Chapter 5

MOLECULAR BIOLOGY IN NUTRITION RESEARCH: MODELING OF FOLATE METABOLISM

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I. GENERAL APPROACHES

The application of molecular biological techniques to the study of nutrient control of metabolism has received increased attention over the past 10 years. The isolation and characterization of genes encoding key enzymes in metabolic pathways has revealed new insights into the processes by which nutrient status influences metabolic fluxes through these pathways. The ability to manipulate enzyme levels by genetic means has allowed the development of various metabolic models for assessing whether particular enzymes exert key kinetic control over metabolic pathways and for assessing the role of particular enzymes in the compartmentalization of nutrients and metabolites in cells and tissues. Transgenic animals expressing elevated enzyme levels in specific tissues can be developed and the effects of reduction or elimination of a specific enzyme can be ascertained using gene knockout techniques.

Introduction of genes or cDNAs into mammalian cells by transfection allows the development of kinetic models expressing various levels of the protein of interest. A major advantage of this system is that it is possible to modify the level or specificity of a single enzyme in a common background so theoretically all other enzyme levels in the cell are unchanged. Care must be taken to ensure that the "modified" system truly represents the normal physiology of the cell. Many enzymes that catalyze steps in a metabolic pathway are associated together as multiprotein complexes. The protein introduced via an expression system may not form part of such a complex, due to insufficient associated proteins for forming the complex or, if a cDNA from a different species is used, because the protein may differ from the endogenous protein in residues required for complex formation. Kinetic data obtained in such cases may not reflect the true kinetics and flux of the pathway that the enzyme is normally associated with. The protein may also have to be directed to the correct localation in the cell and techniques are available to target proteins to subcellular compartments. The cDNA is normally under the control of a eukaryotic promoter. This promoter can be inducible, which allows regulated expression of the encoded protein, or can be constitutive. Depending on the transfection conditions, the expression of the cDNA of interest can be transient or permanent. The vectors used in these studies normally also express an antibiotic resistance gene to allow a simple selection for cells that express the transfected DNA. In this report, we describe the application of some of these techniques to the study of folate metabolism and its homeostasis.

II. MODELING OF FOLATE METABOLISM

A. INTRODUCTION

Tissue folates are metabolized to poly-γ-glutamyl coenzymes (Fig. 1) that function in metabolic cycles involved in amino acid and DNA precursor

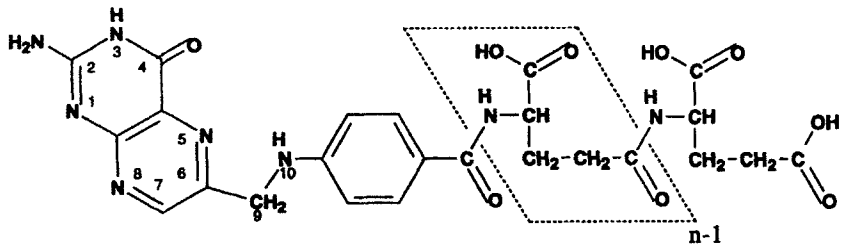

FIG. 1. Structure of folylpoly-γ-glutamate.

synthesis (Shane, 1989). Folate coenzymes are involved in serine–glycine interconversion, methionine synthesis, and choline degradation, histidine catabolism, *de novo* purine synthesis, and thymidylate synthesis. While monoglutamates are the forms absorped across the intestinal mucosa and are the circulating and transport forms of folate, tissue retention and accumulation requires their conversion to the polyglutamate species. The polyglutamates are also the active coenzymatic forms, usually with greatly reduced K_m values for their respective enzymes and affinities that are up to three orders of magnitude greater than the pteroylmonoglutamate forms (Shane, 1989).

