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Partial Purification and Properties of an Enzyme from *Escherichia coli* that Catalyzes the Conversion of Glutamic Acid and 10-Formyltetrahydropteroylglutamic Acid to 10-Formyltetrahydropteroyl-γ-glutamylglutamic Acid†

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ABSTRACT: An enzyme that catalyzes the conversion of L-glutamic acid and 10-formyl-H_4folic acid (also known as 10-formyl-H_4pteroylglutamic acid) to 10-formyl-H_4pteroyl-γ-glutamylglutamic acid has been purified by 74-fold from extracts of *Escherichia coli*. ATP, Mg^{2+}, and a monovalent cation (K^+ or NH_4, but not Na^+) are required for the enzyme to function. Radioactive and bioautographic analyses revealed the formation of a single product. This product was identified as 10-formyl-H_4pteroyl-γ-glutamylglutamic acid from its spectral characteristics, its ability to be used effectively as a growth factor for *Lactobacillus casei* 7469, and from radioactive analysis that indicated the incorporation into the product of 1 mol of glutamate/mol of 10-formyl-H_4pteroylglutamic acid utilized. The enzyme functions optimally at pH 9.0–9.8 and at 50°. Its molecular weight is estimated at 42,000–43,000. The K_m values are 180 μM for L-glutamic acid and less than 2 μM for (−)10-formyl-H_4pteroylglutamic acid. The only other naturally occurring folate compounds with significant activity as substrate are H_4pteroylglutamic acid and 5,10-methylene-H_4pteroylglutamic acid; however, these compounds are not used as effectively (K_m values are 10–12 μM) as 10-formyl-H_4pteroylglutamic acid.

Although it has been recognized for several years that nearly all of the naturally occurring forms of folate[1] are pteroylpolyglutamates (Pfiffner et al., 1946; Rabinowitz and Himes, 1960; Kozloff and Lute, 1965; Clandinin and Cossins, 1972; Houlihan and Scott, 1972), with the number of glutamate residues ranging from two (Usdin, 1959; Roos and Cossins, 1971; Shin et al., 1972) up to 12 (Kozloff and Lute, 1973), depending on the tissue analyzed, very little work has been done on the enzymology of the formation of these polyglutamate compounds. Several years ago, Griffin and Brown (1964) reported that H_4PteGlu could be converted in small quantities to the corresponding diglutamate in the presence of glutamic acid, ATP, and extracts of *Escherichia coli*. In the present paper, we report on our further investigations of this enzymatic process. Since the results to be presented indicate that the probable physiological substrate for the enzyme under investigation is 10-formyl-H_4PteGlu, we have here named the enzyme 10-formyltetrahydropteroyldiglutamic acid synthetase, or 10-formyl-H_4PteGlu$_2$ synthetase, for short.

Materials and Methods

Pteroylglutamic acid, *p*-aminobenzoic acid, dithiothreitol, all nonradioactive amino acids and nucleotides, DEAE-Sephadex A-50, Sephadex G-150, DNase I, RNase A, rabbit muscle aldolase, ovalbumin, and L-γ-glutamyl-L-glutamic acid were purchased from Sigma Chemical Co.;

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[1] In accord with the nomenclature rules recommended by the IUPAC-IUB Commission on Biochemical Nomenclature, the following terminology is used: "folate" as the general term for the family of compounds; PteGlu, pteroylglutamic acid (folic acid); H_2PteGlu and H_4PteGlu, 7,8-dihydropteroylglutamic acid and 5,6,7,8-tetrahydropteroylglutamic acid, respectively; PteGlu$_2$, pteroyl-γ-glutamylglutamic acid; 10-formyl-H_4PteGlu, 10-formyltetrahydropteroylglutamic acid; 10-formyl-H_4PteGlu$_2$, 10-formyltetrahydropteroyl-γ-glutamylglutamic acid; 5,10-methylene-H_4PteGlu, 5,10-methylenetetrahydropteroylglutamic acid; 5,10-methenyl-H_4PteGlu, 5,10-methylidynetetrahydropteroylglutamic acid.

DEAE-cellulose was from Gallard-Schlesinger Chemical Corp.; hydroxylapatite was from Bio-Rad Laboratories; 5-formyl-H_4PteGlu was from Lederle Laboratories; Tris and ammonium sulfate (both ultra pure) were from Schwarz/Mann; Darco G-60 activated charcoal from Atlas Powder Co.; L-[U-^{14}C]glutamic acid, [G-^{3}H]PteGlu, [2-^{14}C]PteGlu, and [8-^{14}C]ATP were from Amersham-Searle; L-[3-^{3}H]glutamic acid was from New England Nuclear; and folic acid assay medium was from Difco Laboratories. Aminopterin, 10-methyl-PteGlu, and pteroyl-α-glutamylglutamic acid were gifts from Lederle Laboratories, and Dr. Roy Kisliuk of Tufts University kindly supplied homofolic acid.

Folate Compounds. H_2PteGlu and H_2pteroic acid were prepared by reduction of folic acid and pteroic acid, respectively, as described by Futterman (1957). H_4PteGlu was synthesized by reduction of H_2PteGlu with sodium borohydride (Mathews and Huennekens, 1963). 10-Formyl-PteGlu and 10-formyl-H_2PteGlu were prepared by treatment of PteGlu and H_2PteGlu, respectively, with 98% formic acid (Silverman et al., 1961). 5,10-Methylene-H_4PteGlu was synthesized as described by Wiberg and Buchanan (1964), and 5-methyl-H_4PteGlu by the method of Sakami and Ukstins (1961).

