GENE-ENZYME RELATIONSHIPS AT THE *am* LOCUS IN *NEUROSPORA CRASSA*

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1. INTRODUCTION

THE amination deficient, *am*, locus in *Neurospora crassa* is of particular interest, since previous work (Fincham, 1954; Pateman, 1957), has shown that mutation at this locus specifically affects the formation of the enzyme glutamic dehydrogenase by the organism. A number of independently induced *am* mutants are available and a genetical and biochemical investigation of four of these is described in this paper.

2. METHODS

The origin of the experimental strains has been previously described (Pateman, 1957; Fincham and Pateman, 1957*b*) with the exception of *am^R* which is an amination deficient mutant kindly supplied by Dr J. L. Reissig. The mutants *am³²*, *am⁴⁷* and *am¹* were induced by ultraviolet light, *am²⁹* by 20-methylcholanthrene and *am^R* by β -propionolactone.

Media. For growth tests and for the keeping of stock cultures, either Fries No. 3 medium or the minimal medium "N" of Vogel and Bonner (1956) was used, with suitable supplements where necessary. Mycelium for enzyme extraction was grown on Fries No. 3 or on N-free buffered medium (Fincham, 1954) supplemented with 0.01 *M* ammonium tartrate and other supplements where necessary. All crosses between strains were made on medium favouring sexual reproduction. (Westergaard and Mitchell, 1947).

Enzyme extraction. Mycelial pads were grown in 50 ml. lots of medium in 250 or 350 ml. conical flasks or in 150 ml. of medium in penicillin flasks for 48 hr. at 25° C. from a heavy inoculum of conidia. The cultures were not agitated during growth. The pads were thoroughly washed with distilled water, blotted to remove excess moisture, ground in 3.5 times their weight of 0.05 *M* pH 8.0 or pH 7.4 phosphate buffer (KH_2PO_4 — Na_2HPO_4) in a chilled mortar with powdered glass, and filtered by suction through a layer of kieselguhr after the volume of buffer had been made up to 20-30 times the weight of mycelium. The filtrates were almost clear and were used without dialysis. Protein contents of extracts were determined by the quantitative biuret procedure described elsewhere (Fincham, 1954).

Glutamic dehydrogenase assay. Glutamic dehydrogenase activities were determined in the following system: 1.0 ml. extract; 1.6 ml. 0.1 *M* phosphate buffer, pH 8.0 or pH 7.4; 0.1 ml. 0.1 or 0.2 *M* sodium α -ketoglutarate; 0.1 ml. 0.1 *N*(NH_4)₂ SO_4 ; 0.2 ml. reduced triphosphopyridine nucleotide (TPNH) solution to give an optical density at 340 $m\mu$ of 0.3-0.4. The rate of oxidation of TPNH was followed by measuring the rate of decrease of optical density at 340 $m\mu$ in a Unicam SP. 500 spectrophotometer at room temperature (18-21° C.). The reaction was usually

started by the addition of the TPNH but, since many extracts oxidise, or otherwise destroy TPNH, at a very low rate independently of the glutamic dehydrogenase reaction, very low activities were measured as the difference between the rate before and after the addition of the ammonium salt. Specific activities are expressed as change in optical density $\times 10^3/\text{min./mg. protein}$. Specific activities obtained were considerably greater than those reported in an earlier paper (Fincham, 1954). This difference is probably due to the more nearly optimal conditions, particularly in respect of pH and α -ketoglutarate concentration (Fincham, 1957), used for enzyme assay in the present work.

3. WILD TYPES AND PSEUDOWILDS FROM $am \times am$ CROSSES

(i) *Crosses between am strains*

It has been shown previously (Pateman, 1957) that the three independently induced mutations which resulted in the strains am^{29} , am^{32} and am^{47} either occurred at the same locus or at very closely linked loci. A further series of crosses between the four strains am^{29} , am^{32} , am^{47} and am^R were set up comprising all six possible combinations of parents taken two at a time. In addition, crosses between identical am parents were up as controls. Ascospores from these crosses were spread on Fries No. 3 medium plus 1.5 per cent. agar, 1.5 per cent. sorbose, 0.75 per cent. sucrose and 0.02 M glycine contained in Petri dishes. The glycine strongly inhibits the growth of am ascospores with relatively little effect on wild-type spores (Pateman, 1957). The sorbose induces a colonial growth habit in any wild types which develop and facilitates isolation. The plates were kept at 60° C. for 35 minutes immediately after spreading and then incubated at 25° C. The total number of live ascospores was estimated by counting the germinated ascospores in twenty sample fields of each plate with a binocular dissecting microscope, after two days' incubation. The plates were examined daily and any colonies that developed were isolated and cultured; in two cases the number of colonies was large and a representative sample only was transferred. The results of these plating experiments are given in Table I.

