# Mammalian Folylpoly-γ-glutamate Synthetase. 1. Purification and General Properties of the Hog Liver Enzyme†

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ABSTRACT: Folylpolyglutamate synthetase was purified 30 000–150 000-fold from hog liver. Purification required the use of protease inhibitors, and the protein was purified to homogeneity in two forms. Both forms of the enzyme were monomers of $M_r$ 62 000 and had similar specific activities. The specific activity of the homogeneous protein was over 2000-fold higher than reported for partially purified folylpolyglutamate synthetases from other mammalian sources. Enzyme activity was absolutely dependent on the presence of a reducing agent and a monovalent cation, of which $K^+$ was most effective. The purified enzyme catalyzed a MgATP-dependent addition of glutamate to tetrahydrofolate with the concomitant stoichiometric formation of MgADP and phosphate. Under conditions that resembled the expected substrate and enzyme concentrations in hog liver, tetrahydrofolate was metabolized to long glutamate chain length derivatives with the hexaglutamate, the major in vivo folate derivative, predominating. Enzyme activity was maximal at about pH 9.5. The high-pH optimum was primarily due to an increase in the $K_m$ value for the L-glutamate substrate at lower pH values, and the reaction proceeded effectively at physiological pH provided high levels of glutamate were supplied.

The physiological roles of folylpolyglutamates, the major intracellular forms of folate, have been recently reviewed (Cichowicz et al., 1981; Kisliuk, 1981; McGuire & Bertino, 1981; McGuire & Coward, 1984; Shane & Stokstad, 1985). Folylpolyglutamates are the preferred coenzymes for the enzymes of one-carbon metabolism and appear to play an important regulatory role as inhibitors of some folate-dependent enzymes. They are also preferentially retained by mammalian tissues, and their synthesis serves as a mechanism by which the cell can concentrate folates, while pteroylmonoglutamates are the transport forms of the vitamin. Mammalian cell mutants which lack folylpolyglutamate synthetase activity are auxotrophic for the products of one-carbon metabolism, such as methionine, glycine, thymidine, and purines, and have low intracellular folate levels (McBurney & Whitmore, 1974; Taylor & Hanna, 1977; Foo & Shane, 1982).

Regulation of folylpolyglutamate synthetase, the enzyme that catalyzes the conversion of folates to polyglutamate derivatives, would be expected to play an important role in the regulation of folate homeostasis in the cell. Folylpolyglutamate synthetase is also a promising target enzyme for chemotherapeutic agents. However, the mammalian enzyme has not been studied in detail as it has proved to be a highly unstable enzyme. After preliminary characterizations of crude enzyme preparations from sheep liver (Gawthorne & Smith, 1973) and Chinese hamster ovary cells (Taylor & Hanna, 1977), McGuire et al. (1980) more extensively characterized a 70-fold-purified preparation from rat liver. Following this, the enzymes from mouse liver (Moran & Colman, 1984), hog liver (Cichowicz et al., 1981), and beef liver (Pristupa et al., 1984) were purified up to 350-fold and partially characterized. The specific activities of these preparations were over 2000-fold lower than those of purified bacterial folylpolyglutamate synthetases, suggesting that the preparations were still very impure or that the catalytic activity of the mammalian enzyme was considerably lower than that of the bacterial enzyme. Further purification of the mammalian preparations resulted in loss of enzyme activity.

Bacterial folylpolyglutamate synthetase, which was first characterized by using partially purified enzyme from *Escherichia coli* (Monsurekar & Brown, 1975), has been purified to homogeneity from *Corynebacterium* (Shane, 1980), *Lactobacillus casei* (Bognar & Shane, 1983), and *Escherichia coli* (Bognar et al., 1985). However, the substrate specificities of the bacterial and mammalian enzymes differ considerably, and the bacterial enzymes are a poor model for assessing the specificity of the mammalian enzyme for folate analogues or for assessing the factors that are involved in the regulation of folate homeostasis in mammalian cells.

In this report, the stabilization, purification, and general properties of homogeneous hog liver folylpolyglutamate synthetase are described. In the accompanying papers, the kinetic properties of the enzyme, its specificity for substrates and analogues, the regulation of the enzyme, and its role in folate homeostasis are discussed (Cichowicz & Shane, 1987; George et al., 1987; Cook et al., 1987).

## Experimental Procedures

*Materials.* [$^3$H]Folic acid (PteGlu)[1] labeled in positions 3′, 5′, 7, and 9 (47.0 or 28.4 Ci/mmol), L-[U-$^{14}$C]glutamic acid (10 mCi/mmol), [8-$^{14}$C]ATP (57 mCi/mmol), and [γ-

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[1] Abbreviations: PteGlu, pteroylmonoglutamic acid (folic acid); $H_4PteGlu_n$, 5,6,7,8-tetrahydropteroylpoly-γ-glutamate, *n* indicating the number of glutamate moieties; *p*AbaGlu$_n$, *p*-aminobenzoylpoly-γ-glutamate; Pte, pteroic acid; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane.

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$^{32}$P]ATP were obtained from Amersham. Nucleotide derivatives, amino acids, amino acid analogues, and protease inhibitors were obtained from P-L Biochemicals, Vega Biochemicals, Chemalog, Sigma Chemical Co., CalBiochem, Aldrich Chemical Co., and Boehringer. Metal ion "free" Tris, Tris-HCl, and ammonium sulfate were obtained from Schwarz/Mann. Fresh hog livers were obtained from the Esskay and Benson Meat Companies in Baltimore.

Folic acid and aminopterin were obtained from Sigma Chemical Co. Methotrexate was obtained from Aldrich Chemical Co. Folic acid polyglutamates ($PteGlu_{2-7}$), (6*RS*)-$H_4PteGlu_{1-7}$, (6*S*)-$H_4PteGlu_{1-7}$, (6*R*)-10-formyl-$H_4PteGlu_{1-7}$, (6*S*)-$H_4$[$^3$H]PteGlu (28.4 Ci/mmol), and (6*S*)-5-methyl-$H_4$[$^3$H]PteGlu (28.4 Ci/mmol) were synthesized, purified, and characterized as described previously (Shane, 1980; Foo & Shane, 1982). 5,10-Methylene-$H_4PteGlu_n$ was prepared by mixing $H_4PteGlu_n$ with formaldehyde.

*Folylpolyglutamate Synthetase Assay.* Enzyme activity was routinely measured by the incorporation of [$^{14}$C]glutamate into folylpolyglutamates using unlabeled (6*RS*)-$H_4$PteGlu as the folate substrate. Reaction mixtures, unless indicated otherwise, contained 100 mM Tris/50 mM glycine/NaOH buffer, pH 9.75 (22 °C), (6*RS*)-$H_4$PteGlu (40 μM), L-[$^{14}$C]glutamate (250 μM; 1.25 μCi), ATP (5 mM), $MgCl_2$ (10 mM), KCl (20 mM), β-mercaptoethanol (100 mM), bovine serum albumin (50 μg), and enzyme in a total volume of 0.5 mL. The reaction tubes were capped and incubated at 37 °C for 2 h. The pH of the assay mixture was 9.0 at 37 °C. The reaction was stopped by the addition of ice-cold 30 mM β-mercaptoethanol (1.5 mL) containing 10 mM glutamate, and the labeled folate product was separated from unreacted labeled glutamate by chromatography on small DEAE-cellulose (Whatman DE52) columns, as described previously (Shane, 1980). One unit of enzyme activity catalyzes the formation of 1 nmol of product/h under these standard assay conditions.

In some studies on the in vitro metabolism of folates to polyglutamate forms, the assay mixture was modified by the use of (6*S*)-$H_4$[$^3$H]PteGlu or [$^3$H]PteGlu (0.1 μM) as the folate substrate, and unlabeled glutamate (5 mM) replaced [$^{14}$C]glutamate. The assay mixtures were sterilized by filtration (0.22-μm filters) and were incubated at 37 °C for 3, 24, or 48 h.

