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FOLATE DERIVATIVES OF PHOTOSYNTHETIC TISSUES

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Key Word Index—*Raphanus sativus; Triticum vulgare; Spinacea oleracea; Pisum sativum*; folate derivatives; aminopterin; illumination.

Abstract—An examination has been conducted of folate derivatives in extracts of spinach, pea, and wheat leaves and the cotyledons of 6-day-old radish seedlings using *Lactobacillus casei*. These tissues contained folate activity equivalent to 28, 15, 14 and 30 μg of pteroylglutamic acid per g dry wt of tissue respectively, and the folate derivatives were shown to consist mainly of formyl and methyl tetrahydropteroylpolyglutamates, conjugated with more than three glutamic acid moieties. During germination the folate content of both green and etiolated radish cotyledons increased rapidly to a maximum at 6–8 days and then decreased. Etiolated tissues contained half the folate levels found in normal tissues, but the proportion of methyl derivatives was higher. A comparison of the levels of folates present in radish cotyledons harvested in the dark and light phases of a 13:11 hr light:dark cycle revealed significant differences in formyl and unsubstituted derivatives. In light-harvested tissues, formyl derivatives decreased by 50% while unsubstituted derivatives increased by 600%, methyl derivatives being unchanged. Treatment of radish cotyledons with aminopterin resulted in a rapid depletion of methyl tetrahydropteroylglutamates while formyl and unsubstituted derivatives declined less abruptly. Pteroylglutamic acid accumulated in these treatment.

INTRODUCTION

THE IMPORTANCE of tetrahydropteroylglutamate derivatives in several unrelated synthetic pathways is now widely appreciated.[1] Knowledge of their metabolism in higher plants has increased rapidly in recent years, but evidence for their involvement in several biosynthetic reactions still remains fragmentary. Enzymes which catalyze the formation and interconversion[2–12] of C-1 substituted derivatives of H_4PteGlu† *in vitro* have been studied and the role of H_4PteGlu in the interconversion of serine in higher plants is well established.[2–6] H_4PteGlu derivatives are also required for the oxidative decarboxylation of glycine,[13,14]

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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. The term 'folate' and 'tetrahydropteroylglutamate' are used synonomously. The subscript *n* denotes an undertermined number of glutamic acid residues.

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the biosynthesis of methionine,[15,16] and indirect evidence suggests an involvement in the reactions of purine ring synthesis.[17–19] Recent studies[20,21] have shown that chloroplast *N*-formylmethionyl-*t*RNA transformylases utilize 10-HCO-H_4PteGlu as a formyl group donor and therefore support earlier conjectures[22,23] that the mechanism for protein biosynthesis in chloroplasts and mitochondria is similar to that of bacterial systems.

The involvement of folates in the reactions outlined above suggests that they are normal constituents of higher plant tissues and detailed microbiological and chromatographic analyses have indeed shown that formyl and methyl derivatives of H_4PteGlu occur in higher plant species.[24–29] As part of a continuing investigation of folate derivatives in higher plants, the levels of these compounds in spinach, pea and wheat leaves and radish cotyledons have been assayed under conditions which permit their determination in as near their naturally occurring states as practicable.

The metabolic roles of folates in photosynthetic plant tissues have to date been mainly studied *in vitro* and consequently the physiological significance of these roles *in vivo* has not been completely evaluated. In order to elucidate possible metabolic interrelationships between the various derivatives of H_4PteGlu and their participation in the metabolism of photosynthetic tissues, analyses were made of the folate pools of, (a) etiolated radish cotyledons and wheat leaves, (b) radish cotyledons harvested in the light and dark periods of a 13:11 hr light:dark cycle, and (c) excised radish cotyledons infiltrated and incubated with 2×10^{-5} M aminopterin, a potent antagonist of folate metabolism.

