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Formation of N-Acetylglutamate by Extracts of Higher Plants

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ABSTRACT

The enzymic synthesis of N-acetylglutamate was studied in extracts of higher plant tissues, especially in sugar beet leaves (*Beta vulgaris* L.). Sugar beet leaves had an enzyme that transferred the acetyl group either from acetyl-CoA or from N^2-acetylornithine to glutamate. The enzyme was so unstable that special precautions were necessary for its detection and appreciable purification was impossible. The *Km* values were 2.5 and 0.025 mM for acetyl-CoA and N^2-acetylornithine, respectively. The *Km* for glutamate was 23 mM with acetylornithine-glutamate transacetylase and 2.7 mM with acetyl-CoA-glutamate transacetylase. The pH optimum for acetyl-CoA-glutamate transacetylase was about 7.2 whereas that for acetylornithine-glutamate transacetylase was about 8.3. Acetylphosphate, N^2-acetyl-2,4-diaminobutyrate, propionyl-CoA, and succinyl-CoA were not substrates.

Arginine inhibited the acetyl-CoA-glutamate transacetylase and acetylglutamate phosphokinase but had no effect on the acetylornithine-glutamate transacetylase. Related compounds had either no effect or much less than arginine. Arginine had no effect on enzyme levels.

Acetyl-CoA-glutamate transacetylase was also found in *Raphanus sativus* L., *Glycine max* L. Merr., *Arachis hypogaea* L., *Brassica rapa* L., and *Pisum sativum* L. Acetylornithine-glutamate transacetylase was found in all of the above species plus *Zea mays* L., *Avena sativa* L., and *Triticum aestivum* L.

N-Acetylglutamate has been shown to be an intermediate in the biosynthesis of arginine in *Escherichia coli* (7), *Saccharomyces cerevisiae* (1), *Chlamydomonas reinhardti* (11), and *Chlorella vulgaris* (9). We have shown (10) that enzymes from higher plants can phosphorylate glutamate and N-acetylglutamate and can reduce the phosphorylated products to their corresponding aldehydes. These results indicate that N-acetylglutamate is formed in higher plants and might be an intermediate in arginine biosynthesis. There is little available information on glutamate acetylation and possible control by arginine in higher plants. This paper presents evidence for acetyl-CoA-glutamate transacetylase (EC 2.3.1.1) (ACGT[1]) and N^2-acetylornithine-glutamate transacetylase (EC 2.3.1.-) (AOGT) occurrence in several higher plants.

$$\text{Acetyl-CoA} + \text{L-glutamate} \rightarrow \text{N-acetyl-L-glutamate} + \text{CoA} \quad (1)$$

$$\text{N}^2\text{-acetyl-L-ornithine} + \text{L-glutamate} \rightleftharpoons \text{N-acetyl-L-glutamate} + \text{L-ornithine} \quad (2)$$

Some effects of arginine on the activity and levels of these enzymes are included.

MATERIALS AND METHODS

Uniformly labeled L-^{14}C-glutamic acid (Amersham/Searle[2]) was purified as previously described (9). Pure N^2-acetyl-L-ornithine was obtained by the acetylation of L-ornithine (12). Hog kidney acylase was purchased from Nutritional Biochemical Co.; DTE and N-acetylglutamate were from Cyclo Chemical Co.; and acetyl-CoA, propionyl-CoA, and succinyl-CoA were from P-L Biochemicals Inc. Schwarz/Mann supplied enzyme grade ammonium sulfate, and Hopkin and Williams Ltd. supplied the MBT. PVP was obtained from GAF Corporation and purified by the procedures of Loomis and Battaile (5).

Sugar beet (*Beta vulgaris* L. cv. Giant Western), radish (*Raphanus sativus* L. cv. Champion), turnip (*Brassica rapa* L. cv. Shogoin), soybean (*Glycine max* L. Merr. cv. Provar), peanut (*Arachis hypogaea* L. cv. Florigiant), spinach (*Spinacia oleracea* L. cv. Winter Bloomsdale), peas (*Pisum sativum* L. cv. Alaska), corn (*Zea mays* L. cv. Cornell M3), oats (*Avena sativa* L.) and wheat (*Triticum aestivum* L. cv. Tascosa) were grown in soil in the greenhouse with a complete nutrient solution. Tissue cultures of soybean, var. Mandarin, were obtained from O. L. Gamborg, Prairie Research Laboratory, Saskatoon, Saskatchewan, Canada.

