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Mammalian Folyl Polyglutamate Synthetase: Partial Purification and Properties of the Mouse Liver Enzyme†

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ABSTRACT: Folyl polyglutamate synthetase has been partially purified from mouse liver, and the general features of this enzyme have been characterized. The purification procedure utilized fractionation with ammonium sulfate, gel filtration, and affinity chromatography on ATP–agarose and resulted in a 350-fold increase in specific activity with 8–20% recovery of enzyme activity. Enzyme could be stabilized by glycerol or by ATP, but stability was not appreciably enhanced by folate. The enzymatic reaction was completely dependent on folate, ATP, and Mg^{2+} while partial reaction rates were observed in the absence of KCl or β-mercaptoethanol. Highest reaction rates were observed at pH 8.2–9.5 at 37 °C. Chromatography of purified enzyme on calibrated gel filtration columns suggested a molecular weight of 65 000. Mouse liver folyl polyglutamate synthetase coupled [^{3}H]glutamic acid to all of the naturally occurring folates studied. Analysis of the reaction products by high-performance liquid chromatography demonstrated that several folyl oligoglutamates were formed at low substrate concentrations but that only folyl diglutamate was formed at substrate concentrations approaching saturation. Dihydrofolate, tetrahydrofolate, 5,10-methylenetetrahydrofolate, 10-formyltetrahydrofolate, and 5-formyltetrahydrofolate were the best substrates. Folic acid and 5-methyltetrahydrofolate were also substrates for this reaction, but much higher concentrations of these compounds were required to saturate the enzyme. These data suggest that all of the tetrahydrofolyl compounds (except 5-methyltetrahydrofolate) are the monoglutamyl substrates for polyglutamation in vivo and that 5-methyltetrahydrofolate is not likely to be a direct precursor for folate polyglutamates in mouse liver.

Folate derivatives present in mammalian cells exist predominantly as polyglutamate conjugates in which the amide linkages involve the γ-carboxyl rather than α linkage typical of peptides (Houlihan et al., 1972; Moran et al., 1976; Noronha & Aboobaker, 1963; Shin et al., 1972b). Several lines of evidence suggest that these folyl polyglutamates are the folate forms used as cofactors in the cell for the folate-dependent biosynthetic reactions. Thus, all of the purified folate-dependent enzymes examined to date utilize the folyl oligoglutamates at least as well as the corresponding monoglutamates [recently reviewed in McGuire & Bertino (1981)]. In addition, L1210 mouse leukemia cells have been shown to contain only polyglutamate forms of the folates even under conditions of growth-rate-limiting folate deficiency (Moran et al., 1976). This same cell line has been shown to accumulate polyglutamyl dihydrofolate but not dihydrofolate itself when exposed to cytotoxic levels of the dihydrofolate reductase inhibitor methotrexate (MTX)[1] (Moran et al., 1975).

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It is now apparent that the widely used chemotherapeutic agent MTX can be metabolized to oligoglutamate forms by normal and neoplastic mammalian tissues (Fry et al., 1982; Galivan, 1980; Jacobs et al., 1977; Jolivet & Schilsky, 1981; Whitehead, 1977). The role of these oligoglutamate forms of MTX in the host toxicity and/or antitumor selectivity of this drug is currently being questioned. However, several mammalian cell types have been shown to selectively retain MTX polyglutamates intracellularly under conditions in which MTX itself is rapidly lost to the medium (Fry et al., 1982; Galivan, 1980). The transport of the naturally occurring folates parallels this behavior: folyl monoglutamates are rapidly transported into and out of mammalian cells by a carrier-mediated system (Lichtenstein et al., 1969). On the other hand, L1210 cells containing folyl polyglutamates do not lose these compounds to their surroundings upon extensive incubation (Moran, 1983). Hence, polyglutamation appears to be a mechanism to trap folate cofactors in the cell as metabolites that are substrates for all of the folate-interconverting enzymes except the membrane-associated permease.

A mutant line of Chinese hamster ovary (CHO) cells has been selected which lacks the enzyme responsible for the synthesis of folyl polyglutamates (McBurney & Whitmore, 1974; Taylor & Hanna, 1977). This cell is auxotrophic for the end products of folate metabolism; i.e., it cannot survive if forced to grow on exogenous folate. Yet, the reduced folate transport system in this mutant was identical with that in wild-type cells as were all of the folate enzymes examined except folyl polyglutamate synthetase (FPGS). Hence, FPGS is essential to the survival of proliferating mammalian cells.

The purification of bacterial FPGS and the first details of the behavior of the mammalian enzyme have been reported (McGuire et al., 1980; Shane, 1980a; Taylor & Hanna, 1977). In this report, we describe the purification and characterization of FPGS from mouse liver. The purification procedure involves the use of substrate stabilization of FPGS and the first reported use of affinity chromatography for the mammalian enzyme.

Materials and Methods

Materials. H_2PteGlu, *l*-H_4PteGlu, and *dl*-5-CH_3-H_4PteGlu were prepared and purified as previously described (Moran et al., 1976). *dl*-5-CHO-H_4PteGlu was purchased as the calcium salt (Sigma Chemical Co.; St. Louis, MO). Initially, $PteGlu_3$ was obtained from Lederle Laboratories (Pearl River, NY) and was purified by DEAE-cellulose (Eastman Chemical Co.; Rochester, NY) column chromatography using gradient elution with ammonium acetate (NH_4OAc) (Moran et al., 1976). This column does not differentiate well between $PteGlu_3$ and $PteGlu_n$ so that the $PteGlu_3$ used in these experiments was contaminated with small quantities of $PteGlu_2$ and $PteGlu_4$ (see Figure 5); pteroic acid, *p*-aminobenzoylglutamate, and pterins were, however, removed during chromatography, and this preparation of $PteGlu_3$ was found to be free of PteGlu by HPLC. Subsequently, folyl oligoglutamates containing two to eight glutamate residues were obtained from the Southern Alabama Medical Research Foundation. These compounds were found to be >95% pure by HPLC. 10-CHO-H_4PteGlu was made by first dissolving 5-CHO-H_4PteGlu in 1 N HCL for 30 min and then adjusting the pH to 9 for an additional 15 min. Crude 10-CHO-H_4PteGlu was purified on a 0.9 × 35 cm column of DEAE-cellulose that was eluted with a convex gradient of NH_4OAc (0.01–1.5 M with a 475-mL mixer volume); all buffers contained 1% β-ME. The 10-CHO-H_4PteGlu peak was brought to pH 9 immediately after elution from this column. 5,10-CH_2-H_4PteGlu was formed by incubating 10 mM *l*-H_4PteGlu with 40 mM formaldehyde at pH 4.5 for 15 min in the presence of 50 mM α-thioglycerol. α-Thioglycerol has been found to be as potent as β-ME for the protection of H_4PteGlu from oxidative degradation (R. G. Moran and P. D. Colman, unpublished results). L-[3,4-^{3}H]Glutamate (40 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Enzyme-grade $(NH_4)_2SO_4$ was purchased from Schwarz/Mann Biochemicals. Agarose–N^6-[[(aminohexyl)carbamoyl]methyl]adenosine 5'-triphosphate (i.e., ATP–agarose) was purchased from Sigma Chemical Co. All other materials were reagent grade and were purchased from Sigma Chemical Co. or Mallinckrodt Chemicals (St. Louis, MO).

A stock charcoal suspension was made by suspending 2 g of acid-washed activated charcoal (Sigma Chemical Co.) and 50 mg of dextran T-70 (Pharmacia Chemicals; Uppsala, Sweden) in 50 mL of water. Prior to use, charcoal was washed once with 5 volumes of 10 mM glutamate, pH 6.8, containing 10 mM β-ME and was resuspended at 40 mg/mL in this same solution containing 0.15 M KH_2PO_4, pH 5.

Mice. Most of the experiments reported here utilize FPGS purified from BDF_1 female mice (Simonsen Laboratories; Gilroy, CA). However, no differences were detected in enzyme from BDF_1, DBA/2J, or CDF_1 mice; some experiments using ammonium sulfate pellets (fraction 2) were performed with enzyme from livers of mixed strains.