RESULTS

Folate Derivatives of Different Photosynthetic Plant Tissues

Naturally occurring folates in leaf extracts of pea, spinach and wheat as well as radish cotyledons were fractionated on DEAE-cellulose[30] and assayed microbiologically with *L. casei* and *P. cerevisiae*. Recoveries of the derivatives after column chromatography were 80–95%. Individual derivatives were identified using the criteria outlined previously.[31] Substantial increases in the microbiological response after chicken pancreas γ-glutamylcarboxypeptidase treatment was taken as an indication that the isolated 'peaks' contained diglutamyl derivatives.[32]

A variety of derivatives were detected in the plant extracts before carboxypeptidase treatment (Table 1). Small amounts of 5-CH_3-H_4PteGlu were present in some of the extracts before enzyme treatment. In contrast such treatment appreciably increased the amounts of

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10-HCO-, 5-HCO-, and 5-CH_3-H_4PteGlu$_2$ present, indicating that most of the derivatives extracted from the tissues were highly conjugated.

Since radish cotyledons contained a high level of folates and could be readily obtained from seedlings grown under controlled conditions, this tissue was the major one used in further studies.

TABLE 1. LEVELS OF FOLATE DERIVATIVES IN PHOTOSYNTHETIC TISSUES

Derivative	Radish cotyledons		Pea leaf		Wheat leaf		Spinach leaf	
	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP	Before γ-GCP	After γ-GCP
10-HCO-H_4PteGlu	294	553	237	160	115	238	191	218
10-HCO-H_4PteGlu$_2$	136	10 090	71	3366	95	6780	48	3566
5-HCO-H_4PteGlu	101	n.d.	134	n.d.	57	n.d.	n.d.	n.d.
5-CH_3-H_4PteGlu	462	698	443	570	153	275	4923	4671
10-HCO-H_4PteGlu$_3$	58	n.d.	n.d.	n.d.	n.d.	861	n.d.	n.d.
H_4PteGlu	57	262	17	91	14	n.d.		n.d.
5-HCO-H_4PteGlu$_2$	62	3255	57	2296	n.d.	3807	191	3207
5-CH_3-H_4PteGlu$_2$	149	15 220	493	5394	22	1397		11 140
H_4PteGlu$_2$	19	n.d.	117	1278	n.d.	n.d.		653
Total recovered from column	1338	30 080	1569	13 160	456	13 360	5353	23 460
Range of total folates in extracts before chromatography	1400 to 2800	32 200	2200 to 7100	14 400	1500 to 2100	15 900	5500 to 7900	28 100

n.d.—Not detected. Data are expressed as ng PteGlu equivalents/g dry wt for *L. casei*. Extracts were prepared from fresh tissue and chromatographed on DEAE-cellulose before and after γ-glutamylcarboxypeptidase (γ-GCP) treatment. Fractions were assayed with *L. casei* and *P. cerevisiae*. The large bracket denotes that the pteroylglutamate activity eluted in the position of these derivatives could not be assigned to any one derivative.

Changes in Folate Content of Radish Cotyledons during Germination, Senescence and Etiolation

The importance of folates in the metabolism of photosynthetic tissues might conceivably be elucidated by a comparison of their levels in green and etiolated tissues. Figure 1 illustrates that a rapid increase in the concentration of total folates occurred in germinating radish cotyledons. Tissues germinated in the dark contained lower levels of folates than cotyledons of comparable age germinated in the 13:11 hr light:dark regime. Maximal levels were reached at 6–7 days, at which time the green cotyledons contained nearly twice the folate content of the etiolated tissues. After 11 days sampling of etiolated tissue was discontinued due to the onset of fungal attack. The level of folates in the green tissue decreased markedly as the cotyledons became progressively senescent. The onset of senescence was apparent by a visible loss of chlorophyll and an increase in the fr. wt/dry wt ratio of the tissue samples.