*Identification of Folate Products.* The eluants from the DEAE columns were treated with $HgCl_2$ to remove mercaptoethanol, and the folate products were cleaved to *p*-aminobenzoylpolyglutamates (*p*$AbaGlu_n$), as described previously (Foo et al., 1980). The labeled *p*$AbaGlu_n$ derivatives were converted to azo dyes of naphthylethylenediamine, purified by chromatography on Bio-Gel P2, reconverted to *p*$AbaGlu_n$, and separated, according to glutamate chain length, by HPLC on a strong anionic exchanger (Whatman SAX), as described previously (Shane, 1982).

The same methodology was used to identify endogenous folates in hog liver. Aliquots of hog liver (approximately 1 g) were homogenized in 3 volumes of 6.7% trichloroacetic acid, and the mixture was centrifuged. The supernatant was extracted with diethyl ether (3 × 2 volumes) to remove most of the trichloroacetic acid, and unlabeled folates were converted to *p*$AbaGlu_n$ and were purified and separated as described above. The derivatives were detected by $A_{280nm}$. Recoveries were assessed by adding [$^3$H]*p*AbaGlu standard to the tissue extracts.

*γ-Glutamyl Hydrolase Activity.* γ-Glutamyl hydrolase was assayed by using $PteGlu_3$[$^{14}$C]GluGlu (0.5 mCi/mmol) as the substrate (Krumdieck & Baugh, 1980; Shane et al., 1983).

*Protein Assays.* Protein concentration was measured by the procedure of Bradford (1976) using bovine serum albumin as the standard.

*Identification of Nucleotide Products.* The folylpolyglutamate synthetase reaction mixture was as described above except 500 μM L-glutamate and 10 μM ATP were used. The labeled substrate was [$^{14}$C]ATP (57 mCi/mmol), [γ-$^{32}$P]ATP (100 mCi/mmol), or [$^{14}$C]glutamate (10 mCi/mmol). Labeled AMP, ADP, ATP, and $P_i$ were separated and measured as described previously (Shane, 1980).

*Electrophoresis.* Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out by using a 5% stacking gel and a 10% separating gel in a slab apparatus using the discontinuous buffer system of Laemmli (1970). Protein bands were visualized by a silver staining procedure (Bio-Rad Laboratories).

*Enzyme Purification.* Livers from freshly killed hogs were transported on ice from the slaughterhouse to the laboratory. All subsequent procedures were carried out at 0–4 °C, and all extracts were stored at 0 °C in an ice bath. All buffer solutions used were adjusted to the indicated pH at room temperature (22 °C).

Livers were cut into large pieces (30–33 g), and each piece was cut into small pieces and suspended in 100 mM Tris–HCl buffer, pH 8.4 (90 mL), containing 50 mM mercaptoethanol, 10 mM benzamidine, type II-0 ovomucoid (500 mg/L), $α_1$-antitrypsin (25 mg/L), and aprotinin (25 000 units/L). The suspension was homogenized for 20 s using a Polytron homogenizer, and the mixture was centrifuged at 27000*g* for 1 h. Floating debris was removed, and the supernatant was decanted through a double layer of cheesecloth and stored on ice until the total liver had been processed. Four liters of homogenization buffer was used to homogenize 1296 g of liver. The supernatants were combined to give the crude extract (fraction 1).

Ten percent streptomycin sulfate (w/v) in 100 mM Tris-HCl buffer, pH 8.4 (367 mL), containing 50 mM mercaptoethanol and 10 mM benzamidine (pH readjusted with NaOH), was added to the crude extract (3300 mL) to give a final concentration of 1%. After stirring for 1 h, the precipitate was removed by centrifugation at 16000*g* for 30 min to give fraction 2 enzyme.

A saturated ammonium sulfate solution in 100 mM Tris-HCl buffer, pH 8.4, containing 50 mM mercaptoethanol and 10 mM benzamidine (pH adjustment not required) was prepared at room temperature. The solution (892 mL) was added to the streptomycin sulfate supernatant (3570 mL) to give a 20% saturated solution. After being stirred for 1 h, the mixture was centrifuged to remove the precipitate, and additional ammonium sulfate solution (1453 mL) was added to the supernatant (4360 mL) to give a 40% saturated solution. After being stirred overnight, the precipitated protein was collected by centrifugation and redissolved in 100 mM Tris-HCl buffer, pH 7.5 (600 mL), containing 50 mM mercaptoethanol, 10 mM benzamidine, and type II-0 ovomucoid (500 mg/L). The preparation was dialyzed overnight against two changes of the same buffer lacking type II-0 ovomucoid (9 L each), and insoluble material was removed by centrifugation (fraction 3).

Fraction 3 enzyme (1050 mL) was applied to a phosphocellulose (P11; Whatman) column (18 × 7.7 cm) that had been equilibrated with 100 mM Tris-HCl buffer, pH 7.5 (3 L), containing 50 mM mercaptoethanol and 10 mM benzamidine. The column was washed with the equilibration buffer (3 L) and eluted with a linear gradient (6 L) of KCl (0–500 mM)

Table I: Purification of Hog Liver Folylpolyglutamate Synthetase[a]

| fraction | volume (mL) | act. (units[b]/mL) | protein (mg/mL) | sp act. (units/mg) | purification (x-fold) | yield (%) |
|---|---|---|---|---|---|---|
| (1) crude extract | 3300 | 25 | 28 | 0.89 | 1.0 | 100 |
| (2) 1% streptomycin sulfate | 3570 | 23 | 21 | 1.1 | 1.2 | 100 |
| (3) 20–40% ammonium sulfate | 1050 | 76 | 27 | 2.8 | 3.1 | 97 |
| (4) phosphocellulose peak 1 | 66 | 581 | 7.6 | 76 | 86 | 46 |
| (4) phosphocellulose peak 2 | 64 | 450 | 22 | 20 | 23 | 35 |
| (5) chromatofocusing peak 1 | 174 | 127 | 0.16 | 796 | 900 | 27 |
| (5) chromatofocusing peak 2 | 290 | 54 | 0.27 | 203 | 230 | 19 |
| (6) phenyl-agarose peak 1 | 122 | 144 | 0.043 | 3360 | 3790 | 21 |
| (6) phenyl-agarose peak 2 | 109 | 120 | 0.097 | 1230 | 1390 | 16 |
| (7) hydroxylapatite peak 1 | 16 | 210 | 0.0071 | 29500 | 33300 | 17[c] |
| (7) hydroxylapatite peak 2 | 16 | 172 | 0.0062 | 27900 | 31500 | 12[c] |

[a] Experimental details are described under Experimental Procedures. [b] Units are nanomoles of glutamate incorporated into folate product per hour. [c] Recoveries adjusted as 30 mL of fraction 6 enzyme was applied to the hydroxylapatite column.

in the same buffer. Enzyme activity was resolved into two major peaks (peaks 1 and 2), and enzyme in each peak was further purified separately by using identical procedures. Fractions containing enzyme activity were combined (1000 mL, 770 mL), and saturated ammonium sulfate in buffer was added (1500 mL, 1155 mL) to give a 60% saturated solution. After being stirred for 1 h, the precipitate was collected by centrifugation, and the pellet was resuspended in 25 mM Tris–acetate buffer, pH 8.3 (25 mL), containing 50 mM mercaptoethanol and dialyzed overnight against the same buffer (3 L). The dialyzed solution was centrifuged at 17000*g* for 1 h to remove insoluble material to give fraction 4 (66 mL, 64 mL).

Fraction 4 enzyme was applied to a chromatofocusing column (PBE 94; Pharmacia; 50 × 1 cm) that had been equilibrated with 25 mM Tris–acetate buffer (pH 8.4)/50 mM mercaptoethanol (500 mL) and layered with elution buffer (5 mL). The elution buffer contained a 1 to 13 dilution of Polybuffer 96 (Pharmacia) adjusted to pH 6.0 with 1 M acetic acid and 50 mM mercaptoethanol. A pH gradient was developed by eluting the column with the elution buffer (500 mL) at a linear flow rate of 30 cm/h. Column fractions containing enzyme activity were pooled (174 mL, 290 mL; fraction 5).

