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STUDIES ON γ-GLUTAMYL CARBOXYPEPTIDASE

I. THE SOLID PHASE SYNTHESIS OF ANALOGS OF POLYGLUTAMATES OF FOLIC ACID AND THEIR EFFECTS ON HUMAN LIVER γ-GLUTAMYL CARBOXYPEPTIDASE

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SUMMARY

A number of analogs of pteroylglutamyl-γ-glutamyl-γ-glutamic acid in which the terminal glutamic acid residue has been replaced by other α-amino acids have been prepared and used to characterize the human liver γ-glutamyl carboxypeptidase. Details of modifications and improvements in the solid phase procedures for the synthesis of pteroylpolyglutamates are described. The compounds prepared are: pteroylglutamyl-γ-glutamyl-γ-serine; pteroylglutamyl-γ-glutamyl-γ-threonine; pteroylglutamyl-γ-glutamyl-γ-aspartic acid; pteroylglutamyl-γ-glutamyl-γ-glycine; pteroylglutamyl-γ-glutamyl-γ-proline; pteroylglutamyl-γ-glutamyl-γ-valine; pteroylglutamyl-γ-glutamyl.γ-luecine; pteroylglutamyl-γ-glutamyl-γ-phenylalanine; and pteroylglutamyl-γ-glutamyl-γ-leucylleucine. These analogs have been examined for their ability to support the folate requiring bacteria *Streptococcus faecalis* and *Lactobacillus casei*. Their effects on partially purified human liver γ-glutamyl carboxypeptidase have been studied. On the basis of these studies certain conclusions concerning the mode of action of γ-glutamyl carboxypeptidase have been made.

INTRODUCTION

In nature folic acid exists in a variety of poly-γ-glutamyl forms[1–7]. Evidence is accumulating to indicate that the number of γ-glutamyl residues may serve as a specific factor of recognition in the formation of certain coenzyme–apoenzyme complexes[8–10]. One such enzyme is the B_{12} independent homocysteine transmethylase[11,12]. In this system a requirement for 5-methyltetrahydropteroyltriglutamate has been demonstrated[12,13], whereas the corresponding monoglutamyl derivative is inhibitory. Recently a methionine-less mutant of *Neurospora crassa* has been discovered in which the defect was demonstrated to be in the biosynthesis of the poly-γ-glutamates of folic acid[14]. These findings suggest a physiological role for the biosynthetic enzymes which give rise to the pteroylpoly-γ-glutamates, and to the pteroylpoly-γ-glutamyl peptidases (γ-glutamylcarboxypeptidases, conjugases) which degrade these substrates.

This latter group of enzymes is very widely distributed in nature. Among the numerous biological forms in which this activity has been identified are: hog kidney[15], rat liver[16], chicken pancreas[17], bacteria[18,19], plants[20], pollen[21], and human tissues[22,23]. Even though the ubiquitous distribution of the γ-glutamyl carboxypeptidases has been established they have been poorly characterized due, in large part, to the lack of a suitable assay and the unavailability of chemically defined substrates. Both of these obstacles have been removed by the development, in this laboratory, of an unambiguous chemical synthesis of the poly-γ-glutamyl derivatives of folic acid[24]. These procedures have permitted the development of a rapid radioactive assay for γ-glutamyl carboxypeptidases[25].

In the course of studies on the human liver γ-glutamyl carboxypeptidase it became desirable to obtain analogs of polyglutamates in order to study the details of substrate requirements for this enzyme. Accordingly, a number of analogs of pteroylglutamyl-γ-glutamyl-γ-glutamic acid in which the carboxyl terminal glutamate has been replaced by other amino acids have been prepared. The study of the effects of these analogs and certain homopolyglutamyl peptides on human liver γ-glutamyl carboxypeptidase activity has provided information as to the mode of action of this enzyme.

HUTCHINGS *et al.*[26] and PLANTE *et al.*[27] have prepared a number of analogs of folic acid in which the glutamyl residue has been replaced by other amino acids. However, no analogs of the general class pteroylglutamyl-γ-glutamyl-γ-R, where R is an amino acid other than glutamic, have been reported. These compounds, aside from their value in the characterization of γ-glutamyl carboxypeptidases, could be of interest as analogs of the coenzyme functioning forms of poly-γ-glutamates of folic acid. For example, as pointed out above, the B_{12} independent pathway to methionine shows an absolute requirement for the triglutamate of folic acid, thus the possibility exists that an analog of the triglutamate could selectively inhibit this pathway.

