COMPLEMENTATION AT THE ADENYLOSUCCINASE LOCUS IN *ASPERGILLUS NIDULANS*[1]

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THE phenomenon of allelic (interallelic) complementation between mutants defective for the same enzyme was discovered in heterokaryons of Neurospora (GILES, PARTRIDGE and NELSON 1957; FINCHAM and PATEMAN 1957). Since no stable diploid is formed in this fungus, there is a problem in the control of nuclear ratios, and thus of gene ratios, in the heterokaryon (PITTENGER and ATWOOD 1956; CASE and GILES 1960). A similar problem arises with *in vivo* studies of allelic complementation in the Enterobacteria which use unstable partial heterozygotes (GAREN and GAREN 1963; LOPER *et al.* 1964). Although yeasts such as Saccharomyces (DORFMAN 1964) or Schizosaccharomyces (MEGNET and GILES 1964) have the advantage that a stable diploid is formed, no heterokaryotic stage is available.

Attention has thus been turned in this laboratory to *Aspergillus nidulans* in which both a heterokaryotic and a stable diploid phase can be obtained (PONTECORVO 1963). A special point of interest in Aspergillus lies in the reports of a few cases in which there is an apparent difference in complementation in heterokaryons and diploids (ROBERTS 1963, 1964; PONTECORVO 1963). A similar phenomenon has also been reported in Coprinus where genes at different loci fail to complement in dikaryons (LEWIS 1961; MORGAN 1961) but complement in diploids (LEWIS unpublished; see PONTECORVO 1963).

It appeared that adenine-specific mutants might provide a suitable gene-enzyme system for study in *A. nidulans*. In Neurospora, adenine-specific mutants are those which are blocked in the reactions after inosine monophosphate (IMP) and consequently fail to grow when adenine is replaced by hypoxanthine (Figure 1). Two major groups have been recognized in Neurospora: mutants at the *ad-8* locus blocked in the reaction IMP to AMPS (adenosine monophosphate succinate) which lack AMPS synthetase and accumulate hypoxanthine (ISHIKAWA 1962a) and mutants at the *ad-4* locus blocked in the two chemically similar reactions 5-amino-4-imidazole (N-succinylo-carboximide) ribotide (SAICAR) to 5-amino-4-imidazole carboximide ribotide (AICAR) and AMPS to AMP (adenosine monophosphate) which lack adenylosuccinase and accumulate SAICAR (GILES, PARTRIDGE and NELSON 1957).

Both types of adenine-specific mutants have been isolated for the first time in

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A. nidulans and this paper describes allelic complementation among the adenylosuccinase mutants. Complementation has been compared in both heterokaryotic and diploid stages and a complementation map based on diploids has been constructed. It has been found that the number of complementing pairs was increased when the test environment was modified (conditional complementation) and unexpected cases of negative interactions (negative complementation) between mutant alleles or between wild-type and mutant alleles have been discovered.

MATERIALS AND METHODS

Origin of strains: Four translocation-free stocks were obtained from DR. ETTA KÄFER, her stock numbers 641: *paba1 y; ve*+; 818: *bi1; meth2; ve*⁻; 149: *bi1; Acr1 w3; ribo2* and 796: *lys5; s3; nic8*. Allele symbols are those of KÄFER (1958). The strains used in this work were derived from crosses between these stocks or their progeny. The standard wild type (A 182) was a *ve*⁻ segregant (KÄFER 1962) from one of the crosses. The two other standard strains were A 52: *bi1 y; Acr1; ribo2 ve*⁻ (designated AR strain) and A 178: *Acr1 w3; lys5; nic8; ve*⁻ (designated ARW strain).

Media: Minimal medium (MM) was the standard medium for Aspergillus (PONTECORVO, ROPER, HEMMONS, MACDONALD and BUFTON 1953), but included a trace element solution (EVERSOLE 1956) as recommended by KÄFER (1958). Complete medium (CA) was the medium of MACKINTOSH and PRITCHARD (1963) modified by the addition of trace elements (EVERSOLE 1956) and DL-histidine HCl (40 μg/ml). Riboflavin (5 μg/ml) or L-lysine HCl (370 μg/ml) were also added when the AR or ARW strains were cultured.

Incubation: Unless stated otherwise, cultures were incubated at 37°C.

Isolation of mutants: Adenine-requiring mutants were isolated following ultraviolet irradiation of conidial suspensions and filtration-concentration of the survivors (WOODWARD, DE ZEEUW and SRB 1954). The AM series of mutants was derived from A 182 by plating in minimal medium (MM) plus adenine (100 μg/ml) and histidine (40 μg/ml). All other mutants were isolated from A 52. The plating medium was supplemented MM plus desoxycholate and the survivors were screened by replica plating with velveteen (MACKINTOSH and PRITCHARD 1963). Adenine-specific mutants were identified by their failure to grow on hypoxanthine (75 μg/ml), histidine (40 μg/ml) and hypoxanthine plus histidine and by their ability to grow on adenine (100 μg/ml). All the adenine-specific mutants used in the allelic complementation studies were obtained in both the AR and ARW backgrounds by the appropriate crosses with either A 52 or A 178. A few of the ARW strains lacked the *nic* marker.

Accumulation studies: (1) Cross-feeding experiments to detect the accumulation of hypoxanthine were done by a modification of the method of NELSON (1963). Conidia of adenine-specific mutants were embedded in MM supplemented with limiting adenine (10 μg/ml) and incubated for 48 hours. Blocks of agar were cut out of the plates and placed on a surface of dialysis membrane covering MM plates similarly seeded with a hypoxanthine-utilizing strain. Growth of the indicator strain was scored after 48 and 72 hours incubation. (2) The accumulation of SAICAR was detected by the chromatographic technique of BERNSTEIN (1961). The mutants were grown in shake culture for 3 to 4 days with limiting adenine (25 μg/ml). Washed mycelium was extracted by grinding with 70% ethanol and the extracts were lyophilized. The residues were dissolved in water and chromatographed. SAICAR accumulation in culture filtrates from mutants grown as above was also detected by Bratton-Marshall tests done by the method of LUKENS and BUCHANAN (1959).

