

GENETIC STUDIES OF *ad-8* MUTANTS IN *NEUROSPORA CRASSA*. I. GENETIC FINE STRUCTURE OF THE *ad-8* LOCUS¹

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IN recent years, evidence has accumulated which indicates that genetic recombination occurs not only between different loci but also between alleles within one locus (PONTECORVO 1958). A precise genetic analysis of allelic relationships within one locus is certainly an essential approach to understanding genes, since studies of allelism can help to elucidate both the structure and function of a particular gene.

The present study will describe the genetic fine structure of the adenine-8 (*ad-8*) locus in *Neurospora crassa*, a locus at which a large number of mutants of independent origin are available. Some of the mutants are of spontaneous origin and others have been induced by various physical and chemical mutagens. Biochemical studies indicate that the *ad-8* locus controls one reaction in the biosynthetic pathway to adenine, i.e., the conversion of inosine monophosphate to adenosine monophosphate succinate and that all *ad-8* mutants lack activity for a single enzyme, adenylosuccinate synthetase. A genetic map has been established for the *ad-8* locus as the result of a series of over 1,000 interallelic crosses. This map indicates the existence of numerous distinct mutational sites within the *ad-8* locus. The map has proved especially useful in studies of interallelic complementation at this locus (ISHIKAWA 1960). In addition, evidence concerning genetic fine structure has also been helpful in elucidating the nature of forward and reverse mutational events at this locus, especially in relation to the types of mutagens used.

MATERIALS AND METHODS

The *ad-8* mutants used in these studies were isolated by the filtration-concentration method described by WOODWARD, DE ZEEUW and SRB (1954). Table 1 indicates the origin of the 308 primary and 77 secondary *ad-8* mutants

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TABLE 1
Origin of primary and secondary *ad-8* mutants

Treatment	Number of mutants	
	Primary*	Secondary†
None (spontaneous)	25	2
X-ray	34	..
Ultraviolet	121	75
Nitrous acid	49	..
Ethyl methanesulfonate	13	..
5-bromodeoxyuridine (BUDR)	5	..
5-fluorodeoxyuridine (FUDR)	4	..
BUDR + FUDR	44	..
BUDR + FUDR + UV	13	..
Total	308	77

* The majority of these mutants came from the stock collection at Yale University and were obtained in experiments performed by Drs. F. J. DE SERRES, N. J. NELSON, M. E. CASE and N. H. GILES.

† Ultraviolet was the only mutagen used in the production of secondary mutants.

induced by various types of physical and chemical treatment of conidia from wild-type strain 74A (or closely related strains). Homocaryotic revertants derived from ultraviolet (UV) treatment of various primary *ad-8* mutants were used to obtain secondary *ad-8* mutants. Opposite mating types were isolated from crosses with wild type or with a yellow (*γlo*) strain which is compatible in heterocaryons with the mutant strain. Mutants at other loci employed as genetic markers were either ones induced in 74A (or in closely related strains) or ones which had been backcrossed at least three times to 74A.

An arbitrary *ad-8* group identification number has been given to each mutant. The symbols E and ES preceding arabic numerals designate primary and secondary *ad-8* mutants, respectively.

Spontaneous and UV-induced reversion experiments were performed under the following standard conditions. A washed macroconidial suspension was prepared from seven-day-old conidia grown on glycerol complete medium at 25°C. Part of the suspension was plated on Fries' minimal media containing 0.2 percent sucrose and 1.0 percent sorbose to detect spontaneous reversions. The other part was exposed to 6000 ergs of UV at 10 ergs/sec/mm² as described in detail by GILES (1951) and plated on the same media. Platings for calculating viability were made at appropriate dilutions on minimal media supplemented with adenine. The number of adenine-independent cultures appearing over a period of seven days was recorded. Many of the *ad-8* mutants used in the reversion experiments were double mutants carrying *γlo* marker as a precaution against contamination.

Interallelic crosses at the *ad-8* locus were made in 125 ml Erlenmeyer flasks containing 20 ml crossing media. WESTERGAARD and MITCHELL's synthetic crossing medium was supplemented with 400 γ /ml adenine sulfate, 0.05 percent acetate, appropriate supplements for markers, and filter paper to improve the fertility of interallelic crosses. Crosses were kept for four or five weeks at 25°C. Ascospores obtained were plated on Fries' minimal media supplemented with

0.1 percent sucrose, 1.0 percent sorbose, and appropriate supplements for markers, by the overplating method modified from NEUMEYER's technique (NEUMEYER 1954). Adenine-independent colonies appearing in two or three days after shocking were isolated and tested to determine marker distribution. Distances on the genetic map are given as percent prototrophs in the total number of viable ascospores. The spacing of sites on the map is based on relative prototroph frequencies obtained in various crosses.