The crosses between different am strains fell into two groups, which were distinct with respect to the frequency, time of development and appearance of the apparent wild-type colonies.

The apparent wild types from the $am^{29} \times am^{32}$ and $am^{32} \times am^{47}$ crosses began to form visible colonies after 5-7 days' incubation and the colonies showed great variation in the rate and compactness of growth. When tested for growth on minimal medium, the apparent wild-type isolates in this group usually gave growth intermediate between that typical for am and am^+ strains. In fact, all these apparent wild types which were tested proved to be pseudowilds (see next section).

The apparent wild types from the other $am \times am$ crosses began to form visible colonies after 3-4 days' incubation; these colonies were uniform in appearance with a more vigorous, compact growth than

the great majority of the pseudowild colonies. When tested for growth on minimal medium, wild-type isolates from these crosses showed growth fully comparable to that of stock wild types. When tested they all proved to be true wild types (see next section).

The frequency of true wild types from crosses between different *am* strains is very low and in the absence of suitable markers it is uncertain whether they are due to crossing-over or some such phenomenon as "gene conversion", or perhaps a combination of both. However, it is clear that the wild types are not due to normal back-mutation or contamination, since no wild types were recovered from the control crosses between identical *am* strains.

TABLE 1
Frequency and nature of wild types from am × am crosses

Cross	Total no. ascospores	Total no. apparent wild types	Frequency (per cent.)	Pseudowild	True wild types
<i>am</i> ²⁹ × <i>am</i> ²⁹	585,000	0	...	0	0
<i>am</i> ³² × <i>am</i> ³²	629,000	0	...	0	0
<i>am</i> ⁴⁷ × <i>am</i> ⁴⁷	464,000	0	...	0	0
<i>am</i> ^R × <i>am</i> ^R	512,000	0	...	0	0
<i>am</i> ²⁹ × <i>am</i> ³²	184,000	426	ca. 0.23	19 *	0
<i>am</i> ²⁹ × <i>am</i> ⁴⁷	651,000	14	ca. 0.002	0	14
<i>am</i> ²⁹ × <i>am</i> ^R	1,377,000	3	ca. 0.0002	0	3
<i>am</i> ³² × <i>am</i> ⁴⁷	156,000	87	ca. 0.06	16 *	0
<i>am</i> ³² × <i>am</i> ^R	1,293,000	10	ca. 0.0008	0	10
<i>am</i> ⁴⁷ × <i>am</i> ^R	715,000	0	<0.0002	0	0

* Only a sample of the apparent wild types was transferred from the *am*²⁹ × *am*³² and *am*³² × *am*⁴⁷ crosses.

(ii) *Nature of apparent wild types from crosses between am strains*

All the apparent wild types isolated were backcrossed to one of the same parental *am* strains that had been used in the cross from which they were derived. Samples of ascospores from each of these backcrosses were plated on the selective medium described above. The germinated ascospores were examined after incubation for 2-3 days at 25° C.

In those backcrosses which gave an approximate 1 : 1 segregation of wild-type and *am* ascospores, the wild-type parent was classified as a "true" wild type (see table 1) since its behaviour was similar to that of the stock wild types which carried the *am*⁺ allele. Crosses between wild types isolated from these backcrosses and *am* strains also gave 1 : 1 segregation for *am* and *am*⁺ ascospores.

In backcrosses of apparent wild types derived from *am*²⁹ × *am*³² and *am*³² × *am*⁴⁷ there were no *am*⁺ ascospores in the progeny, but after about 5 days, colonies developed, similar in frequency and every other respect to those from the original *am*²⁹ × *am*³² and *am*³² × *am*⁴⁷ crosses. Since these apparent wild types did not carry the *am*⁺ allele

and were intermediate in growth between *am* and *am*⁺ strains on minimal and selective media, it seemed most probable that they were pseudowilds (Pittenger, 1954).

