The effect of 3,3-difluoroglutamate (F2Glu) on the reaction catalyzed by rat liver folylpolyglutamate synthetase was investigated. F2Glu was a potent, concentration-dependent inhibitor of poly(γ-glutamylation) using [3H]Glu and either methotrexate (4-NH2-10-CH3PteGlu) or tetrahydrofolate as substrates. It was determined that F2Glu acted as an alternate substrate, but in contrast to the previously characterized alternate substrate 4-fluoroglucurate (McGuire, J. J., and Coward, J. K. (1985) J. Biol. Chem. 260, 6747-6754), it did not terminate polyglutamate chain elongation. Instead, F2Glu promoted chain elongation. Thus, synthesis of products from [3H]methotrexate containing 1 and 2 additional amino acid residues occurred at a substantially higher rate in the presence of F2Glu when compared to identical reactions in the presence of Glu; this was more pronounced for the product containing 2 additional residues. Identities of the products were established by their respective chromatographic elution positions and by limit digestion with γ-glutamyl hydrolases. Ligation of Glu to 4-NH2-10-CH3PteGlu-γ-(3,3-difluoroglutamate) was also enhanced. These results are consistent with F2Glu enhancing the synthesis of poly(γ-glutamate) metabolites at the level of either the incoming amino acid (glutamate analog) or the γ-glutamyl acceptor species. F2Glu is thus the first glutamate analog which enhances chain elongation catalyzed by folylpolyglutamate synthetase.

Folates and classical antifolates such as MTX1 are converted intracellularly to poly(γ-glutamyl) metabolites by the enzyme folylpolyglutamate synthetase (EC 6.3.2.17, Equation 1; for comprehensive reviews of the biochemistry of folyl- and antifolylpolyglutamates, see Refs. 3, 10, and 13).

\[
PteGlu_\gamma, + ATP + L-Glu \rightarrow PteGlu_\gamma, + ADP + Pi (1) \]

Since it is now known that folylpolyglutamates are essential to the proper functioning of folate metabolism (11, 13), and antifolylpolyglutamates are implicated in the cytotoxic action of classical antifolates such as MTX (3, 10), folylpolyglutamate synthetase has become an important enzyme for study in folate biochemistry and biochemical pharmacology. In this regard, the specificity of this enzyme for pteroyl and L-glutamate substrates (Equation 1) has been extensively investigated (13). For pteroyl substrates, the structure of the heterocyclic component can vary considerably, but a terminal L-glutamate has been shown to be absolutely required for substrate activity in all reports to this time. Specificity for the incoming amino acid in Equation 1 is strict but not absolute (13). L-Homocysteic acid (21) and DL-erythro- or DL-threo-4-fluoroglucurate (4, 14) can serve as efficient alternate substrates, but their incorporation causes chain termination. Chain termination by the 4-fluoroglucurate diastereomers demonstrates the stringent specificity for L-glutamate at the γ-glutamyl acceptor site. We now report the effects of 3,3-difluoroglutarate (Scheme 1) on the polyglutamylation of MTX and tetrahydrofolate catalyzed by partially purified folylpolyglutamate synthetase from rat liver. Our results indicate that F2Glu is a more efficient substrate for this enzyme than is L-glutamate and, in addition, that incorporation of F2Glu promotes the further synthesis of polyglutamates.

