A Modified Glutamic Acid Dehydrogenase as a Result of Gene Mutation in *Neurospora crassa*

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Previous papers (Fincham, 1950, 1954) have been concerned with mutant strains of *Neurospora crassa* which appear completely to lack glutamic acid dehydrogenase and which require one of a number of alternative L- α -amino acids for normal growth. More recently, Pateman (1957) has studied ultraviolet-light-induced reverse mutations in these strains. Most of the apparent back-mutants proved to have regained the capacity to synthesize glutamic dehydrogenase in normal amounts, but several strains, although growing more or less like the wild type, gave exceptionally low glutamic dehydrogenase activities in crude extracts. One of these latter strains is the subject of this paper. It produces an abnormal glutamic dehydrogenase distinguished from the normal *Neurospora* enzyme by its capacity for reversible activation; activation can be brought about by mild heat treatment or, to some extent, by incubation with substrates.

The mutation resulting in the production of the abnormal enzyme has been shown to be at the same locus as the original mutation (associated with apparent absence of glutamic dehydrogenase) from which it was secondarily derived (Fincham & Pateman, 1957).

MATERIALS AND METHODS

Neurospora strains. In this paper the term 'mutant' will be used to refer to strains carrying the gene for abnormal glutamic dehydrogenase. Several different mutant and wild-type strains were isolated in the course of the analysis of a series of crosses stemming from the standard wild-type strain St Lawrence A (St.L.A) and the original mutant, isolated by Pateman, no. 689. Strains used for enzyme preparations are listed in Table 1. The detailed derivation of the strains is given elsewhere (Fincham & Pateman, 1957).

Enzyme preparations. Cultures were grown from heavy conidial inocula for 48 hr. at 25° without agitation in 150 ml. lots of N-free buffered medium (Fincham, 1954) supplemented with 0.01 M ammonium tartrate. The mycelium was collected by filtration, thoroughly washed with glass-distilled water, blotted to remove excess of moisture, roughly weighed, and ground in an ice-chilled mortar with powdered glass and three times its wet weight of 0.05 M phosphate ($\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$) buffer, pH 8.0. After grinding, the volume of buffer was increased to 12 times the original wet weight of mycelium and the blend was filtered by suction through a kieselguhr pad (Fincham, 1954). The almost clear opalescent filtrate was either dialysed with

stirring against three changes of the same buffer as used in the extraction (to give 'crude extract', Table 1), or was further fractionated as follows. The filtrate was placed in an ice bath and solid $(\text{NH}_4)_2\text{SO}_4$ was added with stirring over 15 min. to give 50% saturation. The precipitate was collected by centrifuging, taken up in a volume of 0.05 M phosphate, pH 8.0, equal to about ten times the original wet weight of mycelium, and dialysed against three changes of the same buffer at 0–5°, the last change being left in the refrigerator overnight. After dialysis the preparation was centrifuged and any precipitate was discarded. Preparations were usually stored frozen at –15°; under these conditions wild-type preparations were generally fairly stable for many weeks, but most of the mutant preparations lost activity at the rate of about 50% per week. After freezing and thawing, some further precipitate often appeared which could be removed without detriment to the activity of the preparation. Dialysed fractionated preparations contained only traces of ammonia (of the order of 10^{-4} M), and were usually purified about twofold (with respect to protein) as compared with the crude extracts (Table 1). Unlike crude dialysed extracts, they did not reduce triphosphopyridine nucleotide (TPN) appreciably in the absence of glutamate. Some further purification could be achieved by refractionating between 40 and 50% saturation with $(\text{NH}_4)_2\text{SO}_4$, but the resulting preparation lost activity rapidly. A 50–80% saturation fraction had very little activity.

The protein content of enzyme preparations was determined by the biuret procedure previously described (Fincham, 1954).

