a racemic conglomerate (a mixture of individual *d* crystals and *l* crystals in equal proportions) such as may result from ordinary nonvital chemical reactions.

It is only necessary that the individual crystals be separated far enough from each other so that their solutions do not mix when the crystals dissolve. If the crystals are dissolved under these conditions, each optically active crystal will provide a small amount of an optically active solution.

The process might well occur in nature. If a solution of a racemic mixture formed as the result of an ordinary chemical reaction were to crystallize as a racemic conglomerate, and then evaporate to dryness, the crystals might easily be scattered by the wind or some other disturbance. This process would fulfil the required conditions, and each individual crystal, separated from the others, would supply an optically active solution. In this way an optically active solution would be made available for the first vital reaction.

[1] J. D. Bernal, *Physical Basis of Life* (London: Routledge & Kegan Paul, 1951); S. W. Fox, *Am. Scientist*, **44**, 347, 1956; A. I. Oparin, *Origin of Life* (New York: Macmillan Co., 1938).

[2] For a discussion of these problems see G. F. Gause, "Optical Activity and Living Matter," *Biodynamica* (Normandy, Mo.), 1941. E. Schrödinger, *What Is Life?* New York: (Macmillan Co., 1945); E. Schrödinger, in *History of Mathematics*, ed. Newman (New York: Simon & Schuster, 1956), **2**, 985; H. Weyl, in *History of Mathematics*, ed. Newman (New York: Simon & Schuster, 1956), **1**, 871.

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*THE GENETIC CONTROL OF ADENYLOSUCCINASE IN NEUROSPORA CRASSA**

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In order to elucidate the mechanisms by which genes control enzyme formation, an experimental system favorable for both biochemical and genetical investigations is of paramount importance. Although an increasing number of instances are now known in which mutational changes have given rise to specific enzymatic deficiencies (Horowitz[1]), few of these systems have yet been characterized genetically in detail. The present paper will report initial results with a system in *Neurospora crassa* which appears to be particularly well suited for combined biochemical and genetical studies. A series of over twenty adenine-requiring mutants of independent origin has been obtained in one wild-type strain as a result of mutation at a single locus which is quite favorable for genetical studies, having suitable closely linked markers on either side. These mutants are all characterized by being deficient for a single enzyme involved in the terminal step in adenine biosynthesis—adenylosuccinase[2]—for which a convenient assay procedure is available. Furthermore, certain of the mutants are capable of reversion, and the characteristics of the resulting adenine-independent types can be investigated both biochemically and genetically.

Additionally, since all mutants were produced in a single wild-type strain, it

has been possible to test for heterocaryon formation and growth in the absence of adenine with the various strains. The unexpected result has been that certain combinations of mutants are able to form adenine-independent heterocaryons (bicaryons)† in which appreciable adenylosuccinase activity can be demonstrated, even though both components lack detectable adenylosuccinase activity. Thus the initial studies with this system are already proving to be of exceptional interest in relation to the problem of gene action in enzyme formation. Brief reports of certain of these results have been presented previously (Giles, Partridge, and Nelson[3]; Giles and Partridge[4]).

Production and Preliminary Classification of Mutants.—The adenine-requiring mutants used in these studies were obtained in wild-type strain 74A (of St. Lawrence) by application of the filtration-concentration technique of Woodward, deZeeuw, and Srb.[5] Untreated (control) macroconidia and macroconidia exposed to either X-rays or ultraviolet were utilized to obtain mutants. Following filtration, platings were made on minimal agar supplemented with adenine (and other supplements in certain experiments in which additional types of mutants were being sought), and single colonies were isolated for testing. The resulting adenine-requiring mutants (606 in number) were next screened for their response to hypoxanthine. The present studies are concerned with the resulting 47 adenine-specific mutants which do not grow on hypoxanthine.

Further characterization of these mutants was sought by means of heterocaryon tests for complementation resulting in growth in the absence of adenine. Utilizing a single, arbitrarily chosen strain as a standard, all other adenine-specific mutants were tested for heterocaryon complementation with this strain by mixing conidia of the two mutants on a minimal agar plate. On the basis of these tests, two distinct groups of mutants were detected: group E (27 mutants), which gave abundant growth with the tester within 3 days or less after inoculation in the absence of adenine, and group F (20 mutants) which gave no growth with the tester (Table 1).

