Microbial Synthesis of Folate Polyglutamates from Labelled Precursors

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SUMMARY

The uptake and synthesis of folate polyglutamates from [^3H]pteroylglutamic acid and [^{14}C]p-aminobenzoic acid was studied in *Lactobacillus casei*, *Streptococcus faecalis*, *L. plantarum* and *Escherichia coli*. The analytical techniques were based on oxidative or hydrolytic degradation of the folate compounds followed by chromatographic separation of the resulting mixtures of pteroylglutamate derivatives or p-aminobenzoylpoly- γ -L-glutamates and comparison with appropriate synthetic standards. All four organisms synthesized numerous polyglutamate derivatives from the precursors provided. The large majority (85 to 95 %) of folates of lactobacilli were polyglutamates with more than seven glutamyl residues; *L. casei* formed several such unidentified long-chain components. In contrast, *E. coli* and particularly *S. faecalis* synthesized mainly polyglutamates with seven or less glutamate residues; tetra-, hexa- and heptaglutamates were tentatively identified in *E. coli*, and tri-, tetra- and pentaglutamates in *S. faecalis*. While *L. casei* appeared to synthesize a mixture of formyl- and methyltetrahydro derivatives, formyltetrahydro derivatives predominated in *E. coli* and *S. faecalis*.

INTRODUCTION

Understanding of the early steps in the microbial synthesis of folate compounds is largely based on the work of Brown and co-workers with *E. coli* (Brown, Weisman & Molnar, 1961; Reynolds & Brown, 1964; Weisman & Brown, 1964; Griffin & Brown, 1964). While these authors' findings *in vitro* support a path yielding successively dihydropteroate (H_2Pte), dihydropteroylglutamate (H_2PteGlu), tetrahydropteroylglutamate (H_4PteGlu), tetrahydropteroyldiglutamate ($\text{H}_4\text{PteGlu}_2$) and $\text{H}_4\text{PteGlu}_3$ -triglutamate in this species, it is still somewhat uncertain whether H_2Pte is an obligatory intermediate *in vivo*. This pathway is also thought to operate in *L. plantarum* (Shiota & Disraely, 1961), in micrococci (Merola & Koft, 1960) and in yeast (Jaenicke & Chan, 1960). A pathway involving p-aminobenzoylglutamate (p-ABGlu) probably predominates in pneumococci (Ortiz & Hotchkiss, 1966) and perhaps in *Mycobacterium avium* (Katsunuma & Shoda, 1958). Recently Martin & Koft (1972) have observed the formation of rhizopterins (10-HCO-Pte) and formylated pteroyldi- and pteroyltriglutamates during the growth of *Staphylococcus epidermidis*, thus suggesting a novel biosynthetic pathway presumably independent of a dihydropteroate (H_2Pte) or tetrahydropteroate (H_4Pte).

The nature of the folate polyglutamate end products of these biosynthetic routes has been difficult to establish, mainly because of the large number of possible derivatives, their small quantity and unfavourable chromatographic properties (Baugh & Krumdieck, 1971; Shin, Buehring & Stokstad, 1972). Only in yeast and *Clostridium acidurici* have the number of glutamyl residues present in the folate compounds been unequivocally deter-

mined, yeast containing primarily heptaglutamate derivatives (Pfiffner, Calkins, Bloom & O'Dell, 1946) and *C. acidi-urici* pteroyltriglutamates (Curthoys, Scott & Rabinowitz, 1972). In the present study labelled folate polyglutamates synthesized from [^3H]pteroylglutamic acid by *L. casei* and *S. faecalis* and from *p*-amino[^{14}C]benzoic acid by *L. plantarum* and *Escherichia coli* were extracted and analysed following either oxidative or hydrolytic degradation.

METHODS

Organisms. *Streptococcus (lactis) faecalis* R NCIB6459, *L. casei* var. *rhamnosus* NCIB10463, *L. plantarum* (arabinosus 17-5) NCIB6376, and *E. coli* NCIB8109 were obtained from the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.

Media and growth conditions. *Streptococcus faecalis* was carried on stock cultures of Bacto micro assay culture agar (Difco) and inoculated by loop into 50 ml of sterile Bacto folic acid casei medium (Difco) containing a nearly limiting concentration of [^3H]pteroylglutamic acid of 3.2 ng and 0.2 $\mu\text{Ci/ml}$. The culture was incubated for 20 to 24 h at 37 °C to obtain organisms in the stationary phase of growth.

