GENETIC STUDIES OF ad-8 MUTANTS IN NEUROSPORA CRASSA. II. INTERALLELIC COMPLEMENTATION AT THE ad-8 LOCUS[1]

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IN earlier studies with Neurospora, it was believed that with biochemical mutants the heterocaryon test was a satisfactory test for allelism, based on the expectation that only heterocaryons between nonallelic mutants should be capable of growth on nonsupplemented media (BEADLE and COONRADT 1944). However, experiments by MITCHELL and MITCHELL (1956) suggested that a positive heterocaryon test did not constitute acceptable evidence of nonallelism, since some combinations of their allelic mutants at the *pyr-3* locus were capable of complementation. Recently it has become evident that interallelic complementation is a phenomenon of common occurrence, not only at various loci of Neurospora (GILES 1958; CATCHESIDE 1960) but also in other organisms such as Salmonella (HARTMAN, HARTMAN and SERMAN 1960), yeast (MEGNET 1959), Aspergillus (PONTECORVO 1958) and Drosophila (CARLSON 1959; FAHMY and FAHMY 1959; BROSSEAU 1960).

In the present study, evidence will be presented for the occurrence of complementation between adenine-requiring mutants at the *ad-8* locus in Neurospora. A comparison of the complementation map of the locus with the genetic map established in previous studies (ISHIKAWA 1962) provides the basis for a discussion of the structure and function of the *ad-8* gene.

MATERIALS AND METHODS

The 308 primary *ad-8* mutants (also referred to as E mutants) used in this investigation were obtained from the 74A wild-type strain or from other closely related strains derived by inbreeding of 74A. Seventy-seven secondary mutants were obtained from revertant strains of various *ad-8* mutants. The origin and general characteristics of these mutants have been described previously (ISHIKAWA 1962).

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Heterocaryon tests for complementation were made as follows: fresh conidia, grown on a glycerol complete medium, were used to prepare heavy conidial suspensions of the individual *ad-8* mutants in distilled water. Approximately 0.05 ml of a conidial suspension was pipetted onto a spot near the edge of a petri dish containing about 15 ml of minimal agar supplemented with 40 γ/ml DL-histidine. Histidine stimulates the growth of heterocaryons at the *ad-8* locus. Four spots were made when two mutant strains were tested against each other—two of them were control spots, one of each strain, and the two other spots were mixtures of both suspensions. Petri dishes thus inoculated were left in the transfer room overnight and then incubated at 25°C. The results were recorded daily for at least ten days. A positive heterocaryon response was observed as circular mycelial growth from both the spots of mixed conidia which were placed on diametrically opposite edges of the dish. Growth due to reverse mutation was indicated by irregular mycelial growth (particularly in control spots), and was usually identified without difficulty. For convenience in further descriptions, a positive response in heterocaryons between two mutants or between two groups will be denoted by a "+" mark between the two mutant numbers or between the two group numbers.

To test the growth rate of heterocaryons, agar blocks (ca. 2×2 mm) were cut from the heterocaryon cultures and inoculated into 20 ml liquid media in 125 ml Erlenmeyer flasks. Mycelial mats were harvested after a designated time, dried and weighed.

The following general methods, which are modified from LIEBERMAN's procedures in *Escherichia coli* (LIEBERMAN 1956), have been employed to assay adenylosuccinate synthetase (AMP-S synthetase) activity in wild types, mutants, and heterocaryons in Neurospora. Mycelia were grown in Fernbach flasks containing 250 ml Fries' minimal media with appropriate supplements. Inoculation was made by adding one ml of a conidial suspension. Cultures were maintained at 25°C for three days. Mycelial pads were harvested by filtration, washed with chilled water, and frozen quickly. The frozen mycelia were lyophilized overnight and milled to dry powder, or the frozen mycelia were ground with dry ice in a chilled mortar. All the following operations were performed at 4°C. Extracts were prepared by shaking mycelia in 0.005 M potassium phosphate buffer at pH 7.2 for one hour. The supernatant was obtained by centrifugation for 50 min at $30{,}000 \times g$ to remove debris. The supernatant will be called "crude extract." The protein content of each preparation was determined by the Biuret reagent (GORNALL, BARDAWILL and DAVID 1949). The enzyme activity was assayed by a continuous recording of the increase in optical density at 280 mμ as inosine monophosphate (IMP) and aspartic acid were converted to adenosine monophosphate succinate (AMP-S) at 36°C. The reaction mixture of 1.0 ml volume in the sample cell contained IMP, 1.0 μmole; guanosine triphosphate (GTP), 0.15 μmole; L-aspartate, 5.0 μmole; $MgCl_2$, 10.0 μmole; and enzyme preparation, 0.05 ml. L-aspartate was omitted from the reaction mixture in the reference cell. Specific activity, expressed as the optical density change per mg

protein per min per ml at 280 mμ, has been calculated from the linear portion of the absorption curves.

