Mammalian Folylpoly-γ-glutamate Synthetase. 4. In Vitro and in Vivo Metabolism of Folates and Analogues and Regulation of Folate Homeostasis†

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ABSTRACT: The regulation of folate and folate analogue metabolism was studied in vitro by using purified hog liver folylpolyglutamate synthetase as a model system and in vivo in cultured mammalian cells. The types of folylpolyglutamates that accumulate in vivo in hog liver, and changes in cellular folate levels and folylpolyglutamate distributions caused by physiological and nutritional factors such as changes in growth rates and methionine, folate, and vitamin B_{12} status, can be mimicked in vitro by using purified enzyme. Folylpolyglutamate distributions can be explained solely in terms of the substrate specificity of folylpolyglutamate synthetase and can be modeled by using kinetic parameters obtained with purified enzyme. Low levels of folylpolyglutamate synthetase activity are normally required for the cellular metabolism of folates to retainable polyglutamate forms, and consequently folate retention and concentration, while higher levels of activity are required for the synthesis of the long chain length derivatives that are found in mammalian tissues. The synthesis of very long chain derivatives, which requires tetrahydrofolate polyglutamates as substrates, is a very slow process in vivo. The slow metabolism of 5-methyltetrahydrofolate to retainable polyglutamate forms causes the decreased tissue retention of folate in B_{12} deficiency. Although cellular folylpolyglutamate distributions change in response to nutritional and physiological modulations, it is unlikely that these changes play a regulatory role in one-carbon metabolism as folate distributions respond only slowly. 4-Aminofolates are metabolized to retainable forms at a slow rate compared to folates. Although folate accumulation by cells is not very responsive to changes in folylpolyglutamate synthetase levels and cellular glutamate concentrations, cellular accumulation of anti-folate agents would be highly responsive to any factor that changes the expression of folylpolyglutamate synthetase activity.

In the preceding papers (Cichowicz & Shane, 1987a,b; George et al., 1987), the general properties and substrate and analogue specificity of hog liver folylpolyglutamate synthetase have been described. The availability of homogeneous folylpolyglutamate synthetase has allowed the use of this protein as a model for studying the factors involved in the regulation of folate metabolism in mammalian cells.

In this report, the in vitro metabolism of folates and folate analogues by purified folylpolyglutamate synthetase is described. The effects of folylpolyglutamates and inhibitors of the enzyme on folylpolyglutamate synthesis are described and are discussed in relation to the factors involved in the regulation of folate homeostasis and in the retention of anti-folates by mammalian tissues. Preliminary studies on the effects of inhibitors of folylpolyglutamate synthetase on the in vivo metabolism of folates in mammalian cells are reported.

Experimental Procedures

Materials. Substrates and reagents were synthesized or obtained from commercial sources as described in the preceding papers (Cichowicz & Shane, 1987a,b; George et al., 1987). ^{3}H-Labeled folic acid ([^{3}H]PteGlu),[1] labeled in positions 3′, 5′, 7, and 9 (47 Ci/mmol), and [^{3}H]aminopterin, labeled in positions 7 and 9 (22 Ci/mmol), were obtained from Amersham. Minimal essential medium (α modified) (MEM, DM325), which contained methionine (100 μM), serine (240 μM), glycine (666 μM), folic acid (2.2 μM), and vitamin B_{12}, but lacked ribosides, ribotides, deoxyribosides, and deoxyribotides, and MEM lacking glycine and folate (-GAT medium) were obtained from K.C. Biologicals.

In Vitro Metabolism of Folates and Analogues. Folate and analogue metabolism was studied by using the enzyme assay conditions described in the preceding papers (Cichowicz & Shane, 1987a,b). Assay mixtures and incubation times were modified as indicated. All metabolism assay mixtures utilized purified enzyme. The products of the metabolism experiments were cleaved to *p*ABaGlu$_n$ and *p*Aba analogue derivatives and separated by HPLC as described in the preceding papers (Chichowicz & Shane, 1987a,b). Methotrexate derivatives were cleaved to *N*-methyl-*p*AbaGlu$_n$ by reduction with Zn under acidic conditions (Tyerman et al., 1977) and were separated by HPLC without purification via azo dye derivatives.

Cell Culture. The Chinese hamster ovary (CHO) cells (WTT2) used in this study were auxotrophic for proline. CHO and HeLa cells were routinely grown as monolayers at 37 °C in MEM supplemented with 10% dialyzed fetal calf serum,

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[1] Abbreviations: CHO, Chinese hamster ovary; MEM, minimal essential medium, α modified; +GAT medium, MEM containing glycine, adenosine, and thymidine. Other abbreviations are as described in George et al. (1987).

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Table I: In Vitro Metabolism of Folates and Folate Analogues by Folylpolyglutamate Synthetase[a]

substrate	products formed (pmol)[b]							
	total	glutamate chain length						
		2	3	4	5	6	7	8
1 μM (6*RS*)-H_4PteGlu	360	40	20	130	160	18	0	
1 μM (6*S*)-H_4PteGlu	380	19	12	51	250	49	0	
1 μM (6*S*)-H_4PteGlu$_2$	350	0	25	42	240	40	0	
1 μM (6*S*)-H_4PteGlu$_3$	310	0	0	51	220	36	0	
1 μM (6*S*)-H_4PteGlu$_4$	330	0	0	0	280	46	0	
1 μM (6*R*)-10-formyl-H_4PteGlu	370	60	170	130	16	1	0	
1 μM (6*R*)-5,10-methylene-H_4PteGlu	340	24	9	34	240	36	0	
1 μM (6*S*)-5-methyl-H_4PteGlu	330	270	50	12	2	1	0	
1 μM H_2PteGlu	320	15	26	150	120	3	0	
1 μM H_2PteGlu$_2$	420	0	29	240	150	2	0	
1 μM PteGlu	260	53	65	140	7	0		
1 μM aminopterin	260	160	90	11	0			
1 μM dihydroaminopterin	290	270	25	2	1	0		
1 μM methotrexate[c]	190	150	17	18	4	1	0	
1 μM (6*S*)-H_4Pte-4-aminobutyryl-Glu[d]	300	0	180	81	32	0		
1 μM H_2Pte-4-aminobutyryl-Glu[d]	430	0	280	130	15	0		
10 μM (6*RS*)-H_4PteGlu	3420	150	260	1700	1230	67	1	0
10 μM (6*S*)-H_4PteGlu	3480	280	260	700	2110	130	8	0
10 μM (6*S*)-H_4PteGlu$_5$	490	0	0	0	0	490	0	
10 μM (6*S*)-H_4PteGlu$_6$	82	0	0	0	0	0	82	0
10 μM (6*S*)-H_4PteGlu$_7$	32	0	0	0	0	0	0	32
10 μM (6*R*)-10-formyl-H_4PteGlu	3250	680	1840	690	42	0		
10 μM (6*S*)-5-methyl-H_4PteGlu	3400	3000	370	27	12	1	0	
10 μM (6*S*)-5-methyl-H_4PteGlu$_2$	720	0	610	39	8	2	0	
10 μM (6*R*)-5,10-methylene-H_4PteGlu$_5$	580	0	0	0	0	580	0	
10 μM (6*R*)-5,10-methylene-H_4PteGlu$_6$	62	0	0	0	0	0	62	0
10 μM (6*R*)-5,10-methylene-H_4PteGlu$_7$	46	0	0	0	0	0	0	46
10 μM H_2PteGlu	3800	570	630	2150	450	0		
10 μM H_2PteGlu$_2$	3000	0	480	2080	440	0		
10 μM H_2PteGlu$_5$	67	0	0	0	0	67	0	
10 μM PteGlu	2680	510	660	1420	83	0		
10 μM PteGlu$_5$	34	0	0	0	0	34	0	
10 μM aminopterin	3260	2880	320	43	10	6	0	
10 μM dihydroaminopterin	2320	2160	110	33	18	3	0	
10 μM methotrexate[c]	2640	2450	180	0				
10 μM (6*S*)-H_4Pte-4-aminobutyryl-Glu[d]	2250	0	940	1010	300	0		
10 μM H_2Pte-4-aminobutyryl-Glu[d]	3680	0	2880	750	53	0		
10 μM (6*S*)-tetrahydrohomofolate	1150	270	720	140	10	4	0	
10 μM homofolate	840	190	630	20	4	0		