A chloramphenicol-resistant strain of *L. casei* originally described by Millbank, Davis, Rawlins & Waters (1970) and employed in routine microbiological assay in this laboratory (O'Broin, Scott & Temperley, 1973) was carried on stock cultures of folic acid assay PGA broth (BBL, Cockeysville, Maryland, U.S.A.) containing 1 % noble agar (Difco), pteroylglutamic acid (0.1 ng/ml), and chloramphenicol (Chloromycetin, Parke-Davis, Hounslow; 20 $\mu\text{g/ml}$). Inoculation from a similarly composed broth culture was made into 2 l of broth containing [^3H]pteroylglutamic acid (0.2 ng or 5 nCi/ml) and the culture was incubated for 48 h at 37 °C to obtain stationary phase organisms.

A *p*-aminobenzoate-requiring mutant of *E. coli* (Lampen, Jones & Roepke, 1949) was cultured in a basal synthetic medium composed of the following (g/l): NaCl, 2.94; NH_4Cl , 2.66; KH_2PO_4 , 3.4; CaCl_2 , 7.4×10^{-3} ; MgSO_4 , 0.25; glucose, 2.0; *p*-aminobenzoate, 4×10^{-6} ; pH 7.2. Two l (4×0.5 l) sterile basal medium containing 6 μg or 5 μCi *p*-amino[^{14}C]benzoate were inoculated with 0.1 ml from a broth culture and incubated on a reciprocating shaker, at 37 °C for 24 to 36 h to provide stationary phase organisms.

Lactobacillus plantarum, a test organism for the assay of *p*-aminobenzoic acid, was stored on Bacto micro assay culture agar and grown in the assay medium recommended by Lewis (1942) except that it was found necessary to treat 'vitamin-free' casein hydrolysate (enzymic) (Nutritional Biochemicals Corp. Cleveland, Ohio, U.S.A.) four times with 0.45 % charcoal (Norit A; Sigma) at pH 3 for 15 min each at room temperature. The medium (100 ml) containing 0.2 μg or 0.5 μCi *p*-amino[^{14}C]benzoate was inoculated from a broth culture and incubated for 36 to 48 h at 37 °C.

Chemicals. *p*-Aminobenzoic acid (*p*-AB) was purchased from Koch-Light Laboratories Ltd. *p*-Aminobenzoyl-L-glutamic acid (*p*-ABGlu) was obtained from Sigma, and pteroylglutamic acid (PteGlu) from Cyanamid of Great Britain, Gosport, Hampshire. Calcium leucovorin (5-HCO- H_4PteGlu) was a gift from Lederle Division, American Cyanamid, Pearl River, New York, U.S.A. H_4PteGlu was prepared from PteGlu by reduction with NaBH_4 (Blair & Saunders, 1970); 10-HCO- H_4PteGlu was prepared from 5-HCO- H_4PteGlu by acidification to form 5,10-methyldiene tetrahydropteroylglutamic acid (5,10-CH = H_4PteGlu) and subsequent neutralization with alkali (Brown, Scott, Foster & Weir, 1973). Pteric acid (Pte) was prepared and purified as described previously (Houlihan, Boyle & Scott, 1972). Pteroyl poly- γ -L-glutamates (PteGlu_{2-7}) were prepared by the solid-phase method (Krumdieck & Baugh, 1969; Baugh, Stevens & Krumdieck, 1970). Quantitative,

oxidative or reductive cleavage of the C₉–N₁₀ bond (Freed, 1966) yielded the corresponding polyglutamates of *p*-aminobenzoylglutamate (*p*-ABGlu_{2–7}).

[³H]Pteroylglutamic acid, 15 Ci/mmol; [2-¹⁴C]PteGlu, 55 mCi/mmol; 5-¹⁴CH₃ H₄PteGlu, 54 mCi/mmol; and pteroylhexaglutamyl ([U-¹⁴C]glutamic acid; PteGlu₇), 1.2 mCi/mmol, were obtained from the Radiochemical Centre, Amersham, Buckinghamshire. *p*-Amino [¹⁴COOH]benzoic acid (52 mCi/mmol) was obtained from Schwarz/Mann, Orangeburg, New York, U.S.A.

Diethylaminoethylcellulose (Whatman DE52) was purchased from W. & R. Balston Ltd, Maidstone, Kent. QAE (quaternary ammonium exchanger) Sephadex A25 was obtained from Pharmacia. Triton X-100 (Rohm & Haas) was purchased from BDH, and the scintillation chemicals 2,5-diphenyloxazol (PPO) and 2,2'-*p*-phenylen-bis-(4-methyl-5-phenyloxazol) (POPOP) from Intertechnique Ltd, Sussex.

Extraction of folates. The bacteria were collected by centrifugation (6000g, 10 min), suspended in 0.9 % NaCl solution and re-sedimented. The wash was combined with the supernatant medium and samples taken for radioactivity determination. Washed organisms were suspended in 20 ml of 0.1 M-phosphate buffer pH 7.0 containing 0.5 % ascorbate and the suspension was autoclaved at 121 °C for 5 to 10 min. The debris was collected by centrifugation and samples of it, as well as the supernatant extract, were removed for radioactivity counting. The extract was then divided into three or four portions, one of these usually being chromatographed, without further treatment, on QAE Sephadex as described below.