Measurement of enzyme activity. A Unicam SP. 500 spectrophotometer was used to measure oxidation of reduced triphosphopyridine nucleotide (TPNH) or reduction of TPN at 340 m μ . The usual assay system consisted of a suitable dilution of enzyme in 0.08 M phosphate ($\text{Na}_2\text{HPO}_4\text{-KH}_2\text{PO}_4$) buffer, pH 8.0, 6.0 or 6.7×10^{-3} M sodium α -oxoglutarate, 3.3×10^{-3} M ammonium salt (usually $(\text{NH}_4)_2\text{SO}_4$, though NH_4Cl gave the same activity) and $5.0\text{--}6.5 \times 10^{-5}$ M TPNH, giving an E_{340} of 0.32–0.40. These components were mixed in a 1 cm. silica spectrophotometer cell, the final volume being 3 ml. The reaction was started by the addition of either TPNH or ammonium salt, followed by immediate mixing by inversion of the cell. Density readings were taken 30 sec. after the start of the reaction, and at 15 sec. intervals thereafter. With the concentrations of TPNH usually employed the reaction catalysed by the wild-type enzyme was approximately constant in rate during the first change in density of 0.1, so that rates of up to about 0.1/min. could be measured with an error of only a few per cent; initial rate was proportional to enzyme concentration. Mutant-enzyme preparations gave accelerating reaction rates under certain conditions (see the Results section). Assays were performed at room temperature, which was nearly constant

Table 1. *Summary of enzyme preparations*

In all assays the reaction was started by the addition of TPNH; activities of mutant preparations were calculated from maximum rather than initial rates.

No.	Strain	Type of preparation	Concn. in assay system (mm)		Heat treatment	Specific activity	
			α -Oxo-glutarate	NH_4^+		Untreated	Heated
1	St.L.A (wild type)	Fractionated	6.0	2.5	—	1350	—
2	1361-12 (mutant)	Fractionated	6.0	2.5	—	100	—
3	1432-12 (wild type)	Fractionated	8.0	2.5	8 min., 50°	1700	1700
4	1432-34 (mutant)	Fractionated	8.0	2.5	8 min., 50°	76	370
5	St.L.A	Crude extract	6.7	3.3	10 min., 50°	1000	1000
	St.L.A	Fractionated	6.7	3.3	10 min., 50°	2100	2100
6	1432-78 (mutant)	Crude extract	6.7	3.3	10 min., 50°	60	230*
	1432-78 (mutant)	Fractionated	6.7	3.3	10 min., 50°	110	225*
7	1497-34 (mutant)	Crude extract	6.0	3.3	6 min., 50°	20	170
	1497-34 (mutant)	Fractionated	6.0	3.3	6 min., 50°	38	250
8	S5a (wild type)	Fractionated	6.7	3.3	5 min., 50°	1200	1200
9	1435-12 (mutant)	Fractionated	6.7	3.3	5 min., 50°	40	250
10	1435-34 (mutant)	Fractionated	6.7	3.3	6½ min., 38.5°	170	1200

* These values are probably low because of over-prolonged heat treatment.

during any one experiment and which varied between 18° and 21° from one experiment to another. All components of the system except the TPNH were kept in a water bath at about 1° above room temperature before mixing; the TPNH solution, 0.2 ml. of which was added in a final volume of 3 ml., was kept in an ice bath. Enzyme activities are expressed as $10^3 \Delta E_{340}/\text{min.}$ Specific activities are expressed as activity/mg. of protein.

Chemicals. α -Oxoglutaric acid was used as supplied by L. Light and Co. Ltd., Colnbrook, Bucks. TPN (different batches of 81–98% purity, sodium salt) was the product of the Sigma Chemical Co. (U.S.A.) and was obtained through George T. Gurr Ltd. (136, New Kings Road, London, S.W. 6). TPNH was prepared in solution by reducing TPN with a dialysed extract of an acetone-dried powder of pig heart and a slight excess of DL-isocitrate (based on Evans & Nason, 1953) and was stored frozen at -15° .

