Biosynthesis of Protein Amino Acids in Plant Tissue Culture IV
Isotope Competition Experiments using Glucose-U-\(^{14}\)C
and Potential Intermediates\(^1,2,3\)

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Summary. The isotope competition method with glucose-U-\(^{14}\)C as a carbon source has been used to determine whether or not selected compounds contribute carbon to the biosynthesis of protein amino acids in cells derived from Paul's Scarlet Rose. Of 48 compounds tested, 15 contributed carbon to protein amino acids. The results show for the first time that homoserine is an intermediate in threonine biosynthesis; that homoserine, cystathionine and homocysteine are intermediates in methionine biosynthesis and that histidinol is an intermediate in histidine biosynthesis in plant cells.

The results obtained in part I (5) of this series demonstrate that the isotope competition method can be used with cells of Paul's Scarlet Rose tissue cultures grown in suspension. In these experiments protein amino acids provided in the culture medium competed with the amino acids biosynthesized from glucose for incorporation into protein. This type of experiment can also be used to recognize intermediates in biosynthesis. If a compound which is not a protein amino acid causes a decrease in the amount of protein amino acid derived from glucose when it is added to the culture medium then the compound is an intermediate in biosynthesis or readily converted into an intermediate.

In this paper we report the results of extending the use of the isotope competition method to the identification of intermediates in the biosynthesis of protein amino acids in Rose tissue.

Materials and Methods

Hexahomoserine was a gift from Dr. H. P. Broquist of the University of Illinois. Glutamic semi-aldehyde was prepared according to Vogel and Davis (34).

Imidazole glycerol was prepared by the method of Ames (1). The material gave 1 spot which reacted with diazotized sulfanilic acid, on paper chromatography in \(\mu\)-butanol acetic acid-water (120:30:50) or \(\mu\)-butanol-pyridine-water (1:1:1).

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Imidazole glycerol was estimated by the periodate method of Ames (1).

\(O\)-Succinyl-\(DL\)-homoserine was synthesized by the method of Flavin, Delavier-Klutchko, and Slaughter (11). The \(O\)-acetyl derivatives of \(DL\)-homoserine and \(L\)-serine were prepared by acetylation in acid solutions as described by Greenstein and Winitz (16). Each of these compounds gave only 1 ninhydrin reacting spot when 50 \(\mu\)g was chromatographed on paper using \(\mu\)-butanol-acetic acid-water (120:30:50). The acetylated compounds were clearly separated from the parent compounds.

\(DL\)-\(\delta\)-Hydroxy norvaline, \(\delta\)-amino-\(\nu\)-valeric acid, \(\omega\)-amino-\(\nu\)-caproic acid and \(L\)-histidinol were obtained from Cyclo-Chemical Company. The \(N\)-acetyl-\(\delta\)-hydroxy norvaline was prepared using the method described by Greenstein & Winitz (16).

Acetolactate acetate (ethyl ester) was obtained from K and K Laboratories and saponified as described by Kramitz, (19). Other compounds tested were obtained from Nutritional Biochemical Company or Sigma Chemical Company.

Glucose-U-\(^{14}\)C was purchased from New England Nuclear Corporation.

The experiments reported herein were performed as previously described (5) but with the following minor modifications. The amounts of amino acids in the protein hydrolysates were estimated on aliquots of the column eluates. The ammonia was removed from the aliquots which had been made alkaline with a known quantity of NaOH, by drying in vacuo over \(\text{H}_2\text{SO}_4\). The NaOH was neutralized with the calculated quantities of acetic acid prior to the estimation of amino acid.

The sterility of all cultures was checked as described (5) and data from any found not to be sterile, was discarded.
In isotope competition experiments, the carbon source in the culture media was 0.5% glucose supplemented with 5 μc of glucose-U-14C.