**MATERIALS AND METHODS**

**RESULTS**

The Effect of 3,3-Difluoroglutarate on Folylpolyglutamate Synthetase Activity—The effect of F2Glu on incorporation of [3H]Glu into MTX polyglutamate products was compared to that of simple isotopic dilution by nonradioactive L-Glu. At a route (see "Materials and Methods") would yield a 50:50 mixture). The γ-enantiomer of F2Glu was assumed to be inactive since γ-Glu is neither a substrate nor an inhibitor of the rat liver folylpolyglutamate synthetase (16). Although it seems reasonable based on these facts to assume the inactivity of D-F2Glu, validation of this assumption by synthesis and/or resolution and testing of the individual enantiomers will be undertaken; HPLC, high pressure liquid chromatography.
equivalent levels of L-amino acid up to 2.5 mM, FzGlu was the more potent inhibitor of [3H]Glu incorporation (Fig. 1). Inhibition was considered to result from the L isomer of FzGlu only, by analogy with the fact that D-Glu is neither a substrate nor an inhibitor of the rat liver enzyme (16). Pre-incubation of enzyme with FzGlu for 1 h in the absence of [3H]Glu did not enhance inhibition (data not shown). FzGlu displayed similarly increased inhibitory potency relative to Glu when (6R,S)-tetrahydrofolic acid was the pteroyl substrate. FzGlu was also a better inhibitor than L-Glu of [3H]Glu incorporation into aminopterin (4-amino-10-methyl-pteroylglutamylglutamyl moiety) [3H]glutamate (a level shown in this experiment to be subsaturating; 3.3 cpm/pmol), and 865 units of rat liver folylpolyglutamate synthetase. Incubation was for 3 h. Either FzGlu (●) or unlabeled L-glutamate (○) was added at the indicated concentration.

3.3-Difluoroglutamate and Folypolyglutamate Synthetase—FzGlu could decrease incorporation of [3H]Glu by serving either as a simple inhibitor or as an alternate substrate. To assay whether FzGlu could be ligated to the γ-carboxyl of a pteroyl substrate, [3H]MTX was used as a substrate with either Glu or FzGlu in a 6-h incubation; products were analyzed by HPLC. Analysis on strong anion-exchange HPLC (Fig. 2A) showed that the predominant product (50% of original substrate) synthesized from Glu was MTX-γ-Glu with 7% MTX-γ-(Glu)2; longer products were undetectable. With FzGlu there were also two products observed (Fig. 2B): one (25% of original substrate) eluted at about 22.5 min near MTX-γ-(Glu)2 and the other (39%) eluted at about 40.5 min, overlapping with MTX-γ-(Glu)3. Continuation of the HPLC gradient did not elute any other radioactive peaks and recovery of radioactivity from the column was nearly quantitative suggesting that no other products were synthesized from FzGlu. Chemically synthesized MTX-γ-(4-fluoroglutamate) (4-amino-10-methyl-pteroylglutamylyl γ (4 fluoroglutamate)) (14)), which contains a terminal residue with a γ-pKₐ similar to that of FzGlu, eluted at the same position as did the first product eluted in this system (data not shown), suggesting that this first product was MTX-γ-FzGlu. Analysis of the products by reversed-phase HPLC (Fig. 3) at pH 5.5 where the pKₐ difference between Glu and FzGlu would be suppressed and where the polyglutamates eluted in decreasing order of chain length, showed that the products eluted at positions consistent with addition of 1 and 2 residues, respectively. Longer products were not observed with either substrate. As partial confirmation of the suppression of the pKₐ difference at pH 5.5, MTX-γ-(4-fluoroglutamate) eluted with a retention time which was essentially identical to that of MTX-γ-Glu in this HPLC system. Based on the relative amount of radioactivity in each peak on the reversed-phase column compared to the peaks on the anion-exchange column, the first product peak on the anion-exchange column corresponded to the product with one addition and the second product peak corresponded to the product with two additions; these assignments were also consistent with their theoretical order of elution on an anion-exchange column. Further evidence that these radioactive peaks represented MTX-γ-FzGlu and MTX-γ-(FzGlu)₂, respectively, is presented below.

