Pteroylglutamate Derivatives in *Pisum sativum* L. BIOSYNTHESIS OF COTYLEDONARY TETRAHYDROPTEROYLGLUTAMATES DURING GERMINATION

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1. The concentrations of pteroylglutamate derivatives in the cotyledons of pea (*Pisum sativum* L.) seedlings were determined by microbiological assay by using *Lactobacillus casei* (A.T.C.C. 7469), *Streptococcus faecalis* (A.T.C.C. 8043) and *Pediococcus cerevisiae* (A.T.C.C. 8081). During germination the pteroylglutamate content of the cotyledons increased rapidly from 0.2 µg/g dry wt. to 4.0 µg/g dry wt., the maximum values being reached approx. 120 h after imbibition. 2. Approx. 50% of the pteroylglutamate pool of 3-day-old seedlings was accounted for by highly conjugated derivatives. The concentrations of such derivatives were greatest when precautions were taken to inactivate endogenous enzymes before extraction of the tissues. 3. Individual derivatives present in the tissue extracts were separated by chromatography on DEAE-cellulose. Synthesis of the major derivative, 5-methyltetrahydropteroylmonoglutamate, was inhibited by administration of 0.1 mM-aminopterin and -amethopterin solutions during imbibition. Under these conditions pteroylglutamic acid accumulated in the tissues. 4. Feeding experiments employing [2-¹⁴C]pteroylglutamic acid and 5[¹⁴C]-methyltetrahydropteroylmonoglutamic acid revealed that both compounds were incorporated into conjugated and unconjugated derivatives of the pteroylglutamate pool.

With development of refined techniques for microbiological assay (Bakerman, 1961) and ion-exchange chromatography (Silverman, Law & Kaufman, 1961; Sotobayashi, Rosen & Nichol, 1966) considerable knowledge has been gained regarding the natural occurrence of pteroylglutamates. For example, it is now well established that methyl and formyl derivatives of H₄PteGlu* occur widely in mammalian tissues and microorganisms (Blakley, 1969) and enzymes catalysing their synthesis and interconversion have been isolated (Friedkin, 1963). It is also clear that highly conjugated glutamyl derivatives form a significant part of the pteroylglutamate pool of such organisms (Noronha & Aboobaker, 1963; Schertel, Boehne & Libby, 1965; Allfrey & King, 1950; Wright, 1955; Hakala & Welch, 1957; Sirotnak, Donati & Hutchinson, 1963).

Although the pteroylglutamate contents of various plants of dietary importance have been examined (Butterworth, Santini & Frommeyer,

1963; Santini, Brewster & Butterworth, 1964), relatively few detailed studies of these compounds in higher plants have been reported. Several of the investigations that have been conducted with higher plant tissues are incomplete analyses as no determinations of methylated derivatives have been made (Santini *et al.* 1964; Iwai & Nakagawa, 1958*a,b*; Iwai, Nakagawa & Okinaka, 1959). Later work (Roos, Spronk & Cossins, 1968; Rohringer, Kim & Samborski, 1969) has revealed that 5-methyl derivatives are often the principal components of the pteroylglutamate pool of plant species, and enzyme studies (Dodd & Cossins, 1969, 1970; Burton & Sakami, 1969; Shah & Cossins, 1970) have demonstrated their importance as precursors of methionine. Plant species are also known to contain conjugated pteroylglutamate derivatives (Iwai *et al.* 1959; Roos *et al.* 1968; Rohringer *et al.* 1969; Shah & Cossins, 1970) as judged by the effects of γ-glutamyl carboxypeptidases on pteroylglutamate concentrations. However, as several plants contain appreciable activities of such hydrolases (Bird, Brinkley, Bloom, Emmett & Piffner, 1945; Bird, Robbins, Vandenbelt & Piffner, 1946; Iwai, 1957; Iwai & Nakagawa, 1957; Neilson & Holmström, 1957) it is not clear whether the

* The abbreviations used for pteroylglutamic acid and its derivatives are those suggested by the IUPAC-IUB Commission as listed in the *Biochemical Journal* (1967), 102, 15: e.g. 10-HCO-H₄PteGlu = N¹⁰-formyltetrahydropteroylmonoglutamate.

amounts of conjugated derivatives are altered during the extraction process.

In the present work the concentrations of pteroylglutamate derivatives in germinating pea seedlings have been assayed under conditions that permit the determination of the majority of naturally occurring pteroylglutamates. To obtain estimates of conjugated derivatives, it was found necessary to modify the extraction method generally used. Evidence is presented that a substantial synthesis of these compounds occurs in the cotyledons of pea seedlings during the early stages of germination.

