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Complementation and enzyme studies of revertants induced in an *am* mutant of *N. crassa*

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1. INTRODUCTION

It is known that amination deficient, *am*, mutants of *Neurospora crassa* lack normal NADP-linked glutamate dehydrogenase (GDH) (Fincham, 1962) and that several produce abnormal varieties of this protein. Previous work (Pateman, 1957; Fincham, 1957) has shown that further mutation induced in *am* strains could result in phenotypic reversion associated with the production of varieties of NADP-linked glutamate dehydrogenase distinct from that produced by wild-type *N. crassa*. The work reported here is concerned with the genetical and complementation characteristics of revertants which were induced by ultra-violet light in an am^3 strain. An account is also given of some of the biochemical characteristics of the GDH varieties produced by these back mutants.

2. MATERIALS AND METHODS

(i) *Strains and culture conditions*

The stock wild-type strain used was St Lawrence *A* ($74A = STA$). The am^3 strain was originally S2929 induced by Barratt at Stanford using 20-methylcholanthrene as the mutagen. The am^3 strains used in these experiments were obtained by backcrossing a stock am^3 *a* strain to ST*A* for six generations; consequently the *am* and am^+ strains were largely isogenic except for the *am* and mating-type alleles. The minimal medium used was Fries No. 3 (Beadle & Tatum, 1945) or that given by Vogel (1956). When it was convenient to induce a compact colonial growth habit the usual 1·5% sucrose in the medium was replaced by 1·5% sorbose and 0·75% sucrose. Slope cultures and still liquid cultures were made at 25°C. on a medium favouring sexual reproduction (Westergaard & Mitchell, 1947).

(ii) *Induction of revertants*

Cultures of am^3 were grown for 10 days at 25°C. on Fries No. 3 minimal plus 0·005 M DL-alanine agar slopes. The conidia were washed from the slopes with

sterile water, passed through a cotton wool filter and centrifuged at 3000 *g* for 10 min. The pellet of conidia was suspended in sterile water, centrifuged, resuspended in sterile water and counted with a haemocytometer slide. The concentration of the spore suspension was adjusted to 5×10^6 per ml. and then irradiated in an open Petri dish with ultra-violet light from a low-pressure mercury vapour lamp. A predetermined exposure time of 200 sec. killed between 98·5% and 99·5% of the conidia. Before and after irradiation aliquots of the spore suspension were appropriately diluted and spread on minimal medium plus 0·005 M DL-alanine and sorbose, in order to provide estimates of conidial viability and survival rate respectively. The irradiated suspension was centrifuged and the conidia resuspended in sterile water before distribution in 1 ml. aliquots on Petri dishes containing minimal medium, supplemented with 0·02 M glycine to inhibit the growth of non-revertant conidia. Each plate was spread with *ca.* $1{\cdot}5 \times 10^6$ irradiated conidia. All plates were incubated at 25°C.; the viability and survival rate counts were made at 72 hours after incubation. Inspection of the glycine plates between 48 hours and 60 hours after incubation showed occasional hyphae which were growing more strongly than the hypae from the surviving *am* conidia. Pieces of the vigorously growing hyphae were transferred to minimal slopes. In order to purify the apparent revertants each isolate was crossed to an am^3 strain. Ascospores from these crosses were spread on minimal plus 0·02 M glycine since it is possible to distinguish *am* and am^+ ascospores germinating on this medium (Pateman, 1960). It was possible to isolate from the majority of the crosses individual ascospores which grew more strongly than *am* ascospores on the glycine medium. The single ascospore revertant isolates were tested for growth on minimal medium and used as the stock culture for subsequent experiments.

(iii) *Glutamate dehydrogenase assays of revertants*

All the purified revertants were assayed for glutamate dehydrogenase activity in the following routine fashion. Mycelium of each strain which had been grown in liquid still culture for 48 hours at 25°C. on minimal medium was collected and pressed dry in absorbent paper. The blotted mycelium was extracted in 20 ml. per g. of 0·1 M pH 8·0 orthophosphate buffer by grinding with glass in a mortar. The resultant slurry was filtered through Kieselguhr (or 'Celite' filter aid) to give a cell-free extract which was used in the enzyme assay.