Based on its relative inhibition of [3H]Glu incorporation (Fig. 1) and the amounts of each product observed above (Figs. 2 and 3), it appeared that FzGlu was not only a substrate for folypolyglutamate synthetase but that it was a better substrate than Glu itself. Thus, the time course for incorporation of Glu and FzGlu was quantitated using [3H]MTX as the substrate. Regardless of the amino acid substrate present, the total amount of [3H]MTX converted to products was essentially the same at each point over the entire time period (data not shown). In each case, essentially all [3H]MTX was metabolized to products by the end of the assay period (24 h), the distribution of products was, however, markedly different (Fig. 4A). In the presence of Glu, MTX-γ-Glu accumulated rapidly during the first 6 h and reached a plateau; MTX-γ-(Glu)₂ was synthesized slowly throughout the incubation period and its level remained low. With FzGlu as the substrate, MTX-γ-FzGlu predominated at incubation times less than 3 h, but MTX-γ-(FzGlu)₂ was apparent even at 1 h. MTX-γ-FzGlu did not accumulate to high levels because it was rapidly converted to MTX-γ-(FzGlu)₂, which was the predominant product at all times beyond 3 h. The high recovery of the [3H] input in the substrate and observed product peaks suggested that, even at 24 h, no further products were synthesized from FzGlu; however, since on anion-exchange HPLC the second product eluted late in the salt gradient beyond which the high phosphate caused the samples to separate into two phases and to quench, it could not be reliably determined that even longer products were not made. Analysis of the products by reversed-phase HPLC, however, confirmed that no other
The 4-h conditions; partial hydrolysis of [3H]MTX-(F2Glu)2 occurred under the 16-h incubation conditions, but [3H]MTX-r-F,Glu was not affected (Table 1B).

Analysis of the data of Fig. 4A in terms of the amount of amino acid incorporated at each time point (Fig. 4B) showed quantitatively to [3H]MTX-r-Glu in 4 h at 2 pg of protein (Table 1B); [3H]MTX-y-Glu itself was quantitatively converted to [3H]MTX, containing FzGlu were still partially intact (Table 1A). After 5 h, each product was quantitatively converted to [3H]MTX-y-(Glu)2, containing this amino acid should chromatograph on DEAE-cellulose similar to their Glu-containing counterparts. Thus, under identical reaction conditions, 2.7

FIG. 4. Time course of ligation of 3,3-difluoroglutamate or glutamate to [3H]methotrexate catalyzed by rat liver folylpolyglutamate synthetase. Standard folylpolyglutamate synthetase assay conditions (see "Materials and Methods") were used with 0.1 μM [3H]MTX (6 μCi/mmol; 4670 cpm/mmol) as pteroyl substrate, FzGlu or unlabeled L-Glu (2.5 mM), and 565 units of rat liver folylpolyglutamate synthetase. After 9 h of incubation (arrow), an additional 865 units of enzyme were added to the 24-h samples to drive the reaction to completion. Samples incubated for the time periods indicated were analyzed by HPLC as described under "Materials and Methods." Control samples lacking only amino acid showed no significant metabolism of the radioactivity throughout the course of the experiment. A, levels of individual products as a function of time: MTX-y-Glu (○); MTX-y-(Glu)2 (●); MTX-r-FzGlu (□); MTX-r-(FzGlu)2 (■); B, total amount of Glu (○) or FzGlu (■) added.

products were synthesized at 24 h (data not shown).

Effect of Pteroyl Substrate Concentration on the Product Distribution--In some instances (e.g. tetrahydrofolate), the concentration of pteroyl substrate affects the distribution of products synthesized by folylpolyglutamate synthetase (16). Increasing the concentration of [3H]MTX (0.1, 0.3, 1, 3, and 10 mM tested) increased the total product synthesized nearly linearly as expected from concentrations below the Km for this substrate (74 μM, Ref. 12). There was a slight shift in the product distribution toward MTX-r-FzGlu as the concentration increased; however, it only increased from 51% of total product at 0.1 μM to 63% at 10 mM (data not shown).

