carbonate, heated and filtered. The filtrate was then concentrated and cooled, when a precipitate (25 mg.), apparently identical with lead formate, separated. This also had zero activity.

The values of the radioactivities of the fragments obtained in the above degradations are summarized in Table 2.

Attempted incorporation of labelled mevalonic acid lactone

DL-[1-¹⁴C]Mevalonic acid lactone (25 µc) was added in sterile water to a flask containing an 8-day culture of *P. cyclopium*. A second flask was similarly treated with DL-[2-¹⁴C]mevalonic acid lactone (10 µc). After a further 3 days the flasks were harvested and the palitantin was extracted as before but in neither case did it show any significant activity. In addition the mycelium was Soxhlet-extracted with light petroleum (b.p. 40–60°) for 18 hr. and the extract was concentrated to dryness. On assay the crude product from [2-¹⁴C]mevalonic acid lactone was significantly active (147 counts/min.), whereas the product from the [1-¹⁴C]-lactone was only slightly active (26 counts/min.), cf. Table 1. The lipid fraction was assumed to contain steroidal material, e.g. ergosterol (Thomas, 1955).

SUMMARY

1. The biosynthesis of palitantin, C₁₄H₂₂O₄, an alicyclic metabolite of *Penicillium palitans* and also of *Penicillium cyclopium*, has been studied.

2. Degradations of labelled palitantin derived from [1-¹⁴C]acetate strongly support a biosynthetic

mechanism involving the head-to-tail condensation of acetate units.

3. The biosynthetic pathway does not appear to involve mevalonic acid.

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A Further Genetic Variety of Glutamic Acid Dehydrogenase in *Neurospora crassa*

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The amination-deficient mutants of *Neurospora crassa* all appear to be deficient in glutamic dehydrogenase, and are all due to genetic change within a short segment (the *am* locus) of the chromosome corresponding to linkage group 5 (Pateman & Fincham, 1958; Fincham, 1959*a*; R. W. Barratt, personal communication). Treatment of conidia of several of these mutants with ultraviolet light has resulted in the induction of apparent

reversions to the wild-type condition. Some of the 'revertants' were indistinguishable from the standard, wild-type organism, whereas in others there seemed to have been only partial repair of the original mutational damage to the genetic basis of glutamic-dehydrogenase formation (Pateman, 1957). Two of these imperfect revertants have now been investigated in detail. The first, derived from the enzyme-negative allele *am*² [the revised gene symbols adopted in Fincham (1959*a*) are used here], and designated *am*²¹, has been shown to produce an abnormal kind of glutamic dehydrogenase which is almost inactive as normally extracted, but

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can be activated by mild heat treatment (Fincham, 1957). The second revertant is described in this paper and is shown to produce another variety of the enzyme, the abnormality in this case consisting, most conspicuously, of increased Michaelis constants for all substrates.

MATERIALS AND METHODS

Origins of strains of Neurospora. The standard wild-type organism, used throughout, was either St Lawrence A (STA, sometimes referred to in the literature as 74A) or 2a, a strain of the opposite mating type closely related to STA through inbreeding. The original partial revertant was obtained by Pateman (1957) and was given the number 1038. It originated through ultraviolet treatment of a strain of mating-type *a* which was devoid of glutamic-dehydrogenase activity because of the presence of the allele *am*³. Evidence, to be considered below, indicates that 1038 and its descendants carry a new allele, derived from *am*³ and designated *am*^{3a}. The strain 26-7 (*A am*^{3a}) was derived from the cross 1038 × STA, whereas 34-1 (*a am*^{3a} *albino*) came from 26-7 × an albino strain of mating-type *a*, and 1566-78 (*a am*^{3a}) from 34-1 × STA. B317 and 46004 are unlinked arginine-requiring mutants, the first lacking argininosuccinase and the second probably deficient in the enzyme which forms argininosuccinate (Newmeyer, 1957). The double mutant 42-4 (*a am*^{3a} *arg*-10) was isolated from 26-7 × B317 (*a arg*-10).

Reagents. α -Oxoglutaric acid and glutamic acid were obtained from Roche Products Ltd., and were used as the sodium salts. Triphosphopyridine nucleotide (TPN) of 95–100% purity was obtained from the Sigma Chemical Co. through G. T. Gurr Ltd., 136, New Kings Road, London, S.W. 6. Reduced TPN (TPNH) was prepared in solution by the general method of Evans & Nason (1953), with a slight excess of DL-isocitrate and an ammonium sulphate (50–60% saturation) fraction of pig-heart extract, dialysed before use, as source of isocitric dehydrogenase.