In further experiments (Table 2) etiolation of wheat leaves was also associated with a decreased folate content; the level being 7·4 μg/g dry wt as compared to approximately 13·3 μg/g dry wt for the green tissue. In general, the quantities of individual derivatives were lower in the etiolated tissues but the percentage of methylated compounds was higher (Table 2).

The Effects of Light on Levels of Folate Derivatives

Radish seedlings were grown with daily illumination (13:11 hr, light:dark cycle) for 5 days. On the 6th day samples of the cotyledons were excised and immediately frozen in dry ice. Such samples were taken 1 hr before and 5 hr after the lights were turned on. Assay of carboxypeptidase treated extracts (Table 3) showed that a decrease in total folate content

occurred after the period of illumination whether the levels were expressed on a dry wt or chlorophyll basis. The effects of illumination were examined in more detail after column chromatography. Since levels of mono- and di-glutamate derivatives with the same sub-

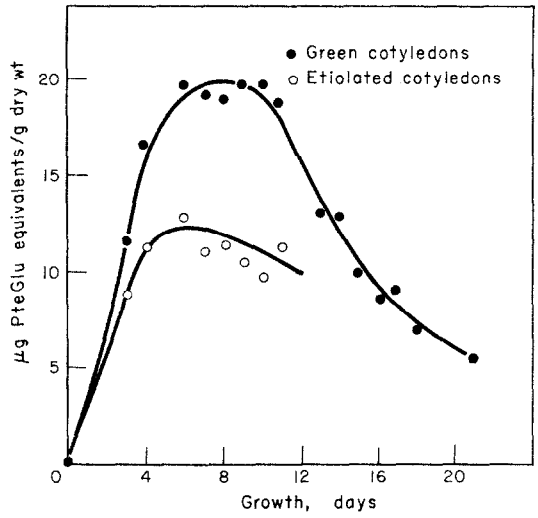

FIG. 1. CHANGES IN FOLATE CONTENT OF RADISH COTYLEDONS DURING GERMINATION. Green and etiolated cotyledons were obtained from seedlings grown in a 13:11 hr light:dark cycle and total darkness respectively. Each point is the average of duplicate extractions. The data are expressed in PteGlu equivalents for *L. casei*.

stituent group were similarly affected, they are included together in Table 3 for clarity. The illuminated tissues were found to contain significantly lower levels of formyl derivatives and considerably greater quantities of unsubstituted derivatives in two separate experiments. The level of methyl derivatives was only slightly affected under these conditions.

TABLE 2. LEVELS OF FOLATE DERIVATIVES IN EXTRACTS OF ETIOLATED AND GREEN RADISH COTYLEDONS AND WHEAT LEAVES

	Radish		Wheat	
Derivative	Etiolated	Green	Etiolated	Green
10-HCO-H_4PteGlu	371	553	879	238
10-HCO-H_4PteGlu$_2$	3110	10 090	1890	6780
5-HCO-H_4PteGlu	88	n.d.	944	n.d.
5-CH_3-H_4PteGlu	236	698	1457	275
10-HCO-H_4PteGlu$_3$	n.d.	n.d.	n.d.	861
H_4PteGlu	n.d.	n.d.	211	n.d.
5-HCO-H_4PteGlu$_2$	2125	3255	938	3807
5-CH_3-H_4PteGlu$_2$	8535	15 220	1119	1397
Total recovered % of total recovered	14 470	30 080	7438	13 360
Methyl derivatives	61%	53%	35%	13%
Other derivatives	39%	47%	65%	87%

n.d.—Not detected. Data are expressed as ng PteGlu equivalents/g dry wt for *L. casei*. Extracts were prepared from 6-day-old radish and 8-day-old wheat seedlings. After γ-glutamylcarboxypeptidase treatment, the extracts were chromatographed and assayed with *L. casei* and *P. cerevisiae*.