Additional evidence for two distinct groups of mutants has been obtained from genetic studies. In crosses to wild type, individual mutants from the two groups

TABLE 1

ORIGIN OF ADENINE-SPECIFIC MUTANTS

GROUP	MUTANTS DERIVED FROM CONIDIA EXPOSED TO: X-rays	Ultraviolet	No Treatment	TOTALS
E	25	0	2 (E11 and E12)	27
F	17	1 (F19)	2 (F6 and F10)	20*

* Does not include mutant 44206, of ultraviolet origin (Barratt *et al.*, *Advances in Genetics*, **6**, 1, 1954).

exhibit generally regular 1:1 segregation in asci. Crosses between mutants in the two groups give approximately 25 per cent wild-type segregants, demonstrating that the two types are not linked. Further studies with the F mutants have shown that these constitute a group of allelic mutants in linkage group III located between *prol*-1 and *leu*-1 (Barratt *et al.*[6]). Heterocaryon and crossing tests have also shown that the F mutants are allelic with the previously known *ad-4* mutant (44206) reported as adenine-specific (and temperature-sensitive) by Mitchell and Houlahan.[7] Group E mutants constitute a new group of allelic, adenine-specific mutants not as yet located in any particular linkage group.

Biochemical Characterization of Mutants.—Biochemical studies of the two groups of mutants were initiated in an effort to determine what step in adenine synthesis is blocked in each type. Culture filtrates of E mutants were found to be active in supporting growth of all other groups of adenine mutants except F, a result which appeared to place E prior to F in the biosynthetic sequence. Ultraviolet absorption spectra of the culture filtrates of both types were obtained, and characteristic accumulation products were then separated by ion-exchange chromatography. The results of these studies, especially with F filtrates, have been described in a preliminary note (Partridge and Giles[8]) and are similar to those obtained independently for the F mutant 44206 by Whitfeld.[9] The evidence indicates that F mutants are blocked in the terminal step in adenine synthesis, involving the splitting of adenosine monophosphate succinate (AMP-S) to adenosine monophosphate (AMP).

Comparative studies of E and F mutants, including examination of the absorption spectra of filtrates from the single and double mutants, as well as identification of the accumulation product (hypoxanthine) in an E mutant responsible for the biological activity of its filtrate, led to the tentative conclusion that E mutants were blocked in the step prior to F in biosynthesis, namely, the conversion of inosine monophosphate (IMP) to adenosine monophosphate succinate (AMP-S). More recent observations (Partridge and Giles, unpublished) indicate that a revision of this view may be required. It now appears that F mutants are blocked in not one, but two, reactions, the second involving the splitting of 5-amino-4-imidazole-(N-succinylo-carboxamide) ribotide (SAICAR) to 5-amino-4-imidazole carboxamide ribotide (AICAR) (Buchanan[10]). The accumulation of hypoxanthine by E-F double mutants appears to place the E block prior to the two F blocks and hence to require a modification of the previous view[3, 4] regarding the step blocked in E mutants. Additionally, preliminary investigations of a third group of mutants (J) indicate that these may be considered basically adenine-specific, although they grow quite well on hypoxanthine when histidine is also present. The absorption spectrum of J-mutant filtrates is much like that of F mutants and suggests a close relationship between the substrates of the reactions under their respective control. Further studies of these relationships are under way. A study of the properties of double mutants involving J, E, and F types should help to decide the question of the relative positions of the compounds whose metabolism they affect, in the path of adenylic acid synthesis or as by-products thereof. All present evidence, however, supports the previous conclusion that F mutants are indeed blocked in the splitting of AMP-S to AMP.

The present experiments have been concerned principally with an analysis of the genetic control of this reaction, based on studies of the AMP-S splitting enzyme, adenylosuccinase, in wild type, F mutants, and revertants induced in F mutants.

Adenylosuccinase in Wild Type, Mutants, and Revertants.—On the basis of the evidence just discussed, indicating that F mutants accumulate AMP-S, it appeared that they might be defective with respect to the AMP-S splitting enzyme, adenylosuccinase (Carter and Cohen[11]). Hence tests for this enzyme were performed on mycelial extracts of wild type, F mutants, and certain other adenine mutants.

The following assay procedure was utilized: Mutant strains were grown with adenine sufficient for submaximal growth; prototrophic cultures were grown rou-

tinely on Fries minimal medium; mycelial pads were harvested by filtration, washed with water at 5° C., dried in the frozen state, weighed, and aliquot portions taken. The aliquots were crushed to powder, extracted by shaking in 0.05 *M* phosphate buffer, pH 7.0, for one hour at 5°, and centrifuged with refrigeration to remove visible sediment. Supernates were separated from lipid layers and sediment and were assayed either immediately or after one or two days' storage in a deep-freeze. Activity was not removed by filtration through a membrane of pore diameter $>1\ \mu$.

