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THE OCCURRENCE AND FUNCTION OF PTEROYLGLUTAMATES IN RADISH COTYLEDONS

by

© ADRIAN MAURICE C. SPRONK

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled 'The occurrence and function of pteroylglutamates in radish cotyledons' submitted by Adrian Maurice C. Spronk in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

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Date Oct. 19, 1971

ABSTRACT

A detailed examination of pteroylglutamate derivatives in extracts of spinach, pea, and wheat leaves, as well as the cotyledons of radish seedlings, has been conducted. Derivatives were assayed microbiologically with *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043) and *Pediococcus cerevisiae* (ATCC 8081) after separation by DEAE-cellulose chromatography and treatment with γ-glutamyl carboxypeptidases. On a dry weight basis, spinach, pea and wheat leaves, and radish cotyledons contained 28, 15, 14 and 30 μg pteroylglutamate activity for *L. casei* per g of tissue, respectively. In general, the constituents of the pteroylglutamate pool were conjugated with more than three glutamic acid residues and were substituted with formyl and methyl groups in the N^{10} and N^5 positions of tetrahydropteroylglutamic acid respectively. Smaller quantities of N^5-formyl and unsubstituted derivatives were also detected. Highly conjugated derivatives were only detected when precautions were taken to destroy endogenous γ-glutamyl carboxypeptidase activity. This was routinely achieved by heating the tissue at 95°C in ascorbate buffer before homogenization.

A comparison of pteroylglutamate contents in cotyledons obtained from seedlings grown in a 13:11 hour light:dark cycle with those of etiolated seedlings revealed that green tissues contained greater quantities of all derivatives, the total levels being approximately twice that of etiolated cotyledons. Illumination of etiolated seedlings for 24 hours increased the pteroylglutamate level by approximately 20%. This increase was inhibited by treatment with aminopterin which also markedly inhibited chlorophyll biosynthesis. Also, levels of pteroylglutamates in cotyledons increased rapidly during the first 6 days of growth and declined with senescence.

It is concluded that tissues with a more complex and intense metabolism require greater amounts of these compounds to mediate their one-carbon metabolism.

An examination of seedlings grown in a 13:11 hour light:dark cycle revealed that levels of pteroylglutamate derivatives were altered by commencement of the illumination period. The major effects were a decrease of 10 μg in formyl derivatives and an increase of 7 μg in unsubstituted derivatives per g dry cotyledons. The significance of these changes are discussed in relation to operation of the glycolate pathway and changes in the levels of serine associated with illumination of these tissues.

Formate-C^{14} was readily incorporated into methyl and formyl pteroylglutamates of illuminated (2000 foot candles) cotyledon disks during a 10 min feeding period, suggesting that some interconversion of these derivatives occurred *in vivo*. No radioactivity was detected in these compounds after administering $HC^{14}O_3^-$ and glycine-2-C^{14}. The reasons for this are discussed in relation to the synthesis of 5,10-methylene-C^{14}-tetrahydropteroylglutamate and its compartmentation.

Aminopterin-treatment of radish cotyledons resulted in a rapid depletion of methyl pteroylglutamates while levels of formyl and unsubstituted derivatives declined less abruptly. Pteroylglutamic acid accumulated in these tissues. Studies of the effects of aminopterin-induced pteroylglutamate deficiency on the ability to fix $HC^{14}O_3^-$ indicated that photosynthesis was partially inhibited.

The results are discussed in relation to earlier investigations which have implicated pteroylglutamate derivatives in the one-carbon metabolism of plants. It is suggested that these derivatives are important in radish cotyledons in the formation of methyl groups and in reactions of the glycolate pathway.

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LIST OF ABBREVIATIONS

PteGlu	:	pteroylglutamic acid
H_2PteGlu	:	dihydropteroylglutamic acid
H_4PteGlu	:	tetrahydropteroylglutamic acid
5-HCO-H_4PteGlu	:	5-formyltetrahydropteroylglutamic acid
5-CH_3-H_4PteGlu	:	5-methyltetrahydropteroylglutamic acid
10-HCO-H_4PteGlu	:	10-formyltetrahydropteroylglutamic acid
5,10-CH_2=H_4PteGlu	:	5,10-methylenetetrahydropteroylglutamic acid
5,10-CH≡H_4PteGlu	:	5,10-methenyltetrahydropteroylglutamic acid
5-HCNH-H_4PteGlu	:	5-formiminotetrahydropteroylglutamic acid
$PteGlu_2$	:	pteroyldiglutamic acid
$PteGlu_3$	:	pteroyltriglutamic acid
$PteGlu_n$	:	pteroylpolyglutamic acid (indeterminate number of glutamic acid moieties)
DCMU	:	3-(3,4-dichlorophenyl)-1,1 dimethyl urea
PPO	:	2,5-diphenyloxazole
Dimethyl-POPOP	:	*p*-bis[2-(4-methyl-5-phenyloxazolyl)]-benzene

All other abbreviations are those commonly used in biochemical literature.

INTRODUCTION AND LITERATURE REVIEW

Biological systems have a special coenzyme, H_4PteGlu or derivatives with additional glutamic acid moieties, associated with the metabolism of one-carbon units. This coenzyme is a reduced form of PteGlu, a vitamin commonly referred to as folic acid. H_4PteGlu serves as a coenzyme in a number of otherwise unrelated pathways by acting as an acceptor or donor of one-carbon units at the oxidation levels of formate, formaldehyde, and methanol. This coenzyme is functional in the biosynthesis and degradation of purines, pyrimidines, and various amino acids. It is also important in other cellular reactions such as protein biosynthesis and the *de novo* generation of methyl groups. The metabolic role of H_4PteGlu will be discussed below with special reference to plant systems. Other aspects of this coenzyme as a vitamin, such as its discovery, its chemical and physical properties, its synthesis, as well as its importance in clinical studies, are not of direct interest here and the reader is referred to a number of excellent reviews (Wagner and Folkers, 1965; Huennekens and Osborn, 1959; Stokstad, 1954; Johns and Bertino, 1965; Nystrom and Nystrom, 1967; Sullivan, 1967; Baker and Frank, 1968).

Pteroylglutamates and the Metabolism of Pyrimidines and Purines

Nutritional studies with bacteria have revealed that pteroylglutamates are involved as coenzymes in the biosynthesis of thymine derivatives (Stokes, 1944). With the advent of compounds enriched with isotopic carbon, it was shown that a one-carbon group, arising from the 3-position

of serine or the 2-position of glycine, was readily incorporated into thymine in bacteria (Crosbie, 1958) and rats (Elwyn and Sprinson, 1950). Formate and formaldehyde were also good precursors in this synthesis (Goldthwait and Bendich, 1952; Hamill *et al.*, 1956). Pteroylglutamate deficiency caused by an aminopterin treatment, markedly decreased incorporation of these precursors in the rat (Goldthwait and Bendich, 1952) and in suspensions of mouse Erlich cells (Prusoff *et al.*, 1956). Aminopterin is a pteroylglutamate antagonist and interferes in the biosynthesis or regeneration of H_4PteGlu from H_2PteGlu. This latter pteroylglutamate derivative is inactive as a coenzyme, *i.e.* H_2PteGlu cannot function as a carrier of one-carbon units in biological systems.

Elucidation of thymine biosynthesis *in vitro* followed, and was culminated by proposal of a sequence of three reactions (reactions 1-3) for the biosynthesis of deoxythymidine and the subsequent regeneration of the coenzyme with a one-carbon substituent (Humphreys and Greenberg, 1958).

$$\text{d UMP} + 5,10\text{-}CH_2\text{=}H_4\text{PteGlu} \rightleftharpoons \text{d TMP} + H_2\text{PteGlu} \qquad (1)$$

$$H_2\text{PteGlu} + \text{NADPH}_2 \rightleftharpoons H_4\text{PteGlu} + \text{NADP} \qquad (2)$$

$$H_4\text{PteGlu} + \text{"}C_1\text{"} \rightleftharpoons 5,10\text{-}CH_2\text{=}H_4\text{PteGlu} \qquad (3)$$

The reaction catalyzed by thymidylate synthetase (reaction 1) is of special interest as the pteroylglutamate coenzyme plays a dual role: firstly, as a donor of the one-carbon group, and secondly, as a reductant. Therefore, before the same pteroylglutamate molecule can be involved again in the transfer of one-carbon groups (reaction 3), its pteridine ring must be reduced at the expense of $NADPH_2$ (reaction 2). It should be noted that the one-carbon group in reaction 3 must enter at the

formaldehyde level of oxidation to allow repetition of the sequence of reactions presented here.

The thymidylate synthetase system has been studied almost exclusively with soluble enzyme preparations from thymus and *E. coli*. Despite the importance of this system in the biosynthesis of a DNA precursor, it is surprising that its presence has been demonstrated in only relatively few tissues. It is of interest to note that to date, no higher plant tissues have been examined for thymidylate synthetase (Blakley, 1969).

Extracts of *E. coli* infected with T-even phage can synthesize another pyrimidine, 5-hydroxymethylcytidylate, according to equation 4 (Flaks and Cohen, 1959).

$$\text{d CMP} + 5,10\text{-CH}_2\text{-H}_4\text{PteGlu} + \text{H}_2\text{O} \rightleftharpoons \text{5-hydroxymethyl-d CMP} + \text{H}_4\text{PteGlu} \qquad (4)$$

Since no oxidation of the pteridine ring occured in this reaction, its mechanism is believed to be quite different from that of thymidylate synthetase.

In purine ring biosynthesis the H_4PteGlu derivatives play an important role as donors of carbons 2 and 8. The reactions involved have been extensively studied with extracts of avian liver, a tissue in which purine synthesis is high, due to the formation of uric acid, a major excretory product. 5,10-CH=H_4PteGlu provides a one-carbon group (reaction 5) which eventually becomes carbon 8 of the purine ring (Buchanan and Hartman, 1959). Carbon-2, the last carbon added before closure of the purine ring, is derived from 10-HCO-H_4PteGlu (reaction 6) (Buchanan and Hartman, 1959).

2-amino-N-ribosyl-acetamide-5'-phosphate + 5,10-CH=H_4PteGlu ⇌ 2-formamido-N-ribosyl-acetamide-5'-phosphate + H_4PteGlu (5)

5-amino-1-ribosyl-4-imidazole-carboxamide-5'-phosphate + 10-HCO-H_4PteGlu ⇌ 5-formamide-1-ribosyl-4-imidazole-carboxamide-5'-phosphate + H_4PteGlu (6)

Studies with bacterial cultures support a similar pathway for purine biosynthesis in microorganisms. Intermediates of the pathway were metabolized as expected and blockage of the pathway by either a genetic deficiency or inhibitor resulted in the accumulation of intermediates preceeding the blocked reaction (Hartman and Buchanan, 1959).

There is rather fragmentary evidence for this pathway of purine biosynthesis in higher plants. For example, Iwai *et al.* (1963) have shown that sulfonamide treated pea seedlings which were folate deficient accumulated 2-amino-N-ribosyl-acetamide-5'-phosphate. In other work, seedling extracts of *Phaseolus radiatus* appeared to utilize 5-amino-imidazole-carboxamide at increased rates as germination proceeded (Giri and Krishnaswamy, 1957). This same intermediate labelled with C^{14} was readily incorporated into purines of excised wheat embryos (Berezniak and Wang, 1961). The involvement of pteroylglutamates in the biosynthesis of both deoxy- and ribonucleic acids in algae is suggested by the work of Scheffrahn (1966). It was found that *p*-aminobenzoic acid and PteGlu could reverse the marked decrease in nucleic acid content of sulfonamide-inhibited *Chlorella* cultures.

Pteroylglutamates also participate in the fermentation of purines

by *Clostridium* and other bacteria (Rabinowitz and Pricer, 1957). Formiminoglycine, an intermediate in this fermentation, was further degraded to glycine in a reaction requiring H_4PteGlu (reaction 7). The 5-HCNH-H_4PteGlu was then oxidized to 10-HCO-H_4PteGlu, which in turn was converted to formate and H_4PteGlu (see later section of this Introduction).

$$\text{formiminoglycine} + H_4\text{PteGlu} \rightleftharpoons \text{5-HCNH-}H_4\text{PteGlu} + \text{glycine} \qquad (7)$$

Metabolism of purines in plants has not been clearly elucidated to date. Scanty evidence supports a pathway in which purines are firstly oxidized to allantoin, which is eventually hydrolyzed to urea and glyoxylate (Reinbothe and Mothes, 1962). For example, radioactivity readily appeared in urea after the administration of adenine-8-C^{14} to leaves of several higher plants (Reinbothe, 1961). Also a proposed route for the metabolism of glycine to urea in wheat seedlings included purines and allantoin as intermediates (Krupka and Towers, 1958). The occurrence of uricase and allantoinase in higher plants may have physiological significance in this connection (Theimer and Beevers, 1971). Thus in higher plants it appears that formiminoglycine is not an intermediate in purine degradation; and hence H_4PteGlu would not be involved in this aspect of plant purine metabolism.

Pteroylglutamates and the Metabolism of Amino Acids

Nutritional studies and radioisotope feeding experiments employing a variety of organisms have revealed that a close biological relationship exists between glycine and serine (for example, see reviews by Blakley, 1969; Huennekens and Osborn, 1959). The freely reversible interconversion of serine and glycine (reaction 8) was found to require H_4PteGlu (Kisliuk

and Sakami, 1954; Blakley, 1954) and pyridoxal-5'-phosphate (Blakley, 1955). The enzyme involved,

$$\text{serine} + H_4\text{PteGlu} \rightleftharpoons \text{glycine} + 5,10\text{-}CH_2\text{=}H_4\text{PteGlu} \qquad (8)$$

commonly named serine hydroxymethyltransferase (E.C. 2.1.2.1 L-serine: tetrahydrofolate-5,10-hydroxymethyltransferase) has been isolated from a wide variety of organisms (Blakley, 1969). Glycine-serine interconversion is readily catalyzed by cell-free plant tissue extracts from many sources, including turnip hypocotyls (Wilkinson and Davies, 1958), castor bean endosperm, pea cotyledons, corn coleoptiles, carrot storage tissue and wheat leaves (Cossins and Sinha, 1966). The enzyme has been partially purified from turnip hypocotyls (Wilkinson and Davies, 1960), cauliflower florets (Mazelis and Liu, 1967), tobacco roots (Prather and Sisler, 1966), and corn leaves (Hauschild, 1959). All the purified enzyme preparations from plant tissues required pyridoxal-5'-phosphate as well as H_4PteGlu for optimal activity. Chloroplast extracts were also capable of forming serine-C^{14} when incubated in the presence of glycine, formaldehyde, H_4PteGlu, and pyridoxal-5'-phosphate (Shah and Cossins, 1970b). Additional evidence for the presence of this enzyme in plants comes from numerous studies of the fate of glycine-C^{14} and serine-C^{14} *in vivo* (for example, see Rabson, Tolbert and Kearney, 1962; Miflin, Marker and Whittingham, 1966; Sinha and Cossins, 1964; Wang and Burris, 1965).

Blakley (1969) is of the opinion that serine (reaction 8) is quantitatively the most important source of "active" one-carbon units in both mammals and microorganisms. If this is so, the fate of the other product, glycine, must also be considered. It can be envisioned that more

glycine would be formed than required for further metabolism, such as protein biosynthesis. It has been argued that the major pathway of glycine catabolism to CO_2 in rat liver is via glyoxylate and formate. These conclusions were based on results obtained by "metabolic-trapping" of products formed from glycine-C^{14} by rat liver extracts (Nakada *et al.*, 1955). The formate could, of course, enter the active one-carbon pool before further oxidation to CO_2 (see later part of this Introduction). On the other hand, in experiments with avian liver slices and homogenates, it was found that free glyoxylate was not an intermediate in the formation of CO_2 from the carboxyl carbon of glycine (Richert *et al.*, 1962). More advanced studies have shown that glycine is readily decarboxylated (reaction 9) by rat liver homogenates and slices, with the enzyme(s) responsible being localized in the mitochondria (Sato *et al.*, 1967, 1969a; Yoshida and Kikuchi, 1970). This is a H_4PteGlu-dependent reaction.

$$\text{glycine} + H_4\text{PteGlu} \rightleftharpoons 5,10\text{-}CH_2\text{=}H_4\text{PteGlu} + CO_2 + NH_3 \qquad (9)$$

If the product glycine from reaction 8 is degraded in this manner the original serine molecule yields two molecules of 5,10-CH_2=H_4PteGlu, plus CO_2 and NH_3. Thus reaction 9 would have a sparing effect on the entrance of serine-3 carbon units into the one-carbon pool. The glycine cleavage reaction is important to glycine fermenting bacteria, which utilize the 5,10-CH_2=H_4PteGlu in a reversal of reaction 8 to generate 3-carbon molecules for anapleurotic metabolism and oxidation to acetate (Klein and Sagers, 1962).

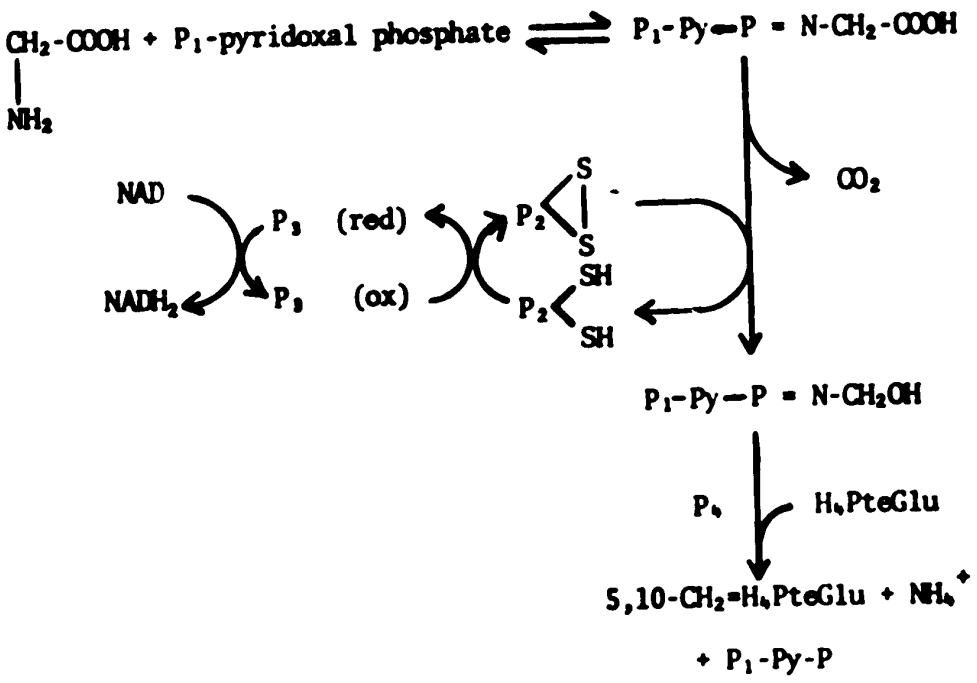
The glycine cleavage reaction has been extensively studied in bacterial systems. It is very complex, involving at least 4 protein factors (P_1, P_2, P_3 and P_4) and 3 cofactors as outlined in the following

scheme (Scheme 1) (Baginsky and Huennekens, 1966). It appears that rat liver has a similar mechanism of glycine cleavage (Motokawa *et al.*, 1969; Sato *et al.*, 1967, 1969a,b). There is some evidence that a glycine splitting reaction may be operative in plant tissues (Cossins and Sinha, 1966). Several other workers have speculated, on the basis of glycine to serine conversion, that such a reaction may occur in plants (for example, see Wang and Waygood, 1962; Rabson, Tolbert and Kearney, 1962). This reaction has also been implicated in the glycolate pathway (Tolbert and Yamazaki, 1969; Bruin *et al.*, 1970) and photorespiration (Kisaki and Tolbert, 1970). The latter two concepts will be described in more detail in a later section of this Introduction.

5-CH_3-H_4PteGlu or its polyglutamate derivatives are instrumental in the biosynthesis of methionine from homocysteine in a reaction catalyzed by 5-CH_3-H_4PteGlu:homocysteine methyltransferase. This enzyme, when isolated from *Neurospora crassa*, *Escherichia coli*, and *Saccharomyces cerevisiae*, was specific for 5-CH_3-H_4PteGlu$_2$ or 5-CH_3-H_4PteGlu$_3$ (Burton *et al.*, 1969). The transmethylase in extracts of bean leaves utilized both mono- and triglutamates of 5-CH_3-H_4PteGlu as methyl donor in methionine synthesis (Burton and Sakami, 1969). A part of this transmethylase activity in leaves is localized in the chloroplasts (Shah and Cossins, 1970a). In work with germinating pea cotyledons, Dodd and Cossins (1969, 1970) have shown the presence of a homocysteine-dependent transmethylase with a high affinity for 5-CH_3-H_4PteGlu as methyl donor. Since these tissues were known to be actively synthesizing 5-CH_3-H_4PteGlu and S-adenosylmethionine, it was suggested that this transmethylase probably has importance in the *de novo* synthesis of methionine, which in turn is converted into S-adenosylmethionine by

SCHEME 1

THE MECHANISM PROPOSED FOR THE CLEAVAGE OF GLYCINE



ATP:L-methionine S-adenosyltransferase (E.C. 2.5.1.6) (Mudd, 1960; Dodd and Cossins, 1970; Cantoni, 1965). S-adenosylmethionine is known to be a major methyl donor in many biosynthetic reactions (Mudd and Cantoni, 1964; Meister, 1965). Considering these reactions, it is clear that pteroylglutamates play an indirect, but key role in a variety of transmethylation reactions.

Another series of reactions dependent on 5-CH_3-H_4PteGlu accounting for the biosynthesis of methionine exists in microorganisms and mammals (see, for example, Blakley, 1969). The reaction mechanism is very complex and is as yet not fully understood. Intensive investigations by various laboratories have shown that the reaction requires cobalamin, S-adenosylmethionine, and certain reducing agents (Taylor and Weissbach, 1967, 1968, 1969a,b; Taylor and Leslie, 1970a,b; Taylor, 1970, Rosales *et al.*, 1970). The occurrence of these reactions in higher plants has not been established to date.

In microorganisms, histidine is synthesized by a pathway which includes purine intermediates. A pteroylglutamate derivative, namely 10-HCO-H_4PteGlu, plays a key role in the reaction sequence by donating a one-carbon unit which becomes the carbon 2 of the imidazole ring (Meister, 1965). All animal species studied required dietary histidine for growth and maintenance of nitrogen equilibrium (Meister, 1965). Plants contain histidine and being autotrophic must of necessity be able to synthesize it. However, to the writer's knowledge, no studies concerning histidine biosynthesis in higher plants have been published.

The catabolism of histidine in mammalian liver and microorganisms occurs via the intermediary formation of formiminoglutamic acid in the urocanoic acid pathway (Meister, 1965). An enzyme from mammalian liver

capable of catalyzing the transfer of the formimino group to H_4PteGlu has been isolated (Tabor and Rabinowitz, 1956). It would be of interest to determine whether plant tissues, particularly senescing tissues, catabolize histidine by involvement of H_4PteGlu. Again no information concerning this aspect of histidine metabolism is available for plant tissues. Other pathways would also have to be considered, for example, those involving a non-enzymic oxidation of imidazolonepropionic acid (Mahler and Cordes, 1965).

Much evidence has been accumulated to indicate that N-formyl methionine plays an important role in the initiation of polypeptide synthesis in bacterial systems (Attardi, 1967; Lengyel and Soll, 1969). In the presence of a transformylating enzyme, the formyl group from 10-HCO-H_4PteGlu is transferred to methionyl-transfer ribonucleic acid (met-$tRNA_f$) (Dickerman, 1967). Present knowledge regarding the mechanism of protein synthesis in chloroplasts and mitochondria of higher plants suggests that it is similar to that of bacterial systems (see review by Boulter, 1970) including chain initiation by N-formyl-methionyl-$tRNA_f$ (Schwartz *et al.*, 1967; Burkard *et al.*, 1969; Merrick and Dure, 1971; Leis and Keller, 1971). However, the mechanisms of protein biosynthesis in the cytoplasm of higher plants and animals appear to be different from that of bacteria in a number of respects (Boulter, 1970). This latter mechanism does not appear to include chain initiation with N-formyl methionine.

Formation and Interconversion of H_4PteGlu Derivatives

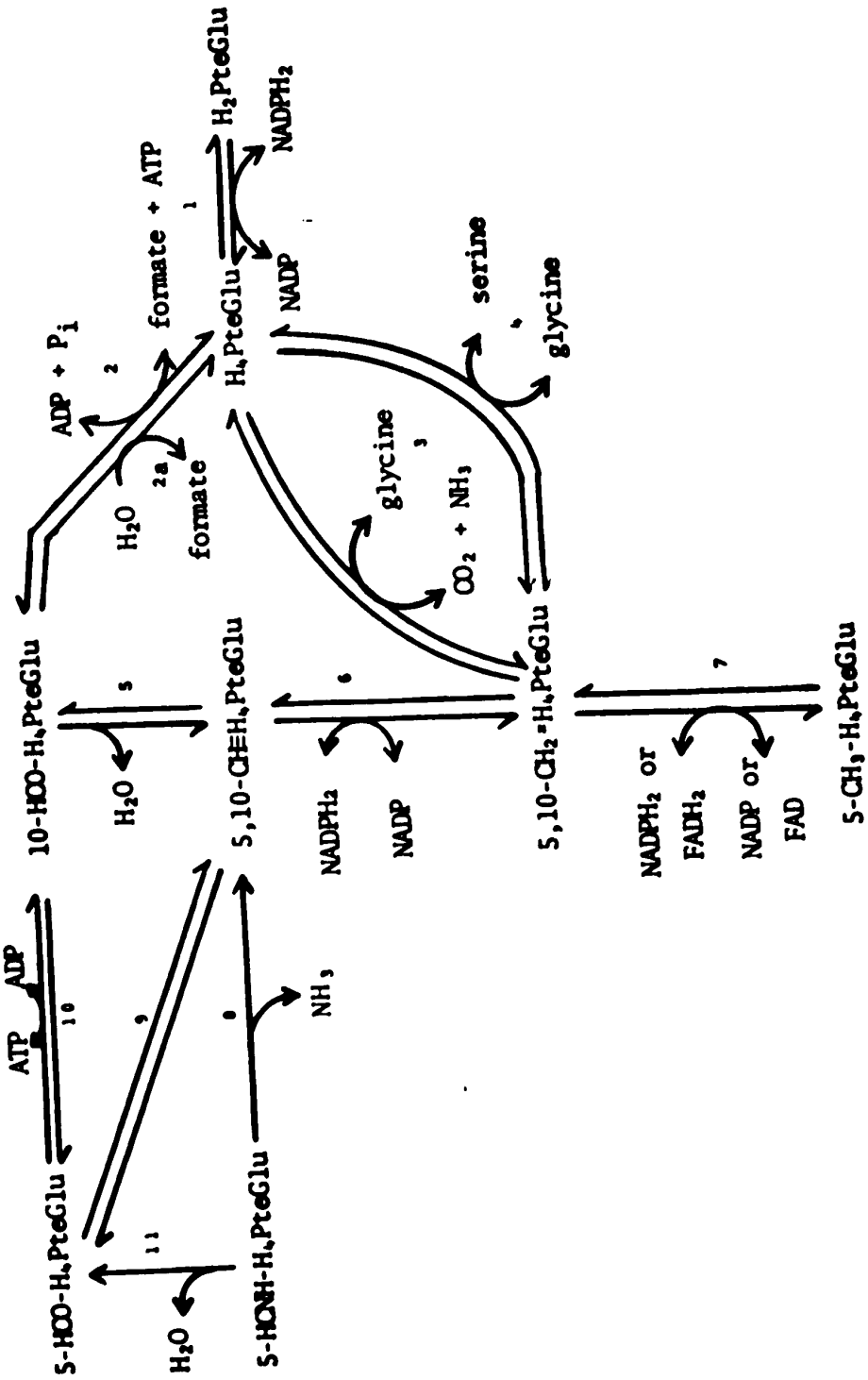
Scheme 2 summarizes the major reactions involved in the formation and interconversion of H_4PteGlu derivatives substituted with a one-carbon

SCHEME 2. FORMATION AND INTERCONVERSION OF TETRAHYDROPTEROYLGLUTAMATE COENZYME DERIVATIVES IN HIGHER PLANTS

Reaction No.	Trivial Name of Enzyme	Systematic Name of Enzyme	E.C. Number
1	H_2PteGlu reductase	5,6,7,8-H_4PteGlu:NADP oxidoreductase	1.5.1.3
2*	10-HCO-H_4PteGlu synthetase	Formate:H_4PteGlu ligase (ADP)	6.3.4.3
2a**	10-HCO-H_4PteGlu deformylase	10-HCO-H_4PteGlu amidohydrolase	3.5.1.10
3	Glycine decarboxylase or "Glycine splitting enzyme"	-------	-------
4	Serine hydroxymethyltransferase	L-serine:H_4PteGlu-5,10-hydroxymethyltransferase	2.1.2.1
5	5,10-CH=H_4PteGlu cyclohydrolase	5,10-CH=H_4PteGlu-5-hydrolase (decyclizing)	3.5.4.9
6	5,10-CH_2=H_4PteGlu dehydrogenase	5,10-CH_2=H_4PteGlu:NADP oxidoreductase	1.5.1.5
7	5,10-CH_2=H_4PteGlu reductase	5,10-CH_2=H_4PteGlu:NAD oxidoreductase	1.1.1.68
8**	5-HCNH_2-H_4PteGlu cyclodeaminase	5-HCNH-H_4PteGlu ammonia-lyase (cyclizing)	4.3.1.4
9**	5-HCO-H_4PteGlu cyclodehydrolase	-------	-------
10**	5-HCO-H_4PteGlu:10-HCO-H_4PteGlu mutase	-------	-------
11**	(Nonenzymic reaction)	-------	-------

* This reaction is reversible in certain purine fermenting bacteria and can be used for ATP formation (for example, see Friedkin, 1963). It also is reversible in spinach leaves (Iwai *et al.*, 1967b).

