Metabolism of Glutamic Acid and N-Acetylglutamic Acid in Leaf Discs and Cell-free Extracts of Higher Plants

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Abstract. Radioactive glutamic acid and *N*-acetylglutamic acid have been incubated with normal and wilted leaf discs and radioactivity recovered in uncombined proline. The discs which had been placed under moisture stress incorporated considerably more label in uncombined proline than did normal discs.

In extracts of swiss chard leaves, both ^{14}C-*N*-acetylglutamic acid and glutamic acid are metabolized to their corresponding semialdehyde as evidenced by a recovery of proline after appropriate treatment. There is also incorporation of label in N_α-acetylornithine from *N*-acetylglutamic acid. These reactions require ATP, Mg^{2+}, and NADH or NADPH.

It has been observed that there is an increase in total proline in shoots and leaves of higher plants under moisture stress (1, 8, 13–15, 19). To understand the mechanism responsible for this increase, one must know the pathways of proline biosynthesis and degradation. The pathways of proline formation are well understood in microorganisms (10, 20–22), and in animals (10), but little is known about the pathway in higher plants (4). If proline biosynthesis is like that of microorganisms, one can expect reactions and intermediates indicated in the following scheme:
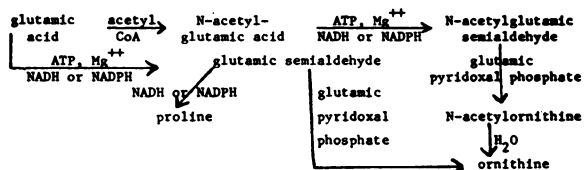
Included in this scheme is the transamination of *N*-acetylglutamic semialdehyde to *N*-acetylornithine and glutamic semialdehyde to ornithine as part of the pathway to arginine.

We present evidence in this paper that glutamic acid and *N*-acetylglutamic acid can be converted to proline *in vivo*. It was also observed that both glutamic acid and *N*-acetylglutamic acid could be converted to their semialdehydes by extracts of swiss chard (*Beta vulgaris* v. cicla) leaves.

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[1] Trade names and company names are included for the benefit of the reader and do not imply any endorsement or preferential treatment of the product listed by the United States Department of Agriculture.

Materials and Methods

Uniformly labeled L-glutamic acid (commercial) was purified by absorbing first on Dowex 50-hydrogen and then on Dowex 1-acetate (12). Labeled *N*-acetylglutamic acid was synthesized (5) from this purified acid and acetic anhydride. Any unreacted glutamic acid was removed by passing the reaction mixture through a column of Dowex 50-hydrogen at 4°.

Experiments were performed on leaves from greenhouse grown turnips (*Brassica rapa* v. Shogoin) and swiss chard (*Beta vulgaris* v. cicla). Turnips were used for *in vivo* leaf disc studies, while swiss chard was used for enzymic experiments.

Turnip leaf discs (2.7 cm in diameter) were weighed, vacuum infiltrated with labeled substrate, blotted dry, reweighed, and dried back to original fresh weight or wilted to 75 % of the fresh weight (2). They were then incubated on moist filter paper to maintain initial incubation weight for from 0 to 6 hr. The discs were extracted with hot 80 % (v/v) ethanol. The alcoholic extracts were dried, and an aliquot chromatographed 2-directionally on paper along with unlabeled amino acid standards (17). The amino acids were detected with ninhydrin or napthoquinone sulfonate (11). The areas containing amino acids were cut out and radioactivity determined in a liquid scintillation counter (11).

Cell-free extracts were prepared by grinding 20 grams of swiss chard leaves (midrib removed) with 40 ml of cold pH 7.5, 0.2 M potassium phosphate buffer, 0.02 ml of 2-mercaptoethanol and 2 grams of water insoluble polyvinyl pyrrolidone (Polyclar AT powder General Aniline Film Corporation[1]) in

a mortar (9). This and subsequent operations were carried out at 0 to 4°. The solids were removed in a refrigerated centrifuge at 12,000g for 20 min. The supernatant was dialyzed for 18 hr against 0.05 M potassium phosphate buffer at a pH of 7.0 containing 10^{-3} M mercaptoethanol.