Preparation of Cell-free Extracts. Tissues (25 g) were ground with 10 g of purified PVP and 50 ml of 100 mM K-phosphate buffer (pH 7) containing DTE (2 mM), MBT (1 mM), and glycerol (20%, v/v) in an Omni-Mixer at top speed for 1 min. This and subsequent operations were carried out at 0 to 4 C. Solids were removed by filtration with several layers of cheesecloth and by centrifugation at 12,000*g* for 30 min. Enzyme grade $(NH_4)_2SO_4$ (472 mg/ml) was dissolved in the supernatant. After centrifugation at 12,000*g* for 20 min, the precipitated protein was dissolved in about 2 ml of pH 8 buffer containing 0.05 M K-phosphate, 0.05 M ammonium acetate, 2 mM DTE, and 10% glycerol. This extract was passed through a column (25 × 1.7 cm) of Sephadex G-25 (coarse) which had been equilibrated with the same buffer. The effluent that contained the protein was collected (≈3 ml).

Assay for ACGT and AOGT. The incubation mixtures (0.25 ml) contained 0.5 μCi of uniformly labeled L-^{14}C-glutamic acid (280 μCi/μmol), 0.5 μmol of acetyl-CoA or 0.025 μmol of acetylornithine, 2.5 μmol of pH 8 K-phosphate, 2.5 μmol of pH 8 ammonium acetate, 0.4 μmol of DTE and enzyme solution. In control incubations, the acetyl donor was omitted. After the mixture had incubated for 20 min at 25 C, 80 μg of unlabeled N-acetylglutamate was added and the solution was immediately applied to a column (3.5 × 1 cm) of Dowex 50-H^+. The incubation tube and column were washed with 15 ml of 0.1 N acetic acid, and the wash was dried at room temperature in an air stream. The residue was dissolved in 2 ml of water, and a 0.5-ml

[1] Abbreviations: ACGT: acetyl-CoA-glutamate transacetylase; AOGT: N^2-acetylornithine-glutamate transacetylase; DTE: dithioerythritol; MBT: 2-mercaptobenzothiazole; *P*CMB: p-chloromercuribenzoate.

[2] Mention of a trademark or proprietary product does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture, and does not imply its approval to the exclusion of other products that may also be suitable.

aliquot chromatographed on paper with phenol-water (100:28, v/v). In critical cases, samples were chromatographed two-directionally with phenol-water and 1-butanol-acetic acid-water (12:3:5, v/v/v). The N-acetylglutamate was then located with ninhydrin after acylase treatment (9), and radioactivity determined by liquid scintillation (8).

Assay of Acetylglutamate Phosphokinase. This enzyme was measured by the method of Hoare and Hoare (3).

Protein was measured by the method of Lowry *et al.* (6) with BSA as the standard.

RESULTS

Reports of acetylglutamate formation in microorganisms (1, 4, 7, 9, 11) indicate that both acetyl-CoA and N^2-acetylornithine might be substrates for glutamate acetylation (equations 1 and 2) in higher plants. Our first results demonstrated that the addition of either acetyl-CoA or acetylornithine to an incubation mixture containing radioactive glutamate increased the radioactivity that was not retained by Dowex 50-H^+ (glutamate is retained but acetylglutamate is not). Two-directional paper chromatography and radioautography revealed that some of the radioactivity in the resin wash was present in compounds other than acetylglutamate. Hence, radioactive acetylglutamate was measured after paper chromatography of the resin effluent.

In spite of the lability of the enzyme and the length of the assay, a satisfactory assay was devised. Figure 1 shows that the amount of acetylglutamate was directly proportional to incubation time up to 40 min at 25 C. The acetylglutamate formed was directly proportional to the amounts of enzyme (Fig. 2).

Preliminary results with leaves of many higher plants showed that most had detectable AOGT and ACGT activities. Sugar beet leaves were selected for detailed study because of their relatively high levels of AOGT and ACGT and because they were readily grown in the greenhouse throughout the year. However, AOGT and ACGT activities of sugar beet leaves fluctuated from day to day, and we were unable to eliminate the variability. The inclusion of DTE, glycerol, and MBT in grinding buffer greatly improved recovery of activity (Table I). However, even with additives, the ACGT activity declined somewhat in 3 hr and drastically in 24 hr (Table I); AOGT was more stable.