** These reactions have not been demonstrated to occur in higher plants, but could possibly occur.


H₂PteGlu
NADPH₂
formate + ATP
1
ADP + Pi
2
H₂O
2a
formate
H₄PteGlu
NADP
serine
3
glycine
4
glycine
CO₂ + NH₃
10-HCO-H₄PteGlu
5
H₂O
5,10-CHEH₄PteGlu
6
NADPH₂
NADP
5,10-CH₂-H₄PteGlu
7
NADPH₂ or
FADH₂
NADP or
FAD
5-CH₃-H₄PteGlu
ATP
ADP
10
9
8
NH₃
5-HCO-H₄PteGlu
11
H₂O
5-HCNH-H₄PteGlu

SCHEME 2

unit at different levels of oxidation. Enzymes catalyzing these reactions are known to occur in microorganisms and various mammalian tissues (see, for example, Friedkin, 1963; Huennekens, 1966). It is interesting to note that concomitant with reduction of the one-carbon group of 10-HCO-H_4PteGlu, transfer of it to the N^5-position occurs.

In plants the presence of enzymes catalyzing the formation of one-carbon derivatives of H_4PteGlu has been substantiated. For example, both 10-HCO-H_4PteGlu synthetase and serine hydroxymethyltransferase from several plant species have been partially purified and studied (Hiatt, 1965; Iwai *et al.*, 1967a,b,c; Cossins and Sinha, 1966; Mazelis and Liu, 1967; Wilkinson and Davies, 1960; Hauschild, 1959; Prather and Sisler, 1966).

The enzymes which interconvert 10-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu have only had limited study in plants. 5,10-CH_2=H_4PteGlu dehydrogenase has been detected in a number of plant tissues (Wilkinson and Davies, 1960; Wong and Cossins, 1966) and that from pea cotyledons has recently been studied in this laboratory (Cossins *et al.*, 1970). Ammonium sulfate fractionated extracts of pea cotyledons have ability to synthesize 5-CH_3-H_4PteGlu from H_4PteGlu when formaldehyde or serine are provided as a source of one-carbon groups. The cofactor requirements for this synthesis, namely FAD, $NADPH_2$, and pyridoxal phosphate, suggested that reactions 4 and 7 of Scheme 2 were catalyzed by the extracts (Roos, 1971). The absence of 5-CH_3-H_4PteGlu among the pteroylglutamate products when $NADPH_2$ was omitted supports this suggestion. To date the occurrence of 5,10-CH=H_4PteGlu cyclohydrolase has not been unequivocally demonstrated in plants.

The enzymes mentioned thus far in this section are functional in the

de novo formation of methyl groups from more oxidized members of the one-carbon pool. It is emphasized that these reactions are especially important to plants for the provision of the methyl groups required for growth. In contrast, mammals can obtain a large part of their methyl group requirement from the diet.

Scheme 2 also shows the interrelationships of 5-HCNH-H_4PteGlu and 5-HCO-H_4PteGlu with other substituted H_4PteGlu derivatives. Since 5-HCO-H_4PteGlu can be utilized by pea seedlings to relieve H_4PteGlu deficiency (Iwai *et al.*, 1962; Suzuki and Iwai, 1970) it is possible that reaction 9 or 10 of Scheme 2 takes place *in vivo*. However, none of the reactions (reactions 8-11) which convert the 5-substituted derivatives to another form have been conclusively demonstrated with plant systems *in vivo* or *in vitro*.

The biosynthetic pathway of H_4PteGlu has not been fully elucidated in any organism. On the basis of limited studies in plants, the pathway appears to be similar to that proposed for other organisms (for example, see Blakley, 1969; Roos, 1971). Work by Mitsuda and Suzuki has shown that leaf extracts convert guanidine-monophosphate to dihydropteridine (Mitsuda *et al.*, 1965, 1966; Mitsuda and Suzuki, 1968) while Iwai's group has demonstrated that the enzymes required for the utilization of a dihydropteridine and formation of H_2PteGlu are present in pea seedling extracts (Iwai *et al.*, 1968). More detailed investigations of the dihydropteroate (Iwai and Okinaka, 1968; Okinaka and Iwai, 1970a,b,c) and the dihydropteroylglutamate synthetases (Ikeda and Iwai, 1970) have confirmed their role in plant tissues. Additional evidence for a pathway similar to that of other organisms comes from inhibitor studies. Sulfonamides, which are metabolic antagonists of *p*-aminobenzoic acid, induced

symptoms of pteroylglutamate deficiency in pea seedlings and *Chlorella* (Iwai *et al.*, 1962; Scheffrahn, 1966). These symptoms could be relieved by administration of *p*-aminobenzoic acid, PteGlu, or 5-HCO-H_4PteGlu. H_2PteGlu reductase (E.C. 1.5.1.3, 5,6,7,8-H_4PteGlu:NADP oxidoreductase) from a higher plant source has been studied in detail (Andreeva, 1968, Suzuki and Iwai, 1970).

Additional Information Regarding Pteroylglutamates in Plants

Mitchell and his associates (1941) discovered that spinach leaves were a rich source of the vitamin PteGlu and accordingly named it *folic* acid. In spite of this, studies of pteroylglutamate metabolism in plant tissues have been neglected in many respects until recently. However, due to the nutritional and clinical interest in this vitamin, plant material of dietary importance has frequently been analyzed for "folic acid" content. Initially, only total levels as assayed with *Lactobacillus casei*, *Streptococcus faecalis*, and *Pediococcus cerevisiae* were determined (Burkholder and McViegh, 1945; Olson *et al.*, 1949; Toepfer *et al.*, 1951; Banerjee *et al.*, 1954; Holloman *et al.*, 1967). As each bacterial species responded to only certain derivatives of PteGlu (for details see Materials and Methods), such assays suggested that the "folic acid" content of plants contained several different derivatives (Stokstad, 1954). The introduction of paper and column chromatography in later analyses of plant extracts confirmed this prediction. Formyl derivatives of H_4PteGlu and derivatives with the pteridine ring partially or fully oxidized have been identified in plant extracts (Rosso, 1958; Iwai *et al.*, 1959; Butterworth *et al.*, 1963; Santini *et al.*, 1964). In addition, the occurrence of derivatives conjugated with one or more glutamic acid residues have been reported in the literature (Toepfer *et al.*, 1951;

Rosso, 1958; Iwai *et al.*, 1959; Santini *et al.*, 1964).

5-CH_3-H_4PteGlu was not recognized as a constituent of the pteroylglutamate pool in higher plants until investigations from this laboratory were reported (Roos *et al.*, 1968). Later studies disclosed that it was the most prevalent derivative in extracts from germinating pea cotyledons and leaves, and it, as well as other derivatives, were also present in extracts of isolated chloroplasts from pea and corn leaves (Shah *et al.*, 1970; Shah and Cossins, 1970a). Other workers have confirmed the presence of 5-CH_3-H_4PteGlu derivatives in wheat plants (Rohringer *et al.*, 1969).

Possible Relationships Between One-carbon Metabolism and Photosynthesis

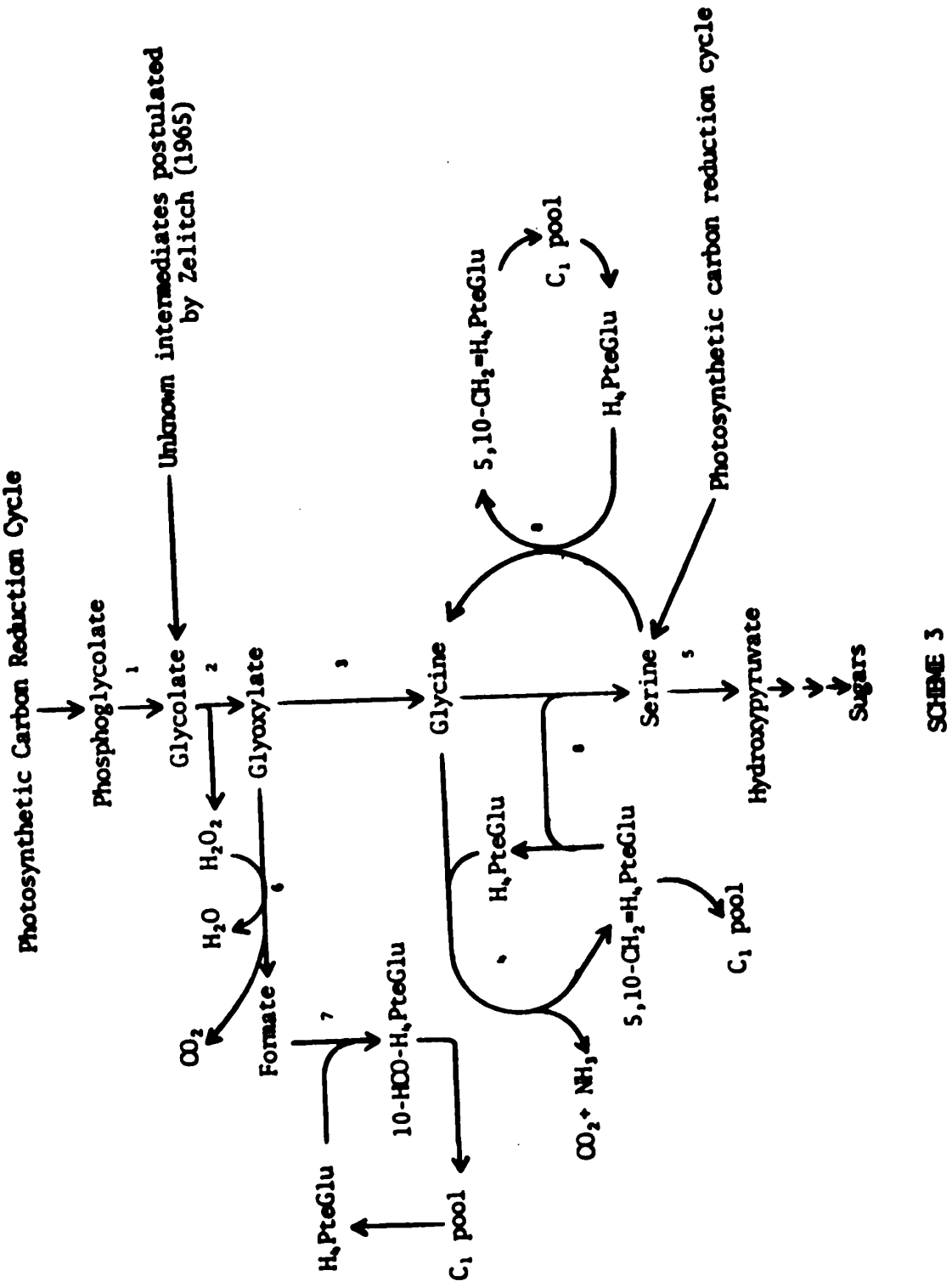
There is now considerable evidence from tracer and enzymological studies that the glycolate pathway (Scheme 3) is an important route in higher plants for flow of photosynthetically fixed CO_2 into hexoses (for example, see review by Tolbert and Yamazaki, 1969). It has been estimated that as much as 50% of the total carbon incorporated by tobacco leaves may flow through the glycolate pathway (Asada *et al.*, 1965; Hess and Tolbert, 1966). Many of the enzymes of the pathway are localized in peroxisomes, although some are associated with the chloroplastic and cytoplasmic fractions (Yamazaki and Tolbert, 1970; Tolbert, 1971). The glycolate pathway has also been found in algae, in a form slightly modified from that of higher plants due to enzymic differences (Bruin *et al.*, 1970).

An essential step of the glycolate pathway is the overall conversion of 2 glycine molecules to serine and CO_2. It is well established (Tolbert and Yamazaki, 1969) that glycine-C^{14} and other early intermediates of the glycolate pathway can be metabolized to serine by

SCHEME 3. THE POSSIBLE RELATIONSHIPS BETWEEN ONE-CARBON METABOLISM AND PHOTOSYNTHESIS.

Reaction No.	Common name of enzyme	Systematic name	E.C. No.
1	Phosphoglycolate phosphatase	Phosphoglycolate phosphohydrolase	3.1.3.18
2	Glycolate oxidase	Glycolate:O_2 oxidoreductase	1.1.3.1
3	Serine-glyoxylate aminotransferase *or* glutamate-glyoxylate aminotransferase	Glycine:2-oxoglutarate aminotransferase	2.6.1.4
4	Glycine decarboxylase	- - - - -	
5	Serine-glyoxylate aminotransferase	- - - - -	
6	Non-enzymic reaction	- - - - -	
7	10-HCO-H_4PteGlu synthetase	Formate:H_4PteGlu ligase (ADP)	6.3.4.3
8	Serine hydroxymethyltransferase	L-serine:H_4PteGlu-5,10-hydroxymethyltransferase	2.1.2.1

The term "C_1 pool" is used to designate one-carbon transfer reactions necessary to regenerate H_4PteGlu. Although "C_1 pool" appears 3 times in the scheme, it does not implicate 3 separate C_1 pools.


Photosynthetic Carbon Reduction Cycle
Phosphoglycolate
1
Glycolate
Unknown intermediates postulated by Zelitch (1965)
2
Glyoxylate
3
Glycine
Serine
5
Hydroxypyruvate
Sugars
Photosynthetic carbon reduction cycle
H_2O_2
H_2O
4
CO_2
Formate
7
10-HCO-H_4PteGlu
H_4PteGlu
C_1 pool
6
H_4PteGlu
C_1 pool
5,10-CH_2-H_4PteGlu
CO_2 + NH_3
H_4PteGlu
5,10-CH_2-H_4PteGlu
C_1 pool

SCHEME 3

photosynthesizing plant tissues. Intramolecular distribution of ^{14}C in the resulting serine suggested that a cleavage of glycine was involved during this synthesis (Rabson, Tolbert and Kearney, 1962; Wang and Waygood, 1962; Wang and Burris, 1963; Sinha and Cossins, 1966; Miflin, Marker and Whittingham, 1966; Bruin *et al.*, 1970). Very recently it was shown that the glycine to serine conversion in spinach preparations can be resolved in two distinct reactions: decarboxylation of glycine and synthesis of serine (Kisaki *et al.*, 1971b). These reactions are mediated by glycine decarboxylase and serine hydroxymethyltransferase respectively. All the activity of the former and most of the activity of the latter was concentrated in the mitochondrial band during isopycnic centrifugation (Kisaki *et al.*, 1971a). The activity of both of these enzymes increased upon the addition of H_4PteGlu to the reaction system (Kisaki *et al.*, 1971b). H_4PteGlu presumably functioned as carrier of the carbon 2 of glycine, the precursor of the carbon 3 of the resulting serine. In support of this, mitochondrial suspensions formed stoichiometric amounts of CO_2, NH_3, and serine from glycine (Kisaki *et al.*, 1971b). The glycine decarboxylase reaction is of interest in that maximum velocity was not attained even when the glycine concentration was 2.5 M. From these studies, it was not established whether 5,10-CH_2-H_4PteGlu formed in this reaction can exchange or be fed into the general one-carbon pool or whether it is preferentially utilized in serine biosynthesis *in vivo*. Considering the products of this reaction, it is tempting to conclude that it may be identical in mechanism to that summarized in Scheme 1.

The last few years have seen a considerable interest in photorespiration, a process which encompasses the uptake of O_2 and release of

CO_2 associated with photosynthesis. On the basis of physiological criteria, the process of photorespiration is different from dark respiration (Tolbert and Yamazaki, 1969). Photorespiration appears to have close relationships with one-carbon metabolism as the CO_2 evolved is thought to arise from the glycine cleavage reaction referred to earlier in this Section (Kisaki and Tolbert, 1970; Tolbert and Yamazaki, 1969; Kisaki *et al.*, 1971a,b). It has been further suggested (Tolbert, 1971) that the one-carbon group arising from this reaction may also be oxidized to CO_2 via 10-HCO-H_4PteGlu and formate. The magnitude of this latter oxidative conversion has not, however, been evaluated.

One-carbon groups may conceivably arise from two other photosynthetic intermediates, glycolate and serine, both of which are members of the glycolate pathway, but which may also arise via other sequences of CO_2 fixation (Zelitch, 1965; Bassham, 1965). First of all, H_2O_2 produced as a result of glycolate oxidase activity, can spontaneously oxidize glyoxylate to formate and CO_2 (Zelitch and Ochoa, 1953). Such formate could enter the one-carbon pool as a result of 10-HCO-H_4PteGlu synthetase activity. Activated one-carbon groups also can be formed by the normal activity of serine hydroxymethyltransferase from the carbon 3 of serine.

A group of compounds chemically related to H_4PteGlu have also been implicated in the process of photosynthesis. Pteridines were, at one time, believed to be involved in the photosynthetic incorporation of $C^{14}O_2$ (Metzner *et al.*, 1957; Metzner *et al.*, 1958; Fuller *et al.*, 1958a; Van Baalen *et al.*, 1957). This belief proved to be fallacious when it was shown that the supporting evidence was, in fact, based on an artifact of paper chromatography (Fuller *et al.*, 1958b). A relationship between pteridine content and photosynthetic capacity has also been noted for

blue-green algae, spinach chloroplasts, (Forrest *et al.*, 1957), and photosynthetic bacteria (Maclean *et al.*, 1966; Kobayashi and Forrest, 1967). Further work led to the suggestion that such pteridines may function as the primary electron acceptor in the photosynthetic reaction centre (Fuller and Nugent, 1969). Such a role in photosynthetic bacteria has, however, been questioned recently as the bulk of extractable pteridines were not associated with isolated chromatophores or preparations of the photosynthetic reaction centre (Reed and Mayne, 1971).

The Present Investigation

Although the prime importance of H_4PteGlu derivatives in biological systems is widely appreciated, it is apparent that relatively little is known about H_4PteGlu-mediated metabolism in plant tissues. It is clear that knowledge in this area has increased rapidly in recent years but relatively few detailed studies of endogenous levels of pteroylglutamates in photosynthetic tissue have been reported. In general such reports are mainly of nutritional interest as such studies have aimed at an evaluation of dietary intake of these vitamin derivatives. Considering the techniques generally used in such work, it is clear that losses of labile compounds occurred during extraction and consequently the quantity of pteroylglutamates in the final extract would be different from that *in vivo*. This would partially explain the large differences in the pteroylglutamate levels reported for the same type of tissue when analyzed in different laboratories (for example, see Table 1 below). Moreover, the occurrence of 5-CH_3-H_4PteGlu in plants had not been reported prior to the start of this investigation, even though it was commonly found in the pteroylglutamate pool of other organisms (Keresztesy and Donaldson, 1961;

Silverman *et al.*, 1961; Bird *et al.*, 1965; Sotobayashi *et al.*, 1966). Clearly, a detailed study of the pteroylglutamates present in photosynthetic tissue was appropriate.

To date, the metabolic roles of H_4PteGlu derivatives in photosynthetic plant tissues have mainly been studied *in vitro* and the importance of these roles *in vivo* have not been well evaluated. In view of suggestions that H_4PteGlu is involved as one-carbon carrier in an important pathway of carbon flow in photosynthesizing tissue, further studies were undertaken to examine the metabolic interrelationships of the H_4PteGlu derivatives in photosynthetic tissue and to evaluate the contribution that the photosynthetically formed intermediates may make to the one-carbon pool. Included in these studies were detailed analyses of the H_4PteGlu derivatives in plant tissues subjected to varying conditions which affected photosynthesis. The effect of aminopterin on the H_4PteGlu derivatives and the incorporation of $HC^{14}O_3^-$ into ethanol soluble fractions by illuminated radish cotyledon disks was also examined in an attempt to obtain a more complete understanding of the role of H_4PteGlu in photosynthetic tissue.

Radish cotyledons were selected as the major tissue of investigation for a number of reasons. Preliminary studies showed that they contained a high level of various H_4PteGlu derivatives, and furthermore, this tissue could be readily grown under controlled conditions. Also, earlier work conducted in this laboratory (Caballero and Cossins, 1970; Cossins and Caballero, 1970) indicated that this tissue readily fixed CO_2 into glycine and serine when illuminated.

MATERIALS AND METHODS

Plant tissues

All plant materials were grown from seed in flats containing a sterilized loam-peat-sand (3:2:1) soil mixture. The seeds were covered with a fine layer of sand to keep the shoot tissue clean when watered with tap water. The flats were routinely illuminated for 13 hours daily by fluorescent and incandescent lighting of 2000 ft. c. intensity. Etiolated tissue was grown in total darkness and handled only in subdued light before use in experiments. Radish (*Raphanus sativus* L. cv. 'Scarlet Globe') and wheat seedlings (*Triticum vulgare* L. cv. 'Thatcher') were grown in growth cabinets at 25°C; spinach (*Spinacea oleracea* L. cv. 'King of Denmark') and pea (*Pisum sativum* L. cv. 'Homesteader') plants were grown at 18°C. The plants, still in their flats, were routinely removed from the growth cabinet to the laboratory 30 minutes before the experimental tissues were rapidly excised and used in experiments.

The laminar portion of the cotyledons from radish seedlings of varying age as noted in the Results were used as the main experimental tissue. Wheat seedlings were grown for 8 days at which time the leaves were excised by cutting 1 cm above the coleoptile sheath. Spinach and pea plants were grown until mature tissue was present, generally 30-40 days. Typical leaves were then excised and used as the experimental material. In some cases, leaf and cotyledon tissues were quickly frozen in an acetone-dry ice bath, lyophilized and stored under vacuum at -20°C. When fresh tissue was used, dry weight equivalents were obtained by use of a dry weight/fresh weight ratio. The dry weight was determined by drying a sample of tissue comparable to that used in the experiment at

105-110°C for 24 hours.

Chlorophyll contents were determined by the method of Arnon (1949). 80% Acetone extracts were prepared, and their absorbancies at 645 and 663 nm were used in calculations of chlorophyll concentration.

Chemicals

General chemicals of the highest quality commercially available were obtained from Fisher Scientific Co., Edmonton, and Nutritional Biochemical Co., Cleveland, Ohio. Fisher Scientific and Schwarz Mann Research Chemicals, Toronto, supplied ascorbic acid.

Various pteroylglutamate derivatives were obtained from commercial sources as follows: PteGlu was obtained from Nutritional Biochemical Co., H_4PteGlu from the Sigma Chemical Co., St. Louis, Mo., *d,l*-5-HCO-H_4PteGlu from Lederle Products, Cyanamid of Canada, Montreal, P.Q. PteGlu$_3$ was a gift from Dr. W. Sakami, Case Western Reserve University, Cleveland, Ohio.

A number of other pteroylglutamate derivatives were synthesized by using one of the above sources as starting material. 10-HCO-H_4PteGlu was formed in two ways. First, a solution of commercially obtained 5-HCO-H_4PteGlu was made to pH 1 by addition of 6 M HCl. After 60 minutes, the pH was rapidly changed to 8.5 by addition of a few drops of 5 M NaOH (Rabinowitz, 1963). The other method required PteGlu for starting material. This derivative was reduced by the method of Davis (1968) and formylated in concentrated (90%) formic acid (Rowe, 1968). 5-CH_3-H_4PteGlu was prepared by borohydride reduction of 5,10-CH=H_4PteGlu (Chanarin and Perry, 1967) or alternatively, by borohydride reduction of the formaldehyde-H_4PteGlu adduct (Sakami, 1963). Triglutamate derivatives were

prepared using PteGlu$_3$ as starting material and proceeding as above. Diglutamate derivatives were routinely prepared from the corresponding triglutamate compounds by use of chicken pancreas γ-glutamyl carboxypeptidase (see below). H_2PteGlu was prepared from PteGlu according to the method of Futterman (1963). In all cases, the synthesized products had spectral (Stokstad and Koch, 1967) and microbiological properties as reported in the literature (see Figure 1; Blakley, 1969; Johns and Bertino, 1965).

Aminopterin was obtained from the Sigma Chemical Co..

Extraction of Pteroylglutamates from Plant Tissues

Samples of tissue (1-2 g fresh weight or 0.1-0.2 g dry weight) were subjected to one of the following extraction procedures.

Method I: Heating prior to homogenization.

The plant tissue was immersed for 10 minutes in 15 ml ascorbate buffer (12 mg/ml ascorbic acid adjusted to pH 6.0 by addition of 5 M KOH) at 95°C. The tissue and buffer were then rapidly cooled in ice and homogenized in a ground glass homogenizer.

Method II: Homogenization followed by heat treatment.

This was essentially the method described by Bird *et al.* (1965). The tissue was homogenized in the ascorbate buffer (described above) at 0°C, then rapidly heated in a 95°C water bath for 10 minutes, and finally cooled in ice.

Method III: Homogenization in the presence of ethanol.

This method was similar to Method II, except that the ascorbate buffer contained either 20% or 50% ethanol (Methods IIIA and IIIB respectively).

Method IV: Homogenization at pH 8.0.

This was also a modification of Method II. The ascorbate buffer contained boric acid (final concentration, 0.05 M) and was adjusted to pH 8.0 with KOH.

After preparation of the homogenate by one of the above Methods, cellular debris and coagulated materials were removed by centrifugation at 18,000 x g for 10 minutes. The pelleted residue was washed twice by resuspension in 10 ml of the ascorbate buffer (described above) and centrifuged again. The supernatants were combined, made to volume, and stored at -20°C. When ethanol was present in the extracts, the pooled supernatants were lyophilized to dryness and the residue redissolved in distilled water.

Microbiological Assay of Pteroylglutamate Derivatives

Lactobacillus casei (ATCC 7469), *Streptococcus faecalis* (ATCC 8043), and *Pediococcus cerevisiae* (ATCC 8081) were the bacteria used for assay of pteroylglutamate derivatives. Stock cultures were maintained on simple yeast extract-agar slants (Freed, 1966). Inoculum bacteria were grown on inoculum broth overnight and washed free of broth with 0.9% saline (Freed, 1966). The basal assay medium for *L. casei* was prepared according to Freed (1966), that for *S. faecalis* according to Jukes (1955) and that for *P. cerevisiae* according to Bakerman (1961). Five ml aliquots of basal media were dispensed into 20x150 mm culture tubes in covered metal racks and sterilized at 121°C for 8 minutes. For details of the composition of the agar slants, inoculum broth, and media, the reader is referred to Appendix I.