Enzyme Assays. (*A*) *FPGS.* The two assay procedures used have been described in detail elsewhere (Moran & Colman, 1984). Enzyme was incubated at 37 °C with PteGlu (0.5 mM), [^{3}H]glutamate (1 mM, 4 mCi/mmol), KCl (30 mM), $MgCl_2$ (10 mM), and ATP (5 mM) in 200 mM Tris-HCl buffer, pH 8.5 at 25 °C, containing 50 mM β-ME. The total volume of the assay incubation was 1.0 or 0.25 mL for mouse liver crude supernatant fractions or fractions 2–5 (as defined in Table I), respectively. The activity of crude undialyzed fractions was determined every 10 min in duplicate for 40 min. The initial slope was determined for any assays which were nonlinear with time. Since the FPGS activity of enzyme purified beyond $(NH_4)_2SO_4$ precipitation was linear for up to 75 min over a fairly wide range of protein concentrations (Moran & Colman, 1984), activity was estimated on fractions 3–5 on 0- and 60-min time points only. Time curves were usually followed for fraction 2, but product formation was linear with time for this fraction. ^{3}H-Labeled product was isolated from incubation mixtures by adsorption onto charcoal (0.5 mL) followed by four washes of the charcoal with 10 mM glutamate containing 10 mM β-ME (first wash, 5 mL; subsequent washes, 10 mL); ethanolic ammonia (1.5 mL of 3 M NH_4OH in 60% ethanol) was used to elute product as described elsewhere (Moran & Colman, 1984). For fractions 2–5, the ethanolic ammonia extracts were counted directly. For crude supernatant fractions, the N_2-dried eluate was chromatographed on 4-cm columns of DEAE-cellulose to avoid

[1] Abbreviations: MTX, methotrexate; FPGS, folyl polyglutamate synthetase; CHO, Chinese hamster ovary; DEAE-cellulose, [(diethylamino)ethyl]cellulose; β-ME, β-mercaptoethanol; Pic A, paired ion chromatography reagent A (Waters Associates, Milford, MA); HPLC, high-performance liquid chromatography; NH_4OAc, ammonium acetate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; HEPES, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; P_i, inorganic phosphate; PP_i, inorganic pyrophosphate; PteGlu, folic acid; H_2PteGlu, dihydrofolate; H_4PteGlu, tetrahydrofolate; 5,10-CH_2-H_4PteGlu, 5,10-methylenetetrahydrofolate; 5-CHO-H_4PteGlu, 5-formyltetrahydrofolate; 10-CHO-H_4PteGlu, 10-formyltetrahydrofolate; 5-CH_3-H_4PteGlu, 5-methyltetrahydrofolate; a folate compound containing multiple glutamic acid residues is indicated by the subscript *n* (if chain length is unspecified) or by an integer subscript, e.g., $PteGlu_2$.

interference by some uncharacterized folate-independent enzyme activity. These columns were washed with 50 mM ammonium acetate (10 mL), and the [^{3}H]PteGlu$_2$ was then eluted with 6 mL of 1 M ammonium acetate. The chromatographic fraction containing ^{3}H-labeled product was collected into a scintillation vial, lyophilized to dryness, and rehydrated with 1 mL of water. Radioactivity was determined after the addition of 10 mL of scintillation cocktail (RIA-II, Research Products International; Elk Grove Village, IL) in a Beckman LS-3155T scintillation counter. Replicate aliquots of the reaction mixture were counted under idential conditions for each set of assays to allow direct conversion of cpm to nanomoles of product. Since it became clear that PteGlu was as good a substrate at saturating concentration as were the fully reduced folates, 0.5 mM PteGlu was used for routine assays.

(*B*) *Conjugase.* Folyl oligoglutamate hydrolase (conjugase) activity was measured by a modification of the method of Krumdieck & Baugh (1970). [^{3}H]PteGlu$_2$, labeled in the terminal glutamic acid, was prepared by incubating 500 μM PteGlu with high specific activity [^{3}H]glutamate (40 mCi/mmol) and fraction 2 FPGS (see Table I) under conditions otherwise identical with standard FPGS assay incubations. ^{3}H-labeled product was isolated by charcoal adsorption as described above, except glutamate was omitted from the washes. The N_2-dried eluates, which contained [^{3}H]PteGlu$_2$ and unreacted PteGlu and ATP, were added to incubations containing authentic PteGlu$_2$ at a final concentration of 100 μM and either fraction 1 or fraction 2 protein (see Table I). Incubation mixtures (final volume 0.1 mL) contained either 100 mM acetate, pH 4.5, and 1 mM $ZnSO_4$ or 1 mM glutamate, 30 mM KCl, 10 mM $MgCl_2$, and 200 mM Tris buffer, pH 8.5. The final specific activity of the PteGlu$_2$ used was 0.4 μCi/μmol. Reaction was stopped by the addition of 0.2 mL of dextran-treated charcoal and 1 mL of H_2O. Radioactivity was determined in a 1-mL aliquot of a 1400*g* supernatant.

Protein Assays. Protein was determined by a linear modification (Hartree, 1972) of the Lowry (Lowry et al., 1951) procedure scaled down to a total volume of 1.67 mL. For protein solutions containing levels of Tris buffer or β-ME that interfere with the Lowry assay, protein was first quantitatively precipitated with deoxycholate and trichloroacetic acid (Bensadoun & Weinstein, 1976).

Preparation of Crude Supernatants. Mice were killed by cervical dislocation, and livers were perfused through the hepatic portal vein with ice-cold 0.25 M sucrose containing 20 mM HEPES buffer, pH 7.4. Approximately 30 mouse livers were used in a typical purification. All steps were performed at 0–4°C. Livers were removed, weighed and minced in two volumes of 0.25 M sucrose buffered with 20 mM HEPES, pH 7.4, containing 50 mM β-ME. The suspended mince was homogenized and centrifuged for 1 h at 160000*g*. Buoyant lipids were removed by aspiration, and the supertant fluid (fraction 1) was immediately assayed for enzyme activity.

$(NH_4)_2SO_4$ Fractionation. Crude supernate was brought to 30% saturation with $(NH_4)_2SO_4$ by the slow addition of 175 g of salt/L of fraction 1. After 1 h, the pellet (fraction 2) was harvested by centrifugation and was dissolved in a minimal volume of 20 mM Tris, pH 7.8, containing 50 mM β-ME, 2 mM $MgCl_2$, and 1 mM ATP (buffer A).

Gel Filtration Chromatography. For batch purifications, a 2.5 × 60 cm column of Ultragel AcA 44 (LKB; Rockville, MD) was packed and equilibrated with buffer A. Fraction 2 enzyme was dissolved in buffer A to a final protein concentration of approximately 15 mg/mL and was applied to this column. The column was eluted with this buffer at a flow rate of 0.4 mL/min, and 5-mL fractions were collected. Fractions containing enzyme but beyond the peak of protein were pooled (fraction 3), and $(NH_4)_2SO_4$ was slowly added to 35% saturation (204 g/L). The suspension was stirred for an additional 60 min, and the precipitate was pelleted by centrifugation at 8000*g* for 30 min. The pellet was dissolved in 2 mL of 50 mM Tris, pH 8.5, containing 50 mM β-ME and 2 mM $MgCl_2$, and the resultant protein solution was desalted on a 0.9 × 35 cm column of Sephadex G-25 previously equilibrated with this same buffer. The protein eluting with the void volume of this Sephadex column was pooled (fraction 4).

Affinity Chromatography on ATP–Agarose. ATP–agarose was washed, suspended in 10 mM Tris, pH 8.0, packed in a 5-mL plastic syringe barrel to a final bed volume of 3 mL, and equilibrated with 50 mM Tris, pH 7.8, containing 50 mM β-ME and 10 mM $MgCl_2$ (buffer B). Fraction 4 enzyme was applied to this column, and the column was washed with 40 mL of buffer B. For maximal retention by the column, the flow-through was reapplied to the column 2 additional times. FPGS was then eluted with buffer B containing 25 mM ATP. The first five fractions (1.2 mL) that contained ATP also contained virtually all the eluted FPGS activity (fraction 5).