Enzyme assays: (1) Adenylosuccinase activity was determined by the decrease in optical density at 280 mμ (OD_{280}) when extracts were incubated with AMPS (GILES, PARTRIDGE and NELSON 1957). Mycelium was grown in suitably supplemented MM plus adenine (100 μg/ml) and histidine (40 μg/ml) for 15 hours with shaking. The washed mycelium was frozen as a pellet in liquid nitrogen, crushed in a mortar and pestle while still frozen and thawed in 0.02 M

potassium phosphate buffer at pH 7.5. Cell debris was spun down in a refrigerated centrifuge for 15 min at 19,000 × *g*. The extracts contained 3 to 5 mg/ml protein as determined by the Folin reagent (LOWRY *et al.* 1951). The assay mixture contained 0.5 ml of 0.1 M Tris(hydroxymethyl)aminomethane buffer (Tris) at pH 8.5; sufficient AMPS solution to yield an initial OD_{280} of 0.8; 0.05 to 0.10 ml of mycelial extract and water to a volume of 1.0 ml. The blank contained the same mixture without AMPS. Assays were done at 36°C with a Beckman DB recording spectrophotometer. The solutions were equilibrated for 3 min and then the reaction was started by addition of equal volumes of extract to blank and assay mixtures. The reaction rate was measured during the linear part of the curve. Qualitative tests for adenylosuccinase activity were done in a similar way but the amounts of the reagents were doubled and they were mixed cold. The OD_{280} was measured at the time of mixing and again after incubation at 30°C. Extracts containing adenylosuccinase showed a marked decrease in OD within 30 min and no change thereafter; extracts lacking adenylosuccinase showed no decrease in OD after at least 3 hours incubation. (2) AMPS synthetase activity was detected by the formation of AMPS when extracts were incubated with IMP and L-aspartic acid (ISHIKAWA 1962b). Mycelium was grown in suitably supplemented MM with a low level of adenine (25 μg/ml) and no histidine. The extracts were prepared by the procedure described above.

Complementation tests: (1) The preliminary intergenic complementation tests of the adenine-specific mutants were done with heterokaryons synthesized by the standard technique for Aspergillus (PONTECORVO *et al.* 1953). However, this technique is time-consuming and a more rapid procedure was devised to synthesize heterokaryons for allelic complementation tests. (2) Allelic complementation was detemined by the growth response of diploids. Forced heterokaryons between pairs of adenine-specific mutants in AR (yellow conidia) and ARW (white conidia) backgrounds were obtained on MM supplemented with adenine (500 μg/ml) and histidine (40 μg/ml). Loops of conidia of each strain were taken from slants and mixed in a small drop (<0.05 ml) of complete (CA) medium on the surface of a thick plate of fresh medium. Heterokaryons appeared as vigorous outgrowth from the background growth after 5 to 7 days incubation. Heterokaryosis was verified by inspection with a dissecting microscope for the presence of both yellow and white conidial heads. The diploids were isolated by plating heavy suspensions of conidia from the heterokaryons in MM plus adenine and histidine (ROPER 1952). They were readily recognized by their green conidia. The heterokaryons used for the isolation of the diploids were tested for reversion to adenine independence by streaking a sample of the conidial suspension onto MM supplemented with all the nutritional requirements of the component strains except adenine. The diploids were transferred to master plates and tested by replicating with a 26 point wire replicator (FORBES personal communication) to MM, MM plus 1M KCl and MM plus 2M KCl and incubating at both 37°C and 25°C for 3 and 4 days respectively.

Location of mutants: A modification of the procedure of FORBES (1959) was used to locate the mutants in linkage groups by mitotic haploidization. Heterozygous diploids were synthesized between the mutants and a multiply marked tester strain D (FORBES personal communication) which contains markers in each of the eight linkage groups of *A. nidulans* (KÄFER 1958). Suspensions of conidia of the diploids were stabbed into thick plates of CA plus 50 μg/ml DL p-fluorophenylalanine (LHOAS 1961) and incubated 2 to 3 weeks. It was found that the numbers of haploid segregants recovered were considerably increased if the poorly conidiated growth on these plates was replicated with velveteen to CA. After incubation, white, yellow or green haploid sectors were readily visible. Mutants were mapped within linkage groups by meiotic analysis (PONTECORVO *et al.* 1953).

RESULTS

Characterization of the adenine-specific mutants: Among the total of 430 adenine mutants isolated, 89 failed to grow on hypoxanthine plus histidine and were classified as adenine-specific. (The histidine was added to the test medium to exclude a hypothetical third class of adenine-specific mutants. These would

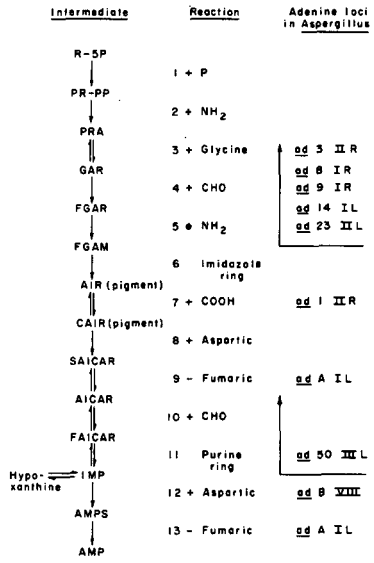

FIGURE 1.—Location of adenine mutants of *Aspergillus nidulans* in the adenine pathway. The mutants shown blocked before reaction 6 did not accumulate imidazoles as indicated by chromatography of mycelial extracts. Chromatography of mycelial extracts of mutant *ad1* revealed an accumulation product which was similar to that of the purple adenine mutants in Neurospora (H. BERNSTEIN, personal communication). The mutant *ad50* (isolated and mapped by G. DORN, unpublished) was not tested for its accumulation but responds to hypoxanthine. Pathway and symbols for intermediates after MAGANSANICK (1962).

correspond to the J (*ad-5*) type of GILES, PARTRIDGE and NELSON (1957) in Neurospora blocked in either reaction 10 or 11 in Figure 1 (T. FRENCH and K. MUNKRES, unpublished) which respond to hypoxanthine plus histidine, but not to hypoxanthine alone.) That the adenine-specific mutants differ from the adenine mutants previously described in *A. nidulans* (PONTECORVO *et al.* 1953; KÄFER 1958) is shown by the finding that representatives of each of the six known loci were found to utilize hypoxanthine plus histidine and are thus blocked before IMP (Figure 1).