Enzyme activities were measured by following the reduction of NADP or the oxidation of $NADPH_2$ by optical density measurements at 340 mμ. In a preliminary survey of the revertants the following reaction mixture was used at 20°C.: 0·1 ml. extract; 0·1 ml. 0·2 M disodium α-oxoglutarate; 0·2 mg. $NADPH_2$ in 0·1 ml. 0·1 M pH 8·0 orthophosphate buffer; 0·1 ml. 0·2 M NH_4Cl; 2·6 ml. 0·1 M pH 8·0 orthophosphate buffer. The mixture was completed and the reaction started by the addition of the NH_4Cl. This assay system will be referred to as the pH 8—20°C. system. The cell housing of the Unicam SP500 spectrophotometer was maintained at 20°C. by circulating water. The reduction in optical density at 340 mμ during the first 30 sec. after completion of the reaction mixture was taken as the measure

of glutamate dehydrogenase activity. Specific enzyme activities are expressed as 1000 times the change in optical density (O.D.) at 340 mμ per min. per mg. protein. Protein concentrations were determined by the method Lowry *et al.* (1951) using a standard curve prepared with casein.

In later more detailed examination of the properties of the mutant enzymes formed in the various partial revertants a number of different assay systems were used, as follows:

(*a*) *Standard reductive amination system*

2·50–2·55 ml. 0·05 M *tris*-(hydroxymethyl) aminomethane–HCl ('Tris') buffer at pH 8·4; 0·10–0·20 mg. $NADPH_2$ in 0·10–0·15 ml. of the same buffer (to give a final increment in O.D. at 340 mμ of about 0·4); 0·15 ml. 0·2 M disodium α-oxoglutarate; 0·10 ml. M NH_4Cl; 0·1 ml. mycelial extract added at zero time. Note that in this system the NH_4Cl concentration is five times that in the pH 8—20°C. system.

(*b*) *Preincubation with substrates*

As (*a*), but with the enzyme incubated with the $NADPH_2$ and α-oxoglutarate and 1 ml. of buffer at the reaction temperature for 2 min. before the addition of the remaining buffer and the NH_4Cl to start the reaction.

(*c*) *Preincubation—low ammonia*

As (*b*), but with 0·1 ml. 0·1 M NH_4Cl in place of 0·1 ml. M NH_4Cl (i.e. a final concentration of 3·3 mM instead of 33·3 mM.)

(*d*) *Standard oxidative deamination system*

2·7 ml. 0·15 M monosodium glutamate in Tris buffer (as above), 4 mg. NADP in 0·2 ml. water, and 0·1 ml. mycelial extract at zero time.

(*e*) *Low glutamate*

As (*d*), but with the final glutamate concentration reduced from 135 mM to 33 mM.

Tests were made in each of the above systems both at 25°C. and at 35°C. The NADP and $NADPH_2$ were obtained as the monosodium salts either from the Sigma Chemical Co. or from the Boehringer Co. (Courtin & Warner, Lewes, Sussex).

3. RESULTS

(i) *Production of revertants*

Several hundred apparent revertant isolates were obtained from the irradiation experiments. A total of 102 apparent revertants which grew more strongly than am^3 on minimal medium were crossed to an am^3 strain. From eighty-seven of these crosses isolates were made of single ascospores which grew significantly faster after germination on glycine medium than typical am^3 ascospores. Although several thousand ascospores were screened from each cross only typical am^3

ascospores were observed in the progeny of the other fifteen crosses. The eighty-seven purified revertants were tested for growth on liquid minimal and minimal plus 0·005 M DL-alanine medium. All the revertants grew better than an am^3 strain on liquid minimal medium from a conidial inoculum in still culture at 25°C. for 72 hours. The majority of the revertants grew as well as am^+, but a few strains made rather less growth than am^+ on minimal. All the revertants, as well as am^3 and am^+, grew in a similar fashion on minimal plus DL-alanine medium.

(ii) *Genetic analysis of revertants*

In order to discover the location of the induced mutation each purified revertant was crossed to an am^+ strain. If a modifier mutation had occurred at some site other than the am^3 site, it was assumed that recombination could result in the segregation of am^3 ascospores from the revertant $\times$ am^+ cross. Since am^3 ascospores could be distinguished from am^+ during germination on glycine medium at 25°C. (Pateman, 1960) a total of 400–1000 random ascospores were screened from each cross. No *am* recombinants were obtained from eighty-four of the revertant $\times$ am^+ crosses. Using the formula suggested by Horowitz (1953) the maximum map distance apart of the hypothetical modifier and the am^3 site could be calculated. The data showed that had any of the 'reversion' sites in these eighty-four been more than 2·3 *c*M. from the am^3 there would have been less than a 1% chance of failing to find at least one am^3 recombinant.

The crosses between am^+ and three of the revertants (am^{3-4}, am^{3-5}, and am^{3-27}) all gave large numbers of slow-growing ascospores amongst the progeny. Subsequent analysis showed that these slow ascospores were not am^3 recombinants. The am^{3-4}, am^{3-5}, and am^{3-27} mutations, when carried in the ascospore nuclei, usually resulted in slow ascospore germination similar to but not as extreme as that induced by am^3 itself.