RESULTS

Properties of the wild-type enzyme

The activity of the wild-type enzyme in 0.08 M orthophosphate ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$) buffer increased with increasing pH up to 8.0. The pH optimum, determined in 0.032 M aminotrihydroxymethylmethane (tris)-acetic acid buffer, was close to 8.5 (Fig. 1). In sodium pyrophosphate-HCl buffer the pH optimum, though less accurately determined, was again about 8.5. The activities of the enzyme in the tris-acetic acid and pyrophosphate-HCl buffers at pH 8.0 were respectively about 60 and 70% of the activity in orthophosphate at the same pH value, and the highest activity obtained was with orthophosphate at pH 8.0; accordingly, this buffer was used in all subsequent experiments both with wild-type and with mutant enzyme (the pH optimum of the mutant enzyme was similar to that of the wild-type enzyme,

Fig. 1. Enzyme activity and pH. Reaction mixtures with 6.7×10^{-3} M α -oxoglutarate, 3.3×10^{-3} N- $(\text{NH}_4)_2\text{SO}_4$, 0.032 M tris-acetic acid buffer, 57 $\mu\text{g.}$ of protein from wild type (preparation 8, Table 1) (●) or 400 $\mu\text{g.}$ of protein from the mutant (preparation 9) (○) in 3 ml., and approx. 5.5×10^{-5} M TPNH. Reactions were started by adding the ammonium salt in 0.2 ml. after 4 min. incubation of the enzyme with the TPNH and α -oxoglutarate; pH values were determined on reaction mixtures with a glass electrode.

Fig. 1). Experiments in which the enzyme was used to reduce TPN with glutamate showed, for this reaction also, a pH optimum of about 8.5 in pyrophosphate, but a higher activity at pH 8.0 in orthophosphate.

The enzyme gave rate-substrate concentration curves of the Michaelis-Menten type for glutamate, α -oxoglutarate and NH_4^+ ion. Michaelis constants,

determined by the methods of Lineweaver & Burk (1934), were as follows: 7.2 mM for glutamate, 3.7 mM for α -oxoglutarate, mM for NH_4^+ ion (Figs. 2-4).

Basis for the distinction between wild-type and mutant enzymes

Wild type and mutant strains were always clearly distinguished by the relatively very low glutamic dehydrogenase activities shown by crude extracts of the latter (Table 1, and Fincham & Pateman, 1957). A second distinction of a qualitative character was revealed by experiments originally designed to test the heat-stability of the two kinds of preparation. It was found that crude extracts or fractionated preparations of mutant strains increased strikingly in activity on being heated to 35–50° for 4–10 min. Except where the heating probably exceeded the optimum, increases in activity of six- to eight-fold were generally obtained (Table 1), although no absolute significance can be attached to these figures since the activity shown by untreated mutant preparations depends on the technique of measurement (see below). Wild-type preparations showed no measurable change in activity when subjected to similar heat treatment. In general, the activity of heat-treated mutant preparations still fell short of typical wild-type activity (Table 1).

Another less immediately obvious difference between wild-type and mutant-enzyme preparations was the presence of a lag phase in the reaction catalysed by mutant enzyme when the reaction was

started by adding TPNH. No such lag has been observed in the wild-type enzyme under the conditions used in this series of experiments, although a lag effect, which has not been further investigated, has been noticed when wild-type enzyme has been tested at pH 7.4. The cause of the lag effect with mutant enzyme is considered below.

Fig. 3. Effect of NH_4^+ ion concentration on activity of wild-type enzyme. Reaction mixtures with 0.08M orthophosphate, pH 8.0, 6.7×10^{-3} M α -oxoglutarate, approx. 3.5×10^{-5} M TPNH and 57 μ g. of protein of preparation 8 (Table 1) in 3 ml. $\circ$, Rate (R) and NH_4^+ ion concentration (C); $\bullet$, C plotted against C/R for determination of the Michaelis constant (K_m , mM).

Fig. 2. Effect of glutamate concentration on activity of wild-type enzyme. Reaction mixtures with 0.06M orthophosphate, pH 8.0, approx. 10^{-4} M TPN, and 0.25 mg. of protein of preparation 1 (Table 1). $\circ$, Rate (R) and glutamate concentration (C); $\bullet$, C plotted against C/R for determination of the Michaelis constant (K_m , 7.2 mM).