5,10-Methenyl-H_4PteGlu was prepared by the action of 0.1 *N* HCl (containing 5 m*M* dithiothreitol) on 5-formyl-H_4PteGlu (May et al., 1951). The formation of 5,10-methenyl-H_4PteGlu can be assessed by measurement of its characteristic absorption maximum at 350–352 nm. The pH (9.6) at which enzymatic activity is measured in the present work is alkaline enough so that 5,10-methenyl-H_4PteGlu is converted quantitatively to 10-formyl-H_4PteGlu (May et al., 1951) (this compound has no absorption maximum at 352 nm). 10-Formyl-H_4aminopterin, 10-formyl-H_4pteroic acid, N^{11}-formyl-H_4homofolic acid, and 10-formyl-H_4pteroyl-α-glutamylglutamic acid were obtained by treatment of the corresponding deformylated tetrahydro compounds with formic acid (Rowe, 1971). The excess formic acid was removed by evaporation under vacuum and each residue was dissolved in 0.1 *N* HCl containing 10 m*M* 2-mercaptoethanol. The tetrahydro compounds used in making these formylated derivatives were made by reduction of the appropriate compounds as described above for the reduction of PteGlu to H_4PteGlu.

(−)5,10-Methenyl-H_4PteGlu was synthesized as follows. H_2PteGlu obtained by reduction of PteGlu (Futterman, 1957) was reduced enzymatically to (−)H_4PteGlu in the presence of an extract of *Streptococcus faecalis* (Zakrzewski and Sansone, 1971). This material was then evaporated to dryness and treated with formic acid (Rowe, 1971). The resulting (−)5,10-methenyl-H_4PteGlu was purified on a cellulose column (Rowe, 1971). This purified compound when added to reaction mixtures at pH 9.6 was converted to (−)10-formyl-H_4PteGlu.

Preparation of Radioactive 5,10-Methenyl-H_4PteGlu. Radioactive [^{14}C or ^{3}H]PteGlu (0.5 m*M*) was reduced to H_2PteGlu (Futterman, 1957), and this material was adsorbed to 20 mg of activated charcoal. After the charcoal was washed with 10 ml of 0.1% 2-mercaptoethanol, H_2PteGlu was eluted with 2 ml of a mixture containing ethanol, concentrated NH_4OH, and 10 m*M* 2-mercaptoethanol (3:1:2, v/v). The eluate was evaporated to near dryness and the residue was dissolved in 2 ml of 10 m*M* phosphate buffer (pH 6.8) containing 1% 2-mercaptoethanol. The H_2PteGlu was reduced to H_4PteGlu with sodium borohydride (Mathews and Huennekens, 1963) and the solution was evaporated to dryness. The residue was treated immediately with 98% formic acid (Rowe, 1971) and the resulting product was adsorbed to 20 mg of activated charcoal. After the charcoal had been washed with 10 ml of 10 m*M* 2-mercaptoethanol, the radioactive 5,10-methenyl-H_4PteGlu was eluted with 2 ml of the ethanol–NH_4OH–mercaptoethanol solution described above. The eluate was evaporated to dryness and the residue was dissolved in 0.1 ml of 0.1 *N* HCl containing 5 m*M* dithiothreitol. It was determined by electrophoresis at pH 3.5 (see a later section for the procedure) that each of these preparations was contaminated by approximately 8% with folic acid and also by a certain amount of radioactive material (presumably, degradation products) that remained at the origin during electrophoresis. However, the major product was 5,10-methenyl-H_4PteGlu, as shown by its migration characteristics during electrophoresis and its absorption spectrum which was identical with that reported previously for 5,10-methenyl-H_4PteGlu (Rabinowitz, 1960).

Enzyme Assay. Enzyme activity was assessed by measurement of the amount of radioactive glutamic acid converted (in the presence of the cosubstrate, 10-formyl-H_4PteGlu) to a form that adsorbs to charcoal. Reaction mixtures were prepared to contain in a total volume of 0.5 ml: 0.1 *M* glycine buffer (potassium salt, pH 9.6), 5 m*M* ATP, 5 m*M* $MgCl_2$, 0.2 *M* KCl, 10 m*M* dithiothreitol, 2 m*M* L-[U-^{14}C]glutamic acid (0.65 Ci/mol), 150 μ*M* (±)10-formyl-H_4PteGlu, 50 μg of bovine serum albumin (to help stabilize enzyme activity), and enzyme preparation. Incubation was at 37° for 90 min, after which 0.1 ml of 1 *M* NaH_2PO_4 (pH 4.25) was added to adjust the pH to 6.25. Activated charcoal, 20 mg, was added and the suspension was shaken briefly to adsorb the aromatic compounds. The suspension was filtered through a cotton plug in a Pasteur pipet and the charcoal was washed free of unreacted glutamic acid with 10 ml of a solution (pH 6.8) containing 10 m*M* glutamic acid and 10 m*M* 2-mercaptoethanol. The aromatic compounds were eluted from the charcoal with 1.5 ml of a solution containing ethanol, 30% aqueous ammonia, and 20 m*M* 2-mercaptoethanol (3:1:2, v/v). The eluate was recovered and analyzed for radioactivity. A unit of enzyme is defined as the amount that allows the conversion of 1 μmol/min of glutamic acid to a product that adsorbs to charcoal.

