STUDIES WITH PURPLE ADENINE MUTANTS IN NEUROSPORA CRASSA. III. REVERSION OF X-RAY-INDUCED MUTANTS[1, 2]

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INTEREST in the problem of reversibility of X-ray-induced mutations has been renewed in recent years, owing to results from a number of experiments on selected loci in Drosophila and maize that conflict with results obtained by earlier investigators. Experiments of PATTERSON and MULLER (1930) and TIMOFÉEFF-RESSOVSKY (1932) demonstrated that X-rays can cause reversion of either spontaneous or X-ray-induced recessive mutations at a number of different loci in Drosophila. Their results seemed consistent with the hypothesis that X-ray-induced and spontaneous gene mutations are essentially similar in nature and are caused by intragenic alteration.

Evidence accumulated by STADLER (1941) in maize, however, suggested clear differences between X-ray-induced and spontaneous mutation. A study of the effects of X-irradiation on the *A1* locus (STADLER 1944; STADLER and ROMAN 1948) indicated that X-ray-induced mutations were caused largely, or wholly, by such extragenic alterations as deletions, duplications, or chromosome rearrangements, which only simulate true gene mutation.

LEFEVRE (1950) attempted to induce reverse mutation with X-rays in both germinal and somatic tissue of Drosophila and got the same negative results as STADLER in his experiments with maize. Since LEFEVRE was unable to obtain evidence for reverse mutation, he questioned the reliability of the early reports of X-ray-induced reverse mutation in Drosophila, and concluded that X-ray-induced mutations in Drosophila, as in maize, are not qualitatively similar to spontaneous mutations and that X-radiation is principally, or perhaps wholly, destructive and results in gene loss in both organisms.

GILES and his collaborators (1951, 1955), however, showed that X-rays *can* induce mutations of positive dominant action at the inositol and purple adenine loci in *Neurospora crassa*. Both these papers present evidence that after X-irradiation, reverse mutations were obtained in which normal adenine or inositol syn-

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thesis, interrupted by the original mutation, was restored as a result of genetic changes localized at the purple adenine or inositol loci. However, the mutants used for these studies were induced by ultraviolet radiation or nitrogen mustard, and there still remained some question about the reversibility of X-ray-induced mutants.

The main purpose of this study was to obtain X-ray-induced purple adenine mutants in Neurospora, and to investigate their mutability. If all X-ray-induced mutations that simulate true gene mutations are actually the result of such extragenic alterations as deletions, these changes should be genetically stable. If, however, some X-ray-induced changes result from gene mutation, they should be capable of subsequent change either spontaneously or with further X-irradiation.

The "filtration-concentration" technique of WOODWARD, DE ZEEUW, and SRB (1952, 1954) was used to obtain a large number of purple adenine mutants both from nonirradiated and X-irradiated conidia of a single wild type strain. Initially it was thought that such mutants represented a series of alleles at the *ad-3* locus (MITCHELL and HOULAHAN 1946; BARRATT *et al.* 1954). Subsequent heterokaryon, crossing, and tetrad analyses, however, showed two separate but closely linked loci in the *ad-3* region, which have been designated *ad-3A* and *ad-3B* (DE SERRES 1956; GILES, DE SERRES and BARBOUR 1957). In this paper, evidence about the origin, linkage relations, and mutability of purple adenine mutants at both these loci is presented in detail.

MATERIALS AND METHODS

The wild type strains used, 74A and 73a, derived by morphological and cytological selection of progeny from crosses of Emerson's strains E-5256A and E-5297a, were obtained from DR. PATRICIA ST. LAWRENCE, of Yale University. The mutant strains used are as follows: *hist*-2 (C94) histidine (HAAS *et al.* 1952) and *nic-2* (43002) nicotinic acid (BEADLE and TATUM 1945).

In general, the media used were those described by BEADLE and TATUM (*op. cit.*). For crosses, synthetic crossing medium (WESTERGAARD and MITCHELL 1947) was used with the appropriate biochemical supplementation. Minimal sorbose agar, which has been used throughout these experiments, consists of 0.1 percent sucrose and 0.8–1.0 percent sorbose to inhibit colony size (TATUM, BARRATT and CUTTER 1949). Random ascospore analyses were made by the overplating technique developed by NEWMEYER (1954).

RESULTS AND CONCLUSIONS

The origin of purple adenine mutants obtained from filtration-concentration experiments

The "filtration-concentration" technique of WOODWARD, DE ZEEUW and SRB (1952, 1954) allows screening for mutant conidia of a particular biochemical type in a population of nonmutant conidia, since only nonmutant conidia will germinate and grow in a minimal medium. When the mycelium formed by the

nonmutant conidia is removed, the mutant conidia can be concentrated and specific mutants obtained by selective plating techniques. When macroconidial strains are used in these experiments, not all of the mutant nuclei present in the total conidial population are recoverable since multinucleate conidia containing viable wild type nuclei germinate and grow in minimal medium and mutant nuclei in such conidia are thus lost. Because of this, the mutants recovered from filtration experiments on untreated macroconidia are those derived solely, or almost entirely, from that proportion of viable uninucleate conidia present initially in the total conidial population. However, the effectively uninucleate proportion of a macroconidial population can be increased artificially with X-irradiation, by inactivation of nuclei in multinucleate conidia (ATWOOD 1952; ATWOOD and MUKAI 1954). Experiments to estimate the nuclear distribution in macroconidia from strains grown on minimal agar have shown that the viable uninucleate proportion for untreated conidia is about 20 percent, whereas, after X-irradiation at 50 percent survival this proportion increases to about 78 percent of the total surviving conidia (ATWOOD, personal communication). Thus the total viable uninucleate population in aliquots of a conidial suspension would differ by a factor of two, depending on whether the aliquots were untreated or were X-irradiated to give 50 percent survival. Because of this effect, it seemed possible that the increased recovery of purple adenine mutants from X-irradiated macroconidia could be caused, at least in part, by filtration and concentration of pre-existing mutants of spontaneous origin in the increased uninucleate population resulting from X-irradiation.

