VMINO ACID BIOSYNTHESIS IN TORULOPSIS UTILIS AND NEUROSPORA CRASSA

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The present investigation is concerned with the biosynthesis of the commonly occurring open chain amino acids and proline. The methods used were tracer techniques, including isotopic competition. Since similar studies were recently carried out with *Escherichia coli* (1–3),[1] the results obtained here with *Torulopsis utilis* and *Neurospora crassa* are discussed from the point of view of comparative biochemistry.

EXPERIMENTAL

Methods—For the isotopic competition experiments, the methods employed were analogous to those described for *E. coli* (1).[2]

In the case of *T. utilis* (ATCC 9950), cultures growing vigorously in unlabeled glucose-salt medium[3] were centrifuged, and the organisms were resuspended in fresh glucose-free salt medium to give an optical density of about 0.075 at 650 mμ. To 20 ml. portions of the resulting suspensions were added supplements of 5 mg. of unlabeled glucose, about 5 to 10 μc. of a C^{14}-labeled tracer[4] (glucose or other tracer as indicated), and 2 mg. of any of the desired unlabeled competitors. The supplemented suspensions were incubated at about 25° with aeration until the mass of organisms approximately doubled. In some instances (as indicated), the organisms were not resuspended in fresh medium, but the tracers and competitors were added simultaneously to cultures growing in unlabeled medium.

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[1] A comprehensive report on tracer studies of amino acid biosynthesis in *E. coli* and other microorganisms is to be published shortly as a Carnegie Institution of Washington monograph.

[2] See this reference for citations of earlier work.

[3] The medium was similar to that of Baddiley *et al.* (4); an initial pH of about 4.5 was employed, and glucose (1 per cent) was substituted for sucrose.

[4] The specific activity of the uniformly labeled tracers used was about 6 mc. per milliatom of carbon; that of the glutamic acid-1-C^{14} and of the acetate-1-C^{14} employed was about 1 mc. per mmole. Unless otherwise indicated, the C^{14} compounds used were uniformly labeled.

In the case of *N. crassa* (ATCC 10336), a similar procedure was followed, except for modifications necessitated by the cultural habits of this organism. An appropriate medium[5] and strong agitation (to insure a dispersed condition of the culture) were employed. About 1 mg. (dry weight) of freshly harvested *N. crassa* was used per 20 ml. of the supplemented mixtures.

In addition to the isotopic competition studies, tracer incorporation experiments were carried out in which unlabeled glucose and a given C^{14} tracer were the only supplements.[6]

After cultivation, the organisms were harvested and subjected to the fractionation, paper chromatography, and radioautography procedures previously described (1). Isotopic competition was determined as before (1). In the tracer incorporation experiments the relative specific activities of various protein amino acids were determined (see Abelson (1)).

Materials—The C^{14}-carbon dioxide used was generated from barium C^{14}-carbonate obtained from the Oak Ridge National Laboratory. Uniformly labeled C^{14}-glucose was prepared from canna leaves (6, 7) which had assimilated C^{14}-carbon dioxide. Uniformly labeled L-amino acids (except ornithine) were isolated from *Chlorella pyrenoidosa*, which had been grown on C^{14}-carbon dioxide as a carbon source (8). Labeled L-ornithine was prepared from uniformly C^{14}-labeled L-arginine by the action of arginase (9). L-Glutamic acid-1-C^{14} was isolated from *E. coli* cultivated in the presence of C^{14}-carbon dioxide (10). Sodium acetate-1-C^{14} was obtained from a commercial source. The unlabeled L-amino acids used were commercial products. Several of the keto acids, including *d*-α-keto-β-methylvaleric acid (the keto analogue of L-isoleucine), were kindly furnished by Dr. Alton Meister. Synthetic N^{α}-acetyl-L-ornithine (11) and DL-glutamic γ-semialdehyde (12), which is in equilibrium with the cyclized Δ^1-pyrroline-5-carboxylic acid, were prepared as previously described.

RESULTS AND DISCUSSION

Classification of Amino Acids—The present results obtained with *T. utilis* and *N. crassa* support the subdivision of the open chain amino acids and proline into five groups on the basis of the biosynthetic origin of their carbon skeletons. As illustrated in Table I for *T. utilis* and *N. crassa*, with aspartate as tracer, the protein amino acids shown fall into three distinct groups with respect to relative specific activity. One group con-

[5] A modification of the medium of Beadle and Tatum (5) was employed. Tartrate was omitted to avoid possible complications, although it is not a carbon source for *N. crassa* (5). The modified medium supported adequate growth for the present purposes.