Measurement of enzyme activity. Enzyme activity was measured spectrophotometrically essentially as described by Fincham (1957), the temperature being controlled by circulating water. Reaction mixtures were buffered with 0.08–0.09 M-orthophosphate at pH 8.0; glass-electrode measurements confirmed that this buffer was effective in maintaining the pH at this value. Normally a Na_2HPO_4 – NaH_2PO_4 (94:6) mixture was used, but where high levels of NH_4^+ ion were required it was partly replaced by $(\text{NH}_4)_2\text{HPO}_4$ at pH 8.0, the phosphate concentration being kept constant. The final component of the reaction mixture (usually enzyme, but sometimes ammonium salt) was added in 0.1 ml., from a depression in a Perspex plunger, which was used to obtain virtually instantaneous mixing of the reactants. The unit of activity is an initial rate of change of $E_{340\text{m}\mu}$ of 0.001/min. Specific activity is expressed as units/mg. of protein and, unless otherwise stated, refers to a system which is 6.7 mM- α -oxoglutarate, 13.3 mM- NH_4Cl and approx. 60 μM -TPNH.

Enzyme preparations. Mycelium was grown, from heavy inocula of conidia, for 48 hr. at 25° without agitation. Small quantities of mycelium for the preparation of crude extracts were grown in 50 ml. of medium in 250 ml. conical flasks, and larger quantities for enzyme purifications were

grown in 100 ml. of medium in Roux bottles. The medium was the liquid minimal medium 'N' of Vogel & Bonner (1956). Moist mycelial pads, after being washed with water and blotted with paper towelling, were ground in a chilled mortar with powdered glass and about five times their weight of 0.05 M-phosphate, pH 8.0. Further buffer was added, after grinding, to give a total of 10–30 times the original weight of moist mycelium, and the homogenate was filtered through a washed pad of kieselguhr on a Büchner funnel. The specific activity of the filtrate depended on the amount of protein in the extract. For small quantities of mycelium, where no further purification was intended, 2 g. of kieselguhr was used for each 50 ml. of culture (about 0.5 g. of mycelium). The filtrates then had specific activities of 1000–1500 for the wild type. Where larger quantities were being processed for enzyme purification, about 1 g. of kieselguhr was used/g. of moist mycelium, and the filtrates then had specific activities, for the wild type, of only about 500, owing to the higher recovery of protein. Further purification was carried out by $(\text{NH}_4)_2\text{SO}_4$ fractionation, followed by adsorption on to, and elution from, calcium phosphate gel, according to the procedure described by Fincham (1959b). The final preparations were purified at least 40-fold as compared with the kieselguhr filtrate, and had specific activities, for the wild type, of 20 000–30 000 or slightly more. Preparations of this type will be referred to as '40-fold purified enzyme'. The recovery of activity tended to vary according to the particular batch of gel being used; the most successful batch gave about 80% recovery of activity in the combined fractions, with about 25% of the original activity in the most active gel eluate. The glutamic dehydrogenase from *am*^{3a} strains appeared to have the same fractionation characteristics as the wild-type enzyme. In a few experiments, $(\text{NH}_4)_2\text{SO}_4$ fractions were used without gel treatment (e.g. Table 2).

Protein determinations. Protein was determined colorimetrically by the method of Lowry, Rosebrough, Farr & Randall (1951), with reference to a standard curve prepared with casein.

RESULTS

Distinguishing properties of wild-type, 2l and 3a enzymes

In Fig. 1 are shown the results of tests which show that the glutamic dehydrogenase produced by *am*^{3a} strains (3a enzyme) behaves differently both from wild-type enzyme, and from the enzyme type characteristic of *am*^{2l} strains (2l enzyme).

Unlike the wild-type enzyme, both the 2l and the 3a enzymes can, in crude kieselguhr filtrates, be activated by a few minutes of mild heat treatment (38° was the temperature generally used in the present experiments). In both cases partial activation can also be achieved as a result of incubation with substrate mixtures. However, whereas with the 2l enzyme both TPNH and α -oxoglutarate must be present before any activation occurs (Fincham, 1957), the 3a enzyme is considerably activated by incubation in reaction mixtures

minus TPNH [cf. curves 3 and 5, Fig. 1 (b) and (c)]. The 3a enzyme resembles the 2l enzyme in showing no further activation by substrates after it has been activated by heat [Fig. 1 (b), curves 4 and 6]. When the 3a enzyme is incubated for 3 min. in the reaction mixture minus TPNH before the start of the reaction, a prior heat-activation of the enzyme increases the activity only by about 50 % [curves 5 and 6, Fig. 1 (b)]. The 2l enzyme, however, shows at least a tenfold increase in initial reaction rate as a result of heat-activation under these conditions [curves 5 and 6, Fig. 1 (c)]. Wild-type enzyme typically shows no response to any of these treatments and appears to be already fully active as extracted from the mycelium.

Decrease in NH_4^+ -ion concentration from 0.1 M to 3.3 mM causes a decrease in reaction rate catalysed by the 3a enzyme by a factor of 8–9 [curves 2 and 4, Fig. 1 (b)], whereas the corresponding decrease for both the wild-type and 2l enzymes is no more than 25 %.

The data shown in Fig. 1 indicate that, whereas the am^{2l} extract when fully activated still had less than half the specific activity of the wild-type extract, the maximum activity (at high NH_4^+ -ion

concentrations) of the am^{3a} extract was actually considerably higher than the wild-type value. These results are quite typical of several experiments other than the one shown.