To test this possibility, reconstruction filtration experiments were performed to determine whether pre-existing purple adenine mutants were recovered from X-irradiated macroconidia subjected to the filtration-concentration procedure in proportion to the increase in the total uninucleate population. Such experiments were performed on heterokaryons with disproportionate mutant: wild type nuclear ratios of about 1:3000 and 1:30,000 to mimic the distribution of mutant nuclei of spontaneous origin in macrocondia. Such heterokaryons were made according to the techniques developed by ATWOOD and PITTENGER (1955) and PITTENGER, KIMBALL and ATWOOD (1955) using conidia from a genetically marked *ad-3A ad-3B* double mutant strain and a genetically marked adenine-independent strain. Heterokaryons with previously established nuclear ratios were grown on complete medium to increase the average nuclear number and to decrease the proportion of uninucleate conidia (HUEBSCHMAN 1952) in the untreated conidial population. Under these conditions, the influence of the production of effectively uninucleate conidia by irradiation is maximized.

The number of purple adenine mutants recovered by direct plating of X-irradiated conidia from these heterokaryons is considerably greater than the number recovered from untreated conidia, and this is accounted for by the increase in the number of effectively uninucleate conidia. However, if the untreated and X-irradiated conidia from these heterokaryons are subjected instead to the filtration-concentration procedure, pre-existing mutants are not recovered in pro-

portion to the increase in the effectively uninucleate population. Moreover, the evidence indicates that fewer pre-existing mutants are recovered from X-irradiated conidia than untreated conidia. In Table 1, the results of reconstruction

TABLE 1

The recovery of pre-existing purple adenine mutants after X-irradiation of macroconidia from heterokaryons with disproportionate nuclear ratios using the filtration-concentration technique

Nuclear ratio wild type:mutant	X-ray dose ($r \times 10^3$)	Total viable conidia incubated ($\times 10^7$)	Percentage survival	Total mutants recovered per ml of filtrate	Percentage recovery
3,000:1	None	18.7	...	43.5	...
	36	11.9	63.6	29.0	66.6
30,000:1	None	22.5	...	2.8	...
	36	14.9	66.2	1.8	64.3

filtration experiments on untreated and X-irradiated aliquots of condial suspensions from two different heterokaryons, show that the recovery of pre-existing mutants from X-irradiated conidia is about 65 percent of the recovery from untreated conidia, where a dose of 36,000r gave a survival of 65 percent for the total conidial population. These results suggest that there is an interaction between viable mutant nuclei and inactivated wild type nuclei in multinucleate conidia sufficient to give limited growth of such conidia incubated in minimal medium. Thus, during the incubation period in minimal medium, such conidia that would produce mutant colonies by direct plating are filtered off along with conidia containing only wild type nuclei and are not recovered. Hence it appears that the filtration-concentration technique provides an adequate measure of the incidence of induced mutations without interference from pre-existing spontaneous mutations, unless the incidence of spontaneous mutations is high.

Origin and numbers of individual mutant strains

A large number of purple adenine mutants derived from X-irradiated conidia were obtained by the filtration-concentration technique. The incidence of spontaneous purple adenine mutants was determined by comparable filtrations with nonirradiated conidia. Sampling from the latter filtrations yielded two mutants, both of which were *ad-3A*. Filtration and equivalent sampling of conidia irradiated with doses of 10, 20, 35, and 200×10^3r yielded eight *ad*-3A mutants and 42 *ad-3B* mutants.

The formulations of STEVENS (1942) were used for determinations of the expected number of spontaneous purple adenine mutants derived from X-irradiated conidia. No *ad-3B* mutants were recovered from untreated conidia; the range in

number of mutants expected (P=0.05) is therefore 0–3.69. Since fewer pre-existing mutants are expected from irradiated than from nonirradiated conidia, two or three of the 42 *ad-3B* mutants may have originated spontaneously. Two *ad-3A* mutants were recovered from untreated conidia; hence the range in expected number of mutants (P=0.05) is 0.242–7.22. Similarly, four or five of the 8 *ad-3A* mutants recovered may have been of spontaneous origin.

The first 24 purple adenine mutants (three *ad-3A* and 21 *ad-3B*) recovered in the filtration-concentration experiments were used in this study; their origin and designation are presented in Table 2. The system of terminology used in pre-

TABLE 2

Origin and mutant numbers of purple adenine mutants derived from wild type strain 74A

Experiment number	X-ray dose ($r \times 10^3$)	Locus designation: Present		Locus designation: Previous*	
		Ad-3A	*Ad-3B*	*Ad-3A*	*Ad-3B*
Y59	200	..	B1	...	M1
Y68	200	A1	....	M2	....
Y83	35	..	B2-B8	...	M3-M9
Y112	none	A2	....	M21	....
	35	A3	B9-B21	M23	M10-M20, M22,M24

* GILES 1956.

liminary reports (GILES, DE SERRES and PARTRIDGE 1955; GILES 1956) of these experiments was changed to a system that gives the locus designation used in the present series of papers.

Growth characteristics of the mutants

Qualitative biochemical tests on the 24 strains showed that all the mutants except B16 and B21 have the same general characteristics as pre-existing purple adenine mutants: (1) no growth on minimal medium, (2) more intense purple pigment formation at 35° than at 25°C, and (3), under the longer wave lengths of ultraviolet light, more intense blue fluorescence of filtrates of cultures grown at 35° than at 25°C. At the latter temperature, mutants B16 and B21 show only partial requirements for adenine, but B16 grows on minimal medium only after prolonged incubation. At 35°C, both mutants exhibit a more extreme mutant phenotype, B16 grows only on supplemented minimal medium and B21 shows only limited growth on minimal medium. Filtrates of both mutants, grown at 25° or 35° C, fluoresce blue; but at 25°, purple pigment is formed only on minimal medium. Supplementation with 50 μg/ml of either adenine or hypoxanthine prevents any detectable pigment accumulation.