RESULTS

Complementation patterns: Heterocaryon tests were made in all possible pairwise combinations between 30 *ad-8* mutants (from E2 to E33) at the initial stage of this investigation. Only two combinations, E6 + E8 and E6 + E32, were found to show a positive heterocaryon response on minimal media after four days. Since it had been found that histidine stimulates the growth of *ad-8* mutants, the possibility of detecting additional cases of complementation was examined by making heterocaryons on histidine-supplemented medium, assuming that histidine might also stimulate the growth of heterocaryons. Twelve additional combinations, E6 + E22, E6 + E25, E6 + E26, E6 + E28, E8 + E22, E8 + E25, E8 + E26, E8 + E28, E32 + E22, E32 + E25, E32 + E26, and E32 + E28, were found to give positive responses. These heterocaryons were detectable after six or seven days at 25°C only on minimal medium supplemented with histidine. After this experiment, more mutant combinations were tested on histidine and the complementation patterns of these additional mutants were determined by making tests against known representatives of each group (E6, E51; E26, E80, E96; E8, E32, E110). If mutants give the same complementation pattern as known representatives and heterocaryon tests between these mutants are negative, these mutants constitute a complementation group. Under these conditions, 107 of 308 primary *ad-8* mutants and 22 of 77 secondary *ad-8* mutants gave a positive heterocaryon response with at least one other group of alleles on minimal medium supplemented with histidine. On the basis of heterocaryon responses, six complementing groups, I, II, III, I–II, II–III and I–III, and a noncomplementing group have been found. Pseudowild type tests gave essentially the same results as obtained by complementation tests. Members of the single groups, I, II, and III can complement each other in all combinations, whereas members of double groups complement only one other group; I–II can complement only III, II–III can complement only I, and I–III can complement only II. It seems significant that the heterocaryons between group I and group III (I + III) in general form more rapidly than any other combinations. However, heterocaryons involving particular mutants in group I (E118 and ES6) and group III (E271, ES3, ES4 and ES61), designated as subgroup I′ and III′ respectively, are more or less exceptional. Subgroup I′ complements group III at a very slow rate and does not complement subgroup III′, whereas subgroup III′ complements group I at a slow rate. There are also several exceptional cases involving heterocaryons or pseudowild types of the double types; i.e., group I-II fails to complement or complements very slowly subgroup III′, and group II-III complements subgroup III′ (with the exception of E271).

Despite these exceptional cases, the complementation relationships among the mutants can be represented in such a manner as to form a general complementation map of this locus, according to the general method presented by GILES (1958). The complementation map finally adopted for the *ad-8* locus is shown

in Figure 1, in which figures above each line refer to the number of mutants exhibiting a particular pattern of complementation. Although the number of double mutants found is very small compared with the number of single types, the existence of three kinds of double types does not permit a unique linear arrangement of the three single types. Other arrangements of the three single types could as easily be made; e.g., a triangular or circular shape, or a branched Y shape. Reasons for favoring the arrangement in Figure 1 will be presented in later sections.

Growth rates of heterocaryons: Growth rates of three interallelic heterocaryons (E6 + E61, E61 + E32, and E6 + E32) as well as the wild type (74A), the single mutant (E6), and the interlocal heterocaryon (i.e., a heterocaryon between mutants at two genetically distinct loci), E6 (*ad-8*) + F12 (*ad-4*), have been obtained from growth on minimal, adenine, or histidine media expressed as mg dry weight (Table 1). The growth rate of the interlocal heterocaryon (E6 + F12) on minimal medium was slightly less than the rate on adenine medium at the earlier stages of growth. However this tendency was most pronounced in interallelic heterocaryons. The heterocaryon, E6 + E32 (I + III) began to grow after about three days on minimal medium, while the heterocaryons, E6 + E61 (I + II) and E61 + E32 (II + III) began to grow only after ten days on minimal medium. Histidine is effective in stimulating the growth of these heterocaryons; with histidine, E6 + E32 began to grow within two days and E6 + E61 and E61 + E32 began to grow within four days. The optimal concentration of L-histidine for stimulating the growth of interallelic heterocaryons is about 20 γ/ml. Higher concentrations of histidine show an inhibiting effect.

In comparing growth rates at the two temperatures, 25°C and 35°C, it was found that E6 + E32 was greatly depressed at 35°C, while E6 + E61 and E61 + E32 showed no growth at 35°C after ten days, even with histidine (Table 1). Since the growth of the wild type and the interlocal heterocaryon was stimulated at 35°C in the earlier stages of growth, temperature sensitivity seems to be a characteristic of all these interallelic heterocaryons at the *ad-8* locus.