Thermal Stability Studies. Aliquots of chromatographically desalted enzyme (fraction 5 or fraction 2) were incubated at 37 °C with or without 500 μM folic acid and 5 mM ATP in 200 mM Tris, pH 8.5, containing 20 mM KCl, 10 mM $MgCl_2$, and 50 mM β-ME in a total volume of 0.25 mL. In some experiments, the incubation mixture contained glycerol at a final concentration of 15% (v/v). After various times, [^{3}H]glutamate (1 mM, 4 mCi/mmol) was added to start the reaction, and incubation was continued for an additional 60 min. Reaction was terminated by the addition of 0.5 mL of charcoal suspension, and the charcoal-adsorbed product was isolated and quantitated as described above.

Estimation of Molecular Weight. Standard proteins or purified (fraction 5) FPGS was applied to a 0.9 × 75 cm column of AcA 44 equilibrated with buffer A containing 15% (v/v) glycerol. The void and total volumes of this column were determined with Blue Dextran 2000 and tritiated water, respectively, and the elution positions of standard purified enzymes were determined individually prior to chromatography of FPGS.

Chromatographic Analysis of Reaction Products and Substrate Purities. Analysis of reaction products on DEAE-cellulose and Sephadex G-15 followed published procedures (Baugh & Krumdieck, 1971; Shin et al., 1972a). Column beds used were 0.9 × 60 cm for DEAE-cellulose and 0.9 × 75 cm for Sephadex G-15. Flow rates were held constant with a Harvard peristaltic pump. NaCl concentrations were determined by conductivity against a standard curve; samples were diluted to 20–80 mM for such determinations to obtain a linear relationship between conductivity and NaCl concentration. The purity of folyl monoglutamates was analyzed by HPLC using a Beckman Ultrasphere 5-μm ODS column, 0.4 cm × 25 cm, eluted with an isocratic mixture of Pic A reagent (5 mM) and methanol at a temperature of 40 °C. Optimal conditions for the resolution of folyl monoglutamates were 27% methanol and 73% Pic A. A flow rate of 1 mL/min was maintained with a Spectra-Physics SP-8000 liquid chromatograph. Purity of monoglutamates was measured by electonic integration of peaks from such chromatograms with detection at 280 nm.

Table I: Purification of Mouse Liver FPGS

fraction	total vol (mL)	total enzyme act. (nmol of product h^{-1})	total protein (mg)	sp act. (nmol of product h^{-1} mg^{-1})	rel purification	% recovery
(1) 160000g supernate	86	245	2480	0.099	1.0	100
(2) 0–30% $(NH_4)_2SO_4$ pellet	16	167	201	0.830	8.4	68
(3) Ultragel AcA 44 pool	108	102	48	2.1	21	42
(4) 0–35% $(NH_4)_2SO_4$ pellet of pool	6.3	94	26	3.6	37	38
(5) ATP-agarose pool	10.1	50	1.44	35	348	20

The identity of ^3H-labeled products formed in FPGS incubations with fraction 2 enzyme was determined by cochromatography with folyl oligoglutamate standards. Replicate (4–6) enzyme assays were performed at each of several PteGlu concentrations, and products were isolated by charcoal adsorption. Duplicate assays were counted directly at each concentration. $PteGlu_n$ (n = 1–8) standards were added to the dried residues eluted from charcoal from the remaining replicates and the resultant solutions injected onto a 5-μm Ultrasphere ODS column. Duplicate tubes were combined prior to HPLC analysis for the lower concentrations used. The column was eluted at 40 °C with a discontinuous methanol–Pic A gradient (initial condition, 30% methanol; linear gradient to 34% methanol over 5 min followed by a linear gradient from 34% to 40% methanol over the next 35 min). Markers were detected at 280 nm, and ^3H-labeled products were localized by scintillation counting using 0.5-min fractions.

Determination of Relative Maximal Velocities and Apparent Michaelis Constants. Prior to use in these experiments, the purity of each folyl compound (except 5,10-CH_2-H_4-PteGlu and 10-CHO-H_4PteGlu) was determined by reversed-phase HPLC (see above). Approximately 95% of the A_{280} seen in chromatograms of PteGlu, H_4PteGlu, and 5-CHO-H_4PteGlu eluted as a single peak while 5-CH_3-H_4PteGlu and H_2PteGlu were about 98% pure by this criterion. Chromatographically purified $PteGlu_3$ was found to contain between 5% and 15% of an impurity that eluted from a reversed-phase HPLC column at the retention time of $PteGlu_2$. 10-CHO-H_4PteGlu was used immediately (<60 min) after purification by dilution of the peak tube from the DEAE-cellulose column used for purification of this material (see above). The concentration of NH_4OAc in this fraction was determined by conductivity, and all experimental tubes for the substrate saturation curves using this compound (and a control curve using PteGlu as substrate) were brought to the same concentration of NH_4OAc (1.5–2 mM). Likewise, in determinations of the substrate activity of 5,10-CH_2-H_4PteGlu, all incubations were brought to the same concentrations of formaldehyde as were carried into the assays as a result of the prior incubation for the synthesis of this compound.

In a typical experiment, $(NH_4)_2SO_4$-precipitated FPGS (fraction 2) was desalted on a 0.9 × 35 cm column of Sephadex G-25, concentrated by centrifugal ultrafiltration using an Amicon CF50A cone filter, and added to duplicate incubation tubes containing various concentrations of test folate or of PteGlu as a standard. Six concentrations of test and standard folates (ranging from 0.25K_m to 3K_m) were used for each K_m determination. Reaction was terminated after 1 h, and products were isolated by charcoal adsorption. After chemiluminescence of the scintillation fluid had subsided, the data were analyzed by nonlinear regression analysis fit directly to a rectangular hyperbola utilizing a standard computer program (Cleland, 1967); kinetic parameters were derived by this method. The data were also plotted using Lineweaver–Burk linearization for visualization of results, and the least-squares regression to a line was calculated.

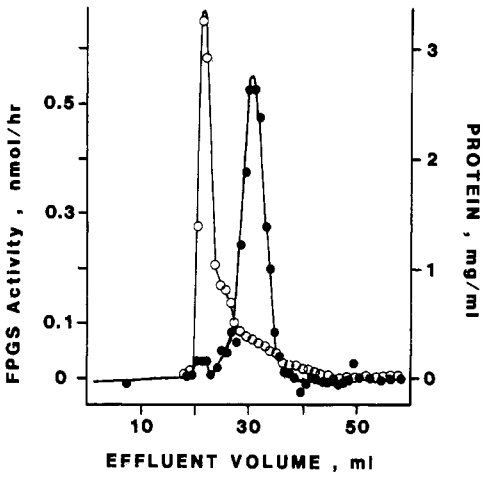

FIGURE 1: Gel filtration chromatography of mouse liver FPGS. Fraction 2 enzyme [14 mg in 1.5 mL; 0.66 nmol h^{-1} (mg of protein)$^{-1}$] was applied to a 0.9 × 70 cm column of Ultragel AcA 44 equilibrated with 50 mM Tris, pH 7.8, containing 50 mM β-ME, 1 mM ATP, and 10 mM $MgCl_2$. Fraction size was 1 mL. Enzyme activity (●) was determined on 200-μL aliquots; protein content (○) was determined as described under Materials and Methods.

Results

Purification. The purification procedure routinely used during this work resulted in a 300–350-fold increase in the specific activity of mouse liver FPGS over that seen in high-speed supernatant fractions, with an overall recovery of 8–20% (Table I). Enzyme stability was the limiting factor in this purification. To maximize enzyme recovery, ATP was present wherever possible past the crude supernatant, and time-consuming steps, e.g., dialysis, were avoided. Typically, this purification procedure involved 48 h from the time mice were sacrificed until fraction 5 enzyme was pooled.

