THE IDENTIFICATION OF PARA-AMINOBENZOYL POLYGLUTAMATES IN EXTRACTS OF *E. COLI*

by

ROBERT ALLAN HENRY FURNESS

The University of Manitoba

A Thesis

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The Faculty of Graduate Studies and Research

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of the requirements for the degree of

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ABSTRACT

A glutamate-containing component of coenzyme A-disulfide prepared from *E. coli* has been isolated and shown to be a co-purifying contaminant. Physical and chemical techniques were used to characterize the contaminant as a mixture of p-aminobenzoyl poly(γ-L-glutamates) containing 5,6,7 and 8 glutamyl residues with the hexa- and heptaglutamyl forms predominating. Similar distributions of these molecules were present in extracts of both *E. coli* strains B23 and K12 grown either in minimal or enriched medium. A preliminary examination of the cellular pteroyl polyglutamate pool, from which the p-aminobenzoyl polyglutamates are derived, has shown a similar distribution. These results have cast some doubt on the reported size distributions of pteroyl polyglutamates in *E. coli*.

TO BARBARA

and

TO MY PARENTS

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I would like to express my gratitude to my advisor, Dr. P.C. Loewen, of the Department of Microbiology, University of Manitoba for his support and guidance during this study and, above all, for his friendship. I am also appreciative of his personal concern and sense of fairness demonstrated in our relationship.

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ABBREVIATIONS

A	absorbance
$A_{275/260}$	ratio of absorbance at 275 nm to that at 260 nm
ADP	adenosine-5'-diphosphate
AMP	adenosine-5'-monophosphate
ATP	adenosine-5'-triphosphate
^{o}C	degree Celsius
$5\text{-}CH_3H_4$pteglu	N^5-methyl-5,6,7,8-tetrahydropteroylglutamate
$5,10\text{-}CH\text{-}H_4$pteglu	N^5,N^{10}-methenyl-5,6,7,8-tetrahydropteroylglutamate
$5,10\text{-}CH_2H_4$pteglu	N^5, N^{10}-methylene-5,6,7,8-tetrahydropteroylglutamate
$5\text{-}CHO\text{-}H_4$pteglu	N^5-formyl-5,6,7,8-tetrahydropteroylglutamate
$10\text{-}CHO\text{-}H_4$pteglu	N^{10}-formyl-5,6,7,8-tetrahydropteroylglutamate
cm	centimeter
CoA	coenzyme A
$(CoA)_2$	oxidized coenzyme A-disulfide
CoASSG	coenzyme A-glutathione mixed disulfide
DEAE	diethylamino-ethyl
dATP	deoxyadenosine-5'-triphosphate
FPGS	folylpolyglutamate synthetase
g	gram
glu	glutamic acid
$glu(\gamma\text{-}glu)_{n-1}$	poly(γ-glutamic acid) of n residues
gsh	glutathione
h	hours

$H_2pteglu_n$	7,8-dihydropteroylpoly(γ-glutamate)
$H_4pteglu_n$	5,6,7,8-tetrahydropteroylpoly(γ-glutamate)
HCl	hydrochloric acid
Km	Michaelis constant
$LiBH_4$	lithium borohydride
M	molar
min	minute
ml	milliliter
mol	mole
NAD^+	oxidized nicotinamide adenine dinucleotide
nm	nanometer
pABA	para-aminobenzoic acid
pABAglu	N-(p-aminobenzoyl)-glutamic acid
$pABAglu_n$	p-aminobenzoylpoly(γ-glutamate) of "n" glutamyl residues
$pteglu_n$	pteroylpoly(γ-glutamate) of "n" glutamyl residues
$PO_4{}^{3-}$	inorganic phosphate ion
Rf	migration relative to the solvent front
RNA	ribonucleic acid
SDS	sodiumdodecylsulfate
TEAB	triethylamine-bicarbonate buffer
Tris	tris-(hydroxymethyl)aminomethane
UTP	uridine-5'-triphosphate
UV	ultraviolet
Vmax	maximum enzymic reaction velocity at saturating substrate concentration
xg	times gravity
Zn	zinc metal

HISTORY

HISTORY

Introduction

During the study of two CoA-containing disulfide compounds found in *E. coli* it became apparent that a co-purifying contaminant existed which contained several glutamyl residues. Several such polyglutamate compounds have been found in nature. Ubiquitous are the pteroyl poly-γ-glutamates, folic acid and its conjugates, and also one of its catabolites, pABA polyglutamate. The polyglutamate portion of $pteglu_n$, which is of variable length (n = 1-7 usually) appears to be of importance in the binding of the folate cofactors to certain enzymes. One exception to this rule is the H_2-$pteglu_6$ found stoichiometrically in bacteriophage T4 tail plate. This molecule is apparently necessary as a structural component for the proper assembly of phage tail plates. The presence of pABA glu_n (in yeast) was first reported in 1946, and it was later established as a breakdown product of $pteglu_n$.