[a] Assay conditions and the cleavage of folate products to *p*AbaGlu$_n$ and their separation by HPLC are described under Experimental Procedures. Assay mixtures contained the indicated unlabeled folate or folate analogue, 500 μM L-[^{14}C]glutamate, 5 mM ATP, and 4 units of enzyme and were incubated for 24 h. [b] Adjusted to account for the increasing specific activities of the products with increasing glutamate chain length. Assay mixtures contained 500 pmol (1 μM) or 5000 pmol (10 μM) substrate. [c] *N*-Methyl-*p*AbaGlu$_n$ derivatives separated by HPLC. [d] Distribution refers to *p*Aba-4-aminobutyryl-Glu$_{n-1}$, which elutes earlier than the corresponding *p*AbaGlu$_n$.

penicillin (10^5 units/L), and streptomycin (0.1 g/L).

To study the effects of folate analogues on cell growth, cells were cultured in MEM lacking folate and glycine (−GAT medium) and supplemented with PteGlu (100 nM) and the analogue under study (10 μM).

In Vivo Metabolism of Folates. Cells were cultured on 100-mm dishes containing MEM (6 mL) lacking folate but supplemented with adenosine (37 μM) and thymidine (41 μM) (+GAT medium; Taylor & Hanna, 1975; Foo & Shane, 1982; Foo et al., 1982). Under these conditions, mammalian cells do not require folate for growth. After culturing for 1 week to deplete intracellular folate levels, the confluent cells were split 1:2 and replated on 100-mm dishes containing the same medium (6 mL). The cells were allowed to attach for 4 h, and [^{3}H]PteGlu (40 μCi, final concentration 142 nM) and unlabeled analogue (10 μM) were added to each plate. After a further 20 h, the plates were washed 5 times with phosphate-buffered saline, the cells were scraped into 20 mM potassium phosphate buffer, pH 7, containing 50 mM mercaptoethanol (1 mL), and the suspension was boiled for 5 min. Cellular debris was removed by centrifugation, and labeled folates in the supernatant were cleaved to *p*AbaGlu$_n$ and separated by HPLC as described above.

Results

In Vitro Metabolism of Unlabeled Folates. The metabolism of unlabeled derivatives was investigated under standard assay conditions modified by the inclusion of 500 μM [^{14}C]glutamate (nonsaturating) and replacing H_4PteGlu with the analogue under study (1 or 10 μM). Assay mixtures contained 4 units of enzyme, and metabolism was allowed to proceed for 24 h (Table I).

With 1 μM analogue as substrate, both (6*S*)-H_4PteGlu and (6*RS*)-H_4PteGlu were metabolized up to the hexaglutamate derivative with the pentaglutamate predominating, although the polyglutamate distribution obtained with (6*RS*)-H_4PteGlu as the substrate was of slightly shorter glutamate chain length. The amount of labeled product formed in each case accounted for approximately 70% of the added substrate, indicating that (6*R*)-H_4PteGlu is a substrate for the reaction, as was suggested by earlier kinetic analyses (Cichowicz & Shane, 1987b), and that chain elongation with this compound proceeds slightly less effectively than with (6*S*)-H_4PteGlu. The major product with (6*S*)-H_4PteGlu$_{2,3,4}$ as the substrates was also the pentaglutamate derivative. (6*R*)-10-Formyl-H_4PteGlu was metabolized to shorter chain length derivatives with tri- and

Table II: Effect of Incubation Conditions on the in Vitro Metabolism of Folates and Folate Analogues[a]

folate	assay/addition or modification	polyglutamate distribution (%)						
		1	2	3	4	5	6	7
[^{3}H]PteGlu	none	10	4	20	54	10	0.4	0
[^{3}H]PteGlu	50 mM Glu	14	4	31	44	6	0.3	0
[^{3}H]PteGlu	pH 8.0	10	8	22	53	7	0.5	0
[^{3}H]PteGlu	50 mM Glu, pH 8.0	12	4	23	51	7	0.5	0
(6*S*)-5-methyl-H$_4$[^{3}H]PteGlu	none	11	70	18	1	0.1	0	0
(6*S*)-5-methyl-H$_4$[^{3}H]PteGlu	pH 8.0	7	75	15	2	0.8	0	0
(6*S*)-5-methyl-H$_4$[^{3}H]PteGlu	50 mM Glu, pH 8.0	5	75	18	2	0.3	0	0
[^{3}H]aminopterin	none	11	65	18	5	1	0.2	0
[^{3}H]aminopterin	50 mM Glu, pH 8.0	10	62	21	5	2	0.3	0
(6*S*)-H$_4$[^{3}H]PteGlu	none	6	2	2	3	23	54	9
(6*S*)-H$_4$[^{3}H]PteGlu	5 mM spermine	7	2	2	5	32	45	5
(6*S*)-H$_4$[^{3}H]PteGlu	5 mM spermidine	8	2	2	4	30	47	8
(6*S*)-H$_4$[^{3}H]PteGlu	200 mM KCl	7	2	2	5	41	38	5
(6*S*)-H$_4$[^{3}H]PteGlu	pH 7 at 3 h	6	1	2	3	52	33	3
(6*S*)-H$_4$[^{3}H]PteGlu	5 mM L-aspartate	7	2	2	5	22	52	9

[a] Assay conditions and the cleavage of folate products to *p*AbaGlu$_n$ and their separation by HPLC are described under Experimental Procedures. The standard assay mixtures (pH 9) contained the indicated labeled folate or folate analogue (0.1 μM), 5 mM L-glutamate, 5 mM ATP, and 4 units of enzyme and were incubated for 24 h. Modifications of the standard assay conditions are indicated.

tetraglutamates predominating while (6*S*)-5-methyl-H$_4$PteGlu was metabolized primarily to the diglutamate. Tetra- and pentaglutamate products predominated with H$_2$PteGlu$_{1,2}$ as the substrate while tetraglutamate was the major product of PteGlu metabolism.

Similar results were seen when 10 μM substrates were used (Table I). Pentaglutamate was still the major product of (6*S*)-H$_4$PteGlu metabolism while tetraglutamate predominated with (6*RS*)-H$_4$PteGlu as the substrate. Tetraglutamates were still the major products of H$_2$PteGlu$_{1,2}$ and PteGlu metabolism while triglutamate predominated with (6*R*)-10-formyl-H$_4$PteGlu as substrate and diglutamate with (6*S*)-5-methyl-H$_4$PteGlu. (6*S*)-5-Methyl-H$_4$PteGlu$_2$ was metabolized mainly to the triglutamate derivative with lesser amounts of tetraglutamate, but the activity observed with this substrate was low.

Only one glutamate moiety was added to (6*S*)-H$_4$PteGlu$_{5,6,7}$, and the substrate activity of these compounds dropped off considerably as the glutamate chain length increased. The total product formed from (6*S*)-H$_4$PteGlu$_7$ was less than 1% that observed with (6*S*)-H$_4$PteGlu as the substrate. The relative difference in substrate effectiveness between these compounds is greater than suggested here, as most, if not all, of the monoglutamate substrate was converted to product in these experiments. Similarly, H$_2$PteGlu$_5$ and PteGlu$_5$ were substrates but demonstrated only low levels of activity. No product was detected with PteGlu$_{6,7}$ as substrates. The products obtained with (6*R*)-5,10-methylene-H$_4$PteGlu$_n$ were similar to those observed with (6*S*)-H$_4$PteGlu$_n$, which presumably reflected conversion of the former compounds to the latter during incubation.