[6] The differentiation between competition and incorporation is made to emphasize differences in experimental design and for purposes of exposition.

sists of glutamate, proline, and arginine (glutamic family), a second of aspartate, methionine, threonine, and isoleucine (aspartic family), and the third of lysine. The results with carbon dioxide, acetate, or glutamate as tracer are in accord with this classification.

As shown in Table II, with alanine as tracer, heavy incorporation into protein alanine, valine, and leucine (pyruvic family) was observed, along

TABLE I

*Incorporation of Tracers into Protein Amino Acids of T. utilis and N. crassa (As Relative Specific Activity)**

Tracer	Glu	Arg	Pro	Asp	Thr	Iso	Met	Lys
T. utilis								
C^{14}-Carbon dioxide†	100	500	95	230	230	240	220	0
Acetate-1-C^{14}†	100	250	129	33	30	30	35	200
C^{14}-Aspartate	100	100	105	167	160	170	170	70
C^{14}-Glutamate	100	80	67	26	25	24	22	40
Glutamate-1-C^{14}	100	90	70	5	0	0	0	0
N. crassa								
C^{14}-Carbon dioxide†	100	440	100	160	160	160	160	0
Acetate-1-C^{14}	100	85	90	90	90	90		93
C^{14}-Aspartate	100	85	100	250	250	250	250	50
C^{14}-Glutamate	100	70	90	51	49	48		47
Glutamate-1-C^{14}	100	60	80	5	0	0		0

* The specific activity figures shown are relative to the respective protein glutamic acid values. See the text for details and foot-note 4. Glu, glutamic acid; Arg, arginine; Pro, proline; Asp, aspartic acid; Thr, threonine; Iso, isoleucine; Met, methionine; Lys, lysine.

† The high relative specific activity of arginine obtained with this tracer is presumably due to incorporation of C^{14} into the amidine carbon atom.

with light incorporation into certain amino acids. The labeling of valine and leucine presumably is introduced via pyruvate. With serine as tracer, approximately equal relative specific activities are obtained in protein serine, glycine, and cysteine (serine family).

Of the remaining protein amino acids, histidine is in a group by itself, as indicated by biosynthetic studies with *N. crassa* (13) and a yeast (14), and the aromatic amino acids form another group in these organisms (15, 16). Thus, the fungal protein amino acids may be classified as follows: (*a*) glutamic acid, proline, arginine; (*b*) aspartic acid, methionine, threonine, isoleucine; (*c*) lysine; (*d*) alanine, valine, leucine; (*e*) serine, glycine,

cysteine (cystine); (*f*) histidine; and (*g*) phenylalanine, tyrosine, tryptophan.

A similar subdivision applies to *E. coli*, except for lysine which in this organism is derived from aspartic acid, as is α,ε-diaminopimelic acid (1, 2). The latter amino acid has not been detected in fungi (17).

The results shown in Table I are in accord with the existence of a Krebs cycle in *T. utilis* and *N. crassa*.

Glutamic Family—The data in Table I are consistent with the conclusion that in *T. utilis* and *N. crassa* the 5-carbon skeletons of proline and arginine

TABLE II

*Incorporation of Tracers into Protein Amino Acids of T. utilis and N. crassa (As Relative Specific Activity)**

C^{14} tracer	Ala	Val	Leu	Ser	Gly	Cys
T. utilis						
α-Alanine	100	40	60	5	5	
Serine	0	0	0	100	100	100
Glycine	0	0	0	15	100	15
N. crassa						
α-Alanine	100	63	60	10	10	10
Serine	0	0	0	100	100	100

* With tracer α-alanine, serine, and glycine, the specific activity figures shown are relative to the respective protein α-alanine, serine, and glycine values. See the text for details. Ala, α-alanine; Val, valine; Leu, leucine; Ser, serine; Gly, glycine; Cys, cysteine.

are derived from glutamate. Table III summarizes the results of isotopic competition experiments with *T. utilis*, members of the glutamic family and presumable intermediates being used as competitors. Analogous experiments with *N. crassa* gave qualitatively very similar results. Ornithine, citrulline, and arginine are seen to compete effectively as sources of protein arginine and of protein proline.[7] Proline as competitor is seen to suppress the radioactivity of protein proline but not that of glutamate and arginine. This result does not support the possible major rôle of proline