Encapsulated strains of certain *Bacillus spp.* (notably *B. subtilis*, *B. licheniformis* and *B. anthracis*) have as part of their capsule a very large homopolymer of glutamic acid. Like the $pteglu_n$ and pABA glu_n the molecule contains γ-amide links but unlike them, it contains D-glu instead of the naturally predominant form L-glu. It is also much larger, being polymerized to the degree of up to several thousand residues as compared to the short oligopeptides of $pteglu_n$.

Pteroylpolyglutamates

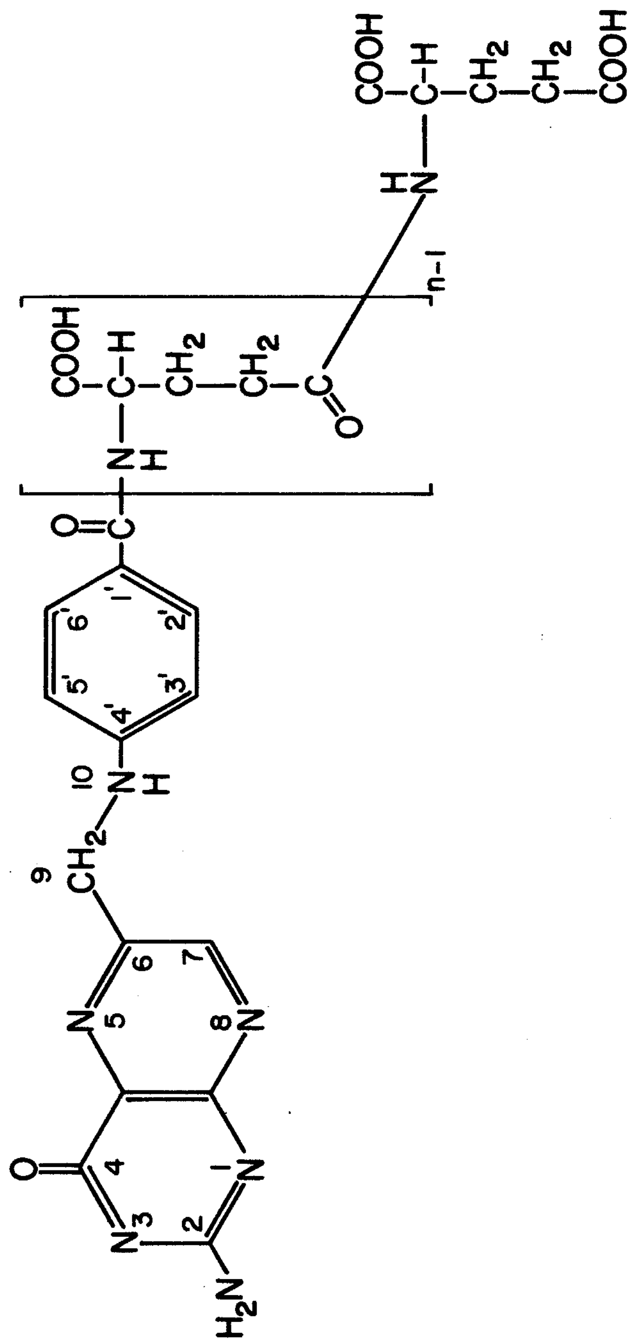
1) Occurrence

The structure of the pteroyl polyglutamates, shown in Figure 1, was elucidated by Angier *et al* (1946). There has been some confusion over nomenclature as folic acid is composed of the pteridine-pABA portion of the molecule and one glutamyl residue and is hence pteroylglutamate. This is the reason for the use of the terminology pteroylpolyglutamates rather than "folyl polyglutamates" or "folic acid conjugates" as was used earlier. The term conjugates referred to the fact that the growth factor folic acid was conjugated to a chain of several glutamyl residues.

