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PTEROYLGLUTAMATES OF HIGHER PLANT TISSUES

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Abstract—The levels of pteroylglutamate derivatives in whole leaf extracts and non-aqueously isolated chloroplasts of pea and maize seedlings have been determined by differential microbiological assay. A number of derivatives capable of supporting the growth of *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043) and *Pediococcus cerevisiae* (ATCC 8081) were separated by DEAE-cellulose column chromatography. The principal derivatives present in all extracts were methyl and formyl compounds of tetrahydropteroylglutamic acid. Increases in the levels of these derivatives following treatment with mammalian and avian γ-glutamyl carboxypeptidases suggested that additional highly conjugated pteroylglutamates were present in all extracts. The levels of pteroylglutamate derivatives in isolated chloroplasts were affected by the isolation method employed. The pteroylglutamate contents of non-aqueously isolated chloroplasts were approximately four times greater than the levels found in chloroplasts isolated in aqueous media. Approximately 96% of the pteroylglutamates in non-aqueously isolated chloroplasts were solubilized after a brief sonication treatment in the presence of ascorbate.

INTRODUCTION

CONSIDERABLE evidence is now available regarding the participation of tetrahydropteroylglutamate derivatives in the one-carbon metabolism of higher plants.[1-6] The presence of these compounds in plants is also well established. Detailed microbiological analyses have now confirmed that higher plants, in common with other organisms, contain both formyl and methyl derivatives of H_4PteGlu.[7-12]†

In recent work from this laboratory,[13] evidence was obtained for the presence of serine hydroxymethyltransferase (L-serine:tetrahydrofolate 5,10-hydroxymethyltransferase, E.C. 2.1.2.1) in isolated pea chloroplasts. This observation, together with the recent finding[14]

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† The abbreviations used for pteroylglutamic acid and its derivatives are those suggested by the IUPAC–IUB Commission as listed in *Biochem. J.* **102,** 15 (1967): e.g. 10-HCO–H_4PteGlu = N^{10}-formyltetrahydropteroylmonoglutamate.

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that such chloroplasts have ability to synthesize methionine from 5-CH_3-H_4PteGlu and homocysteine, suggests that H_4PteGlu derivatives play important roles in the amino acid metabolism of these organelles. It follows that chloroplasts should contain pools of various H_4PteGlu derivatives.

In the present investigations, isolated pea and maize chloroplasts have been examined for pteroylglutamate compounds using a sensitive microbiological procedure. Individual derivatives have been identified and their levels compared with those present in whole-leaf extracts. Preliminary reports of this work have already appeared.[15,16]

RESULTS

Chromatography of Authentic Pteroylglutamates

Samples of several pteroylglutamates, known to occur in biological materials, were subjected to DEAE-cellulose column chromatography as described by Sotobayashi *et al.*[17] The elution sequence of these compounds and their ability to support the growth of the three assay organisms are summarized in Table 1. Although the elution sequence of the derivatives remained unaltered in a number of separate experiments, the elution volume for individual compounds was found to vary slightly. These minor variations could be largely attributed to the bed volume of the columns used. In agreement with earlier work (for review see Blakley[18]), *L. casei* was found to respond to all of the compounds tested while *S. faecalis* and *P. cerevisiae* did not grow significantly on the methylated derivatives. In addition, *P. cerevisiae* gave no measurable growth in the presence of PteGlu.

Pteroylglutamates in Whole-leaf Extracts and Isolated Chloroplasts

Pea tissues. When pea leaf extracts were subjected to column chromatography and the fractions assayed for pteroylglutamates, a number of such derivatives were separated (Table 2). Four of the five derivatives which supported the growth of *L. casei* also gave growth of *S. faecalis*. The growth response of *P. cerevisae* was similar to that observed for