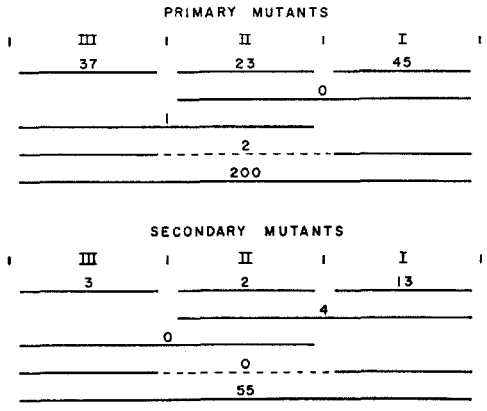

FIGURE 1.—Complementation map of the *ad-8* locus. Roman numerals show complementation groups. Figures above each line refer to mutants of each group.

TABLE 1

Growth in mg dry weight of interlocal and interallelic heterocaryons, an ad-8 *mutant, and a wild-type strain*

Strain or heterocaryon	Medium*	25°C					35°C				
		24 hr	48 hr	72 hr	112 hr	240 hr	24 hr	48 hr	72 hr	112 hr	240 hr
E6+F12	min	1.2	24.6	46.6	72.4	95.2	6.8	33.6	53.6	57.4	70.0
(*ad-8*+	his	1.2	32.0	54.0	77.4	88.0	11.6	38.2	50.2	80.2	62.8
ad-4)	ad	1.2	35.0	62.0	90.4	93.4	7.0	39.4	63.0	69.6	52.8
E6+E61	min	0	0	0	0	1.2	0	0	0	0	0
(I+II)	his	0	0	0	5.6	15.4	0	0	0	0	0
	ad	1.0	19.8	42.0	67.6	107.8	4.6	31.2	55.8	54.1	53.8
E61+E32	min	0	0	0	0	0.6	0	0	0	0	0
(II+III)	his	0	0	0	3.4	13.8	0	0	0	0	0
	ad	1.0	32.0	45.8	62.2	99.4	6.4	43.0	55.0	59.4	51.8
E6+E32	min	0	0	8.2	15.4	47.2	0	0	0	5.2	17.6
(I+III)	his	0	6.0	21.2	35.6	76.2	0	0	5.6	11.6	27.0
	ad	1.0	22.0	43.8	65.2	108.4	7.4	29.8	58.2	67.4	54.4
E6	min	0	0	0	0	0	0	0	0	0	0
(*ad-8*)	his	0	0	0	0	0	0	0	0	0	0
	ad	2.2	28.8	47.4	63.4	84.8	5.4	38.0	57.0	60.2	52.3
74A	min	3.6	37.6	70.0	94.8	88.4	13.4	45.4	69.8	87.6	61.4
(wild)	ad	1.6	36.0	77.2	95.0	73.2	7.2	41.6	70.6	96.8	55.1

* Min, Fries' minimal medium; his, min+DL-histidine 40 γ/ml; ad, min+adenine sulfate 100 γ/ml.

This characteristically slow growth and temperature sensitivity similar to that indicated in Table 1 have been proved by additional extensive growth tests to be of general occurrence in interallelic heterocaryons at the *ad-8* locus. Among the combinations involving the six complementation groups, it was found that heterocaryons I + III give the highest dry weight, while heterocaryons I + II grow faster than II + III. A highly significant correlation has been noted between the growth rate of interallelic heterocaryons as measured in mg dry weight and the time required to detect a positive heterocaryon response in plate tests.

The relationship between complementation and mutagenic origin of ad-8 *mutants:* As pointed out by other workers (CASE and GILES 1960; DE SERRES 1960), the percentage of complementing mutants appears to be related to the kind of mutagen used. This relationship can be clearly demonstrated at the *ad-8* locus, since a significantly large number of mutants of diverse origins has been obtained at this locus (Table 2). More than half of the ultraviolet-induced (UV) primary mutants (56 percent) but only 15 percent of X-ray-induced mutants are capable of complementation. Among the mutants obtained by chemical treatment, all mutants induced by base analogues were noncomplementing, whereas 38 percent of mutants induced by either nitrous acid or ethyl methanesulfonate exhibited complementation, a figure of which is similar to that obtained with mutants of spontaneous origin. Thus, it seems possible to conclude that for *ad-8*