Folylpolyglutamate synthetase catalyzes the addition of glutamate residues to cellular folates and antifolates to form the physiological active coenzymatic forms of the vitamin and more potent anti-folate agents (Shane, 1989). Bacterial folylpolyglutamate synthetase enzymes normally only metabolize folate to the triglutamate or tetraglutamate forms while mammalian folylpolyglutamate synthetase will metabolize folate to longer derivatives. Chinese hamster ovary (CHO) cell mutants that lack folylpolyglutamate synthetase activity (AUXB1) have greatly reduced folate pools, due to an inability to retain folates, and are auxotrophic for methionine, glycine, purines, and thymidine (Taylor and Hanna, 1977; Osborne *et al.,* 1993; Lowe *et al.,* 1993). The auxotrophy can not be relieved by elevating intracellular pteroylmonoglutamate to folate levels normally found in wild type CHO cells (Osborne *et al.,* 1993), demonstrating that the auxotrophy is not due to low folate levels but is due to an inability to synthesize folylpolyglutamates. Wild-type CHO cells normally contain hexa- and heptaglutamates and mammalian tissues contain a range of polyglutamates varying in glutamate chain length from the pentaglutamate to the decaglutamate. To understand why mammalian tissues synthesis these long chain polyglutamate species, and to define the role of folylpolyglutamate synthetase in cellular folate accumulation, we have developed a number of mammalian cell models transfected with various folylpolyglutamate synthetase genes (human and *Escherichia coli*) and containing altered folate coenzyme distributions. These cells have also been used to study the role of different folate derivatives in the various metabolic cycles of one-carbon metabolism.

B. ROLE OF FOLYLPOLYGLUTAMATE CHAIN LENGTH

1. Folate Retention

CHO AUXB1 mutants transfected with the *E. coli* folylpolyglutamate synthetase gene (AUX-*coli*) express the *E. coli* protein in the cytosol and metabolize folates primarily to triglutamates rather than the hexa- and

heptaglutamates normally found in wild-type CHO cells (CHO-WT) (Osborne *et al.*, 1993). In pulse chase studies with cells preincubated in medium containing labeled folinate, or the anti-folate methotrexate, the trace amounts of pteroylmono- and diglutamate in CHO-WT and AUX-*coli* cells are rapidly lost from the cells, while the triglutamates that accumulate in AUX-*coli* are retained approximately as effectively as the longer polyglutamate derivatives in CHO-WT cells. Metabolism of folate or antifolates to the triglutamate appears to be sufficient for effective intracellular retention and accumulation of the vitamin.

2. *Metabolic Effectiveness*

To evaluate the metabolic effectiveness of different polyglutamate chain length folates in the various metabolic cycles of one carbon metabolism, CHO cells were cultured in medium lacking products of one carbon metabolism, such as purines, thymidine or glycine, and the levels of intracellular folate that supported half maximal growth rates were assessed (Lowe *et al.*, 1993). No significant differences were found in folate requirements between CHO-WT cells and AUX-*human* transfectants expressing various levels of human folylpolyglutamate synthetase activity and containing predominant folylpolyglutamates of chain length varying from the tetra- to the octaglutamate (Lowe *et al.*, 1993). In AUX-*coli* cells, which contain triglutamates, similar intracellular folate concentrations to CHO-WT supported growth in thymidine and purine-free medium, but the folate requirement for growth in medium lacking glycine was increased about 100-fold (Table I).

TABLE I

SUBCELLULAR FOLATE CONCENTRATIONS SUPPORTING HALF-MAXIMAL GROWTH RATES IN MEDIA LACKING PURINES, THYMIDINE, OR GLYCINE

	-Glycine		-Purines		-Thymidine	
Cell line	Cytosol	Mitochondria	Cytosol	Mitochondria	Cytosol	Mitochondria
	pmol/10^6 cells[a]					
WTT2	0.81	0.49	0.43	0.27	0.12	0.08
AUXB1	—[b]	—	—	—	~20	—
AUX-*coli*	~100	—	0.60	—	0.20	—
AUX-*mcoli*	1.1	0.45	0.56	0.24	0.14	0.06
AUX*coli-mcoli*	4.8	0.66	0.60	0.10	0.17	0.03

[a] 1 pmol/10^6 cells is equivalent to an intracellular concentration of approximately 1 μM.
[b] No growth or mitochondrial pool negligible or absent.

Glycine is synthesized from serine via the serine hydroxymethyltransferase reaction. Mammalian cells contain two isozymes of this enzyme, one cytosolic and one mitochondrial. Cells defective in the mitochondrial isozyme require glycine for growth (Garrow *et al.*, 1993). Although the data shown in Table I suggested that longer polyglutamates than pteroyltriglutamates were required for glycine synthesis, further studies showed that AUX-*coli*, which expresses *E. coli* folylpolyglutamate synthetase in the cytosol, lacked mitochondrial folates despite possessing normal cytosolic folate pools (Lin *et al.*, 1993). The inability of these transfectants to grow in the absence of glycine could have been due to lack of mitochondrial folate rather than a metabolic ineffectiveness of pteroyltriglutamates. Additional studies indicated that CHO-WT and AUX-*human* transfectants expressed folylpolyglutamate synthetase activity in both the cytosol and the mitochondria and accumulated normal folate pools in these subcellular compartments (Lin *et al.*, 1993). An *E. coli* protein would not be expected to enter the mitochondrion due to lack of a mitochondrial leader sequence (Fig. 2).