TABLE 1. ELUTION SEQUENCE AND RESPONSE OF ASSAY ORGANISMS TO AUTHENTIC PTEROYLGLUTAMATES

Compound	Fractions	Growth response		
		L. casei	*S. faecalis*	*P. cerevisiae*
10-HCO-H_4PteGlu	32–40	+	+	+
10-HCO-H_4PteGlu$_2$	43–52	+	+	+
5-HCO-H_4PteGlu	54–60	+	+	+
5-CH_3-H_4PteGlu	58–68	+	−	−
H_4PteGlu	70–78	+	+	+
5-CH_3-H_4PteGlu$_2$	81–90	+	−	−
5-CH_3-H_4PteGlu$_3$	110–120	+	−	−
PteGlu	132–145	+	+	−

Note. Negative response indicates less than 5% of response shown by *L. casei.*

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TABLE 2. LEVELS OF PTEROYLGLUTAMATE DERIVATIVES IN WHOLE LEAF EXTRACTS AND ISOLATED CHLOROPLASTS OF PEA SEEDLINGS

	Leaf extract				Chloroplast extract			
	Initial		After γ-GCP		Initial		After γ-GCP	
Derivatives	μg/g dry wt.	%	μg/g dry wt.	%	μg/g dry wt. Chloroplasts	%	μg/g dry wt. Chloroplasts	%
10-HCO–H_4PteGlu	2·85	25	3·07	17·4	5·49	30	6·42	29
10-HCO–H_4PteGlu$_2$	1·42	12	1·55	8	2·82	16	0·63	2
5-HCO–H_4PteGlu	1·30	11	2·74	16	0·73	3	6·75	30
5-CH_3–H_4PteGlu	5·18	45	9·13	51·5	4·57	26	7·56	35
H_4PteGlu	0·74	7	0·10	0·1	1·63	8	0·74	4
5-CH_3–H_4PteGlu$_2$	n.d.		1·25	7	3·02	17	n.d.	
Totals	11·50		17·84		18·26		22·10	

n.d.—not determined.

S. faecalis. Routinely 150 fractions were collected from the columns, however, no response was observed with the assay organisms beyond fraction 80. For identification of the separated derivatives the following commonly used criteria[18] were applied: (a) the position of the derivative in the elution sequence (Table 1) and co-chromatography with the authentic compound, (b) differential growth response of the three assay organisms, (c) treatment with γ-glutamyl carboxypeptidases, rechromatography and consideration of (a) and (b).

The major single derivative in the whole-leaf extracts, 5-CH_3–H_4PteGlu, accounted for 45% of the total pteroylglutamates recovered after chromatography (Table 2). Formyl derivatives accounted for the bulk of the remainder. When these extracts were treated with hog kidney γ-glutamyl carboxypeptidase before chromatography, the pteroylglutamate content, as measured with *L. casei*, was increased from 11·5 to 17·8 μg on a dry wt. basis. This increase was reflected by increases in the levels of several individual compounds (Table 2). Greatest increases were evident for 5-HCO–H_4PteGlu and 5-CH_3–H_4PteGlu together with appearance of a new derivative identified, on the basis of the above criteria, as 5-CH_3–H_4PteGlu$_2$.

Initial studies of pteroyglutamates in pea chloroplasts showed that the level of these compounds was affected by the isolation technique used. Chloroplasts isolated in aqueous media[19] in the presence of 0·1 M potassium ascorbate were found to contain 5·8 μg of pteroylglutamates/g dry wt. whereas chloroplasts isolated in non-aqueous media contained 22·0 μg pteroylglutamates/g dry wt. Such differences were observed in several isolations and presumably resulted from partial leaching of the derivatives in aqueous media. As a result of these studies, all subsequent assays of chloroplastic pteroylglutamates were carried out with chloroplasts isolated by the non-aqueous technique. The bulk (96%) of the pteroylglutamate content of chloroplasts isolated non-aqueously was readily solubilized after sonication, the remainder being associated with material sedimenting when the extracts were centrifuged.

