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THE UNIVERSITY OF ALBERTA

THE SYNTHESIS OF PTEROYLGLUTAMATES IN GERMINATING PEA COTYLEDONS

by

© ALFRED JOHAN ROOS

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE

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UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies for acceptance, a thesis entitled 'THE SYNTHESIS OF PTEROYL-GLUTAMATES IN GERMINATING PEA COTYLEDONS' submitted by ALFRED JOHAN ROOS in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

Roland Kohvinge
External Examiner

Edwin A. Gastmans

Brad Shred

Paul R. Gaston

Date. Feb. 23. 1971.

ABSTRACT

The synthesis of pteroylglutamates in pea seeds has been examined over a seven day germination period. It was observed that a rapid synthesis occurred 40-90 hours after the seeds started to imbibe water.

The constituents of the pteroylglutamate pool present in the cotyledons were subjected to DEAE-cellulose column chromatography. Identification of individual derivatives was based upon differential microbiological response and co-chromatography with authentic derivatives before and after treatment with γ-glutamyl carboxypeptidases from various sources. The three assay bacteria employed in this study were *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043) and *Pediococcus cerevisiae* (ATCC 8081). The data show that the major constituents of the pteroylglutamate pool were 10-formyltetrahydropteroylglutamate, 5-formyltetrahydropteroylglutamate and 5-methyltetrahydropteroylglutamate, together with smaller quantities of their glutamyl conjugated forms.

Synthesis of these derivatives was inhibited by use of pteroylglutamic acid antagonists such as aminopterin and amethopterin. These compounds also drastically inhibited germination of the seeds and resulted in the appearance of pteroylglutamic acid which was not detected in the absence of these antagonists.

Feeding experiments employing pteroylglutamic acid-2-C^{14}

and 5-[Methyl-C^{14}]tetrahydropteroylglutamic acid indicated that these derivatives were readily utilized in the synthesis of other pteroylglutamates during germination. In contrast, the only pteroylglutamate containing C^{14} following administration of formate-C^{14} was 10-formyltetrahydropteroylglutamate.

Despite the rapid synthesis of pteroylglutamates during germination, the levels of 5,10-methylenetetrahydrofolate dehydrogenase remained relatively uniform during the first 7 days of germination. This enzyme, in common with the corresponding dehydrogenases from other sources, was shown to have a requirement for NADP, formaldehyde and tetrahydropteroylglutamic acid and was stable only when isolated in the presence of ethylenediamine tetraacetate and 2-mercaptoethanol. The pteroylglutamate product of the reaction was chromatographed on DEAE-cellulose and found to be 10-formyltetrahydropteroylglutamate.

In experiments with ammonium sulphate fractionated extracts, ability to synthesize the principal endogenous derivatives was determined in reaction systems in which formaldehyde, formate and serine served as 1-carbon donors. In all cases, formyltetrahydropteroylglutamic acid derivatives were formed. This synthesis displayed requirements for the 1-carbon donors. In addition, 5-methyltetrahydropteroylglutamic acid was also produced in these experiments. This synthesis required the presence of a reduced pyridine nucleotide but did not display a requirement for the 1-carbon donor. The results are interpreted as indicating that 5,10-

methyltetrahydropteroylglutamic acid, formed from the donors, was oxidized and reduced in these reaction systems. The significance of these derivatives in the 1-carbon metabolism of these germinating tissues is discussed.

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LIST OF ABBREVIATIONS

The abbreviations used are those commonly found in biochemical literature. Pteroylglutamate derivatives are designated by the abbreviations suggested by the IUPAC-IUB Commission as listed in the Biochemical Journal 102 (1967) 15, as follows:

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-methylenetetrahydropteroyl-glutamic acid
5,10-CH≡H_4PteGlu	:	5,10-methenyltetrahydropteroyl-glutamic acid
5-HCNH-H_4PteGlu	:	5-formiminotetrahydropteroyl-glutamic acid
$PteGlu_2$	:	Pteroyldiglutamic acid
$PteGlu_3$	:	Pteroyltriglutamic acid
P-P	:	Pyridoxal-5'-phosphate
EDTA	:	Ethylenediaminetetraacetic acid
POPOP	:	1,5-*bis*-[2-(4-methyl-5-phenoxazolyl)]-benzene
PPO	:	2,5-diphenyloxazole

*This abbreviation is used in the figures to designate pteroylglutamate derivatives in general and is, therefore, synonymous to the term 'folate' used in the early literature.

INTRODUCTION

EARLY STUDIES OF PTEROYLGLUTAMATE DERIVATIVES

Pteroylglutamic acid, more commonly known as folic acid, was first discovered as an antianemia agent in animals (Wills *et al.*, 1931; Hogan and Parrott, 1939) and as a growth factor for various microorganisms (Snell and Peterson, 1940; Mitchell *et al.*, 1941; Stokstad, 1943; Keresztesy *et al.*, 1943). Because of the many natural forms in which this vitamin exists, considerable confusion arose in the literature regarding its possible identity. By the 1940's, it was realized that a variety of names: vitamin M (Day *et al.*, 1938), *L. casei* factor (Day *et al.*, 1945), vitamin U (Stokstad and Manning, 1938), norite eluate factor (Snell and Peterson, 1940) and vitamin B_c (Hogan and Parrott, 1940), had been applied to designate this closely related group of compounds.

Folic acid was the name given to a substance present in the leaves of spinach (Mitchell *et al.*, 1941) which stimulated the growth of *Streptococcus faecalis* R. and *Lactobacillus casei*. Mitchell and co-workers (Mitchell *et al.*, 1944; Frieden *et al.*, 1944; Mitchell and Williams, 1944; Mitchell, 1944) then described some of the chemical and physiological properties of PteGlu together with methods for its concentration from spinach.

On the basis of microbiological assay five compounds essential for the growth of *L. casei* and *S. faecalis* R.

were isolated from various tissues (Stokstad, 1943; Pfiffner *et al.*, 1943; Keresztesy *et al.*, 1943; Day *et al.*, 1945; Hutchings *et al.*, 1944; Pfiffner *et al.*, 1945; Totter *et al.*, 1944). By 1948, the chemical composition of PteGlu had been determined by Stokstad and his associates (Stokstad *et al.*, 1948; Mowat *et al.*, 1948).

TECHNIQUES FOR PETEROYLGLUTAMATE ASSAY

a) Microbiological assay

Three microorganisms have been commonly employed for pteroylglutamate assays, namely *Lactobacillus casei*, *Streptococcus faecalis* and *Pediococcus cerevisiae*. Landy and Dicken (1942) proposed the use of *L. casei* for the microbiological assay of PteGlu. Their media contained all of the factors known to stimulate *L. casei* with the exception of PteGlu. Growth obtained on addition of tissue extracts was taken as a measure of PteGlu contents. Interference by other compounds were shown by several workers (Stokstad, 1941; Stokes, 1944; Snell, 1944; Hutchings and Peterson, 1943) and modifications of the media were subsequently made (Luckey *et al.*, 1944; Teply and Elvehjem, 1945).

S. faecalis media were successfully used for the microbiological determination of PteGlu after the effect of compounds upon the media were studied (Stokes, 1944; Snell and Mitchell, 1941) and necessary modifications had been made (Luckey *et al.*, 1944). Additional modifications

were made by Teply and Elvehjem (1945) permitting its use in titrimetric procedures.

The development of a medium for *P. cerevisiae* and its use as a tool for PteGlu assay progressed much slower than the studies of *L. casei* and *S. faecalis* media (Sauberlich and Baumann, 1948). Originally known as *Leuconostoc citrovorum*, its identity was finally established as a typical strain of *P. cerevisiae* (Felton and Niven, 1953). This organism was shown to grow on PteGlu but only when supplied in microgram quantities. However, it was also shown that 5-HCO-H_4PteGlu could support growth when supplied in millimicrogram quantities. Ability to utilize 5-HCO-H_4PteGlu rather than PteGlu for growth is probably due to inability to assimilate physiological concentrations of PteGlu (Wood and Hitchings, 1960).

b) Chromatographic separation

Prior to the availability of TEAE or DEAE cellulose, pteroylglutamates were commonly isolated and assayed by a combination of paper chromatography and a bioautographic technique (Winsten and Eigen, 1950; Wieland *et al.*, 1952). With subsequent use of substituted celluloses more satisfactory fractionations of pteroylglutamate derivatives were achieved (Silverman *et al.*, 1961; Bird *et al.*, 1965; Noronha and Silverman, 1962; Noronha and Aboobaker, 1963; Schertel *et al.*, 1965). One of the main obstacles encountered in such work was the susceptibility of the tetrahydro forms to atmospheric oxygen. Silverman *et al.* (1961)

outlined a procedure where pteroylglutamates were eluted from DEAE-cellulose by a gradient of phosphate buffer in the presence of ascorbate. Ascorbate was included in the buffer to protect labile derivatives from oxidation. Silverman's technique has been subsequently modified by Sotobayashi *et al.* (1966). This latter technique allows a more uniform mixing of the buffer in a mixing chamber, compensates for differences in temperature and density of the solutions and gives a much superior separation of naturally occurring pteroylglutamates.

NATURAL OCCURRENCE OF PTEROYLGLUTAMATE DERIVATIVES

With development of the techniques for microbiological assay and column chromatography considerable knowledge was gained regarding the distribution of pteroylglutamates in various organisms. To obtain information regarding total pteroylglutamate content, it has become common practice to allow extracts to undergo autolysis or to treat them with a γ-glutamyl carboxypeptidase from chicken pancreas (Mims and Laskowski, 1945) or hog kidney (Bird *et al.*, 1946; Doctor and Couch, 1953). Autolysis, attributed to an endogenous γ-glutamyl carboxypeptidase, like the enzymes from chicken pancreas and hog kidney, released the derivatives from polyglutamates thereby increasing the bacterial assay. It has been clearly shown that many natural materials contain such an enzyme which will release pteroylglutamates at appropriate pH values and temperatures (Bird *et al.*, 1945; Mims *et al.*, 1944; Laskowski *et al.*, 1945; Simpson and Schweigert, 1949).

a) Animal tissues, Microorganisms, and Foods

The majority of investigations on the distribution of pteroylglutamate derivatives have concentrated on animal tissues with emphasis on total contents (Frank *et al.*, 1963; Bird *et al.*, 1965; Iwai *et al.*, 1964; Grossowicz *et al.*, 1963; Cropper and Scott, 1966; Noronha and Aboobaker, 1963; Matoth *et al.*, 1965). In some instances, total pteroylglutamate contents were not determined after carboxypeptidase treatment and in others precautions were not taken to protect the derivatives from oxidation. In most tissues, the principal derivative present was 5-CH_3-H_4PteGlu (Cropper and Scott, 1966; Noronha and Aboobaker, 1963) with lesser amounts of 5- and 10-HCO-H_4PteGlu present mainly as polyglutamates. Polyglutamate derivatives are also present in various microorganisms (Schertel *et al.*, 1965; Allfrey and King, 1950; Wright, 1955, 1956; Hakala and Welch, 1957; Sirotnak *et al.*, 1963). The isolation of *p*-aminobenzoylglutamic acid containing 10-11 glutamic acid residues suggests the occurrence of these very highly conjugated forms in yeast (Ratner *et al.*, 1946).

In foods of dietary importance, the pteroylglutamate content before and after conjugase treatment has been reported by several workers (Herbert, 1963; Santini *et al.*, 1962; Butterworth *et al.*, 1963). From various clinical studies it has been established that approximately 50-100 µg of PteGlu are adequate for the daily requirements of man (Sullivan and Herbert, 1964; Fleming *et al.*, 1963; Sullivan

et al., 1966) an amount readily supplied by the average North American diet.

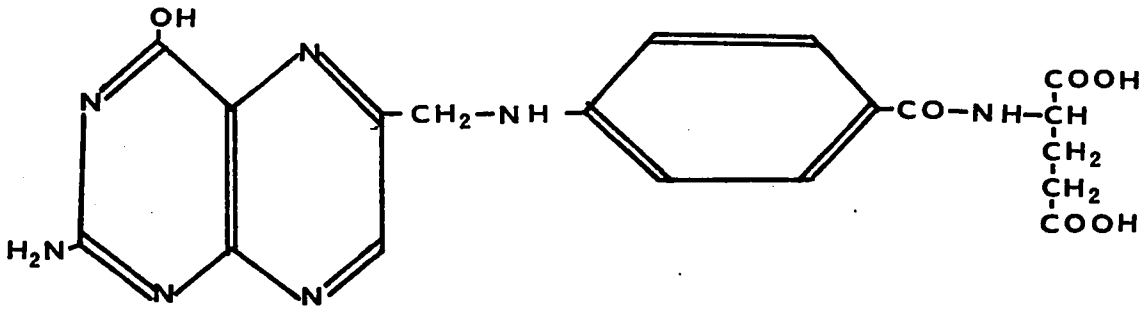
b) Plants

Relatively few detailed investigations of the endogenous levels of PteGlu derivatives in plants have been reported. Iwai and his associates (Iwai and Nakagawa, 1958; 1958a; Iwai *et al.*, 1959) have investigated PteGlu derivatives in green leaves of a number of species. Unfortunately, the microbiological determinations employed only *S. faecalis* and *P. cerevisiae*, consequently no information was obtained concerning the concentration of 5-CH_3-H_4PteGlu. They demonstrated however that most of the assayed derivatives existed as labile reduced forms which were readily converted to 5-HCO-H_4PteGlu on heating under argon in the presence of ascorbate at alkaline pH (Iwai *et al.*, 1959). 10-HCO-H_4PteGlu therefore was probably the principal derivative detected in these assays. They also demonstrated that these reduced formyl derivatives were present, in a large part, as glutamate-containing peptides. Although 10-HCO-H_4PteGlu was later recognized as being present in pea cotyledons, evidence based on differential microbiological assays and co-chromatography showed that 5-CH_3-H_4PteGlu was the major pteroylglutamate present (Roos *et al.*, 1968). This derivative is also present in other higher plant species (Rohringer *et al.*, 1969; Shah *et al.*, 1970). Rohringer *et al.* found an increase in 5-CH_3-H_4PteGlu in wheat leaves after infection with rust.

On the basis of these reports it appears that 5-CH_3-H_4PteGlu and its conjugates may be the principal components of the pteroylglutamate pool of higher plant tissues.

CHEMICAL AND PHYSICAL PROPERTIES OF PTEGLU DERIVATIVES

PteGlu consists of a pteridine linked to *p*-aminobenzoic and L-glutamic acids as shown by the following structural formula:



The names, pteroic acid and pteroylglutamic acid, were suggested by two groups of workers (Angier *et al.* and Cosulich *et al.*, 1946) because of the presence of pteridine and glutamic acid moieties in the molecule. PteGlu can be cleaved in acid or alkaline solutions at elevated temperatures yielding *p*-aminobenzoylglutamic acid and either pterin-6-carboxylic acid (Stokstad *et al.*, 1948) or pterin-6-carboxaldehyde (Waller *et al.*, 1950; Blair, 1957; Roberts, 1961). In addition to halogenation of the benzene ring (Cosulich *et al.*, 1951; 1953; Angier and Curran, 1959; Loo *et al.*, 1965), it will undergo other reactions (Cosulich and Smith, 1949; Angier *et al.*, 1952; Gordon *et al.*, 1948). A change in PteGlu by untraviolet light was first reported by Bloom *et al.* (1944) and photodecomposition of PteGlu

solution by sunlight was reported by Stokstad *et al.* (1947). The decomposition products are *p*-aminobenzoylglutamic acid and pterin-6-carboxaldehyde (Lowry *et al.*, 1949; Raven and Waldmann, 1950).

PteGlu has a limited solubility in water (10 mg/litre at 0°C, 500 mg/litre at 100°C) and very low solubility in most organic solvents. At pH 7.0, it has absorption peaks at 282 and 346 mμ (Rabinowitz, 1960) which readily changes upon reduction to dihydro and tetrahydro forms of the vitamin (Jaenicke and Kulzbach, 1963).

An important chemical feature of PteGlu is the reducibility of the pyrazine portion of the pteridine ring. The catalytic reduction of PteGlu to form either the dihydro or the tetrahydro derivative was first demonstrated by O'Dell *et al.* (1947). H_2PteGlu can be prepared in 0.1 N NaOH in the presence of a catalyst prepared from palladium or platinum oxide (O'Dell *et al.*, 1947) but excess hydrogen uptake occurs, suggesting that considerable amounts of H_4PteGlu are also formed in this reaction (Blakley, 1957; Miller and Waelsch, 1957). A purer sample of H_2PteGlu can be obtained when PteGlu is reduced by dithionite ($Na_2S_2O_4$) (Futterman, 1957). The product can be isolated by precipitation at low pH in the presence of ascorbate (Futterman, 1957; Futterman, 1963) or mercaptoethanol (Friedkin *et al.*, 1962).

In glacial acetic acid (O'Dell *et al.*, 1947) or in neutral aqueous solutions (May *et al.*, 1951; Blakley, 1957)

catalytic hydrogenation of PteGlu to form H_4PteGlu occurs, platinum oxide being an effective catalyst (O'Dell *et al.*, 1947). Reduction of PteGlu by borohydride yields H_4PteGlu together with smaller amount of H_2PteGlu, the proportion of the latter depending upon the reaction conditions, particularly the borohydride concentration and the temperature employed (Hillcoat and Blakley, 1964; Scrimgeour and Vitols, 1966). Reduction with $Na_2S_2O_4$ at 75°C giving a 68% yield after a 90 minute reaction period, has also been described (Silverman and Noronha, 1961). These procedures are, however, time consuming, and impure material is obtained. A recent communication (Davis, 1969) describes a new technique for H_4PteGlu synthesis. It involves the use of $Na_2S_2O_4$ and PteGlu in a solid state reaction, absorption of the H_4PteGlu into glacial acetic acid and precipitation with ether containing mercaptoethanol. The purity of the compound was found to be 90% based on the quantitative conversion of H_4PteGlu to 5,10-CH≡H_4PteGlu by the method of Zakrzewski (1966), and the yield on a weight basis was 95% of the theoretical. Because of the asymetric centre at the 6 position, chemically prepared H_4PteGlu consists of 2 diastereoisomers, the 1,L-H_4PteGlu is active in enzymatic reactions (Mathews and Huennekens, 1960) and for microbiological assay (Rabinowitz, 1960).

Derivatives of PteGlu act as coenzymes in many metabolic reactions. These PteGlu coenzymes are primarily concerned with the transfer of one-carbon units

at the oxidation levels of formate, formaldehyde and methanol and in transforming these from one oxidation state to another. These derivatives may be present as such or in conjugated form with various numbers of glutamic acid residues attached in γ-peptide linkage.

The first metabolically active PteGlu derivative to be recognized, due to its very stable nature, was 5-HCO-H_4PteGlu (Sauberlich and Baumann, 1948; Broquist *et al.*, 1949). 10-HCO-H_4PteGlu, like H_4PteGlu, was not recognized until measures were taken to prevent atmospheric oxidation, and it was found that either ascorbate (Bakerman, 1961) or mercaptoethanol (Blakley, 1960) may be used to protect these oxygen labile derivatives.

5-CH_3-H_4PteGlu can be prepared by borohydride reduction of 5,10-CH_2=H_4PteGlu (Sakami and Ukstins, 1961; Keresztesy and Donaldson, 1961; Gupta and Huennekens, 1967). It was first isolated from fresh liver (Donaldson and Keresztesy, 1959) as a substance which did not support the growth of *S. faecalis* or *P. cerevisiae* and which was designated as prefolic A. 5,10-CH_2=H_4PteGlu, however, was recognized as a product of the enzymatic reaction of serine with H_4PteGlu (Sharadchandra *et al.*, 1955; Jaenicke, 1956) and it is also formed by the non-enzymatic reaction of H_4PteGlu and formaldehyde (Kisliuk, 1957; Blakley, 1957; 1959; 1960). It can be purified by column chromatography at pH 9.5 because of its higher stability than at lower pH values.

5-HCNH-H_4PteGlu was discovered during investigations

on the biological degradation of purines (Rabinowitz and Pricer, 1956) and of histidine in animals (Raven and Jaenicke, 1953; Tabor and Rabinowitz, 1956; Miller and Waelsch, 1956) and in microorganisms (Tabor and Mehler, 1954; Magasanik and Bowser, 1955; Wachsman and Barker, 1955). The half life of the compound in solution over the pH range 5-9 is 1 hour at 37°C, and less than 1 minute at 100°C.

ENZYMATIC CONVERSION OF PTEROYLGLUTAMIC ACID TO TETRAHYDRO-PTEROYLGLUTAMIC ACID.

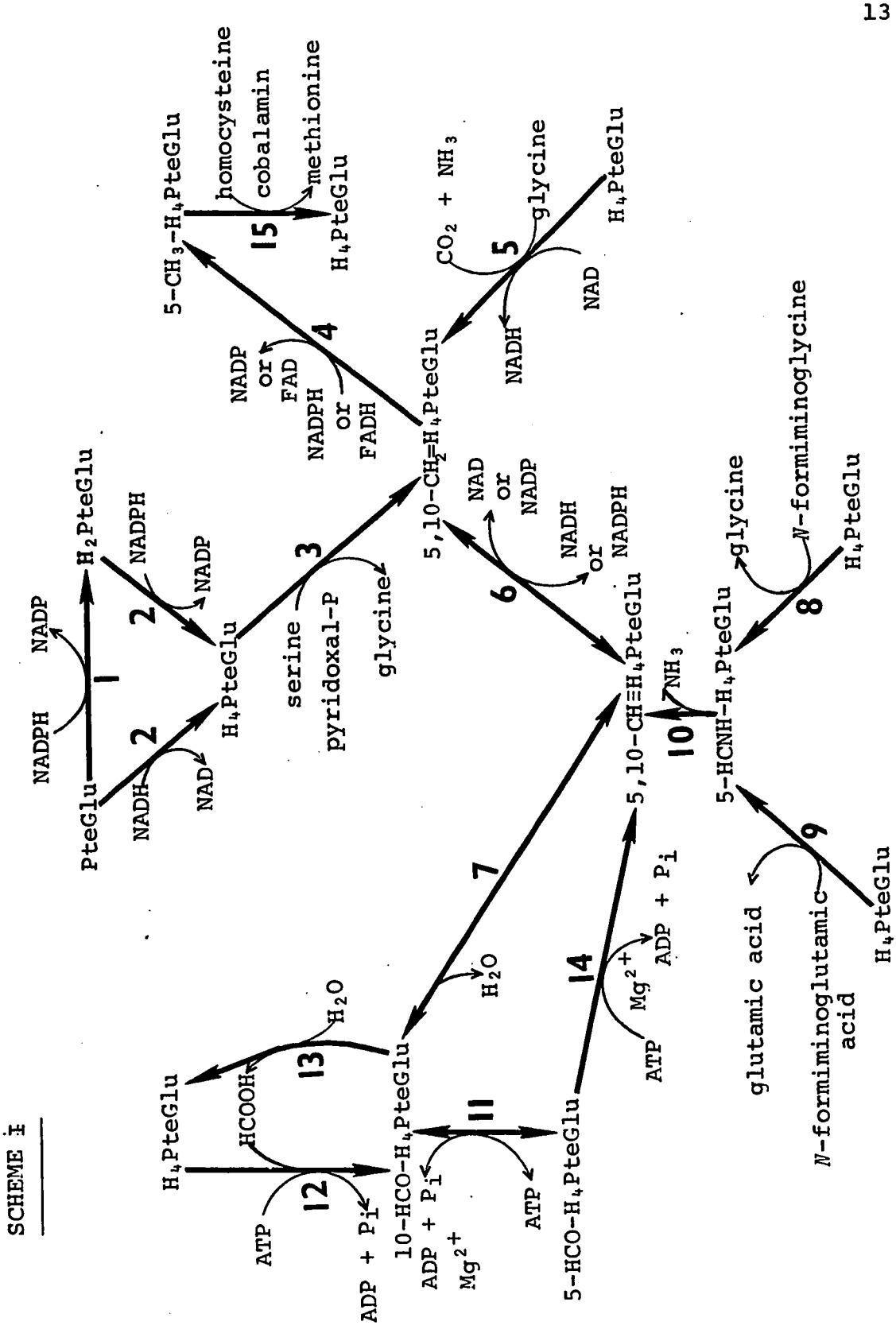
The one-carbon units of these metabolically active derivatives can be oxidized or reduced through enzyme mediated reactions. PteGlu is enzymatically reduced to H_2PteGlu by dihydrofolate dehydrogenase (Scheme 1, reaction 1). This enzyme has been found in *Clostridium sticklandii* (Bertino *et al.*, 1963), is NADH independent and is coupled to the conversion of pyruvate to carbon dioxide and acetyl CoA with the concurrent formation of H_2PteGlu. It is further distinguished from tetrahydrofolate dehydrogenase (Scheme 1, reaction 2) in not being inhibited by aminopterin.