Some of the preparations of commercial radioactive glutamic acid were contaminated with significant quantities of ^{14}C material that adsorbs to charcoal and thus led to relatively high "blanks" (i.e., radioactivity adsorbed in the absence of the cosubstrate, 10-formyl-H_4PteGlu or in the absence of enzyme). These "blanks" could be reduced to a relatively low level by treatment of the radioactive glutamate with activated charcoal prior to using it to measure enzyme activity. In all of the data to be reported, radioactivity adsorbed to charcoal in the absence of enzyme has been subtracted from the values reported. In most cases this represented less than 10% of the total radioactivity measured.

Growth of E. coli. E. coli K_{12} AB 3292 (Huang and Pittard, 1967), a mutant that requires *p*-aminobenzoic acid, was used as a source of enzyme. This organism was used because the cells could be grown on limiting *p*-aminobenzoate to reduce the amount of endogenous folate substrate present in the crude extracts. The cells were grown overnight at 37° in 16-l. amounts in 20-l. carboys. The medium contained, per liter, the following: NH_4Cl, 1 g; $MgSO_4 \cdot 7H_2O$, 0.6 g; KH_2PO_4, 10.5 g; $Ca(NO_3)_2 \cdot 4H_2O$, 15 mg; $FeSO_4 \cdot 7H_2O$,

0.25 mg; thiamine, 2 mg; L-isoleucine, 150 mg; DL-valine, 300 mg; L-arginine, 150 mg; L-histidine, 50 mg; L-proline, 150 mg; *p*-aminobenzoic acid, 2.5 mg; and glucose, 20 g. This strain of *E. coli* requires all of the above amino acids and vitamins for growth. Each 16-l. portion of medium was inoculated with a 500-ml culture of the organism, grown in the same medium. The cells were harvested by centrifugation, and the moist cell cake was stored at −20° until used to prepare extracts.

Miscellaneous Procedures. Bioautography to locate the migration zones of folate compounds on paper chromatograms was conducted as described earlier (Usdin et al., 1954) with *Lactobacillus casei* 7469 as the test organism. Absorbance measurements were made with a Zeiss spectrophotometer (PMQ II). Ultraviolet spectra were recorded with a Perkin-Elmer 202 spectrophotometer. Radioactivity was measured with a Packard Model 3320 scintillation spectrometer. Protein was estimated by the method of Lowry et al. (1951) with bovine serum albumin as the standard.

Results

Purification of Enzyme. E. coli cells were disrupted in a Hughes press (Griffin and Brown, 1964). The broken cells were mixed with an equal volume of 50 m*M* Tris-HCl buffer (pH 8.3) containing 5 m*M* 2-mercaptoethanol and 5 m*M* EDTA (hereafter this solution will be referred to as "buffer"). DNase and RNase (1 mg of each per 40 g of cells) and $MgCl_2$ (final concentration of 10 m*M*) were added and the mixture was incubated at 37° for 30 min. Insoluble material was removed by centrifugation at 40,000*g* for 2 hr. The resulting supernatant solution will be referred to as the "crude extract". All the operations described below were carried out at 4°.

STEP 1. FRACTIONATION WITH AMMONIUM SULFATE. A saturated solution of ammonium sulfate was added slowly, with stirring, to give a 50% saturated solution. The resulting precipitate was recovered by centrifugation and dissolved in a minimum amount of buffer. This solution was dialyzed for 3 hr each against two changes of 100 volumes of buffer that also contained 0.1 *M* KCl.

STEP 2. CHROMATOGRAPHY ON DEAE-SEPHADEX A-50. The dialyzed 0–50% ammonium sulfate fraction (57 ml, 2.6 g of protein) was subjected to chromatography on a column (4.8 × 66.5 cm) of DEAE-Sephadex A-50 that had been equilibrated with buffer containing 0.1 *M* KCl. The column was developed with 1 l. of the same buffer, followed by a linear gradient (5 l.) of KCl (0.1–0.5 *M*) in buffer. Fractions of 12 ml each were collected at a rate of 108 ml/hr. A portion (0.1 ml) of each fraction was analyzed for enzymatic activity. The fractions (310–340) containing activity were combined and dialyzed for 4 hr against 6 l. of buffer. The protein was precipitated by adding solid ammonium sulfate to 70% saturation. The precipitate was recovered by centrifugation and dissolved in a minimal amount of buffer. This solution was dialyzed (6 hr) against 100 volumes of buffer and concentrated to approximately 7 ml by dialysis against 50% polyethylene glycol in buffer containing 10 m*M* EDTA. The concentrated solution was redialyzed (12 hr) against 1000 volumes of buffer containing 50 m*M* KCl.

STEP 3. FILTRATION ON SEPHADEX G-150. The dialyzed protein solution (7 ml, 285 mg of protein) from step 2 was subjected to filtration on a column (2.8 × 61 cm) of Sephadex G-150 that had been equilibrated with buffer containing 50 m*M* KCl. The column was developed with the same solution. Fractions (5 ml) were collected at a rate of 30 ml/hr and 0.05-ml portions were analyzed for enzyme activity. The fractions (48–56) that contained activity were combined and dialyzed (6 hr) against 50 volumes of 1 m*M* potassium phosphate buffer (pH 7.0). This dialysis was repeated twice with fresh buffer.

Table I: Summary of Purification of 10-Formyl-H_4PteGlu$_2$ Synthetase.