Degradation and chromatography of extracts. (i) Permanganate oxidation. To one portion of the extract was added 2.0 % (w/v) KMnO₄ (4:1, v/v) and the pH was adjusted to 9.0 with dilute alkali (Houlihan & Scott, 1972). The mixture was shaken intermittently for 15 min and neutralized with dilute HCl. The brown precipitate was removed by centrifugation (1000g, 10 min) and the supernatant liquid was diluted with 5 mM-phosphate buffer pH 7.0 until the conductivity was less than 3 mS. This solution was applied to a 20 × 0.7 cm glass column containing DEAE cellulose equilibrated with 5 mM-phosphate buffer pH 7.0 and the cellulose washed with two or three volumes of the same buffer. Elution (10 to 15 ml/h) was effected with a convex gradient constructed as in Fig. 2.

(ii) γ -Glutamylcarboxypeptidase treatment. Another portion of the extract was subjected to the action of guinea-pig intestinal mucosa or rat liver 'conjugase' (γ -glutamylcarboxypeptidase). The extract was made 0.1 M with respect to a sodium ascorbate buffer pH 4.5 and the 'conjugase' preparation either from guinea-pig mucosa (Bernstein, Gutstein & Weiner, 1969) or from rat liver (Brown, Davidson & Scott, 1974) was added 4:1 (v/v). Incubation was carried out overnight at 37 °C under a thin layer of toluene. The toluene was removed and the pH of the extract adjusted to 7.5 with dilute alkali. The extract was then centrifuged (1000g, 10 min) and diluted with 50 mM-tris-HCl buffer pH 7.5 containing 0.2 % 2-mercaptoethanol (MET) until the conductivity was less than 2.0 mS. This solution was applied to a 30 × 0.8 cm glass column of QAE Sephadex equilibrated with 50 mM-tris-HCl pH 7.5 and eluted with a linear gradient composed of 300 ml each of 50 mM-tris-HCl, 0.2 % MET pH 7.5 and 0.6 M-NaCl in the same buffer.

Radiochemical methods. The toluene-Triton X-100 scintillant fluid recommended by Turner (1969) was used throughout at a ratio of 1 part sample to 10 parts scintillation fluid. Radioactivities were determined using a Packard Tri-Carb liquid scintillation spectrometer model 3375.

RESULTS

Radioactive incorporation and extraction

The uptake of the labelled precursors [^3H]PteGlu, or [^{14}C]p-AB by the test organisms and the radioactivity subsequently extracted from the stationary phase organisms are summarized in Table 1. While *L. casei* showed the greatest percentage absorption of labelled precursor from the medium, *E. coli* with nearly as much absorption gave by far the greatest ease of extraction of biosynthetic folate compounds. The lactic acid bacteria were quite variable in these respects: *Streptococcus faecalis*, which exhibited good uptake, gave the worst extraction under the conditions used.

Table 1. *Uptake and extraction of radioactivity from [^3H]pteroylglutamate or [^{14}C]p-aminobenzoic acid by different bacteria*

Micro-organism	...	<i>Streptococcus faecalis</i>	<i>Escherichia coli</i>	<i>Lactobacillus casei</i>	<i>Lactobacillus plantarum</i>
Labelled compound	...	PteGlu	p-AB	PteGlu	p-AB
Label extracted from bacteria (%)		22	74	46	26
Label remaining in medium (%)		27	24	19	40
Label in debris (%)		51	1.5	35	30

Streptococcus faecalis folates from [^3H]PteGlu

Anion exchange chromatography of [^3H]folates before and after treatment with guinea-pig mucosal 'conjugase' showed that almost all the folate from stationary phase *S. faecalis* was conjugated by the addition of further glutamyl residues and that they were primarily formylated derivatives (i.e. 5-CHO- $\text{H}_4\text{PteGlu}_x$, 10-CHO- $\text{H}_4\text{PteGlu}_x$) (Fig. 1). After KMnO_4

Fig. 1. QAE Sephadex chromatography of folate compounds extracted from the folate-requiring strain of *S. faecalis* (NCIB6459). Organisms were grown in folate-deficient medium supplemented with [^3H]pteroyl-L-glutamate. ●, Extract; ○, extract subjected to guinea-pig mucosal 'conjugase' preparation. Letters indicate approximate positions of standard compounds on linear gradient: (a) p-AB, (b) 10-CHO- H_4PteGlu , (c) p-ABGlu, (d) 5-CHO- H_4PteGlu , (e) 5-CH $_3$ - H_4PteGlu , (f) PteGlu, (g) PteGlu $_7$. Gradient composed of 0 to 0.6 M-NaCl (2×300 ml) in 50 mM-tris-HCl pH 7.5. Flow rate 20 ml/h at 20 °C. Standards of (b), (c) and (d) were detected by extinction at 280 nm; the other standards were radioactive.