Tetrahydrofolate dehydrogenase has been the subject of extensive investigations, because of its involvement in the synthesis of H_4PteGlu and thymidylate (Wahbe and Friedkin, 1961). The enzyme is distributed in various vertebrate tissues (Kenkare and Braganca, 1963; Bertino *et al.*, 1964; Grignani *et al.*, 1967; Bertino *et al.*, 1960; 1963) and microorganisms (Burchall and Hutchings, 1965; Sirotnak

SCHEME I. MAJOR REACTIONS INVOLVING PTEROYLGLUTAMATE DERIVATIVES

REACTION NUMBER	TRIVIAL NAME	SYSTEMATIC NAME	E.C. NO.
1	Dihydrofolate dehydrogenase	7,8-dihydrofolate:NADP oxido-reductase	1.5.1.4
2	Tetrahydrofolate dehydrogenase	5,6,7,8-tetrahydrofolate: NADP oxidoreductase	1.5.1.3
3	Serine hydroxymethyl-transferase	L-serine:tetrahydrofolate 5,10-hydroxymethyl-transferase	2.1.2.1
4	5,10-methylenetetrahydro-folate reductase	5-methylenetetrahydrofolate: NAD oxidoreductase	1.1.1.68
5	Glycine "Splitting Enzyme"		
6	5,10-methylenetetrahydro-folate dehydrogenase	5,10-methylenetetrahydrofolate: NADP oxidoreductase	1.5.1.5
7	Methenyltetrahydrofolate cyclohydrolase	5,10-methenyltetrahydrofolate 5-hydrolase (decyclizing)	3.5.4.9
8	Glycine formiminotrans-ferase	*N*-Formiminoglycine:tetrahydro-folate 5-formimino transferase	2.1.2.4
9	Glutamate formimino transferase	*N*-Formimino-L-glutamate:tetrahydro-folate 5-formiminotransferase	2.1.2.5
10	Formiminotetrahydrofolate cyclodeaminase	5-Formiminotetrahydrofolate ammonia-lyase (cyclizing)	4.3.1.4
11	Formyltetrahydrofolate mutase		
12	Formyltetrahydrofolate synthetase	Formate:tetrahydrofolate ligase (ADP)	6.3.4.3
13	Formyltetrahydrofolate deformylase	10-Formyltetrahydrofolate amidohydrolase	3.5.1.10
14	5-Formyltetrahydrofolate cyclodehydrase		
15	5-Methyltetrahydrofolate: Homocysteine methyl transferase		

SCHEME I


PteGlu
NADPH
NADP
NADH
NAD
H2PteGlu
H4PteGlu
NADPH
NADP
serine
pyridoxal-P
glycine
5,10-CH2=H4PteGlu
NADP or FAD
NADPH or FADH
5-CH3-H4PteGlu
homocysteine
cobalamin
methionine
H4PteGlu
CO2 + NH3
NADH
NAD
glycine
H4PteGlu
NAD or NADP
NADH or NADPH
5,10-CH≡H4PteGlu
NH3
5-HCNH-H4PteGlu
glycine
N-formiminoglycine
H4PteGlu
glutamic acid
N-formiminoglutamic acid
H4PteGlu
ATP
ADP + Pi
Mg2+
H4PteGlu
HCOOH
10-HCO-H4PteGlu
H2O
H2O
5-HCO-H4PteGlu
ATP
ADP + Pi
Mg2+
1
2
2
3
4
5
6
7
8
9
10
11
12
13
14
15

and Hutchison, 1966; Kessel and Roberts, 1965) and was first partially purified from chicken liver (Futterman and Silverman, 1957; Futterman, 1957, Zakrzewski and Nichol, 1958; Zakrzewski, 1960; Osborn and Huennekens, 1958). Later it was extensively purified in calf thymus (Nath and Greenberg, 1962), sheep liver (Morales and Greenberg, 1964), mouse tumour (Bertino *et al.*, 1964; 1965) and *S. faecalis* (Blakley and McDougall, 1961). A 2000 fold purification of the enzyme from guinea pig liver (Bertino *et al.*, 1966) and a 8000 fold purification from chicken liver (Kaufman and Gardiner, 1965; 1966) have been obtained. H_2PteGlu reductases are strongly inhibited by the PteGlu antagonists, aminopterin and amethopterin (Blakley, 1969). These compounds compete with the substrates for the active site. The enzymes studied to date generally display an extremely high affinity for these inhibitors thus permitting titration of the enzyme with the inhibitor to be used as a quantitative measure of enzyme activity (Werkheiser, 1961).

SOURCES OF ONE-CARBON UNITS FOR PTEROYLGLUTAMATE COENZYMES

Although H_4PteGlu, which is formed after reduction of either PteGlu or H_2PteGlu is usually present in very small amounts in tissues, one-carbon derivatives of this acid are commonly the principal pteroylglutamates. H_4PteGlu accepts a carbon one unit which becomes attached through the N^5 and/or N^{10} nitrogens. The major biological source of this carbon one unit is believed to be serine.

Serine hydroxymethyltransferase (Scheme 1, reaction 3)

has been isolated from many sources, for example Alexander and Greenberg (1956), Huennekens *et al.* (1957), Schirch and Mason (1962), Wang and Burris (1965), Cossins and Sinha (1966), DeBoiso and Stoppani (1967), and catalyzes the reversible formation of 5,10-CH_2=H_4PteGlu and glycine from serine. In *Escherichia coli* glycine is split by a reaction (Scheme 1, reaction 5) involving H_4PteGlu and pyridoxal phosphate, giving as products 5,10-CH_2=H_4PteGlu, NADH, CO_2 and NH_3 (Greenberg *et al.*, 1955). Tracer studies involving glycine-2-C^{14} feedings indicate a similar reaction may occur in plants (Sinha and Cossins, 1964; Cossins and Sinha, 1966; McConnell, 1964) and in the glycollate pathway a similar fate for glycine may exist (Wang and Burris, 1965; Miflin *et al.*, 1966).

Other sources of the one-carbon unit include formate (Sakami, 1948; Kruhøffer, 1951; Siekevitz and Greenberg, 1950) formaldehyde (Siegel and Lafaye, 1950; Mitoma and Greenberg, 1952), the methyl groups of choline (Kruhøffer, 1951; Sakami, 1949; Siekevitz and Greenberg, 1950), methionine (Siekevitz and Greenberg, 1950), acetone (Sakami, 1950), dimethylglycine and sarcosine (Mackenzie, 1950; Mackenzie and Abeles, 1956; Mackenzie and Frisell, 1958) and the formimino groups of formiminoglutamic acid (Tabor and Wyngarden, 1959) and formimino glycine (Rabinowitz and Pricer, 1956).

Formiminotransferases (Scheme 1, reactions 8 & 9) are instrumental in transferring the formimino group of formimino-

glycine or formiminoglutamic acid, intermediates in the metabolism of purines (Rabinowitz and Pricer, 1956) and histidine, (Borek and Waelsch, 1953) to H_4PteGlu. Glycine formiminotransferase (Scheme 1, reaction 8) present in bacteria, has been isolated and purified about 20 fold from *Clostridium cylindrosporum* (Uyeda and Rabinowitz, 1965). The enzyme responsible for the removal of the formimino group from formiminoglutamic acid has been studied extensively in mammals (Tabor and Wyngarden, 1959; Tabor, 1957; Miller and Waelsch, 1955; Miller, 1956; Miller and Waelsch, 1956; Miller and Waelsch, 1957).

Although formaldehyde reacts non-enzymatically with H_4PteGlu, this reaction apparently will cease when very small quantities of H_4PteGlu are present (Osborn *et al.*, 1957). Using such reaction conditions, evidence has been obtained for an enzyme which will assist in the condensation reaction (Osborn *et al.*, 1957). Also an enzymic reaction involving formic acid, H_4PteGlu and ATP has been clearly demonstrated (Goldthwait and Greenberg, 1955; Greenberg *et al.*, 1955; Whiteley *et al.*, 1958; 1958; Rabinowitz and Pricer, 1958; Revel and Magasanik, 1958; Koch and Levy, 1955).

METABOLIC INTERCONVERSION OF THE TETRAHYDROPTEROYLGLUTAMIC DERIVATIVES

Since PteGlu participates in metabolism as tetrahydro derivatives this account will be limited to those enzymes which require as substrates the various derivatives of

H_4PteGlu. Enzymes referred to in this section and the reactions they catalyze are also summarized in Scheme 1.

5,10-CH_2=H_4PteGlu formed from free formaldehyde, and from serine or glycine metabolism is readily oxidized by an enzyme system (Scheme 1, reactions 6 and 7) to 10-HCO-H_4PteGlu (Jaenicke, 1956; Hatefi *et al.*, 1957). It was later found that two enzymes were involved in this reaction with the intermediary formation of 5,10-CH≡H_4PteGlu (Osborn and Huennekens, 1957; Uyeda and Rabinowitz, 1967). 5,10-Methylenetetrahydrofolate dehydrogenase (Scheme 1, reaction 6) has been purified from several sources (Ramasastri and Blakley, 1964; Donaldson *et al.*, 1965; Yeh and Greenberg, 1965; Dalal and Gots, 1967; Cossins *et al.*, 1970) and its properties have been investigated. The enzyme catalyzes a reaction which is sensitive to a number of metabolic inhibitors (Taylor *et al.*, 1966; Dalal and Gots, 1966). Investigation of this enzyme in plant tissues has been carried out in this laboratory (Cossins *et al.*, 1970) by following increases in absorbance at 340 mμ, under conditions which allowed the formation of 10-HCO-H_4PteGlu (Ramasastri and Blakley, 1964). Methenyltetrahydrofolate cyclohydrolase (Scheme 1, reaction 7) which catalyses the hydrolysis of 5,10-CH≡H_4PteGlu to 10-HCO-H_4PteGlu has been demonstrated in extracts of several species (Rabinowitz and Pricer, 1956; Tabor and Rabinowitz, 1956; Tabor and Wyngarden, 1959).

5,10-CH_2=H_4PteGlu can be readily reduced by N^5,N^{10}-methylenetetrahydrofolate reductase (Scheme 1, reaction 4)

to 5-CH_3-H_4PteGlu. In contrast to 5,10-methylenetetrahydrofolate dehydrogenase, the properties of this enzyme have not been so extensively investigated and some uncertainty regarding the source of hydrogen for the reaction is noted in the literature. The reverse reaction can be greatly accelerated by electron accepting compounds such as menadione (Donaldson and Keresztesy, 1962). These presumably reoxidize $FADH_2$ which is produced in the reaction.

It has been shown that 5-CH_3-H_4PteGlu can be formed from 5,10-CH_2=H_4PteGlu provided NADH or NADPH is added to act as the source of hydrogen (Donaldson and Keresztesy, 1962), although NADPH appears to be the more effective coenzyme (Kutzbach and Stokstad, 1967). In this latter report a requirement for FAD was not demonstrated although it is conceivable that sufficient quantities of this compound may have been bound to the enzyme. Other reports on the source of hydrogen have appeared in the literature (Kisliuk, 1963; Foster *et al.*, 1964). Where the enzyme has been purified up to 100 fold it was shown that $FADH_2$ is specifically required as reductant and NADH can serve only when present with FAD and a lipoamide dehydrogenase (Katzen and Buchanan, 1965).

The formation of 5,10-CH≡H_4PteGlu from 5-HCO-H_4PteGlu was first reported by Peters and Greenberg (1957). The enzyme has been called 5-formyltetrahydrofolate cyclodehydrase (Scheme 1, reaction 14). It has since been highly purified from extracts of acetone-dried sheep liver (Greenberg *et al.*, 1965) and some of its properties were studied.

A similar enzyme has also been briefly reported in *Micrococcus aerogenes* (Kay *et al.*, 1960).

A mutase catalyzing the transfer of the formyl group of 5- or 10-HCO-H_4PteGlu was first reported by Greenberg (1954) although it was investigated further in chicken liver (Kay *et al.*, 1960) no detailed investigations with highly purified enzyme have been carried out to date.

Formyltetrahydrofolate synthetase which was first described by Greenberg *et al.* (1955) catalyzes the formation of 10-HCO-H_4PteGlu from H_4PteGlu and formate (Scheme 1, reaction 12). The enzyme has been purified from several sources (Whiteley *et al.*, 1959; Bertino *et al.*, 1962; Rabinowitz and Pricer, 1962; Lansford *et al.*, 1964; Hiatt, 1965; Iwai *et al.*, 1967) and highly purified from *Micrococcus aerogenes* (Whiteley *et al.*, 1958; Albrecht *et al.*, 1966). Its activity has been determined by measuring the incorporation of formate-C^{14} on incubation with H_4PteGlu and ATP (Goldthwait and Greenberg 1955) or by conversion of the product 10-HCO-H_4PteGlu to 5,10-CH≡H_4PteGlu and determining the absorption at 350 mμ (Goldthwait and Greenberg, 1955). Several studies on the reaction mechanism of this enzyme have been carried out (Whiteley and Huennekens, 1962; Uyeda and Rabinowitz, 1964; Joyce and Himes, 1966) but the actual sequence of reaction is as yet not fully understood.

The hydrolytic cleavage (Scheme 1, reaction 13) of the formyl group of 10-HCO-H_4PteGlu yielding formate has been shown to occur by an enzyme catalyzed reaction (Osborne *et al.*,1957;

Huennekens and Scrimgeour, 1963). A similar enzyme which has a specific requirement for 10-HCO-H_4PteGlu and NADP has been purified 200 fold from pig liver (Kulzbach and Stokstad, 1968).

5-HCNH-H_4PteGlu formed by pathways mentioned earlier, is readily deaminated and converted to 5,10-CH≡H_4PteGlu by formiminotetrahydrofolate cyclodeaminase (Scheme 1, reaction 1). It has been purified from *Clostidium cylindrosporum* (Rabinowitz and Pricer, 1956; Uyeda and Rabinowitz, 1967) from rabbit liver (Tabor and Rabinowitz, 1956) and hog liver (Tabor and Wyngarden, 1959). The cyclodeaminase activity from *C. cylindrosporum* could not be separated from cyclohydrolase activity by ultracentrifugation or starch gel electrophoresis, suggesting the two activities may be functions of the same protein (Uyeda and Rabinowitz, 1967). In an earlier study, however, Tabor and Wyngarden (1959) reported that two separate enzymes were necessary to catalyze the formation of 10-HCO-H_4PteGlu and 5-HCNH-H_4PteGlu.

These several enzymic interconversions result in the synthesis of pteroylglutamates which are involved in many metabolic reactions, the more important of which are those concerned with purine and pyrimidine synthesis and the biosynthesis of amino acids and proteins.

INVOLVEMENT OF H_4PTEGLU DERIVATIVES IN PURINE AND PYRIMIDINE SYNTHESIS

Pteroylglutamate derivatives have been shown to be

involved in the introduction of carbons 8 (Giri and Krishnoswamy, 1957; Rabinowitz and Pricer, 1957) and 2 (Stokes, 1944; Lampen and Jones, 1947) into the purine ring. These derivatives do not participate in the synthesis of the pyrimidine ring. They are, however, involved in the introduction of a one-carbon unit into the pyrimidine ring of thymine (Whittaker and Blakley, 1961; Friedkin, 1959), 5-hydroxymethylcytosine (Flaks and Cohen, 1959) and 5-methyluridine (Maley, 1962).

PTEROYLGLUTAMATES AND THE BIOSYNTHESIS OF AMINO ACIDS AND PROTEINS

The intermediary formation and participation of 5,10-CH_2=H_4PteGlu during the enzymatic interconversion of serine to glycine was suggested by Blakley (1954). The enzyme has since been extensively investigated as noted in an earlier section of this thesis. Another derivative 5-CH_3-H_4PteGlu is instrumental in the synthesis of methionine in a wide variety of organisms (for example, Weissbach *et al.*, 1963; Kerwar *et al.*, 1966; Dodd and Cossins, 1970; Burton and Sakami, 1969). A very important biological discovery was the role that formyl methionine plays in initiating the synthesis of polypeptide chains. The formyl donor is 10-HCO-H_4PteGlu and the enzyme responsible for catalytic formylation of met-$tRNA_F$ has been investigated (Marcker, 1965; Horikoshi and Doi, 1967) and successfully purified (Dickerman *et al.*, 1967).

BIOSYNTHESIS OF PTEROYLGLUTAMIC ACID

That pteridines acted as precursors of PteGlu was demonstrated when Korte *et al.* (1958) reported that C^{14}-α-anthopterin (2-amino-4,6-dihydroxypteridine) could be incorporated into PteGlu by several bacterial species. Furthermore, Weygand *et al.* (1949) found that certain strains of bacteria could be readily adapted to utilize 2-amino, 4-hydroxy-6-formylpteridine for PteGlu synthesis (see Scheme 2).

Studies of PteGlu synthesis *in vitro* were first performed by Katunuma (see Blakley, 1969) who showed that either anopterin or 2-amino-4-hydroxy-6-carboxypteridine and *p*-aminobenzoylglutamic acid could be converted to PteGlu by extracts of *Mycobacterium avium*. It was later shown that extracts of *Lactobacillus arabinosus* (Shiota, 1959) and *E. coli* (Brown, 1959; Brown *et al.*, 1960) contained enzyme systems utilizing 2-amino-4-hydroxy-6-formylpteridine or 2-amino-4-hydroxy-6-hydroxymethylpteridine (H_2-pteridine CH_2OH) for the synthesis of pteroic acid or PteGlu. This reaction is now widely known to be inhibited by sulphonamides (see Blakely, 1969).

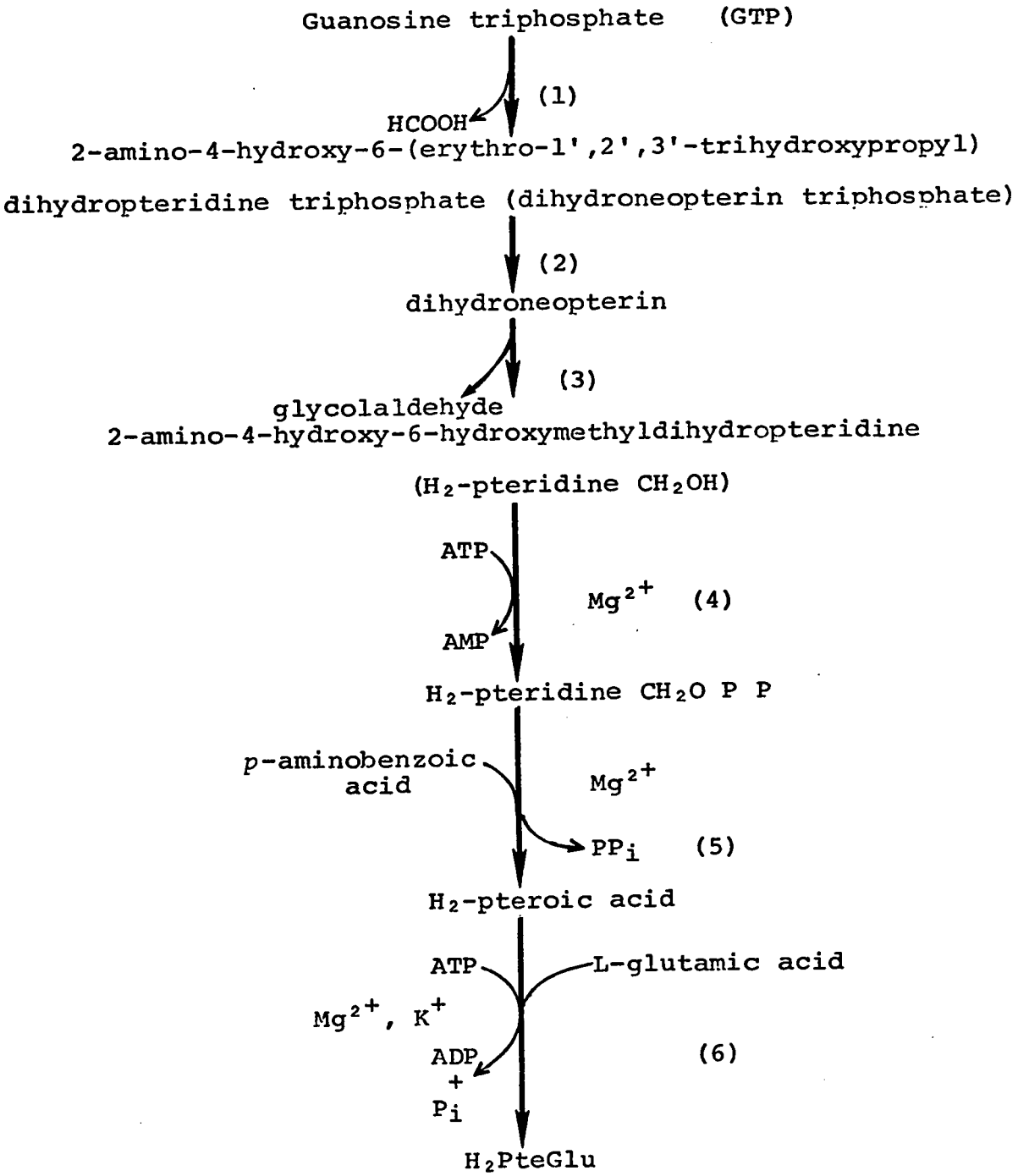
Because *p*-aminobenzoic acid was a much more effective substrate for pteroic acid synthesis than *p*-aminobenzoylglutamic acid was for PteGlu synthesis, it was postulated that pteroic acid (or a reduced form of pteroic acid) was an intermediate in the biosynthesis of PteGlu in *E. coli* (Brown *et al.*, 1960). The reduced froms of pteridines appeared to be used more effectively as substrate than the oxidized forms, a finding that led Brown *et al.* (1960) to suggest that either dihydro or tetrahydropteroic acid, rather

SCHEME II

REACTIONS INVOLVED IN THE BIOSYNTHESIS OF DIHYDROPTEROYL-GLUTAMIC ACID FROM GUANOSINE TRIPHOSPHATE

Reaction Number	Enzyme
1	GTP cyclohydrolase
2	a phosphatase
3	not fully characterized
4	H_2-pteridine-CH_2OH pyrophosphokinase
5	H_2-pteroate synthetase
6	dihydrofolate synthetase

SCHEME II


Guanosine triphosphate (GTP)
(1)
HCOOH
2-amino-4-hydroxy-6-(erythro-1',2',3'-trihydroxypropyl)
dihydropteridine triphosphate (dihydroneopterin triphosphate)
(2)
dihydroneopterin
(3)
glycolaldehyde
2-amino-4-hydroxy-6-hydroxymethyldihydropteridine
(H_2-pteridine CH_2OH)
ATP
Mg^{2+} (4)
AMP
H_2-pteridine CH_2O P P
p-aminobenzoic acid
Mg^{2+}
PP_i (5)
H_2-pteroic acid
ATP
L-glutamic acid
Mg^{2+}, K^+
ADP
+
P_i
(6)
H_2PteGlu

than pteroic acid is the true intermediate in PteGlu biosynthesis. The enzyme system for the formation of dihydropteroic acid from 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine has subsequently been shown to exist in extracts of a number of other microorganisms (Brown *et al.*, 1961; Shiota and Disraely, 1961; Weisman and Brown, 1964; Shiota *et al.*, 1964). Very firm evidence for the two enzymes necessary for this biosynthesis was presented by Ricky and Brown (1969) in *E. coli* and by Shiota (1969) in *Lactobacillus plantarum*.

