REVERSION OF THE *am* LOCUS IN NEUROSPORA: EVIDENCE FOR NONSENSE SUPPRESSION

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THE *am* (amination) locus in *Neurospora crassa* codes for the primary structure of the NADP-linked enzyme glutamate dehydrogenase. Because the enzyme is well characterized (FINCHAM 1957, 1958), stable, and the forward and reverse reactions catalyzed by the enzyme can easily be measured, it provides a good system for the analysis of gene-enzyme relationships. The oxidative deamination and reductive amination reactions are shown in Figure 1.

The seven *am* mutants used in this study were chosen because of the different types of altered glutamate dehydrogenase they produce. They include complementing, and noncomplementing mutants, both CRM[2] and non-CRM formers (CRM$^-$) induced by ultraviolet light (UV) and nitrous acid. The frequencies of reversion of the mutants and the types of revertants obtained by nitrous acid treatment have been compared to determine whether these could be correlated with the original lesions. One hundred and ten revertants have been examined with regard to the qualitative properties of their glutamate dehydrogenase. Striking differences of response are seen between different alleles. Two different CRM$^-$ mutants reverted by changes at unlinked loci. Evidence is presented that at least one of these is analogous to the nonsense mutants in bacteriophage T4 (BENZER and CHAMPE 1962) and in *E. coli* (GAREN and SIDDIQI 1962; WEIGERT and GAREN 1965). Suppressors of this mutant are able to suppress CRM$^-$, noncomplementing mutants in two other genes physiologically unrelated to the *am* locus.

MATERIALS AND METHODS

Description of mutants: The *am* mutants used were obtained from DR. J. R. S. FINCHAM (*am*1, *am*4, *am*5; UV induced) and from DR. D. R. STADLER (*am*14, 15, 17, 19; nitrous acid induced). All were crossed several times to the standard wild types (74A or 3.1a) and carried

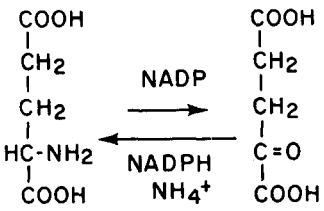

FIGURE 1.—The reactions catalyzed by glutamate dehydrogenase (GDH).

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[2] CRM: "crossreacting material," an antigen that reacts with neutralizing antiserum to a specific protein (here, glutamate dehydrogenase).

the inbred ST. LAWRENCE genetic background. Other stocks were supplied by the Fungal Genetics Stock Center, Dartmouth College.

Induction and isolation of revertants: Conidia from cultures of *am* mutants grown on complete slants for about 7 days at 33°C were treated with nitrous acid as described by FINCHAM and STADLER (1965). 0.4 ml of freshly prepared 0.5M $NaNO_2$ was added to 11.6ml of conidial suspension in 0.1M acetate buffer, pH 4.4. The suspension was incubated at 37°C with occasional shaking. Aliquots were removed at 4 minute intervals, pipetted into an equal volume of sterile 0.1M Na_2HPO_4, and mixed on a Vortex mixer. This effectively neutralized the action of nitrous acid. An aliquot of the original suspension treated identically except for omission of $NaNO_2$ was included as the untreated control. The conidia were sedimented by centrifugation, and the pellet was washed and resuspended in sterile distilled water. The washed material was pipetted into minimal sorbose medium (LESTER and GROSS 1959) supplemented with 0.02M glycine. (Glycine is added to inhibit the slow growth of *am* mutants on minimal medium). The medium was kept liquid at 45°C until the suspension was added, then immediately poured into plates. These were incubated at 25°C and all revertant colonies were isolated until the background growth became too dense (usually after 8 to 10 days).

Suspected revertants were transferred to minimal slants supplemented with glycine and grown up at 25°C. The presumptive revertant was discarded if it failed to grow faster than the *am* mutant from which it was derived. For each revertant retained a vegetative isolate was picked from a streaked plate of the selective medium and crossed to its mutant parent of the opposite mating type to produce a homocaryotic, genetically pure revertant. A single am^+ ascospore was cultured for the genetic and biochemical studies of the glutamate dehydrogenase produced by the revertant.

Preparation of extracts and enzyme assays: Crude extracts were prepared by the method of FINCHAM and STADLER (1965) from 48-hour mycelial pads grown in liquid minimal medium (VOGEL 1964) at 25°C without shaking. Pads were washed in ice cold glass-distilled water, blotted dry and ground with sand in 6 to 8 times their weight of 0.05M sodium phosphate buffer, pH 8.0, containing 0.001M ethylene diamine tetracetic acid (EDTA). All operations were carried out on ice with ice cold solutions. Protein concentration was determined by the method of LOWRY *et al.* (1951) and extracts were diluted to a concentration of 0.2 mg/ml with the extracting buffer.

Glutamate dehydrogenase activity in the "forward" reaction, the oxidative deamination of glutamate, and in the "reverse" reaction, the reductive amination of alpha-ketoglutarate, was measured spectrophotometrically with the standard assay systems of FINCHAM and CODDINGTON (1963). All assays were carried out in a water-jacketed Beckman DB spectrophotometer at 37°C. One unit of specific activity is defined as 1000 times the change in absorbance at 340 $m\mu$ per minute per mg protein. The optical path length was 1 cm.