Since the distinction between am^3 ascospores and those carrying am^{3-4}, am^{3-5}, or am^{3-27} is not clear-cut, it was not practicable to screen effectively large numbers of ascospores from these three revertant $\times$ am^+ crosses for rare am^3 recombinants. However the twenty ascospores which grew most slowly after germination in a sample of 300 ascospores were isolated from each of the three revertant $\times$ am^+ crosses. None of these slow-growing isolates proved to be am^3 strains with the exception of one apparent *am* strain from the $am^{3-5} \times am^+$ cross. Further investigation indicated that this strain was probably a poorly growing variant of am^{3-5}. It is difficult to estimate the probability that am^3 recombinants were segregating in these particular crosses, but it seems unlikely that any 'modifier' mutation could be more than a few centimorgans from the *am* locus. The slow germination of am^{3-4}, am^{3-5} and am^{3-27} ascospores was obviously the result of the abnormal glutamate dehydrogenase activity which these partial revertants possessed (see later section).

To conclude, it seems likely that the 'reversion' sites all fell within the *am* gene, and we regard the non-wild-type revertants as new alleles of the *am* series.

(iii) *Glutamate dehydrogenase activities in the revertants*

The results of the preliminary survey of GDH activity in revertant strains are given in Table 1. If the first extract of a revertant gave less than 50% of the usual wild-type activity two further batches of mycelium were grown and assayed. Only those revertants, numbering fifteen out of the total of eighty-eight, which showed less than 50% of typical wild-type activity in each of the three tests are recorded in Table 1. In spite of their relatively low GDH activity all these partial revertants grew as well or nearly as well as wild-type in minimal medium from conidial inocula. Three of them, am^{3-4}, am^{3-5} and am^{3-27} grew more slowly than am^{+} during the early stages of ascospore germination on minimal medium.

Table 1. *Glutamate dehydrogenase activities of revertants*

Genotype	GDH activity
am^{+}	1200–2400*
am^{3}	10, 32, 20
am^{3-2}	330, 620, 230, 216
am^{3-3}	120, 105, 43
am^{3-4}	10, 124, 16, 26
am^{3-5}	10, 150, 10
am^{3-14}	84, 60, 28
am^{3-17}	12, 10, 16
am^{3-18}	12, 8, 12
am^{3-22}	130, 260, 200
am^{3-27}	15, 60, 20
am^{3-35}	38, 150, 108
am^{3-50}	36, 37, 54
am^{3-80}	48, 80, 26
am^{3-83}	44, 55, 48
am^{3-88}	126, 48, 64
Remaining 73 revertants	800—2300

The activity values are specific activities determined in the pH 8—20°C. assay system, as described in the text. Values lower than fifty are of dubious significance, being based on barely measurable rates.

* Range of eight independent determinations.

The results obtained from tests of extracts of partial revertants in the differential assay systems (described under Methods) are summarized in Table 2 and some of the original data are shown in Fig. 1. In Table 2 the enzyme characteristics of strain 3–12 are included. This strain was originally thought to be a partial revertant, but it subsequently behaved like am^{3} itself. It is included in Table 2 as an example of the am^{3} enzyme. There is a good deal of variation in the estimates of the specific activity (P) in the reductive amination system for some of the strains, notably 3–27. This is probably largely due to the difficulty of achieving the same degree of activation in different extracts using a standard preincubation system. The tests served to subdivide the revertants into six qualitatively distinct classes.

(1) The revertants 3–3, 3–14, 3–35, 3–80, 3–83 and 3–88 all had GDH very similar to that produced by a previously described *am*[3] revertant, called *am*[3b] (Fincham, 1962). At 25°C. their extracts had only very low initial activity in the standard reductive amination assay but showed a progressive activation. This

Table 2. *Results of tests distinguishing between different glutamate dehydrogenase varieties*

	Reductive amination				Oxidative deamination		
	Activated by preincubation				Standard system		
	Specific activity (35°) (*P*)	35°/25°	33·3 mM / 3·3 mM NH_4Cl	Activation factor at 25°*	Specific activity (35°) (*Q*)	35°/25°	*P*/*Q*
Wild	4600	1·6	1·3	1·0	1900	1·75	2·4
3–12**	0	—	—	—	1630	6·3	∞
3b	4900	—	3·3	—	450	—	10·9
3–3	5300	1·65	2·6	5	500	2·3	10·6
3–14	4800	2·3	3·0	6	—	—	—
	5500	—	3·3	—	415	—	13
3–35	5100	2·05	2·4†	5	410	1·9	12·5
3–80	3300	2·55	3·4	5	440	2·6	8
	4900	—	3·9	—	—	—	—
3–83	5200	1·7	3·0	3	375	2·2	14
3–88	7000	1·7	2·8	6	700	1·9	10
3–5	1000	2·5	5	∞	140	3·5	7·1
	1270	—	4	—	200	—	6·3
3–4	4750	2·8	7	19	255	2·4	19
3–27	4500	3·6	high	∞	200	⩾6	22
	2050	—	8	—	150	—	14
	1750	—	5	—	—	—	—
3–22	5850	1·35	3·3	1·5	535	3·0	11
3–17							
3–18	0	—	—	—	0	—	—
3–50							