In Vitro Metabolism of Unlabeled Folate Analogues. Under the conditions described above, 4-aminofolates, such as aminopterin, dihydroaminopterin, and methotrexate, were metabolized primarily to the diglutamate derivative (Table I). Although (6*S*)-H$_4$Pte-4-aminobutyryl-Glu and H$_2$Pte-4-aminobutyryl-Glu were effective substrates for the enzyme, the major products formed with these substrates contained one additional glutamate moiety, equivalent to a triglutamate product, which compares to the penta- and tetraglutamate products obtained with the equivalent PteGlu$_2$ derivatives. (6*S*)-Tetrahydrohomofolate and homofolate were poor substrates and were metabolized primarily to the triglutamate product. No products were detected with oxidized and reduced Pte-kainate and Pte-2-methyl-Glu derivatives.

Metabolism of Labeled Folates and Analogues. To drive the metabolism of folates and analogues as far as possible, the in vitro metabolism studies were repeated with low levels of labeled folate or analogue (0.1 μM), and unlabeled glutamate (5 mM) replaced the labeled glutamate (500 μM). Under these conditions, (6*S*)-H$_4$PteGlu was metabolized primarily to the hexaglutamate derivative, the major endogenous folate in hog liver (Table II). PteGlu was metabolized mainly to the tetraglutamate while diglutamate was still the major product obtained with (6*S*)-5-methyl-H$_4$PteGlu and aminopterin. Increasing the glutamate concentration to 50 mM and/or decreasing the pH to a more physiological value (pH 8) had little effect on the metabolism of these compounds.

(6*S*)-H$_4$PteGlu metabolism was unaffected by the addition of 5 mM L-aspartate to the assay mixture, and all products eluted at the positions of authentic *p*AbaGlu$_n$ standards, indicating that aspartate, which cannot replace glutamate as a substrate with pteroylmonoglutamate substrates, has no apparent affinity for the enzyme with pteroylpolyglutamate substrates and is not an alternate glutamate substrate when long-chain folates are used (Table II). Polycations and high levels of K^+ caused a slight inhibition of (6*S*)-H$_4$PteGlu metabolism. Adjustment of the pH to 7 after 3 h of incubation slowed down the metabolism of (6*S*)-H$_4$PteGlu, demonstrating that metabolism of long glutamate chain length folates was not favored at the lower pH.

Inhibition of Folate Metabolism by Folylpolyglutamates. To assess the ability of folylpolyglutamates to regulate folylpolyglutamate synthesis, the effects of unlabeled folate (usually 50 μM) on the metabolism of labeled (6*S*)-H$_4$PteGlu (0.1 or 1 μM) or PteGlu (1 μM) were investigated. The unlabeled folate concentration was similar to the intracellular folate concentration in hog liver while the labeled folate was present at a concentration considerably below its K_m for folylpolyglutamate synthetase (Cichowicz & Shane, 1987a,b) and at a similar concentration to the in vivo level of pteroylmonoglutamate in hog liver. Under these conditions, inhibition of metabolism should reflect the apparent affinity of the unlabeled folate for the enzyme (see Discussion).

Unlabeled (6*S*)-H$_4$PteGlu caused a large inhibition of (6*S*)-H$_4$[^{3}H]PteGlu metabolism such that the major labeled product formed after 3 h was reduced from the pentaglutamate to the triglutamate derivative and by 24 h from the hexaglutamate to the pentaglutamate derivative (Table III). Unlabeled (6*S*)-H$_4$PteGlu$_{2,3,4}$ were slightly more effective than (6*S*)-H$_4$PteGlu as inhibitors of labeled folate metabolism while (6*S*)-H$_4$PteGlu$_5$ was slightly less effective. Inhibitory action

Table III: Inhibition of (6*S*)-H_4[^{3}H]PteGlu Metabolism by Folates and Folate Analogues[a]

addition (50 μM)	polyglutamate distribution (%)						
	1	2	3	4	5	6	7
	1 μM (6*S*)-H_4[^{3}H]PteGlu, 3 h						
none	3	2	17	37	41	1	0
(6*S*)-H_4PteGlu	3	39	52	7	0	0	0
(6*S*)-H_4PteGlu$_2$	3	42	49	5	0	0	0
(6*S*)-H_4PteGlu$_3$	3	37	54	6	0	0	0
(6*S*)-H_4PteGlu$_4$	3	32	57	5	0	0	0
(6*S*)-H_4PteGlu$_5$	3	22	64	11	1	0	0
(6*S*)-H_4PteGlu$_6$	2	4	43	37	13	0	0
(6*S*)-H_4PteGlu$_7$	3	3	34	40	20	0	0
(6*S*)-H_4PteGluNH$_2$	3	29	54	13	2	0	0
(6*S*)-H_4Pte-ornithine	4	53	39	4	0	0	0
PteGlu-[ψ,CH_2-NH]-DL-Glu	2	2	26	43	27	0	0
(6*S*)-H_4Pte-homocysteate	2	2	22	40	34	1	0
(6*S*)-H_4Pte-homocysteine sulfonamide	2	2	22	40	33	1	0
4-NH_2-Pte-sulfanilyl-Glu[b]	4	7	48	31	10	0	0
4-NH_2-Pte-phosphonate[b]	3	2	21	41	35	1	0
	0.1 μM (6*S*)-H_4[^{3}H]PteGlu, 24 h						
none	2	2	2	2	25	61	6
none[c]	2	2	2	2	36	57	0
(6*S*)-H_4PteGlu	2	2	11	41	43	1	0
(6*S*)-H_4PteGlu$_2$	3	2	19	46	30	1	0
(6*S*)-H_4PteGlu$_3$	3	2	22	46	26	0	0
(6*S*)-H_4PteGlu$_4$	3	2	15	46	34	1	0
(6*S*)-H_4PteGlu$_5$	2	2	6	33	55	2	0
(6*S*)-H_4PteGlu$_6$	2	2	2	2	76	17	0
(6*S*)-H_4PteGlu$_7$	3	2	2	2	62	29	1
(6*S*)-H_4PteGluNH$_2$	3	2	2	11	76	6	0
(6*S*)-H_4Pte-ornithine	4	2	16	42	36	0	0
PteGlu-[ψ,CH_2-NH]-DL-Glu	3	1	2	2	45	45	2
4-NH_2-Pte-sulfanilyl-Glu[b]	4	2	2	4	61	29	1

[a] Experimental conditions are described under Experimental Procedures. The indicated unlabeled analogues (50 μM) were added to reaction mixtures containing labeled H_4PteGlu (0.1 or 1 μM), unlabeled L-glutamate (5 mM), ATP (5 mM), and 5 units of enzyme. The labeled products were identified after a 3- or 24-h incubation. [b] 200 μM. [c] 1 μM (6*S*)-H_4[^{3}H]PteGlu as substrate.

Table IV: Effect of Substrate Concentration on Folate Metabolism[a]

[folate] (μM)	time (h)	polyglutamate distribution (%)						
		1	2	3	4	5	6	7
0.1	2	15	1	8	20	53	3	0
1.0	2	12	6	39	28	15	0	0
10.0	2	18	67	9	5	0	0	0
0.1	24	6	2	2	3	23	54	9
1.0	24	7	0	2	4	67	20	0

[a] Experimental Conditions are described under Experimental Procedures. Assay mixtures contained unlabeled L-glutamate (5 mM), ATP (5 mM), enzyme (4 units), and labeled H_4PteGlu at the indicated concentrations. The labeled products were identified after a 2- or 24-h incubation.

dropped off between the penta- and hexaglutamate derivatives although (6*S*)-H_4PteGlu$_7$ still caused significant inhibition of folate metabolism. Similar effects were seen on the metabolism of [^{3}H]PteGlu (Table V). Increasing the labeled H_4PteGlu concentration from 0.1 to 1.0 μM, which is still considerably below the K_m for the enzyme, significantly inhibited the formation of heptaglutamate (Tables III and IV).

