Ornithine Transaminase in *Neurospora* and its Relation to the Biosynthesis of Proline

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The mould *Neurospora crassa* is normally able to interconvert proline and ornithine, since either proline or ornithine will satisfy the growth requirement of certain mutant strains (Srb, Fincham & Bonner, 1950). Another mutant requires proline specifically, while numerous other strains, representing at least four different genetic loci, respond to ornithine but not to proline (Srb & Horowitz, 1944). A knowledge of the enzymic mechanisms involved in the interconversion of these two amino-acids is clearly necessary for a fuller understanding of the effects of the mutations concerned.

The present study was suggested by the possibility that one step in the conversion of ornithine to proline might be the transfer of one of the amino groups of ornithine to an amino acceptor such as α -ketoglutaric acid. Quastel & Witty (1951) have reported the presence of an ornithine transaminase in animal tissues, and the author (Fincham, 1951) found that ornithine was one of a number of amino-acids to be transdeaminated by *Neurospora* extracts. This paper deals in more detail with the *Neurospora* enzyme and with the nature of the products of the reaction.

MATERIALS AND METHODS

Chemicals

α -Ketoglutaric acid and L-ornithine dihydrochloride were used as supplied by L. Light and Co. and Roche Products, respectively, and were neutralized with NaOH for use. DL-Glutamic γ -semialdehyde diethyl acetal was synthesized and kindly supplied by Dr N. Good (Good & Mitchell, 1952). It was converted to the free aldehyde by dissolving in 0.3N-HCl at room temperature and neutralizing with NaOH 15–30 min. later, when it could be demonstrated chromatographically that all the acetal had disappeared. A specimen of *o*-aminobenzaldehyde was kindly supplied by Dr H. J. Vogel. For use it was made up in 0.01M aqueous solution with the addition of about 2% (v/v) of ethanol to aid dissolution.

Enzyme preparations

The *Neurospora crassa* strains used most extensively were the wild-type strain E5297 (originally isolated by Dr S. Emerson) and a recently re-isolated albino amination-deficient strain (Fincham, 1950) 47305–1088/34, which will

be referred to as strain A. The latter strain, which requires exogenous α -amino N for growth, gave about 5–6 times as much ornithine transaminase activity/mg. N in extracts as did E5297 or any other strain tested. Accordingly, strain A was used in most experiments. Mycelium was grown from a heavy conidial inoculum in Roux bottles each containing 150 ml. Fries no. 3 medium supplemented with 4 μ g. (+)-biotin/l. and, usually, L-arginine to a concentration of 0.0025M. Each litre of Fries medium contains 5 g. ammonium tartrate, 1 g. NH_4NO_3 , 1 g. KH_2PO_4 , 0.5 g. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.1 g. NaCl, 0.1 g. CaCl_2 , trace elements and 15 g. sucrose (Ryan, Beadle & Tatum, 1943). The high level of enzyme in strain A is not dependent on the presence of arginine in the medium, since the substitution of L-glutamic acid for arginine resulted in little diminution in the yield of enzyme; the enzyme yield of E5297 is likewise unaffected by the addition of arginine to the growth medium. After incubation without agitation for 36–60 hr. at 25°, the mycelium was harvested on a large Büchner funnel, washed with about 1 l. distilled water/Roux bottle, sucked fairly dry, pressed between layers of absorbent paper, weighed and ground in an ice-cooled mortar for 15 min. with 2 vol. of 0.0067M- Na_2HPO_4 - KH_2PO_4 buffer (pH 7.3) with the addition of powdered glass. The resulting cream was centrifuged for 5 min. at the top speed of an M.S.E. 'Minor' centrifuge, and the almost clear supernatant was dialysed at 1–5° for 1.5–2 hr. against three changes of the same buffer as was used in the extraction. During dialysis the fluid both inside and outside the cellophan membrane was kept constantly stirred. The bag contents were usually used at once, but in a few experiments it was found that the activity was scarcely impaired by a further 20 hr. dialysis at 5°. Extracts prepared in this manner contained about 1.5 mg. N/ml.

