The Biosynthetic Route from Ornithine to Proline*

(Received for publication, June 7, 1978)

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It is shown by tracer experiments with DL-[2-2H,5-14C]- and DL-{(RS)-5-3H,5-14C}ornithine, that the metabolic conversion of ornithine into proline, in three plant species (Nicotiana tabacum, Datura stramonium, and Lupinus angustifolius), takes place with maintenance of the δ-hydrogen atoms but with loss of the α-hydrogen atom. This indicates a route via α-keto-δ-aminovaleric acid (5-amino-2-oxopentanoic acid) and disproves the accepted route via glutamic γ-semialdehyde (2-amino-5-oxopentanoic acid).

Standard textbooks of biochemistry (1, 2), authoritative reviews of amino acid metabolism (3-6), and metabolic charts (7) are unanimous in the view that the major route to proline (7) in mammals and in microorganisms proceeds from glutamic acid (1) by way of glutamic γ-semialdehyde (3) and Δ¹-pyrroline-5-carboxylic acid (5), and that ornithine (2) is linked to this pathway via glutamic γ-semialdehyde.

Direct derivation of proline from ornithine, if mentioned at all, is assumed to involve transformation into glutamic γ-semialdehyde, catalyzed by ornithine δ-transaminase (EC 2.6.1.13), but is regarded as of minor or questionable significance (3, 8).

Yet, it is known from tracer experiments employing samples of ornithine labeled with 14C (9-19), 15N (14, 20-24), and 2H (25), that ornithine can serve as a precursor of proline in mammals (mice) (20, 21, 25) and microorganisms (12, 15, 16, 18, 24), as well as in higher plants (9-11, 13, 14, 17, 19, 22, 23).

The existence of this metabolic relationship tends to be overlooked also in textbooks of plant biochemistry and reviews of amino acid metabolism in plants (26-29). When mentioned (30-33), it is assumed that conversion of ornithine (2) into proline (7) in higher plants proceeds with loss of the α-amino group, i.e. by the route corresponding to that accepted as occurring in mammals, bacteria, and fungi, in which glutamic γ-semialdehyde (3) constitutes the pivot in the inter-relationship of the members of the glutamic acid family of amino acids. The possibility of an alternative route from ornithine (2) to proline (7), with loss of the α-amino group, is mentioned occasionally (31-33), but supporting evidence for its existence is never presented.

Yet, the existing evidence in support of this alternative route, by way of α-keto-δ-aminovaleric acid (5-amino-2-oxopentanoic acid) (4) and Δ¹-pyrroline-2-carboxylic acid (6), although inconclusive, is at least as strong, if not stronger, than the evidence favoring the accepted pathway.

We have examined the mode of incorporation of DL-[2-2H,5-14C]- and of DL-{(RS)-5-3H,5-14C}ornithine into proline in three plant species, Nicotiana tabacum, Datura stramonium, and Lupinus angustifolius. In each case, the proline which was isolated maintained the 3H/14C ratio of the administered ornithine when [5-3H,5-14C]ornithine was the substrate, but lost more than 90% of the tritium, relative to 14C, when [2-2H,5-14C]ornithine served as precursor. This constitutes conclusive evidence that, in these plants, conversion of ornithine into proline takes place largely, if not entirely, by way of α-keto-δ-aminovaleric acid (4), and that the hitherto accepted route, via glutamic γ-semialdehyde (3), is, at best, a minor pathway.

**SCHEME 1.** Two possible routes (A and B) from ornithine to proline.

**MATERIALS AND METHODS**

**Labeled Compounds**

DL-{(RS)-5-3H,5-14C}Ornithine—The samples of intermolecularly doubly labeled DL-{(RS)-5-3H,5-14C}ornithine hydrochloride were prepared by mixing DL-[5-14C]ornithine and DL-{(RS)-5-3H}ornithine. The 14C-labeled material is commercially available (CEA, France). The 3H-labeled samples were prepared (Amersham/Searle) by catalytic reduction of ethyl α-acetamido-α-carbethoxy-γ-cyanobutyrate with tritium gas, followed by acid hydrolysis and chromatographic purification to constant activity. The distribution of tritium within the material was established by chemical degradation. A small sample of the [5-3H]ornithine monohydrochloride (approximately 50 µCi) was oxidized with acid permanganate (see below), and γ-aminobutyric...
acid and succinic acid were isolated. The molar specific activity of the former was 97 ± 2%, and of the latter 10 ± 3% of that of the original ornithine. This indicates that no more than 3% of the label was present at C-2.