Additions to the sterilized basal assay medium were made aseptically and consisted of ascorbate solution (final concentration, 6 mg/ml, pH 6.8), 0.1-0.5 ml aliquot of the sample to be assayed, water to a volume of 10 mls and finally two drops of diluted bacterial inoculum.

The growth response of the bacteria was measured by titration of the lactic acid produced during 70 hours of incubation at 37°C, at which time growth was essentially complete. A Beckman Expandomatic pH meter, fitted with a combination electrode, was used to monitor the back-titration with standard 0.1 M NaOH to the original pH of the medium (pH 6.8). Reference curves were prepared for each assay using freshly prepared, standardized PteGlu solution for *L. casei* and *S. faecalis* and *d,l*-5-HCO-H_4PteGlu solution for *P. cerevisiae* (see Appendix I). The stock PteGlu solution which was used with dilution for setting up reference curves, was prepared by careful weighing of PteGlu. It was dissolved with the aid of a few drops of 5 M NaOH. The concentration was tested colorimetrically using para-aminobenzoic acid as standard (Schiaffino *et al.*, 1959) and it was found to be 98.2 ± 1.3% of the calculated concentration. As the difference from calculated concentration was smaller than deviation normally associated with microbiological assay, no correction was made. However, the concentration of stock solution of *d,l*-5-HCO-H_4PteGlu was only 66.9 ± 1.0% of the concentration calculated from the manufacturer's specifications. Equimolar amounts of PteGlu and *l*-5-CHO-H_4PteGlu were found to give equal growth response of *L. casei* when the concentration of the latter was based on the colorimetric assay. Accordingly, the corrected value for the concentration of *d,l*-5-HCO-H_4PteGlu was used when *P. cerevisiae* standard curves were constructed. It was assumed that *d*-5-HCO-H_4PteGlu did not promote the

growth of the microorganisms (May *et al.*, 1951). The stock solutions of standard PteGlu and *d,l*-5-HCO-H_4PteGlu were stored under toluene at 4°. They were stable for at least 3 years as no decrease in the slopes of standard curves was noticed during the course of this investigation.

All samples were routinely assayed in duplicate. For determination of levels of total pteroylglutamates, at least three aliquot sizes were employed. All dilutions to bring the pteroylglutamate concentration into the range of linear growth response were made using 6 mg/ml ascorbate solution, pH 6.8.

Enzymatic Hydrolysis of Pteroylpolyglutamates

Pteroylpolyglutamates cannot be quantitatively assayed using conventional microbiological assay without prior hydrolysis of the γ-glutamyl peptide bonds (Blakley, 1969). Hydrolysis is normally accomplished by incubation of extracts with a γ-glutamyl carboxypeptidase. In this investigation, γ-glutamyl carboxypeptidase was prepared from 3 g of Difco-Bacto chicken pancreas extract (Difco-Laboratories, Detroit, Mich.) as outlined by Eigen and Shockman (1963). Activity of the partially purified enzyme was ascertained by incubation with commercial yeast extract (Difco Laboratories). 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. The final volume was 10 mls. After incubation for 5 hours at 37°C, the reaction was terminated by heating the tubes in a water bath at 95°C for 10 minutes. Controls with boiled enzyme were routinely included. It should be noted that chicken pancreas γ-glutamyl carboxypeptidase treatment of a pteroylpolyglutamate results

in the formation of a pteroyldiglutamate (Dabrowska *et al.*, 1949).

Enzymatic hydrolysis of pteroylpolyglutamates in fractions obtained by DEAE-cellulose chromatography (see below) was carried out with a γ-glutamyl carboxypeptidase from pea cotyledons. Chicken pancreas γ-glutamyl carboxypeptidase was not used here since the presence of high phosphate concentrations in the fractions made it desirable to avoid calcium ions which are required for its activity (Mims and Laskowski, 1945). The choice of pea cotyledons as enzyme source was also influenced by the fact that it has considerable activity at pH 6.0 and was readily obtainable (Appendix II). The enzyme was prepared by homogenization of 10 g of 3-day-old pea cotyledons in 100 ml of citrate-phosphate buffer. This buffer contained 42 mmoles of citric acid and 108 mmoles of K_2HPO_4 and had a pH value of 5.5 (Gomori, 1955). Cellular debris was removed by centrifugation at 20,000 x g for 10 minutes. The supernatant proteins were then fractionated by precipitation with $(NH_4)_2SO_4$ (Green and Hughes, 1955). The 0-25% and 25-80% fractions were precipitated by slow addition of $(NH_4)_2SO_4$ with stirring for 30 minutes and collected by centrifugation at 20,000 x g for 10 minutes. A small amount of the 25-80% fraction (approximately 250 mg) was dissolved in 5 ml of the citrate-phosphate buffer described above and passed through a 1.5x25 cm of Sephadex G-10 (Pharmacia, Montreal) to desalt the solution. Finally, the desalted protein solution was diluted with additional buffer to obtain a protein concentration of 2.5 mg/ml. Aliquots (0.2 ml) of this diluted protein solution were used to hydrolyze the pteroylpolyglutamates in the fractions obtained by DEAE cellulose chromatography. In order to expedite microbiological assay, only alternate fractions received enzyme solution while the remaining fractions served essentially as controls. The

The hydrolyses were allowed to proceed for 16 hours at 37°C and were terminated by steaming for 10 minutes before assay using *L. casei*.

Protein determinations were by the Folin-Ciocalteu or Biuret methods (Layne, 1957). Egg albumin (5 x crystalline; Nutritional Biochemical Co.) was used as the reference standard.

DEAE-Cellulose Column Chromatography of Pteroylglutamates

Pteroylglutamate derivatives were separated using columns (final size 1.5 x 20.0 cm) of DEAE-cellulose (Nutritional Biochemical Co.) containing Hyflo-Supercel (Fisher Scientific). The columns were prepared as described by Sotobayashi *et al.* (1966). Up to 20 ml of plant extracts, containing pteroylglutamates, were applied to the columns and washed into the bed with 2 x 2 ml aliquots of 6 mg/ml ascorbate solution (pH 6.0). Elution of the derivatives was achieved with a 0 to 0.5 M KH_2PO_4 gradient (pH 6.0) in the presence of ascorbate (Sotobayashi *et al.*, 1966). Fractions of 3 ml were collected in tubes containing 0.3 ml of 60 mg/ml ascorbate solution (pH 6.0) using an LKB Ultrarac fraction collector (LKB Produckter AB, Stockholm). The fractions were used immediately for microbiological assay or stored at -20°C until required. Recoveries of authentic pteroylglutamates and of derivatives present in plant extracts after chicken pancreas γ-glutamyl carboxypeptidase treatment were normally in the range of 90 ± 10%, as found by others (Sotobayashi *et al.*, 1966; Rohringer *et al.*, 1969). The only exception was authentic H_4PteGlu which had 60-80% recovery after DEAE-cellulose column chromatography (Roos, 1971).

Figure 1 is a representative elution of pteroylglutamates from DEAE-cellulose. The elution shown is that of the derivatives present in

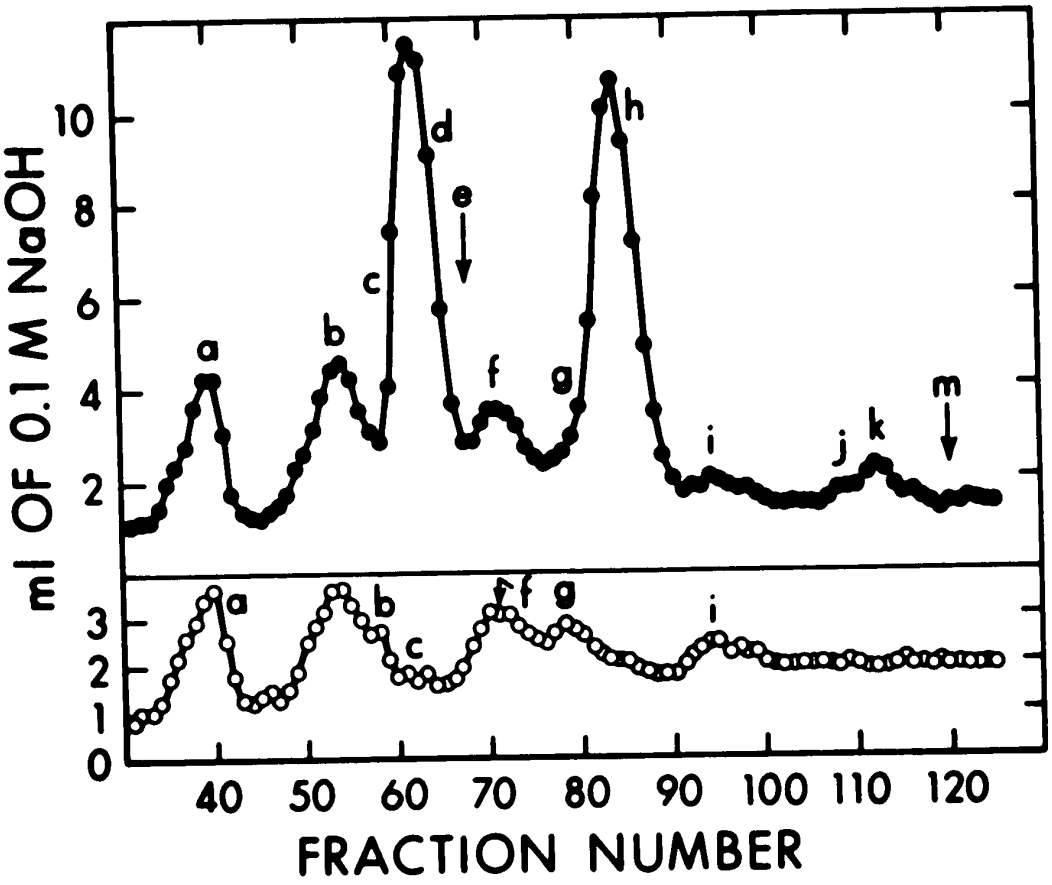
FIGURE 1

DEAE-cellulose chromatography of plant pteroylglutamate derivatives

Pteroylglutamate extracts of pea leaves were prepared by Method II and chromatographed on DEAE-cellulose. The location and measurement of the derivatives was achieved by differential assay using *L. casei* (● - ●) and *S. faecalis* (o - o). Assays involving *P. cerevisiae* were essentially similar to those using *S. faecalis*.

Peak	Derivative	Growth response *L. casei*	*S. faecalis*	*P. cerevisiae*
a	10-HCO-H_4PteGlu	+	+	+
b	10-HCO-H_4PteGlu$_2$	+	+	+
c	5-HCO-H_4PteGlu	+	+	+
d	5-CH_3-H_4PteGlu	+	-	-
e	10-HCO-H_4PteGlu$_3$*	+	-	-
f	H_4PteGlu	+	+	+
g	5-HCO-H_4PteGlu$_2$	+	+	+
h	5-CH_3-H_4PteGlu$_2$	+	-	-
i	H_4PteGlu$_2$	+	+	+
j	5-HCO-H_4PteGlu$_3$	+	-	-
k	5-CH_3-H_4PteGlu$_3$	+	-	-
m	PteGlu*	+	+	-

* The positions of elution of 10-HCO-H_4PteGlu$_3$ and PteGlu are indicated in the Figure, but these derivatives were not detected in the extract chromatographed in this case.


ml OF 0.1 M NaOH
10
8
6
4
2
3
2
1
0
a
b
c
d
e
f
g
h
i
j
k
m
40
50
60
70
80
90
100
110
120
FRACTION NUMBER

pea leaves when extracted by Method II. Also given, are the positions of two derivatives which were not found in this particular extract, but which were encountered in plant extracts during the course of these investigations.

Identification of pteroylglutamate derivatives in natural extracts was based on a number of criteria. Firstly, the position of elution during DEAE-cellulose column chromatography (Figure 1). For comparison the elution was standardized using 11 authentic pteroylglutamate derivatives. The elution pattern obtained was generally consistent with that reported earlier in work where identical or similar conditions had been employed (Sotobayashi *et al.*, 1966; Silverman *et al.*, 1961; Noronha and Silverman, 1962; Bird *et al.*, 1965; Rohringer *et al.*, 1969). One exception was the elution of H_2PteGlu in the fractions (120-140) which also contained PteGlu. The reason for this has not been clarified, but it was suspected that the H_2PteGlu became oxidized to PteGlu before or during chromatography, since under similar conditions H_2PteGlu was eluted before PteGlu (Silverman *et al.*, 1961). A second criterion was the ability of the derivative to support the growth of *L. casei*, *S. faecalis* and *P. cerevisiae*. The latter two bacteria did not respond to 5-CH_3-H_4PteGlu derivatives nor any triglutamyl derivatives. They were particularly valuable in the identification and measurement of 5-HCO-H_4PteGlu derivatives and H_4PteGlu because in the chromatographic procedure employed their peaks overlapped with the 5-CH_3-H_4PteGlu derivative and 10-HCO-H_4PteGlu, peaks, respectively. Other criteria used in the identification of unknown derivatives included chicken pancreas peptidase treatment of individual peaks followed by rechromatography and co-chromatography with an authentic C^{14}-labelled derivative. Formyl

derivatives were also confirmed by demonstrating the interconversion of 10-HCO-H_4PteGlu$_{1,2 \text{ or } 3}$ and 5-HCO-H_4PteGlu$_{1,2 \text{ or } 3}$ under acidic and alkaline conditions (May *et al.*, 1951).

In order to compare the quantities of individual derivatives in plant extracts after chromatography, all quantities are expressed in 'PteGlu equivalents for *L. casei*'. *L. casei* was the microorganism selected because it responded to the widest range of derivatives (Figure 1) and according to the literature responded equally to mono- and diglutamates, whereas *P. cerevisiae* and *S. faecalis* did not (Noronha and Silverman, 1962; Silverman and Wright, 1956). In the present study there was evidence which suggested unequal response of the latter two organisms to pteroylmono- and diglutamates, but a careful study could not be made due to lack of sufficient synthetic diglutamate compounds. It was also observed that the 10-HCO-H_4PteGlu peaks contained more (20-50%, reason for variation unknown) PteGlu equivalents when assayed with *S. faecalis* than when assayed with *L. casei*. Thus it was necessary to make adjustments for these differential growth responses when *S. faecalis* or *P. cerevisiae* were used to assay one of two derivatives in overlapping peaks, such as 5-HCO- and 5-CH_3-H_4PteGlu$_2$. Since the microorganisms responded equally to N^{10} and N^5-formyl derivatives and the N^{10}-formyl derivatives were well separated from other *L. casei* growth promoting compounds, the ratio of the growth responses of *L. casei* and *P. cerevisiae* (or *S. faecalis*) to the N^{10}-formyl derivatives was conveniently employed to convert the response of *P. cerevisiae* (or *S. faecalis*) to N^5-formyl derivatives into PteGlu equivalents for *L. casei*. The amount of 5-HCO-H_4PteGlu$_2$ in terms of PteGlu equivalents for *L. casei* would equal:

$$\frac{\mu g\ 10\text{-HCO-R}\ (\textit{L. casei}\ \text{response})}{\mu g\ 10\text{-HCO-R}\ (\textit{P. cerevisiae}\ \text{response})} \times \mu g\ 5\text{-HCO-R}\ (\textit{P. cerevisiae}\ \text{response})$$

where R = $H_4PteGlu_2$. The amount of 5-CH_3-$H_4PteGlu_2$ in the extract was then obtained by difference of the total in the peak as determined with *L. casei* and the calculated quantity of 5-HCO-$H_4PteGlu_2$ after assay with *P. cerevisiae* (or *S. faecalis*).

Treatment of Radish Cotyledons with Aminopterin

In some experiments, samples of 25 etiolated cotyledons were excised from 6-day-old seedlings and allowed to take up 2 ml of aminopterin (pH 6.5) solution with concentrations in the range 2×10^{-8} to 2×10^{-4}M. This was followed by uptake of five 2 ml aliquots of half strength White's solution (Weston, 1970) and finally by sufficient 10x diluted White's solution to prevent dessication. The cotyledons were then illuminated for 27 hours by a mercury vapour lamp (2000 ft. c.) at 23 ± 2°C.

In experiments employing cotyledons from normal (i.e. light/dark grown) 6-day-old seedlings, the tissues were washed with distilled H_2O and vacuum infiltrated with 2×10^{-5}M aminopterin (pH 6.5) to ensure rapid uptake of the inhibitor. The cotyledons were then placed, adaxial side up, in covered 9 cm petri dishes containing 10 ml of the infiltrating solution and illuminated by the mercury lamp (2000 ft. c.) for various time periods up to 6 hours at room temperature (23 ± 2°).

Some modifications were introduced in 24 hour experiments. In these experiments, cotyledons infiltrated as already described, were supported on a sterile pad consisting of 4 layers of filter paper (Whatman, No.1) and 2 layers of cheesecloth in 13 cm petri dishes. The

support was moistened with 30 ml of a sterile nutrient medium (adjusted to pH 6.5 with KOH) containing 80 mM KNO_3, 1% sucrose, 5 mM KH_2PO_4 (Nieman and Poulsen, 1967). Aminopterin (10^{-5}M) was present in the nutrient medium supplied to such tissues. The petri dishes with cotyledons were then placed in a growth cabinet at 23°C and illuminated with fluorescent-plus tungsten lamps at 1500 ft. c. Cotyledons infiltrated with distilled water served as control tissues in these experiments.

Radioisotope Feeding Experiments

Labelled compounds were obtained from Amersham/Searle Corp., Toronto, and were used without dilution of specific radioactivity, except in one experiment as noted below. Sterile distilled water was used to dilute the solutions from the supplier so that the radioisotope solution could be accurately administered.

Two types of experiments were performed. In one set of experiments, the labelling of pteroylglutamate derivatives was examined. For these experiments, tissue disks were obtained from cotyledons of 6-day-old radish plants. Twenty disks (186 mg) were cut from the cotyledons using a sharp cork borer. These were then carefully placed in a 20 ml Warburg flask containing 0.5 ml of 0.1 M KH_2PO_4 (pH 6.0). The flasks were illuminated from below (2000 ft. c.) with a mercury lamp for a 10 minute equilibration period at 23°C to allow the disks to achieve steady state metabolism. Then 0.2 ml of $NaHC^{14}O_3$ (10 µc, 57 µc/µmole), glycine-2-C^{14} (10 µc, 57 µc/µmole) and sodium formate-C^{14} (10 µc, 59 µc/µmole) were added respectively to the main compartments from the side arms. The flasks were incubated for a further 10 minute period under the

same conditions. In the case of the $NaHC^{14}O_3$ experiment, the disks were killed by pipetting boiling 12 mg/ml ascorbate solution (pH 6.0) into the flask. In the other two cases, the disks were rapidly tipped into a small funnel and rinsed with distilled water to remove the excess radioactive feeding solution before being dropped into 12 mg/ml ascorbate (pH 6.0) at 95°C. Pteroylglutamate derivatives were extracted by Method I as described earlier.

In other experiments, sodium bicarbonate-C^{14} was also supplied to disks of 6-day-old radish cotyledons which had been infiltrated with aminopterin or distilled water. All parameters of these experiments, excepting those noted in the Results, were identical to those described above. At the completion of the feeding period, the tissue disks were killed by pipetting 5 ml of hot 80% ethanol into the main compartment and boiling for 5 minutes. The ethanol soluble materials were fractionated into amino acid, organic acid, and sugar fractions by use of ion exchange resins. Details of these procedures have been described elsewhere (Caballero and Cossins, 1970; Cossins and Beevers, 1963).

Chromatography of Labelled Amino Acids

A Beckman Amino Acid Analyzer (Model 121C) (Beckman Instruments, Inc., Palo Alto, California) was used to separate individual acids of the amino acid fraction. A standard calibrated amino acid mixture containing 0.2 μmole of 20 different amino acids was added to an aliquot (5 - 15 x 10^4 cpm) of the labelled amino acid fraction. Fractions (1.9 ml) were collected from the analyzer during elution using an LKB Ultrorac Fraction Collector. Aliquots (0.2 ml) of each fraction were assayed for radioactivity. The remaining part of each fraction was then reacted

with ninhydrin in order to confirm the elution sequence of the carrier amino acids (Spies, 1957).

Degradation of Labelled Glycine and Serine Samples

In order to determine the intramolecular distribution of C^{14} in glycine and serine, samples of these compounds from the amino acid analyzer were decarboxylated with ninhydrin and then further oxidized with $Na_2S_2O_8$ to obtain the residual carbons, as described by Chang and Tolbert (1965). The degradation apparatus consisted of a 50 ml round bottom flask connected to a short water condenser with a side arm to allow aeration and introduction of reactants in solution. The carbon dioxide produced was trapped in a 15 x 0.5 cm Vigreaux column containing 10 ml of 2 M NaOH. Radioactivity was determined as described later. Approximately 90% of the initial counts in all degradations were found in the two traps. Degradation of commercially obtained glycine-1-C^{14}, glycine-2-C^{14} and aspartic acid-1-C^{14} showed greater than 95% of the label was in the expected position. This indicated minimal randomization, if any, occurred during this degradation procedure.

Determination of Radioactivity

All radioactivity measurements were made in a Unilux II Liquid Scintillation Counter (Nuclear Chicago Corp., Des Plains, Ill.). Aqueous samples (0.1 or 0.2 ml) were counted in vials containing 15 ml of scintillation fluor (0.4 g of dimethyl POPOP, 9.6 g of PPO, 100 ml of anisole, 100 ml of dimethoxymethane, and 600 ml of dioxane). The counting efficiency, as determined by the channels-ratio method, was

found to be 75-80%.

The $C^{14}O_2$ collected in 2 M NaOH was counted by pipetting 1 ml samples into vials containing Cab-o-sil (Cabot Corp., Boston, U.S.A.) and 15 ml of the scintillation fluor. Radioactivity of solutions to be degraded were also measured in the presence of 2 M NaOH and Cab-o-sil. Counting efficiencies were in the range of 65-70%.

All counts were corrected for background.

Extraction of Free Amino Acids from Radish Cotyledons for Determination of Endogenous Pool Sizes

Lyophilized radish cotyledons (approximately 0.2 g) obtained as described in the Results, were placed in 15 ml boiling 80% ethanol for 5 minutes and then cooled. The amino acid fractions were obtained in the manner already described for the isolation of the radioactive amino acid fractions. The dry amino acid residue was redissolved in 5 ml of 0.2 M citrate buffer (pH 2.2) and 500 µl aliquots were chromatographed in the amino acid analyzer. Amino acid levels were calculated by reference to peak areas (H x W values) obtained when a standard mixture of amino acids was separated.

RESULTS

At the initiation of this investigation, a survey of various plant tissues was undertaken to determine pteroylglutamate levels and the nature of the individual derivatives present. This information then had bearing on the final selection of the main experimental material to be used. Pea, spinach, and wheat leaves, as well as radish cotyledons, were rapidly excised, weighed and extracted by a method considered initially to be the best available for natural materials (Bird *et al.*, 1965). This method included homogenization in ascorbate buffer at 0°C to prevent oxidation of labile tetrahydropteroylglutamates and to minimize possible changes caused by endogenous enzymes. Rapid heating in a water bath at 95°C, cooling, and centrifugation completed extract preparation. This method was subsequently designated Method II and is described in greater detail in the Materials and Methods section.

Two anomalies appeared in the results obtained from preliminary work using this method of extraction. The first was that only very low levels of polyglutamates were encountered in the tissue extracts. In some analyses these compounds were apparently absent. Yet, earlier literature had reported that polyglutamates are prevalent in plant tissues (Santini *et al.*, 1964; Iwai *et al.*, 1959; Toepfer *et al.*, 1951; Rosso, 1958). Scrutiny of data obtained by other workers for spinach leaves revealed that a considerable variability in polyglutamate content had been encountered before (Table 1). A second anomaly occurred when different radish cotyledon extracts were chromatographed. Considerable variation was found in the pteroylglutamate activity of certain peaks. For example, the peak consisting of 10-HCO-H_4PteGlu$_3$ contained 5.4 µg

TABLE 1. *Levels of pteroylglutamates of spinach leaves as reported in the literature.*

Authors	Assay Organism	Pteroylglutamate level*		% pteroyl-polyglutamate	Extraction conditions
		Before Peptidase	After Peptidase		
Toepfer *et al.*, 1951	*L. casei*	0.310	0.574	45	Homogenized in buffer, pH 7.2; autoclaved at 15 lbs. for 15 min.
	S. faecalis	0.360	0.654	45	
Toepfer *et al.*, 1951	*L. casei*	0.330	0.486	32	*Same as above*
	S. faecalis	0.344	0.482	29	
Toepfer *et al.*, 1951	*L. casei*	1.100	1.053	0	*Same as above*
	S. faecalis	1.010	0.943	0	
Santini *et al.*, 1964	*L. casei*	0.12	1.40	92	Homogenized in buffer, pH 7.2; autoclaved at 15 lbs. for 15 min.
	S. faecalis	0.07	0.92	93	
Iwai *et al.*, 1959	*P. cerevisiae*	0.55	2.65	79	Homogenized in cold buffer, pH 6.8; autoclaved with ascorbate at 10 lbs. for 30 min.
Present work	*L. casei*	3.11	3.50	11	Homogenized in cold ascorbate buffer, pH 6.0; heated for 10 min. at 95°C.

*Data are expressed as µg/g f. wt.

and 2.2 µg PteGlu equivalents respectively in two different determinations. Generally, when the percentages of monoglutamates were low, the recoveries from the column were also low and triglutamate levels were high, indicating that possibly other, more conjugated derivatives remained on the column in these cases. It was conceivable that uncontrolled hydrolysis of polyglutamyl derivatives could have been occurring during extraction, since it was known that plants contained γ-glutamyl carboxypeptidases which hydrolyzed pteroylpolyglutamates (Iwai, 1957; Bird *et al.*, 1946). To the author's knowledge, no attempts have previously been made to determine whether such enzymes cause hydrolysis of pteroylpolyglutamates during extraction of plant tissues. Since the presence of γ-glutamyl carboxypeptidase activity during extraction could not be excluded, an investigation to elucidate this possibility was undertaken.

A cell-free extract of radish cotyledons was prepared in phosphate buffer and tested for ability to hydrolyze the conjugated pteroylglutamates present in commercial yeast extract. It is clear, from Figure 2, that considerable hydrolysis of polyglutamates occurred in all three pH values examined. Although peptidase activity could be demonstrated under these conditions, it could not be concluded that significant hydrolysis of polyglutamates occurred when method II was implemented. This method of extraction was, in fact, purposely selected because it appeared to minimize hydrolysis of rat liver pteroylpolyglutamate derivatives (Bird *et al.*, 1965). Clearly, it was necessary to verify that no enzymatic hydrolysis of polyglutamates occurred during extraction of the present plant tissues. This was examined by the addition of commercial yeast extract during the extraction of pteroylglutamates from radish cotyledons (Table 2). The levels of

FIGURE 2

The hydrolysis of yeast pteroylpolyglutamates by a cell-free extract of radish cotyledons

The cell-free extract was prepared by homogenization of 6.1 g of 6-day-old radish cotyledons in 50 ml of 0.1 M KH_2PO_4 buffer adjusted to pH 7.1 with NaOH. Cellular debris was removed by centrifugation of the homogenate at 20,000 x g for 10 min. 2.5 ml of the supernatant (6.2 mg protein) were then incubated at 37°C with 300 mg yeast extract (potentially 3700 mμg PteGlu equivalents when hydrolyzed) and 120 mg ascorbic acid. The solutions were buffered with 1 mmole of sodium acetate (● - ●, final pH 4.6), 0.25 mmole KH_2PO_4 (▲ - ▲, final pH 7.1), and 1 mmole of sodium borate (■ - ■, final pH 7.7) respectively. Control tubes with boiled extract are represented in each case by the corresponding open symbols. The data are expressed in PteGlu equivalents for *L. casei*.