It is known that pseudowilds owe their origin to the presence of a disomic nucleus in an ascospore ; if the homologous chromosomes carry mutant alleles which are complementary then the resultant strain may show a semi-wild phenotype. With consequent growth and nuclear division in the pseudowild, the disomic nuclei usually break down and a heterocaryon with two types of haploid nuclei, each type carrying a different mutant allele, is formed. In order to confirm the supposition that the apparent wild types were in fact pseudowilds, it was necessary to show that they originated as single ascospores and that *am* strains of two different types could be derived from them.

This was done as follows. From *am*²⁹ × *am*³² and *am*³² × *am*⁴⁷ crosses, a number of single ascospores which showed rather strong initial growth in the selective medium, were isolated. When tested for growth on minimal and supplemented medium, cultures from these single ascospore isolates gave intermediate growth (see table 4). All the single ascospore cultures behaved like pseudowilds in back-crosses to *am* strains.

Representative strains, 1281 from *am*²⁹ × *am*³² and 1214 from *am*³² × *am*⁴⁷, were used in the following experiments. A number of single conidium isolates were made from sorbose plates streaked with conidial suspensions. All of these isolates were tested for growth on minimal medium and many behaved like typical *am* strains. A number of *am* isolates from 1281 were tested in pairs for heterocaryon formation by superimposing drops of thick conidial suspensions on glycine sorbose plates. These *am* isolates fell into two groups ; combinations involving one strain from each group gave comparatively vigorous colonies, presumably by heterocaryon formation. All the colonies which originated from mixed conidial suspension were cultured and tested for growth on minimal medium. In a similar fashion complementary heterocaryons were formed between *am* isolates from 1214. The dry weights of mycelia from tests of the *am* isolates and complementary heterocaryons in growth flasks are given in table 4. It seems clear from this evidence that the intermediate wild types from *am*²⁹ × *am*³² and *am*³² × *am*⁴⁷ were pseudowilds which gave rise to complementary heterocaryons.

It did not appear to make any difference whether the heterocaryons originated from disomic nuclei or by vegetative hyphal fusion, though pseudowild formation provided an easy, effective method of overcoming incompatibility difficulties in heterocaryon formation. The possibility remains that the semi-wild phenotype of these heterocaryons was, in fact, due wholly or in part to the presence of some disomic or diploid nuclei. This question is not settled, but evidence presented in a later section suggests that the possibility is unlikely. It is of some

interest that from no one type of cross were both true wild types and pseudowilds recovered. In those types of cross from which only true wild types were obtained, it is certain that no pseudowilds were formed, since all colonies were transferred and analysed. But it is possible that rare true wild types occurred in those crosses which produced large numbers of pseudowilds. The great majority of pseudowild and true wild-type colonies are distinct in appearance and time of development on the plates, but there is some overlap. Consequently, a rare, true wild type, which developed late, might not have been obviously different from surrounding pseudowild colonies and might not, by chance, have been included in the small sample of those transferred for testing. However, the experiments

TABLE 2
Enzyme activities of true wild types

Strains	Specific glutamic dehydrogenase activities						
14 wild types from $am^{29} \times am^{47}$. . .	410 590 710	715 1062	505 820	600 320*	550 350*	850 420	
3 wild types from $am^{29} \times am^R$. . .	540	470	535				
10 wild types from $am^{32} \times am^R$. . .	665 897	977 472	825 824	650 768	594	595	

* Assayed at pH 7.4.

described and other work on the recovery of true wild types from $am^{29} \times am^{32}$ and $am^{32} \times am^{47}$ crosses (Pateman, 1956), suggests that if they occur, the frequency is at least no greater than that found for the other *am* intercrosses.

4. ENZYME ACTIVITIES OF WILD TYPES AND PSEUDOWILDS

(i) All the true wild types from $am^{29} \times am^{47}$, $am^{29} \times am^R$ and $am^{32} \times am^R$ were assayed for glutamic dehydrogenase activity. The results are given in table 2. There was some variation between the specific enzyme activities of the different wild types, but there was no evidence to suggest that any of them was significantly different from the stock wild types carrying the am^+ allele.