The assay of N-acetyl glutamate-γ-semialdehyde dehydrogenase was performed in 3 ways: A) by the production of labeled orthoaminobenzaldehyde (OAB)[2]-glutamic semialdehyde complex (16), B) by the production of labeled proline, or C) by the production of labeled $N\alpha$-acetylornithine.

A) When the OAB-GSA complex was measured, the total volume of the incubation mixture was 1.7 ml, and contained 1 ml of dialyzed extract, 400 μmoles of K phosphate buffer pH 7.5, 10 μmoles MgCl$_2$, 5 μmoles NaATP, 5 μmoles NADH, 1 mg OAB, and 1 μc of N-acetyl-U-^{14}C-L-glutamic acid (2 μmoles). The mixture was incubated for 2 hr at 30°, and then hog kidney acylase (5 mg, Nutritional Biochemical Company, activity 5500 μmoles of acetyl-DL-methionine hydrolyzed per mg per hr) was added to the incubation mixture and it was again incubated for 2 hr at 37° to remove acetyl groups. After inactivation of the mixture by boiling and subsequent centrifugation, a 0.1 ml aliquot of the supernatant was chromatographed with added standards 1-directionally on paper in phenol:water. Standard $\triangle$-pyrroline-5-carboxylate (GSA) was prepared by oxidation of delta-hydroxy-alpha-aminovaleric acid with chromic acid (7). The OAB-GSA complex was prepared and chromatographed on paper in phenol:water. The area containing the colored complex was cut out and radioactivity determined in a scintillation counter.

B) When the N-acetyl-^{14}C-glutamic semialdehyde was to be measured by conversion to proline, the initial reaction mixture was the same as for the OAB-GSA complex assay except that no OAB was included. After the initial 2-hr incubation at 30°, 5 mg acylase, 5 μmoles NADH, and an additional 1 ml of extract (containing pyrroline carboxylate reductase) were added, and the mixture (2.8 ml) incubated 2 hr at 37°. A 0.1 ml aliquot of this supernatant, obtained after boiling and centrifugation, was chromatographed with standard proline, 1-directionally on paper in phenol:water. The proline was visualized with ninhydrin, and the radioactivity determined in a scintillation counter.

C) The incubation mixture for the $N\alpha$-acetylornithine assay was the same as for the proline determination, but in addition, contained 5 μmoles of pyridoxal phosphate and 10 μmoles of glutamic acid. This reaction mixture was incubated for 2 hr at 30°, boiled for 2 min, and centrifuged. The super-

natant was decanted and heated for 2 hr at 120° in 1 N HCl. The HCl was removed, the mixture neutralized, transferred onto a 7 × 1 cm column Dowex 50-ammonium (18), and the column washed thoroughly. Basic amino acids were eluted from the column with 2 N NH$_4$OH and chromatographed, with added standards. 1-directionally on paper (17). The amino acids were detected with ninhydrin, and the radioactivity in ornithine determined in a liquid scintillation counter.

The product of the glutamic acid-γ-semialdehyde dehydrogenase assay was measured as proline. The reaction mixture for this assay contained 1 ml extract, 400 μmoles K phosphate buffer pH 7.5, 10 μmoles MgCl$_2$, 5 μmoles NaATP, 5 μmoles NADH, and 1 μc U-^{14}C-glutamic acid in a total volume of 1.6 ml.

Two-directional paper chromatograms (17) were made from desalted extracts of the proline assay mixture (6) containing added standard amino acids. Radioautographs were obtained from these chromatograms.