RESULTS

Biochemical and genetic characterization of ad-8 mutants: Previous studies by GILES, PARTRIDGE and NELSON (1957) provided evidence that *ad-8* mutants constitute one of the two adenine-specific mutant groups in *Neurospora crassa* and that culture filtrates of *ad-8* mutants are active in supporting growth of all other groups of adenine-requiring mutants except those in the other adenine-specific (*ad-4*) group. As a consequence, the reaction controlled by the *ad-8* locus was considered to be prior to one of the two reactions controlled by the *ad-4* locus in the biochemical sequence. Ultraviolet absorption spectra of culture filtrates of *ad-8* mutants demonstrated that these mutants accumulate hypoxanthine or its derivatives (PARTRIDGE and GILES 1957). These results indicated that *ad-8* mutants are blocked in the reaction immediately prior to the last one of the two reactions blocked in *ad-4* mutants, namely the conversion of inosine monophosphate (IMP) to adenosine monophosphate succinate (AMP-S). The enzyme responsible for this reaction is AMP-S synthetase (LIEBERMAN 1956). AMP-S synthetase activity in the wild-type strain of *Neurospora* has been assayed successfully (ISHIKAWA, unpublished). Nine *ad-8* mutants tested have no detectable AMP-S synthetase activity.

The *ad-8* locus is located on the left arm of linkage group VI, approximately 26 crossover units from the centromere based on the frequency of second division segregations in 158 asci obtained from four independent crosses. Analyses of crosses between *ad-8* mutants and three linked marker strains, lysine (*lys*), unknown (*un*), and *ylo*, indicate that the *ad-8* locus is located about 20 crossover units distal to the *lys* locus on the left arm of linkage group VI.

Sixty primary *ad-8* mutants and ten secondary *ad-8* mutants were examined for spontaneous and UV-induced reversion frequencies. Although the majority of mutants tested were able to revert after UV treatment, 11 of 60 primary mutants and three of ten secondary mutants failed to give revertants, even with UV treatment. The nonreverting primary mutants were distributed as follows: five of 12 spontaneous mutants, three of 12 X-ray-induced mutants, two of 27 UV-induced mutants, and one of nine mutants induced by nitrous acid. One of the three nonreverting secondary mutants was of spontaneous origin and the two others were UV-induced. It is interesting to note that in this sample a larger percentage of spontaneous mutants and X-ray-induced mutants were non-revertible, than were mutants induced by UV or by nitrous acid.

Interallelic crosses: As a biochemical marker for interallelic crosses *lys* was used, since this mutant is the one most closely linked to the *ad-8* locus. In

addition, *ylo* was used independently or in combination with the *lys* marker on the basis of the superior viability of *ylo* mutants in crosses and the technical convenience arising from easy phenotypic detection of this marker in progeny tests. Since no markers were known distal to the *ad-8* locus, it was not possible to employ the preferred technique of having markers on both sides of the locus. The distribution of a given marker among prototrophs derived from crosses of *ad-8* mutants has almost always shown a significant asymmetry, and reciprocal crosses resulted in a reciprocal relationship in marker distribution. The order of mutants on the map based on marker distribution coincided exactly with the order based on additivity relationships derived from relative prototroph frequencies. Representative crossing data used to establish these relationships are presented in Table 2.

Selfings were made of representative strains as controls to detect the possibility of elevated reverse mutation frequencies which would yield prototrophs during crossing. Some examples of selfings are shown in Table 2. In most selfings tried, no prototrophs were found, plating a significant number of ascospores. In a few instances, however, an occasional prototroph appeared in repeated selfings. For example, despite the fact that the frequency of spontaneous reversion in conidia of mutant E6 had been determined as 1.8×10^{-8} , one revertant which had a marker of one of the parents was found in a total of 4.8×10^6 viable ascospores obtained from 12 independent selfings in E6. Such a frequency of prototrophs in selfings sets a lower limit for recombination analyses between allelic mutants. The map distances reported in the following experiments are usually highly significant beyond this kind of limitation.