For assay, a fraction of 1 ml. of extract was brought to 3.3 ml. with the extraction buffer, containing AMP-S at a final concentration of 30μg/ml, in a 10-mm. silica cell. The absorption of the substrate-product mixture was followed continuously or at fixed intervals by comparison with an identical mixture, lacking substrate, in a matched reference cell. Activity was recorded as rate of change of absorbance at 280 mμ during the period of constant reaction rate, on a mycelial dry-weight basis.

A study of variation of activity in wild-type and E-mutant extracts with culture times from 32 to 60 hours at 35° indicated a relatively constant activity for both strains, with a maximum between 36 and 44 hours (approximately 30 per cent greater than earlier or later samples). Times of harvesting in subsequent experiments were chosen to correspond to a stage of development in the range of optimal activity. Similar activities were found in 25° and 35° cultures at equivalent stages of development, except in temperature-sensitive strains. High levels of activity were found in wild type (equal in both mating types) and in mutant strains of groups E, J, and B. However, the B strain, when grown on hypoxanthine in place of adenine, developed an extractable purple pigment in the mycelium and was found to be inactive. When the pigmented extract was mixed with the colorless adenine-grown extract of the same strain, it removed the activity of the latter to a large extent. No activity (not more than 0.1 per cent that of wild type) could be detected in most of the nineteen F strains tested, even under conditions of double the usual extract concentration and a fivefold increase in substrate concentration with prolonged incubation. The exceptions were a strain (F16) which showed a trace of growth on minimal medium and a barely detectable activity and strain 44206, which is prototrophic and enzymically active at temperatures up to about 32°. No significant inhibition of wild-type activity was noted upon addition of crude extracts of any of the F strains. Therefore, the enzyme is presumed to be absent or grossly altered in F. Tests with extracts of both wild type and 44206 showed that activity was proportional to extract concentration over the experimental range, giving no evidence of intrastrain inhibition in crude extracts. It is not impossible that fractionation or special treatments of the mutant extracts, or growth of mutants under different conditions, will reveal latent activity (Wagner[12]).

On the basis of the results just discussed, it can be concluded that forward mutation at the *ad-4* locus in the wild type to give adenine-requiring mutants results in the loss of activity of the enzyme adenylosuccinase. It thus became of interest to determine whether reversions in such mutants to adenine independence would result in the restoration of adenylosuccinase activity. For these studies a series of 15 revertants was produced by ultraviolet irradiation of F12 macroconidia. The

revertants were backcrossed to an F12 strain of opposite sex, and homocaryotic adenine-independent isolates were obtained for genetical and biochemical analysis. On the basis of backcrosses to wild type, all 15 revertants test as reverse mutations rather than as suppressor mutations.

Tests were next performed for adenylosuccinase activity, and these demonstrated that activity is present in all 15 revertants. Preliminary quantitative comparisons indicate, however, that the level of enzyme activity is markedly less (averaging about 50 per cent) in the revertant extracts than in those of wild type (Fig. 1). Further tests will be necessary to establish the reproducibility of these

