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[8] The terminology which we have been using for some years to describe the various hierarchies of fibrous structures is as follows: the histologically observable fibrous unit of connective tissue is called the "fiber"; the structures observable in the electron microscope as the unit which is cross-striated and usually 500–1,000 A wide is called the "fibril"; when fibrils of collagen are treated with acid, they fray into thinner bundles, the "filaments"; the smallest fibrous units which show all the properties of collagen are called "protofibrils"—they may be one or only a few polypeptide chains in thickness.

[9] We are indebted to Dr. Karl Schmid for the preparation of purified α-1 acid glycoprotein obtained from human plasma.

[10] Glycine was determined by Christenson, Riggs, and Ray's (*Anal. Chem.*, **23**, 1521, 1951) modification of the method of Alexander, Landwehr, and Seligman (*J. Biol. Chem.*, **160**, 51, 1945), and hydroxyproline by Martin and Axelrod's (*Proc. Soc. Exptl. Biol. Med.*, **83**, 461, 1953) modification of the method of Neuman and Logan (*J. Biol. Chem.*, **184**, 299, 1950).

[11] The experiment was performed in this manner because lyophilized SLS is only partially soluble in neutral or slightly alkaline salt and buffer solutions and essentially insoluble in dilute acid. An aliquot of lyophilized SLS served to indicate the weight of the starting material. The conversion was performed with the second aliquot of wet SLS. It should be noted that excessive packing of an SLS precipitate by centrifugation will reduce its solubility.

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*ON THE GLUTAMATE-PROLINE-ORNITHINE INTERRELATION IN NEUROSPORA CRASSA**

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The metabolic relationship involving glutamate, proline, and ornithine has attracted the attention of a number of investigators working with a variety of species. With respect to mammals, the details of this relationship have been lucidly analyzed by Stetten.[1] In *Escherichia coli* an interrelation has been demonstrated[2] which differs substantially from that described by Stetten for mammals. A relationship involving these three amino acids has also been recognized in *Penicillium* and *Neurospora;* however, in these organisms the details are still obscure. Thus, Bonner suggested that proline and ornithine are formed from glutamate via a com-

mon precursor,[3] and Srb, Fincham, and Bonner proposed that the common precursor may be α-amino-δ-hydroxyvaleric acid or a chemical relative.[4] Fincham postulated that in *Neurospora* proline gives rise to ornithine and also considered the possibility that ornithine may form proline.[5] It was also suggested[6] that in *Neurospora* glutamic γ-semialdehyde (which is in equilibrium with the cyclized Δ¹-pyrroline-5-carboxylate) is a precursor of proline, since certain *Neurospora* mutants respond alternatively to proline or to glutamic γ-semialdehyde.[2, 6, 7]

The present investigation was undertaken to clarify the glutamate-proline-ornithine interrelation in *Neurospora* and to furnish information on the comparative biochemistry of these amino acids. The results obtained provide detailed evidence for metabolic paths leading from glutamate to proline, from glutamate to ornithine, and from exogenous ornithine to proline. In these three paths glutamic γ-semialdehyde is a key intermediate, as illustrated in Figure 1.