The two labeled samples of ornithine were dissolved in glass-distilled water, the solutions were mixed and the doubly labeled sample purified by ion exchange chromatography. The solution was applied to a column (Dowex 50W-X8 200 (H+)). The column (5 x 0.5 cm) was washed, in successions, with water and with 2 M hydrochloric acid. Ornithine was eluted with 4 M hydrochloric acid, and the eluate was repeatedly evaporated with water in vacuo, and then dissolved in glass-distilled water (5 ml) for administration to intact plants. Paper chromatography (propan-1-ol:0.880 ammonia, 7:3, Rf = 0.15) revealed only one peak on a scanning Geiger counter (Packard Radiochromatogram Scanner model 7201) and one spot on spraying with ninhydrin.

The total activity of each of the radionuclides in the purified sample was determined by liquid scintillation counting (Nuclear Chicago Mark I liquid scintillation spectrometer) of triplicate samples, dissolved in "Aqualor" (New England Nuclear) using the channels ratio method, with a 14C external standard. The total activity due to 14C and 3H in the ornithine solutions used in individual experiments is given in Table I.

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**Table I**

| Doubly labeled ornithines used as starting materials |
|-----------------------------------------------------|
| **Experiment No.** | **plant species** | **DL-[2-3H,5-'4C]Ornithine monohydrochloride** | **DL-[5-14C]Ornithine monohydrochloride** |
| | | Nominal specific activity | Nominal total activity | Source | Nominal specific activity | Nominal total activity | Source | Nominal \[^{14}C\]/[^{3}H\] ratio |
| --- | --- | --- | --- | --- | --- | --- | --- | --- |
| 1 | (N. tabacum) | 2.1 | 1.2 | Amersham/Searle | 8.3 | 0.12 | CEA France | 10 |
| 2 | (N. tabacum) | 5 | 0.7 | Amersham/Searle | 11.5 | 0.1 | CEA France | 7 |
| 4 | (D. stramonium) | 2.1 | 0.7 | Amersham/Searle | 8.3 | 0.08 | CEA France | 9 |
| 5 | (L. angustifolius) | 2.1 | 1.0 | Amersham/Searle | 12.3 | 0.07 | CEA France | 15 |

**Administration of Labeled Ornithine to Intact Plants**

The solution (5 ml) containing the doubly labeled samples of ornithine was administered to intact plants by infusion into the stems through cotton wicks. Approximately 100 μCi of 3H (see 3H/\[^{14}C\] ratios, Table I), was used in each experiment. The plants were allowed to grow in contact with the tracer for 2 to 3 days and were then harvested. Aerial parts as well as roots were collected. The plant material was dried for 2 days at 45-50°C and was then ground in an Osterizer blender.

The details of the feeding experiments are summarized in Table II.

**Proline and Ornithine from N. tabacum**

The dry coarsely powdered plant material (tops and roots) was extracted 3 h under reflux with aqueous acetic acid (4%, ν/v, 200 ml) with occasional shaking. The mixture was kept overnight, filtered through glass wool, and the solid residue re-extracted. The combined extract (400 ml) was cooled and centrifuged. The supernatant was decanted and the residue washed with water (2 x 30 ml). The clear brown solution was extracted with ether (3 x 30 ml). The clear brown solution was extracted with ether (3 x 30 ml), the extract was discarded and the aqueous solution was basified to pH 12 with 2 M ammonia. The solution was extracted with ether (4 x 50 ml). Nicotine was isolated from this ether extract.

The aqueous phase yielded proline and ornithine. In early experiments, the aqueous phase was acidified with acetic acid, concentrated in vacuo to 50 ml and dialyzed 24 h in a cellulose dialysis bag (nominal pore size 2.5 nm, Canlab), suspended in a glass cylinder containing 150 ml of distilled water. The dialysate was removed and replaced with distilled water three times in succession. The combined dialysates were evaporated under reduced pressure to a volume of 20 ml and the solution was applied to a column (42 x 2 cm) of Dowex 50W-X8 200 (H+). The column was washed with 50 ml of water, then eluted in succession with hydrochloric acid as follows: 250 ml of 1.5 M, 750 ml of 2.5 M, and 250 ml of 4 M. Fractions (approximately 15 ml) were collected and monitored for radioactivity. Samples (about 0.2 ml) of the radioactive fractions were chromatographed on paper (aqueous ethanol (77%, ν/v, d 0.872) containing 1% v/v diethylamine). Proline (Rf 0.59) was detected by isatin, ornithine (Rf 0.39) by ninhydrin. Activity on the strip was monitored on a scanning Geiger counter. Proline emerged from the ion exchange column with 75 to 125 ml of 2.5 M hydrochloric acid, while ornithine emerged with 270 to 325 ml of 2.5 M hydrochloric acid.