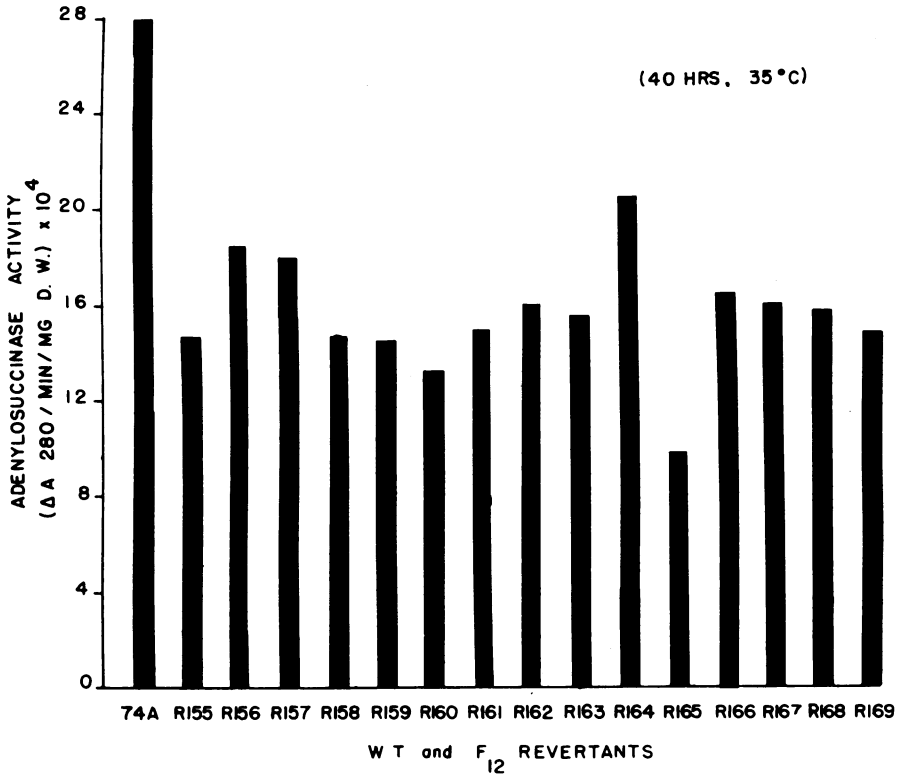

FIG. 1.—Comparative adenylosuccinase activity in wild type (74A) and 15 ultraviolet-induced revertants in the adenylosuccinase-deficient mutant F12.

quantitative comparisons, and to determine whether the observed quantitative differences among revertants arise exclusively from differences in the kinds of reverse mutational changes occurring at the F locus. A brief report of these studies has already been made (Giles and Partridge[4]), and additional detailed observations with these and other revertants will be published subsequently. Certain very recent observations indicate that the lower enzyme activities in at least some of the revertants may result from a more rapid loss of activity after extraction as compared with wild type.

Preliminary studies have also been made of adenylosuccinase in the temperature-sensitive mutant 44206 which grows in the absence of adenine at 25° but has an

absolute requirement at 35°. Mycelial extracts of this strain grown at 25° have about 25 per cent of wild-type activity, whereas those grown at 35° have substantially no activity. When the strain is grown at 32°, the upper limit of growth in the absence of adenine, extract activity is about 2 per cent that of wild type. An examination has been made of the relative thermostabilities of adenylosuccinase activity in extracts obtained from the temperature-sensitive mutant and from wild type. On the basis of comparative residual activities following incubation at 35° and at 48°, adenylosuccinase activity is much less stable in extracts from the temperature-sensitive mutant than in those from wild type. Neither the activities nor the stability in frozen storage of extracts of wild type or of mutant 44206 were altered significantly by insertion of biochemical mutant markers with their concomitant nutritional supplements in growth at 25°, nor was there any effect upon activity of any prototrophic strain upon supplementation of growth medium with adenine.

Further detailed comparisons of the enzymes from the two allelic strains are in progress and will be reported later. In recent experiments temperature-sensitive revertants have been obtained in one F strain, and these are also being studied.

Heterocaryon Complementation between Adenylosuccinase-deficient Mutants.—The previously reported results indicate that all F mutants are biochemically similar in being blocked in the same step in adenine biosynthesis, and all either lack or have modified adenylosuccinase activity. Hence, on the basis of previous studies in *Neurospora* which have indicated that prototrophic bicaryons are regularly formed (in heterocaryon-compatible strains) between biochemically unlike (nonallelic) mutants, but not between biochemically similar (allelic) mutants (Beadle and Coonradt[13]), it was anticipated that F mutants would form adenine-independent heterocaryons with other biochemically dissimilar (nonallelic) adenine-requiring mutants, but not with one another. However, tests of this hypothesis have indicated that it is incorrect. Although F mutants do regularly form prototrophic heterocaryons with biochemically and genetically distinct groups of mutants (as, for example, with E), certain combinations of F mutants are also capable of forming bicaryons which grow in the absence of adenine. Regular complementation with the prompt initiation of growth is obtained in combinations involving mutant F12 with three other mutants, F1, F4, and F5, although the latter three mutants do not complement one another. The growth rates in the absence of adenine of such heterocaryons compared to those of wild type and of a heterocaryon between F12 and an E mutant (E4), as measured at two temperatures, are shown in Table 2. It will be noted that at both 25° and 35° all bicaryons grow at rates comparable to wild type and are not markedly stimulated by adenine, with one exception, F12 + F1, which grows much more slowly at 35° and responds to exogenous adenine. Instances of complementation involving F mutants other than F12 have also been obtained, but these are characterized by delayed initiation of growth and much slower rates on minimal. For the present, attention has been centered on the three heterocaryons cited. In certain of the numerous combinations of F mutants which do not complement, forced heterocaryons have been made, and in all instances these have been found to require exogenous adenine for growth. Attempts to modify these negative results by employing heterocaryons containing various nuclear ratios have been unsuccessful.