Chain Elongation of a Substrate Containing a Terminal 3,3-Difluoroglutamate—To compare the abilities of compounds containing a terminal FzGlu or Glu to serve as substrates for the folylpolyglutamate synthetase catalyzed addition of L-Glu, [3H]MTX-y-Glu and [3H]MTX-y-FzGlu were prepared and their reaction products with L-Glu were analyzed (see "Materials and Methods"). Utilizing gradient elution on DEAE-cellulose (pH 8.0), 2.7 μM [3H]MTX-r-Glu underwent 52% conversion during a 6.5-h incubation period to a product (Fig. 5A) that was identified as [3H]MTX-r-(Glu)2, since it eluted at a position between the unreacted substrate and a chemically synthesized MTX-r-(Glu)2 marker. No radioactivity eluted in this position if L-Glu was omitted from the incubation (Fig. 5B) and there was no radioactivity under the MTX-r-Glu, standard in either case (Fig. 5, A and B). At the pH of elution (pH 8.0), the lower pK values of the α- and γ-carboxyls of FzGlu would be suppressed and materials containing this amino acid should chromatograph on DEAE-cellulose similar to their Glu-containing counterparts. Thus, under identical reaction conditions, 2.7 μM [3H]MTX-r-FzGlu was converted quantitatively (Fig. 6A) to a product which eluted slightly later than the product obtained from [3H]MTX-r-Glu, but just prior to MTX-r-(Glu)2. In the absence of L-Glu, no radioactivity eluted in this position (Fig. 6B) and the unreacted [3H]MTX-y-FzGlu eluted just after the position of [3H]MTX-y-Glu. A 0.1 N HCl wash of the column following the normal elution procedure eluted <2% of the total radioactivity applied demonstrating that all major products were eluted previously. If the reaction mixtures (Fig. 6A) were admixed with [3H]MTX-y-FzGlu just prior to chromatography two radioactive peaks corresponding to those in Fig. 6, A and B, were resolved indicating that the appearance of the single product peak was not an artifact of the chromatography (data not shown).

Based on this evidence, it appeared that the FzGlu-containing compound was a better substrate for the addition of L-Glu as well as for FzGlu. The kinetics of addition of L-Glu to these substrates was examined under conditions (shorter time, lower enzyme level, and lower substrate concentration) where complete conversion to product of the FzGlu-containing substrate would not occur. The results (Fig. 7) clearly demonstrate that in the initial phase of the reaction, the FzGlu-containing substrate was at least 9-fold more active as a
polyglutamylation either as the amino acid to be ligated or as the acceptor species.

one and two additional y-linked glutamates. Elution slightly later than chemically synthesized MTX polyglutamates containing position as MTX--r-(4-fluoroglutamate), i.e. just after the a chain terminator (Figs. 2 and 3). In fact, ligation of either a faster rate when FgGlu was the previously incorporated amino acid than when L-Glu was present. FzGlu thus promoted slightly later (DEAE-cellulose) or slightly earlier (HPLC) largely masked, the two products from FzGlu eluted only after the F2Glu-containing substrate. No alteration in either substrate was detected at 5 h in the absence of L-Glu.

substrate for the addition of Glu than was the Glu-containing peptide.

DISCUSSION

The first fluorinated glutamate analog tested for its effects on polyglutamate synthetase, 4-fluoroglutamate, inhibited incorporation of [3H]Glu into polyglutamates because it acted as an alternate substrate for the enzyme; its incorporation, however, precluded further incorporation of either 4-fluoroglutamate or L-Glu, i.e. it was a chain terminator (4, 14). Based on the similarity of γ-COOH pKₐ values of FzGlu and 4-fluoroglutamate, it was anticipated that FzGlu would act in an analogous manner. FzGlu was readily ligated to a pteroyl substrate and appeared to be an even better substrate than L-Glu (Fig. 4, A and B). However, FzGlu did not act as a chain terminator (Figs. 2 and 3). In fact, ligation of either a second FzGlu (Fig. 4A) or a Glu (Figs. 6 and 7) occurred at a faster rate when FzGlu was the previously incorporated amino acid when than L-Glu was present. FzGlu thus promoted polyglutamylamidation either as the amino acid to be ligated or as the acceptor species.