All of the adenine-specific mutants are slow growing and inhibited by the standard complete medium for Aspergillus (PONTECORVO *et al.* 1953). This is probably why they had not been recovered previously. The optimum adenine supplement for their growth is 100 μg/ml and that for the formation of heterokaryons 500 μg/ml. L-histidine (40 μg/ml) was routinely added to the medium since it has been found in Neurospora that *ad-4* mutants grow better in the presence of this supplement (GILES, unpublished). The mutants do not grow on histidine alone.

The mutants isolated in early experiments were separated into two groups on the basis of accumulation and complementation studies. One group, *ad-A* was found to accumulate SAICAR but not hypoxanthine and the second group, *ad-B*, accumulated hypoxanthine but not SAICAR. All the mutants in group A comple-

mented all those in group B. One representative of each group was selected as a tester (*ad-A*, AM55; *ad-B*, AM57) and used to classify, by complementation, the new adenine-specific isolates obtained in later experiments. Also culture filtrates of these new mutants were examined by the Bratton-Marshall test for SAICAR. A colored product was detected in the filtrates of all of the *ad-A* group, and in none of the *ad-B* group. Of the 89 adenine-specific mutants, 40 were characterized as *ad-A* and 25 as *ad-B*. The remaining 24 were not classified by the above methods and were not used in the allelic complementation studies.

Adenylosuccinase activity was not detectable in 14 *ad-A* mutants tested and was present in 19 *ad-B* mutants tested. Since the test for AMPS synthetase depends on the accumulation of AMPS, this assay can only be done conveniently in adenylosuccinaseless strains. Thus it was possible to show that the five *ad-A* mutants tested had AMPS synthetase, but demonstration that the *ad-B* mutants lack this enzyme would require the synthesis of double (*ad-A*, *ad-B*) mutants.

The characteristics of the *ad-A* and *ad-B* mutants are summarized in Table 1. Their location in linkage groups IL and VIII respectively was determined by mitotic haploidization. Location of *ad-A* in linkage group I has been confirmed by meiotic analysis and the locus mapped 10 units distal to *gal5* (ROBERTS 1963).

None of the *ad-A* mutants grow in 5 days incubation on adenineless medium, and all are recessive to wild type to the extent that *ad-A*/*ad-A*$^+$ diploids are adenine independent. They were tested for phenotypic reversal by growth at a lower temperature (25°C) and by inclusion of 1M or 2M KCl in the test medium (HAWTHORNE and FRIIS 1964). Four of the mutants (16, 38, 55 and 75) were found to be osmotic remedials, i.e. capable of adenine-independent growth in the presence of KCl, and two of these (16 and 38) are also temperature sensitive. All four were later found to be complementers. Adenylosuccinase was detected in extracts of 16 and 38 after growth at 25°C but not after growth at 37°C. The enzyme was not detected in any of the osmotic remedial strains after growth at 37°C with 1M KCl. Mycelial extracts were prepared both in the absence and presence of 1M KCl. The enzyme from wild type was inhibited by high concentrations of KCl.

Eighteen of the 24 unclassified adenine-specific mutants not included in the complementation tests were later examined for adenylosuccinase and all were found to lack the enzyme. Most of these adenylosuccinaseless mutants were com-

TABLE 1

Properties of adenine specific mutants in Aspergillus nidulans

Locus	Number of mutants isolated	Growth response*				Accumulation	Enzymes		Location
		hx	hx+hist	ad	ad+hist		AMPS-synthetase	Adenylo-succinase	
ad-B	25	—	—	+	++	hypoxanthine	not tested	+	VIII
ad-A	40	—	—	+	++	SAICAR	+	—	I L

* hx = hypoxanthine; hist = histidine; ad = adenine.

bined in diploids with an *ad-A* tester (AM3) with which all 16 tested did not complement.

Comparison of allelic complementation in heterokaryons and diploids: The first 140 heteroallelic combinations synthesized were tested for their ability to complement in both heterokaryons and diploids. Of these, 110 did not complement in either system. Twenty-eight combinations complemented strongly in the diploids but showed weak complementation in the heterokaryons while two combinations which complemented weakly in the diploids did not complement in the heterokaryons.

Like the mutants themselves, the noncomplementing heterokaryons and diploids show no growth at all without adenine. The complementing diploids grow like the haploid wild type and like *ad-A*$^+$/*ad-A*$^+$ or *ad-A*/*ad-A*$^+$ dippoids. The complementing heterokaryons typically grow poorly on test medium, although the corresponding control heterokaryons (*ad-A*$^+$ + *ad-A*$^+$ and *ad-A* + *ad-A*$^+$) grow normally, as do the heterokaryons themselves on supplemented medium. Thus there is a generally good qualitative agreement between tests done in both systems but there is a marked quantitative difference in the efficiency of complementation in heterokaryons and diploids. This is illustrated by the growth experiment shown in Table 2. The growth of the complementing heterokaryons on MM is usually about 10% that of the corresponding diploids, but occasionally as high as 50%.

Complementation at the ad-A *locus:* Because of the difficulties in scoring the heterokaryons, the complementation map was constructed from data obtained from heteroallelic diploids. Growth on MM at 37°C within 3 days was taken as a positive response. Preliminary tests indicated that only 16 of the 40 *ad-A* mutants complemented and diploids of these mutants were synthesized in all possible pairs, including reciprocal arrangements and homozygous diploids (256 diploids). An additional 217 nonreciprocal combinations of the remaining possible 660 were also synthesized. Two weakly complementing combinations (42/55 and 57/72), not included in the map, were found but all other 215 combinations were negative.

In the grid for the 16 complementing alleles, reciprocal combinations were in complete agreement. Altogether 66 combinations were complementing and 54 noncomplementing, and their relationships can be represented in the linear complementation map shown in Figure 2 (Left).

A brief survey of the adenylosuccinase from complementing diploids was undertaken. In six diploids the enzyme was not detectable but the assay is only sensitive to 5% or more of the wild-type enzyme. In nine other diploids the enzyme was present with activities ranging up to about 30% of the wild type (Table 3). Some of the complementation enzymes were found to be markedly less stable than the wild-type enzyme when stored in the cold.