The Effect of Aminopterin on Folate Contents of Radish Cotyledons

In these experiments excised radish cotyledons were infiltrated with 2×10^{-5} M aminopterin and incubated in the light for periods up to 24 hr. The effect of such treatment on the levels of individual derivatives is shown in Table 4. Within 3·75 hr the level of methyl

TABLE 3. THE EFFECT OF LIGHT ON THE LEVELS OF FOLATE DERIVATIVES IN RADISH COTYLEDONS

Folate	Experiment 1				Experiment 2			
	Dark		Light		Dark		Light	
	Level	%	Level	%	Level	%	Level	%
Total folate μg/g dry wt								
L. casei	32·0		25·0		32·4		25·2	
S. faecalis	21·9		18·5		24·3		18·5	
L. casei μg/mg chlorophyll	3·21		2·53		3·49		2·63	
Derivatives by group μg/g dry wt								
Formyl derivatives	16 980	66	6200	33	16 270	62	5250	28
Methyl derivatives	8050	32	5180	27	8260	32	7250	37
Unsubstituted derivatives	630	2	7480	40	1530	6	6910	35
Total recovered from column	25 660		18 860		26 060		19 410	

The seedlings received 13 hr of illumination daily, starting at 10 a.m. On the 6th day samples of the cotyledons were quickly excised at 9 a.m. (dark) and 3 p.m. (light) and immediately killed by freezing in an acetone-dry ice bath. After lyophilization of the tissue samples, derivatives were extracted, treated with γ-glutamyl-carboxypeptidase and assayed with *L. casei* and *S. faecalis*. Data, which are averages of duplicate samples, are expressed in PteGlu equivalents per g dry wt and per mg chlorophyll.

Data for individual classes are expressed as ng PteGlu equivalent/g dry wt for *L. casei* and as percentages of total folates recovered from the column.

derivatives was substantially decreased while a derivative not detected in extracts of the control tissues and identified as PteGlu on the basis of elution position and co-chromatography with PteGlu-2-^{14}C was present. After 6 hr of aminopterin treatment a further decrease in the level of methyl derivatives occurred accompanied by large increases in the PteGlu peak. The latter derivative accounted for 62% of the total folate activity recovered after chromatography of these extracts. Analysis of the various derivatives (Table 4) after extending the period of aminopterin treatment confirmed the earlier observations that the

TABLE 4. EFFECT OF AMINOPTERIN ON FOLATE DERIVATIVES OF EXCISED RADISH COTYLEDONS

Derivatives	3·75 hr				6 hr				24 hr			
	Aminopterin		Control		Aminopterin		Control		Aminopterin		Control	
	Level	%	Level	%	Level	%	Level	%	Level	%	Level	%
Formyl derivatives	4970	25	5390	23	6240	19	6140	25	5730	34	7120	29
Methyl derivatives	8180	41	14 250	60	1600	5	12 900	52	390	2	13 510	55
Unsubstituted derivatives†	4230	22	3940	17	4520	14	5960	23	1200	9	4100	17
PteGlu	2360	12	n.d.		19 730	62	n.d.		7690	55	n.d.	
Total recovered from column	19 740		23 580		32 090		25 000		14 010		24 730	

n.d.—Not detected. Data are expressed as ng PteGlu equivalents/g dry wt for *L. casei* and as percentages of total folates recovered from the column. † Excluding PteGlu. Excised 6-day-old radish cotyledons were infiltrated with 2×10^{-5} M aminopterin or water (controls) and incubated for the periods shown as described in the Experimental Section. After lyophilization, extracts were prepared, treated with γ-glutamyl-carboxypeptidase, and chromatographed. *L. casei* and *S. faecalis* were used to assay the derivatives.

levels of methyl derivatives were most affected while large amounts of PteGlu accumulated. On a dry wt basis the aminopterin-treated tissues contained only approximately 58% of total pteroylglutamates of the control tissues.