During the preparation of these analogs, a number of modifications and improvements in the solid phase peptide synthesis of pteroylpoly-γ-glutamates have been made. These modifications, the use of the analogs in the study of human liver γ-glutamyl carboxypeptidase, and the evaluation of their ability to support the growth of the folic acid requiring bacteria *Streptococcus faecalis* and *Lactobacillus casei* are the subject of this report.

MATERIALS

Reagents were purchased from the following supply houses: poly-L-α-glutamic acid (molecular weight 5400) from Miles-Yeda, Ltd., Rehovoth, Israel; Boc*–L-amino acid resin esters from Mann Research Laboratories, Inc., of New York, quantitative data received from the supplier on the mmoles of Boc–L-amino acids esterified per g of resin were used for subsequent calculations; Boc–glutamate-α-benzyl ester from Cyclo Chemical Co., and Schwarz BioResearch, Inc.; isobutylchloroformate from K and K Laboratories, Inc.; *N*-methylmorpholine, trifluoroacetic acid, and trifluoroacetic anhydride from Eastman Chemical Products, Inc.; folate free bacterial media from Difco Laboratories. The *N*-methylmorpholine and isobutylchloroformate

* Boc–, *tert.*-butyloxycarbonyl.

were purified just prior to use by distillation. Care was exercised to ensure that all reagents and solvents were pure and free of water.

Radioactivity measurements were made with a Unilux (Nuclear Chicago) liquid scintillation counter using *p*-dioxane, naphthalene, 2,5-diphenyloxazole, 1,4-bis-[2-(5-phenyloxazolyl)]benzene counting fluid.

EXPERIMENTAL AND RESULTS

The various Boc–L-amino acid-resin esters were used as received from the supplier. Approx. 3.0 g of the resin-esters were weighed into a reaction vessel[28] attached to a mechanical mixer that rotated the flask 60 times per min. The resin was allowed to swell by washing it three times with 30-ml aliquots of methylene chloride with mixing periods of 3 min. The deprotection of the amino group was accomplished as described before, treating with 20% (v/v) trifluoroacetic acid in methylene chloride for 30 min at room temperature. The deprotection solvent was removed by suction filtration, and the resin washed three times with 30-ml portions of methylene chloride. Neutralization of the resin was accomplished by mixing it with 30 ml methylene chloride and 3 ml triethylamine for 10 min, followed by three additional methylene chloride washings as described above.

Peptide forming steps

A 2.2-fold molar excess (all calculations based on the available resin bound NH_2 groups) of dry Boc–glutamate-α-benzyl ester was weighed into a carefully dried, glass stoppered, 50-ml graduated cylinder. To this vessel a 2.5-fold excess of *N*-methylmorpholine in 30 ml methylene chloride was added and the solution placed in an ice bath for 15 min. The mixed anhydride of the free γ-carboxyl group of Boc–glutamate-α-benzyl ester and isobutylformate was formed by the addition of a 2.0-fold excess of freshly distilled isobutylchloroformate to the chilled solution above. After 15 min at 0° the anhydride was added to the deprotected resin and the vessel rotated for 2 h. At the end of this period the solvent was removed by filtration, and the resin washed 3 times with 30-ml aliquots of methylene chloride.

Acetylation

In order to improve the purity of the final product it was considered desirable to terminate the peptide formation of those residues which had failed to react with the entering mixed anhydride. To accomplish this end, acetylation was employed after each peptide forming step, as described by MERRIFIELD[29]. Following peptide formation and washing, 30 ml of methylene chloride and 5.0 ml triethylamine were added to the resin and the reaction vessel rotated. After 10 min, 3.0 ml of acetic anhydride was added and mixing continued for 2 h. The resin was then filtered and washed three times with methylene chloride.

The Boc–glutamyl-L-amino acid-resin ester was now ready for a second cycle of deprotection, peptide formation, and acetylation as described.

Termination: coupling of pteroic acid

The resin-bound, deprotected tripeptides were terminated by coupling the mixed anhydride of N^2,N^{10}-ditrifluoroacetylpteroic acid and isobutylformate as previously described. The reaction vessel, after final coupling of pteroic acid and

washing, was placed in a desiccator over P_2O_5 and dried in *vacuo* for at least 4 h prior to cleavage from the resin.