Heat inactivation of enzyme activity: Two-ml aliquots of ice cold extract were pipetted into test tubes and immersed in a rapidly circulating, accurately controlled constant temperature bath (Haake thermoregulator) at 65°C for a time sufficient to destroy 50% of the wild-type (74A or 3.1a) glutamate dehydrogenase activity. Aliquots were withdrawn and rapidly pipetted into test tubes placed in an ice-water bath to stop inactivation. While the time to reach half inactivation was extremely sensitive to small temperature changes, it was possible to achieve reasonably reproducible 50% inactivations after 12 minutes of heating. Maximum variation was less than $\pm 10\%$ on repeated experiments. A second heat sensitivity test was carried out by diluting each extract 1:1 with ice cold 0.2M NaH_2PO_4. This lowered the pH to 6.2. Under these conditions and protein concentration (0.1 mg/ml), the enzyme is considerably more sensitive to thermal inactivation. Six minutes of exposure to 62°C gave reproducible 50% loss of wild-type GDH activity. All heat inactivations reported in this paper were either 12 or 6 minute treatments as indicated by the conditions of exposure (pH). Enzyme activity in both cases was assayed at pH 8.5 in Tris-HCl buffer by measuring the reduction of alpha-ketoglutarate.

RESULTS

The maximum reversion frequencies of the various *am* alleles by nitrous acid

is shown in Table 1. Only revertant isolates that gave ascospores growing significantly faster than the mutant when crossed back to their parent *am* have been included in this table. More than 80% of the isolates selected as revertants by preliminary screening tests gave genetically pure revertants. No revertants were detected for *am*15 in over 2×10^8 untreated and an equal number of treated viable conidia. The reversion of the other six *am* mutants was enhanced 10- to 20-fold over the spontaneous frequency and in nearly all cases an increase in the absolute number of revertants above background was obtained. It was difficult to estimate precisely the amount of stimulation caused by nitrous acid because of the limited accuracy in measuring the observed low spontaneous reversion frequencies. Two mutants, *am*14 and *am*19, reverted five to ten times more readily than the other alleles examined.

Seventy revertants have been assayed for their vegetative growth response on three liquid media—minimal, minimal supplemented with alanine, and minimal supplemented with glycine. Mycelial pads from 2-day old cultures grown at 25° were dried and weighed. *am* mutants respond well to alanine-supplemented medium, grow slowly on minimal, and are inhibited by glycine. Wild type 74A is slightly inhibited by glycine but grows equally well on the other two. None of the 32 revertants from *am*1, *am*4, *am*5 tested to date has shown significant differences from the wild type in growth response. However, three of seven *am*14 revertants grew poorly on glycine and were stimulated by alanine; several revertants of *am*17 (3/18) and *am*19 (2/13) showed a similar response.

Because the *am* mutants grow slowly even on glycine-supplemented medium (although very slowly and after a considerable lag), it was important to determine if the GRIGG effect (the suppression of prototrophs by a dense, slowly metabolizing mutant background—GRIGG 1965) was operating in these experiments.

TABLE 1

Summary of reversion analyses at the am *locus*

Mutant	Inducing agent	Complementation*	CRM†	Revertants induced by UV‡			Revertants induced by N.A.		
				Reversion frequency	Classes of GDH	Fraction identical to W.T.	Reversion frequency	Classes of GDH	Fraction identical to W.T.
*am*1	UV	+	+	2.9	1	15/15	0.29	1	11/11
*am*4	UV	—	+	. .	.	. . . . .	0.15	1	17/17
*am*5	UV	—	—	. .	.	. . . . .	0.10	3	5/17
							1 *su* class		
*am*14	N.A.	+	—	13.2	4	1/23	0.91	7	1/20
*am*15	N.A.	—	—	0	.	. . . . .	0	.	. . . . .
*am*17	N.A.	—	—	9.5	3	10/17	0.33	3	15/25
							1 *su* class		
*am*19	N.A.	+	+	20	5	0/17	1.3	6	1/20

* Complementation reported by FINCHAM and STADLER (1965).
† CRM studies reported by ROBERTS and PATEMAN (1964).
‡ UV reversion data from STADLER (1966).
Symbols: UV, ultraviolet light; N.A., nitrous acid; CRM, serological crossreacting material; *su*, a class of revertants due to unlinked suppressors; W.T., wild type. Reversion frequency is the maximum reversion frequency per 10^8 survivors. Classes of GDH refers to the qualitative classes of glutamate dehydrogenase (GDH) from revertants based on catalytic and heat stability properties of the enzyme as described in the text. Fraction identical to W.T. refers to the fraction of revertants whose GDH is indistinguishable from the wild-type enzyme.