The occurrence of adenine-independent heterocaryons between adenine-requiring, adenylosuccinase-deficient mutants immediately raised the question whether enzyme activity is present in such heterocaryons. Tests of mycelial extracts have indeed shown that adenylosuccinase activity is present. As shown in Figure 2, heterocaryons between F12 and F4 and F12 and F5 have about one-quarter the activity of wild type and about one-half that of a heterocaryon between F12 and E4.

TABLE 2

GROWTH RATES OF HETEROCARYONS BETWEEN F MUTANTS COMPARED WITH THOSE OF WILD TYPE AND AN E + F HETEROCARYON*

MUTANT COMBINATION	GROWTH RATE (MM/HR)			
	25°		35°	
	Minimal	Adenine	Minimal	Adenine
Wild type (74A)	3.0	3.2	4.0	4.2
F12 + E4	3.2	3.5	4.5	4.9
F12 + F1	3.0	3.5	2.0	4.4
F12 + F4	3.1	3.6	3.9	4.5
F12 + F5	2.8	3.5	4.3	4.6

* Tubes in duplicate; adenine supplement, 50 μg/ml.

A later experiment has shown that the heterocaryon between F1 and F12, which is temperature-sensitive, provides an extract only about 6 per cent as active as that of wild type, when grown at 25° either with or without exogenous adenine. Its activity relative to that of the non-temperature-sensitive intra-F heterocaryons is then 25 per cent, a ratio similar to that of strain 44206 compared to wild type. The fact that a heterocaryon of F1 and F12 grows at wild-type rate on minimal medium, whereas it produces only 6 per cent of wild-type activity in its extract, might suggest that at least one step (the terminal) in the production of an essential cellular constituent (AMP) may be greatly oversupplied in terms of its specific enzyme in a normal organism.

These results indicate that the heterocaryons, even those within the F group, can synthesize AMP by the same route as wild type. The spectra of the heterocaryon filtrates are indistinguishable from those of wild type and thus provide no indication of an alternative pathway in these special cases.

Recent evidence that F mutants also lack activity for another reaction in adenine biosynthesis, the splitting of SAICAR to AICAR (Buchanan[10]; Partridge and Giles, unpublished), appeared to reopen the possibility that complementation might arise on the basis of these two reactions—one mutant (e.g., F12) lacking activity for one, the other three mutants lacking activity for the other. However, tests of mycelial extracts of the four mutants indicate that all lack enzyme activity for this second substrate, an activity which can be demonstrated in wild type, in F revertants, and also in heterocaryons of the complementary F mutants. No significant differences in relative activities for the two reactions have been found in any instance.

Genetic tests have been made to determine whether the adenine-independent cultures obtained from the various F-mutant combinations are indeed heterocaryons with mutant nuclei only and do not contain wild-type nuclei arising as a result of reversion or some other genetic change. In all instances tested, such