Measurement of enzyme activity

In most experiments Warburg manometers were used. Each manometer vessel contained, in the main cup, 0.8 ml. dialysed mycelial extract, 0.3 ml. 0.05M- NaHCO_3 and 0.1 ml. 0.2M-sodium α -ketoglutarate, and, in the side arm, 0.1 ml. 0.05M- NaHCO_3 , 0.1 ml. 0.2M-ornithine hydrochloride and 0.2 ml. distilled water (with distilled water in place of either or both reactants in control vessels). The gas atmosphere was 5% CO_2 :95% N_2 (by vol.) giving a theoretical pH of 7.31. The reaction was started by tipping in the contents of the side arm; it was stopped, and most of the protein precipitated, after dismantling the manometers, by either adding to each flask 0.05 ml. of 5% (w/v) aqueous acetic acid and heating to 100° for 15–30 sec. or adding 0.05 ml. of 10% (w/v) aqueous Na_2WO_4 and 0.05 ml. 10N-HCl. The bath temperature was 35°.

Determination of amino-acids

Paper chromatography by the ascending method was used for the identification and approximate quantitative determination of amino-acids. For quantitative work the solvent *n*-propanol-acetic acid-water (75:5:20 by vol.), which gave a good separation of ornithine and glutamic acid with R_F values 0.06 and 0.19 respectively, was used. Standard-sized (0.8 μ l.) spots of deproteinized digests were applied 9 mm. apart along a line 15 mm. from the edge of a sheet of Whatman no. 1 filter paper. Series of ornithine and glutamic acid standards were applied to the same sheet, and, to ensure the maximum possible uniformity in spot size, colour and R_F value between standards and experimentals, the standard spots were generally superimposed on dried spots of the appropriate control digest. After overnight equilibration with the solvent vapour, chromatograms were run for 1–2 hr. (corresponding to a solvent rise of 8–12 cm.), dried, sprayed uniformly with 0.25% (w/v) ninhydrin in *n*-butanol, and allowed to dry at room temperature. For critical comparisons, unknown and standard spots were labelled on one side in pencil, cut out, shuffled, and arranged in order of intensity by inspection of the reverse side. The arrangement of the standards in the right order served as a check on the reliability of each chromatogram. With duplicated spots, amino-acid concentrations of from 1 to 12 mm could generally be determined to within 1 mm and, at the lower concentrations, determination to within 0.5 mm was sometimes possible depending on the evenness of the spots.

Determination of carbonyl compounds

Substances giving a yellow product with 2,4-dinitrophenylhydrazine were determined qualitatively by chromatography as follows. First a spot of saturated dinitrophenylhydrazine in *N*-HCl and then a spot of the unknown solution were applied to the same position on the paper. Three further superimpositions of each solution, with drying between each application, sufficed to give spots of a reasonable intensity. *n*-Butanol saturated with excess 6% (w/v) aqueous NH_3 was used as the solvent, and chromatograms were run upwards for 2–3 hr. after overnight equilibration against the solvent vapour. The α -ketoglutarate derivative gave a well defined spot at R_F 0.13. In chromatograms of experimental digests the only other spots which appeared were one at R_F 0.81 which corresponded to unreacted dinitrophenylhydrazine, and another, rather faint, at R_F 0.45–0.50 (depending on the amount of protein in the test solution) which apparently corresponded to glutamic semialdehyde (see Results). The latter substance has been found to give only 1–2% of the colour yield with dinitrophenylhydrazine and alkali given by an equimolar amount of α -ketoglutaric acid. The following method was accordingly used for quantitative determination of α -ketoglutarate. To 1 ml. of deproteinized digest (diluted 1:20 or 1:40 with water) 1 ml. of half-saturated dinitrophenylhydrazine in *N*-HCl was added, followed, after 15 min. at room temperature, by 5 ml. *N*-NaOH. The red colour was read relative to a water blank on an 'Eel' photoelectric colorimeter (Evans Electroselenium Ltd., Harlow, Essex) with a green filter, and the reading compared with a linear standard curve for α -ketoglutarate.