Fig. 4. Effect of α -oxoglutarate concentration on activity of wild-type enzyme. Reaction mixtures with 0.09M orthophosphate, pH 8.0, 3.3×10^{-3} N $(\text{NH}_4)_2\text{SO}_4$, approx. 4.5×10^{-5} M TPNH, and 29 μ g. of protein of preparation 5 (Table 1) in 3 ml. $\circ$, Concentration (C) and rate (R); $\bullet$, C plotted against C/R for determination of the Michaelis constant (K_m , 3.7 mM).

In general, mutant-enzyme preparations were less stable at -15° than wild-type preparations (see Methods section). It was also found that whereas wild-type preparations were quite stable at 50° for periods of up to 20 min. (preparations 3, 5 and 8, Table 1, were tested), most of the mutant preparations tested (preparations 4, 6 and 7, Table 1) showed a pronounced falling off in activity at 50° after the initial activation effect. Preparation 7, for example, lost about half its activity between 2 and 12 min. at 50° . On the other hand, preparation 9 was stable during 20 min. at 50° . Thus although there is a general indication that the mutant enzyme is less stable than the wild-type enzyme, there is much variation in this respect between different preparations. Ageing of mutant preparations did not result in any apparent qualitative change in the properties of the enzyme; in particular, the heat-activation effect was similar in fresh and old preparations.

Since the enzyme fractions used in these experiments were relatively crude it was thought possible that a difference in respect of an impurity, rather than a difference in the enzymes themselves, might account for the difference in properties of wild-type and mutant preparations. This possibility was rendered less plausible by a number of experiments on mixed mutant and wild-type preparations. Table 2 shows the results of a representative experiment. There was no inhibition of wild-type enzyme activity by the mutant preparation, nor stimulation of mutant-enzyme activity by the wild-type preparation. The effect of heat on the mixture was such as would be expected if the mutant enzyme alone was responding to the treatment.

Table 2. *Absence of interaction between wild-type and mutant-enzyme preparations*

Reaction mixtures (3 ml.) contained: 1 ml. of enzyme solution in 0.1M phosphate, pH 8.0, containing 0.025 mg. of wild-type protein or 0.11 mg. of mutant protein or both, and final concentrations of 0.09M phosphate, pH 8.0, 6.7×10^{-3} M α -oxoglutarate, 3.3×10^{-3} M $(\text{NH}_4)_2\text{SO}_4$, and approx. 6×10^{-5} M TPNH. Reactions were started by adding the TPNH. Fractionated enzyme preparations 5 and 6 were used.

Nature of enzyme	$10^3 \times \text{Activity } (\Delta E_{340}/\text{min.})$	
	Enzyme solution not heated	Enzyme soln. heated to 50° for 10 min. immediately before testing
Wild type	51	51
Mutant	8.6*	55†
Mixture	60	110

* Showing a slightly accelerating rate; initial rate, as measured over the first 90 sec., is shown.

† Enzyme tested at half strength, and the resulting activity doubled.

Nature of the temperature activation of mutant enzyme

A complication which arose in assessing the effect of temperature treatment was due to the fact that mutant-enzyme preparations which were kept at or below 21° showed an initial lag in reaction rate when the reaction was started by adding TPNH. After temperature activation this lag disappeared (Fig. 8). The best measure of the effect of the temperature treatment appeared to be the difference in initial rates, although the initial rate catalysed by untreated mutant enzyme was difficult to assess and may, in fact, have been zero. In Figs. 5-7 rates measured over the first 90 sec. are shown, although this procedure probably overestimates true initial rates in the untreated or deactivated mutant enzyme.

It was found that the rapid increase in mutant-enzyme activity obtained by brief treatment at $35-50^{\circ}$ was reversed, though relatively slowly, on returning the preparation to 21° . On repeating the heat treatment there was a second reversible activation (Fig. 5). In one experiment the cycle of operations was repeated three times, the degree of activation being the same on each occasion. Fig. 6 shows the time course of activation at different temperatures. At temperatures of 35° or more activation was very rapid, being complete in 90 sec. At 30° activation was very much slower and the final level of activity attained was somewhat lower than at 35° (although the activity may still have been slowly increasing at the end of the experiment). At 26° the rate of activation was very low. The data give the impression that the final activity obtained,