In more recent experiment, membrane ultrafiltration instead of dialysis was used to separate the amino acids from high molecular weight compounds (34). Unlabeled L-proline (5 mg) was added to the concentrated aqueous plant extract (10 ml), the pH was adjusted to pH 8.5 with ammonia and the solution was placed in a mechanically stirred ultrafiltration cell (Amicon model U.M.M) fitted with a mem-
brane having a nominal pore size of 1.1 nm (Amicon UM-2). This membrane allows passage to molecules with molecular weights less than approximately 1000. The cell was pressurized with nitrogen to 35 p.s.i. When the volume of solution in the cell was reduced to 2 ml, 8 ml of water were added to the cell, three times in succession.

The combined ultrafiltrates were applied to a column (42 x 2 cm) of Dowex 50W-X8 200 (H+), the column was eluted in succession with 250 ml each of 1.5 M, 2 M, 2.5 M, 3 M, and 4 M hydrochloric acid. The activity of the eluted fractions was monitored by liquid scintillation counting. Radioactive fractions whose elution volumes corresponded to those of proline and ornithine were spotted on paper. The presence of proline was confirmed by spraying with ninhydrin (yellow) and with isatin (blue). The presence of ornithine was confirmed by spraying with ninhydrin (purple) and with vanillin (green-yellow fluorescence in ultraviolet light).

The column fraction containing ornithine was repeatedly evaporated with water in vacuo to remove excess hydrochloric acid. Ornithine hydrochloride was repeatedly recrystallized from aqueous ethanol until paper chromatography (aqueous ethanol (77% v/v, d 0.872) containing 1% v/v diethylamine (Rf 0.39)) showed a single radioactive ninhydrin-positive spot.

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2-Phenyl-1-oxopyrrol[1,2-c]imidazolidine-3-thione (Proline Phenylthiohydantoin)

Proline hydrochloride (23 mg) was dissolved in aqueous pyridine (50% v/v, 3 ml) and the pH of the solution adjusted to pH 8.6 with sodium carbonate. Phenylisothiocyanate (250 mg) was dissolved in 25% (v/v) sulfuric acid (4 ml). Potassium permanganate (1.5 M) was added dropwise at room temperature until the solution remained pink for some time. It was allowed to stand at room temperature for 24 h. A few drops of potassium permanganate solution were added to the pink color and the mixture was allowed to stand at room temperature for a further 24 h. The mixture was warmed and a hot saturated solution of barium hydroxide was added with stirring until the pH was 6.5. The solution was filtered through a fine sintered glass funnel and the residue washed with warm distilled water.

The filtrate and washings were concentrated and applied to a Dowex 50-X4 (H+) column. After washing the column with water, the amino acids were eluted with 2.5% ammonium hydroxide. The eluate following column chromatography. "H/C ratios of the products are given in Tables III and IV.

Degradation of [1H]Ornithine to Locate the Position(s) of Label

Oxidation of Ornithine to γ-Aminobutyric Acid Ornithine hydrochloride (250 mg) was dissolved in 25% (v/v) sulfuric acid (4 ml). Potassium permanganate (1.5 M) was added dropwise at room temperature until the solution remained pink for some time. It was allowed to stand at room temperature for 24 h. A few drops of potassium permanganate solution were added to the pink color and the mixture was allowed to stand at room temperature for a further 24 h. The mixture was warmed and a hot saturated solution of barium hydroxide was added with stirring until the pH was 6.5. The solution was filtered through a fine sintered glass funnel and the residue washed with warm distilled water.

The filtrate and washings were concentrated and applied to a Dowex 50-X4 (H+) column. After washing the column with water, the amino acids were eluted with 2.5% ammonium hydroxide. The eluate following column chromatography. "H/C ratios of the products are given in Tables III and IV.

