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The Solid-Phase Synthesis of Polyglutamates of Folic Acid*

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ABSTRACT: Details of an unambiguous chemical synthesis by which pteroyl-(γ-L-glutamyl)$_n$-L-glutamic acid of any desired chain length (up to $n = 6$) may be prepared are reported. The procedures employed are modifications of the Merrifield solid-phase peptide synthetic system. Glutamic acid was attached to the resin by esterification of *t*-butyloxycarbonyl-L-glutamic acid α-benzyl ester (I) to the benzyl chloride groups on the resin. Deprotection was carried out by treatment for 30 min with 20% v/v trifluoroacetic acid in methylene chloride at room temperature. Peptide formation was accomplished by reaction of the mixed anhydride of I and isobutyl formate with the amino-deprotected aminoacyl-resin. After cycles of deprotection and coupling to the desired chain length, the peptide was terminated by coupling *N*-2,*N*-10-bis(trifluoroacetyl)-pteroic acid (II). The mixed anhydride of II and isobutyl formate was used for the peptide formation. The synthesis of II is described. Cleavage of the product from the resin was carried out by gassing the resin-bound product suspended in trifluoroacetic acid with HBr. Deprotection of the cleaved product was carried out in 0.5 N NaOH under N_2, after removal of the trifluoroacetic acid. Details of the chromatographic behavior and biological activity of the products are reported.

Since the discovery and characterization of folic acid (Snell and Peterson, 1940; Mitchell *et al.*, 1941; Hutchings *et al.*, 1948; Stokstad *et al.*, 1948), more than 20 years ago, a great deal of experimental evidence has accumulated indicating that most natural sources contain a large fraction of their total folate activity in the form of polyglutamyl derivatives (see Figure 1). Polyglutamates of folic acid have been demonstrated in algae (Ericson *et al.*, 1953; Banhidi and Ericson, 1953), lichens (Sjøstrom and Ericson, 1953), yeast (Pfiffner *et al.*, 1945–1947), a number of species of bacteria (Hutchings *et al.*, 1948; Hakala and Welch, 1955; Vora and Tamband, 1966; Wright, 1955; Wood and Wise, 1965; Sirotnak *et al.*, 1963), as well as in animal tissues such as blood (Noronha and Aboobaker, 1963) and liver (Noronha and Silverman, 1962; Wright and Welch, 1943). This widespread distribution led Rabinowitz to state that the *in vivo* functioning form of this coenzyme must be a polyglutamyl derivative of pteroylglutamic acid, and that the latter should be considered as a convenient synthetic analog of the natural cofactor (Rabinowitz *et al.*, 1960). The study of the biological functions of the glutamyl side chain has been, however, limited to a few but very intriguing reports, all showing that the polyglutamates function as either better coenzymes than pteroylglutamic acid (Wright, 1955, 1956; Large and Quayle, 1963) or are indeed absolute requirements for enzyme activity (Guest and Jones, 1960). The main obstacle to further investigations on the biological functions of the polyglutamates of folic acid has been their unavailability. In fact, all the studies conducted until now have made use of the triglutamate, pteroyl-di-γ-glutamylglutamic acid, synthesized by the Lederle group in 1948 (Boothe *et al.*, 1948) and/or of polyglutamate preparations of uncertain structure and

* From the Nutrition Division and the Department of Biochemistry, University of Alabama Medical Center, Birmingham, Alabama 35233. *Received December 2, 1968.* Principal support for these investigations was received from American Cancer Society Grant T-67 I. Contributing support was received from National Institute of Health Grants AM-11333 and AM-08644, and Army Contract DA-49-193-MD-2299.

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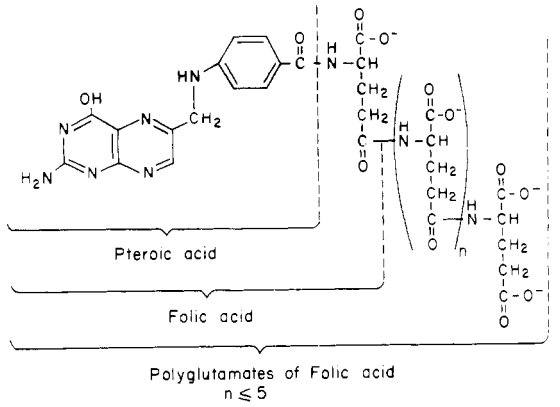

FIGURE 1: Structural formula of pteroyl-γ-glutamylglutamic acids.

purity isolated from natural sources (Wright, 1955, 1956).