This could cause reversion frequencies to be underestimated and might lead to nonrandom recovery of different revertant types. A reconstruction experiment with conidia from wild type 3.1a and *am*4a was carried out. Mutant and prototrophic conidia were mixed in various proportions, plated in molten agar and incubated exactly as in the reversion experiments. No decrease in the recovery of wild-type colonies was observed even at mutant concentrations five times the maximum density used in the reversion tests. While the possibility of selectively losing revertants growing at less than wild-type rates has not been ruled out, the isolation of a number of these suggests that this is not a serious problem.

A survey of glutamate dehydrogenase (GDH) activity and heat stability in

TABLE 2

Summary of glutamate dehydrogenase analyses of am *revertants*

Mutant	Class of revertant	Number of revertants	Relative Specific Activity‡			Heat stability‖	
			Reduction	Oxidation	Ratio§	pH 8.0	pH 6.2
*am*1 (11 revertants)	I	11	.8–1.0	.8–1.0	.8–1.0	(91–119%)	(91–110%)
*am*4 (17 revertants)	I	17	.8–1.3	.8–1.3	.8–1.1	(85–117%)	(85–110%)
*am*5 (17 revertants)	I	5	.9–1.5	.8–1.5	.8–1.1	(91–105%)	(94–114%)
	II	11	.4–.6*	.8–1.3	.4–.6	0	(14–40%)*
	III†	1	0	0	0	. . .	. . .
*am*14 (20 revertants)	I	1	1.0	1.1	.9	(114%)	(100%)
	IV	5	.08–.15	.08–.11	.9–1.2	0	0
	V	2	.08	.09–.1	.8–.9	(120–144%)	0
	VI	6	.2–.6	.12–.4	1.5–1.9	(130–178%)	0
	VIa	2	.4–1	.4–1	1.1	(144–176%)	0
	VII	3	.1–.2	.2–.3	.4–.6	0	0
	VIII	1	.6	.4	1.5	19	0
*am*17 (25 revertants)	I	15	.8–1.2	.8–1.4	.8–1.3	(90–135%)	(80–119%)
	IX	3	1.3–1.6	.07–.1*	16–19	(46–69%)	(111–146%)*
	X†	7	.3–.5	0	. . .	(29–47%)	(120–145%)*
*am*19 (20 revertants	I	1	1.0	.9	1.1	(91%)	(94%)
	XI	7	.7–1.2	.7–1.2	.9–1.2	0	0
	XIa	3	.6	.6	1	0	0
	XIb	1	.4	1.0	.4	0	0
	XII	3	1.2–1.3	.5–.7	2–2.6	(4–6%)	0
	XIII	3	.2	.16–.2	1.3–1.6	0	0
	XIV	1	.5*	.9	.6	(67%)	(156%)*
	XV	1	1.4	1.0	1.4	(29%)	(18%)

* Exact activity difficult to measure owing to activation. For heat stabilities, measurements were compared over a similar incubation time.

† This class has been shown to be due to unlinked suppressors.

‡ Specific activity relative to a wild-type control extracted at the same time; no heat treatment. See METHODS for a more detailed description.

§ Ratio of the revertant reductive:oxidative activity divided by the wild-type ratio.

‖ Value determined from the percent revertant activity retained divided by that retained by a wild-type extract treated in an identical manner. All treatments at pH 8.0 were for 12 minutes; those at pH 6.2 were heated for 6 minutes.

crude mycelial extracts of revertants has been made. Since this enzyme catalyzes the oxidation of glutamate and the reverse reaction at appreciable rates, it was possible to compare revertants by the ratio of the forward to the reverse reaction. Wild-type specific activities for the reductive amination of alpha-ketoglutarate were about 3.5 times those for the oxidation of glutamate and were consistently in the range of 40 to 60 units. While many revertant GDH activities fell in this range, there were marked exceptions (Table 2). On this basis *am*5, 14, 17, 19 each yielded at least two classes of revertants with enzymes clearly distinguishable from wild type. A wide spectrum of types has emerged in which either the specific activity or the velocity ratio of the two reactions, or both, are altered. This is shown especially well in revertants of *am*14 and *am*19 (Table 2). Specific activities range from 100% to about 10% of the wild-type level in different revertants. There appears to be little correlation of total activity to the reduction: oxidation ratio. With the exception of a single revertant from *am*5 discussed below, all revertants possessed at least 10% of the wild-type reductive catalytic activity. This is in contrast to the mutants from which they were derived, which showed zero or negligible activity under the same assay conditions. Oxidative deamination relative to reduction was greater than wild type in a small number of revertants. In others it was much lower than the wild type, and in one class of revertants from *am*17 no oxidative deamination of glutamate was detected. Enzyme activation in the presence of the reaction mixture was also observed in extracts from certain revertants. This too was used as a criterion in classifying GDH of revertants. The fluctuations in GDH specific activity of different extracts from any revertant were similar to those encountered in the wild-type extracts and in general were in the same direction as the wild-type control. Replicate assays of a single extract consistently had a variation of less than ± 10%.

