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# The Identification of Poly-γ-glutamyl Chain Lengths in Bacterial Folates†

Charles M. Baugh,* Eleanor Braverman, and M. G. Nair

ABSTRACT: Mild procedures have been developed for the extraction of folyl polyglutamates in 90% yield. These have been applied to three strains of folic acid requiring bacteria (*Lactobacillus casei* 7469, *Streptococcus faecium* 8043, and a methotrexate-resistant strain of 8043) grown on a defined medium supplemented with folic acid labeled in the glutamyl moiety with $^{14}C$. To minimize the heterogeneity of the folates conferred by the pteridine ring, the state of reduction, and position or nature of single carbon substituents, the crude extracts have been subjected to a reductive procedure which cleaves the $C^9$–$N^{10}$ bond. This generated a homologous series of *p*-aminobenzoylglutamyl poly-γ-glutamates which have been identified by DEAE-cellulose cochromatography with authentic compounds synthesized in this laboratory. In summary, the following distributions have been found (the number in parentheses refers to the total number of glutamates): *L. casei:* (1) 3.2%; (2) 0%; (3) 9.3%; (4) 59.4%; (5) 23%; (6) 5.1%; (7) 0%. *S. faecium:* (1) 16.5%; (2) 8.5%; (3) 20%; (4) 54.5%; (5) 0%; (6) 0%; (7) 0%. *S. faecium*-MR: (1) 1%; (2) 5.7%; (3) 6.9%; (4) 81.3%; (5) 5.1%; (6) 0%; (7) 0%. The derivative with four glutamates predominates in all the strains examined.

The folates are a family of closely related chemical structures whose number may exceed 140 members (Baugh and Krumdieck, 1971). Of this number perhaps 30 structures have been identified by one criterion or another (Shiota, 1970). In recent years, the availability of the poly-γ-glutamates of folic acid by chemical synthesis (Krumdieck and Baugh, 1969; Meienhofer and Jacobs, 1970) and increasing evidence of a functional coenzymatic role for them (Large and Quayle, 1963; Guest and Jones, 1960; Whitfield and Weissbach, 1968; Burton *et al.*, 1969) have focused considerable attention upon these naturally occurring peptides. Numerous reports have appeared in the recent literature assigning structures to the poly-γ-glutamates of folic acid from a variety of biological specimens (Buehring *et al.*, 1974; Shin *et al.*, 1972a,b; Osborne-White and Smith, 1973; Corrocher *et al.*, 1972). The majority of these reports describe techniques of Sephadex gel filtration and/or DEAE-cellulose chromatography, and differential microbiological assays before and after deconjugation with a variety of crude pteroylglutamyl-γ-polyglutamyl hydrolases to identify and assign structures to the various folates. At least two investigators have employed more direct methods (Houlihan and Scott, 1972; Curthoys *et al.*, 1972). In this laboratory we have repeatedly observed that the folates behave abnormally on Sephadex gels, with chain lengths below the tri- eluting much later than one would predict based on molecular weight, apparently due to adsorptive properties conferred by the pteridine. In addition, their unusual behavior decreases as the polyglutamyl chain is lengthened in the fully oxidized folates. It also appears unlikely that details of structure such as the state of reduction and nature of single-carbon substituents could be assigned unequivocally by this technique since the molecular weight changes are minimal and the changes in adsorptive properties due to reduction and substitution of single-carbon units on the pteridine are not predictable. Similarly, the use of DEAE-cellulose to identify the natural folates has not been feasible in this laboratory. For example, cirtovorum factor ($N^5$-formyl-5,6,7,8-tetrahydrofolic acid) is eluted along with pteroylglutamyl-(glutamyl)$_3$-glutamic acid (Pte-$G_5$)[1] from columns of DEAE-cellulose under a variety of elution conditions. Similar unexpected elution characteristics have also been noted to exist between the fully and partially reduced pteroyl polyglutamates (Kisliuk *et al.*, 1974). These workers showed that adding five glutamyl residues to tetrahydrofolic acid does not alter its affinity for DEAE-cellulose as much as does converting it to dihydrofolic acid.