The following compounds were tested at the concentrations (mM) given in parenthesis: β-alanine (1.1, 1.1, 1, 0.5, 10, 20), γ-amino-butyric acid (1.0, 0.5, 10, 20, 30), δ-amino-n-valeric acid (1, 2, 5), ω-amino-n-caproic acid (2, 5), ω-amino-n-caproic acid (1, 1, 1.2, 2), l-α-amino-transadipic acid (1.25, 1.25), DL-α-amino-adipic acid (5, 10, 20), hexahomoserine (1.3, 1.3, 2.0), l-Homocitrulline (1.0, 1.6), LL+ meso-diamino-pimelic acid (0.5, 0.5), DL-glutamic-semialdehyde (0.75, 1.5, 3.0), l-homoserine (0.8, 1.7, 3.4), O-succinyl-dl-homoserine (1.2, 5, 5), O-acetyl-dl-homoserine (0.5, 1.2, 5), α:γ-diaminobutyric acid (1, 2, 2), DL:α:α-cystathionine (0.9, 0.9, 1.3, 2.0), l-histidinol (1, 2, 5, 5), imidazoleglycerol (2, 4, 10, 20), DL-α:δ-hydroxy-nor-valine (1.1, 2), DL-α:α-cystathionine (2, 5), N-acetyl-l-threonine-4-hydroxy-nor-valine (5, 10, 20), N-acetyl-l-threonine (1.1, 2), O-acetyl-l-threonine (2, 5) with l-arginine (5), O-acetyl-l-serine (0.5, 1.2, 5), succinic acid (5, 8.5, 20, 40, 60), l-malic acid (3.75, 7.5, 20, 40, 60), pyruvic acid (2.25, 5.7, 10, 20, 50), lactic acid (2, 2, 2.2, 20, 20), DL-glyceric acid (1, 2, 10, 20, 50), dihydroxyaceton (1.1, 20), glyoxal (1, 2, 2), glycerol (50, 100), ethyl alcohol (4, 3, 40), citric acid (1, 5, 5), α-keto-glutaric acid (0.68, 5, 10, 20, 40), acetic acid (1, 1), formic acid (0.5, 1), formamide (4, 0.4, 5, 10), D-ribose (0.66, 6.6, 10, 16.5, 33), D-xylene (0.66, 6.6, 10, 33), DL-arabinose (10, 20), D-erythrose (0.84, 1.7), sucrose (14.6, 29.2), α-keto-isovaleric acid (0.1, 0.2, 0.4, 0.43, 0.5, 0.85), α-keto-isocaproic acid (0.1, 0.2, 0.4, 0.5, 0.77), phenyl-pyruvic acid (0.1, 0.2, 0.4, 0.6, 1.0), p-hydroxy-phenyl-pyruvic acid (0.5, 1.0), aceto-lactic acid (0.75, 1, 2, 4), shikimic acid (0.58, 1.0). Where the numbers are italicized, the compound was brought to pH 6.0 with NaOH, the other acids were brought to pH 6.0 with NH4OH before addition to the medium.

Results

Inhibition of Growth. Some of the compounds tested gave definite inhibition of growth. Growth of tissue was regarded as inhibited if the fresh weight was less than 90% of the controls at the end of the experiment (6). The lowest concentration (mM) at which growth inhibition was observed, was: D-ribose 16.5, n-xylene 33, δ-hydroxy-nor-valine 1, N-acetyl-δ-hydroxy-nor-valine 5, N-acetyl-aspartic acid 10, N-acetyl-asparagine 20, α:γ-diaminobutyric acid 1, O-acetyl-l-serine 2.0, O-acetyl-l-serine 5 with l-arginine 5, O-acetyl-homoserine 2, O-succinyl-homoserine 5, β-alanine 5, α-amino-adipic acid 5, α-keto-isovaleric acid 0.5, α-keto-isocaproic acid 0.5, formic acid 1, formamide 10, erythrose 0.84, pyruvic acid 5.7, succinic acid 40, malic acid 20, α-ketogluatric acid 40, α-amino-n-caproic acid 1, α-aceto-lactic acid 2, phenyl-pyruvic acid 0.4. The results of competition experiments in which growth inhibition was observed have not been included in the results.