Pseudowild types (PWT), which are phenotypically wild but behave as mutants in crosses, have previously been found in the progeny of crosses between allelic mutants as well as in crosses between mutants at different loci (MITCHELL, PITTENGER and MITCHELL 1952; PITTENGER 1954). At the *ad-8* locus, PWT's were found, without exception, in crosses between complementing mutants after overplating on minimal media supplemented with histidine. The recovery of PWT's is apparently stimulated by the presence of histidine and suppressed partially or completely at 35°C. The frequency of PWT formation varies from cross to cross, but appears to average about 0.1 percent of the total viable ascospores. In interallelic crosses at the *ad-8* locus, PWT colonies are easily distinguished from true wild-type colonies because of their much smaller colony size on Fries' minimal medium containing sorbose at 35°C.

To prepare a basic map, 19 representative mutants were crossed in various combinations, and a recombination map was constructed, as shown in Figure 1. On this map, distances are presented in terms of percent prototrophs obtained in viable ascospores plated. For most combinations repeated crosses were plated and in Figure 1 average distances are given, together with the number of times a particular cross was repeated. The order of alleles is based both on additivity relationships (in terms of map distances) and on the asymmetrical distribution of markers among prototrophs. For convenience in further descriptions and dis-

TABLE 2

Representative crosses indicating the relationship of ad-8 mutants

Cross*	No. viable ascospores × 10 ⁵	No. adenine prototrophs	Percent adenine prototrophs†	Percent <i>lys</i> or <i>yl</i> o in adenine prototrophs		Indicated mutant order or relationship
				<i>lys</i>	<i>yl</i> o	
E6 × E32 <i>yl</i> o	5.25	385	0.073	..	65	
E32 × E6 <i>yl</i> o	5.55	415	0.075	..	30	E32-E6
E6 <i>yl</i> o × E32 <i>lys</i>	2.78	223	0.080	59	34	
E6 <i>yl</i> o × E80	1.66	65	0.039	..	35	
E6 × E80 <i>yl</i> o	7.96	347	0.044	..	66	E80-E6
E80 × E6 <i>lys yl</i> o	7.16	286	0.040	23	36	
E32 × E80 <i>yl</i> o	7.91	290	0.037	..	29	
E80 × E32 <i>yl</i> o	2.75	84	0.031	..	66	E32-E80
E80 <i>yl</i> o × E32 <i>lys</i>	8.12	220	0.027	60	33	
E6 <i>yl</i> o × E42	2.19	20	0.009	..	21	
E42 <i>lys</i> × E6 <i>yl</i> o	1.55	3	0.002	100	0	E42-E6
E42 × E6 <i>lys yl</i> o	4.45	24	0.005	37	40	
E110 × E32 <i>yl</i> o	3.96	42	0.011	..	26	
E32 <i>lys</i> × E110 <i>yl</i> o	7.66	140	0.018	30	65	E110-E32
E35 × E6 <i>yl</i> o	4.69	0	
E70 × E6 <i>yl</i> o	2.34	0	E6, E35, E70; identical
E6 × E70 <i>yl</i> o	3.60	0	
E35 × E70 <i>yl</i> o	5.11	0	
E34 × E6 <i>yl</i> o	4.78	0	E34; multisite
E34 × E80 <i>yl</i> o	7.43	0	
E34 × E110 <i>yl</i> o	3.90	0	
ES8 × E6 <i>yl</i> o	3.57	51	0.014	..	35	
ES8 × E134 <i>yl</i> o	5.19	0	
ES8 × E118 <i>yl</i> o	7.05	0	ES8; multisite
ES8 × E118 <i>yl</i> o	6.16	0	
ES8 × E145 <i>lys</i>	4.82	2	0.0004	100	..	
ES8 × E110 <i>yl</i> o	5.41	64	0.012	..	64	
E6 × E6 <i>lys yl</i> o	6.95	0	
E32 <i>yl</i> o × E32 <i>lys</i>	5.58	0	selfings
E80 × E80 <i>yl</i> o	7.20	0	

* The first strain is *A* mating type and the second *a* mating type.

† All prototrophs tested if less than 100, otherwise a sample of 100 was tested.

cussions, the entire map is divided into nine arbitrary subregions designated as a, b, . . . , i (Figure 1).