The first isolation of $pteglu_n$ was by Pfiffner *et al* (1946), who isolated the pteroyl heptaglutamate from yeast. Chemical analysis along with microbiological assay of the folates gave the ratio of seven γ-glutamyl residues per pteridine. The presence of $pteglu_n$ was established in several organisms, but studies were complicated by several factors. The presence of folate compounds was determined by microbiological assay as with *L. casei*, *S. faecalis* and *P. cerevisiae*, organisms incapable of *de novo* synthesis of folic acid but able to utilize different forms of the pteroyl monoglutamate (i.e. pyrazine oxidation state and N^{5-}, N^{10-} substituents), $pteglu_2$ or $pteglu_3$ for growth (Schertel *et al* 1965; Johns and Bertino 1965). It is not possible with bioassay to distinguish all forms of these folates without modification; for example $5\text{-}CH_3H_4$ pteglu and $pteglu_3$ yield the same results. Pteroyl polyglutamates were assayed by cleavage to $pteglu_2$ by

Figure 1. The structure of $pteglu_n$.

The molecule consists of a pteridine ring (1-8) joined to a p-aminobenzoyl (1'-6',10) residue. This is linked by an amide bond to an oligopeptide of (γ-L-glutamic acid) of length n. Co-enzyme activity is determined by oxidation state at $C^5 - C^8$, the one-carbon constituent carried at N^5 or N^{10} and also the number of glutamyl residues present.



H2N
N
O
CH2
COOH
C-H
CH2
n-1

a γ-glutamyl carboxypeptidase ("conjugase") contained in chicken pancreas (Bird *et al* 1945, 1946). Chromatographic properties of natural folate derivatives do not allow easy separation of $pteglu_n$ by size or charge, probably due to interaction of the pteridine moiety with chromatography matrices (Shin *et al* 1972). Also, chemical determinations of glutamate chain length require isolation of the peptide in pure form. For these reasons determinations of the distributions of $pteglu_n$ chain lengths in different organisms was not practical. *Corynebacterium* excreted a $pteglu_3$ into its growth medium (Hutchings *et al* 1948) while *Streptococcus pneumoniae* contained small amounts of pteglu and $pteglu_2$, as well as large amounts of $pteglu_3$ and higher conjugates (Sirotnak *et al* 1963). The presence of $pteglu_n$ was also established in tissues from rat (Bird *et al* 1965), chicken liver (Noronha and Silverman 1962) and human whole blood (Herbert *et al* 1962). Schertel *et al* (1965) identified $pteglu_2$ and $pteglu_3$ in yeast, as well as a polyglutamate form they assumed to be $pteglu_7$ on the basis of the work of Pfiffner *et al* (1946).

Characterization of glutamyl chain length was simplified by cleavage of the C^9–N^{10} bond, either oxidatively using alkaline permanganate (Houlihan and Scott 1972) or reductively with Zn/HCl (Baugh *et al* 1974). Cleavage removes the chromatographically troublesome pteridine portion, leaving pABA glu_n, which can be separated more easily on the basis of glutamyl chain length. The results are not entirely quantitative, as discussed at length by Maruyama *et al* (1978), Baugh *et al* (1979) and Lewis and Rowe (1979), but are much clearer

than those previously obtained. As can be seen from Table 1, the distribution of $pteglu_n$ chain length varies from organism to organism, but the polyglutamate group is apparent in all organisms studied and one chain length predominates in each.

There is still much confusion in the literature over the cellular distribution and even the predominant form of $pteglu_n$ in some organisms. Much of this is a result of inconsistency and inadequacy of techniques, as discussed above. This is true especially in mammalian systems, where most of the folate pool exists as 5-CH_3H_4 $pteglu_n$, due to the failure of both oxidative and reductive cleavage to hydrolyse this form (Lewis and Rowe 1979).

In bacterial systems, however, some of the variation appears to be due to culture conditions. There is, for example *L. casei*, for which Baugh *et al* (1974) reported $pteglu_4$ as the predominant folate, but other authors found mostly $pteglu_8$ (Buehring *et al* 1974; Brody *et al* 1979; Brown *et al* 1974). Bassett *et al* (1976b) attributed this shift in chain length to exogenous folate availability as the only significant difference between the work of Baugh *et al* (1974) and the other groups was the former's use of almost twice the medium folate concentration. This they confirmed.