TABLE 2

Complementation group and origin of ad-8 *mutants**

Complementation group	Primary mutants							Secondary mutants		
	No. of mutants	Origin†						No. of mutants	Origin†	
		Sp	X	UV	NA	EMS	AN		Sp	UV
I	45	2	1	39	2	1	0	13	0	13
II	23	5	3	9	3	3	0	2	1	1
III	36	1	1	20	13	1	0	3	0	3
I–II	0	0	0	0	0	0	0	4	0	4
II–III	1	0	0	0	1	0	0	0	0	0
I–III	2	1	0	0	1	0	0	0	0	0
Complementing	107	9	5	68	20	5	0	22	1	21
Noncomplementing	188	16	29	53	29	8	53	55	1	54
Total no.	295	25	34	121	49	13	53	77	2	75
Percent complementing	36	36	15	56	38	38	0	29	50	28

* Thirteen mutants induced by combined treatment with UV and base analogues are omitted from this table.
† Following abbreviations are used: Sp, spontaneous; X, X-rays; UV, ultraviolet; NA, nitrous acid; EMS, ethyl methanesulfonate; AN, 5-bromodeoxyuridine and 5-fluorodeoxyuridine.

mutants the relative frequency of complementing mutants is related to the origin of mutants in such a way that a larger percentage of complementing mutants is produced by UV treatment than by other treatments, whereas a smaller percentage of complementing mutants is produced by X rays than by other agents (with the exception of base analogues). The perhaps unexpected nature of mutants induced by base analogues is explained by the fact that all these mutants are located at one specific genetic site on the fine structure map of the *ad-8* locus (ISHIKAWA 1962).

An examination of the relationship between the origin of mutants and their complementation patterns indicates that more than half of complementing UV-induced mutants and of the chemically induced mutants classified in groups I and III, respectively. At present, however, these data do not appear to be sufficient to permit the conclusion that there is preferential induction by certain mutagens of mutants classifiable into certain complementation groups.

Studies with the secondary *ad-8* mutants indicate that 29 percent of the mutants were complementing types, whereas 35 percent of the primary *ad-8* mutants were complementing (Table 2). The relationship between the complementation pattern and the background of the secondary mutants is analyzed in Table 3. Thirteen of 21 complementing secondary mutants of group I background were classified as group I. Only one complementing secondary mutant of group II background was classified as group II. Four I-II double mutants were obtained from a group I background. Three group III secondary mutants were also obtained from a group I background, but these mutants were placed in subgroup III′, which complements group I at an exceptionally slow rate. All secondary mutants derived from revertants of noncomplementing primary mutants were noncomplementing. These results appear to suggest a tendency for

TABLE 3

Relationship between complementation pattern and derivation of secondary ad-8 *mutants*

Background			Complementation pattern							
Mutant	Complementation group	Origin	I	II	III	I–II	II–III	I–III	—	Total
E51	I	UV	7	0	3	0	0	0	6	16
E118	I	UV	6	0	0	4	0	0	24	34
		Sp	0	1	0	0	0	0	1	2
E60	II	UV	0	1	0	0	0	0	7	8
E96	II	UV	0	0	0	0	0	0	2	2
E32	III	UV	0	0	0	0	0	0	1	1
E108	—	UV	0	0	0	0	0	0	10	10
E124	—	UV	0	0	0	0	0	0	4	4
Total			13	2	3	4	0	0	55	77

secondary mutations to involve, or show some interaction with, the complementation group of the primary mutants from which they were derived.

Adenylosuccinate synthetase in the wild type, ad-8 *mutants, and heterocaryons:* Whenever crude extracts of the wild-type strain were assayed for AMP-S synthetase, no change of optical density at 280 mμ was observed. It is expected that AMP-S synthesized by this enzyme is split further to adenosine monophosphate (AMP) by the adenylosuccinase contained in the wild-type extract. To separate the two enzymes the wild-type extract was subjected to an ammonium sulfate fractionation at pH 7.2. A large amount of adenylosuccinase activity was found in the 40–50 percent fraction, whereas AMP-S synthetase activity was strongest in the 50–60 percent fraction. However, the complete separation of the two enzymes was not possible in this experiment. An effort to separate the two enzyme activities by differential inhibition of adenylosuccinase was made, but no satisfactorily specific inhibitor for this enzyme has been found so far. For the present, the best method to exclude interference of adenylosuccinase activity in the AMP-S synthetase assay is to use as the source of AMP-S synthetase *ad-4* mutants which lack adenylosuccinase. In all tests employing *ad-4* mutants, the strain F12 was utilized.