Map distances are additive in most crosses. In some cases where crosses were repeated more than five times statistical analyses have shown that the additivity of map distances as plotted is highly significant within five percent error on the basis of percent prototrophs in viable ascospores. Most of the exceptional cases in which map distances are not additive consist of crosses involving two primary mutants, E121 and E96, (in subregion g), and two secondary mutants, ES3 (in

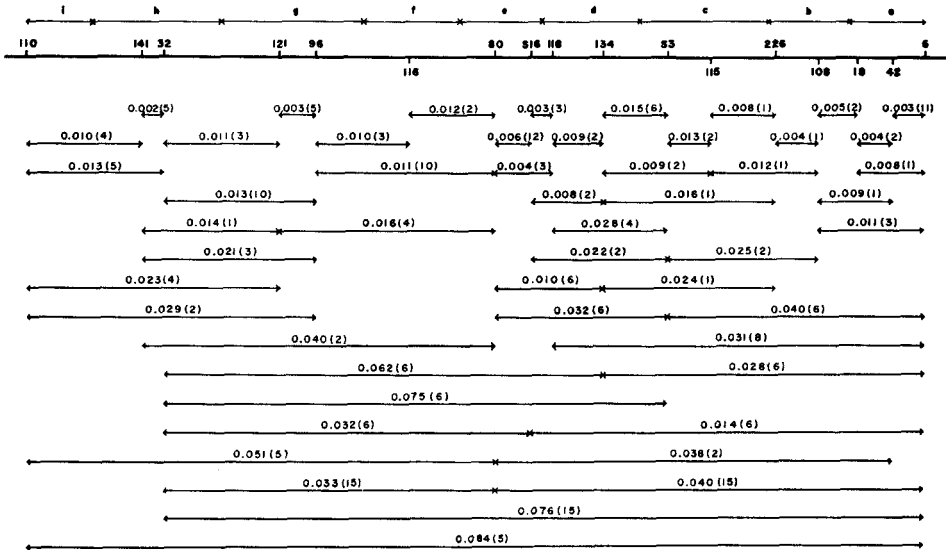


FIGURE 1.—Linkage relationships of some *ad-8* mutants. Capital E of the mutant number is omitted. Map distances are presented in terms of percent prototrophs obtained in viable ascospores plated. Number in bracket shows number of times cross was repeated. a, b, . . . , i indicate subregions.

subregion c), and ES16 (in subregion e). For example, the two crosses, E96 × E80 and E96 × E32 give a smaller sum of distances than is expected on the basis of the cross, E80 × E32; and map distances obtained from crosses involving ES3 are always larger than expected on the basis of other related crosses.

In order to construct a more precise map, additional mutants were intercrossed within each subregion. As a result, 162 out of 308 primary mutants and 13 out of 77 secondary mutants have been located on the map. On the final map presented in Figure 2, only one representative mutant has been indicated (instead of showing all mutants obtained from the same filtration flask), since the possibility of isolating duplicate mutants in the course of filtration-concentration experiments, although presumably small, cannot be neglected. In all, 37 different genetic sites have been found at this locus.

Distribution of genetic sites as related to the origin of mutants: Possible relationships between the distribution of mutants on the genetic map and the mu-

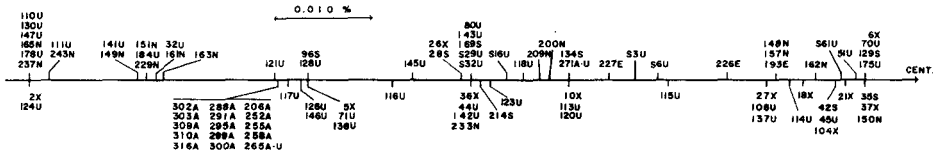


FIGURE 2.—Genetic map of the *ad-8* locus. On the map capital E of the mutant number is omitted. Origin of mutants is abbreviated as follows: A, base analogues; E, ethyl methane-sulfonate; N, nitrous acid; S, spontaneous; U, ultraviolet; X, X rays.

tagens used have been investigated using data presented in Figure 2. It appears clear that mutants induced either by X rays or by UV are distributed all over the map with no evident specificity for one particular region. However, no mutants of spontaneous origin are located on the left one third of the map and a considerable number of mutants induced by nitrous acid occur on the left-hand half of the map.

In experiments with 5-bromodeoxyuridine (BUDR) and 5-fluorodeoxyuridine (FUDR), 60 mutants were obtained from 15 different filtration flasks, all being located at one particular genetic site linked very closely with E121 (subregion g). The question may be raised whether these analogue-induced mutants are duplicates of one spontaneous mutation which occurred in the original population. The actual procedure in screening for these mutants was as follows (CASE, unpublished). Cultures were grown on media containing BUDR and FUDR, singly or in combination, with or without thymidine, by inoculating a small amount of wild-type conidia. These cultures were harvested separately and subjected to filtration-concentration experiments in different flasks. Thus mutants were obtained from four different filtration-concentration experiments which were conducted on different dates. These mutants should be different from each other, since different populations of the wild-type strain were used. In one particular experiment, the wild-type population used for each flask grew from a separately isolated culture of 74A. And the mutants obtained from the untreated population were not located at the genetic site in question. Furthermore, preliminary reversion experiments show that under the standard conditions there are differences in spontaneous and UV-induced reversion frequencies of mutants obtained from the same filtration-concentration experiment. Therefore, although mutants obtained from the same filtration flask may well be duplicates of one mutation, mutants from different flasks in one experiment should result from different mutational events. On the basis of these considerations, one may conclude that BUDR and FUDR induced at least 15 independent mutations at one specific site in the *ad-8* locus. A more detailed account of the effect of BUDR and FUDR in *Neurospora* will be published elsewhere.