Proline from D. stramonium and L. angustifolius

Extraction of the dried ground plant material and isolation of proline was performed essentially as described for the corresponding experiments with N. tabacum, except that carrier proline (approximately 100 mg) was added to the eluate following column chromatography. "H/C ratios of the products are given in Tables III and IV.

Table II

| Experiment | Species | Plants No. | Age (weeks) | Duration of experiment (days) | Harvested plant material fresh (dry weight (g)) | Specific activity (dpm/mmol x 10^5) |
|------------|---------|------------|-------------|-------------------------------|-----------------------------------------------|----------------------------------|
|            | N. tabacum | 1 | 2 | 12 | 3 | 250(7) | 90 |
|            | N. tabacum | 2 | 6 | 15 | 11 | 2 | 190 | 45 |
|            | D. stramonium | 4 | 6 | 15 | 10 | 2 | 22 |
|            | L. angustifolius | 6 | 15 | 12 | 2 | 21 |
|            | N. tabacum | 3 | 7 | 11 | 2 | 220 | 60 |
|            | D. stramonium | 5 | 19 | 15 | 3 | (3.5) |
|            | L. angustifolius | 7 | 16 | 13 | 2 | 22 |

Table III

Incorporation of dl-[RS]-5-H,5-14C]ornithine into proline

| Experiment | Plant species | "H/14C ratio of administered dl-[RS]-5-H,5-14C]ornithine | "H/14C ratio (% retention of "H) | Specific activity (dpm/mmol x 10^5) |
|------------|--------------|------------------------------------------------------|----------------------------------|----------------------------------|
| 1 | N. tabacum | 11.9 ± 0.6 | 12.0 ± 0.8 (101 ± 9) | 11.4 ± 0.1 (68 ± 5) |
| 2 | N. tabacum | 6.6 ± 0.1 | 6.4 ± 0.04 (97 ± 2) | 6.2 ± 0.04 (94 ± 1) |
| 4 | D. stramonium | 9.6 ± 0.1 | 9.6 ± 0.1 (100 ± 2) | 10.5 ± 0.2 |
| 6 | L. angustifolius | 14.5 ± 0.3 | 14.6 ± 0.1 (101 ± 2) | 9.1 ± 0.1 |

Table IV

Incorporation of dl-[2-14C]ornithine into proline

| Experiment | Plant species | "H/14C ratio of administered dl-[2-14C]ornithine | "H/14C ratio (% retention of "H) | Specific activity (dpm/mmol x 10^5) |
|------------|--------------|--------------------------------------------------|----------------------------------|----------------------------------|
| 3 | N. tabacum | 9.6 ± 0.6 | 10.0 ± 0.06 (104 ± 7) | 0.66 ± 0.007 (7 ± 1) |
| 5 | D. stramonium | 8.4 ± 0.1 | 8.50 ± 0.001 (60 ± 0.1) | 3.3 ± 0.1 |
| 7 | L. angustifolius | 6.3 ± 0.1 | 6.30 ± 0.003 (14 ± 1) | 5.5 ± 0.2 |
170-180°C and 1.5 x 10⁻³ mm pressure. γ-Aminobutyric acid (80 mg) (53%) melting at 195-196°C was obtained.

**Oxidation of Ornithine to Succinic Acid—**Ornithine monohydrochloride (293 mg) was dissolved in 10% (v/v) sulfuric acid (10 ml). Powdered potassium permanganate (1 g) was added and the mixture stirred and refluxed for 1 h. It was cooled and sulfur dioxide was passed until a colorless solution was obtained. This was extracted with ether in a continuous extractor for 24 h. The ether extract was dried and evaporated and the residue was crystallized from benzene. Succinic acid (65 mg) (53%), melting at 181-182°C, was obtained.

**RESULTS**

In separate experiments, samples of DL-[2-¹⁴C]ornithine, obtained by mixing DL-[RS]-5-¹³C]ornithine, with DL-[2-¹⁴C]ornithine, and with DL-[RS]-5-¹³C]ornithine, respectively, were administered by the wick method to in each of the tracer experiments, and those of the products isolated from these experiments, are shown in Tables III and IV. The per cent retention of tritium, relative to ¹⁴C, within each of the products, with reference to the administered ornithine, is also shown. Little difference between the ¹³C/¹⁴C ratios of the administered and the re-isolated ornithine was observed. The samples of proline isolated from the experiments using DL-[RS]-5-¹³C]ornithine as the substrate maintained the ¹³C/¹⁴C ratio of the administered ornithine (Table III). The samples of proline isolated from the plants which had been kept in contact with DL-[2-¹⁴C]ornithine on the other hand, contained little tritium while remaining rich in carbon-14 (Table IV).