GENETIC ANALYSIS

Effect of individual purple adenine mutant strains on crossing over in the hist-2—nic-2 *interval*

To determine whether any of the purple adenine mutants had resulted from genetic alteration restricted to the *ad-3* region, we first had to find out whether the linkage relations (with closely linked markers) of the individual mutants were different from "standard" or "normal values" for these same intervals. Previous studies showed that the *ad-3* region is located between the closely linked *hist-2* and *nic-2* loci, and that *ad-3A* and *ad-3B* loci are probably less than 0.15 crossover unit apart (DE SERRES 1956). Since it seemed reasonable to assume that a spontaneous mutant was less likely to have resulted from extragenic alteration than any of the X-ray-induced mutants, the recombination frequencies obtained for the *hist-2—ad-3* and *ad-3—nic-2* regions with such a mutant were used as "standard values" for these regions.

Colony counts were used for estimating the number of adenine-independent segregants in parental and recombinant classes when ascospores were plated (overplating methods of NEWMEYER 1954) from crosses of the purple adenine mutants with a *hist-2—nic-2* double mutant strain. Experiments with 6 to 8 week-old crosses of mutant A2 gave evidence for equivalent recovery of complementary members of each of the three principal segregation classes, indicating comparable viability of the adenine-independent segregants under the plating conditions used.

Results of the analyses of these crosses are presented in Table 3. Since the number of colonies obtained on the individually supplemented plates represent the number of single crossovers plus any double crossovers, and the number of colonies on the doubly supplemented plates represent the total number of ascospores of all these genotypes, recombination frequencies for the individual intervals may be calculated directly (e.g., for B1 the percentage recombinatoin for the *hist-2—ad-3* interval = $58/2703 \times 100 = 2.1$ percent).

Three mutants (B3, B7, B20) were omitted since too few ascospores for a plating analysis could be obtained from these crosses. Evidence from crosses of these mutants to wild type strain 73a suggests the presence of gross chromosome rearrangements in these strains as indicated by marked abortion patterns, low viability of ascospores, and marked deviation of the progeny from the expected ratio of 1:1. B21 was omitted since it grows extensively on minimal at 25° C.

Data from these experiments on the 19 crosses remaining indicate that one of the *ad-3A* mutants (A1) and 11 of the *ad-3B* mutants (B2, B5, B6, B8, B10, B11, B14, B15, B16, B17, and B18) have linkage relations similar to the mutant of known spontaneous origin, A2, and thus appear to have resulted from mutational changes restricted to either the *ad-3A* or *ad-3B* locus. With mutants A3, B1, and B13 crossing over in at least one interval was slightly or substantially reduced. These and unpublished data on the crosses of mutants B4 and B12 suggest the presence of chromosome rearrangements associated with each mutant, which result in an increase recovery of histidine or apparent niacin-requiring segregants,

TABLE 3

Recombination frequencies obtained for the hist-2—ad-3—nic-2 *regions from crosses of purple adenine mutants with a* hist-2 nic-2 *double mutant strain*

Cross:	a	hist-2	+	nic-2
	A	+	ad-3	+

Mutant number	Total colonies per plating series				Recombination percentage	
	M*	M + niacin	M + histidine	M + histidine + niacin	*hist-2—ad-3*	*ad-3—nic-2*
A1	0	44	59	2233	2.0±0.6†	2.6±0.6
A2	1	50	80	2810	1.8±0.4	2.8±0.6
A3	0	38	5	661	5.7±1.8	0.8±0.6
B1	0	58	4	2703	2.1±0.6	0.2±0.2
B2	1	52	67	2450	2.1±0.6	2.7±0.6
B4	11	170	21	517	32.9±4.1	4.1±1.8
B5	1	11	33	1015	1.1±0.6	3.3±1.2
B6	2	29	62	2322	1.2±0.4	2.7±0.6
B8	1	58	47	2162	2.7±0.8	2.2±0.6
B9	1	45	18	1221	3.7±1.0	1.5±0.6
B10	0	53	53	2215	2.4±0.6	2.4±0.6
B11	1	47	63	2143	2.2±0.4	2.9±0.8
B12	33	53	1385	2677	2.0±0.4	51.7±2.0
B13	0	27	0	1399	1.9±0.8	0.0±0.0
B14	3	31	61	1875	1.7±0.6	3.3±0.8
B15	0	2	8	274	0.7±1.0	2.9±2.0
B16	2	47	45	2365	1.9±0.6	1.8±0.6
B17	0	37	43	1829	2.0±0.6	2.4±0.8
B18	1	67	67	2497	2.7±0.6	2.7±0.6
B19	1	33	31	2428	1.4±0.4	1.3±0.4

* M = minimal.
† 95 percent confidence limits.

thus drastically altering the estimates of recombination in one of the regions adjacent to the *ad-3B* locus.

Comparative reversion experiments

Mutants resulting from gene mutation were expected to be capable of further change, either spontaneously or as a result of X-irradiation. Those mutations in the direction of wild type should grow on a minimal medium without the growth factor required by the parental strain. Thus, if any of the X-ray-induced or spontaneous mutants resulted from gene mutation, they should give rise to reversions under experimental conditions suitable for detection of these types.

The reversion experiments were performed on conidial isolates of each strain. (B21 was included by performing the experiment at 37° C.) The procedure was adapted from that used by GILES (1951). Centrifuged conidial suspensions of each mutant were irradiated with a dose of 25,000r (ca. 4000r/min, 6¼ min, 250 kv, 30 ma, 1 mm of aluminum filter) and then plated.