(ii) A representative sample of pseudowilds from $am^{29} \times am^{32}$ and $am^{32} \times am^{47}$ were assayed for glutamic dehydrogenase activity. The results are given in table 3. There was a good deal of variation in the enzyme activities of the pseudowilds, but this was probably due to uncontrollable factors such as varying nuclear ratio. In no case, so far, has the enzyme activity of a pseudowild extract approached that of the stock wild types.

(iii) Table 4 shows the results of the enzyme assays of *am* isolates and reconstituted heterocaryons derived from 1281 and 1214, which are single ascospore pseudowilds from $am^{29} \times am^{32}$ and $am^{32} \times am^{47}$ respectively. These results illustrate the remarkable fact that certain *am* alleles, individually incapable of detectable glutamic dehydrogenase activity, are complementary in function in heterocaryons with the resultant production of enzyme. Moreover, it seems probable that this complementary action is between alleles in different nuclei in the heterocaryon.

TABLE 3
Growth on minimal medium and enzyme activities of pseudowilds

Strains	Dry weight of mycelium in mg.		Specific glutamic dehydrogenase activity
	Fries minimal	Fries plus M/200 DL-alanine	
1348 <i>am</i> ⁺	43	42	325 *
Pseudowilds from $am^{29} \times am^{32}$			
1279	28	36	49 *
1280	28	31	77 *
1281	26	34	40 *
1283	29	43	57 *
1284	31	47	97 *
Pseudowilds from $am^{32} \times am^{47}$			
1214	3	33	60
1215	1	28	99
1216	9	30	67
1217	14	30	114

Cultures for growth tests were grown from small conidial inocula for 72 hours at 25° C. in 100 ml. flasks containing 15 ml. of medium.

* Assayed at pH 7.4.

5. PROPERTIES OF FORCED HETEROCARYONS

In order to extend the information derived from study of pseudowild types, heterocaryons carrying various combinations of *am* alleles were synthesised. The different *am* strains were each crossed to two arginine-requiring mutants, 46004 (*arg*-1, linkage group I) and B317 (*arg*-10, linkage group VII). These mutants were supplied by Dr Dorothy Newmeyer, who has established their linkage relationships (Newmeyer, 1957). Double mutants were isolated from the crosses, and heterocaryon formation was forced by inoculating mixtures of conidia from *arg*-1 and *arg*-10 strains on to agar slants lacking arginine but supplemented with alanine. Only double mutants which formed heterocaryons readily with the original *arg*-1 and *arg*-10 strains were used for heterocaryon formation; all were of mating type *a*.

Table 5 shows the results of enzyme assays on heterocaryons formed between strains which, individually, were incapable of forming detectable glutamic dehydrogenase. The combinations $am^{32} + am^{47}$

and $am^{32} + am^{29}$ were found to produce the enzyme though at considerably less than the usual wild-type level. All other types of heterocaryons formed no detectable glutamic dehydrogenase in repeated experiments; the minimum activity which should have been detectable

TABLE 4

Growth on minimal medium and enzyme activities of am isolates and reconstituted heterocaryons from pseudowilds

Strain	Dry weight of mycelium in mg.		Specific glutamic dehydrogenase activity
	Fries minimal	Fries plus M/200 DL-alanine	
Pseudowild 1281 from $am^{29} \times am^{32}$	26	34	40 *
<i>am</i> isolates from pseudowild 1281			
1	0	29	0 (<3)
2	0	24	0 (<3)
3	0	23	0 (<3)
4	0	25	0 (<3)
5	0	24	0 (<3)
6	0	22	0 (<3)
Heterocaryons between <i>am</i> isolates from 1281			
1+4	26	33	80
1+5	30	37	49
2+3	29	37	96
2+6	37	38	64
Pseudowild 1214 from $am^{32} \times am^{47}$	3	33	60
<i>am</i> isolates from pseudowild 1214			
1	0	30	0 (<3)
2	0	34	0 (<0.6)
3	0	33	0 (<2)
4	0	34	0 (<1)
Heterocaryons between <i>am</i> isolates from 1214			
1+4	10	35	75
2+3	2	33	82
3+4	9	29	84
1346 am^+	35	33	550

* Assayed at pH 7.4.

in each experiment is indicated in table 5 and varied according to the protein content of the extract and the stability of the spectrophotometer at the time. These results were checked by growth experiments. The heterocaryons which produced glutamic dehydrogenase grew almost as well as the wild type on minimal medium, while those which formed no detectable glutamic dehydrogenase grew no better on minimal medium than did homocaryotic *am* strains (*i.e.* no weighable growth after 3 days at 25° C.), although they grew at approximately wild-type rates on medium supplemented with DL-alanine at $\frac{1}{2}$ mg. per ml.