The three enzyme types can also be quite sharply distinguished on the basis of thermostability. In one experiment in which crude kieselguhr filtrates were compared directly, 3a enzyme lost about 35 % of its activity during 3 min. at 60.5° (in phosphate buffer at pH 8.0), whereas 2l enzyme was at least 98 % inactivated under the same conditions. Similar preparations from wild type are typically almost completely stable at this temperature.

In the following sections the properties of the 3a enzyme will be described in more detail.

Conditions for activation of the 3a enzyme

The activation behaviour of crude 3a filtrates has been described in the preceding section. Preparations of 3a enzyme purified 40-fold have given rather variable results. Two such preparations, one from strain 26–7 and one from strain 1566–78, behaved as if already fully activated in that they gave no increase in reaction rate as a result of incubation with substrates. Other preparations, however, showed pronounced activation effects (Table 1). Although a certain amount of activation occurred as a result of incubation in reaction mixture minus α -oxoglutarate, the presence of this substrate is necessary for the maximum effect. In another experiment, α -oxoglutarate in phosphate buffer, in the absence of both TPNH and NH_4^+ ion, could activate this enzyme preparation.

Purified preparations, which were activated by exposure to substrates, were activated even more by warming to 38° for a few minutes. After heat-activation no further activation could be achieved as a result of incubation with substrates. Unlike the activated form of the 2l enzyme, which has a half-life of about 30 min. at 21° (Fincham, 1957, 1960), the activated form of the 3a enzyme

Fig. 1. Enzyme assays showing the different properties of wild, 3a- and 2l-type glutamic dehydrogenases. Kieselguhr-filtered extracts of the strains STA (am^+) (a), 1566–78 (am^{3a}) (b) and 1361–12 (am^{2l}) (c) were used, the amounts of protein per assay being 85, 79 and 90 μg . respectively. Reaction mixtures contained, in 3.0 ml., 20 μmoles of α -oxoglutarate and 0.18 μmole of TPNH. Temp., 21°. Numbers on the curves indicate the following conditions: 1 and 2, 10 μmoles of NH_4^+ ions; 3–6, 300 μmoles of NH_4^+ ions; 2 and 4, enzyme warmed to 38° for 5 min. and cooled rapidly to 21° immediately before adding to the complete reaction mixture; 5, enzyme incubated for 5 min. in the reaction mixture minus TPNH, and the TPNH added (in 0.2 ml.) at zero time; 6, enzyme heat-treated as in 2 and 4, and then incubated with the substrate mixture as in 5. In this experiment procedures 4, 5 and 6 were omitted for the wild-type enzyme, other experiments having shown 3–6 to give identical results with the wild type.

Table 1. Effects of incubation with substrate mixtures on the activity of a preparation of enzyme 3a

Mixtures at approx. 20° contained 0.8 M-sodium phosphate, pH 8.0, 6.7 mM- α -oxoglutarate, 3.3 mM-ammonium chloride, approx. 45 μM -TPNH and 60 μg . of 40-fold purified enzyme from strain 26–7. All the components except one were mixed in 2.8 or 2.9 ml., and the last component was added 3 min. later to bring the volume to 3.0 ml.

Component added last	NH_4^+ ions	α -Oxo-glutarate	TPNH	Enzyme
Initial rate of reaction	78	39	75	13
(10 ³ $\Delta E_{340} \text{ m}\mu/\text{min.}$)				

Table 2. *Thermostabilities of wild-type and 3a-type enzyme preparations separately and in mixture*

Reaction mixtures contained 6.7 mM- α -oxoglutarate, 3.3 mM-ammonium chloride, approx. 55 μ M-TPNH and 0.12 mg. of protein from *am*^{3a} (26-7), or 0.05 mg. of protein from wild type, or both. The fractions used were precipitated from crude extracts with 45% saturated ammonium sulphate (added as the solid salt). Before testing, enzyme samples were heated at 58° for various times in a water bath. 'Zero' time of heating was taken as 1 min. after placing in the bath, corresponding approximately to the time at which temperature equilibrium was attained.

	Wild type			Type 3a			Mixture		
Time of heating (min.)	0	3	9	0	3	9	0	3	9
Activity (units/min.; initial rate)	40	40	40	28	7	2	70	48	42
Theoretical for no interaction	—	—	—	—	—	—	68	47	42

appeared to be quite stable at room temperature. In one experiment with a 40-fold-purified preparation from strain 1566-78, almost no activity was lost during 2 hr. at 21° after activation at 38°.

Similarly purified wild-type preparations normally show no activation, either through warming or through incubation with substrates at pH 8.0, though activation as a result of incubation with an α -oxoglutarate-phosphate mixture has been observed with one rather old and partially inactivated wild-type preparation.

Thermostability

As already mentioned, glutamic dehydrogenase of type 3a is considerably more thermolabile than the wild-type enzyme, though less so than the 21 type. Thermostability varies with the stage of purification (more purified preparations being less stable), and probably also with other factors which have not been systematically investigated, such as protein concentration and the volume of the solution heated. Consequently it is not possible to give generally meaningful values for half-lives at different temperatures. Nevertheless, comparable preparations of wild-type and 3a-type enzymes have always shown large differences in thermostability when tested under the same conditions.