Metabolism experiments using [^{3}H]PteGlu utilized lower amounts of enzyme to limit the extent of metabolism of the labeled folate. Under these conditions, unlabeled PteGlu (200 μM) was slightly less effective than (6*S*)-H_4PteGlu (50 μM) at inhibiting folate metabolism (Table VI) while inhibitory activity dropped off considerably with PteGlu$_3$, and PteGlu$_6$ was only slightly inhibitory. No inhibition was observed with H_2Pte while H_2PteGlu$_{1,2,5}$ were very good inhibitors of labeled folate metabolism.

Inhibition of Folate Metabolism by Folate Analogues. The ability of folate analogues to inhibit the in vitro metabolism of folate was studied in an analogous manner. (6*S*)-H_4Pte-ornithine was a potent inhibitor of labeled (6*S*)-H_4PteGlu (Table III) and PteGlu (Table V) metabolism, demonstrating much higher apparent affinity than any of the naturally occurring folate derivatives. PteGlu-[ψ,CH_2-NH]-DL-Glu was less effective, demonstrating similar apparent affinity to (6*S*)-H_4PteGlu$_7$, while 4-NH_2-Pte-sulfanilyl-Glu was slightly more effective, demonstrating similar apparent affinity to (6*S*)-H_4PteGlu$_{5,6}$ (Table III). (6*S*)-H_4PteGluNH$_2$ was a good inhibitor with slightly less apparent affinity than (6*S*)-H_4PteGlu while other pteroyl amino acid analogues, including the homocysteate, homocysteine sulfonamide, methionine sulfone, kainate, 2-aminobutyrate, and 2-methylglutamate derivatives, were only very weak inhibitors of labeled folate metabolism or were without any apparent effect (Tables III, V, and VI).

Aminopterin inhibited PteGlu metabolism with similar apparent affinity to (6*S*)-H_4PteGlu$_4$, and its dihydro derivative was even more potent with better apparent affinity than (6*S*)-H_4PteGlu (Tables V and VI). Methotrexate and its dihydro derivative were weak inhibitors or inactive (Tables V and VI).

Pterin derivatives such as 4-NH_2-Pte-phosphonate (Table III) and the dihydro- forms of pterin-6-carboxyglutamate, Pte-carboxamide, Pte-sulfonamide, and pterin-6-hydroxymethyl-pyrophosphate (Table VI) had only slight or no effect

Table V: Inhibition of [^{3}H]PteGlu Metabolism by Folates and Folate Analogues[a]

addition[b] (50 μM)	polyglutamate distribution (%) 1	2	3	4	5	6
none	17	26	33	24	1	0
(6*S*)-H_4PteGlu	56	31	8	4	0	0
(6*S*)-H_4PteGlu$_2$	52	33	12	4	0	0
(6*S*)-H_4PteGlu$_3$	57	30	9	4	0	0
(6*S*)-H_4PteGlu$_4$	46	35	14	5	0	0
(6*S*)-H_4PteGlu$_5$	35	45	12	8	1	0
(6*S*)-H_4PteGlu$_6$	25	44	24	6	1	0
(6*S*)-H_4PteGlu$_7$	25	39	27	8	1	0
aminopterin	46	35	18	1	0	0
methotrexate	23	34	31	12	0	0
(6*S*)-H_4Pte-ornithine	81	15	3	0	0	0
(6*S*)-H_4Pte-kainate	23	29	30	17	1	0
(6*S*)-H_4Pte-2-NH_2-butyrate	23	28	30	19	1	0
DTBSF	62	33	4	1	0	0

[a] Experimental conditions are described under Experimental Procedures. The indicated unlabeled analogues (50 μM) were added to reaction mixtures containing labeled PteGlu (1 μM), unlabeled L-glutamate (5 mM), ATP (5 mM), and 1 unit of enzyme. The labeled products were identified after a 24-h incubation. [b] (6*S*)-H_4Pte-Met-sulfone and (6*S*)-H_4Pte-2-methyl-Glu had no effect.

Table VI: Inhibition of [^{3}H]PteGlu Metabolism by Oxidized and Dihydrofolate Analogues[a]

addition[b] (50 μM)	polyglutamate distribution (%) 1	2	3	4	5
none	63	29	6	1	0
(6*S*)-H_4PteGlu	84	12	4	1	0
PteGlu[c]	81	15	3	1	0
PteGlu$_3$[c]	73	18	7	3	0
PteGlu$_6$[c]	71	24	4	1	0
DTBSF	94	5	1	0	0
Pte-DL-homocysteine sulfonamide[c]	68	24	5	2	1
PteGluNH$_2$[c]	84	12	2	1	0
H_2PteGlu	89	9	2	0	0
H_2PteGlu$_2$	85	12	3	0	0
H_2PteGlu$_5$	84	12	5	0	0
dihydroaminopterin	91	6	3	0	0

[a] Experimental conditions are described under Experimental Procedures. The indicated unlabeled analogues (50 μM) were added to reaction mixtures containing labeled PteGlu (1 μM), unlabeled L-glutamate (5 mM), ATP (5 mM), and 1 unit of enzyme. The labeled products were identified after a 3-h incubation. [b] H_2Pte, dihydromethotrexate, dihydropterin-6-carboxy-Glu, 4-NH_2-H_2Pte-carboxamide, 4-NH_2-H_2Pte-sulfonamide, and dihydropterin 6-hydroxymethyl-pyrophosphate had no effect. [c] 200 μM analogue.

on folate metabolism. DTBSF, a possible covalent inactivator of folylpolyglutamate synthetase (George et al., 1987), was a potent inhibitor of [^{3}H]PteGlu metabolism (Tables V and VI).

Effect of Analogues on the in Vivo Metabolism of Folate. The effect of analogues on the net uptake and metabolism of labeled PteGlu by mammalian cells was studied in cells cultered in +GAT medium. Under these conditions, the cells did not require folate for growth. Addition of unlabeled PteGlu (10 μM) to the culture medium increased the folate concentration in the medium 70-fold and caused a 34-fold increase in the accumulation of folate by CHO cells and a 51-fold increase in HeLa cells (Table VII). The increased substrate concentration in the CHO and HeLa cells caused a shortening of the glutamate chain lengths of the major labeled folate derivatives, although, in both cell lines, only low proportions of short glutamate chain length folates were detected. Increased substrate also caused an almost complete loss of very long glutamate chain length derivatives.

Pte-ornithine and PteGlu-[ψ,CH_2-NH]-Glu had little or no effect on net folate uptake and no significant effect on the metabolism of labeled folate by these cells. These compounds had no apparent effect on CHO and HeLa cell growth when the cells were cultured in –GAT medium, i.e., under conditions where the cells were dependent on exogenous folate for growth, suggesting that mammalian cells are unable to transport these analogues.

4-NH_2-Pte-sulfanilyl-Glu was a potent inhibitor of cell growth and reduced the net accumulation of labeled folate by the cells and inhibited folylpolyglutamate synthesis such that the major derivatives were two glutamate moieties shorter than in the absence of this analogue. The polyglutamate distribution in the CHO cells appeared to be bimodal with peaks at the pentaglutamate and heptaglutamate. This type of distribution is consistent with 4-NH_2-Pte-sulfanilyl-Glu inhibition of dihydrofolate reductase causing an accumulation of oxidized folates in the cell, rather than direct inhibition of folylpolyglutamate synthetase activity, per se. Oxidized folate would be expected to be metabolized to shorter glutamate chain length folates, and the slower metabolism of oxidized derivatives to polyglutamate forms would be expected to lead to decreased retention of folate by the cells.

Modeling of in Vitro and in Vivo Metabolism. Table VIII shows a simplified model for the metabolism of folates, derived by using kinetic constants for individual folates obtained previously (Cichowicz & Shane, 1987b). With 100 nM H_4PteGlu$_n$ and saturating L-glutamate and MgATP concentrations, the enzyme would turn over about 100 times per hour with the monoglutamate substrate but only once every 8 h with the heptaglutamate substrate.