Another instance of a shift in $pteglu_n$ size profile is in *E. coli* B upon infection with bacteriophage T4D, which contains $H_2pteglu_6$ as a component of its tail plate (Kozloff and Lute 1965). The $H_2pteglu_6$ is a stoichiometric component of the wedge-shaped structures comprising

TABLE I

Size distributions of pteroyl polyglutamates in various organisms.

Organism	Glutamyl Residues in $pteglu_n$ (% of Total)										Reference
	1	2	3	4	5	6	7	8	9	>9	
Strep. faecalis ATCC 8043	16.5	8.5	20	54.5							Baugh *et al* (1974)
ATCC 8043 methotrexate resist	1	5.7	6.9	81.3	5.1						Baugh *et al* (1974)
8043		1	10	86	2.6						Brody *et al* (1979)
8043			4	61	23						Buehring *et al* (1974)
Lactobacillus casei ATCC 7469	3.2	0	9.3	59.4	23	5.1					Baugh *et al* (1974)
								100			Brody *et al* (1979)
						8	14	42	19		Buehring *et al* (1974)
Corynebacterium spp.				>95							Shane (1980a)
Clostridium acidi-urici			>95								Curthoys *et al* (1972)
Escherichia coli B			>90								Wood *et al* (1968) Kozloff & Lute (1973)
K12			~20	~20	~50						Powers and Snell (1976)
K12	13	14.5	36	12	16	8	1.5				Bassett *et al* (1976b)
NCIB 8109	13	18	30	13	20	5	1				Bassett *et al* (1976b)
Saccharomyces cerevisiae						16	70	12			Bassett *et al* (1976a)
Neurospora crassa					14.5	80	0	5.6			Chan & Cossins (1980)
Rat liver					>95						Shin *et al* (1972) Brody *et al* (1979)
Bacteriophage T_4						100					Kozloff & Lute (1965)

the outer portion of the hexagonal baseplate and is found in close association with the apically located phage gene 11 product (Kozloff *et al* 1975, 1979). Kozloff's group has further shown that the H_2pteglu$_6$ is required as a structural component involved in attachment of the long tail fibers at or near the apices (Kozloff *et al* 1979). This was accomplished using the inhibition of *in vitro* tail fiber attachment with oligo (γ-glutamyl) chains, γ-glutamyl carboxypeptidase and antisera directed against a polyglutamate hapten. They proposed that the presence of the seven anionic charges allows strong tail fiber attachment yet allows the necessary rotation freedom. This does not explain, however, the absolute requirement for H_2pteglu$_6$. Kozloff and Lute (1973) noted a shift from predominantly pteglu$_3$ to pteglu$_6$. This shift was chloramphenicol-sensitive and apparently due to a late phage gene product. T4D gene 28^--infected cells accumulate larger conjugates (pteglu$_9$-pteglu$_{12}$) but little pteglu$_6$. Viable phage are not formed. It appears that pteglu$_6$ is formed from a larger conjugate by cleavage due to a phage gene product. The successful complementation of T4D gene 28^--infected bacterial extracts with the addition of pteglu$_6$ confirmed this (Kozloff *et al* 1973). The mechanism of the shift to larger conjugates is still unclear (Nakamura and Kozloff 1978).

2) Synthesis

The first report of a folylpolyglutamate synthetase (FPGS) was by Griffin and Brown (1964), who described such an activity in *E. coli*.

Subsequent work (Brown *et al* 1974) described folate synthesis in the succession H_2pte, H_2pteglu, H_4pteglu and H_4pteglu$_2$ and reported the extraction of an ATP-dependent FPGS catalyzing the formation of 10 CHO-H_4pteglu$_2$ only (Masurekar and Brown 1975). This led to the postulation of two enzymes for polyglutamate synthesis.

Sakami *et al* (1973) described two FPGS activities in *Neurospora crassa*, one specific for the conversion of H_4pteglu to H_4pteglu$_2$ and one specific for elongation of H_4pteglu$_2$ to H_4pteglu$_3$. These activities could be chemically fractionated and were genetically characterized. Two methionine auxotrophs, *met*-6 and *mac* each were found to be lacking one of these activities (Chan and Cossins 1980).