Results and Discussion

Leaf Disc Experiments. These experiments again demonstrate a large increase in uncombined proline under moisture stress as contrasted with normal moisture conditions (table I). In 1 treatment, the recovery of label in proline was 10 % of the radioactive glutamic acid infiltrated, indicating that glutamic acid is probably a direct precursor of proline in higher plants as it is in microorganisms (20–22) and animals (10). The data do not reveal a marked difference in the efficiency of conversion of glutamic acid or N-acetylglutamic acid to proline. The results could indicate that both compounds are converted to proline by independent pathways, or that one is converted to the other by acetylation or deacetylation. Evidence for deacetylation of N-acetylglutamic acid was obtained from the recovery of considerable glutamic acid. Because of the lack of

Table I. *The Effect of Water Content of Leaf Discs on the Recovery of Radioactivity in Uncombined Proline From Labeled Glutamic Acid or N-Acetylglutamic Acid*

Incubation time	Radioactivity in uncombined proline	
	Unwilted	Wilted
	dpm	
Glutamic acid, 1.3×10⁶ DPM		
2 hr	3000	49,000
6 hr	1800	140,000
N-Acetylglutamic acid, 8×10⁵ DPM		
2 hr	1100	14,000
6 hr	300	30,000

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[2] The abbreviations used are: ortho-aminobenzaldehyde (OAB), glutamic-γ-semialdehyde (GSA), and N-acetylglutamic semialdehyde (N-Ac-GSA).

definitive evidence that either glutamic acid or N-acetylglutamic acid was preferentially converted to proline *in vivo*, it was decided to seek evidence that the enzymes for converting these acids to the corresponding semialdehydes are present in higher plants. The semialdehydes are likely intermediates in proline and arginine biosynthesis (10).

Cell-free Experiments. We decided to determine whether either glutamic acid or N-acetylglutamic acid could be converted to GSA or N-Ac-GSA in the presence of higher plant extracts. Since these products are unstable, they were converted to more stable compounds, *i.e.*, OAB-GSA complex, proline, or N-acetylornithine. In all 3 cases, the compounds were identified so that it was determined that GSA or N-Ac-GSA were products of the initial reaction.

The results presented in table II demonstrate that both glutamic acid and N-acetylglutamic acid were converted to the corresponding semialdehyde in the presence of a plant extract and the appropriate substrates. The results also indicate that N-acetylglutamic acid is more active than glutamic acid, but the significance of this cannot be assessed at present.

Table II. *Semialdehyde Formation From* ^{14}C*-Glutamic Acid and* N-*Acetylglutamic Acid by Swiss Chard Leaf Extracts*

Incubation mixture		Substrate	Radioactivity recovered in product	Product recovered as
			dpm	
Complete		Glutamic	31,100	Proline
”	(boiled)	”	700	”
— ATP		”	9,600	”
— Mg²⁺		”	8,100	”
— NADH		”	2,600	”
Complete		N-Ac-Glu	197,800	”
”	(boiled)	”	11,400	”
— ATP		”	19 200	”
— Mg²⁺		”	19,800	”
— NADH		”	16,500	”
— NADH + NADPH		”	201,200	”
Complete		”	207,700	GSA-OAB
”	(boiled)	”	12,300	”
”		”	70,300	N-Ac-Orn
”	(boiled)		0	”

The absence of counts in the product, in the presence of boiled enzyme, showed that the reaction was enzymatic. The requirement for ATP, Mg²⁺, and NADH demonstrate that the reactions were comparable to those of microorganisms (20–22). Here, also, phosphorylation appears to have been a necessary step before reduction of the carboxyl group of either glutamic acid or N-acetylglutamic acid. When N-acetylglutamic was the substrate,
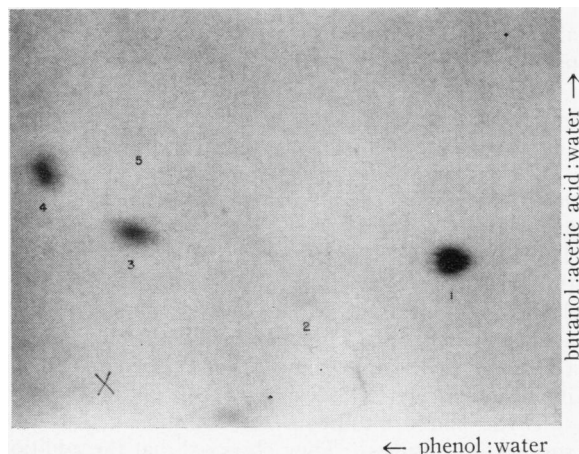