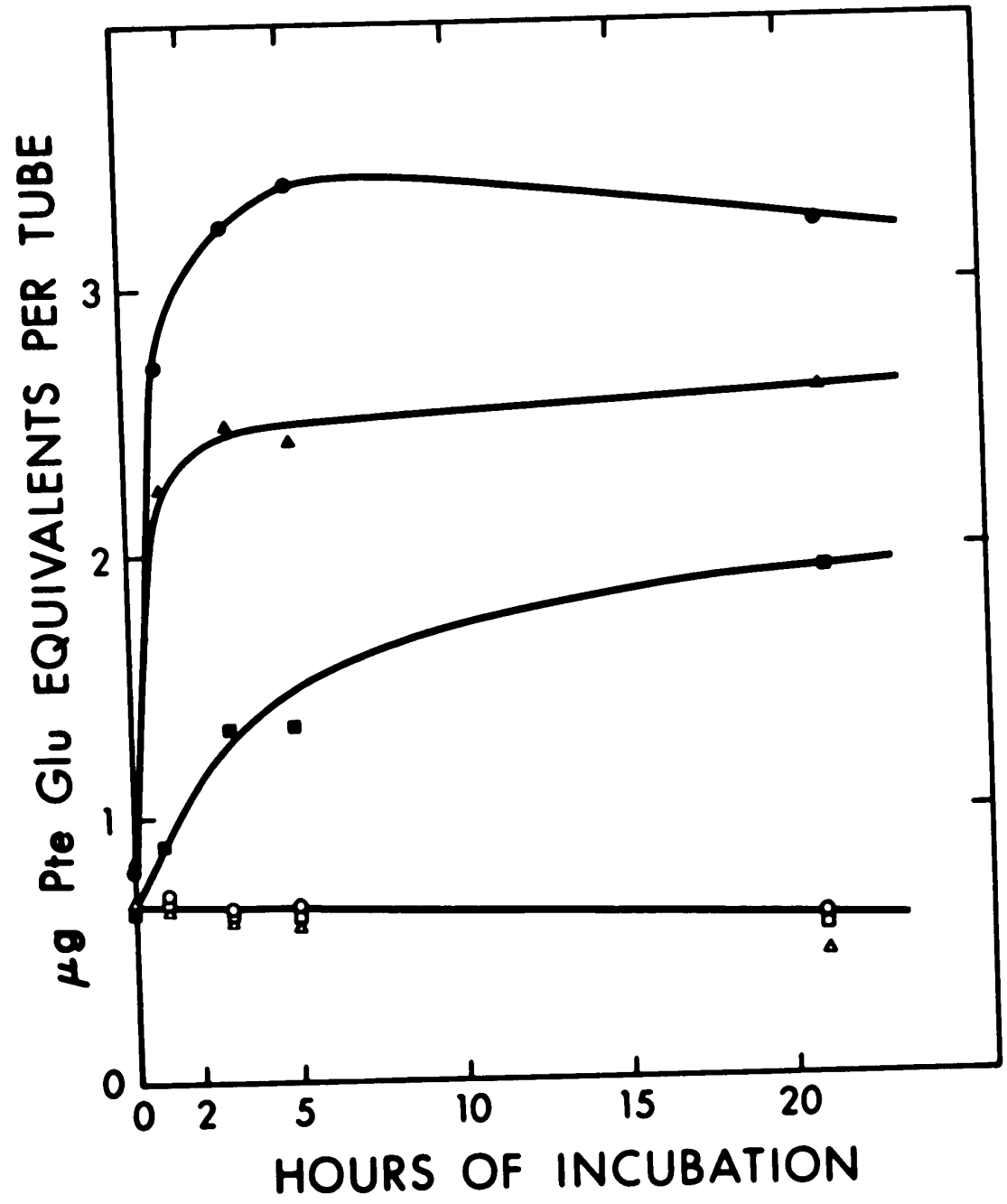

µg Pte Glu EQUIVALENTS PER TUBE
0
1
2
3
0
2
5
10
15
20
HOURS OF INCUBATION

TABLE 2. *Endogenous γ-glutamyl carboxypeptidase activity during extraction of pteroylglutamates from radish cotyledons*

Sample	Yeast Extract	µg PteGlu equivalents/g d. wt.			
		Before peptidase		After peptidase	
		Uncorrected	Corrected*	Uncorrected	Corrected**
1	absent	17.0	17.0	20.1	20.1
2	absent	18.4	18.4	22.2	22.2
3	present	30.0	28.8	42.4	20.0
4	present	32.8	31.2	49.1	20.0

* Corrected for 0.2 µg pteroylglutamates present in added yeast extract.

** Corrected for 3.7 µg pteroylglutamates present in added yeast extract.

Pteroylglutamates in radish cotyledons were extracted by Method II in the presence or absence of 1 ml of 30% yeast extract. The levels of pteroylglutamates were assayed with *L. casei* before and after chicken pancreas γ-glutamyl carboxypeptidase treatment. The data are in PteGlu equivalents.

pteroylglutamates found in the samples were calculated with and without correction for the added derivatives present in the yeast extract. For the purpose of calculation, it was assumed that no γ-glutamyl carboxypeptidase activity was present and the correction for added pteroylglutamates would be equal to the level found in the yeast extract before chicken pancreas γ-glutamyl carboxypeptidase treatment. If this assumption were valid, the corrected levels of samples 3 and 4 before peptidase activity (column 2) should have approximated those of samples 1 and 2 as was the case after peptidase treatment (column 4). The results did not show this and it must be concluded that some hydrolysis of the derivatives occurred during extraction. The hydrolytic activity was not, however, sufficient to hydrolyze all the pteroylpolyglutamates present, even when additional polyglutamates were omitted (samples 1 and 2). Considering these results, it was of importance to modify the extraction procedure so that endogenous levels of polyglutamate derivatives could be assessed.

The Effect of Extraction Procedure on the Pteroylglutamate Levels of Various Plant Tissues

An investigation of procedures to obtain extracts of pteroylglutamates from plant tissues was undertaken in order to find a method which limited hydrolysis of endogenous pteroylpolyglutamates. As it is well known that a number of reduced pteroylglutamate derivatives are labile to oxidation and degradation (Blakley, 1969), reducing conditions were a prerequisite considered in modifying Method II. Three innovations were incorporated into the methodology, namely, heat denaturation of enzymes before homogenization (described in detail in Materials and

Methods as Method I), homogenization in the presence of ethanol (Method III), and homogenization at pH 8.0 (Method IV) since it was known that radish cotyledon γ-glutamyl carboxypeptidase activity at this pH was very low (see Appendix II). Using these methods, it was found that the levels of total pteroylglutamates in extracts of radish cotyledons before chicken pancreas γ-glutamyl carboxypeptidase treatment were markedly affected by the extraction method (Table 3).

Before proceeding further, it should be noted that it was impossible to determine accurately total pteroylglutamate levels in extracts with large amounts of polyglutamates, such as occurred before peptidase treatment (Table 3). The *L. casei* growth response did not increase linearly when aliquot size was increased and surprisingly a growth response greater than expected was found (Figure 3). For example, calculations based on such assays gave pteroylglutamate levels which varied from 1.1 μg to more than 7 μg per g d. wt. of tissue. Averaging this 7-fold difference could clearly not be justified, and therefore a range of values is reported in Table 3 where this was encountered. After chicken pancreas peptidase treatment this variability was not observed (Figure 3, upper line) and therefore it was suspected that polyglutamates were responsible for the variable growth response. However, it is also possible that other factors which were also affected by peptidase treatment might be associated with this effect. In order to obtain a better estimate of levels of less conjugated pteroylglutamates in extracts before peptidase treatment, these derivatives were separated from the polyglutamates by DEAE-cellulose column chromatography.

Figure 3 also demonstrates that the quantities of total pteroylglutamates in duplicate samples prepared by Method I and treated with

TABLE 3. *The effect of extraction method and peptidase treatment on the levels of pteroylglutamates in radish cotyledons*

Type of tissue	Method of Extraction	Mono- and diglutamates*	µg PteGlu equivalents/g d.wt.	
			Before peptidase**	After peptidase**
Fresh	I	1.3	1.4 - 2.8	32.2
Fresh	I †	n.a.	1.1 - 7.4	29.4
Fresh	II	10.4	25.5	30.0
Fresh	IIIA	7.6	23.7	30.2
Lyophilized	IIIB	n.a.	1.0 - 8.2	27.2
Fresh	IV †	n.a.	3.0 - 12.3	23.7
Lyophilized	I	1.0	1.1 - 8.3	31.7

n.a. - not assayed

Data are expressed as PteGlu equivalents for *L. casei*.

* Mono- and diglutamate levels were obtained after separation of individual derivatives by DEAE-cellulose chromatography.

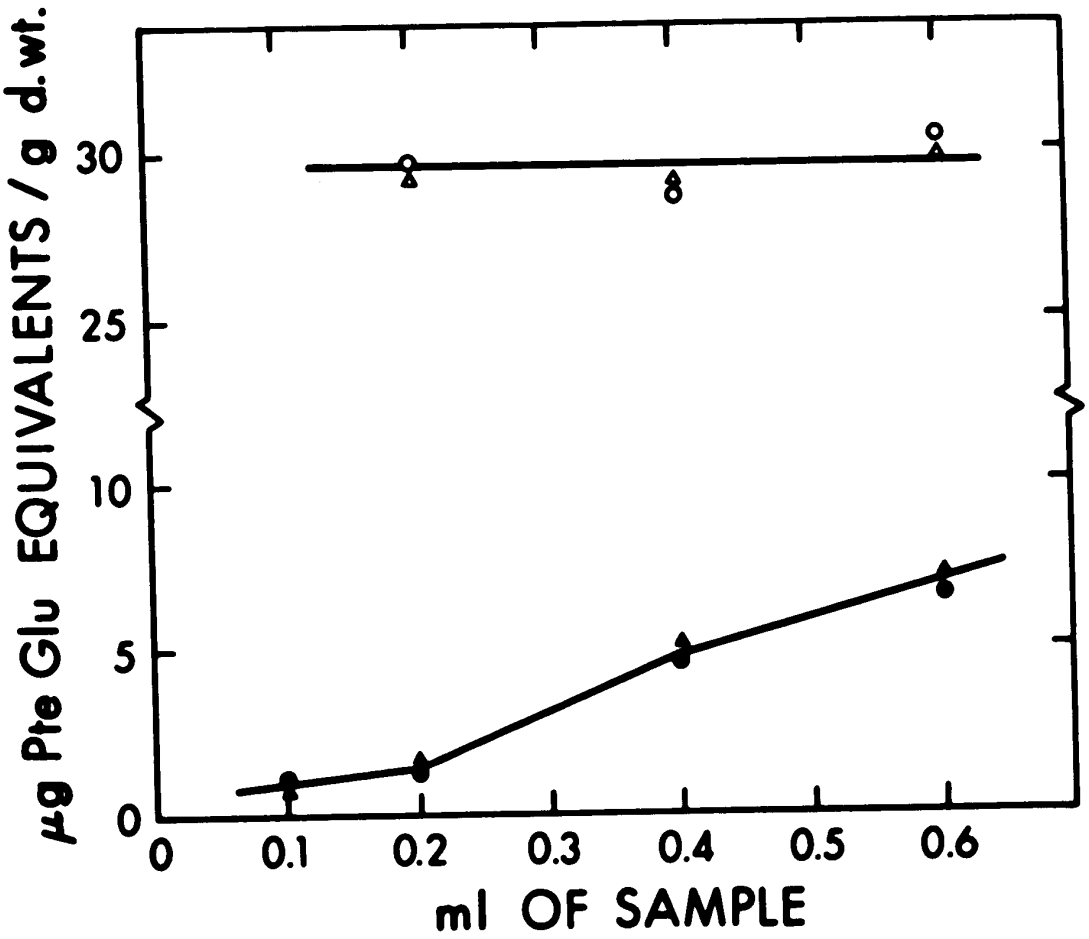
** Data are the ranges or averages of duplicate extractions.

† The tissue sampled in these extractions was grown at a different time than that used for other extractions.

FIGURE 3

The effect of sample size on the calculated level of pteroylglutamates in radish cotyledons

Pteroylglutamates were extracted by Method I from duplicate samples of 6-day-old radish cotyledons. An appropriate dilution was made of extracts before (closed symbols) and after (open symbols) chicken pancreas γ-glutamyl peptidase treatment. Duplicate samples, symbolized by the triangle and circle, were assayed with *L. casei*. Data are expressed in terms of PteGlu equivalents.


µg Pre Glu EQUIVALENTS / g d.wt.
30
25
10
5
0
0
0.1
0.2
0.3
0.4
0.5
0.6
ml OF SAMPLE

chicken pancreas γ-glutamyl carboxypeptidase agree very well.

Of the different methods used for extraction, Methods I and IIIB reduced endogenous peptidase activity most effectively, since the lowest levels of *L. casei* active pteroylglutamates were found (Table 3). Method IV was nearly as effective, while Method IIIA was the least effective in this regard. However, only in the cases of Method I and IIIA were total pteroylglutamate contents similar to those found with Method II. Thus, it appears that Method I was superior to the others. Lyophilization of tissue had no significant effect on the total levels of pteroylglutamates before or after chicken pancreas γ-glutamyl carboxypeptidase treatment (Table 3), and thus this treatment could be safely used before extraction of pteroylglutamates, a step routinely employed in later analyses.

Chromatography of radish cotyledon extracts, prepared by Methods I, II, and IIIA, confirmed that hydrolysis of conjugated derivatives occurred if no precautions were taken to inactivate endogenous enzymes (Figure 4). Extracts from Method I had very low levels of mono- and diglutamates as compared to the levels present when Method II and IIIA were applied. It is also evident (Figure 4) that inclusion of low ethanol concentrations (Method IIIA) partially inhibited hydrolysis of conjugated derivatives to monoglutamates.

In order to confirm that Method I was a valid extraction technique for other plant tissues, leaves of wheat, spinach, and pea plants were extracted by this method and the results compared with those for Method II (Table 4). It was found that in these extractions hydrolysis due to endogenous enzymes occurred to a greater extent than in those of radish cotyledons since the levels of mono- and diglutamates in extracts

FIGURE 4

The effect of extraction method on the chromatographic pattern of radish cotyledon pteroylglutamates

Extracts of 6-day-old radish cotyledons were prepared by Methods I, II, and IIIA, chromatographed on DEAE-cellulose and assayed with *L. casei*. Data are in terms of mμg PteGlu equivalents/g d. wt. The peaks are designated as:

Peak *a*, 10-HCO-H_4PteGlu; Peak *b*, 10-HCO-H_4PteGlu$_2$;

Peak *c*, 5-HCO-H_4PteGlu; Peak *d*, 5-CH_3-H_4PteGlu;

Peak *e*, 10-HCO-H_4PteGlu$_3$; Peak *f*, H_4PteGlu;

Peak *g*, 5-HCO-H_4PteGlu$_2$; Peak *h*, 5-CH_3-H_4PteGlu$_2$.

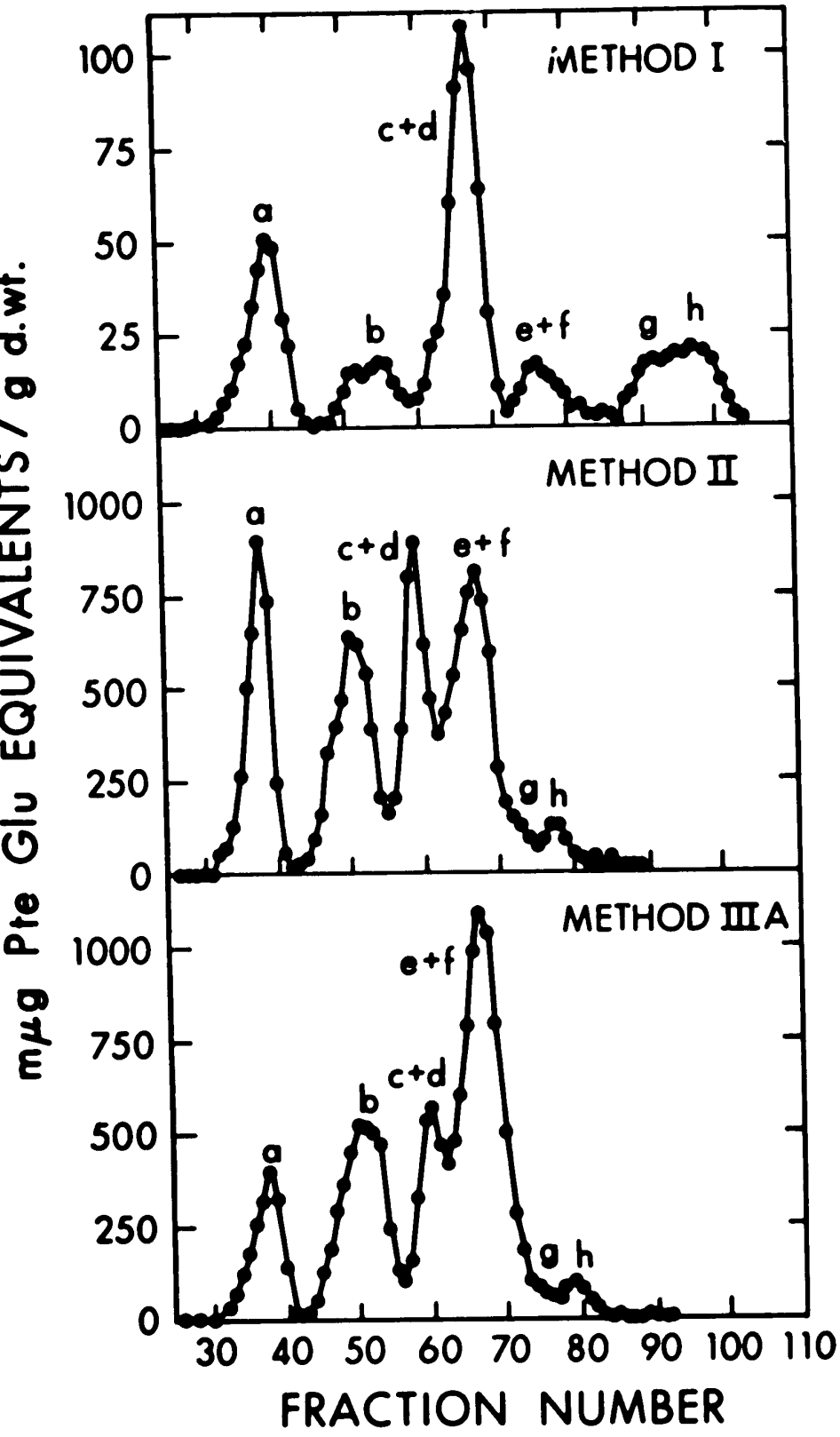

METHOD I
METHOD II
METHOD IIIA
a
b
c+d
e+f
g h
100
75
50
25
0
1000
750
500
250
0
30 40 50 60 70 80 90 100 110
FRACTION NUMBER
mμg Pte Glu EQUIVALENTS / g d.wt.

TABLE 4. *The effect of extraction method and peptidase treatment on the levels of pteroylglutamates in wheat, spinach, and pea leaves*

Leaf tissue	Method of Extraction	µg PteGlu equivalents/g d.wt.		
		Mono- and diglutamates*	Before peptidase**	After peptidase**
Wheat	I	0.5	1.5 - 2.1	15.9
	II	12.3	13.3	15.7
	I †	n.a.	n.a.	13.2
Spinach	I	5.4	5.5 - 7.9	28.1
	II	21.6	24.3	27.3
	I †	n.a.	4.5 - 17.0	28.7
Pea	I	1.6	2.2 - 7.1	14.4
	II	13.7	15.2	14.5
	I †	n.a.	16.2	16.9

n.a. - not assayed.

Data are expressed as PteGlu equivalents/g d.wt. for *L. casei*.

* Mono- and diglutamate values were obtained after DEAE-cellulose chromatography.

** Data are the ranges or averages of duplicate extractions.

† The tissue sampled in these extractions was grown at a different time than that used in other extractions.

prepared by Method II approached the 'before peptidase' and 'after peptidase' values. After peptidase treatment of extracts prepared by Method I and II, the levels of pteroylglutamates were similar. Total pteroylglutamate contents of plant tissues grown and analyzed in separate experiments were found to be within 15% of each other (Tables 3 and 4).

The Nature of Pteroylglutamate Derivatives in Different Plant Tissues

The four photosynthetic plant tissues examined in the previous section were found to contain readily detectable levels of pteroylglutamate derivatives. To obtain more knowledge regarding the occurrence and levels of individual derivatives, extracts of these species prepared by Method I were fractionated by DEAE-cellulose chromatography. Fractions collected from the columns were subjected to differential microbiological assay using *L. casei* and *P. cerevisiae*.

Recoveries of pteroylglutamates from the columns ranged from 80-95% in these analyses and individual derivatives were identified on the basis of (a) their position of elution from a standardized column and (b) their ability to promote bacterial growth in the microbiological assay (see Figure 1). In addition, certain other criteria based on the properties of individual peaks, such as co-chromatography with authentic compounds and the interconversion of 10-HCO- and 5-HCO-H_4PteGlu derivatives, were considered. Substantial increases in the size of peaks after chicken pancreas γ-glutamyl carboxypeptidase treatment of the extract was taken as indicative of their being diglutamyl derivatives. A wide variety of derivatives were detectable in extracts before peptidase treatment (Table 5). After such treatment, considerably increased amounts of 10-HCO-,

TABLE 5. *Levels of pteroylglutamate derivatives in extracts of four plant tissues before and after peptidase treatment*

Derivative	Radish cotyledons		Pea leaf		Wheat leaf		Spinach leaf	
	Before	After	Before	After	Before	After	Before	After
10-HCO-H_4PteGlu	294	553	237	160	115	238	191	218
10-HCO-H_4PteGlu$_2$	136	10093	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.	191	n.d.
5-HCO-H_4PteGlu$_2$	62	3255	57	2296	n.d.	3807		3207
5-CH_3-H_4PteGlu$_2$	149	15217	493	5394	22	1397		11141
H_4PteGlu$_3$	19	n.d.	117	1278	n.d.	n.d.		653
Total	*1338*	*30078*	*1569*	*13155*	*456*	*13358*	*5353*	*23456*

Extracts were prepared from fresh tissue by Method I and chromatographed on DEAE-cellulose before and after chicken pancreas γ-glutamyl carboxypeptidase treatment. Fractions were assayed with *L. casei* and *P. cerevisiae*. Data are expressed in mμg PteGlu equivalents/g d. wt. for *L. casei*. The large bracket denotes that the pteroylglutamate activity eluted in the position of these derivatives was not assigned to any one derivative because no distinct peaks were prevalent. n.d. - not detected.

5-HCO-, and 5-CH_3-H_4PteGlu$_2$ were found. Since preliminary investigations with pea leaves revealed differences of less than 10% in the quantity of major individual derivatives (those greater than 10% of the total from the column), it was considered acceptable to perform only one chromatographic run to obtain a representative pteroylglutamate pattern of these tissues. The quantities of the major individual derivatives in extracts prepared from tissues grown at different times commonly displayed larger variations (up to 30%). These variations are, perhaps, not excessive when one considers the inherent variability of biological materials. The derivatives which were minor constituents in the tissue extracts showed considerably more variation, occasionally being different by a factor of 2. However, as these derivatives were present in such small amounts, it is likely that a large part of this variability was associated with the sensitivity of the assay. Since these analyses were rather laborious, and it was clear that different plant tissues contained varying amounts of individual derivatives, more extensive analyses were not performed. In conclusion, the data in Table 5 are considered typical of the pteroylglutamates in extracts of plant tissues which were grown and treated as described.

In considering the results of these analyses it must be emphasized that 5,10-CH=H_4PteGlu, 5,10-CH_2=H_4PteGlu and 5-HCNH-H_4PteGlu may also be present *in vivo* but would be detected as 10-HCO-H_4PteGlu, H_4PteGlu and 5-HCO-H_4PteGlu respectively, due to their instability in neutral aqueous solutions (Blakley, 1969). Also as a result of preliminary experiments it was known that a certain amount of 10-HCO-H_4PteGlu derivatives were converted to 5-HCO-H_4PteGlu derivatives concomitant with chicken pancreas peptidase treatment. Thus values reported for the latter derivatives are

are probably greater than occurred *in vivo*. Although the occurrence of H_2PteGlu (or H_2PteGlu$_2$ after chicken pancreas γ-glutamyl carboxypeptidase treatment) might be expected in the extracts (see Introduction), no evidence to support this was found. The possibility exists that such compounds occur in amounts below the level of detection after chromatography. In this connection it is also of interest to add that these observations imply that the endogenous pool of PteGlu must also be very small.

The increase in the level of diglutamates after peptidase treatment indicated the presence of polyglutamates (Table 5). In order to examine this point further, the polyglutamates in extracts of radish cotyledons were chromatographed on DEAE-cellulose. Pteroylglutamates were eluted with the usual gradient for 150 fractions and then by 0.5 M and 1.0 M KH_2PO_4 buffers containing potassium ascorbate, pH 6.0. Even numbered fractions were treated with pea cotyledon γ-glutamyl carboxypeptidase. This resulted in finding at least 4 additional peaks which supported the growth of *L. casei* and a significant increase in the size of a fifth peak (Figure 5).

The Effect of Light on the Levels of Pteroylglutamates in Plant Tissues

As outlined in the Introduction, the metabolism of pteroylglutamate derivatives *in vivo* has received only limited study in plants. Also, several possible relationships between these compounds and photosynthetic metabolism were suggested. Since it has been established in the present work that a number of pteroylglutamate derivatives occur in photosynthetic tissues, further investigations to elucidate their possible roles were undertaken.

FIGURE 5

The effect of peptidase treatment on individual fractions isolated by DEAE-cellulose chromatography of radish cotyledon extracts

Extracts from 6-day-old radish cotyledons were chromatographed by gradient elution to fraction 150 as outlined in Materials and Methods. This was followed by sequential elution using 25 ml of 0.5 M KH_2PO_4 and 1.0 M KH_2PO_4 buffers containing 6 mg/ml K-ascorbate acid, pH 6.0. These latter buffers appeared in the effluent at fractions 168 and 176 respectively, the void volume of column being 54 ml. Even numbered fractions (● - ●) were treated with pea cotyledon peptidase and then assayed using *L. casei*. Odd numbered fractions (o - o) were assayed without enzyme treatment. Data are expressed as PteGlu equivalents/g d. wt.