Yield of completed resin-bound products

The yields of the various completed resin-bound products were determined by the spectrophotometric quantitation of the uncoupled N^2,N^{10}-ditrifluoroacetylpteroic acid after converting it to pteroic acid. The yields of the various analogs were as follows: pteroylglutamyl-γ-glutamyl-γ-serine (57%); pteroylglutamyl-γ-glutamyl-γ-threonine (62%); pteroylglutamyl-γ-glutamyl-γ-aspartic acid (71%); pteroylglutamyl-γ-glutamyl-γ-glycine (61%); pteroylglutamyl-γ-glutamyl-γ-proline (19%); pteroylglutamyl-γ-glutamyl-γ-valine (24%); pteroylglutamyl-γ-glutamyl-γ-leucine (53%); pteroylglutamyl-γ-glutamyl-γ-phenylalanine (57%); and pteroylglutamyl-γ-glutamyl-γ-leucylleucine (43%). The yields were determined at this stage for convenience, losses encountered in cleavage and final deprotection are not great.

Cleavage and deprotection of product

Cleavage of the product from the resin was conducted as previously described using trifluoroacetic acid and gaseous HBr. Protecting groups were removed by dissolving the washed product in about 50 ml of N_2 saturated 0.1 M NaOH under a constant stream of N_2. The 0.1 M NaOH suspension was placed in a 50° water bath. The end point for deprotection was complete solution. The final product was obtained as a solid by adjusting the golden yellow alkaline solution to pH 2 with HCl, followed by chilling and centrifugation. The instability of the product in 0.1 M NaOH in the presence of O_2 should be noted; accordingly, those steps involving base treatment were carried out as rapidly as possible.

Purification of products

The precipitated products were suspended in a small volume of water (20–40 ml) and the pH brought to 7.0 with 0.1 M NaOH. Up to 600 μmoles of the compounds were applied to a 2 cm × 27 cm DEAE cellulose column containing 10 g dry weight DEAE cellulose chloride. The columns were eluted with a linear gradient consisting of 1 l of 0.005 M phosphate buffer (pH 7.0) in the mixing chamber and 1 l of 0.5 M NaCl in 0.005 M phosphate buffer (pH 7.0) in the second vessel. The range of molarities of NaCl required for elution of the analogs reported here varied from 0.32 M for the serine analog to 0.34 M for the aspartic acid analog, pteroic acid is eluted at 0.23 M.

Ultraviolet spectra of purified analogs

An aliquot from the peak tube of the DEAE cellulose column eluate was diluted into 0.1 M NaOH and the ultraviolet spectrum taken. The analogs reported here have the identical spectral properties of teropterin (pteroyl-γ-glutamyl-γ-glutamyl-glutamic acid). In 0.1 M NaOH: $\lambda_{max_1} = 256$ nm; $\lambda_{max_2} = 285$ nm; $\lambda_{max_3} = 365$ nm; $\lambda_{min_1} = 235$ nm; $\lambda_{min_2} = 269$ nm; $\lambda_{min_3} = 333$ nm. It should be noted that the spectral contribution of phenylalanine is negligible over these wavelengths[30]. Although the molar extinction coefficients were not determined, this laboratory had earlier demonstrated the molar absorbance of the triglutamate and the heptaglutamate derivatives of pteroic acid to be identical. Calculations of concentrations have been made under the assumption that these analogs have a molar extinction coefficient equal to that of teropterin in 0.1 M NaOH at 365 nm.

Partial purification of γ-glutamyl carboxypeptidase from human liver

200 g of fresh human liver obtained at autopsy was homogenized in 600 ml of N_2 (the enzyme is unstable in the presence of O_2 at this stage) saturated water for 4 min at 0°. The homogenate was centrifuged at 13 200 × *g* for 30 min. A 55–90% saturated $(NH_4)_2SO_4$ fraction was obtained from the supernatant. The $(NH_4)_2SO_4$ precipitate was redissolved in about 100 ml water and 5% protamine sulfate was added to a final concentration of 0.5% (v/v). After centrifugation at 13 200 × *g* for 30 min the solution was dialyzed overnight against 25 l of water in the cold. The dialyzed solution was centrifuged at 27 000 × *g* for 30 min, the precipitate was discarded, and the supernatant was fractionated with absolute ethanol at −8 to −10°. The γ-glutamyl carboxypeptidase activity was obtained in the ethanol precipitate (0.15:0.60, v/v). This precipitate was applied at pH 7.5 to a 2 cm × 15 cm column of DEAE cellulose in the chloride form. The γ-glutamyl carboxypeptidase did not bind and was recovered in the column effluent and water wash. The enzyme was concentrated, by dialyzing against solid carbowax (polyethylene glycol), to about 10 ml. The concentrated enzyme was reprecipitated by ethanol as before and the precipitate redissolved in about 10 ml of water. The 24-fold purified γ-glutamyl carboxypeptidase so obtained was stable in the refrigerator for up to three weeks.