Fig. 5. Reversible temperature activation of the mutant enzyme. Enzyme from preparation 9 (Table 1) in 0.1M phosphate buffer, pH 8.0 (0.4 mg. of protein/ml.), was kept in water baths at the temperatures indicated. Samples (1 ml.) were withdrawn at the times indicated and assayed in a system containing 0.08M orthophosphate, pH 8.0, 3.3×10^{-3} M $(\text{NH}_4)_2\text{SO}_4$, 6.0×10^{-3} M α -oxoglutarate and approx. 5.4×10^{-5} M TPNH after 3 min. at 21° . Reactions were started by addition of the TPNH. Untreated and deactivated samples gave accelerating reaction rates with initial rates close to zero; the mean rate over the first 90 sec. has been taken in each case.

as well as the rate of activation, is dependent on temperature in the range 25–35°, although this point is not fully established. The simplest explanation of these observations appeared to be that the

Fig. 6. Time course of activation of mutant enzyme at different temperatures. Enzyme from preparation 9 (kept frozen for 1 week) in 0.01M phosphate buffer, pH 8.0 (0.24 mg. of protein/ml.). Samples (1 ml.) were withdrawn at the times indicated, equilibrated to 21° (room temp.) for 3 min., and immediately assayed as described under Fig. 5. The curve for 29° was obtained from a different experiment with the same enzyme (Fig. 7) by correcting for difference in enzyme concentration.

Fig. 7. Absence of effect of dilution on reversible temperature activation of mutant enzyme. Curve A: activation at 29° of prep. 9 (kept frozen for 9 days) in 0.1M phosphate buffer, pH 8.0; 0.3 mg. of protein/ml. (×) and 0.15 mg. of protein/ml. (○). Curve B: decay of activity of the same preparation (0.4 mg. of protein/ml.) in the same buffer, after activation at 38° for 10 min. and (at time 0) transferring to 21° without further dilution (+) or diluted with an equal volume of buffer (●). Activity was assayed as described under Fig. 5.

mutant enzyme existed in two forms in temperature-dependent equilibrium, with an inactive form predominating at 21° and an active form predominating at 30°. Other more complicated explanations were not excluded however.

It was conceivable either (a) that activation was due to the dissociation of the enzyme from an inhibitor and decay of activity to the recombination of the inhibitor with the enzyme, or (b) that activation was due to the combination of the enzyme with an activating impurity in the preparation, with decay in activity the reverse of this process. In (a) the rate of decay in activity (expressed as rate of change of specific activity) should decrease with increasing dilution of the enzyme preparation, and in (b) the same should apply to the rate of activation. To test these possibilities the effects of dilution on the rates of activation and decay in activity were investigated, 29° being selected as giving a suitably slow activation rate. Neither of the two possible dilution effects was found (Fig. 7), and it was concluded that a reversible change in the enzyme molecule itself was involved.

Effects of substrates on the activity of the mutant enzyme

The relation between activity of mutant enzyme (preparation 10, Table 1) and NH_4^+ ion concentration was found to be of the usual Michaelis type, with a Michaelis constant of close to 0.6 mM. In this experiment the enzyme was pre-incubated with α -oxoglutarate and TPNH to overcome the lag effect (see below). The relation between activity and α -oxoglutarate concentration was more complicated (Fig. 10), and will be discussed in relation to the lag effect already mentioned.

The lag in reaction rate, which was observed when the reaction catalysed by untreated mutant enzyme was started by the addition of TPNH, was not shortened by more prolonged incubation of the enzyme with α -oxoglutarate and ammonium salt before the start of the reaction. Nor was the lag altered appreciably by incubation of the enzyme with TPNH and ammonium salt, the addition of α -oxoglutarate being used to start the reaction. However, when the enzyme was incubated for 4.5 min. with TPNH and α -oxoglutarate and the reaction started by the addition of the ammonium salt, not only was the lag abolished but the maximum rate attained was considerably increased (Fig. 8). Fig. 9 shows that during incubation of the mutant enzyme with TPNH and α -oxoglutarate its activity increases up to a maximum achieved (under the conditions of this experiment) in about 6 min. An experiment similar to that illustrated in Fig. 8, but with wild-type enzyme, showed no such effect; the reaction rate was the same irrespective of the order in which the substrates were added.