Substrate turnover time, the time required for the complete conversion of PteGlu$_n$ to PteGlu$_{n+1}$, was calculated for 4 nM enzyme (saturating glutamate) and for 50 nM enzyme (half-saturating glutamate), the latter approximating expected in vivo conditions. The values can only be considered approximate as initial rate conditions were assumed. No allowance has been made for competition between substrates or for in vivo binding of substrates to folate-dependent enzymes, both of which might be expected to increase the substrate turnover time (see Discussion). The estimated substrate turnover times are in good agreement with the observed in vitro metabolism of folates reported in this study. For instance, in 24-h incubation studies with 4 nM enzyme (Table II), H_4PteGlu was metabolized primarily to the hexaglutamate, and little mono-to tetraglutamate was detected. The calculated turnover times indicate that initial metabolism to the triglutamate should be fairly rapid and that 24 h should be sufficient for metabolism to the hexaglutamate but not for complete conversion of hexa- to heptaglutamate. The very long substrate turnover time for octaglutamate formation (8 days) would explain the absence of octa- and nonaglutamate products in the in vitro metabolism experiments.

Discussion

The retention, and concentration, of folates by mammalian tissues requires their conversion to polyglutamate derivatives. In the metabolism studies described in this paper, hog liver folylpolyglutamate synthetase was used as a model in vitro system for studying factors that may regulate folate homeostasis. Using assay conditions that approximated the substrate concentrations expected in mammalian tissues and using relatively large amounts of purified enzyme, although still only about 10% of the in vivo concentration, we found that similar folate derivatives accumulated to those found in vivo. Products that accumulated were very poor substrates for the

Table VII: Effect of Analogues on [^{3}H]PteGlu Uptake and Metabolism by Chinese Hamster Ovary and HeLa Cells[a]

cell line	analogue	net folate uptake (cpm × 10^{-3})	polyglutamate distribution (%)									
			1	2	3	4	5	6	7	8	9	10
CHO	none	56	8	1	2	3	13	27	29	16	1	0
CHO	PteGlu	26[b]	16	0.5	3	8	43	28	2	0	0	0
CHO	4-NH_2-Pte-sulfanilyl-Glu	15	24	0	1	11	30	11	14	8	0.5	0
CHO	Pte-ornithine	58	7	0.4	2	3	11	26	31	20	1	0
CHO	PteGlu-[ψ,CH_2-NH]-Glu	47	13	0.3	2	3	9	22	29	21	2	0
HeLa	none	87	20	0.2	0.9	2	2	7	23	37	7	0.7
HeLa	PteGlu	62[b]	23	0.4	1	1	9	34	26	6	0.3	0
HeLa	4-NH_2-Pte-sulfanilyl-Glu	41	20	0.6	2	2	22	39	12	3	0.5	0
HeLa	Pte-ornithine	56	11	0.1	0.8	1	2	7	24	43	8	0.8
HeLa	PteGlu-[ψ,CH_2-NH]-Glu	42	29	0.4	1	1	2	5	15	36	10	1

[a] Experimental conditions are described under Experimental Procedures. Folate-depleted cells were cultured in +GAT medium containing [^{3}H]-PteGlu (0.14 μM) and the indicated unlabeled analogue (10 μM) for 22 h, and intracellular labeled folates were extracted and cleaved to *p*AbaGlu$_n$ derivatives which were separated, according to glutamate chain length, by HPLC. [b] Total folate accumulation increased 34-fold (CHO) and 51-fold (HeLa).

Table VIII: Estimated Turnover Times under Various Reaction Conditions[a]

substrate	catalytic turnovers/h	substrate turnover time (h)	
		4 nM enzyme[b]	50 nM enzyme[c]
H_4PteGlu	98	0.26	0.04
H_4PteGlu$_2$	99	0.25	0.04
H_4PteGlu$_3$	57	0.44	0.07
H_4PteGlu$_4$	16	1.5	0.25
H_4PteGlu$_5$	4.4	5.7	0.90
H_4PteGlu$_6$	*0.38*	*66*	*11*
H_4PteGlu$_7$	*0.13*	*192*	*30*
H_2PteGlu	153	0.16	0.03
H_2PteGlu$_2$	145	0.17	0.03
H_2PteGlu$_5$	*0.55*	*45*	*7.3*
5-methyl-H_4PteGlu	5.5	4.5	0.72
PteGlu	6.3	4.0	0.63
PteGlu$_2$	7.3	3.4	0.55
PteGlu$_3$	2.1	12	1.9
PteGlu$_4$	*0.15*	*167*	*27*
PteGlu$_5$	*0.005*	*>5000*	*>800*
aminopterin	30	0.83	0.13
methotrexate	3.4	7.4	1.2

[a] Kinetic constants were from Cichowicz and Shane (1987b) or were estimated from data in Table I (italicized values). All turnover values are for 100 nM folate substrate and saturating L-glutamate, except where noted. Substrate turnover is the time required for the addition of one glutamate residue to the indicated folate substrate (100 nM) with the indicated enzyme concentration, assuming initial rate conditions. [b] Similar to conditions described in Tables II and III. [c] Physiological conditions, i.e., 20–70 nM enzyme and assuming half-saturating glutamate concentration.

enzyme, and the distributions of products obtained in metabolism studies could be modeled by using kinetic constants obtained with individual folate substrates (Cichowicz & Shane, 1987b). The accumulation of hexaglutamate derivatives in vivo and in vitro is due to the very slow catalytic turnover of the enzyme with pteroylhexaglutamate substrates. The absence of very long chain length derivatives such as octa- and nonaglutamates in in vitro studies, although small amounts of these compounds are found in vivo, was due to the lower enzyme concentration used in vitro and the 24-h incubation time. The formation of these derivatives in vivo would be expected to be a very slow process. The primary factor in determining the predominant folylpolyglutamate in cells is the substrate specificity of folylpolyglutamate synthetase. Similarly, mutant CHO cell transfectants expressing the human (Sussman et al., 1986) or *Escherichia coli* (C. Osborne and B. Shane, unpublished results) folylpolyglutamate synthetase genes contain folylpolyglutamate distributions characteristic of human cells or *E. coli*, respectively, provided the enzyme activity expressed is similar to the normal level in CHO cells.

The distribution of labeled products in metabolism and competition studies was consistent with the ordered kinetic mechanism determined with aminopterin as a substrate, in which the folate product is released from the enzyme after addition of a single glutamate moiety (Cichowicz & Shane, 1978b). There was no indication of multiple glutamate residue addition to enzyme-bound folate with folylpolyglutamate derivatives, suggesting that the kinetic mechanism does not differ for folylpolyglutamate substrates.

Enzyme Concentration. As multiple glutamate residues are added to pteroylmonoglutamates to generate folylpolyglutamates, a reduction in enzyme concentration would be expected to have a larger effect on the formation of long-chain derivatives than on short-chain derivatives. This effect was noted in in vitro metabolism studies. However, these studies also showed that with substrates that turn over at a rapid rate, such as H_4PteGlu, relatively large reductions in enzyme concentration cause relatively minor changes in the predominant folate that accumulates, i.e., hexaglutamate, although the appearance of longer chain derivatives, which are formed at a very slow rate, is greatly suppressed. This observation, which can be modeled by using kinetic parameters obtained for individual substrates, suggests that folate concentration by tissues may not be very responsive to differences in enzyme levels provided that the pteroylmonoglutamate substrate, which is present at a constant level, can be metabolized rapidly to a polyglutamate derivative that is of sufficient chain length to be retained by the tissue. Recent studies indicate that mono- and diglutamates of 4-aminofolates are rapidly lost from mammalian cells, while the efflux rate for triglutamate is greatly decreased or negligible and negligible efflux of tetraglutamate occurs (Samuels et al., 1985). Longer glutamate chain length methotrexate derivatives are preferentially retained by mammalian cells (Jolivet et al., 1982; Fabre et al., 1984; McGuire et al., 1985).