Saturated ammonium sulfate in buffer (20 mL, 32 mL) was added to fraction 5 enzyme to give a 10% saturated solution, and the solution was applied to a phenyl-agarose column (Bethesda Research Laboratories; 15 × 1 cm) that had been equilibrated with 100 mM Tris-HCl buffer (pH 8.4)/50 mM mercaptoethanol containing 10% ammonium sulfate. The column was washed with the equilibration buffer (300 mL), and a decreasing linear gradient of ammonium sulfate in the same buffer (10–0%; 250 mL) was used to elute the column. Elution of enzyme activity was completed by washing the column with buffer/mercaptoethanol alone (200 mL). Active fractions were pooled (122 mL, 109 mL) to give fraction 6.

An aliquot (30 mL) of fraction 6 enzyme was dialyzed overnight against 10 mM potassium phosphate buffer, pH 7.5, containing 50 mM mercaptoethanol, and the solution was applied to a hydroxylapatite column (Bio-Rad; 5 × 1 cm) that had been equilibrated with the dialysis buffer (20 mL). The column was washed with buffer (2 × 4 mL), and enzyme was eluted with 30 mM potassium phosphate buffer (pH 7.5)/50 mM mercaptoethanol (6 × 4 mL) and 50 mM potassium phosphate buffer (pH 7.5)/50 mM mercaptoethanol (2 × 5 mL). The second to fifth 30 mM potassium phosphate buffer eluents were combined to give fraction 7.

## Results

*Purification of Folylpolyglutamate Synthetase.* A typical purification of the hog liver enzyme is summarized in Table I. Streptomycin sulfate was used to remove nucleic acids and other polyanionic species, which interfered with the subsequent ammonium sulfate and phosphocellulose fractionation steps. A 3-fold enrichment of specific activity was obtained after ammonium sulfate fractionation, a step that was primarily used to concentrate the extract.

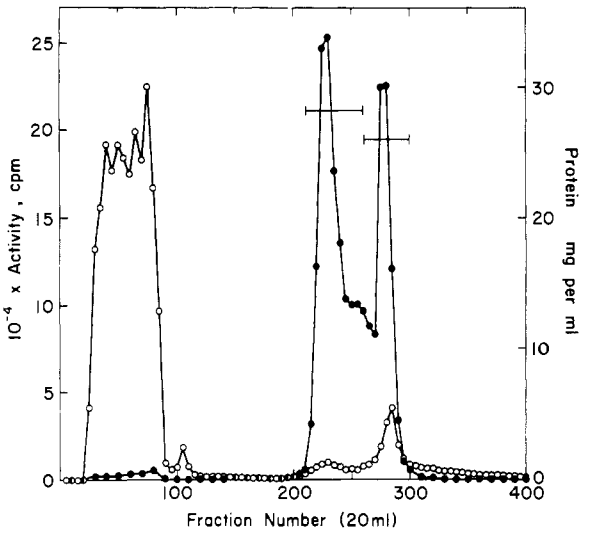


FIGURE 1: Phosphocellulose P-11 chromatography of hog liver folylpolyglutamate synthetase. Experimental details are given under Experimental Procedures. Fraction 3 enzyme (1050 mL) was applied to a P-11 column (18 × 7.7 cm) equilibrated with 100 mM Tris-HCl buffer (pH 7.5)/50 mM mercaptoethanol/10 mM benzamidine. The column was washed with the equilibration buffer (3 L) and eluted with a 6-L gradient of KCl (0–500 mM) in buffer. Fractions were assayed for enzyme activity [100 μL; (●)] and for protein concentration (○).

Enzyme activity was resolved into two major peaks after chromatography on phosphocellulose (Figure 1). The specific activity of the major peak (peak 1) was enhanced about 30-fold as the bulk of the protein was not retained by the column while enzyme activity (peak 1) was eluted by a KCl gradient at approximately 90 mM KCl. Enzyme in the second peak was enriched to a lesser extent. For reasons discussed below, enzymes in the two activity peaks were purified separately.

γ-Glutamylhydrolase activity was monitored during the early steps of the purification. Twelve percent of the total hydrolase activity was found in the 20–40% ammonium sulfate fraction and the remainder in the 40% ammonium sulfate supernatant. No detectable hydrolase activity was found in the two folylpolyglutamate synthetase peaks obtained after phosphocellulose chromatography.

The enzyme was purified about 10-fold by chromatofocusing chromatography (Figure 2) as the bulk of the protein did not

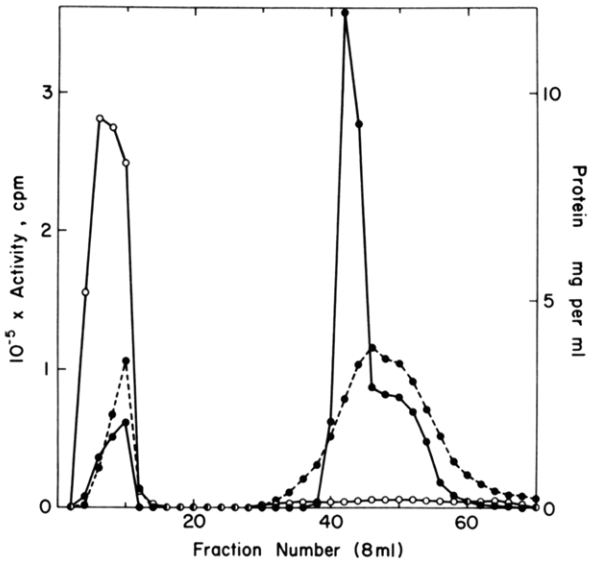


FIGURE 2: Chromatofocusing chromatography of folylpolyglutamate synthetase. Experimental details are given under Experimental Procedures. Fraction 4 enzyme (peak 1, 66 mL) was applied to a PBE 94 column (50 × 1 cm) equilibrated with 25 mM Tris–acetate buffer (pH 8.4)/50 mM mercaptoethanol. The column was eluted with Polybuffer 96, pH 6.0 (1 to 13 dilution), containing 50 mM mercaptoethanol. Fractions were assayed for enzyme activity [50 μL; (●—●)] and for protein concentration (○). The enzyme activity elution profile obtained with fraction 4 peak 2 enzyme is also indicated (●---●).

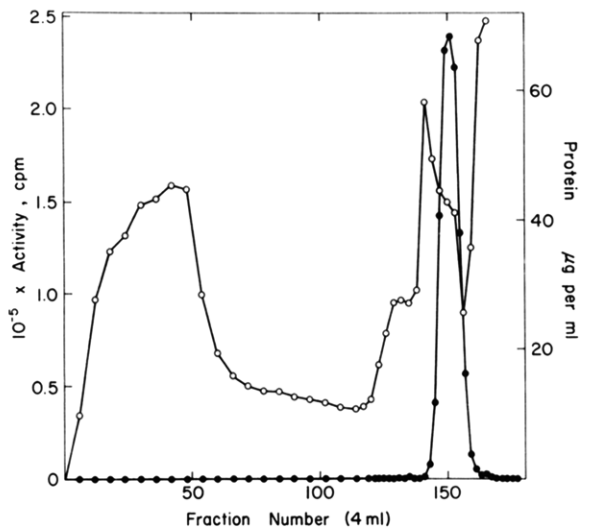


FIGURE 3: Phenyl-agarose chromatography of folylpolyglutamate synthetase. Experimental details are given under Experimental Procedures. Fraction 5 enzyme was applied to a phenyl-agarose column (15 × 1 cm) equilibrated with 100 mM Tris-HCl buffer (pH 8.4)/50 mM mercaptoethanol containing 10% ammonium sulfate. The column was washed with 300 mL of the equilibration buffer and eluted with a linear gradient (250 mL) of ammonium sulfate (10–0%) in buffer and then buffer alone (200 mL). Fractions were assayed for enzyme activity [30 μL; (●)] and protein concentration (○). The ammonium sulfate gradient started at fraction 110 and ended at fraction 160.

bind to the column. Enzyme activity was eluted from the column by a decreasing pH gradient, from pH 8.3 to pH 6.0, with the major activity peak eluting around an apparent pH of 7.6. Peak 2 enzyme consistently eluted later than peak 1 enzyme.