It has also been shown that dihydropteroic acid was a possible intermediate in the biosynthesis of pteroylglutamate compounds in plants which thereafter combined with L-glutamic acid to form dihydropteroylglutamic acid (Iwai *et al.*, 1968). In a later publication, it was subsequently shown that dihydrofolic acid synthetase and dihydropteroic acid synthetase occurred in pea seedlings (Iwai *et al.*, 1969, 1970a, 1970b). However, these workers proposed that the dihydropteroic acid synthetase catalyzed steps 4 and 5 (Scheme II) and was a homogeneous enzyme on the basis of chromatographic behavior and ultracentrifugal analysis.

The formation of 2-amino-4-hydroxy-6-hydroxymethylpteridine from guanine-2-C^{14} has been demonstrated by *Cornynebacteria* (Krumdieck *et al.*, 1964). Radioactive guanine was also found to label pteridines found in the skin of amphibia (Levy, 1964) and when injected into the pupae of the butterfly (*Colias eurythem*), labelled pteridines

were isolated from the adults (Watt, 1967).

In cell-free extracts of *E. coli*, Burg and Brown (1966) have shown that guanosine triphosphate is the only guanine compound that can be utilized directly as a precursor of PteGlu. This has also been established by other workers (Shiota and Palumbo, 1965; Dalal and Gots, 1965). During this synthesis carbon 8 of GTP does not become incorporated into the pteridines but appears as formic acid (Burg and Brown, 1966; 1968). The other product of the reaction is 2-amino-4-hydroxy-6-(erythro-1', 2',3'-trihydroxypropyl) dihydropteridine triphosphate (Burg and Brown, 1968). Evidence was also presented that an arsenate-sensitive phosphatase, present in *E. coli*, is involved in the dephosphorylation of dihydroneopterin triphosphate, a step apparently necessary for this compound to be converted to dihydropteroic acid.

The conversion of dihydroneopterin to 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine involves the loss of a two carbon unit as proposed by Reynolds and Brown (1964) and occurs consequently without the intermediary formation of 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine (Jones and Brown, 1967). The enzyme has been purified some 500 fold, and the products of the reaction identified as 2-amino-4-hydroxy-6-hydroxymethyldihydropteridine and glycolaldehyde (Brown, 1969).

THE PRESENT INVESTIGATIONS

The isolation and identification of pteroylglutamate from animal tissues and microorganisms and their involvement in many metabolic reactions have been briefly outlined in an earlier part of this Introduction. To date, there is also considerable evidence for involvement of these derivatives in the one-carbon metabolism of higher plant tissues (Hiatt, 1965; Iwai *et al.*, 1967; Wong and Cossins, 1966; Prather and Sisler, 1966; Burton and Sakami, 1969; Dodd and Cossins, 1969; Cossins *et al.*, 1970). However, as mentioned earlier, relatively few detailed studies of the endogenous levels of these compounds in plants have been reported.

Because various aspects of the C_1 metabolism of pea seeds have been investigated in this laboratory (Cossins and Sinha, 1965; Wong and Cossins, 1966; Dodd and Cossins, 1968), this tissue was selected for the present investigations. In addition, an active 5,10-methylenetetrahydrofolate dehydrogenase indicated that there may be a rapid synthesis of 10-HCO-H_4PteGlu in this tissue (Wong and Cossins, 1966) relating to an increasing demand upon one-carbon units in the germinating peas for synthetic reactions discussed earlier. It was also necessary to employ *L. casei* in microbiological determinations on plant tissue as previous workers failed to do so, not enabling them to detect methylated derivatives.

Although the synthesis of pteroylglutamic acid has now been studied in detail, the relationships between various conjugated forms of H_4PteGlu are still not clear. In the

present work, attempts were, therefore, made to assay levels of such polyglutamyl derivatives. In addition, their synthesis from labelled precursors administered during the early stages of germination was examined.

MATERIALS AND METHODS

MATERIALS

Chemicals were purchased from Lederle Products, Cyanamid of Canada, Limited; Sigma Chemical Company; Eastman Organic Chemicals and Nutritional Biochemicals, Corp. Scintillation grade 2,5-diphenyloxazole (PPO) and 1,4-*bis*-[2-(5-phenyloxazolyl)] benzene (POPOP) were purchased from Nuclear-Chicago. Special enzyme grade ammonium sulfate was obtained from Mann Research Laboratory, Inc. 10-Formyltetrahydropteroylglutamic acid was synthesized from 5-formyltetrahydropteroylglutamic acid (Rabinowitz, 1963). 5-Methyltetrahydropteroylglutamic acid was prepared by borohydride reduction of 5,10-methylene-tetrahydropteroylglutamic acid (Sakami, 1963). Tetrahydropteroylglutamic acid was prepared by reduction of $Na_2S_2O_4$ and pteroylglutamic acid using $Na_2S_2O_4$ in a solid state reaction (Davis, 1969).

Pteroylglutamic acid-2-C^{14} and 5-methyl-C^{14}-tetrahydropteroylglutamic acid was purchased from the Radiochemical Centre, Amersham, England. For the present studies, pteroylglutamic acid-2-C^{14} was dissolved in sterile demineralized water to give a final specific radioactivity of 1 µc/0.02 µmole/1.7 ml of solution. 5-methyl-C^{14}-tetrahydropteroylglutamic acid was dissolved in 0.5% ascorbate solution to give a final specific radioactivity of 1 µc/0.016 µmole/0.8 ml of solution and formate-C^{14} was dissolved in sterile demineralized water to give a final specific radioactivitv of 1 µc/0.023 µmole/0.2 ml of solution.

METHODS

(i) *Plant Material and Germination Procedure*

Seeds of *Pisum sativum* L. cv. Homesteader were obtained from Steele Robertson Ltd., Edmonton. They were grown in darkness in moist sterile vermiculite at 25°C for periods up to 7 days. Initially the seeds were soaked in distilled water for 24 hr (Roos *et al.*, 1968). When cotyledons were required, the testas and embryos were carefully removed and in some cases the embryos were also retained for experimental work.

(ii) *Methods for Isolation of Pteroylglutamate Derivatives*

After removal of testas and embryos, pteroylglutamate derivatives were extracted from pea cotyledons by one of the following three methods.

METHOD I - extraction was carried out by grinding 10 g samples of cotyledons in a mortar followed by use of a hand blender. Tissues were ground in an ice cold mixture of 2.2 mls 10% (w/v) potassium ascorbate (pH 6.0) and 9.8 mls demineralized water. This homogenate was diluted with 28 mls of 0.5% (w/v) potassium ascorbate, pH 6.0 (Bird *et al.*, 1965).

METHOD II - slices approximately 1 mm in thickness, were prepared from germinating pea cotyledons using a sharp razor blade and 10 g samples were immediately immersed in a boiling solution containing 2.2 mls 10% (w/v) potassium ascorbate (pH 6.0) and 9.8 mls demineralized water. After boiling for 10 min., the samples were cooled rapidly in an ice bath and 10 mls of ice cold 0.5% (w/v) potassium

ascorbate (pH 6.0) were added.

METHOD III - pea cotyledons (10 g) were lyophilized (Virtis Automatic Freeze-Dryer Model 10-010) and ground to a fine powder using a mortar and pestle. The powder was then added to a boiling solution of 2.2 mls 10% (w/v) potassium ascorbate (pH 6.0) and 9.8 mls demineralized water. After 10 min. at 95°C, the extract was rapidly cooled as in Method II. Additional homogenization of the extract was accomplished with a hand blender followed by dilution with ascorbate solution as in Method II.

Homogenates prepared by these methods were routinely heated for 10 min. by immersion in a water bath maintained at 95°C, cooled rapidly in an ice bath and diluted with an additional 10 mls of 0.5% (w/v) cold potassium ascorbate as described by Bird *et al.* (1965).

(iii) *Chromatography of Pteroylglutamate Derivatives*

Extracts prepared by the methods outlined above were finally centrifuged at 18,000 x g for 20 min. in a Servall Refrigerated Automatic Centrifuge operated at 2°C to remove denaturated protein. The clear supernatants were stored in a frozen state until required for microbiological assay. Microbiological assays were carried out either on dilutions of the supernatants or on fractions separated by column chromatography. Columns of DEAE-Cellulose were prepared by the method of Sotobayashi *et al.* (1966). A mixture consisting of 18 g N,N-DEAE-cellulose and 22.5 g Hyflo Supercel was suspended in 1500 mls distilled water and packed into glass

columns by gravity flow to a depth of 28 cms. The columns were washed with 50 mls 0.5 N KOH followed by distilled water until the rinse had a pH value 7.0 - 7.5. Fifty mls of 0.5 M potassium phosphate buffer (pH 6.0) were then added followed by distilled water until the rinse was free of phosphate. Aliquots 1-5 mls, of the extracts, containing 0.05 - 0.8 µg of pteroylglutamates were applied to the columns and the derivatives eluted using a gradient of phosphate buffer in the presence of 0.6% (w/v) potassium ascorbate (pH 6.0) (Sotobayashi *et al.*, 1966). The phosphate gradient was altered following collection of fraction 130. The 10 ml head on the column was removed and replaced with 10 mls potassium phosphate (pH 6.0) containing 0.6% (w/v) ascorbate, and an equal molar concentration allowed to drop onto this head. The effluent from the columns was collected in 3 ml fractions at room temperature using an LKB Ultra Rac Fraction Collector, Type 7000 (LKB-Produckters AB, Stockholm, Sweden). Each collecting tube contained an additional 0.1 ml of 10% (w/v) potassium ascorbate solution (pH 6.0) (Silverman *et al.*, 1961). The fractions were either subjected immediately to a differential microbiological assay with *L. casei*, *S. faecalis* and *P. cerevisiae* or were stored in a frozen state until required. The elution sequence of several common pteroylglutamate derivatives was determined by chromatographing 0.05 - 0.10 µg samples of the authentic derivatives under these conditions. Reference curves were constructed using authentic PteGlu for *L. casei* and *S. faecalis* and 5-HCO-H_4 PteGlu for *P. cerevisiae*.

(iv) *Microbiological assay*

The tissue extracts in potassium ascorbate or fractions collected during column chromatography were assayed for pteroylglutamates after appropriate dilutions were made. The assay procedure was the 'aseptic plus ascorbate' method of Bakerman (1961). Three bacteria were used, namely *Lactobacillus casei* (ATCC 7469), *Streptococcus faecalis* (ATCC 8043) and *Pediococcus cerevisiae* (ATCC 8081). *L. casei* requires relatively low levels of folic acid for minimal growth and consequently is more sensitive than *S. faecalis* (Jukes and Stokstad, 1948). *P. cerevisiae*, on the other hand, has a specific requirement for the tetrahydro derivatives and will consequently not grow when H_2PteGlu or PteGlu are supplied. Bacteria were in all cases purchased from the American Type Culture Collection, Rockville, Maryland, U.S.A. The nutritional requirements of these bacteria are well documented (Hansen, 1964; Freed, 1966).

In the present work, cultures were maintained on agar slants and transfer to new slants was made at weekly intervals to maintain active cultures. The inoculum was prepared the evening before the start of an assay and the bacteria from The inoculum were washed twice in sterile saline and diluted approximately eleven times for inoculation of the assay tubes. These assay tubes contained 10 mls of solution consisting of 5 mls of basal assay media with varying amounts of standard and unknown samples, the final volume of each tube being adjusted to 10 mls by addition of 0.5% (w/v)

potassium ascorbate (pH 6.0). The assay organisms were found to respond linearly to PteGlu within the range of 0.1 - 1.0 mμg for *L. casei* and 0.5 - 5.0 mμg for *S. faecalis*. *P. cerevisiae* gave good response over the range of 0.1 - 1.0 mμg for 5-HCO-H_4PteGlu, however a linear response was not obtainable for this latter organism. Duplicates were routinely run for both unknown and standard samples. Each assay was replicated three times.

The inoculated assay tubes were briefly shaken and incubated at 37°C for 72 hr. After the incubation period was completed, the amount of growth of bacteria was determined by titration of the lactic acid formed (Freed, 1966). From the standard curve obtained with each experiment the amount of pteroylglutamate in the test solutions was calculated by interpolation.

(v) *Incubation of Plant Extracts with γ-Glutamyl Carboxypeptidase*

In order to assay levels of polyglutamylderivatives, cotyledon extracts were routinely incubated with γ-glutamyl carboxypeptidases known to hydrolyze such derivatives to compounds capable of supporting growth of the assay organisms (Blakley, 1969). In addition to the commonly employed enzymes from chicken pancreas (Mims and Laskowski, 1945) and hog kidney (Doctor and Couch, 1953) a third enzyme, partially purified from three-day-old cotyledons was frequently used in the present studies. Each enzyme was partially purified from the source materials as follows. All procedures were carried out at 2°C.

(i) Chicken pancreas γ-glutamyl carboxypeptidase: This enzyme was isolated from acetone powders as described by Mims and Laskowski (1945). 3 g of acetone powder (Difco-Bacto) were suspended in 15 mls of 0.1 M phosphate buffer (pH 8.0). This suspension was stirred for 4.5 hr at 2°C. The pH was checked periodically and readjusted to 8.0 by addition of 0.1 N NaOH. After this period the extract was squeezed through 4 layers of cheesecloth and centrifuged at 18,000 x g for 20 min. in a Servall refrigerated automatic centrifuge model RC-2 operated at 2°C. The supernatant was collected. Following 40-80% fractionation with ammonium sulfate and recentrifugation as described above, the small brownish precipitate was dialyzed overnight with approximately 2 mls of 0.1 M phosphate buffer (pH 8.0) against a large volume of the same buffer. After removal of the dialysis sac it was made up to 7 mls and stored in a frozen state until required. Before use a 10-fold dilution was prepared using 0.2 M sodium borate buffer (pH 7.8).

(ii) Hog kidney γ-glutamyl carboxypeptidase: The hog kidney enzyme (Doctor and Couch, 1953) was prepared by stirring 1.5 g of hog kidney extract purchased from Difco Laboratories with 15 mls of 0.32% L-cysteine·HCl (pH 4.5) for 2 hr at 2°C. The extract was clarified by centrifugation at 18,000 x g for 20 min. in the Servall centrifuge operated at 2°C and then treated with Dowex AG1-X10, 200-400 mesh anion exchange resin (pH 4.5) for 1 hr. After removal of the resin by centrifugation the supernatant containing the enzyme was diluted to 50

mls with 0.32% L-Cysteine·HCl (pH 4.5) and stored in a frozen state until required.

(iii) *Pea Cotyledon Hydrolase:* The enzyme was prepared by grinding 10 g of 3-day-old cotyledons in 20 mls 0.1 M potassium phosphate buffer (pH 6.0). The homogenate was squeezed through four layers of cheesecloth and centrifuged at 18,000 x g for 20 min. in the Servall centrifuge operated at 2°C. Saturated $(NH_4)_2SO_4$ solution was then added to the supernatant to give a final concentration of 80% of saturation. After standing for 30 min. at 2°C the precipitated protein was removed by centrifugation. The precipitate was then redissolved in 40 mls of the same buffer and stored in a frozen state until required. For demonstration of enzyme activity a 10-fold dilution of the enzyme extract was made in 0.1 M sodium acetate buffer (pH 4.5) containing 10% (w/v) ascorbate.

Demonstration of Enzyme Activity from these Sources

Enzyme activities were demonstrated using Difco-Bacto yeast extract as substrate. In each experiment 1 ml of the enzyme solution was incubated with 1 ml of yeast extract and buffer containing 10% (w/v) ascorbate. The yeast extract was prepared by dissolving 20 g of Difco-Bacto yeast extract in 100 mls of 1% (w/v) potassium ascorbate (pH 6.0). The solution was heated in a water bath maintained at 95°C for 10 min., cooled rapidly and centrifuged at 18,000 x g for 20 min. in the Servall centrifuge operated at 2°C. The solution was dispensed into 10 test tubes and stored frozen

until required.

The avian enzyme was incubated with 7 mls of 0.2 M sodium borate buffer (pH 7.8) while the two other enzymes were incubated with 0.1 M sodium acetate buffer (pH 4.5). The avian enzyme required, in addition, 1 ml of 0.1 M calcium chloride in a final volume of 10 mls. When tissue extracts (1-3 mls) were tested for polyglutamate contents they were previously adjusted to pH 7.8 with 0.5 N NaOH for the avian enzyme and to 4.5 with 0.5 N HCl for use with the other two enzymes. In all cases 1 ml of the enzyme solution was incubated with the substrate in a final volume of 10 mls. Incubations at 35°C were performed in a constant temperature bath for 5 hr (avian enzyme) and 2-3 hr for the other enzymes. Enzyme activity was confirmed in each experiment by including reaction systems containing boiled enzyme.

Experiments Involving Pteroylglutamate Antagonists

Air dried pea seeds (60 g) were soaked for 24 hr in 300 mls of 10^{-4} M aminopterin or amethopterin. They were then transferred to moist vermiculite and allowed to germinate as described in an earlier part of this thesis. Samples (10 g) were taken after 1 and 3 days of germination. Pteroylglutamates which were extracted by Methods I and II were assayed with *L. casei*. Aliquots (3-5 ml) were subjected to DEAE-cellulose column chromatography and differential microbiological assays with *L. casei* and *P. cerevisiae* were carried out.

Feeding Experiments Involving C^{14}-labelled Pteroylglutamates and $HC^{14}OOH$

The labelled compounds from Amersham/Searle Corp. were diluted with sterile distilled water and supplied to the tissues in the following amounts: PteGlu-2-C^{14}, 0.067 µmoles containing 3.3 µc of C^{14}; 5-$C^{14}H_3$-H_4PteGlu, 0.082 µmoles containing 5.0 µc of C^{14}; $HC^{14}OOH$, 0.23 µmoles containing 10 µc of C^{14}.

In all feeding experiments 30 dry pea seeds were allowed to imbibe 2 mls of a solution containing the radioactive isotope under sterile conditions. Additional distilled water was added after complete imbibition of the radioactive solution. The seeds were then transferred to sterile petri dishes lined with filter paper and allowed to germinate at 25°C in darkness. Pteroylglutamate derivatives were extracted as described under Methods I and II followed by chromatography on DEAE-cellulose. Radioactivity was measured in a liquid scintillation counter (Nuclear Chicago Corp., Unilux II Model) using a fluor containing dioxane 600 mls; anisole 100 mls; dimethoxyethane 100 mls; PPO 4 gms; and POPOP 0.5 gms. The counting efficiency was 75% and all counts were corrected for background. Aliquots of the same fractions assayed for radioactivity were also assayed for pteroylglutamates using *L. casei* and *P. cerevisiae*.

Experiments Involving Cell-Free Extracts

(a) 5,10-CH_2=H_4PteGlu dehydrogenase activity

Isolation of 5,10-methylenetetrahydrofolate dehydrogenase

from pea cotyledons was performed at various stages of germination. After removal of embryos and testas, 10 gm of the cotyledons were ground in 0.01 M potassium phosphate buffer (pH 7.5) containing 0.001 M EDTA and 0.01 M 2-mercaptoethanol (Cossins, Wong and Roos, 1970). The resulting slurry was squeezed through four layers of cheesecloth and further clarified by centrifugation at 18,000 x g for 20 min. in the Servall Centrifuge operated at 2°C. The supernatant was then fractionated by addition of saturated ammonium sulfate solution adjusted to pH 7.5 with ammonium hydroxide. Protein which percipitated between 60-80% saturation was collected by centrifugation and redissolved in 20 mls of the phosphate buffer.

Enzyme activity was assayed spectrophotometrically using a Beckman DB spectrophotometer equipped with a potentiometric recorder as outlined by Wong and Cossins (1966). The assay mixture, in a total volume of 3 mls, contained 0.01 M phosphate buffer (pH 7.5), 12.5 µmoles formaldehyde, 0.8 µmoles H_4PteGlu and 50 µmoles mercaptoethanol. This mixture was preincubated for 4 min. at 37°C to allow for formation of 5,10-CH_2=H_4Pt·Glu. Varying amounts of the enzyme preparation were then added, followed by a further incubation of 2 min. at 37°C. 1.8 µmoles of NADP were finally added to the reaction cuvette to initiate the reaction. The reference cuvette routinely contained all of the above components with the exception of NADP which was replaced by distilled water. The amount of phosphate buffer in the reaction system was

varied to give a final volume of 3 mls. After initiation of the reaction with NADP, changes in absorbance at 340 mμ were followed continuously for 4 minute periods. One unit of 5,10-CH_2=H_4PteGlu dehydrogenase activity is defined as the amount of enzyme causing a change in absorbancy of 0.01 per minute (Yeh and Greenberg, 1965).

(b) 5,10-CH_2=H_4PteGlu reductase activity

Isolation of 5,10-CH_2=H_4PteGlu reductase from 3-day-old pea cotyledons was accomplished by grinding 10 g samples of the cotyledons in 0.01 M potassium phosphate buffer (pH 6.7) containing 0.001 M EDTA, 0.01 M 2-mercaptoethanol and 10 mg/litre FAD. The resulting slurry was squeezed through four layers of cheesecloth and further clarified by centrifugation at 18,000 x g for 20 min. The supernatant was then fractionated by addition of saturated ammonium sulfate solution (pH 6.7). Protein which precipitated between 50-80% saturation was collected by centrifugation and redissolved in 20 mls of the phosphate buffer. 0.3 ml of this extract was then incubated with 0.089 μmole H_4PteGlu 12.5 μmole formaldehyde, 50 μmole 2-mercaptoethanol, 1.0 μmole FAD, 3.5 μmole $NADPH_2$ and 0.01 M potassium phosphate buffer (pH 6.7) to give a final volume of 4 mls.