DISCUSSION

Derivatives of H_4PteGlu *in Photosynthesis Plant Tissue*

In agreement with earlier studies,[24,27] photosynthetic plant tissues were found to contain readily detectable quantities of folate derivatives which principally differed in the C-1 substituent group and the number of glutamic acid moieties. The present results also emphasize that the folates of these photosynthetic tissues are predominately highly conjugated (Table 1) with more than three glutamic acid residues. As these tissues contain appreciable levels of γ-glutamylcarboxypeptidase this predominance of conjugated derivatives was only found when precautions were taken to inactivate such hydrolase activity.[26,28,33] The high levels of conjugated folates and the finding that this class of derivatives was affected by light (Tables 2 and 3) and aminopterin treatment (Table 4) suggests that they represent more than a storage pool of C-1 units.[29] Indeed, it is possible that some reactions of C-1 metabolism in such tissues may involve highly conjugated folates. In support of this suggestion is the finding[15] that the rate of methionine formation by 5-CH_3-H_4PteGlu : homocysteine methyltransferase from plant leaves is increased 5–10 times when 5-CH_3-H_4PteGlu$_3$ replaces 5-CH_3-H_4PteGlu as methyl donor. There may be other such enzymes in plants which preferentially utilize conjugated folates, a situation already documented for microorganisms.[34–37] However as the majority of studies with plant extracts have been carried out almost exclusively with monoglutamate derivatives it is clear that conjugation is not an absolute or general requirement for enzyme activity *in vitro*.

From Tables 1 and 2 it is apparent that different tissues contained varying proportions of individual derivatives with respect to the C-1 substituent group. For example, 53% of the C-1 moieties bound to the pteroylglutamates of radish cotyledons consisted of methyl groups, while in wheat leaves the corresponding value was only 13%. Unsubstituted derivatives were also detected in the extracts but were generally only minor constituents. The occurrence of these compounds was, however, affected by prior illumination of the cotyledons (Tables 3 and 4).

Folates of Etiolated and Green Tissues

The rapid increase in folate content of radish cotyledons which accompanied germination (Fig. 1), like that occurring in pea cotyledons,[28,38] probably reflects an increased demand for C-1 units as the metabolic activities of the developing seedling increase. Furthermore, green cotyledons had higher folate levels than etiolated tissues of comparable age (Fig. 1, Table 2). The possibility that a correlation exists between folate content and metabolic intensity is further suggested by the higher levels of these derivatives in tissues with photosynthetic capacity (Table 2) and the decline in folate content which accompanied senescence (Fig. 1). The higher levels present in green tissue may also be related to a requirement for C-1 units

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in the biosynthesis of chlorophyll in addition to involvement in other aspects of chloroplast metabolism.[39,40]

Possible Functions of H_4PteGlu *Derivatives in Photosynthetic Metabolism*

After illumination of radish cotyledons a large part of the folate pool originally at the formyl level of oxidation occurred as H_4PteGlu. This change could either reflect a net loss of formyl groups or the synthesis of 5,10-methylene H_4PteGlu derivatives as the latter would appear as H_4PteGlu in the neutral, aqueous extracts employed for analysis. The apparent inverse relationship which existed between these derivatives on transfer to light could conceivably be related to the light-dependent flow of carbon in the glycolate pathway as methylene derivatives would be generated and utilized in the glycine decarboxylase and serine hydroxymethyltransferase reactions.[41] Recent studies have indicated that glycine decarboxylation within the glycolate pathway may proceed at a rate greater than that catalyzed by serine hydroxymethyltransferase *in vitro*.[14] If this situation prevailed *in vivo*, 5,10-methylene-H_4PteGlu derivatives would be expected to accumulate, resulting in the observed higher levels of unsubstituted H_4PteGlu derivatives. In order to account for the changes in the levels of formyl derivatives in this proposal, it is further suggested that in darkness these derivatives may represent a storage pool of C-1 units which tend to be rapidly reduced on illumination via methylene tetrahydrofolate dehydrogenase and $NADPH_2$ and are, therefore, available to enter the glycolate pathway at the point of serine synthesis. Such formyl derivatives could logically be included in the small unlabeled C-1 pool which Hess and Tolbert[42] have postulated to account for the lower than expected levels of ^{14}C in the 3 position of serine formed by tobacco leaves after 4 sec of photosynthesis in $^{14}CO_2$. Alternatively, H_4PteGlu could enter the pathway at the point of glycine decarboxylation after loss of the formyl group in transformylation reactions.