Mouse liver FPGS precipitated at low concentrations of $(NH_4)_2SO_4$. As a result, fractionation of crude extract with $(NH_4)_2SO_4$ resulted in a substantial purification of enzyme activity (7–15-fold in different experiments). Moreover, this step removed an uncharacterized enzyme activity that interfered with the use of charcoal adsorption per se as a method of isolating product (Moran & Colman, 1984). Assay of fractions 2–5 was, thus, considerably simplified.

FPGS eluted from analytical columns of Ultragel AcA 44 as a single sharp, symmetrical band that was well separated from the bulk of protein precipitated with 30% $(NH_4)_2SO_4$ (Figure 1). Highly purified enzyme (fraction 5) behaved similarly. However, preparative gel filtration on this matrix was not as efficient, and the purification of FPGS attained appeared dependent on protein concentration. When fraction 2 enzyme was applied to an Ultragel AcA 44 column in concentrated solution (>20 mg/mL), FPGS activity eluted as a broad peak only slightly separated from the bulk of excluded protein. Concentration of FPGS-containing fractions by $(NH_4)_2SO_4$ precipitation increased the specific activity, usually by a factor of 2, without significant loss of enzyme activity. Recovery of FPGS from Ultragel AcA 44 was poor

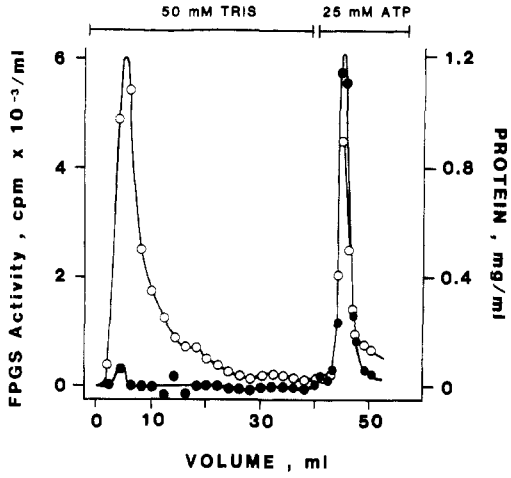

FIGURE 2: Affinity chromatography of FPGS on ATP–agarose. Fraction 2 enzyme (13.2 mg) was applied to a 5-cm column of ATP–agarose packed to a volume of 3 mL in a 5-mL syringe. Enzyme activity (●) and protein content (○) were determined as described under Materials and Methods.

(<5%) if ATP was not added to the elution buffer.

Fraction 4 enzyme was well adsorbed by an agarose column to which ATP was covalently linked via an eight-carbon spacer to the N-6 amino group (Figure 2). Enzyme was desalted before application to this column because $(NH_4)_2SO_4$ interfered with binding to the column as well as with assay of enzyme activity in the breakthrough volume. Recovery of FPGS from ATP–agarose was variable: although 50% of applied enzyme activity usually eluted as a sharp peak coincident with ATP (Figure 2), as little as 20% and as much as 100% have been recovered from this column in different purifications. FPGS was not eluted from ATP–agarose by high-salt buffers (up to 0.2 M phosphate) but was eluted by as little as 0.5 mM ATP. High concentrations of ATP (25 mM) were routinely used for elution to obtain a concentrated protein solution. The specific activity of ATP–agarose-purified FPGS was not increased by high-salt washes of the column prior to removal of enzyme with ATP nor by elution of FPGS with lower ATP concentrations.

Conjugase Activity. Conjugase was low but measurable in fractions 1 and 2 when assayed under conditions optimal for beef liver conjugase [pH 4.5 in the presence of 1 mM Zn^{2+} (Silink et al., 1975)]. Fraction 2 conjugase activity [0.31 nmol h^{-1} (mg of protein)$^{-1}$] was only slightly different from fraction 1 enzyme activity [0.23 nmol h^{-1} (mg of protein)$^{-1}$], although 90% of the total conjugase activity was discarded with the 30% $(NH_4)_2SO_4$ supernatant fraction. However, conjugase activity was not detectable even in fractions 1 and 2 [<0.02 nmol h^{-1} (mg of protein)$^{-1}$] when assayed under the conditions of the FPGS assay (pH 8.5 Tris buffer without added Zn^{2+}).

Stability. The activity of fraction 1 FPGS was completely lost at 4 °C overnight; likewise, extracts prepared from frozen mouse livers had specific activities that were 10–15% of those of freshly prepared extracts. Fraction 2 enzyme that was stored at 4 °C as a solution or as an $(NH_4)_2SO_4$ suspension lost activity with a half-time of approximately 0.7 day. However, fraction 2 enzyme could be stored as a suspension in 50% $(NH_4)_2SO_4$ at −25 °C for at least 1 month with little or no loss of activity. Fraction 5 enzyme was not stable to further manipulations; activity was completely lost within a few hours at 4 °C in the absence of ATP and glycerol.

Mouse liver FPGS was found to be quite sensitive to thermal denaturation. The activity of fraction 2 enzyme was lost with a half-time of 7 min when incubated at 37 °C and pH 8.5 in

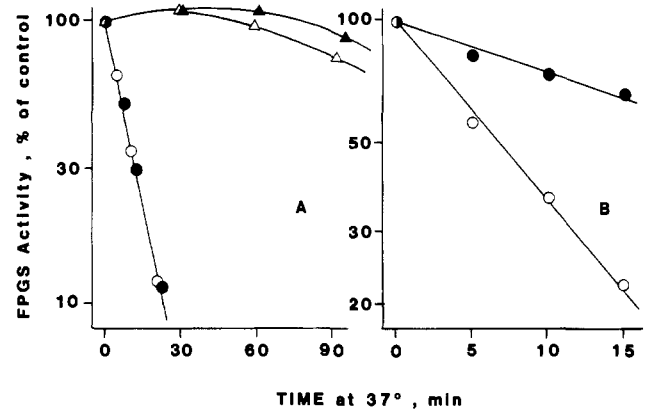

FIGURE 3: Stabilization of mouse liver FPGS by substrates (A) and by glycerol (B). (A) Fraction 2 enzyme was incubated in 0.2 M Tris, pH 8.5, containing 50 mM β-ME, 30 mM KCl, and 10 mM $MgCl_2$ for the indicated periods of time (○) or in the same mixture containing, in addition, 500 μM PteGlu (●), 5 mM ATP (△), or both compounds (▲). [^{3}H]Glutamate (1 mM, 4 mCi/mmol) was added, incubation was continued for 1 h, and product was isolated by charcoal adsorption. (B) Similar incubations were performed using fraction 5 FPGS in assay mixtures lacking ATP, PteGlu, and glutamate in the absence (○) or presence (●) of 15% (v/v) glycerol. Reaction was begun after the indicated periods of preincubation by the addition of substrates. The half-time ($t_{1/2}$) for the loss of activity was 7.0 (A) and 6.8 (B) min for the absence of substrates, 7.0 min in the presence of PteGlu (A), >90 min in the presence of ATP (A), and 23 min in the presence of 15% glycerol (B). Each point represents the mean activity of duplicate incubation tubes.

Table II: Requirements for the FPGS Reaction[a]

	pmol of [^{3}H]PteGlu$_2$ formed per h			
			fraction 5	
	fraction 1	fraction 2	expt 1	expt 2
complete system	62	306	197	61
−folate	5	8	0.8	5
−ATP	3	2	13	8
−$MgCl_2$			0	7
−KCl			53	22
−β-ME				14

[a] Crude enzyme (fraction 1) was added to the various reaction mixes without dialysis; dissolved 0–30% $(NH_4)_2SO_4$ pellet (fraction 2) and ATP–agarose-purified enzyme (fraction 5) were desalted before use by passage through a 0.9 × 35 cm column of Sephadex G-25.

reaction buffer without substrates (Figure 3). ATP (5 mM) greatly enhanced the thermal stability of FPGS, with no detectable loss of activity for at least 60 min. However, saturating concentrations of folic acid (500 μM) did not detectably alter the rate of loss of enzyme activity at 37 °C (Figure 3A). The thermal stability of purified (fraction 5) enzyme in the presence and absence of ATP and folate was identical with that of fraction 2 enzyme (data not shown). FPGS was also stabilized by glycerol; the half-time of thermal denaturation was increased to 23 min in the presence of 15% glycerol (Figure 3B).

