Effects of Amino Acids on Transaminase Production in *Neurospora crassa*: Evidence for Four Different Enzymes

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A previous note (Fincham, 1951) reported that cell-free dialysed extracts of *Neurospora crassa* mycelium would catalyse the formation of glutamic acid from α -oxoglutaric acid and a number of different α -amino acids. The present study was undertaken with the objects of determining whether the production of transaminases by *Neurospora* is increased by the addition of amino acids to the growth medium, and of gaining information as to the number of enzymes involved in the transaminations catalysed by crude extracts.

METHODS

Chemicals. α -Oxoglutaric acid was obtained from L. Light and Co. Ltd. Colnbrook, Bucks. Amino acids, which were purchased from L. Light and Co. Ltd., British Drug Houses Ltd., Poole, Dorset, or Roche Products Ltd. Welwyn, were all pure as judged by homogeneity on paper chromatograms. The DL-isomers of α -aminobutyric acid, norvaline, norleucine and methionine, and the L-isomers of all other amino acids were used throughout; concentrations are given in terms of the L-isomer, except where otherwise stated. Pyridoxal phosphate was a gift from Roche Products Ltd. The buffer used throughout in enzyme experiments was 0.067 M phosphate ($4\text{Na}_2\text{HPO}_4:1\text{KH}_2\text{PO}_4$), pH 7.4.

Organism. In all experiments to be reported in detail the strain used was a wild-type *Neurospora crassa* of mating-type A, number 126-1, which had been isolated in this laboratory by Mr J. A. Pateman.

Culture methods. A basal nitrogen-free medium buffered with 0.067 M phosphate at pH 5.6 (Fincham, 1954), and containing 1.5% (w/v) sucrose and 5 $\mu\text{g./l.}$ of D-biotin, was used throughout. This is referred to as 'N-free medium' or, when supplemented with 0.005 M ammonium tartrate, as 'minimal medium'. Amino acid supplements were added as indicated. Various sizes of culture flask were used to give a depth of medium of approx. 1 cm. Inocula consisted of approx. 1 ml. of a turbid suspension of conidia (taken from a fresh agar slant culture) for each 100 ml. of medium. Growth was continued without agitation for 48 hr. at 25°. When minimal medium was used without amino acid supplement about 75% of the ammonia supplied was used up during the period of growth; added amino acids appeared to reduce ammonia utilization where comparable amounts of growth were made. The pH values of the media varied between fairly narrow limits during growth; in the experi-

ment illustrated in Table 1 the pH at the end of the growth period varied between 4.85, in the culture with no added amino acid, and 5.35, in the culture with added sodium glutamate.

Preparation of extracts. Mycelial mats were washed, blotted, roughly weighed and ground in chilled phosphate buffer, as previously described (Fincham, 1953). After grinding with 4-5 ml. buffer/g. blotted wet weight, additional buffer was added to give a total of 12-14 ml./g. before centrifuging for 5 min. at 4000 rev./min. in an M.S.E. 'Medium' centrifuge. The supernatants were dialysed for 2-3 hr. against two changes of mechanically stirred phosphate buffer at 0-5°. Extracts were usually found to contain 3-5 mg./ml. protein, which was determined by the quantitative biuret procedure of Stickland (1951), adapted as previously described (Fincham, 1954).

Transaminase assay procedure. 75 $\times$ 9 mm. test tubes contained: 0.075 ml. 0.1 M Na α -oxoglutarate; 0.075 ml. 0.1 M L-amino acid (Na salt in the cases of acidic amino acids) or water as control; 0.15 ml. extract added after temperature equilibration. Incubation was for 60 min. at 35°, after which the reactions were stopped, and protein was precipitated by the addition to each tube of 0.015 ml. 5% (v/v) acetic acid and brief heating to 100°.