To overcome these difficulties an unequivocal chemical synthesis for this family of compounds seemed highly desirable. A procedure developed for this purpose (Baugh and Krumdieck, 1968) is now available and is the subject of this report.

Experimental and Results Section

The polyglutamates of folic acid (pteroylpoly-γ-glutamylglutamic acids) may be viewed as unusual polypeptides in which the glutamyl residues are in peptide linkage through their γ-carboxyl groups, and the N-terminal residue is pteroic acid (Figure 1). When viewed in this manner, the chemical synthesis of these molecules logically falls within the realm of peptide chemistry. The solid-phase peptide synthetic procedures as first described by Merrifield (Merrifield, 1963) appeared to offer a convenient approach to the synthesis of these products.

Preliminary experiments were conducted initiating the synthesis by esterification of *t*-butyloxycarbonyl-L-glutamic acid α-benzyl ester (I) through its free γ-carboxyl group to the chloromethylated resin. Successive glutamyl residues were added in peptide linkage through their γ-carboxyl ends by going through cycles of deprotection of the amino group of the resin-bound moiety, followed by coupling of an additional molecule of I. All of the experimental conditions adhered strictly to those described by Merrifield (1963) and Marshall and Merrifield (1965). Two major difficulties became apparent in these preliminary studies. First, it was found that the resin-ester linkage through the γ-carboxyl group of glutamic acid was weaker than the usual α-carboxyl-resin-ester bond. As a consequence, the conventional deprotection procedure, namely treatment with 1 N HCl in glacial acetic acid for 30 min at room temperature, resulted in significant loss of product from the resin with unacceptable low yields. Second, it was observed that the peptide-forming step catalyzed by dicyclohexylcarbodiimide, in either dimethylformamide or methylene chloride, resulted in incomplete coupling reactions even when a fourfold excess of the entering amino acid was used. A milder deprotection procedure and a more efficient peptide forming reaction were therefore required.

Using *N*-(2-^{14}C-*t*-butyloxycarbonyl)-L-glutamic acid α-benzyl ester,[1] which allowed a rapid and accurate determination of both the extent of deprotection and the yield of the peptide-forming steps, a procedure was perfected employing a 20% v/v solution of trifluoroacetic acid in methylene chloride as the deprotecting agent and the mixed anhydride of isobutyl formate and I as the activated entering amino acid. A detailed description of the procedure follows.

Radioactive I of known specific activity labeled with ^{14}C in position 2 of the *t*-butyloxycarbonyl group was esterified to the Merrifield resin by refluxing in ethanol in the presence of triethylamine for 48 hr. All three reagents were used in equimolar amounts. The calculation of the amount of resin needed was based on the capacity value given by the manufacturer. Batches of 3.0–5.0 g of resin were esterified at a time. At the end of the refluxing period, the resin was sequentially washed with ethanol, a 50:50 v/v mixture of 0.1 N HCl and glacial acetic acid (to free the resin of any ionically bound I), water, and methanol. The amount of unreacted I was determined by measuring ^{14}C in the combined washings, and the yield of the esterification was calculated by difference. Yields in the neighborhood of 0.45 mmole of I/g of esterified resin were consistently obtained.