MATERIALS AND METHODS

Chemicals. All chemicals were of the highest quality commercially available. Tetrahydropteroylglutamic acid was purchased from Sigma Chemical Company, St Louis, Mo., U.S.A., or was alternatively synthesized from PteGlu in a solid state reaction by using dithionite (Davis, 1969). The 10-HCO-H₄PteGlu was synthesized from 5-HCO-H₄PteGlu (Lederle Products, Cyanamid of Canada Ltd., Montreal, Canada) by the method of Rabinowitz (1963) and 5-CH₃-H₄PteGlu was prepared from 5,10-CH₂-H₄PteGlu by borohydride reduction (Sakami, 1963). PteGlu was purchased from Nutritional Biochemicals, Cleveland, Ohio, U.S.A., and H₂PteGlu was synthesized as described by Blakley (1960). [2-¹⁴C]PteGlu and 5[¹⁴C]-CH₃-H₄PteGlu were purchased from The Radiochemical Centre, Amersham, Bucks., U.K. The radioactively labelled PteGlu was dissolved in sterile water to a final specific radioactivity of 1 μ Ci/20 nmol per 1.7 ml of solution. Radioactively labelled 5-CH₃-H₄PteGlu was dissolved in sterile 0.5% potassium ascorbate solution to give a final specific radioactivity of 1 μ Ci/16 nmol per 0.8 ml of solution. Aminopterin and amethopterin were purchased from Sigma Chemical Co. and Lederle Products respectively.

Plant material. Seeds of *Pisum sativum* L. cultivar Homesteader were soaked initially in water and, after being surface sterilized, were germinated in darkness at 25°C for periods of up to 7 days (Roos *et al.* 1968). At intervals during this period, samples of the seedlings were harvested and the cotyledons, after removal of the testas and embryos, were retained for pteroylglutamate analyses.

Methods for extraction of pteroylglutamate derivatives. Endogenous pteroylglutamates were extracted from the cotyledons in the presence of ascorbate by one of three methods. Method 1. Samples of the cotyledons (10 g) were finely homogenized in an ice-cold mixture of 2.2 ml of 10% (w/v) potassium ascorbate (pH 6.0) and 9.8 ml of water by using a Ten Broeck homogenizer. The homogenate was then diluted by addition of 28 ml of 0.5% potassium ascorbate (pH 6.0). This method is essentially that described by Bird, McGlohon & Vaitkus (1965). Method 2. Slices approx. 1 mm thick were prepared from the cotyledons (Cossins & Turner, 1963) and 10 g samples of these were immediately immersed in a boiling solution of 2.2 ml of 10% (w/v) potassium ascorbate (pH 6.0) and 9.8 ml of water. After boiling for 10 min the mixture was

rapidly cooled in an ice bath and 10 ml of ice-cold 0.5% potassium ascorbate (pH 6.0) was added followed by thorough homogenization as before. Method 3. Samples of the cotyledons (10 g) were frozen in a solid CO₂ bath and freeze-dried. The dried tissue was then ground to a fine powder by using a pestle and mortar and added immediately to the boiling ascorbate solution described in method 2. After cooling, the homogenate was diluted with 10 ml of 0.5% potassium ascorbate (pH 6.0) as in the other methods.

Homogenates prepared by these methods were immersed for 10 min in a water bath at 95°C to complete pteroylglutamate extraction and denature soluble protein. All extracts were finally diluted by addition of a further 10 ml of 0.5% ice-cold potassium ascorbate (pH 6.0) and centrifuged at 18000g for 20 min at 2°C. The supernatants were stored in a frozen state.

Chromatography of pteroylglutamate derivatives. Columns (20 cm $\times$ 1.8 cm) of DEAE-cellulose were prepared as described by Sotobayashi *et al.* (1966). Samples (1–5 ml) of the tissue extract, containing 0.05–0.8 μ g of pteroylglutamates, were applied to the columns and the derivatives were eluted at room temperature with a gradient of phosphate buffer in the presence of 0.6% potassium ascorbate (pH 6.0) (Sotobayashi *et al.* 1966). Fractions (3 ml) of the column effluent were collected by using an LKB Ultra Rac Fraction Collector, model 7000 (LKB-Produkter AB, Stockholm, Sweden). Each collecting tube contained 0.1 ml of 10% (w/v) potassium ascorbate solution (Silverman *et al.* 1961). After collection of fraction 130, the gradient was altered by washing the column with 1M-phosphate buffer containing 0.6% potassium ascorbate (pH 6.0). Pteroylglutamate derivatives in fractions collected from the columns were identified by using the basic criteria described by Sengupta & Cossins (1971). These included a consideration of the position of elution of the derivative compared with that of an authentic derivative, co-chromatography with authentic derivatives, differential response shown by the three assay organisms, and a consideration of chromatographic behaviour after treatment with γ -glutamyl carboxypeptidases from three sources (see below). Recovery of derivatives after DEAE-cellulose chromatography was found to be 100 $\pm$ 10% of values obtained before this step.