The various assay systems are described in the text. Different rows represent independent determinations.

* Activated/non-activated rate measured over first ½ min. of reaction.
** 3–12 is identical in its glutamate dehydrogenase to the original *am*[3].
† Tested at 25°—others in this column at 35°.

activation was much more rapid (though still observable) at 35°C. The enzyme could in each case be fully activated to approximately the normal wild-type level by preincubation for 2 min. with α-oxoglutarate and $NADPH_2$ at either temperature. Reduction of the ammonium chloride concentration from 33·3 to $3{\cdot}3 \times 10^{-3}$ M gave about a threefold decrease in reaction rate (following activation), indicating a

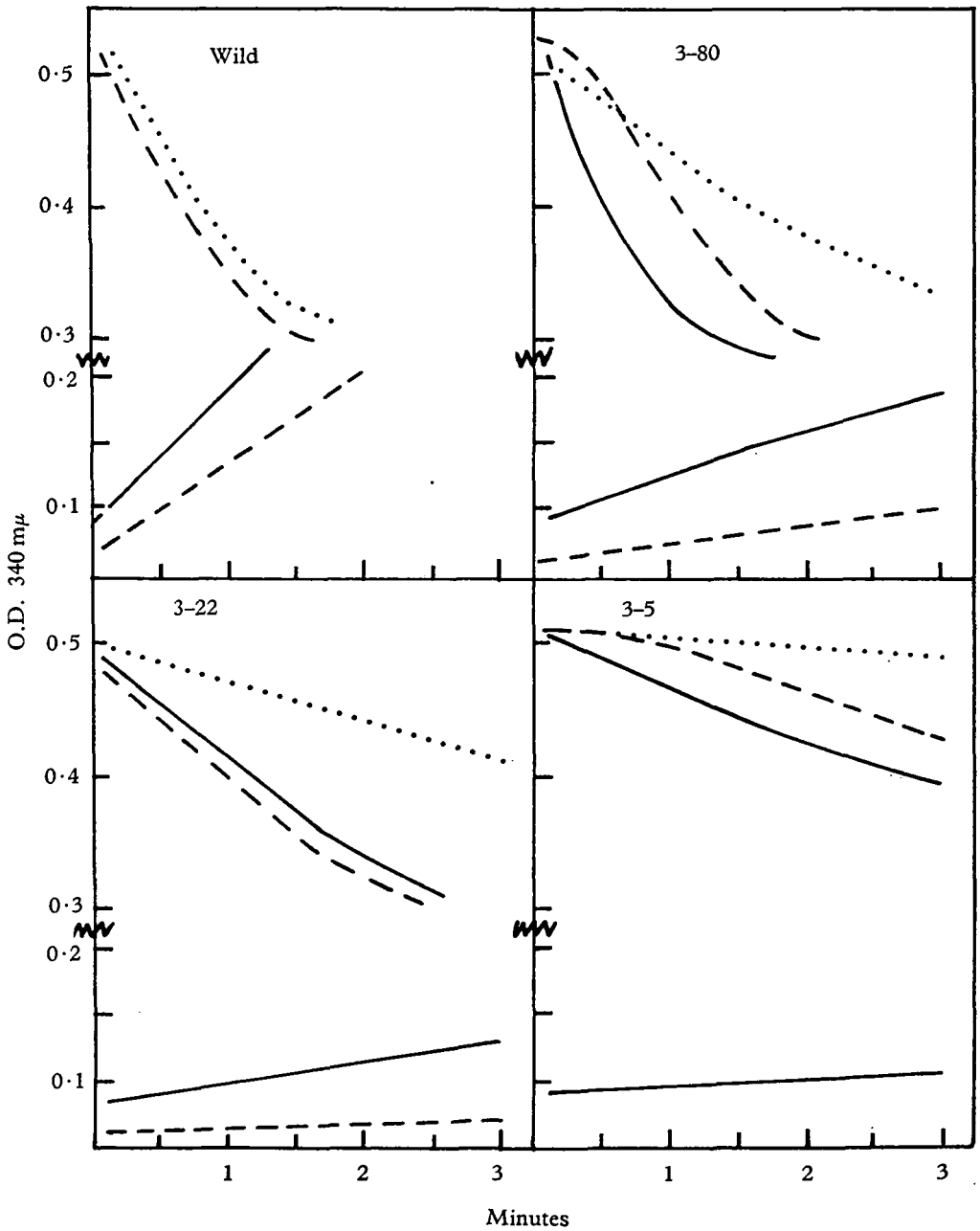

Fig. 1. Enzyme assays showing the different kinds of glutamate dehydrogenase activity present in extracts of wild-type and three of the partial revertants. The assay systems, each at 35°C. were as follows:

Reductive amination ($NADPH_2$ oxidation descending curves): standard system —broken line; enzyme preincubated with substrates—full line; enzyme preincubated with substrates but with low ammonium chloride—dotted line. Oxidative deamination (NADP reduction ascending curves): standard system—full line; low glutamate system—broken line (see Materials and Methods).

In the case of the wild-type enzyme preincubation with substrates makes no difference to the activity and this particular test was not made in the experiment illustrated. The amounts of protein in each assay were 50, 80, 30 and 40 μg. for wild-type, 3-80, 3-22 and 3-5 respectively.

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Michaelis constant for ammonium ion in the region of 10^{-2} M, about 5–10 times the wild-type value. The enzyme was relatively poorly activated in the oxidative deamination reaction mixtures, even with 135 mM. glutamate; the reaction rate in this direction is more strongly dependent on glutamate concentration in the range 33–135 mM. than is the case with wild-type GDH.

(2) The revertant 3–5 produced GDH similar in many respects to that found in the preceding group. It differed in showing a lower maximum activity, even after activation, though whether this is due to an intrinsically less active enzyme or to a reduced production of enzyme protein is not known. It was also less rapidly activated in the reductive amination system and its activity showed an even more extreme dependence on ammonium chloride concentration consistent with Michaelis constant for ammonium ion in the region of 2×10^{-2} M. In general the 3–5 enzyme seemed to show the same abnormalities as the enzyme of the am^{3b} type but in more extreme form.