Identification of the FzGlu reaction products was made by a combination of chromatographic and enzymatic techniques. On conventional DEAE-cellulose chromatography at pH 8.0 (Fig. 6) and reversed-phase HPLC at pH 5.5 (Fig. 3), where effects of the different pKₐ values of Glu and FzGlu would be largely masked, the two products from FzGlu eluted only slightly later (DEAE cellulose) or slightly earlier (HPLC) than chemically synthesized MTX polyglutamates containing one and two additional γ-linked glutamates. Elution slightly later than MTX-γ-Glu on DEAE-cellulose chromatography has been observed (14) with chemically synthesized MTX-γ-(4-fluoroglutamate); this γ-dipeptide co-elutes with MTX-γ-Glu on reversed-phase HPLC. On anion-exchange HPLC at pH 3.3 where the pKₐ differences are not suppressed, the least retained product synthesized from FzGlu eluted at the same position as MTX-γ-(4-fluoroglutamate), i.e. just after the MTX-γ-(Glu)₂ marker. The hog kidney γ-glutamyl hydrolase data suggest that both products synthesized from FzGlu contain residues in γ-linkage. Hydrolysis of the products by chicken pancreas γ-glutamyl hydrolase can be used to assign a MTX-γ-(amino acid) structure (Ref. 14). The fact that the FzGlu product which eluted first on anion-exchange HPLC was not hydrolyzed by this enzyme while the later eluting product was hydrolyzed to yield the first product (Table 1) is consistent with the first product being MTX-γ-FzGlu. From analyses performed before limit digestion was reached (Table 1), it appeared that FzGlu-containing peptides were less susceptible to enzymatic hydrolysis than Glu-containing peptides. Resistance of chemically synthesized 4-NH₂-10-Glu-pteroyl-(4-fluoroglutamyl)-γ-glutamate to hydrolysis by hog kidney γ-glutamyl hydrolase has been observed in related studies (9) suggesting low hydrolyzability may be a general feature of γ-peptide bonds in the vicinity of fluorine-containing residues. Detailed kinetic studies with FzGlu-containing structures will be undertaken to verify this point. The data on the kinetics of addition, chromatographic properties, and hydrolysis suggest that two products were formed by polyglutamate synthetase from MTX and FzGlu, viz. MTX-γ-FzGlu and MTX-γ-(FzGlu).

In all analytical systems employed, only two ligation products of MTX and FzGlu were detected. This is similar to our observation that purified rat liver folypolyglutamate synthetase only synthesizes MTX-γ-Glu and MTX-γ-(Glu)₂ (17), while it is capable of making pentaglutamate derivatives of the natural folate, tetrahydrofolate (16). The basis for this length specificity is not known, but the fact that no further ligation occurred even when MTX was quantitatively converted to MTX-γ-(FzGlu) (Fig. 6) argues that it is not simple substrate limitation as might be suggested for MTX (Fig. 5).

The exact mechanism by which FzGlu promotes synthesis of polyglutamates, either as the acceptor or incoming species, is unclear presently. Based on a kinetic analysis, FzGlu had a lower K⁺ and a higher Vmax than did Glu, as the incoming species. Even though these apparent K⁺ values were determined at 0.1 μM MTX (K⁺ for MTX = 74 μM), the value for Glu was close to that (0.65 ± 0.09 mM) previously determined (16) for the rat liver enzyme at saturating MTX. This suggests that the K⁺ and Vmax values determined in the present work may accurately reflect the true values for these two amino acids. As shown by Cichowicz and Shane (4), the polyglutamyl synthetase reaction rate increases with increasing pH, consistent with the hypothesis that the unprotonated amine form of Glu is the actual substrate for the polyglutamate synthetase reaction. Thus, more efficient ligation of FzGlu could be the result of the lower pKₐ of its α-amino group relative to that of Glu which would mean that a higher proportion of FzGlu would be unprotonated relative to Glu at the reaction pH. Initial experiments designed to determine the effect of pH on the synthetase reaction with either FzGlu or Glu as substrates were ambiguous, however, because of the limited stability of the enzyme at lower pH values.