It appeared that this multiplicity of problems could be greatly simplified if the heterogeneity of this large group of

† From the Department of Biochemistry, College of Medicine, University of South Alabama, Mobile, Alabama 36688. *Received June 24, 1974.* Principal support for these investigations was received from the American Cancer Society Grant CI-86-O and National Institutes of Health Grant 1-RO1-CA-16048.

[1] Abbreviations used are: Pte-$G_5$, pteroylglutamyl-(glutamyl)$_3$-glutamic acid; pABG$_n$, *p*-aminobenzoyl polyglutamate.

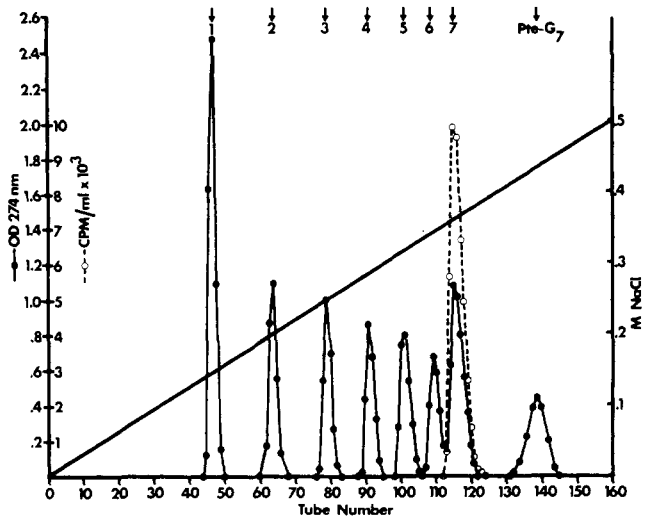


FIGURE 1: Elution pattern of the reductive cleavage product of Pte-G*-$G_6$, cochromatographed with nonradioactive *p*-aminobenzoyl polyglutamates with from one to seven glutamates and Pte-$G_7$.

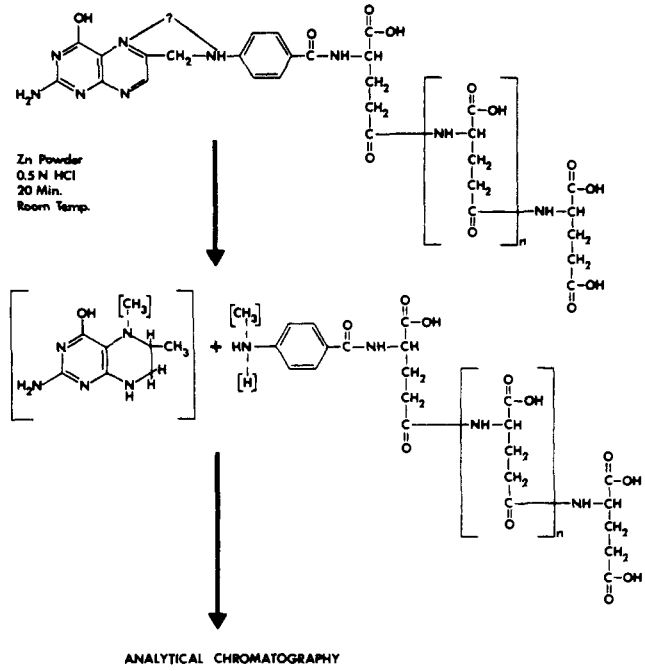


FIGURE 2: Schematic representation of the reductive cleavage procedure. The question mark at positions 9–10 indicates the multiplicity of single carbon substituents which may occur in the natural folates. The bracketed methyl and hydrogen denote the two possible forms of the cleaved $pABG_n$. The bracketed fully reduced pteridine may be expected to possess a methyl group at position 6 and a second methyl or hydrogen at position 5 depending on the single carbon substituent. The structure of this pteridine is presumptive and since this detail was not pertinent to the present studies, it was not examined.

compounds could be reduced (Baugh and Krumdieck, 1971). An obvious approach would be to remove the pteridine portion, thus generating a family of as few as 7 or as many as 14 structures, assuming a maximum of 7 glutamyl residues and depending upon the nature and amount of substituents at positions $N^{10}$ and $N^5$–$N^{10}$ in the natural folates. Seven of these possible structures could arise by reduction and ring opening to give the *N*-methyl-*p*-aminobenzoyl polyglutamates. The expectation of a maximum of 14 structures also assumes that the acid treatment employed in both the extraction and cleavage would deformylate any $N^{10}$-formyltetrahydrofolates that were present. To this end, the *p*-aminobenzoylglutamyl polyglutamates with from one to seven glutamyl residues and the corresponding family of *N*-methyl-*p*-aminobenzoyl glutamates have been synthesized and their chromatographic properties studied. A quantitative nondestructive procedure for the cleavage of the $C^9$–$N^{10}$ bond in folate has also been developed.