(3) The enzyme formed in the revertant 3–4 is again not very different in its main peculiarities from GDH of the am^{3b} type. It seems somewhat less active in the reductive amination assay systems, and is slower to become activated in the standard system. It is markedly less active in the oxidative deamination system and is almost inactive at the lower glutamate concentration. The dependence of activity on ammonium ion concentration is very high, with a Michaelis constant in the region of 4×10^{-2} M.

(4) Revertant 3–27 produces an enzyme with properties not clearly distinct from that formed in 3–4 except in relatively lower activity, even after preincubation with substrates, at 25°C. A difference between 3–4 and 3–27 is more clearly indicated by the complementation tests (see next section).

(5) 3–22 enzyme differed from the others just considered in showing a much more rapid activation during incubation with α-oxoglutarate and $NADPH_2$, the difference from the am^{3b} type being especially marked at 25°C. It was, however, clearly different from wild-type GDH in its high dependence on concentration of ammonium salt (Michaelis constant about 10^{-2} M, similar to that of the am^{3b} type). Of all the revertants examined in these experiments, 3–22 approaches most nearly in its properties to the previously described revertant am^{3a} (Fincham & Bond, 1961; Fincham, 1962). The Michaelis constant for ammonium ion does not appear to be so high as in am^{3a}, however, and the 3–22 enzyme was not found to have the characteristically low thermostability of am^{3a} GDH. Indeed none of the revertant enzymes described in the present paper appeared to be markedly unstable at 60°C. in 0·05 M phosphate, pH 8·0.

(6) Three revertants, 3–17, 3–28 and 3–50, were remarkable in showing practically no GDH activity on first test in any of the assay systems. Subsequently, however, significant and sometimes quite high activity was detected in apparently similar extracts of 3–18 and 3–50. The only difference in the extraction procedure lay in the presence of 0·001 M ethylenediamine tetra-acetic acid (EDTA) in the phosphate buffer in the later experiments. Extracts of 3–18 and 3–50 were then made with 0·02 M EDTA in the phosphate buffer. Both mutants then gave GDH activity in

the wild-type range (using the standard reductive amination assay system) with no evident lag in activity when the extract was added as the final addition of the reaction mixture. Extracts made at the same time with phosphate buffer lacking EDTA were almost inactive. An extract of a 3–18 strain, made in the absence of EDTA, could however be activated by the addition of EDTA, the degree of activity after 1 hour at room temperature being dependent on the EDTA concentration (Fig. 2). Similar tests on 3–17 have not been performed but it is pre-

