurospora minimal (BEADLE and TATUM 1945). The growth tubes used for the determination of growth rates of the heterokaryons are essentially the same as the tubes closed at one end described by RYAN *et al.* (1943). Crosses were made on synthetic crossing medium (WESTERGAARD and MITCHELL 1947) with the appropriate biochemical supplementation. Random ascospore analyses on crosses were made by the plating methods of NEWMEYER (1954). Heterokaryon tests were made with heavy conidial suspensions of the individual mutant strains. Approximately 0.03 ml of the individual conidial suspensions was placed in spots near the periphery of petri plates previously prepared with 15–20 ml of minimal medium solidified with 1.0 percent agar. Each plate contained control spots of the two strains being tested with each other, and two spots where conidia were mixed and heterokaryon formation could take place. The spots where the conidia were mixed were diametrically opposed so that when a positive heterokaryon test was obtained, the two heterokaryons grew toward each other, and did not rapidly overgrow the control spots. When heterokaryon tests were made on "leaky" strains, which grow to some extent on unsupplemented minimal medium, heterokaryon formation could be detected only by the difference in size of the colony formed on the control spot of the "leaky" strain versus the spots where conidia from both strains were mixed. All plates were incubated at 25°C and results recorded after 24–36 hours.

EXPERIMENTAL

Preliminary studies

Linkage studies with purple adenine mutant 35203 and with genetic markers known to be linked to this mutant (see BARRATT *et al.* 1954) have demonstrated that the *ad-3* locus is located 2 to 3 units to the right of the *hist-2* locus and 2 to 3 units to the left of the *nic-2* locus. Additional crosses of the 24 purple adenine strains (derived from the St. Lawrence wild type strain 74A) with the double mutant strain *hist-2 nic-2*, have shown that, in general, these purple adenine strains have linkage relationships similar to strain 35203. This evidence was interpreted as indicating that the 24 purple adenine strains and strain 35203 represented alterations of the same locus and were, therefore, presumably allelic.

However, it was not feasible to obtain more direct evidence for the allelism of these strains from intercrosses, for, as demonstrated in the original studies of MITCHELL and HOULAHAN (1946) and later studies by G. KØLMARK (unpublished) for mutants 35203, 38701, 38709, 45601, and 68306, various intercrosses and selfings of purple adenine mutants were highly sterile. While different methods were being tried to increase the numbers of viable ascospores from these crosses, attempts were made to obtain preliminary evidence for allelism from heterokaryon tests.

Heterokaryon tests with purple adenine strains derived from strain 74A

Since the 24 purple adenine strains are essentially isogenic, it seemed reasonable to assume that preliminary evidence for allelism of these mutants could be obtained if it

could be demonstrated that, under comparable experimental conditions, the individual purple adenine strains would give positive heterokaryon tests with the non-purple adenine-specific strain, E1, which has an identical genetic background, and negative tests with one or all of the other purple adenine strains.

When such tests were made by the methods described previously, positive heterokaryon tests were obtained not only between each of the purple adenine mutants and the non-purple adenine-specific strain, but also in certain combinations of the purple adenine mutants. In the initial experiment, when all 24 of the purple adenine mutants were tested against one member of this group, all the mutant combinations except three gave positive heterokaryon tests. It appeared that there were two groups of purple adenine mutants, a small group of alleles containing three of the 24 mutants (group A), and a larger group of alleles with 21 mutants (group B). Since the positive heterokaryon tests among the purple adenine mutant strains were unanticipated, an experiment was performed to confirm and extend these observations by testing all 24 purple adenine mutants for heterokaryon formation with each other in all possible combinations. Within twenty-four hours, all the group A mutants had formed heterokaryons with all the group B mutants, and no evidence was obtained for heterokaryon formation with any of the other mutant combinations. In all cases where heterokaryon formation was observed, the duplicate spots where conidia were mixed on each plate were in agreement. With one of the "leaky" purple adenine mutants, which has only a partial requirement for adenine at 25°C, the lag period was sufficient for heterokaryon formation with the three group A mutants to take place at a time when the control spots of the "leaky" mutant showed only slight growth.

On the basis of the results of these heterokaryon tests, the 24 purple adenine strains derived from the St. Lawrence wild type strain 74A can be divided into two physiologically distinct groups: group A—strains A1 through A3; group B—strains B1 through B21.

Heterokaryon tests with the original purple adenine strains

Since no evidence for heterokaryon formation between mutant 35203 and its presumed alleles was obtained in the experiments of MITCHELL and HOULAHAN (1946), backcrosses of these strains were made to obtain isolates that would form heterokaryons with biochemical mutants derived from the St. Lawrence wild type strain 74A. Originally, such isolates were available only for mutant 35203. Heterokaryon experiments on these isolates demonstrated that 35203 gave positive heterokaryon tests only with group A mutants, indicating that strain 35203 is in group B. F_1 isolates of strains 38701, 38709, 45601, and 68306 have since been used to group these mutants. Subsequent heterokaryon experiments on these strains have shown that strains 38701, 38709, and 68306 are in group A, and strain 45601, in addition to strain 35203, is in group B.