We report here the details of this methodology and its application to the assignment of structure and distribution with respect to the number of γ-glutamyl residues in the folates of three different bacterial strains.

## Materials and Methods

Cyclo Chemical Co. and Bachem supplied *tert*-butyloxycarbonyl glutamate α-benzyl ester; New England Nuclear Corp. was the supplier of the *tert*-butyloxycarbonyl [U-$^{14}$C]glutamate α-benzyl ester. All other chemicals, solvents, and supplies used in the solid phase synthesis of the various poly-γ-glutamyl derivatives were obtained and treated as previously reported (Krumdieck and Baugh, 1969; Nair and Baugh, 1973; Baugh *et al.*, 1970). Lyophilized cultures of the folate-requiring bacteria *Lactobacillus casei* ATCC 7469 and *Streptococcus faecium* ATCC 8043 were purchased from the American Type Culture Collection. The methotrexate-resistant strain of *S. faecium* ATCC 8043 was developed and isolated by Dr. Roy Kisliuk, Department of Biochemistry, Tufts University; this gift is gratefully acknowledged. Bacterial media were purchased from Difco Laboratories.

*The Synthesis of Poly-γ-glutamates of p-Aminobenzoylglutamyl Polyglutamates and N-Methyl-p-aminobenzoylglutamyl Polyglutamates.* The various poly-γ-glutamyl peptides were prepared as previously reported (Krumdieck and Baugh, 1969; Nair and Baugh, 1973; Baugh *et al.*, 1970). The completed, protected resin-bound peptides were terminated by coupling with *N*-trifluoroacetyl-*p*-aminobenzoic acid or *N*-methyl-*p*-aminobenzoic acid as their mixed anhydrides formed with isobutyl chloroformate. The details of this coupling were identical with those previously reported for the synthesis of polyglutamates of folic acid (Krumdieck and Baugh, 1969). Cleavage of the completed product from the resin was carried out as reported for the synthesis of the poly-γ-glutamates of methotrexate (Nair and Baugh, 1973). [$^{14}$C]Poly-γ-glutamates and pteroyl[U-$^{14}$C]glutamic acid were prepared by insertion of *tert*-butyloxycarbonyl[U-$^{14}$C]glutamate α-benzyl ester at the desired places in the standard synthesis procedure.

*Chromatographic Procedures.* The standard analytical procedure used throughout these studies employed a 1.2 × 40 cm column of DEAE-cellulose in the chloride form. These columns were eluted by linear gradients of 1 l. of 0.005 M phosphate buffer (pH 7) in the mixing chamber and 1 l. of 0.5 M NaCl in the same buffer in the reservoir.

When these chromatographic procedures were applied to *p*-aminobenzoyl glutamate or *p*-methylaminobenzoyl glutamate they were noted to have virtually identical elution patterns. Mixtures of the two derivatives of corresponding glutamyl chain lengths could not be resolved. For example, $pABG_4$ and *p*-methyl-$ABG_4$ elute as a single symmetrical peak. Spectra taken on either side of the peak fracton indicate that *p*-methyl-$ABG_n$ ($\lambda_{max}$ 289, pH 7.0) moves slightly behind $pABG_n$ ($\lambda_{max}$ 278, pH 7.0). These data indicate normal behavior of these derivatives on DEAE-cellulose chromatography, in contrast to the folates discussed above. Their separation appears to be directly related to the number of glutamyl residues and therefore to the number of