The details of the seven tracer experiments are summarized in Tables I and II.

**DISCUSSION**

Two alternative routes to proline (7) from ornithine (2), with loss, respectively, of the δ-amino group (Route A) or of the α-amino group (Route B) are shown in Scheme 1. The link between ornithine (2) and proline (7) which is accepted (1-7), implicitly or explicitly, as existing in mammals and microorganisms, corresponds to Route A, via glutamic γ-semialdehyde (3).

Such a pathway is believed to operate also in higher plants, and it is relevant to trace the development of this notion. It’s seeds are to be found in two papers, published some 20 years ago.

These papers (10, 11) report the finding that after administration of DL-[2-¹⁴C]ornithine to various tissues of several higher plants (barley (10, 11), clover (10, 11), pine (11) watermelon (11), walnut (11), and maize (11)), followed by extraction and paper chromatography of the plant extracts, radioactivity was detectable in spots attributed, i.a. to proline and glutamic acid. The authors speculated, without further experimental justification, that (10) “this close association of proline with ornithine metabolism points to a pathway of proline formation from ornithine in higher plants, presumably via glutamic γ-semialdehyde and Δ¹-pyrroline-5-carboxylate, a metabolic process known to operate in N. crassa (36)” and that (11) “these results can be interpreted to mean that ornithine is utilized via glutamic γ-semialdehyde through either ring closure leading to proline, or dehydrogenation yielding glutamic acid, . . . a behavior pattern similar to that described by Vogel (8) for Neurospora crassa and Torulopsis utilis”.

The interrelationship in plants of ornithine and proline was subsequently studied by L. Fowden and his associates. Label from DL-[2-¹⁴C]ornithine was found to enter proline in mung bean seedlings (13) and in pumpkin cotyledons (17). An enzyme preparation, obtained (37) from peanut cotyledons converted ornithine into proline, in the presence of α-ketoglutarate and NADH or NADPH. It was assumed that the transaminase component of this enzyme preparation was an ornithine-6-transaminase, by analogy, and on the basis of a colorimetric assay with α-aminobenzaldehyde, regarded (38) as specific for Δ¹-pyrroline-5-carboxylic acid. This assay (39-43) is based on the formation of yellow dihydroquinazolinium salts when cyclic imines react with α-aminobenzaldehyde (44-46). Thus, a positive test is obtained not only with Δ¹-pyrroline-5-carboxylic acid (5) (due to formation of 3a,4-(2H) dihydropropyrrolidino-[2,1-b]quinazolinium-1-carboxylic acid (47) but also with Δ¹-pyrroline-2-carboxylic acid (6) (due to formation of 3a,4-(2H) dihydropropyrrolidino-[2,1-b]quinazolinium-3a-carboxylic acid) (48, 49) as well as with other cyclic imines (44-46, 48). Even though it has been pointed out repeatedly (41, 48, 50) that this test is not specific for Δ¹-pyrroline-5-carboxylic acid, it was assumed (38) either on the basis of this inadequate assay, or without supporting evidence altogether, other than analogy, that the ornithine transaminase activity demonstrated in extracts obtained from mung bean (51), pea (52), wheat (52), sunflower (53), pumpkin (54), and squash (38) is due to ornithine Δ₆-transaminase (EC 2.6.1.13).

The only direct attempt to establish which one of the two amino groups of ornithine is lost in the transamination reaction, catalyzed by an ornithine transaminase from a plant tissue, was carried out by Seneviratne and Fowden (55). A sample of DL-[2-¹⁴C]ornithine was incubated with a mitochondrion enzyme preparation from mung bean seedlings. The labeled reaction products were separated chromatographically and subjected to chemical oxidation (hydrogen peroxide) and reduction (borohydride). Hydrogen peroxide oxidation of α-keto-δ-aminovalerate (4), in equilibrium with Δ¹-pyrroline-2-carboxylate (6), yields α-amino acid in the presence of a-ketoglutarate and pyridoxal phosphate yielded labeled products which “included α-keto-δ-aminovaleric acid (oxidized to γ-aminobutyric acid and reduced to α-hydroxy-δ-aminovaleric acid) and glutamic γ-semialdehyde (oxidized to glutamic acid), together with a compound tentatively identified as Δ¹-pyrroline-2-carboxylic acid” (55). Apparently unwilling to entertain the possibility of enzymic α-transamination of ornithine, the authors maintained (55) that the formation of α-keto-δ-aminovaleric acid (4) and Δ¹-pyrroline-2-carboxylic acid (6) was due entirely to a chemical reaction (cf. Ref. 48) between pyridoxal phosphate and ornithine. They supported this view