The esterified resin was transferred to a reaction vessel (Merrifield *et al.*, 1966) which in turn was attached to a mechanical mixer designed to rotate the flask 60 times/min. All subsequent manipulations were conducted in the same vessel. All the washings mentioned below were carried out using 30-ml aliquots of solvent and a mixing time of 3 min. The esterified resin was allowed to swell by washing it three consecutive times with methylene chloride after which the *t*-butyloxycarbonyl groups were removed by treatment with 30 ml of 20% v/v trifluoroacetic acid in methylene chloride for 30 min at room temperature. Completeness of deprotection was ascertained by counting[2] small aliquots of the resin and finding total loss of the radioactive label. Ninhydrin determinations of the deprotecting fluid showed only 1.0–1.5% cleavage of product from the resin. The aminoacyl-resin was then washed three times with ethanol and three times with dimethylformamide and neutralized by treating with 30 ml of dimethylformamide containing 3.0 ml of redistilled triethylamine for 10 min. After three addi-

[1] Reagents were purchased from the following supply houses: *N*-(2-^{14}C-*t*-butyloxycarbonyl)-L-glutamic acid α-benzyl ester from Mann Research Laboratories; *N*-(*t*-butyloxycarbonyl)-L-glutamic acid α-benzyl ester and the Merrifield resin from Cyclo Chemical Co.; isobutyl chloroformate from K & K Laboratories, Inc.; *N*-methylmorpholine, trifluoroacetic acid, and trifluoroacetic anhydride from Eastman Chemical Products, Inc.; solvents, *N*-methylmorpholine, and isobutyl choroformate were purified by distillation.

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

tional washings with dry purified (Thomas and Rochow, 1957) dimethylformamide, the aminoacyl-resin was ready for the next step. Coupling of the next glutamic acid residue was accomplished by preparing the mixed anhydride of I and isobutyl formate as follows. A 1.65-fold molar excess (all calculations based on the available resin-bound NH_2 groups) of carefully dried I was dissolved in purified dimethylformamide, and a 1.80 molar excess of *N*-methylmorpholine (Anderson *et al.*, 1966) was added. The mixture was chilled to 0° and a 1.5-fold excess of freshly distilled isobutyl chloroformate was added. The mixed anhydride was allowed to form for 15 min at 0° and then added to the reaction vessel containing the resin. The relative molar proportions of isobutyl chloroformate, I, and *N*-methylmorpholine were chosen to assure that no unreacted isobutyl chloroformate, which could attack the amino groups of the growing peptide resulting in a peptide-terminating reaction, would remain at the end of the anhydride-forming step. The excess amine was found to be desirable to provide the proper conditions of pH required for the formation of the mixed anhydride.

The coupling reaction was allowed to proceed for 2 hr with continuous mixing. The unreacted excess materials were collected by filtration and washing of the resin with dimethylformamide for three consecutive times. By counting the nonincorporated ^{14}C at this stage, the over-all yield of the procedure, from deprotection through peptide formation, was determined. It was found to be consistently between 95 and 100%. The presence of moisture significantly lowers the yield. In this regard, the use of methylene chloride as a more suitable anhydrous solvent for the peptide-forming step is being investigated and appears promising. It should be noted also that freshly distilled isobutyl chloroformate with a boiling range of 126–130° must be used to secure high yields.

The mixed-anhydride approach to the formation of peptide bonds through the γ-carboxyl end of glutamic acid was found to be far superior to the dicyclohexylcarbodiimide-catalyzed reaction. When the latter was tried using a 2.0-fold excess of I over the amount of resin-bound NH_2 groups a yield of only 21.0% was obtained.

Washing three times with absolute ethanol and three with methylene chloride brought the synthesis back to the deprotection stage. Successive cycles of deprotection and coupling were carried out as described until the desired number of glutamic acid residues had been attached in γ-peptide linkage.

The coupling of a molecule of pteroic acid to the amino end of the peptide was conducted using pteroic acid (Levi, 1967) protected and made soluble in dimethylformamide by conversion into its *N*-2,*N*-10-bistrifluoroacetyl derivative (II). Derivative II was prepared by stirring carefully dried pteroic acid as a suspension in trifluoroacetic anhydride (2.0 g in 20 ml) for 24 hr at room temperature. Excess anhydride and by-products of the reaction were removed by filtration. The solid white product was washed with 1% v/v aqueous trifluoroacetic acid for 30 min and finally with water. The product was then dried *in vacuo* over phosphorus pentoxide. When the chromatographically homogenous material thus obtained was heated to 50° in 0.1 N NaOH for 10 min, a quantitative removal of the protecting groups was achieved. Spectrally and chromatographically pure pteroic acid was recovered.