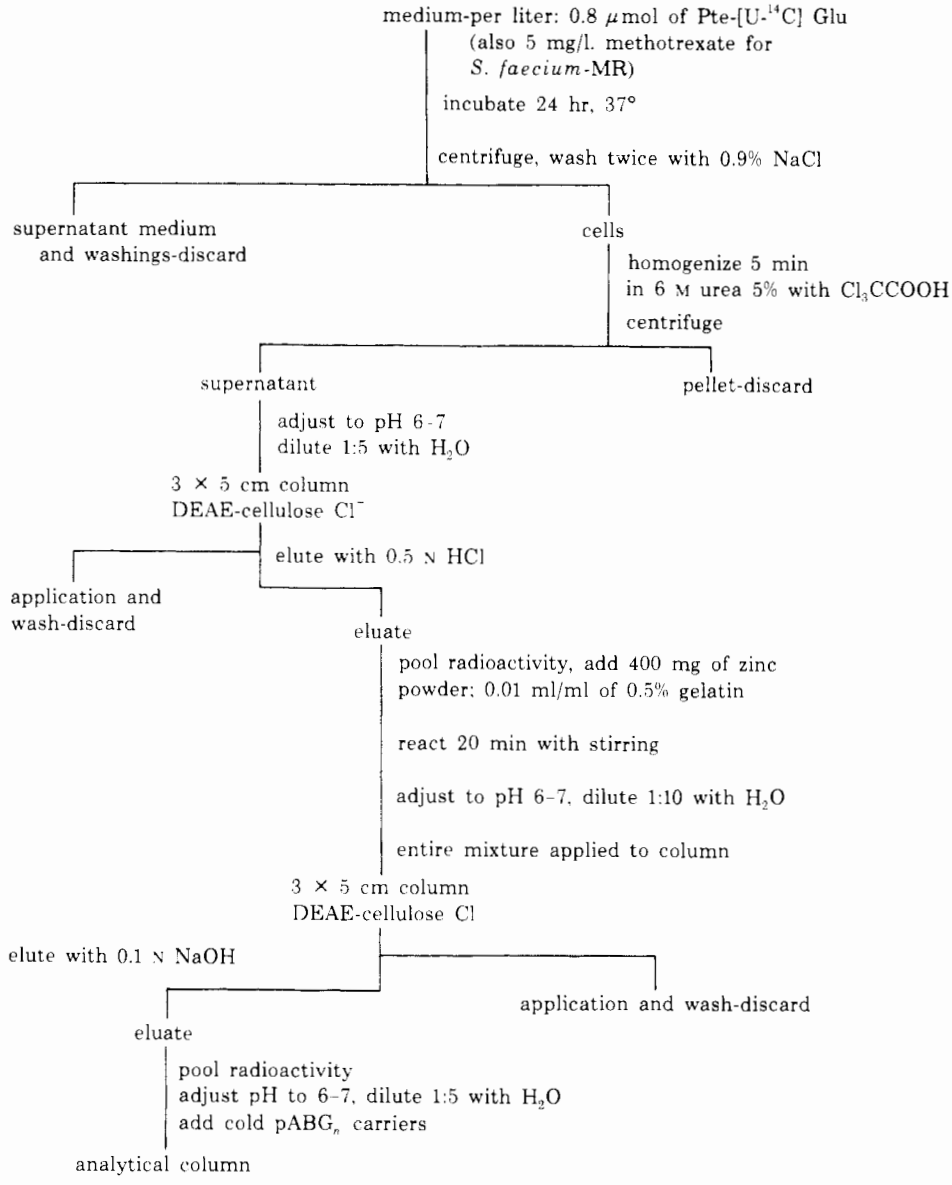


FIGURE 3: A flow diagram summarizing the details of bacterial growth, folate extraction, and reductive cleavage.

negative charges. Adsorptive properties conferred by the aminobenzene moiety appear to be negligible.

*Reductive Cleavage of the $C^9$–$N^{10}$ Bond in the Folates.* The basis for the cleavage procedure was adopted from the established methods for the determination of folic acid by the colorimetric procedure of Bratton and Marshall (Hutchings *et al.*, 1947). Initially this approach was attractive because of its proven ability to cleave quantitatively the $C^9$–$N^{10}$ bond in folic acid, leaving the diazotizable amino group intact. A primary concern centered around the lability of the γ-peptide bonds when subjected to the rigorous conditions of acid and heat. Nondestructive conditions have been developed.