[7] Added ornithine appears to contribute to the amidine carbon of arginine, possibly via carbon dioxide liberated through decarboxylation. Added ornithine also contributes to glutamate, probably via glutamic γ-semialdehyde. Citrulline and arginine presumably contribute to proline via ornithine, formed through the "ornithine cycle" (18); the ornithine formed may then yield proline via glutamic γ-semialdehyde.

as ornithine precursor proposed for *T. utilis* (19) and *N. crassa* (20). Glutamic γ-semialdehyde contributes to protein proline and to some extent to glutamate and arginine.

Evidence has been obtained with *N. crassa* mutants that glutamic γ-semialdehyde is a precursor not only of proline but also of ornithine and hence arginine (21).[2] The present finding that the semialdehyde contributes to arginine is consistent with a rôle of the semialdehyde as ornithine precursor in *T. utilis* as well as *N. crassa*. However, in view of the contribution of added semialdehyde to glutamate, it seems possible that at least part of the exogenous semialdehyde did not directly form ornithine.

TABLE III

*Effect of Competitors on Radioactivity of Amino Acids of Glutamic Family in T. utilis (As Per Cent Radioactivity of Protein Amino Acids Relative to Control)**

C^{12} competitor	Glu	Arg	Pro
None (control)	100	100	100
Ornithine†	75	12	4
Citrulline†	95	25	25
Arginine†	95	15	15
Glutamic γ-semialdehyde	65	70	6
Proline	100	100	0

* The tracer employed was C^{14}-glucose. See the text for details. Glu, glutamic acid; Arg, arginine; Pro, proline.

† The tracer and competitor were added simultaneously to a culture growing in unlabeled medium.

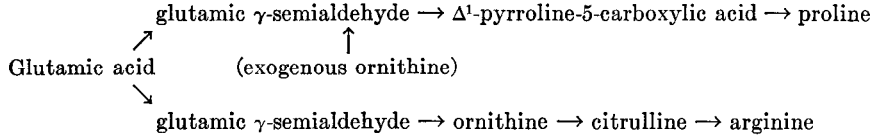
In contrast to the results with the fungi, the semialdehyde does not compete with glutamate and arginine in *E. coli* under analogous conditions (1).

Studies with *N. crassa* mutants have also shown that exogenous, but not *endogenous*, ornithine is a major source of proline, and that glutamic γ-semialdehyde as proline precursor may be distinct from the semialdehyde as ornithine precursor (21). These results have been interpreted as reflecting a physical separation of the proline and ornithine paths (21). This separation was ascribed to a spatial organization of relevant enzyme systems[8] with a resulting more or less restrictive "channeling" of metabolites (21).

[8] Thus the enzyme system involved in the formation of glutamic γ-semialdehyde as proline precursor may differ, with respect to organization and possibly to other properties, from that thought to be involved in the formation of the semialdehyde as ornithine precursor. Also, the enzyme catalyzing the ready conversion of exogenous ornithine to glutamic γ-semialdehyde may differ, as to organization and perhaps as to other properties, from the enzyme thought to be involved in the biosynthetic conversion of glutamic γ-semialdehyde to ornithine. Enzyme organization resulting

The present tracer studies point to a distinction between endogenous and exogenous ornithine in *T. utilis* also. With ornithine (at a concentration of about 1 γ per ml.) as tracer, the specific activities of proline and arginine were in the ratio, 100:6, respectively; when the labeled ornithine was diluted with carrier and used at a concentration of 50 γ per ml., this ratio was 100:80, respectively. These results indicate that the exogenous ornithine as a source of proline does not equilibrate with the endogenous ornithine as a source of arginine. On the other hand, the separation of endogenous from exogenous ornithine is not complete, as illustrated especially by the result with the diluted tracer ornithine.[9]

The present results relating to the glutamic family support the accompanying scheme.[10]



The glutamate-proline-ornithine interrelation shown above differs from the one found in *E. coli* in which ornithine is formed via several acetylated intermediates (23, 24).[11] In isotopic competition experiments with *T. utilis* and *N. crassa*, N^{α}-acetylornithine, a precursor of ornithine in *E. coli* (11, 23), did not act as a source of arginine. This finding is in harmony with the failure of N^{α}-acetylornithine to satisfy the growth requirement of *N. crassa* mutants which respond to ornithine (23), and with the lack of detectable acetylornithinase activity in extracts of wild type *N. crassa* (21).