Bioassays

Bioassays for proline and for Δ^1 -pyrroline-5-carboxylic acid were carried out using two mutant strains of *Escherichia coli*, 55-1, which requires L-proline for growth, and 55-25, which will grow with either proline or pyrrolinecarboxylic acid (Vogel & Davis, 1952). The cultures of these two strains were kindly supplied by Dr B. D. Davis. Maintenance of the cultures and the bioassay methods were essentially as described by Vogel & Davis. Deproteinized digests which were found to be active in promoting growth of the *Esch. coli* mutant 55-25 were acidified with HCl and applied to a line 15 mm. from the edge of a filter-paper sheet in a row of 0.8 μ l. spots, 2.5 mm. apart. Three superimposed applications along the same line gave a sufficiently high concentration of growth-promoting substance. The chromatograms were developed with three different solvents (Table 2) for a time sufficient for the solvent to rise 10–11 cm. above the starting line in each case. After drying, the chromatograms were cut into strips 15 mm. wide, parallel to the direction of solvent flow, and each strip was cut into sections 5 mm. wide from the starting line upwards. These sections were dropped into small test tubes each containing 1 ml. of the medium of Davis & Mingioli (1950) ('Medium A': Vogel & Davis, 1952) from which glucose had been omitted; the media so produced were autoclaved to 1 atmosphere excess pressure and inoculated with strain 55-25 as single drops of cell suspension in 20% (w/v) glucose. After incubation for 24 hr. at 30°, cultures were diluted to 6 ml. and their turbidities measured in the 'Eel' colorimeter.

RESULTS

Preliminary results concerning this and other transaminase systems in *Neurospora* have already been reported (Fincham, 1951). The pH optimum for the ornithine transaminase system in phosphate buffer was found to be about 8.

Fig. 1. Displacement of CO_2 from CO_2 -bicarbonate buffer during ornithine transamination. For conditions, see text. This is Exp. 1 of Table 1.

Acid production during the reaction

It was found that when enzyme preparations were incubated with ornithine and α -ketoglutarate in bicarbonate- CO_2 buffer in a Warburg manometer there was a rapid evolution of CO_2 (Fig. 1). In two

experiments in which excess sulphuric acid was added from a second side arm after the completion of the reaction it was found that the total CO₂ liberated agreed closely with the amount of bicarbonate added to the flask; thus no extra CO₂ had been produced and the liberation of CO₂ during the transamination reaction must have been due to acid production. CO₂ was evolved at a low rate from control digests lacking both reactants, and this 'blank' rate was unaffected by the presence of ornithine alone, but was generally more or less depressed by the presence of α -ketoglutarate alone. Thus the appropriate control for the complete system was taken to be the digest lacking ornithine but not α -ketoglutarate.

Stoichiometry and the equilibrium position of the reaction. In Table 1 results of two typical experiments using the relatively very highly active extracts from strain A are given. Allowing for the

seemed unlikely to be due to inactivation of enzyme, since, when preparations of lower activity were used, it continued at a lower rate for 4 hr. or more. Furthermore, in one experiment fresh substrates were tipped in from a second side arm after this apparent equilibrium had been reached, and CO₂ liberation recommenced at about the rate expected if no enzyme activity had been lost. Accepting that the data given in Table 1 give the equilibrium position of the reaction, the equilibrium constant

$$\frac{[\text{Glutamic acid}] \cdot [X]}{[\text{Ornithine}] \cdot [\alpha\text{-Ketoglutarate}]} \text{ is about } 20,$$

assuming that ornithine is transformed to a single product X. As will be shown in the next section, this assumption is almost certainly not true, and the further transformation of the initial product probably accounts for the one-sidedness of the equilibrium found.

Table 1. Results of two experiments using dialysed extract of strain A grown on arginine-supplemented medium

(Incubation in Warburg manometers at 35° for 125 min. (Exp. 1) and 170 min. (Exp. 2). For other conditions see text.)