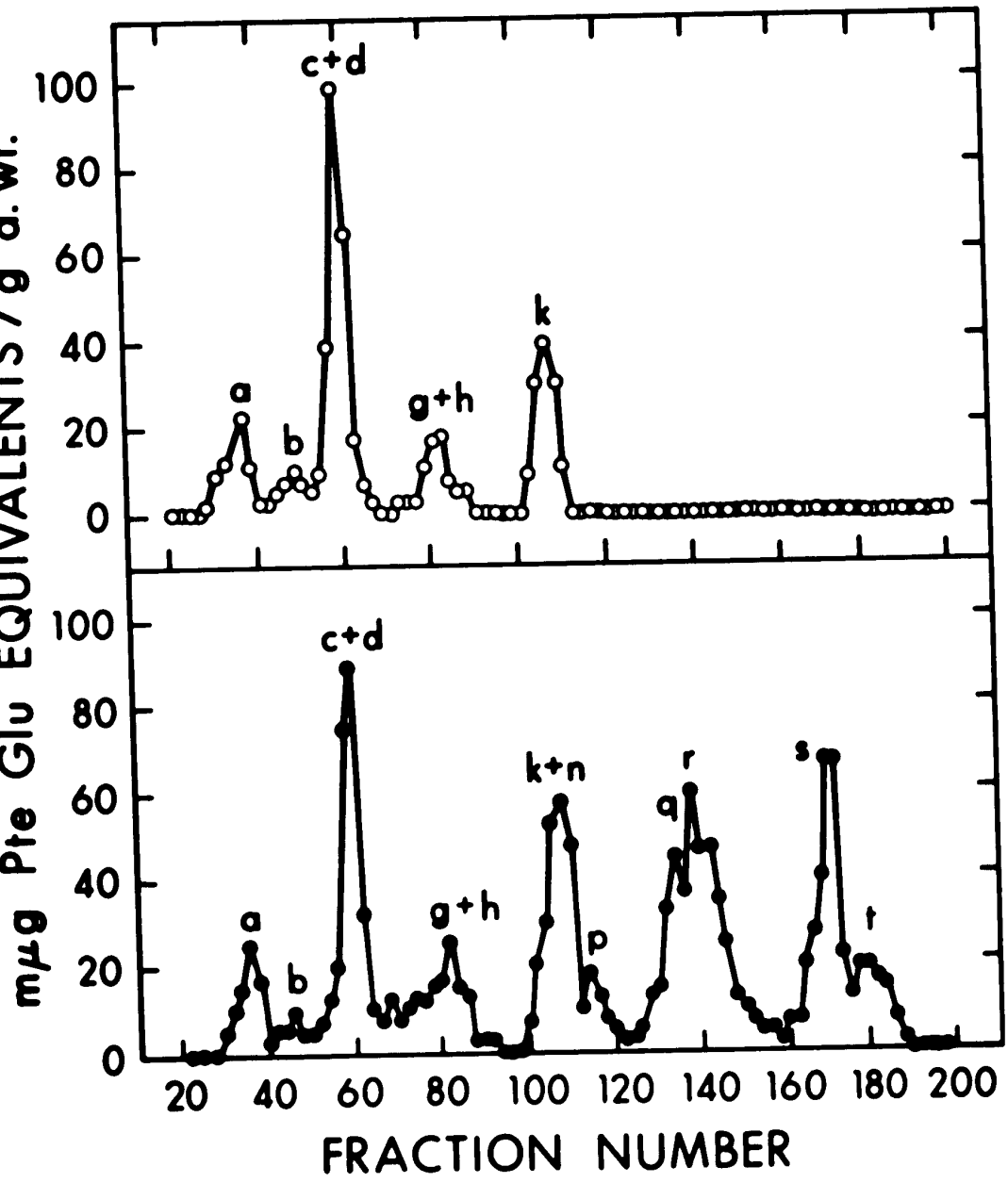
The peaks are designated as:

Peak *a*, 10-HCO-H_4PteGlu; Peak *b*, 10-HCO-H_4PteGlu$_2$;

Peak *c*, 5-HCO-H_4PteGlu; Peak *d*, 5-CH_3-H_4PteGlu;

Peak *g*, 5-HCO-H_4PteGlu$_2$; Peak *h*, 5-CH_3-H_4PteGlu$_2$;

Peak *k*, 5-CH_3-H_4PteGlu$_3$; Peaks *n* - *t*, unidentified pteroylglutamate derivatives.


mμg Pte Glu EQUIVALENTS / g d. wt.
FRACTION NUMBER
c+d
a
b
g+h
k
k+n
q
r
s
p
t
0
20
40
60
80
100
120
140
160
180
200

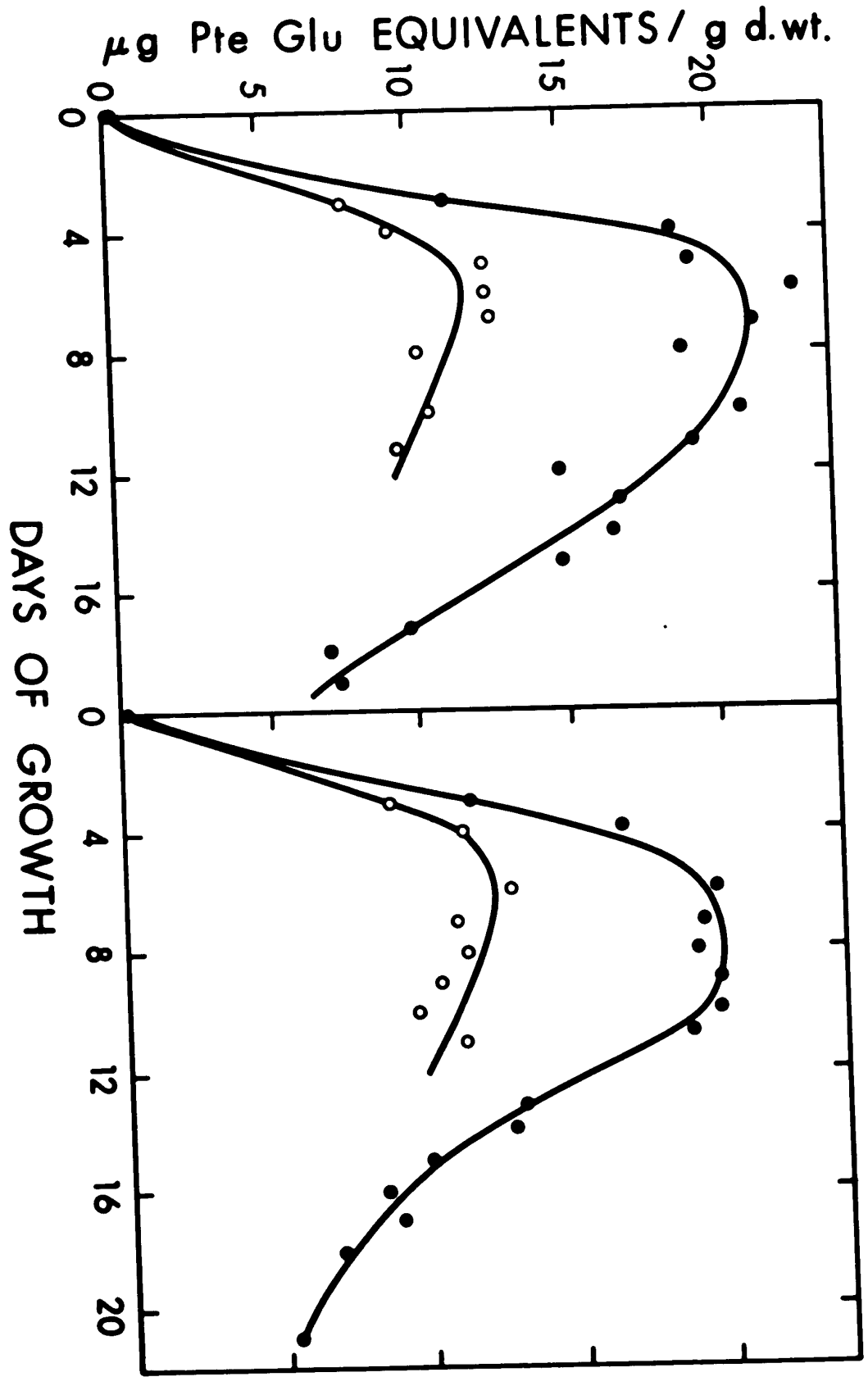
The importance of certain pteroylglutamate derivatives in the metabolism of photosynthetic tissues might conceivably be revealed by a comparison of the levels of these derivatives in green and etiolated tissues. Changes in levels of total pteroylglutamates in germinating radish cotyledons grown in similar regimes except for illumination are illustrated in Figure 6. A very rapid increase in pteroylglutamate levels was observed during germination in both normal and etiolated tissue. Maximal levels were reached between 6 and 7 days under the conditions employed, at which time the normal cotyledons had nearly twice the pteroylglutamate content of etiolated tissue. The levels present in the latter, however, decreased only slightly until sampling had to be discontinued at 11 days because the tissue was subject to fungal attack and had a generally unhealthy appearance. The level of pteroylglutamates in normal tissue decreased as the cotyledons became progressively more senescent. Senescence in this case was obvious by a visible loss of chlorophyll from the cotyledons and an increase in the fresh weight/dry weight ratios calculated in conjunction with these studies.

This work (Figure 6) was carried out before need for an improved extraction technique was realized and thus the tissue samples were extracted by Method II. Endogenous peptidase activity was present during extraction of the etiolated tissue and, therefore, it is not a lack of it that accounted for the reduced pteroylglutamate levels found in extracts of etiolated tissue. The period of maximal pteroylglutamate content corresponded to the time when the primary leaves were starting to appear. It is also of interest to add that 7-day-old cotyledons are known to exhibit an active intermediary metabolism in which pteroylglutamate coenzymes have been implicated (Caballero and Cossins, 1970). For these

FIGURE 6

Changes in pteroylglutamate content of radish cotyledons during germination and senescence

Radish seedlings were grown as described in the Materials and Methods, to obtain normal (● - ●) and etiolated (o - o) cotyledons. At 11 a.m. daily, the cotyledons were excised and rapidly extracted by Method II. Data for two independent experiments are given. Each point is the average of duplicate extractions. The data are expressed in PteGlu equivalents for *L. casei*.


μg Pte Glu EQUIVALENTS / g d.wt.
0
5
10
15
20
DAYS OF GROWTH
0
4
8
12
16
20

reasons, cotyledons of this age were routinely used in later experiments.

In further experiments, data were also obtained from etiolated wheat leaves. In this plant, as in radish, etiolation was associated with a lower pteroylglutamate content, being only 8.2 µg/g.d.wt. as compared to approximately 14.2 µg/g d. wt. in the normal green tissue. In general, the quantities of individual derivatives, as well as the totals eluted from the columns, were lower in the etiolated tissues (Table 6). The proportions of methyl derivatives were also higher in the etiolated tissues.

As the preceeding studies showed that green tissue had a higher concentration of pteroylglutamates than etiolated tissue, the question arises whether it would be possible to increase levels of pteroylglutamates by illuminating etiolated seedlings. The results of such experiments are summarized in Table 7. Illumination of etiolated radish seedlings for 24 hours increased the pteroylglutamate content by about 20%. The proportion of pteroylglutamates which promoted the growth of *S. faecalis* also increased by illumination. Since *S. faecalis* does not respond to methyl derivatives, it can be concluded that levels of formyl and unsubstituted derivatives increased more than methyl derivatives. This latter finding is consistent with the results of Table 6.

Since aminopterin inhibits the formation of reduced pteroylglutamates in germinating pea cotyledons (Roos and Cossins, 1971), it is possible that a similar effect may occur in radish cotyledons during the early stages of seedling growth. In order to investigate this, samples of 25 cotyledons were allowed to take up small quantities (4×10^{-11} to 4×10^{-7} mole) of aminopterin through the cut hypocotyls at the beginning of an illumination period. The results of one such experiment (Table 8)

TABLE 6. *Levels of pteroylglutamate derivatives in extracts from etiolated and normal radish cotyledons and wheat leaves*

Derivative	Radish		Wheat	
	Etiolated	Green	Etiolated	Green
10-HCO-H_4PteGlu	371*	553	879	238
10-HCO-H_4PteGlu$_2$	3110	10093	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	15217	1119	1397
Total recovered	14465	30078	7438	13358
% of total recovered				
Methyl derivatives	61%	53%	35%	13%
Other derivatives	39%	47%	65%	87%

n.d. - not detected

* Data are in terms of mug PteGlu equivalents/g d. wt. for *L. casei*.

Extracts were prepared by Method I from 6-day-old radish and 8-day-old wheat seedlings. After chicken pancreas peptidase treatment, the extracts were chromatographed and assayed with *L. casei* and *P. cerevisiae*.

TABLE 7. *The effect of illumination on the pteroylglutamate content of etiolated radish cotyledons*

Experiment	Etiolated			Illuminated		
	*L. casei**	*S. faecalis**	Ratio**	*L. casei**	*S. faecalis**	Ratio**
1	14.7	6.5	0.44	17.8	8.7	0.50
2	13.9	6.0	0.43	16.6	9.1	0.55

* Data are expressed as µg PteGlu equivalents/g d.wt.

** *S. faecalis*:*L. casei* ratio.

Samples of radish cotyledons were from 6-day-old etiolated seedlings or etiolated seedlings which were illuminated for an additional 24 hours with a mercury lamp (2000 ft. c.) at 23° ± 2°. After lyophilization of tissue samples, derivatives were extracted by Method I, treated with chicken pancreas γ-glutamyl carboxypeptidase and assayed with *L. casei* and *S. faecalis*.

TABLE 8. *Levels of individual pteroylglutamate derivatives after illumination of etiolated radish cotyledons in the presence of aminopterin*

Derivatives	mμg PteGlu equivalents/g d.wt.		
	Control	Light	Light + AME
10-HCO-H_4PteGlu	1780	3340	662
10-HCO-H_4PteGlu$_2$	1650	2956	1323
5-HCO-H_4PteGlu	4340	1004	
5-CH_3-H_4PteGlu		2927	
10-HCO-H_4PteGlu$_3$	320	640	186
H_4PteGlu		220	
5-HCO-H_4PteGlu$_2$	360	590	
5-CH_3-H_4PteGlu$_2$		395	
H_4PteGlu$_2$	n.d.	54	
Polyglutamates*	1830	1146	
PteGlu	n.d.	n.d.	6793
Total	*10280*	*13272*	*8964*

n.d. - not detected.
* A peak in fractions 110-120 active for *L. casei* only; the derivatives in this peak were not identified, but probably includes 5-CH_3-H_4PteGlu$_3$.

Samples of 25 etiolated radish cotyledons (6-day-old) were illuminated with a mercury lamp (2000 ft. c.) for 27 hr in the presence or absence of 0.4 μmole aminopterin(AME) as described in the Materials and Methods. The control data are from etiolated cotyledons killed at the initiation of the treatments. The cotyledons were lyophilized and the laminar portion extracted by Method II. The chromatographed extracts were assayed with *L. casei* and *P. cerevisiae*. Data are expressed as PteGlu equivalents for *L. casei*. The brackets indicate the sum of pteroylglutamate peaks not differentially assayed (control) or very low levels of microbiological response not associated with distinct peaks (light + AME).

clearly show that aminopterin affected the levels of tetrahydropteroyl-glutamate derivatives, especially 5-CH_3-H_4PteGlu, as well as the ability of these tissues to increase their total pteroylglutamate content. PteGlu accumulated in the aminopterin-treated tissue.

In these investigations, the effect of aminopterin on the chlorophyll formation of etiolated radish cotyledons was readily visible. Quantitative measurement of chlorophyll content showed that a logarithmic relationship (Figure 7) existed between chlorophyll production and quantity of aminopterin in the feeding solution.

The effect of light on the pteroylglutamate pattern in photosynthetic tissue was also examined in another manner. Radish seedlings were grown as usual for 5 days with 13 hours of illumination daily. On the 6th day, cotyledons were excised and immediately frozen at periods 1 hour before and 5 hours after the lights were turned on. Assay of peptidase-treated extracts showed that a considerable decrease in total pteroyl-glutamate content occurred after this period of illumination whether the levels were expressed on a dry weight or chlorophyll basis (Table 9). The effects of illumination were examined in more detail after chromatography (Table 10). In order to facilitate the examination of the results obtained, the data are tabulated according to the substituent groups of the derivatives. The illuminated tissues were found to contain significantly lower levels of formyl derivatives and considerably greater amounts of unsubstituted derivatives in two separate experiments. The level of methyl derivatives in illuminated tissues were also slightly lower.

Radish cotyledons are known to metabolize glycine and serine rapidly in light and in darkness (Cossins and Caballero, 1970), and as this

FIGURE 7

Effect of aminopterin on chlorophyll biosynthesis of etiolated radish cotyledons

Twenty-five 6-day-old etiolated radish cotyledons were excised under water and the cut ends placed in 2 ml of aminopterin solution of varying concentrations. This was followed by 5 ml of half strength White's solution and finally sufficient 10x diluted White's solution until the end of the experimental period of 27 hours, when the cotyledons were lyophilized.

Chlorophyll was determined by the method of Arnon (1949); extracts of etiolated cotyledons were calculated to have 0.55 mg "chlorophyll"/g d. wt.; normal tissues had 9.5 - 11 mg/g d. wt.

The data shown is the average of 2 determinations. The vertical bars indicate the variation encountered in these duplicates.

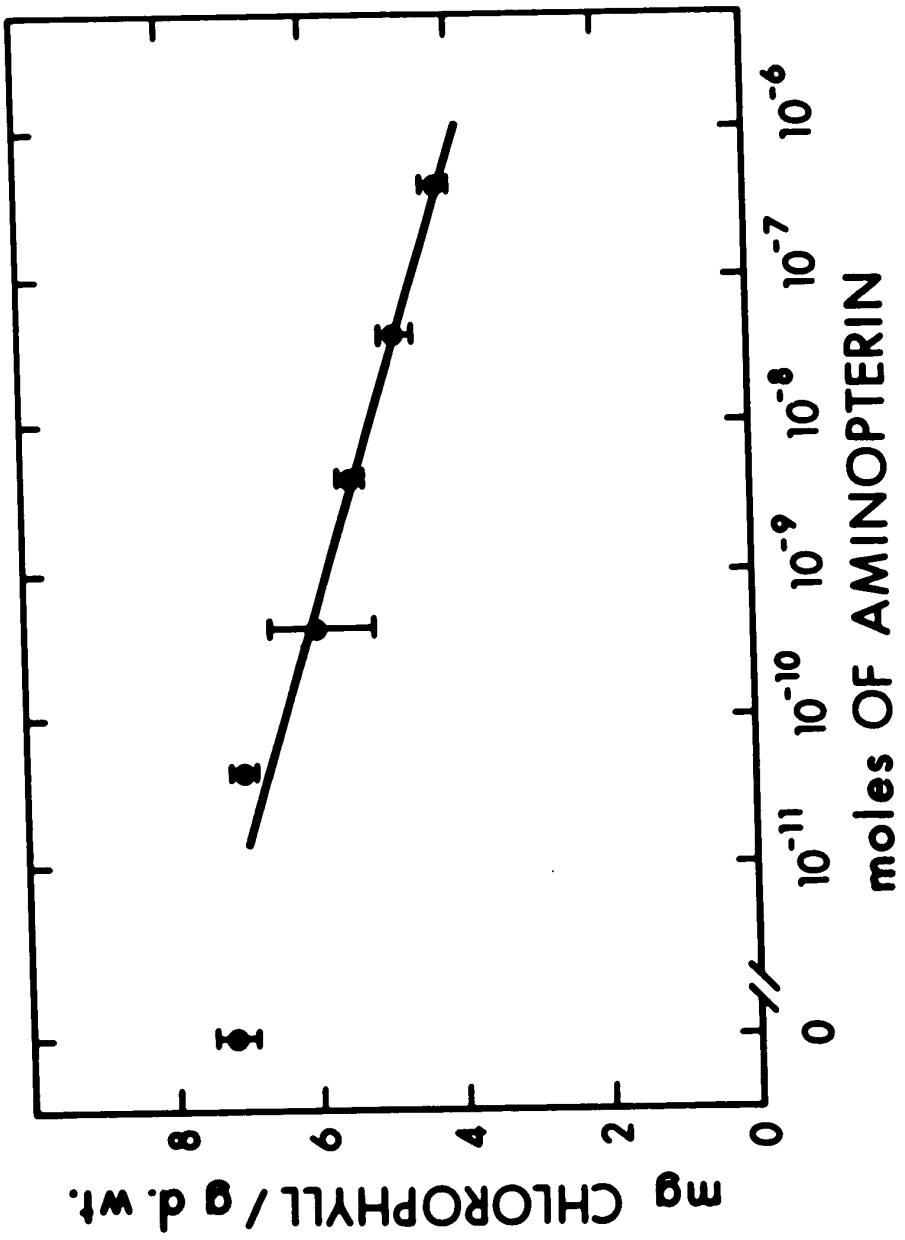

mg CHLOROPHYLL / g d. wt.
0
2
4
6
8
0
10^{-11}
10^{-10}
10^{-9}
10^{-8}
10^{-7}
10^{-6}
moles OF AMINOPTERIN

TABLE 9. *The effect of light on the total pteroylglutamate content of radish cotyledons*

Experiment	Dark			Light		
	µg/g d. wt.		µg/mg chlorophyll	µg/g d. wt.		µg/mg chlorophyll
	L. casei	*S. faecalis*	*L. casei*	*L. casei*	*S. faecalis*	*L. casei*
1	32.0	21.9	3.21	25.1	18.5	2.53
2	32.4	24.3	3.49	25.2	18.5	2.63

Radish seedlings were grown normally with 13 hours of illumination daily, starting at 10 a.m. On the 6th day of growth, 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 by Method I, treated with chicken pancreas 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 d. wt. and per mg chlorophyll.

TABLE 10. *The effect of light on the levels of pteroylglutamate derivatives of radish cotyledons*

Derivatives by substituent group	Experiment 1				Experiment 2			
	Dark		Light		Dark		Light	
	Level*	%**	Level*	%**	Level*	%**	Level*	%**
Formyl derivatives	16980	66	6200	33	16270	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	*25660*		*18860*		*26060*		*19410*	

* Data are expressed as mμg PteGlu equivalents/g d. wt. for *L. casei*.

** Denotes % of total eluted from the column.

This data was obtained by chromatography of one of the duplicate extracts described on Table 9.

L. casei and *S. faecalis* were used to assay the fractions for pteroylglutamates.

TABLE 11. *The effect of light on the levels of the major free amino acids of radish cotyledons*

Amino acid	Experiment 1			Experiment 2		
	Dark*	Light*	Light/Dark ratio	Dark*	Light*	Light/Dark ratio
Aspartic acid	19.15	16.70	0.87	17.55	24.86	1.43
Threonine	1.97	3.68	1.85	1.83	3.05	1.66
Serine	4.46	11.76	2.65	4.29	8.71	2.03
Glutamic acid	26.22	36.10	1.37	31.99	42.08	1.32
Proline	2.22	2.53	1.14	2.92	3.01	1.03
Glycine	1.70	10.09	5.96	4.37	10.36	2.37
Alanine	3.36	11.01	3.29	3.76	7.13	1.89
Valine	0.86	1.90	2.19	0.88	2.15	2.45
Isoleucine	0.35	0.10	0.28	0.34	0.11	0.32
Total recovered	*60.29*	*93.96*	*1.56*	*67.94*	*101.47*	*1.49*

* Data are expressed in μmoles amino acid/g d. wt.

Amino acid extracts were prepared as described in the text from 6-day-old radish cotyledons treated as described in Table 10. The amino acids were separated quantitatively using an amino acid analyzer.

conceivably involves one-carbon metabolism, it was of interest to examine the levels of these amino acids in further experiments (Table 11). In general the quantities of the major amino acids were greater on a dry weight basis in the light-harvested cotyledons. Total amino acid content increased approximately 50%. Of the individual amino acids, glycine, serine, and alanine, showed the greatest percentage increase.

The Origin of the One-carbon Group of H_4PteGlu Derivatives in vivo

Further study of possible relationships between photosynthesis and H_4PteGlu mediated one-carbon metabolism was continued by a brief examination of the origins of the one-carbon moieties of methyl and formyl tetrahydropteroylglutamates in radish cotyledons. In the Introduction, it was noted that the one-carbon groups of H_4PteGlu derivatives can arise from the carbon-2 of glycine, the carbon-3 of serine and formate. These compounds are also known to be actively metabolized by illuminated radish cotyledon disks (Cossins and Caballero, 1970; Caballero and Cossins, 1970; Cossins and Sinha, 1965). Accordingly, such disks were allowed to take up solutions of sodium bicarbonate-C^{14}, glycine-2-C^{14} and sodium formate-C^{14}, respectively, which had high specific radioactivities. The feeding period selected was 10 minutes, mainly to limit incorporation of C^{14} to the one-carbon position of the H_4PteGlu derivatives rather than general incorporation into the pteroylglutamate moiety. Pteroylglutamates were extracted by Method I, treated with peptidase and chromatographed (Figures 8-10). No radioactive peaks corresponding to peaks with pteroylglutamate activity could be detected after either the sodium bicarbonate-C^{14} (Figure 8) or glycine-2-C^{14} feeding (Figure 9). In contrast, radioactivity from formate-C^{14} was

FIGURE 8

Chromatography of pteroylglutamate derivatives in illuminated radish cotyledon disks after incubation in $HC^{14}O_3^-$

Samples of 20 disks (186 mg) from 6-day-old radish cotyledons were placed in a Warburg flask containing 0.5 ml of KH_2PO_4 buffer (pH 6.0). After equilibration in the light for 10 min at 23°C, 10 µc of $NaHC^{14}O_3$ (57 µc/µmole) in 0.2 ml H_2O were tipped from the side arm. The incubation was terminated after 10 min by addition of boiling 12 mg/ml ascorbate (pH 6.0). Derivatives were extracted by Method I, treated with chicken pancreas γ-glutamyl carboxypeptidase and then chromatographed. Aliquots (0.2 ml) of the 3 ml fractions were assayed for C^{14} (● - ●) and for pteroylglutamates with *L. casei* (o - o).

The peaks are designated as follows:

Peak *b*, 10-HCO-H_4PteGlu$_2$; Peak *d*, 5-CH_3-H_4PteGlu;

Peak *g*, 5-HCO-H_4PteGlu$_2$; Peak *h*, 5-CH_3-H_4PteGlu$_2$.

Total incorporation of C^{14} in the experimental period was 16.3 x 10^6 cpm. The complete extract was chromatographed and the data presented are for 0.2 ml aliquots of the fractions. Most of the radioactivity was eluted before fraction 40 and none was detected after fraction 100.

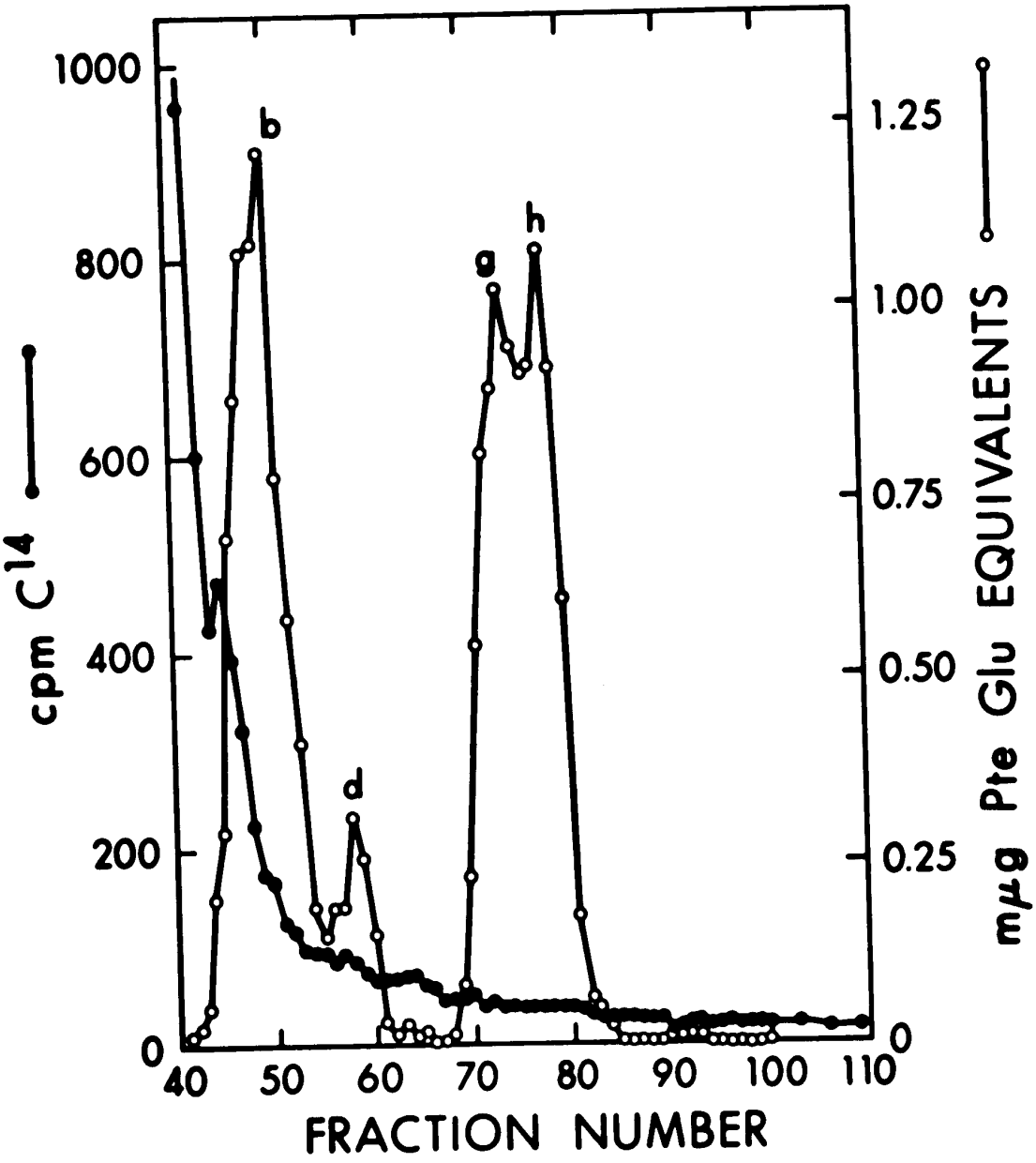

1000
800
600
400
200
0
cpm C14
b
a
h
d
40
50
60
70
80
90
100
110
FRACTION NUMBER
1.25
1.00
0.75
0.50
0.25
0
mμg Pte Glu EQUIVALENTS

FIGURE 9

Chromatography of pteroylglutamate derivatives in illuminated radish cotyledon disks after incubation in glycine-2-C^{14}

Samples of 20 disks (186 mg) from 6-day-old radish cotyledons were placed in a Warburg flask containing 0.5 ml of KH_2PO_4 buffer (pH 6.0). After equilibration in the light for 10 min at 23°C, 10 µc of glycine-2-C^{14} (59 µc/µmole) in 0.2 ml H_2O were pipetted into the flask. The incubation was terminated after 10 min by tipping the disks into a funnel and rapidly washing them with distilled H_2O to remove excess glycine-2-C^{14}, followed by heating at 95°C in 12 mg/ml ascorbate (pH 6.0). Derivatives were extracted by Method I, treated with chicken pancreas γ-glutamyl carboxypeptidase and chromatographed. Aliquots (0.2 ml) of the 3 ml fractions were assayed for C^{14} (● - ●) and pteroylglutamates with *L. casei* (o - o).