These suggestions however assume that radish cotyledons form glycine and serine via the reactions of the glycolate pathway. To examine this, cotyledon disks[43] were allowed to fix $^{14}CO_2$ for periods of 45 sec and 5 min. In such experiments 45–50% of the label was found in the amino acid fraction, 50% of this being in glycine and serine after 45 sec and 85–90% after 5 min. Glycine and serine were both randomly labeled in these samples as determined by ninhydrin and persulfate oxidation.[44] From such data it follows that glycine and serine are both formed from carbon dioxide in this tissue by the reactions of the glycolate pathway.

The Effect of Aminopterin on Folate Derivatives

The present investigations have also shown that treatment of excised radish cotyledons with aminopterin decreased the levels of H_4PteGlu derivatives and led to an accumulation of PteGlu (Table 4). In such experiments the levels of methyl derivatives and PteGlu were most affected and showed an inverse relationship. This apparently selective effect of aminopterin on methyl derivatives implies that the folate metabolism of these tissues is to some extent compartmented so that the bulk of the methylated derivatives occupy a pool distinct from that shared by the formyl and unsubstituted derivatives. Of particular interest in this

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respect are studies regarding the compartmentation of folates in rat liver[45] and pea cotyledons[46] which suggest that mitochondria contain mostly formyl derivatives while the bulk of the methylated derivatives occur in the cytoplasm. A similar association of formyl derivatives with the mitochondria of radish cotyledons would also be consistent with their postulated role in the reactions of the glycolate pathway and would logically explain why the levels of methyl derivatives were least affected by light. The rapid depletion of methyl derivatives in these experiments further suggests that turnover of methyl groups was appreciable in the light. The accumulations of PteGlu in extracts of aminopterin-treated tissues may be related to such turnover or could reflect *de novo* synthesis from guanosine triphosphate before folate deficiency prevented the formation of this precursor.[1] Considering the changes in the levels of PteGlu and methyl derivatives after aminopterin treatment (Table 4), it is suggested that the accumulated PteGlu is derived mainly from the tetrahydropteroylglutamate pool involved in C-1 transfer, methyl group biogenesis, etc. During aminopterin treatment this pool will become depleted of reduced folates as a result of thymidylate synthetase activity and blockage of H_2PteGlu reductase by aminopterin.[1] PteGlu might then be formed from H_2PteGlu either enzymically or non-enzymically. Nieman and Poulsen[47] have presented evidence that DNA synthesis commences within 6 hr after radish cotyledons have been excised and incubated under the conditions employed in the present work. Thus it follows that some turnover of H_4PteGlu would occur through the thymidylate synthetase reaction.

It is of interest to note that PteGlu, and not $PteGlu_n$, accumulated in the tissues treated with aminopterin. In this regard it is possible that there is normally a continuous, slow hydrolysis of all pteroylpolyglutamates to monoglutamates *in vivo* by γ-glutamylcarboxypeptidases. Eventually all conjugated derivatives would be transformed to H_4PteGlu, which alone appears to act as the substrate for pteroylpolyglutamate synthetase.[48] In the presence of aminopterin there would however be no recycling of $H_2PteGlu_n$ and H_2PteGlu to metabolically active derivatives, and subsequent hydrolysis of $H_2PteGlu_n$ would favour accumulation of the monoglutamate derivative.