Microbiological assay of pteroylglutamates. The tissue extracts and fractions collected from ion-exchange chromatography were assayed for pteroylglutamate derivatives by using the 'aseptic plus ascorbate' technique of Bakerman (1961). *Lactobacillus casei* (A.T.C.C. 7469), *Streptococcus faecalis* (A.T.C.C. 8043) and *Pediococcus cerevisiae* (A.T.C.C. 8081) were used to inoculate the assay tubes in triplicate. After incubation at 37°C for 72 h the lactic acid formed was titrated and used as a measure of bacterial growth (Freed, 1966). The concentration of derivatives was then determined from standard curves prepared by using authentic PteGlu and 5-HCO-H₄PteGlu for each assay. The results are expressed in PteGlu equivalents when *L. casei* and *S. faecalis* were used and in 5-HCO-H₄PteGlu equivalents when *P. cerevisiae* was the assay organism.

γ -Glutamyl carboxypeptidase treatments. The enzymes from hog kidney and chicken pancreas (Difco Laboratories, Detroit, Mich., U.S.A.) were extracted and partially

purified by Dowex AG1-X10 treatment (Doctor & Couch, 1953) and ammonium sulphate fractionation (Mims & Laskowski, 1945) respectively. A third hydrolase, having ability to hydrolyse polyglutamyl derivatives to monoglutamates, was isolated from 3-day-old pea cotyledons. In this isolation, the cotyledons (10g) were homogenized in 20 ml of 0.1M-potassium phosphate buffer (pH 6.0) at 2°C. After passing the homogenate through fine cheese-cloth, it was centrifuged at 18000g for 20 min at 2°C. Saturated ammonium sulphate solution was then slowly added to the supernatant to give a final saturation of 80%. After being left for 30 min at 2°C the precipitated protein was recovered by a further centrifugation, redissolved in 40 ml of the buffer and stored in a frozen state until required. This extract was diluted tenfold before use with 0.1M-sodium acetate buffer (pH 4.5) containing 10% (w/v) potassium ascorbate.

The activities of all three hydrolases were routinely assessed by using a substrate prepared from commercial yeast extract (Difco Laboratories). Polyglutamyl derivatives in the yeast extract were extracted by stirring it with 5 parts of 1% (w/v) potassium ascorbate (pH 6.0), then heating the mixture at 95°C for 10 min. After rapid cooling, the extract was clarified by centrifugation and the supernatant used as the substrate for the hydrolases. Reaction systems containing the chicken enzyme were incubated in 0.1M-sodium borate buffer (pH 7.8) as described by Mims & Laskowski (1945). The other enzymes were incubated in potassium acetate buffer containing 0.085M-acetic acid adjusted to pH 4.5 with 5M-KOH (Doctor & Couch, 1953). Incubations were carried out at 35°C for 5 h (chicken enzyme) and for 2–3 h for the other hydrolases.

Experiments with pteroylglutamate antagonists. Samples (60g) of air-dried pea seeds were allowed to imbibe 0.1 mM-aminopterin and -amethopterin for 24 h at 20°C. After thorough rinsing in sterile water the seeds were transferred to sterile vermiculite and incubated in darkness at 25°C for periods of up to 3 days. Pteroylglutamate derivatives were then extracted by methods 1 and 2 and samples (3–5 ml) of the extracts were subjected to DEAE-cellulose chromatography as before. This treatment effectively separated the antagonists from the endogenous pteroylglutamates.

¹⁴C-feeding experiments. In all feeding experiments, samples (30 surface-sterilized seeds) (Wong & Cossins, 1969) were allowed to imbibe the labelled solution in sterile petri dishes at 20°C. Additional water was added at intervals during the first 6 h of imbibition. Under these conditions, the labelled substrates were rapidly and completely taken up by the seeds. The seeds were then transferred to sterile petri dishes lined with filter paper and allowed to germinate at 25°C in darkness. Pteroylglutamate derivatives were extracted by methods 1 and 2 from the cotyledons during germination. Samples of the extract containing 0.3–0.8 μ Ci of ¹⁴C were then subjected to DEAE-cellulose chromatography. Portions (1 ml) of the fractions collected were then assayed for radioactivity by scintillation counting (Dodd & Cossins, 1969). Under these feeding conditions it was found that the amounts of ¹⁴C in individual fractions containing the derivatives were at least 20 times above the background count of 30 c.p.m. All counts were corrected for background radiation. The counting efficiency was approxi-

mately 75%. Samples of the column effluents were simultaneously assayed for pteroylglutamates by using *L. casei* and *P. cerevisiae*.