A further 4–6-fold purification was obtained by phenyl-agarose hydrophobic chromatography using a reverse ammonium sulfate gradient (Figure 3). The bulk of the non-specific protein did not bind to the column under the conditions

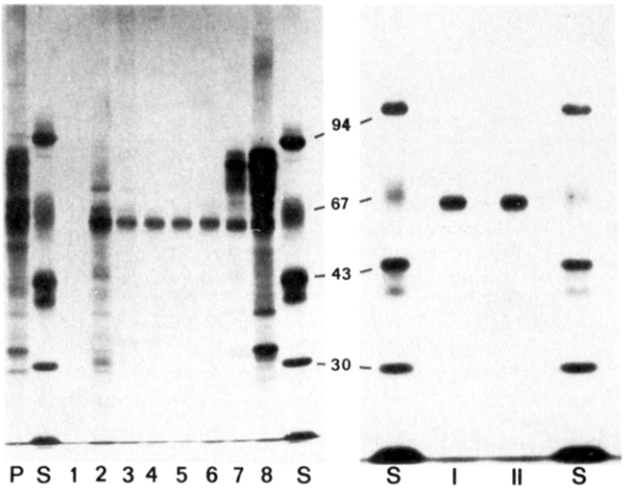


FIGURE 4: Sodium dodecyl sulfate gel electrophoresis of folylpolyglutamate synthetase fractions after chromatography on hydroxylapatite. Experimental details are described under Experimental Procedures. Protein was detected by a silver staining procedure. The electrophoretic mobilities of phosphorylase *b* ($M_r$ 94K), albumin (67K), ovalbumin (43K), and carbonic anhydrase (30K) standards (lanes S) are indicated. Soybean trypsin inhibitor (20K) and lactalbumin (14.4K) standards chromatographed at, or close to, the dye front. (Left gel) Polypeptide profile obtained with proteins in the phenyl-agarose extract that was applied to the hydroxylapatite column is shown in lane P. Polypeptides in the 30 mM potassium phosphate buffer (pH 7.5)/50 mM mercaptoethanol eluents are shown in lanes 1–6, and those in the 50 mM potassium phosphate buffer (pH 7.5)/50 mM mercaptoethanol wash are shown in lanes 7 and 8. 3.75 units of enzyme were applied to lanes P, 7, and 8 and 10 units of enzyme to lanes 2–6. Peak enzyme activity eluted in fractions 4 and 5, and no activity or polypeptides were detected in fraction 1. (Right gel) Polypeptide profiles of purified peak 1 and peak 2 enzyme (25 units) are shown in lanes I and II.

used or bound more tightly than folylpolyglutamate synthetase.

A final 9–20-fold purification of the enzyme was achieved by chromatography on hydroxylapatite. Practically all the applied protein was retained by this column, and folylpolyglutamate synthetase was specifically eluted with 30 mM potassium phosphate buffer (pH 7.5)/50 mM mercaptoethanol with about an 80% recovery (Figure 4). In some cases, the initial and final 30 mM buffer eluents contained traces of contaminating proteins. An additional 10% percent of the applied activity was recovered after elution with 50 mM potassium phosphate buffer, pH 7.5, but the bulk of the applied protein was also eluted under these conditions. The specific activities of enzyme purified from the two phosphocellulose peaks were identical, within the limits of experimental error, and both preparations were homogeneous as judged by a single band after sodium dodecyl sulfate gel electrophoresis (Figure 4). In each case, a molecular weight of 62 000 was obtained. Overall, the enzyme was purified about 33 000-fold with a recovery of 29%.

The specific activity of enzyme in crude hog liver extracts varied considerably, ranging from 0.25 to 0.89 nmol $h^{-1}$ (mg of protein)$^{-1}$. It was noted that smaller livers, presumably from younger animals, had the highest specific activities. Homogeneous protein was also obtained from the lower specific activity extracts by using the procedures described above. In these cases, purifications of over 100 000-fold and specific activities up to 40 μmol $h^{-1}$ (mg of protein)$^{-1}$ were achieved. Slight differences in specific activities of purified protein were probably due to the difficulty in accurately measuring the dilute protein concentrations in these preparations. The additional enrichment required was mainly achieved in the hydroxylapatite chromatography step.

Table II: Stability of Folylpolyglutamate Synthetase under Various Storage Conditions[a]

| addition | activity remaining (%) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | days at 0 °C | | | | days at −20 °C | | | |
| | 4 | 18 | 48 | 215 | 4 | 18 | 48 | 215 |
| none[b] | 88 | 85 | 45 | 9 | 44 | 2 | 0 | [c] |
| protease inhibitors[d] | 97 | 89 | 79 | 17 | 91 | 56 | 9 | |
| 10% dimethyl sulfoxide | 89 | 84 | 52 | 4 | 89 | 54 | 36 | 10 |
| 30% dimethyl sulfoxide | 94 | 83 | 57 | 7 | 101 | 85 | 72 | 46 |
| 12.5% ethylene glycol | 90 | 83 | 81 | 49 | 90 | 75 | 75 | 38 |
| 10% sucrose | 92 | 94 | 87 | 25 | 89 | 71 | 41 | 12 |
| 0.02% NP40 | 93 | 30 | 2 | 0 | 2 | 0 | 0 | |
| 0.02% Triton X-100 | 98 | 84 | 82 | 37 | 1 | 1 | 0 | |
| 0.1 M glycine buffer, pH 10 | 85 | 79 | 65 | 8 | 79 | 37 | 0 | |
| 0.1 M Tris–phosphate, pH 8.4 | 88 | 81 | 68 | 14 | 75 | 25 | 0 | |
| 0.5 M KCl | 84 | 70 | 35 | 1 | 56 | 16 | 0 | |
| 0.5 M KCl (plastic) | 95 | 49 | 8 | | 64 | 42 | 4 | |
| 5 mM dithiothreitol | 92 | 73 | 50 | 2 | 8 | 0 | 0 | |

[a] Fraction 6 enzyme in 100 mM Tris-HCl buffer, pH 8.4, containing 50 mM mercaptoethanol and the indicated additions, was stored in glass vials at 0 °C in an ice bath or at −20 °C for the indicated times, and enzyme activity remaining was determined. [b] Addition of glutamate (1 mM), ADP (1 mM), MgATP (1 mM), or MgATP plus $H_4$PteGlu (10 μM) had no significant effect. [c] Not determined. [d] 10 mM benzamidine, type II-0 ovomucoid (500 mg/L), $\alpha_1$-antitrypsin (25 mg/L), and aprotinin (25 000 units/L).

In initial studies, attempts were made to purify phosphocellulose-purified enzyme by using Sephadex G-150 chromatography. When Tris-HCl buffer (pH 8.4)/50 mM mercaptoethanol was used as the eluent, no activity was recovered from the column. However, with buffer plus 500 mM KCl, 60% of the activity applied to the column was recovered with a 2–3-fold increase in specific activity. Enzyme activity eluted at an apparent molecular weight of approximately 66 000, indicating a monomeric protein. A molecular weight of 30 000 was assessed after HPLC of the phenyl-agarose fraction on a TSK 3000 SW column. However, the enzyme was retarded on this column, and its elution position was dependent on the elution buffer used. Near-homogeneous protein was obtained after binding of phenyl-agarose-purified enzyme to ATP (type 4)–agarose and its subsequent elution with 1 mM MgATP. However, enzyme activity was unstable in the presence of MgATP. In all attempted purifications of the phenyl-agarose fraction, the intensity of the $M_r$ 62 000 band obtained after sodium dodecyl sulfate gel electrophoresis of column fractions mirrored the elution profile of enzyme activity.