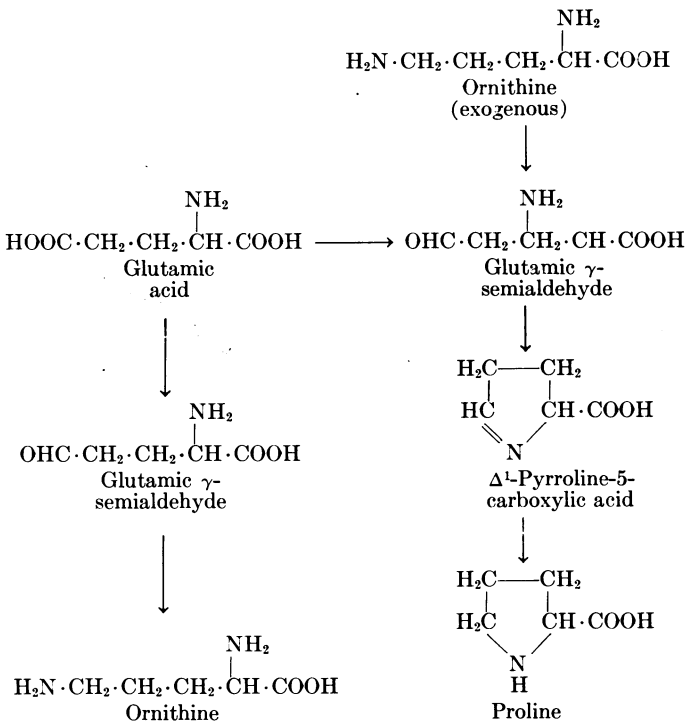

FIG. 1.—Glutamate-proline-ornithine interrelation in *Neurospora*. Glutamic γ-semialdehyde is shown twice to emphasize the possibility that the semialdehyde as proline precursor does not form a common pool with the semialdehyde as ornithine precursor. See text. The arrows shown are in the direction of biosynthesis.

These studies were primarily based on the ability of mycelial pads of proline- or ornithine-requiring mutants to excrete relevant products upon incubation with appropriate supplements, and also on growth responses of such mutants. The characteristics of the mutant strains used are summarized in Table 1.

For the mycelial-pad experiments, the mutants were grown in 125-ml. Erlenmeyer flasks containing 20 ml. of minimal medium[8] and an appropriate supplement (1 mg. of L-arginine hydrochloride for strain B or 2 mg. of L-proline for strain A; see

Table 1). After inoculation with conidia of the desired strain and incubation at 30° C. for about 40 hours without shaking, the mycelial pads obtained were gently lifted out of the flasks, rinsed by suspending in distilled water, resuspended in 3.5 ml. of 0.1 *M* phosphate at pH 7 (or pH 4.5, as indicated), and incubated with shaking at 30° C. for about 3 hours to permit depletion of endogenous substrates. Without renewal of the medium, the desired substrates were then added in a volume of 0.5–1 ml., and incubation with shaking at 30° was continued for an additional 8 hours. The mycelia were then removed, and the supernatant media were tested qualitatively and quantitatively for specific products formed.

TABLE 1

MUTANT STRAINS OF *Neurospora crassa*

Present Designation	Source Designation	Reference*	Source†	Growth Requirement‡
Strain A	21863-49A	Re of 21863§	I	p
Strain B	29997R1	Re of 29997‖	II	o, c, a
Strain C	35401R1A	Re of 35401♯	II	g, p, o, c, a
Strain D	44207-P787-4a	Re of 44207♯	II	g, p, o, c, a
Strain E	51506-T6a	Original strain♯	I	g, p, o, c, a

* Re = reisolate.
† I = kindly furnished by Mrs. M. Bonner; II = kindly furnished by Mrs. M. B. Mitchell.
‡ *p* = proline; *o* = ornithine; *c* = citrulline; *a* = arginine; *g* = glutamic γ-semialdehyde. The growth requirements are satisfied by any one of the substances indicated. The requirement of strain A is not satisfied by *g*, *o*, *c*, or *a*; that of strain B, not by *g* or *p*.
§ G. W. Beadle and E. L. Tatum, *Am. J. Botany*, **32**, 678–686, 1945.
‖ A. M. Srb and N. H. Horowitz, *J. Biol. Chem.*, **154**, 129–139, 1944.
♯ A. M. Srb, J. R. S. Fincham, and D. Bonner, *Am. J. Botany*, **37**, 533–538, 1950.

Ornithine to Proline.—In order to test for a possible conversion of ornithine to proline, mycelial-pad experiments were carried out with strain B. In a typical experiment a mycelial pad was incubated at pH 7, as described, with 7.5 micromoles of L-ornithine per milliliter. It was found that 0.9 micromole of L-proline per milliliter had been excreted into the medium. The presence of proline was recognized by bioautography, using the proline-requiring mutant strain 55-1 of *E. coli*.[6] The same strain was used for quantitative assay in liquid medium.[6] In control experiments without added ornithine, only traces of proline were found in the supernatant medium.