The effect of changes in in vivo enzyme levels has recently been studied by using CHO mutants transfected with the human folylpolyglutamate synthetase gene (Sussman et al., 1986). A 14-fold decrease in enzyme activity results in only a 2-fold decrease in folate accumulation, and the major folate derivatives (hexa- and heptaglutamates) remain unchanged although the proportion of longer chain length derivatives is greatly diminished. In cases where enzyme activity is very low (<2% of wild type), folate accumulation is still only reduced by 4-fold, and the major polyglutamate present is slightly shorter (pentaglutamate) and short chain length derivatives do not accumulate. Although folate accumulation is not directly proportional to enzyme levels in mammalian

cells expressing mammalian enzyme, a linear relationship between folate accumulation and enzyme activity is seen in CHO mutants expressing the *E. coli* folylpolyglutamate synthetase (Osborne and Shane, unpublished results). These cells metabolize folate only to the triglutamate form, a chain length that is sufficient to overcome their auxotrophic requirement for glycine, purines, and thymidine (see below). It appears that only low levels of enzyme are required to metabolize folates to derivatives that are retained by mammalian cells but that higher levels are needed to generate the long chain length derivatives that are normally observed in these cells.

Intracellular Folate Levels. The retention of apparent affinity for the enzyme by reduced folylpolyglutamates has led to the suggestion that folylpolyglutamate synthesis may be regulated by a product inhibition type mechanism (Cichowicz & Shane, 1987b; McGuire et al., 1980; Foo & Shane, 1982). Although this phenomenon would actually represent competition between substrates, the extremely low k_{cat} values with the longer glutamate chain length derivatives imply, in effect, that they can be considered as inhibitors of the reaction. Under conditions where labeled H_4PteGlu is metabolized to long chain length folylpolyglutamate derivatives, physiological concentrations of unlabeled folylpolyglutamates cause significant inhibition of this process. Although significant metabolism of the unlabeled folate would also be expected under these conditions, it is noteworthy that the apparent affinities displayed by unlabeled folates as inhibitors of labeled folate metabolism are inversely proportional to their K_m values as substrates. Intracellular folylpolyglutamates would slow the rate of polyglutamate synthesis by a factor of $1 + I/K_m$. Under physiological conditions, the maximum rate reduction would be about 2-fold, and probably considerably less as the bulk of intracellular folate would be bound to folate-dependent enzymes. This would have the same effect as reducing folylpolyglutamate synthetase levels by a factor of 2, which, as discussed above, would not significantly affect folate accumulation unless the folate substrate is a form that cannot be rapidly metabolized to the tri- or tetraglutamate derivative (see below).

Increasing the medium PteGlu concentration 70-fold results in 35- and 50-fold increases in the net accumulation of labeled folate by CHO and HeLa cells, respectively. Other studies have demonstrated that net folate and methotrexate accumulation by cultured cells is approximately proportional to the medium folate or methotrexate content (Hilton et al., 1979; Foo & Shane, 1982; Foo et al., 1982; Watkins & Cooper, 1983; Steinberg et al., 1983; McGuire et al., 1985). Mammalian cells can transport high nonphysiological levels of folate because the K_t for transport is very high (Sirotnak, 1985; Henderson et al., 1986). The cell's capacity to accumulate high levels of folate reflects that the conversion of high levels of folate to retainable polyglutamate derivatives is not limited under these conditions. For instance, the estimated turnover time for the complete conversion of 100 nM H_4PteGlu to the triglutamate derivative under physiological conditions in hog liver is about 5 min (Table VIII). Increasing the folate concentration to 1 or 10 μM would increase this turnover time to 6 and 15 min, respectively. Over the same 100-fold concentration range, the turnover time for the conversion of H_4PteGlu$_6$ to the heptaglutamate would increase from 11 to 25 h. Although polyglutamate distributions are slightly shortened in mammalian cells cultured in the presence of high folate, and short-chain derivatives do not accumulate, there is an almost complete loss of very long chain length derivatives. This effect is also observed in in vitro metabolism studies when the folate concentration is increased and can be explained by the slower metabolic turnover of folates when folate levels are high, which becomes significant with derivatives that turn over at a slow rate. In addition, any reduction in turnover rate would have a greater affect on the formation of long-chain derivatives as more catalytic turnovers are required for their synthesis. Competition with medium chain length derivatives that are increased in concentration, and have higher apparent affinity for the enzyme, may also play a role in the loss of long-chain derivatives.

Endogenous folylpolyglutamate distributions are slightly longer in folate-depleted cells (Priest et al., 1983) and in the livers of folate-depleted animals (Cassady et al., 1980). Although this could suggest an increase rate of polyglutamate synthesis with very long glutamate chain length derivatives, which may be explained by relief of inhibition by the decreased concentration of the higher affinity shorter glutamate chain length forms and the shorter time required for turnover of long chain length derivatives when the folate concentration is reduced, competition effects and changes in substrate turnover time are unlikely to be significant at low cellular folate levels. As the predominant folates in cells are synthesized at a relatively rapid rate while long chain length derivatives are formed only slowly and represent "old" folate, depletion or removal of entering pteroylmonoglutamate would be expected to gradually change the distribution of folylpolyglutamates to longer chain length derivatives.

Although folates do not appear to turn over at an appreciable rate in cultured mammalian cells (Steinberg et al., 1983), folates in slow growing mammalian tissues do turn over. The half-life of hepatic folate in the rat has been reported to be 4 days (Thenen et al., 1973). The major endogenous folate in rat liver is the pentaglutamate, and labeled pentaglutamate predominates 24 h after administration of a labeled folate dose to the rat (Leslie & Baugh, 1974; Shane, 1982). However, 4 weeks after the injection, the small amount of labeled folate still present is primarily hexa- and heptaglutamate (Leslie & Baugh, 1974). This overshoot of the endogenous polyglutamate distribution after a lengthy time interval represents the same phenomenon as the appearance of longer polyglutamate distributions in folate-depleted cells and tissues and is consistent with the very poor substrate activity of longer chain length folylpolyglutamates. This overshoot, and the ability to mimic in vivo folate metabolism using in vitro conditions, strongly suggests that extension of the polyglutamate chain is a one-way process in cells and implies that folate turnover in tissues does not involve hydrolysis of the polyglutamate chain or, alternately, that any hydrolyzed folate is released and does not equilibrate with the folylpolyglutamate pool. A slow efflux of folylpolyglutamates with efflux rates inversely proportional to polyglutamate chain length, turnover by direct cleavage to non-folate derivatives (Murphy et al., 1976), or direct hydrolysis to short chain length folates and consequent release from the cell would be consistent with the data. Mammalian γ-glutamylhydrolases and transpeptidases that can hydrolyze folylpolyglutamates directly to the diglutamate derivative have been described (Rosenberg & Neumann, 1974; Silink et al., 1975; Brody & Stokstad, 1982; Elsenhans et al., 1984; McGuire & Coward, 1984).

Folate and methotrexate accumulation by mammalian cells is increased by prior folate depletion (Foo & Shane, 1982; Nimec & Galivan, 1983; Galivan et al., 1983), and polyglutamate formation is more rapid in dividing cultures (Ninec & Galivan, 1983). Increased net accumulation of a labeled folate dose occurs in the regenerating rat liver (Marchetti et al., 1985) while endogenous folate levels drop slightly and

slightly longer endogenous polyglutamates are found (Eto & Krumdieck, 1982). A reduction in competing folate may explain increased methotrexate polyglutamate formation under these conditions, as the conversion of methotrexate to polyglutamates is a relatively slow process (see below). However, for reasons discussed above, reduction of competition by intracellular folate is unlikely to be responsible for the increased folate accumulation. These data probably reflect the increased capacity of mammalian cells cultured in the presence of limited folate to transport folate (Kane et al., 1986; Kamen & Capdevila, 1986).