The pattern of pteroylglutamate derivatives in pea chloroplasts after chromatography on DEAE-cellulose (Table 2), was, in many respects, different from that found in the whole-leaf extracts. The major derivative was 10-HCO–H_4PteGlu, accounting for 30% of the

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pteroylglutamates separated. 5-CH_3-H_4PteGlu was also conspicuous (26% of total) and 5-CH_3-H_4PteGlu$_2$, although not detected in whole-leaf extracts before γ-glutamyl carboxypeptidase treatment, was present in the initial chloroplast extracts. The pteroyglutamate content of pea chloroplasts was increased by approximately 21% following treatment with hog kidney γ-glutamyl carboxypeptidase, indicating that appreciable levels of derivatives were associated with the isolated chloroplasts. This increase was largely accounted for by elevations in the levels of 5-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu (Table 2).

Maize tissues. Young maize leaves contained several pteroylglutamate derivatives which were readily separated by the chromatographic procedure (Table 3). As in the whole pea leaf extracts, 5-CH_3-H_4PteGlu was the major derivative accounting for 39% of the pteroylglutamates isolated. The other derivatives were those found in pea leaf extracts with the exception of 5-CH_3-H_4PteGlu$_2$. Treatment of these extracts with γ-glutamyl carboxypeptidase gave

Table 3. Levels of pteroylglutamate derivatives in whole leaf extracts and isolated chloroplasts of maize

	Leaf extract				Chloroplast extract			
	Initial		After γ-GCP		Initial		After γ-GCP	
Derivatives	μg/g dry wt.	%	μg/g dry wt.	%	μg/g dry wt. Chloroplasts	%	μg/g dry wt. Chloroplasts	%
10-HCO-H_4PteGlu	0·99	15	0·64	25	0·84	18	1·14	13
10-HCO-H_4PteGlu$_2$	0·96	14	0·65	10	0·27	6	0·49	6
5-HCO-H_4PteGlu	0·15	2	1·28	19	0·21	5	2·11	24
5-CH_3H_4PteGlu	2·63	39	2·29	35	2·93	64	3·96	46
H_4PteGlu	1·09	16	0·35	5	0·36	7	0·24	3
5-CH_3-H_4PteGlu$_2$	0·92	14	0·43	6	n.d.		0·74	8
Totals	6·74		6·64		4·61		8·68	

n.d.—not determined.

little change in the total level of tissue pteroylglutamates. The decrease in H_4PteGlu observed in the other assays (Table 2) suggests that this compound was partially destroyed during the enzyme treatment. The increases observed in the levels of 10-HCO-H_4PteGlu and 5-HCO-H_4PteGlu (Table 3) suggest that polyglutamyl forms of these derivatives are present in young maize leaves.

The pattern of pteroylglutamates in isolated maize chloroplasts (Table 3) was characterized by a predominance of 5-CH_3-H_4PteGlu. This compound represented 64% of the total derivatives present. Smaller amounts of formyl derivatives and H_4PteGlu were also found. An 88% increase was observed in total pteroylglutamates when the extracts were treated with γ-glutamyl carboxypeptidase (Table 3). Again the levels of H_4PteGlu were decreased by this enzyme treatment. The very substantial increase in the levels of formyl and methyl derivatives as a result of this treatment indicates that highly conjugated forms of these compounds are present in the pteroylglutamate pool of these chloroplasts.

DISCUSSION

When the proportions of individual pteroylglutamates in the whole leaf extracts are compared with those found in extracts of the isolated chloroplasts, it is evident that sig-

nificant differences exist. For example, the contribution of 5-CH_3-H_4PteGlu to the total pteroylglutamate pool in the whole leaf and in the chloroplasts was different in both of the species examined. Similarly, differences existed with respect to the distribution of polyglutamyl derivatives. Although the present experiments were not designed to determine the proportion of total leaf pteroylglutamates that were associated with the chloroplasts, the differences outlined above suggest that these derivatives are compartmented in these tissues. The derivatives associated with the chloroplasts do not appear to be membrane bound as judged by their ready solubilization and their loss from chloroplasts isolated in aqueous media.