The allele am^1 , which is described in detail elsewhere (Fincham

and Pateman, 1957*b*; Fincham, 1957) was isolated as an apparent back-mutant in an experiment in which conidia of an *am*⁴⁷ strain were irradiated with ultraviolet light. The available evidence (Fincham, 1957) supports the hypothesis that *am*^l produces a glutamic dehydrogenase qualitatively different from that of the wild type. Extracts of *am*^l strains have low glutamic dehydrogenase activity which can be increased to a level approaching that of wild type by warming to 35° C. or higher for a few minutes. This extra activity is slowly lost on returning the activated extract to room temperature, but can be regained on repeated warming. Treatment at 35-50° C. does not affect the activity of wild-type extracts, nor does it induce

TABLE 5
Specific glutamic dehydrogenase activities from forced heterocaryons

	<i>arg-1</i>		
	<i>am</i> ⁴⁷ (1509-78)	<i>am</i> ³² (1526-78)	<i>am</i> ^R (1538-78)
<i>arg-10</i> { <i>am</i> ⁴⁷ (1507-56)	0 (<2) 0 (<2)	60	0 (<3) 0 (<0.5)
<i>am</i> ³² (13) .	67 *	0 (<5)	0 (<3)
<i>am</i> ²⁹ (14) .	0 (<2) 0 (<1)	46 150	0 (<2) 0 (<5)
<i>am</i> ^R (27) .	0 (<1)	0 (<4) 0 (<0.3)	0 (<4) 0 (<0.5)

* Grown on Vogel and Bonner's medium "N" instead of the usual N-free buffered medium with ammonium tartrate.

The medium was supplemented with DL-alanine in all cases, either ½ mg. or 1 mg./ml. Wild type typically gave specific activities of 500 or more grown under the same conditions.

activity in extracts of other *am* mutants lacking glutamic dehydrogenase. The factor by which the activity of *am*^l extract is increased by warming varies from experiment to experiment, but has always been at least six in the present series of experiments (table 6). The variation is thought to be due to differences in room temperature; the effect of warming has generally been less at higher room temperatures at which *am*^l enzyme is probably already partly activated or rapidly becomes so on mixing with substrates.

Results of experiments on heterocaryons carrying *am*^l in combination with other *am* alleles are shown in table 6. Extracts of heterocaryons of the type *am*^l+*am*⁴⁷ were qualitatively similar to *am*^l homocaryons, although their activity was generally rather less. Thus there is no indication of any complementary interaction between these two alleles. On the other hand, extracts of *am*^l+*am*³² heterocaryons behaved as if they contained a mixture of wild-type and

am^l-type glutamic dehydrogenases (Fincham, 1957); their activity before heat treatment was very much greater than that of typical

TABLE 6
Enzyme activities of heterocaryons involving am^l

Strain	Experiment number	Specific glutamic dehydrogenase activity	
		Extract untreated	Extract treated at 38.5° C. for 5 min.
Homocaryons			
<i>arg-10 am</i> ⁺ (B317-9-9) *	1	580	590
<i>arg-1 am</i> ^l (1519-34) *	1	30	320
<i>arg-1 am</i> ^l (1517-56) *	1	39	420
<i>albino am</i> ^l (1432-78)	3	23	250
	5	36	460
	8	45	340
Heterocaryons			
<i>arg-10 am</i> ⁺ + <i>arg-1 am</i> ^l B317-9-9 + 1517-56	1	570	800
<i>arg-10 am</i> ⁴⁷ + <i>arg-1 am</i> ^l 1505-56 + 1517-56	1	14	200
	6	38	220
	7	24	160
1507-56 + 1519-34	1	20	330
	2	9	165
	5	26	330
	6	63	390
<i>arg-10 am</i> ³² + <i>arg-1 am</i> ^l 11 + 1519-34	2	160	250
13 + 1519-34	2	205	270
	5	205	340
<i>arg-10 am</i> ²⁹ + <i>arg-1 am</i> ^l 14 + 1517-56	7	120	220
14 + 1519-34	3	14	50
	4	11	54
	5	84	235
		143	290
	6	100	180
		130	180
	7 †	95	240
<i>arg-10 am</i> ^R + <i>arg-1 am</i> ^l 27 + 1517-56	7	20	20
	8	28	83
27 + 1519-34	2	36	140
	5	37	160
	6	110	330
		21	34
	7 †	60	115
	8 †	73	110

* Grown in medium supplemented with $\frac{1}{2}$ mg./ml. L-arginine hydrochloride.

