A STRUCTURAL GENE FOR THE DPN-SPECIFIC GLUTAMATE DEHYDROGENASE IN NEUROSPORA

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A PART from the TPN (triphosphopyridine nucleotide)-specific glutamate dehydrogenase (T-GDH) first described by FINCHAM (1950, 1951), *Neurospora crassa* possesses a DPN (diphosphopyridine nucleotide)-specific glutamate dehydrogenase (D-GDH) which is present (SANWAL and LATA 1961a) both in wild-type strains (SANWAL and LATA 1961b) and in mutants lacking T-GDH (*am-1* mutants). The presence of D-GDH provides an explanation for the commonly observed leakiness of all *am-1* mutants.

It has been demonstrated (SANWAL and LATA 1962a,b) that during growth on minimal medium supplemented with glutamate (or other nitrogenous compounds which are easily converted *in vivo* to glutamate) active T-GDH of wild-type cells or an antigenically cross-reacting material corresponding to T-GDH of *am-1* mutants (SANWAL and LATA 1962c) is repressed and D-GDH is derepressed. Indeed, all environmental conditions which lead to a repression of T-GDH invariably also lead to a derepression of D-GDH. This coupled regulation of two enzymes has been labelled "concurrent regulation" to distinguish it from other types of regulation, such as coordinate (AMES and GARRY 1959) and multivalent (FREUNDLICH, BURNS and UMBARGER 1962) regulation. The concurrent regulation of T-GDH and D-GDH of *Neurospora* is strikingly similar to the so-called 'compensatory' regulation of γ - and β -chains of hemoglobin during fetal development. Thus, it has been demonstrated that as hemoglobin-F levels fall in the growing embryo the hemoglobin-A levels increase correspondingly till, soon after birth, it becomes the only major component of the normal blood (CHERNOFF and SINGER 1952; ZUCKERKANDL 1964). Based on the operon theory of JACOB and MONOD (1961), a genetic model for the regulation of the hemoglobin varieties has been recently proposed (ZUCKERKANDL 1964), which with certain modifications may equally well be applicable to the regulation of glutamate dehydrogenases of *Neurospora*.

As a first step in our efforts to understand the genetic basis of the concurrent regulation of D-GDH and T-GDH, we present in the following report the isolation and characterization of mutants producing catalytically defective varieties of D-GDH.

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MATERIALS AND METHODS

Strains: The strains of *Neurospora crassa* used were *am-1*¹⁶ (donated by Dr. D. R. Stadler, Washington State University), *am*⁴, and the standard wild-type strain STA4. Other strains obtained by mutation and crossing are described later.

Media: The minimal medium of VOGEL (1956) was used throughout. For the growth of *am-1* and mutants producing an altered D-GDH (*d* mutants), the minimal medium was supplemented with 1 mg L-alanine/ml. Crosses were made on Bacto cornmeal agar or the crossing medium of WESTERGAARD and MITCHELL (1947) supplemented with 1 mg alanine per ml. For the induction of D-GDH VOGEL's medium was supplemented with 0.05 M L-alanine. All of the media contained 2% sucrose as the carbon source. Colonial growth was induced by the inclusion of 1% sorbose in the minimal media solidified with 1.5% agar.

Isolation and characterization of mutants: Conidia (suspended in distilled water) of a 5 day old culture of *am*¹⁶ were subjected to ultraviolet (UV) irradiation from a 15 w germicidal lamp to a survival of 10^{-3} to 10^{-4} . The irradiated suspension was added to 100 ml of minimal medium contained in a 500 ml capacity Erlenmeyer flask. The flask was shaken vigorously on a rotary shaker at 28°C for approximately 72 hours. Mutant enrichment (WOODWARD, DEZEEUW and SIBB 1954) was achieved by filtering the suspension through four layers of cheesecloth periodically (at 10, 18, 24, 48, 58 and 70 hours after treatment). The remaining conidia were concentrated by centrifugation and plated on sorbose-agar medium supplemented with L-alanine. Colonies appearing after 3 days were tested on minimal medium to identify the *am-1*; *am-2* mutants. Only those mutants were studied further which did not grow on minimal medium for 5 days.

Complementation: All tests were carried out on solidified minimal medium. Conidial suspensions of the *am-1*; *am-2* mutants were prepared in distilled water. Suspensions of two mutants at a time were separately mixed and about 0.2 ml of the mixture was dropped in a well (7 mm diameter) in the solid medium. Growth observations were made within 72 hours.

Enzyme assays: Both D-GDH and T-GDH were assayed in extracts by following the disappearance of the appropriate reduced coenzymes in the reductive amination assay described earlier (SANWAL and LATA 1961b). One unit of either D-GDH or T-GDH is defined as the amount of enzymes which causes a decrease of 0.001 in optical density at 340 m μ .