The free amino acid, FzGlu, is unlikely to be a specific tool for increasing polyglutamate synthesis in cell culture or in vivo because Glu is utilized by many metabolic pathways most of which are more important quantitatively than polyglutamylation. As an illustration, 4-fluoroglutamate is toxic, but the mechanism is unrelated to polyglutamylation (6). The data presented here suggest, however, that new homologs of natural folates and classical antifolates containing FzGlu instead of Glu (e.g. 5-formyl-tetrahydropteroyl-(FzGlu) and 4-amino-10-methyl-pteroyl-(FzGlu)) might display properties useful in studies of folate/antifolate biochemistry and biochemical pharmacology, and in therapeutics. Specifically, there might be increased intracellular accumulation of the
homolog relative to the parent compound as a result of two effects. The first effect is the potentially increased activity of the homolog as a substrate for folylpolyglutamate synthetase (based on the data of Figs. 6 and 7). At least the first addition of Glu to the homolog may be more rapid than for the Glu-containing compound. Since addition of even a single γ-glutamyl residue dramatically decreases the ability of a compound to efflux from cells (1), greater folylpolyglutamate synthetase substrate activity should lead to greater retention. The second effect is the apparently decreased sensitivity to hydrolysis by γ-glutamyl hydrolase (see text; Table 1). In the case of MTX for example, this could be important because intracellular MTX polyglutamates can be hydrolyzed to MTX which may efflux from the cell before re-glutamylation; presumably, this hydrolysis is catalyzed by γ-glutamyl hydrolase. In contrast, based on the data of Table 1 and recent work on enzymatic hydrolysis of 4-amino-10-methylpteroyl(4-fluoroglutamate)-γ-glutamate (9), polyglutamates of an FzGlu-containing homolog might be hydrolyzed slowly and then only to the FzGlu-containing dipeptide structure which should efflux from cells much less readily than MTX (1) and thus would have both a continued toxic effect and a greater potential for re-conversion to longer polyglutamates. Increased accumulation of the FzGlu-containing homologs of natural folates and antifolates could thus be mediated through increased polyglutamate synthesis and/or decreased turnover.

If, as has been suggested in the cases of antitumor compounds such as MTX (10) and natural folates such as leucovorin (19), polyglutamate accumulation leads to increased therapeutic benefit in chemotherapy regimens, then the FzGlu-containing homologs potentially could be more effective agents in these regimens. The above conjectures implicitly assume that substitution of F2Glu would not cause a significant change in the transport properties of a homolog or in its interaction with its intracellular target(s). Based on our demonstration that an MTX homolog containing 4 fluoroglutamate instead of Glu (15) has transport properties and dihydrofolate reductase inhibitory potency similar to MTX, this assumption seems reasonable. Synthesis of FzGlu-containing homologs of natural folates and antifolates is currently in progress in order to determine their properties in vitro and in vivo.

Radiochemicals. 3′,5′,7′-3H-MTX (20 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Partial purity of 3′,5′,7′-3H-MTX (20 Ci/mmol) was determined by anion exchange as described previously (18, 19). [γ-3H]Glu- and [4-14C]Glu-containing MTX polyglutamates were prepared as described in Materials and Methods.

Enzymic assay. Folylpolyglutamate synthetase was assayed (16) using [γ-3H]Glu as a substrate and DEAE-cellulose mini-columns to separate free from bound radioactivity. Folylpolyglutamate synthetase activity was assayed in a 20-μl reaction mixture containing 10 μM [γ-3H]Glu, 10 mM MgCl₂, and 5 units of enzyme. The reaction was stopped by the addition of 20 μl of a 2×4 M Tris-HCl buffer (pH 7.5). The reaction mixture was treated as described in Materials and Methods. The enzyme activity was expressed as nanomoles of substrate incorporated into folylpolyglutamates per minute per milligram of protein. In some experiments, enzyme activity was determined in the presence of the dihydrofolate reductase inhibitor, methotrexate (MTX).


Materials and Methods

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Enzymas. Rat liver polyglutamate synthetase was prepared as previously described (17) and contained 89 units of enzymatic activity. Big kidney and chicken pancreas prolidase were purified by precipitation of homogenates with 34% (v/v) ethanol.