TABLE 4

Comparisons of spontaneous and X-ray-induced reversion in the individual purple adenine mutants (25° C)

Mutant number	Experiment number	Percentage survival	Control			X-irradiated with 25,000r		
			Viable conidia tested ($\times 10^6$)	Number of revisions	Reversions per 10^6 viable conidia	Surviving conidia tested ($\times 10^6$)	Number of reversions	Reversions per 10^6 surviving conidia
B1	Y90	69.0	160.3	0	0.0	127.8	0	0.0
A1	Y90	77.0	138.2	0	0.0	111.3	14	0.13
	Y121	76.5	193.5	0	0.0	148.2	13	0.09
B2	Y90	76.5	127.2	20	0.16	98.5	30	0.30
B3	Y90	91.8	123.0	0	0.0	126.7	0	0.0
B4	Y90	77.4	154.9	0	0.0	118.7	0	0.0
B5	Y90	89.0	209.4	0	0.0	184.5	0	0.0
B6	Y90	83.5	160.0	0	0.0	133.6	0	0.0
B7	Y90	64.0	194.9	0	0.0	124.2	0	0.0
B8	Y90	88.4	117.0	1	0.01	126.0	9	0.07
B9	Y121	74.5	191.0	0	0.0	142.8	0	0.0
B10	Y121	76.2	185.0	0	0.0	141.2	4	0.03
B11	Y121	86.5	226.0	0	0.0	196.0	17	0.09
B12	Y121	78.2	243.2	0	0.0	190.1	0	0.0
B13	Y121	80.0	245.0	0	0.0	194.0	0	0.0
B14	Y121	76.5	206.6	0	0.0	163.9	0	0.0
B15	Y121	74.0	215.0	0	0.0	159.1	0	0.0
B16	Y121	65.0	197.8	0	0.0	128.5	0	0.0
B17	Y121	72.5	212.0	0	0.0	149.9	63	0.42
B18	Y121	70.6	214.0	0	0.0	151.1	0	0.0
B19	Y121	83.4	166.5	0*	0.0	137.5	0*	0.0
A2	Y121	60.9	177.5	42	0.24	109.0	102	0.93
B20	Y121	80.4	166.0	0	0.0	134.1	0	0.0
A3	Y121	65.5	246.0	0	0.0	159.9	0	0.0
B21†	Y157	31.3	194.0	781	3.6	60.9	1485	22.2

* A few colonies resulting from contamination with *ad-8A* conidia have been eliminated from the tabulation.
† 35,000r.

Results of these experiments are presented in Table 4. In this series, nine of the mutants were mutable. A1 was used as a control in both sets of experiments and the agreement in survival and reversion frequencies indicates that all mutants were tested under comparable conditions.

Irradiated and control suspensions were plated at the original concentration and at a tenfold dilution to test for inhibition of prototrophs at these concentrations of conidia (GRIGG 1952). There was no evidence for any marked inhibition of this type at the conidial concentrations used.

The X-ray-induced reversion frequencies include the frequencies of spontaneous reversion. X-irradiation increases the frequency of reversion in mutants B2, B8, B21, and A2; but with A1, B10, B11, and B17, reversions were obtained only with X-irradiation. The reversion frequencies of mutant B21, however, are the highest of any mutants tested. The slightly higher dose (35,000r) accounts

for part of this increase, but the basically higher reversion frequencies of this strain undoubtedly are a reflection of a greater instability of this partial mutant.

Analysis of reversions

Biochemical and genetic studies on revertants produced in a number of inositol mutants (GILES *et al.* 1955) showed that these reversions fall into two categories: (1) partial or complete reverse mutations at the inositol locus, and (2) those caused by mutation at a suppressor locus.

Preliminary studies on purple adenine mutant 38701 (ultraviolet induced) (GILES *et al.*, *op. cit.*; KØLMARK and GILES 1955) showed that reversions at this locus (*ad-3A*) behave as alleles and are not caused by suppressor mutation. In general, reversions arising spontaneously or induced by ultraviolet, X-rays, or chemicals have resulted in the simultaneous restoration of adenine independence and loss of pigment formation. In addition, it has been shown that partial reversions can be obtained in which adenine independence is not restored completely. We wanted to know whether the same type of effects could be found for X-ray-induced reversions in X-ray-induced mutants and in the spontaneous mutants. Revertants of individual mutants were therefore tested to determine the level of adenine independence, and to find out whether reversion was caused by suppressor or reverse mutation.

Origin of homokaryotic strains

Since all the revertants were induced in macroconidial strains, they may be heterokaryotic when first isolated, as were those induced in the macroconidial strain of mutant 38701 (KØLMARK and GILES, *op. cit.*). Although adenine independence is dominant to adenine dependence in these heterokaryotic reversions, the heterokaryotic condition does not permit an accurate analysis for the level of adenine independence when the fluorescence and pigmentation tests are used.

Revertants from X-irradiated conidia of the mutable strains were selected initially, on the basis of qualitative biochemical tests, for obtaining strains ranging from those appearing almost completely adenine dependent to those appearing to be adenine independent. These revertants were crossed to wild type strain 73a or to a marked purple adenine strain, and serial or random ascospore isolations were made, respectively, to recover homokaryotic strains. Some of the homokaryotic revertants recovered and used in subsequent tests are: A1-R73, A1-R75, A1-R77, A1-R78, A2-R132, B2-R49, B10-R2, B10-R3, B10-R4, B11-R9, B11-R12, B11-R18, B17-R30, B17-R38 and B17-R63 (the first symbol is the mutant number, the second the isolation number of the revertant induced in that mutant).

Biochemical analyses on homokaryotic reversions

To determine the degree of adenine independence in X-ray-induced reversions, the homokaryotic strains were tested for adenine stimulation, fluorescence, and purple pigment formation at 25° and 33° C. These tests are the same as those used on the original purple adenine mutants and all the possibly heterokaryotic revertants recovered from the mutable strains.