Glutamic γ-Semialdehyde to Proline.—Since glutamic γ-semialdehyde had been proposed as a precursor of proline,[6] analogous experiments were performed with the semialdehyde as a supplement instead of ornithine. Again using strain B, 8 micromoles of synthetic DL-glutamic γ-semialdehyde[6] per milliliter yielded 0.3 micromole of L-proline per milliliter, as determined by bioassay.[6]

Glutamate to Glutamic γ-Semialdehyde.—Experiments were also carried out to explore the possible conversion of glutamate to glutamic γ-semialdehyde. Strain A was used, because it does not give a growth response to glutamic γ-semialdehyde and might therefore be expected to excrete this compound. A mycelial pad of this strain was incubated at pH 4.5 with 50 micromoles of L-glutamate (previously adjusted to pH 4.5) per milliliter and 4 micromoles of *o*-aminobenzaldehyde per milliliter. The *o*-aminobenzaldehyde was employed as a trapping agent for glutamic γ-semialdehyde. In the supernatant medium glutamic γ-semialdehyde indeed accumulated, as indicated by the appearance of the previously described yellow color with the trapping agent.[6] Bioassay with *E. coli* mutant 55-25[6] showed that 0.5 micromole of L-glutamic γ-semialdehyde had been excreted per milliliter of super-

natant medium. No significant amounts of proline were found in the medium on testing with strain 55-1. Only traces of the semialdehyde were detected when either glutamate or the trapping agent was omitted.

Ornithine to Glutamic γ-Semialdehyde.—Since both ornithine and glutamic γ-semialdehyde were found to yield proline, the possible conversion of ornithine to glutamic γ-semialdehyde was examined. That this conversion can occur was indicated by Fincham's finding of an ornithine δ-transaminase.[5] In resting-pad experiments with strain A, 7.5 micromoles of L-ornithine per milliliter gave rise to 0.4 micromole of L-glutamic γ-semialdehyde per milliliter.

Selective Inhibition of Glutamic γ-Semialdehyde Formation.—In so far as both glutamate and ornithine can yield glutamic γ-semialdehyde, experiments were conducted to determine whether or not the conversion of glutamate to glutamic γ-semialdehyde involves ornithine as an intermediate. Again, using mycelial pads of strain A, the inhibitory effect of semicarbazide was examined, since it seemed possible that this effect might be selective (in view of the likelihood that ornithine δ-transaminase is pyridoxal phosphate–dependent and hence semicarbazide-sensitive). As seen in Table 2, semicarbazide at a concentration of 1 micromole per

TABLE 2

SELECTIVE EFFECT OF SEMICARBAZIDE ON GLUTAMIC γ-SEMIALDEHYDE FORMATION BY MYCELIAL PADS*

Substrate†	Semicarbazide†	Glutamic γ-Semialdehyde†
Glutamate, 50	0	0.5
Glutamate, 50	1	0.5
Glutamate, 50	4	0.2
Ornithine, 7.5	0	0.4
Ornithine, 7.5	1	0.1
Ornithine, 7.5	4	0.0

* Proline-requiring *Neurospora* mutant strain A was used. See text for details.
† All the figures in this table represent concentrations in micromoles per milliliter.

milliliter does not affect semialdehyde formation from glutamate; however, semialdehyde formation from ornithine is inhibited to the extent of 75 per cent. At a semicarbazide concentration of 4 micromoles per milliliter, the conversion of ornithine is completely inhibited, while semialdehyde is still formed from glutamate, albeit in reduced amounts. These data thus suggest that the conversion of glutamate to semialdehyde does not proceed via ornithine.

Evidence against Proline as Ornithine Precursor.—Since in mycelial-pad experiments no evidence could be obtained for a conversion of proline to ornithine, experiments were done to determine whether such evidence could be adduced by means of another technique. Accordingly, growth tests (cf. Srb *et al.*[4]) were performed with strain A, using mixed supplements of proline and ornithine. When this strain was grown on limiting amounts (0.03–0.1 micromole per milliliter) of proline, addition of L-ornithine hydrochloride (0.03–0.1 micromole per milliliter) had no sparing or stimulating effect on growth. These findings suggest that proline does not readily yield ornithine and therefore probably is not a quantitatively important ornithine precursor. These conclusions thus render unlikely as a major path the cyclic scheme (with proline as ornithine precursor) which had been proposed for mammals by Shemin and Rittenberg[9] and for *Neurospora* by Fincham.[5]