The most extensive work has been that of Shane (1980a, 1980b, 1980c) on FPGS from *Corynebacterium spp*. He characterized the cellular folate content as to glutamyl chain length, one-carbon substituent and oxidation-reduction state (Shane 1980a) and isolated an FPGS enzyme to greater than 95% purity. This enzyme (Shane 1980b) has a molecular weight of 53 000 by SDS gel electrophoresis a pH optimum of 9.5 or greater, an absolute requirement for L-glutamate and for a monovalent cation (K^+, 200 mM). The activity was Mg ATP-dependent, but dATP or even UTP would suffice to an extent. Folate requirement was variable but monoglutamates were much more effective substrates than higher forms, with H_4pteglu the most effective (Km = 2 μM). Despite the much lower affinity of his enzyme for di- and triglutamates, Shane showed that there was no appreciable difference in activities between the purified enzyme and crude extract, implying that only one and not

two enzymes were present. He speculated that formation of longer glutamyl chains had different optimum conditions. In support of the one-enzyme hypothesis he referred to "preliminary studies" indicating the utilization of $5,10CH_2H_4pteglu_{2-4}$, but this reference was unsupported by data.

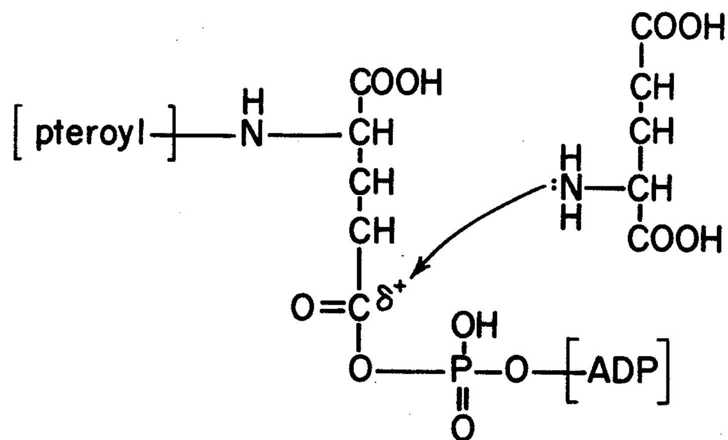
Kinetic studies (Shane 1980c) indicate the reaction proceeds by an Ordered Ter Ter mechanism with substrates joining the enzyme complex in the order MgATP, H_4pteglu, glutamate, followed by release of products in the order ADP, $H_4pteglu_2$, PO_4^{3-}. Two speculative mechanisms consistent with this sequence are presented, both involving nucleophilic attack of the free glutamyl α-amine, which would not be ionized at the pH for optimum activity (pH 9.5 or greater). One mechanism proposes nucleophilic attack on the mixed anhydride of a pteroyl (γ-glutamyl-phosphate) intermediate (Figure 2a), while the other proposes a concerted nucleophilic attack of the glutamyl α-amine on a γ-carboxyl carbonium ion of the pteglu coupled with a similar attack of one of the γ-carboxyl oxygens on the γ-phosphorus of ATP (Figure 2b). Both mechanisms are plausible but the evidence at present (Shane 1980c) is at best indirect.

There have been several studies of mammalian FPGS. Gawthorne and Smith (1973) showed H_4pteglu, 5-CHO-H_4pteglu and 5-CH_3H_4pteglu to be substrates for enzyme extracts of sheep liver. Taylor and Hanna (1977) showed much lower activity for 5-CH_3H_4pteglu using enzyme from Chinese hamster ovary (CHO) cells. Spronk (1973) and McGuire *et al* (1980) found rat liver FPGS to be inactive with 5-CH_3H_4pteglu, as were 10-CHO-H_4pteglu and H_2pteglu. Studies with non-mammalian enzymes (Sakami *et al* 1973,

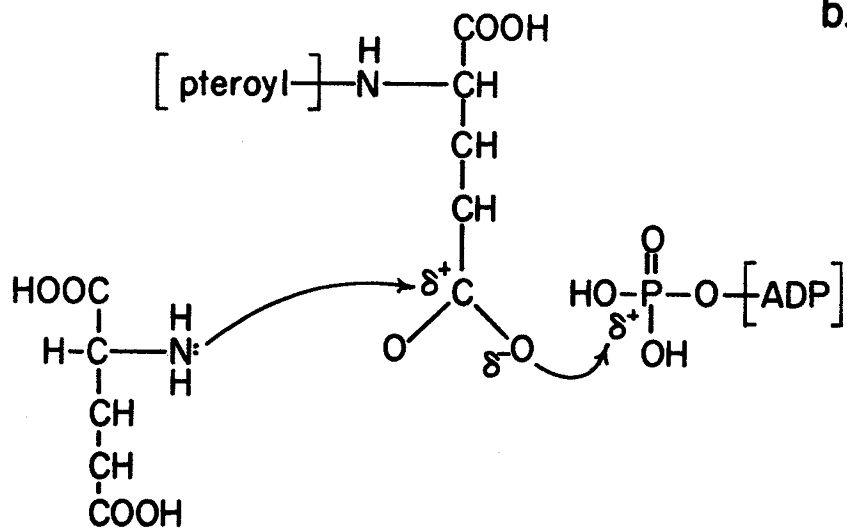
Figure 2. Possible mechanisms of chain elongation by folylpoly-glutamate synthetase.