Effects of One-Carbon Substitution. In vitro incubation studies with different folate derivatives were potentially complicated by the lability of some of these compounds, and the extent of metabolism in some of the experiments was affected by the use of subsaturating glutamate concentrations. However, the types of polyglutamates that accumulated are consistent with the substrate specificities noted for individual folates (Cichowicz & Shane, 1987a,b; George et al., 1987) and can be modeled by using kinetic constants derived from individual folates. The data again demonstrate that the substrate effectiveness of pteroylmonoglutamate derivatives has little bearing on the catalytic efficiency with polyglutamate derivatives.

Under conditions where H_4PteGlu is metabolized primarily to penta- or hexaglutamate derivatives, H_2PteGlu is metabolized to tetra- and pentaglutamates, PteGlu to tetraglutamate, 10-formyl-H_4PteGlu to tri- and tetraglutamates, homofolate and tetrahydrohomofolate to triglutamates, and 5-methyl-H_4PteGlu, methotrexate, aminopterin, and dihydroaminopterin to diglutamate derivatives. The final distribution of folylpolyglutamates in vivo in hog liver is governed by the substrate effectiveness of H_4PteGlu$_n$ rather than by other one-carbon forms of folate. The lack of substrate activity of relatively short polyglutamate chain length derivatives of some folate one-carbon forms does not preclude further chain length elongation, but these compounds have to be converted to the preferred substrate form, H_4PteGlu$_n$ before chain extension occurs. Because of this, the retention of folate by tissues and folate homeostasis would be expected to be regulated by physiological and nutritional factors, and metabolic disturbances, which affect the proportion of folate present as the H_4PteGlu$_n$ form.

If the interconversion of different folate one-carbon forms via the metabolic pathways of one-carbon metabolism under normal physiological conditions occurs at a faster rate than the addition of glutamate moieties to folate, a decrease in the proportion of cellular folate derivatives that are effective substrates for the enzyme would have the same effect on the rate of polyglutamate synthesis as an equivalent decrease in enzyme level. As discussed above, this would not be expected to significantly affect folate accumulation. However, metabolic interconversion of different pteroylmonoglutamates may be quite slow as most of the enzymes of one-carbon metabolism possess much higher affinity for polyglutamate substrates than monoglutamates (see below). Although H_2PteGlu and 10-formyl-H_4PteGlu are metabolized to shorter chain length products than H_4PteGlu, their initial metabolism in vivo to retainable polyglutamates would be quite rapid, and an increase in the proportion of these forms of the vitamin is unlikely to have a significant effect on the accumulation of cellular folate. However, metabolism of PteGlu to the triglutamate and 5-methyl-H_4PteGlu to the diglutamate is fairly slow (Table VIII), and conversion of the latter compound to the triglutamate is extremely slow, suggesting that an inability to convert these compounds to other folate one-carbon forms would result in their loss from the cell and consequently a decrease in cellular folate levels.

In man and experimental animals, B_{12}-dependent methionine synthase activity is reduced under conditions of vitamin B_{12} deficiency. A functional folate deficiency results due to accumulation of 5-methyl-H_4PteGlu$_n$, a substrate for methionine synthase, at the expense of other folate one-carbon forms, including H_4PteGlu$_n$ (Shane & Stokstad, 1985). In addition, tissue levels of folate are reduced up to 60%, which is due to an impaired ability to retain folate rather than impaired tissue uptake of the vitamin (Shane et al., 1977; Shane & Stokstad, 1985). The impaired retention of folate can be entirely explained by the decreased level of H_4PteGlu$_n$ under these conditions and the poor substrate activity of 5-methyl-H_4PteGlu for folylpolyglutamate synthetase, and the almost complete lack of substrate activity with polyglutamate forms of this compound. It has been suggested that 10-formyl-H_4PteGlu rather than H_4PteGlu is the substrate for folylpolyglutamate synthetase in mammalian tissues and that decreased levels of 10-formyl-H_4PteGlu$_n$ cause the impairment of folate retention in vitamin B_{12} deficiency (Perry et al., 1983). However, the data presented here clearly indicate that, although 10-formyl-H_4PteGlu is an effective substrate for the enzyme, H_4PteGlu$_n$ is the preferred substrate especially at the polyglutamate level.

Similarly, variations in the methionine concentration in the media of tissue culture cells, which affect the proportion of 5-methyl-H_4PteGlu$_n$ and H_4PteGlu$_n$, cause changes in the polyglutamate chain length distribution of intracellular folates, with longer polyglutamate derivatives occurring under conditions where the proportion of H_4PteGlu$_n$ is increased, and affect the net accumulation of labeled folate by these cells (Foo & Shane, 1982; Foo et al., 1982). Addition of purines to bacterial cell cultures also causes a decrease in the polyglutamate chain length distribution of intracellular folates which results from a purine-induced rearrangement of folate one-carbon forms favoring 10-formyl-H_4PteGlu$_n$, an ineffective substrate for bacterial folylpolyglutamate synthetases (Shane et al., 1983).

Other Regulatory Factors. The high K_m for the glutamate substrate of folylpolyglutamate synthetase at physiological pH, which is similar to the concentration of glutamate in hepatic tissue and higher than that in peripheral tissues, suggests that folylpolyglutamate synthesis in vivo can be regulated in part by intracellular glutamate levels. The ability of Ehrlich ascites tumor cells to accumulate methotrexate polyglutamates is dependent on the glutamate or glutamine concentration in the culture medium (Fry et al., 1983). Insulin and dexamethasone stimulate methotrexate polyglutamate formation by cultured mammalian cells, while cAMP has the opposite effect (Kennedy et al., 1983; Galivan, 1984). Although the mechanism by which these changes occur has not been established, it is possible that these hormones act indirectly by modifying cellular glutamate levels.

Regulation of One-Carbon Metabolism. It has been suggested that one-carbon metabolism may be regulated under different physiological and nutritional conditions by changing the glutamate chain length of folates in the cell, thus affecting the flux of one-carbon units through the different metabolic reactions of one-carbon metabolism (Krumdieck et al., 1977). As indicated above, modulations of the glutamate chain length of folates have been observed in tissue culture cells, animals, and bacteria in response to folate, methionine, vitamin B_{12}, and purine status and to changes in growth rates. Folylpolyglutamate synthetase levels are unaffected by nutritional modulation (Taylor & Hanna, 1977), and the data discussed above suggest that γ-glutamylhydrolase plays little, if any, role in modulating the folylpolyglutamate distribution in the cell.

All of the observed changes in folylpolyglutamate distributions in vivo can be explained by the substrate specificity and affinities of folates for folylpolyglutamate synthetase and can be mimicked in vitro using homogeneous enzyme. Although the changes in polyglutamate distribution reflect a secondary effect, due to variations in the folate one-carbon distribution and/or the folate level in the cell, it is possible that these changes may still modulate the reactions of one-carbon metabolism. However, these changes would occur at a slow rate, and such a regulatory mechanism would be incapable of responding to immediate changing needs for specific products of one-carbon metabolism.

The substrate effectiveness and affinity of folylpolyglutamates for a number of hog liver enzymes involved in one-carbon metabolism have been investigated (Lu et al., 1984; MacKenzie & Baugh, 1980; Matthews & Baugh, 1980; Matthews et al., 1982; Ross et al., 1984). In all cases, increased affinities are observed with polyglutamate derivatives, and in most cases, but not all, hexaglutamates are the most effective substrates. Methylenetetrahydrofolate reductase, the committed enzyme in methionine biosynthesis, exhibits the highest degree of specificity for the hexaglutamate derivative (Matthews & Baugh, 1980), and kinetic modeling suggests that the relative one-carbon flux through thymidylate synthesis vs. methionine synthesis would be increased 2–3-fold by changing the folylpolyglutamate substrates from the hexaglutamate to the penta- or heptaglutamate derivatives (Lu et al., 1984). Initial studies with mutant CHO cell transfectants expressing the *E. coli* folylpolyglutamate synthetase gene suggest that pteroyltriglutamates are sufficient to enable normal retention of folate by these cells and are sufficient to overcome the auxotrophic requirement for purines, thymidine, and glycine (Osborne and Shane, unpublished results). The metabolism of folates to long chain length derivatives in mammalian cells may reflect a requirement for methionine synthesis.