Thermal denaturation of D-GDH: Crude cell-free extracts were prepared by alumina grinding and centrifugation as described by SANWAL and LATA (1961b). An approximate 40-fold purified D-GDH was prepared by ammonium sulfate precipitation and alumina C γ adsorption as outlined earlier (SANWAL and LATA 1961b). Thermal inactivation kinetics of the partially purified D-GDH was studied at 50.8°C. Tubes containing the enzyme were rapidly brought to 50.8°C and aliquots were removed at 5-minute intervals thereafter and chilled immediately to 0°C. The chilled samples after appropriate dilution in buffer were assayed later. The first-order rate-constant of inactivation k was calculated as an average value from the equation $k = 1/t (\ln (x_0/x_1))$, where x_0 = activity at zero time and x_1 = activity remaining at time, t . Representative first-order plots of the heat inactivation data are given in Figure 1.

RESULTS

*Induction of *am*¹⁶, *d* mutants:* Out of over 900 colonies isolated after UV irradiation and filtration enrichment, only 14 were unable to grow on minimal medium for a period of 3 days. Some of these mutants, however, produced thereafter a very sparse mycelial growth much like that produced by the wild-type under starvation conditions on solidified agar without added nutrients. Out of these 14 presumed D-GDH less mutants, three (794, 868 and 912) turned out to be relatively non-leaky and failed to grow on minimal medium for a period of 6 days at 28°C. The growth characteristics and the amount and nature of the D-GDH

FIGURE 1.—Thermal denaturation of D-GDH from strains 16a and 794. Experimental conditions are described in the text. The slopes of these plots yield the value of k .

produced by these mutants were studied. Results of this study are given in Table 1.

It will be noted that all three *am-1*¹⁶; *am-2* mutants produced a defective D-GDH which is heat labile. It is likely that under uninduced conditions the amount of D-GDH produced is so small that growth is not possible on minimal medium.

Cross of D-GDH-less mutants with am⁴: Strain 868 (*am-1*¹⁶; *am-2*) was crossed with an amination-deficient mutant (*am*⁴+) and 97 random ascospores were isolated on alanine medium and tested for growth on minimal medium. Those isolates which did not show any growth for 5 days were scored as *am-1*; *am-2* mutants. Out of 97 ascospores, 53 failed to grow on minimal plates. This shows that strain 868 has a gene defect also for D-GDH.

Linkage relationship of mutant 868: One of the major difficulties in the analysis of a cross of 868 (*am-1*; *am-2*) with wild type (+ +) is the scoring for the recombinants in growth tests. Thus, the recombinant class (+ *am-2* and *am-1* +)

TABLE 1
Some characteristics of the am¹⁶d mutants

Strain	Genotype	Specific activity		$k(\text{min}^{-1})$ 50.8°C	Growth (minimal medium) 2 to 5 days	Growth (alanine medium) 2 days
		D-GDH*	T-GDH			
<i>am-1</i> ¹⁶	<i>am-1</i> +	500	0	.012	+++	+++
794	<i>am-1</i> ; <i>am-2</i>	17	0	.040	—	++
868	<i>am-1</i> ; <i>am-2</i>	120	0	.027	—	++
912	<i>am-1</i> ; <i>am-2</i>	250	0	.027	—	++

* Measured under induced conditions (SANWAL and LATA 1962b).
+++ , copious growth; ++ good growth; — no growth.
Further explanation in the text.

would both be expected to grow on the minimal medium after a lag period. Fortunately, however, in the analysis of an ordered tetrad from a cross of 868 with wild type STA4, a new phenotype ('patchy') was represented amongst the ascospores which on the basis of enzyme analysis and thermal denaturation turned out to have the genotype (+ *am*-2), i.e., it had normal levels of T-GDH but possessed a D-GDH which was heat labile like the parental strain 868 (Table 1). It grew like the wild type on minimal medium (without any lag), but after 3 to 5 days typical water-soaked areas appeared in the middle of the plate and spread outwards. Like the wild type, its growth was not inhibited by 0.02 M glycine or any of the 20 common amino acids (tested at a concentration of 0.02 M each). With the finding of the 'patchy' phenotype, the following method was adopted to score the different genotypes of the cross (+ +) × (*am*-1; *am*-2): Discharged ascospores were picked up by a sterile swab and suspended in distilled water. They were then heat shocked at 60°C for 30 minutes and plated on sorbose medium supplemented with alanine. Each colony was tested on minimal medium and minimal medium containing 0.02 M glycine. Growth was scored after 2 and 5 days. After 2 days on minimal medium colonies of the following genotypes grew: (+ +), (+ *am*-2) and (*am*-1 +). Colonies not growing till 5 days on minimal are (*am*-1; *am*-2), and colonies showing 'patchy' phenotype after this time are (+ *am*-2); the numbers of recombinants in these two classes are thus easily scored. Colonies not growing on glycine supplemented minimal medium for 5 days are of the genotype (*am*-1 +) and (*am*-1; *am*-2). The number of the (*am* d) class being known, (*am* +) recombinants can be scored. Similarly, colonies which grow on glycine supplemented medium have the genotype (+ +) or (+ *am*-2). The number of the (+ *am*-2) class being known ('patchy' phenotype in minimal medium), the number having the genotype (+ +) can be calculated. That the method of scoring recombinants, discussed above, was correct was verified by testing at random colonies (five from each group) which had been assigned a particular genotype, for the presence of T-GDH and heat labile (50.8°C) D-GDH. In every case strict correspondence between the assigned genotype and enzymic make-up was obtained. It may be mentioned that colonies not growing on glycine were all non-patchy and that the 'patchy' cultures all grew on glycine. In Table 2 are given the results of an analysis of a cross between 868 (*am*-1; *am*-2) and STA4 (+ +). Since there is a 1:1:1:1 ratio of segregation among the