Pteroyl[U-$^{14}$C]glutamyl-γ-(glutamyl)$_5$-glutamic acid (Pte-G*-$G_6$), 0.5 μmol, was added to 5 ml of 0.5 N HCl and made 0.05% with gelatin. Zinc powder, 400 mg, was added and the suspension stirred at room temperature for 20 min. The reaction was neutralized and diluted with 10 volumes of water. Nonradioactive *p*-aminobenzoyl poly-γ-glutamates and Pte-$G_7$ were added as carriers. pABG and $pABG_7$ were added in larger amounts than those with intermediate chain lengths to serve as boundary markers for identification. The members $pABG_2$ through $pABG_6$ were added in decreasing amounts as the number of glutamates increased. The reaction mixture, still containing the zinc, was then applied to a 3 × 5 cm column of DEAE-cellulose $Cl^-$. The column was washed with water and eluted in 10-ml fractions with 0.1 N NaOH. The tubes containing radioactivity, usually two, were pooled, neutralized with HCl, diluted with five volumes of water, applied to a standard analytical column, and eluted as described above. These data are shown in Figure 1 and validate this methodology. The confinement of radioactivity to the $pABG_7$ peak and the absence of label from the shorter pABG members demonstrate that the method is nondestructive toward γ-peptide bonds. The absence of label in the Pte-$G_7$ peak indicates that the cleavage was quantitative. This experiment also serves to substantiate our ability to resolve these complex mixtures of closely related structures by DEAE-cellulose chromatography. The reductive cleavage is illustrated in Figure 2.

*Growth of Bacteria.* The respective folate-free assay media were used to culture *L. casei* and *S. faecium*. The methotrexate-resistant *S. faecium* was grown in the same

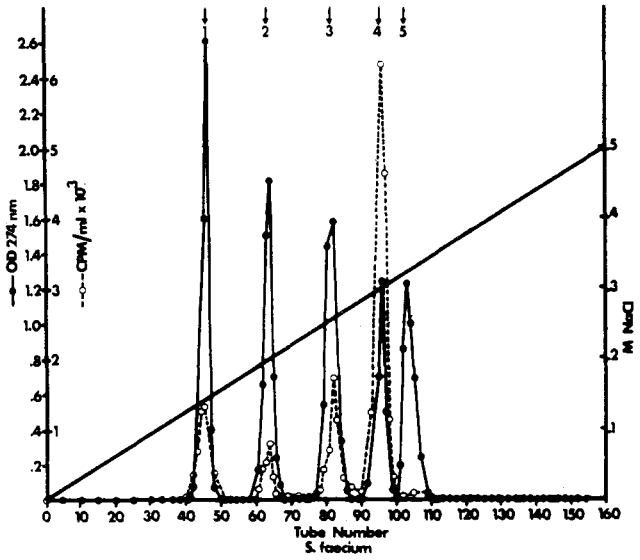


FIGURE 4: Elution pattern of the analytical DEAE-cellulose cochromatography of the cleaved folates from *S. faecium* grown on Pte-G*. Cariers are nonradioactive $pABG_n$. The chain lengths are noted by arrow and number at the top of the figure.

Difco medium as the parent strain, but supplemented with 5 μg/ml of methotrexate. In all cases the incubations were carried out at 37° in a total volume of 1 l. distributed equally between two 1-l. erlenmeyer flasks and supplemented with 0.8 μmol of pteroyl[U-$^{14}$C]glutamic acid ($6 \times 10^6$ total cpm)/l. The flasks were inoculated by aseptic addition of a 5-ml log growing culture to each flask.

*Extraction of Folates.* The use of 8 M urea was employed by Kozloff *et al.* (1970) to release pteroylpolyglutamates from T2L *Escherichia coli* bacteriophage ghosts. We have extended this method to incorporate the simultaneous denaturation of protein by the use of 5% trichloroacetic acid to provide a clear, protein-free extract, containing the folates. The bacteria were harvested after 24 hr by centrifugation and washed twice with ice-cold isotonic saline. The supernatant medium and washings were combined, their total radioactivity was determined, and then they were discarded. The washed, packed cells were transferred to a 100-ml Virtis homogenization cup and a volume of 6 M urea containing 5% trichloroacetic acid estimated to be five times the volume of the packed cells was added. The vessel was placed in an ice bath and homogenized for 5 min at maximum speed. The homogenate was centrifuged and the precipitate washed with 1% trichloroacetic acid. The precipitate was determined to be free of radioactivity. The supernatant and washings were combined, neutralized, diluted with five volumes of water, and applied to a 3 × 10 cm column of DEAE-cellulose $Cl^-$. The column was washed with water and stripped with 0.5 N HCl. The tubes containing radioactivity were pooled and subjected to the reductive cleavage as described. Recovery of label at this point was consistently between 85 and 90% based on the calculated uptake of radioactive folic acid by the bacteria. The overall method, from cultivation of bacteria to the analytical column, is schematically summarized in Figure 3.