The product was first identified as the *N*-2,*N*-10-ditrifluoroacetyl derivative of pteroic acid when it was observed that quantitations based on the molecular weight of the doubly substituted derivative, *i.e.*, 504, agreed to within 2% with spectral quantitations carried out using the molar extinctions coefficients of pteroic acid[3] after removal of the trifluoroacetyl groups. Elemental analysis has since confirmed this tentative identification.

The coupling of II to the poly-γ-glutamyl-resin complex was carried out using the mixed anhydride of II and isobutyl formate as the activated molecule. The formation of the anhydride followed essentially the same procedure used for the coupling of the glutamyl residues. The same molar ratios of isobutyl chloroformate, protected amino acid (in this case II), and *N*-methylmorpholine were used. The anhydride formation was carried out in dimethylformamide at 0° for 30 min. Gentle mixing of the anhydride solution and the resin-peptide complex for 18 hr resulted in a satisfactory coupling of II. The yield of this step was always in the vicinity of 77%. This figure is obtained by converting the unreacted II into pteroic acid and quantitating it spectrally. The amount of incorporated II was then ascertained by difference from the total.

After washing the resin-bound product with ethanol, the reaction vessel was placed in a desiccator over phosphorus pentoxide and dried under vacuum. Deprotection and cleavage of the product from the solid support was conducted by treating the pteroyl-γ-glutamyl-resin suspended in trifluoroacetic acid with gaseous HBr under anhydrous conditions for two 3-min and one 6-min periods. Nearly 70% of the product was recovered from the first 3-min period of HBr treatment. The trifluoroacetic acid was immediately removed in a flash evaporator under reduced pressure. To the syrup remaining, water was added whereupon a clear, yellow, amorphous precipitate formed. The liquid phase was decanted and the yellow precipitate treated under nitrogen with nitrogen-saturated 0.5 N NaOH at 50°. Complete solution occurred rapidly with loss of the base-labile protecting groups. Since the product is unstable in alkali (Stokstad *et al.*, 1948), every effort was made to shorten the duration of this last step. The golden yellow solution was therefore rapidly adjusted to pH 7.0 with HCl. At this point, the products give an ultraviolet spectrum indistinguishable from that of teropterin.

The over-all yield of the synthesis was calculated for the octapeptide, pteroylhexa-γ-glutamylglutamic acid, by quantitating the material spectrally using a molar extinction coefficient of 9.26×10^3 at 365 mμ in 0.1 N NaOH (*vide infra*). A 28% yield was obtained.

[3] Dr. Robert Angier in a personal communication provided the following values: pteroic acid in 0.1 N NaOH, λ_{max} 255 (ϵ 26.3×10^3), 275 ($23.4 \times 10_3$), and 365 mμ (8.9×10^3).

The polyglutamates can be desalted by simply increasing the acidity of the solution to pH 2.0 which precipitates them as an amorphous yellow solid. An alternative is the use of Sephadex G-10 columns with water as eluent.

Column purification was carried out using a 1 × 150 cm column of DEAE-cellulose in the phosphate form. The column was developed with a linear gradient constructed with 1 l. of 0.1 M NaCl in the mixing chamber and 1 l. of 0.8 M NaCl in the second vessel. Both NaCl solutions were in 0.005 M phosphate buffer (pH 7.0). Only one major peak appeared in the elution pattern.

In order to ascertain by degradation the number of glutamyl residues present in the synthetic product, it was desirable to quantitate spectrally the synthetic polyglutamates. To this effect a molar extinction coefficient for the heptaglutamate was obtained using [2-^{14}C]pteroic acid of carefully determined specific activity to terminate the molecule. Accurate quantitations based on radioactivity could thus be made. Based on these quantitations a molar extinction coefficient at 365 mμ in 0.1 N NaOH of 9.26 × 10^3 was obtained.