(c) Synthesis of pteroylglutamates *in vitro*

Experiments were designed to assess ability of cell-free extracts to synthesize various pteroylglutamates from serine and formate. The reaction systems (see Results

section) routinely included pyridoxal phosphate and adenosine triphosphate when the one-carbon sources were serine and formate respectively. After incubation the reactions were terminated by placing the incubation tubes in a boiling water bath for 10 min. Pteroylglutamate derivatives were then isolated and assayed by DEAE-cellulose chromatography and differential microbiological assay.

Protein Estimation

The method used in the present studies was that of Lowry *et al.* (1951). Folin-Ciocalteau reagent was obtained from the Fisher Scientific Company and diluted three times using distilled water. A reference curve was prepared using crystalline egg albumin (200 µg/ml). All determinations were made at least in duplicate and absorbancies were measured at 500 mµ using the Beckman DB spectrophotometer.

RESULTS

I. *PTEROYLGLUTAMATE CONTENTS OF GERMINATING PEA COTYLEDONS*

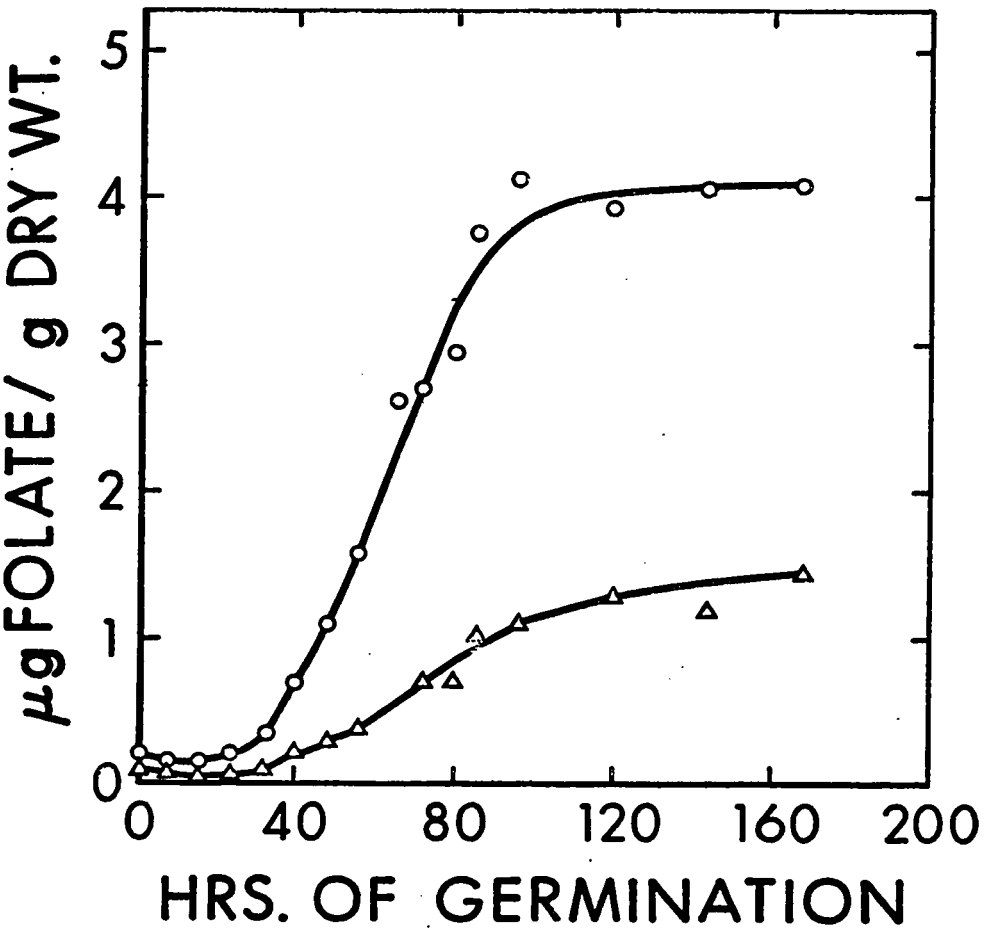
At frequent intervals during a seven day germination period, 10 g samples of the cotyledons were extracted as outlined by Method I. After centrifugation, the extracts were routinely assayed for pteroylglutamates employing *L. casei* and *S. faecalis*. The results of such assays are illustrated in Figure 1.

The pteroylglutamate content of the cotyledons remained very low during the first 30 hr of germination. This is in contrast to the considerable increase that occurred between 40 and 90 hr. After approximately 100 hr of germination, the pteroylglutamate content reached maximal levels which were more than 15 times the initial values obtained. The difference in response by the two bacteria indicated the possible presence of methylated and polyglutamyl derivatives. The possibility exists, therefore, that the observed increases may represent either a net synthesis of pteroylglutamate or a progressive hydrolysis of polyglutamates to yield derivatives capable of supporting the growth of *L. casei* and *S. faecalis*. However, when extracts of 1-day-old cotyledons were treated with hog kidney (Doctor and Couch, 1953) and chicken pancreas (Mims and Laskowski, 1945) γ-glutamyl carboxypeptidases, no increase in the growth of either assay organism was observed. This indicated that no significant level of polyglutamates occurred in the cotyledons at this stage of germination.

FIGURE 1

CHANGES IN PTEROYLGLUTAMATE CONTENTS OF GERMINATING PEA COTYLEDONS

After extraction of derivatives by Method I, assays were made using *L. casei* (o - o) and *S. faecalis* (Δ - Δ). Treatment with hog kidney and chicken pancreas γ-glutamyl carboxypeptidases failed to increase the growth response by either assay organism. The value illustrated in this figure represents the averages of several determinations. The data are in the PteGlu equilavents.


µg FOLATE/ g DRY WT.
0
1
2
3
4
5
0
40
80
120
160
200
HRS. OF GERMINATION

In order to determine the nature of the pteroylglutamates present in germinating pea cotyledons, extracts were subjected to chromatographic fractionation (Sotobayashi *et al.*, 1966). To standardize the procedure, authentic samples (0.05 - 0.10 µg) of 10-HCO-H_4PteGlu, 5-HCO-H_4PteGlu, 5-CH_3-H_4PteGlu, H_4PteGlu and PteGlu were individually applied to the columns. Table 1 summarizes the separation of these compounds and the growth response obtained by the three bacteria. In agreement with published data (Stokstad and Koch, 1967) the formyl derivatives and H_4PteGlu supported the growth of all three assay organisms. Similarly, *P. cerevisiae* did not respond to PteGlu and together with *S. faecalis* did not grow in the presence of 5-CH_3-H_4PteGlu.

There is considerable confusion in the published data regarding the response of the three bacteria to di-, tri- and polyglutamyl derivatives. The subject is discussed in detail by Blakley (1969). In general, *L. casei* is known to respond to pteroylglutamates with as many as three glutamic acid residues. It is also agreed that *P. cerevisiae* and *S. faecalis* respond to 5-HCO-H_4PteGlu, but response to di- and triglutamyl forms of this derivative has not been thoroughly elucidated (Blakley, 1969).

Pteroylglutamates (0.3 - 0.8 µg) extracted from 1- and 3-day-old cotyledons by Method I were next subjected to DEAE-cellulose column chromatography. The collected fractions were assayed with all three assay organisms. The results of such assays are illustrated in Figures 2-5.

TABLE 1

SEPARATION OF AUTHENTIC PTEROYLGLUTAMATE DERIVATIVES BY DEAE-CELLULOSE COLUMN CHROMATOGRAPHY

Compound	Fractions	Growth Response		
		L. casei	*S. faecalis*	*P. cerevisiae*
10-HCO-H_4PteGlu	38-45	+	+	+
5-HCO-H_4PteGlu	60-70	+	+	+
5-CH_3-H_4PteGlu	64-75	+	-	-
H_4PteGlu	70-82	+	+	+
PteGlu	125-145	+	+	-

Authentic samples (0.05 - 0.10 µg) were chromatographed by DEAE-cellulose chromatography followed by assay employing *L. casei*, *S. faecalis* and *P. cerevisiae*. The recovery of H_4PteGlu was usually 60-80% while the recovery of the other derivatives was approximately 100% of the original sample as determined by *L. casei*. The position of elution was not altered by treatment with chicken pancreas γ-glutamyl carboxypeptidase. The data are in PteGlu equilavents for *L. casei* and *S. faecalis* and 5-HCO-H_4PteGlu equilavents for *P. cerevisiae*.

FIGURE 2

CHROMATOGRAPHY OF PTEROYLGLUTAMATES FROM 1-DAY-OLD PEA COTYLEDONS

Derivatives were separated by DEAE-cellulose chromatography followed by assay using *L. casei*. The peaks are designated as:

Peak *a*, 10-HCO-H_4PteGlu; Peak *b*, 10-HCO-H_4PteGlu$_2$;
Peak *e*, 5-CH_3-H_4PteGlu and 5-HCO-H_4PteGlu; and
Peak *g*, 5-CH_3-H_4PteGlu$_2$.

No growth response was obtained before fraction 30 or after fraction 100. Recoveries were 100±10% of values obtained before chromatography. The data are in PteGlu equivalents.

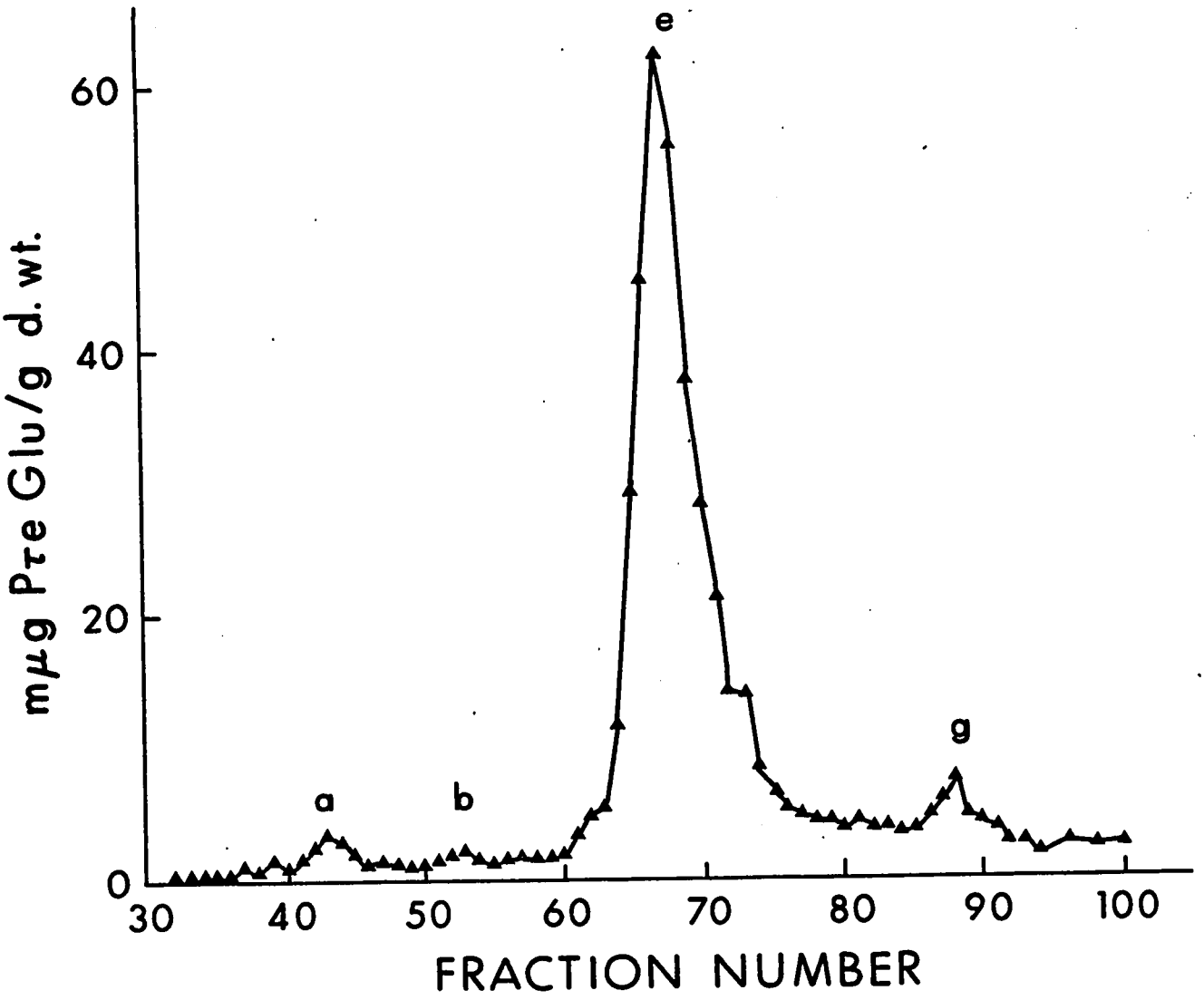

e
60
40
20
0
mμg Pτe Glu/g d. wt.
a
b
g
30
40
50
60
70
80
90
100
FRACTION NUMBER

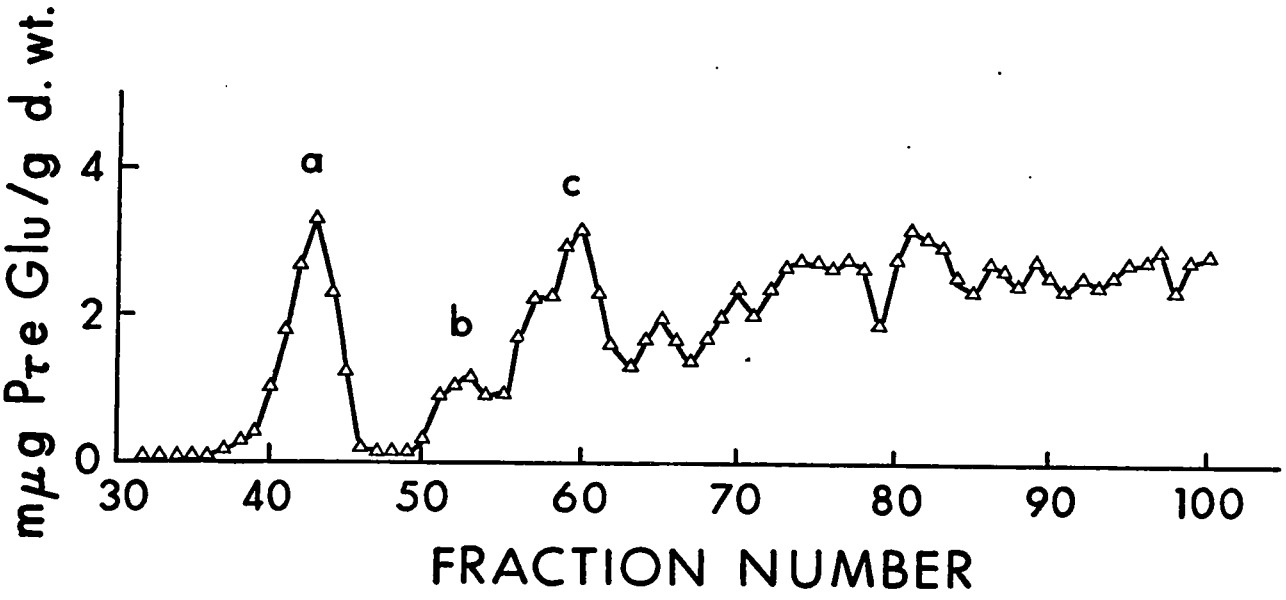
FIGURE 3

DIFFERENTIAL ASSAY OF PTEROYLGLUTAMATES FROM 1-DAY-OLD PEA COTYLEDONS

Derivatives were separated by DEAE-cellulose chromatography followed by assay using *S. faecalis* (Δ - Δ) and *P. cerevisiae* (o - o). The peaks are designated as:

Peak *a*, 10-HCO-H_4PteGlu; Peak *b*, 10-HCO-H_4PteGlu$_2$; Peak *c*, a pteroic acid derivative; Peak *d*, 5-HCO-H_4PteGlu.

No growth response was obtained before fraction 30 or after fraction 100. The data are in PteGlu equilavents for *S. faecalis* and 5-HCO-H_4PteGlu equilavents for *P. cerevisiae*.


mμg Pτe Glu/g d. wt.
4
2
0
a
b
c
30
40
50
60
70
80
90
100
FRACTION NUMBER

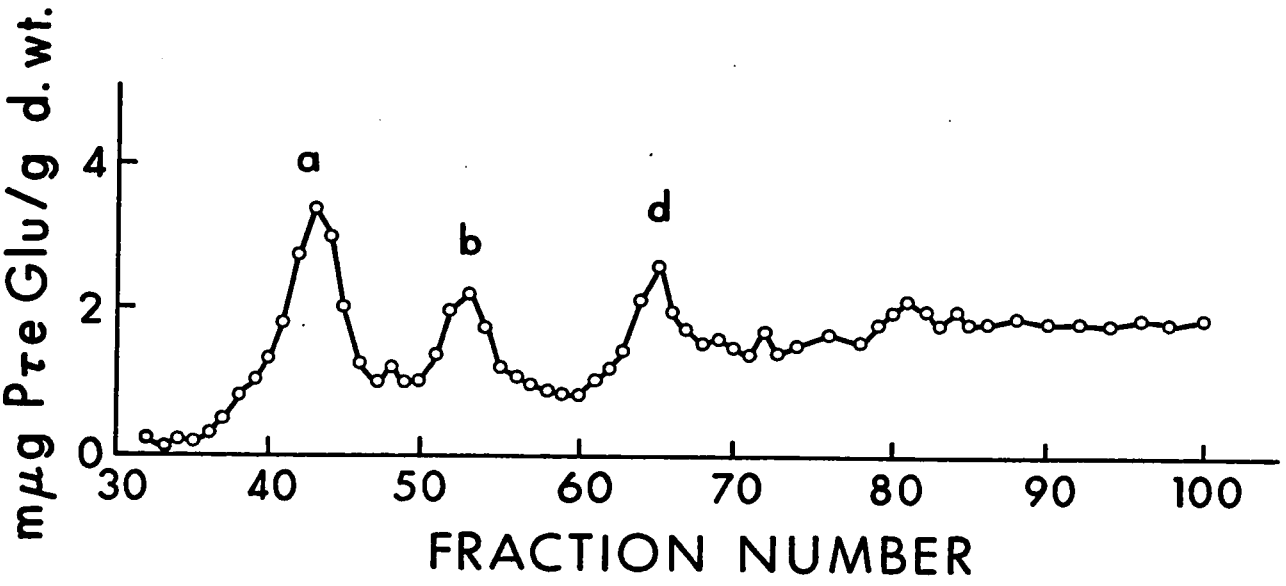

mμg Pτe Glu/g d. wt.
4
2
0
a
b
d
30
40
50
60
70
80
90
100
FRACTION NUMBER

FIGURE 4

CHROMATOGRAPHY OF PTEROYLGLUTAMATES FROM 3-DAY-OLD PEA COTYLEDONS

Derivatives were separated by DEAE-cellulose chromatography followed by assay using *L. casei*. The peaks are designated as:

Peak *a*, 10-HCO-H_4PteGlu; Peak *b*, 10-HCO-H_4PteGlu$_2$;
Peak *e*, 5-CH_3-H_4PteGlu and 5-HCO-H_4PteGlu; and
Peak *g*, 5-CH_3-H_4PteGlu$_2$.

No growth response was obtained before fraction 30 or after fraction 100. Recoveries were 100±10% of the values obtained before chromatography. The data are in PteGlu equivalents.

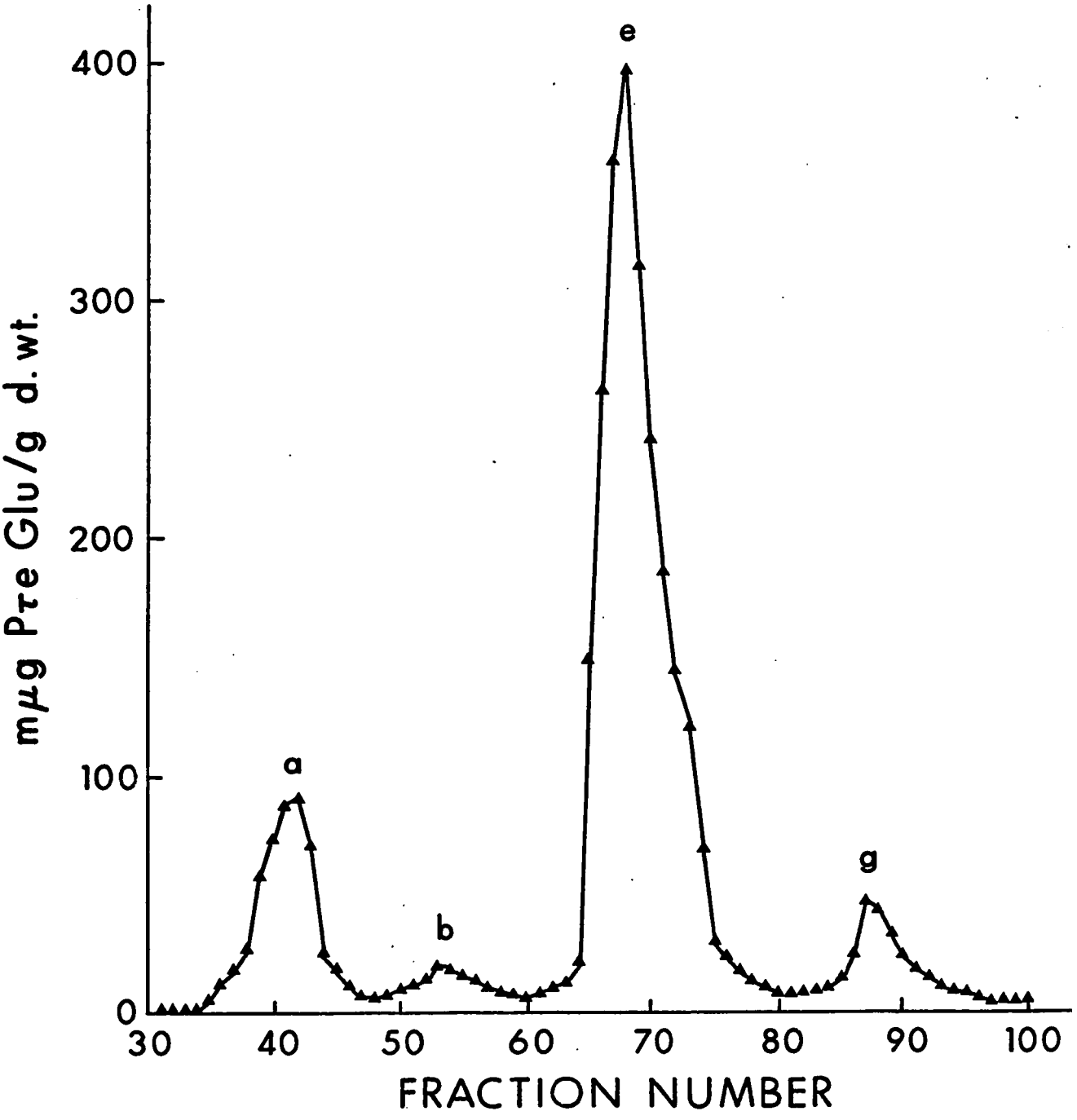

400
300
200
100
0
mμg Pre Glu/g d.wt.
a
b
e
g
30
40
50
60
70
80
90
100
FRACTION NUMBER