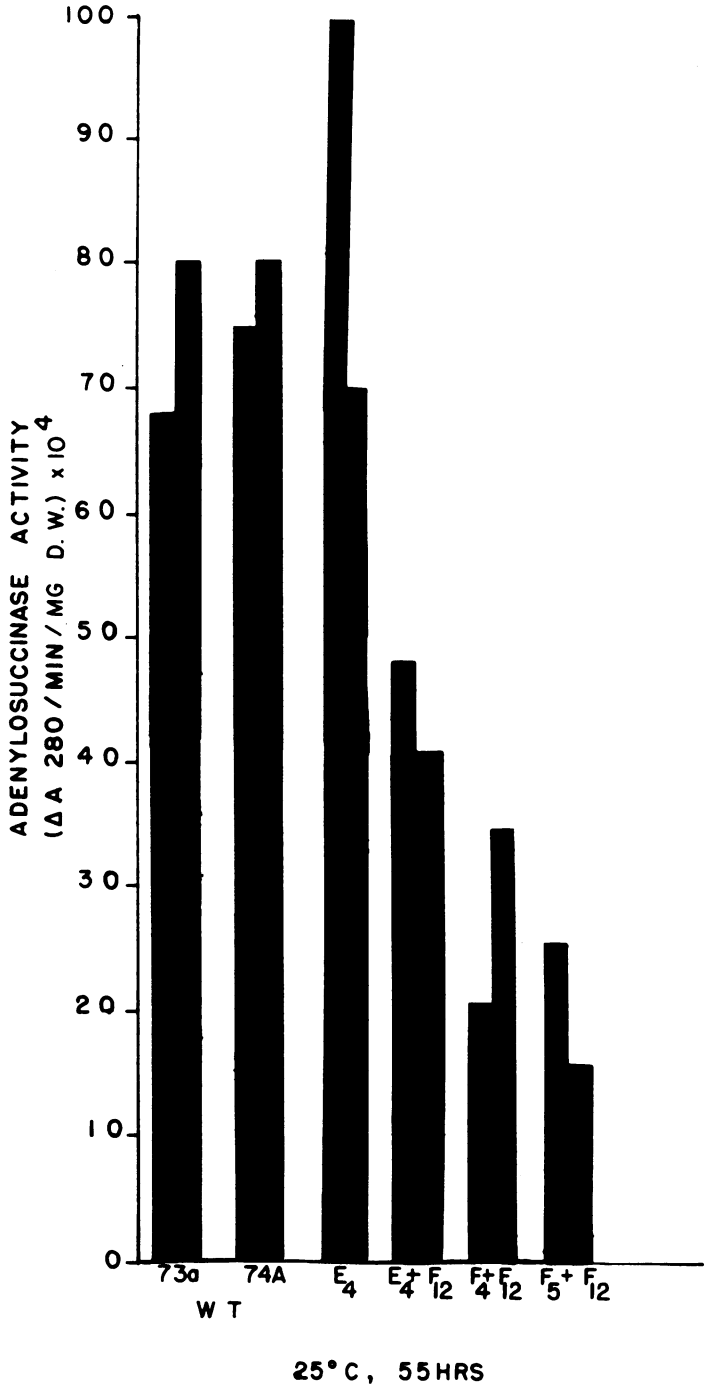

FIG. 2.—Comparative adenylosuccinase activities in mycelial extracts of wild types (74A and 73a), an E mutant (E4), and adenine-independent heterocaryons between F mutants (F4 + F12; F5 + F12). Results of duplicate tests are indicated.

presumptive heterocaryons can be resolved by hyphal-tip isolations and conidial platings to recover the two original mutant types, which can be identified by their pattern of heterocaryon formation with F testers. Despite extensive tests, no evidence has been obtained for the presence of other than mutant nuclei in these heterocaryons. In addition, preliminary tests using genetically marked strains have given no indication of mitotic recombination between the two types of mutant nuclei in such heterocaryons.

In view of the complementation in vivo between certain F mutants, resulting in the restoration of adenylosuccinase activity in heterocaryons, preliminary attempts have been made to detect such activity in an in vitro system in which mycelial extracts from two complementary mutant types were mixed. To date, such tests have given only negative results.

Genetic Relationships of Adenylosuccinase-deficient Mutants.—As already indicated, all F mutants are located in linkage group III between the same two markers. Intensive genetic analyses of these apparently allelic mutants have only recently been initiated, now that satisfactorily marked stocks are available. Analyses of crosses between various mutants are proving to be difficult because of sterility and poor ascospore viability, especially in serial isolations. However, it is already clear that crosses of certain mutants yield a low frequency of adenine-independent progeny, whereas selfings of the parents give only adenineless progeny.

To date, most adenine-independent segregants have proved to be pseudo-wild[14] rather than true wild types. Pseudo-wild types have resulted only from crosses of mutants which complement in heterocaryons. Only a few instances of true wild types have been detected as yet. In crosses where markers have been present, the origin of these adenine prototrophs has been associated with marker recombination. However, present crossing results are not extensive enough to determine what type of recombination (whether "orthodox" or involving "conversion") occurs between various mutants. It is expected that current studies will clarify further the genetic relationships of the various F mutants.

A particular study has been made of the inheritance of the pattern of heterocaryon complementation in F mutants exhibiting this behavior. This analysis involved crosses of such F mutants to wild type and tests of the heterocaryon responses of these F mutants in the first and subsequent generations. In all tests to date, F segregants which are heterocaryon-positive (capable of forming heterocaryons on minimal with compatible, biochemically unrelated strains) have shown the same pattern of heterocaryon response as the parental strain. Further tests have involved crosses of heterocaryon-positive strains of two F mutants (capable of complementation), one strain carrying a closely linked marker gene. Tests of segregants from such crosses indicate that a given pattern of heterocaryon complementation is linked with the marker, as is expected if this response is a regular characteristic of a particular adenine mutant.

In summary, in all tests to date, it has not been possible to separate the pattern of heterocaryon complementation from the particular adenine mutant with which it was originally associated. Hence it appears highly probable that this pattern is a result of the particular type of mutational event producing a given adenine mutant.

Discussion.—The preceding evidence indicates that one group of allelic, adenine-specific mutants in *N. crassa* (group F—or *ad-4*—containing 21 mutants of independent origin) can be characterized biochemically as being blocked in the terminal step in adenine biosynthesis, involving the splitting of AMP-S to AMP, as a result of the absence or impairment in these mutants of the AMP-S splitting enzyme, adenylosuccinase. This enzyme is present in wild type and in other biochemically distinct mutants. One of the *ad-4* mutants is temperature-sensitive and, when grown at 25° in the absence of adenine, contains appreciable adenylosuccinase activity. There is preliminary evidence that the enzyme from this mutant is much more thermolabile than that from wild type.