Exp. no.	...	...	1					2	
Manometer no.	...	...	1	2	3	4	5*	1	2
μ Moles added:									
L-Ornithine			0	20	20	20	20	0	20
α -Ketoglutarate			20	0	20	20	20	20	20
μ Moles after incubation:									
Glutamate			—	0	14-15	14-15†	0	3-4	18-20
Ornithine			0	Approx. 20	6-8	6-8	Approx. 20	0	3-5-5
α -Ketoglutarate			15.2	0	2.1	2.4	21.0	+++	+
CO ₂ displaced from buffer			1.6	2.1	7.3	7.2	0.3	1.3	8.2

* Extract boiled in no. 5.

† Glutamate in 3 and 4 determined as excess over concentration in 1.

‡ α -Ketoglutarate determinations semiquantitative in Exp. 2.

relatively small transformation of α -ketoglutarate to glutamate in the absence of ornithine, close to 1 mole of α -ketoglutarate is transformed to glutamate for every mole of ornithine disappearing. However, CO₂ was liberated only to the extent of 0.4-0.5 mole/mole of ornithine disappearing. This relation held true in a number of experiments in which the transamination reaction proceeded to various degrees of completion. Although the mechanism of the acid production is not entirely clear, the constancy of this relation to ornithine disappearance made it seem probable that the CO₂ liberation was a good index of the progress of the transamination reaction. Using the most active enzyme preparations the rate of CO₂ output in the experimental vessel fell to that in the 'no ornithine' control in about 90 min. after the start of the reaction. This apparent equilibrium had been attained in the experiments, the results of which are given in Table 1. The cessation of CO₂ production

The nature of the product of ornithine transamination

Preliminary observations. Glutamic acid was the only amino-acid which could be demonstrated chromatographically to have been formed in the reaction. Also, the ornithine transaminase system was unique among several transaminase systems in yielding no new keto acid detectable as its dinitrophenylhydrazon on a chromatogram after an experiment in which mycelial extract and substrates were incubated aerobically in phosphate buffer. No ammonia (determined in Conway units by nesslerization) was liberated during the reaction. These observations suggested that the carbonyl group initially formed condensed with the remaining amino group to form a pyrroline ring. This would provide a possible explanation for the apparent acid production since the pyrroline nitrogen might be expected to have diminished basic strength as compared with the free amino group.

2:4-Dinitrophenylhydrazone derivative. It seemed possible that a keto or aldehyde compound might be formed which was too unstable to survive the usual deproteinization procedure. Accordingly, digests from experiments of the type illustrated in Fig. 1 were applied directly to paper with dinitrophenylhydrazine without any deproteinization. The kind of result obtained is shown in Fig. 2. From the digest containing both ornithine and α -ketoglutarate a yellow spot with R_f 0.45 was obtained. There was

Fig. 2. Chromatograms of 2:4-dinitrophenylhydrazones from (A) digests from a manometric experiment of the standard type (L. to R.: complete system, no ornithine, no α -ketoglutarate) and (B) a solution containing 0.025 M-DL-glutamic γ -semialdehyde and heated enzyme preparation to give the same protein concentration as in A. The fast-running spot is unreacted dinitrophenylhydrazine.

no trace of such a spot from the control lacking ornithine, and a much fainter spot in a similar position from the control without α -ketoglutarate may have been due to streaking of the large excess of reagent present in this case. Control solutions containing the same concentration of mycelial extract and synthetic glutamic semialdehyde gave a strong yellow spot with a similar R_f value. Both the new spot in the experimental digests and the spot of the glutamic semialdehyde derivative turned orange-brown on spraying with N-NaOH (in this respect they resembled the fast-running spot of unreacted dinitrophenylhydrazine and differed from the spots of the derivatives from α -ketoglutaric acid and α -keto- δ -aminovaleric acid, which

remained yellow under these conditions). Contrary to expectation, both failed to give any colour with ninhydrin. A solution containing α -keto- δ -aminovaleric acid, the reasonable alternative to glutamic semialdehyde as the product of ornithine transamination, was obtained by oxidizing DL-proline in a manometer vessel with sheep-kidney acetone powder (Krebs, 1939). The dinitrophenylhydrazone of this compound showed a chromatographic behaviour markedly different from that of the glutamic semialdehyde derivative. In the solvent *n*-butanol-water-glacial acetic acid (77:18.5:4.5 by volume) it had R_f 0.47, while the derivatives of the product of ornithine transamination and glutamic semialdehyde ran overlapping with the excess reagent at the solvent front.