Transaminase activities were measured by estimating the glutamate formed. Samples (2 $\mu\text{l.}$) of deproteinized reaction mixtures were applied 15 mm. apart along a line 2 cm. from the long edges of 28 $\times$ 46 cm. Whatman no. 1 filter paper sheets. Three spaces were left blank on each sheet. The sheets were stapled into cylinders and the chromatograms were developed with phenol saturated with water (ascending) for 7-8 hr. after being allowed to equilibrate overnight with the solvent vapour. In cases where the mixtures to be analysed included ornithine a small beaker containing a 1:1 concentrated ammonia solution-water mixture was present in the chromatography vessel during equilibration and development of the chromatograms. The chromatograms were thoroughly dried at room temperature, sprayed uniformly with 0.25% (w/v) ninhydrin in *n*-butanol and left at room temperature until the amino acid spots just became visible, when the glutamic acid area was sprayed a second time to ensure excess of reagent. After leaving in the dark for approx. 20 hr. colour development was completed by warming for 30 min. at 40-50°. A strip 3 cm. wide, cut parallel to the starting line to include glutamic acid but to exclude aspartic acid spots, was read in an 'Eel' Scanner (Evans Electroselenium Ltd., Harlow, Essex) with the slit partially screened so as to scan a 25 mm. width. The strip from each sheet was read in three 15 cm. sections, each section including one of the blank lanes. For each section the instrument was readjusted to give the arbitrary reading of 5 with the blank lane in front of the slit; this figure was subtracted from all experimental

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readings. Only the maximum reading given by each spot was recorded (the 'maximum density' method of Block, LeStrange & Zweig, 1952). Glutamate standards containing the same concentrations of phosphate and acetic acid as the experimental mixtures were run for each set of

Fig. 1. Typical standard curve for glutamate. Papers developed with phenol saturated with water in atmosphere containing ammonia vapour. The standard errors of the means of quadruplicate determinations are indicated.

Fig. 2. Reaction-time curves for various transaminase systems. Mycelium, blotted wet weight 1.65 g., was grown in 150 ml. minimal medium supplemented with $0.01M$ DL-methionine. Digests contained, in 0.3 ml., 0.6 mg. protein, $7.5 \mu\text{moles}$ α -oxoglutarate, $7.5 \mu\text{moles}$ L-amino acid (or water in control) and $7 \mu\text{g.}$ pyridoxal phosphate. Control values have been subtracted from glutamate determinations throughout.

determinations, generally on the same sheets. Experimental determinations were made in quadruplicate, the readings were averaged, and the glutamate concentrations were calculated by reference to a standard curve. A typical standard curve is shown in Fig. 1. It will be seen that the relationship between scanner reading and glutamate concentration is linear only at low concentrations. Above a concentration of approx. $0.004M$ glutamate the method becomes less sensitive and accurate; below $0.002M$ the error is about ± 0.05 – $0.10 \mu\text{M}$ glutamate in an 0.3 ml. digest, while at the higher concentrations it may be as great as $\pm 0.2 \mu\text{M}$. Above a concentration of $0.01M$ glutamate the method has been quite unreliable.

Fig. 2 shows progress curves for glutamate formation from α -oxoglutarate and a variety of amino acids by an extract prepared from mycelium grown on a medium containing DL-methionine. It will be seen that in most cases the rate of glutamate formation decreases with time, and it is evident that in many of the experiments the 60 min. incubation period was too long for precise evaluation of initial rates of reaction and thus of the amounts of the various transaminases present. The conclusions which can be drawn from such experiments are at best only semi-quantitative. The tendency will be to underestimate rather than to exaggerate the stimulatory effect of any given amino acid on transaminase formation.

RESULTS

Transaminase activities of mycelium grown on minimal medium

The transaminase activities detected in extracts of minimal-grown mycelium in the present study were the same as those previously reported (Fincham, 1951). No appreciable glutamate was formed in the standard assay system from glycine, serine, threonine, lysine or histidine, even when pyridoxal phosphate was added. Nor could any convincing evidence be obtained for the participation of pyruvate in any transamination reaction other than the one with glutamate. Small amounts of alanine, barely in excess of the control value, were formed in one experiment from pyruvate and, respectively, aspartate, valine, leucine, isoleucine, phenylalanine and methionine, in the presence of pyridoxal phosphate. Such small activities might well be due to the functioning of glutamate as an intermediate since this amino acid is always present in traces in mycelial extracts. In particular, pyruvate is quite inactive in the ornithine transaminase system, as judged by the very sensitive colorimetric test with *o*-aminobenzaldehyde (Fincham, 1953).