To further investigate the effectiveness of pteroyltriglutamates in glycine synthesis, *E. coli* folylpolyglutamate synthetase was targeted to the mitochondria of AUXB1 and AUX-*coli* cells using a modified *E. coli* folylpolyglutamate synthetase gene construct preceded by a mammalian mitochondrial leader sequence (Lin and Shane, 1994). The leader sequence was obtained from a human ornithine transcarbamoylase cDNA. Cells expressing the *E. coli* enzyme in their mitochondria (AUX-*coli*-m*coli* and AUX-m*coli*, Table I) accumulated mitochondrial folate. In these cells, pteroyltriglutamates functioned as effectively as the longer glutamate chain length

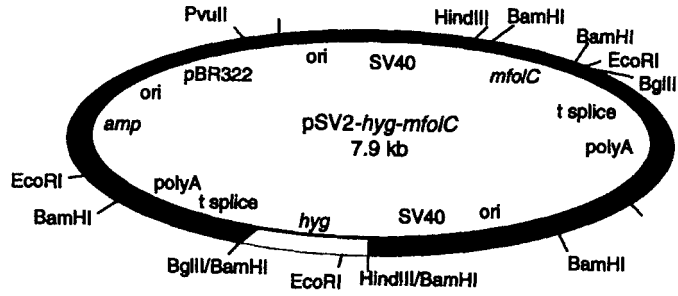

FIG. 2. Mammalian expression plasmid. This plasmid contains a modified *E. coli* FPGS gene (m*folC*) and a bacterial hygromycin resistance gene bordered by mammalian expression sequences. Each is preceded by a SV40 promoter and followed by a poly(A) signal and a splice region from t antigen. The vector also contains an *ori* region from pBR322 and an ampicillin resistance gene to allow for growth and selection in bacteria.

folates found in wild type CHO cells in the metabolic cycle of glycine synthesis provided they were located in the mitochondria (Table I).

Preliminary studies have been carried out on the ability of these transfectants to synthesize methionine via the B_{12}-dependent 5-methyltetrahydrofolate:homocysteine transmethylase (methionine synthase) reaction. CHO-WT cells will grow in the absence of methionine provided sufficient homocysteine and vitamin B_{12} are provided. CHO cell transfectants expressing *E. coli* folylpolyglutamate synthetase grow more slowly or do not grow under these conditions, suggesting that the longer glutamate chain length folates typically found in mammalian tissues are required for methionine synthesis (Lowe *et al.*, 1993).

C. COMPARTMENTATION OF CELLULAR FOLATE

AUX-*coli* cells lacked mitochondrial folate despite possessing high levels of cytosolic folate and folylpolyglutamates can not enter the mitochondria of mammalian cells. As indicated above, targeting of the *E. coli* folylpolyglutamate synthetase to the mitochondria of these cells (AUX-*coli*-m*coli*) restored mitochondrial folate pools, indicating that mitochondrial folate accumulation is dependent on mitochondrial folylpolyglutamate synthetase activity. However, AUX-m*coli* cells, which express *E. coli* folylpolyglutamate synthetase activity in their mitochondria but not in their cytosol, also possessed normal cytosolic and mitochondrial folate pools. Pulse chase experiments indicated that mitochondrial folylpolyglutamates can be released without prior hydrolysis and CHO transfectants expressing *E. coli* folylpolyglutamate synthetase activity solely in the mitochondria possessed normal cytosolic folylpolyglutamate pools (Fig. 3). In these cells mitochondrial folate accumulation did not appear to be limited by mitochondrial folate transport but was governed by competition between mitochondrial

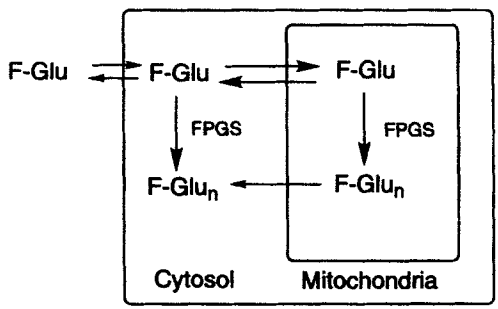

FIG. 3. Model for mitochondrial and cytosolic accumulation of folylpolyglutamates.

and cytosolic folylpolyglutamate synthetase activity. Elevated expression of cytosolic folylpolyglutamate synthetase activity led to a mitochondrial folate deficiency which could only be overcome by expression of very high levels of mitochondrial folylpolyglutamate synthetase.