The optimum pH for AMP-S synthetase in Neurospora was about 7.5 for phosphate buffer and about 7.8 for glycine buffer. The optimum concentrations for the two buffers were about 0.02 M and 0.10 M respectively. These results indicate that inorganic phosphate partially inhibits the AMP-S synthetase of Neurospora at higher concentrations, as was also reported in *Escherichia coli* (LIEBERMAN 1956) and in mammalian tissues (DAVEY 1959). Tests for the optimum concentration of each component in the reaction mixture show that IMP, 0.5 μmole, GTP, 0.1 μmole and L-aspartate, 3.0 μmole are enough to saturate the enzyme reaction. Excess GTP appears to inhibit the reaction. Even with saturating levels in the reaction mixture, a gradual decrease in the reaction rate was observed within a short time (about four min). Addition of more substrate and GTP to the reaction mixture was not effective in restoring the rate. When

phosphoglycerate (PGA) was added in the reaction mixture to regenerate GTP, the reaction was kept at a constant rate for at least 20 min at 36°C. The decrease in reaction rate may be due to an inhibition by guanosine diphosphate (GDP) produced in the reaction mixture, as suggested by LIEBERMAN (1956). AMP-S synthetase can be stored for at least one month in a deep freeze with little loss of activity.

The mitochondrial fraction of the wild-type strain prepared by the method described by HASKINS, TISSIERES, MITCHELL and MITCHELL (1953) showed no AMP-S synthetase activity. Since appreciable activity was obtained in the supernatant fraction, it seems reasonable to conclude that AMP-S synthetase is not located in the mitochondria.

Using *ad-4* mutants as the source of wild-type AMP-S synthetase, it was found that the amount of adenine supplement in the culture media has a great effect on enzyme activity. For example, mycelia grown in 100 γ/ml adenine showed 13 percent of activity of culture with 10 γ/ml adenine. When mycelial growth was stimulated by the addition of histidine, enzyme activity was decreased compared with cultures grown with the same amount of adenine alone. These results are interpreted as indicating the occurrence of enzyme repression (or negative feedback) (cf. VOGEL 1957) in which the product of a biosynthetic pathway, adenine in this case, inhibits a prior stage of synthesis. Histidine seems to affect the AMP-S synthetase activity indirectly by sparing adenine. Adenine has been shown to be a feedback inhibitor of purine biosynthesis in other organisms (GOTS and GOLDSTEIN 1959).

Ethylenediaminetetraacetate (EDTA) showed a stimulating effect at a low concentration, but a marked inhibition of enzyme activity at higher concentrations. This inhibition was partly overcome by the addition of a large amount of $MgCl_2$. Mercaptoethanol appears to stabilize the enzyme, since assay results were more reproducible with it. When adenosine triphosphate (ATP) was added in the reaction mixture instead of GTP, no evidence was obtained to support the idea that ATP alone or both ATP and GTP are required for the reaction (YEFIMOCHKINA and BRAUNSTEIN 1959; DAVEY 1959). GDP was a strong inhibitor for this enzyme. The addition of PGA can partially reverse the inhibition by GDP. This may be due to the regeneration of GTP from GDP in the presence of PGA. Parachloromercuribenzoic acid and NaF inhibit the reaction partially.

To assay extracts of *ad-8* mutants for AMP-S synthetase, double mutants (*ad-8* and *ad-4*) have been prepared to avoid complications with adenylosuccinase activity. No AMP-S synthetase activity was detected in nine *ad-8* mutants tested, including the slightly leaky mutant, E80, even when double the usual concentration of extract was used. No significant inhibition of the wild-type AMP-S synthetase activity was observed upon the addition of crude extracts of *ad-8* mutants.

The occurrence of adenine-independent heterocaryons within the *ad-8* locus as well as between *ad-8* and other loci raises the question whether AMP-S synthetase activity can be detected in these heterocaryons. To eliminate complications

with adenylosuccinase activity, forced heterocaryons, in which the *ad-4* marker was introduced in both strains, were prepared, using *pan-2* and *lys* as markers. Mycelia were usually grown in minimal medium supplemented with 10 γ/ml adenine, which is known to give a partially derepressed condition. The enzyme activity of interlocal heterocaryons appears to average 50 percent of the wild-type AMP-S synthetase activity. All interallelic heterocaryons assayed failed to give detectable AMP-S synthetase activity. Unsuccessful attempts were made to obtain enzyme activity, using the maximal amount of protein possible in assays, by the addition of various substances to the usual reaction mixture (e.g. PGA, EDTA, mercaptoethanol, ATP), or by assaying at lower temperatures. Negative results were also obtained using different extraction procedures, trying ammonium sulfate fractionation, and using mycelia cultured with a different level of adenine, or mycelia of different ages. Unmarked interallelic heterocaryons were also assayed after ammonium sulfate fractionation, but again no enzyme activity was detected. The lower limit of the specific activity which could be detected by the present procedure without purification is approximately one percent of the wild-type enzyme activity. Therefore, on the basis of this investigation, one possible conclusion is that interallelic heterocaryons yield less than one percent of the wild-type enzyme or a grossly altered (perhaps unstable) enzyme which cannot be detected by the present procedure.