High pressure Liquid Chromatography. The products of reactions were analyzed by HPLC. Fractionated HPLC was employed to determine the positions at 280 mλ in 1.0 ml fractions were included in each sample to provide detection at 280 mλ. The samples were suspended in 5% (v/v) ethanol in 0.1 M Tris HCl buffer, pH 7.4, 0.1 M NaCl. An aliquot of each sample was injected into a HPLC system with a Hitachi L-6200 spectrophotometric detector equipped with a Hitachi L-6200 integrator and a Hitachi L-6200 recorder. The elution was performed on a C18 column (Shimadzu, 8 µmol as described by Sato and Gallivan 16). The elution was achieved with a gradient of 10% isocratic elution of 1 ml min⁻¹ at 5 min, 2 ml min⁻¹ at 5 min, 3 ml min⁻¹ at 5 min. The column was equilibrated with 10% isocratic elution of 1 ml min⁻¹ at 5 min, 2 ml min⁻¹ at 5 min, 3 ml min⁻¹ at 5 min. The column was equilibrated with 25% isocratic elution of 1 ml min⁻¹ at 5 min, 5 ml min⁻¹ at 5 min. The column was equilibrated with 50% isocratic elution of 1 ml min⁻¹ at 5 min, 10 ml min⁻¹ at 5 min. Radioactivity was then detected directly with a Finch-Beckman radiometric flow monitoring system (Beckman Instruments, Fullerton, Calif.).

Preparative magnetic separation and isolation of 3D products containing glutamate to 3-D-furfurylglutamate. Standard polyglutamate synthetase reaction mixture (1.0 ml) was mixed with 5% (v/v) ethanol in 0.1 M Tris HCl buffer, pH 7.4, 0.1 M NaCl. An aliquot of each sample was injected into a HPLC system with a Hitachi L-6200 spectrophotometric detector equipped with a Hitachi L-6200 integrator and a Hitachi L-6200 recorder. The elution was performed on a C18 column (Shimadzu, 8 µmol as described by Sato and Gallivan 16). The elution was achieved with a gradient of 10% isocratic elution of 1 ml min⁻¹ at 5 min, 2 ml min⁻¹ at 5 min, 3 ml min⁻¹ at 5 min. The column was equilibrated with 10% isocratic elution of 1 ml min⁻¹ at 5 min, 2 ml min⁻¹ at 5 min, 3 ml min⁻¹ at 5 min. The column was equilibrated with 25% isocratic elution of 1 ml min⁻¹ at 5 min, 5 ml min⁻¹ at 5 min. The column was equilibrated with 50% isocratic elution of 1 ml min⁻¹ at 5 min, 10 ml min⁻¹ at 5 min. Radioactivity was then detected directly with a Finch-Beckman radiometric flow monitoring system (Beckman Instruments, Fullerton, Calif.).

Chromatographic analysis of products enzymatically synthesized from L-Val-Glu and L-Glu. Analyses were performed on a reversed-phase HPLC, as described above, or on a DEAE-cellulose column. The samples were dissolved to a final concentration of 2.0 µM in 0.1 M Tris HCl buffer, pH 7.4, 0.1 M NaCl. The samples were then mixed with 5% (v/v) ethanol in 0.1 M Tris HCl buffer, pH 7.4, 0.1 M NaCl. An aliquot of each sample was injected into a HPLC system with a Hitachi L-6200 spectrophotometric detector equipped with a Hitachi L-6200 integrator and a Hitachi L-6200 recorder. The elution was performed on a C18 column (Shimadzu, 8 µmol as described by Sato and Gallivan 16). The elution was achieved with a gradient of 10% isocratic elution of 1 ml min⁻¹ at 5 min, 2 ml min⁻¹ at 5 min, 3 ml min⁻¹ at 5 min. The column was equilibrated with 10% isocratic elution of 1 ml min⁻¹ at 5 min, 2 ml min⁻¹ at 5 min, 3 ml min⁻¹ at 5 min. The column was equilibrated with 25% isocratic elution of 1 ml min⁻¹ at 5 min, 5 ml min⁻¹ at 5 min. The column was equilibrated with 50% isocratic elution of 1 ml min⁻¹ at 5 min, 10 ml min⁻¹ at 5 min. Radioactivity was then detected directly with a Finch-Beckman radiometric flow monitoring system (Beckman Instruments, Fullerton, Calif.).
3.3-Difluoroglutamate and Folyylpolyglutamate Synthetase