1 Borohydride reduction of (4) (ζ=6(0)) at pH 8 yields proline as the sole product (56, 57). At lower pH some proline, together with another product, postulated (56), but not proven to be α-hydroxy-δ-aminovaleric acid, is obtained. Borohydride reduction of (5) (ζ=5(3)) yields proline (40, 57, 58).
by the observation that if, prior to incubation with [2-\(^{14}\)C]-
orlthine, "the enzyme preparation was subjected to pro-
longed dialysis (namely overnight at 0-4°C) before use, glu-
tamic \(\gamma\)-semialdehyde formation was negligible," i.e. only \(\alpha\)-keto-\(\delta\)-aminovalerate and \(\Delta^1\)-pyrroline-2-carboxylyc were
formed. This, the authors imply, was due to the lability of the
ornithine transaminase which, "therefore catalyzed \(\delta\)-trans-
amination from ornithine" (55).

A similar reluctance to face the possibility that the product
of the enzymic transamination of ornithine might be a com-
 pound other than \(\Delta^1\)-pyrroline-5-carboxylyc acid (5) is to be
found in a closely related study (59) on the reduction of (5),
catalyzed by an L-stereospecific proline dehydrogenase from
wheat germ. Two samples of substrate were used in this study.
One, DL-(5), was obtained by chemical synthesis from DL-\(\alpha\)-
amino-\(\delta\)-hydroxyvaleric acid (60), the other (assumed to be
L-(5)), by enzymic transamination from L-ornithine, employ-
ing the ornithine transaminase from squash cotyledons
(39). The two substrate samples showed qualititative dif-
ferences in the enzymic reaction. The authors commented (59)
that "strangely enough F5C," (i.e. (5)) which is formed by
transamination from ornithine, is reduced equally well to
proline with either NADPH or NADH; however, chemically
synthesized F5C uses NADH much more effectively than
FADPH\(^{\text{H}}\).

It should be noted in this context that enzyme preparations
from pea (61, 62) and from bean (61) seedlings are known to
catalyze the reduction to proline of \(\Delta^1\)-pyrroline-2-carboxylyc
acid.

In the cited papers by Fowden and his associates (13, 17,
37, 38, 54, 55, 59, 60) only two experiments are mentioned
which suggest loss of the \(\alpha\)-amino group in the course of the
enzymic transamination of ornithine in plant tissues. One is
the detection of a chromatographic spot, corresponding in \(R_F\)
value to that of an authentic sample of \(\alpha\)-keto-\(\delta\)-aminovaleric
acid (4), amongst the products resulting from the incubation
of ornithine with an extract of bean callus tissue, in the
presence of pyridoxal phosphate (63). This is dismissed as a
chemical artifact (55). The other is the work of Hasse et al.
(49) whose experiments paralleled, but whose results contra-
dicted those of Seneviratne and Fowden (50). This work is
 cited (37) without discussion or comment. Hasse et al. (49)
showed that transamination of ornithine, catalyzed by enzyme
preparations from mung bean and from a lupine species (L.
angustifolius) yielded a product whose \(\alpha\)-aminobenzaldehyde
adduct was electrophoretically identical with that of authentic
\(\Delta^1\)-pyrroline-2-carboxylyc acid (6) but distinct from that of
authentic \(\Delta^1\)-pyrroline-5-carboxylyc acid (5). Thus, \(\alpha\)-trans-
amination of ornithine was demonstrated.

Further evidence for the loss of the \(\alpha\)-amino group and the
retention of the \(\delta\)-amino group in the conversion of ornithine
into proline in plant tissues comes from studies with tissue
cultures from Jerusalem artichoke tubers (22, 23). Incubation
of these cultures with DL-[\(5\)-\(^{3}\)H,\(^{15}\)N]ornithine yielded proline en-
riched in \(^{15}\)N. Incubation with DL-\([\alpha\)-\(^{15}\)N]ornithine yielded proline
which was not significantly enriched in \(^{15}\)N. Similarly, proline isolated (14) from intact Jimsonweed (D. stramonium)
which had been grown in contact with DL-[2,\(^{14}\)C,\(^{15}\)N]orni-
 thine, maintained the \(^{14}\)C/\(^{15}\)N ratio of the precursor, whereas-
proline from a parallel experiment with DL-[2,\(^{14}\)C,\(\alpha\)-\(^{15}\)N]orni-
thine contained only little \(^{15}\)N in excess of natural abundance but
was rich in \(^{14}\)C.