Enzyme Preparation	Specific Activity (munits/mg of Protein)	Overall Yield (%)
Crude extract	0.31	100
0–50% ammonium sulfate fraction	0.56	80
DEAE-Sephadex eluate	2.46	47
Eluate form Sephadex G-150	13.70	36
Fraction from hydroxylapatite column	23.10	30

STEP 4. CHROMATOGRAPHY ON HYDROXYLAPATITE. The preparation from step 3 (46 ml, 47 mg of protein) was applied to a column (1.6 × 15 cm) of hydroxylapatite that had been washed with 1 m*M* potassium phosphate buffer (pH 7.0). The column was developed by passing through, successively, 100 ml each of potassium phosphate buffer (pH 7.0) at the following concentrations (m*M*): 1, 10, 25, 40, 80, 100, 200, and 300. Most of the enzyme activity was eluted with 24 and 40 m*M* buffer and most of the protein was eluted with 80–300 m*M* buffer. The fractions containing enzyme activity were combined and dialyzed (12 hr) against 100 volumes of 0.1 *M* potassium glycinate buffer (pH 9.6) that also contained 5 m*M* 2-mercaptoethanol. The dialyzed material was stored in 0.5-ml amounts at −20°.

Table I contains a summary of the purification of the enzyme. The purification achieved was 74-fold with approximately 30% overall recovery of the enzyme. At this stage of purification the enzyme preparation was free from activities that result in the degradation of glutamate and 10-formyl-H_4PteGlu, but was contaminated somewhat with phosphatases.

Properties of Enzyme. Product formation is linear with incubation time up to 2 hr and linear with enzyme concentration at 90-min incubation periods. The activity of the enzyme is optimal at 50°. The enzyme functions maximally between pH 9.0 and 9.8, with half-maxima at pH 8.2 and pH 10.4. Little or no activity was evident at pH 7.0. The molecular weight of the enzyme was estimated at 42,000–43,000 by its behavior on a column of Sephadex G-200. Proteins of known molecular weight used as standards to calibrate the column were pancreatic ribonuclease A, chymotrypsinogen, ovalbumin, and rabbit muscle aldolase. The data were plotted and the molecular weight was estimated as described by Andrews (1964).

ATP, Mg^{2+} (supplied as $MgCl_2$), and K^+ all have been shown to be absolute requirements for product formation. Dithiothreitol is also needed for maximal activity of the enzyme, although it is not clear whether this compound is needed to maintain activity of the enzyme or to keep the substrate, 10-formyl-H_4PteGlu, in the reduced state (or perhaps both).

CATION REQUIREMENTS. Further investigations have shown that Mn^{2+} can partially replace the requirement for Mg^{2+}, but Ca^{2+} cannot. A concentration of 1 m*M* $MgCl_2$ is needed for maximal product formation (in the presence of

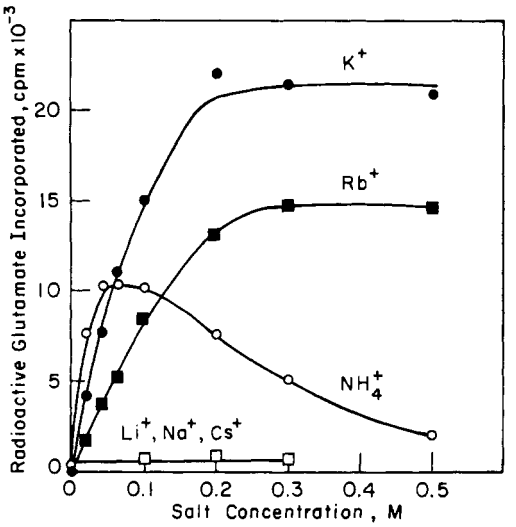

FIGURE 1: Effect of monovalent cations on enzyme activity. Reaction mixtures were prepared with 25 mM sodium glycinate buffer instead of 0.1 M potassium glycinate. Varying amounts of chloride salts were added as shown. Each reaction mixture contained 10.1 μg of purified enzyme protein. Incubation was for 60 min. Enzyme activity is presented as radioactive glutamate incorporated into the product.

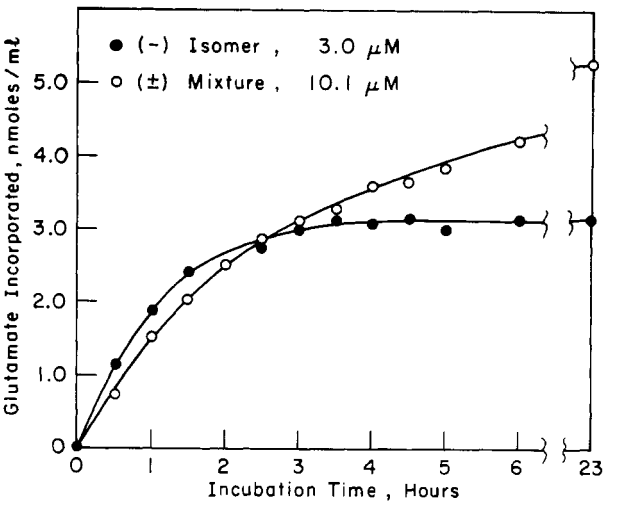

FIGURE 2: Comparison of (−)10-formyl-H_4PteGlu and (±)10-formyl-H_4PteGlu as substrate. Reaction mixtures of 10 ml each were prepared to contain 10-formyl-H_4PteGlu added as either the (−) isomer (3.0 μM) or the (±) mixture (10.1 μM). Purified enzyme was added as 4.26 μg of protein. All other components were added at the concentrations given in Materials and Methods for a typical reaction mixture. Portions (0.5 ml) were withdrawn at various times during incubation and analyzed for incorporation of glutamate.

Table II: Folate Compounds Used as Substrate.