Requirements for Enzyme Activity. Product formation by FPGS (fraction 1, 2, or 5) was completely dependent on the presence of a folate compound, ATP, and $MgCl_2$ (Table II). The low rate of reaction detected for fraction 5 enzyme in the absence of added ATP probably reflects incomplete separation of enzyme from ATP by Sephadex G-25 chromatography. Reaction rates in the absence of KCl or β-ME were 30% or 22%, respectively, of that seen with the complete system. Since these reactions were performed with PteGlu as a substrate, the requirement of β-ME for optimal product formation reflects a property of the protein rather than the stability of the folate substrate.

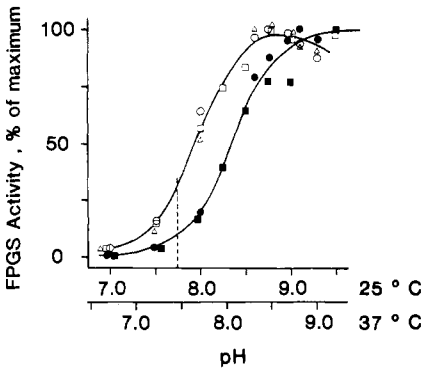

FIGURE 4: pH dependency of the FPGS reaction. Fraction 2 mouse liver enzyme was incubated with either 500 μM PteGlu (closed symbols) or 25 μM *dl*-5-CHO-H_4PteGlu (open symbols) for 1 h in 200 mM Tris buffer. The indicated pH values were determined at room temperature; however, the corresponding pH values at 37 °C are indicated for comparison. The vertical dashed line indicates pH 7.4 at 37 °C. Each point represents the mean of duplicate assays; each symbol represents a separate experiment.

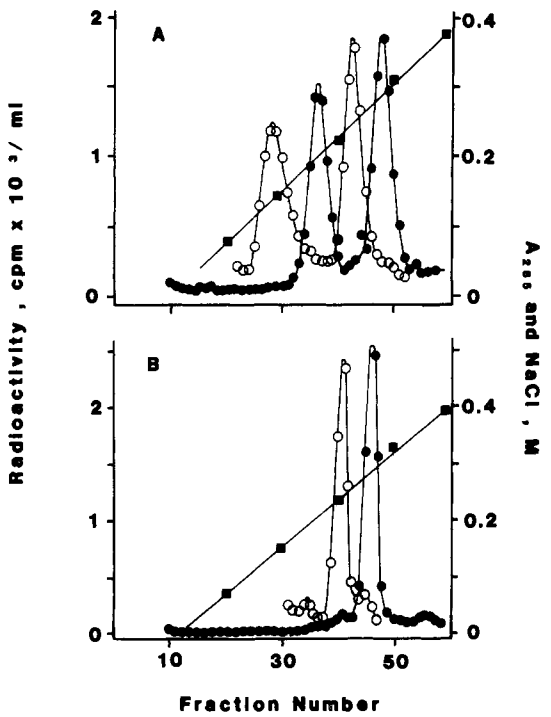

FIGURE 5: Characterization of the product of the mouse liver FPGS reaction on columns of DEAE-cellulose. The enzyme used in these experiments was purified by $(NH_4)_2SO_4$ fractionation and ATP–agarose chromatography as described in the text. FPGS was incubated with PteGlu (2 mM) or $PteGlu_3$ (0.5 mM) for 18 h under standard conditions in a total volume of 0.5 mL per reaction. ^{3}H-Labeled products were isolated by charcoal adsorption, as described in the text, mixed with standards, and then chromatographed on columns of DEAE-cellulose eluted with a linear gradient of NaCl in 5 mM phosphate, pH 7.1. Fractions were 3.1 mL. The product of the reaction using $PteGlu_3$ as substrate was chromatographed on DEAE-cellulose in the experiment shown in (B) while both products were mixed and chromatographed in (A). The standards (1 μmol) used were PteGlu (A) and $PteGlu_3$ (A and B). (O) A_{285}; (●) radioactivity; (—) NaCl concentration.

The pH dependency of the reaction was determined by using 500 μM PteGlu and 25 μM *dl*-5-CHO-H_4PteGlu as substrates (Figure 4). The rate of product formation was maximal only at pH values at 25 °C >8.5 for both compounds; however, reaction rates increased rapidly above pH 8.0 at 25 °C. The rate of reaction with PteGlu reached half of maximal values at a pH (8.4, at 25 °C) that was significantly higher than the equivalent pH value (at 25 °C) for 5-CHO-H_4PteGlu (7.9).

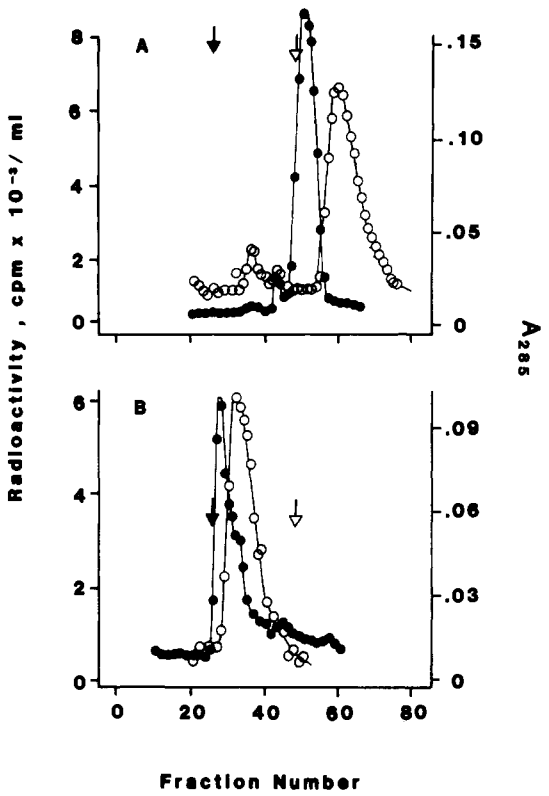

FIGURE 6: Characterization of the products of the mouse liver FPGS reaction on Sephadex G-15. Enzyme was prepared and products were isolated as described in Figure 5. The product of the FPGS reaction using PteGlu as a substrate was mixed with PteGlu (1 μmol) and chromatographed on a 0.9 × 75 cm column of Sephadex G-15 equilibrated with 20 mM ammonium bicarbonate (A). Similarly, the product isolated from a FPGS reaction using $PteGlu_3$ as substrate was chromatographed with a marker of $PteGlu_3$ (1 μmol) (B). Fractions were 1.6 mL. (O) A_{285}; (●) radioactivity; (open and closed arrows) total volume and exclusion volume of the Sephadex G-15 column, as determined by the elution positions of NaCl and Blue Dextran, respectively.

Enzyme activity at a pH of 7.4 at 37 °C was less than 5% of maximum with PteGlu but was about one-third of maximum with 5-CHO-H_4PteGlu. Fraction 5 enzyme showed a similar pH dependency.

Chromatographic Characterization of the Products of the Mouse Liver FPGS Reaction. The products of the mouse liver FPGS reaction were analyzed on several column chromatographic systems that have previously proven useful for the characterization of folyl polyglutamates. Development of a DEAE-cellulose column with a linear gradient of NaCl has been shown to allow the separation of PteGlu and its oligoglutamate derivatives as a series of well-resolved peaks eluted in order of the length of the glutamate chain (Baugh & Krumdieck, 1971). The ^{3}H-labeled product of the FPGS reaction formed from the linkage of [^{3}H]glutamate with $PteGlu_3$ eluted at the position expected for [^{3}H]$PteGlu_4$ on this DEAE-cellulose column (Figure 5A) while the product formed from PteGlu eluted at a NaCl concentration midway between the PteGlu and $PteGlu_3$ standards (Figure 5B). By this criterion, then, the major products of the FPGS reaction utilizing saturating concentrations of PteGlu and $PteGlu_3$ as substrates were $PteGlu_2$ and $PteGlu_4$, respectively.