The activating effect of α -oxoglutarate, in the presence of TPNH, on the mutant enzyme may help to explain the observations shown in Fig. 10. When the activity of untreated mutant enzyme was plotted against α -oxoglutarate concentration a sigmoid curve was obtained instead of the usual Michaelis relationship. The activity of the enzyme at the lowest α -oxoglutarate concentrations was almost negligible. After temperature activation of the enzyme a curve approximating to the Michaelis-Menten type was obtained, with a Michaelis constant of approximately 2 mM (rather lower than for the wild-type enzyme). It is noteworthy that the higher the α -oxoglutarate concentration, the less was the effect of temperature treatment. In a separate experiment it was found that mutant enzyme incubated with TPNH and 2×10^{-2} M α -oxoglutarate for 4-5 min. had as much as 70% of the activity of the same preparation after temperature activation. It thus seems reasonable to suggest that the mutant enzyme can be converted into the active form either by temperature treatment or by incubation with saturating concentrations of α -oxoglutarate in the presence of TPNH. This hypothesis is further developed in the Discussion.

Fig. 8. Effect of pre-incubation with substrates on activity of mutant enzyme. Reaction mixtures with 6.67×10^{-3} M α -oxoglutarate, 3.3×10^{-3} M $(\text{NH}_4)_2\text{SO}_4$, 0.09 M orthophosphate, pH 8.0, approx. 6×10^{-5} M TPNH, and 1 mg. of protein of preparation 9 (Table 1) in 3 ml. Curve A: enzyme mixed with buffer, α -oxoglutarate and ammonium salt (2.8 ml.) 4 min. before addition of TPNH (0.2 ml.) at zero time. Curve B: enzyme mixed with buffer, TPNH and ammonium salt (2.9 ml.) 4 min. before the addition of α -oxoglutarate (0.1 ml.) at zero time. Curve C: enzyme mixed with buffer, TPNH and α -oxoglutarate (2.9 ml.) 4 min. before the addition of ammonium salt at zero time. Curve D: enzyme activated by 3 min. at 41° , returned to room temp. (3 min. at 21°), and treated as for curve A.

Fig. 9. Time course of substrate activation of mutant enzyme. Reaction mixtures with 0.09 M orthophosphate, pH 8.0, 10×10^{-3} M α -oxoglutarate, 3.3×10^{-3} M $(\text{NH}_4)_2\text{SO}_4$, approx. 5.3×10^{-5} M TPNH and 0.40 mg. of mutant protein from preparation 9 (Table 1), kept frozen for $3\frac{1}{2}$ weeks. The ammonium salt (in 0.2 ml.) was added to the other components (2.8 ml.) at different times after mixing, except for the zero-time assay where the enzyme was added last. The non-pre-incubated enzyme gave an increasing reaction rate; the mean rate over the first 90 sec. is plotted.

Fig. 10. Effect of α -oxoglutarate concentration on activity of mutant enzyme. Reaction mixtures with 0.09 M orthophosphate, pH 8.0, 3.3×10^{-3} M $(\text{NH}_4)_2\text{SO}_4$, approx. 4.7×10^{-5} M TPNH and (curve A) 0.64 mg. of protein from preparation 9 (Table 1); (curve B) 0.21 mg. of protein of the same preparation activated by 3 min. at 41° , or (curve C) 0.59 mg. of the same preparation activated by 6 min. at 38.5° after 3 weeks of frozen storage. Reactions were started by adding the ammonium salt (in 0.2 ml.) $4\frac{1}{2}$ min. (curve A) or 2 min. (curve B) after mixing the other components, or (curve C) by adding the TPNH. The difference in maximum activity between curves B and C is attributed to ageing of the enzyme, and the difference in shape to the different order of addition of substrates (see text).