The results of these tests have shown that X-ray-induced reversions were obtained in mutants A1, B10, B11, and B17, which appear to be completely adenine independent. The evidence also indicates that X-ray-induced reversion in two of the mutants (and possible four, since there is always some question about the origin of reversions recovered from X-irradiated conidia in mutants that revert spontaneously) resulted in partial adenine independence: A1-R77, A1-R78, B10-R2, B10-R3 (and B2-R49, and A2-R132, if they are of X-ray origin), all show varying degrees of adenine independence, most simply interpreted as resulting from varying levels of restoration of the normal adenine synthesis. These results are comparable to those of GILES *et al.* (1955) in which a partial restoration of inositol synthesis was demonstrated for an X-ray-induced revertant in mutant 89601 (induced by nitrogen mustard).

To determine whether the partial revertants were synthesizing enough adenine to permit growth at wild type rate, an experiment was performed on four revertants that showed varying degrees of partiality in the previous tests. The growth tube method (RYAN *et al.*, 1943) was used for comparisons of the linear growth rates of the revertants with that of wild type strain 74A and one of the parental purple adenine mutants, B10, at 33–34° C on minimal agar and minimal agar supplemented with adenine (Figure 1). All tubes were in duplicate and all experimental points represented averages of the measurements of total linear growth made over a period of four days.

Growth curves obtained on minimal agar for each of the revertants provide evidence for partial restoration of adenine synthesis in these mutants. The length of the lag period varies considerably from revertant to revertant, and the maximum growth rates achieved by each of the revertants in the period covered by the experiment are all less than that of wild type strain 74A. On minimal agar supplemented with adenine, all the revertants, in addition to B10, grow at wild type rate.

Genetic tests on reversions

Although the evidence presented by GILES *et al.* (1955) indicates that revertants induced by X-radiation in purple adenine mutant 38701 are not the result of suppressor mutation, additional evidence on the reversion of mutants, such as that on inositol mutants (GILES and PARTRIDGE 1953) or on tryptophan mutants at the *td* locus (YANOFSKY 1952), indicates that even within a series of alleles some of the mutants may revert by means of suppressor mechanisms and others may not. Since these same types of effects might apply to mutants in the *ad-3* region, revertants from each of the mutable strains were analyzed genetically to determine the nature of the reversion.

Crosses were made of each of the homokaryotic revertant strains with wild type strain 73a. Since all revertants except A2-R132 show no purple pigmentation at 25° C, the presence of the suppressor can be detected by incubating the isolates from these crosses at 25° C and scoring for the presence or absence of purple pigment. Accumulation of purple pigment in any of the isolates would indicate adenine dependence and would provide presumptive evidence for reversion