Effects in Isotope Competition Experiments.

No reduction in the radioactivity of any protein amino acid relative to the control was detected.
when the following compounds were provided in the culture medium: β-alanine, γ-amino-butyric acid, δ-amino-n-valeric acid, ω-amino-n-caproic acid, α-amino-adipic acid, hexahomoserine, homocitulline, glutamic-semialdehyde, α: γ diamino-butyric acid, δ-hydroxy-nor-valine, N-acetyl-δ-hydroxy-nor-valine, O-acetyl-serine, N-acetyl-aspartic acid, N-acetyl-asparagine, succinic acid, pyruvic acid, glyceric acid, malic acid, lactic acid, dihydroxy-acetone, glycerol, glyoxylic acid, glyoxal, ethyl alcohol, α-keto-glutaric acid, citric acid, acetic acid, formic acid, formamide, xylose, ribose, arabinose, imidazole-glycerol or α-acetolactic acid.

The results of experiments in which reduction of radioactivity in protein amino acids occurred when unlabelled compounds were present in the culture medium of Rose tissue are shown in Table I.

In addition, in the presence of unlabeled sucrose the radioactivity of each protein amino acid in the hydrolysate is decreased in the ratio of concentration of glucose to total sugar concentration in the medium.

These results together with those previously reported for Paul’s Scarlet Rose tissue cultures (5,6) indicate that the pathways for the biosynthesis of protein amino acids shown in Figure 1 are functioning during the growth of these cells.

**Discussion**

The basis for the selection of the compounds tested in these experiments was: A) they have been isolated from plants or B) they are known to be intermediates in amino acid biosynthesis, and they were readily available.

Many compounds at concentrations that were not inhibitory had no effect on the labeling of protein amino acids. This behavior was not expected since many of the compounds tested are intermediates of glycolysis or the tricarboxylic acid cycle. Several different mechanisms exist whereby a compound which is expected to, does not contribute carbon to amino acid biosynthesis in isotope competition experiments. These include compartmentation, major usage by an alternative pathway, restricted entry into the cells or the compound may not be an intermediate. It is not possible from data in this paper to determine which mechanism accounts for the negative results.

The results presented in this paper indicate that many of the intermediates in the biosynthesis of lysine, threonine, isoleucine, methionine, histidine, phenylalanine, and tyrosine in Paul’s Scarlet Rose cells are the same as those in microorganisms (22). Some of the compounds recognized as intermediates in this study have been recognized as intermediates in amino acid biosynthesis in other plants (9). The biosynthesis of arginine was discussed in an earlier paper (7).

The results obtained with shikimic acid, phenylpyruvic acid and p-hydroxy-phenylpyruvic acid are comparable to those obtained by Gamborg and Neish (13). The data show that the keto-acids are not interconverted. Earlier, tyrosine and phenylalanine were shown not to be interconverted (5). The keto-acids however do have a common origin; shikimic acid being one common intermediate. Gamborg and co-workers (12, 14) have evidence that prephenic acid is the precursor of p-hydroxy-phenylpyruvic acid and phenylpyruvic acid in wax bean and in mung beans.

Participation of diaminopimelic acid in the biosynthesis of lysine as demonstrated in these experiments is additional evidence to that provided by Vogel (35), Shimura and Vogel (32), Finlayson and McConnell (10), and by Griffith and Griffith (17) for the presence of the diaminopimelic acid pathway of lysine biosynthesis in plants.