To differentiate revertants further into enzymatically distinct classes, heat stability tests were performed on the glutamate dehydrogenase activity in extracts of revertants and wild type. Figure 2 shows the retained activity of the wild-type

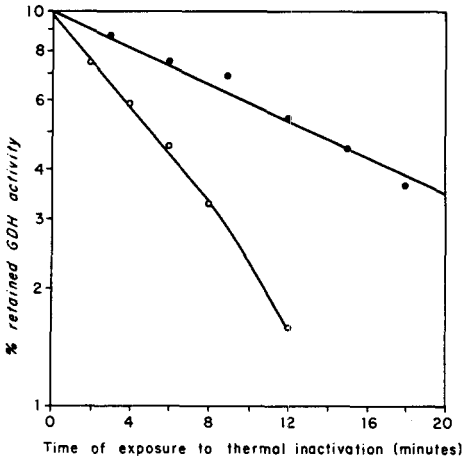

FIGURE 2.—The thermostability of wild type 74A glutamate dehydrogenase. (●) Treated at 65°, pH 8.0, 0.2 mg protein/ml. (○) Treated at 62°, pH 6.2, 0.1 mg protein/ml.

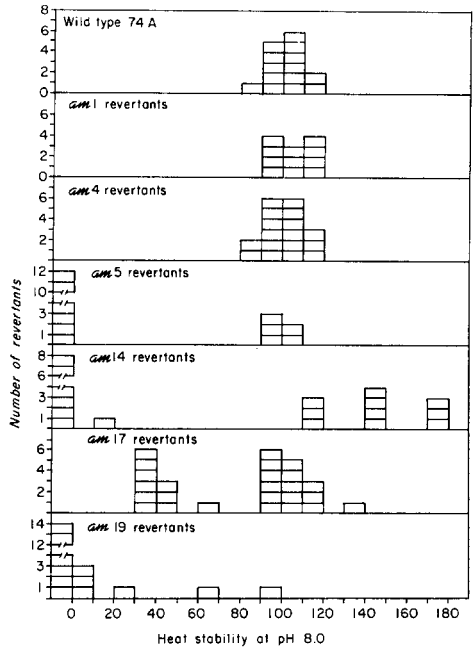

FIGURE 3.—Heat stability at pH 8.0 of glutamate dehydrogenase in extracts of *am* revertants. See text for a description of calculations.

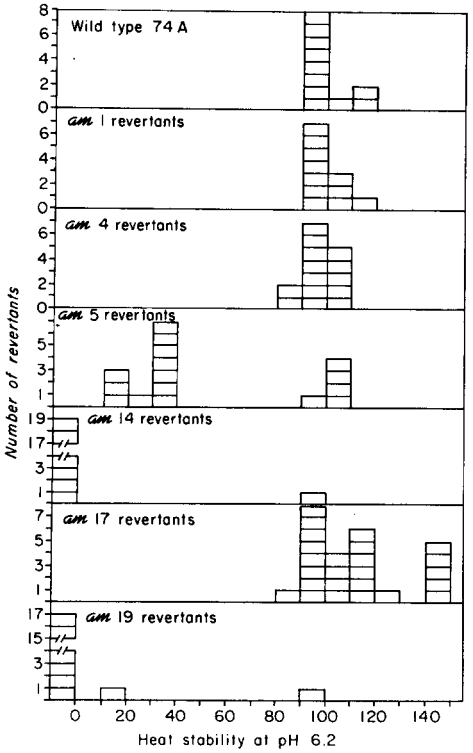

FIGURE 4.—Heat stability at pH 6.2 of glutamate dehydrogenase in extracts of *am* revertants. See text for a description of calculations. One revertant with heat stability greater than 150% of wild type is omitted.

enzyme after treatment under two standard conditions. Reasonably reproducible half-inactivation could be obtained if all conditions were rigidly controlled. The retained activity values of wild-type GDH, included in Figures 3 and 4 were obtained in experiments done over a period of several months. The heat stability of revertant GDH (Table 2) is expressed as a fraction of the retained revertant activity divided by the remaining activity of a wild-type control after simultaneous heat treatment. A value of 100% indicates that the revertant enzyme retained the same fraction of activity as the wild-type control.

Table 2 presents a summary of the qualitative characteristics of GDH from the 110 revertants investigated. The revertants may be divided into at least four main classes on the basis of their heat stabilities: equally stable, intermediate, labile, and more stable than wild type. There is generally a good correlation of classes defined on this basis with those defined on the basis of unique qualitative and quantitative catalytic activity. All mutants which reverted gave at least some revertants that were indistinguishable from the wild type. Class 1 includes the 50 revertants of this type. *am*1 and *am*4 revertants are entirely of this class as are the majority derived from *am*17. Mutants *am*14 and *am*19 each gave only a single revertant identical to wild type. The one originating from *am*19 was of spontaneous origin. The other 60 revertants are subdivided into at least 14 distinct groups.