Aspartic Family—Isotopic competition results with members of the as-

in "channeling" may be expected to promote ornithine synthesis via glutamic γ-semialdehyde by counteracting the tendency of the semialdehyde to cyclize, or by facilitating the removal of ornithine formed, or by both.

[9] Furthermore there may be a distinction between endogenous and exogenous glutamate in both *T. utilis* and *N. crassa*, as suggested by the results given in Table I. When the glutamic family is labeled via endogenous glutamate, *e.g.* with aspartate as tracer, the specific activities of the members of this family are generally less variable than with exogenous glutamate as tracer. The relative specific activities of the members of the glutamic family, moreover, vary with cultural conditions, such as washing of the cells in the case of *T. utilis*. In this organism, as well as in *N. crassa*, the extent of labeling of the glutamic family may well be affected by the nature and size of possible glutamate reservoirs (22).

[10] Glutamic γ-semialdehyde is shown twice to emphasize the possibility that glutamic γ-semialdehyde as proline precursor and glutamic γ-semialdehyde as ornithine precursor do not form a common pool. The quantitative significance of the step from glutamic γ-semialdehyde to ornithine has not been ascertained.

[11] In *E. coli*, a minor path connects the main routes to ornithine and proline (24).

partic family, and intermediates are given in Table IV for *N. crassa*. Very similar competition results were obtained with *T. utilis* with C^{14}-carbon dioxide as tracer. In addition, α-aminobutyrate was found to contribute

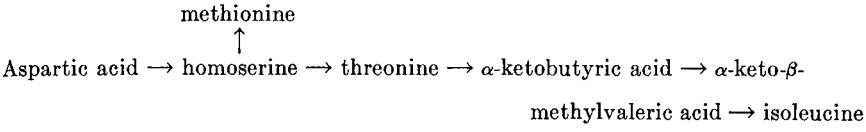
TABLE IV

*Effect of Competitors on Radioactivity of Amino Acids of Aspartic and Pyruvic Families in N. crassa (As Per Cent Radioactivity of Protein Amino Acids Relative to Control)**

C^{14} tracer	C^{12} competitor	Asp	Thr	Iso	Met	Ala	Val	Leu
Carbonate	None (control)	100	100	100	100			
"	Homoserine	100	7	7	6			
"	Threonine	90	8	6				
"	Isoleucine	100	100	12				
Glucose	None (control)	100	100	100		100	100	100
"	α-Ketobutyrate	100	100	35				
"	α-Keto-β-methylvalerate	100	100	5				
"	Pyruvate					75	63	58
"	α-Ketoisovalerate					100	64	55
"	α-Ketoisocaproate					100	100	25
"	Valine					100	43	62
"	Leucine					100	100	10

* See the text for details. Asp, aspartic acid; Thr, threonine; Iso, isoleucine; Met, methionine; Ala, α-alanine; Val, valine; Leu, leucine.

to protein isoleucine in both organisms. These results support the paths in the accompanying diagram.

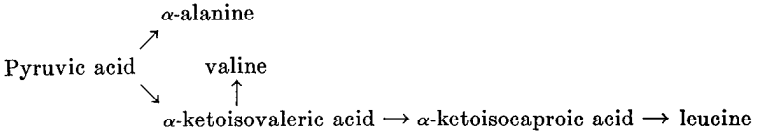


The function of aspartate as threonine precursor has previously been shown for a yeast (25), but apparently not for *N. crassa*. In the latter organism homoserine has been found to yield threonine and methionine (26), and α,β-dihydroxy-β-methylvalerate (27) forms isoleucine via its α-keto analogue (28). In *E. coli* similar interrelations among metabolites derived from aspartate have been found, except that in this organism lysine and α,ε-diaminopimelic acid are also aspartate derivatives (2).

Lysine—The data in Table I are consistent with the view that in *T. utilis* the carbon chain of lysine is built up through a combination of the succinate moiety of α-ketoglutarate with acetate (or a related compound), in confirmation of earlier results (29, 30). The results in Table I indicate

that *N. crassa* also utilizes the succinate moiety of α-ketoglutarate for lysine synthesis, but (in contrast to *T. utilis*) derives the remaining 2 carbon atoms of lysine from a precursor which does not appear to equilibrate with exogenous acetate or with Krebs cycle acetate.[12] In neither fungus does aspartate serve as a major lysine precursor, as it does in *E. coli*.