In order to confirm that these differences were due to an intrinsic difference between two kinds of enzyme molecule, rather than to other factors such as protein concentration or the presence in one or other type of preparation of stabilizing or destabilizing substances, a heat-inactivation experiment was performed in which wild-type enzyme and 3a-enzyme preparations were heated separately and in a mixture. The results (Table 2) showed that the stability of each kind of glutamic dehydrogenase was unaffected by mixture with the other kind of preparation.

Michaelis constants

Michaelis constants for all five substrates were determined for 40-fold-purified preparations from both wild type and strain 1566-78 (*am*^{3a}). The preparations of the 3a enzyme were heated to 38°

for 4 min. immediately before addition to reaction mixtures in order to exclude activating effects of substrates.

Figs. 2-4 show that the Michaelis constant for glutamate (K_{GA}) and that for TPN (K_{TPN}) are both very much higher for 3a enzyme than for wild-type enzyme. Fig. 2 shows, in agreement with the finding of Frieden (1959c) for ox-liver glutamic dehydrogenase, that with the wild-type *Neurospora* enzyme the apparent Michaelis constant for each of these two substrates decreases as the concentration of the other is increased. The constants estimated from the data of Fig. 2 for wild-type enzyme are: 7.0 mM for K_{GA} , 26 μ M for K_{TPN} and 1.1×10^{-6} M² for the complex constant $K_{GA \cdot TPN}$ (Alberty, 1953). For the 3a enzyme, both K_{GA} and K_{TPN} are so high that it has not been possible to determine the Michaelis constant of either substrate in the presence of nearly saturating concentrations of the other. Consequently, the values obtained from the data in Figs. 3 and 4 (0.1 M for K_{GA} and 0.4 mM for K_{TPN}) may be over-estimates. However, the fact that nearly the same estimate of K_{GA} was obtained at two rather widely different TPN concentrations (Fig. 3) suggests that any such over-estimation was not very great and that, for 3a enzyme, $K_{GA \cdot TPN}$ is approximately equal to $K_{GA} \cdot K_{TPN}$.

The 3a enzyme also had abnormally high Michaelis constants for all three substrates involved in the reverse reaction (glutamate formation). The wild-type Michaelis constant for α -oxoglutarate (K_{OGA}) has been determined, both during the present work and by Fincham (1957, 1960a), and is about 3.5 mM, and that for NH_4^+ ion ($K_{NH_4^+}$) is between 1.0 and 1.5 mM. The wild-type Michaelis constant for TPNH (K_{TPNH}) appeared, from repeated experiments (e.g. Fig. 5), to be close to 16 μ M. This is a somewhat lower value than that reported by Fincham (1952b), the difference being probably due to a tendency in the earlier work to under-estimate initial rates of reaction at lower levels of TPNH. Each of these Michaelis constants was determined at nearly saturating concentrations of the non-varying substrates. For the 3a enzyme, however, the relatively high Michaelis

constants made saturation of the enzyme with α -oxoglutarate and TPNH impossible to achieve without making $E_{340\text{ m}\mu}$ undesirably high. Frieden (1959c) found that, for the ox-liver enzyme, the apparent Michaelis constant for each of the substrates α -oxoglutarate and TPNH increased with the concentration of the other, provided that the concentration of NH_4^+ ion was high. This effect has not, however, been found for the *Neurospora* 3a enzyme, and the indications are that the constant for each substrate is nearly independent of the concentrations of the other two. Thus Fig. 5

Fig. 2. Relationship between glutamate and TPN concentrations, and velocity of the forward reaction catalysed by 40-fold-purified wild-type enzyme. Reaction mixtures at 25.5° each contained $8\text{ }\mu\text{g.}$ of protein from wild type (ST4) in 3.0 ml. Lines fitted to the experimental points are for the following concentrations of glutamate, reading from top to bottom: 3, 6, 15, 40 and 100 mM.

Fig. 3. Determinations of K_{GA} for 3a enzyme at two concentrations of TPN: 0.2 mM (upper line) and 0.8 mM (lower line). Each reaction mixture contained $30\text{ }\mu\text{g.}$ of protein of 40-fold-purified 3a enzyme. Temp., 21° . The indicated Michaelis constant is approx. 0.1M at both TPN concentrations.

shows nearly the same estimate of K_{TPNH} obtained at quite widely different α -oxoglutarate and NH_4^+ -ion concentrations, and Fig. 6 shows nearly the same estimate of K_{OGA} obtained at two different concentrations of ammonium salt. It thus seems likely that the estimates of Michaelis constants indicated by Figs. 5-7, and tabulated in Table 3, are not far from the true values. K_{OGA} and K_{TPNH} are each about three times and $K_{\text{NH}_4^+}$ about 30 times as high for 3a enzyme as for wild-type enzyme. The large difference in respect of $K_{\text{NH}_4^+}$ is also evident in the results of the experiments on crude extracts shown in Fig. 1.