More than half the *ad-8* mutants tested have produced no prototrophs in crosses with at least one other mutant in the present analyses. Two mutants which produce no prototrophs when crossed are interpreted as occupying the same site and will be called "identical site alleles" (see Table 2 for examples). Some identical site alleles may be too close to be separated with the resolving power presently feasible in interallelic *ad-8* crosses, whereas other alleles may involve defects covering a small continuous region on the genetic map. Identical site alleles have been found at 15 of the 37 sites presently identified at the *ad-8* locus. The distribution of these sites based on numbers of identical site alleles is shown in Table 3 and compared with examples of the Poisson distribution. The chi-square test shows that the distribution of the total number of observed sites does not give a good fit with the theoretical Poisson distribution because of the existence of four particularly mutable sites. The distributions of observed sites with regard to the origin of the mutations follow roughly the theoretical Poisson

TABLE 3

Distribution of genetic sites at which identical site alleles have been found

Distribution	Origin	m^*	No. of identical site alleles															
			0†	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Observed sites	(1) Total	2.24	.22	5	4	0	2	0	1	1	1	0	0	0	0	0	0	1
	(2) UV	1.00	.18	3	1	0	1	1
	(3) Nitrous acid	0.50	.8	3
	(4) X-ray	0.22	.8	1
	(5) Spontaneous	0.33	.5	1
	Ethyl methane-sulfonate	3
	Base analogues	...	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1
Examples of Poisson‡	(1)	2.24	5	10	11	8	5	2	1
	(2)	1.00	14	14	7	2	1
	(3)	0.50	17	9	2
	(4)	0.22	37	8	1
	(5)	0.33	15	5	1

* The mean number of mutations calculated from the following equation (DAVID and JOHNSON 1952): $m/(1-e^{-m}) = x$, where x is an average number of alleles per site.

† The number of sites at which no allele has yet been found.

‡ The five examples of the Poisson distribution are given using means and numbers of sites equivalent to observed distributions.

distribution with the one clear exception of base analogue-induced alleles. These results suggest that one of the exceptionally mutable sites is specific for mutants arising after treatments with BUDR and FUDR, whereas the other three sites show no mutagen specificity.

Considerable variation has been found in reversion frequencies among identical site alleles. For example, at the site involving mutant E6, all mutants indicated above the line in Figure 2 (complementing mutants) show a reversion frequency of about 1×10^{-6} with UV irradiation, whereas among mutants indicated below the line (noncomplementing mutants at the same site) E37 shows a frequency of 1×10^{-8} while E35 and E150 have yielded no revertants with UV treatment. In general, these data suggest that noncomplementing mutants are more stable than complementing ones, even when the two types are located at the same site.

Multisite mutations: In the course of crossing experiments, 12 mutants failed to produce prototrophic progeny when crossed with other mutants located at two or more different genetic sites (Figure 3). Representative data of such crosses have been presented in Table 2. These mutants will be referred to here as "multisite mutations" (SUYAMA, MUNKRES and WOODWARD 1959). Of these 12 mutants, five primary mutants (E34, E41, E93, E103 and E127) and two secondary mutants (ES1 and ES30) failed to produce prototrophs in crosses with mutants at any other site at this locus even when a significant number of ascospores was plated. E14 produced no prototrophs in crosses with E209 or with mutants located to the left of it. E24 and E43 produced no prototrophs in crosses with E115 or with mutants located to the left of it. In addition, ES8 and ES23 produced no prototrophs in crosses with mutants located between ES3 and E145, and between E96