Two mechanisms of glutamate addition to the free γ-carboxyl of $pteglu_n$ have been proposed by Shane (1980c). One invokes a nucleophilic attack on an active intermediate (a) while the other involves a concerted nucleophilic attack without prior formation of an active form of pteglu (b).

a.



b.



Shane 1980b) also reported relative inactivity with 5-CH_3H_4pteglu. These data are not necessarily in conflict with the fact that 5-CH_3H_4pteglu$_n$ are present as a large proportion of mammalian folate pools (Lewis and Rowe 1979) as they may only indicate that conversion to 5-CH_3H_4pteglu$_n$ takes place after polyglutamation.

McBurney and Whitmore (1974) isolated several auxotrophic mutants of CHO cells showing defects in intracellular folate metabolism. Among these were AUX B1 (a glycine, adenosine and thymidine auxotroph) and AUX B3 (a glycine and adenosine auxotroph). Not only did these lines have reduced folate concentrations but AUX B1 contained only monoglutamates while AUX B3 also contained some di- and triglutamates as opposed to the pentaglutamate of the parent line. The authors decided on genetic bases that these mutants had defects in one single locus. Using extracts of these lines Taylor and Hanna (1977) demonstrated that AUX B1 and AUX B3 lacked the parental folylpolyglutamate synthetase activity. Interestingly, they also showed that the number of glutamyl residues added to the substrate is dependent upon the H_4pteglu concentration. At low levels (1 μM) mostly H_4pteglu$_3$ and some H_4pteglu$_4$ are formed while at higher levels (5 μM) mostly H_4pteglu$_2$ is made. At very high levels (100 μM) only H_4pteglu$_2$ results. Taylor and Hanna (1979) further studied FPGS in extracts of revertant lines of AUX B1 and found increased heat sensitivity over wild-type in FPGS activity. This increased lability was apparent when either H_4pteglu$_2$ or H_4pteglu$_4$ synthesis was monitored. This led the authors to conclude that both diglutamate and tetraglutamate activities were on one enzyme.

McGuire *et al* (1980) have partially purified the rat liver FPGS

which shows similarities to the *Corynebacterium* enzyme (Shane 1980b). It requires a monovalent cation, of which K^+ (20 mM) is optimal, has a high pH optimum (8.4), exhibits an absolute requirement for L-glutamate and a less stringent requirement for MgATP that can be alleviated by other nucleotide triphosphates. In contrast, the substrate specifity for folates is broader in that H_2pteglu and 5,10CH-H_4pteglu are utilized much more easily (compared to the primary substrate H_4pteglu) than is the case with the *Corynebacterium* enzyme. Also, the rat liver enzyme catalyses *in vitro* the formation of $pteglu_4$ in large amounts and $pteglu_5$ (the predominant form in rat liver Table I) in an almost equal quantity. As with Taylor and Hanna's (1977) CHO extract the longer products are formed at low H_4pteglu concentrations while at higher concentrations mostly $pteglu_2$ is formed. It was found that $pteglu_5$ was not effective as a substrate and also was a strong inhibitor of the enzyme. This led McGuire *et al* to propose that, on binding of $pteglu_5$ to the enzyme, the length of the glutamyl chain places the terminal γ-carboxyl out of the active site. This suggests that, at least in rat liver, the predominant length of $pteglu_n$ is controlled solely by the size of the active site of FPGS. The binding of the end product $pteglu_5$ to the active site also indicates that feedback inhibition of polyglutamation may be one form of regulation of intracellular folate levels.