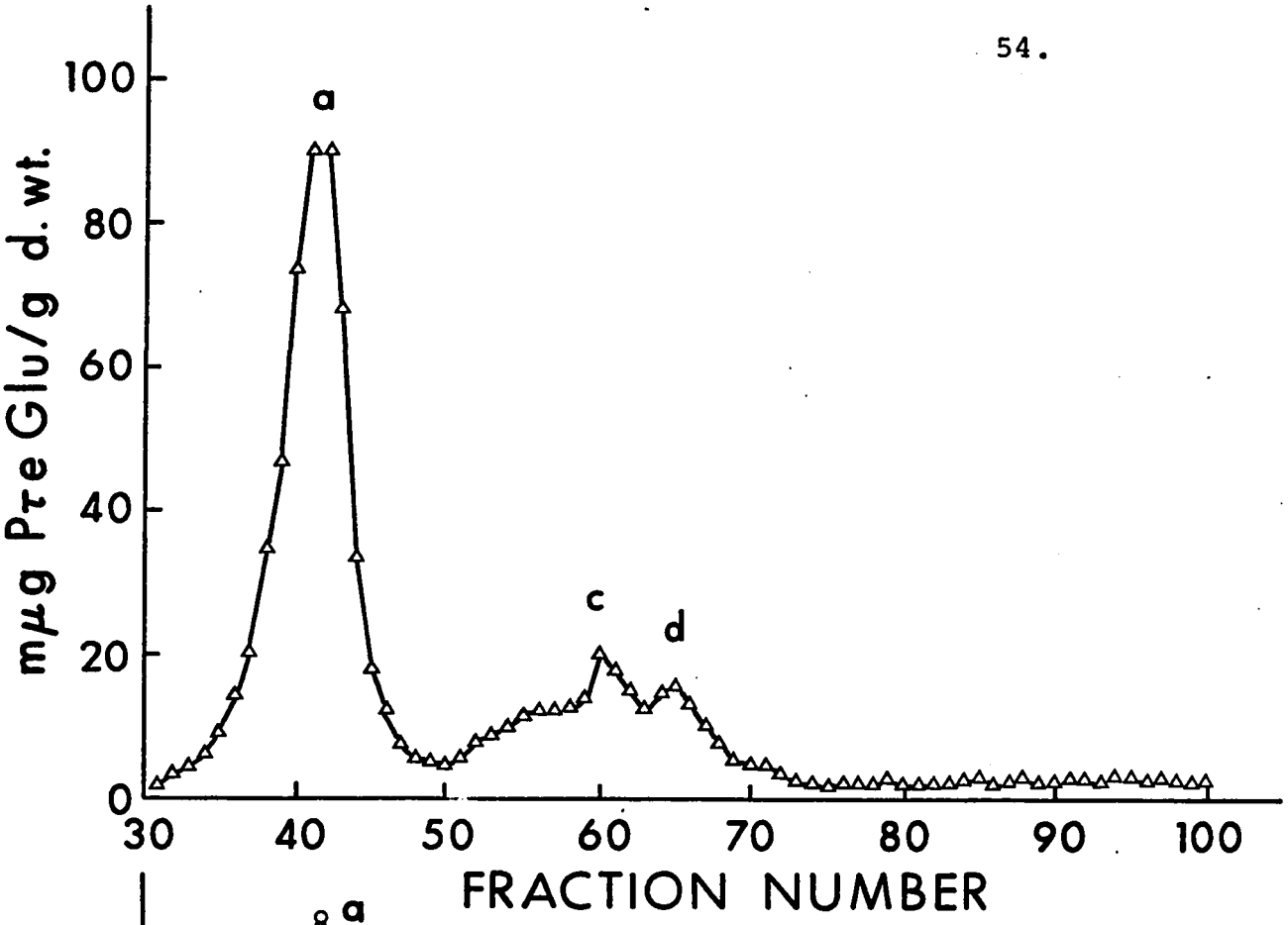
FIGURE 5

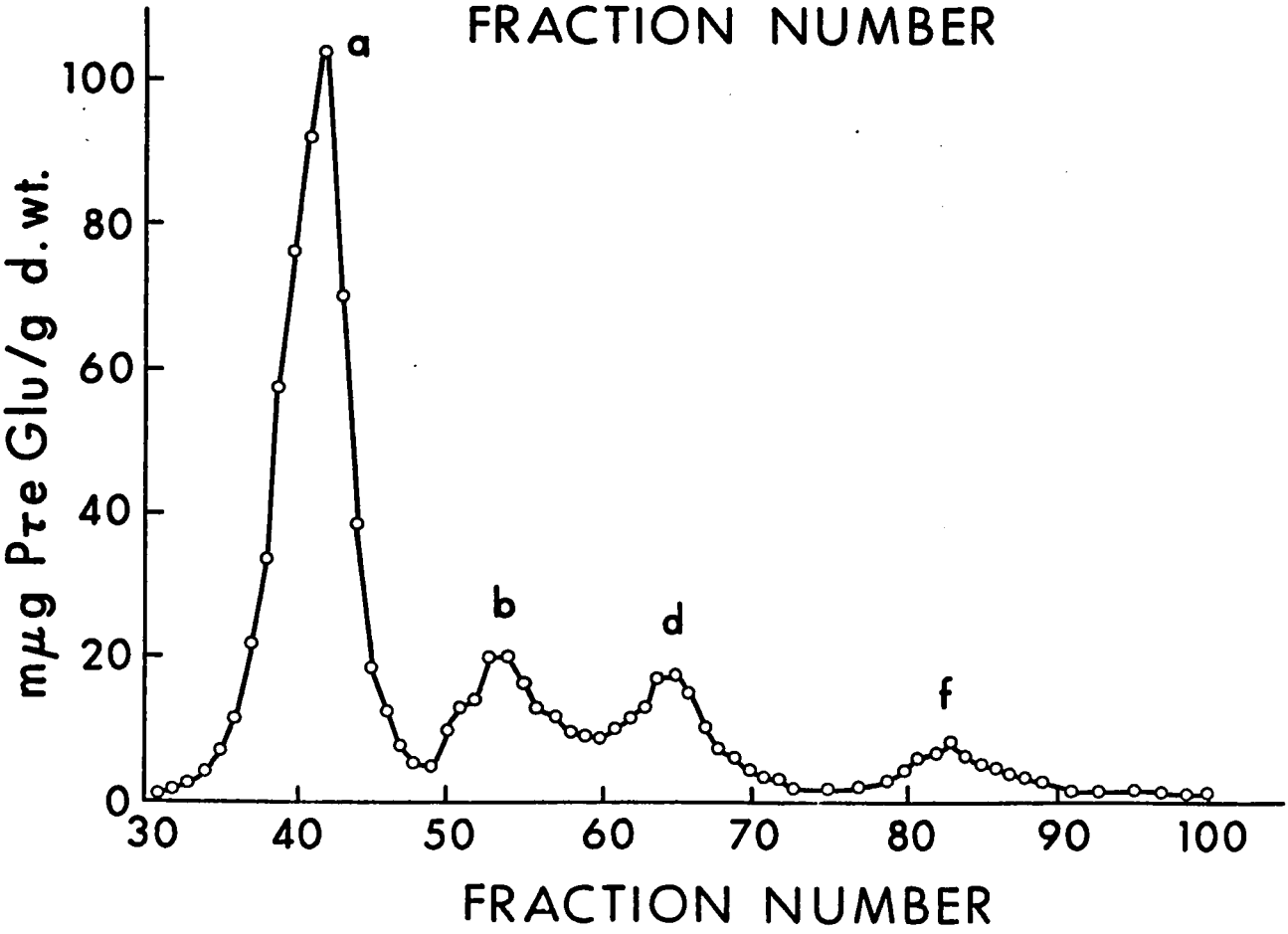
DIFFERENTIAL ASSAY OF PTEROYLGLUTAMATES FROM 3-DAY-OLD PEA COTYLEDONS

Derivatives were separated by DEAE-cellulose chromatography followed by assay using *S. faecalis* (Δ - Δ) and *P. cerevisiae* (o - o). The peaks are designated as:

Peak *a*, 10-HCO-H_4PteGlu; Peak *b*, 10-HCO-H_4PteGlu$_2$; Peak *c*, a pteroic acid derivative; Peak *d*, 5-HCO-H_4PteGlu and Peak *f*, 5-HCO-H_4PteGlu$_2$.

No growth response was obtained before fraction 30 or after fraction 100. The data are in PteGlu equivalents for *S. faecalis* and 5-HCO-H_4PteGlu equivalents for *P. cerevisiae*.


100
80
60
40
20
0
mμg Pτe Glu/g d.wt.
a
c
d
30
40
50
60
70
80
90
100
FRACTION NUMBER


100
80
60
40
20
0
mμg Pτe Glu/g d.wt.
a
b
d
f
30
40
50
60
70
80
90
100
FRACTION NUMBER

The identification of the separated pteroylglutamates was based upon (i) differential microbiological response (Table 1); (ii) a comparison of the position of elution with that of the authentic derivative (Table 1); (iii) co-chromatography with the authentic derivative; and (iv) the effect of chicken pancreas γ-glutamyl carboxypeptidase treatment followed by rechromatography. Several 'peaks' were separated (Figures 2-5). Peak *a* (Figures 2-5) supported the growth of the three bacteria and co-chromatographed with 10-HCO-H_4PteGlu. Autoclaving (115°C, 15 psi for 10 min.) a 5 ml sample of the 3-day-old cotyledon extract, in the presence of 0.5% (w/v) potassium ascorbate (pH 6.0) (Stokstad and Koch, 1967), resulted in loss of this peak and approximately 80% recovery of it in the 5-HCO-H_4PteGlu position (Fractions 60-70). On the basis of the criteria mentioned above, peak *a* was identified as 10-HCO-H_4PteGlu. This derivative was present in very small quantities after 1 day of germination (Figures 2 and 3), but increased substantially after 3 days of germination (Figures 4 and 5).

The presence of a very small growth response (peak *b*) by *L. casei* and *P. cerevisiae* occurred when fractions 50-57 were assayed (Figures 2-5). These fractions gave little growth of *S. faecalis*. This peak decreased in size and was largely converted to a derivative eluting with fractions 79-87 when autoclaved in the presence of ascorbate as described earlier. The effect of autoclaving suggested isomerization from 10-HCO-H_4PteGlu$_2$ to 5-HCO-H_4PteGlu$_2$.

Co-chromatography with authentic 10-HCO-H_4PteGlu$_2$ (Cossins and Shah, 1971, in preparation) gave a single peak of *L. casei* growth, comparable in position, to peak *b*. On the basis of these findings, peak *b* is tentatively identified as 10-HCO-H_4PteGlu$_2$.

A compound located in peak *c* was instrumental in supporting the growth of only *S. faecalis* (Figures 3 and 5) and presumably is a pteroic acid derivative (Stokstad and Koch, 1967). A comparison of Figures 3 and 5 shows that 3-day-old cotyledons contained appreciably higher levels of this derivative than 1-day-old seedlings. However, this increase was in no way as significant as that observed for 10-HCO-H_4PteGlu.

The major pteroylglutamate derivative (peak *e*) gave no detectable growth response with *S. faecalis* and *P. cerevisiae* (Figures 2-5). When co-chromatography was carried out with authentic 5-CH_3-H_4PteGlu, peak *e* increased in size and by the theoretical amount. On the basis of these findings and the other criteria mentioned earlier, it was concluded that the major pteroylglutamate present in pea cotyledons was 5-CH_3-H_4PteGlu.

Because 5-HCO-H_4PteGlu and 5-CH_3-H_4PteGlu were not clearly separated on the basis of *L. casei* growth (Figures 2-5) levels of the former compound were routinely measured using *P. cerevisiae* and *S. faecalis*. Confirmation of this derivative (peak *d*) was accomplished by co-chromatography with authentic 5-HCO-H_4PteGlu. Whether the presence of this compound represents its occurrence in pea cotyledons as such or isomerization of endogenous 10-HCO-H_4PteGlu is not certain.

H_4PteGlu, H_2PteGlu and PteGlu, although not detected in the extracts, may be present in very small quantities. Theoretically one would expect the former two derivatives to occur in tissues synthesizing pteroylglutamates as they have been clearly implicated as intermediates in the formation of reduced derivatives (Shiota, 1969; Iwai *et al.*, 1969; Mitsuda *et al.*, 1969). Authentic H_4PteGlu, synthesized by the method of Davis (1969), was readily detected in fractions 70-82 after chromatography (Table 1). This chemically synthesized compound was active in the assay of 5,10-CH_2=H_4PteGlu dehydrogenase (Ramasastri and Blakley, 1964; Cossins *et al.*, 1970) and co-chromatographed with samples of H_4PteGlu purchased from Nutritional Biochemical Corporation and Sigma. Although PteGlu was collected in fractions 125-145 (Table 1) and was readily assayed with *L. casei*, no evidence was obtained for an endogenous derivative in these fractions when the extracts were chromatographed.

A derivative (peak *g*) which supported the growth of *L. casei* but not of *P. cerevisiae* or *S. faecalis* was eluted later than H_4PteGlu (Figures 2 and 4). Tentative identification of this peak will be discussed later in this thesis.

Peak *f* was present in very small quantities (Figures 1 and 5) and was found to vary considerably with each replication of extraction. Although its position of elution is fairly similar to that of H_4PteGlu, a consideration of microbiological growth response suggested that it was a different derivative. For example, the response of *S. faecalis* to

authentic H_4PteGlu was 80-90% of the response by *L. casei*, however, *S. faecalis* did not appear to respond to peak *f* (Figures 3 and 5). It was also observed that after autoclaving the tissue extracts at 115°C and 15 lbs. for 10 minutes peak *f* increased slightly in size. Furthermore, treatment of the extracts with the plant peptidase resulted in considerable decrease in the levels of peak *f*. In contrast authentic H_4PteGlu was extensively oxidized by autoclaving and its quantity was not appreciably affected by peptidase treatment. The differential microbiological response, stability to autoclaving and peptidase treatment, suggest that peak *f* may be 5-HCO-H_4PteGlu$_2$.

Derivatives such as 5,10-CH_2=H_4PteGlu, 5,10-CH≡H_4PteGlu or 5-HCNH-H_4PteGlu although implicated in one-carbon metabolism (Blakley, 1969) would not occur in the present extracts for the following reasons: 1) 5,10-CH_2=H_4PteGlu would not be chromatographed at pH 6.0 as it is unstable at this pH value, dissociating into formaldehyde and H_4PteGlu (Blakley, 1969). Chromatography of this derivative has been successful at higher pH values; for example, Ramasastri and Blakley (1964) carried out their separations at pH 8.0. 2) 5,10-CH≡H_4PteGlu is stable at very low pH values and would be converted to 10-HCO-H_4PteGlu in our extracts under conditions employed in the present work (Blakley, 1969). 3) 5-HCNH-H_4PteGlu would also be lost during the present extractions as its half life in solution over the pH range 5-9 is 1 hour at 37°C and less than 1 minute at 100°C (Blakley, 1969). Clearly any quantity

of this derivative remaining after extraction by the present technique would be difficult to detect.

Determinations of the total quantity of 5-CH_3-H_4PteGlu and 10-HCO-H_4PteGlu in pea cotyledons were extended to cover a seven day germination period. The pteroylglutamates were determined by differential microbiological assay of fractions collected after DEAE-cellulose chromatography. As suggested by the data in Figure 1, there is a net synthesis of both these derivatives during this period (Table 2). However, the increases in the levels of these compounds were much less between 5 and 7 days of germination.

TABLE 2

LEVELS OF 10-HCO-H_4PTEGLU AND 5-CH_3-H_4PTEGLU IN GERMINATING PEA COTYLEDONS

Age	mμg derivative/g.d.wt.	
	10-HCO-H_4PteGlu*	5-CH_3-H_4PteGlu**
1	Trace	345
3	440	2180
5	580	2760
7	640	2920

Pteroylglutamates were extracted by Method I and samples (1-5 mls) of the extracts were subjected to DEAE-cellulose chromatography. Assays were performed on the fractions using *L. casei*, *S. faecalis* and *P. cerevisiae*.

* average of assays with all three bacteria.

** average of assays with *L. casei*.

The data are in PteGlu equilavents for *L. casei* and *S. faecalis* and 5-HCO-H_4PteGlu equilavents for *P. cerevisiae*.

II. *THE EFFECT OF EXTRACTION PROCEDURE ON THE TOTAL PTEROYLGLUTAMATE CONTENT OF GERMINATING PEAS.*

When pteroylglutamates were extracted by Method I subsequent treatment with hog kidney γ-glutamyl carboxypeptidase failed to increase the level of derivatives as measured by *L. casei*. Although in the present investigation attempts were made to minimize any endogenous γ-glutamyl carboxypeptidase activity, the possibility remains that such an enzyme could be present and could hydrolyze polyglutamates during the isolation procedure. The presence of such an active enzyme in the extracts, while not necessarily altering the total levels of pteroylglutamates assayed, could conceivably alter the levels of polyglutamates detected after extraction. Examination for such activity in pea cotyledon extracts was carried out using Difco Bacto yeast extract as substrate.

It is clear from Table 3 that in the presence of the plant extract, considerable hydrolysis of polyglutamates occurred. The extent of this hydrolysis was very similar to that observed when hog kidney γ-glutamyl carboxypeptidase was used (Table 3).

The presence in cotyledons of an enzyme capable of hydrolyzing polyglutamyl derivatives might therefore explain why these compounds were not detected when Method I was employed. To examine this point in more detail precautions were taken to kill the tissues before extraction (Method II). For the sake of comparison other samples of tissues were

TABLE 3

HYDROLYSIS OF YEAST EXTRACTS BY PEPTIDASES FROM PEA COTYLEDONS AND HOG KIDNEY

Incubation	Pteroylglutamate Content as determined by *L. casei* (mμg)	
	Pea cotyledon enzyme	Hog kidney enzyme
Without enzyme	173	175
With enzyme	1580	1640
Pteroylglutamates released	1407	1465

Pteroylglutamates were assayed after incubation of 1 ml of yeast extract in reaction systems containing 0.1 M sodium acetate buffer (pH 4.7) and 1% (w/v) ascorbate. 1 ml of enzyme solution was added in a final volume of 10 mls and incubated for 2 hr at 35°C. Reaction systems containing boiled enzyme failed to increase the pteroylglutamate content as assayed with *L. casei*. The data are in PteGlu equilavents.

rapidly freeze dried (Method III) before inactivation and extraction. The results of these experiments are summarized in Table 4.

When Method I was employed the levels of derivatives were not appreciably affected by treatment with hog kidney γ-glutamyl carboxypeptidase. In contrast significant increases were observed after enzyme treatment of extracts prepared by Methods II and III. As the increase in response by *P. cerevisiae* was greater than that observed with *L. casei* it is clear that the polyglutamates which were released were in a large part compounds other than 5-CH_3-H_4PteGlu. It is also of interest to note that the data obtained when Methods II and III were employed (Table 4) show that the increase in response by *L. casei* was 15% in 1-day-old cotyledons and approximately 50% in 3-day-old cotyledons. The corresponding increase for *P. cerevisiae* was 50% in 1-day-old cotyledons and 66% in 3-day-old cotyledons.

The differences observed when Methods I and II were employed (Table 4) were further evident when such extracts were subjected to DEAE-cellulose chromatography (Figures 6 and 7, Table 5). As data obtained from extracts isolated by Method III were essentially similar to data obtained from Method II, they are not included in Table 5.

All of the derivatives present in extracts isolated by Method II were present after using Method I with the exception of three conjugated derivatives. These latter compounds designated as poly 1-3 in Table 5 gave only a slight growth response with *L. casei*. After individual carboxypeptidase

TABLE 4

THE EFFECTS OF EXTRACTION METHOD AND PEPTIDASE TREATMENT ON THE LEVELS OF PTEROYLGLUTAMATES IN PEA COTYLEDONS
(expressed as µg/g.d.wt.)

		Method I		Method II		Method III	
Treatment	Age	*L. casei*	*P. cerevisiae*	*L. casei*	*P. cerevisiae*	*L. casei*	*P. cerevisiae*
Without enzyme	1	0.27	0.14	0.24	0.06	0.24	0.06
With enzyme		0.27	0.13	0.28	0.11	0.29	0.12
Without enzyme	3	2.60	0.81	1.24	0.23	1.23	0.23
With enzyme		2.71	0.82	2.74	0.78	2.70	0.79

Pteroylglutamates were determined after incubation with and without enzyme. The reaction system contained, in addition to the enzyme, 0.1 M sodium acetate buffer (pH 4.7) and 1% (w/v) ascorbate in a final volume of 10 mls. After incubation for 2 hr at 35°C the reaction was stopped by boiling. The data are in PteGlu equilavents for *L. casei* and 5-HCO-H_4PteGlu equilavents for *P. cerevisiae*.

FIGURE 6

CHROMATOGRAPHY AND DIFFERENTIAL ASSAY OF PTEROYL-GLUTAMATES FROM 1-DAY-OLD COTYLEDONS

Derivatives were separated by DEAE-cellulose chromatography followed by assay using *L. casei* (▲ - ▲) and *P. cerevisiae* (● - ●). The peaks are designated as:

Peak *e*, 5-CH_3-H_4PteGlu; Peak *i*, a polyglutamate derivative.

No growth response was obtained before fraction 30 or after fraction 160. The recoveries for *L. casei* were 100±10% of the values obtained before chromatography. The data are in PteGlu equilavents for *L. casei* and 5-HCO-H_4PteGlu equilavents for *P. cerevisiae*.

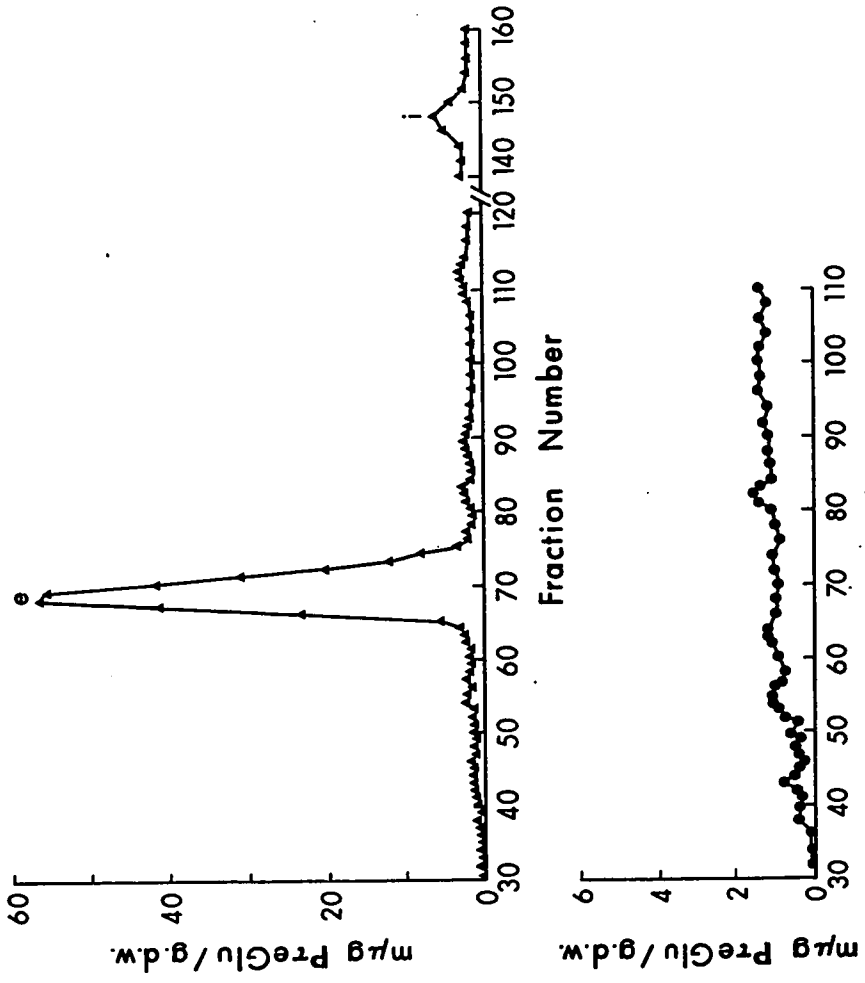

mμg PreGlu / g.d.w.
60
40
20
0
30
40
50
60
70
80
90
100
110
120
140
150
160
e
i
Fraction Number
mμg PreGlu / g.d.w.
6
4
2
0
30
40
50
60
70
80
90
100
110
Fraction Number

FIGURE 7

CHROMATOGRAPHY AND DIFFERENTIAL ASSAY OF PTEROYL-GLUTAMATES FROM 3-DAY-OLD COTYLEDONS

Derivatives were separated by DEAE-cellulose chromatography followed by assay using *L. casei* (▲ - ▲) and *P. cerevisiae* (● - ●). The peaks are designated as:

Peak *a*, 10-HCO-H_4PteGlu; Peak *b*, 10-HCO-H_4PteGlu$_2$; Peak *d*, 5-HCO-H_4PteGlu; Peak *e*, 5-CH_3-H_4PteGlu; Peak *f*, 5-HCO-H_4PteGlu$_2$; Peak *g*, 5-CH_3-H_4PteGlu$_2$; and Peak *h*, *i* and *j* as polyglutamyl derivatives.