Table 1. Linuc digestion by y-glutamyl hydrolases of the reaction products synthesized by rat liver folylpolyglutamate synthetase from 14C-MTHF and glutamic acid or 3,3-difluoroglutamic acid. Reactions were performed and products were analyzed by NPLC as described in "Materials and Methods." The 14C-MTHF/(Glu)4 and putative 14C-MTHF/(Glu)5 substrates were biosynthetically prepared and purified as described in "Materials and Methods." Each hydrolase reaction contained substrate at 0.4 μg hog kidney y-glutamyl hydrolase or chicken pancreas y-glutamyl hydrolase in a 100 mM ammonium phosphate, pH 7.2, 30 μl reaction volume. The 14C-MTHF/(Glu)4 substrates were not sufficiently separated to rule out the possibility that the small percentage of radioactivity which co-eluted with MTHF/(Glu)4 did not result from "tailing" of the 14C peak rather than from complete hydrolysis of the products. In support of this rainbosity, using MTHF rather than MTHF/(Glu)4, it was observed that this also appeared in the F.1 substrate containing samples and that NPLC in an isocratic system (15 μM ammonium phosphate, pH 7.2) where the 2 species were well resolved showed that the Radioactivity was detected by 30% was the product MTHF/(Glu)4. Presented are the averages of duplicate, except that all values in the table are single determinations. Reactions using a 3 h incubation with hog kidney hydrolase and reactions using 7 μg of chicken pancreas hydrolase for 6 h were run in duplicate (data not shown; see text).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Activity</th>
<th>MTHF/(Glu)4</th>
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<tr>
<td>MTHF/(F)Glu</td>
<td>+</td>
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A. Hog Kidney y-Glutamyl Hydrolase (2.5 h incubation)

B. Chicken Pancreas y-Glutamyl Hydrolase (16 h incubation)

Figure 5. DEAE-cellulose chromatography analysis of the products of reaction of glutamate and MTHF/(Glu)4 and MTHF/(Glu)5 with rat liver folylpolyglutamate synthetase. Reaction conditions as in Figure 1, except 1.7 μM MTHF/(Glu)4 or (Glu)5 (11.12 μCi) were the starting material in the presence (Panel A) or absence (Panel B) of 5 μM L-Glu and 170 units of rat liver folylpolyglutamate synthetase during a 6.5 h incubation. Duplicate or triplicate reactions were analyzed by DEAE-cellulose chromatography (Materials and Methods) and a representative chromatograph of each condition is shown. Standards for each reaction were run and NPLC eluted (unlabeled) to 150 or 300 μM (Δ) nm. Radioactivity is indicated by solid circles (●).

Figure 6. DEAE-cellulose chromatography analysis of the products of reaction with glutamate and MTHF/(Glu)4 and MTHF/(Glu)5 with rat liver folylpolyglutamate synthetase. Reaction conditions as in Figure 1, except 1.7 μM MTHF/(Glu)4 or (Glu)5 (11.12 μCi) were the starting material in the presence (Panel A) or absence (Panel B) of 5 μM L-Glu and 170 units of rat liver folylpolyglutamate synthetase during a 6.5 h incubation. Duplicate or triplicate reactions were analyzed by DEAE-cellulose chromatography (Materials and Methods) and a representative chromatograph of each condition is shown. Standards for each reaction were run and NPLC eluted (unlabeled) to 150 or 300 μM (Δ) nm. Radioactivity is indicated by solid circles (●).