3) Significance

The polyglutamate conjugates of folic acid (with the exception of bacteriophage T4 as previously mentioned) appear to be the active coenzyme forms of the vitamin. Kisliuk and Gaumont (1974) showed that H_4pteglu$_3$ and H_4pteglu$_6$ were much better substrates for *L. casei* thymidylate synthetase than was H_4pteglu. Polyglutamate forms of folate analogs were strong inhibitors of the enzyme, while pABAglu$_6$ and glu(γ-glu)$_5$ were not bound by the enzyme, indicating that the poly(γ-glu) portion of the molecule enhanced binding but did not determine it. Coward *et al* (1974) demonstrated that dihydrofolate reductase from various mammalian sources has a higher affinity for H_2pteglu$_3$ H_2pteglu$_5$ or H_2pteglu$_7$ than for H_2pteglu. Powers and Snell (1976) found that *E. coli* ketopantoate hydroxymethyl transferase (involved in the synthesis of pantothenic acid) had much lower Km values for 5,10-CH_2H_4pteglu$_4$ and 5,10-CH_2H_4pteglu$_5$ than for other glutamyl chain lengths of the coenzyme. Baggott and Krumdieck (1979) obtained similar results with chicken liver 10-formyltetrahydrofolate:5'-phosphoribosyl-5-amino-4-imidazole-carboxamide formyl transferase, an enzyme of purine synthesis. The enzyme had a specificity (as defined by the ratio $^{Vmax}/_{Km}$) for the tetraglutamyl form 250-fold of that for the monoglutamyl form of the coenzyme. In their estimation this would make the tetraglutamate the only active form at the intracellular folate concentration found (1-2 μM).

The work of McBurney and Whitmore (1974) and that of Taylor and

Hanna (1977, 1979) provides both genetic and biochemical evidence that the polyglutamyl forms of folate are necessary for the biosynthesis of glycine, purines and pyrimidines in mammalian cells. Moreover, the ability of AUX B3 FPGS to make H_4pteglu$_3$ but not H_4pteglu$_4$ (Taylor and Hanna 1979) indicates the requirement of the larger oligo(γ-glutamyl) coenzymes.

The work of McBurney and Whitmore (1974) still leaves open the possibility that polyglutamation of folic acid is a means of ensuring cellular retention of the vitamin as the AUX B1 and AUX B3 FPGS mutants contain 10% and 40% of the assayable folic acid of the parental wild-type. This is supported by the observation (Herbert *et al* 1962) that serum contains only pteroyl monoglutamate, indicating it may be only a transport form of the vitamin as suggested by Rabinowitz (1960). However, in light of the enzyme kinetic studies cited above, this must be regarded as an auxiliary mechanism in cellular folate metabolism. As noted in both the bacterial (Bassett *et al* 1976; Powers and Snell 1976) and mammalian (McGuire *et al* 1979; Taylor and Hanna 1977) systems, the degree of polyglutamation is in inverse proportion to available folate levels. This relationship may be a manifestation of metabolic efficiency. When folate is limiting, both retention and reactivity are enhanced at the expense of ATP and glutamic acid. With excess folate, enhancement of activity and retention are not paramount, and ATP and glutamate are conserved. Such controls are quite feasible in the control of so important a metabolite.

Para-aminobenzoylpolyglutamates

At about the same time as the isolation of $pteglu_n$ Ratner *et al* (1946) isolated a $pABAglu_n$ from autolysed yeast. This was identified by the spectral characteristics of the pABA portion, especially the disappearance of the absorption at 273 nm on exposure to acid (pH 1) and by the presence of glutamic acid. The absorption drop is due to ionization of the arylamine, which indicates the peptide link to glutamate is through the pABA carboxyl. This was confirmed by diazotization of the free aryl amine, as opposed to the unreactiveness of blocked amines (e.g. N-acetyl pABA). Chemical analysis yielded a ratio of 10 or 11 glutamates per pABA, all being the L-isomer (bioassay). On the basis of a difference between estimates of glutamic acid nitrogen and α-amino nitrogen the presence of one dicarboxylic amino acid other than glutamic acid was postulated but not determined. The structural analogy between the pABA peptide and folic acid was noted by the authors, but no confirmation was attempted.

Stokstad *et al* (1947) noted that pABA glu was a product of UV photolysis of pteglu. Enzymic degradation of pteglu to pABAglu was demonstrated by Keresztesy and Silverman (1953). Futterman and Silverman (1957) showed the reaction to be dependent upon NAD^+ and ATP and thought it to be non-enzymic oxidation of an unstable reduction product, probably H_4pteglu.