*Stabilization of Folylpolyglutamate Synthetase.* Initially, attempts at purifying folylpolyglutamate synthetase yielded enzyme preparations that were very unstable. Phosphocellulose chromatography yielded a single activity peak, but attempted further purification, using a variety of chromatographic procedures, resulted in total loss of enzyme activity.

Increased enzyme stability was observed when the protease inhibitor benzamidine was added to the homogenization buffer. Chromatography of this preparation on phosphocellulose resulted in two activity peaks. One peak eluted at the same position as the single activity peak obtained after chromatography of the enzyme preparation that was homogenized in the absence of protease inhibitors, and at a similar position to peak 2 described above. The other peak eluted slightly earlier from the column at the position of peak 1 (described above). These observations suggested that during homogenization, and the initial purification steps, significant proteolysis was occurring. When additional protease inhibitors were added to the homogenization buffer (Experimental Procedures), and to the chromatography buffers, an increased proportion of enzyme activity eluted from the phosphocellulose column at the position of peak 1, and, in some cases, virtually all the activity was chased from the peak 2 position to the peak 1 position. In these cases, a single homogeneous activity peak was obtained after chromatofocusing chromatography. It has not been established whether the peak 2 enzyme obtained in the presence of protease inhibitors is identical with peak 2 enzyme obtained in their absence. It was noted that protease inhibitor treatment greatly stabilized the enzyme and allowed the further purification of activity in peak 2. Although the further purification of peak 1 and peak 2 enzyme was identical, peak 2 enzyme consistently displayed heterogeneity in its elution from the chromatofocusing column, while peak 1 eluted as a single species (sometimes contaminated with peak 2 enzyme), and was more unstable during storage than peak 1 enzyme. This heterogeneity, and the later elution of peak 2 enzyme from the chromatofocusing column, is consistent with deamidation of the protein. Although no apparent differences were observed in the molecular weight and final specific activities of peak 1 and peak 2 enzyme, the data suggest that peak 2 contains a modified protein. Consequently, all characterizations and metabolic studies described in this report, and the accompanying papers, were carried out with enzyme purified from peak 1.

The conditions required for the stabilization of highly purified (fraction 6) enzyme are shown in Table II. Enzyme in buffer plus 50 mM mercaptoethanol was fairly stable for several months when stored at 0 °C in an ice bath but was labilized by freezing at −20 °C. Although the buffer used had a very low metal ion content, the reducing agent was slowly oxidized such that it had to be replenished every 4 weeks. Although not carefully studied, it appeared that enzyme could be stored in the absence of reducing agents for short periods of time and either was not oxidized or could be reactivated with mercaptoethanol. However, prolonged storage in the absence of reducing agents appeared to lead to irreversible inactivation. Various agents, such as Triton X-100 and metal ion free ethylene glycol (Washabaugh & Collins, 1983), increased the stability of the preparation when stored at 0 °C. Addition of dimethyl sulfoxide or ethylene glycol, and to some extent sucrose, allowed the storage of the enzyme at −20 °C, but no increase in stability over that achieved at 0 °C was obtained. The enzyme was unstable when frozen. It appears likely that the low protein concentration was a major contributing factor to the instability observed.

Homogeneous protein (fraction 7) was more unstable than the phenyl-agarose extract (fraction 6) and could only be stored for several weeks before appreciable inactivation occurred. Inactivation was accompanied by loss of the $M_r$ 62 000 band on sodium dodecyl sulfate gel electrophoresis and the

appearance of discrete bands at $M_r$ 35 000 and 40 000, approximately. It was not clear whether this proteolysis was due to contaminating proteases carried through the purification procedure or to contamination of the glassware. It should be noted that the protein concentration in these purified preparations was very low (typically 0.3–10 μg/mL). Because of this problem, enzyme was normally stored after phenyl-agarose purification, and aliquots were purified to homogeneity on hydroxylapatite as required.

*General Properties.* Enzyme activity was dependent upon a folate substrate, ATP, $Mg^{2+}$, and glutamate and was decreased about 50% in the absence of bovine serum albumin. Reducing agent requirements were assessed by using aminopterin (100 μM) as the folate substrate as $H_4$PteGlu is unstable in the absence of reducing agents. Enzyme activity was reduced 93% in the absence of a reducing agent (assay mixtures contained approximately 200 μM mercaptoethanol derived from the enzyme extract). The monovalent cation requirement was assessed by using the standard assay mixture modified by the exclusion of all monovalent cations with the exception of Tris. The buffer was replaced with Tris-HCl buffer, pH 9.75, and the Tris and Mg salts of $H_4$PteGlu and ATP were used. Enzyme was dialyzed against Tris buffer and contributed less than 20 μM $K^+$ to the assay mixture. Under these conditions, enzyme activity was reduced greater than 98%.

Under the standard assay conditions (40 μM $H_4$PteGlu, 5 mM MgATP, and 250 μM L-glutamate), all substrates with the exception of glutamate were near-saturating. With a 1-h incubation, and holding the fixed substrates at the standard assay concentrations, kinetic plots for ATP and glutamate exhibited typical Lineweaver–Burk kinetics, while substrate inhibition was observed at $H_4$PteGlu concentrations above 50 μM. $K_m$ values of 10 μM for ATP ($V_{max}$ = 31 μmol $h^{-1}$ $mg^{-1}$) and 332 μM for glutamate ($V_{max}$ = 78 μmol $h^{-1}$ $mg^{-1}$) were calculated under these conditions, the differences in $V_{max}$ values reflecting the low glutamate concentration in the standard assay.

*Reducing Agent Requirements.* The enzymatic requirement for a reducing agent was investigated with aminopterin as the folate substrate. Dithiothreitol was a more effective activator than mercaptoethanol and activated to 10% higher levels. Half-maximal stimulation was observed with 0.9 mM dithiothreitol and 3.2 mM mercaptoethanol, while maximal stimulation was observed with 2.5 and 25 mM reagents, respectively. High levels of dithiothreitol (>10 mM) and mercaptoethanol (>100 mM) were inhibitory.

*Monovalent Cation Requirement.* The hog liver enzyme had an absolute requirement for monovalent cations which could be met by $K^+$, $Rb^+$, or $NH_4^+$ (Figure 5A). The $K_d$ values for activation were 1.71 mM for $K^+$, 3.2 mM for $Rb^+$, and 3.5 mM for $NH_4^+$, and the relative maximal activities obtained with each ion were 1.00, 0.41, and 0.78, respectively. A slight activation was observed with high levels of $Na^+$ which could have been due to contamination with trace levels of $K^+$. $Li^+$ and $Cs^+$ did not activate the enzyme. Monovalent cation activation was restricted to ions of ionic radii 1.33–1.47 Å. The $K_d$ for $K^+$ was unaffected by increasing the glutamate concentration in the assay mixture from 250 μM to 10 mM, suggesting that the monovalent cation was not required to form salts with the free carboxyl group(s) of the glutamate substrate.