Enzyme assay

γ-Glutamyl carboxypeptidase was assayed by a non-microbiological procedure developed in this laboratory[25], based on the quantitation of enzymatically liberated uniformly ^{14}C-labeled glutamate from synthetic pteroylglutamyl-γ-glutamyl-γ-[$^{14}C_5$]-glutamic acid used as substrate. The standard assay used throughout this report is described below: 0.5 ml 0.1 M acetate buffer (pH 4.5); 0.25 ml of 0.1 mM pteroylglutamyl-γ-glutamyl-γ-[$^{14}C_5$]glutamic acid (specific radioactivity 662 800 disint./min per μmole); and 0.65 ml water were added to a 13 mm × 100 mm test tube. The tube was brought to thermal equilibrium by placing it in a 37° water bath for 3 min. The reaction was initiated by the addition of 0.1 ml of enzyme and mixing. After 10 min incubation in a 37° water bath, the reaction was terminated by the addition of 0.1 ml of 50% (w/v) trichloroacetic acid. To the tube was then added 0.1 ml of a 5% activated charcoal (Merck) suspension in 0.1 M acetate buffer (pH 3.0). The reaction mixture was then filtered through a disposable plastic syringe fitted to a disposable Millipore filter. An aliquot of the filtrate containing the liberated [^{14}C]glutamate was measured into a counting vial followed by 10 ml of scintillation fluid. Efficiency of radioactive measurements were determined by the channel ratio method. Radioactivity is reported after extrapolation to 100% efficiency, as disint./min. Reagent blanks obtained by the addition of trichloroacetic acid prior to addition of enzyme were invariably zero.

Microbiological assays

Microbiological assays were carried out with *S. faecalis* ATCC No. 8043, and *L. casei* ATCC No. 7469, by previously described procedures employed in this laboratory for a number of years. The compounds were assayed in triplicate at a concentration (determined spectrophotometrically) midpoint on the standard curve ($5 \cdot 10^{-10}$ g/assay tube for *L. casei* and $3 \cdot 10^{-9}$ g/assay tube for *S. faecalis*). The standard curve was established with DEAE-cellulose column purified folic acid. Growth was monitored at 650 nm after 18 h incubation at 37°.

TABLE I

THE MICROBIOLOGICAL ACTIVITY OF ANALOGS FOR *S. faecalis* AND *L. casei* COMPARED TO FOLIC ACID

*Compound assayed**	*Relative microbiological activity (%)*	
	S. faecalis	*L. casei*
R–γ-L(+)-serine	0	80
R–γ-L(+)-threonine	0	67
R–γ-L(+)-aspartic acid	0	29
R–γ-glycine	0	25
R–γ-L(+)-proline	0	18
R–γ-L(+)-valine	0	18
R–γ-L(+)-leucine	0	12
R–γ-L(+)-phenylalanine	0	5
R–γ-L(+)-leucylleucine	0	0
R–γ-OH	100	100
R–γ-L(+)-glutamic acid	0	100
R–γ-L(+)-glutamyl-γ-glutamic acid	0	0
Folic acid	100	100

* R = pteroylglutamyl-γ-glutamyl.

The microbiological activities for the analogs and the mono-, di-, tri-, and tetra-γ-glutamyl derivatives of pteroic acid are presented in Table I. These data indicate no response by *S. faecalis* but a variable response by *L. casei* to these analogs.

Effects of analogs on γ-glutamyl carboxypeptidase activity

The analogs were tested for their ability to inhibit the action of γ-glutamyl carboxypeptidase on pteroylglutamyl-γ-glutamyl-γ-[$^{14}C_5$]glutamic acid. All of the

TABLE II

THE INHIBITION OF HUMAN LIVER γ-GLUTAMYL CARBOXYPEPTIDASE BY ANALOGS OF POLYGLUTAMATES OF FOLIC ACID

The various analogs were added to the standard assay mixture in place of water to give a final level equal to the substrate concentration. $[A]/[S] = 1$. Approx. 10% of the substrate present was hydrolyzed during the 10-min incubation period in the control reaction; the reaction is first order over this interval.