† Heterocaryon made afresh from the original stocks for this experiment.

Values derived from the same experiment are more closely comparable than those from different experiments, owing to differences in room temperature.

am^l extracts, but their response to heat was very much less. This result was not unexpected in view of the complementary interaction already shown between *am*⁴⁷ and *am*³². More surprisingly, both *am*^l + *am*²⁹ and *am*^l + *am*^R heterocaryons gave evidence of producing

some enzyme resembling wild-type glutamic dehydrogenase in not responding to the temperature treatment. Extracts of heterocaryons of these two types sometimes had rather low activity before warming, but in no case was the stimulation of activity by warming as great as in extracts of am^l or $am^l + am^{47}$ cultures tested in the same experiment. The rather variable behaviour of these heterocaryons in replicated experiments is unexplained; it could perhaps be due to fluctuations in nuclear ratio. The data as a whole suggest that am^l is capable of interacting with either am^{32} , am^{29} or am^R (but not with am^{47}) to produce glutamic dehydrogenase of the normal type. There is a strong indication that the combination $am^l + am^R$ is less effective in this respect than $am^l + am^{32}$, with $am^l + am^{29}$ perhaps occupying an intermediate position. These provisional conclusions could be better tested by experiments in which nuclear ratios were controlled. It remains possible that some of the heterocaryons involving am^l may produce still further varieties of glutamic dehydrogenase, but the investigation of this possibility depends on the development of other methods for distinguishing types of enzyme.

6. IS THE FORMATION OF DIPLOID NUCLEI THE BASIS OF COMPLEMENTARITY IN HETEROCARYONS?

Perhaps the most surprising aspect of the complementarity shown by certain pairs of am alleles is the fact that it apparently operates with the different alleles in separate nuclei. The question arises whether the interaction is actually due to the occasional spontaneous fusion of nuclei such as is known to occur in *Aspergillus nidulans* (Pontecorvo *et al.*, 1953). If diploid nuclei are responsible for the enzyme production in $am^x + am^y$ heterocaryons they must presumably be present in the mycelium in fair frequency to account for the levels of enzyme found, since a previous study (Fincham, 1954) indicated that the level of glutamic dehydrogenase in heterocaryons was not far from being proportional to the percentage of am^+ nuclei. On the other hand, stable diploids have never been demonstrated in *Neurospora*, although it is known that they have been looked for without success by several investigators. Thus it seems probable that diploid nuclei, if they occur, are highly unstable and become readily reduced to haploids (perhaps by a process analogous to that by which a haploid heterocaryon arises from the presumably originally disomic nucleus of a pseudowild ascospore). Such diploidisation and subsequent reduction would be accompanied by free recombination of unlinked markers. To test this possibility a search was made for conidia, produced by a heterocaryon of type $arg-1\ am^{47} + arg-10\ am^{32}$, which had become homocaryotic for one or other arg mutant, while remaining heterocaryotic with respect to the am alleles. Conidia were spread on agar plates supplemented with 0.3 mg. of L-arginine hydrochloride per ml., a concentration of arginine which satisfies an arginine requirement without permitting optimal growth of am

conidia. A total of 102 well-grown sporelings were isolated. All except 3 were arginine-independent, and the three which required arginine all proved to be homocaryotic for *am*. A further 75 sporelings which appeared to be growing relatively poorly on the original plate were isolated. Of these 73 required arginine, and all were homocaryotic for *am*; the other two were arginine-independent and behaved as if heterocaryotic for $am^{32}+am^{47}$. Thus there was no evidence for somatic recombination such as might be expected if unstable diploid nuclei were being formed. A further possibility is that diploid nuclei are formed which are effective in promoting enzyme formation but undergo no further division; such diploid nuclei could only be detected by cytological methods which have not yet been developed.