Conditional complementation: Modifications of the test regime to include incubation at 25°C and addition of high concentrations of KCl to MM resulted in an increased number of positive responses among the diploids. The particular diploids showing such conditional complementation did not contain alleles which themselves exhibited the same conditional behavior in haploids. In all cases where

TABLE 2

Comparative growth of heterokaryons and diploids on minimal and on adenine-supplemented medium

Heterokaryon AR/ARW	Complementation reaction	Dry weight in mg: Diploid, MM + 500 μg/ml ad and 40 μg/ml hist	Diploid, MM	Heterokaryon, MM + 500 μg/ml ad and 40 μg/ml hist	Heterokaryon, MM
+/+	....	220	300	214	271
AM74/+	....	212	266	191	251
+/AM74	....	225	308	213	186
4/AM74	Negative	148	0	54	0
AM74/4	Negative	175	0	90	0
16/AM74	Weak Positive	243	5	195	0
AM74/16	Weak Positive	228	5	174	0
38/AM74	Weak Positive	237	35	215	0
AM74/38	Weak Positive	250	63	219	0
41/AM74	Positive	211	298	173	3
AM74/41	Positive	254	308	198	15
55/AM74	Positive	199	271	232	16
AM74/55	Positive	225	382	181	33
60/AM74	Positive	218	306	275	31
AM74/60	Positive	276	301	188	21
61/AM74	Positive	217	210	238	2
AM74/61	Positive	251	232	245	1
75/AM74	Positive	194	279	207	138
AM74/75	Positive	250	264	194	76

Each figure represents the dry weight in mg of five colonies grown on separate agar plates covered with a disc of cellophane dialysis membrane. The plates were inoculated with 1 mm cubes cut from the growing mycelial front of colonies and incubated at 37°C for 6 days. The resulting colonies were scraped from the surface of the membrane and dried for 40 hours at 55°C.

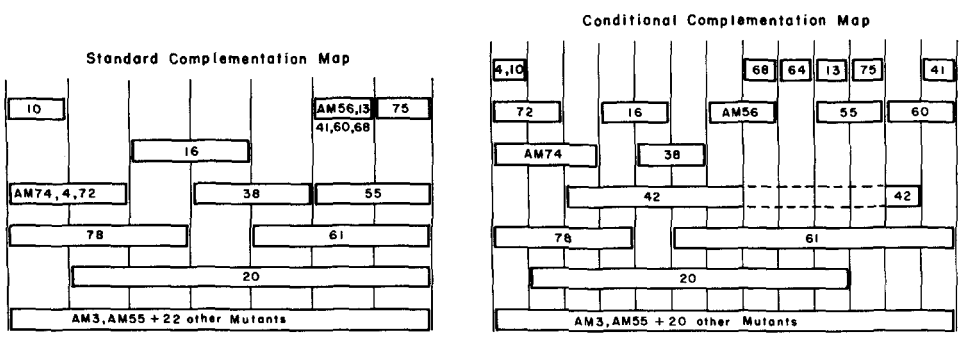

FIGURE 2.—Complementation maps for the adenylosuccinase locus (*ad-A*). Left: Standard complementation map; Right: Conditional complementation map.

reciprocal diploids were available the conditional responses were the same. The data are summarized in Table 4. It is interesting that mutants 42, which complements weakly, and 64, which is noncomplementing under the standard test regime, show many instances of conditional complementation.

TABLE 3

Adenylosuccinase from complementing diploids

Diploid	Adenylosuccinase activity	Stability of enzyme at pH 7.5, 0°C
+/+	2.6–3.2	....
13/AM74	0.06	Wild-type
16/AM74	0.35	Unstable
38/AM74	0.87, 0.95	Unstable
41/AM74	0.22, 0.37	Wild-type
60/AM74	<0.05	....
61/AM74	0.43	Wild-type
68/AM74	0.78	Intermediate stability
75/AM74	<0.05	....
AM56/AM74	0.84	Wild-type
68/AM55	<0.05	....
16/AM56	<0.05	....
55/AM56	<0.05	....
38/AM56	<0.05	....
72/AM56	0.20	Not determined
78/AM56	0.14	Not determined

Specific activity = ΔOD_{280}/10 min/mg protein.

The standard complementation map (Figure 2 Left) can be redrawn to include the conditional responses and the resulting conditional complementation map is shown in Figure 2 (Right). The complexity of the map is considerably increased both in the number of complementation units (complons) and in the number of complementation groups. Note particularly the change in arrangement of mutants in the groups containing AM 56, 55 and 75 in the standard map.

TABLE 4

Conditional complementation between alleles at the ad-A *locus*

	Heteroallelic diploids			
Complementation	20–	55–	42–	64–
Strong at 37°C	....	AM74 4 10 72	....	....
Weak at 37°C, enhanced at 25°C	10	68 38 78	55	....
None at 37°C, positive at 25°C	4 41 60 75	AM56 41 60 68	4 10 13 41 68 72 75	AM56 AM74 4 10 13 41 55 60 68 72 75 78
Positive at 37°C only with KCl	....	No test since 55 osmotic remedial	....	AM74 4 13 20 41 60 72
None under any test condition	AM56 AM74 13 55 61 68 72 78	13 20 61 75	AM56 AM74 20 60 61 78	61
Heteroallelic diploids	13	15	14	14
Reciprocal diploids	13	15	0	0

AM 56 and 68 were redistributed to the left, and 41 and 60 to the right of 55 and 75 in the conditional map. The map can still be drawn in linear form although one inconsistency is now present (mutant 42).

Negative complementation (1). Modification of the response of conditional ad-A *mutants by combination in diploids with certain other alleles:* During the course of the complementation tests it was noted that noncomplementing diploids containing a conditional mutant as one allele showed two sorts of response. In one type, the expected responses to lowered temperature or to KCl were observed and the diploids behaved like the haploid conditional mutants or the homozygous conditional diploids. That is, the diploids grew on MM at 25°C or on MM plus KCl at 37°C but not on MM at 37°C. In the other type the expected responses were delayed by two or three days and the growth considerably diminished. Thus some mutants behave as recessive to the conditional mutants but others as semidominant. These modified responses are shown in Table 5. Again the available reciprocal diploids behaved identically.