Two attempts to isolate the dinitrophenylhydrazone of the product of ornithine transamination have been unsuccessful. Large-scale digests were deproteinized with tungstic acid and added to excess dinitrophenylhydrazine in hydrochloric acid. The α -ketoglutaric acid derivative was extracted from the acidic solution with ether. There was no appreciable crystallization from the aqueous residue, although, had α -keto- δ -aminovaleric acid been present, it should have been readily isolated by this method. Qualitative observations on synthetic glutamic semialdehyde indicate that its dinitrophenylhydrazone is much more soluble in dilute hydrochloric acid than that of α -keto- δ -aminovaleric acid.

Reaction with *p*-dimethylaminobenzaldehyde. When portions of the experimental digest were heated with an equal volume of 2% (w/v) *p*-dimethylaminobenzaldehyde in N-hydrochloric acid (Ehrlich's reagent), a red colour developed. The colour was fully developed by 4–5 min. heating in a boiling-water bath, tended to fade on cooling, and became reintensified on reheating. Control digests lacking either ornithine or α -ketoglutarate did not give the test; neither did digests with both reactants but with the enzyme boiled. Solutions containing synthetic glutamic semialdehyde gave the test, but a solution containing α -keto- δ -aminovaleric acid (prepared enzymically from DL-proline) did not.

Reaction with *o*-aminobenzaldehyde. This reagent gave a strong yellow colour with ornithine transaminase reaction mixtures, but no colour with control digests lacking ornithine or α -ketoglutarate or with boiled enzyme. It was found that the enzyme reaction could be carried out in the presence of the reagent and the development of the yellow colour followed in a photoelectric colorimeter (Fig. 3). No attempt has been made to determine whether the relation between colour development and ornithine disappearance is a direct one, but it will be seen that this method provides a simple and sensitive test for the enzyme, which might be made

quantitative. A point of special interest which emerges from this type of experiment is that when the reaction proceeds in the presence of *o*-aminobenzaldehyde it goes to completion, there being no detectable ornithine remaining at the end of the reaction.

The colour reaction with *o*-aminobenzaldehyde is characteristic of Δ^1 -pyrroline and related compounds (Vogel & Davis, 1952), and, taken in conjunction with the data on the reaction with dinitrophenylhydrazine given above, it suggests strongly that the primary product of ornithine transamina-

Fig. 3. Reaction of the product of ornithine transamination with *o*-aminobenzaldehyde. Open colorimeter tubes contained: 0.0016 M-L-ornithine, 0.0016 M-sodium α -ketoglutarate, 0.0008 M-*o*-aminobenzaldehyde, 0.02 M-phosphate buffer (pH 7.3), and 0.3 ml. enzyme preparation (from strain A grown on Fries medium supplemented with glutamate) in a total vol. of 6.25 ml. The α -ketoglutarate was added at time 0. Room temp., about 18.5°. ●, complete system; ○, enzyme boiled; •, no ornithine; +, no α -ketoglutarate.

tion is glutamic γ -semialdehyde which gives Δ^1 -pyrroline-5-carboxylic acid by spontaneous ring closure. The latter compound would also be expected to react with Ehrlich's reagent, which is used as a test for pyrroles and related compounds.

Promotion of growth of bacterial mutants. Ornithine transaminase reaction mixtures, deproteinized by acidification with acetic acid and brief heating, supported the growth of *Esch. coli* mutant strain 55-25 (which responds to either proline or Δ^1 -pyrrolinecarboxylic acid, according to Vogel & Davis), but were inactive for strain 55-1 (which requires proline specifically). In Fig. 4 are shown the results of an experiment in which the growth-promoting activity for strain 55-25 of the transamination product is compared with that of L-proline. There is some indication that the shapes of

the growth-concentration curves for the two compounds are somewhat different, but the data are consistent with ornithine having been converted into a product having approximately the same activity for 55-25 as L-proline. The appreciable activity of the control digest lacking ornithine is possibly due to the presence of some residual ornithine in the enzyme preparation, which was made from mycelium grown on medium containing arginine.