It seemed of interest to determine whether substrates had any effect on the rate of decay of activity of temperature-activated enzyme. α -Oxoglutarate alone, at a concentration of $1.67 \times 10^{-2} M$, was found to have no effect on the rate of decay of activity at 22° . The addition of TPNH, or

TPNH plus ammonium salt, to temperature-activated enzyme caused a very much more rapid loss of activity than occurred in the absence of these substrates (Table 3). The activity was much more stable when both TPNH and α -oxoglutarate were present than when TPNH was added alone. These results are consistent with the observation, made in experiments on the effect of α -oxoglutarate concentration on the activity of temperature-activated mutant enzyme, that enzyme activity fell off rapidly with time in reaction mixtures containing TPNH and low α -oxoglutarate concentrations, but that this did not occur to the same extent at higher α -oxoglutarate concentrations. This probably explains the difference in shape of the two curves for activated enzyme shown in Fig. 10; unduly low activities were obtained where the enzyme had been briefly incubated with TPNH and low concentrations of α -oxoglutarate.

A few experiments were made to determine the effect of glutamate concentration on the rate of reduction of TPN by mutant enzyme. It was found that in this case also there was an appreciable lag (after starting the reaction by addition of TPN) before the maximum rate of reduction was attained. This lag, which was not observed in similar experiments with the wild-type enzyme, was abolished, and the maximum rate was much increased, by temperature activation of the enzyme. The activated enzyme gave a concentration-activity curve for glutamate of the Michaelis-Menten type, with a Michaelis constant of about 2.5 mM (less than the value found for the wild-type enzyme). In the corresponding experiment with untreated enzyme the activities at low glutamate concentrations seemed unduly low (Fig. 11), and the Michaelis constant was apparently considerably higher than for the activated enzyme. These observations suggest that glutamate plus TPN, like α -oxoglutarate plus TPNH, tends to activate the enzyme, though the activation seems never to be complete in this case; even at the highest glutamate concentrations the activities of temperature-activated and untreated enzymes show no tendency to converge. It is possible that higher concentrations of TPN were required for complete activation, but this point has not been investigated.

DISCUSSION

It seems clear that the mutant enzyme must be qualitatively different from the wild-type enzyme, and that the mutant enzyme exists in two readily interconvertible forms.

While it is possible that temperature activation and substrate activation may be quite unrelated, it seems simpler to suppose that they represent two different ways of bringing about the same change in

Table 3. *Effect of substrates on the rate of decay of activity of heat-activated mutant enzyme*

Mutant enzyme, preparation 9, 0.31 mg. of protein/ml. in 0.1M phosphate, pH 8.0, was activated by 5 min. at 38° , and used at room temperature (21.5°) 3 min. later. Final concentrations in reaction mixtures: $3.3 \times 10^{-3} N$ - $(NH_4)_2SO_4$, $6.7 \times 10^{-3} M$ α -oxoglutarate, 0.08M phosphate, pH 8.0, approx. $6 \times 10^{-5} M$ TPNH, and 0.31 mg. of protein/3 ml.

Treatment of enzyme	Activity ($\Delta E_{340}/\text{min.} \times 1000$)
Mixed with NH_4^+ ions and α -oxo-glutarate in 2.8 ml.; TPNH in 0.2 ml. added after $3\frac{1}{2}$ min.	60
Mixed with TPNH and α -oxoglutarate in 2.8 ml.; NH_4^+ ions in 0.2 ml. added after $3\frac{1}{2}$ min.	58
Mixed with TPNH and NH_4^+ ions in 2.8 ml.; α -oxoglutarate in 0.2 ml. added after $3\frac{1}{2}$ min.	11, rising to 20
Mixed with buffer only in 2.4 ml.; other reactants in 0.6 ml. added after 5 min.*	45
Mixed with buffer and TPNH in 2.6 ml.; other reactants in 0.4 ml. added after 5 min.*	20, rising to 25

* Not closely comparable with the first three tests since the enzyme was one day older.