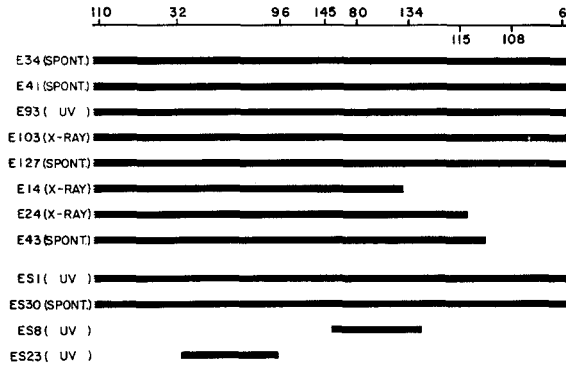


FIGURE 3.—Multisite mutations at the *ad-8* locus. Origin of multisite mutants is indicated in bracket.

and E163, respectively. Map distances obtained with crosses involving ES8 and ES23 appeared to be smaller than expected, although the distribution of a marker among adenine prototrophs was normal (Table 2). All multisite mutants except ES8 and ES23 were stable in reversion experiments with UV-irradiation.

Genetic analyses of secondary ad-8 mutants: An attempt has been made to determine whether the genetic location of secondary mutants is influenced by residual effects of the primary mutation. Among 53 secondary mutants crossed, ten have been located at the same genetic site as the original primary mutant in which the subsequent reversion had been induced, whereas 19 have been located at different genetic sites (Table 4). Four multisite secondary mutants have also been found. Two (ES1 and ES30) involved a defect covering the entire locus; one (ES8) involved the genetic site in which the primary mutant was located, and the fourth (ES23) did not. In the most striking case, seven out of 24 secondary mutants derived from one particular revertant, E118 R4 (which has five percent of the wild-type AMP-S synthetase activity; ISHIKAWA, unpublished), have been located at the same genetic site as the primary mutant (E118) and in addition two of the three multisite secondary mutants induced in this revertant include the original E118 site. Although these results are not extensive, they make it

TABLE 4

Genetic analyses of some secondary ad-8 mutants

Primary mutant used	Origin*	Genetic location				
		Same as primary	Different from primary	Multisite mutation	Not yet decided†	Not yet tested
E51	UV	0	8	1	1	6
E118	UV	7	7	2	6	12
	Sp	0	1	1	0	0
E60	UV	1	1	0	6	0
E108	UV	1	2	0	7	0
E124	UV	1	0	0	0	3

* UV, ultraviolet; Sp, spontaneous.

† Because of too high reversion frequency or leakiness.

appear highly probable that in some instances secondary mutations tend either to be induced preferentially at (or near) the genetic site where the original primary mutation was located, or to involve larger defects in the genetic material.

DISCUSSION

A genetic map of the *ad-8* locus has been constructed on the basis of relative prototroph frequencies observed in repeated crossing experiments with various *ad-8* mutants. The resolving power in the present crossing analyses is approximately 1×10^{-5} (estimated from the number of viable ascospores obtained and the standard errors of prototroph frequencies among viable ascospores in repeated crosses).

One hundred and seventy-five mutants have been crossed in various combinations and 37 genetic sites have been distinguished within the *ad-8* locus. A linear arrangement of the genetic sites is possible on the basis of the two following procedures which agree in giving a single consistent order: (1) prototroph frequencies show (with very few exceptions) additive relationships and (2) the distribution among prototrophs of a linked marker carried by one of the parents is usually asymmetrical. Although the general additivity of prototroph frequencies in interallelic crosses is not in disagreement with the view that prototrophs may arise as a result of reciprocal exchanges, other mechanisms, e.g., gene conversion, may be involved (CASE and GILES 1958; STADLER 1959; SUYAMA *et al.* 1959). A few exceptions to the additivity of map distances have been noted in the statistical analyses of the crossing data; e.g., map distances in some crosses involving E96, E121 and ES16 were smaller than expected from additive relationships. On the other hand, crosses involving ES3 resulted in larger map distances than expected. These exceptions suggest the existence of negative and positive interference, respectively. However, further experimentation would be required to substantiate such interference in interallelic crosses, since standard errors found in repeated crosses have been relatively large and three factor crosses (CHASE and DOERMANN 1958) have not been possible at this locus. In addition, the possibility that small rearrangements may be present in some of these exceptional mutants has not yet been excluded.