Similar results were observed when AUXB1 cells were transfected with a human folylpolyglutamate synthetase cDNA (Garrow *et al.*, 1992). Expression of the human cDNA encoding a mature folylpolyglutamate synthetase protein restored cytosolic folylpolyglutamate synthetase activity in these cells and overcame the cell's requirement for thymidine and purines but the cells remained auxotrophic for glycine, reflecting the absence of a folate pool in the mitochondria (Table II). Expression of a human cDNA encoding a folylpolyglutamate synthetase with a mitochondrial leader sequence restored folylpolyglutamate synthetase activity in the mitochondria and the cells contained normal mitochondrial folate pools and were prototrophic for glycine (Table II). Although cells expressing human folylpolyglutamate synthetase solely in the mitochondria are prototrophic for thymidine and purines, which are synthesized in the cytosol, their cytosolic foate pools are quite small, which contrasts to the normal cytosolic folate pools observed in cells expressing *E. coli* folylpolyglutamate synthetase solely in the mitochondria. This probably reflects the slower movement of longer chain length folylpolyglutamates out of the mitochondria. Although a cytosolic folylpolyglutamate synthetase isozyme is not absolutely required, it is needed for establishment of normal cytosolic folate pools.

D. ROLE OF FOLYLPOLYGLUTAMATE SYNTHETASE LEVEL IN FOLATE ACCUMULATION

Table III shows the effect of folylpolyglutamate synthetase activity on the accumulation of folate and methotrexate, an antifolate drug, by CHO cell transfectants expressing levels of human folylpolyglutamate synthetase ranging from 2 to 1400% of wild-type CHO cells. Tissue culture cells express

TABLE II

FOLATE AND FPGS DISTRIBUTION AND GLYCINE REQUIREMENT IN CHO TRANSFECTANTS

FPGS DNA transfected	FPGS activity in		Folate in		Glycine auxotrophy
	Cytosol	Mitochondria	Cytosol	Mitochondria	
None	No	No	No	No	Yes
Human cDNA	Yes	No	Yes	No	Yes
Human cDNA(mito)	No	Yes	Yes	Yes	No

TABLE III

FOLATE AND METHOTREXATE ACCUMULATION BY CHO CELL TRANSFECTANTS

Cell	FPGS activity (pmol/hr/10^6 cells)	Folate or analog accumulation (pmol/10^6 cells)		
		5 n*M* folinate	20 μM folinate	5 μM methotrexate
CHO-WT	90	2.3	22.9	4.5
AUX-*human*-0.02[a]	>2	0.4	5.5	3.7
AUX-*human*-0.07	6	1.3	11	3.9
AUX-*human*-0.21	19	1.7	19	4.4
AUX-*human*-0.79	71	2.2	44	9.7
AUX-*human*-14x	1400	3.7	430	135

[a] AUX-*human-x* are CHO cells expressing human FPGS activity, x representing activity relative to wild-type CHO cells.

higher levels of folylpolyglutamate synthetase than are normally found in mammalian tissue. The lower range of activity levels shown in this study are similar to enzyme levels found in mammalian tisues while the higher levels are more typical of some of the levels that have been observed in leukemia cells. At low to physiological medium folate concentrations (5 n*M* folinate), there was little effect of folylpolyglutamate synthetase activity on accumulation except in cells expressing very low levels of activity.

Kinetic constants obtained with purified human folylpolyglutamate synthetase (unpublished data) were used to model folate accumulation and the derived data are compared with actual accumulation rates in Table IV. The data assume no competition between folate mono- and diglutamate

TABLE IV

FOLINATE (2 n*M*) ACCUMULATION BY CHO CELLS AND THEORETICAL TRANSPORT AND POLYGLUTAMYLATION RATES

Cell	FPGS (pmol/hr)	Folate uptake (actual) (fmol/hr)	Polyglutamylation rate[a] (calculated) (fmol/hr)
CHO-WT	90	35	640
CHO-*human*-0.02	>2	12	>27
CHO-*human*-0.07	6	30	81
CHO-*human*-0.21	19	47	256
CHO-*human*-0.79	71	40	957
Influx rate (calc)		30 fmol/hr	