## Results

These techniques have been used to study the relative distribution of pteroyl polyglutamates in three bacterial strains. The elution profile of the analytical column for *S. faecium* may be seen in Figure 4. These data clearly indicate that the major and highest pteroyl polyglutamate in this organism is the Pte-$G_4$. The absence of longer chain lengths is striking as is the relatively minor amounts of those derivatives possessing fewer than four glutamates.

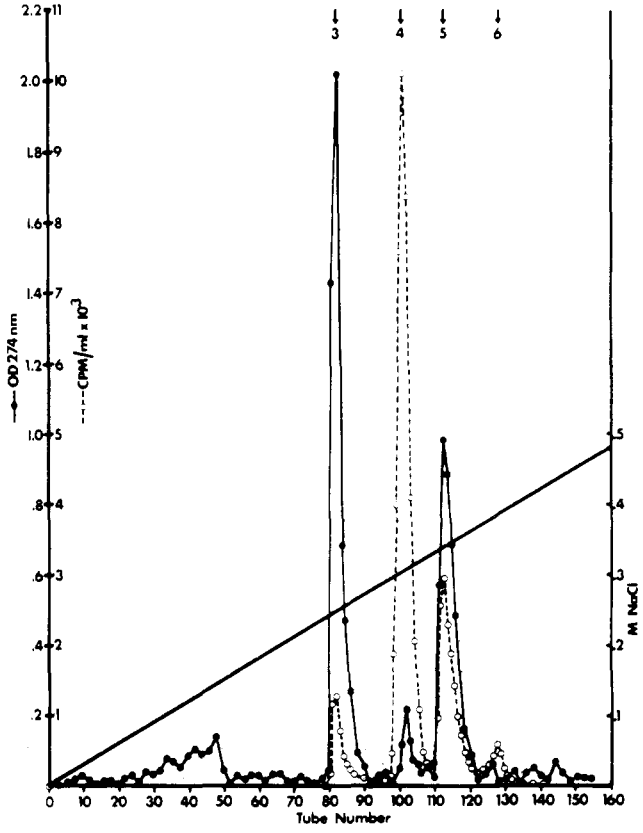


FIGURE 5: Elution pattern of the analytical DEAE-cellulose chromatography of the cleaved folates from *L. casei* grown on Pte-G*. Markers and notations are the same as those for Figure 4.

Figure 5 illustrates the results for *L. casei*. Again the major pteroyl polyglutamate was found to be Pte-$G_4$. However, two significant differences from *S. faecium* can be pointed out. First is the appearance of a significant amount of Pte-$G_5$ and a small amount of Pte-$G_6$; second is the total absence of Pte-$G_2$ in this organism.

The methotrexate-resistant mutant of *S. faecium* provided the data shown in Figure 6. Qualitatively, these data are quite similar to those obtained with the parent strain (see Figure 4). Quantitatively, however, there is a large increase in the amount of Pte-$G_4$ relative to the other forms. The Pte-$G_4$ in this strain accounts for more than 80% of the total folates in contrast to 55 and 60% for the parent strain and *L. casei*, respectively.

The thought that the quantitative differences between the distribution of pteroyl polyglutamates seen with *S. faecium* and *S. faecium*-MR might have a bearing on methotrexate resistance led to an experiment in which methotrexate labeled with [U-$^{14}$C]glutamate and nonradioactive folate were employed. No radioactivity from methotrexate was detectable in the trichloroacetic acid–6 M urea extracts, nor in the protein precipitate. The data strongly suggest that the methotrexate resistance in the mutant is conferred by a transport alteration which excludes methotrexate.