Tracer experiments with \(^{15}\)N in intact plants suffer from the
defect that the amount of \(^{15}\)N-labeled material which must be
administered to obtain detectable \(^{15}\)N enrichment in the target
molecules may be large enough to swamp the nitrogen pool,
if the amino group of the tracer is biochemically labile. Since,
in the above experiments, \(^{15}\)N enrichment was not determined
in any amino acid other than proline, the level of \(^{15}\)N-enrich-
ment of the general nitrogen pool is unknown, and the results,
while strongly suggesting that proline is generated from orni-
thine via \(\Delta^1\)-pyrroline-2-carboxylyc acid (6) (i.e. via Route B,
Scheme 1) are therefore not entirely conclusive.

This difficulty is avoided by the use of substrates doubly
labeled with \(^{3}\)H and \(^{14}\)C. The results of the present study,
employing such substrates, confirm the conclusions of the
\(^{15}\)N studies. Unequivocal evidence is obtained that in three plant
species, tobacco (N. tabacum), Jimsonweed (D. stramonium),
and lupines (L. angustifolius), ornithine is converted into
proline with loss of the \(\alpha\)-amino group (Route B). The view
that in these plants proline arises from ornithine via glutamic
\(\gamma\)-semialdehyde (3) (Route A) is rendered untenable.
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Scheme 2. Predicted $^3$H/$^{14}$C ratios of proline formed from [(RS)-5-$^3$H,5-$^{14}$C]ornithine by Routes A and B.

Scheme 3. Predicted $^3$H/$^{14}$C ratios of proline formed from [2-$^3$H,5-$^{14}$C]ornithine by Routes A and B.
such a result was not observed.

In Table V, the predicted values for the retention of tritium, relative to $^{14}$C, in samples of proline obtained from $^3$H,$^5$C-labeled ornithine by the two different pathways (Schemes 2 and 3) are compared with the experimental values (Tables III and IV). The conclusion is inescapable that conversion of ornithine into proline in the three plant species under investigation takes place largely, if not entirely, by Route B, i.e., via $\Delta^1$-pyrroline-2-carboxylic acid (6).

On the basis of the data here presented, and the evidence available in the literature (14, 22, 23, 49-63) but largely ignored, it must be concluded that the conversion of L-ornithine into L-proline in the following plant species takes place via $\alpha$-ketoadaminovaleric acid (4) and $\Delta^1$-pyrroline-2-carboxylic acid (6) (Route B, Scheme 1) rather than via glutamic $\gamma$-semialdehyde (3) and $\Delta^1$-pyrroline-5-carboxylic acid (5) (Route A, Scheme 1): D. stramonium (14) (Jimsonweed), N. radiatus (61) (bean), tissue culture of P. vulgaris (common artichoke), L. angustifolius (49) (narrow-leaved lupine), N. subtilissima (22) (chicory), and P. sativum (61, 62) (garden pea).

The question arises whether this route is to be found in other plants, in general.

It is interesting to note that the same pathway has been demonstrated to operate in several Clostridia (16, 24), that there is evidence for a pathway between ornithine and proline in Escherichia coli which does not involve $\Delta^1$-pyrroline-2-carboxylic acid (12), that strains of Neurospora crassa and Aerobacter aerogenes, genetically blocked in the synthesis of ornithine into L-proline in the following plant species takes place largely, if not entirely, by Route B, i.e., via $\alpha$-ketoadaminovaleric acid (4) and $\Delta^1$-pyrroline-2-carboxylic acid (6) (Route B, Scheme 1): D. stramonium (14) (Jimsonweed), tissue culture of Helianthus tuberosus (22, 23) (Jerusalem artichoke), L. angustifolius (49) (narrow-leaved lupine), N. radiatus (61) (bean), tissue culture of P. vulgaris (common bean) callus (63) and Pisum sativum (61, 62) (garden pea).

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J. Biol. Chem. 1979, 254:640-647.

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