Fig. 11. Effect of glutamate concentration on activity of mutant enzyme. Reaction mixtures with 0.075M orthophosphate, pH 8.0, approx. $1.3 \times 10^{-4} M$ TPN and 0.2 mg. of protein of preparation 10 (Table 1). In curve A the enzyme was untreated and in curve B it was activated by 6 min. at 38.5° , cooled to 21° (room temp.) for 2 min., and used immediately. Reactions were started by the addition of the TPN. In curve A the reactions showed a slight initial lag; maximum rates are plotted.

the enzyme. This supposition is encouraged by the fact that at high α -oxoglutarate concentrations substrate activation tends to give nearly the same activity as temperature activation.

The following general hypothesis is offered to cover the two types of activation and also the anomalous relation between rate of reaction and α -oxoglutarate concentration. The mutant enzyme can exist in an inactive form *Ei*, or an active form *Ea*, the former predominating at 20° and the latter at 30° or higher. In the enzyme-TPNH complex, the position of the equilibrium at 20° again strongly favours the inactive form, and the attainment of this equilibrium is evidently more rapid than in the free enzyme. The TPNH-enzyme- α -oxoglutarate complex, however, is, at equilibrium, mainly in the active form at 20°, this implying that the affinity of *Ea*-TPNH for α -oxoglutarate must be much greater than that of *Ei*-TPNH. When untreated mutant enzyme is mixed at 20° with TPNH and α -oxoglutarate, the initial rate of activation will be proportional to the concentration of the TPNH-*Ei*- α -oxoglutarate complex, which, since the affinity of TPNH-*Ei* for α -oxoglutarate must be relatively very low, will be nearly proportional to α -oxoglutarate concentration over all but the upper part of the experimental concentration range. Thus the activity attained after a given period of incubation will be a function of α -oxoglutarate concentration (and possibly of TPNH concentration as well, though there is no evidence bearing on this point). Since the activity of the active form of the enzyme will itself be dependent on substrate concentration in the usual way (Fig. 10, curve C), the activity found will tend to increase with the square of the α -oxoglutarate concentration at low concentrations. Thus the sigmoid form of curve A (Fig. 10) seems explicable on the basis of the substrate-activation effect.

It would be surprising if such a difference in enzyme properties as exists between mutant and wild-type strains did not result in any difference in growth. In fact, though the mutant can scarcely be distinguished from wild type by growth experiments at 25°, it grows poorly on the usual minimal medium at 20° and is strongly stimulated by added α -amino acids at this temperature. The experiments are reported fully elsewhere (Fincham & Pateman, 1957). Thus there seems to be a good correlation between the *in vivo* and *in vitro* characteristics of the mutant.

From the point of view of physiological genetics the present case is of interest as an example of a qualitative change in a specific enzyme brought about by gene mutation. Such cases have hitherto been rare (but see Horowitz & Fling, 1953; Maas & Davis, 1952), although many mutations are known, both in *Neurospora* and in other micro-organisms,

which have caused an apparently complete loss of an enzyme. With glutamic dehydrogenase of *N. crassa* a series of three alleles, corresponding to normal enzyme, altered enzyme, and apparent absence of enzyme, is now known (Fincham & Pateman, 1957).

SUMMARY

1. Glutamic acid dehydrogenase has been extracted and somewhat purified from wild-type *Neurospora crassa*, and some of its properties have been determined.

2. A mutant type, obtained secondarily by ultraviolet-light-induced mutation from a strain lacking detectable glutamic dehydrogenase, has been shown to produce a glutamic dehydrogenase different from the wild-type enzyme. The mutant enzyme has very little activity at 20°, but becomes active after a few minutes at 35–50°. This temperature activation was completely reversible; all the extra activity was lost after 2 hr. at 21°, but was quantitatively regained on repeated heat treatment. Experiments on the effect of dilution on activation and on loss of activity indicated that neither of these processes was due to a reaction with an impurity in the preparation.

3. Some activation of the mutant enzyme also resulted from incubation with substrates; a high concentration of α -oxoglutarate plus reduced triphosphopyridine nucleotide gave almost as much activation as did temperature treatment, whereas glutamate plus the oxidized coenzyme appeared to be less effective. The interpretation of these results is discussed.

4. The activity of the wild-type enzyme was quite unaffected by the procedures which increased the activity of the mutant enzyme. Experiments in which mixtures of wild-type and mutant enzymes were tested showed no interaction between the two types of preparation.

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