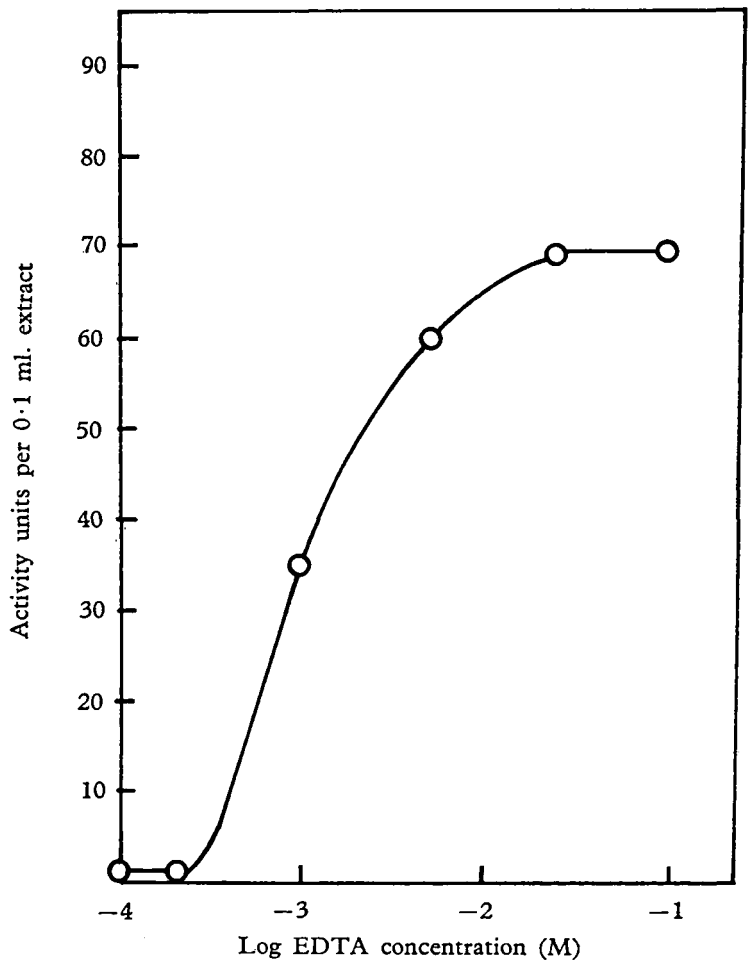

Fig. 2. Activation of glutamate dehydrogenase from revertant 3–18 by EDTA. Samples of mycelial extract (0·2 mg. protein/ml.) made with 0·05 M pH 8·0 phosphate without EDTA were supplemented with EDTA (pH 8·0) to give the concentrations shown, incubated 1 hour at room temperature, and assayed in the standard reductive amination system (for composition see text).

sumed that this revertant may be similar to 3–18 and 3–50. In this connexion it is of interest that the mutant enzyme produced by a partial revertant derived from *am*^{19} is activated by EDTA as well as by some other polycarboxylic acids (Sundaram & Fincham, in press).

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Extracts of all the partial revertants were tested to determine whether they could be activated simply by warming, as had been found to be the case with the partial revertant am^{21}, derived from am^2 (Fincham, 1957). None was found to be activated by such treatment.

(iv) *Enzyme complementation between partial revertants and* am *mutants*

It has been shown (Fincham, 1959) that of the first eleven *am* mutants, am^1 to am^{11}, only two pairs (am^1+am^2) and (am^1+am^3) form complementary heterocaryons, although a few more complementing pairs are now known involving more recently isolated *am* mutants (Fincham & Stadler, 1964). The complementary heterocaryon am^1+am^3 grows on minimal medium more strongly than either am^1 or am^3 alone and possesses 10–25% of the usual am^+ GDH activity.

It is not possible to test revertants from am^3 in pairwise combinations with other *am* mutants in heterocaryons for complementary growth, since all the revertants are capable of independent growth on minimal medium. However, both the *am* mutants and a number of the partial revertants possess very little GDH activity, as measured by $NADPH_2$ oxidation in the reductive amination assay at pH 8 and 20°C., without prior incubation of the enzyme with substrates. Consequently it is possible to test forced heterocaryons between the *am* mutants and the partial revertants for GDH activity resulting from complementation. Strains were prepared which contained an *arg-10* allele in addition to one of the *am* alleles, e.g. am^1 *arg-10*. Another series of strains which contained an *arg-1* allele in addition to one of the partial revertant alleles, e.g. am^{3-X} *arg-1* were also made. Forced heterocaryons, e.g. (am^1 *arg-10* + am^{3-X} *arg-1*) were made from mixed inocula of the two double mutants on minimal medium. Growth on minimal medium from such a mixed inoculum is necessarily heterocaryotic, since neither component strain can grow alone in the absence of an arginine supplement.