Accumulation of α-Ketoisovalerate by Alternative Proline or Ornithine Responders.—During an investigation of the characteristics of strains C, D, and E (see Table 1),

which respond alternatively to proline, ornithine, citrulline, or arginine,[4] or glutamic γ-semialdehyde,[2,6,7] it was noted that these strains accumulate in their culture filtrates unusually large amounts of keto acids. Among these, α-ketoisovalerate was identified and estimated to occur in concentrations of 50–100 mg. per liter of culture filtrate. The identification of α-ketoisovalerate was based on paper chromatography,[10] combined with inhibition of the K-12 strain of *E. coli*,[11] and on the properties of the 2,4-dinitrophenylhydrazone of this keto acid, including melting point (199° C.), mixture melting point, and infrared spectrum.[12] The relationship, if any, between α-ketoisovalerate accumulation and the growth requirements of these mutants has not yet been analyzed.

Conclusions and Discussion.—The foregoing mycelial-pad experiments provide evidence for the following pathways:

a) Glutamate → glutamic γ-semialdehyde → Δ^1-pyrroline-5-carboxylate → proline.

b) Ornithine → glutamic γ-semialdehyde → Δ^1-pyrroline-5-carboxylate → proline.

c) Glutamate → glutamic γ-semialdehyde → ornithine.

Path *c* is indicated by the present finding that glutamate can form glutamic γ-semialdehyde, together with the earlier one by Fincham[5] that *Neurospora* has an enzyme which can convert the semialdehyde to ornithine.

In an effort to ascertain the physiological significance of these three paths, the characteristics of various relevant mutant strains were considered.

One class of mutants (strains C, D, and E) was shown to respond alternatively to proline, ornithine, citrulline, or arginine[4] and was subsequently found to respond also to glutamic γ-semialdehyde.[2, 6, 7] It was previously suggested[4] that such mutants are blocked in the synthesis of a common precursor of proline and of ornithine (which, in turn, is known to form arginine via citrulline[13]). The alternative response to proline or ornithine was thought to be due to a ready interconvertibility of these two amino acids via the common precursor, so that either amino acid could satisfy the requirement for both. The present results, which show that ornithine is readily converted to proline but not proline to ornithine, suggest that the interpretation of the behavior of these mutants needs reconsideration. Moreover, these results indicate that the deficiency in mutants such as strains C, D, and E is specifically in the synthesis of proline and not in that of arginine. Consistent with this conclusion are the growth responses of these and other mutants, as recorded by Srb *et al.*[4] Their data show that for mutants such as strain B (which respond to ornithine, citrulline or arginine but not to proline, and hence appear to have an arginine requirement) citrulline is a much more effective growth factor than ornithine. In contrast, for mutants such as strains C, D, and E, ornithine is a far better growth factor than citrulline. In the latter class of mutants, added ornithine therefore probably satisfies a requirement other than one for arginine. In view of the known convertibility of ornithine to glutamic γ-semialdehyde, the requirement satisfied by ornithine is presumably one for proline, with intermediate formation of glutamic γ-semialdehyde. The response to added glutamic γ-semialdehyde is also consistent with this interpretation, and so is the response to citrulline or arginine, since the latter two amino acids are known to give rise to ornithine via the "ornithine cycle".[13] The ornithine so formed may function as a source of glutamic γ-semialdehyde, as mentioned before.

It thus appears that mutants such as strains C, D, and E can make ornithine for arginine synthesis but nevertheless have a requirement for proline. Consequently, this endogenous ornithine apparently is not available for adequate proline synthesis. Similarly, if path *c* is a quantitatively substantial one, glutamic γ-semialdehyde as ornithine precursor does not seem to be available for adequate proline formation. It may therefore well be that *Neurospora* is organized in such a manner that the paths of proline and ornithine synthesis are physically separated. It is suggested that such a separation may be caused by a spatial organization of relevant enzyme systems,[14] with a resulting more or less restrictive "channeling" of metabolites.