No growth response was obtained before fraction 30 or after fraction 200. The recoveries for *L. casei* were 100±10% of the values obtained before chromatography. The data are in PteGlu equilavents for *L. casei* and 5-HCO-H_4PteGlu equilavents for *P. cerevisiae*.

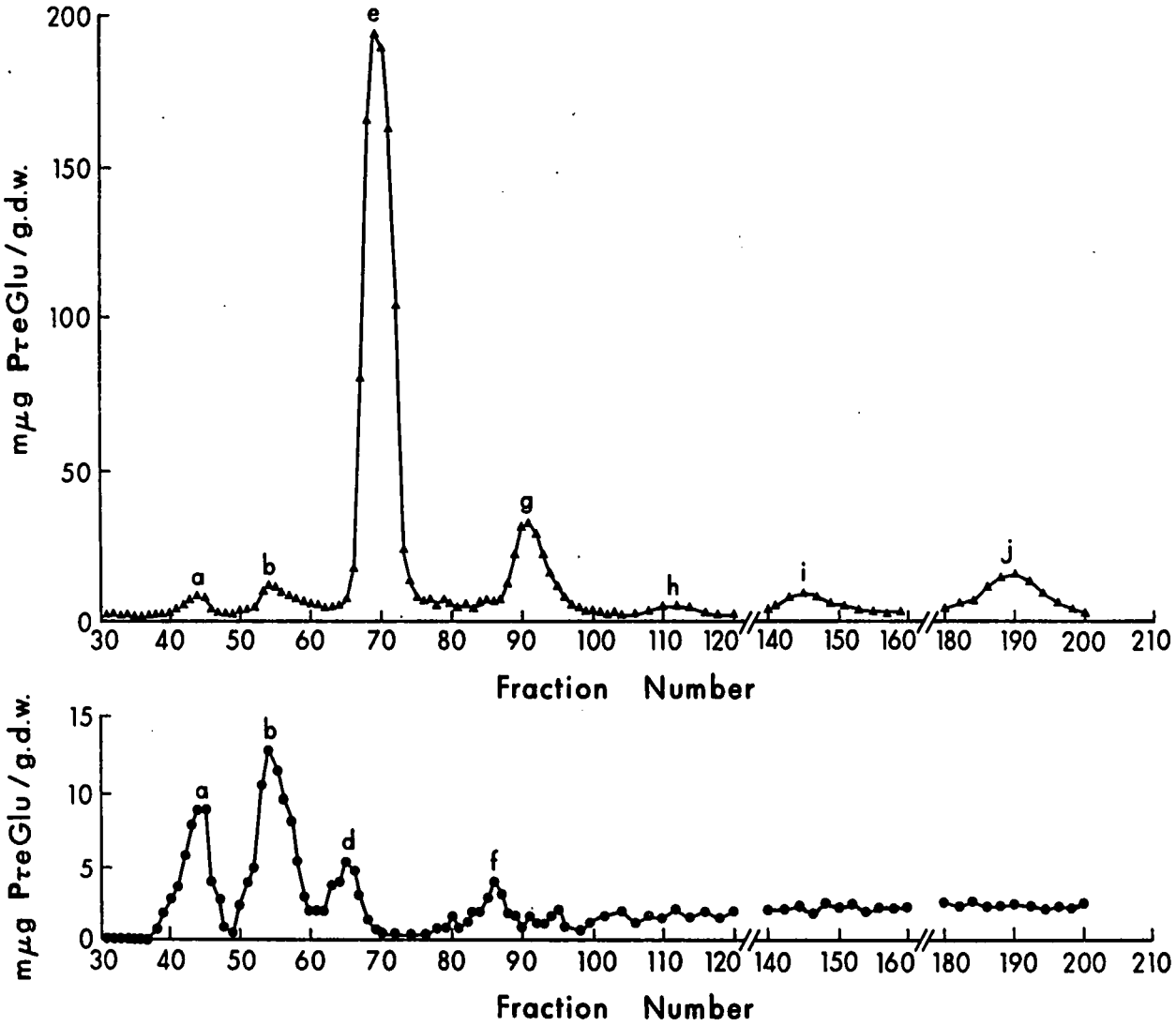

mμg PτeGlu / g.d.w.
200
150
100
50
0
a
b
e
g
h
i
j
30 40 50 60 70 80 90 100 110 120 140 150 160 180 190 200 210
Fraction Number
mμg PτeGlu / g.d.w.
15
10
5
0
a
b
d
f
30 40 50 60 70 80 90 100 110 120 140 150 160 180 190 200 210
Fraction Number

TABLE 5

LEVELS OF INDIVIDUAL PTEROYLGLUTAMATE DERIVATIVES IN 3-DAY-OLD PEA COTYLEDONS

	Method I mμg/g.d.wt.	Method II mμg/g.d.wt.	
Derivative	Before Peptidase Treatment	Before Peptidase treatment	After Peptidase treatment
10-HCO-H_4PteGlu	470	50	258
10-HCO-H_4PteGlu$_2$	131	80	36
5-HCO-H_4PteGlu	110	22	535
5-CH_3-H_4PteGlu	2150	895	2065
5-HCO-H_4PteGlu$_2$	52	18	9
5-CH_3-H_4PteGlu$_2$	225	204	315
Poly 1	*n.d.*	50 (80)*	*n.d.*
Poly 2	*n.d.*	87 (190)	*n.d.*
Poly 3	*n.d.*	172 (1200)	*n.d.*
TOTAL	3138	1578 (2739)	3218

* Values obtained after peptidase treatment of individual peaks.

n.d. - no detectable growth response with *L. casei*.

Data illustrating distribution of PteGlu derivatives following DEAE-cellulose column chromatography of extracts prepared by Methods I and II. Included in the Table are the increased growth responses obtained with *L. casei* after peptidase treatment of peaks identified as Poly 1, 2 and 3. Recoveries after peptidase treatment in column 3 were 100±10% of the values obtained when samples of the extracts were assayed with *L. casei*. The recovery of pteroylglutamates indicated in column 2 was 15% lower than expected, however, no additional peaks were eluted. The recovery of pteroylglutamates in column 1 were 100±10%. The data are in PteGlu equivalents.

treatments an increased growth response was observed for each of these compounds. Differential assay of these compounds after carboxypeptidase treatment revealed that poly 1 and 2, although supporting the growth of *L. casei*, failed to support the growth of *P. cerevisiae*. Poly 3 after such treatment effectively supported the growth of *L. casei* and to a lesser extent that of *P. cerevisiae*. The response of *P. cerevisiae* to poly 3 was 40% that of *L. casei*.

Carboxypeptidase treatment before chromatography of extracts prepared by Method II gave considerable increases in the levels of 5-CH_3-H_4PteGlu (Table 5). Although this treatment gave levels of 10-HCO-H_4PteGlu which were significantly lower than obtained in Method I the total formyl derivatives were increased. The discrepancy observed in 10-HCO-H_4PteGlu levels can be largely explained on the basis of isomerization to the 5-formyl derivative during incubation with the enzyme at pH 4.7. Consequently the levels of total formyl derivatives are very similar in both methods. Another noteworthy effect of carboxypeptidase treatments was the disappearance of the three conjugated derivatives. A compound, tentatively designated as 5-CH_3-H_4PteGlu$_2$ (Table 5) was present in all extracts. This peak appeared to contain a methylated derivative on the basis of its inability to support the growth of *S. faecalis* and *P. cerevisiae*. A similar derivative has been observed in pea root tips after avian carboxypeptidase treatment (Sengupta and Cossins, 1971). However the present peak may contain more than one derivative as treatment with

the carboxypeptidase from pea cotyledons actually increased the size of this peak rather than decreasing it as would be expected if it were entirely 5-CH_3-H_4PteGlu$_2$.

III. *THE EFFECT OF PTEROYLGLUTAMATE ANTAGONISTS ON GERMINATION AND SYNTHESIS OF PTEROYLGLUTAMATES IN PEA COTYLEDONS*

In the present studies, failure to detect PteGlu, H_2PteGlu and H_4PteGlu in the cotyledons could conceivably be due to their presence in extremely small pools or their absence from the tissues. The formation of H_2PteGlu and its reduction to H_4PteGlu by tetrahydrofolate dehydrogenase has been outlined in an earlier section of this thesis (Literature Review; Scheme 1, reaction 2). As H_4PteGlu is established as the acceptor of one-carbon units in a variety of biological systems it seems most unlikely that the present tissues are an exception to this. Blockage of the pathway for H_4PteGlu synthesis with aminopterin or amethopterin, both competitive inhibitors of the tetrahydrofolate dehydrogenase enzyme, should result in the accumulation of H_2PteGlu. It also follows that failure to synthesize H_4PteGlu should have pronounced effects on germination.

Pea seeds which had been imbibed in 10^{-4}M aminopterin or amethopterin solution followed by transfer to moist vermiculite failed to germinate (Table 6). On examination it was observed that the levels of pteroylglutamate derivatives were considerably lower than those of control peas of comparable age. DEAE-cellulose chromatography revealed that aminopterin and amethopterin treatments led to decreases in the quantity of

TABLE 6

THE EFFECT OF AMINOPTERIN AND AMETHOPTERIN ON LEVELS OF PTEROYLGLUTAMATE DERIVATIVES DURING GERMINATION OF PEA SEEDS

Treatment	Days of Germination	Radicle Length (mm)	Pteroylglutamates µg/g.d.wt. of cotyledons		
			10-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	PteGlu
Control	1	4.5	Trace	0.35	*n.d.*
	3	30.0	0.32	2.68	*n.d.*
Aminopterin	1	4.0	*n.d.*	0.15	0.11
	3	6.5	*n.d.*	*n.d.*	1.36
Amethopterin	1	4.0	Trace	0.14	0.12
	3	6.5	*n.d.*	*n.d.*	1.48

n.d. - not detected.

Pea seeds were allowed to imbibe in 10^{-4}M aminopterin or amethopterin for 24 hr, washed with distilled water and planted in vermiculite. Pteroylglutamates were extracted by Method I, separated by DEAE-cellulose column chromatography and assayed employing *L. casei*. The recoveries were 100±10% of those expected from values obtained with *L. casei* before chromatography. The data are in PteGlu equivalents.

5-CH_3-H_4PteGlu in one-day-old seedlings. Furthermore this compound although the major derivative in the control seeds after 3 days could not be detected in the samples treated with the antagonists (Table 6). Similarly no formylated derivatives were detected in the treated seeds. However a compound identified as PteGlu was readily detected in the treated seeds. The levels of PteGlu increased as the seeds were kept in moist vermiculite but repeated attempts to detect this derivative in the control seeds failed.

It was rather surprising that PteGlu, rather than H_2PteGlu, was isolated from the treated seeds. This observation appears to indicate that synthesis of H_2PteGlu and H_4PteGlu may proceed following the formation of PteGlu in these tissues. In other words a large part of pteroyl-glutamate synthesis may occur by a pathway involving addition of glutamic acid to pteroic acid.

IV. *FEEDING EXPERIMENTS EMPLOYING C^{14} LABELLED PTEROYL-GLUTAMATES AND FORMATE-C^{14}.*

The accumulation of PteGlu in cotyledons whose growth was inhibited with aminopterin or amethopterin (Table 6) suggested that this derivative may be an intermediate in synthesis of more highly reduced compounds such as 5-CH_3-H_4-PteGlu. On the other hand, tetrahydro derivatives may be synthesized by a pathway not involving PteGlu (Brown, 1969; Iwai *et al.*, 1969; Shiota, 1969). In order to examine these possibilities micromolar quantities of C^{14} labelled PteGlu were supplied as shown in Table 7. After chromatography the

TABLE 7

INCORPORATION OF PTEGLU-2-C^{14} INTO PTEROYLGLUTAMATES OF GERMINATING PEA COTYLEDONS

Age (Days)	Derivative	Quantity mμg/g.d.wt.	Specific Activity cpm/mμg
1	10-HCO-H_4PteGlu	34	*n.d.*
	10-HCO-H_4PteGlu$_2$	20	*n.d.*
	5-CH_3-H_4PteGlu	285	18
	5-CH_3-H_4PteGlu$_2$	25	*n.d.*
	PteGlu	1040	149
3	10-HCO-H_4PteGlu	345	*n.d.*
	10-HCO-H_4PteGlu$_2$	38	*n.d.*
	5-CH_3-H_4PteGlu	2830	21
	5-CH_3-H_4PteGlu$_2$	300	23
	PteGlu	450	147

n.d. - no radioactivity detected.
Cotyledons were allowed to imbibe a solution containing 66 mμmoles of PteGlu-2-C^{14} and then germinated for periods up to 2 days. Pteroylglutamates were extracted by Method I, and separated by DEAE-cellulose column chromatography. Radioactivities were determined from the same fractions which were assayed for pteroylglutamates. The data are in PteGlu equilavents.

bulk of the radioactivity recovered from the columns was associated with pteroylglutamates as determined by microbiological assay. A significant amount of C^{14} was, however, associated with fractions which failed to support the growth of the three assay organisms. This radioactivity occurred in distinct 'peaks', the bulk being collected in fractions 19-28. No evidence was obtained for labelling of 10-HCO-H_4PteGlu which, nevertheless, was readily detected by microbiological assay.

As pteroylglutamates were extracted by Method I in the present experiment, little information was obtained pertaining to incorporation of the radioactivity into polyglutamyl derivatives. In order to examine this point, pteroylglutamates were extracted from the cotyledons by Method II. After chromatography a significant amount of radioactivity was found to be associated with the polyglutamyl derivatives of the tetrahydro form. This is in agreement with the findings of Sengupta and Cossins (1971) who found that radioactivity from PteGlu-2-C^{14} was incorporated into polyglutamyl derivatives in the root tips of germinating peas. It is of interest that in this latter experiment very little if any PteGlu-2-C^{14} became conjugated. Microbiological assay and specific activities of PteGlu-2-C^{14} were very similar to the data reported for Method I.

As conjugated derivatives of PteGlu-2-C^{14} were detected in the feeding experiments, it is conceivable that PteGlu is converted to more highly reduced derivatives such as

5-CH_3-H_4PteGlu before additional glutamic acid moieties are added. To consider this possibility, micromolar quantities of 5-$C^{14}H_3$-H_4PteGlu were supplied as shown in Table 8. Chromatography revealed that the bulk of the radioactivity was associated with pteroylglutamates as determined by microbiological assay. Again a considerable amount (5-10%) of the radioactivity was associated with fractions which failed to support the growth of the three assay organisms. This latter radioactivity increased in quantity as the peas germinated. It occurred in distinct 'peaks' with the bulk being collected in fractions 13-18. Less than 5% of this radioactivity (fractions 13-18) represented impurities present in the 5-$C^{14}H_3$-H_4PteGlu. Chromatography on DEAE-cellulose columns and differential assay employing *L. casei* and *P. cerevisiae* suggested the major impurity present was 5-HCO-H_4PteGlu and representing about 7% of the growth response obtained by *L. casei*. Table 8 illustrates that the polyglutamate derivatives (poly 1-3) contained a considerable amount of radioactivity after supplying 5-$C^{14}H_3$-H_4PteGlu. However, radioactivity was not detected in 5-CH_3-H_4PteGlu$_2$. Furthermore, no radioactivity was found to be associated with the peaks of 10-HCO-H_4PteGlu$_2$ or its monoglutamate. The association of radioactivities with poly 1-3 clearly illustrated that these tetrahydro derivatives can be readily synthesized from 5-$C^{14}H_3$-H_4PteGlu in the tissues.

One-carbon units which enter the pteroylglutamate pool are believed to be derived mainly from precursors such as

TABLE 8

INCORPORATION OF 5-$C^{14}H_3$-H_4PTEGLU INTO PTEROYLGLUTAMATES OF GERMINATING PEA COTYLEDONS

Age (Days)	Derivative	Quantity mμg/g.d.wt.	Specific Activity cpm/mμg
1	10-HCO-H_4PteGlu	36	*n.d.*
	10-HCO-H_4PteGlu$_2$	12	*n.d.*
	5-CH_3-H_4PteGlu	874	228
	5-CH_3-H_4PteGlu$_2$	45	*n.d.*
	Poly 2	15	*n.d.*
3	10-HCO-H_4PteGlu	85	*n.d.*
	10-HCO-H_4PteGlu$_2$	60	*n.d.*
	5-CH_3-H_4PteGlu	1060	179
	5-CH_3-H_4PteGlu$_2$	156	*n.d.*
	Poly 1	55	10
	Poly 2	112	39
	Poly 3	195	25

n.d. - no radioactivity detected.
Cotyledons were allowed to imbibe a solution containing 80 mμmoles of 5-$C^{14}H_3$-H_4PteGlu, and then germinated for periods up to 3 days. Pteroylglutamates were extracted by Method I, and separated by DEAE-cellulose column chromatography. Radioactivities were determined from the same fractions which were assayed for pteroylglutamates. The data are in PteGlu equilavents.

serine or glycine as illustrated in Scheme 1 of the Literature Review. This situation was difficult to verify *in vivo* as such compounds are still not available with sufficiently high specific radioactivities.

Attempts were made, however, to label the pteroylglutamate pool with formate-C^{14}. As pea cotyledons are known to contain high levels of 10-HCO-H_4PteGlu synthetase (Iwai *et al.*, 1969), this substrate should be readily incorporated *in vivo*. The results of such an experiment are illustrated in Table 9. Radioactivity was found to be associated with only 10-HCO-H_4PteGlu. Repeating this experiment gave considerable variation in the specific activities obtained for 10-HCO-H_4PteGlu. In this experiment, greater than 95% of the radioactivity was found associated with peaks, chromatographing earlier than the 10-HCO-H_4PteGlu position. These other labelled compounds probably included serine which is readily formed from formate-C^{14} by plant tissues (Cossins and Sinha, 1965).

Pteroylglutamates were also extracted from the embryos of 3-day-old seedlings which had been allowed to imbibe a solution of PteGlu-2-C^{14}. The extraction procedure did not involve prior killing of the tissues (Method I) and following chromatography a number of labelled derivatives were separated (Table 10). Radioactivity was present in 5-CH_3-H_4PteGlu, the specific activity of this derivative being 16 cpm/mμg. In contrast, 5-CH_3-H_4PteGlu$_2$ had a much lower specific activity of 7 (Table 10). These specific

TABLE 9

INCORPORATION OF FORMATE-C^{14} INTO PTEROYLGLUTAMATES OF GERMINATING PEA COTYLEDONS

Age (Days)	Derivative	Quantity mμg/g.d.wt.	Specific Activity cpm/mμg
1	10-HCO-H_4PteGlu	16	*n.d.*
	10-HCO-H_4PteGlu$_2$	6	*n.d.*
	5-CH_3-H_4PteGlu	365	*n.d.*
	5-CH_3-H_4PteGlu$_2$	38	*n.d.*
3	10-HCO-H_4PteGlu	520	25
	10-HCO-H_4PteGlu$_2$	146	*n.d.*
	5-CH_3-H_4PteGlu	2384	*n.d.*
	5-CH_3-H_4PteGlu$_2$	274	*n.d.*

n.d. - no radioactivity detected.

Cotyledons were allowed to imbibe a solution containing 230 mμmoles of formate-C^{14} and then germinated for periods up to 3 days. Pteroylglutamates were extracted by Method I, and separated by DEAE-cellulose column chromatography. Radioactivities were determined from the same fractions which were assayed for pteroylglutamates. The data are in PteGlu equilavents.

TABLE 10

INCORPORATION OF PTEGLU-2-C^{14} INTO PTEROYLGLUTAMATES OF 3-DAY-OLD EMBRYOS

Derivative	Quantity mμg/g.d.wt.	Specific Activity cpm/mμg
10-HCO-H_4PteGlu	890	*n.d.*
10-HCO-H_4PteGlu$_2$	984	*n.d.*
5-CH_3-H_4PteGlu	9521	16
5-CH_3-H_4PteGlu$_2$	2329	7
PteGlu	1515	36

n.d. - not detected.

Pea cotyledons were allowed to imbibe a solution containing 66 mμmole of PteGlu-2-C^{14}. After 3 day germination period, the embryos were carefully removed and extracted by Method I, and fractionated by DEAE-cellulose column chromatography. Pteroylglutamates were assayed using *L. casei*. The radioactivities were determined from the same fractions which were assayed for pteroylglutamates. The data are in PteGlu equilavents.

activities were, therefore, lower than those calculated for the earlier experiment (Table 7) with pea cotyledons. The specific activity of the PteGlu isolated from the embryos was only 36, considerably lower than that obtained from the cotyledons (Table 7). This difference, therefore, suggests that PteGlu present in the embryos is not entirely derived from the cotyledonary pool.

V. *STUDIES INVOLVING CELL-FREE SYSTEMS*

The results of the previous feeding experiments indicate that the principal pteroylglutamates in pea cotyledons are synthesized during the early stages of germination. When considered in the light of studies on the occurrence of enzymes catalyzing the synthesis and interconversion of these compounds in plants (Cossins *et al.*, 1970), it is logical to conclude that the one-carbon metabolism of pea seedlings can, in large part, be summarized as in Scheme 1, page 13. To ascertain whether this conclusion was valid, the following experiments were conducted using cell-free extracts of 3-day-old pea cotyledons.

a. *Synthesis of Pteroylglutamate Derivatives in vitro*

In these experiments, cell-free extracts which had been fractionated with ammonium sulphate (see Methods and Materials) were examined for ability to synthesize formyl and methyl derivatives of H_4PteGlu. As pea cotyledons are known to contain high levels of 5,10-CH_2=H_4PteGlu dehydrogenase activity (Cossins *et al.* 1970), reaction systems containing formaldehyde were examined, in addition to those containing

serine and formate respectively. After incubation the products formed were separated by DEAE-cellulose chromatography and assayed microbiologically with *L. casei*. The results of such experiments are summarized in Tables 11-13.