7. DISCUSSION

There are two main points of interest in the results described. First, the complementary action of certain pairs of *am* mutants in the production of glutamic dehydrogenase. Second, the genotypic status of the different *am* mutants and what information can be deduced from them concerning the organisation of that genetic material called the *am* locus, which apparently plays some specific role in the formation of glutamic dehydrogenase in *Neurospora*. These two points are of course inter-related, but for convenience will be discussed separately.

If, as seems probable, glutamic dehydrogenase synthesis may be promoted by the joint action of two kinds of separate nuclei, neither of which is capable of forming the enzyme by itself, a chromosome-enzyme relationship of unexpected complexity seems to be indicated. There is little doubt that *Neurospora* glutamic dehydrogenase is a single enzyme; as reported elsewhere (Fincham and Pateman, 1957a) neither mixtures of am^{32} and am^{47} extracts nor extracts of mixtures of am^{32} and am^{47} mycelium have been found to possess any activity, so these two mutants evidently do not produce different components of a complex enzyme system. Moreover, *Neurospora* glutamic dehydrogenase has recently been purified 40-fold (Fincham, unpublished) without great loss of total activity. The data could be interpreted as suggesting that glutamic dehydrogenase is formed in a series of distinct steps under the control of different parts of the *am* locus. In the positive heterocaryons one can imagine an intermediate in enzyme synthesis diffusing from one nucleus to another, if the enzyme is synthesised in the nucleus, or from one gene product in the cytoplasm to another, if it is synthesised in the cytoplasm. The difficulty with such a picture, apart from the current trend of biochemical opinion against multi-step enzyme synthesis (see, for example, Spiegelman, with added comment by Velick, 1956), is that it provides no obvious reason why the genes concerned with the various steps should be tightly organised in a complex locus in the wild type. As an alternative we suggest that the whole *am* locus may, in the wild type, act as a

unit in producing a single product (or plasmid, in Catcheside's (1956) terminology) which passes into the cytoplasm and becomes an essential part of the glutamic dehydrogenase-forming system. In the heterocaryons nuclear products with non-overlapping defects can be imagined as becoming organised in such a way, presumably through being built in to the same structure, that they can form a synthetic functional unit. A similar suggestion has been made by Giles *et al.* (1957) for their parallel case involving adenylosuccinase formation. Such a hypothesis requires that nuclear products be incorporated at specific cytoplasmic sites with rather high efficiency, since it is otherwise difficult to explain how the complementation could work as well as it apparently does, even though the heterocaryons have not been found to approach the wild type in efficiency of enzyme production.

The present information on the physiological relationships of the *am* mutants can be represented by a linear diagram (fig. 1). Mutations

FIG. 1.—A diagrammatic representation of the physiological relationships between hetero-alleles at the *am* locus.

which are mutually complementary may be envisaged as affecting non-overlapping regions of the complex locus, while mutations which are non-complementary may affect overlapping regions. Whether such a diagrammatic representation bears any relationship to the actual spatial organisation of the locus is doubtful.

Any tentative conclusions that might be made concerning the genetic status of the *am* mutants investigated, depend to a great extent on the mode of origin postulated for the wild types recovered from certain *am* intercrosses. In the absence of tetrad analysis with suitable markers, it is not possible to say whether these wild types arise as a result of orthodox crossing-over, gene conversion, or a combination of both. But it is probably significant that the wild-type frequencies from *am* intercrosses do not support any postulated linear arrangement of *am* "alleles". For example, in fig. 2 some of the wild-type frequencies from *am* intercrosses are represented in a linear diagram. This is one of the more plausible possible arrangements, yet there are two discrepancies with the rest of the data, (1) the frequency of wild types from *am*^R × *am*⁴⁷ is less than 0.0002 per cent.; (2) there were no true wild types from *am*³² × *am*⁴⁷. Furthermore, any attempt to

incorporate the data on complementarity and wild-type recovery in a single linear scheme, raises a number of anomalies. Thus it seems unlikely that all of the recovered wild types can have been the result of orthodox crossing-over, at least a proportion and possibly all of them may have been due to gene conversion. This is in line with other work on yeast (Lindgren, 1955; Winge, 1955; Roman, 1956), *Neurospora* (Mitchell, 1955*a, b*; St Lawrence, 1956; Giles, 1951) and possibly *Aspergillus* (Pritchard, 1955). The fact that gene conversion must at least be taken into account makes it vastly more difficult to relate wild-type formation to the actual topography of the *am* locus. It has already been pointed out by St Lawrence and Bonner (1957) that the demonstration of gene conversion in organisms such as fungi, makes the interpretation of recombinant frequencies in terms of detailed spatial organisation of the genetic material a somewhat hazardous procedure. The elegant and refined analysis of the genetic material of a bacteriophage by Benzer (1957) may only be possible

FIG. 2.—A linear diagram based on wild-type frequencies obtained from some of the *am* intercrossovers.

with this type of organism and not applicable to more complex forms of life.