Of the transaminase activities which were detectable, aspartic acid transaminase activity was always relatively very high, ornithine transaminase activity was usually fairly high, while alanine transaminase activity was quite variable but usually low. The other activities were low, with the different amino acids showing consistently the

following order of effectiveness as amino donors: phenylalanine = valine = isoleucine > leucine > norleucine > norvaline > methionine > α -aminobutyric acid > tryptophan (methionine and tryptophan were tested in only two experiments).

Effects of amino acids on transaminase production

There were two main series of experiments. In the first a number of different amino acids were substituted for ammonium tartrate as sole N-source in the minimal medium. The results of this series are not reported in detail, since glutamate was estimated less accurately than in later experiments. The main effects, however, were clear and quite consistent with the results of later experiments and will be referred to in the text. The second series was concerned with the investigation

of the effects of amino acids added as supplements to ammonium tartrate. The results are shown in Table 1. The results of a number of additional experiments are shown in Table 2 and Fig. 3. The effects of valine, leucine and phenylalanine on transaminase production seemed to be greater when they were supplied as sole source of nitrogen (Table 2), but in general the effects were qualitatively the same whether the amino acids were supplied as sole or as supplementary N-sources. The effects may be summarized as follows:

Alanine transaminase activity was generally low but rather variable from one preparation to another. There seemed to be no consistent effect of added amino acid on this enzyme activity, which fluctuated independently of all other transaminase activities which were investigated.

Table 1. *Effects of amino acids on production of transaminases*

Media 50 ml., with amino acid supplements to 5×10^{-3} M concentration. Digests contained, in 0.3 ml., 0.025 M α -oxoglutarate, 0.025 M L-amino acid as indicated, 0.15 ml. buffered mycelial extract. Incubation at 35° for 60 min. except in cases of 'phenylalanine' and 'isoleucine' extracts where it was 105 and 120 min. respectively.

Supplement to 'minimal medium'	Blotted wt. mycelium (g.)	Protein in extract (mg./ml.)	Glutamate formed							In water control
			From alanine	From aspartate	From valine	From leucine	From iso- leucine	From phenyl- alanine	From ornithine	
			(μ moles)							
None	0.30	7.5	0.2	1.1	0.3	0.3	0.3	0.4	0.7	0.1
Glutamic acid*	0.35	4.0	0.5	1.0	0.3	0.3	0.3	0.4	0.4	0.1
Aspartic acid*	0.45	4.0	0.3	1.0	0.2	0.2	0.3	0.3	0.5	0.1
Arginine HCl	0.44	5.0	0.5	1.0	0.5	0.4	0.5	0.6	1.0	0.1
Valine	0.22	4.0	0.7	1.3	2.2	1.6	1.9	1.6	1.4	0.1
Leucine	0.20	4.0	0.2	1.4	1.4	1.0	1.3	1.0	1.0	0.1
Isoleucine	0.10	1.0	0.2	1.0	1.1	0.8	1.1	1.0	0.8	0.1
Phenylalanine	0.12	2.0	0.2	1.3	2.7	2.5	2.5	1.9	1.6	0.1
Alanine	0.30	3.5	0.3	1.3	0.5	0.4	0.6	0.6	1.0	0.1

* Half-neutralized with NaOH.

Table 2. *Comparison of effects of amino acids as sole N-sources and as supplements to ammonium tartrate*

Volumes of media: Expt. 1, 150 ml.; Expt. 2, 50 ml.; Expt. 3, 75 ml. Concentrations of N-sources: ammonium tartrate 0.005 M, valine and leucine 0.01 M, arginine HCl and phenylalanine 0.005 M. Transamination reaction mixtures as for Table 1; incubation at 35° for 60 min.