It was desirable to purify the activities to see if they could be separated. The extract from a G-25 column was subjected to chromatography on DEAE-cellulose under several conditions and on hydroxyapatite, but the recovery of activity of ACGT was so poor (<5%) that it was impossible to detect any separation of activities or to establish whether any purification was accomplished. Apparently, the ACGT is much more labile when adsorbed than in solution (Table I). No attempt was made to purify AOGT even though it is more stable because the main objective of further purification would have been to establish whether ACGT and AOGT activities were due to one enzyme or two.

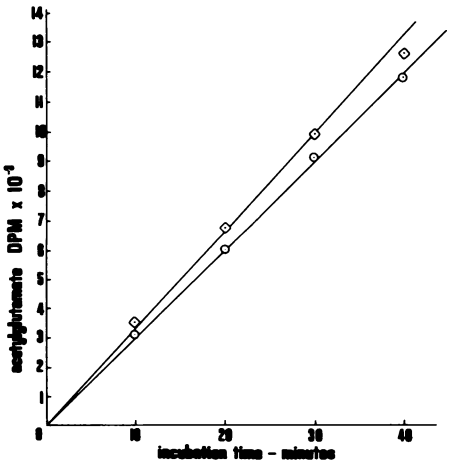

FIG. 1. Effect of incubation time on ACGT (○) and AOGT (□) activity. Incubation conditions were as in text.

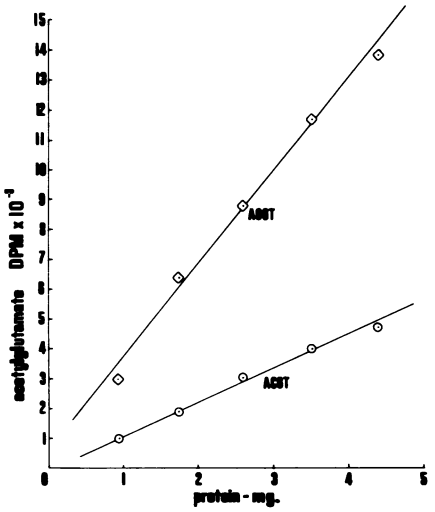

FIG. 2. Effect of amount of enzyme on ACGT and AOGT activity.

Table I. *Effect of Various Factors on ACGT and AOGT Activities in Sugar Beet Leaf Extracts*

Deviations from normal treatment	Relative ACGT activity	Relative AOGT activity
None	100[a]	100[b]
DTE, glycerol, MBT and PVP omitted during extract preparation	0	0
DTE, MBT and glycerol omitted during extract preparation	58	96
DTE omitted during extract preparation	75	96
Glycerol omitted during extract preparation	75	97
MBT omitted during extract preparation	86	100
Held at 0 C for 3 hr before assay	93	99
Held at 25 C for 3 hr before assay	80	99
Held at 0 C for 24 hr before assay	48	90
Held at -20 C for 24 hr before assay	69	99

[a] 13.8 pmoles of N-acetylglutamate were formed per gram fresh weight in 20 min at 25 C.

[b] 52.0 pmoles of N-acetylglutamate were formed per gram fresh weight in 20 min at 25 C.

The formation of acetylglutamate was enzymic since none was formed in the absence of plant extract or in the presence of heated extracts (Table II). Without an acetyl donor, only a small amount of acetylglutamate was formed. The specificity of the acetyl donor was shown by the inability of N^2-acetyl-2,4-diaminobutyrate and acetylphosphate to acetylate glutamate. Propionyl-CoA and succinyl-CoA did not promote the formation of an acyl-glutamate.

Tables I and II also demonstrate that glycerol, DTE, and ammonium acetate reduced loss of ACGT activity. The effect of PCMB indicates that a thiol group is essential for activity of ACGT but not AOGT.

The *Km* value for glutamate was 23 mM with AOGT and 2.7 mM with ACGT. The *Km* values for acetyl-CoA and acetylornithine were 2.5 mM and 0.025 mM, respectively.

The activity of sugar beet leaf ACGT was optimum at pH 7.2 (Fig. 3) in phosphate buffer and that of AOGT at about pH 8.3 (Fig. 4). The stabilities of both ACGT and AOGT were maximal and unaffected between pH 6.5 and 8.5.