Since a revertant could be produced by mutation at the site of the original lesion, at a second site in the *am* gene, or by a suppressor mutation, it was of interest to determine the locus of the reversion event. Genetically pure revertants were crossed to wild type 74A or 3.1a and random spores screened for the presence of am^- mutants arising by recombination. Ascospores were germinated on minimal medium supplemented with glycine. One thousand germinated spores were scanned from each cross. According to the formulation of HOROWITZ (1953) the failure to observe a recombinant by chance alone between two genes one map unit apart is reduced to the 1% probability level when this many spores are examined. All ascospores which germinated but failed to grow were isolated, cultured on complete medium, and subsequently tested for *am* growth characteristics. A majority of the revertants 96 of 110 whose GDH had been characterized were examined in this way. Failure to recover amination-deficient recombinants indicates that the reversion event occurred at or in close proximity to the *am* gene. Eighty-eight strains examined were of this type. No genetic evidence for the presence of second-site alterations within the *am* locus has been obtained because of the difficulty of recovering rare auxotrophic spores among large numbers of prototrophs. However, the production of at least six qualitatively distinct revertant-enzyme classes at the *am* locus from single alleles (*am*14 and *am*19) implies that second-site revertants are included.

Eight crosses of revertants by wild type (seven revertants of *am*17 and one from *am*5) did yield *am* mutant segregants. In each case the frequency of mutant ascospores was about 25%. Such a frequency indicates an unlinked suppressor and this was verified by ordered tetrad analysis (Table 3). Asci containing 6 am^+ : 2 am^- spores (6 normal amination to 2 amination-deficient spores) are unambiguous evidence for an external suppressor. A cross of revertant by wild

TABLE 3

Tetrad analysis of am *suppressed mutants × wild type*

Strain crossed × wild type	Ascus type		
	8*am*+ : 0*am*−	6*am*+ : 2*am*−	4*am*+ : 4*am*−
*am*17 (*su*+)	0	0	10
*am*17 RN56 (*su*+)	0	0	3
*am*17 RN6 (*su*−)	0	3	1
RN8 (*su*−)	2	7	4
RN22 (*su*−)	2	4	0
RN30 (*su*−)	4	7	3
RN33 (*su*−)	1	3	1
RN48 (*su*−)	0	2	0
RN68 (*su*−)	2	5	1
*am*5 (*su*+)	0	0	5
*am*5 RN35 (*su*−)	1	6	3

am+ alanine independent; *am*− alanine dependent. The numbers in this table refer to the number of asci scored of each type.

type is expected to give $8^+ : 0^-$ asci. An ascus containing $4^+ : 4^-$ ascospores obtained from such a cross may indicate assortment of a suppressor, but also could indicate the presence of mutant nuclei contaminating the revertant. However, an ascus of the $6^+ : 2^-$ type arising from this cross in appreciable frequencies can only be explained by the assortment of a suppressor from the allele upon which it acts.

Suppression and the qualitative characteristics of the revertant enzyme are clearly correlated in revertants of *am*17 (Table 2). Isolates (*am*17RN6, 8, 22, 30, 33, 48, 68) which are suppressed have a GDH activity clearly different from wild-type and from a second distinct type derived from *am*17. (This latter type has been shown genetically to occur at the *am* locus.) The suppressors lead to the production of about one-half wild-type specific activity for the reduction reaction but fail to restore detectable activity for the oxidative deamination reaction. Heat stabilities of the enzyme from suppressed strains are similar to each other but clearly different from wild type. On the basis of their enzymatic properties the suppressed *am*17 strains appear to be identical. However, the suppressor of *am*5 is clearly different. No measurable GDH activity has been found under the conditions of assay in spite of the fact that the suppressed strain has an enhanced growth rate compared to the original *am*5 mutant or *am*5 segregants derived from the suppressed strain.

It is important to demonstrate that the qualitative properties of the enzyme in a suppressed strain are controlled by the suppressor gene and are correlated with prototrophic growth. Conversely, when a reversion event has occurred at the *am* locus, the qualitative properties of GDH should segregate with the *am* locus in crosses. These expectations were tested in the following way. Suppressed and unsuppressed revertants of *am*17 were crossed to a strain that was wild-type at the *am* locus but carried the inositol mutant 89601. This mutant which is closely

linked to the *am* gene was used to mark the wild-type (am^+) allele genetically. Ordered asci from these crosses were dissected and tetrads analyzed for growth response and segregation of unique GDH activity. Table 4 shows that the qualities of the glutamate dehydrogenase segregate with the *am* locus for a revertant shown genetically to have occurred at the *am* locus (*am*17RN56). Additional tetrads from this cross confirm that no other loci determine or modify the unique properties of GDH from this revertant. Table 4 also shows that two of the suppressors modify the *am*17 mutant in such a way as to produce qualitatively identical enzyme activities in segregants. Enzyme activity of a type distinctly different from wild type is formed by the *am* mutant in the presence of the suppressor. Asci of the $6am^+$: $2am^-$ type provide evidence that the qualitative properties and catalytic activity restored by the suppressors are controlled by a single locus. The locus is clearly separable from the *am* gene. Prototrophic growth response is shown to be related to the production of a functional *am* gene product in these asci. The spore pair receiving the *am*17 allele without the suppressor showed the am^- phenotype and produced no detectable GDH activity.