Negative complementation (2). Adenylosuccinase activity in heterozygous ad-A/ad-A$^+$ *diploids:* Diploids were synthesized between each of the *ad-A* mutants in the AR background and the wild-type ARW strain, and assayed for adenylosuccinase activity. The results are shown in Figure 3. All the diploids involving noncomplementing mutants showed about 50% of the enzyme activity of the homozygous (*ad-A*$^+$/*ad-A*$^+$) wild-type diploid. However, those diploids involving complementing mutants showed a variety of activities. One group gave about 50% of the activity of the wild-type homozygote, another

TABLE 5

Modification of the response of conditional ad-A *mutants by combination with certain other alleles in diploids*

	Heteroallelic diploids			
Conditional response	16–	38–	55–	75–
Of conditional mutant*	TS KCl	TS KCl	KCl	KCl
Response to lower temperature (25°C) delayed	AM3 **20**† 22 28 35 52 56	....	None expected	None expected
Response to media with KCl delayed	AM3 **20** 22 28 35 52 56	**20** 7 52	AM3 **AM56** 6 7 **13** 17 **20** 33 37 **41** 56 57 **60** **61** 65 **68**	65 66
Expected responses to lower temperature and/or high salt media	....	**61**	27 64 **75**	6 33 35 37 **55** 57 **61** 79
Heteroallelic diploids	8	5	19	10
Reciprocal diploids	2	3	7	2

* TS = temperature-sensitive mutants; KCl = osmotic remedial mutants.
† Mutants in bold type (**16**) are known to complement.

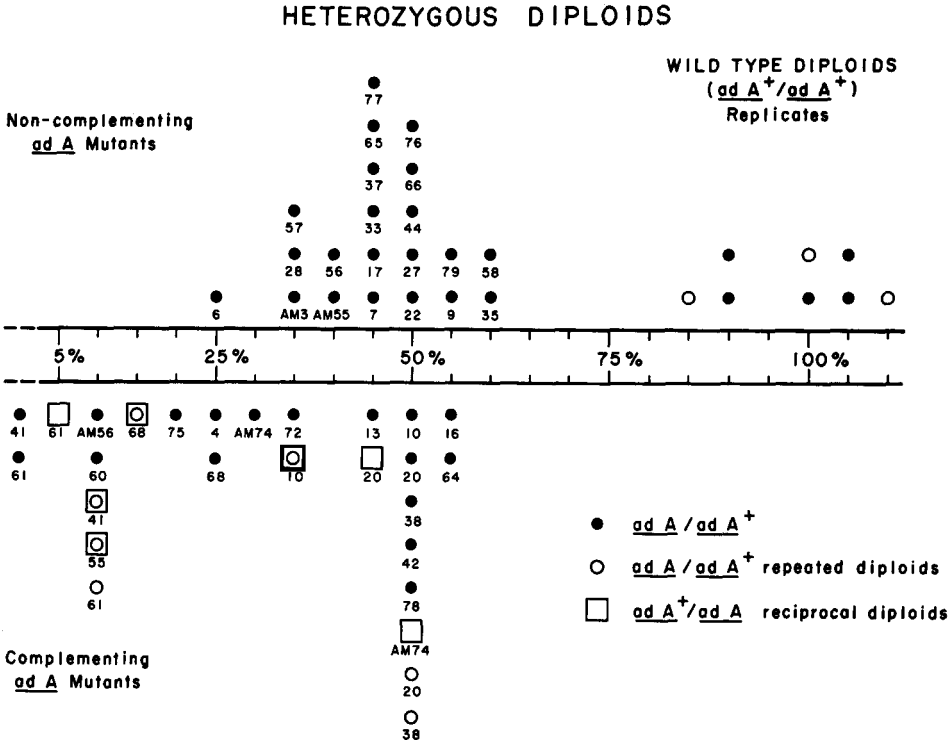

FIGURE 3.—Adenylosuccinase activity in diploids grown in liquid minimal medium. The extraction and assay procedures are described in the text. The numbers indicate the mutant alleles.

about 25% and a third group 10% or less. The assays were done at least twice for the diploids of the complementing mutants, and three times for those with very low activities. There were no obvious differences in the growth of any of the heterozygotes either on solid or in liquid minimal medium.

New diploids of eight of the complementing mutants were synthesized in the same arrangement (*ad-A/ad-A*$^+$) and also the reciprocal arrangement (*ad-A*$^+$/*ad-A*) was synthesized for six of them. In all cases, the enzyme activities showed the same proportional relationships to the wild-type diploid as in the first experiment, and the amounts of enzyme in the reciprocal pairs were not significantly different (Figure 3). The phenomenon is therefore consistent and the good agreement of the reciprocals suggests that the altered enzyme levels are not due to the presence of modifier mutations in the heterozygotes. It is notable that most of the mutants which produce low enzyme activities in the heterozygous diploids are at the right hand side of the complementation maps (Figure 2).

Four of the heterozygotes with very low enzyme activities as well as one from each of the other two groups involving the complementing mutants were broken down by mitotic haploidization. In all cases the *ad-A* and *ad-A*$^+$ alleles were recovered among the haploids in their expected linkage with either the *y bi* or the

y^+ bi^+ markers. Thus, the above results cannot be attributed to the error of isolating the wrong diploids.

Mixing experiments with crude mycelial extracts of diploids having low or normal (50% of wild type) enzyme activities yielded no evidence for an enzyme inhibitor specifically associated with the low activity extracts. The reaction could be inhibited if sufficiently large volumes of any crude extract, including wild type, were added to the assay mixture. The inhibition also occurred after the crude extract had been heated to 100°C for 3 minutes and probably results from the presence of nucleotides. Similar results were obtained in mixing experiments with extracts of haploid organisms.

DISCUSSION

These results indicate that the structure of adenylosuccinase is determined by one gene, *ad-A*, located in the left arm of linkage group I, and linked to *gal5*. Mutants classified by the criteria of accumulation product and noncomplementation with an *ad-A* tester were found, in all the cases examined, to lack adenylosuccinase or, in the case of the conditional mutants, to produce an altered enzyme. Similarly, another group of mutants which lack adenylosuccinase, and were obtained in an independent survey of previously unclassified adenine-specific mutants, proved noncomplementary to an *ad-A* tester in all cases examined.