In complete reaction systems containing formaldehyde, $NADPH_2$ and $H_4PteGlu$ (Table 11), it is clear that a number of pteroylglutamate derivatives were formed. Control reaction systems, containing all of the above components, but without addition of the plant protein, were found to contain only the substrate $H_4PteGlu$ after this incubation period. Pteroylglutamates formed in the complete reaction system included both 10- and 5-formyl derivatives as well as a significant amount of $5\text{-}CH_3\text{-}H_4PteGlu$. In addition to these identified derivatives, a major 'peak' of *L. casei* growth was associated with fractions 47-55. As this 'peak' was not detected in reaction systems which did not contain plant protein, the compound responsible for this growth response cannot be an impurity in the $H_4PteGlu$ used. It is interesting to note that this compound was also absent from reaction systems which did not contain formaldehyde (Table 11). The possibility, therefore, remains that this compound may be an oxidation product of a formylated $H_4PteGlu$ derivative. This latter possibility is, to some extent, borne out by the observation (Table 11) that reaction systems which did not contain added $NADPH_2$ produced smaller amounts of the formyl derivatives and the unidentified compound. Such reaction systems also failed to produce any $5\text{-}CH_3\text{-}H_4PteGlu$ and

TABLE 11

SYNTHESIS OF PTEROYLGLUTAMATES BY CELL-FREE EXTRACTS CONTAINING FORMALDEHYDE

Omission from reaction system*	Quantity of pteroylglutamates (µg/reaction system)					
	10-HCO H_4PteGlu	Fractions 47-55	5-HCO- H_4PteGlu	5-CH_3- H_4PteGlu	H_4PteGlu	Total
NONE	9.5	13.5	5.9	6.3	1.4	36.6
$NADPH_2$	1.7	2.8	4.4	absent	13.9	22.7
FORMALDEHYDE	0.9	absent	absent	19.8	7.9	28.6

* The complete reaction system contained in a final volume of 4 mls; 22 µmoles potassium phosphate buffer (pH 6.7); 50 µmoles mercaptoethanol; 1.0 µmole FAD; 12.5 µmoles formaldehyde; 89 mµmoles H_4PteGlu, 3.5 µmoles $NADPH_2$ and the tissue extract (20 mg of protein in 0.3 ml of solution). The reaction systems were incubated at 32°C for 90 min. The reaction was then terminated by boiling in the presence of ascorbate (pH 6.7) and the products separated by chromatography on DEAE-cellulose. Derivatives were assayed microbiologically. The data are in PteGlu equilavents.

considerable amounts of H_4PteGlu remained in the system after the incubation period.

On the basis of these experiments, it is logical to conclude that the cell-free extracts contained enzymes capable of oxidizing 5,10-CH_2=H_4PteGlu to formyl derivatives. Furthermore, the dependence on $NADPH_2$ for production of 5-CH_3-H_4PteGlu suggests that such extracts also contained 5,10-CH_2=H_4PteGlu reductase activity. However, when the products formed in reaction systems not containing formaldehyde were examined (Table 11) some inconsistencies were apparent. For example, the omission of formaldehyde from the reaction system was not associated with a decrease in the production of 5-CH_3-H_4PteGlu. Clearly, production of this pteroylglutamate under the present conditions did not require the addition of formaldehyde. In contrast, synthesis of the formyl derivatives and the unidentified compound were drastically affected by this omission. The possibility, therefore, remains that such extracts contained endogenous substrates capable of donating 1-carbon units for production of 5-CH_3-H_4PteGlu.

In order to examine these possibilities, further experiments were conducted with formate and serine as potential donors of 1-carbon units. The results of such experiments are summarized in Tables 12 and 13. It is clear from Table 12 that the production of 10-HCO-H_4PteGlu and 5-HCO-H_4PteGlu was affected when formate was not included in the reaction system. As in the earlier

TABLE 12

SYNTHESIS OF PTEROYLGLUTAMATES BY CELL-FREE EXTRACTS CONTAINING FORMATE

Omission from Reaction system*	Quantity of Pteroylglutamates (µg/reaction system					
	10-HCO-H_4PteGlu	Fractions 47-55	5-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	H_4PteGlu	Total
None	3.1	absent	2.4	18.0	7.3	30.8
$NADPH_2$	2.2	absent	2.0	absent	18.1	22.3
Formate	1.0	absent	absent	19.3	7.6	27.9

* The complete reaction system contained in a final volume of 4 mls: 17 µmoles of potassium phosphate buffer (pH 6.7); 50 µmoles mercaptoethanol; 1.0 µmole FAD; 12.5 µmoles formate; 89 mµmole H_4PteGlu; 15 µmoles ATP; 3.5 µmoles $NADPH_2$ and 20 mg of protein from the tissue extract. The reaction systems were incubated at 32°C for 90 minutes. The reactions were terminated by heating in a boiling water bath and in the presence of 280 µmoles potassium ascorbate (pH 6.7). The products were separated by chromatography on DEAE-cellulose and the derivatives assayed microbiologically. The data are in PteGlu equilavents.

TABLE 13

SYNTHESIS OF PTEROYLGLUTAMATES BY CELL-FREE EXTRACTS CONTAINING SERINE

Omission from reaction system*	Quantity of pteroylglutamates (µg/reaction system)					
	10-HCO-H_4PteGlu	Fraction 47-55	5-HCO-H_4PteGlu	5-CH_3-H_4PteGlu	H_4PteGlu	Total
None	5.6	4.3	3.8	13.5	7.8	35.0
$NADPH_2$	1.4	absent	2.0	absent	19.5	22.9
Serine and pyridoxal phosphate	0.9	absent	absent	19.8	7.9	28.6

* The complete reaction system contained in a final volume of 4 mls; 21 µmoles potassium phosphate buffer (pH 6.7); 50 µmoles mercaptoethanol; 1.0 µmole FAD; 12.5 µmoles serine; 89 mµmoles H_4PteGlu; 100 mµmoles P-P; 3.5 µmoles $NADPH_2$ and 20 mg of protein from the tissue extract. The reaction systems were incubated at 32°C for 90 min. The reactions were then terminated by heating in a boiling water bath and in the presence of 250 µmoles potassium ascorbate (pH 6.7). The products were separated by chromatography on DEAE-cellulose and the derivatives assayed microbiologically. The data are in PteGlu equilavents.

experiment (Table 11) levels of 5-CH_3-H_4PteGlu were drastically affected by omission of $NADPH_2$. However, omission of formate did not decrease production of this derivative. Similar results were obtained for reaction systems containing serine (Table 13). As in the earlier experiments, the production of formyl derivatives appeared to be related to the presence of serine in the reaction system, but synthesis of 5-CH_3-H_4PteGlu again did not require the addition of this 1-carbon precursor. On the basis of these experiments, it appears that the 1-carbon precursors were utilized in the formation of formyl derivatives but the possibility remains that some other precursor present in the extract was capable of donating 1-carbon units for synthesis of 5-CH_3-H_4PteGlu.

b. *5,10-CH_2=H_4PteGlu Dehydrogenase in Cell-free Extracts*

As the earlier experiments *in vitro* suggested that 5,10-CH_2=H_4PteGlu, generated from formaldehyde and H_4PteGlu, was extensively oxidized under the conditions used, it was concluded that this conversion would be in large part mediated by 5,10-CH_2=H_4PteGlu dehydrogenase (Scheme 1, page 13). In order to examine this possibility, extracts were prepared from pea cotyledons at various stages of germination and examined for this enzyme as described in the Materials and Methods.

(i) *Reaction requirements and nature of products formed.*

The reduction of NADP by the plant protein was found to have absolute requirements for formaldehyde and H_4PteGlu. The formation of 10-HCO-H_4PteGlu by

5,10-methylenetetrahydrofolate dehydrogenase was demonstrated to require NADP, H_4PteGlu and HCHO. Omission of any of these compounds from the cuvettes resulted in no significant changes in absorbance over a period of 10 min. Changes in absorbance at 340 mμ were in large part due to the reduction of NADP as addition of glutathione and purified yeast glutathione reductase (Sigma Chemical Company) led to rapid decreases in absorbance (Figure 8).

The pteroylglutamate products formed under these conditions were investigated by chromatography on DEAE-cellulose followed by differential microbiological assay. Table 14 shows that the main pteroylglutamate product was 10-HCO-H_4PteGlu. In addition, significant amounts of 5-HCO-H_4PteGlu and the unidentified compound (fractions 47-55) were also formed. No evidence was obtained for the production of 5-CH_3-H_4PteGlu. In this connection, it should be noted that these extracts were prepared in the absence of FAD, a compound known to be essential for protection of 5-CH_3-H_4PteGlu reductase activities from *Escherichia coli* during isolation (Katzen and Buchanan, 1965).

(ii) *Changes in enzyme activity during germination*

Data presented in an earlier part of this thesis shows that a pronounced biosynthesis of pteroylglutamates occurs during germination of pea cotyledons. In addition, extracts of these organs have been shown to be

FIGURE 8

PRODUCTION OF $NADPH_2$ BY 5,10-CH_2=H_4PTEGLU DEHYDROGENASE

The reaction cuvette contained in a total volume of 3 mls, 13.5 µmoles potassium phosphate buffer (pH 7.5); 12.5 µmoles formaldehyde; 0.8 µmole H_4PteGlu; 50 µmoles mercaptoethanol; 3.5 units of the enzyme and 1.8 µmole NADP. The reaction was followed at 340 mµ for 12 min. then 3 µmoles of glutathione (GSSG) and 0.04 mg yeast glutathione reductase were added as indicated by the arrow.

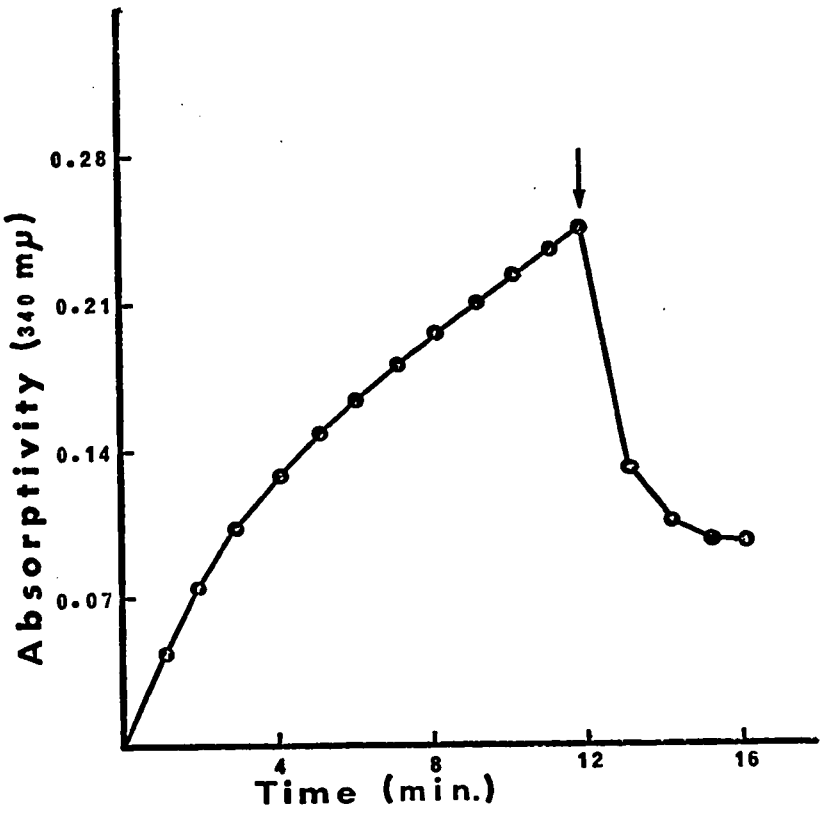

0.28
0.21
0.14
0.07
Absorptivity (340 mμ)
4
8
12
16
Time (min.)

TABLE 14

PTEROYLGLUTAMATE PRODUCTS FORMED FROM EXTRACTS CONTAINING 5,10-CH_2=H_4PTEGLU DEHYDROGENASE ACTIVITY

Derivatives	Quantity (µg/reaction system)
10-HCO-H_4PteGlu	125
Unidentified 'peak' Fractions 47-55	100
5-HCO-H_4PteGlu	57
H_4PteGlu	43
Total pteroylglutamates recovered	325

The reaction cuvette contained a total volume of 3 mls; 16.5 µmoles potassium phosphate buffer (pH 7.5); 12.5 µmoles formaldehyde; 0.8 µmole H_4PteGlu; 50 µmoles mercaptoethanol and the tissue extract containing 15 mg of protein. After addition of 1.8 µmole NADP changes in absorbance at 340 mµ were followed at 32°C for 20 min. The reaction was then terminated by immersion in a boiling water for 10 min. followed by cooling at 0°C. After dilution aliquots containing 120 mµg of pteroylglutamates were applied to DEAE-cellulose columns. Derivatives were assayed using *L. casei* and *P. cerevisiae*. The data are in PteGlu equilavents for *L. casei* and 5-HCO-H_4PteGlu equilavents for *P. cerevisiae*.

capable of producing the major endogenous pteroylglutamate derivatives *in vitro*. As synthesis of these derivatives would conceivably be catalyzed by the appropriate enzymes shown in Scheme 1, page 13 the possibility remains that such synthesis may be regulated by the levels of these enzymes in the tissues during germination. To examine this point, levels of 5,10-CH_2=H_4PteGlu dehydrogenase were examined in pea cotyledons during various stages of germination. In addition, cotyledons which were made pteroylglutamate deficient by treatment with aminopterin, were also examined for levels of this enzyme. Similarily, seeds treated with chloramphenicol and cycloheximide were also examined to determine whether these inhibitors of protein synthesis would modify the levels of this enzyme during germination. The results of such experiments are summarized in Table 15. In each case, levels of enzyme activity in crude homogenates were assayed using the standard reaction system (see Materials and Methods).

It is clear from Table 15 that seeds which had imbibed the pteroylglutamate antagonist and the protein inhibitors showed considerable inhibition of seedling development. Levels of enzyme activity were not, however, appreciably altered by these treatments. Similarily, the levels of enzyme activity in the controls were not altered appreciably during the period

TABLE 15

THE EFFECT OF CHLORAMPHENICOL, CYCLOHEXIMIDE AND AMINOPTERIN ON SEEDLING GROWTH AND ENZYME ACTIVITY DURING EXAMINATION

Seeds imbibed in	1	2	3	4	5	6	7
Distilled water:							
units/g f.wt.	62.2	69.7	54.1	61.9	60.5	54.9	58.2
units/mg protein	0.8	1.01	0.90	1.29	1.55	2.03	2.53
radicle length (mm)	4.5	11.5	35.0	55.0	70.0	86.0	100.0
plumule length (mm)	<2.0	<2.0	12.0	24.0	35.0	52.0	70.0
Chloramphenicol ($2x10^{-3}M$):							
units/g f.wt.		74.5		74.2		63.7	
units/mg protein		1.15		1.37		2.28	
radicle length (mm)		6.0		30.0		80.0	
plumule length (mm)		<2.0		11.0		35.0	
Cycloheximide ($2x10^{-4}M$):							
units/g f.wt.		77.9		69.8		71.0	
units/mg protein		1.11		1.20		2.15	
radicle length (mm)		5.5		14.0		33.0	
plumule length (mm)		<2.0		<2.0		15.0	
Aminopterin ($10^{-4}M$):							
units/g f.wt.		68.4		75.8			64.9
units/mg protein		0.88		1.40			1.70
radicle length (mm)		4.4		7.0			7.7
plumule length (mm)		<2.0		<2.0			<2.0

Levels of enzyme activity in crude homogenates were assayed using the following standard reaction system. The reaction cuvette contained in a total volume of 3 mls; 15.5 µmoles potassium phosphate buffer (pH 7.5); 12.5 µmoles formaldehyde; 0.8 µmole H_4PteGlu; 50 µmoles mercaptoethanol and the tissue extract containing 15 mg of protein. After addition of 1.8 µmole NADP changes in absorbance were followed at 340 mµ for 20 min. at 32°C.

of germination examined. In contrast, specific activity increased over the period of germination examined.

DISCUSSION

1. *SYNTHESIS OF PTEROYLGLUTAMATES DURING GERMINATION*

The present studies clearly illustrate that a net synthesis of pteroylglutamates occurs in the cotyledons of germinating pea seeds (Figure 1). The levels after 4 days of germination were approximately 15 times greater than the levels found in the dormant seeds. This physiological event may be characteristic of many germinating tissues and may apply also to other vitamins. Its relative importance from a nutritional standpoint should, therefore, be readily appreciated. In earlier studies, with monocotyledonous seeds, Burkholder (1943) presented evidence for synthesis of pteroylglutamic acid during germination of oats, wheat, barley and maize. In contrast, however, later work by Ghanekar and Braganca (1960) on the levels of pteroylglutamates in *Cicer arientinum* reported a marked decrease during germination. However, as peroxidase activity was high in the experimental material, it is not clear whether this enzyme, in oxidizing pteroylglutamic acid to 2-amino-4-hydroxy-6-formyl-pteridine and *p*-aminobenzoylglutamic acid, could account for the decreases observed under natural conditions. In agreement with the present work, Erismann and Schopfer (1959) showed that germinating pea seedlings synthesize pteroylglutamates. Synthesis of PteGlu and HCO-H_4PteGlu was more pronounced when the seedlings were illuminated. However, as these workers did not use all three assay organisms and did not report separations of the individual derivatives, the exact nature

of these syntheses is difficult to ascertain.

Studies with animal tissues (Noronha and Silverman, 1962; Silverman *et al.*, 1961) and yeast cultures (Schertel *et al.*, 1965) clearly illustrate that 5-CH_3-H_4PteGlu and its polyglutamyl derivatives are the major components of the pteroylglutamate pool. In *Bacillus subtilis*, however, little or no 5-CH_3-H_4PteGlu or polyglutamyl derivatives have been found (Sirotnak *et al.*, 1963). As indicated in the Literature Review, 5-CH_3-H_4PteGlu had not been detected in higher plant tissues before the present work.

It is clear from the present investigations that 5-CH_3-H_4PteGlu and its polyglutamyl derivatives accounted for approximately 80% of the pteroylglutamate pool of pea cotyledons with formyl derivatives of H_4PteGlu accounting for much of the remainder (Table 5). Conceivably this tissue must be highly dependent upon 5-CH_3-H_4PteGlu as a source of methyl groups. Several workers have, in fact, shown that 5-CH_3-H_4PteGlu is an active donor of methyl groups to homocysteine forming methionine in a wide variety of tissues (Woods *et al.*, 1965; Taylor and Weissbach, 1966; Dodd and Cossins, 1970). In earlier work from this laboratory, Dodd and Cossins (1970) concluded that this reaction was of major importance as a route for the *de novo* synthesis of methionine. Clearly, this amino acid, besides being incorporated into protein, may itself act as a source of methyl groups for a variety of transmethylation reactions mediated by S-adenosyl-methionine (Dodd, 1969).

Considering these interrelationships, the present studies suggest that the metabolic fate of 5-$C^{14}H_3$-H_4PteGlu would be worthy of close examination. Of particular interest would be an investigation of incorporation of the methyl group into nucleic acids and a determination of the rate of turnover of this pteroylglutamate during various stages of germination.

Very recently, Okinaka and Iwai (1970) also presented data showing an increase of pteroylglutamate content in germinating pea cotyledons. The increase they reported appears less than that reported in the present studies. As *S. faecalis* was the only assay organism used, these workers would actually not have assayed approximately 80% of the pteroylglutamates present *in vivo*. Iwai's group have contributed considerable information to this field, particularly their elucidation of the biosynthesis of dihydropteroylglutamic acid in plants (Iwai *et al.*, 1969) and the biosynthesis of dihydropteroic acid from 2-amino-4-hydroxy-6-hydroxymethyl dihydropteridine (Okinaka and Iwai, 1970a, 1970b). It is of considerable interest to point out that these workers and others (*e.g.* Brown, 1969; Shiota, 1969) have shown that the enzymes involved utilize only the dihydro derivatives as substrates. As this had been generally accepted for some time, it was felt, in the present studies, that the presence and accumulation of H_2PteGlu in tissue extracts would be demonstrable, particularly where the biosynthetic pathway had been blocked by aminopterin. As

pea cotyledons which were allowed to imbibe this inhibitor (Table 6) showed no significant signs of germination, the necessity for the fully reduced forms of this vitamin was suggested. Examination of the pteroylglutamates present under these conditions (Table 6) clearly illustrated that no tetrahydro forms of the vitamin were synthesized. In agreement with the above workers, the biosynthesis appeared to occur via reduction of H_2PteGlu and PteGlu and did not involve the addition of glutamic acid to tetrahydropteroic acid. One anomaly, however, was the isolation of PteGlu rather than H_2PteGlu in these experiments. It may be that H_2PteGlu was oxidized to PteGlu during the extraction procedure. Enzymes catalyzing the oxidation of H_2PteGlu and H_4PteGlu have been detected in pea cotyledon extracts (Cossins, unpublished work). However, it has been shown that H_2PteGlu can be isolated by DEAE-cellulose column chromatography as readily as H_4PteGlu. Its position of elution fell between that of H_4PteGlu and PteGlu as noted earlier by Silverman *et al.* (1961). In the present studies, H_4PteGlu added to crude tissue extracts before boiling was successfully detected later after column chromatography so there is reason to believe, on this basis, that a significant amount of H_2PteGlu should still have been present in extracts of the aminopterin treated peas.

When peas, which had been allowed to imbibe aminopterin, were extracted by Method II, no conjugation of PteGlu was observed, so it appears that this process occurs after

hydrogenation in this tissue. Labelled PteGlu when fed to aminopterin treated cotyledons was subsequently isolated as PteGlu in the tissue extracts prepared by Method II. However, in the control seeds, radioactivity was also associated with polyglutamyl methylated derivatives of H_4PteGlu. Apart from labelling of PteGlu, the bulk of the radioactivity occurred in 5-CH_3-H_4PteGlu. This indicates that 5-CH_3-H_4PteGlu was the direct precursor of the conjugated derivatives found in germinating cotyledons. Additional support for this suggestion came from the 5-$C^{14}H_3$-H_4PteGlu feedings experiments. Radioactivity from this derivative readily appeared in the conjugated compounds (Table 8).