TABLE 4

Segregation of the qualitative properties of GDH in ordered asci from suppressed and unsuppressed revertants of am17

Cross	Spore pair No.	Genes segregating			Enzyme specific activity		Class
		Color	*am*	*inos*	Reduction	Oxidation	
Unsuppressed revertant							
*am*17RN56 *a* × *al-2 inos A*							
	1–1	+	+	—	50	30	*am*+
	1–3	+	+	—	51	30	*am*+
	1–5	al	+	+	65	3.0†	R
	1–7	al	+	+	65	3.5†	R
Suppressed revertant							
*am*17RN22 *a* × *al-2 inos A*							
	1–1	al	+	+	30	0	*am*− *su*−
	1–3	+	+	+	30	0	*am*− *su*−
	1–5	al	+	—	60	15	*am*+ *su*+
	1–7	+	+	—	55	14	*am*+ *su*+
	2–1*	+	+	+	20	0	*am*− *su*−
	2–3	+	+	—	42	21	*am*+ *su*+
	2–5	al	—	—	0	0	*am*− *su*+
	2–7	al	+	+	42	22	*am*+ *su*−
*am*17RN8 *a* × *al-2 inos A*							
	1–1*	+	+	+	25	0	*am*− *su*−
	1–3	al	+	—	45	13	*am*+ *su*+
	1–5	al	+	+	20	0	*am*− *su*−
	1–7	+	+	—	46	12	*am*+ *su*+

Enzyme assays and specific activity calculations are described in the text. *al-2* is a gene on linkage group I causing albino conidia.

* Mycelial pads from these two asci were grown on minimal medium supplemented with alanine in addition to inositol which was included in all cultures.

† Enzyme activated in the reaction mixture.

R means revertant for the *am* locus; *su*− refers to the functionally detectable allele of the suppressor, i.e., the one which suppresses *am*−; *su*+ refers to the nonsuppressing, wild-type allele of the suppressor.

To confirm genetically the supposed presence or absence of the suppressor gene in particular spore pairs of one $8am^+$: $0am^-$ ascus analyzed in Table 4, a member of each spore pair was crossed to wild type. Random spores from these crosses were scanned for am^- segregants. Only the spore pairs expected to carry the suppressed *am* mutant on the basis of their enzymatic qualities were indeed found to yield mutant recombinants.

To test for site specificity and in a general way to gain more insight into the mechanism of suppression, the suppressors were crossed to other *am* alleles. Advantage was again taken of *inos* as a marker for a particular *am* allele. am^- $inos^-$ double mutants of *am*14, *am*4 and *am*15 (described in Table 1) were crossed to $am17^-su^-$ strains and asci were dissected in order. A $6am^-$: $2am^+$ ascus is unambigous evidence for the failure of the suppressor to act on the allele being tested. (In this paper suppressor minus (su^-) refers to the functionally detectable suppressor; su^+ refers to the non-suppressing wild-type allele.) It was possible to show that most prototrophic, i.e. suppressed, (am^+) ascospores carried the $inos^+$ allele and therefore almost certainly carried the *am*17 mutant allele. At least six asci were scored from each cross and in every case some of the $6am^-$: $2am^+$ type were found, indicating that the suppressors were site specific and had no general effect on the *am* locus or on an alternate metabolic pathway. Because of the technical difficulties in working with the suppressor of *am*5, it has not been further characterized. Analysis of prototroph to auxotroph frequency in random ascospores from crosses of $am5^-su^-$ by $am17^-$ and $am17^-su^-$ by $am5^-$ do indicate that both suppressors are site specific and fail to act on the heteroallele.

The CRM-less noncomplementing properties of the two *am* alleles reverting by suppressor mutations suggested an analogy to the super-suppressor system of yeast (MANNEY 1965) and to the nonsense mutants in *E. coli* alkaline phosphatase (GAREN and SIDDIQI 1962; WEIGERT and GAREN 1965) as do various features of the suppression itself. For this reason it was of great interest to test for the effects of the suppressors on mutants at other loci. Mutants known to be CRM^- and noncomplementing were obviously of particular interest. When each of the suppressors of *am*17 was crossed to a CRM^-, noncomplementing *tryp-3* mutant, *td*140, clear genetic evidence was found for suppression of *td*. TERRY (1966) showed that this mutant could revert by site-specific suppressors and proposed that it might be of the nonsense type. Random ascospores from a cross of $am17^-su^-$ by *td*140 are expected to yield a frequency of 50% growers and 50% nongrowers (td^+: td^-) when plated on alanine supplemented medium if the suppressor has no effect on the tryptophan mutant. If the *td* mutant is suppressed by an unlinked gene, the ratio is expected to be 75% td^+: 25% td^-. The latter result was observed with each *am*17 suppressor; a class of prototrophs growing slower than wild type was recognizable in each case. Because repeated attempts to obtain complete ordered asci failed, several $tryp^+$ ascospores from each of the two classes were grown up and crossed back to wild type to demonstrate the presence of the suppressed *td*140 allele. The results of random-spore analysis shown in Table 5 confirmed the suppression of this *tryp-3* mutant. A few representative tetrads from these crosses were examined for the segregation of the tryptophan requirement.