Mono- and polyglutamyl folates have been previously shown to behave anomalously on Sephadex G-15 (Shin et al., 1972a). Thus, several monoglutamyl folates are retained on this resin beyond the total volume of the column while polyglutamates are excluded to a higher degree than would be expected from their molecular weights. As shown in Figure 6A,B, the FPGS

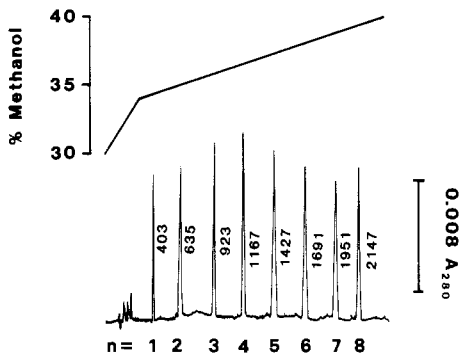

FIGURE 7: Separation of folate oligoglutamates by reverse-phase HPLC. A mixture of standard folate oligoglutamates ($PteGlu_n$, n = 1–8; 175–400 pmol of each) was injected onto an Ultrasphere 5-μm ODS column and eluted with a gradient of methanol and Pic A reagent. The top panel depicts the methanol concentration used for elution. The bottom panel shows the absorbance at 280 nm of the HPLC effluent. The numbers next to each peak represent the retention time in seconds. For details, see Materials and Methods.

reaction product using PteGlu as substrate eluted from G-15 immediately before PteGlu while that formed from conjugation of [^{3}H]glutamate with $PteGlu_3$ eluted at the breakthrough volume of this column; this behavior would be expected for [^{3}H]$PteGlu_2$ and [^{3}H]$PteGlu_4$, respectively (Shin et al., 1972a).

The oligoglutamate forms of PteGlu can be easily separated by paired-ion chromatography on a 5-μm Ultrasphere ODS column (Figure 7). Separation of three sequential compounds of this series was possible with isocratic elution, but adequate resolution of all of these compounds with acceptable peak shapes required a discontinuous gradient of methanol. The separation achievable under these conditions (Figure 7) allows sufficient space between standards for the detection of unexpected peaks or side products. When this chromatographic system was used to analyze the products of the FPGS reaction, essentially all radioactivity chromatographed coincident with $PteGlu_n$ standards (Figure 8A). At low PteGlu concentrations, an appreciable proportion of the ^{3}H-labeled products were $PteGlu_3$ and $PteGlu_4$ while higher proportions of the products were $PteGlu_2$ as the PteGlu concentration approached saturation (Figure 8B).

Substrate Specificity for Folate Compounds. The activity of a number of naturally occurring folates as substrates for FPGS was studied by using fraction 2 enzyme. The maximal velocity of reaction showed little variation among the folate monoglutamates tested (Table III). *dl*-5-CH_3-H_4PteGlu, *dl*-10-CHO-H_4PteGlu, H_2PteGlu, and *l*-H_4PteGlu were found to have a 25–50% higher V_{max} than PteGlu, while the V_{max} of *dl*-5-CHO-H_4PteGlu was comparable to that of PteGlu. The major difference in the substrate activities of the various monoglutamyl folates studied was in the concentrations required to produce half-maximal reaction velocities. On this basis, PteGlu was a poor substrate, with an apparent K_m on the order of 140 μM while H_2PteGlu, *l*-H_4PteGlu, *l*-5,10-CH_2-H_4PteGlu, *dl*-10-CHO-H_4PteGlu, and *dl*-5-CHO-H_4PteGlu had apparent K_m values in the range of 4–9 μM. 5-CH_3-H_4PteGlu was also a poor substrate by this criterion. The ratio of the apparent K_m to the V_{max} was 15–45 times higher for the monoglutamyl-reduced folate derivatives studied (with the exception of 5-CH_3-H_4PteGlu) than for PteGlu. The activity of $PteGlu_3$ and $H_2PteGlu_3$ showed a relatively low apparent K_m but a correspondingly low V_{max}.

The substrate–velocity curves for all of the folates studied were well described by a rectangular hyperbola, showing no measurable tendency to faster velocities at lower substrate concentrations than would be expected for such a relationship. This fact is easily seen in the close fit of the data for PteGlu,

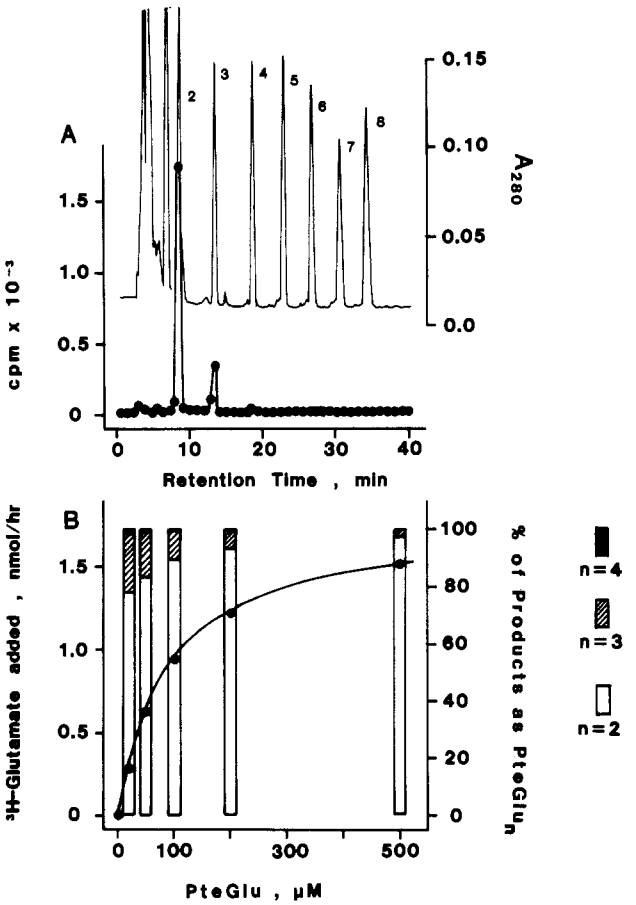

FIGURE 8: Analysis of FPGS reaction products by HPLC. Fraction 2 enzyme was incubated with the indicated concentrations of PteGlu for 1 h at 37 °C, and the products were isolated by charcoal adsorption. (A) HPLC analysis of the reaction products following incubation of FPGS with 100 μM PteGlu. The numbers next to the peaks in panel A represent the number of glutamates in the corresponding standard. The solid symbols represent radioactivity (A). (B) Distribution of the total ^{3}H incorporated among the $PteGlu_2$ (open bar), $PteGlu_3$ (hatched bar), and $PteGlu_4$ peaks (solid bar). The total charcoal-adsorbed radioactivity (●) is also graphed as a function of PteGlu concentration. For details, see the text.

Table III: Substrate Activity of the Naturally Occurring Folates for Mouse Liver FPGS

compd	app K_m[a] (μM)	rel V_{max}[b]	$V_{max}/K_{m,app}$[c]
PteGlu	137 ± 20 (10)	1.00	1.0
H_2PteGlu	8.6 ± 0.01 (2)	1.57 ± 0.29	28 ± 7
l-H_4PteGlu	7.0 ± 1.4 (2)	1.31 ± 0.07	30 ± 7
l-5,10-CH_2-H_4PteGlu	4.8 ± 0.7 (2)	1.17 ± 0.05	26 ± 0.8
dl-5-CHO-H_4PteGlu	8.1 ± 1.0 (2)	0.97 ± 0.15	16 ± 5
dl-10-CHO-H_4PteGlu	3.9 ± 0.3 (3)	1.25 ± 0.05	44 ± 1
dl-5-CH_3-H_4PteGlu	87.0 ± 2.7 (2)	1.30 ± 0.04	2.0 ± 0.2
$PteGlu_3$	68 (1)	0.61	1.6
$H_2PteGlu_3$	17 (1)	0.32	3.4

[a] V_{max} and apparent K_m values were determined by fitting data to a rectangular hyperbola according to a nonlinear regression computer program (Cleland, 1967). The number in parentheses indicates the number of experiments that were pooled for each value, which is expressed as the means ± the standard deviation. Each experiment had a minimum of six concentration points with duplicate assays per point. The least-squares correlation coefficients for the data for these experiments, when linearized by a double-reciprocal transformation (Lineweaver & Burk, 1934), were >0.98. [b] The ratio of V_{max} for a substrate in a given experiment to the V_{max} for PteGlu in that same experiment. [c] Relative to the same ratio for PteGlu as measured in the same experiment.