Metabolism of Anti-folates. Although 4-aminofolates are fairly good substrates of folylpolyglutamate synthetase, metabolism studies indicate that diglutamate derivatives are extremely poor substrates. The diglutamate of methotrexate is also the major metabolite detected in in vitro metabolism studies with the rat liver (McGuire et al., 1983) and beef liver enzymes (Schoo et al., 1985). Diglutamate is the major product of methotrexate metabolism in primary rat hepatocyte cultures (Rhee & Galivan, 1986) and of aminopterin metabolism in a human leukemia cell line (Samuels et al., 1985) which is consistent with the in vitro derived data. However, these data contrast with other studies in which the 4-aminofolate derivatives that accumulate in cultured mammalian cells are of longer glutamate chain length, ranging from tri- to pentaglutamates (Jolivet et al., 1982; Fabre et al., 1984; McGuire et al., 1985; Samuels et al., 1985). The longer derivatives, which are still of shorter glutamate chain length than folates in these cells, are more predominant after prolonged exposure to the drug or after further incubation of cultured cells in drug-free medium. In the latter case, the final distribution is generated partly by loss of shorter chain length derivatives and partly by further metabolism of retained derivatives. Differences in anti-folate metabolism may reflect differences in substrate specificity of folylpolyglutamate synthetases from different sources but could also be caused by differences in efflux rates of short-chain 4-aminofolate polyglutamates from the different cell types.

The metabolism of methotrexate to potentially retainable polyglutamate derivatives would be a relatively slow process in mammalian cells compared to reduced folates. Consequently, minor differences in substrate specificity of synthetases from different sources, especially with di- and/or triglutamate derivatives which are very poor substrates for the enzyme, could have a large effect on the retention of anti-folates. In addition, differences in efflux rates for di- and triglutamates of 4-aminofolates between different cell types have been observed (Samuels et al., 1985). If a combination of these factors allows a cell to accumulate the drug in a retainable form, with prolonged exposure further chain extension of the drug would be expected, even if the rate of metabolism is very slow. With increased culture time, the longer chain length derivatives formed would become more prominent as the levels of shorter chain derivatives would be expected to reach a steady state based on their rates of synthesis and efflux. The longer polyglutamate distributions found after further incubation in drug-free medium (McGuire et al., 1985) would be predicted as shorter derivatives would be lost from the cell, and only chain extension of retainable forms would occur.

The slow metabolism of 4-aminofolates to retainable polyglutamates also suggests that any reduction in polyglutamate synthesis rates, caused by, for example, differences in enzyme levels in different tissues, would have a greater effect on the retention of methotrexate derivatives than on folate derivatives. A methotrexate-resistant human breast cancer cell line has been described in which the major factor involved in drug resistance is an almost complete inability to accumulate polyglutamates of methotrexate (Cowan & Jolivet, 1984). K_m and V_{max} values of folate and methotrexate for the synthetase appear normal, and the cells metabolize folate to similar, if not slightly longer, folylpolyglutamate derivatives than the parent cell line. However, labeled folate accumulation is significantly decreased and the proportion of labeled intracellular pteroylmonoglutamate increased, indicating a reduced rate of polyglutamate synthesis with folates as well. Although the lesion in these cells has not been established, a decreased affinity of MgATP or glutamate for the enzyme, decreased cellular glutamate, or changes in efflux rates could explain the data.

Pte-ornithine derivatives are potent inhibitors of folylpolyglutamate synthetase and of folate metabolism in vitro but Pte-ornithine is not cytotoxic to CHO or HeLa cells, and has no apparent effect on intracellular folate metabolism, suggesting that the compound is not transported by mammalian cells. 4-Fluoroglutamate and homocysteate analogues of methotrexate are effective inhibitors of dihydrofolate reductase but are considerably less cytotoxic than methotrexate (Rosowski et al., 1984; Galivan et al., 1985), which is probably due to their inability or poor ability to be converted to retainable polyglutamate derivatives. Some anti-folates are only effective enzyme inhibitors in the polyglutamate form while methotrexate polyglutamates are more effective inhibitors of folate-dependent enzymes, other than dihydrofolate reductase, than methotrexate itself (Fernandes et al., 1983; Chabner et al., 1985). Modifications of anti-folates that increase their substrate effectiveness for folylpolyglutamate synthetase as polyglutamate derivatives should result in more effective cytotoxic agents. Modifications of Pte-ornithine that retain affinity for folylpolyglumate synthetase and allow transport of the derivative into mammalian cells should result in a cytotoxic agent that is specific for folylpolyglutamate synthetase.

Registry No. DTBSF, 31362-25-1; H_4PteGlu, 135-16-0; (6*S*)-H_4PteGlu, 71963-69-4; (6*S*)-H_4PteGlu$_2$, 105879-64-9; (6*S*)-H_4PteGlu$_3$, 88903-89-3; (6*S*)-H_4PteGlu$_4$, 105816-60-2; (6*R*)-10-formyl-H_4PteGlu, 74644-66-9; (6*R*)-5,10-methylene-H_4PteGlu, 31690-11-6; (6*S*)-5-methyl-H_4PteGlu, 31690-09-2; H_2PteGlu, 4033-27-6; H_2PteGlu$_2$, 52723-92-9; PteGlu, 59-30-3; (6*S*)-H_4Pte-4-aminobutyryl-Glu, 105662-63-3; H_2Pte-4-aminobutyryl-Glu, 105618-70-0; (6*S*)-H_4PteGlu$_5$, 88903-90-6; (6*S*)-H_4PteGlu$_6$,

88904-13-6; (6*S*)-$H_4PteGlu_7$, 105816-61-3; (6*S*)-5-methyl-$H_4PteGlu_2$, 105816-62-4; (6*R*)-5,10-methylene-$H_4PteGlu_5$, 83679-35-0; (6*R*)-5,10-methylene-$H_4PteGlu_6$, 93452-47-2; (6*R*)-5,10-methylene-$H_4PteGlu_7$, 93452-48-3; $H_2PteGlu_5$, 53749-53-4; $PteGlu_5$, 33611-85-7; (6*S*)-$H_4PteGluNH_2$, 105618-74-4; (6*S*)-H_4Pte-ornithine, 105618-90-4; Pte-Glu-[ψ,CH_2-NH]-DL-Glu, 105879-46-7; 4-NH_2-Pte-sulfanilyl-Glu, 64920-89-4; $PteGlu_3$, 89-38-3; $PteGlu_6$, 35409-55-3; $PteGluNH_2$, 81672-03-9; $H_4PteGlu_2$, 38968-11-5; $H_4PteGlu_3$, 4227-85-4; $H_4PteGlu_4$, 50998-24-8; $H_4PteGlu_5$, 41520-73-4; $H_4PteGlu_6$, 50998-25-9; $H_4PteGlu_7$, 50998-26-0; 5-methyl-$H_4PteGlu$, 134-35-0; $PteGlu_2$, 19360-00-0; $PteGlu_4$, 29701-38-0; K, 7440-09-7; L-glutamic acid, 56-86-0; spermine, 71-44-3; spermidine, 124-20-9; folylpoly-γ-glutamate synthetase, 63363-84-8; aminopterin, 54-62-6; dihydroaminopterin, 6814-97-7; methotrexate, 59-05-2; (6*S*)-tetrahydrohomofolate, 105816-63-5; homofolate, 3566-25-4.

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