Comparison of the complementation and genetic maps: With the establishment of complementation and genetic maps at the *ad-8* locus (cf. ISHIKAWA 1962), a comparison of the order of mutants on both maps can be made. Such a comparison is critical for a better understanding of the structure and action of a gene on the basis of current hypothesis (CRICK 1958). A comparison of a complementation map with a genetic map made previously at the *pan-2* locus (CASE and GILES 1960) and at the *pyr-3* locus (WOODWARD 1962) indicated that the two maps were colinear with the significant exceptions. The terms "colinear" or "isomorphic" are employed in the sense that corresponding alleles are in the same relative order on the two maps. Although it will be noticed at first glance that there are exceptions in the order of the two maps at the *ad-8* locus, there is also considerable agreement as shown in Figure 2 in that most mutants in group I of the complementation map tend to be located in the right-hand region of the genetic map, and most group III mutants are located in the left-hand region, whereas group II mutants lie between the two regions. On the other hand, all double type mutants and several exceptional single type mutants are located in a particular region (designated region X) which occupies a position between regions I and II on the genetic map. Mutants in region X appear to be arranged randomly with regard to complementation grouping but their genetic sites are grouped very closely on the genetic map even when their behavior in complementation is different. Moreover, mutants located in this particular region show more or less exceptional behavior in complementation (including pseudowild type tests) as described previously.

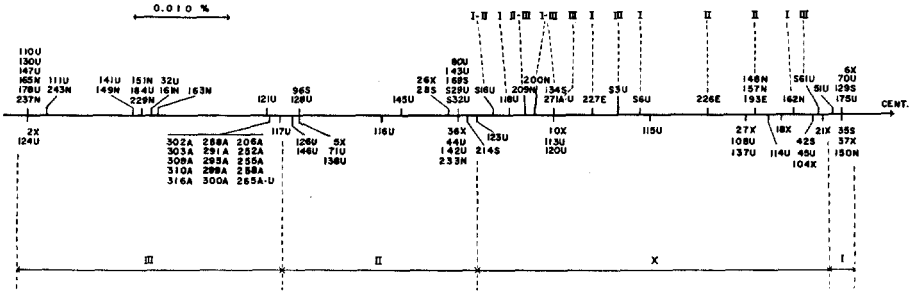

FIGURE 2.—Comparative complementation and genetic maps at the *ad-8* locus. Roman numerals show complementation groups as described in the text. On the genetic map capital E of the mutant number is omitted and complementing mutants are placed above the line, non-complementing ones below. Origin of mutants is abbreviated as follows: A, base analogues; E, ethyl methanesulfonate; N, nitrous acid; S, spontaneous; U, ultraviolet; X, X rays.

DISCUSSION

It is becoming more evident that the pattern of interallelic complementation is probably characteristic for a particular locus. For example, the number of complementation units found within one locus of Neurospora depends on the locus. Some loci have been reported to have three or four units (CATCHESIDE and OVERTON 1958; GILES 1958), and others more than five (GILES 1958). In the course of the screening of *ad-8* mutants only three complementation units were discovered in the first 22 mutants tested and thereafter no additional single types were found in testing a total of 385 *ad-8* mutants to date. Double types appeared sporadically in comparison with single types, since only seven mutants (four of them possibly identical) out of 385 mutants obtained were double types. The spreading effect in such double types, which is interpreted to mean the extension of the effect of localized genetic damage over two or more complementation units, may be intrinsically more infrequent or exceptional at this locus than at other loci, where various multiple overlapping types have been observed frequently (cf. GILES 1958).

The construction of a complementation map depends on the existence of multiple overlapping types, based on the assumption that apparent non-functional regions in such overlapping types are continuous (GILES 1958). The arrangement of the three complementation units at the *ad-8* locus by such a method need not be a linear one. Because of the existence of three different double types, a closed or a branched map can be constructed. The absence of a unique linear complementation map at the *ad-8* locus may be exceptional. However, this situation raises a possible question as to the significance of the linearity of complementation maps at other loci, which are constructed only by the employment of overlapping types.