RESULTS

Changes in concentrations of derivatives in cotyledons during germination. We reported (Roos *et al.* 1968) that the total pteroylglutamate contents of 3-day-old pea cotyledons were considerably higher than the concentrations found in the seeds after 24 h of imbibition. To examine these changes in greater detail, a number of assays were made over a 7-day germination period (Fig. 1). The pteroylglutamate contents of the cotyledons remained very low during the first 30 h of germination. This is in contrast with the considerable increase that occurred between 40 and 90 h of germination. After this, the pteroylglutamate concentration reached a maximum value which was more than 15 times the initial values obtained. As in earlier work with this species (Roos *et al.* 1968), the assays with *L. casei* gave results that were higher than those obtained when *S. faecalis* was used. This suggests that the derivatives present were largely methylated, and possibly also conjugated, forms of PteGlu. Considering the inability of the assay organisms to respond to highly conjugated derivatives (Blakley, 1969), the increases observed (Fig. 1) may be caused either by net synthesis or by an extensive hydrolysis of conjugated derivatives to compounds capable of supporting the growth of

Fig. 1. Changes in pteroylglutamate content of pea cotyledons during germination. Cotyledon extracts were prepared by method 1 and assayed with *L. casei* (○) and *S. faecalis* (▲). Each point represents the average of at least three separate determinations.

L. casei and *S. faecalis*. These possibilities were examined in further experiments.

Effect of extraction technique on concentrations of conjugated derivatives. To determine whether significant amounts of highly conjugated derivatives were present in germinating pea cotyledons, the extracts were treated with mammalian and avian γ -glutamyl carboxypeptidases as described in the Materials and Methods section. As reported by Roos *et al.* (1968), no significant increases in the growth response of the assay organisms occurred because of this treatment. Clearly, conjugated derivatives are either not present in these tissues or are rapidly hydrolysed during preparation of the extract. The latter alternative appears to be correct, as evidence for the presence of conjugated derivatives was only obtained if precautions were taken to inactivate endogenous enzymes during the extraction process (Table 1). Significant increases in the results given by the microbiological assay were evident after hog kidney carboxypeptidase treatment of the extracts prepared by methods 2 and 3. The increases observed in the assays employing *P. cerevisiae* suggest that the conjugated derivatives included compounds other than polyglutamates of 5-CH₃-H₄PteGlu. In 3-day-old seedlings approx. 50% of the pteroylglutamates in the cotyledons are highly conjugated, i.e. inactive in supporting growth of *L. casei*. In contrast, these derivatives accounted for only 15% of the total pteroylglutamates extracted from 1-day-old cotyledons.

The absence of conjugated derivatives in extracts prepared by method 1 (Table 1) is explained by the observation that such derivatives, present in yeast extract, were extensively hydrolysed when incubated with cell-free cotyledon extracts (Table 2). The extent of this hydrolase activity was comparable to that exhibited by the γ -glutamyl carboxypeptidase isolated from hog kidney.

Chromatography of pteroylglutamate derivatives. As reported by Roos *et al.* (1968), the method

of Sotobayashi *et al.* (1966) was found to give excellent resolution of a large number of naturally occurring pteroylglutamates. The recoveries of these derivatives in tissue extracts after carboxypeptidase treatments were generally in the 90–110% range.

The differences observed in the amounts of pteroylglutamates when methods 1 and 2 were employed (Table 1) were further evident after DEAE-cellulose chromatography (Figs. 2 and 3). All of the derivatives present in extracts prepared by method 1 were present after employing method 2, and in addition three conjugated derivatives were found in extracts prepared by method 2. Peaks *a*, *b* and *d* although not clearly resolved (Fig. 2*b*) were readily detectable on increasing the size of sample assayed by *L. casei*. The principal derivative, identified by Roos *et al.* (1968) as 5-CH₃-H₄PteGlu, was accompanied in both extracts by smaller amounts of its diglutamate (peak *d*). 10-HCO-H₄PteGlu and its diglutamate were also present, the amounts of these derivatives being substantially higher in the extracts prepared by method 1. The conjugated derivatives (peaks *e*–*g*) gave relatively little growth response of *L. casei* (Fig. 3*b*). However, after individual treatment of these fractions with carboxypeptidase, the growth response of *L. casei* was in each case increased. Differential assay of these fractions after such treatment showed that the derivatives present in peaks *e* and *f*, while supporting the growth of *L. casei*, failed to show activity for *P. cerevisiae*. Both organisms responded to the third derivative (peak *g*) after carboxypeptidase treatment, the response shown by *P. cerevisiae* being 40% of that shown by *L. casei*.