Fifteen out of 37 sites within the *ad-8* locus are occupied by more than two alleles which do not produce any prototrophs in intercrosses (identical site alleles). The number of genetic sites within a locus can be estimated on certain assumptions (PONTECORVO 1958). The maximum estimate of the number of sites is given by dividing the total recombination distance for a locus by the smallest recombination value between two alleles (PONTECORVO 1958). This procedure is based on the assumptions that the frequency of recombination between adjacent sites is the same over the whole region and that the distribution of the observed sites is at random. The maximum number of sites within the *ad-8* locus is thus estimated to be 84 in the present analysis. The minimum number of sites can be estimated by the following equation published by ROMAN (1956), which is based on the assumptions that the number of alleles crossed is significantly large and that alleles located at the same site are absolutely identical: $n = N/I$, where n is

the estimated number of sites, N is the total number of allelic combinations and I is number of allelic combinations giving no prototrophs. Since there is a possibility that identical site alleles obtained from the same filtration flask may be due to a duplication of one original mutation, such mutants were counted as one to make sure of obtaining a minimum estimate. One site which appears to be specific for mutants induced by base analogues has been excluded from this calculation. The value n thus estimated for the *ad-8* locus is 25. The actual number of sites found at this locus, 37, is between the maximum (84) and the minimum (25) estimates. If the assumptions used to determine the maximum estimate are true, then 47 more sites should be detected on the map in the course of further extensive crossing experiments. The result that the number of observed sites is larger than the minimum estimate indicates that more identical site alleles were observed than the estimate and may suggest the existence of a nonrandom distribution of sites at the *ad-8* locus.

The distribution of identical site alleles within the *ad-8* locus has been found to deviate from a Poisson distribution. Four sites have been detected where mutations are preferentially induced. Three of these sites appear to be highly mutable without mutagen specificity. One other site is occupied exclusively by identical site alleles induced in experiments with BUDR and FUDR.

The incorporation of 5-bromouracil (BU) into deoxyribonucleic acid (DNA) has been published by several investigators (WEYGAND, WACKER and DELLWEG 1952; DUNN and SMITH 1954; ZAMENHOF and GRIBOFF 1954). Severe distortion of the sequence characteristics of bacterial DNA by BUDR incorporation has been also postulated (SHAPIRO and CHARGAFF 1960). Mutable sites which are specific for base analogues have been reported at the *rII* region of bacteriophage T4 (FREESE 1959a,b,c; BENZER 1961). 5-fluorouracil (FU) has been shown to block the conversion of uracil and orotic acid to DNA thymine (EIDINOFF, KNOLL and KLEIN 1957; BOSCH, HARBERS and HEIDELBERGER 1958; DANNEBERG, MONTAG and HEIDELBERGER 1958). HOROWITZ, SANKKONEN and CHARGAFF (1960) reported that FU and FUDR interfere with the biosynthesis of DNA in *E. coli*. However, no report has been published to date indicating the incorporation of FUDR or FU into DNA. Current hypotheses hold that genetic information is determined by the precise sequence of purine pyrimidine bases in DNA and that mutations may be due to changes in such base sequences of DNA (CRICK 1958). The question arises whether the mutagenic effect of base analogues is due to the incorporation of these substances into DNA or to metabolic disturbances which may result in a mutant configuration for a particular sequence of DNA. The assumption has been made that phage mutations may arise as a direct result of the incorporation of BUDR into DNA (FREESE 1959a,b,c). It is, however, difficult to believe that mutations observed at the specific site of the *ad-8* locus are due to the incorporation of both BUDR and FUDR into DNA, since no evidence has been yet presented to show the incorporation of FUDR into DNA. Although there is no evidence to show that both analogues inhibit the same reaction, it may be assumed that these analogues have a specific effect on the biosynthesis of DNA resulting in a specific change in base sequence. A possible

general mechanism would involve an inhibition of thymidine synthesis by these analogues permitting the replacement of thymine by another base.

Mutations obtained spontaneously or induced by X rays, UV, or nitrous acid are distributed almost randomly over the *ad-8* locus. This result is not in agreement with the more extensive data obtained in bacteriophage (BENZER 1961) which indicate that spontaneous mutants are not randomly distributed but occur frequently at certain genetic sites within the *rII* region.

Mutants which produce no prototrophs in intercrosses with other mutants located at two or more different sites within the *ad-8* locus have been detected in these studies. There has long existed a term, "position effect," to describe phenotypic effects resulting not only from rearrangements of chromosomes, such as translocations and inversions, but also from deficiencies and duplications (GOLDSCHMIDT 1955). According to this definition, the mutants described above are due to a position effect of some kind. Another term "multisite mutation" has been introduced recently to describe similar kinds of mutants without discussing the mechanism of mutation (SUYAMA *et al.* 1959; HARTMAN, LOPER and SERMAN 1960). This term appears to be unsuitable for some cases, such as small deletions including only one site on the map. The term "extended mutation" has also been used (CRICK and ORGEL, unpublished). If no back mutation occurs with such mutants, they may be considered to be extended mutations. If a mutant shows true back mutation (not due to a suppressor), it will be assumed to be a point mutation.