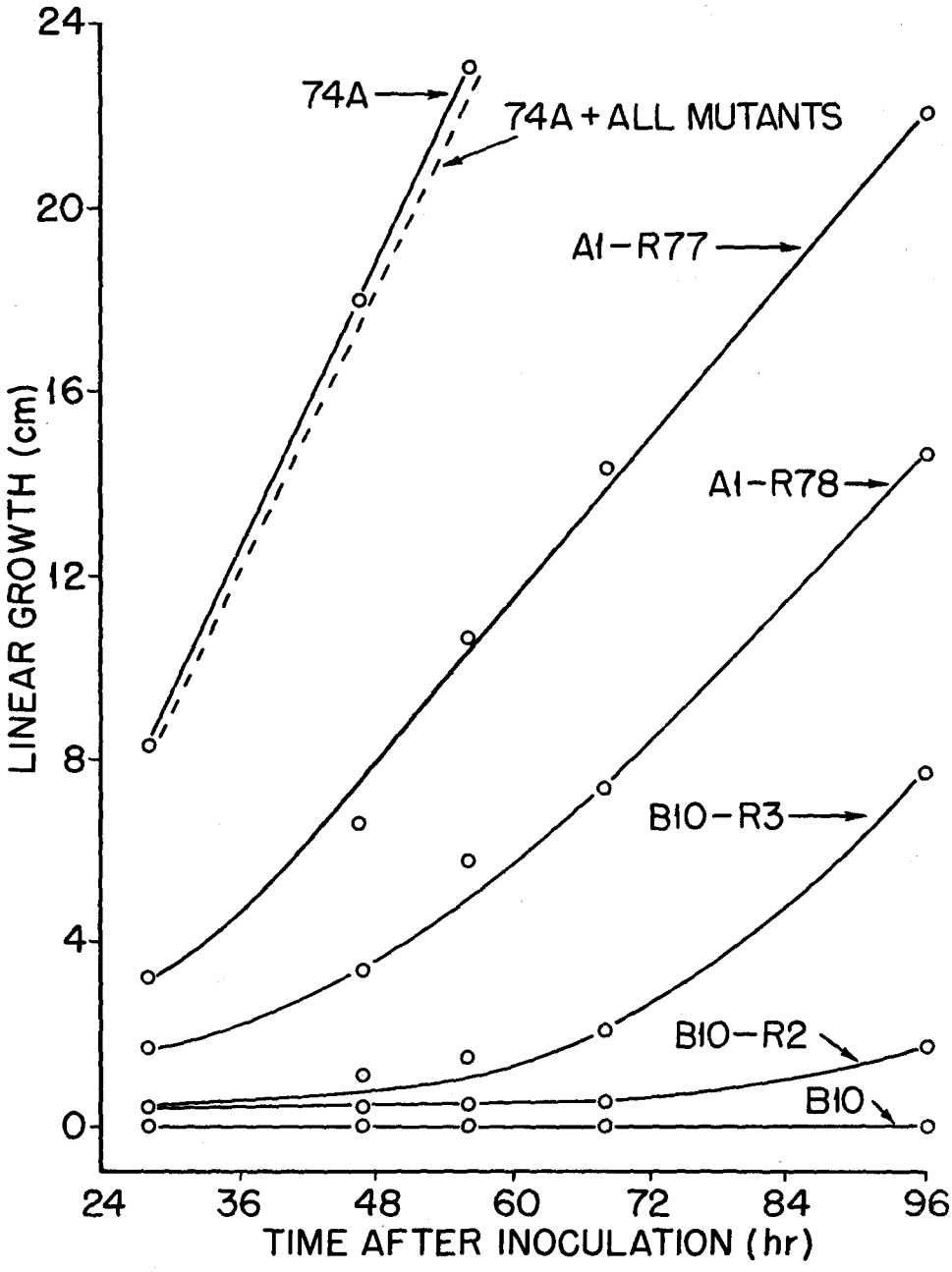

FIGURE 1.—Linear growth of homokaryotic partial revertants as compared with wild type strain 74A and purple *adenine* mutant B10 in growth tubes of minimal agar (solid lines) and minimal agar supplemented with 100 μg/ml of adenine (broken line) at 33–34° C.

caused by mutation at a suppressor locus. At least 200 random ascospore isolations were made from each of these crosses, and no evidence for the presence of suppressor mutation has been obtained, as indicated by the absence of purple pigment accumulation in the F_1 progeny.

Analysis of a partial reversion

The difficulty in obtaining more precise linkage relations for individual reversions was overcome to some extent by the recovery of a temperature-sensitive reversion from mutant B10, which exhibits a mutant phenotype more extreme in the 35–37° C range than indicated in Figure 1. Growth tube experiments on this revertant showed that heterokaryotic combinations of B10-R2 and A1 or A2 grow at less than wild type rate and show marked stimulation by the addition of exogenous adenine to the medium, whereas heterokaryons between the parental strain B10 and A1 or A2 show no exogenous adenine stimulation and grow at wild type rate (GILES 1956). Two alternatives seemed possible to explain the behavior of this revertant: (1) that mutational change confined to the *ad-3B* locus could result in interference with the complementary biochemical relation between the *ad-3A* and *ad-3B* loci in a heterokaryon, or (2) that the reverse-mutational event occurring in B10 was not confined to the *ad-3B* locus but modified the structure of the *ad-3A* locus as well.

Attempts were made to differentiate between these alternatives by crossing the revertant to a marked strain of A2, since, if B10-R2 was accompanied by mutation at the *ad-3A* locus, the frequency of adenine-independent recombinants recovered from such a cross would differ from the frequency recovered from a cross of the parental strain B10 with A2. In addition, crosses were made to a *hist-2 nic-2* double mutant to compare the linkage relations of the revertant with those of the parental strain in the *hist-2—ad-3* and *ad-3—nic-2* intervals.

The results of plating analyses, performed at 35–37° C, on these crosses are presented in Tables 5 and 6. Data from crosses of B10-R2 and B10 indicate no difference in the linkage relations of these two strains either with the *hist-2* or *nic-2* markers or with the *ad-3A* mutant A2. From the data available, the mutational changes that occurred in B10, resulting in the appearance of B10-R2, appear to have been confined solely to the *ad-3B* locus.

TABLE 5

Comparative linkage relations of a partial ad-3B *reversion and the parental strain in the* hist-2—nic-2 *region*

Mutant number	Total colonies per plating series				Recombination percentage	
	M*	M + niacin	M + histidine	M + histidine + niacin	*hist-2—ad-3*	*ad-3—nic-2*
B10-R2	2	167	357	6560	2.5±0.4†	3.9±0.4
B10	3	126	202	4952	2.5±0.4	4.1±0.6

* M = minimal.
† 95 percent confidence limits.

TABLE 6

Distribution of markers in adenine-independent isolates from crosses of a partial ad-3B *reversion and the parental strain with a marked strain of* ad-3A *mutant A2*

Cross:	a	hist-2	ad-3A	+	nic-2
	A	+	+	ad-3B	+

Mutant number	Total viable ascospores	Number of adenine-independent isolates	Recombination percentage	Genotypes of adenine-independent isolates			
				H+++	*+++N*	*H++N*	*++++*
B10-R2	23,180	22	0.19±.06*	0	21	0	1
B10	12,840	11	0.17±.08	0	10	1	0

* 95 percent confidence limits.

Mutability of a partial reversion

The extreme mutant phenotype of B10-R2 in the 35-37° C range permitted further tests for mutability on this X-ray-induced revertant. Since the genetic tests suggest that this revertant resulted from some form of intragenic alteration, it appeared desirable to determine whether this strain was mutable and could give rise to wild type revertants spontaneously or after X-irradiation. Reversion experiments were performed in the same manner as described previously, except that plates were incubated at 35–37° C, and conidia were irradiated with a dose of 35,000r. Reversion frequencies of 0.22 per 10^6 viable conidia and 0.86 per 10^6 surviving conidia were obtained for untreated and X-irradiated conidia, respectively. The partial revertant thus differs from the parental strain in that it is more unstable either spontaneously or after X-irradiation. No evidence is as yet available as to whether this strain mutates in the other direction, to adenine dependence.

DISCUSSION

The data from comparable forward-mutation experiments in which the filtration concentration technique was used to recover spontaneous and X-ray-induced purple adenine mutants demonstrated that substantially all the *ad-3B* mutants are of X-ray origin. Although the origin of any particular mutant could never be proved conclusively, the data do indicate that the incidence of purple adenine mutants of spontaneous origin at this locus must be extremely low and that X-irradiation has resulted in a substantial increase in the recovery of biochemical mutants of this type.

The origin of the *ad-3A* mutants recovered from X-irradiated conidia in these same experiments was not established with certainty since a number of *ad-3A* mutants recovered from untreated conidia. Two such mutants of uncertain origin, A1 and A3, were used in the present study.