*Compound added**	*Disint./min liberated*	*Inhibition (%)*
No additions	1404	—
R–γ-L(+)-serine	777	45
R–γ-L(+)-threonine	744	47
R–γ-L(+)-aspartic acid	436	69
R–γ-glycine	944	33
R–γ-L(+)-proline	1213	14
R–γ-L(+)-valine	728	48
R–γ-L(+)-leucine	732	48
R–γ-L(+)-phenylalanine	551	61
R–γ-L(+)-leucylleucine	574	59

* R = pteroylglutamyl-γ-glutamyl.

analogs were tested at an $[Ao]/[S]$ ratio of 1, ($[A]$ = molar concn. of analog, $[S]$ = molar concn. of substrate), and all proved to inhibit the cleavage of radioactive glutamate from teropterin (Table II). These data do not distinguish between the possibility that the analogs were substrates, and therefore were competitive inhibitors or whether they inhibited in some other manner. Therefore, the analogs were tested as substrates. 0.1 ml of a 1 mmolar solution of the analog was added to 0.5 ml of 0.1 M acetate buffer (pH 4.5), 0.25 ml of γ-glutamyl carboxypeptidase was added, and the tube incubated for 30 min. The reaction was terminated by the addition of 0.5 ml 5% trichloroacetic acid. Dilutions were made for *S. faecalis* assay as described in the legend to Table III.

The effect of pteroylglutamyl-γ-glutamyl-γ-leucine on γ-glutamyl carboxypeptidase (Table II) and the fact that this analog proved to be a poor γ-glutamyl carboxypeptidase substrate led to the preparation of pteroylglutamyl-γ-glutamyl-γ-leucylleucine. This analog proved to be incapable of supporting the growth of *S. faecalis* or *L. casei* before or after γ-glutamyl carboxypeptidase treatment, and was inhibitory to γ-glutamyl carboxypeptidase. The fact that pteroylglutamyl-γ-glutamyl-

TABLE III

THE MICROBIOLOGICAL ASSAY OF ANALOGS WITH *S. faecalis* AFTER PRETREATMENT WITH HUMAN LIVER γ-GLUTAMYL CARBOXYPEPTIDASE

Compounds to be assayed were added (0.1 μmole) to 0.5 ml of 0.1 M acetate buffer (pH 4.5). The reactions were initiated by the addition of 0.25 ml of partially purified γ-glutamyl carboxypeptidase and stopped by the addition of 0.5 ml of 5% trichloroacetic acid. The reactions were diluted and bioassayed with *S. faecalis* at three levels, in triplicate. The values reported are averages of all three levels. Controls were established by the addition of water in place of enzyme. The controls gave no growth of *S. faecalis* at any level tested.

Compound assayed	*% of control*
R–γ-L(+)-glutamic acid	100
R–γ-L(+)-serine	74
R–γ-L(+)-threonine	47
R–γ-L(+)-aspartic acid	19
R–γ-glycine	91
R–γ-L(+)-proline	46
R–γ-L(+)-valine	60
R–γ-L(+)-leucine	42
R–γ-L(+)-phenylalanine	58
R–γ-L(+)-leucylleucine	0

* R = pteroylglutamyl-γ-glutamyl.

γ-leucylleucine is not a γ-glutamyl carboxypeptidase substrate was confirmed by quantitative ninhydrin determinations on the charcoal filtrates. No ninhydrin positive material was liberated by γ-glutamyl carboxypeptidase action on this analog, whereas 0.59 μmole of ninhydrin active material was liberated in a simultaneous assay using an equivalent amount of pteroylglutamyl-γ-glutamyl-γ-glutamyl-γ-glutamic acid as substrate.