TABLE 5

Genetic evidence from random spores for suppression of td*140 by suppressors of* am*17*

Isolate* crossed to wild type 74A	Plated on alanine			Plated on glycine tryptophan		
	tryp+	tryp−	%tryp−	am+	am−	%am−
Putative wild-type						
1	156	0	0%	160	0	0%
2	189	0	0%	200	0	0%
3	232	0	0%	183	0	0%
Putative suppressed *td*140						
*td*140 *su*-17RN68 (1) *a*	300	112	27%	221	0	0%
*td*140 *su*-17RN68 (2) *a*	160	46	22%	153	60	28%
*td*140 *su*-17RN68 (3) *a*	244	88	26%	218	0	0%

* Tryptophan independent isolates arising from a cross of *td*140a × *am*17RN68 *A* (*su*-) were judged to be of two types on the basis of different growth rate as described in the text.

Three types of asci were found: 8 *tryp*+: 0 *tryp*−, 4 *tryp*+: 4 *tryp*− and 6 *tryp*+: 2 *tryp*−. The last class demonstrates rather clearly that *td*140 is suppressible by the *am* suppressor. (The interpretation of these three types of asci has been discussed previously.) The suppressor of *am*5 has no demonstrable effect on *td*140.

Strong evidence for the generalized suppressing ability of these suppressors is provided by *am*17RN22. It also suppresses a noncomplementing polarity mutant, M54, which lacks essentially five enzyme activities and maps in the *arom-2* region of the *arom* cluster (CASE, GILES, and PARTRIDGE 1967). In addition, suppressors have been crossed to 35 mutants at 15 loci on all seven linkage groups and scored for ability to suppress the test allele. Two other suppressible alleles have been found—a new *am* mutant (isolated by me) and an allele of *tryp-1* (supplied by T. I. BAKER). No suppressible alleles were discovered among 6 other noncomplementing *am* mutants, 7 noncomplementing *his-1* mutants (supplied by D. CATCHESIDE) or 5 other *tryp-1* mutants.

DISCUSSION

The seven *am* mutants examined in this study were chosen from the 17 available alleles because of the different types of altered glutamate dehydrogenase they produced. It was of interest to compare the frequency of nitrous acid induced reversion and types of revertants obtained from qualitatively different mutants in the same gene and to see what correlations could be made between reversion and the type of lesion involved. Complementing, noncomplementing, CRM and non-CRM formers of both ultraviolet and nitrous acid origin were examined. The complementing mutants, *am*1, *am*14, *am*19, produce distinctly different forms of altered GDH. FINCHAM and CODDINGTON (1963) have proposed that the enzyme produced by *am*1 has an amino acid substitution at the active site. This conclusion is based on the properties of the hybrid complementing enzyme and the isolated mutant enzyme. While catalytically inactive, it still possesses stable folding properties. They have suggested that other mutants complementing *am*1

do not have alterations involved directly in the active site but that substitutions elsewhere lead to misfolding and failure to generate the active site. Lesions of the latter type might be corrected by *in vitro* treatments leading to alteration of conformation and correct juxtaposition of residues involved in catalysis. Such findings have been reported for *am*19 by SUNDARAM and FINCHAM (1964). Some second-site amino acid substitutions in such a mutant also might be expected to restore partial enzymatic activity by establishing a more nearly native tertiary structure. From the 40 revertants of *am*14 and *am*19 induced by nitrous acid a number of distinct enzyme classes were observed and only one of the 20 revertants for each allele was found to be indistinguishable from wild-type. Secondary alterations are clearly favored. The different classes observed are a minimum estimate of the number of possible revertant alterations but if they represent unique genetic sites, the frequency of mutagenic events at different sites is roughly equal. The clear exception to this is the back-mutation event which is induced by nitrous acid rarely if at all for these alleles. Genetic tests established that all reversions of *am*14 and *am*19 which were examined occurred at the *am* locus. Not all of the distinct classes of revertants from either allele can be accounted for by changes within the same codon if nitrous acid causes only transitions by oxidative deamination of adenine and cytosine. Such action is expected to produce a class identical to wild type (back-mutation) and a maximum of two other classes which may or may not be qualitatively distinguishable from this type. While no genetic evidence is yet available to support this internal suppressor hypothesis, it is clearly implied by the enzyme data. In contrast *am*1 gave only a single class of revertants identical to wild type. This is consistent with its proposed alteration.