All monovalent cations inhibited enzyme activity at high concentrations (Figure 5A,B), and a linear relationship was observed between ionic radius and extent of inhibition (Figure 5B), suggesting that the inhibition was due to an interaction at a separate site from the activation site. The departure from

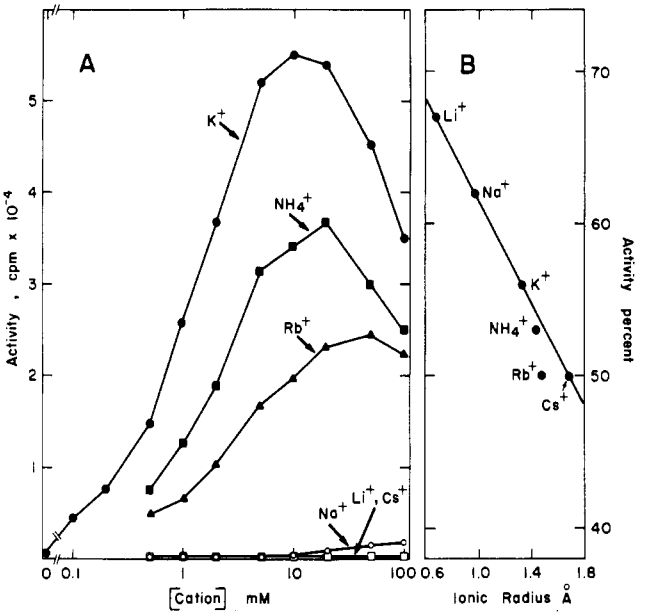


FIGURE 5: Effect of monovalent cations on folylpolyglutamate synthetase activity. The assay mixture, containing 1.4 units of purified folylpolyglutamate synthetase, was as described under Experimental Procedures except KCl was omitted, Tris-HCl buffer replaced the Tris/glycine/NaOH buffer, and the Tris and Mg salts of $H_4$PteGlu and ATP were used. (A) Monovalent cations as enzyme activators. Chloride salts of the indicated cations were added as indicated. (B) Monovalent cations as enzyme inhibitors. Assay mixtures contained 20 mM KCl and the indicated cations (200 mM). Activity is expressed relative to that obtained in the presence of 20 mM KCl alone.

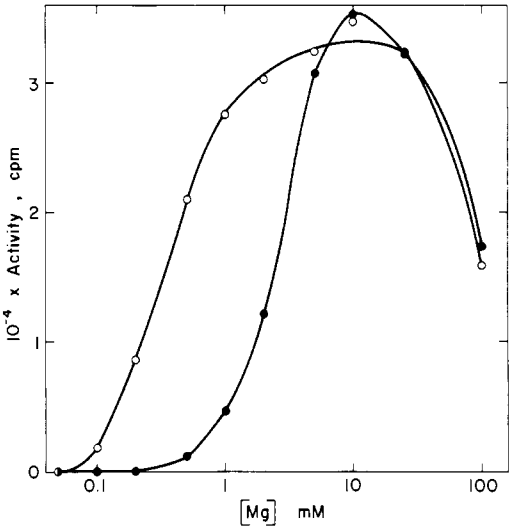


FIGURE 6: Effect of $Mg^{2+}$ concentration on folylpolyglutamate synthetase activity. The assay mixture, containing 0.9 unit of purified enzyme, was as described under Experimental Procedures except the $Mg^{2+}$ concentration was varied, as indicated. The assay mixture contained 0.1 mM ATP (○) or 5 mM (●) ATP.

linearity with $NH_4^+$ and $Rb^+$ (Figure 5B) would be expected as these ions also compete with $K^+$ for the activation site and activate the enzyme to a lesser extent.

*Divalent Cation Requirement.* The effect of $Mg^{2+}$ concentration on enzyme activity is shown in Figure 6. In the presence of 100 μM ATP, enzyme activity increased with increasing $Mg^{2+}$ concentration to a maximum at about 2 mM, while higher levels were inhibitory. Using an approximate association constant for $MgATP^{2-}$ in the presence of 20 mM $K^+$ of 50 000 $M^{-1}$, the $MgATP^{2-}$ concentration would increase from 64 to 99 μM as the $Mg^{2+}$ concentration was increased from 100 μM to 2 mM, and free $ATP^{4-}$ (and $KATP^{3-}$) de-

Table III: Characterization of Folate Products of the Hog Liver Folylpolyglutamate Synthetase Reaction[a]

| folate | glutamate | time (h) | polyglutamate distribution (%)[b] 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 40 μM (6*RS*)-$H_4$PteGlu | 250 μM L-[$^{14}$C]glutamate | 2 | | 84.6 | 14.4 | 1.1 | 0 | 0 | 0 | 0 | 0 |
| 10 μM (6*RS*)-$H_4$PteGlu | 250 μM L-[$^{14}$C]glutamate | 2 | | 64.2 | 29.7 | 5.9 | 0.3 | 0 | 0 | 0 | 0 |
| 10 μM $H_2$PteGlu | 250 μM L-[$^{14}$C]glutamate | 2 | | 72.1 | 23.4 | 4.6 | 0 | 0 | 0 | 0 | 0 |
| 10 μM aminopterin | 250 μM L-[$^{14}$C]glutamate | 2 | | 100 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 0.1 μM (6*S*)-$H_4$[$^3$H]PteGlu | 5 mM L-glutamate | 3 | 8.0 | 2.0 | 3.3 | 3.4 | 57.6 | 23.2 | 2.5 | 0 | 0 |
| 0.1 μM (6*S*)-$H_4$[$^3$H]PteGlu | 5 mM L-glutamate | 24 | 6.1 | 2.3 | 2.2 | 3.3 | 22.6 | 54.3 | 9.2 | 0 | 0 |
| 0.1 μM (6*S*)-$H_4$[$^3$H]PteGlu | 5 mM L-glutamate | 48 | 5.7 | 1.9 | 1.9 | 3.4 | 21.1 | 55.8 | 10.2 | 0 | 0 |
| 0.1 μM [$^3$H]PteGlu | 5 mM L-glutamate | 24 | 14.3 | 5.5 | 12.8 | 48.4 | 15.1 | 3.9 | 0 | 0 | 0 |
| 0.1 μM [$^3$H]PteGlu | 5 mM L-glutamate | 48 | 6.6 | 4.4 | 8.4 | 56.5 | 21.0 | 3.1 | 0 | 0 | 0 |
| endogenous | | | | 0.4 | 0.4 | 1.7 | 15.9 | 49.7 | 24.5 | 6.3 | 1.2 |

[a] Assay conditions and the cleavage of folate products to $p$AbaGlu$_n$ and their separation by HPLC are described under Experimental Procedures. Folate and glutamate substrates and the incubation time were modified as indicated. Assay mixtures (0.5 mL) contained 1.3 units of enzyme (unlabeled folate substrates) or 4 units of enzyme (labeled folate substrates). In extended incubation studies, additional enzyme (4 units) was added at 3 h, and the complete reaction mixture minus labeled folate (0.5 mL) was added at 24 h. [b] The distribution of products obtained with labeled glutamate as the substrate has been adjusted to account for the increasing specific activity of the folate product with increasing glutamate chain length.

creases from 36 to 1 μM over the same concentration range. As the $K_m$ for ATP in the presence of excess $Mg^{2+}$ is about 10 μM, these data indicate that $MgATP^{2-}$ is the nucleotide substrate for the reaction and that free ATP is a potent inhibitor of the reaction. Similarly, in the presence of 5 mM ATP, 5 mM $Mg^{2+}$ was required for maximal activity (Figure 6), and potent inhibition by free ATP was observed at lower $Mg^{2+}$ concentrations. At lower $Mg^{2+}$ concentrations, practically all the $Mg^{2+}$ was bound to ATP, with the $MgATP^{2-}$ concentration increasing from 99.6 μM at 100 μM $Mg^{2+}$ to 4.69 mM at 5 mM $Mg^{2+}$. Again, excess $Mg^{2+}$ was inhibitory.

$Mn^{2+}$ was the only other divalent cation tested that supported enzyme activity. At 1 mM cation concentration (100 μM ATP), $Mn^{2+}$ exhibited 68% of the activity obtained with $Mg^{2+}$, while no activity was observed with $Co^{2+}$, $Ca^{2+}$, or $Zn^{2+}$.

*pH Optimum.* The pH optimum of the enzyme is shown in Figure 7. Tris–glycine buffers of pH 7.5–11.5 (20 °C) were used to generate the pH curve. In the standard assay mixture, the pH range generated at 37 °C was 6.75–9.5, as indicated in Figure 7. Under standard assay conditions (250 μM L-glutamate), activity increased with pH, reaching an optimum around 9.4, and was about 20% of maximal at physiological pH (7.4).