FIG. 1. Radioautogram of a proline assay with N-acetylglutamic acid as substrate. Chromatography was done in phenol :water and butanol :acetic acid :water. 1. Glutamic acid (released by added acylase). 2. Glutamine. 3. N-acetylornithine. 4. Proline. 5. γ-aminobutyric acid.

NADPH was as effective a reducing agent as NADH. The lower recovery of product in N-acetylornithine or ornithine probably reflected a lower activity of the transaminase under these reaction conditions. The possibility that the activity of N-acetylglutamic acid was due to a conversion of this compound to glutamic acid was discounted because when N-acetylglutamic acid was the substrate, no radioactivity could be detected in glutamic acid or in proline, indicating the absence of an endogenous acylase.

Radioautographs (Fig. 1) show that when N-acetylglutamic acid is the substrate and proline the desired product, there is considerable activity in Nα-acetylornithine. The small amount of activity in glutamine and γ-aminobutyric acid was probably formed from glutamic acid released after the acylase was added. The recovery of activity in Nα-acetylornithine after acylase treatment indicates inactivity of the hog kidney acylase toward the acetylornithine. The incubation of 1 μc of Nα-acetyl-L-ornithine with acylase under normal incubation conditions resulted in the release of only 1 % of the radioactivity to ornithine. The label in acetylornithine indicated the presence of an active acetylornithine δ-transaminase (3), and is probably made possible by transamination from amino acids produced by proteolysis and in the presence of endogenous bound pyridoxal phosphate.

Turnip leaf extracts actively reduced N-acetylglutamic acid to its semialdehyde, but this activity was much less than with swiss chard leaf extracts. In the preparation of cell-free extracts from swiss chard leaves, it was necessary to use polyvinylpyrrolidone in the grinding mixture to protect against inactivation by polyphenols (9); subsequent dialysis or passage through Sephadex G-25 also resulted in greatly increased activity. The enzymes were active

after remaining frozen for several weeks in the presence of 2-mercaptoethanol.

The glutamic acid-to-ornithine and the ornithine-to-proline conversions have been shown to utilize acetylated intermediates in some organisms (6, 21, 22). Glutamate-to-ornithine and glutamic acid-to-proline conversions involving nonacetylated intermediates in *N. crassa* have been investigated (20). The 2 pathways appear to be spatially separated in this organism. *Eschirichia coli* utilizes acetylated derivatives for arginine biosynthesis and nonacetylated compounds to proline (16). Dougall and Fulton (3) have used isotope competition experiments which implicated acetylated derivatives of glutamic semialdehyde and ornithine in the arginine pathway of rose tissue cultures. They observed that the addition of *N*-acetylornithine limited incorporation of label into arginine. It was deduced that *N*-acetylglutamic acid was also on the pathway, although the latter compound had no effect in their assay (presumably because of nonabsorption).

Neither our experiments with turnip leaf discs nor with swiss chard extract shows whether glutamic acid, *N*-acetylglutamic acid, or both are the normal precursor of proline in higher plants. However, it is clear from these experiments that glutamic acid and *N*-acetylglutamic acid are converted to the corresponding semialdehyde by plant extracts. It is also clear that enzymes are present which will convert *N*-acetylglutamic acid to *N*α-acetylornithine. *N*α-acetylornithine has been shown, by isotope competition experiments in higher plants, to be on the path to arginine biosynthesis (3).

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