Allelic complementation tests among the *ad-A* mutants done under standard conditions may be summarized in the form of a standard complementation map (Figure 2 Left) which comprises ten complementation groups (patterns) defining seven complementation units (complons). A linear map may be drawn, although the number of groups found is too few to distinguish critically between linear, circular or more complex maps (CARLSON 1961).

It is of interest to compare the complementation map for the adenylosuccinase locus in Aspergillus, Neurospora, and Schizosaccharomyces (Table 6). In each case, the map is relatively simple and can be drawn in linear form. The numbers of complementation groups and units are also similar, which may reflect a basic similarity in the structure of the enzyme from these three organisms.

In the past few years considerable success has been achieved with *in vitro*

TABLE 6

Complementation at the adenylosuccinase locus in three Ascomycetes

Organism	Adenylosuccinase mutants			Complementation map		
	Noncomp.	Comp.	Total	Form	Groups	Units
*Neurospora crassa**	60	49	109	Linear	18	11
Schizosaccharomyces pombe†	6	10	16	Linear	7	5
Aspergillus nidulans						
Standard map	24	16	40	Linear	10	7
Conditional map	22	18	40	Linear	17	13

* WOODWARD, PARTRIDGE and GILES 1958; CASE and GILES unpublished.
† MEGNET and GILES 1964.

studies in Neurospora (WOODWARD 1959; FINCHAM and CODDINGTON 1963; GROSS and WEBSTER 1963) and in Enterobacteria (LOPER 1961; SCHLESINGER 1964) aimed at the elucidation of the mechanism of allelic complementation. It is now generally accepted that this involves the aggregation of differently defective but otherwise identical polypeptide chains to yield a multimeric molecule (a homogenous aggregate) which differs from the wild-type enzyme but possesses some activity (CRICK and ORGEL 1964).

The results of a limited study of complementation enzymes from diploids in the present system agree with the general findings from other systems. The amount of enzyme varies from about 30% of wild type down to below the limit of detection, about 5% of the wild type. That growth occurs with less than 5% of the wild-type enzyme is not surprising since a study of revertants of adenylosuccinase mutants in Neurospora had shown that this organism will grow well with as little as 2% of the wild-type enzyme (WOODWARD, PARTRIDGE and GILES 1960). Qualitative differences between the complementation and wild-type enzyme in *A. nidulans* were also detected. There is at present no suitable technique for the growth of heterokaryons of Aspergillus for the extraction of complementation enzyme (ROBERTS 1965).

Three interesting findings on allelic complementation have emerged from this study: first, the quantitative difference in complementation in heterokaryons and diploids; second, the occurrence of conditional complementation; and, third, interactions between alleles which are best described as negative complementation.

There is generally good qualitative agreement between the results of the complementation tests done in heterokaryons and in diploids. However, combinations which complement well in diploids grow far less vigorously in heterokaryons, and do not grow at all in the cases of pairs which complement weakly in diploids. The low efficiency of heterokaryon complementation in Aspergillus raises the question of the proportion of cells in a forced heterokaryon of this organism which are truly heterokaryotic. There are indications that this proportion may be small and that the forced heterokaryon consists of a mosaic of homokaryotic and heterokaryotic cells (CLUTTERBUCK 1963). This suggests, as does the early observation, that heterokaryons arise as sectors (PONTECORVO *et al.* 1953), that there is little or no nuclear migration in the mycelium of Aspergillus.

Factors which may contribute to a low efficiency of complementation in heterokaryotic cells are (1) those which affect the absolute local concentrations of interacting polypeptides and (2) those which affect their relative concentrations. The first case might result because gene products are required to diffuse from different nuclei in heterokaryotic cells, whereas they diffuse from the same nucleus in diploid cells. The second case might result from imbalance in nuclear ratios. In Neurospora, CASE and GILES (1960) have observed that heterokaryons derived from unstable disomic pseudo-wild types formed in crosses between alleles at the *pan-2* locus, in which there is presumably an initial 1:1 nuclear ratio, provide a more sensitive test for complementation than do heterokaryons formed by conidial mixing.

PONTECORVO (1963) describes and discusses several cases where differences in intergenic complementation between heterokaryons and diploids have been found in Aspergillus and also in Coprinus. His examples involve interactions between nonallelic loci at which recessive mutations determine either nutritional deficiencies or the suppression of nutritional mutants. He advances the hypothesis that the absence of complementation in heterokaryons results from the intranuclear localization of the interacting components. However, in the case of the nutritionally deficient mutants this could also result from a low efficiency of heterokaryon complementation particularly in cases which involve an enzyme composed of two or more *unlike* polypeptides (a heterogenous aggregate). The examples cited by PONTECORVO (APIRION's acetate mutants, unpublished) and by ROBERTS (1964) involve leaky mutants, and the poor growth of heterokaryons may have been misinterpreted as no complementation rather than as weak complementation. It may also be significant that the acetate mutants could involve the heterogenous pyruvic dehydrogenase system (KOIKE, REED and CARROLL 1963; HENNING and HERZ 1964).

The increase in interallelic complementation and the resulting expansion of the complementation map owing to modification of the test regime, that is, conditional complementation, is not surprising. Mutation at different sites within the *ad-A* locus would result in a variety of altered polypeptides. It is evident that changes in the environment could affect the formation or structure of some polypeptides, the processes of aggregation, or the structures of the aggregates. It is likely that further modification of the test system, such as change of pH, would reveal more conditional responses.

The third novel aspect of allelic complementation emerging from this study is the finding of two classes of negative complementation. In the first, the expected responses of conditional mutants were modified when they were combined with certain other mutants in diploids. In the second, the amount of adenylosuccinase formed in *ad-A*/*ad-A*$^+$ diploids with 9 of the 16 complementing mutants was considerably less than the commonly reported value (MCKUSICK 1962) of approximately 50% of the *ad-A*$^+$/*ad-A*$^+$ diploid. This value had been found with the remaining complementing mutants and all of the noncomplementing mutants. The converse situation of a positive effect has been described in the alkaline phosphatase (*P*) system in *E. coli* in which a P^+/P^- merozygote produced more than 50% of the enzyme of the P^+/P^+ merozygote (GAREN and GAREN 1963).