In order to overcome certain difficulties involved in the classical concept of the "gene", Benzer (1957) proposed the acceptance, in its place, of three fundamental genetic units, the "recon", the "muton" and the "cistron". The cistron is the functional unit and two apparently similar mutants which are non-complementary in a heterocaryon are considered to be located in the same cistron. Thus *am*³² would be in a different cistron to *am*²⁹ and *am*⁴⁷. The position of *am*^R is not so clear, since it is not complementary with *am*²⁹, *am*³² or *am*⁴⁷; it might be a deletion or defect which overlaps the other *am* sites, but this seems improbable since true wild types have been recovered from *am*²⁹ × *am*^R and *am*³² × *am*^R crosses. The case of the *am* mutants raises questions of terminology not covered by Benzer's scheme. The entity which we have referred to in this paper as the *am* "locus" is clearly some kind of significant unit since (a) it is concerned as a whole with the synthesis of a single specific enzyme, (b) its parts are extremely closely linked (and perhaps hardly separable by orthodox crossing-over), and (c) it is not analysable into two or

more discrete cistrons. One may ask whether the cistron is the significant functional unit in all cases and whether some other term may not be necessary to cover such cases as the present. Until more information is available on the generality of this kind of situation, we propose to continue to use the term "locus".

In view of these considerations and with the data at present available, it seems best to regard the different *am* mutants as carrying different "heteroalleles" (Roman, 1956, and Beadle, 1957) at the *am* locus. This terminology recognises the basic fact that the *am* mutants carry demonstrably different components of the *am* locus, between which some form of gene conversion (transmutation, Beadle, 1957) can probably occur. Whether or not crossing-over can take place between these heteroalleles has yet to be satisfactorily demonstrated. The fact that certain pairs of heteroalleles are physiologically complementary provides a further criterion for distinguishing between them. It is obvious that more experimental work requires to be done with, if possible, a further group of independently induced *am* mutants, in order to extend our understanding of the gene-enzyme relationships at the *am* locus.

8. SUMMARY

(1) It has been shown that wild types can be recovered from crosses between different *am* mutants. From certain intercrosses only true wild types with a very low frequency were obtained, while from the other crosses only pseudowilds with a comparatively high frequency were recovered.

(2) The true wild types grew normally on minimal medium and possessed glutamic dehydrogenase activity fully comparable to stock wild types. The frequencies of these wild types from the various intercrosses are not readily interpretable in terms of crossing-over within a linear arrangement of "sites", and it is suggested that at least some of the wild types may be due to gene conversion (transmutation).

(3) The growth of pseudowilds on minimal medium was intermediate between that usual for *am* and stock wild-type strains, and they possessed low glutamic dehydrogenase activity.

(4) *am* strains with no detectable glutamic dehydrogenase activity were isolated from pseudowilds and heterocaryons were reconstituted from them. These heterocaryons were shown to possess low enzyme activity similar to the pseudowilds. Thus certain *am* heteroalleles, which individually in homocaryons are incapable of producing detectable glutamic dehydrogenase activity, are complementary in function when present in different nuclei in a heterocaryon, with the resultant production of enzyme.

(5) Forced heterocaryons were made between the various *am* mutants in all possible combinations. Pairs of heteroalleles which

had given pseudowilds in sexual crosses formed glutamic dehydrogenase in heterocaryons; pairs of heteroalleles which had given no pseudowilds in sexual crosses formed no glutamic dehydrogenase in heterocaryons.

(6) Homocaryotic *am*¹ strains produce a qualitatively different glutamic dehydrogenase from the wild type, but *am*¹ is complementary with certain other *am* heteroalleles, since forced heterocaryons produce greater or smaller amounts of apparently normal glutamic dehydrogenase in addition to the *am*¹ enzyme.

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