The very strikingly distinct values of $K_{\text{NH}_4^+}$ provided a good opportunity for an additional check of the hypothesis of an intrinsic difference between the wild-type enzyme and 3a enzyme. The results of an experiment on mixed 3a and wild-type preparations (Fig. 7) show that each type of enzyme retains its own characteristic affinity for NH_4^+ ion (or ammonia) in the presence of all the components of the other type of enzyme preparation.

Comparative maximum velocities

Comparison of 40-fold-purified 3a and wild-type glutamic-dehydrogenase preparations showed that the ratio of the rate of the back reaction (glutamate synthesis) to that of the forward reaction was very much lower with the wild-type enzyme than with the 3a enzyme. The comparison, at relatively high concentrations of all substrates, is shown in Table 4. At these substrate concentrations the reaction velocities obtained with the wild-type enzyme must

Fig. 4. Determination of K_{TPN} for 3a enzyme. Each reaction mixture contained $60\text{ }\mu\text{g.}$ of protein of 40-fold-purified 3a enzyme, with a glutamate concentration of 0.1M. Temp., 21° . The indicated Michaelis constant is 0.4 mM.

have been fairly close to maximal in both reaction directions. For the 3a enzyme, however, and on the basis of the Michaelis constant listed in Table 3, the observed forward and backward velocities should be multiplied by 3.0 and 2.6 respectively to give maximal velocities. Thus the ratio $V_{\text{max. (back)}}/V_{\text{max. (forward)}}$ is about 5 for wild-type and about 45 for 3a-type enzyme.

This difference is quite consistent with the differences in Michaelis constants already noted. According to Frieden (1959c), the equilibrium constant for the reaction system should be obtainable from the expression

$$K = \frac{V_{\text{max. (forward)}}}{V_{\text{max. (back)}}} \cdot \frac{K_{\text{TPNH} \cdot \text{OGA} \cdot \text{NH}_4^+}}{K_{\text{TPNH} \cdot \text{GA}}} \cdot [\text{H}^+].$$

Taking the value of the complex constant $K_{\text{TPNH} \cdot \text{GA}}$ for wild-type enzyme as that given by the data of Fig. 2, and making the assumptions (which are at least consistent with the data of Figs. 3, 5 and 6) that, for 3a enzyme, $K_{\text{TPNH} \cdot \text{GA}}$ is approximately

equal to $K_{\text{TPNH}} \cdot K_{\text{GA}}$, and that, for both enzyme types, the complex constant $K_{\text{TPNH} \cdot \text{OGA} \cdot \text{NH}_4^+}$ is approximately equal to $K_{\text{TPNH}} \cdot K_{\text{OGA}} \cdot K_{\text{NH}_4^+}$, the equilibrium constant at 21° is calculated to be $1.0 \times 10^{-13} \text{ M}^2$ from the 3a data and $1.2 \times 10^{-13} \text{ M}^2$ from the wild-type data. The agreement with the value of $1.0 \times 10^{-13} \text{ M}^2$ determined from the equilibrium measurements of Olsen & Anfinsen (1953) (which were, however, made at 25°) is closer than could have been expected, in view of the various uncertainties involved in the calculations.

The maximum specific activity for wild-type enzyme in the forward reaction is greater than that measured for crystalline ox-liver glutamic dehydrogenase at a slightly less favourable pH (7.6), but otherwise under very similar conditions (Strecker, 1955). The wild-type enzyme preparation used in the experiment recorded in Table 4 had been stored frozen at -10° for 6 weeks, and was more active when freshly made.

Fig. 5. Determinations of Michaelis constants for TPNH for 3a and wild-type enzymes. In the experiment with 3a enzyme, 1.6 $\mu\text{g.}$ of a 40-fold-purified preparation was present in each reaction mixture, with the following concentrations of NH_4^+ ion and α -oxoglutarate respectively: 97 and 19.4 mM (○); 19.4 and 19.4 mM (●); 97 and 3.9 mM (○). In the experiment with wild-type enzyme (●), 1.2 $\mu\text{g.}$ of protein of a 40-fold-purified preparation was present in each reaction mixture, with 26.7 mM- NH_4^+ ion and 16.7 mM- α -oxoglutarate. Temp., 21°. The indicated values of K_{TPNH} are 16 μM for wild-type and 56 μM for 3a enzyme.

Fig. 6. Determinations of Michaelis constant for α -oxoglutarate for 3a enzyme at two concentrations of NH_4^+ ion [added as $(\text{NH}_4)_2\text{HPO}_4$]: upper line, 0.02 M; lower line, 0.1 M. The TPNH concentration was 77 μM ; 2 $\mu\text{g.}$ of 40-fold-purified enzyme was present in each reaction mixture. Temp., 21°.