TABLE 2

Growth characteristics of random ascospores from the cross: 868 (am d) × STA4 (+ +)

No. of ascospores not growing on Medium N (<i>am</i> -1; <i>am</i> -2)	No. of ascospores not growing on Medium N + 0.02 M glycine (<i>am</i> -1 +)	No. of ascospores growing on Medium N + 0.02 M glycine (+ +)	No. of ascospores giving rise to 'patchy' growth (+ <i>am</i> -2)	Total number of ascospores isolated and tested
31	32	39	32	134

Equal numbers of the four classes are expected in the absence of linkage.

progeny of the cross, the conclusion seems inevitable that there is either no linkage between *am-1* and *am-2* or that they are very far apart on the same chromosome. This conclusion is also supported by the phenotypic and biochemical analysis of ascospores from two asci of this cross. In both asci, 1:1:1:1 segregation was obtained for the parental and recombinant classes.

Complementation tests: Nine *am-1*¹⁶; *am-2* mutants (667, 794, 798, 836, 868, 893, 912, 921 and 932) were tested in pairwise combinations for complementation on minimal medium. No complementation was observed.

Michaelis constants of wild-type and mutationally altered D-GDH: Partially purified D-GDH (after induction) from strain 868 and *am*¹⁶ did not show substantial differences in the *K_m* values for glutamate and DPN. Thus, *K_m* values for glutamate for D-GDH from 868 and *am*¹⁶ were 0.60 μ M and 1.48 μ M, and for DPN, 0.36 μ M and 0.15 μ M, respectively.

DISCUSSION

The main aim of this work was to find whether the structure of D-GDH was determined by a genetic locus distinct from *am* (linkage group V), which has been shown by other workers (FINCHAM 1950, 1951) to be the structural gene for T-GDH. This aim has been accomplished. The two enzymes are structurally controlled by the loci *am-1* (T-GDH) and *am-2* (D-GDH), which are not linked. It is surprising that none of the *am-2* mutants isolated here showed a complete absence of D-GDH; all the mutants studied in any detail produced a catalytically active enzyme which differed from the wild-type D-GDH in being heat-labile. This heat lability perhaps explains the lower levels of D-GDH in all *am-2* mutants under induced conditions, and also the absence of growth of (*am-1*¹⁶; *am-2*) mutants in the minimal medium. Conceivably, the altered enzymes, because of their lability, are produced in such low concentrations under uninduced conditions that amination of α -ketoglutarate cannot occur.

It is interesting to note that while strains of the genotype (*am-1*¹⁶; *am-2*) do not grow on ammonia, and (*am-1* +) strains are inhibited by 0.02 M glycine (PATEMAN 1957), the strains (+ *am-2*) behave exactly like the wild type (++) for the first 3 or 4 days of growth. Only after this time (+ *am-2*) strains develop a 'patchy' phenotype. Whatever, then, the function of D-GDH is, it is likely that this finds an expression in the late growth phase of the organism and may even be involved in a transition from the mycelial to conidial differentiation.

In our earlier deliberations of the possible mechanism of concurrent regulation of D-GDH and T-GDH (SANWAL and LATA 1962b) out of a number of possibilities one of the points considered was that in the presence of the inducer, the T-GDH may be converted to D-GDH at the cytoplasmic level. With the demonstration here of a gene locus for D-GDH this possibility can be discarded and a hypothesis of concurrent regulation at the primary template level must be sought. As a pure speculation it can be suggested that the regulation of the two GDH of *Neurospora* is due to the presence of regulatory genes (JACOB and MONOD 1961) which act in a 'cascade' manner (PONTECORVO 1963; ZUCKERKANDL 1964).

Theoretically, concurrent regulation could be achieved exactly in the same manner as postulated for the regulation of hemoglobin synthesis (ZUCKERKANDL 1964).

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SUMMARY

A few mutants of *Neurospora* have been obtained which differ from the wild-type strain in producing a DPN-specific glutamate dehydrogenase which is heat labile. The locus determining this enzyme is not linked with the gene *am*, which is responsible for the synthesis of a TPN-specific glutamate dehydrogenase.

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