Furthermore, it has been demonstrated that certain of the F mutants lacking detectable adenylosuccinase activity can revert either spontaneously or following ultraviolet irradiation to give adenine-independent isolates testing as reverse mutations. Fifteen such revertants have been shown to possess adenylosuccinase activity, although their average activity is only about 50 per cent that of wild type, and none is equal to wild type in activity, at least after frozen storage of their extracts.

The foregoing results establish that in this instance forward mutation in the wild type to adenine requirement results in the loss of activity of a specific enzyme involved in adenine biosynthesis, and that reverse mutation results in the restoration of this activity. Additionally, it appears, on the basis of qualitative (temperature-sensitivity) and quantitative enzyme activity tests, that changes at the *ad-4* locus arising from mutation in either the forward or the reverse direction may produce diverse mutant and revertant types. Hence this particular situation provides an exceptionally favorable system for further combined biochemical and genetical studies of mutants arising by both forward and reverse mutational events at a single gene locus.

Of particular interest in the present investigation has been the unexpected finding that certain combinations of *ad-4* mutants are able to form heterocaryons (bicaryons) which can grow in the absence of adenine, and that adenylosuccinase activity, although absent from the mutants, is present in the heterocaryons. This type of complementation takes place between certain mutant combinations only. It is also clear that the absence of complementation does not result from a failure of heterocaryon formation, since heterocaryons have been forced with several such combinations, and these require adenine for growth. Several possible interpretations of these observations have been considered.

The occurrence between *ad-4* mutants of complementation essentially similar to that observed between biochemically unrelated types, in which different reactions are known to be blocked, raises the question as to whether the presumed single-step reaction from AMP-S to AMP may actually consist of two steps. However, all presently available evidence tends to refute this possibility. Biochemical studies with yeast and pigeon liver favor the view that a single-step reaction is involved, catalyzed by a single enzyme (Carter and Cohen[11]). In the present material no qualitative differences were detected among the various *ad-4* mutants, such as the accumulation of intermediates other than AMP-S. Furthermore, in vitro mixing of extracts of the F mutants capable of complementation

in heterocaryons failed to produce any activity for the over-all reaction. In fact, no spectral changes were observed in AMP-S in the presence of such extracts, either separate or mixed, whereas it might have been anticipated that, since the enzyme is soluble in active strains, an indication of one or both of the hypothetical component reactions would have been observed in the presence of separate extracts, and certainly an indication of the over-all reaction should have appeared in a mixture of such hypothetical soluble component enzymes.

The possibility that complementation might arise on the basis of differential activities for the splitting of SAICAR and AMP-S also appears to have been eliminated by the failure to demonstrate any activity for either substrate in any of the four F mutants concerned. At present it appears likely that a single enzyme, deficient in all the *ad-4* mutants, is able to catalyze both splitting reactions, which are chemically quite similar.

Additional general evidence against the view that two reactions may be involved comes from the fact that the *ad-4* mutants constitute not two classes, but three, with respect to their complementation. It is thus necessary to assume that the majority of the mutants lack both postulated activities. However, it is clear that the majority of these mutants must have arisen by single mutational events, which would require a remarkably close juxtaposition of the loci presumably controlling the distinct reactions. In the absence of any evidence to the contrary, it seems reasonable to assume that the splitting of AMP-S involves a single reaction catalyzed by a single enzyme. Furthermore, all available data support the view that mutation at only one locus results in the absence or modification of adenylosuccinase.

Another possibility considered was that heterocaryons able to grow in the absence of adenine could do so because they contained wild-type nuclei. However, the following evidence is against this view. (1) Such heterocaryons can be resolved by hyphal-tip isolations and conidial platings, with the recovery of adenine-requiring colonies containing either one or the other of the two original types of mutant nuclei as determined by their heterocaryon responses with the parental tester stocks. Adenine-independent colonies are also recovered from macroconidial platings, but all such colonies tested have proved to be heterocaryons, undoubtedly arising from conidia containing both types of mutant nuclei. Extensive tests have failed to produce any evidence that true wild-type nuclei are present in these heterocaryons. (2) Preliminary tests for mitotic recombination involving linkage group III markers have given negative results. (3) Quantitative comparisons of adenylosuccinase activity in E-F heterocaryons with that in intra-F heterocaryons would appear to require that about one-quarter of the nuclei in the latter heterocaryons be wild type in genotype, which is obviously not the case. Thus these results appear to eliminate the possibility that mitotic recombination or some type of nuclear interaction analogous to transformation or transduction is occurring to produce permanently wild-type nuclei in these adenine-independent heterocaryons.