Treatment of extracts prepared by method 1 with avian carboxypeptidase did not alter the amounts of derivatives isolated. However, similar treatments of extracts prepared by method 2 considerably increased the total amounts of the derivatives separated (Table 3). Much of this increase was accounted for by increased amounts

Table 1. *Effects of extraction technique and γ -glutamyl carboxypeptidase treatment on concentrations of pteroylglutamates*

Cotyledon extracts prepared by methods 1, 2 and 3 were assayed for pteroylglutamate derivatives before and after incubation with hog kidney γ -glutamyl carboxypeptidase (γ -GCP). The results are expressed in PteGlu and 5-HCO-H₄PteGlu μ g equiv./g dry wt. in assays involving *L. casei* and *P. cerevisiae* respectively.

γ -GCP treatment	Age of cotyledons (days)	Method 1		Method 2		Method 3	
		<i>L. casei</i>	<i>P. cerevisiae</i>	<i>L. casei</i>	<i>P. cerevisiae</i>	<i>L. casei</i>	<i>P. cerevisiae</i>
Before assay	1	0.27	0.14	0.24	0.06	0.24	0.06
After assay	1	0.27	0.13	0.28	0.11	0.29	0.12
Before assay	3	2.60	0.81	1.24	0.23	1.23	0.23
After assay	3	2.71	0.82	2.74	0.78	2.70	0.79

of 5-CH₃-H₄PteGlu. Although this enzyme treatment resulted in amounts of 10-HCO-H₄PteGlu significantly less than those obtained when method 1 was employed, the amount of the total formyl derivatives was only slightly increased. This may be largely due to an isomerization of 10-HCO-H₄PteGlu to the 5-formyl derivative, a change favoured by incubation of the extract at pH 4.7. Another notable change associated with carboxypeptidase treatment was the absence of peaks *e-g* when such extracts were chromatographed.

Effects of pteroylglutamate antagonists. As the microbiological assays summarized in Fig. 1 suggest that pteroylglutamate derivatives may be synthesized during germination, the effects of aminopterin and amethopterin were examined to elucidate this point. Seeds which had been imbibed in 0.1 mM solutions of these inhibitors did not show any extension of the radicle after 3 days (Table 4). When extracts of these tissues were subjected to DEAE-cellulose chromatography and assayed with *L. casei*, the amounts of pteroylglutamate derivatives in the treated seeds were considerably less than those in control seeds of comparable age. Aminopterin and amethopterin treatments decreased the quantity of 5-CH₃-H₄PteGlu in 1-day-old cotyledons. Further, this compound, although the major derivative in the control seeds after 3 days, could not be detected in the samples treated with the antagonist. In addition, formylated derivatives were not detected in the treated seeds. When similarly treated seeds were extracted by method 2 it was also clear that aminopterin and amethopterin prevented synthesis of con-

jugated derivatives. Another major difference between the controls and the treated seeds was the appearance in the latter of PteGlu. The amounts of this compound were considerably increased after 3 days. Repeated attempts to detect PteGlu in the control seeds were unsuccessful. No evidence was obtained for an accumulation of H₂PteGlu or H₄PteGlu in the controls or treated seeds over this period of investigation.

¹⁴C experiments. The accumulation of PteGlu in cotyledons treated with aminopterin and amethopterin (Table 4) suggested that this derivative may be an intermediate in the synthesis of more reduced compounds such as 5-CH₃-H₄PteGlu. On the other hand, tetrahydro derivatives may be synthesized in these tissues by a pathway not involving PteGlu, as is well documented for several organisms (Brown, 1959; Iwai, Okinaka, Ikeda & Suzuki, 1969; Shiota, 1969). To examine these possibilities, micromolar quantities of ¹⁴C-labelled PteGlu were supplied during imbibition as described in the Materials and Methods section. After periods of germination of 24 and 48 h respectively, samples of the cotyledons were extracted by method 1 and examined for pteroylglutamate derivatives as summarized in Table 5. After chromatography, the bulk of the radioactivity recovered from the columns was associated with pteroylglutamate derivatives as determined by microbiological assay. Approx. 10% of the ¹⁴C recovered was, however, associated with fractions that did not support the growth of the three assay organisms. This radioactivity occurred in distinct 'peaks', most being in fractions 19–28. No evidence was obtained for radioactive labelling of formyl derivatives, which were, nevertheless, readily detected by microbiological assay. Radioactive labelling of methylated derivatives was, however, detected; the diglutamate of 5-CH₃-H₄PteGlu did not contain ¹⁴C after 24 h of germination, but did after 48 h. As the derivatives summarized in Table 5 were extracted by method 1, little information was obtained about the possible incorporation of ¹⁴C into highly conjugated derivatives. To examine this point, we performed an experiment identical with that summarized in Table 5, except that the derivatives were extracted by method 2. Chromatography of these extracts revealed that approximately 5% of the ¹⁴C present was associated with reduced polyglutamyl derivatives. No evidence was obtained for conjugation of PteGlu despite the fact that large amounts of [¹⁴C]PteGlu remained in the tissues after 48 h. As before (Table 5), ¹⁴C was also detected in methylated derivatives when method 2 was employed.