The conversion of threonine to isoleucine occurred in Rose tissue (5). α-Amino-butyric acid also contributed carbon to isoleucine (table I). α-Amino-butyric acid was probably deaminated to α-keto butyric acid before incorporation into isoleucine. This hypothesis is supported by evidence for the presence of the enzymes synthesizing α-aceto-α-hydroxy-butyric acid, α: β dihydroxy-β-

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**Figure 1.** Summary scheme for the biosynthesis of protein amino acids based on data from isotope competition studies. A single arrow is not intended to represent a single enzyme catalyzed reaction.
methyl-valeric acid, \(\alpha\)-keto-\(\beta\)-methyl-valeric acid and isoleucine in a number of plants (20) indicating that \(\alpha\)-keto butyric acid is an intermediate in the biosynthesis of isoleucine. The hypothesis is strengthened by the brief report of Umbarger (33) that threonine deaminase was found in extracts of cantaloupe and tobacco and by the demonstration of a threonine deaminase which was inhibited by isoleucine in vitro in extracts of Paul's Scarlet Rose tissue (8).

Bilinski and McConnell (2), Vogel (35), and Naylor, Rabson and Tolbert (24) concluded that threonine was synthesized from aspartate in plants. In part II of this series (6) evidence was presented for a metabolic relationship between threonine and aspartate. The conversion of aspartate to homoserine has been studied by Sasaoka (27, 28), Sasaoka and Inagaki (29, 30), and by Larson and Beevers (21). The demonstration that homoserine was converted to threonine in Paul's Scarlet Rose provides further evidence for this pathway.

The conversion of homoserine to methionine in higher plants has not, to the authors' knowledge, been previously reported. Cystathionine and homocysteine also contributed carbon to methionine thus outlining the pathway of methionine biosynthesis. Rowbury and Wood (26) and Delavier-Klutckho and Flavin (4) showed that O-succinyl homoserine is an intermediate in methionine biosynthesis in bacteria but not fungi. Nagai and Flavin (23) have presented evidence for O-acetyl homoserine being an intermediate in methionine biosynthesis in fungi. Giovanelli and Mudd (15) showed that extracts of spinach convert O-acetyl and O-succinyl-homoserine to cystathionine in the presence of cysteine. These compounds were tested at a series of concentrations to see if they contribute carbon selectively to methionine in Rose tissue cultures. The 2 compounds could not be distinguished from homoserine in these experiments. Hydrolysis of O-succinyl homoserine to homoserine by cell free extracts from Paul's Scarlet Rose was observed (unpublished). Such hydrolysis could account for the lack of preferential use of these compounds in methionine biosynthesis.

Knowledge of the histidine biosynthetic pathway has been obtained by studies on yeasts and bacteria (22). Siegel and Gentile (31), and Casselton (3) provided evidence indicating that imidazole-glycerol is produced when the growth of Chlorella vulgaris or Prototheca zopfii is inhibited by 3-amino-1,2,4-triazole. Histidine reverses this inhibition. They concluded that imidazole-glycerol phosphate is an intermediate in histidine biosynthesis. The data provided here show for the first time, that histidinol is an intermediate in histidine biosynthesis in higher plants.

Our results show that during growth of the Rose tissue, \(\alpha\)-keto-isovaleric acid is an intermediate in the biosynthesis of valine and leucine. This complements the demonstration of the enzymes for the synthesis of leucine from \(\alpha\)-keto isovaleric acid in maize embryos (25). The data in this paper also supplement studies showing that \(\alpha\)-keto isovaleric acid and \(\alpha\)-keto isocaproic acids are transaminated to valine and leucine, respectively, in a variety of plants (20).

The results in this paper together with those in the previous parts of this series (5, 6, 7) indicate that in the actively growing cells of Paul's Scarlet Rose in tissue culture many of the intermediates used in the biosynthesis of the protein amino acids are the same as those used by other organisms (22). The outstanding exception to this generalization is the pathway of biosynthesis of lysine which in most cells involves diamino-pimelic acid but in yeasts involves \(\alpha\)-amino adipic acid.

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