## Discussion

A mild (0°), nearly quantitative (90%) extraction procedure to remove the folates from their protein binders has

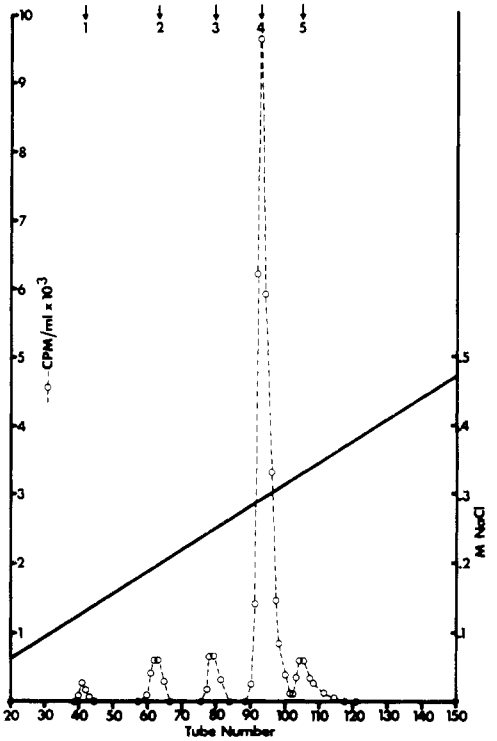


FIGURE 6: Radioactive profile of the cleaved folates from the methotrexate resistant mutant of *S. faecium*. Broad experience with the chromatographic procedures and confidence in the reproducibility of the system obviated the necessity of nonradioactive markers during the latter stages of these studies.

been developed. This procedure, in contrast to the generally employed methods of boiling or autoclaving, does not lead to destruction of the compounds whose identity is being sought (Shin *et al.*, 1972b; Osborne-White and Smith, 1973; Corrocher *et al.*, 1972; Houlihan and Scott, 1972). In addition, since the natural folates coexist with enzymes capable of their alteration, the simultaneous denaturation and cell disruption should provide a true reflection of the *in vivo* distribution of folates.

The methods reported here reduce the heterogeneity of this large family of compounds to a level readily manageable by conventional chromatographic techniques. In addition, the methods reported circumvent the problems associated with reliance upon crude hydrolases of uncertain specificity plus ambiguous and inaccurate microbiological assays for the assignment of structure. The report of Houlihan and Scott (1972) on the pteroylpolyglutamates of rat liver recognized and circumvented these problems using alkaline permanganate oxidation to cleave the $C^9$-$N^{10}$ bond. This work is further discussed in the following article (Leslie and Baugh, 1974). Curthoys *et al.* (1972) isolated and identified the major folates of *Clostridium acidi-urici* as pteroyltriglutamates, without degradation. However, this approach does not appear applicable to those biological systems which possess more complex mixtures of folates. The report by Shin *et al.* (1972a) is perhaps more relevant to the present report. These workers, employing techniques of Sephadex gel chromatography and differential microbiological assay before and after enzymatic glutamyl hydrolysis, concluded that all folates of *L. casei* possess more than six glutamyl residues. These results are incompatible with the data presented here, since only 5.1% of the folates in this organism were found to have six glutamates and no forms with more than six were discovered. The major form in *L. casei* as determined by the methods described here is the Pte-$G_4$, appearing as 60% of the total.

The folates of *L. casei* and *S. faecium* have also been examined by Buehring *et al.* (1974) using techniques of Sephadex gel filtratration, DEAE-cellulose chromatography, and differential microbiological assay applied to the intact folates. These workers report that 75% of the folates of *L. casei* have more than seven glutamyl residues. The data reported here are in serious conflict with those interpretations since we find no folate derivatives in this organism with more than six glutamic acid residues. Similar, but less extensive disagreement is also apparent between our data and that reported by the same group for *S. faecium*. Although there is reasonable agreement that the major polyglutamate for this organism is Pte-$G_4$, these workers report 24% as the Pte-$G_5$, where we find none. These differences may be reconciled in the light of the unusual and nonpredictable adsorptive characteristics conferred on the folates by reduction and one-carbon substitution, or they might also be due to differential growth conditions as well as the state of growth of the bacteria. We feel this behavior justifies the conversion of the natural folates to derivatives which behave normally as anions when glutamyl chain length is the parameter being examined.