As conjugated derivatives of H₄PteGlu were labelled in the above experiment, it is conceivable that PteGlu is reduced and converted into

Table 2. *Hydrolysis of conjugated pteroylglutamates present in yeast extract*

Pteroylglutamate concentrations were assayed after incubation of 1 ml of yeast extract in reaction mixtures containing 0.1 M-sodium acetate buffer (pH 4.5) and 1% (w/v) potassium ascorbate. A 1 ml sample of hog kidney or pea cotyledon γ -glutamyl carboxypeptidase preparation was added as indicated. The final volume was 10 ml and incubations were performed at 35°C for 2 h. Reaction mixtures containing boiled enzyme gave no increase in the amounts of pteroylglutamates as assayed by *L. casei*.

Incubation conditions	Pteroylglutamate content as determined by <i>L. casei</i> (ng)	
	Pea cotyledon enzyme	Hog kidney enzyme
Without enzyme	173	175
With enzyme	1580	1640
Conjugated derivatives hydrolysed	1407	1465

Fig. 2. Separation of pteroylglutamate derivatives in extracts of 1-day-old seedlings. Extracts were prepared by method 1 (a) and method 2 (b). After DEAE-cellulose chromatography, the fractions were assayed for pteroylglutamates by using *L. casei*. The assay organisms gave no detectable growth response when incubated with samples of fractions 100–210 in elution (a). The derivatives shown are: a, 10-HCO- H_4 PteGlu; b, 10-HCO- H_4 PteGlu₂; c, 5-CH₃- H_4 PteGlu and 5-HCO- H_4 PteGlu; d, 5-CH₃- H_4 PteGlu₂ and 5-HCO- H_4 PteGlu₂; f, unidentified conjugated derivative. The 5-formyl derivatives were detected when peaks c and d were assayed with *P. cerevisiae*.

derivatives such as 5-CH₃- H_4 PteGlu, before the addition of glutamyl residues. To examine this possibility, micromolar quantities of 5-[¹⁴C]-CH₃- H_4 PteGlu were supplied to imbibing pea seeds. After suitable periods of germination, the pteroylglutamates were extracted by method 2 and separated as before (Table 6).

As in the earlier feeding experiments, most of the radioactivity recovered was associated with pteroylglutamates as determined by microbiological assay. Also, approx. 5–10% of the ¹⁴C eluted from the columns was associated with fractions that did not support the growth of the three assay organisms. These radioactivity was mainly associated with compounds not exchanging with the cellulose bed

and it increased in quantity as the seeds germinated. The major labelled pteroylglutamate detected in these experiments was the compound that had been supplied, which decreased in specific radioactivity as the seedlings germinated. No ¹⁴C was detected in the formyl derivatives separated or in 5-CH₃- H_4 PteGlu₂. The conjugated compounds (peaks e–g) were, however, significantly labelled in this experiment after 3 days of germination.

DISCUSSION

The large increase observed in the pteroylglutamate content of pea cotyledons (Fig. 1) suggests that net synthesis of these derivatives

Fig. 3. Separation of pteroylglutamates in extracts of 3-day-old seedlings. Extracts prepared by method 1 (a) and method 2 (b) were subjected to DEAE-cellulose chromatography. The fractions collected were assayed with *L. casei*. No growth response was observed for fractions 100–210 in elution (a). Peaks are designated as in Fig. 2; e and g, unidentified conjugated derivatives.

Table 3. Amounts of individual pteroylglutamate derivatives in 3-day-old pea cotyledons

Results are expressed in PteGlu equivalents (ng/g dry wt. of cotyledons). The values in parentheses were obtained after hog kidney γ -glutamyl carboxypeptidase (γ -GCP) treatment of individual peak fractions. n.d., no detectable growth response with *L. casei*.

Derivative	Method 1	Method 2	
	Before and after γ -GCP treatment	Before γ -GCP treatment	After γ -GCP treatment
10-HCO-H ₄ PteGlu	470	50	258
10-HCO-H ₄ PteGlu ₂	131	80	36
5-HCO-H ₄ PteGlu	110	22	535
5-CH ₃ -H ₄ PteGlu	2150	895	2065
5-HCO-H ₄ PteGlu ₂	52	18	9
5-CH ₃ -H ₄ PteGlu ₂	225	204	315
Peak e	n.d.	50 (80)	n.d.
Peak f	n.d.	87 (190)	n.d.
Peak g	n.d.	172 (1200)	n.d.
Total	3138	1578 (2739)	3218

Table 4. *Effect of aminopterin and amethopterin on amounts of cotyledonary pteroylglutamate derivatives during germination*

Pteroylglutamate derivatives were extracted by method 1, separated by chromatography on DEAE-cellulose and then assayed with *L. casei*. n.d., no detectable growth response.