Qualitative biochemical tests showed that purple adenine mutants at both loci have quite similar properties of pigment accumulation, fluorescence, and complete requirements for adenine or related compounds. Two of the mutants differ from

the others, however, in that they have only partial growth factor requirements. The recovery of this type of mutant indicates that, in the forward-mutation experiments, intermediate alleles were produced at the *ad-3B* locus in addition to those with complete requirements for adenine. The fact that two of the 21 *ad-3B* mutants were of this type is certainly no measure of the frequency of intermediate alleles at this locus, since the majority of mutant conidia of this type should germinate, grow, and be filtered off during the incubation period in minimal medium in the filtration-concentration experiments.

The linkage data on the individual mutant strains indicate the marked heterogeneity of genetic alteration obtained with X-irradiation. At least half the mutants appear to have resulted from such extragenic alterations as chromosomal rearrangements or deficiencies. Although the linkage data are not precise enough to indicate the presence of two separate loci in the *ad-3* region, since the *ad-3A* and *ad-3B* loci are probably less than 0.15 crossover units apart (DE SERRES 1956), in the current analysis, *ad-3A* and *ad-3B* mutants should have essentially the same linkage relations with the *hist-2* and *nic-2* markers. From the data available, 12 purple adenine mutants have linkage relations similar to the mutant of known spontaneous origin as shown in columns 2 and 3 in Table 7. These data are interpreted as indicating that these 12 mutants have resulted from intragenic alterations equivalent to point mutation.

Comparative reversion experiments with all the mutants showed that there is a marked correlation between irreversibility and abnormal linkage relations (Table 7). Of the 11 *ad-3B* mutants with apparently "normal" linkage relations, five are reversible either spontaneously or after X-irradiation, and six such mutants (B5, B6, B14, B15, B16, and B18) are stable. Present data are not adequate to determine whether any one of the latter group of mutants is mutable, but reverts at rates too low to have been detected in these experiments, or whether all the mutants are stable and thus may have resulted from such small deletions or rearrangements that no major alteration in linkage relations were observed with the markers used. In no case were reversions obtained in a mutant with linkage relations markedly different from mutant A2.

The genetic data for most of the reversions are not so precise as those for the parental strains, but the general genetic evidence available may be interpreted as indicating that the strains have resulted from alterations localized to the *ad-3* region, which at least in many instances may be considered intragenic. Data from more extensive analysis on a partial *ad-3B* revertant indicated that reversions do occur that are indistinguishable from the parental strain, on the basis of linkage relations with closely linked markers. The occurrence of such alterations on an intragenic level is substantiated by the subsequent mutability of such revertants either spontaneously or as a result of X-irradiation.

The results obtained in these experiments on the X-ray-induced reversibility of X-ray-induced mutants thus are in agreement with the findings of the early work in Drosophila and differ from the results of experiments reported by STADLER

TABLE 7

Correlation between linkage relations with hist-2 *and* nic-2 *markers and mutability of purple adenine mutants*

Mutant number	Recombination percentage		Reversions/10^6 survivors	
	hist-2—ad-3	*ad-3—nic-2*	Spontaneous	X-ray-induced
A1	2.0±0.6*	2.6±0.6	0.00	0.11
A2	1.8±0.4	2.8±0.6	0.24	0.93
B2	2.1±0.6	2.7±0.6	0.16	0.30
B5	1.1±0.6	3.3±1.2	0.00	0.00
B6	1.2±0.4	2.7±0.6	0.00	0.00
B8	2.7±0.8	2.2±0.6	0.01	0.07
B10	2.4±0.6	2.4±0.6	0.00	0.03
B11	2.2±0.6	2.9±0.6	0.00	0.09
B14	1.7±0.6	3.3±0.8	0.00	0.00
B15	0.7±1.0	2.9±2.0	0.00	0.00
B16	1.9±0.6	1.8±0.6	0.00	0.00
B17	2.0±0.6	2.4±0.6	0.00	0.42
B18	2.7±0.6	2.7±0.6	0.00	0.00
A3	5.7±1.8	0.8±0.6	0.00	0.00
B1	2.1±0.6	0.2±0.2	0.00	0.00
B3	....	....	0.00	0.00
B4	32.9±4.0	4.1±1.8	0.00	0.00
B7	....	....	0.00	0.00
B9	3.7±1.0	1.5±0.6	0.00	0.00
B12	2.0±0.4	51.7±2.0	0.00	0.00
B13	1.9±0.8	0.0±0.0	0.00	0.00
B19	1.4±0.4	1.3±0.4	0.00	0.00
B20	....	....	0.00	0.00

* 95 percent confidence limits.

(1941, 1944) and STADLER and ROMAN (1948) in maize, and by LEFEVRE (1950) in Drosophila.

There is evidence that the *a1* allele used for the experiments on the effect of X-irradiation on dominant mutation (STADLER 1944) may not have been so suitable for such a study as it was once thought. The *a1* allele is ordinarily quite stable and mutable only in the presence of the *Dt* gene (RHOADES 1941), or by the breakage-fusion-bridge cycle (MCCLINTOCK 1951) of chromosome 9. MCCLINTOCK suggests that reversions of *a* and *A* are ascribable to changes in an inhibited state of *a* controlled by a type of dissociator-activator system. Evidence presented by STADLER (1951) indicates that the *A′* alleles that arise from the *a* allele under the influence of *Dt* are qualitatively different from normal *A* alleles, since the *A′* alleles are mutable in the presence of *Dt* whereas normal *A* alleles are not. The evidence from all these studies suggests that mutation of the *a1* allele may occur *only* under the influence of *Dt* or "Dt-like" factors. If this is the case, then *a1*

allele does not meet the requirements discussed by STADLER (1941, 1944), since critical evidence of failure to mutate to a dominant allele can only be obtained from recessive alleles capable of spontaneous mutation. In addition, alternative explanations are possible for the results of the experiments on X-ray-induced mutation of the *A1* allele (STADLER and ROMAN 1948). In choosing *A* losses from a total of about 415 anthocyanin-free seedlings for cytogenetic study, selection was made for those F_1 plants with nondefective pollen. A cytogenetic analysis on two of these plants, and one with segregating subnormal pollen, demonstrated that these *A* losses were caused by deficiencies and not gene mutation of the *A1* allele. However, the possibility also exists, as was pointed out by these authors, that there were instances of gene mutation of *A1* among the population of *A* losses observed, but because of coincident but independent chromosomal alterations, they were not haploviable, or else gave such reduced gametophytic transmission that they were eliminated from the cytogenetic analysis.