EXPERIMENTAL

Plant material. All plant material was grown from seed in flats containing a sterilized loam–peat–sand (3:2:1) mixture. Radish (*Raphanus sativus* L. cv. Scarlet Globe) and wheat seedlings (*Triticum vulgare* L. cv. Thatcher) were grown in cabinets at 25° for 6 and 8 days respectively. Spinach (*Spinacea oleracea* L. cv. King of Denmark) and pea (*Pisum sativum* L. cv. Homesteader) plants were grown at 18° for 30–40 days. Illumination (185 lx at soil level) was provided by fluorescent and incandescent lighting. The flats were moved from the growth cabinet to the laboratory 30 min before excision of tissues unless stated otherwise in the Results. In some cases radish cotyledons were quickly frozen in an acetone dry ice bath, lyophilized, and stored under vacuum at −20°. When fresh tissue was used, dry wt equivalents were obtained by use of a dry wt/fr. wt ratio. The ratio was determined by drying a sample of weighed tissue at 105–110° for 24 hr.

Infiltration of radish cotyledons with aminopterin. Excised cotyledons were washed with H_2O and vacuum infiltrated with 2×10^{-5} M aminopterin (pH 6·5 with KOH) to ensure rapid uptake of inhibitor. The cotyledons were placed in covered Petri dishes (9 cm) containing 10 ml of the infiltrating solution and illuminated with a Hg lamp (185 lx), for up to 6 hr at 23 ± 2°. Some modifications were introduced in the 24-hr experiments. The infiltrated cotyledons were supported on a sterile pad consisting of 4 layers of filter paper (Whatman, No. 1) and 2 layers of cheese cloth in 13 cm Petri dishes. The support was moistened with 30 ml of a sterile nutrient medium containing 80 mM KNO_3, 1% sucrose, 5 mM KH_2PO_4, 20 μM aminopterin and adjusted to pH 6·5 with KOH. The Petri dishes with the cotyledons were then placed in a growth

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cabinet at 23° and illuminated with fluorescent-plus-tungsten lamps at 140 lx. Cotyledons infiltrated with H_2O and incubated without aminopterin served as controls in these experiments. On completion of the experiments the tissues were lyophilized.

Extraction of pteroylglutamates from plant tissues. Samples of tissue (1–2 g fr. wt or 0·1–0·2 g dry wt) were immersed for 10 min in 15 ml of 12 mg/ml ascorbic acid buffer (pH 6·0 with KOH) maintained at 95°. Aftee cooling in ice the tissue was ground in a glass homogenizer. Cellular debris and insoluble materials werr removed by centrifugation at 18 000 *g* for 10 min. The pellet was washed by resuspending it twice in 10 ml of the ascorbate buffer.

Determination of chlorophyll. Chlorophyll concentrations were determined by preparing extracts of lyophilized radish cotyledons with 80% acetone and reading their absorbancies at 645 and 663 nm[49] in a spectrophotometer.

Microbiological assay and column chromatography of folate derivatives. The assay of folates with *L. casei* (ATCC 7469) *S. faecalis* (ATCC 8043) and *P. cerevisiae* (ATCC 8081) and separation of individual derivatives on DEAE-cellulose have been described earlier.[26,28,31] All samples were routinely assayed in duplicate and for determination of total folates at least three aliquot sizes were employed.

Enzymatic hydrolysis of conjugated derivatives. In this investigation, γ-glutamylcarboxypeptidase was prepared from 3 g of Difco Bacto chicken pancreas extract (Difco Laboratories, Detroit, U.S.A.).[50] The incubation mixture, routinely contained 0·2 ml enzyme (0·8 mg protein), 0·1 ml of 0·25 M $CaCl_2$, 2·5 ml of 0·2 M boric acid adjusted to pH 7·8 with NaOH, and 5 ml of extract containing pteroylpolyglutamates in a final volume of 10 ml. After incubation for 5 hr at 37° the reaction was terminated by heating the tubes at 95° for 10 min.

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