Growth rates of group A–group B heterokaryons

No evidence was obtained in the experiments on the purple adenine strains derived from 74A for marked growth rate differences in any of the group A–group B heterokaryons. All the group-A combinations with group-B mutants were similar in behav-

TABLE 1

Growth rates of selected heterokaryons between group A and group B strains as compared with the individual purple adenine strains and with wild type strain 74A. (Linear growth rates on minimal medium and minimal medium supplemented with 0.1 mg of adenine sulfate per ml)*

Strain	Medium	
	Minimal (mm/hr; 25°C)	Minimal + adenine (mm/hr; 25°C)
A1	0	3.4
A2	0	3.3
A3	0	3.2
B10	0	3.6
B10 + A1	3.5	3.6
B10 + A2	3.5	3.5
B10 + A3	3.6	3.6
74A	3.4	3.5

* Each growth rate based on the linear growth made in a 40.5-hour period in triplicate tubes.

ior, the individual heterokaryons were all established at about the same time, and all the plates were overgrown by the heterokaryotic colonies at the same rate. Since the growth rates of these heterokaryons were essentially the same, experiments were performed to compare the growth rates of selected heterokaryons with the parental wild type strain 74A, to determine whether these heterokaryons grew at wild type rate. If the group A–group B mutants were not completely complementary in the heterokaryons formed between them, then the growth rates of these heterokaryons might be less than wild type rate, and approach or equal wild type rate only by supplementation with exogenous adenine or a comparable growth factor.

By the growth tube method described previously, growth rates of individual heterokaryons between mutants A1, A2, and A3 with mutant B10 were compared on minimal medium, and minimal medium supplemented with adenine with the growth rates of the individual mutants and that of wild type. The results of this experiment are presented in table 1. None of the individual strains from either group grew on minimal medium, but heterokaryons formed with these mutants grew at rates comparable to wild type. No evidence was obtained for adenine stimulation of any of the heterokaryons, since the growth rates on minimal medium supplemented with adenine were comparable to those obtained on unsupplemented minimal medium.

Random ascospore analyses of group A × group B mutant crosses

From the evidence obtained in the heterokaryon experiments, it was apparent that purple adenine mutants did not arise from mutation of a single functional unit, but rather from mutation of two functionally distinct units. Evidence from recombination studies, discussed previously, indicated that these mutants were located between the *hist-2* and *nic-2* loci on linkage group I. Since the linkage relationships of the 24 purple adenine strains did not indicate consistent closer linkage of one group of mutants with either of the markers, it appeared possible that the functional units

might correspond to two genes so closely linked that random fluctuations in linkage values obscured the order.

If this were the case, the order of the genes with reference to the markers could be either *hist-2 ad-A ad-B nic-2* or *hist-2 ad-B ad-A nic-2*. Hence, crosses were made with appropriately marked strains to determine whether the two groups were separable by crossing over.

Preliminary attempts with corn meal agar (BEADLE and TATUM 1945) or the Westergaard-Mitchell synthetic crossing medium supplemented with low levels of adenine sulfate (0.05–0.15 mg/ml) to intercross or self purple adenine mutants were not consistent. When ascorpores were formed, they exhibited a low viability. Supplementation of the Westergaard-Mitchell synthetic medium with higher levels of adenine sulfate (0.6–1.0 mg/ml) gave large numbers of viable ascospores when intergroup crosses were made (group A × group B). Intragroup crosses (group A × group A or group B × group B) and selfings, however, did not respond to high levels of adenine or numerous other supplementations that were tried. Even when large numbers of ascopores were obtained in these crosses, under optimal crossing conditions, the viability of the ascospores ranged from three to ten percent.

Crosses of all the individual group B purple adenine strains (except strain 45601) were made, initially, with the triple mutant strain *hist-2* A2 *nic-2* to determine whether adenine-independent isolates could be obtained from such crosses, and to determine whether the origin of adenine-independent isolates was associated with crossing over between the *hist-2* and *nic-2* markers. If adenine-independent isolates occur and are associated with normal crossing over they should be almost entirely of genotypes $+++nic\text{-}2$ or $hist\text{-}2+++$ depending on the linear order of the two groups. When these crosses were analyzed, the majority gave rise to adenine-independent isolates in the limited number of ascospores analyzed in each cross (ca 6,000). Most, or all, adenine-independent isolates from any particular cross were of genotype $+++nic\text{-}2$. No isolates of genotype $hist\text{-}2+++$ were obtained in any of these crosses.