Compound[a]	Glutamate Incorporated (nmoles)
(±)10-Formyl-H_4PteGlu	7.64
(±)H_4PteGlu	1.08
(±)5,10-Methylene-H_4PteGlu	1.75
PteGlu	0.17
(±)10-Formyl-H_4aminopterin	3.74
(±)11-Formyl-H_4homofolic acid	4.20

[a] The compounds were added at 150 μM to reaction mixtures, except for PteGlu which was added at 70 μM. Each reaction mixture contained 9.8 μg of purified enzyme protein.

Table III: Folate Compounds that Inhibit Enzymatic Incorporation of Glutamate.

Compound[a]	Inhibition (%)
H_2PteGlu	80.8
(±)10-Methyl-H_4PteGlu	85.2
(±)5-Methyl-H_4PteGlu	66.2
(±)10-Formyl-H_4pteroic acid	47.4
H_2pteroic acid	36.2

[a] The mixtures of (±) isomers were added at 150 μM; H_2PteGlu and H_2pteroic acid at 70 μM. Each reaction mixture also contained 150 μM (±)10-formyl-H_4PteGlu as substrate.

0.2 mM ATP). The data of Figure 1 show that the monovalent cation requirement can be satisfied by K^+, NH_4^+, or Rb^+, but Li^+, Na^+, and Cs^+ are ineffective. NH_4^+ is more effective than K^+ at low concentrations, but becomes inhibitory at higher concentrations, probably because of accompanying changes in pH at high concentrations of NH_4^+. Rb^+ is less effective than either K^+ or NH_4^+.

AMINO ACIDS AND NUCLEOTIDES USED AS SUBSTRATES. The K_m for glutamic acid was determined to be 0.18 mM from data plotted according to the Lineweaver-Burk method (Lineweaver and Burk, 1934). The following compounds were not used as substrate in place of glutamic acid: glutamine, aspartic acid, γ-L-glutamyl-L-glutamic acid, and L-α-aminoadipic acid. Since the addition of D-glutamic acid did not dilute out the incorporation into the product of radioactivity from ^{14}C-labeled L-glutamate, we conclude that the enzyme is specific for the L stereoisomer.

The following nucleotides could not replace the ATP requirement: TTP, CTP, UTP, XTP, ITP, and ADP. GTP and dATP were used, respectively, approximately 20 and 54% as well as ATP. With the purified enzyme, maximal synthesis of product was achieved in the presence of 0.2 mM ATP.

FOLATE COMPOUNDS USED AS SUBSTRATE. The folate compounds used as substrate are listed in Table II. The data show that 10-formyl-H_4PteGlu is by far the best substrate. The naturally occurring compounds H_4PteGlu and 5,10-methylene-H_4PteGlu can be used, but not as well as 10-formyl-H_4PteGlu. PteGlu has only slight activity as substrate. The analogs, 10-formyl-H_4aminopterin and 11-formyl-H_4homofolic acid, are active as substrates but these compounds do not occur naturally. Other folate compounds that we determined are not substrates are: H_2PteGlu, 5-methyl-H_4PteGlu, 5-formyl-H_4PteGlu, 10-formyl-H_4pteroic acid, H_2pteroic acid, p-aminobenzoic acid, N-formyl-p-aminobenzoic acid, 10-methyl-H_4PteGlu, 10-methyl-PteGlu, 10-formyl-H_2PteGlu, and aminopterin. All of the compounds listed above were also tested as inhibitors and those that did inhibit, along with the degree of inhibition, are listed in Table III.

K_m values for the folate compounds that are substrates were difficult to obtain with accuracy because of the limitations of the radioactive assay at low substrate concentrations owing to a reasonably high amount of radioactivity present in the blank (i.e., no folate substrate). However, rough estimations have indicated that the K_m for (−)10-formyl-H_4PteGlu is less than 2 μM and that for (−)H_4PteGlu or (−)5,10-methylene-H_4PteGlu is 10–12 μM.

The results presented in Figure 2 show that the (−) isomer of 10-formyl-H_4PteGlu is used as substrate and that in-

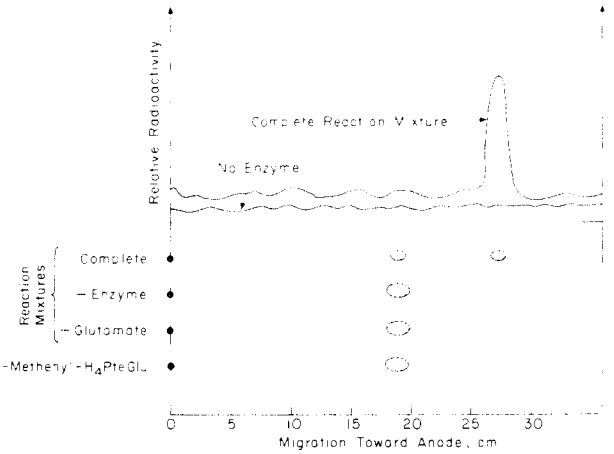

FIGURE 3: Bioautogram of an ionopherogram showing a single enzymatic product that is radioactive and is active as a growth factor for *Lactobacillus casei* 7469. Reaction mixtures were prepared to contain purified enzyme (9.8 μg) and either radioactive (^{14}C) or nonradioactive glutamic acid. The mixtures were processed in the routine manner and the resulting eluates from the charcoal were spotted, along with standard 5,10-methenyl-H_4PteGlu (at pH 3.5 10-formyl-H_4PteGlu exists as the methenyl compound), on Whatman No. 3 paper. These materials were subjected to electrophoresis at pH 3.5 (pyridine, 30 ml; glacial acetic acid, 300 ml; and water to make a total volume of 6 l.) in a Varsol-cooled Savant tank at 4 kV for 2.5 hr. The ^{14}C-labeled product was located by analyzing the paper strip with a Packard (Model 7200) strip scanner. That portion of the ionopherogram that contained nonradioactive product (i.e., produced in the reaction mixture to which nonradioactive glutamate was added) was subjected to bioautography with the use of *Lactobacillus casei* 7469 as the test organism. Shaded areas represent growth zones on the bioautogram. Although the results shown were obtained with the purified enzyme, we have repeated the experiment with the use of crude extract as a source of enzyme and the results were the same in that only a single radioactive product that was also active for *L. casei* was evident.