These findings suggest that in order to be acted upon by γ-glutamyl carboxypeptidase, substrates must have a terminal γ-peptide bond. To further examine the bond requirements L-glutamyl-(α-glutamyl)$_{\simeq 40}$-α-glutamic acid and L-glutamyl-(γ-

glutamyl)$_2$-γ-glutamic acid were tested as substrates. Incubations were carried out as follows: 0.5 ml of 0.1 M acetate buffer (pH 4.5) and 1.0 ml of substrate at a concentration of 0.1 μmole/ml were added to 13 mm $\times$ 100 mm test tubes. The tubes were brought to thermal equilibrium and the reactions initiated by the addition of 0.3 ml of partially purified γ-glutamyl carboxypeptidase. The tubes were incubated for 30 min, then terminated by the addition of 0.1 ml 50% (w/v) trichloroacetic acid. Duplicate control assays were run simultaneously in which the trichloroacetic acid was added prior to enzyme. Quantitative ninhydrin determinations were carried out on all tubes. The amount of glutamic acid liberated was determined by difference. In the case of L-poly-α-glutamic acid no glutamic acid was liberated, whereas glutamyl-(γ-glutamyl)$_2$-γ-glutamic acid was cleaved to the extent of 93% (0.28 μmole of glutamate liberated).

DISCUSSION

The present report is a continuation of earlier studies in which poly-γ-glutamyl derivatives of pteroic acid were prepared. A significant simplification is introduced here with the utilization of a single solvent system, methylene chloride, for all of the steps in the formation of the peptide chain. Methylene chloride is particularly well suited for solid phase peptide chemistry in so far as the solvent is stable and easily dried with $CaCl_2$. Reagent grade methylene chloride, in contrast to dimethylformamide, required no prior purification.

Glutamic acid was successfully coupled in γ peptide linkage to eight different resin-bound α-amino acids. It would appear, therefore, that the procedures described are readily applicable to solid phase peptide synthesis in general, whenever a γ glutamyl peptide linkage is desired.

In spite of the high yields in the peptide forming steps, the difficulty of separating small amounts of closely related contaminating products makes intermediate acetylation steps attractive and convenient. Acetylation following each peptide bond-forming step leads to the formation of small quantities of *N*-acetyl terminated peptides that are readily separated by ion-exchange chromatography from the desired products.

Teropterin has four free carboxyl groups and has full microbiological activity for *L. casei* but no activity for *S. faecalis*. The diglutamate of pteroic acid (pteroyl-glutamyl-γ-glutamate) has three free carboxyl groups and is equally active for both organisms. The analogs reported here all possess three free carboxyl groups (equivalent to the di-) except the aspartate compound which has four (equivalent to the tri-). The fact that none of the analogs showed folate activity for *S. faecalis* suggests that parameters other than the number of carboxyl groups or charge must be considered for penetration. Discrimination could conceivably be on the basis of size, analogous to the *Escherichia coli* system studied by PAYNE AND GILVARG[31].

The variable ability of the analogs tested to support the growth of *L. casei* cannot be interpreted with the data on hand. There is no parallelism between their growth supporting ability for *L. casei* (Table I) and their ability to serve as γ-glutamyl carboxypeptidase substrates (Table II). It remains to be established whether or not the analogs are functioning as coenzymes without prior cleavage of the carboxy terminal acids. It is interesting to point out in this regard that WRIGHT[8] has reported the existence of amino acids other than glutamic acid in natural folate compounds.

The study of the various analogs for their ability to serve as substrate for human liver γ-glutamyl carboxypeptidase allows certain conclusions to be drawn relative to enzyme specificity and mode of action. The fact that all of the analogs of the general structure pteroylglutamyl-γ-glutamyl-γ-R could serve as substrate indicates a low degree of specificity with regards to the nature of the –R group. Pteroylglutamyl-γ-glutamyl-γ-leucine was about 40% as effective a substrate as the triglutamate under the experimental conditions used. However, when the carboxy terminal amino acid was linked through an α peptide bond, as in the case of pteroylglutamyl-γ-glutamyl-γ-leucylleucine, the ability to serve as substrate was lost. These findings indicated that the enzyme functions as a carboxypeptidase, and suggests a strict requirement for the γ peptide bond. To further examine this latter point, the ability of α and γ linked L-polyglutamic acids to serve as substrate was studied. The ability of the γ polyglutamate and the inability of the α polyglutamate to serve as substrate confirm the requirement for a terminal γ peptide bond.

In summary, several new analogs of polyglutamates of pteroic acid have been prepared by a modified solid phase procedure. These analogs have been assessed for their ability to support the folic acid requiring bacteria *S. faecalis* and *L. casei*, and used to study the structural requirements of substrates for human liver γ-glutamyl carboxypeptidase.