The final hypothesis to be considered, and the one that seems most probable on the basis of present evidence, is that heterocaryon complementation arises from an interaction involving two different types of mutant nuclei present in a common cytoplasm, resulting in the synthesis of adenylosuccinase, and that this complementation occurs without any permanent genetic change having taken place in the

nuclei involved. As yet there is no evidence as to what the nature of such an interaction may be. It is possible that the presence of one type of nucleus may so modify cytoplasmic conditions that the other nucleus becomes capable of effecting adenylosuccinase synthesis. A more likely possibility would appear to be that some type of nuclear product interaction is involved. The present genetic evidence indicates that a given pattern of complementation is a genetic characteristic of a particular mutant and inseparable from the mutant locus. Thus the presence of a suppressor in one adenine strain, specific for another strain, and hence permitting growth in a heterocaryon, is ruled out (unless the suppressor locus is exceedingly closely linked with the *ad-4* locus). A reasonable possibility would seem to be that, in cases where complementation occurs, the two mutants involved have arisen as a result of mutational changes at different sites in the *ad-4* locus. Where complementation fails, the two mutants may have a certain portion of the locus damaged in common. Thus the mechanism could be visualized as involving cytoplasmic interaction between two types of imperfect templates resulting in the formation of perfect templates for enzyme formation, or a co-operative functioning of two imperfect templates to produce a normal enzyme. It will be of interest to determine whether this hypothesis can be correlated with the behavior of these mutants on crossing, especially with respect to the origin of wild types. It is evident, of course, that other interaction mechanisms can also be hypothesized.

The occurrence of prototrophic heterocaryons between *ad-4* mutants provides further evidence that the heterocaryon test is not a consistent one for detecting allelism (Mitchell[15]). Furthermore, although it has not been possible to obtain stable diploid heterozygotes in *Neurospora*, the evidence that adenine-independent pseudo-wild types arise from crosses of mutants which complement, strongly supports the view that the characteristics of such heterozygotes would be similar to those of heterocaryons with respect to enzyme formation. Thus the present results appear to make more difficult the general application of the *cis-trans* position effect test to delimit a locus as a functional unit (Benzer[16]), at least if functional unity is defined as control over the synthesis of a single enzyme.

Summary.—Combined genetic and biochemical studies have been performed on a series of adenine-specific mutants in *N. crassa*. Twenty-one mutants of independent origin have arisen as a result of mutation at a single locus (*ad-4*) in linkage group III between two closely linked marker genes. Initial crossing analyses with certain of these mutants indicate that adenine-independent isolates arise with a low frequency in some crosses, and that the majority of these types are pseudo-wilds, although true wild types occur in certain combinations. The recombination mechanism associated with the origin of wild types has not yet been established.

Biochemically, all the mutants have been found to be blocked in the terminal step in adenine biosynthesis, involving the splitting of adenosine monophosphate succinate (AMP-S) to adenosine monophosphate (AMP), and to lack or have impaired activity for the AMP-S splitting enzyme, adenylosuccinase. One of the mutants is temperature-sensitive and produces an adenylosuccinase which in crude extracts is much more thermolabile than that from wild type. Certain of the *ad-4* mutants are capable of reverse mutation to adenine-independent phenotypes, and these have been shown to possess restored adenylosuccinase activity, although at levels (or of stabilities) below that of wild type.

These results establish that in this instance forward mutation at a single locus in the wild type to adenine requirement results in the loss of activity of a specific enzyme involved in adenine biosynthesis and that reverse mutation to adenine independence results in the restoration of this activity. Additionally, changes at this locus, arising from either forward or reverse mutational events, may produce diverse mutant and revertant types as judged by qualitative and quantitative tests of enzyme activity.

Certain combinations of *ad-4* mutants have been found to form heterocaryons (bicaryons) able to grow in the absence of adenine. Although the individual mutants lack detectable adenylosuccinase activity, this enzyme is synthesized by the bicaryons. Possible mechanisms for this unexpected type of complementation between alleles in enzyme formation are discussed, along with its implications for biochemical genetic theory in general.

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† This term, rather than dicaryon which generally has a more restricted meaning, is used to designate a heterocaryon having only two kinds of mutant nuclei. Heterocaryons having more than two kinds of mutant nuclei, e.g., tricaryons, have not yet been investigated in these studies.

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