cubation for 3–4 hr results in the incorporation into the product of 1 mol of glutamate/mol of (−)10-formyl-H_4PteGlu added. Incubation for longer periods did not result in the further incorporation of glutamate, a fact that suggests that the (−)10-formyl-H_4PteGlu was used up after 3–4 hr and that the product is a diglutamate. When (±)10-formyl-H_4PteGlu was provided as substrate at approximately three times the concentration of the (−) (or natural) isomer, the rates of glutamate incorporation were approximately the same for the first 3 hr (Figure 2). However, further incubation of the mixture containing the (±) substrate resulted in increased incorporation of glutamate until finally, after a prolonged period (23 hr), the amount of glutamate incorporated equalled approximately half of the (±) mixture provided. This observation indicates that only the (−) isomer can be used as substrate, and, furthermore, that the (+) isomer inhibits somewhat the utilization of the (−) compound. The fact that after a long incubation period apparently all of the (−) isomer of the (±) mixture was used up suggests that the inhibition by the (+) isomer is competitive.

Products. NUMBER OF GLUTAMATE RESIDUES IN THE PRODUCT. The results presented in the preceding sections indicate that a folate product (or products) is formed that contains one or more radioactive glutamate residues. The experiment described in Figure 3 was devised to show how many folate products are formed. The results of the electrophoretic analysis shown in Figure 3 clearly show that only one radioactive product was formed and that it can be used as a growth factor by *Lactobacillus casei* 7469. The results also show that the product migrates much faster (27 cm) than standard 5,10-methenyl-H_4PteGlu (18 cm) during electrophoresis at pH 3.5.

Since 5,10-methenyl-H_4PteGlu$_2$ and the corresponding triglutamate compound were not available as standards, the electrophoretic results summarized in Figure 3 do not establish the number of glutamate residues in the product. To obtain information bearing on this point, two incubation mixtures were prepared, each containing 9.8 μg of purified enzyme, in which (a) (±)10-formyl-H_4[G-^{3}H]PteGlu (238 Ci/mol) and L-[U-^{14}C]glutamic acid (20.9 Ci/mol) were supplied as substrate, and (b) (±)10-formyl-H_4[2-^{3}H]PteGlu (21.4 Ci/mol) and L-[G-^{3}H]glutamic acid (378 Ci/mol) were added as substrates. All other components of the reaction mixtures were as described under Materials and Methods. The incubations were carried out and the mixtures were processed and subjected to electrophoresis as described in Figure 3. In each case the radioactive product was eluted from the paper with a solution containing 10 m*M* 2-mercaptoethanol and 0.1 *N* HCl. The ^{3}H and ^{14}C contents of the eluted materials were determined by analysis in a scintillation counter. From the radioactivity incorporated and from the specific radioactivity of each of the radioactive substrates it was possible to calculate the number of moles of glutamate incorporated into the product per mole of (−)10-formyl-H_4PteGlu utilized. The results showed that these ratios were close to one for each experiment (1.08 for the experiment in which [^{14}C]glutamate and 10-formyl-H_4[^{3}H]PteGlu were used and 0.99 when [^{3}H]glutamate and 10-formyl-H_4[^{14}C]PteGlu was used) and thus that the product must have been a diglutamate.

IDENTIFICATION OF THE PRODUCT AS 10-FORMYL-H_4PTEGLU$_2$. Since the substrate for the enzyme is 10-formyl-H_4PteGlu, the logical conclusion is that the product is 10-formyl-H_4PteGlu$_2$, although the results presented above provide no evidence that the formyl group is retained or that the oxidation state remains at the tetrahydro level. In order to obtain evidence bearing on these points, enough of the product had to be obtained to conduct spectrophotometric analyses. For this purpose, a 100-ml reaction mixture containing (±)10-formyl-H_4PteGlu, [U-^{14}C]glutamate (1 Ci/mol), and other components at the concentrations used for the routine assays (196 μg of enzyme) was prepared and the mixture was incubated for 10 hr at 37°. After incubation, 1 ml of 2-mercaptoethanol and 20 ml of 1 *M* phosphate buffer (pH 4.25) were added and the mixture was treated with charcoal to adsorb the aromatic compounds, and the materials were eluted from the charcoal by the procedures described under Materials and Methods. The eluate was evaporated to dryness in vacuo and the residue was dissolved in 5 ml of 5 m*M* phosphate buffer containing 2% 2-mercaptoethanol. This material was subjected to chromatography on DEAE-cellulose as described in Figure 4. Three peaks were evident from the resulting elution profile (Figure 4). Peak I contained material that, after acidification, exhibited an absorption band at 352 nm, but no radioactive material was evident. Peak II contained a substance (or substances) that was both radioactive and absorbed uv light at 352 nm. 10-Formyl-H_4PteGlu (and the corresponding polyglutamates) is known to be converted to 5,10-methenyl-H_4PteGlu at low pH values and this substance exhibits an absorption maximum at 352 nm in acid, but H_4PteGlu (and the corresponding polyglutamates) and dihydrofolate compounds do not possess this property. The absorption spectra (at pH 1.0) of materials from peak I and peak II were recorded and both were identical with stan-

dard 5,10-methenyl-H_4PteGlu (i.e., maximum at 352 nm). Fractions 80–90 from peak III were combined and concentrated, in vacuo, by sevenfold. The spectrum of the concentrated material at pH 1.0 exhibited a maximum at 325 nm, a property that is characteristic of 10-formyl-H_2PteGlu (Houlihan and Scott, 1972).