[a] Calculated from kinetic constants of purified human FPGS.

substrates for chain elongation, and none would be expected at the intracellular folate concentrations obtained under these conditions (low intracellular folate). Triglutamate synthesis is required for folate retention and, at low intracellular folate concentrations, rates of conversion of pteroylmono- to diglutamate and diglutamate to triglutamate are similar. Pteroylmonoglutamate concentrations were calculated from that found in AUXB1 cells or were analyzed directly. Theoretical polyglutamylation rates (cellular capacity to synthesize pteroyltriglutamates) exceeded accumulation rates except in cells expressing very low levels of folylpolyglutamate synthetase, and the actual accumulation rates were similar to the calculated folate influx rates under these conditions. Folate accumulation was limited by influx and was not responsive to folylpolyglutamate synthetase activity except in cells expressing lower levels of folylpolyglutamate synthetase, i.e., enzyme levels found in some mamalian tissues. As folate accumulation rates mirrored calculated influx rates, essentially all transported folate was metabolized to retained polyglutamate derivatives (Lowe *et al.*, 1993).

When cells were incubated with pharmacological levels of folinate (20 m*M*), which mimics the dosage used in some chemotherapeutic treatments, or with the antifolate methotrexate, which is a poor substrate for folylpolyglutamate synthetase, cellular accumulation was highly dependent on folylpolyglutamate synthetase activity (Table III). CHO-WT cells accumulated about 3 to 5% as much methotrexate and folinate as cells overexpressing human folylpolyglutamate synthetase, indicating that, at a maximum, only 5% of transported folate or methotrexate was retained by the wild type cell. Modeling of folate accumulation under these conditions is shown in Table V. Although folinate accumulation was dependent on folylpolyglutamate synthetase activity, accumulation was significantly lower than the calculated polyglutamylation rates for conversion of monoglutamate to diglutamate and diglutamate to triglutamate. Folate accumulation requires conversion to the triglutamate and with high intracellular levels of folate, competition between entering pteroylmonoglutamate and cellular polyglutamates and diglutamate formed should decrease the rate of triglutamate synthesis, and this competition would be expected to increase as cellular folate levels increase. When this competition is modeled by using kinetic parameters for human folylpolyglutamate synthetase and cellular folate content at the start and end of the incubation with folinate, a range of rates for conversion of diglutamate to triglutamate, which should represent cellular accumulation rates, is obtained (Table V). For example, for cells expressing folylpolyglutamate synthetase at 20% of that in CHO-WT cells (CHO-*human*-0.21, Table V), the initial pteroyltriglutamate formation rate would be 2.4 pmol/hr/10^6 cells and would drop to 0.5 pmol/hr/10^6 cells after the 22-hr incubation. This compares with an average accumulation rate of

TABLE V

FOLINATE (20 μM) ACCUMULATION BY CHO CELLS AND THEORETICAL TRANSPORT AND POLYGLUTAMYLATION RATES

Cell	FPGS (pmol/hr)	Folate uptake (actual) (pmol/hr)	Theoretical polyglutamylation rate (pmol/hr)		
			1 to 2[a]	2 to 3[a]	2 to 3 (inhib)[b]
CHO-WT	90	0.58	60	30	
CHO-*human*-0.2	>2	0.03	>1.6	>0.16	0.04–0.03
CHO-*human*-0.7	6	0.21	4.8	1.4	0.39–0.14
CHO-*human*-0.21	19	1.10	15.3	7.3	2.4–0.5
CHO-*human*-0.79	71	2.18	57.3	37.2	14.5–2.0
CHO-*human*-17	1600	22.2			
Influx rate (calc)		28 pmol/hr			

[a] Calculated from kinetic constants of purified human FPGS.

[b] Triglutamate synthesis rate obtained from kinetic parameters if rate modified by inhibition by entering pteroylmonoglutamate and cellular folylpolyglutamate stores.

1.1 pmol/hr/10^6 cells over this period. Folate accumulation under these conditions can be modeled entirely by using kinetic parameters for folylpolyglutamate synthetase and is totally dependent on the level of folylpolyglutamate synthetase activity. Influx rates under these conditions are greater than 28 pmol/hr/10^6 cells, and transport does not limit accumulation with high doses of folinate.