A number of forced heterocaryons were grown on minimal medium and assayed for GDH activity using the pH 8—20°C. system. The results of the enzyme assays are given in Table 3. It can be seen that strain am^{3-22} shows significant complementation only with am^1 and is therefore similar in its complementation characteristics to am^3, the strain from which it was derived. Strains am^{3-18} and am^{3-27} do not complement with any of the *am* strains tested; as a result of the second mutation they have lost the ability to complement with am^1. Strains am^{3-3} and am^{3-4} complement both with am^1 and with am^7 and am^{13}. It is probable that am^{13} is in fact a repeat mutation of am^7 since am^+ recombinants are not produced in $am^{13} \times am^7$ crosses (Pateman, unpublished data). Therefore the revertants am^{3-3} and am^{3-4} have gained the ability to complement with am^7, which is not possessed by am^3. It is not possible to give precise quantitative estimates of the amount of complementary GDH activity, but it is of the order of 10–15% of the usual am^+ activity in all the complementary combinations. The complementation relationships of the partial revertants and the *am* mutants are represented diagrammatically in Fig. 3.

Not all the partial revertants were tested for their complementation charac-

Table 3. *Glutamate dehydrogenase activities of forced heterocaryons between* am *mutants and partial revertants*

am allele	–	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*11*	*13*
–	–	11	24	22	25	20	24	30	21	10	26	32
+	2006	—	—	—	—	—	—	—	—	—	—	—
3–3	64	520	10	60	12	8	32	190	17	17	25	300
		270						300				
		400						215				
3–4	10	112	16	32	10	24	36	160	12	16 30		114
		180						170				
		200						200				
3–18	15	60	25	55	4	12	40	20	4	30	40	4
3–22	95	377	39	45	30	14	8	63	15	24	18	75
		380						50				
		410										
3–27	40	74	20	30	10	24	12	12	64	40	28	56
		40										
		35										
3–80	70	180	20	38	26	19	47	150	35	20	34	185
		260						210				
		210						190				

The figures refer to specific activities measured in the pH 8—20°C. system (see text). Values lower than fifty are of dubious significance.

teristics in forced heterocaryons. Therefore it is possible that some strains at present classified as similar on biochemical criteria (e.g. 3–1A and 3–18) could be distinguished by complementation. This means that the estimate of six qualitatively distinct groups of partial revertants is a minimum one.

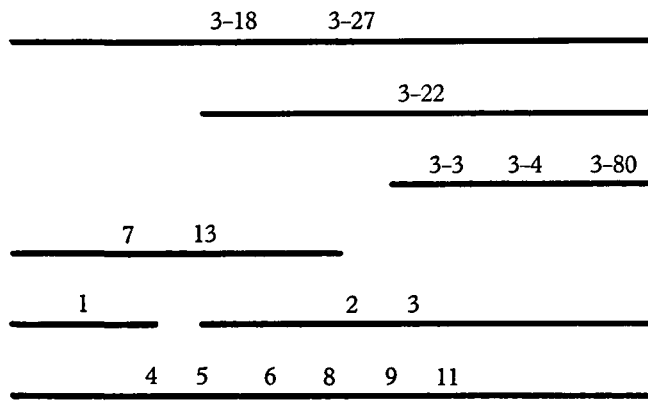

Fig. 3. Complementation map of partial revertants and *am* mutants. Any pair of mutants which are represented by the same line or two overlapping lines do not complement. Mutants which are represented by non-overlapping lines show enzyme complementation.

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4. DISCUSSION

It has been shown that at least thirteen out of a total of eighty-seven revertants produced a variety of glutamate dehydrogenase which is recognizably different from that of the wild-type. Furthermore, the thirteen partial revertants fall into six distinct groups on the basis of their enzyme and complementation characteristics. Since four of the groups of partial revertant contain only one member it is probable that in a larger sample of induced revertants additional new types of partial revertant would be found. In fact previous experiments (Pateman, 1957; Fincham & Bond, 1961) had detected a revertant type, am^{3a}, which was not represented in the present sample of revertants. It is not possible to form any estimate of the possible number of different ways in which further mutation can restore some GDH activity in an am^3 strain. However it does seem that the group (1) (am^{3b}) type—if it is an homogeneous group—is the most easily induced. It seemed unlikely that the preponderance of group (1) mutants was just due to a selective advantage in the mutation experiments, since several of the rarer types grew as strongly on minimal medium.