Peaks are designated as follows:

Peak *b*, 10-HCO-H_4PteGlu$_2$; Peak *d*, 5-CH_3-H_4PteGlu;

Peak *g*, 5-HCO-H_4PteGlu$_2$; Peak *h*, 5-CH_3-H_4PteGlu$_2$.

Total incorporation of C^{14} in the experimental period was 2.30 x 10^6 cpm. The complete extract was chromatographed and the data presented are for 0.2 ml aliquots of the fractions. Most of the radioactivity was eluted before fraction 40 and none was detected after fraction 100.

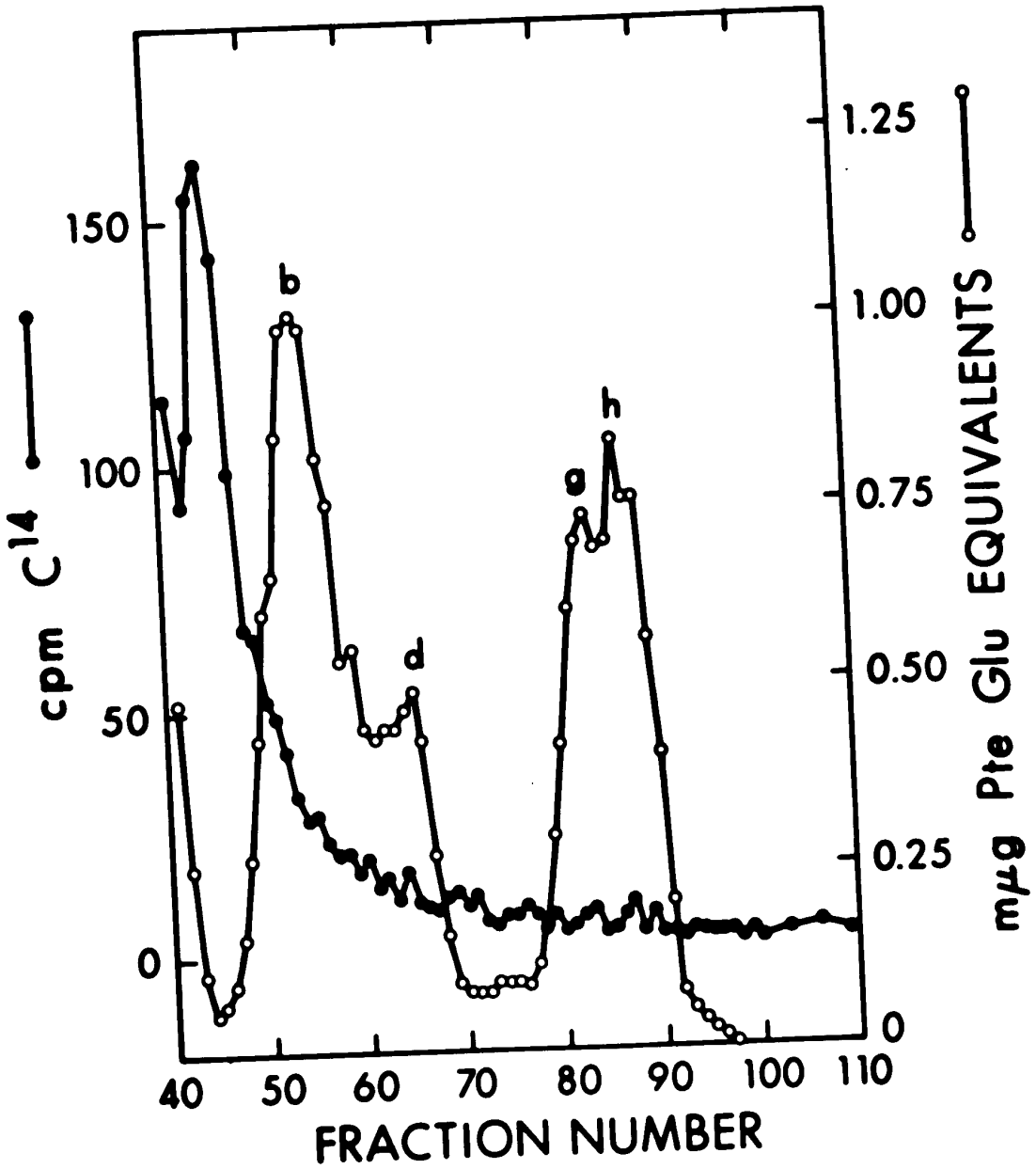

cpm C14
150
100
50
0
b
d
a
h
1.25
1.00
0.75
0.50
0.25
0
mμg Pte Glu EQUIVALENTS
40
50
60
70
80
90
100
110
FRACTION NUMBER

FIGURE 10

The incorporation of formate-C^{14} into tetrahydropteroylglutamates by illuminated radish cotyledon disks

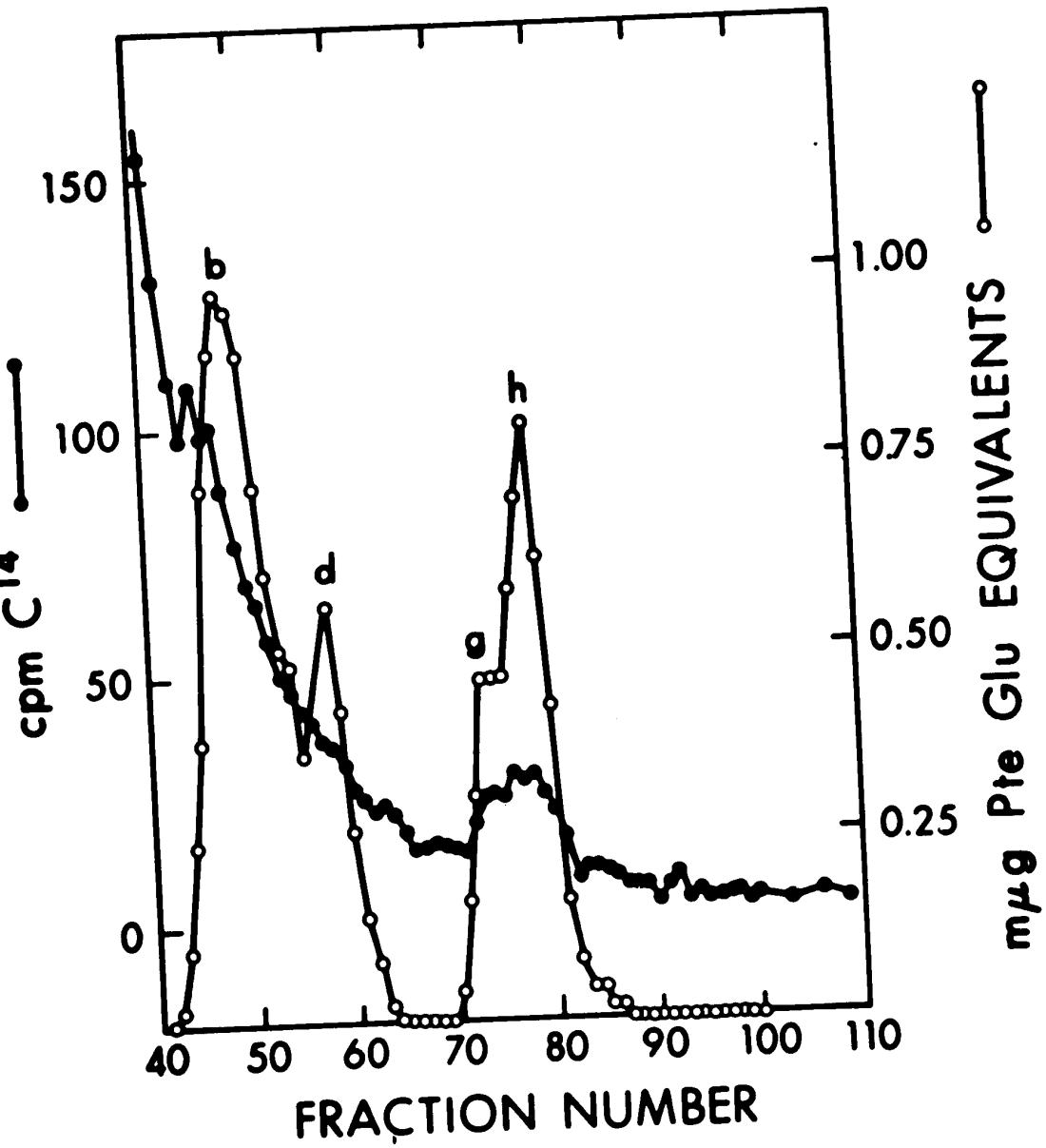
Samples of 20 disks (186 mg) from 6-day-old radish cotyledons were placed in Warburg flasks containing 0.5 ml KH_2PO_4 buffer (pH 6.0). After equilibration in the light for 10 min, 10 μc of formate-C^{14} (59 μc/μmole) in 0.2 ml H_2O were pipetted into the flask. The incubation was terminated after 10 min by tipping the disks into a funnel, washing them rapidly with distilled H_2O to remove excess formate-C^{14}, followed by heating at 95°C in 12 mg/ml ascorbate (pH 6.0). Derivatives were extracted by Method I, treated with chicken pancreas γ-glutamyl carboxypeptidase, and then chromatographed. Aliquots (0.2 ml) of each 3 ml fraction were assayed for C^{14} (● - ●) and pteroylglutamate activity with *L. casei* (o - o).

The peaks were designated as follows:

Peak *b*, 10-HCO-H_4PteGlu$_2$; Peak *d*, 5-CH_3-H_4PteGlu;

Peak *g*, 5-HCO-H_4PteGlu$_2$; Peak *h*, 5-CH_3-H_4PteGlu$_2$.

Total incorporation of C^{14} in the experimental period was 3.28×10^6 cpm. The complete extract was chromatographed and the data presented are for 0.2 ml aliquots of the fractions. Most of the radioactivity was eluted before fraction 40 and none was detected after fraction 100.


150
100
50
0
cpm C14
1.00
0.75
0.50
0.25
mμg Pte Glu EQUIVALENTS
b
d
a
h
40
50
60
70
80
90
100
110
FRACTION NUMBER

associated with the 5-HCO- and 5-CH_3-H_4PteGlu$_2$ peak (Figure 10). Although this peak contained only 0.065% of the total radioactivity taken up by the disks, calculations revealed that the pteroylglutamates in this peak had 12% of the specific radioactivity of the formate-C^{14} supplied.

Since the $HC^{14}O_3^-$ feeding experiments did not show any trace of C^{14} in the pteroylglutamates, further investigations using this approach to study the possible relationship between photosynthetic carbon flow and H_4PteGlu derivatives were not carried out. Formate-C^{14} appeared to enter the pteroylglutamate pool by a direct route without oxidation to $C^{14}O_2$, a pathway presumably distinct from that followed by carbon dioxide during photosynthesis in this species.

The Effects of Aminopterin on Pteroylglutamate Contents and Ability to Fix $HC^{14}O_3^-$ in the Light

The metabolic importance of a coenzyme in intermediary metabolism can often become apparent from a comparison of the metabolism of normal tissue with that of tissue deficient in the coenzyme. In studies employing animals, tissue cultures, and microorganisms, (for example, see Blakley, 1969) aminopterin and methotrexate, two well known PteGlu analogues, have been used to induce H_4PteGlu deficiency. These analogues are known to inhibit dihydrofolate reductase activity *in vitro*, and are generally thought to act similarly *in vivo* (Blakley, 1969). This means that in aminopterin- or methotrexate-treated tissues, H_2PteGlu will not be fully reduced to H_4PteGlu. As a consequence, metabolic reactions dependent on H_4PteGlu will be severely inhibited *in vivo*.

In the present investigation, aminopterin did not interfere in the microbiological assay of pteroylglutamates after DEAE-cellulose chromatography and was, therefore, used in the following experiments. The effect of aminopterin on the photosynthesis of higher plants has not, to the writer's knowledge, been examined in detail. To investigate possible relationships between photosynthesis and one-carbon metabolism, an initial short-term aminopterin experiment was carried out.

Excised radish cotyledons were infiltrated with 2×10^{-5}M aminopterin; and incubated for periods up to 6 hours. The treated cotyledons were then analyzed for pteroylglutamate levels. In other cases, disks (5 mm diameter) were cut from the treated cotyledons and used to assess their ability to fix $HC^{14}O_3^-$ in the light.

Aminopterin clearly affected the levels of pteroylglutamate derivatives (Table 12). Within 3 3/4 hours from the start of the treatment, differences between the control and treated samples were evident. The levels of methyl derivatives were substantially lower while a derivative, not found in extracts of the control tissues, was collected in fractions 125-140. The latter derivative was eventually identified as PteGlu (see below). Since the mono- and diglutamate derivatives with the same substituent groups were similarly affected by aminopterin, for clarity they were included together in Table 12. After 6 hours of aminopterin treatment, a further decrease in the level of methyl derivatives was observed. The unsubstituted derivatives were affected similarly but to a lesser degree. The level of PteGlu in these treated tissues was very high, accounting for 62% of the total pteroyl-glutamate activity recovered from the column. In contrast to experiment 1, the total amount of pteroylglutamate in this experiment was higher

TABLE 12. *The effect of short-term aminopterin treatments on levels of pteroylglutamate derivatives in excised radish cotyledons*

Derivatives	Experiment 1				Experiment 2			
	Aminopterin		Control		Aminopterin		Control	
	Level*	%**	Level*	%**	Level*	%**	Level*	%**
Formyl derivatives	4970	25	5390	23	6240	19	6140	25
Methyl derivatives	8180	41	14250	60	1600	5	12900	52
Unsubstituted derivatives †	4230	22	3940	17	4520	14	5960	23
PteGlu	2360	12	n.d.		19730	62	n.d.	
Total recovered from column	*19740*		*23580*		*32090*		*25000*	

n.d. - not detectable.

* Data are expressed as mμg PteGlu equivalents/g d. wt. for *L. casei*.

** Denotes % of total eluted from the column. † excluding PteGlu.

Excised 6-day-old radish cotyledons were infiltrated with 2×10^{-5}M aminopterin or water and incubated for 3 3/4 hours (Experiment 1) or 6 hours (experiment 2) as described in the Materials and Methods. After lyophilization of the cotyledons, extracts were prepared by Method I, treated with chicken pancreas γ-glutamyl carboxypeptidase, and chromatographed. *L. casei* and *S. faecalis* were used to assay the fractions.

than the control, largely due to the presence of PteGlu, which was not observed in the control.

The identity of PteGlu produced in these experiments was established as follows. Firstly, fractions 129-139 were pooled and co-chromatographed with PteGlu-2-C^{14}. The result, illustrated in Figure 11, was the elution of smooth, overlapping peaks of microbiological- and radio-activity which coincided closely. Secondly, similar levels of this compound were found in extracts before treatment with chicken pancreas γ-glutamyl carboxypeptidase indicating that this peak was not a naturally occurring conjugated derivative. Lastly, assay of the peak with *S. faecalis* gave a growth response essentially equal to that of *L. casei*, while *P. cerevisiae* did not respond at all. Collectively, these results suggest that this derivative was PteGlu.

Besides the study of the effect of aminopterin on pteroylglutamate levels, its possible effect on the photosynthetic fixation of $HC^{14}O_3^-$ was examined. Treatment of radish cotyledons with 2×10^{-5}M aminopterin for 3 1/4 hours did not appear to have any adverse effects on ability to incorporate the label into the ethanol soluble compounds (Table 13). Because of their possible involvement in one-carbon metabolism, the levels of C^{14} associated with individual amino acids were examined in detail (Table 14). Aminopterin had no affect on the percentage of C^{14} incorporated into individual amino acids. All of the radioactivity could be accounted for in the five amino acids shown, with 90% of it being concentrated in glycine and serine. Radioactivity was not detected in methionine in any of these experiments.

Since 6 hours of aminopterin treatment did not fully deplete the H_4PteGlu derivatives of excised radish cotyledons (Table 12), another

FIGURE 11

Co-chromatography of fractions 129-139 with PteGlu-2-C^{14}

Fractions 129-139 (65 mμg PteGlu activity) were pooled and co-chromatographed with 50 mμg of PteGlu-2-C^{14} (31.4 μc/μmole). Total C^{14} (Δ - Δ) and PteGlu equivalents as assayed with *L. casei* (● - ●) in each fraction are shown.

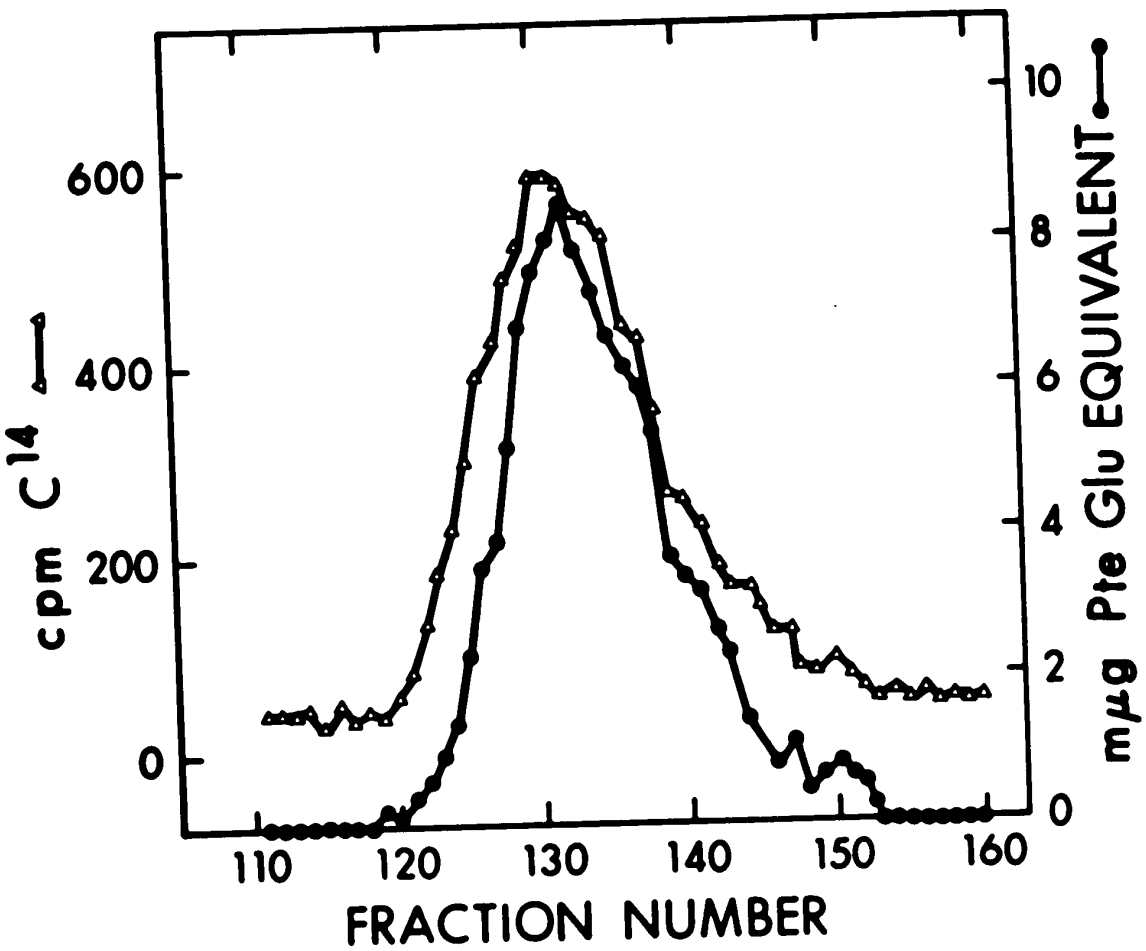

600
400
200
0
cpm C14
10
8
6
4
2
0
mμg Pte Glu EQUIVALENT
110
120
130
140
150
160
FRACTION NUMBER

TABLE 13. *The effect of short-term aminopterin treatment on the incorporation of $HC^{14}O_3^-$ into ethanol soluble compounds of radish cotyledons*

Sample No.	1	2	3	4
Fraction	+ AME	+ AME	Control	Control
Amino acids	736,100 (43.9)	563,600 (44.7)	561,400 (45.2)	491,800 (43.4)
Organic acids	481,500 (28.7)	375,000 (29.7)	355,000 (28.6)	347,400 (30.6)
Sugars	445,600 (26.6)	321,100 (25.5)	314,200 (25.3)	284,400 (25.0)
Ether solubles	13,300 (0.8)	12,550 (1.0)	10,425 (0.8)	12,320 (1.1)
Total C^{14} in fractions	1,676,500	1,272,250	1,241,025	1,135,920

Data are expressed in cpm incorporated in the experiment; numbers in brackets denote the percentage of the recovered radioactivity in each fraction.

Cotyledons of 6-day-old radish seedlings were excised and infiltrated with $2x10^{-5}$M aminopterin (+AME) or H_2O (control) and illuminated from below with mercury lamp (2000 ft. c.) for 3 1/4 hours. Samples of 10 disks (5 mm diameter) cut from these cotyledons were placed in Warburg flasks containing 0.5 ml of KH_2PO_4 buffer at pH 6.0. 0.2 ml of $NaHC^{14}O_3$ (10 µc/0.5 µmole/0.2 ml) was administered as described in the Materials and Methods. At the end of the 5 minute feeding period, the tissues were killed and fractionated.

TABLE 14. *Distribution of radioactivity in individual amino acids following $HC^{14}O_3^-$ fixation by radish cotyledons treated with aminopterin for 3 1/4 hours*

Amino acid \ Sample No.	1 + AME	2 + AME	3 Control
Aspartic acid	14,600 (2.1)	18,500 (3.5)	15,200 (3.0)
Serine	248,900 (36.5)	191,900 (36.4)	197,700 (38.7)
Glutamic acid	6,000 (0.9)	8,100 (1.5)	4,900 (0.9)
Glycine	390,600 (57.3)	284,400 (53.9)	275,200 (53.3)
Alanine	21,700 (3.2)	24,900 (4.7)	2,100 (4.1)
Total recovered after chromatography	681,800	527,800	495,100

Data are expressed in cpm incorporated by 10 tissue disks in the experiment. Bracketed numbers indicate the percentage of total C^{14} recovered after chromatography in each compound. Sample number 4 was lost.

Aliquots containing 5-10x10^4 cpm of the amino acid fraction obtained from disks of radish cotyledons which had been pre-treated with aminopterin (+AME) for 3 1/4 hours (Table 13) were chromatographed with carrier amino acids.

experiment with prolonged aminopterin treatment was undertaken in the hope that the formyl and unsubstituted tetrahydropteroylglutamates would be affected in addition to the methyl derivatives. The conditions of incubation were modified to include sucrose, KNO_3, and KH_2PO_4, which are known to promote DNA synthesis in excised radish cotyledons (Nieman and Poulsen, 1967). This would presumably increase losses of H_4PteGlu through the thymidylate synthetase reaction. Analysis of the various derivatives by column chromatography extended the earlier observations that levels of 5-CH_3-H_4PteGlu derivatives were most affected by aminopterin treatment (Table 15), while a large amount of PteGlu accumulated. Aminopterin-treated tissue extracts contained approximately 58% of the total pteroylglutamates in the control extracts. The pteroylglutamate levels were calculated to be 102 mμg and 192 mμg PteGlu equivalents per cotyledon of the aminopterin-treated and control samples, respectively.

As before, disks of the 24 hour treated tissues were examined for ability to carry out photosynthesis in $HC^{14}O_3^-$. To better assess the effect of aminopterin on photosynthesis, the $HC^{14}O_3^-$ was administered for periods of 45 seconds and 5 minutes, respectively. The data for both feeding periods (Table 16) show that the 24 hour aminopterin treatment decreased the total incorporation of radioactivity into the ethanol soluble fractions. However, in the 45 second feeding, the distribution of label among the fractions was not altered appreciably, while after 5 minutes a greater percentage of label accumulated in the sugar fraction of the aminopterin-treated samples, apparently at the expense of the amino acids.

Further analysis of the amino acid fractions showed that aminopterin decreased the incorporation of C^{14} into the amino acids (Table 17).

TABLE 15. *The effect of a 24-hour aminopterin treatment on the levels of pteroylglutamate derivatives in excised radish cotyledons*

Derivative by substituent group	Aminopterin		Control	
	Level*	%**	Level*	%**
Formyl derivatives	5730	34	7120	29
Methyl derivatives	390	2	13510	55
Unsubstituted derivatives †	1200	9	4100	17
PteGlu	7690	55	n.d.	
Total recovered from column	14010		24730	

* Data are expressed as mμg PteGlu equivalents/g d. wt. for *L. casei*.

** Denotes % of total recovered from the column.

† Excludes PteGlu. n.d. - not detected.

Excised 6-day-old radish cotyledons were infiltrated with 2×10^{-5}M aminopterin or water and incubated in nutrient medium as described in Materials and Methods. After lyophilization of the cotyledons, derivatives were extracted by Method I, treated with chicken pancreas γ-glutamyl carboxypeptidase and chromatographed. *L. casei* and *S. faecalis* were used to assay the fractions.

TABLE 16. *The effect of 24 hour aminopterin treatment on the incorporation of $HC^{14}O_3^-$ into ethanol soluble compounds of radish cotyledons*

Sample No.	45 sec in $HC^{14}O_3^-$				5 min in $HC^{14}O_3^-$			
Fraction	1 +AME	2 +AME	3 Control	4 Control	5 +AME	6 +AME	7 Control	8 Control
Amino acids	390,300 (48.7)	358,600 (46.4)	1,447,270 (49.8)	936,430 (48.6)	1,058,680 (35.1)	1,125,640 (38.6)	2,837,920 (51.6)	2,004,160 (48.9)
Organic acids	363,900 (45.3)	354,300 (45.9)	1,338,850 (46.0)	903,900 (46.9)	985,050 (32.7)	883,450 (30.3)	1,714,460 (31.2)	1,269,210 (31.0)
Sugars	45,700 (5.6)	56,400 (7.3)	111,650 (3.8)	83,290 (4.3)	949,410 (31.5)	886,680 (30.4)	911,520 (16.6)	808,230 (19.7)
Ether solubles	2,600 (0.3)	2,800 (0.4)	8,060 (0.3)	2,990 (0.2)	21,500 (0.7)	17,170 (0.6)	33,260 (0.6)	18,560 (0.5)
Total C^{14} in fractions	802,500	772,100	2,905,830	1,926,610	3,014,640	2,912,940	5,497,160	4,100,160

Data are expressed as cpm incorporated by the disks. The numbers in brackets are the percentages of the total C^{14} recovered in the fractions.