These negative effects can be interpreted on the basis of interactions at the protein level in an extension of the general model of complementation (CRICK and ORGEL 1964; GAREN and GAREN 1963). The hypothesis is that adenylosuccinase in *A. nidulans* is a multimer resulting from the random association of polypeptides, and the negative effects occur because the enzyme formed between mutant and wild-type polypeptides has very little or no activity. There would by a similar diminution of conditional responses if the hybrid enzyme formed between the polypeptides of a conditional mutant and those from a nonconditional mutant did not respond to the conditional environment. The occurrence of some heterozygotes (*ad-A*/*ad-A*$^+$) which have activities of about 10% of

the homozygous wild-type diploid indicate that the enzyme may be a tetramer, because the lowest possible value predicted for this association is 1/16 of wild type. Evidence has recently been obtained that adenylosuccinase in Neurospora is a multimer of eight identical units (BRAYMER and WOODWARD 1965).

The above hypothesis is based on the assumption that the ratio of polypeptides in the diploids is 1:1. However, the negative effects described could also result if the ratio were not 1:1 as a result of differential production or degradation of polypeptides. This would affect the amount of enzyme by changing the relative proportions of the association products.

Further experiments are now required to determine whether adenylosuccinase in Aspergillus is a multimer, and, if so, the number of units involved. The hypothesis developed above will be open to verification if it proves possible to produce the negative effects *in vitro*.

There are already indications that negative complementation may not be uncommon. It has recently been described for allelic complementation with some T4 phage mutants (BERNSTEIN, EDGAR and DENHARDT 1965). It is also possible that the complex series of negative interactions between alleles of structural genes in Tetrahymena (ALLEN 1965) may have a similar basis. Since there have been few studies on enzyme levels in diploid microorganisms, and studies on higher organisms have generally involved small numbers of alleles, it is possible that relatively few systems in which negative complementation might be observed have been examined. Interactions of this kind may be an important aspect of the dominance relationship between alleles of some structural genes (GAREN and GAREN 1963; BERNSTEIN, EDGAR and DENHARDT 1965). In this connection, the results presented here illustrate that a series of alleles can exhibit degrees of dominance, the specification of which changes at different levels of analysis of the phenotype. Thus a conditional mutant which responds normally to the conditional environment when combined with another mutant in a diploid is dominant to that mutant, but is incompletely dominant to a mutant if the diploid shows a diminished response to the conditional environment. In the *ad-A/ad-A*$^+$ diploids, all of the mutants are recessive to wild type in terms of the growth of the heterozygotes, but the decreased enzyme levels in some diploids indicate that certain mutant alleles are incompletely recessive to wild type by this criterion. It seems probable that for some types of biochemical mutants enzymic activities as low as 10% of wild type would be inadequate to support growth. In such situations, diploids heterozygous for mutant and wild-type alleles comparable to those found in the present studies would exhibit complete "dominance" of the auxotrophic mutant allele as judged by the usual phenotypic criterion of growth on a minimal medium.

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SUMMARY

Adenine-specific mutants have been isolated and found to represent two new loci. One locus, *ad-A*, is in linkage group I and determines the structure of the enzyme adenylosuccinase. Conditional mutants which are temperature sensitive or osmotic remedial occur at the locus: *ad-B* is in linkage group VIII and probably determines AMPS synthetase. A detailed study has been made of allelic complementation at the *ad-A* locus using 40 mutants of which 16 were able to complement. A comparison was made of complementation in heterokaryons and in diploids and it was found that although the qualitative results were in close agreement, there were marked quantitative differences which suggest a low efficiency of heterokaryon complementation. A linear complementation map has been constructed using the data from diploids and found to be similar to the maps of the adenylosuccinase loci in Neurospora (*ad-4*) and in Schizosaccharomyces (*ad-8*). Testing the heteroallelic diploids for temperature sensitives and osmotic remedials resulted in an increased number of complementation responses which have been called conditional complementation. The diploids did not contain alleles which were themselves conditional mutants. The complementation map redrawn to include the conditional responses remained linear although it became more complex.

Two sorts of negative interaction (negative complementation) between alleles at the locus have been observed. In one, the responses of the conditional mutants were considerably delayed and diminished in some heteroallelic diploids. In the other, the amount of enzyme formed in *ad-A*/*ad-A*$^+$ diploids with 9 of the 16 complementing mutants was decreased to between 5 and 25% of the *ad-A*$^+$/*ad-A*$^+$ diploid, whereas diploids with the remaining complementing mutants and all 24 noncomplementing mutants produced 50% of the enzyme of the homozygous wild-type diploid. These findings may reflect the types of multimer formed in the diploids.

LITERATURE CITED

ALLEN, S., 1965 Genetic control of enzymes in Tetrahymena. Brookhaven Symp. Biology **18** (in press).

BERNSTEIN, H., 1961 Imidazole compounds accumulated by purine mutants of *Neurospora crassa*. J. Gen. Microbiol. **25**: 41–46.

BERNSTEIN, H., R. S. EDGAR, and G. H. DENHARDT, 1965 Intragenic complementation among temperature sensitive mutants of bacteriophage T4D. Genetics **51**: 987–1002.

BRAYMER, H. D., and D. O. WOODWARD, 1965 Relationship between quaternary structure of adenylosuccinate AMP-lyase and interallelic complementation (Abstr.) Fed. Proc. **24**: 469.

CARLSON, E. A., 1961 Limitations of geometrical models for complementation mapping of allelic series. Nature **191**: 788–790.

CASE, M. E., and N. H. GILES, 1960 Comparative complementation and genetic maps of the *pan-2* locus in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **46**: 659–676.

CLUTTERBUCK, A. J., 1963 Studies in heterokaryosis. Aspergillus News Letter **4**: 21–22.

CRICK, F. H. C., and L. E. ORGEL, 1964 The theory of interallelic complementation. J. Mol. Biol. **8**: 161–165.

DORFMAN, B., 1964 Allelic complementation at the *ad-5/7* locus in yeast. Genetics **50**: 1231–1243.

EVERSOLE, R. A., 1956 Biochemical mutants of *Chlamydomonas reinhardi*. Am. J. Botany **43**: 404–407.

FINCHAM, J. R. S., and A. CODDINGTON, 1963 The mechanism of complementation between *am* mutants of *Neurospora crasa*. Cold Spring Harbor Symp. Quant. Biol. **28**: 517–527.