Electrophoretic analyses of the materials in the three peaks were conducted as described in Figure 3. The results showed that the migration characteristics of the material in peak I resembled the standard monoglutamate compound (Figure 3); the material in peak II (fractions 60–75) was a mixture consisting of compounds that migrated as the standard monoglutamate and as the diglutamate enzymatic product; and peak III material migrated as a diglutamate. From these observations and the spectra, we conclude that the radioactive material in peak III was 10-formyl-H_2PteGlu$_2$ formed by oxidation of the putative 10-formyl-H_4PteGlu$_2$, the enzymatic product. Since the material in peak I is not radioactive and exhibits spectral characteristics, after acidification, of 5,10-methenyl-H_4PteGlu, we conclude that before acidification this substance was the unreacted (+) isomer (plus possibly some of the unreacted (−) isomer) of the (±)10-formyl-H_4PteGlu added as substrate. When the fractions comprising peak II were analyzed separately, we found that fractions 60–68 contained two compounds active as a growth factor for *L. casei,* but only one, which migrated as a diglutamate, that was radioactive. The nonradioactive substance migrated as a monoglutamate and did not absorb uv light at 352 nm after acidification. Thus, this substance was probably 10-formyl-H_2PteGlu, an oxidation product of the substrate. Fractions 70–75 of peak II contained only one component that could be used as a growth factor by *L. casei* and since this substance was radioactive, migrated as a diglutamate, and absorbed at 352 nm after acidification, we conclude that it was 10-formyl-H_4PteGlu$_2$ and that this compound was the enzymatic product.

NATURE OF THE GLUTAMYLGLUTAMATE LINKAGE IN THE PRODUCT. The best way to decide whether the enzymatic product contains a γ-glutamylglutamate or an α-glutamylglutamate residue would be to compare the properties of the product with the standards, 10-formyltetrahydropteroyl-α-glutamylglutamate and 10-formyltetrahydropteroyl-γ-glutamylglutamate. However, since the latter compound was not available and the α-glutamylglutamate compound was, we designed an experiment to eliminate the possibility that the enzymatic product is an α-glutamylglutamate. For this purpose, the growth-promoting potency for *L. casei* of the enzymatic product was compared with that of standard (±)10-formyltetrahydropteroyl-α-glutamylglutamate. The latter compound (29 nmol) and an amount of an incubated reaction mixture equivalent to 1.5 nmol of the (±)10-formyl-H_4PteGlu added as substrate were subjected to electrophoresis and analyzed by bioautography with *L. casei* as described in Figure 3. Two growth spots of approximately equal size and intensity were evident from the reaction mixture (one was unreacted substrate and the other the product) and each of these was approximately equal in size to the spot obtained from the standard compound. The growth zone resulting from the enzymatic product was produced by a maximum of approximately 0.75 nmol (PteGlu and PteGlu$_2$ compounds are known to be used equally well). An amount of 30 nmol of the standard (10-formyl-H_4pteroyl-α-glutamylglutamate) was needed to elicit an equal response. These observations show that the growth-promoting potency of the product is several times greater than that of

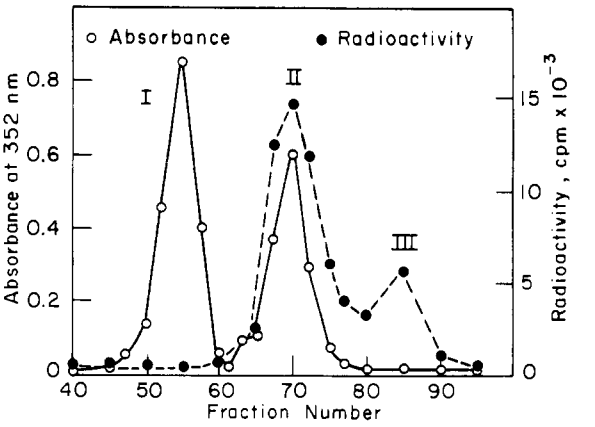

FIGURE 4: Purification of enzymatic product by chromatography on DEAE-cellulose. The material described in the text was applied to a column (1.6 × 7.5 cm) of DEAE-cellulose that had been equilibrated with 5 m*M* potassium phosphate buffer (pH 6.8). The column was developed first with 70 ml of the same buffer followed by a KCl gradient prepared by initially placing 100 ml of 5 m*M* of the phosphate buffer in the mixing chamber and 100 ml of 0.1 *M* phosphate buffer, that also contained 1 *M* KCl, in the reservoir. All of the buffers used for development also contained 2% 2-mercaptoethanol. Fractions of 2 ml each were collected at a rate of 20 ml/hr. Radioactivity was determined on 0.025-ml portions. Another 0.025-ml portion of each fraction was acidified with 0.5 ml of 0.12 *N* HCl (containing 10 m*M* 2-mercaptoethanol) and the absorbance at 352 nm was determined.

the standard α-glutamylglutamate compound and, therefore, indicate that the product is not an α-glutamylglutamate compound. The only reasonable alternative is that the product is 10-formyltetrahydropteroyl-γ-glutamylglutamic acid.