Cotyledons of 6-day-old radish seedlings were excised and infiltrated with $2x10^{-5}$M aminopterin (+AME) and incubated in the nutrient medium for 24 hours as described in the Materials and Methods. Disks (samples of 15 and 10 in the 45 sec and 5 min experiments, respectively) were placed on 0.5 ml of KH_2PO_4 buffer at pH 6.0. 0.2 ml of $NaC^{14}O_3^-$ (42.7 µc/µmole/0.2 ml) was administered by the standard procedure. At the end of the 45 sec and 5 min feeding periods, the tissues were killed and fractionated.

TABLE 17. *Distribution of radioactivity in individual amino acids following $HC^{14}O_3^-$ fixation by radish cotyledons pre-treated with aminopterin for 24 hours*

Sample No. / Amino acid	45 sec in $HC^{14}O_3^-$				5 min in $HC^{14}O_3^-$			
	1 +AME	2 +AME	3 Control	4 Control	5 +AME	6 +AME	7 Control	8 Control
Aspartic acid	164,300 (43.7)	115,500 (33.0)	343,100 (26.3)	242,400 (27.0)	127,700 (12.8)	100,200 (9.1)	200,100 (7.1)	217,100 (10.9)
Serine	48,900 (13.3)	62,300 (17.8)	264,800 (20.3)	177,100 (19.6)	491,500 (49.5)	550,500 (48.3)	1,220,400 (43.6)	877,800 (43.9)
Glutamic acid	15,100 (4.1)	12,500 (3.6)	16,500 (1.3)	12,100 (1.3)	34,500 (3.5)	35,800 (3.2)	80,900 (2.9)	74,900 (3.7)
Glycine	70,800 (19.3)	92,700 (26.5)	401,900 (30.8)	243,100 (27.1)	273,100 (27.5)	345,800 (31.6)	1,049,700 (37.5)	651,400 (32.6)
Alanine	68,000 (18.5)	66,900 (19.1)	276,800 (21.3)	224,000 (25.0)	67,000 (6.7)	87,800 (7.9)	247,300 (8.8)	177,300 (8.9)
Total C^{14} recovered after chromatography	367,100	349,900	1,303,100	898,700	993,800	1,120,100	2,798,400	1,998,500

Data are expressed in cpm incorporated by the tissue disks during the feeding period. Bracketed numbers indicate the percentage of total C^{14} recovered after chromatography in each compound.

Aliquots containing 5-15x10⁴ cpm of the amino acid fractions from the earlier experiments (Table 16) were chromatographed with carrier amino acids.

However, when compared to the total C^{14} incorporated into the amino acid fraction, the radioactivities in individual amino acids were not equally affected by aminopterin. For example, after 45 seconds of $HC^{14}O_3^-$ fixation by the aminopterin-treated tissue, the percentage of C^{14} in aspartic acid was higher than in the control but lower in glycine and serine. After 5 minutes of photosynthesis, serine and glycine contained approximately 48% and 29%, respectively, of the total C^{14} incorporated into the ethanol soluble fractions of aminopterin-treated tissue, as compared to approximately 43% and 34%, respectively, in the controls.

Since $HC^{14}O_3^-$ was readily incorporated into glycine and serine during photosynthesis (Tables 14 and 17) but not into the formyl and methyl groups of H_4PteGlu (Figure 8), it was of interest to determine the intramolecular distribution of C^{14} in these amino acids. Samples of glycine and serine, isolated after chromatography, were degraded to examine this as summarized in Table 18. In all of the degradations, approximately 90% of the initial radioactivity was recovered as $C^{14}O_2$. The results clearly show that after 5 minutes of photosynthesis both glycine carbons were essentially equally labelled, while the carboxyl carbon of serine contained 35% of C^{14} present in this amino acid. The intramolecular distribution of C^{14} in the amino acids from aminopterin-treated tissues was essentially like that of the control.

Degradations were also performed on the glycine and serine labelled after 45 seconds of photosynthesis. Random labelling of the glycine molecule was again observed and approximately one-third of the radioactivity in serine was present in the carboxyl position (Table 18).

TABLE 18. *The intramolecular distribution of C^{14} in glycine and serine after photosynthesis of $HC^{14}O_3^-$*

Amino acid		5 min of $HC^{14}O_3^-$ + AME † cpm C^{14}	5 min of $HC^{14}O_3^-$ + AME † % *	5 min of $HC^{14}O_3^-$ Control †† cpm C^{14}	5 min of $HC^{14}O_3^-$ Control †† % *	45 sec of $HC^{14}O_3^-$ Control ††† cpm C^{14}	45 sec of $HC^{14}O_3^-$ Control ††† % *
GLYCINE	COOH	1241	49.1	966	50.5	1870	48.2
	CH_2NH_2	1285	50.9	949	49.5	2011	51.8
	% C^{14} recovered**	*93.8*		*85.6*		*90.8*	
SERINE	COOH	693	34.1	676	35.3	1033	35.0
	$CHNH_2$	1328	65.9	1235	64.7	1754	65.0
	CH_2OH						
	% C^{14} recovered**	*86.2*		*91.8*		*91.8*	

† from sample No.1 of Table 14.
†† from sample No.3 of Table 14.
††† from sample No. 3 of Table 17.

* the percentage of recovered C^{14} in the indicated position.
** The percentage of C^{14} present before degradation that was recovered as CO_2.

The degraded amino acids were purified by means of an amino acid analyzer from radish cotyledon disks which had photosynthesized $HC^{14}O_3^-$ for 5 min or 45 sec. Prior to cutting the disks, the cotyledons had been treated with aminopterin (+AME) or water as described in Tables 14 and 17. The data are the average of two degradations.

DISCUSSION

The significance of the data presented in the Results will now be discussed in separate sections which deal with various aspects of the present study of pteroylglutamates in photosynthetic plant tissues.

The Extraction of Pteroylglutamates from Plant Tissues

The present studies have clearly demonstrated that the observed levels of pteroylmono- and polyglutamates in plant extracts were strongly dependent on the extraction technique used (Tables 3 and 4, Figure 4). An enzymic factor, or factors, was responsible for hydrolysis of polyglutamates to monoglutamates during extraction by Method II (Figure 2, Table 2). This technique had been recommended earlier (Bird *et al.*, 1965) for determining blood and liver pteroylglutamates in as near their naturally occurring states as practicable. Due to this hydrolysis, it was necessary to modify Method II in a number of ways. The procedure of heating the tissue before homogenization (Method I) was found to be the most satisfactory and preferred method of extraction of radish cotyledon pteroylglutamates for the following reasons.

(a) Only very low levels of mono- and diglutamate derivatives were present in extracts prepared by Method I. This indicated that hydrolytic activity was non-existent or minimal.

(b) Method I ensured that any other indiscriminate enzyme reactions which might transform pteroylglutamates from their naturally occurring states would also be minimal.

The importance of this latter precaution is in fact emphasized by later work of Bird *et al.* (1969) who demonstrated that a significant formylation of H_4PteGlu occurred during preparation of pteroylglutamate extracts from rat liver. As a consequence they recommended enzyme denaturation at 95°C in ascorbate before homogenization of the liver slices.

(c) The levels of total pteroylglutamates extracted by Method I were equal to those obtained with Method II (Table 3).

In further investigations with other plant species (Table 4) these general advantages of Method I were also apparent. In each case it was clear that extensive hydrolysis of conjugated derivatives occurred (Method II) unless precautions were taken to inactivate endogenous enzymes.

In other investigations (Iwai *et al.*, 1959; Santini *et al.*, 1964) such precautions were not taken in the extraction of leaf tissues; nevertheless high levels of pteroylpolyglutamates were found. As the buffers used in these investigations had pH values close to 7, it is likely (Appendix II) that the endogenous hydrolases were less active and conjugated derivatives remained in the final homogenate. The endogenous γ-glutamyl carboxypeptidase activity responsible for the hydrolysis of pteroylpolyglutamates in extraction Method II may be due to a number of enzymes. One of these could conceivably be a specific pteroylpolyglutamate hydrolase having importance in the interconversion of pteroylpolyglutamates *in vivo*.

The present investigations have also shown that lyophilization of

radish cotyledons before extraction did not affect the total level of pteroylglutamates (Table 3). The value of lyophilization as a technique in the study of pteroylglutamates in plants was readily apparent when experiments involving sampling of illuminated tissues were undertaken (Table 10). In such studies it was desirable to kill the tissue as quickly as possible as this work revealed that pteroylglutamate levels were markedly affected by light. Another advantage to lyophilization was that a large, relatively homogeneous, sample of tissue could be rapidly and conveniently preserved for later analysis. Furthermore, a number of other determinations, such as free amino acid levels and chlorophyll contents, could be made with such a tissue sample.

Derivatives of H_4PteGlu in Photosynthetic Plant Tissues

In agreement with earlier studies (Toepfer *et al.*, 1951; Iwai *et al.*, 1959; Santini *et al.*, 1964; Roos *et al.*, 1968; Rohringer *et al.*, 1969), the photosynthetic plant tissues used in the present work were found to contain readily detectable amounts of pteroylglutamates (Tables 3 and 4). Tissues from four different species were found to contain amounts ranging from approximately 14 µg per g d. wt. to 30 µg per g d. wt. for wheat leaves and radish cotyledons, respectively. It is interesting to note that the level of pteroylglutamates in spinach leaves (Table 1) exceeded the levels reported for this tissue by others. The main reasons for these discrepancies may be related to the use, in the present studies, of an improved extraction technique which employed ascorbate and heating at 95°C, ensuring protection of the labile derivatives. However, these differences could also be due to differences in the physiological condition of the tissue analyzed. It is also of interest

to note that the quantities of pteroylglutamates in the plants examined in the present investigations are comparable with those reported by others for yeast cells, housefly, wheat leaves and wheat rust spores extracted in the presence of ascorbate or dithiothreitol to protect labile derivatives (Table 19). In contrast, plant tissues are not as rich in pteroylglutamates as rat liver or *Physalia* (Table 19).

A detailed examination of individual pteroylglutamate derivatives present in plant tissues showed that they contain a complex pattern of derivatives. The derivatives differed from one another with respect to the one-carbon substituent group and the number of glutamic acid moieties (Table 5). The bulk of these derivatives were found to be conjugated when precautions were taken to prevent their hydrolysis during extraction. When the degree of conjugation was examined (Figure 5) it appeared to be variable and included both methyl and formyl derivatives. Variations in the number of glutamic acid residues of such polyglutamyl derivatives have also been observed in chicken liver extracts by Noronha and Silverman (1962).

The results of the present investigation also show that large amounts of the H_4PteGlu derivatives in plant extracts were substituted with formyl groups in the N^{10} and N^5 positions (Table 5). The presence of considerable amounts of formylated pteroylglutamates in extracts of green plant tissues is consistent with earlier work (Iwai *et al.*, 1959; Santini *et al.*, 1964). Formyl derivatives have also been found in extracts of dormant and germinating seeds by others (Rosso, 1958; Roos *et al.*, 1968). All of the plant extracts assayed in this investigation (Table 5) also contained significant quantities of methyl derivatives. As these compounds have been detected in animal tissues (Noronha and

TABLE 19. *Quantities of pteroylglutamates in different tissues.*

Source	Quantity of pteroylglutamate† µg/g f. wt.	µg/g d. wt.	Reference
Rat liver	21.35	--	Bird *et al.*, 1969
Physalia	40	--	Wittenberg *et al.*, 1962
Yeast	--	30.0	Schertel *et al.*, 1965
Housefly*	--	29.1	Miller, 1969
Wheat rust spores	--	24.5	Jackson *et al.*, 1970
Pea cotyledons (6-day-old)	--	3.9	Shah *et al.*, 1970
Wheat leaves	1.66	--	Rohringer *et al.*, 1969
Wheat leaves	1.76	15.8	Present work
Pea leaves	1.99	14.4	Present work
Spinach leaves	3.59	28.1	Present work
Radish cotyledons	3.50	32.2	Present work

† *L. casei* assay, after peptidase treatment.

* Acetone powder preparation extracted with dithiothreitol as protective agent.

Silverman, 1962; Silverman *et al.*, 1961; Bird *et al.*, 1965) mammalian blood (Bird *et al.*, 1965) wheat leaves (Rohringer *et al.*, 1969) pea seedlings (Roos *et al.*, 1968), and yeast cells (Schertel *et al.*, 1965), their occurrence is no doubt ubiquitous. Unsubstituted H_4PteGlu derivatives were also detected in the extracts, usually as very minor constituents of the pteroylglutamate pool (Table 5). It is noted, however, that the level of these derivatives appeared to be dependent upon the illumination of the tissue immediately prior to its being killed (Tables 10, 12 and 15).

The presence of these differently substituted derivatives in extracts of plant tissues is consistent with the involvement of pteroylglutamate coenzymes in the one-carbon metabolism of plants *in vivo* as outlined in the Introduction. The absence of 5,10-CH=H_4PteGlu and 5,10-CH_2=H_4PteGlu derivatives does not preclude their occurrence *in vivo*, since in aqueous extracts they would be found as 10-HCO-H_4PteGlu and H_4PteGlu derivatives respectively. In this connection, the incorporation of formate-C^{14} into methyl derivatives (Figure 10) may indicate that these two unstable derivatives were intermediates in the formation of the methyl derivatives.

The Possible Importance of Conjugated Pteroylglutamates in the Metabolism of Higher Plants

The present results show that the pteroylglutamate derivatives present in plant tissues were highly conjugated (Tables 3, 4 and 5; Figures 4 and 5). It appears that these derivatives contain more than three glutamic acid residues as only low levels of mono-, di-, and triglutamate derivatives were found. The predominance of conjugated

derivatives and the finding that these were affected by aminopterin treatment (Tables 12 and 15) and light (Table 10) suggest that conjugated derivatives are of physiological importance.

Many reactions mediated by pteroylglutamates may involve the conjugated coenzyme forms. In support of this it is known that the rate of methionine formation by 5-CH_3-H_4PteGlu:homocysteine methyltransferase preparations from plant leaves is 5-10 times greater when 5-CH_3-H_4PteGlu$_3$ replaces 5-CH_3-H_4PteGlu as methyl donor (Burton and Sakami, 1969). There may be other enzymes in plants which preferentially bind conjugated pteroylglutamates as has been demonstrated in microorganisms. Rabinowitz and associates, in their study of 10-HCO-H_4PteGlu synthetase and 5,10-CH_2=H_4PteGlu reductase from *Clostridium* species, found the K_m's for the triglutamyl derivatives to be 10- and 3-fold lower respectively than that for the corresponding monoglutamates (Himes and Rabinowitz, 1962; Uyeda and Rabinowitz, 1967). Also, the conversion of serine to glycine by *Clostridium* extracts occurred more rapidly with pteroylpolyglutamate coenzymes (Wright, 1956). Rabbit liver preparations readily utilized H_4PteGlu$_3$ as coenzyme in the formation of serine from glycine and formaldehyde (Blakley, 1957). It is interesting to note that the 5-CH_3-H_4PteGlu:homocysteine methyltransferases of *N. crassa* and *S. cerevisiae* appear to be specific for conjugated methyl derivatives (Burton *et al.*, 1969).

Despite these findings regarding pteroylpolyglutamates, one must cognize that nearly all the plant enzymes dependent on pteroylglutamate coenzymes have been studied almost exclusively with monoglutamate derivatives (for references see Introduction). Thus conjugation is not an absolute or general requirement *in vitro*. Clearly, further

investigations are necessary to elucidate the significance of conjugation in the functioning of pteroylpolyglutamates *in vivo*.

Selected Aspects of the Metabolism of Pteroylglutamates in Radish Cotyledons

(a) *Pteroylglutamates in etiolated and green tissues.*

The rapid increase in the pteroylglutamate content of radish cotyledons accompanying germination (Figure 6) suggests that these derivatives are important in the metabolism of this tissue. Green cotyledons had a much higher level of these compounds than etiolated tissues (Figure 6). In view of their known catalytic role in one-carbon metabolism such a result would be expected, as germinated tissues have a much more intense and complex metabolism than ungerminated tissue. Consistent with the interpretation of a correlation between pteroylglutamate content and metabolic intensity are the lower levels of pteroylglutamates in senescing cotyledons (Figure 6). Other studies (Shah *et al.*, 1970; Okinaka and Iwai, 1970) have demonstrated that pea cotyledons also contained much larger quantities of pteroylglutamates after a germination period of 4 to 8 days. Green wheat leaves also contained higher levels of pteroylglutamates than their etiolated counterparts (Table 6).

Illumination of etiolated radish cotyledons for 24 hours increased the pteroylglutamate content by approximately 20% (Table 7). These results again support the concept that more of these compounds occur in tissues with a more intense and complex metabolism. In this particular instance, part of this increase may be related to an involvement of pteroylglutamates in the biosynthesis of chlorophyll (Figure 7) as well as other aspects of chloroplast metabolism (Shah and Cossins, 1970a, b). Earlier reports support the conclusion regarding pteroylglutamates and

chlorophyll biosynthesis in pea seedlings (Schopfer and Grob, 1954) and *Chlorella* (Scheffrahn, 1966). On the other hand, the higher levels in green tissues may reflect a special role of pteroylglutamates in the autotrophic metabolism of these tissues as discussed below.

(b) *The function of pteroylglutamates in photosynthetic metabolism.*

The present work illustrates that the pteroylglutamate derivatives which occur in illuminated radish cotyledons are markedly different from those found in darkened tissues (Table 10). An inverse relationship apparently exists between the levels of the formyl and unsubstituted derivatives in the extracts of tissues obtained from these different physiological conditions. Thus it appeared that after illumination of the cotyledons, a large part of the pteroylglutamates originally with a one-carbon group at the formyl level of oxidation now occurred as H_4PteGlu and 5,10-CH_2-H_4PteGlu derivatives. Two proposals can be advanced to account for these light-induced changes. Firstly, the high levels of H_4PteGlu found may be involved in the light dependent flow of carbon through the glycolate pathway. In this connection the present studies (Table 18) suggest that reactions of this pathway are instrumental in the synthesis of the glycine and serine, as judged by the random labelling of these amino acids after only a very short period of photosynthesis of $HC^{14}O_3^-$ (Rabson *et al.*, 1962; Hess and Tolbert, 1966; Bruin *et al.*, 1970). Although the 2 and 3 carbons of serine were not analyzed separately, the data argues for serine formation via the glycolate pathway. Recent studies have indicated that the glycine decarboxylation reaction of the glycolate pathway can proceed at a rate greater than the serine hydroxymethyltransferase reaction *in vitro* (Kisaki *et al.*, 1971b). If this reflects the situation *in vivo*,

5,10-CH_2=H_4PteGlu derivatives would be expected to accumulate, resulting in high levels of unsubstituted H_4PteGlu derivatives in the aqueous extracts. In order to account for the changes in the levels of formyl derivatives (Table 10) in this proposal, it is further suggested that in darkness these derivatives represent a storage pool of coenzyme which, after a rapid reduction of the one-carbon group by $NADPH_2$ (Scheme 2), can enter the glycolate pathway at the point of serine synthesis. These derivatives would be the small unlabelled one-carbon pool which Hess and Tolbert (1966) have postulated to account for the lower than expected levels of C^{14} in the serine-3 position after 4 seconds of $C^{14}O_2$ photosynthesis by tobacco leaves. Alternatively, the coenzyme could enter the pathway at the point of glycine decarboxylation after transfer of the formyl group in other synthetic reactions.

Secondly, the higher levels of serine found in illuminated radish cotyledons (Table 11) may also be responsible for the high levels of H_4PteGlu$_n$ being found in extracts of light-harvested tissue. As the serine hydroxymethyltransferase reaction is a readily reversible reaction, the higher levels of serine would increase the tendency of reaction to proceed in the reverse direction (*i.e.* to glycine formation), trapping relatively large amounts of H_4PteGlu$_n$ as 5,10-CH_2=H_4PteGlu$_n$. This hypothesis is, however, less plausible than the first because it necessitates that the oxidation of the one-carbon groups of 5,10-CH_2=H_4PteGlu$_n$ be restricted in the light.

Other mechanisms are also possible either instead of, or in addition to, the two already suggested. In recent years, considerable evidence has accumulated which indicates that the activity of certain enzymes may be regulated by light (Hatch and Slack, 1970). Perhaps pteroylglutamate

dependent enzymes are among those subject to this type of regulation. It may also be speculated that 5,10-CH≡H_4PteGlu$_n$ and 5,10-CH_2=H_4PteGlu$_n$ are functional in light-driven electron transport with each molecule rapidly alternating between the two forms as it picks up electrons, possibly from photochemically generated $NADPH_2$, and transfers them to an as yet unknown acceptor.

Clearly, it would be desirable to elucidate the effects of light on the constituents of the pteroylglutamate pool with further experiments using radish cotyledons. The changes in the levels of the pteroylglutamates in response to varying periods of light and darkness is one aspect which requires more detailed examination. A number of experiments involving inhibitors also come to mind. For example, experiments with DCMU (Miflin *et al.*, 1966) could confirm the light dependent nature of the pteroylglutamate pattern found in the present results (Table 10). Inhibition of the glycolate pathway by α-hydroxysulfonates (Zelitch, 1965) could conceivably prevent the accumulation of H_4PteGlu related to operation of the glycolate pathway but not that affected by levels of serine, light-activated enzymes or electron transport.

The effect of light on the levels of individual pteroylglutamate derivatives should also be examined in other plant tissues. In such investigations, comparisons might be made of tissues which differ in their ability to photorespire. As the CO_2 produced in photorespiration is believed to be derived from the glycine decarboxylase reaction (Kisaki *et al.*, 1971a, b), a correlation between the rates of photorespiration and levels of unsubstituted H_4PteGlu derivatives might be found.

The photosynthesis of $HC^{14}O_3^-$ did not result in labelling of the

formylated and methylated pteroylglutamate derivatives (Figure 8). Without a reasonably direct flow of photosynthetically fixed $HC^{14}O_3^-$ into these derivatives, a considerable dilution of specific radioactivity could be expected. Low specific activities of an immediate precursor of the one-carbon group of the derivatives would make it difficult to measure the levels of C^{14} associated with the latter. As the glycolate pathway is highly compartmented (Tolbert, 1971), it could provide a reasonably direct route for the entrance of $HC^{14}O_3^-$ into the pteroylglutamate bound one-carbon pool via 5,10-$C^{14}H_2$=H_4PteGlu$_n$. Unfortunately, this derivative dissociates into formaldehyde-C^{14} and H_4PteGlu$_n$; therefore, it is impossible to assess the amount of radioactivity associated with it. It is likely that compartmentation causes the one-carbon groups to be preferentially used in serine biosynthesis and hinders the entry of the derivative into the compartment in which methylated forms predominate (see below). After glycine-2-C^{14} feeding, the pteroylglutamates were also not labelled. Theoretically, glycine-2-C^{14}, as a substrate for glycine decarboxylase, would be a good precursor of 5,10-$C^{14}H_2$=H_4PteGlu$_n$. But again, compartmentation could restrict the labelling of the formylated and methylated pteroylglutamate derivatives even if high specific activity glycine-2-C^{14} would enter the decarboxylase reaction, as this reaction appears to be strictly localized in the mitochondria (Kisaki *et al.*, 1971b). However, as levels of free glycine were high in illuminated radish tissues (Table 11), the dilution of the glycine-2-C^{14} must also have been considerable.

Formate-C^{14} was rapidly incorporated into formyl and methyl pteroylglutamate derivatives (Figure 10). As the feeding period was only 10 minutes, it is likely that the substituent groups, rather than the rest

of the molecule, contained the C^{14}. In the light of the results with the $HC^{14}O_3^-$ feeding (Figure 8), it can be concluded that the formate was not oxidized to CO_2 by formate dehydrogenase (Cossins and Sinha, 1965) and refixed. 10-HCO-H_4PteGlu synthetase was probably the enzyme which initially metabolized the formate-C^{14}. This enzyme, at present, has not been demonstrated to occur in radish cotyledons but there is no reason to doubt its occurrence as it has been found in a wide variety of plant species and tissues (Hiatt, 1965; Iwai, 1967a, b). Although both non-photosynthetic and photosynthetic plant tissues readily metabolize formate (Cossins and Sinha, 1965), the physiological importance of free formate in plant one-carbon metabolism has not been ascertained to date.

(c) *The effect of aminopterin on pteroylglutamate metabolism and photosynthesis.*

The present investigation has also shown that the treatment of excised radish cotyledons with aminopterin decreased their levels of tetrahydropteroylglutamate derivatives and caused PteGlu to accumulate (Tables 8, 12 and 15). Levels of methyl derivatives and PteGlu were the most markedly affected and showed an inverse relationship. The highly selective effect of aminopterin on methyl derivatives implies that the pteroylglutamate metabolism of these tissues is to some extent compartmented. In this respect it appears that the bulk of the methylated derivatives occupy a pool distinct from that shared by the major part of the formyl and unsubstituted derivatives. Of particular interest here are studies regarding the compartmentation of pteroylglutamates in rat liver (Wang *et al.*, 1967) and pea cotyledons (Clandinin and Cossins, 1971) which have revealed that mitochondria contained mostly formyl derivatives while the bulk of the methyl derivatives occur in the

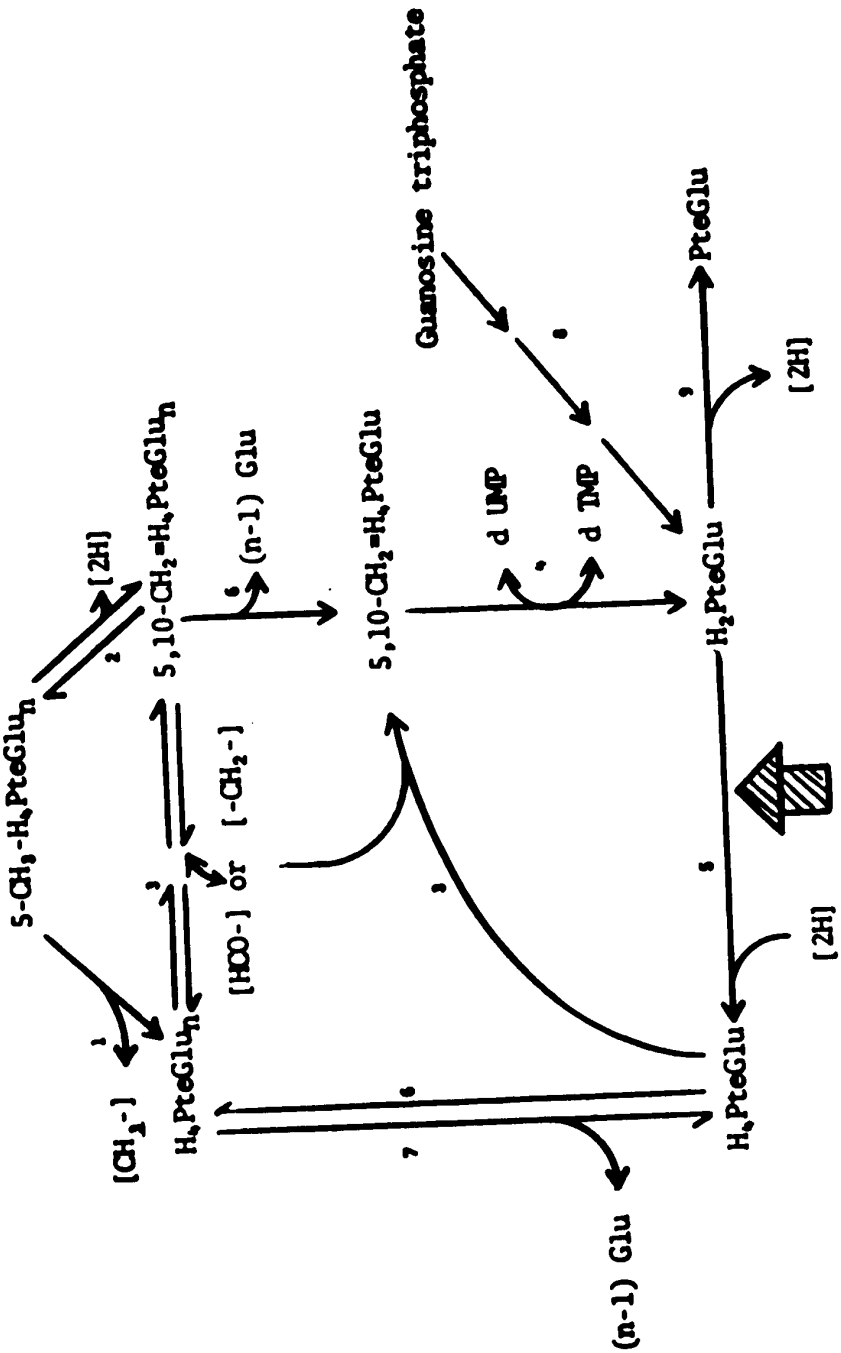
cytoplasm. Perhaps radish cotyledons also have a concentration of formyl derivatives in the mitochondria. The concept of a large pool of formyl derivatives in the mitochondria would link well with their postulated role in the reactions of the glycolate pathway and logically explain why levels of methyl derivatives were hardly affected by light. (Table 10). As nearly all the methyl derivatives were depleted within 6 hours of aminopterin treatment (Table 12) it is reasonable to assume that those of the chloroplastic pool (Shah *et al.*, 1970; Shah and Cossins, 1970a) were also drastically reduced. Thus it appears that the chloroplastic pool of pteroylglutamates is more susceptible to aminopterin then the pool containing the bulk of the formyl derivatives.