Table 3. *Michaelis constants for 3a and wild-type glutamic dehydrogenases*

Enzyme type	Substrate				
	L-Glutamate (mM)	α -Oxoglutarate (mM)	NH_4^+ ion (mM)	TPN (μM)	TPNH (μM)
Wild	7	3.5	1.2	26	16
3a	100	9	35	400	56

Characteristics of the glutamic dehydrogenase formed in am^+ + am^{3a} heterocaryons

One might expect a heterocaryon containing a mixture of am^+ and am^{3a} nuclei in a common cytoplasm to produce a mixture of the two kinds of enzyme characteristic of the respective homocaryons. To check this point, a forced heterocaryon was made from strain 42-4 (am^{3a} *arg*-10) and strain 46004 (am^+ *arg*-1). Growth on minimal-agar medium, resulting from simultaneous inoculation of these two strains, was necessarily heterocaryotic, since neither homocaryon is capable of growing in the absence of arginine. The results of an experi-

ment on an extract of this heterocaryon, grown in liquid minimal medium, are shown in Fig. 8. The relation between reaction velocity and NH_4^+ -ion concentration was of the same type as obtained with an artificial mixture of wild-type and 3a-type preparations (cf. Fig. 7). A mixture of wild-type and 3a enzymes in the proportion 1:3.4 (in terms of their respective maximum velocities) would account for the heterocaryon data. When the extract was heated to 60.5° for 30 min. (a treatment known to destroy most of the activity of am^{3a} extracts and scarcely affecting wild-type activity), the effect was such as would be expected if 70% of the 3a-enzyme component, but none of the wild-type component, had been destroyed. Thus the results are in accordance with the

Fig. 7. Plots of reciprocal velocities versus reciprocal NH_4^+ ion concentration for: ●, 3 μ g. of 40-fold-purified 3a enzyme (from strain 1566-78); ○, 2.5 μ g. of 40-fold-purified wild-type enzyme (from STA); (◐) 1.5 μ g. of the 3a enzyme mixed with 1.25 μ g. of the wild-type enzyme, each in 3.0 ml. Concentrations of the other reactants were: α -oxoglutarate, 6.67 mM; TPNH, approx. 65 μ M. Temp., 21°. Lines drawn are theoretical for $K_{NH_4} + 1.18$ mM and V_{max} 72.5 for the wild-type enzyme, $K_{NH_4} + 35$ mM and V_{max} 125 for the 3a enzyme, and no interaction in the mixture.

Table 4. Forward and back reaction velocities catalysed by wild-type and 3a enzyme preparations (40-fold purified)

Reaction mixture I contained: 0.12M- NH_4^+ ion [as $(NH_4)_2HPO_4$]; 0.02M- α -oxoglutarate; 0.15 mM-TPNH; 0.07M-orthophosphate. Reaction mixture II contained: 0.1M-L-glutamate; 0.85 mM-TPNH; 0.08M-orthophosphate. Both were at pH 8.0.

Source of enzyme	Specific activities (ΔE_{340} m μ /min./mg. of protein)		Ratio of velocities (back/forward)
	Mixture I	Mixture II	
STA (am^+)	32 000	6520	4.9
1566-78 (am^{3a})	62 000	1220	51

Fig. 8. Relation between NH_4^+ ion concentration and velocity of reaction catalysed by a kieselguhr-filtered extract of the heterocaryon 42-4 (am^{3a} *arg*-10) + 46004 (am^+ *arg*-1). ○, Extract unheated (except for preliminary activation at 38°); ●, extract heated at 60.5° for 30 min. before testing. Concentrations of the other reactants were: α -oxoglutarate, 6.67 mM; TPNH, approx. 65 μ M; approx. 35 μ g. of extract protein was present in each reaction mixture. Temp., 21°. The curves drawn are theoretical on the assumptions that the unheated extract contained a mixture of wild-type enzyme, with $K_{NH_4} + 1.2$ mM and V_{max} 27, and 3a enzyme, with $K_{NH_4} + 35$ mM and V_{max} 92; and that the 3a component was 72% inactivated by the heat treatment whereas the wild-type component was unaffected.

expectation that an am^+ + am^{3a} heterocaryon will produce two types of glutamic dehydrogenase, one with a high affinity for NH_4^+ ion and high thermostability and one with much lower affinity for NH_4^+ ion and lower thermostability.

Inheritance of am^{3a}

The evidence that the formation of the distinct 3a type of enzyme is due to a new allele (am^{3a}) at the am locus is derived principally from the analysis of crosses between wild and 3a-type strains. Cultures grown from ascospores from such crosses were scored by determination of their glutamic-dehydrogenase type, since no reliable method has been devised for distinguishing 3a types on the basis of growth characteristics. Two criteria were used for distinguishing the two enzyme types in crude kieselguhr filtrates: the relatively low activity shown by the 3a extracts at low concentrations of NH_4^+ ion and the relatively low thermo-

stability of this activity. The two criteria were always found to agree (Fig. 9).

Seventeen asci from crosses between 3a strains and wild type (six from 1038 $\times$ STA, five from 26-7 $\times$ *albino a*, four from 26-7 $\times$ B317 and two from 34-1 $\times$ STA) were each dissected into the four spore pairs; the ascospores were germinated and the resulting cultures were scored for glutamic-dehydrogenase type. A typical result is shown in Fig. 9. In each case, two of the spore pairs gave cultures producing enzyme of the 3a type whereas the other two yielded wild-type enzyme. In addition, strain 1038 was crossed to a strain of mating-type A, which carried the 'enzyme-negative' allele am^1 . Four asci from this cross each contained two spore pairs which gave cultures of the typical 3a type, and two which gave cultures apparently devoid of any glutamic-dehydrogenase activity.