The assay system used in the preliminary screening of the revertants measured the ability of the extracts to synthesize glutamate at 20°C. This was probably the best single criterion for detecting intermediate levels of enzyme activity in the revertants, since am^3 itself has no detectable activity in this system. Extracts of the am^3 strain itself and of all the partial revertants show a considerable degree of GDH activity when activated by incubation with certain substrates of the enzyme or by EDTA. It is probable that many of this type of partial revertant would have been overlooked if the revertants had been screened using an assay system which brought about a higher degree of activation. However, it is possible that a different assay system would have brought to light enzyme abnormalities which were missed in the present study. In fact the spectrum of revertant types observed must depend on the variety of criteria used to detect them. A more refined enzymatic analysis would probably demonstrate further revertant types amongst the large group of apparently normal revertants. It is obvious that growth rate is an insensitive criterion for detecting heterogeneity in a sample of revertants. It is doubtful if any of the partial revertants discussed here would have been detected by growth tests of revertant strains.

The am^3 mutant variety of GDH is chemically and physically very similar to the normal enzyme (Fincham, 1962; Fincham & Coddington, 1963) and has latent activity. Its inactivity *in vivo* and in some *in vitro* assay systems must be due to an abnormal folding structure rather than to the absence of any essential component of the active centre. The several different types of partial revertant obtained from am^3 presumably represent several different ways of modifying the folding of the protein so as to permit the conformation change necessary for activation to occur more readily. The fact that there are so many different kinds of partial revertant makes it seem very unlikely that all could be due to a change at the same position in the am^3 polypeptide chain. Some at least, must almost certainly be due to

changes of amino acid residues in the chain other than the one changed in am^3, and hence, by hypothesis, to mutations at different genetic sites within the gene other than the primary am^3 site. Thus, though genetic evidence is lacking, we can be fairly confident that at least some of the partial revertants are intragenic double mutants, with the second mutation acting as a partial suppressor of the effect of the first.

A striking fact established by the work on primary and secondary mutants at the *am* locus in *N. crassa* is the number of ways in which mutation can result in an altered GDH protein which still possesses significant catalytic activity. Three primary *am* mutants, am^2, am^3, am^{19} (Fincham, 1961; Fincham & Stadler, 1964), at least six different types of secondary mutant reported here, and at least six different kinds of revertant from am^{19} (Stadler, in press) all produce abnormal but somewhat active glutamate dehydrogenase of one kind or another. So a total of at least eighteen different mutant varieties of *Neurospora* GDH are now known of which fifteen show some activity under some conditions. It is reasonable to assume that this is only a small proportion of the possible genetic modifications of the enzyme which could be induced. It is remarkable that the precise structural and spatial configuration of amino acids thought to be necessary for an enzymatic 'active site' can be affected directly or indirectly in so many ways to result in altered and not complete loss of catalytic activity. Furthermore, many of the partial revertants with abnormal GDH protein apparently grow as well as the wild-type. Consequently it is possible that natural populations of *N. crassa* may contain a variety of genetic variants, or be polymorphic, with respect to GDH protein.

SUMMARY

A total of eighty-seven revertants were induced by ultra-violet light in an am^3 strain. All of these revertants appear to be the result of mutation at sites in or close to the *am* locus. Fourteen of the eighty-seven revertants were partial revertants in that under some conditions of assay they possessed low glutamate dehydrogenase activity compared with the wild-type although their growth rate was similar to that of the wild-type. Enzyme extracts of thirteen of the partial revertants were assayed for glutamate dehydrogenase in various ways in order to establish qualitative distinctions between different kinds of mutant enzyme. On the basis of these tests six different groups were established, of which one contained six revertants, one three and the others one. All except one of the mutant enzyme types showed a marked activation when incubated with α-oxoglutarate plus $NADPH_2$, and all of these had Michaelis constants for ammonium ion much higher than is found for the wild-type enzyme. The remaining group of three revertants gave, at first, no enzyme activity in any of the assay systems. Two of these (the third was not tested) were shown to produce an enzyme variety which becomes quite inactive in phosphate buffer at pH 8·0 but can be fully activated by the addition of ethylenediamine tetra-acetic acid. Forced heterocaryons between each of six partial revertants and eleven *am* mutants were made and the resultant

sixty-six heterocaryons assayed for glutamate dehydrogenase activity. The partial revertants differed among themselves in their complementation characteristics. Some complemented with none of the *am* mutants, some with am^1 only, and some with am^1 or with am^7. The complementation tests confirmed the differences established by the enzyme studies. The data presented here, together with previous work, demonstrate that ultra-violet light induced mutation in an *am* strain can result in at least eight types of revertant differing from each other in respect of the glutamate dehydrogenase variety which each can produce.

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