With respect to localization and stability, the following four different categories of mutants have been observed within the *ad-8* locus: (1) revertible mutants occupying one genetic site, (2) revertible mutants occupying more than one genetic site, (3) nonrevertible mutants occupying one genetic site, and (4) nonrevertible mutants occupying more than one genetic site. On the basis of the previous discussion, mutants in these four categories are considered to represent the following kinds of mutations: category (1) is a point mutation (no suppressors have been found for mutants at this locus); (2) is a multisite mutation; (3) is an extended mutation, assuming that such a mutation occupies a small but continuous region which covers only one genetic site on the present genetic map; (4) is a multisite mutation as well as an extended mutation. It is most likely, *a priori*, that nonrevertible mutants occupying more than one site involve a deletion. However, the possibility that a rearrangement such as an inversion or a translocation may be involved must also be considered. Although revertible mutants occupying more than one site (category 2) have also been reported at the *pyr-3* locus in *Neurospora* (SUYAMA *et al.* 1959), the mechanism of such multisite mutation is not known, but it may be suggested in view of a reduction of map distances where prototrophs occurred that some kind of rearrangement of the genetic material may be involved.

The present investigations are also of interest in connection with the question whether mutants produced by particular types of mutagens show any correlation in their characteristics with respect to reversion and recombination. X rays appear to be capable of inducing a considerable number of revertible *ad-8* mutants,

as has been demonstrated at several other loci (DE SERRES 1958; CASE and GILES 1958; WOODWARD, PARTRIDGE and GILES 1960). However, three out of 12 X-ray-induced primary mutants tested have proved to be multisite mutants and may well involve such drastic alterations as deletions or chromosomal rearrangements, while only one out of the 27 UV-induced primary mutants tested was a multisite mutation. These results are similar to those found by STADLER (1941) in suggesting that UV produces more apparent point mutations than do X rays. The initially unexpected result that four out of 12 spontaneous mutants tested were multisite mutants may indicate that spontaneous mutation can also involve extensive damage similar to that caused by X rays. This result is not in agreement with the earlier finding by STADLER (1941, 1946) that spontaneous mutations are largely point mutations similar to UV-induced ones, but it is in agreement with the results obtained with *rII* mutants in bacteriophage T4 which indicate that a significant number of spontaneous mutants are "deletion" types (BENZER 1959). Mutations induced by nitrous acid are similar to UV-induced mutants in that no multisite mutations have been obtained among 11 such mutants tested.

The data from genetic analyses of induced secondary *ad-8* mutants indicate that in a considerable number of such secondary mutants the genetic change giving rise to these mutants has occurred at (or included) the same site at which the primary mutation and subsequent reverse mutation had been induced. These results suggest that some apparent reverse mutations (particularly partial reverse mutations) do not constitute a genuine return to the complete wild-type condition but may retain residual genetic effects of the primary mutation such that subsequent secondary mutations are more likely to be associated with such residual genetic effects and may, in fact, sometimes extend such effects over large regions of the genetic map.

SUMMARY

A new group of adenine-requiring mutants in *Neurospora crassa*—at the *ad-8* locus—has been located 26 map units to the left of the centromere in linkage group VI. A total of 308 primary *ad-8* mutants (obtained from wild-type 74A or other closely related wild-type strains) and 77 secondary *ad-8* mutants (obtained from revertant strains at this locus) have been isolated. These mutants are blocked in the conversion of inosine monophosphate to adenosine monophosphate succinate (AMP-S) and lack AMP-S synthetase activity.

Interallelic crosses using 175 primary and secondary mutants have shown that this locus is composed of at least 37 mutational sites arranged in a linear sequence—the intersite distances being generally additive. Three sites appear to be especially mutable without exhibiting mutagen specificity while at one other site occurred at least 15 independent mutations derived from experiments with 5-bromodeoxyuridine and 5-fluorodeoxyuridine.

Fourteen out of 70 mutants tested failed to yield reverse mutants even after ultraviolet irradiation. Ten of these 14 mutants were multisite mutants which

produced no prototrophs in intercrosses with two or more other *ad-8* mutants located at different sites.

Genetic analyses of induced secondary *ad-8* mutants indicate that some apparent reverse mutants do not constitute a genuine return to the wild-type condition but tend to retain residual effects of the primary mutational extent.

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