Two categories of experimental results appear to support one of the three possible orders of the groups on the complementation map at the *ad-8* locus. One is the quantitative analysis (by growth rates) of interallelic heterocaryons and the other is the comparison between possible complementation maps and the

genetic map. It has been suggested that the amount of enzyme activity, or the degree of expression of the wild phenotype (as, for example, the growth rate in the absence of supplement) resulting from complementation is positively correlated with the distance apart of a given pair of complementing mutants on a complementation map (WOODWARD, PARTRIDGE and GILES 1958; WOODWARD 1959). It has been found in the present data at the *ad-8* locus that complementation between single groups I and III, based on both the time required for a positive response and the growth rate of heterocaryons, is more efficient than is that of any combinations of mutants from other complementation groups (with some exceptions including subgroups I′ and III′). This evidence then suggests, in the light of the above hypothesis, that the distance between groups I and III on the complementation map may be greater than that of any other combinations of groups, indicating the linear order of the three single groups to be "I–II–III." Furthermore, a comparison of possible complementation and genetic maps at the *ad-8* locus has revealed that most single types can be arranged in a linear order "I–II–III" on the genetic map. However, all double types and some mutants classified in single groups are exceptional in the sense that they are all located within a single particular region of the genetic map. If all overlapping types found at the *ad-8* locus are assumed to be exceptions of some kind, because of their rare occurrence and their exceptional location on the genetic map, it must be concluded that the three complementation units at the *ad-8* locus are independent of each other. Thus a unique complementation map cannot be constructed without accepting the hypothesis that the degree of complementation is positively correlated with distance apart of complementation groups on the complementation map.

The gene as a functional unit has been defined by the use of the term "cistron" (BENZER 1957). In BENZER's definition, any two mutants are considered to be defective in the same functional unit (cistron) if the trans configuration, in which both mutant genomes are present, but separated, within the same cell, does not exhibit a wild phenotype. The cis configuration of such mutants produces a wild phenotype. Cistrons thus defined are independent of each other in function. Furthermore, the cis and trans configurations between mutants in different cistrons result in equal degrees of restoration of the wild phenotype. It has been assumed that two complementing alleles at the *ad-8* locus are different in function, showing the existence of different functional (complementation) units. Thus, the question can be raised whether such complementation units are equivalent to cistrons. Evidence from the enzyme assays at the *ad-8* locus has shown that although the cis configuration (simulated by interlocal heterocaryons since no double *ad-8* mutants are available) produces approximately 50 percent of the wild-type enzyme activity, all trans configurations (interallelic heterocaryons) tested produce less than one percent of the wild-type enzyme activity. The growth rates of all interallelic heterocaryons have been found to be significantly lower than that of interlocal heterocaryons. In addition, growth tests have shown that all interallelic heterocaryons are temperature sensitive; i.e., at 35°C some of them grow at very much reduced rates and others fail to

grow, a result unlike that obtained with interlocal heterocaryons. These results indicate that the degree of restoration of function in the trans configuration is always much less than that in the cis configuration at the *ad-8* locus and that the partially restored function may be altered. The conclusion can be drawn from these results that the functional units defined by complementation tests at the *ad-8* locus are not comparable with the functional unit, the cistron, as defined by BENZER. This conclusion has also been suggested in the case of the *pan-2* locus (CASE and GILES 1960). The whole *ad-8* locus may be considered equivalent to a cistron and the complementation map may then be taken to indicate some other type of functional heterogeneity within a cistron.

Since no nuclear exchange has been established as occurring in complementing heterocaryons in Neurospora, it appears certain that complementation involves the interaction of gene products in the cytoplasm of such heterocaryons (WOODWARD *et al.* 1958). Although a detailed mechanism of complementation is still hypothetical, several possible models can be postulated for the type of interactions involved in complementation. They are: (a) The exchange between or union of two differentially defective, nonfunctional units occurring in an earlier stage of protein synthesis for example, exchange between ribonucleic acid molecules, which are believed to serve as templates for protein synthesis (cf. CRICK 1958); (b) Interaction occurring between protein molecule(s) fully or partially synthesized: (1) Interaction involving union of or exchange between two differentially defective parts of a single polypeptide chain, as reported in the study of subtilisin-modified ribonucleases (RICHARDS 1958); (2) Interaction involving dissociation and exchange of two more differentially defective polypeptide chains of a single protein, as known to occur in the case of hemoglobin (RAPER, GAMMACK, HUEHNS and SHOOTER 1960; SINGER and ITANO 1959); (3) Interaction involving dissociation and reaggregation of differentially defective aggregates (enzyme polymers) as suggested by BRENNER (1959), CASE and GILES (1960), and CRICK and ORGEL (unpublished).