In the experiments of LEFEVRE (1950), the requirements established by STADLER (1941, 1944) for obtaining critical evidence on the effects of X-rays on dominant and recessive mutation have not been fulfilled. No evidence is presented demonstrating that the recessive alleles used for study of X-ray effects on various loci in the X chromosomes are capable of mutation spontaneously. Although attempts to induce reversions in white-eye mutants of three different origins were unsuccessful, in none of these three stocks was a spontaneous reversion observed.

Although the present studies showed that a large number of X-ray-induced *ad-3B* mutants are stable, they have also shown that a substantial portion (25 percent in the present sample) are capable of either spontaneous or X-ray-induced reversion. Furthermore, the evidence suggests that the X-ray-induced alterations that have been observed are caused by gene mutation, or to localized alterations that simulate gene mutation.

The genetic nature of X-ray-induced changes that simulate gene mutation has been the subject of much discussion. In a review of the gene concept, and the role of radiation studies in forming that concept, STADLER (1954) concludes that "we have no positive criterion to identify mutations caused by a change within the gene and that the alterations interpreted as gene mutations in experiments are merely the unclassified residue that cannot be proved to be due to other causes."

The work of MCCLINTOCK (1951, 1955) shows quite clearly that, in maize, instability at a locus may be attributable to a number of factors. Among those cases that she has studied, there are loci at which the instability is under autonomous control, and others at which the instability is under the control of mutators or suppressors such as *Ac* or *Spm*. There appear to be no adequate criteria for distinguishing between those loci at which mutation is caused by alteration of the genetic material within a locus and those at which mutation is caused by alterations that do not directly alter the structure of the genes themselves but that actually affect the functioning of controlling elements located at or near such genes.

Further evidence presented by MCCLINTOCK (1951) would suggest specific

control of certain types of genic action by heterochromatin. Transposed to new locations by the breakage-fusion-bridge cycle, these heterochromatic elements continue their specific control, but affect the action of genic components at the new locations. Radiation-induced position effects in Drosophila (see LEWIS 1950, for a general review) resulting from transposition of heterochromatin, give evidence that some of the same general types of effects, obtained with the breakage-fusion-bridge cycle, can be induced by X-irradiation.

Apparently, then, even in cases where the absence of any detectable disturbance in linkage relations and failure to obtain evidence for suppressors would suggest that reverse mutation in a particular mutant resulted from an "intragenic alteration," other interpretations are possible. Such results could be obtained either by mutation of a very closely linked "suppressor" which restored the synthesis interrupted by the original mutation, or by the removal of transposed heterochromatin, which could have produced the original "mutant" phenotype and by its very removal restores the normal phenotype. We have no simple or generally useful criteria for distinguishing between these alternatives.

In light of these observations, it is perhaps not surprising that STADLER (1954) commented that "in the study of gene mutation, we are for the present in an anomalous position. A mutant may meet every test of gene mutation, and yet, if it is not capable of reverse mutation there is ground for the suspicion that it may be due to gene loss, while, if it is capable of reverse mutation, there is ground for the suspicion that it may be due to an expression effect. The only escape from this dilemma is through a more intensive study of the mutations of specific genes selected as best suited to detailed genetic analysis, in hope of developing more sensitive criteria for the identification of gene mutation."

SUMMARY

(1) A series of 24 purple adenine mutants obtained in forward-mutation experiments by the "filtration-concentration" technique were used to investigate the reversibility of X-ray-induced mutants in the *ad-3* region in *Neurospora crassa*. Three are *ad-3A* mutants and 21 are *ad-3B* mutants.

(2) Comparable experiments with untreated and X-irradiated conidia showed that substantially all *ad-3B* mutants recovered from filtration of X-irradiated conidia are of X-ray origin, whereas the origin of *ad-3A* mutants recovered in the same experiments is questionable.

(3) Two of the X-ray-induced *ad-3B* mutants have partial requirements for adenine; B21 grows extensively, and B16 shows limited growth on minimal medium at 25° C, whereas the remaining mutants show complete requirements for adenine.

(4) Linkage data from crosses of each of the *ad-3B* mutants have shown that 11 of the *ad-3B* mutants have "normal" linkage relations in the *hist-2—ad-3* and *ad-3—nic-2* intervals, and thus appear to have resulted from intragenic alteration; however, since recombination in one or both of these intervals was altered in crosses of the remaining mutants these appear to have resulted from some form of extragenic alteration.

(5) Comparative reversion experiments showed that 5 of the 11 *ad-3B* mutants with "normal" linkage relations are mutable and revert back to wild type either spontaneously or after X-irradiation, whereas all the mutants with "abnormal" linkage relations are stable.

(6) Biochemical analysis of X-ray-induced *ad-3B* mutants showed that partial revertants were obtained in addition to those appearing to be complete revertants to wild type.

(7) Genetic analyses of X-ray-induced *ad-3B* revertants showed that partial and complete reversion to wild type results, in many instances, from localized changes in the *ad-3B* region, indicating that X-rays are capable of inducing changes most simply interpreted as reverse (gene) mutations. No evidence was obtained indicating that reversion occurs through suppressor mutation.

(8) Reversion tests on an X-ray-induced partial *ad-3B* revertant showed that this strain is mutable and gives rise to wild type reversions spontaneously and at higher rates after X-irradiation.

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