The pH profiles were repeated by using various concentrations of glutamate ranging from 50 μM to 5 mM, and approximate $K_m$ and $V_{max}$ values were obtained for glutamate at the different pH values. $K_m$ values were above 5 mM at pH 6.75 and ranged from about 2.3 mM to 170 μM as the pH increased from 7.5 to 9.5. Relative $V_{max}/K_m$ values (Figure 7), which represent the effectiveness of glutamate as a substrate at low glutamate concentrations, demonstrated a steep pH profile with an optimum at pH 9.4 and about 4% maximal activity at pH 7.4. At saturating glutamate concentrations (Figure 7), the same pH optimum of 9.4 was observed, but the variation with pH was much less, such that 80% of maximal activity was observed at physiological pH values. These data suggest that the high-pH optima observed for folylpolyglutamate synthetases (McGuire et al., 1980; Shane, 1980; Bognar & Shane, 1983; Moran & Colman, 1984; Bognar et al., 1985) are a result of the free amine of glutamate ($pK$ = 9.7 at 25 °C) being the substrate for the reaction. At lower pH values, activity is only slightly reduced provided sufficient unprotonated glutamate is provided. The relative $V_{max}$ values shown in Figure 7 can only be considered approximate as the accuracy of the folylpolyglutamate synthetase assay is reduced when very high levels of labeled glutamate substrate are used and the $K_m$ values were high at the lower pH values. Because

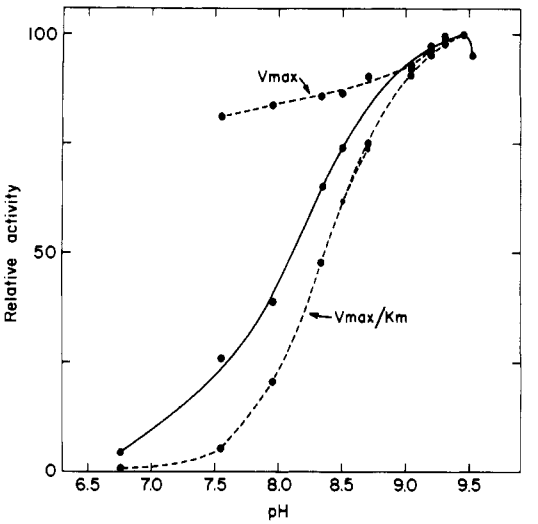


FIGURE 7: Effect of pH on folylpolyglutamate synthetase activity. The complete assay mixture was as described under Experimental Procedures except the buffer used was 100 mM Tris/50 mM glycine adjusted to various pH values with NaOH or HCl. The pHs indicated are the pHs of the complete assay mixture at 37 °C. Relative activities under standard assay conditions [250 μM L-glutamate; (●—●)] and the calculated relative $V_{max}$ and $V_{max}/K_m$ values for L-glutamate (●- - -●) are shown. All values are normalized to that obtained at pH 9.4 (equals 100).

of this, the slightly lowered $V_{max}$ values in the lower pH range may be artifactual. The data suggest that protonated glutamate is not a substrate and has little, if any, affinity for the enzyme.

*Products of ATP Cleavage.* The stoichiometry of the reaction was investigated by using [$^{14}$C]glutamate, [$^{14}$C]ATP, or [$\gamma$-$^{32}$P]ATP as the labeled substrate. Only very small amounts of hydrolysis of [$^{14}$C]ATP to ADP and AMP, and [$\gamma$-$^{32}$P]ATP to phosphate, were observed in the absence of enzyme or individual substrates. In the complete assay mixture, containing all substrates and enzyme, the ratio of the [$^{14}$C]$H_4$PteGlu$_2$, [$^{14}$C]ADP, and [$^{32}$P]$P_i$ products formed was 1.0:0.9:0.8, and only trace amounts of [$^{14}$C]AMP were found, demonstrating that for every glutamate moiety added to the folate molecule, one ATP molecule was hydrolyzed to ADP and phosphate.

*Folate Distribution in Hog Liver and Products of the Reaction.* The endogenous distribution of folylpolyglutamates in hog liver, assessed by $A_{280nm}$ of their $p$AbaGlu$_n$ cleavage products after HPLC separation, is shown in Table III. Hexaglutamates were the predominant derivatives followed

by hepta- and pentaglutamates. Derivatives of glutamate chain length up to nine were detected. The total folate content of hog liver was 18.1 nmol/g wet weight, equivalent to an intracellular folate concentration of about 25 $\mu$M.

Under the standard assay conditions, 40 $\mu$M (6*RS*)-$H_4$PteGlu was metabolized primarily to the diglutamate derivative although small amounts of tri- and tetraglutamate were also formed (Table III). Reducing the folate concentration to 10 $\mu$M caused an increase in the average glutamate chain length of the products, although diglutamate still predominated, and a trace of pentaglutamate was detected. Under identical conditions, 10 $\mu$M $H_2$PteGlu was metabolized up to the tetraglutamate with the diglutamate predominating, while 10 $\mu$M aminopterin was metabolized solely to the diglutamate derivative.

Long-chain polyglutamate derivatives were formed when the reaction mixture was modified by replacing [$^{14}$C]glutamate (250 $\mu$M) with 5 mM unlabeled glutamate and (6*RS*)-$H_4$PteGlu with low levels of (6*S*)-$H_4$[$^3$H]PteGlu and increasing the enzyme concentration and incubation time (Table III). Under these conditions, the major folate product formed was the hexaglutamate, and appreciable amounts of heptaglutamate were also observed. Increasing the incubation time from 24 to 48 h, with addition of the complete incubation mixture minus labeled folate at 24 h, did not significantly increase the glutamate chain length of the products, suggesting that the labeled folate derivatives had broken down by 24 h. Under similar reaction conditions, PteGlu was metabolized primarily to the tetraglutamate derivative in 24 h, and increasing the incubation time to 48 h resulted in further metabolism although the tetraglutamate still predominated.

## Discussion

Mammalian folylpolyglutamate synthetase is a potentially important target enzyme for antifolate drugs, and its specificity for folate analogues is expected to play a role in defining the cytotoxic action of some of these compounds. Some antifolates are effective enzyme inhibitors only when metabolized to polyglutamate forms, and the parent compounds are in effect pro-drugs (Fernandes et al., 1983; Fabre et al., 1984; Chabner et al., 1985). Regulation of the enzyme is also expected to play an important role in the regulation of folate homeostasis. The mammalian enzyme has been characterized to some extent (McGuire et al., 1980; Bognar et al., 1983; Pristupa et al., 1984; Moran & Colman, 1984), but detailed studies have been hampered by the low degree of purity of these preparations. Previous attempts at purifying this protein have been unsuccessful due to the marked lability of the enzyme and the low yields obtained after limited purification.

In this report, the purification of the hog liver enzyme is described. Enzyme was purified 30 000–150 000-fold with yields of 20–29%, depending on the initial specific activity of the preparation, and was homogeneous as judged by a single band of $M_r$ 62 000 after sodium dodecyl sulfate gel electrophoresis. The final specific activity of the preparation (78 $\mu$mol $h^{-1}$ $mg^{-1}$), when assayed under conditions comparable to previous studies (2–5 mM glutamate as substrate), was over 2000-fold higher than the highest value reported for partially purified mammalian enzyme in the literature (Moran & Colman, 1984).

The enzyme was purified in two forms which could be separated on phosphocellulose. Both forms had similar properties during purification and apparently identical relative molecular masses and specific activities, but one form displayed heterogeneity in elution from a chromatofocusing column and appeared to be modified, possibly by proteolysis and/or deamidation. The marked lability encountered in initial purification attempts appears to be due to proteolysis of the protein and, possibly, its oxidation. Enzyme activity was stabilized by the inclusion of protease inhibitors and mercaptoethanol during purification, and by avoiding freezing of the preparation. Highly purified preparations, which contained low protein concentrations, were additionally stabilized by the inclusion of Triton X-100, which probably prevented the absorption of the protein to glassware and could be stored for several months without appreciable loss of activity. Homogeneous preparations, which typically contained from 1 to 10 $\mu$g/mL protein, were less stable and were susceptible to proteolysis. The ability of protease inhibitors to stabilize homogeneous enzyme has not been investigated.