Since most of the group B mutants did give rise to adenine-independent isolates in crosses with the marked A2 strain, and since they were associated in origin with crossing over in one direction only between the linked markers, these data were interpreted as indicating (1) that the two physiological units that give rise to group A and group B purple adenine mutants in the *ad-3* region correspond to two separate, but closely linked genes, and (2) that the linear order of these genes with reference to other selected markers in this region of linkage group I is: *sex hist-2 ad-A ad-B nic-2 me*(35809) (see BARRATT *et al.* 1954).

To confirm the foregoing conclusions, and to obtain estimates of linkage between these genes, additional crosses were made by use of multiple marked strains. Since the crosses of group B strains with the doubly marked A2 strain gave adenine-independent isolates of genotype $+++nic\text{-}2$, it was anticipated that crosses of group A strains with a doubly marked 35203 (group B) strain would give rise to adenine-independent isolates of genotype $hist\text{-}2+++$. The results of more extensive analyses on intergroup crosses of these types are presented in table 2. In each cross there is a marked correlation between the origin of adenine-independent isolates with crossing over in

TABLE 2

Genotypes of adenine-independent isolates obtained from intergroup crosses of purple adenine mutants and estimates of linkage between mutants in each group

Cross	Viable ascospores tested**	No. of adenine-independent isolates	Genotypes of adenine-independent isolates				Percent recombination
			Crossovers		Noncrossovers		
			hist-2 +++	+++ *nic-2*	*hist-2*++ *nic-2*	++++	
A2 × *hist-2* 35203 *nic-2*	16,783	44	44	0	0	0	0.52 ± 0.06
35203 × *hist-2* A2 *nic-2*	38,220	130	0	128	1	1	0.68 ± 0.04
	7,668*	27	0	27	0	0	
A1 × *hist-2* 35203 *nic-2*	16,433	61	61	0	0	0	0.74 ± 0.07
A3 × *hist-2* 35203 *nic-2*	11,883	76	76	0	0	0	1.28 ± 0.10
B10 × *hist-2* A2 *nic-2*	12,840	11	0	10	1	0	0.15 ± 0.02
	21,120*	15	0	12	1	2	

* Data from a different cross of the same mutant strains.

** Viability ranged from 80–90 percent.

one direction between the closely linked markers. In the reciprocal crosses of A2 with 35203, the association of one or the other marker with the origin of adenine-independent isolates is consistent with the order of genes postulated from previous crosses.

In these crosses, as in some of the crosses discussed previously, a few adenine-independent isolates have been obtained of "noncrossover" genotypes (table 2). Each one of these exceptional types was examined genetically, since apparent adenine-independent isolates of these genotypes can originate in a number of ways. A certain fraction of those of genotype ++++ proved to be heterokaryons, which arose presumably either as pseudo-wilds (MITCHELL *et al.* 1952; PITTENGER 1954) or through heterokaryon formation between group A and group B ascospores on the plates. These have been eliminated in the tabulation. In the cross 35203 × A2, the low frequency of these exceptional types is low enough to have arisen from multiple crossover events. In the cross of B10 × 35203, however, other phenomena may be responsible. Some or all of the exceptional adenine-independent isolates from this cross could represent instances of reverse-mutation since these strains are known to be mutable. In addition, the possibility of gene conversion must be considered in view of the recent results of LINDEGREN (1955) and MITCHELL (1955a, b).

Estimates of linkage were made from the total number of adenine-independent isolates obtained in each cross. It might be postulated that some of the apparent differences in linkage between group A and group B mutants result from small chromosome rearrangements or deficiencies. However, if it is assumed that each of the genes in the *ad-3* complex extends over a short segment of the chromosome, the estimates of linkage between mutants in certain intergroup crosses can be interpreted as indicating that the individual mutant strains have arisen by mutation occurring at different sites in each gene. On this basis, if these genes are made up of closely linked, inter-dependent units that share a common function biochemically, each of which can mutate independently of one another, selected interallelic crosses might give rise to adenine-independent isolates.

Although the viability of ascospores obtained from such crosses is still very low owing to crossing difficulties, various interallelic crosses within each of the two groups of physiological alleles have been attempted by using marked strains of A2 or 35203. Preliminary analyses on four of these crosses show that adenine-independent isolates do arise. However, since the numbers of adenine-independent isolates from any particular cross have been small, and since they are of both "parental" and "crossover" genotypes, the mechanism (or mechanisms) by which adenine-independent isolates originate in specific interallelic crosses in the *ad-3* complex remains obscure.