PRODUCTS OF ATP CLEAVAGE. In an effort to determine the products formed from ATP during the synthesis of 10-formyl-H_4PteGlu, [U-^{14}C]ATP was added to a reaction mixture and the products were analyzed as described in Table IV. The data presented in the table show that the enzyme preparation contained a significant amount of contaminating enzyme activity for the conversion of ATP to ADP in the absence of the other substrate, 10-formyl-H_4PteGlu. However, the difference between the amount of ADP produced in the presence of 10-formyl-H_4PteGlu and in its absence (8.68 − 7.30 = 1.38 nmol) was equal to the amount of glutamate incorporated into the product (1.37 nmol). In addition, the data of Table IV show that only an insignificant amount of AMP was produced. These observations provide evidence that the reaction involves the conversion of ATP to ADP and P_i and that these compounds and the other product, 10-formyl-H_4PteGlu$_2$, are formed in equimolar quantities.

Discussion

Since pure pteroyl-γ-glutamylglutamate (PteGlu$_2$) was not available to us in large enough quantities to synthesize 5,10-methenyl-H_4PteGlu$_2$ or 10-formyl-H_4PteGlu$_2$, we could not compare directly the properties of the enzymatic product and these synthetic compounds. However, the indirect evidence strongly suggests that the product is 10-formyl-H_4PteGlu$_2$. The spectral evidence that the product, upon acidification, contains an absorption band at 352 nm leaves little doubt about the presence of the formyl group in the product. The conclusion that the product contains two glutamate residues is based primarily on the experiments with radioactive substrates which indicate that 1 mol of glutamate is incorporated into the product per mol of 10-for-

Table IV: Production of ADP from ATP during Enzymatic Reaction.[a]

Reaction Mixture	Adenine Nucleotide Present after Incubation: ATP (nmoles)	ADP (nmoles)	AMP (nmoles)	Glutamate Incorporated (nmoles)
Complete	11.6	8.68	0.145	1.37
Omit 10-formyl-H_4PteGlu	12.9	7.30	0.125	0
Omit enzyme	20.9	0	0	0

[a] Reaction mixtures of 0.1 ml were prepared to contain 200 μM [U-^{14}C] ATP (2.2×10^4 cpm/nmol), 1.1 μg of purified enzyme protein, and other components at concentrations described under Materials and Methods for routine assays (with the use of nonradioactive glutamate). Incubation was at 37° for 1 hr after which the reaction was stopped by the addition of 0.01 ml of 0.5 *M* EDTA. A portion (0.02 ml) of each mixture (along with standards of ATP, ADP, and AMP) was spotted on Whatman No. 3 paper. The materials were subjected to chromatography (descending technique) with isobutyric acid, 30% aqueous ammonia, and water (66:1:33, v/v) as the developing solvent. The areas on the paper corresponding to the zones of migrations of ATP, ADP, and AMP were extracted with 0.5 ml of 10 m*M* sodium phosphate buffer (ph 7.0) and the radioactivity of each was determined. Parallel reaction mixtures were prepared, with [U-^{14}C] glutamate and nonradioactive ATP, to determine how much glutamate had been incorporated into the product. This experiment has been repeated three times with results virtually identical with those shown in the table. That is, no significant amount of AMP was evident and the amount of ADP formed in the presence of 10-formyl-H_4PteGlu over that formed in its absence in each determination equalled the amount of glutamate incorporated.

myl-H_4PteGlu utilized. Our evidence rules out the possibility of the product containing an α-glutamylglutamate group and thus strongly supports the alternative possibility; i.e., that the two glutamate residues are connected as γ-glutamylglutamate. This is not a surprising finding, since pteroyl-α-glutamylglutamate compounds have not been reported to occur naturally.

The finding that a single product is formed indicates that the enzyme is concerned only with the formation of a diglutamate compound. This observation suggests that another enzyme (or enzymes) is needed to add the other glutamate residue (or residues) to form pteroylpolyglutamates. The fact that only a diglutamate was formed from 10-formyl-H_4PteGlu in the presence of crude extracts is not evidence against the occurrence of such enzymes. It may be, for example, that the substrate for the addition of a third glutamate residue is different from the product of the addition of the second glutamate, or that the pH of the reaction mixture was not conducive for the addition of the third glutamate.

An important question that arises is whether 10-formyl-H_4PteGlu is the true physiological substrate for the addition of a second glutamate, since both H_4PteGlu and 5,10-methylene-H_4PteGlu are also used. Although accurate K_m values have not been determined, estimations suggest that the K_m for 10-formyl-H_4PteGlu is less than 2 μM, whereas the K_m for either H_4PteGlu or 5,10-methylene-H_4PteGlu is approximately 10 μM. This, along with the knowledge that 10-formyl-H_4PteGlu is a recognized metabolite, strongly indicates that this compound is the physiological substrate in *E. coli*.

Our results confirm previous work from this laboratory (Griffin and Brown, 1964) that H_4PteGlu can be used as substrate in *E. coli* for the formation of a diglutamate, although the yield of the diglutamate was very low in the previous work, probably because the wrong substrate was used (10-formyl-H_4PteGlu was not tested) and the pH (7.8) of the reaction mixture was not high enough.

Whether 10-formyl-H_4PteGlu is the physiological substrate for diglutamate formation in other microbial systems and in animal systems remains a subject for further investigation. Preliminary reports indicate that H_4PteGlu can be converted to the diglutamate in *Neurospora* (Sakami et al., 1973) and in rat liver (Spronk, 1973); 10-formyl-H_4PteGlu was not tested in the rat system, but it was reported not to be a substrate in *Neurospora*.

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