The noncomplementing mutants as a class are expected to be heterogenous in nature since a number of different kinds of lesions might lead to the failure to form partially active enzyme hybrids. In addition to drastic conformational alterations or failure of subunits to associate because of single amino acid substitutions, gross structural change in the protein resulting from extended deletions, a shift in reading frame (CRICK *et al.* 1961) or chain terminating codons (BENZER and CHAMPE 1962; SARABHAI *et al.* 1964) would cause noncomplementing mutants. ROBERTS and PATEMAN (1964) have performed CRM determinations on a number of *am* alleles including the noncomplementers used in this study—*am*4, 5, 15, 17. Only *am*4 was found to produce an antigenically related protein. This mutant yields revertants with a single class of glutamate dehydrogenase which is indistinguishable from wild type. The other three noncomplementers are non-CRM formers. Failure to recover revertants of *am*15 after treatment with UV (STADLER 1966), nitrous acid, or spontaneously, is consistent with there having been a reading-frame shift or deletion. TESSMAN (1962) has presented evidence for the production by nitrous acid of large deletions covering nearly the entire *rII* region of phage T4. In Neurospora, MALLING (1965) has shown that about 10% of the nitrous acid-induced *ad-3* mutants revert only spontaneously while another 6% do not revert at all. *am*5 and *am*17 are especially interesting because both of these mutants revert by changes at unlinked loci in addition to events at the *am* locus, and both are CRM-less, noncomplementing mutants. These characteristics

suggest that *am*5 and *am*17 are nonsense mutants; that is, their mutant codons no longer code for amino acids but instead are recognized as chain termination codons. In addition, the following observations support this hypothesis:

(1) No general suppressors for the *am* locus have been picked up in extensive reversion analyses by this author, by STADLER (1966) or by PATEMAN and FINCHAM (1965). (2) The suppressors of *am*5 and *am*17 have no detectable effect on a number of independently isolated *am* alleles of both CRM^+ and CRM^- types. In particular the suppressors fail to act on a nonreverting mutant believed to be a deletion (*am*15), a complementing mutant (*am*14) and a CRM^+, non-complementer (*am*4). This clearly demonstrates the site specificity of the suppressors. (3) The two nonsense codons proposed by BRENNER *et al.* (1965), UAA and UAG, could lead to only two amino acid substitutions by single transitions expected to be induced by nitrous acid. However, the previously unassigned codon UGA appears also to be nonsense (BRENNER *et al.* 1967) and ochre (UAA) mutants should therefore yield only one revertant class by transition. Thus, there can be a maximum of two classes of revertants arising from mutational events in the amber (UAG) nonsense codon because alterations elsewhere in the locus are expected to be without effect in restoring GDH activity. One class should arise by back-mutation and be indistinguishable from wild type. The other event might or might not result in a distinguishable or even functional protein. Only two qualitatively distinct classes of revertants arising from nitrous acid-induced events at the *am* locus have been observed for both *am*5 and *am*17. One class of revertant GDH is identical to wild-type and the other clearly different. *am*5 and *am*17 probably are both amber mutants since they yield two revertant classes by base transitions. The potential to demonstrate subtle differences in heat stability and catalytic activity has been shown earlier in this paper. (4) Preliminary evidence indicates a suppressor of *am*17 to be dominant in heterocaryons. This is consistent with the proposed mechanism of action of nonsense suppressors. (5) The suppressors of *am*17 while site specific also suppress mutants in genes physiologically unrelated to *am*. A CRM^- *tryp-3* mutant, *td*140, reported to be suppressible by TERRY (1966), a polar mutant in the *arom 2* gene, M 54, which lacks essentially five enzymes produced by the genes in the *arom* cluster (CASE, GILES, and PARTRIDGE 1967), a *tryp-1* mutant and an independently isolated *am* mutant are suppressed by one or more of the suppressors of *am*17.

Characterization of a number of newly isolated spontaneous and UV-induced suppressors is in progress. In addition the effect of other mutagens on the *am*17 allele is being analyzed.

Table 1 shows a rather good correlation between the relative frequencies of reversion for different *am* alleles produced by UV and nitrous acid, two non-specific mutagens. The approximate tenfold difference in UV:NA frequencies has also been reported by KAKAR *et al.* (1964) in reversion experiments with the isoleucine-valine mutants of *S. cerevisiae*. Maximum reversion frequencies induced by nitrous acid show a surprisingly good correspondence to the number of distinct revertant classes obtained from a particular allele. With the exception of the true back-mutants, the individual classes based on qualitative properties of GDH

appear to occur in about equal proportions in revertants of *am*14 and *am*19. This is clearly not the case for revertants of *am*5 and *am*17 occurring at the *am* locus. Although differences in frequencies of this type are usually attributed to neighboring base effects, a completely satisfactory explanation is unavailable.

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SUMMARY

Six allelic mutants lacking NADP-linked glutamate dehydrogenase (GDH) were induced to revert with nitrous acid. Analysis of the catalytic and heat stability properties of revertant GDH showed that 60 of the 110 revertants were clearly distinguishable from wild type. Two CRM-negative, noncomplementing alleles, *am*17 and *am*5, were shown to revert by site specific suppressors. (CRM: "crossreacting material", an antigen that reacts with neutralizing antiserum to glutamate dehydrogenase) The suppressors of *am*17 possess properties similar to the nonsense suppressors in *E. coli* including the ability to suppress specific CRM$^-$, noncomplementing mutants in three other genes. Extracts from the suppressed *am*17 strains contained a GDH activity qualitatively different from wild-type or from a second type of revertant of the same allele occurring at the *am* locus. Ultraviolet and nitrous acid reversion of five different alleles was compared.

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