Fig. 4. Promotion of growth of *Esch. coli* mutant 55-25 by deproteinized digests. C contained initially 0.012 M-L-ornithine and 0.012 M- α -ketoglutarate; B contained α -ketoglutarate only, and A received neither reactant. After incubation C contained about 0.003 M-ornithine (estimated from CO_2 displacement). The solutions were incorporated into minimal medium at 1/200 their original concentration, and further diluted with medium containing solution A, all media thus receiving equal amounts of any residual protein. 5 ml. lots autoclaved (without glucose) to 1 atmos. and inoculated with 55-25 cells in sterile glucose solution. Turbidities read on the colorimeter after 21 hr. at 34°. The results indicate growth-promoting substance equivalent to about 0.002 M- and 0.01 M-L-proline in B and C respectively.

Chromatographic behaviour. In Table 2 are given approximate R_F values both for growth-promoting substances in the digests and for synthetic glutamic semialdehyde applied to the paper in 0.3 N-hydrochloric acid. The R_F 's given by the latter substance agree well with the values reported by Vogel & Davis (1952) for Δ^1 -pyrrolinecarboxylic acid; presumably the semialdehyde is largely converted into Δ^1 -pyrroline carboxylic acid under these conditions. With 50% (v/v) aqueous ethanol as solvent the transaminase digest gives a single growth-promoting band agreeing in position with that given

Table 2. R_F values of substances promoting growth of *Esch. coli* mutant 55-25

Solvent	Glutamic γ -semialdehyde	Ornithine transaminase digest	Δ^1 -Pyrroline carboxylic acid
Phenol sat. with water-glacial acetic acid (16:1, v/v)	0.82	0.49, 0.80*	—
<i>n</i> -Butanol-glacial acetic acid-water (77:4.5:18.5, v/v)	0.24	0.06, 0.21*	0.24†
Aqueous ethanol (50%, v/v)	0.76	0.79	0.77†

* Two bands of approximately equal potency.

† Values from Vogel & Davis (1952).

by glutamic semialdehyde. With the two solvents phenol-acetic acid and *n*-butanol-acetic acid, however, the digest gave two growth-promoting bands, one of them (the faster-running in each case) agreeing in position with glutamic semialdehyde. When glutamic semialdehyde was superimposed on deproteinized digest along the starting line it augmented only the faster-running band in each case. It thus appears that Δ^1 -pyrroline-5-carboxylic acid may be partially converted to another, related, proline precursor by the *Neurospora* extract, although it is not excluded that the change takes place during the deproteinization procedure.

Reversibility of the reaction

The fact that the reaction between ornithine and α -ketoglutarate does not go to completion implies that it should be possible to demonstrate its reversibility. If glutamic semialdehyde is the primary product of the reaction, *Neurospora* extracts should convert it partially to ornithine in the presence of glutamate. Evidence that this is so was obtained from two experiments. A preliminary experiment showed an apparent formation of ornithine (detected chromatographically) whether glutamate was added to the digest or not, and it could not be said that glutamate increased the small yield of ornithine. Using mycelial extracts which had been subjected to an extra 20 hr. dialysis against phosphate buffer, however, ornithine formation was extremely small in the absence of added glutamate and considerably greater in its presence. A chromatogram illustrating the second experiment is shown in Fig. 5. The amino-acid spot presumed to be ornithine had the same R_F value as ornithine standards on both phenol-water-ammonia and *n*-propanol-acetic acid chromatograms. It will be seen from Fig. 5 that only about half as much ornithine was formed from glutamic semialdehyde as would be expected from the equilibrium position of the reverse reaction. This discrepancy can be ascribed to two causes. First, there was considerable disappearance of glutamic semialdehyde (as shown by reduced intensity of the dinitrophenylhydrazone derivative on a chromatogram) even in the absence of glutamate in this experiment, and the concentration of this substance which was available for conversion to ornithine was certainly less than the concentration added. Secondly, in the imperfectly