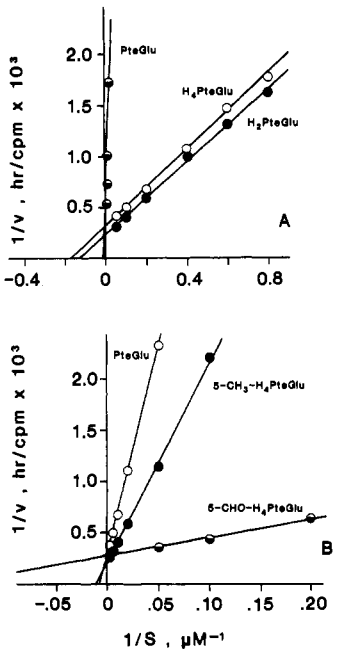

FIGURE 9: Activity of mouse liver FPGS with naturally occurring folate monoglutamates. Fraction 2 enzyme was incubated for 1 h with the indicated concentrations of purified folate compounds as described under Materials and Methods. Each symbol represents the mean of duplicate determinations from a representative experiment.

H_2PteGlu, H_4PteGlu, 5-CHO-H_4PteGlu, and 5-CH_3-H_4PteGlu to Lineweaver–Burk plots (Figure 9).

Inhibition of FPGS. During studies on the fractional precipitation of FPGS, it became apparent that enzyme was inhibited by $(NH_4)_2SO_4$. Enzyme activity was decreased by 50% in the presence of 40 mM $(NH_4)_2SO_4$ (data not shown). Fraction 2 FPGS was also inhibited by ADP, AMP, P_i, and PP_i.

Molecular Weight. The apparent molecular weight of fraction 5 enzyme was determined on a calibrated column of Ultragel AcA 44. FPGS eluted from this column immediately after *Lactobacillus casei*/MTX thymidylate synthase (M_r 70000) (Figure 10). The molecular weight of mouse liver FPGS estimated by this method was 65000.

Discussion

We herein describe the partial purification of an enzyme activity from mouse liver that has the characteristics of a folyl polyglutamate synthetase. In the presence of folate and ATP, this enzyme incorporated [^{3}H]glutamic acid into a product that was aromatic (i.e., charcoal adsorbable) and that had the chromatographic behavior of a folyl oligoglutamate on three column systems (Sephadex G-15, DEAE-cellulose eluted with a NaCl gradient, and reverse-phase paired-ion HPLC). In addition, we have also found that the product of this reaction chromatographed at the position of $PteGlu_n$ on columns of DEAE-cellulose eluted with a gradient of ammonium acetate (Moran & Colman, 1984).

The addition of [^{3}H]glutamic acid to a number of folate compounds was catalyzed by this enzyme. Product formation was not observed in the absence of a folate compound or of ATP for either crude or partially purified enzyme. The maximum rate of reaction was quite similar for folate derivatives of substantially different apparent K_m values, suggesting that the rate of the catalytic step depends only on the presence of the structural feature common to these folates, namely, the glutamic acid side chain. The one fully oxidized folate studied, PteGlu, was a poor substrate when compared to the reduced folates examined. This seems to indicate that the conformation

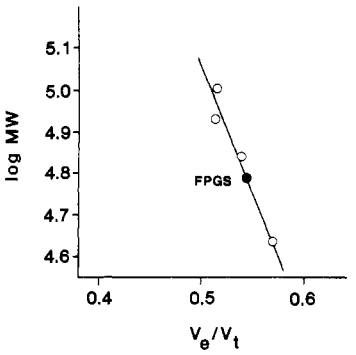

FIGURE 10: Estimation of the molecular weight of mouse liver FPGS by gel filtration. Fraction 5 enzyme was chromatographed on a 0.9 × 70 cm column of Ultragel AcA 44 that was equilibrated and eluted with 50 mM Tris containing 10 mM $MgCl_2$, 1 mM ATP, 30 mM KCl, 50 mM β-ME, and 15% glycerol. The standards used were yeast glucose-6-phosphate dehydrogenase (102000 daltons), *Escherichia coli* alkaline phosphatase (86000 daltons), *L. casei*/MTX thymidylate synthase (70000 daltons), and ovalbumin (46000 daltons). Standards and FPGS were chromatographed individually under identical conditions.

of the pteridine ring was a more important determinant of the initial binding of substrate than was the presence or absence of formyl or methylene substituents at N-5 or N-10. However, a methyl substituent at N-5 clearly diminished substrate binding, although it appeared to be of little significance to the efficiency of the catalytic step. In other experiments to be reported elsewhere, we have observed that a methyl substituent at the N-10 position of a 4-oxo- or 4-aminopteridine had a similarly detrimental effect on the apparent K_m for the FPGS reaction. These results indicate that differences in the substrate activity of various folyl monoglutamates are primarily due to the interaction of the folates with those amino acids involved in the initial binding step, rather than with those involved in catalysis.

The study of the activity of various folate compounds as substrates for this enzyme was complicated by the fact that FPGS converted the reaction product, a folyl diglutamate, to longer chain derivatives [see Figure 8 and McGuire et al. (1980)]. Under our standard assay conditions (i.e., a saturating folate concentration and consumption of less than 5% of substrate in a 1-h incubation), only the formation of folyl diglutamate was measured. However, in the course of saturation analysis experiments, FPGS converted high concentrations of a monoglutamate exclusively to a diglutamate product while, at low concentrations, several products were formed (Figure 8). In order to interpret the apparent K_m values listed in Table II, one must consider not only that higher polyglutamates were formed at low substrate concentrations but also that these experiments were performed on an impure protein preparation. However, fraction 2 was found to be optimal for such studies since it (1) did not contain the enzyme activity(ies) that interfered with use of the charcoal adsorption assay (Moran & Colman, 1984), (2) was not as labile as fraction 5 enzyme, and (3) was sufficiently active to allow accurate determinations of reaction velocity at low substrate concentrations. It should also be noted that fraction 2 enzyme did not contain detectable levels of conjugase activity. We believe that the data of Table II indicate the order of activity of various folates as substrates for FPGS. It is relevant to our choice of fraction 2 for these studies that the substrate specificity of the Corynebacterium enzyme was identical for crude and homogeneous preparations (Shane, 1980a). Experiments to be described elsewhere demonstrated that the reaction of multiple substrates with fraction 2 enzyme was mutually ex-

clusive. This suggests that only one enzyme is involved in the broad substrate specificity reported here.

A most striking aspect of FPGS is its low specific activity in mammalian tissues. We (Moran & Colman, 1984) and others (McGuire et al., 1979) have determined that, among rodent tissues, FPGS is most abundant in liver. Crude cytosol fractions of mouse liver were found to convert PteGlu to oligoglutamates at such a slow rate [100 pmol h^{-1} (mg of protein)$^{-1}$] that it is questionable whether this enzyme activity would be sufficient to account for the cellular formation of folyl polyglutamates. It has been calculated that the maximum velocities observed in homogenates of enzyme-rich tissues under optimal assay conditions would be about twice that required by a rapidly growing cell population (Moran, 1983). However, the rates of mammalian FPGS activity at neutral pH (Figure 4; Taylor & Hanna, 1977; McGuire et al, 1980) and physiologic concentrations of folates might be too low to account for the polyglutamation of the naturally occurring folates. This may suggest either that FPGS exists in the cell in a microenvironment of mild alkalinity or that the pH optimum of FPGS is modified by the intracellular environment. The low specific activity of FPGS in mammalian tissues also has practical implications for the purification of this enzyme. If the turnover number recently determined for the pure Corynebacterium enzyme (Shane, 1980b) is similar to that of mammalian FPGS, liver enzyme would have to be purified 4×10^5-fold in order to obtain a homogeneous preparation. If this calculation is correct, 100 g of liver would contain only 10 μg of FPGS.