The rapid rate of loss of methyl derivatives suggests that the turnover of methyl groups, themselves, must be high. This is to be expected as autotrophic tissues are probably highly dependent on the pteroylglutamate coenzymes for methyl groups. Theoretically, a high rate of turnover could be assessed by rapid incorporation of exogenous 5-$C^{14}H_3$-H_4PteGlu into metabolic products. However, in practice, studies of this type by others have been inconclusive as neither wheat leaves (Rohringer *et al.*, 1969) nor pea cotyledons (Roos and Cossins, 1971) appeared to extensively metabolize the methyl group of 5-$C^{14}H_3$-H_4PteGlu.

The PteGlu occurring in extracts of aminopterin-treated tissues could have originally been the skeleton of the methyl pteroylpolyglutamates or it could have formed *de novo* from guanosine triphosphate before pteroylglutamate deficiency prevented the formation of this precursor of the pteridine nucleus (Blakley, 1969). The reactions are outlined in Scheme 4. Considering the inverse relationship between levels of PteGlu and methyl derivatives (Tables 8, 12, and 15) and the rapid rate of accumulation of PteGlu (Table 12), it is proposed that

SCHEME 4. THE POSSIBLE MECHANISMS FOR THE FORMATION OF PTEGLU IN AMINOPTERIN-TREATED RADISH COTYLEDONS

Reaction No.	Description of Reaction(s)
1	5-CH_3-H_4PteGlu:homocysteine methyltransferase
2	5,10-CH_2=H_4PteGlu reductase
3	See Scheme 2 (on page 12) for details
4	Thymidylate synthetase
5	H_2PteGlu reductase
6	Pteroylpolyglutamate hydrolase
7	Pteroylpolyglutamate synthetase
8	A series of 6 reactions involved in the *de novo* synthesis of H_2PteGlu from GTP
9	Enzymatic or non-enzymatic oxidation reaction


5-CH3-H4PteGlun
[CH2-]
H4PteGlun
[HCO-] or [-CH2-]
5,10-CH2=H4PteGlun
[2H]
(n-1) Glu
5,10-CH2=H4PteGlu
d UMP
d TMP
Guanosine triphosphate
H2PteGlu
PteGlu
[2H]
[2H]
H4PteGlu
(n-1) Glu
Inhibition by aminopterin

SCHEME 4

PteGlu is formed mainly from the tetrahydropteroylglutamate pool which is involved in one-carbon transfer, methyl group formation, etc. (Scheme 4, reactions 1, 2, and 3). The pool becomes depleted of reduced coenzyme because of the thymidylate synthetase reaction (reaction 4) and the blockage of H_2PteGlu reductase by aminopterin (Blakley, 1969). Then H_2PteGlu is oxidized enzymically or non-enzymically (reaction 9) to account for PteGlu in the extracts. Nieman and Poulsen (1967) have presented evidence that DNA synthesis commences within 6 hours after radish cotyledons have been excised and incubated under appropriate conditions; thus one can argue that in the present investigations an active formation of the DNA precursor thymidylate was actively being formed before that time.

It is interesting to note that PteGlu, and not $PteGlu_n$, accumulated. This is interpreted as meaning that the thymidylate synthetase has a specific requirement for the monoglutamate, making it necessary to postulate pteroylglutamate hydrolase reactions (reaction 6) in Scheme 4. It is not likely that $H_2PteGlu_n$ would be hydrolyzed while the bulk of the other derivatives remain as polyglutamates.

The quantities of PteGlu formed *de novo* may also have contributed significantly to the total levels of PteGlu assayed. The reason for arriving at this conclusion is as follows.

(1) It is reasonable to assume that *de novo* synthesis of H_2PteGlu ceases 6-12 hours after the initiation of aminopterin treatment because the pteroylglutamate pool, active in the general one-carbon metabolism of the cell, was essentially depleted in 6 hours, preventing the biosynthesis of purines. On the basis of this and

the difference in total levels between aminopterin-treated and control tissues (Table 15) the loss of pteroylglutamate skeletons is estimated to be 0.6-0.8 μg/g d. wt. per hour.

(2) It is also known that levels of total pteroylglutamates in 6- and 7-day-old radish cotyledons were steady (Figure 6), meaning rates of synthesis and degradation of pteroylglutamate skeletons must be equal in tissues of this age. Thus the rate of synthesis *de novo* must also be 0.6-0.8 μg/g d. wt. per hour in normal tissue, which is up to 40% of the rate of PteGlu accumulation in the aminopterin-treated tissues (Table 12).

In this connection, it would be of considerable interest to obtain more precise information of the turnover of pteroylglutamate skeletons, which could possibly be assessed by a study of the effect of sulfonamides on their levels. Such information would allow a better estimation of the rate of conversion of methyl derivatives to PteGlu, and might cast additional light on the relationship between functionally compartmented pools of pteroylglutamates. It is intriguing to speculate that a rapid rate of turnover may be part of the regulatory mechanism controlling levels of pteroylglutamates in plant tissues which in turn could affect other synthetic pathways by occurring in rate-limiting quantities.

The study of the effects of aminopterin on photosynthesis was conducted primarily to identify reactions of photosynthetic metabolism which are dependent on tetrahydropteroylglutamates. Aminopterin was found to affect photosynthesis of cotyledons treated with this analogue for a 24 hour period (Tables 16 and 17). In contrast tissues which had

been treated for only 3¼ hours were not inhibited in this respect (Tables 13 and 14). After a 24 hour aminopterin treatment, illuminated radish cotyledons had impaired ability to fix $HC^{14}O_3^-$ into the ethanol soluble compounds, especially those present in the organic acid and amino acid fractions. It is important to consider how these results might be related to the levels of individual pteroylglutamate derivatives present after 24 hours of aminopterin treatment.

Theoretically, the reduced levels of formyl and unsubstituted derivatives may have limited the rate of glycine-serine conversion and consequently the flow of C^{14} through the glycolate pathway. This does not, however, appear to be the case. In the 45 second $HC^{14}O_3^-$ feeding experiments, the ratios of labelled glycine and serine in the aminopterin-treated cotyledons were not significantly different from those of the controls, while in the 5 minute experiments the ratios for the treated tissues were actually lower than those of the controls. Since the methyl derivatives did not appear to be important intermediates in the fixation or subsequent metabolism of $HC^{14}O_3^-$ (Figure 8), it is doubtful that the depletion of these derivatives was directly responsible for the different labelling pattern observed. On the other hand, the loss of methyl derivatives, which no doubt affects ability of the inhibited tissues to generate methyl groups, may have had far reaching consequences on the photosynthetic, as well as the general, metabolism of these tissues. For example, in a complex process such as photosynthesis there are conceivably enzymes which have a short half-life. In normal tissues these are constantly synthesized so the rate of the reactions which they catalyze do not become rate-limiting as a result of low enzyme concentrations. Since an absence of methyl groups will probably result in

shortages of both methionine and t-RNA's which are dependent on methylation for activity (Yarus, 1969), it is likely that protein synthesis is restricted in aminopterin-treated tissue and consequently deficiencies of enzymes with a short half-life will ensue. As purine biosynthesis depends on pteroylglutamate coenzymes, a pteroylglutamate deficiency could also result in a depletion of NAD, NADP, and ADP and specific RNA's, which in turn could limit the rate of $HC^{14}O_3^-$ fixation as well as play a regulatory role in the flow of C^{14} into various products. As no detailed examinations were made of the secondary effects due to tetrahydropteroylglutamate deficiency, it is only possible to give general explanations without singling out the critical secondary effect, if one exists. More detailed research is necessary to elucidate the effects of pteroylglutamate deficiency on the incorporation of $HC^{14}O_3^-$ in radish cotyledons.

CONCLUSIONS

On the basis of the present work, the following major conclusions can be drawn regarding the occurrence of pteroylglutamate derivatives in photosynthetic plant tissues and their metabolism in radish cotyledons.

(1) All the photosynthetic plant tissues examined contained readily detectable levels of pteroylglutamate derivatives. They were recovered as highly conjugated formylated, methylated, and unsubstituted derivatives in aqueous extracts.

(2) Levels of individual derivatives recovered in extracts of radish were dependent on the physiological state of the tissue.

(3) The methylated derivatives were in a rapid state of turnover and appear to be compartmented from the bulk of the formylated and unsubstituted derivatives.

(4) There was evidence for a rapid formation of glycine and serine via the reactions of the glycolate pathway. The higher levels of $H_4PteGlu_n$ found in illuminated cotyledons may have been due to their involvement in this pathway.

(5) Evidence which might have indicated that one-carbon groups for general one-carbon metabolism arise directly from the glycolate pathway or its intermediates could not be obtained.

Unfortunately, the lack of detailed experimental evidence makes many of the statements regarding the function of pteroylglutamates in

the metabolism of radish cotyledons speculative. A number of experiments which may confirm some of these possibilities have been suggested.

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APPENDIX I

MICROBIOLOGICAL ASSAY - MATERIALS AND METHODS

Microbiological assay was used extensively for the assay of pteroylglutamates in plant tissue extracts. The composition of the media for *Lactobacillus casei*, *Streptococcus faecalis*, and *Pediococcus cerevisiae* was based on publications of Freed (1966), Jukes (1955), and Bakerman (1961) respectively. These publications were also gleaned for general methodology of microbiological assay. The medium for *P. cerevisiae* had some minor modifications which improved the reproducibility of the growth response of this microorganism to *d,l*-5-HCO-H_4PteGlu. The modifications were a decrease in acid casein, an increase in sodium citrate and potassium phosphate and the use of Mg-, Fe-, $MnSO_4$ solution and $MnSO_4$ solution instead of solids. To avoid preparing and storing two vitamin stock solutions, no special vitamin solution as recommended by Bakerman (1961) was made for *P. cerevisiae*. The only vitamin in this solution not common to that which was already used for *L. casei* and *S. faecalis* was pteroylglutamate, which, therefore, was added separately to *P. cerevisiae* media. The compositions of the media are given in Table 20.

After mixing the reagents, the pH was adjusted with NaOH. Toluene was removed prior to the addition of Tween 80 to prevent uncontrollable frothing. Aliquots (5 ml) of basal assay media were dispensed in 20x150 mm culture tubes in covered metal racks and autoclaved for 8 min at 121°C.

The stock solutions prepared as follows were made according to Freed (1966) or Hansen (1965) and stored under toluene in a refrigerator.

Stock solution 1. Acid casein. Acid casein solution was made

TABLE 20. *Composition of media used in microbiological assay.*

	L. casei	*S. faecalis*	*P. cerevisiae*
Stock solutions (ml):			
1. Acid casein	100	100	100
2. Enzymatic casein	--	--	96
3. *l*-Tryptophan	20	20	40
4. Adenine-Guanine-Uracil	50	50	100
5. Xanthine	100	100	100
6. Asparagine	60	60	60
7. Vitamins	100	100	100
8. Mg-, Fe-, Mn-, SO_4	10	10	50
9. $MnSO_4$	20	20	20
10. Pteroylglutamic acid	--	--	10
Tween 80	--	--	0.5
Dry Reagents* (g):			
Cysteine-HCl	0.5	0.5	0.5
Glucose (Dextrose)	40	40	40
Sodium acetate	40	--	20
Sodium citrate·H_2O	--	40	30
KH_2PO_4	1	--	4
K_2HPO_4	1	6.2	4
Glutathione (reduced)	0.005	0.005	0.005

* Dry reagents were dissolved in 250 ml H_2O and then added to the bulk of the medium. Final volume of media was made to 1 liter.

according to Freed (1966), but the pH was not adjusted back to 7 because precipitation of amino acids would occur with certain batches of casein. 100 g of acid hydrolyzed casein was dissolved in 700 ml H_2O. The pH was adjusted to 3.5 and 20 g of Norit A were added. After 20 minutes, this was filtered and the filtrate was made to 1 *l*. A second charcoal treatment was necessary in some cases to remove all compounds with pteroylglutamate activity.

Stock solution 2. Enzymatic casein. This solution was made by dissolving 25 g of the enzymatically hydrolyzed casein in 800 ml of H_2O with adjustment of pH to 3.5. The solution was stirred with 10 g Norit A for 20 minutes and filtered. The filtrate was made to 1 *l*.

Stock solution 3. *l*-Tryptophan. (Hansen, 1965). 5 g *l*-tryptophan were suspended in 50 ml 1 M HCl, heated till dissolved, and diluted to 500 ml.

Stock solution 4. Adenine-Guanine-Uracil. (Hansen, 1965). 0.2 g of each nucleotide was dissolved in 10 ml 6M HCl, and diluted to 1 *l*.

Stock solution 5. Xanthine. (Hansen, 1965). 0.2 g xanthine was suspended in 8 ml concentrated ammonium hydroxide, heated until dissolved and diluted to 1 *l*.

Stock solution 6. Asparagine. (Hansen, 1965). 5 g of *l*-asparagine $\cdot H_2O$ was dissolved with heating in 500 ml H_2O.

Stock solution 7. Vitamins. (Freed, 1966). The following were dissolved in approximately 300 ml H_2O: 10 mg *p*-aminobenzoic acid, 40 mg pyridoxine HCl, 4 mg thiamine HCl, 8 mg calcium pantothenate, 8 mg niacin and 0.2 mg biotin. Then 10 mg riboflavin was dissolved separately in 200 ml H_2O with the aid of 0.25 ml concentrated acetic acid. Finally, a buffer containing the following was prepared: 1.9 g sodium acetate, 1.6 ml

concentrated acetic acid and 40 ml water. The three solutions were added together and made to 1 *l*.

Stock solution 8. Mg-, Fe-, Mn- SO_4 solution. (Freed, 1966). The following were dissolved in 40 ml H_2O with the aid of 10 drops concentrated HCl, and made to 500 ml: 10 g $MgSO_4 \cdot 7H_2O$, 0.5 g $FeSO_4 \cdot 5H_2O$, and 0.5 g $MnSO_4 \cdot H_2O$.

Stock solution 9. $MnSO_4$ solution. 2 g $MnSO_4 \cdot H_2O$ were dissolved and diluted to 200 ml.

Stock solution 10. Pteroylglutamate solution. This solution was the same as used for the standard curve for *L. casei* and was 2 mμg/ml.

The bacteria were maintained by monthly transfer to fresh agar slants made according to the method of Freed (1966). 20 Tubes with approximately 10 ml agar were made from: 5 g Difco Bacto yeast extract, 1 g dextrose, 1 g sodium acetate, 3 g agar, and 200 ml water. The mixture was heated with stirring to dissolve the agar, dispensed into tubes which were plugged with cotton and sterilized 20 minutes at 121°C.

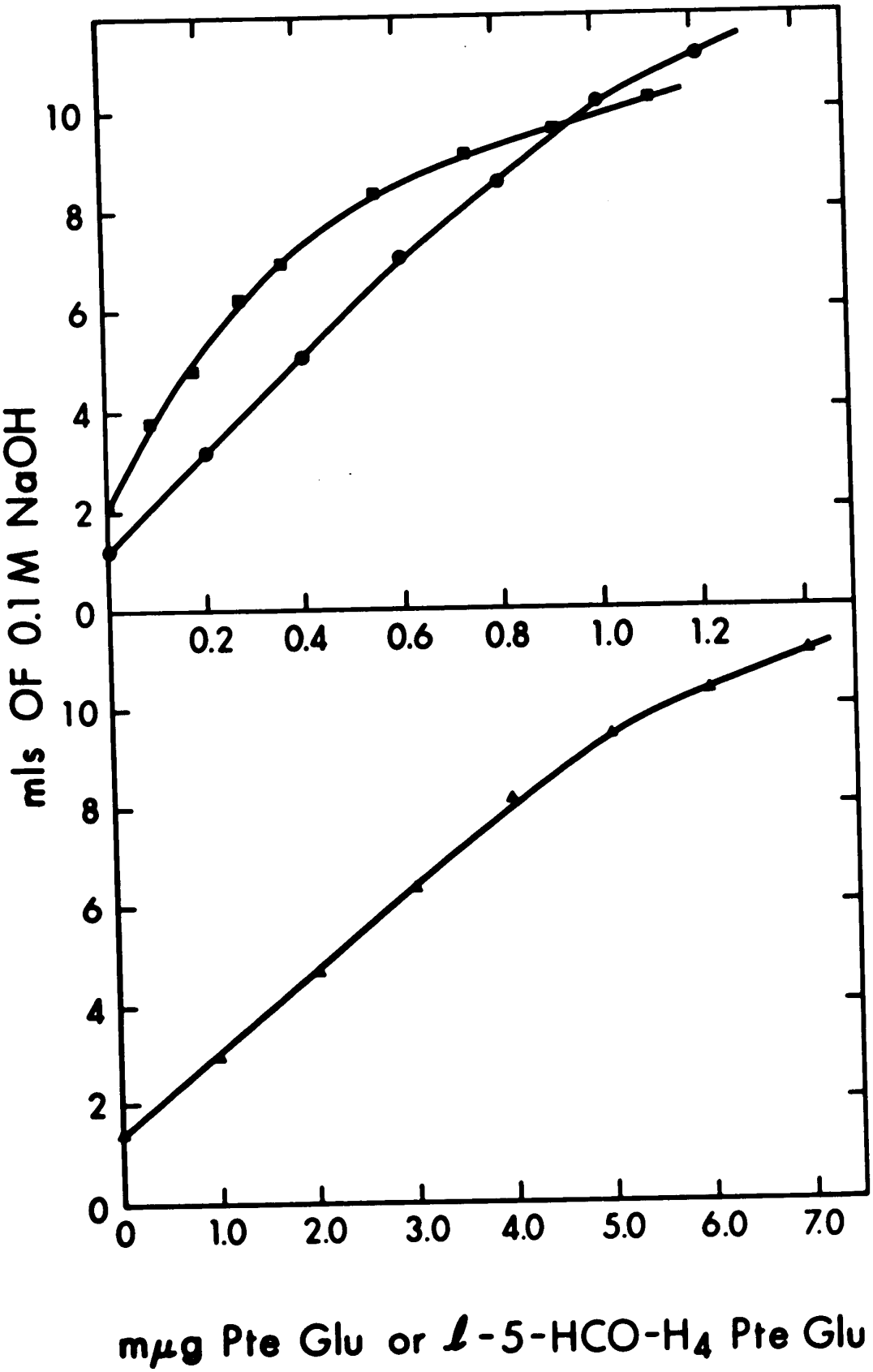
Bacterial inoculum broth had the following composition (Freed, 1966): 5 g Difco peptone, 1 g Difco yeast extract, 10 g dextrose, 10 g sodium acetate, 5 ml K-phosphate solution (25 g KH_2PO_4 and 25 g K_2HPO_4 in 500 ml) and 5 ml Mg-, Fe-, Mn- SO_4 solution. The broth was adjusted to pH 6.8 and made to 500 ml. 7 ml of broth were dispensed in 15 x 120 mm test tubes and sterilized 15 minutes at 121°C.

Growth response to standardized quantities of synthetic PteGlu or *d,l*-HCO-H_4PteGlu was routinely measured with all assays of unknowns. When titer value was plotted against the amount of synthetic PteGlu or 5-HCO-H_4PteGlu, a linear plot was obtained for *L. casei* and *S. faecalis*; a smooth curve was obtained for *P. cerevisiae*. Figure 12 shows typical curves.

FIGURE 12

Growth response of L. casei, S. faecalis, *and* P. cerevisiae *to PteGlu and l-5-HCO-H₄PteGlu*

The growth response of *L. casei* (● - ●) and *S. faecalis* (▲ - ▲) to standardized authentic PteGlu, and of *P. cerevisiae* (■ - ■) to *l*-5-HCO-H_4PteGlu, was measured by back-titration of the lactic acid produced during 70 hours of growth.


mls OF 0.1 M NaOH
0
2
4
6
8
10
0.2
0.4
0.6
0.8
1.0
1.2
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
mμg Pte Glu or l-5-HCO-H4 Pte Glu

APPENDIX II

The Effect of pH on Radish Cotyledon and Pea Cotyledon γ-glutamyl Carboxypeptidase Activity

Cell-free extracts were prepared from the cotyledons of 6-day-old radish seedlings (grown as described in Materials and Methods) and peas germinated in vermiculite for 3 days at 22°C. Tissue samples (10 g) were homogenized in 100 ml of citrate-phosphate buffer (pH 5.5). This buffer contained 42 mmoles of citric acid and 108 mmoles of K_2HPO_4 per liter. The homogenates were centrifuged at 20,000 x g for 10 minutes to remove the cellular debris. The supernatants were used for assay of γ-glutamyl carboxypeptidase activity.

Radish cotyledon γ-glutamyl carboxypeptidase activity was assayed by incubation of 0.25 ml of the cell-free preparation (0.93 mg protein), 100 mg yeast extract (1.25 µg PteGlu equivalents after chicken pancreas γ-glutamyl carboxypeptidase treatment) and 5 ml of citrate-phosphate buffer in a final volume of 10 ml. Variation in pH of the incubation mixture was obtained by varying the composition of the citrate-phosphate buffer, *i.e.* differing volumes of 0.2 M citric acid and 0.4 M K_2HPO_4 were used (Gomori, 1957). After an incubation period of 40 minutes at 37°C, the reaction was terminated by boiling for 10 minutes.

The pea cotyledon γ-glutamyl carboxypeptidase activity was assayed by incubation of 0.25 ml of the cell free preparation (0.6 mg protein), 300 mg yeast extract (3.7 µg PteGlu equivalents) and 5 ml of citrate-phosphate buffer in a final volume of 10 ml. After an incubation period of 60 minutes at 37°C, the reaction was terminated by boiling for 10

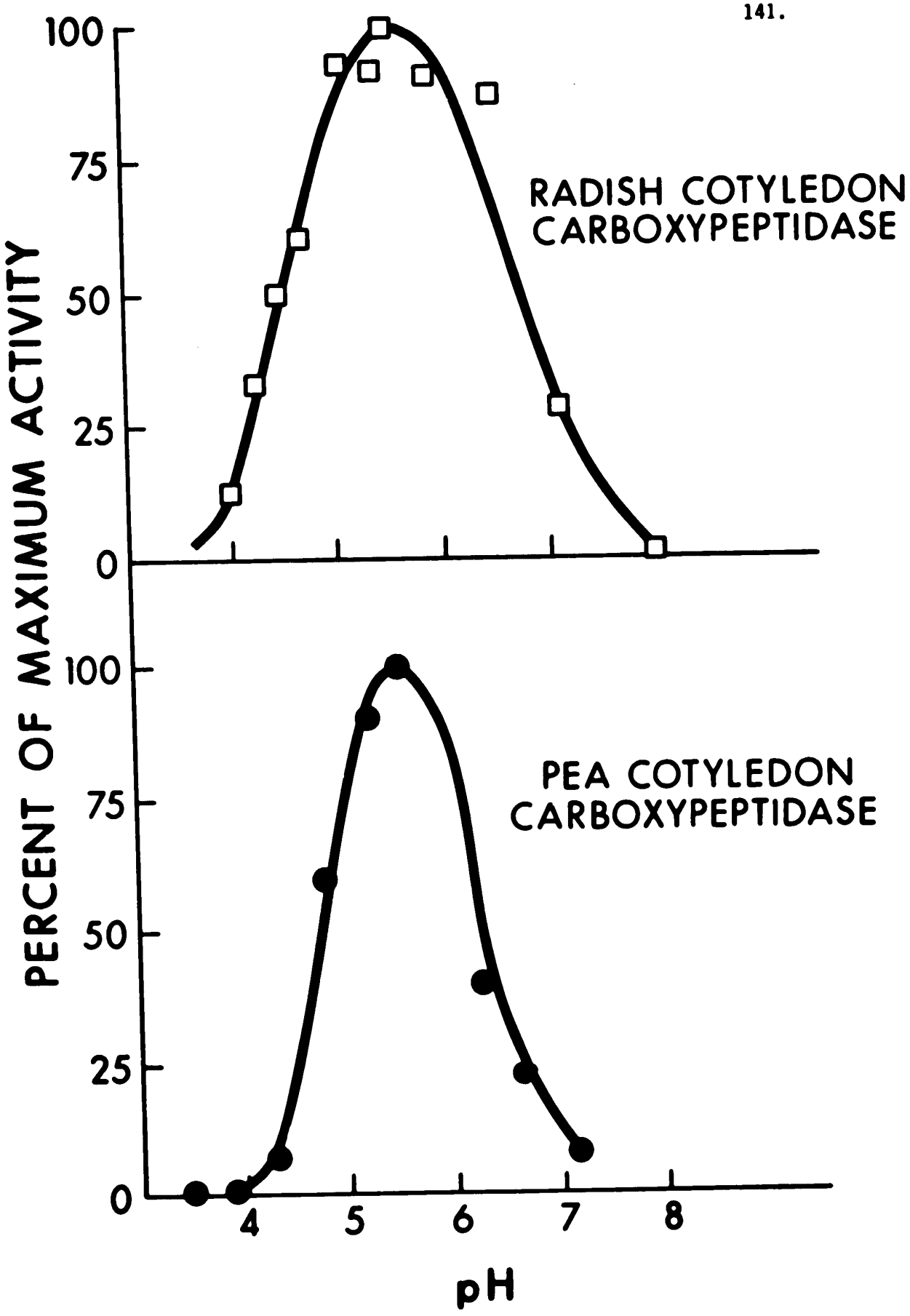
minutes.

Hydrolytic activities of the radish and pea cotyledon preparations were measured by the increase in growth response of *L. casei* and *S. faecalis* respectively. Net activity was calculated by subtraction of PteGlu equivalents determined for reaction systems which included boiled enzyme. The results of these experiments are illustrated in Figure 13.

FIGURE 13

The effect of pH on radish cotyledon and pea cotyledon γ-glutamyl carboxypeptidase activity

The assay of radish and pea cotyledon γ-glutamyl carboxypeptidase activity is described in the text. Data are reported as percentages of the activity measured at pH 5.5. The final pH was measured after termination of the reaction. At the completion of the incubation, the radish and pea cotyledon preparations incubated at pH 5.5 had hydrolyzed 35% and 55% of the added yeast extract pteroylpolyglutamates respectively.


100
75
50
25
0
RADISH COTYLEDON
CARBOXYPEPTIDASE
PERCENT OF MAXIMUM ACTIVITY
100
75
50
25
0
PEA COTYLEDON
CARBOXYPEPTIDASE
4
5
6
7
8
pH