Since completion of the present studies, Kim[20] has reported analysis of pteroylglutamate derivatives in isolated wheat chloroplasts. In agreement with the species examined in the present work, wheat chloroplasts were found to contain derivatives capable of supporting the growth of the three assay bacteria and large increases in their levels occurred after treatment with an avian γ-glutamyl carboxypeptidase. Although complete resolution of the chloroplastic derivatives was not achieved in this later work,[20] the data suggest that the compounds identified in pea and maize chloroplasts may be of widespread occurrence in chloroplasts.

In agreement with analyses of other biological materials[18] the principal derivatives occurring in the leaf tissues studied, were formyl and methyl derivatives of H_4PteGlu. It is of interest that derivatives at a higher level of oxidation were not detected. Such compounds have been reported[7] to occur in leaf tissue extracts. However, the experimental conditions employed in these earlier studies may have allowed some oxidation of the naturally occurring derivatives.

Considering the importance of formyl and methyl derivatives of H_4PteGlu in the C-1 metabolism of diverse species, it is likely that the pools of these compounds in chloroplasts have similar roles. In an earlier report[14] it was suggested that the pteroylglutamates associated with chloroplasts could serve to provide C-1 units for synthesis of serine[13] and methionine.[14] Serine has recently been suggested as an intermediate in the formation of acetyl CoA in chloroplasts[21] and methionine could provide methyl groups for transmethylation reactions leading to synthesis of several important chloroplastic constituents.[22,23] If these conclusions are correct, it is conceivable that the 10-HCO–H_4PteGlu pool may have importance in both purine and protein biosynthesis within the chloroplast.

Although the metabolic inter-relationships between pteroylglutamates in different compartments within the leaf remain to be elucidated, it is highly likely that the associated one-carbon units are derived, in large part, from photosynthetic pathways. The presence of serine hydroxymethyltransferase in chloroplasts[13] indicates that C-1 units at the oxidation level of formaldehyde could arise indirectly from C_3 intermediates. C-1 units at the oxidation level of formate might also arise from glyoxylate formed from photosynthetic intermediates within the chloroplast. Formate is known to be formed spontaneously from glyoxylate in the presence of hydrogen peroxide[24] and the enzymic decarboxylation of glyoxylate by chloroplasts[25] may also have importance in this respect.

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EXPERIMENTAL

Chemicals. Pteroylglutamic acid and H_4PteGlu were obtained from Nutritional Biochemicals. Dr. W. Sakami of Case Western Reserve University, Cleveland, U.S.A., kindly supplied a sample of $PteGlu_3$. $H_4PteGlu_3$ was prepared by reduction of $PteGlu_3$ using dithionite.[26] 5-HCO–H_4PteGlu and [Me-^{14}C]5-CH_3–H_4PteGlu were purchased from Lederle, Cyanamid, and Amersham-Searle, respectively. 5-CH_3–H_4PteGlu and 5-CH_3–$H_4PteGlu_3$ were prepared by borohydride reduction[27] of the corresponding methylene derivatives.

Plant material. Seeds of pea (*Pisum sativum*, L. var. Homesteader) and maize (*Zea mays*, L. var. Alta Gold) were soaked in distilled H_2O for 24 hr and germinated in moist vermiculite at 25°. The seedlings received 16 hr of light and 8 hr of darkness in growth cabinets for a total period of 12 and 15 days for maize and pea respectively. The pea seedlings were then placed in darkness for 36 hr to deplete their starch content. Young expanding leaves of both species were harvested and after removal of large veins, were lyophilized. Dried tissue was then stored over P_2O_5 *in vacuo* at −20°.

Chloroplast isolation. Chloroplasts were isolated by the non-aqueous technique of Rogers, Shah and Goodwin,[28] modified to include CCl_4/*n*-hexane gradients (sp.gr. 1·31 and 1·30). The final chloroplast pellet was washed 3 times with *n*-hexane and dried *in vacuo* at −4°.