		Glutamate formed								
Expt. no.	N-source	Blotted wt. mycelium (g.)	Protein in extract (mg./ml.)	From	From	From	From	From	From	In water control
				alanine	aspartate	leucine	valine	phenyl- alanine	ornithine	
(μmoles)										
1	NH ₄ ⁺	1.43	3.5	0.2	1.1	0.3	0.4	0.4	0.5	0.1
	Phenylalanine	0.10	3.7	0.1	1.0	1.5	—	1.4	1.3	0.0
	Phenylalanine + NH ₄ ⁺	1.02	4.2	0.2	1.0	1.1	1.3	1.1	1.3	0.1
2	NH ₄ ⁺	0.35	4.0	0.1	0.6	0.1	—	0.1	—	0.1
	Valine	0.12	4.0	0.1	0.9	1.8	—	1.4	—	0.1
	Leucine	0.10	2.7	0.1	0.7	1.5	—	1.2	—	0.1
	Valine + NH ₄ ⁺	0.24	4.5	0.4	1.0	1.0	—	0.9	—	0.1
	Leucine + NH ₄ ⁺	0.20	4.5	0.2	1.1	1.1	—	1.3	—	0.1
3	NH ₄ ⁺	0.40	4.8	0.2	1.2	—	0.3	—	0.7	0.1
	Arginine	0.40	3.2	0.3	1.3	—	0.4	—	1.7	0.2
	Arginine + NH ₄ ⁺	0.52	4.6	0.4	1.3	—	0.5	—	1.6	0.2

Aspartic acid transaminase activity was usually relatively high and showed no consistent relation to the composition of the medium. In one case mycelium grown on minimal medium gave unusually low aspartic acid transaminase activity (Table 2), but this did not seem to be a consistent effect.

Ornithine transaminase activity appeared to be stimulated to some extent by a number of apparently unrelated amino acids. Valine, leucine, isoleucine, phenylalanine, alanine and arginine all caused an increase in glutamate formation in the ornithine transaminase system of the order of 2- to 3-fold (Tables 1 and 2). Allowance must be made for the relatively high protein content of the extract of 'minimal medium'-grown mycelium recorded in Table 1).

The most striking responses to added amino acids were shown by the transaminase activities for the group of amino acids consisting of valine, norvaline, leucine, isoleucine, norleucine, α -aminobutyric acid, phenylalanine, tryptophan and methionine. Transamination of all these amino acids, with the exceptions of methionine and tryptophan, were investigated in the preliminary series of experiments using amino acids as sole sources of nitrogen as well as in the experiment illustrated in Fig. 3. Transamination of tryptophan was studied in only two experiments, and that of methionine in three. All these transaminase activities have varied together in a parallel fashion. Valine, leucine, isoleucine and phenylalanine all caused striking increases in this group of activities. The increase in the amount of glutamate formed in the test system was usually about 4- to 5-fold and sometimes more; this must correspond to at least a 5-fold increase in enzyme concentration. In two experiments, including the one from which Fig. 2 was derived, methionine had a similar effect. There was also a suggestion (Table 1) that alanine might stimulate this group of activities, but the effect, if any, was relatively small. The result shown in Fig. 3 is typical of a number of experiments where the whole range of transaminase activities was investigated. The different amino acids showed consistently the following order of effectiveness as amino donors: phenylalanine $\approx$ isoleucine $\approx$ valine $>$ leucine $>$ norleucine $>$ norvaline $>$ methionine $>$ α -aminobutyric acid $>$ tryptophan. The data as a whole are consistent with the hypothesis of a single enzyme acting on alternative substrates at the following approximate relative rates: phenylalanine, isoleucine and valine 100, leucine 75, norleucine 65, norvaline 50, methionine 40, α -aminobutyric acid 30, tryptophan 15.

The growth of mycelium on valine, leucine, isoleucine and phenylalanine as sole N-sources was only 10-20% of that on 'minimal medium' in 48 hr. Even as supplements to 'minimal medium',

these amino acids inhibited growth, usually to the extent of about 30%, but sometimes more, depending, probably, on the size of the inoculum. DL-Methionine, which appeared to have a similar effect on transaminase production, was not inhibitory.