Duplicate samples of column-purified pteroylheptaglutamate were quantitated spectrally and the number of glutamic acid residues per mole determined after complete hydrolysis of the molecule. The hydrolysis was carried out in sealed ampoules containing 5 ml each of 6 N HCl and glacial acetic acid heated to 100° for 3 hr. More prolonged hydrolysis did not increase the amount of glutamic acid liberated. The hydrolyzed glutamic acid was determined by quantitative ninhydrin. A small correction was introduced to account for the ninhydrin-positive material resulting from cleavage of the C^9–N^{10} bond of the pteroyl moiety and from possible deamination of the amino group in position 2. This was accomplished by hydrolyzing, in separate ampoules but under identical conditions, equimolar amounts of pteroic acid. The small amount of ninhydrin-positive material liberated from pteroic acid was subtracted from that of the heptaglutamate. After converting the corrected reading into micromoles of glutamic acid, 6.9 residues of glutamic acid per mole of pteroylhexa-γ-glutamylglutamic acid were found.

Using the procedure described, all of the polyglutamates of folic acid from the di to the hepta have been prepared. The chromatographic properties of the various pteroylpoly-γ-glutamyl glutamic acids have been studied. Approximately 20 μmoles of each derivative possessing from one to seven glutamyl residues has been chromatographed on DEAE-cellulose columns. Aliquots of DEAE-cellulose in the chloride form were collected in a Buchner funnel and the excess water was removed by suction. The DEAE-cellulose (20 g) was deaerated and placed in a 1 × 40 cm column. The sample was applied at pH 7.0 in a volume of from 5 to 15 ml. The column was eluted by 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 (pH 7.0) in the second chamber. The column was monitored at 365 mμ 15.5-ml; fractions

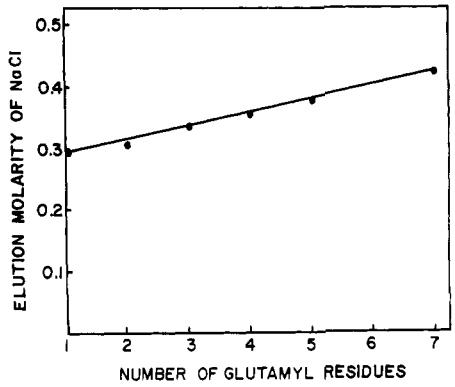

FIGURE 2: Molarity of NaCl at which polyglutamates of increasing length are eluted from a standardized DEAE-cellulose column (Cl form)

were taken. The chromatographic behavior of these compounds is summarized in Figure 2. It can be seen from these data that the molarity of NaCl required for elution is directly proportional to the number of free carboxyl groups.

The ability of the synthetic polyglutamates to sustain growth of the assay organisms *Lactobacillus casei* and *Streptococcus faecalis* and to serve as substrates for plasma conjugase (*vide infra*) has been studied with the following results. The diglutamate has been shown to support growth of *S. faecalis*. The di- and triglutamates are fully active for *L. casei*. Polyglutamates of folic acid containing more than three glutamyl residues show no microbiological response with either *S. faecalis* or *L. casei*. An enzyme present in human plasma (Wolff *et al.*, 1949) known by the generic name of "conjugase" hydrolyzes γ-glutamyl residues from polyglutamates of folic acid. When synthetic polyglutamates containing more than three glutamyl residues were incubated with human plasma, microbiological activity equivalent to the monoglutamate was obtained with either *L. casei* or *S. faecalis*.

The above synthetic procedures have been shown to be readily adaptable to the synthesis of analogs of the polyglutamates having other amino acid residues in the polypeptide chain. The inclusion of ^{14}C-labeled glutamyl residues at any point of the chain is, of course, easily attainable and should greatly facilitate the study of the biochemical and nutritional roles of these molecules. These matters are presently under investigation.

Acknowledgments

We thank Dr. C. E. Butterworth, Jr., for his interest in this work. We also thank Mrs. Barbara Hudson and Miss Eleanor Braverman for their expert technical assistance. The gift of a culture of a Pseudomonad (that produces pteroic acid from folic acid) from Dr. Carl Levi is gratefully acknowledged.

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