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REFERENCES

1 J. J. PFIFFNER, S. B. BINKLEY, E. S. BLOOM AND B. L. O'DELL, *J. Am. Chem. Soc.*, 69 (1947) 1466.
2 B. L. HUTCHINGS, E. L. R. STOKSTAD, N. BOHONOS, N. H. SLOANE AND Y. SUBBAROW, *J. Am. Chem. Soc.*, 70 (1948) 1.
3 L. E. ERICSON, E. WIDOFF AND Z. G. BANHIDI, *Acta Chem. Scand.*, 7 (1953) 974.
4 J. M. NORONHA AND M. SILVERMAN, *J. Biol. Chem.*, 237 (1962) 3299.
5 J. M. NORONHA AND V. S. ABOOBAKER, *Arch. Biochem. Biophys.*, 101 (1963) 445.
6 F. M. SIROTNAK, G. J. DONATI AND D. J. HUTCHINSON, *J. Bacteriol.*, 85 (1963) 658.
7 R. C. WOOD AND M. F. WISE, *Texas Rept. Biol. Med.*, 23 (1955) 512.
8 B. E. WRIGHT, *J. Am. Chem. Soc.*, 77 (1955) 3930.
9 B. E. WRIGHT, *J. Biol. Chem.*, 219 (1956) 873.
10 P. J. LARGE AND J. R. QUAYLE, *Biochem. J.*, 87 (1963) 386.
11 J. R. GUEST AND K. M. JONES, *Biochem. J.*, 75 (1960) 120.
12 C. D. WHITFIELD AND H. WEISSBACH, *Biochem. Biophys. Res. Commun.*, 33 (1968) 996.
13 J. R. GUEST, S. FRIEDMAN, M. A. FOSTER, G. TEJERINA AND D. D. WOODS, *Biochem. J.*, 92 (1964) 497.
14 J. SELHUB, E. BURTON AND W. SAKAMI, *Federation Proc.*, 28 (1969) 533.
15 O. D. BIRD, M. ROBBINS, J. M. VANDERBELT AND J. J. PFIFFERN, *J. Biol. Chem.*, 163 (1946) 649.
16 V. MIMS, J. R. TOTTER AND P. L. DAY, *J. Biol. Chem.*, 155 (1944) 401.
17 V. MIMS AND M. LASKOWSKI, *J. Biol. Chem.*, 160 (1945) 493.
18 B. E. VOLCANI AND P. MARGALITH, *J. Bacteriol.*, 74 (1957) 646.
19 A. G. PRATT, E. J. CRAWFORD AND M. FRIEDKIN, *J. Biol. Chem.*, 243 (1968) 6367.
20 K. IWAI, *Memoirs Res. Inst. Food Sci. Kyoto Univ.*, 13 (1957) 1.
21 N. NIELSEN AND B. HOMSTROM, *Acta Chem. Scand.*, 11 (1957) 101.
22 R. WOLFF, P. L. DROUET AND R. KARLIN, *Bull. Soc. Chim. Biol.*, 31 (1949) 1439.

23 C. M. BAUGH AND C. L. KRUMDIECK, *Lancet*, ii (1969) 519.
24 C. L. KRUMDIECK AND C. M. BAUGH, *Biochem.*, 8 (1969) 1568.
25 C. L. KRUMDIECK AND C. M. BAUGH, *Anal. Biochem.*, in the press.
26 B. L. HUTCHINGS, J. H. MOWAT, J. J. OLESON, E. L. R. STOKSTAD, J. H. BOOTHE, C. W. WALLER, R. B. ANGIER, J. SEMB AND Y. SUBBAROW, *J. Biol. Chem.*, 170 (1947) 323.
27 L. T. PLANTE, E. J. CRAWFORD AND M. FRIEDKIN, *J. Biol. Chem.*, 242 (1967) 1466.
28 R. B. MERRIFIELD, J. M. STEWART AND N. JERNBERG, *Anal. Chem.*, 38 (1966) 1905.
29 R. B. MERRIFIELD, *J. Am. Chem. Soc.*, 85 (1963) 2149.
30 D. B. WETLAUFER, *Advan. Prot. Chem.*, 17 (1962) 310.
31 J. W. PAYNE AND C. GILVARG, *J. Biol. Chem.*, 243 (1968) 6291.