These results are consistent with the hypothesis that the 3a-type enzyme is determined by a new allele at the am locus. They do not, however, rule out the alternative possibility of the 3a phenotype's being due to the unchanged am^3 allele together with a 'suppressor' mutation in a distinct, though linked locus. Unless such a suppressor was extremely closely linked to am , one would expect enzyme-negative am^3 ascospores to be produced as a result of genetic recombination in 3a $\times$ wild-type crosses, if the suppressor hypothesis were true. Such ascospores can be distinguished both from 3a type and from wild-type ascospores by their poor growth when germinated on minimal-agar medium. Nearly 1200 ascospores from the cross 26-7 $\times$ *albino a* were germinated on minimal agar and examined, but none was seen that looked like a typical enzyme-negative type. Thirty-nine sporelings which showed weak growth were transferred for further testing. Of these, all except one proved to be inviable (quite unlike typical am mutants), and the other was a wild type. These data, combined with earlier results on the same lines obtained by Pateman (1957), make it reasonable to regard the original mutant 1038 as having acquired a new allele am^{3a} in place of the original mutant allele am^3 , 'allele' being used to mean a distinct inheritable state of a locus, and 'locus' to mean a small discrete chromosomal segment differentiated from neighbouring loci by function (Fincham, 1959a).

DISCUSSION

The present study brings the number of alleles at the am locus, known to determine distinct varieties of glutamic dehydrogenase, to three. This is in addition to 11 alleles associated with absence of any glutamic-dehydrogenase activity (Fincham, 1959a). The allele am^{21} , as reported by Fincham

Fig. 9. Segregation of am^+ from am^{3a} in an ascus from the cross STA (am^+) $\times$ 34-1 (am^{3a}). The curves represent glutamic-dehydrogenase assays on the kieselguhr-filtered extracts of cultures grown from the four spore pairs of ascus no. 1565. The assay system contained, in 3.0 ml., 20 μ moles of α -oxoglutarate, 40 μ moles of ammonium chloride (added to start the reaction), approx. 0.15 μ mole of TPNH and 0.2 ml. of mycelial extract containing 0.30, 0.28, 0.18 and 0.18 mg. of protein in 1565-12, -34, -56 and -78 respectively. O, Extracts unheated; ●, extracts heated to 61.5° for 15 min. immediately before assay.

(1957), determines the production of a form of the enzyme which tends to be inactive at 20°, but which can be reversibly activated, perhaps through a refolding of the enzyme molecule with unmasking of the active centre, by raising the temperature. The active form of the 2l enzyme seems to be more or less similar to the wild-type enzyme in its Michaelis constants, so far as information is available. The glutamic dehydrogenase determined by *am^{3a}* bears some resemblance to the 2l enzyme in being activated by mild heat; however, the activation is much less readily reversible, and can be partially accomplished through incubation with α -oxoglutarate in the absence of TPNH, a procedure which does not activate the 2l enzyme. When fully activated, the 3a enzyme still differs sharply from the wild-type and 2l varieties in showing abnormally high Michaelis constants for all substrates. It may be significant that the increases in Michaelis constants, as compared with the wild-type values, are much greater for glutamate, oxidized TPN and NH_4^+ ion, substrates which all carry a positive charge, than for TPNH and α -oxoglutarate, which do not. This may suggest that the peculiarity of the 3a enzyme may consist in an alteration in the charge on the enzyme molecule in the vicinity of the active centre. It should be remembered, however, that there is no evidence which would exclude the possibility that unionized ammonia, rather than NH_4^+ ion, is the immediate substrate of the enzyme.

If it is assumed that the amount of glutamic dehydrogenase, expressed as a proportion of the total cell protein, is the same in *am^{3a}* strains as it is in the wild type, we must suppose that the 3a enzyme has a considerably higher turnover number for the back reaction (glutamate synthesis) than has the wild-type enzyme, even though much higher substrate concentrations are required to realize the maximum reaction velocity. It thus seems possible that, under conditions of high nutrition, *am^{3a}* might rank as a progressive mutant allele, in the sense of having a selective advantage over the original wild-type gene *am⁺*. Rather surprisingly, it has not so far been possible to demonstrate any deleterious effect of *am^{3a}* on growth even under conditions of limited nitrogen supply.