Preliminary evidence for complexity from a serial ascospore analysis on a group A × group B cross

Although analyses of various intergroup crosses by plating methods have indicated that adenine-independent isolates do arise, this method of analysis does not permit the recovery of the presumed complementary crossover product, the double purple adenine mutant. In order to determine whether the double mutant could be recovered from an ascus in which one of the ascospore pairs was adenine-independent, serial ascospore isolations were made from the cross ++ 35203 (group B) + × *hist-2* A2 (group A) + *nic-2* (GILES and DE SERRES unpublished). Although only a portion of this analysis has been completed, some of the results are pertinent to the present analysis. In a total of 325 asci giving complete germination (at least one member of each ascospore pair germinated), five asci have been obtained containing adenine-independent ascospore pairs. In each of these asci, three pairs of ascospores are adenine requiring and the remaining pair is adenine independent. In each case, the genotype of the adenine-independent pair of ascospores is +++*nic-2*, as might be anticipated from the results obtained from ascospore platings of this same cross (table 2).

The objectives of the current analysis of these five asci are to determine whether the presence of both singles and the double mutant can be demonstrated in each ascus by genetic and biochemical techniques.

CONCLUSIONS

Previous evidence from experiments on purple adenine mutants 35203, 38701, 38709, 45601, and 68306 by MITCHELL and HOULAHAN (1946) suggested that these mutants were allelic, since only purple progeny were obtained when these mutants were crossed among themselves. On the basis of this evidence, this locus was subsequently designated the *ad-3* locus by BARRATT *et al.* (1954). Combined evidence from the present study, however, indicates that the *ad-3* locus is not unitary, as was once thought, but rather a complex consisting of phenotypically similar purple adenine mutants that fall into two physiologically distinct groups.

The results of the heterokaryon experiments on 24 purple adenine strains derived from the St. Lawrence wild type strain 74A, and the original purple adenine strains 35203, 38701, 38709, 45601, and 68306, have demonstrated that these mutants can be assigned to one of two groups on the basis of these tests. The analyses of selected heterokaryons between members of each of these groups have shown that these two

groups of mutants have mutually complementary biochemical functions. Furthermore, such heterokaryons grow on minimal medium at the same rate as the parental wild type strain 74A.

The behavior of purple adenine mutants in crosses also indicates the presence of two physiologically distinct groups. Selfings of purple adenine mutants are characteristically highly sterile under all crossing method variations that have been tried. Few ascospores are formed and usually over 90 percent of these ascospores fail to germinate. When various crosses are made among purple adenine mutants, the high sterility characteristic of the selfings is observed only when intragroup crosses are made. Intergroup crosses show the same high germination of ascospores generally found in crosses between unrelated biochemical mutants. It appears, then, that the sterility and low germination of ascospores from crosses of purple adenine mutants is indicative of the physiological identity of the parental strains.

The analysis of intergroup crosses has shown that the two physiologically distinct groups are separable by crossing over. Moreover, in such crosses, the origin of adenine-independent isolates is associated with crossing over in one direction only between closely linked markers. In these crosses, the origin of "wild type" segregants is the same as would be expected from crosses of any two closely linked genes.

Since the purple adenine mutants fall into two groups, which give positive heterokaryon tests and which give rise to adenine-independent type predominantly by crossing over in one direction between closely linked markers, the data seem best interpreted on the basis of two functionally different but closely linked genes. Since mutation of either gene gives rise to phenotypically similar purple adenine mutants, and since these genes are closely linked, it is proposed that the gene loci be designated *ad-3A* and *ad-3B*. Group A mutants, then, are at the *ad-3A* locus and group B mutants at the *ad-3B* locus.

SUMMARY

(1) Heterokaryon experiments have shown that 24 purple adenine mutants derived from the St. Lawrence wild type strain 74A fall into two physiologically distinct groups: group A—A1 through A3; group B—B1 through B21. Additional heterokaryon experiments have shown that of the five original purple adenine strains, 38701, 38709, and 68306 are in group A, and 35203 and 45601 are in group B.

(2) In general, group A and group B strains are mutually complementary in intergroup heterokaryons, and that such heterokaryons grow at wild type rate.

(3) Random ascospore analyses of intergroup crosses have demonstrated that the origin of adenine-independent isolates is associated with crossing over in one direction only between closely linked markers. The physiological groups thus correspond to two functionally different, but closely linked genes. Since mutation of both of these genes gives rise to phenotypically similar purple adenine mutants, it is proposed that the gene loci be designated *ad-3A* and *ad-3B*. Group A mutants result from mutation at the *ad-3A* locus and group B mutants from mutation at the *ad-3B* locus.

(4) The order of these loci in relation to selected markers in linkage group I is as follows: *sex hist-2 ad-3A ad-3B nic-2 al-2*.

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