Metabolic compartments have now been shown for many metabolites (*e.g.* Oaks and Bidwell, 1970). Compartmentalization may also be of considerable importance in the biosynthesis of the pteroylglutamate derivatives. Iwai *et al.* (1968) reported that dihydrofolate synthetase in pea seedlings which catalyzes the formation of dihydropteroylglutamic acid from dihydropteroic acid and L-glutamic acid, is located in the mitochondrial fraction. On the other hand, the dihydropteroate synthetase was found to be associated with the mitochondrial as well as the soluble fraction (Okinaka and Iwai, 1970). Earlier, Iwai *et al.* (1967) reported that formyl tetrahydrofolate synthetase in pea seedlings was located in the soluble fraction. The conclusion finally drawn by Iwai's group was that most pteroylglutamates probably up to H_2PteGlu are synthesized in the mitochondria

with synthesis of more reduced derivatives occurring in the cytoplasm. If this is the case, then the question arises of why no radioactivity could be detected in the 10-HCO-H_4PteGlu pool following PteGlu-2-C^{14} feedings when labelling of the 5-CH_3-H_4PteGlu pool was appreciable (Table 7). Considering the specific radioactivity (18-21 cpm/mμg) of 5-CH_3-H_4PteGlu, it is clear that saturation of the 10-HCO-H_4PteGlu pool with C^{14} would have given a detectable peak of C^{14} after chromatography. The present data are, however, readily explained if one assumes that compartmentalization of pteroylglutamates occurs in pea cotyledons. Although PteGlu-2-C^{14} must have ready access to the cytoplasmic pool, its accessibility to other compartments may be restricted. In the light of this observation, more recent studies in this laboratory (Clandinin and Cossins, unpublished data) have shown that isolated plant mitochondria contain significant levels of a pteroic acid derivative(s) as well as significant amounts of the formylated and methylated tetrahydro derivatives. In addition, such mitochondria have ability to catalyze the interconversion of several pteroylglutamates. In this connection, it is of interest to note that mitochondria appear to have considerable ability to form 10-HCO-H_4PteGlu but less ability to synthesize 5-CH_3-H_4PteGlu. Considering the many reactions in which pteroylglutamates participate, an understanding of the metabolic interrelationship between these compartments may have an important bearing on the regulation of biosynthetic

pathways during germination.

2. *TURNOVER AND BIOSYNTHESIS OF ONE-CARBON UNITS DURING GERMINATION*

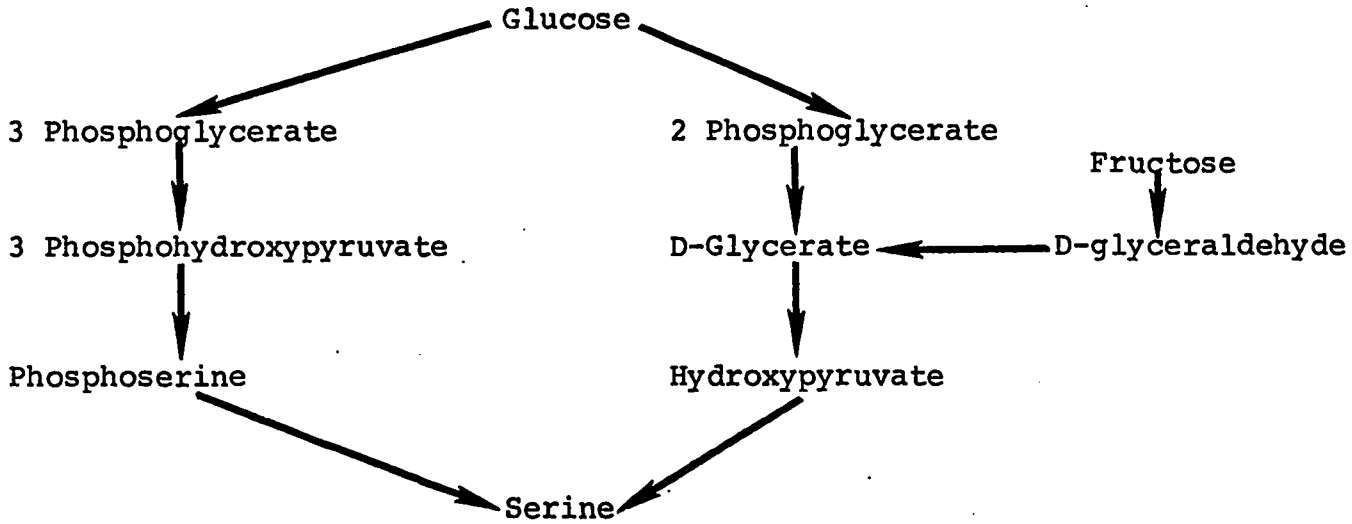
Some of the important donors of one-carbon units in tissues of various organisms are illustrated in Scheme I, page 13 of the Literature Review. At the present time, however, the quantity of one-carbon units transferred to other metabolites is unknown. It might be assumed that turnover rates would be quite high. Theoretically tracer studies utilizing 5-$C^{14}H_3$-H_4PteGlu would be expected to give a good indication of this, based upon the rate of disappearance of the label from the methyl carbon. The experiments which were conducted (Table 8), indicated, however, that the disappearance of ^{14}C was nearly negligible during the three day period studied. The specific radioactivities in Table 8 are based upon assays using *L. casei* and include conjugated as well as the monoglutamate forms. When the total radioactivities recovered are expressed as cpm/mμg of pteroylglutamate following hydrolysis of the polyglutamate forms, values of 228 cpm/mμg and 179 cpm/mμg of total pteroylglutamate are obtained which illustrate that essentially none of the radioactivity left the methyl pteroylglutamate pool. The slight lowering of specific activity conceivably represents a dilution effect rather than disappearance of ^{14}C from the pool. This possibility may be more readily studied by supplying smaller quantities of the 5-$C^{14}H_3$-H_4PteGlu derivative. Although physiological quantities were supplied,

the labelled solution contained quantities of the metabolically inactive isomer. Thus appreciable levels of 'excess' 5-CH_3-H_4PteGlu will remain in the tissues after these relatively long experimental periods. These problems make interpretation of turnover very difficult. If turnover rates are low then why do the levels of these compounds build-up during germination? Perhaps a very small metabolic pool is involved and the supplied radioactive derivative cannot enter this pool, but instead becomes incorporated with a large storage pool in the cytoplasm. This storage pool may be metabolically inert, but may serve as a translocation pool to supplement the derivatives in the roots and shoots, perhaps at a later stage of germination (Okinaka and Iwai, 1970; Sengupta and Cossins, 1971).

It would appear that in storage tissues, such as cotyledons, serine and glycine may serve as the major precursors of one-carbon units required to support different biosynthetic reactions. Germinating tissues could produce serine through synthesis from carbohydrates. Two pathways (Scheme III) for the formation of serine from glucose in vertebrate tissue has been demonstrated (Ichihara and Greenberg, 1957). There is evidence for the enzymes of both pathways in tissue preparations (Walsh and Sallach, 1966; Fallon and Byrne, 1965) and also for a pathway leading from fructose to serine via oxidation of D-glyceraldehyde (Katterman *et al.*, 1961). It has been proposed that in plants, the second pathway is active since an NAD-dependent

SCHEME III

PATHWAYS FOR SYNTHESIS OF SERINE FROM GLUCOSE



D-glycerate dehydrogenase is widely distributed and is particularly active in green leaves (Stafford *et al.*, 1954). Also the alanine-hydroxypyruvate transaminase of this pathway has been demonstrated in the leaves of 10 species (Willis and Sallach, 1962) and its occurrence coincided with that of the D-glycerate dehydrogenase.

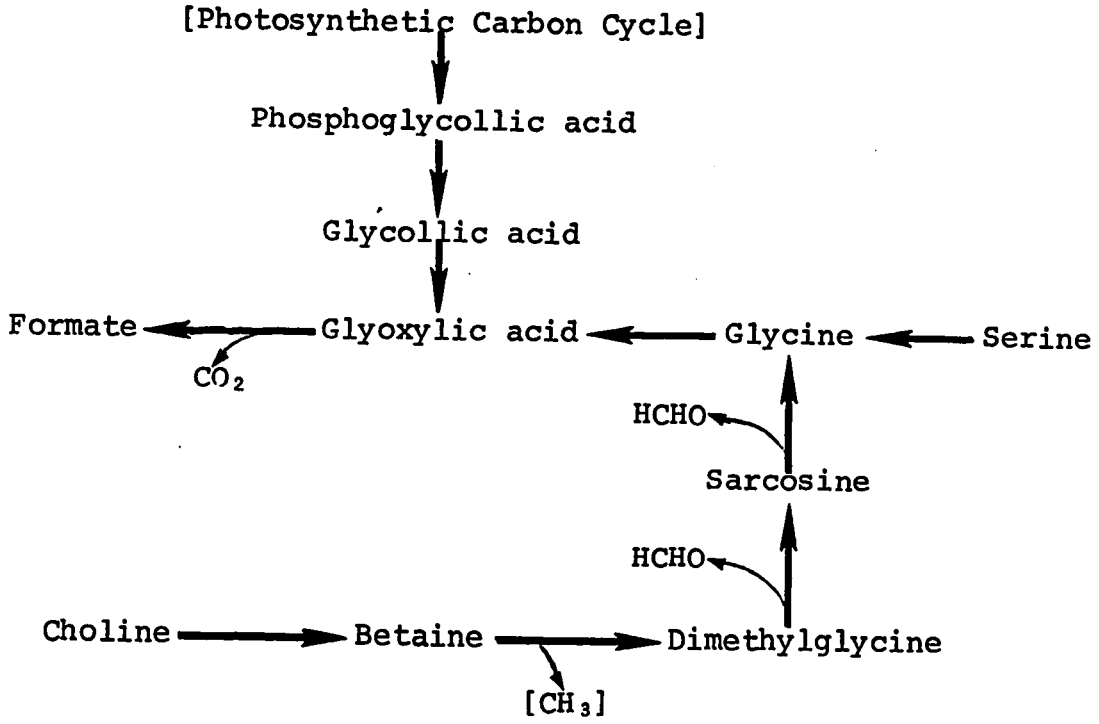
In photosynthetic plants (Scheme IV) another pathway has been proposed from evidence based on C^{14} labelling (Rabson *et al.*, 1962). When glycollate-2-C^{14} was fed to plants, serine-2,3-C^{14} and glycerate-2,3-C^{14} were major products. According to this scheme, D-glycerate dehydrogenase and alanine-hydroxypyruvate transaminase would function in the formation of glycerate from serine and not *vice versa*. There is other evidence supporting this pathway (*e.g.* Wang and Burris, 1965). The importance of this pathway may, however, lie in the formation of glycine.

Although a photosynthetic glycollate pathway, as outlined in Scheme IV, would not operate in germinating pea cotyledons, serine could be formed essentially by this route as various investigators have reported the ability of many plant tissues to convert glycollate into this amino acid (*e.g.* Cossins and Sinha, 1967; Tanner and Beevers, 1965). Similarly, significant quantities of serine could be produced as a result of protein hydrolysis, an event known to be extensive in pea cotyledons during germination (Lawrence and Grant, 1963).

One-carbon units may also be generated from compounds

SCHEME IV

POSSIBLE ROUTE FOR SYNTHESIS OF ONE-CARBON UNITS FROM CHOLINE AND PHOTOSYNTHETIC INTERMEDIATES



like sarcosine and dimethylglycine (Scheme IV). Betaine catabolism is known to follow a pathway involving these compounds as intermediates (Fruton and Simmonds, 1960). Betaine may alternatively be involved in a transmethylation reaction with homocysteine (Dubnoff, 1949; Muntz, 1950). Dimethylglycine and sarcosine are known to produce one-carbon units at the formaldehyde level of oxidation by rat liver mitochondria (Mackenzie and Frisell, 1958), but the possibility of similar reactions occurring in plants remains to be determined. Betaine is a common constituent of many plant tissues and its synthesis and role as a methyl group donor are now fairly well documented (Sribney and Kirkwood, 1954; Byerrum *et al.*, 1956; Bowman and Rohringer, 1970). It would be of some interest to determine whether this compound had a physiological role as a one-carbon donor to the pteroylglutamate pool during germination.

On the basis of published work, it would appear that one-carbon units, at the oxidation level of formate, can enter the pteroylglutamate pool of pea cotyledons. The high levels of 10-HCO-H_4PteGlu synthetase in these tissues (Iwai *et al.*, 1967), the rapid incorporation of formate into serine (Cossins and Sinha, unpublished data) and the observed labelling of 10-HCO-H_4PteGlu following administration of formate-C^{14} (Table 9) support this contention.

At the present time, much that can be said regarding the generation of one-carbon units for pteroylglutamate mediated reactions must rest on indirect evidence such as the

occurrence of the necessary enzymes and the formation of an appriopriate end-product such as serine or methionine. Direct evidence for these reactions occurring *in vivo* is lacking in most cases because of the difficulty in demonstrating that a theoretical one-carbon precursor is in fact incorporated into the pteroylglutamates of intact living tissues. Clearly, the quantities of pteroylglutamate derivatives in living tissues are extremely small and in the dynamic sense must be catalytic. The present commercial specific radioactivities of C^{14}-labelled precursors such as serine, etc., restrict incorporation of label, even if the pools were in equilibrium, to levels not significantly above those of background. The earlier classical approach to metabolic studies of determining fluctuations in levels of a product when supplying a suspected precursor while of some importance in dietary experiments (Blakley, 1969) may have limited value in higher plant systems. Concentrations of added precursors which exceed the physiological range may profoundly alter the pattern of pteroylglutamates as occurs in *Saccharomyces* under changing culture conditions (Combepine, Cossins and Kim, in preparation).

The results of the experiments with cell-free extracts (Tables 11-13) while of a preliminary nature indicate that the principal pteroylglutamates of pea cotyledons can be synthesized to some extent. Evidence was obtained for 5,10-CH_2=H_4PteGlu dehydrogenase activity (Table 14, Figure 8) and the synthesis of 5-CH_3-H_4PteGlu when the extracts

were prepared in the presence of FAD, suggests that 5,10-CH_2=H_4PteGlu reductase was also present. Although these enzyme preparations were not purified, requirements for reduced pyridine nucleotide and H_4PteGlu show that oxidation and reduction of 5,10-CH_2=H_4PteGlu was taking place.

In conclusion, the results of the present studies by demonstrating the net synthesis of several metabolically important pteroylglutamates during germination draw attention to an important area of plant biochemistry which is, to date, far from fully understood. The basic regulatory mechanisms controlling production and utilization of the individual derivatives in higher plants are unknown as are many of the interrelationships which presumably exist between the pteroylglutamates and key cellular constituents.

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APPENDIX I

GENERAL PROCEDURE FOR MICROBIOLOGICAL ASSAY OF PTEROYL-GLUTAMATES

The procedure followed was that of Hansen (1964) and Freed (1966) slightly modified as follows.

The bacteria used were those commonly employed for the microbiological determination of pteroylglutamates. They were purchased from American Type Culture Collection, and suspended in physiological sterilized saline (0.9% w/v NaCl). With the aid of a transfer loop (sterilized by passing through a flame) a small quantity of bacteria were transferred onto agar slants. The bacteria were allowed to grow at 37°C overnight, or until a visible line of growth appeared. Transfer was then made onto new slants and the incubation repeated. Three or four transfers were made initially and subsequently once or twice weekly to maintain active bacteria.

The culture medium was prepared the evening before the start of an assay by transferring cells from the agar slant to the inoculum broth and incubating overnight at 37°C. The culture medium unlike the agar slants was a liquid medium but like the slants had all the requirements for growth including the vitamin being assayed. For inoculation of the basal assay media it was necessary that as little nutrient material as possible be carried over from the culture medium, as any such carry over would have resulted in high blanks. To overcome this problem, the culture medium containing the bacteria was centrifuged at 2000-4000 r.p.m.

(Bench Top International Centrifuge, Model HN), the supernatant was discarded and the packed cells resuspended in sterile isotonic saline. This process was repeated twice more and then sufficient dilutions of the bacteria were made. This latter procedure was very important in assaying for pteroylglutamate derivatives because the vitamin is effective for bacterial growth in extremely low concentration (10^{-10}g - 10^{-9}g). The dilution made was usually about 1:11 depending however on the amount of growth originally in the culture medium. Inoculation was accomplished by partly filling a 10 ml syringe fitted with a 12 gauge needle and allowing 1 drop of the bacteria, diluted in physiological sterile saline, to fall in each tube of the assay medium.

The basal assay medium (Table 1) was semisynthetic including some natural materials such as casein hydrolyzate from which contaminating vitamins had been removed, together with vitamins, purines, salts, some amino acids, fermentable sugar, and buffer. Some of the ingredients were kept in the form of stock solutions in the refrigerator at 2°C under toluene and over chloroform. The other ingredients were weighed out as needed. For this basal medium plain test tubes, (20 x 150 mm), were used and were kept in aluminium racks (60 tubes/rack) fitted with aluminium covers.

All sterilizations were performed in an autoclave at 15 lbs. per square inch. The tubes were maintained at this pressure, 15 min. for the solid and liquid media and 12 min. for the basal assay media. A longer period of time resulted

TABLE I. COMPOSITION OF BASAL ASSAY MEDIA

COMPOUND	QUANTITY (mls)		
	L. casei	*S. faecalis*	*P. cerevisiae*
Acid hydrolyzed casein	100	100	100
Enzymatic hydrolyzed casein	--	--	100
Adenine-Guanine-Uracil	50	50	50
Asparagine	60	60	60
Manganese Sulfate	20	20	20
Salt B	10	10	80
Tryptophan	20	20	40
Vitamin	100	100	100
Xanthine	100	100	100
Tween 80	--	--	1
	QUANTITY (gms)		
L. cysteine $HCl \cdot H_2O$	0.5	0.5	0.76
Glucose, anhydrous	40.0	40.0	40.0
Glutathione	0.005	0.005	0.005
K_2HPO_4, anhydrous	1.0	6.2	4.0
KH_2PO_4, anhydrous	1.0	--	4.0
Sodium citrate $\cdot 2H_2O$	--	40.0	35.0
Sodium acetate	40.0	--	--

in excessive darkening of the media and resulted in higher blank values during titration of the assay media. The sterilized tubes were allowed to cool to room temperature (25°C) and stored at this temperature until required. Ideally they should be stored at 2°C to prevent excessive evaporation. Darkening of the assay media occurred slowly at room temperature and was of little consequence unless tubes were stored for periods in excess of two weeks.

PREPARATION OF STOCK SOLUTIONS

Acid-hydrolyzed casein: - 100 g of the hydrolyzate were dissolved in about 700 mls of water and the pH adjusted to 3.5 with concentrated HCl. The solution was decolorized by stirring with 20 g of activated charcoal (Norite A) at room temperature until it had a light straw color. This normally required 20 min. of stirring. This step removed any contaminating vitamins which may still remain in the casein. The solution was filtered by suction to remove the Norite A and the filtrate adjusted to pH 6.8 with concentrated NaOH. After dilution to 1 litre it was stored under toluene in the refrigerator. Occasionally a precipitate formed in this solution on standing. This was mainly due to insolubility of tyrosine. The casein solution was, however, always thoroughly stirred before use.

Enzymatic and hydrolyzed casein: - 25 g of the hydrolyzate were dissolved in about 700 mls of water by adjusting the pH to 3.5 with concentrated HCl and by heating to 45°C. The solution was decolorized with 5 g of Norite A as described

for the acid hydrolyzed casein. The filtrate was adjusted to pH 6.8 with concentrated NaOH made up to 1 litre, and stored under toluene in the refrigerator.

Adenine-Guanine-Uracil: - Samples (0.2 g) of adenine sulfate, guanine hydrochloride and uracil were carefully heated in a 500 ml Erlenmeyer flask containing approximately 150 mls of water and 4 mls of concentrated HCl. When the solids were dissolved, the solution was cooled and transferred to a 1000 ml volumetric flask and made to volume with water.

Salt solution A: - 25 g of K_2HPO_4 and 25 g of KH_2PO_4 were dissolved in water and subsequently diluted to 500 mls.

Salt solution B: - 10 g of $MgSO_4 \cdot 7H_2O$ and 0.5 g each of $FeSO_4 \cdot 7H_2O$ and $MnSO_4 \cdot 4H_2O$ were dissolved in water, 0.5 ml concentrated HCl was added and the solution made up to 500 mls. Both salt solutions were stored under toluene.

Asparagine: - 5 g of L-asparagine monohydrate were dissolved in water and the solution made up to 500 mls.

Maganese sulfate: - 2.0 g of $MnSO_4 \cdot H_2O$ were dissolved in water and made up to 200 mls. The solution was stored under toluene.

Tryptophan: - 3 g of L-tryptophan were dissolved in 30 mls of 1N HCl. The solution was stirred and heated to 80°C until the amino acid was fully dissolved. The solution was then adjusted to 300 mls and stored under toluene.

Vitamins: - 10 mg of *p*-aminobenzoic acid, 40 mg of pyridoxine·HCl, 4 mg of thiamine·HCl, 8 mg calcium pantothenate,

8 mg of nicotinic acid and 0,2 mg of biotin were dissolved in about 300 mls of water. To this solution 10 mg of riboflavin were added dissolved in about 200 mls of 0.02 N acetic acid. Then a solution containing 1.9 g of anhydrous sodium acetate and 1.6 ml of acetic acid in about 40 mls of water was added. This vitamin solution was diluted with water to 1 litre, and stored under toluene and over chloroform.

Xanthine: - 0.2 g of xanthine were suspended in a small volume of water and 4 mls of concentrated ammonium hydroxide were added. The solution was heated until all xanthine had dissolved and then diluted with water to 1 litre. The solution was stored at 2°C.

COMPOSITION OF SOLID MEDIA FOR MAINTAINING CULTURES

5 g of Difco Bacto yeast extract were dissolved in 200 mls of water and then 1 g of anhydrous glucose, 1 g of anhydrous sodium acetate and 3 g of agar were added. The suspension was heated in a beaker with occasional stirring until the ingredients were dissolved. An automatic pipetting syringe was used to pipette the agar solution 10 mls into each of 20 tubes and was initially prewarmed by passing through hot water. The tubes were subsequently plugged with cotton. Following sterilization as described earlier, they were cooled in a slanted position, and stored at 2°C.

COMPOSITION OF CULTURE MEDIUM

5 g of Difco Bacto Peptone, 1 g of Difco yeast extract, 10 g of anhydrous glucose, and 10 g of anhydrous sodium

acetate were dissolved in 5 mls of salt solution A and 5 mls of salt solution B in 200 mls of water. The pH was adjusted to 6.8 with 0.1 N NaOH and made up to 500 mls with water. 10 mls were pipetted into each of 50 tubes with the automatic pipetting syringe. The tubes were plugged with cotton and sterilized as described earlier. Following sterilization they were stored at 2°C.

COMPOSITION OF THE BASAL ASSAY MEDIA

The solution was prepared by mixing in the stock solutions (Table 1) in the following order: casein hydrolyzate(s), tryptophan, adenine-guanine-uracil, xanthine, asparagine, vitamin and salts from salt solution B. The solid ingredients were dissolved in 200 mls of water/litre of assay media and added to the several stock solutions already mixed. The mixture was adjusted to pH 6.8 (*L. casei, S. faecalis*), or pH 7.0 (*P. cerevisiae*) using concentrated NaOH. Then a manganese sulfate solution was added (Table 1). In the *P. cerevisiae* media, 1.0 mls of Tween 80 (Polysorbate 80) were also added/litre of media. 5 mls were pipetted into each of 200 tubes and sterilized as described earlier.

Table II summarizes the major steps in the subsequent microbiological assay of pteroylglutamate derivatives.

TABLE II. MAJOR STEPS IN THE MICROBIOLOGICAL ASSAY OF PTEROYLGLUTAMATE DERIVATIVES

1.	Agar slants transferred weekly.
2.	Liquid culture on complete media for 18 hr at 37°C.
3.	Sedimentation of cells by centrifugation
4.	Resuspension of cells in sterile saline
5.	Resedimentation and resuspension in sterile saline
6.	Inoculation of assay medium supplemented with folate standards or unknowns
7.	Growth at 37°C for 72 hr
8.	Titration of lactic acid produced