We have attempted to develop affinity columns based on the binding of FPGS to ATP and also to a folate ligand in order to attain rapid purification of this unstable protein. ATP–agarose did retain FPGS (Figure 2) and seemed to act as an affinity column. Thus, ATP levels sufficient to saturate enzyme binding sites eluted FPGS, while high salt concentrations did not do so. We presume that the low degree of purification of FPGS with this column (Table I) was a reflection of the large number of enzymes that could use ATP as substrate and, hence, could bind to ATP–agarose. Our attempts to develop a folate-based affinity column have been unsuccessful to date. Columns containing high amounts of PteGlu, MTX, or $PteGlu_3$ covalently linked to agarose through α- and/or γ-carboxyls did not retain mouse liver FPGS (data not shown).

The pathway that monoglutamyl folates follow in vivo during their incorporation into the polyglutamyl cofactor pools is of considerable importance. Although the present study cannot answer this question unequivocally, the data of Table III are germane to the related question of which folate(s) is (are) the substrate(s) for FPGS in vivo. It seems clear that the low levels of enzyme and the low activity of PteGlu at physiological concentrations (Table III) preclude PteGlu as a candidate. Likewise, it seems likely that $H_2PteGlu$ is not the physiological substrate in spite of its activity as a substrate (Table III), since both mono- and polyglutamyl forms of $H_2PteGlu$ are present at low concentrations in growing cells (<0.1 μM; Jackson et al., 1977; Moran et al., 1976). The equivalent activity of $H_4PteGlu$, 5,10-CH_2-$H_4PteGlu$, 10-CHO-$H_4PteGlu$, and 5-CHO-$H_4PteGlu$ (Table III) suggests that any or all of the fully reduced folate forms (except 5-CH_3-$H_4PteGlu$) could serve as substrates for FPGS, in vivo. It is particularly interesting, however, that 5-CH_3-$H_4PteGlu$, the folate used as a serum transport form in mammals (Bird et al., 1965), was a poor substrate for mouse liver FPGS. This may imply that 5-CH_3-$H_4PteGlu$ must be converted to $H_4PteGlu$ within the hepatocyte before it can contribute to the folyl polyglutamate pool in liver.

Mouse liver FPGS had some similarities to the enzyme from rat liver (McGuire et al., 1980) and from CHO cells (Taylor & Hanna, 1977) and even to the purified Corynebacterium enzyme (Shane, 1980a,b). The stability of enzyme from all of these sources was similar. In addition, in our studies as in previous studies of the CHO enzyme, stability was dramatically improved by ATP. FPGS from all sources studied to date precipitated at low degrees of saturation with $(NH_4)_2SO_4$. Gel chromatography of the bacterial enzyme showed skewing of the peak of FPGS activity at low salt concentrations and high protein concentrations (Shane, 1980a). We have found an anomalously broad, skewed peak of FPGS activity on gel filtration columns at high protein concentrations but not at low protein concentrations. The molecular weight of rat liver FPGS (68 000) (McGuire et al., 1980) was the same as our value for the mouse enzyme, and both were similar to that of the bacterial enzyme (53 000) (Shane, 1980a). Likewise, the pH profiles of FPGS from all sources studied to date show a distinctly alkaline pH optimum. The ability of ATP (but not PteGlu) to stabilize mouse liver FPGS and the utility of ATP as an affinity ligand suggest that ATP is the first substrate to bind to FPGS from mouse liver. This postulated order of binding of substrates is also supported by the fact that mouse liver FPGS binds to ATP–agarose in the absence of either folate or glutamate. Kinetic studies indicated that the enzymes isolated from both Corynebacterium species and *Lactobacillus casei* have an ordered sequential mechanism with ATP binding to enzyme first (Shane, 1980b; Bognar & Shane, 1983). The similarities found to date between bacterial FPGS and FPGS from mammalian sources suggest to us that the function of this enzyme may be the same in prokaryotes and mammalian tissues.

Acknowledgments

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Registry No. PteGlu, 59-30-3; $H_2PteGlu$, 4033-27-6; $H_4PteGlu$, 71963-69-4; 5,10-CH_2-$H_4PteGlu$, 31690-12-7; 5-CHO-$H_4PteGlu$, 58-05-9; 10-CHO-$H_4PteGlu$, 2800-34-2; 5-CH_3-$H_4PteGlu$, 134-35-0; $PteGlu_3$, 89-38-3; $H_2PteGlu_3$, 52723-91-8; FPGS, 63363-84-8.

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Analysis of the Stable End Products and Intermediates of Oxidative Decarboxylation of Indole-3-acetic Acid by Horseradish Peroxidase†

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ABSTRACT: A study of the in vitro oxidation of indole-3-acetic acid (IAA) by horseradish peroxidase (HRP) has been carried out by isolating and characterizing the products. Under our experimental conditions, indole-3-acetic acid was converted to oxindole-3-carbinol, 3-methyleneoxindole, indole-3-carbinol (IC) (novel product), and indole-3-carboxaldehyde at pH values between 5.0 and 7.0. The oxygen atom in indole-3-carbinol (IC) arises from O_2. A reaction sequence for peroxidase-catalyzed oxidation of IAA is proposed in which the peroxidase functions as a one-electron oxidizing agent and indol-3-ylmethyl hydroperoxide is the first product of the interaction between oxygen and IAA. Chemiluminescence produced in HRP-catalyzed oxidation of IAA at pH 7.0 is probably derived from the enzymatic degradation of indol-3-ylmethyl hydroperoxide to IC.

Activation of indole-3-acetic acid (IAA)[1] in plants involves two reactions: (1) the formation of physiologically active intermediate products via the oxidation of IAA by peroxidase and (2) the binding of an intermediate oxidation product of IAA to a receptor molecule in the plant cell (Meudt & Galston, 1962).

The oxidation of IAA by HRP can occur in the absence of added hydrogen peroxide and in the presence of catalytic amounts of catalase (Kenten, 1955; Ray, 1962; Fox et al., 1965). Little is known, however, of the detailed metabolic pathway of HRP-catalyzed oxidation of IAA, probably because of the instability of the metabolites and the lack of a precise detection method for the products. It has long been suggested that the initial step in the HRP-catalyzed reaction is a one-electron transfer from IAA to ferriperoxidase. The resulting IAA radical could then be attacked by O_2, producing a β-hydroperoxyindolenine analogue (Hinman & Lang, 1965; Morita et al., 1967; Ricard & Job, 1974). Hinman & Lang (1965) have proposed that β-hydroperoxyindolenine-3-acetic acid is nonenzymatically converted to two major end products, MOI and IA, via an indolenine epoxide. On the other hand, Nakajima & Yamazaki (1979) have recently suggested the formation of another hydroperoxide of IAA, probably indol-

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[1] Abbreviations: IAA, indole-3-acetic acid; MOI, 3-methyleneoxindole; OIC, oxindole-3-carbinol; IC, indole-3-carbinol; IA, indole-3-carboxaldehyde; DBAS, 9,10-dibromoanthracenesulfonate; PBN, α-phenyl-*N*-*tert*-butylnitrone; Me_3Si, trimethylsilyl; HRP, horseradish peroxidase; HPLC, high-performance liquid chromatography; GC, gas chromatography; TLC, thin-layer chromatography; UV, ultraviolet; IR, infrared; NMR, nuclear magnetic resonance; ESR, electron spin resonance; RQY, relative quantum yield; RTLI, relative total intensity; MS, mass spectrometry; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; Me_2SO, dimethyl sulfoxide.

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