E. ROLE OF FOLYLPOLYGLUTAMATE SYNTHETASE IN ANTI-FOLATE CYTOTOXICITY

Methotrexate accumulation was also dependent on folylpolyglutamate synthetase activity in these cells (Table III). The cytotoxicity of methotrexate does not require its conversion to polyglutamates but cellular accumulation of the drug is highly dependent on folylpolyglutamate synthetase activity (Kim *et al.*, 1993). There was no difference in sensitivity of the different transfectants to the drug when the cells were continuously exposed to the drug. However, cells expressing higher levels of folylpolyglutamate synthetase were much more sensitive to pulse exposure (4 hr) to methotrexate than cells with lower levels (Table VI). These studies clearly show that the level of folylpolyglutamate synthetase can be a major determinant of methotrexate cytotoxicity even though its target in the cell is dihydrofolate reductase and effective inhibition of the reductase does not require polyglutamylation of methotrexate.

TABLE VI

EFFECT OF FPGS ACTIVITY ON SENSITIVITY OF CELLS TO METHOTREXATE

Cells	ED_{50}	
	72-hr methotrexate exposure (n*M*)	4-hr methotrexate exposure (μM)
CHO-WT	3–10	10–33
AUX-*human*-0.02	1–3	33–100
AUX-*human*-0.07	3–10	10–33
AUX-*human*-0.21	3–10	3–10
AUX-*human*-0.79	3–10	1–3

F. SUMMARY

Model CHO cells obtained by transfecting CHO mutants with the *E. coli* and human folylpolyglutamate synthetase genes have proven useful for assessing the role of folylpolyglutamates in one carbon metabolism and for delineating how folate intracellular stores are regulated. Cells expressing enzymes in specific subcellular compartments, expressing enzymes with different substrate specificity's, and expressing enzyme activity at different levels, all in a common background, in this case the CHO cell, has allowed the development of kinetic models for assessing the role of folylpolyglutamate synthetase in folate retention and in the cytotoxicity of antifolates.

REFERENCES

Garrow, T. A., Admon, A., and Shane, B. (1992). Expression cloning of a human cDNA encoding folylpoly-γ-glutamate synthetase and determination of its primary structure. *Proc. Natl. Acad. Sci. U.S.A.* **89,** 9151–9155.

Garrow, T. A., Brenner, A., Whitehead, V. M., Chen, X.-N., Duncan, R. G., Korenberg, J. R. and Shane, B. (1993). Cloning of human cDNAs encoding mitochondrial and cytosolic serine hydroxymethyltransferases and chromosomal localization. *J. Biol. Chem.* **268,** 11910–11916.

Kim, J.-S., Lowe, K. E., and Shane, B. (1993). Regulation of folate and one carbon metabolism in mammalian cells. 4. Role of folylpolyglutamate synthetase in antifolate metabolism and cytotoxicity. *J. Biol. Chem.* **268,** 21680–21685.

Lin, B.-F., Huang, R.-F. S., and Shane, B. (1993). Regulation of folate and one carbon metabolism in mammalian cells. 3. Role of mitochondrial folylpolyglutamate synthetase. *J. Biol. Chem.* **268,** 21674–21679.

Lin, B.-F., and Shane, B. (1994). Expression of *Escherichia coli* folylpolyglutamate synthetase in the Chinese hamster ovary cell mitochondrion. *J. Biol. Chem.* **269,** 9705–9713.

Lowe, K. E., Osborne, C. B., Lin, B.-F., Kim, J.-S., Hsu J.-C., and Shane, B. (1993). Regulation of folate and one carbon metabolism in mammalian cells. 2. Effect of folylpolyglutamate

synthetase substrate specificity and level on folate metabolism and folylpolyglutamate specificity of metabolic cycles of one carbon metabolism. *J. Biol. Chem.* **268,** 21665–21673.

Osborne, C. B., Lowe, K. E., and Shane, B. (1993). Regulation of folate and one carbon metabolism in mammalian cells. 1. Folate metabolism in Chinese hamster ovary cells expressing *Escherichia coli* or human folylpoly-γ-glutamate synthetase activity. *J. Biol. Chem.* **268,** 21657–21664.

Shane, B. (1989). Folylpolyglutamate synthesis and role in the regulation of one carbon metabolism. *Vitam. Horm. (N.Y.)* **45,** 263–335.

Taylor, R. T., and Hanna, M. L. (1977). Folate-dependent enzymes in cultured Chinese hamster ovary cells: Folylpolyglutamate synthetase and its absence in mutants auxotrophic for glycine + adenosine + thymidine. *Arch. Biochem. Biophys.* **181,** 331–344.