Fig. 5. Diagram of ninhydrin-coloured chromatogram showing enzymic formation of ornithine from glutamic γ -semialdehyde and glutamate. Tubes (60 x 9 mm.) contained α -ketoglutarate (KGA), L-ornithine (O), sodium L-glutamate (GA) (all at initial concentration 0.0125 M) and 0.025 M-DL-glutamic γ -semialdehyde (GSA) as indicated. All contained 0.08 ml. enzyme preparation in 0.05 M-phosphate buffer at pH 7.3 in a total vol. of 0.16 ml., were layered with 1 ml. liquid paraffin to exclude air and incubated 3 hr. at 35°. The chromatogram was developed with phenol saturated with water with NH_3 vapour in the atmosphere. Digests 3 and 6 gave spots in the ornithine position corresponding in intensity to, respectively, 0.001–0.002 M- and 0.003–0.004 M-ornithine standards. The faint streak indicated below the ornithine region in 2, 3, 4 and 6 is due to glutamic semialdehyde or trailing of ornithine, or both.

anaerobic conditions, there was some oxidation of glutamate to α -ketoglutarate in the absence of glutamic semialdehyde, and α -ketoglutarate so formed would tend to stop ornithine synthesis.

Presence of the enzyme in various mutant strains of Neurospora

Ornithine transaminase activity was detected in extracts of mycelium of the genetically different mutant strains 27497, 21502, 34105 and 29997, all of which require ornithine, citrulline or arginine for growth (Srb & Horowitz, 1944). The enzyme was also obtained from strain 21863, which has a specific requirement for L-proline, and from strains E5041, 44207 and 51506T6 which will grow with any one of the above amino-acids and also with α -amino- δ -hydroxyvaleric acid or glutamic γ -semialdehyde (Srb, Fincham & Bonner, 1950; also unpublished results of Dr A. M. Srb and the author). The amount of enzyme activity in each case was about the same as that obtained from the wild-type E5297. Washed pads of intact mycelium of strains E5297, 21863, 27947, 21502, 34105 and 29997 were all found to be active in carrying out the reaction.

DISCUSSION

There seems to be good evidence for the following picture of the ornithine transaminase reaction. The δ -amino group of ornithine is transferred to α -keto-glutaric acid to give glutamic acid and, initially, glutamic γ -semialdehyde. The last compound is, at the pH of the experiments, in equilibrium with Δ^1 -pyrroline-5-carboxylic acid through spon-

extracts into another substance which must be very closely related to it since it also will serve as a proline precursor for *Esch. coli*. Possibly this substance might be Δ^2 -pyrroline-5-carboxylic acid, which might give the same addition product with ethanol (Vogel & Davis, 1952) as the Δ^1 compound and thus be indistinguishable from it on an ethanol chromatogram. This point needs further investigation. At least one of the substances into which ornithine is transformed must have acidic properties to account for the displacement of carbon dioxide from bicarbonate buffer during the reaction.

The existence of an enzyme system transdeaminating ornithine in the δ -position was to be expected from the work of Stetten (1951) who fed DL-ornithine, labelled with ^{15}N in the α - or δ -amino groups, to mice. It was found that much of the α -nitrogen appeared in the proline and hydroxyproline of the tissue proteins, and less in glutamic and aspartic acids. The δ -nitrogen, on the other hand, contributed more to glutamic acid and little to proline.

It is interesting to consider the *Neurospora* ornithine transaminase system in relation to the following scheme, put forward by Shemin & Rittenberg (1945), to account for the practically equal labelling of the α - and δ -amino groups of the ornithine derived from the tissue arginine of rats fed with [^{15}N] glycine.

taneous, though evidently reversible, ring closure. The further transformation of a large part of the initial product of the reaction accounts for the observed equilibrium position being towards the glutamate side and away from ornithine. When *o*-aminobenzaldehyde is added to trap the Δ^1 -pyrroline-5-carboxylic acid, probably by formation of the dihydroquinazolinium compound (see Vogel & Davis, 1952), the transfer of the δ -amino group of ornithine goes to completion. The same enzyme preparations will catalyse the formation of ornithine from glutamic γ -semialdehyde in the presence of glutamate, and it seems highly probable that the same enzyme is involved as in the reverse reaction. The chromatographic behaviour of reaction mixtures suggests that the Δ^1 -pyrroline-5-carboxylic acid may be partially transformed by *Neurospora*