Control of Glutamate Acetylation. According to current concepts of regulation, acetylation of glutamate would be an appropriate step for arginine to control its own formation (9). Consequently, the effects of arginine and related compounds were

Table II. *Effect of Various Substances on the Formation of Acylglutamate*

Incubation mixture	Acetylglutamate formed	
	ACGT	AOGT
	% of control	
Complete	100[a]	100[b]
Complete-extract heated 5 min at 100 C	0	0
Extract omitted	0	0
Acetyl donor omitted	5.8	1.4
NH_4 acetate omitted	79	80
PCMB added (1 mM)	18	100
EDTA added (1 mM)	100	100
	Acylglutamate formed	
	% of AOGT control	
Minus acyl donor plus succinyl CoA	2	
Minus acyl donor plus propionyl CoA	3	
Minus acyl donor plus acetylphosphate	2	

[a] 12.2 pmoles of acetylglutamate were formed per gram fresh weight in 20 min at 25 C.

[b] 47.5 pmoles of acetylglutamate were formed per gram fresh weight in 20 min at 25 C.

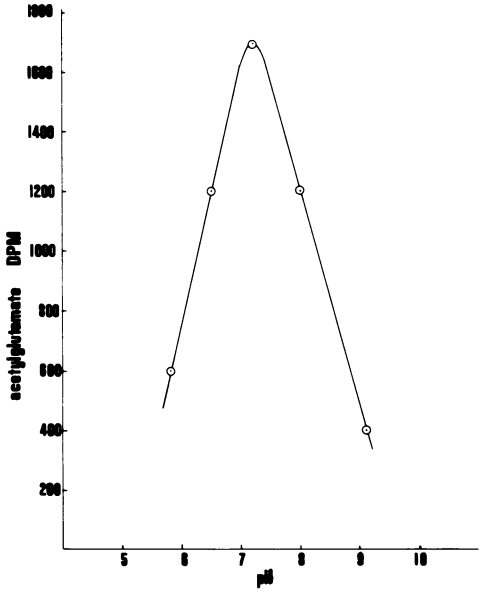

FIG. 3. Effect of pH of incubation mixture on activity of ACGT. Phosphate buffer was used throughout pH range tested.

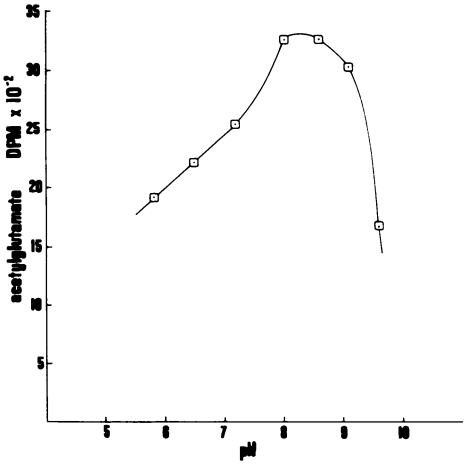

FIG. 4. Effect of pH of incubation mixture on activity of AOGT. Phosphate buffers were used throughout the pH range tested.

Table III. *Effect of Various Substances on ACGT and AOGT Activities*

Compound	Concentration	Inhibition	
		ACGT*	AOGT*
	mM	%	%
L-arginine	0.1	56	0
L-ornithine	1.0	87	91
DL-citrulline	20	44	0
L-homocitrulline	10	0	0
L-homoarginine	10	9	0
L-indospicine	10	7	0
L-proline	100	0	0

* Without inhibitor ACGT formed 10.6 pmoles and AOGT formed 49.2 pmoles of acetylglutamate per gram fresh weight in 20 min at 25 C.

tested on activity and levels of ACGT and AOGT in higher plants.

Table III shows that 0.1 mM-arginine inhibited ACGT by more than 50% but had no effect on AOGT. On the other hand, ornithine inhibited both ACGT and AOGT. This inhibition was undoubtedly an artifact due to the reversal of the formation of acetylglutamate (equation 2) (9). Citrulline, homocitrulline, and indospicine inhibited ACGT activity somewhat but had no effect on AOGT. Homocitrulline and proline were not inhibitory. The absence of any inhibition by proline supports the present concept that acetylglutamate is not on the pathway of proline biosynthesis.

Since arginine did not inhibit AOGT, it could not control its own biosynthesis by inhibiting glutamate acetylation. The arginine inhibition of ACGT provides a mechanism for the limitation of the "acetyl derivative" pool. In the absence of any arginine inhibition of AOGT, arginine might control its own synthesis by inhibiting acetylglutamate phosphokinase, the next enzyme of arginine biosynthesis. Inhibition of acetylglutamate phosphokinase by 65% with 1 mM L-arginine indicates that some control of arginine biosynthesis occurs at this step.