The first mechanism (a) seems to be unlikely, since complementation has been found *in vitro* under conditions where little or no protein synthesis could occur (WOODWARD 1959). The first and second types of interactions in mechanism (b) would be likely if the active protein resulting from complementation were identical with that in the wild type. However, evidence at the *am* and *ad-4* loci indicates that the enzyme from complementing heterocaryons is often different from the wild type (FINCHAM 1959; PARTRIDGE 1960). The last type of interaction of mechanism (b) would be attractive if an enzyme molecule proves to be a polymer whenever complementation is observed. In fact, in recent years, increasing evidence has been obtained for the occurrence of dissociation and/or aggregation of protein molecules under certain conditions; e.g., in glutamic dehydrogenase (FRIEDEN 1958; SOYAMA 1958; VALENTINE 1959), in alcohol dehydrogenase (KÄGI and VALLEE 1960), in thetin-homocysteine methylpherase (DURELL and CANTONI 1959; KLEE and CANTONI 1960), in phosphorylase (MADSEN and CORI 1956), in haptoglobin (ALLISON 1959), in macroglobulin

(DEUTSCH and MORTON 1958) and in β-lactoglobulin (TIMASHEFF and TOWNEND 1961).

In attempting an explanation of complementation at the *ad-8* locus by this mechanism, the assumption may be made that AMP-S synthetase is a polymer, (e.g., dimer) which contains an active site around an axis of symmetry. Further assumptions would then be made that the active site would be formed by the cooperation of three segments arranged in an order around the axis corresponding to the three complementation units, and that damage within any one segment would be extended along the whole segment. Then dissociation and reaggregation of differentially defective aggregates in the course of complementation would result in pairs of the segments which compensated each other's defects at the active site. The prediction would be that such a mixed aggregate would be altered in nature as compared to enzyme from wild type because of the particular situation permitting a compensation of two different defects. If the compensation at the active site were imperfect, then the enzyme activity of the mixed aggregate could be very low.

It seems probable that an alteration in the base sequence of deoxyribonucleic acid (DNA) would result in a corresponding change of a specific structure (amino acids) in the protein, since it is assumed that DNA carries the information that determines the specificity of the protein molecule (CRICK 1958). Therefore, it is suggested that the arrangement of complementation units should tend to correspond to the linear order of mutations on the genetic map. If there is an exceptional segment of the enzyme molecule which is not critical for activity but from which can extend an effect of damage to one or more critical segments by some kind of structural relationship (such as crosslinks), the complementation map will be nonlinear and will not be isomorphic with the genetic map. The actual comparison of the complementation and genetic maps at the *ad-8* locus appears to be in good agreement with such an expectation, since the complementation map of the *ad-8* locus may be considered nonlinear in that all double types and some mutants classified as single types involve exceptions to a complete isomorphism of the two maps. In particular, these exceptional mutants are all located within a single distinct region of the genetic map, which may correspond to the particular segment of the enzyme molecule discussed above.

An interpretation of the occurrence of a large group of noncomplementing mutants should also be provided in describing the genetic map of the *ad-8* locus. Evidence has been presented previously (ISHIKAWA 1962) for the existence of two classes of noncomplementing mutants, point mutants and extended mutants. It may be appropriate to suggest that noncomplementing, extended mutations result in such severe damage to the whole active site or other part on the protein molecule that it is incapable of complementation. However, a reasonable interpretation should be given for the fact that the majority of noncomplementing mutants are point mutants and are located almost randomly over the whole extent of the genetic map. An interesting assumption has been made that in at least some noncomplementing mutants no proteins related to the normal enzyme

activity are formed, as may be true for non-CRM forming mutants in Neurospora and *Escherichia coli* (CASE and GILES 1960). On the basis of the present interpretation of complementation between *ad-8* mutants, an alternative interpretation would be that noncomplementing mutants may be so damaged that the damage cannot be corrected because of the failure of proper pairing with another differentially defective polypeptide.

SUMMARY

130 of 385 *ad-8* mutants gave a positive complementation response with at least one other group of alleles. The resulting complementation pattern has been interpreted as indicating the existence of three distinct functional units at this locus. Interallelic heterocaryons at 25°C never attain a growth rate in the absence of adenine equivalent to the wild-type rate. In addition, all interallelic heterocaryons are temperature sensitive at 35°C, exhibiting little or no growth.

Enzyme assays for adenosine monophosphate succinate (AMP-S) synthetase in wild type have yielded a significant amount of activity using guanosine triphosphate and Mg^{++} with the substrates, inosine monophosphate and aspartic acid. All *ad-8* mutants tested have no detectable AMP-S synthetase activity. Heterocaryons between *ad-8* mutants and mutants at other loci possess approximately 50 percent of wild-type AMP-S synthetase activity, but enzyme assays of interallelic heterocaryons (between *ad-8* mutants) failed to show detectable activity.

A comparison of the complementation map (as finally drawn in the light of quantitative data) with the genetic map of the *ad-8* locus shows a certain parallelism between the two maps with several significant exceptions. These exceptions suggest the existence of a special genetic region of this locus related to each of the three basic complementation units.

Possible interpretations of interallelic complementation and of the relationship between the complementation and genetic maps at the *ad-8* locus have been discussed.

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