Extraction of H_4PteGlu *derivatives.* (a) *Whole leaf tissue.* Freeze-dried pea and maize leaves (100–300 mg) were rapidly homogenized at 0° in 10 ml of 1% (w/v) potassium ascorbate (pH 6·0) using a glass blender. The homogenates were then heated for 10 min at 95° to complete extraction of H_4PteGlu derivatives,[29] rapidly cooled to 0° and centrifuged for 10 min at 15,000 *g*. These extraction conditions employing ascorbate did not cause significant reduction of oxidized derivatives. Similarly the heat treatment did not cause extensive isomerization of 10-HCO–H_4PteGlu to 5-HCO–H_4PteGlu. The supernatants were retained for microbiological assay. (b) *Isolated chloroplasts.* Samples of freeze-dried chloroplasts (approx. 10 mg) were suspended in 5 ml of 1% (w/v) potassium ascorbate solution (pH 6·0) and immediately heated to 95° for 5 min. After rapid cooling to 0° the suspension was subjected to ultrasonic treatment for 3 × 5 sec, at maximum intensity. The suspension was incubated at 95° for 5 min, cooled to 0° and centrifuged at 6000 *g* for 10 min. The supernatants were then assayed for pteroylglutamate derivatives.

Chromatographic separation of H_4PteGlu *derivatives.* Aliquots of whole-leaf and chloroplast extracts, having pteroylglutamate contents of approximately 250 mμg, were applied to columns (20 × 1·8 cm) of DEAE-cellulose using a phosphate gradient in the presence of ascorbate (Sotobayashi *et al.*[17]). Column effluents were collected in 3 ml fractions in tubes containing 0·3 ml of 0·6% (w/v) potassium ascorbate solution (pH 6·0). After collection, the fractions were stored at − 20°. The position of specific pteroylglutamates in the elution sequence was established by use of authentic derivatives as summarized in Table 1.

Treatment of pteroylglutamate extracts with γ-glutamyl carboxypeptidases. In order to determine levels of polyglutamyl pteroylglutamates in whole-leaf and chloroplast extracts, incubations were routinely carried out using hog kidney and chicken pancreas γ-glutamyl carboxypeptidases. These enzymes were isolated from commercial preparations (Difco Laboratories, Detroit, U.S.A.) as described by Doctor and Couch[30] and Mims and Laskowski.[31] Incubations with the mammalian enzyme were carried out at 35° for 4 hr using reaction systems containing 2·5 ml of plant or chloroplast extract, 1 ml of carboxypeptidase preparation and 6·5 ml of 0·15 M sodium acetate buffer (pH 4·5). The reaction was terminated by boiling for 2 min and the pH of the supernatants was adjusted to 6·5 prior to column chromatography. The pancreatic enzyme was incubated at 32° for 4 hr in reaction systems containing 2·5 ml of plant or chloroplast extract, 1 ml of carboxypeptidase, 1 ml of 0·1 M $CaCl_2$ and 5·5 ml of 0·2 M sodium borate buffer (pH 7·3). The reaction was terminated and the pH adjusted as before. For identification of diglutamates the corresponding 'peak' fractions were pooled. Phosphate was removed by addition of EtOH (final conc. 50% v/v). After drying *in vacuo*, the extract was taken up in a minimum volume of distilled water and incubated with the kidney enzyme as before. Activities of both carboxypeptidases were confirmed by incubations in which the plant extracts were replaced by commercial yeast extract (Difco Laboratories, Detroit, U.S.A.).

Microbiological assays of pteroylglutamate derivatives. Whole leaf and chloroplast extracts were assayed for pteroylglutamates before and after column chromatography using the 'aseptic plus ascorbate' technique of Bakerman.[32] *L. casei* (ATCC 7469), *S. faecalis* (ATCC 8043) and *P. cerevisiae* (ATCC 8081) were used as assay organisms. Aliquots of the solutions to be assayed were added to pteroylglutamate-free media,[32] inoculated with the assay organism and incubated at 37°. Lactic acid produced after 72 hr of incubation was

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then titrated with 0·1 N NaOH and used as a measure of bacterial growth. Standard curves were constructed for *L. casei* and *S. faecalis* using authentic PteGlu; for *P. cerevisiae*, 5-HCO-H_4PteGlu was used.

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Key Word Index—Pisum sativum; Leguminosae; *Zea Mays*; Gramineae; chloroplasts; pteroylglutamates