If an ornithine transaminase similar to that of *Neurospora* occurs in animals, which seems likely from the work of Quastel & Witty (1951), the part of the cycle from ornithine to pyrrolinecarboxylic acid is accounted for. The postulated mechanism for the conversion of proline to ornithine is still hypothetical. α -Keto- δ -aminovaleric acid is known as the oxidation product of D-proline in the presence of mammalian D-amino-acid oxidase (Krebs, 1939), but has not yet been implicated in the metabolism of L-proline. It seems probable, however, that there is some mechanism for the biosynthesis of ornithine other than the reversal of the ornithine transaminase reaction, since the *Neurospora* mutants which require ornithine, citrulline or arginine for growth show a normal amount of ornithine transaminase activity. This seems to imply that the

ornithine transaminase system alone is not adequate as an ornithine-synthesizing mechanism *in vivo*. Possibly one of the steps in the hypothetical part of the above cycle is deficient in these mutants.

The conversion of Δ^1 -pyrroline-5-carboxylic acid to proline certainly occurs in *Esch. coli* and *Neurospora* since either of these compounds will satisfy the growth requirements of certain mutant strains of both organisms. The *Esch. coli* mutant 55-1 and the *Neurospora crassa* mutant 21863, both of which require proline specifically, are presumably unable to carry out this conversion. Taggart & Krakaur (1949) have concluded that L-proline is oxidized by the mammalian cyclophorase system by way of glutamic semialdehyde, and the reactions connecting these two compounds may be freely reversible.

SUMMARY

1. An enzyme present in extracts of *Neurospora crassa* mycelium transfers the δ -amino group of L-

ornithine to α -ketoglutaric acid with the formation of glutamic acid and glutamic γ -semialdehyde. The latter compound is partly converted, probably spontaneously, to Δ^1 -pyrroline-5-carboxylic acid, and there is some evidence that this is in enzyme-catalysed equilibrium with another closely related compound.

2. If *o*-aminobenzaldehyde is added to trap the Δ^1 -pyrrolinecarboxylic acid the reaction goes to completion; otherwise, under the conditions of the experiments, it comes to equilibrium with about 80% of the ornithine deaminated, starting with equimolar amounts of ornithine and α -ketoglutarate.

3. Evidence has been obtained for the synthesis of ornithine from glutamic γ -semialdehyde and glutamate by the same enzyme preparations.

4. Both mycelial extracts and intact mycelium of mutant strains of *Neurospora* having a growth requirement for ornithine showed a normal amount of ornithine transaminase activity.

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Partition Chromatography of Insulin and Other Proteins

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A method for partition chromatography of ribonuclease has been described in which use is made of two-phase mixtures prepared from ammonium sulphate, ethylene glycol monoethyl ether (cellosolve) and water with kieselguhr as support for the stationary phase (Martin & Porter, 1951). Further investigation of phase mixtures of a similar type, but using other glycol ethers and a variety of inorganic and organic solutes, has shown that a considerable number of such systems can be produced. Many of these are suitable for the partitioning of proteins and in some cases they may be adapted for successful partition chromatography. This paper describes the difficulties inherent in the technique and its particular application as a method of

isolation or assay for insulin. Brief descriptions of the method have already been given (Porter, 1952*a*, *b*).

EXPERIMENTAL

Materials

All the glycol ethers used were redistilled after addition of SnCl_4 using a good fractionating column. The only criterion of purity used was that there should be minimal absorption at 280 $\text{m}\mu$.

The kieselguhr was Hyflo Super-Cel obtained from Johns-Manville Co. Ltd., Artillery Row, London, S.W. 1. It was allowed to stand overnight in 2N-HCl, washed with water until free of chloride and dried at 110°. Silane-treated