FINCHAM, J. R. S., and J. A. PATEMAN, 1957 Formation of an enzyme through complementary action of mutant alleles in separate nuclei in a heterokaryon. Nature **179**: 741–742.

FORBES, E., 1959 Use of mitotic segregation for assigning genes to linkage groups in *Aspergillus nidulans*. Heredity **13**: 67–80.

GAREN, A., and S. GAREN, 1963 Complementation *in vivo* between structural mutants of alkaline phosphatase from *E. coli*. J. Mol. Biol. **7**: 13–22.

GILES, N. H., C. W. H. PARTRIDGE, and N. J. NELSON, 1957 The genetic control of adenylosuccinase in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **43**: 305–317.

GROSS, S. R., and R. E. WEBSTER, 1963 Some aspects of interallelic complementation involving leucine biosynthetic enzymes of Neurospora. Cold Spring Harbor Symp. Quant. Biol. **28**: 543–547.

HAWTHORNE, D. C., and J. FRIIS, 1964 Osmotic-remedial mutants. A new classification for nutritional mutants in yeast. Genetics **50**: 829–839.

HENNING, U., and C. HERZ, 1964 Ein Strukturgen-Komplex für den Pyruvat-Dehydrogenase-Komplex von *Escherichia coli* K 12. Z. Vererb. **95**: 260–275.

ISHIKAWA, T., 1962a Genetic studies of *ad-8* mutants in *Neurospora crassa*. I. Genetic fine structure of the *ad-8* locus. Genetics **47**: 1147–1161. —— 1962b Genetic studies of *ad-8* mutants in *Neurospora crassa*. II. Interallelic complementation at the *ad-8* locus. Genetics **47**: 1755–1770.

KÄFER, E., 1958 An 8-chromosome map of *Aspergillus nidulans*. Advan. Genet. **9**: 105–145. —— 1962 Aspergillus News Letter **3**: 3–4.

KOIKE, U., L. J. REED, and W. R. CARROLL, 1963 α-keto acid dehydrogenation complexes. IV. Resolution and reconstitution of the *Escherichia coli* pyruvate dehydrogenation complex. J. Biol. Chem. **238**: 30–39.

LEWIS, D., 1961 Genetical analysis of methionine suppressors in Coprinus. Genet. Res. **2**: 141–155.

LHOAS, P., 1961 Mitotic haploidization by treatment of *Aspergillus niger* diploids with *para*-fluoro-phenylalanine. Nature **190**: 744.

LOPER, J. C., 1961 Enzyme complementation in mixed extracts of mutants from the Salmonella histidine B locus. Proc. Natl. Acad. Sci. U.S. **47**: 1440–1450.

LOPER, J. C., M. GRABNAR, R. C. STAHL, Z. HARTMAN, and P. E. HARTMAN, 1964 Genes and proteins involved in histidine biosynthesis in Salmonella. Brookhaven Symp. Biol. **17**: 15–52.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.

LUKENS, L. N., and J. M. BUCHANAN, 1959 Biosynthesis of the purines. XXIII. The enzymatic synthesis of N-(5-amino-1-ribosyl-4-imidazolyl carbonyl)-L-aspartic acid 5′ phosphate. J. Biol. Chem. **234**: 1791–1798.

MACKINTOSH, N. E., and R. H. PRITCHARD, 1963 The production and replica plating of microcolonies of *Aspergillus nidulans*. Genet. Res. **4**: 320–322.

MAGASANIK, B., 1962 Biosynthesis of purine and pyrimidine nucleotides. pp. 295–334. *The Bacteria*, Volume 3. Edited by I. C. GUNSALUS and R. Y. STANIER. Academic Press, New York.

MCKUSICK, V. A., 1962 On the X chromosome of man. Quart. Rev. Biol. **37:** 69–175.

MEGNET, R., and N. H. GILES, 1964 Allelic complementation at the adenylosuccinase locus in *Schizosaccharomyces pombe*. Genetics **50:** 967–971.

MORGAN, D. H., 1961 Recombination and complementation between suppressor genes in *Coprinus lagopus*. Heredity **16:** 239.

NELSON, N., 1963 Cross-feeding experiments to test for biological activity. Neurospora Newsletter **3:** 10.

PITTENGER, T. H., and K. C. ATWOOD, 1956 Stability of nuclear proportions during growth of Neurospora heterokaryons. Genetics **41:** 227–241.

PONTECORVO, G., 1963 Microbial genetics: restrospect and prospect. Proc. Roy. Soc. Lond. B **158:** 1–23.

PONTECORVO, G., J. A. ROPER, L. J. HEMMONS, K. D. MACDONALD, and A. W. J. BUFTON, 1953 The genetics of *Aspergillus nidulans*. Advan. Genet. **5:** 141–238.

ROBERTS, C. F., 1963 The genetic analysis of carbohydrate utilization in *Aspergillus nidulans*. J. Gen. Microbiol. **31:** 45–58. —— 1964 Complementation in balanced heterokaryons and heterozygons diploids of *Aspergillus nidulans*. Genet. Res. **5:** 211–229. —— 1965 Failure of the membrane technique for growth of mycelium on solid media. Aspergillus News Letter **6:** 17.

ROPER, J. A., 1952 Production of heterozygous diploids in filamentous fungi. Experentia **8:** 14–15.

SCHLESINGER, M. J., 1964 *In vitro* complementation and the subunit structure of *E. coli* alkaline phosphatase. Brookhaven Symp. Biol. **17:** 66–79.

WOODWARD, D. O., 1959 Enzyme complementation *in vitro* between adenylosuccinaseless mutants of *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **45:** 846–850.

WOODWARD, D. O., C. W. H. PARTRIDGE, and N. H. GILES, 1958 Complementation at the *ad-4* locus in *Neurospora crassa*. Proc. Natl. Acad. Sci. U.S. **44:** 1237–1244. —— 1960 Studies of adenylosuccinase in mutants and revertants of *Neurospora crassa*. Genetics **45:** 535–554.

WOODWARD, V. W., J. R. DE ZEEUW, and A. M. SRB, 1954 The separation and isolation of particular biochemical mutants of Neurospora by differential germination of conidia followed by filtration and selective plating. Proc. Natl. Acad. Sci. U.S. **40:** 192–200.