Many of the properties of the hog liver enzyme are similar to those reported for the *Corynebacterium*, *Lactobacillus*, and *E. coli* folylpolyglutamate synthetases (Shane, 1980; Bognar & Shane, 1983; Bognar et al., 1985). All these proteins are monomeric, have high pH optima, hydrolyze 1 mol of $MgATP^{2-}$ substrate to $MgADP^-$ and phosphate for every mole of glutamate added to the folate substrate, are inhibited by $ATP^{4-}$ and high levels of $Mg^{2+}$, and have an absolute requirement for a monovalent cation, which can be met by $K^+$, $NH_4^+$, or $Rb^+$. The $K_d$ for monovalent cations is about 30-fold lower for the hog liver enzyme than for the bacterial enzymes. Monovalent cations also appear to have weak affinity for a separate site on the enzyme, and high concentrations are inhibitory. The mechanism of enzyme activation by monovalent cations is unknown. However, $K^+$ protects the *Corynebacterium* enzyme from inactivation by a variety of proteases, and this protection does not require the presence of substrates, suggesting that the monovalent cation induces a conformational change in the protein or stabilizes a particular conformation (Shane, 1983). Unlike the bacterial enzymes, the hog liver enzyme has an absolute requirement for a reducing agent.

The high-pH optimum is most likely due to the free amine of glutamate being the form of the substrate that binds to the enzyme, and the reaction proceeds effectively at physiological pH values provided high levels of glutamate are provided. The $K_m$ for glutamate at pH 7.5 (2.3 mM) is similar to the glutamate level in mammalian liver and is higher than the glutamate concentration in peripheral tissues (Krebs et al., 1976). Although the possibility that the pH optimum is due to the titration of a functional group on the protein, also with a p$K$ around 9.5, cannot be completely excluded, the ability of high concentrations of glutamate to overcome the low activity at the lower pH values suggests that this is unlikely or that the functional group on the protein plays a role in the binding of glutamate.

In extended incubation experiments, the hog liver enzyme metabolized (6*S*)-$H_4$PteGlu to polyglutamates of chain length up to seven with the hexaglutamate, the major intracellular form of the vitamin, predominating. As the in vitro pattern of metabolites obtained closely mirrored the in vivo distribution of folates in hog liver, a single enzyme can account for the metabolism of folates to polyglutamate forms in this tissue. It should be noted that the concentrations of folate, MgATP, and glutamate used in the extended incubation experiment approximated the expected in vivo concentrations of these substrates in mammalian liver while the enzyme concentration (8–16 units/mL of assay mixture) was slightly lower then the calculated physiological concentration (40–140 units/mL of intracellular water, 22–77 nM). The buildup of hexaglutamate derivatives in vitro and in vivo suggests that polyglutamates

of chain length six and above are very poor substrates for the enzyme. Although the small amounts of octa- and nonaglutamates found in vivo were not detected in the in vitro experiments, it is unlikely that a second enzyme is required for their synthesis. If the long chain length folate derivatives are very poor substrates for the enzyme, lengthy incubations would be required before they would be detected. Further extension of the incubation time was not possible in the in vitro experiments as reduced folate derivatives were unstable and were degraded within 24 h. Experiments described in an accompanying paper (Cook et al., 1987) confirm that the purified enzyme can metabolize heptaglutamate to the octaglutamate derivative. The ability of the hog liver enzyme to metabolize $H_4$PteGlu to long-chain polyglutamate forms differs from results seen with bacterial enzymes, as the only effective polyglutamate substrates for bacterial enzymes are 5,10-methylene-$H_4$PteGlu$_n$ derivatives.

The specificity of folate, glutamate, and nucleotide substrates and analogues for the hog liver enzyme and the role of the enzyme in the regulation of folate homeostasis are discussed in the accompanying papers (Cichowicz & Shane, 1987; George et al., 1987; Cook et al., 1987).

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**Registry No.** Folylpoly-γ-glutamate synthetase, 63363-84-8; L-glutamic acid, 56-86-0.

REFERENCES

Bognar, A. L., & Shane, B. (1983) *J. Biol. Chem. 258*, 12574–12581.

Bognar, A. L., Cichowicz, D. J., & Shane, B. (1983) in *Chemistry and Biology of Pteridines* (Blair, J. A., Ed.) pp 627–632, de Gruyter, Berlin.

Bognar, A. L., Osborne, C., Shane, B., Singer, S. C., & Ferone, R. (1985) *J. Biol. Chem. 260*, 5625–5630.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Brody, T., Shane, B., & Stokstad, E. L. R. (1979) *Anal. Biochem. 92*, 501–509.

Chabner, B. A., Allegra, C. J., Curt, G. A., Clendeninn, N. J., Koizumi, S., Drake, J. C., & Jolivet, J. (1985) *J. Clin. Invest. 76*, 907–912.

Cichowicz, D. J., & Shane, B. (1987) *Biochemistry* (second of four papers in this issue).

Cichowicz, D. J., Foo, S. K., & Shane, B. (1981) *Mol. Cell. Biochem. 39*, 209–228.

Cook, J. D., Cichowicz, D. J., George, S., Lawler, A., & Shane, B. (1987) *Biochemistry* (fourth of four papers in this issue).

Fabre, G., Fabre, I., Matherly, L. H., Cano, J.-P., & Goldman, I. D. (1984) *J. Biol. Chem. 259*, 5066–5072.

Fernandes, D. J., Bertino, J. R., & Hynes, J. B. (1983) *Cancer Res. 43*, 1117–1123.

Foo, S. K., & Shane, B. (1982) *J. Biol. Chem. 257*, 13587–13592.

Foo, S. K., Cichowicz, D. J., & Shane, B. (1980) *Anal. Biochem. 107*, 109–115.

Gawthorne, J. M., & Smith, R. M. (1973) *Biochem. J. 136*, 295–301.

George, S., Cichowicz, D. J., & Shane, B. (1987) *Biochemistry* (third of four papers in this issue).

Kisliuk, R. L. (1981) *Mol. Cell. Biochem. 39*, 331–346.

Krebs, H. E., Hems, R., & Tyler, B. (1976) *Biochem. J. 158*, 341–353.

Krumdieck, C. L., & Baugh, C. M. (1980) *Methods Enzymol. 66*, 663–666.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Masurekar, M., & Brown, G. M. (1975) *Biochemistry 14*, 2424–2430.

McBurney, M. W., & Whitmore, G. F. (1974) *Cell (Cambridge, Mass.) 2*, 173–182.

McGuire, J. J., & Bertino, J. R. (1981) *Mol. Cell. Biochem. 38*, 19–48.

McGuire, J. J., & Coward, J. K. (1984) in *Folates and Pterins* (Blakley, R. L., & Benkovik, S. J., Eds.) Vol. 1, pp 135–190, Wiley, New York.

McGuire, J. J., Hsieh, P., Coward, J. K., & Bertino, J. R. (1980) *J. Biol. Chem. 255*, 5776–5788.

Moran, R. G., & Colman, P. D. (1984) *Biochemistry 23*, 4580–4589.

Pristupa, Z. B., Vickers, P. K., Sephton, G. B., & Scrimgeour, K. G. (1984) *Can. J. Biochem. Cell Biol. 62*, 495–506.

Shane, B. (1980) *J. Biol. Chem. 255*, 5655–5662.

Shane, B. (1982) *Am. J. Clin. Nutr. 35*, 599–608.

Shane, B. (1983) in *Chemistry and Biology of Pteridines* (Blair, J. A., Ed.) pp 621–626, de Gruyter, Berlin.

Shane, B., & Stokstad, E. L. R. (1985) *Annu. Rev. Nutr. 5*, 115–141.

Shane, B., Bognar, A. L., Goldfarb, R. D., & LeBowitz, J. H. (1983) *J. Bacteriol. 153*, 316–325.

Taylor, R. T., & Hanna, M. L. (1977) *Arch. Biochem. Biophys. 181*, 331–344.

Washabaugh, M. W., & Collins, K. D. (1983) *Anal. Biochem. 134*, 144–152.