Treatment	Days of germination	Radicle length (mm)	Concn. of pteroylglutamate (PteGlu equivalents $\mu\text{g/g}$ dry wt.)		
			10-HCO-H ₄ PteGlu	5-CH ₃ -H ₄ PteGlu	PteGlu
Control	1	4.5	Trace	0.35	n.d.
	3	30.0	0.32	2.68	n.d.
Aminopterin	1	4.0	n.d.	0.15	0.11
	3	6.5	n.d.	n.d.	1.36
Amethopterin	1	4.0	Trace	0.14	0.12
	3	6.5	n.d.	n.d.	1.48

Table 5. *Incorporation of [2-¹⁴C]PteGlu into pteroylglutamates of germinating pea cotyledons*

Groups of 30 surface-sterilized seeds were allowed to imbibe 66 nmol of [2-¹⁴C]PteGlu (1 $\mu\text{Ci}/20$ nmol per 1.7 ml) under sterile conditions. After germination for 1 and 2 days, respectively, samples of the cotyledons were extracted by method 1. The extracts were subjected to DEAE-cellulose chromatography followed by assay of their radioactivity and pteroylglutamate content. n.d., no radioactivity detected.

Age of cotyledons (days)	Derivative	Concn. of pteroylglutamate (ng/g dry wt.)	Specific radioactivity (c.p.m./ng of derivative)
1	10-HCO-H ₄ PteGlu	34	n.d.
	10-HCO-H ₄ PteGlu ₂	20	n.d.
	5-CH ₃ -H ₄ PteGlu	285	18
	5-CH ₃ -H ₄ PteGlu ₂	25	n.d.
	PteGlu	1040	149
2	10-HCO-H ₄ PteGlu	345	n.d.
	10-HCO-H ₄ PteGlu ₂	38	n.d.
	5-CH ₃ -H ₄ PteGlu	2830	21
	5-CH ₃ -H ₄ PteGlu ₂	300	23
	PteGlu	450	147

occurs during germination. This conclusion is supported by the results of experiments involving pteroylglutamate antagonists (Table 4) and the demonstration (Table 5) that at this stage of germination [¹⁴C]PteGlu was incorporated into more reduced derivatives. This biosynthesis, also found in other species (Burkholder, 1943; Erismann & Schopfer, 1959), probably reflects an increased demand for one-carbon units as the metabolic activities of the developing seedling increase. It is logical to conclude that pteroylglutamate-mediated reactions will be of central importance during these early stages of germination and in this connexion the complete inhibition of seedling development by aminopterin and amethopterin treatments is notable.

In common with other tissues, the pteroylglutamate pool of pea cotyledons was found to contain appreciable amounts of highly conjugated derivatives. These compounds appear to be very readily hydrolysed during extraction unless pre-

cautions are taken to inactivate endogenous enzymes. The enzymes responsible for such hydrolyses could conceivably be specific pteroylpolyglutamate hydrolases or less specific peptidases capable of degrading a variety of peptides. In mammalian tissues, detailed studies (Hoffbrand & Peters, 1969) of such enzymes have led to the suggestion that they may have importance in the absorption of highly conjugated derivatives present in the diet. Their physiological significance in plants is, however, difficult to ascertain. The metabolic roles of conjugated pteroylglutamates in plants will have to be elucidated before a function can be ascribed to these enzymes.

The marked inhibition of tetrahydropteroylglutamate biosynthesis by aminopterin and amethopterin (Table 4) suggests that such reduced derivatives are formed via H₂PteGlu. Enzymes catalysing the synthesis of this latter compound have been extensively studied in pea seedlings by Iwai *et al.* (1969). Highly purified cell-free

Table 6. *Incorporation of 5[¹⁴C]-CH₃-H₄PteGlu into pteroylglutamates of germinating pea cotyledons*

Groups of surface-sterilized seeds were allowed to imbibe 80 nmol of 5[¹⁴C]-CH₃-H₄PteGlu (1 μ Ci/16 nmol per 0.8 ml) under sterile conditions. After periods of germination, samples of the cotyledons were extracted by method 2. Derivatives were then isolated and assayed as in Table 5. n.d., no radioactivity detected.