It seems very unlikely that glutamic dehydrogenases from different sources act through basically different mechanisms. However, the compulsory substrate binding order of TPNH, NH_4^+ ion, α -oxoglutarate, which seems to follow from Frieden's (1959c) data for the ox-liver enzyme, does not seem very consistent with some of the substrate-activation effects which have been noted in the various *Neurospora*-enzyme types. Thus the 2l enzyme is activated by incubation with TPNH and α -oxoglutarate (both being necessary) in the absence of

more than a trace of ammonium salt (Fincham, 1957), and the present study shows that α -oxoglutarate in the absence of either TPNH or ammonium salt can have a marked activating effect on the 3a enzyme. It seems possible, but unlikely, that these activating effects of TPNH and α -oxoglutarate have nothing to do with their substrate function. The lack of any strong effect, with the 3a enzyme, on the apparent Michaelis constant for one substrate, of variations in the concentrations of the other substrates, is more consistent with a random substrate-binding order than with the type of compulsory binding order deduced by Frieden. It may well be that random substrate binding, on the one hand, and a compulsory binding order, on the other, represent limiting cases which are seldom perfectly realized in practice; the combination of an enzyme with one substrate may be weakly or strongly affected by its combination with other substrates without the binding order being completely determined.

In addition to the three known alleles (*am⁺*, *am^{2l}* and *am^{3a}*) which produce distinct active varieties of glutamic dehydrogenase, it is probable that several of the other known alleles produce inactive forms of the enzyme molecule. That this may be so is suggested particularly by the fact that, in two cases, pairs of *am* alleles which are individually incapable of promoting the formation of any sort of active glutamic dehydrogenase, can form active, though abnormal, forms of the enzyme when present together in a heterocaryon (Fincham, 1959b). There is evidence, both from ultracentrifuge data (Frieden, 1959a, b) and from electron microscopy (Valentine, 1959), that ox-liver glutamic dehydrogenase consists of dissociable and re-associable subunits. If the same applies to the *Neurospora* enzyme, complementary interaction between individually enzyme-negative alleles can be pictured as occurring through association of different varieties of defective subunit which have defects which are functionally non-overlapping.

It is clearly very desirable, where genetically determined variations in the properties of an enzyme are found, that the structural bases of the variations should be determined. Information of this sort would not only lead to a fuller understanding of the problem of the genetic determination of protein structure, but might also, through a correlation of chemical changes in the enzyme molecule with changes in kinetic properties, illuminate the detailed mechanism of the enzyme action.

SUMMARY

1. A new *Neurospora crassa* mutant allele of the *am* locus, a chromosome segment already known to be concerned with glutamic-dehydrogenase forma-

tion, causes the formation of a distinct variety of this enzyme. The new allele, which was derived by mutation of the mutant allele am^3 , itself associated with apparent complete absence of glutamic dehydrogenase, is called am^{3a} .

2. The glutamic dehydrogenase associated with am^{3a} (the 3a enzyme) shows abnormally high Michaelis constants for all substrates, particularly for glutamate, triphosphopyridine nucleotide and NH_4^+ ion.

3. The ratio of maximum velocity in the reverse reaction (glutamate synthesis) to that in the forward reaction is about ten times as great for the 3a enzyme as for the wild-type enzyme. Extracts of am^{3a} strains have much higher maximum velocities in the reverse reaction than have wild-type extracts.

4. The 3a enzyme is less stable to heat than wild-type glutamic dehydrogenase.

5. In many preparations the 3a enzyme differs from the wild-type enzyme in not showing full activity until it has been given a mild heat treatment (e.g. 38° for a few minutes). This activation can also be brought about, to some extent, by incubation of the enzyme in the presence of α -oxoglutarate. Heat-activated 3a enzyme does not revert readily to the inactive form when returned to room temperature.

6. The main differences between the 3a and wild-type enzymes are just as marked in 40-fold-purified preparations as in crude extracts, though it was occasionally found that purified 3a preparations did not require activation. The two kinds of enzyme retained their respective properties when mixed together.

7. A heterocaryon containing both am^+ (i.e. wild type at the am locus) and am^{3a} nuclei produced glutamic dehydrogenase, which behaved as if it were a mixture of the 3a and wild-type enzymes.

8. Two mutant alleles at the am locus, each associated with a quite distinct abnormal type of glutamic dehydrogenase, are now known. Tests are described which distinguish the two mutant enzymes from each other, and from the wild type.

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Pyrimidine Metabolism in Parasitic Flatworms

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β -Alanine and β -aminoisobutyric acid have previously been reported as prominent free amino acids in several species of parasitic flatworms (Campbell, 1960a, b). These two β -amino acids were not detected in the free-living flatworms which were examined. Because of these findings it was thought that an investigation of the metabolic origin of these compounds might contribute to a better understanding of the biochemistry of parasitism.

When β -alanine and β -aminoisobutyric acid were first reported (Campbell, 1960a) from cestodes, it was suggested that they might arise from uracil and thymine respectively via the reductive pathway of pyrimidine degradation which has been established *in vivo* by Fink and co-workers (Fink, Henderson & Fink, 1952; Fink, Cline, Henderson & Fink, 1956; Fink, McGaughey, Cline & Fink, 1956; Fink, 1956) and *in vitro* by Grisolia and co-workers (Grisolia & Wallach, 1955; Wallach & Grisolia, 1957; Grisolia & Cardoso, 1957; Caravaca & Grisolia, 1958) and others (Canellakis, 1956;

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