Pyruvic Family—The incorporation data in Table II with alanine as tracer show labeling of alanine, valine, and leucine. As mentioned above, the labeling of valine and leucine presumably is introduced via pyruvate. In Table IV isotopic competition data for the pyruvic family in *N. crassa* are presented. Closely similar results were obtained in analogous experiments with *T. utilis*.[13] These data support the scheme shown below.



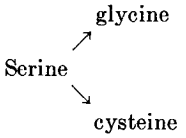
The rôle of pyruvate as valine precursor has previously been shown in *E. coli* (1) and in *T. utilis* (31). In *N. crassa*, α,β-dihydroxyisovalerate (32) and α-ketoisovalerate (28) are known to yield valine. The function of α-ketoisovalerate as a precursor of α-ketoisocaproate and leucine has thus far been demonstrated in *E. coli* only (1). The present results show that the two fungi synthesize leucine in the same manner as does *E. coli*. The conversion of α-ketoisovalerate to α-ketoisocaproate is suggested to involve the loss of a 1-carbon fragment (presumably the carboxyl carbon of α-ketoisovalerate) and addition of a 2-carbon fragment which is probably derived from acetate or a related compound, in view of the finding by Ehrensvärd *et al.* (29) that in *T. utilis* the carboxyl group of leucine appears to come from acetate carboxyl.

Serine Family—As illustrated in Table II for both *T. utilis* and *N. crassa*, tracer serine labels protein serine, glycine, and cysteine at equal specific activity. However, with tracer glycine in *T. utilis*, serine and cysteine were found to have only 15 per cent each of the specific activity of protein glycine.

These results indicate the following metabolic relationship among members of the serine family.

[12] Thus, in *N. crassa*, the results with C^{14}-glutamate as tracer are consistent with a contribution of 4 carbon atoms to lysine via α-ketoglutarate; with glutamate-1-C^{14} as tracer, lysine does not become labeled; and with acetate-1-C^{14} as tracer, the specific activity of the lysine is equal to only about that of the protein glutamate. In contrast, in *T. utilis*, with acetate-1-C^{14} as tracer the specific activity of lysine is twice that of protein glutamate.

[13] In incorporation experiments with *T. utilis* and *N. crassa*, with C^{14}-valine as tracer, significant labeling was observed only in protein valine and leucine.



It has previously been concluded (*e.g.* by Ehrensvärd *et al.* (33)) that glycine can form serine in yeast. Apparently, under the cultural conditions used here, serine is a major precursor of both glycine and cysteine in the two fungi. The same relationship has been observed in *E. coli* (1).[14]

There are, however, at least three differences in serine metabolism between *E. coli* (1) and the two fungi when these organisms are grown on glucose-salt medium. In *E. coli*, but not in the fungi, exogenous threonine contributes to serine, and serine contributes to members of the pyruvic family; in *T. utilis*, but not in *E. coli*, glycine can yield serine and cysteine, as shown in Table II.

SUMMARY

The biosynthesis of open chain amino acids and proline in *Torulopsis utilis* and *Neurospora crassa* was studied by means of tracer techniques. The results are in harmony with the existence of a Krebs cycle in these organisms; they permit the classification of the amino acids studied into groups on the basis of biosynthetic origin, and they support the following biosynthetic sequences (in both *T. utilis* and *N. crassa*): (*a*) glutamic acid, glutamic γ-semialdehyde, Δ^1-pyrroline-5-carboxylic acid, proline; (*b*) glutamic acid, glutamic γ-semialdehyde, ornithine, citrulline, arginine; (*c*) aspartic acid, homoserine, threonine, α-ketobutyric acid, α-keto-β-methylvaleric acid, isoleucine; (*d*) homoserine, methionine; (*e*) α-ketoglutaric acid, lysine; (*f*) pyruvic acid, α-alanine; (*g*) pyruvic acid, α-ketoisovaleric acid, α-ketoisocaproic acid, leucine; (*h*) α-ketoisovaleric acid, valine; (*i*) serine, glycine; (*j*) serine, cysteine. *T. utilis* and *N. crassa* apparently differ from each other in details of lysine formation. Both organisms differ from *E. coli* in several respects, including the glutamate-proline-ornithine relationship, lysine synthesis, as well as aspartate and serine metabolism.

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