Prescott and Affronti (1968) reported that a methotrexate-resistant strain of *S. faecium* possessed a 1000-fold increase in conjugase over the parent strain and suggested that this elevated enzyme could be responsible for the methotrexate resistance. This laboratory recently provided evidence of the existence in nature of polyglutamates of methotrexate. Although these studies were carried out in rats, they give some credence to this hypothesis (Baugh *et al.*, 1973). However, the clear lack of penetration of methotrexate into the methotrexate-resistant *S. faecium* used in our experiments appears to require another explanation for this resistance.

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# The Uptake of Pteroyl[$^{14}$C]glutamic Acid into Rat Liver and Its Incorporation into the Natural Pteroyl Poly-γ-glutamates of that Organ†

Garth I. Leslie‡ and Charles M. Baugh*

ABSTRACT: The uptake of a dose of pteroyl[U-$^{14}$C]glutamic acid (Pte-G*) into rat liver and its incorporation into hepatic folates were examined at a number of time intervals from 1 hr to 28 days. The folates were extracted from the liver and then converted quantitatively to the *p*-aminobenzoyl glutamates of corresponding poly-γ-glutamyl chain length. These derivatives were then separated by chromatography on a DEAE-cellulose column using a NaCl gradient in phosphate buffer. Uptake was found to reach a maximum between 6 and 24 hr after intraperitoneal injection of Pte-G*. There was rapid addition of glutamyl residues in the liver so that after 3 hr, 75% of the folates had been converted to the tetra- and pentaglutamate forms. After 24 hr, presumably a steady-state relationship was reached and over 90% of the labeled folates were in the penta- and hexaglutamyl forms, with the remainder consisting of tetra- and heptaglutamates.

The polyglutamate derivatives of pteroylglutamic acid have for some time been known to be quantitatively important (Pfiffner *et al.,* 1946; Hutchings *et al.,* 1948; Noronha and Silverman, 1962; Noronha and Aboobaker, 1963; Sirotnak *et al.,* 1963; Bird *et al.,* 1965; Schertel *et al.,* 1965), and a number of workers (Wright, 1956; Blakley, 1957; Guest and Jones, 1960; Kisliuk and Woods, 1960; Large and Quayle, 1963; Wang *et al.,* 1967; Whitfield and Weissbach, 1968; Burton *et al.,* 1969; Kozloff *et al.,* 1970) have been able to demonstrate the functional importance of some of these derivatives. Since methods have been developed for the solid phase synthesis of poly-γ-glutamates (Krumdieck and Baugh, 1969), it has become possible to consider more exact determination of poly-γ-glutamate chain lengths and their functions in different tissues.

Most workers who have attempted to determine the natural polyglutamates (Shin *et al.,* 1972; Osborne-White and Smith, 1973; Thenen *et al.,* 1973) have undertaken to identify them with regard to both the poly-γ-glutamyl chain length and the one-carbon substituents. However, due to the instability of the folates, their coexistence with many enzymes capable of modifying them, and the theoretically large number of possible molecular modifications, separation and identification of individual molecules become a very complex problem. Quantitative conversion of pteroylglutamyl derivatives to their corresponding *p*-aminobenzoylglutamate derivatives, which are relatively stable molecules, provides a solution to these problems.

Two approaches have been employed for cleavage of the $C^9$–$N^{10}$ bond of pteroyl glutamates to yield the corresponding *p*-aminobenzoyl glutamates. One of these, in which the molecules are cleaved by an oxidative procedure, was used by Houlihan and Scott (1972) and Brown *et al.* (1974) in studies of mammalian folates. The other approach, which involves reductive cleavage of the molecules, has recently been developed in this laboratory (Baugh *et al.,* 1974).

The method employed by Houlihan and Scott (1972) makes use of heating and autoclaving to stop conjugase activity, procedures which may well result in alteration of the folate molecules. Additionally, they do not document any control experiments to establish the validity of the results obtained by the procedure used. On the other hand, the method employed in the previous paper (Baugh *et al.,* 1974) used 5% trichloroacetic acid in 6 M urea at low temperatures to stop conjugase activity, precipitate the proteins, and leave the folates in solution. It is also established by the appropriate control experiments that the procedure used for cleavage of the $C^9$–$N^{10}$ bond does in fact cleave only that bond, leaving the γ-peptide linkages intact.

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