The present interpretation of mutants such as strains C, D, and E also has a bearing on the evaluation of the significance of path *a*. It has been concluded that in these mutants glutamic γ-semialdehyde satisfies a proline requirement. This conclusion and the fact that proline-requiring strain A does not give a growth response to glutamic γ-semialdehyde but can excrete this substance in pad experiments indicate that *a* is a quantitatively major endogenous pathway.

The available information does not permit an evaluation of the quantitative significance of path *c*, leading to ornithine. The known genetically different ornithine-requiring mutants such as strain B and others[13] have been of little help in clarifying ornithine synthesis; on the other hand, the present results may aid in understanding the nature of these mutants. In so far as ornithine has been concluded to participate in a channeled pathway, the possibility merits consideration that these mutants are blocked through a disturbance in enzyme organization rather than through the actual absence (cf. Fincham[5]) of an enzyme. Such a disturbance could effect either the formation of ornithine or its utilization in the synthesis of arginine via citrulline.

The conclusions drawn here, from a study of *Neurospora* mutants, with respect to pathways as well as to channeling are fully consistent with metabolic relationships deduced from tracer experiments with *N. crassa* and *Torulopsis utilis* wild-type strains.[15]

From the point of view of comparative biochemistry, the picture of the glutamate-proline-ornithine interrelation now proposed for *Neurospora* is different from that found in *E. coli*[2] but is strikingly similar to that reported for mammals.[1] In *E. coli* ornithine is formed via several acetylated intermediates[2] and, when supplied exogenously, is converted to proline only to a minor extent.[16] In *Neurospora*, on the other hand, no evidence could be found for the participation of such acetylated intermediates,[2,17] and exogenous ornithine is readily converted to proline. Thus, although all these organisms utilize glutamate, proline, and ornithine as metabolites, the pathways associated with these metabolites show differences. Such instances of biochemical diversity should prove useful in the study of taxonomical and evolutionary relationships. In this connection it seems especially interesting that the fungus *Neurospora* more closely resembles mammals than it does the bacterium *E. coli*.

Summary.—In *N. crassa* proline is mainly synthesized from glutamate via glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylate. Another route to proline proceeds from exogenous ornithine via glutamic γ-semialdehyde and Δ^1-pyrroline-5-carboxylate.

Evidence for the sequence glutamate–glutamic γ-semialdehyde–ornithine has been obtained, but the quantitative significance of this path is unknown.

Neurospora mutants responding alternatively to proline, ornithine, or glutamic γ-semialdehyde appear to be blocked between glutamate and glutamic γ-semialdehyde as proline precursor. Such mutants accumulate keto acids, among which α-ketoisovaleric acid has been identified.

A mutant responding to proline but not to glutamic γ-semialdehyde presumably is blocked in the conversion of the semialdehyde to proline.

Evidence has been presented which indicates that certain of the metabolites involved in the glutamate-proline-ornithine interrelation may be "channeled" as a result of the spatial organization of relevant enzyme systems.

The possibility has been considered that certain *Neurospora* mutants responding to ornithine but not to proline may be blocked through a disturbance in enzyme organization.

The glutamate-proline-ornithine interrelation in *Neurospora* is strikingly similar to that in mammals but differs from that in *E. coli*.

* This investigation was supported in part by a fellowship (to H. J. V.) from the Damon Runyon Memorial Fund, and in part by a grant (to D. M. B.) from the Atomic Energy Commission, Contract No. AT-(30-1)-1017. Preliminary note: H. J. Vogel, *Bacteriol. Proc.*, p. 101, 1954.

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[12] Infrared spectra of the 2,4-dinitrophenylhydrazone derived from the natural product and of α-ketoisovaleric acid 2,4-dinitrophenylhydrazone were kindly compared and found identical by Drs. H. H. Wasserman and D. D. Grassetti.

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[15] P. H. Abelson and H. J. Vogel (to be published).

[16] H. J. Vogel (to be published).

[17] No acetylornithinase activity could be detected in extracts of wild-type *N. crassa* by means of experiments analogous to those which permitted the demonstration of this enzyme in *E. coli*; cf. Vogel (1953), *op. cit.*