Fig. 3. Glutamate formation by transamination catalysed by two extracts with (not cross-hatched) and without (cross-hatched) pyridoxal phosphate at a concentration of 47 μ g./ml. reaction mixture. Ornithine and aspartic acid transaminase activities are practically unaffected by the pyridoxal phosphate.

Effects of pyridoxal phosphate

In one experiment extracts of two cultures grown, respectively, on 'minimal medium' and 'minimal medium' plus valine were tested for transaminase activities with and without the addition of pyridoxal phosphate to a final concentration of 47 μ g./ml. of reaction mixture. The results are shown in Fig. 3. Aspartic acid and ornithine transaminase activities were not appreciably affected by the addition of pyridoxal phosphate. All the other activities tested for were increased by the addition of the coenzyme with the doubtful exception of tryptophan transaminase activity of the extract of valine-grown mycelium. The data are consistent with increases of activity of the order of 2-fold, or rather more in the case of alanine transaminase. Since coenzyme was limiting to about the same extent in both extracts, it seems that the stimulation of some transaminase activities by exogenous valine must be an effect on apoenzyme, rather than on coenzyme production.

DISCUSSION

It seems clear that the transaminase activities shown by *Neurospora* extracts must be due to at least four different enzymes.

Aspartic acid transaminase production does not seem to be markedly dependent on the composition of the medium. Assuming that this transaminase, like all others so far investigated in detail, has pyridoxal phosphate as coenzyme, the apoenzyme-coenzyme complex must be relatively stable.

The alanine transaminase, judging from its very variable, but generally low, activity, is a rather unstable enzyme. The striking response of alanine transaminase activity to pyridoxal phosphate suggests that dissociation of the coenzyme in dilute extracts is the general cause of low alanine transaminase activities. In previous studies (Fincham, 1951, 1954) alanine was found to be more rapidly transaminated by *Neurospora* extracts than any other amino acid. This apparent discrepancy is probably to be accounted for by the fact that extracts were tested in these previous experiments at about four times the concentration used in the present work. It seems likely that in extracts of mycelium grown without exogenous amino acid alanine transaminase is always the most active transaminase provided that adequate concentrations of pyridoxal phosphate are present.

The ornithine transaminase differs from the other transaminases in its pattern of response to added amino acids. Valine, leucine, isoleucine, phenylalanine, arginine and alanine all appear to stimulate its production, though the increases in activity were sometimes fairly small. Like the aspartic acid transaminase, it had no appreciable requirement for pyridoxal phosphate in the one experiment where this possibility was tested. It was to be expected that ornithine would be transaminated by a special enzyme since the δ -amino, rather than the α -amino group, is involved in the ornithine transaminase reaction (Fincham, 1953).

The other transamination reactions to be considered may all be catalysed by the same enzyme. The main reason for supposing that only one enzyme is involved is that the efficiencies of all nine amino acids as amino donors have varied together in a parallel fashion over as much as a 20-fold range of activity. Although this argument has less force in the cases of methionine and especially tryptophan, which have been tested less extensively, all the data at present available are consistent with the hypothesis of a single transaminase acting on these amino acids. The second reason for supposing that a single enzyme is involved is that, so far as the experiments go, they suggest that all nine activities can be induced by the addition to the growth medium of any one of

the nine amino acids. This would not be conclusive by itself since it is well known that adaptive enzyme formation may sometimes be induced by substances which are not substrates of the enzyme in question (Cohn & Monod, 1953). Taken as a whole, however, the data seem to suggest rather strongly the existence in *N. crassa* of a transaminase of rather wide specificity having pyridoxal phosphate as coenzyme. It remains possible that fractionation procedures will reveal that more than one enzyme is involved.

There is no doubt that the growth of *Neurospora* in the presence of certain amino acids permits the recovery of certain transaminases in relatively high concentration. The mechanism may be similar to that involved in the well-known cases of induction of adaptive enzymes. The fact that most of the amino acids which appear to promote transaminase formation are also somewhat inhibitory may, however, suggest that restriction of growth, as such, may be the more immediate cause of the phenomenon. This seems unlikely for two reasons. First, some of the highest activities have been obtained from cultures which were inhibited rather little (e.g. the valine-supplemented culture, Table 1) or not at all (methionine-supplemented cultures in two experiments, cf. Fig. 2). Secondly, other kinds of culture conditions (e.g. growth on aspartate as sole N-source) may restrict growth without leading to the recovery of high transaminase activities.