Age of cotyledons (days)	Derivative	Concn. of pteroylglutamate (ng/g dry wt.)	Specific radioactivity (c.p.m./ng of derivative)
1	10-HCO-H ₄ PteGlu	36	n.d.
	10-HCO-H ₄ PteGlu ₂	12	n.d.
	5-CH ₃ -H ₄ PteGlu	874	228
	5-CH ₃ -H ₄ PteGlu ₂	45	n.d.
	Peak <i>f</i>	15	n.d.
3	10-HCO-H ₄ PteGlu	85	n.d.
	10-HCO-H ₄ PteGlu ₂	60	n.d.
	5-CH ₃ -H ₄ PteGlu	1060	179
	5-CH ₃ -H ₄ PteGlu ₂	156	n.d.
	Peak <i>e</i>	55	10
	Peak <i>f</i>	112	39
	Peak <i>g</i>	195	25

extracts were found to catalyse the synthesis of dihydropteroate, which then combined with L-glutamate to form H₂PteGlu. The presence of dihydrofolate reductase (5,6,7,8-tetrahydrofolate-NADP oxidoreductase; EC 1.5.1.3) in these tissues (Iwai *et al.* 1969) (A. J. Roos and E. A. Cossins, unpublished work) would account for the subsequent synthesis of H₄PteGlu and the sensitivity of tetrahydropteroylglutamate biosynthesis to aminopterin and amethopterin. Considering these reactions, it is, however, surprising that H₂PteGlu did not accumulate when the tissues were treated with the antagonists. Further, PteGlu, although not an intermediate in the proposed pathway for tetrahydropteroylglutamate biosynthesis (Iwai *et al.* 1969), was an effective precursor as judged by the incorporation of ¹⁴C into 5-CH₃-H₄PteGlu (Table 5). It should be emphasized, however, that during this incorporation a considerable dilution of ¹⁴C occurred, which suggests that 5-CH₃-H₄PteGlu was also being synthesized from unlabelled precursors. Several attempts to detect accumulations of H₂PteGlu were made without success. Authentic H₂PteGlu was, however, readily separated and detected by the chromatographic procedure. In all cases, treatment of the tissues with the aminopterin and amethopterin was accompanied by marked accumulations of PteGlu. In common with other plant tissues (Andreeva & Efimova, 1966), pea cotyledons can reduce PteGlu (E. A. Cossins, unpublished work). Although it is not clear, at present, whether this activity is distinct from that of dihydrofolate reductase, it is possible that some synthesis of reduced derivatives may proceed via PteGlu and that the PteGlu reductase (7,8-dihydrofolate-NADP oxidoreductase; EC 1.5.1.4)

possibly in this tissue is sensitive to the antagonists supplied.

The apparent lack of conjugated derivatives of PteGlu in the aminopterin and amethopterin experiments is notable. The diglutamate derivative of 5-CH₃-H₄PteGlu was, however, formed from [2-¹⁴C]PteGlu (Table 5) in the absence of these inhibitors which suggests that the labelled conjugated derivatives may be synthesized via 5-CH₃-H₄PteGlu. However, this suggestion was not supported by results from the experiments with ¹⁴C-labelled 5-CH₃-H₄PteGlu (Table 6). After adding this derivative the endogenous pool of 5-CH₃-H₄PteGlu₂ was not labelled, although some radioactivity was detected in the conjugated compounds present in peaks *e*, *f* and *g* (Table 6). Since the amount of radioactivity in these peaks represented only a small proportion of that supplied the possibility cannot be excluded that only the labelled methyl group, and not the pteroylglutamate moiety, was transferred to these derivatives. In *E. coli* (Griffin & Brown, 1964), glutamyl residues appear to be added to tetrahydro derivatives in a stepwise manner. However, in these studies, 5-HCO- and 5-CH₃-tetrahydropteroylglutamates were not effective substrates in the conjugation reaction. In contrast, little is known regarding the synthesis of conjugated derivatives in higher organisms. The present investigations suggest that conjugated derivatives are formed in higher plants by conjugation of tetrahydropteroylglutamates. However, as the 5-CH₃-H₄PteGlu feeding experiments were inconclusive in this respect, it is clear that further investigations are required to elucidate the mechanism of this synthesis in higher plant tissues.

The predominance of methylated derivatives in the pteroylglutamate pool of this tissue (Table 3) is also characteristic of many other species. For example, studies of maize and pea leaves (Cossins & Shah, 1971) have shown that such compounds account for 35 and 51% of the total pteroylglutamate pool respectively. These derivatives are also the principal pteroylglutamates of various animal tissues (Blakley, 1969) and of *Saccharomyces* cultures (Schertel *et al.* 1965; Combepine, Cossins & Lor, 1971). Considering the importance of 5-methylpteroylglutamates as donors of methyl groups (Blakley, 1969), it is conceivable that in the tissues under consideration these compounds have similar roles. Several workers have, in fact, confirmed that this is so in plants, it now being clear that 5-CH₃-H₄PteGlu and its di- and tri-glutamates can act as precursors of methionine (Dodd & Cossins, 1970; Burton & Sakami, 1969; Shah & Cossins, 1970). In pea seedlings, such methyl groups appear to participate, via the intermediary formation of *S*-adenosylmethionine (Dodd & Cossins, 1969), in several transmethylation reactions and it is likely that this involvement will be found in other plant tissues.

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