Since arginine lowers the glutamate acetylase level in bacteria (4), arginine could control its own synthesis by lowering the levels of ACGT and AOGT in higher plants. To investigate this possibility, soybean tissue cultures were grown on several levels of arginine, but even in the presence of 20 mM arginine, ACGT and AOGT levels were not decreased.

Glutamate Acetylation in Other Plants. If glutamate acetylation is a normal step in arginine biosynthesis in higher plants, then ACGT or ACGT plus AOGT should be present in all higher plants. AOGT was also found in radish leaves, soybean leaves, peanut seedlings, turnip leaves, spinach leaves, pea seedlings, corn seedlings, oat seedlings, and wheat seedlings. Except for the last three plants, ACGT was also detected. These results indicate that acetylation of glutamate is a normal process in most higher plants. The failure to find ACGT in three species may be due to an extraordinary lability of the enzymes.

DISCUSSION

In microorganisms, the acetyl donor for glutamate acetylation may be acetyl-CoA or acetylornithine (13). Only in *Chlorella* (9) was it clear that both acetyl-CoA and acetylornithine were substrates of glutamate acetylase. The presence of both ACGT and AOGT in seven higher plant species may mean that glutamate is acetylated by both acetyl-CoA and acetylornithine in all higher plants. The fact that we were unable to detect ACGT in monocots could mean that this enzyme is not present in monocots but

extreme lability would appear to be a more reasonable explanation.

Since both ACGT and AOGT were present in *Chlorella* (9), both may also be present in other lower plants. Udaka (13) has suggested that the presence of ACGT is a characteristic of "primitive" bacteria and that the presence of AOGT is characteristic of "advanced" bacteria. However, the mechanism of acetylornithine formation in advanced bacteria is not known. If ACGT is as labile in microorganisms as in higher plants, its lability rather than its absence may account for inability to detect ACGT in many bacteria. The probable presence of both ACGT and AOGT in *Pseudomonas aeruginosa* supports this idea (2). An organism might be expected to have both ACGT and AOGT activities since ACGT would provide the *de novo* formation of acetylated intermediates and AOGT would promote arginine formation while preserving the energy required for acetylation.

It was essential to use paper chromatography as well as resin columns in the assay for acetylglutamate formation because in the presence of both acetyl-CoA and acetylornithine, radioactive compounds other than acetylglutamate were formed that were not retained on Dowex 50-H^+. One of the contaminating compounds was radioactive α-ketoglutarate, probably formed by transamination. Attempts to reduce the formation of α-ketoglutarate to an acceptably low level by the inclusion of alanine (9) and transamination inhibitors in the incubation mixture were unsuccessful.

The lability (Table I) of ACGT and AOGT made purification of the activities impossible and necessitated the preparation of a new extract daily. Attempts to stabilize the enzyme by the use of MBT, thiols, PVP, and glycerol and by partial purification permitted assay of enzymic ativity but were inadequate to permit purification. We were unable to obtain any evidence for the separation of ACGT from AOGT and therefore cannot state whether one or two enzymes are responsible for the activities.

Another barrier to the purification of the enzyme activities was the low level of activity in the leaves tested. The extract from about 0.5 g of sugar beet leaves was required for a valid assay. Hence, the logistics for purification were very difficult.

The marked variability of ACGT and AOGT activities of sugar beet leaves from day to day was a source of frustration, as occasionally no activity was detected. The fluctuations were probably due to variations of levels in the leaves since they were handled identically each time. The effects of time of day, light intensity, temperature, and use of insecticides were not correlated with enzyme levels. Older plants always had lower ACGT and AOGT activities (even in young leaves) presumably due to high polyphenol formation.

The specificity of the enzyme (Table II) indicates that acetylglutamate is the normal product of acylation and that acetyl-CoA and acetylornithine are the normal acetyl donors *in vivo*.

This work establishes how arginine formation is controlled in higher plants. Arginine did not repress the formation of ACGT and AOGT. Arginine was a potent inhibitor of ACGT and acetylglutamate phosphokinase but had no effect on AOGT (Table III). Related compounds had so little effect that they would be unimportant in regulating arginine biosynthesis. Arginine appears to control the net flow of carbon from glutamate to arginine via inhibition of acetylglutamate phosphokinase. Arginine then controls the level of acetylated intermediates by inhibiting ACGT.

The presence of AOGT in all higher plant species tested and the presence of ACGT in most of them indicates that acetylglutamate is probably a normal constituent of all higher plants and, presumably, a normal precursor of arginine.

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