It should be emphasized that the data given in this paper apply only to a single strain of the mould. Preliminary experiments indicate that other wild-type strains may show rather different patterns of transaminase production.

It is of some interest to compare the transaminases found in *Neurospora crassa* with those which have recently been reported to occur in a number of other organisms. There are few obvious homologies. Rudman & Meister (1953) report two transaminases in *Escherichia coli* catalysing transamination between glutamate and (a) phenylalanine, tyrosine, tryptophan and aspartate and (b) isoleucine, norleucine, leucine, valine and norvaline. The amino acids in each group transaminate with others of the same group as well as with glutamate, a possibility which has not been tested with *Neurospora*. The same authors also detected a valine-alanine transaminase in *Esch. coli*. The situation in *Esch. coli* thus seems to be quite different from that in *Neurospora*, a difference which might be expected to be reflected in differences in the types of amino acid-requiring mutants obtainable in the two organisms (cf. Myers & Adelberg, 1954). Wilson, King & Burris (1954) present data on transaminases in higher plants which suggest that, at least in the white lupin, the leucine-glutamate, valine-glutamate and phenyl-

alanine-glutamate transaminases are all distinct and separable enzymes. In animal tissues specific alanine-glutamate transaminases are well known (Green, Leloir & Nocito, 1945) and may be similar in their properties to the corresponding *Neurospora* enzyme, but ornithine transaminase from rat liver (Meister, 1954) is more active with pyruvate than with α -oxoglutarate, while the *Neurospora* enzyme does not react with pyruvate. Transaminases acting specifically on glutamine and asparagine (Meister, Sober, Tice & Fraser, 1952) have not, so far as the authors are aware, been looked for in *Neurospora*.

A number of transamination reactions (e.g. histidine-glutamate, glycine-glutamate, threonine-glutamate, lysine-glutamate) have been reported to be catalysed by extracts of both *Esch. coli* (Feldman & Gunsalus, 1950) and higher plants (Wilson *et al.* 1954), although they have not been found to be catalysed appreciably by *Neurospora* extracts. In view of the rather striking effects on enzyme production reported in the present paper, it seems possible that evidence for additional transaminases in *Neurospora* might be obtained, if special media were used.

Observations parallel in many respects to those reported here were made by Roberts, Ayengar & Posner (1953) in the case of β -alanine-glutamate and γ -aminobutyric acid-glutamate transaminase activities in *Aspergillus fumigatus*. Equal stimulation of both activities was observed when either β -alanine or γ -aminobutyric acid was used as sole source of nitrogen for growth of the mould.

SUMMARY

1. The addition of certain amino acids to the growth medium of wild-type *Neurospora crassa*, mating-type A, number 126-1, results in increased concentrations of certain transaminases in mycelial extracts. At least four different enzymes must be postulated to explain the effects observed.

2. The alanine-glutamate transaminase (in the absence of added pyridoxal phosphate) has variable and generally low activity and is not markedly affected in its production by exogenous amino acids.

3. The aspartate-glutamate transaminase generally showed rather high activity which was not consistently affected by added amino acids.

4. The ornithine-glutamate transaminase activity appears to be stimulated 2- to 3-fold by a variety of amino acids including arginine.

5. Nine other transaminase activities appeared to be correlated, and it is suggested that they may be properties of a single enzyme which is capable of catalysing glutamate formation from, in decreasing order of activity, phenylalanine, isoleucine, valine, leucine, norleucine, norvaline, methionine, α -aminobutyric acid and tryptophan. The production of this enzyme, or correlated group of enzymes, is increased usually at least 5-fold by the presence in the medium of valine, leucine, isoleucine, phenylalanine or methionine.

6. The activities of both the alanine-glutamate transaminase and the transaminase referred to in (5) were stimulated by fortification of the reaction mixture with pyridoxal phosphate. The other two enzymes showed no response to pyridoxal phosphate.

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