Fig. 2. DEAE-cellulose chromatography of *p*-amino[3'- ^3H]benzoyl-poly- γ -L-glutamate derivatives obtained by alkaline KMnO_4 oxidation of folates from the folate-requiring strain of *S. faecalis* (NCIB6459). Numerals indicate approximate elution positions ($\pm 3\%$) of chemically synthesized standard compounds, i.e. *p*-ABGlu $_x$ ($x = 0, 1, \dots, 7$). Gradient composed of 0 to 1.0 M-KCl (550 ml + 235 ml) in 5 mM-phosphate buffer pH 7.0. Flow rate 10 to 15 ml/H at 20 °C.

Fig. 3. QAE Sephadex chromatography of folate compounds extracted from a *p*-AB-requiring mutant of *E. coli* (NCIB8109). Organisms were grown in defined medium supplemented with [^{14}C]*p*-AB. ●, Extract; ○, extract treated with rat liver 'conjugase' preparation; for other symbols see Fig. 1.

oxidation of these folate compounds, the tritiated *p*-aminobenzoyl-poly- γ -L-glutamates produced were mainly derivatives of tetra- and pentaglutamate folates (Fig. 2).

Escherichia coli folates from [^{14}C]*p*-AB

The elution profile of the [^{14}C]folates extracted is shown in Fig. 3 together with compounds released after treatment with a conjugase preparation from rat liver. After

Fig. 4. DEAE-cellulose chromatography of *p*-aminobenzoyl¹⁴C poly- γ -L-glutamates obtained by alkaline KMnO_4 oxidation of native folates of a *p*-AB-requiring mutant of *E. coli* (NC1B8109). Other details as in Fig. 2.

Fig. 5. QAE Sephadex chromatography of folate compounds extracted from the folate requiring strain of *L. casei* (NC1B10463). Organisms were grown in folate-deficient medium supplemented with [3',5'- n]- ^3H pteroyl-L-glutamate K. ●, Extract; ○, extract subjected to guinea-pig mucosal 'conjugase' preparation. Other details as in Fig. 1.

'conjugase' treatment the primary products were 5-CHO- H_4PteGlu and 10-CHO- H_4PteGlu . Chromatography of permanganate-oxidized extracts (Fig. 4) revealed that the predominant *p*-aminobenzoyl¹⁴C poly- γ -glutamate derivatives were probably *p*-AB $\text{Glu}_{4,6,7}$ with lesser quantities of triglutamate and compounds with greater than seven glutamate residues.

Lactobacillus casei folates from [^3H]PteGlu

As with *S. faecalis* and *E. coli* the pteroylglutamates resulting from [^3H]PteGlu metabolism in *L. casei* are all conjugated. The major product of guinea-pig 'conjugase' action

Fig. 6. DEAE-cellulose chromatography of tritiated *p*-aminobenzoylpoly- γ -L-glutamates from the folate requiring strain of *L. casei* (NCIB10463) obtained by alkaline oxidation of native folates with KMnO_4 . ●, Extract; ○, extract after partial hydrolysis with exogenous guinea-pig 'conjugase' preparation. Other details as in Fig. 2.

appears to be 5-CHO- H_4PteGlu with smaller amounts of 10-formyl- and 5-methyltetrahydropteroylglutamates (Fig. 5). Chromatography on the DEAE-cellulose before and after 'conjugase' treatment showed that polyglutamate derivatives with less than seven glutamyl residues (i.e. eluting before 300 ml) were absent. The predominant compounds are considerably more acidic and likely to have nine or more glutamate residues, though there are as yet no synthetic standards available for comparison.

Lactobacillus plantarum folates from [^{14}C]*p*-AB

A preliminary study of the folate compounds synthesized by this organism indicated that they were essentially similar to those of *L. casei*, most of the ^{14}C activity being associated with derivatives having more than seven glutamyl residues. Small quantities of *p*-ABGlu_{6,7} were detected but nothing of shorter chain length.

DISCUSSION

While numerous workers have reported the occurrence of polyglutamate folates in yeast (Piffner *et al.* 1946; Schertel, Boehne & Libby, 1965) and various bacteria, e.g. *Staphylococcus* (Martin & Koft, 1972), *Bacillus* (Wright, 1956), *Diplococcus* (Sirotnak, Donati & Hutchinson, 1963), *Coxiella* (Matthews, Silverman & Paretsky, 1963), *Clostridium* (Parker, Wu & Wood, 1971) and *Escherichia* (Kozloff & Lute, 1973), few have employed microbes to synthesize radioactively-labelled folate compounds. Radioactively-labelled biologically-active pteroyltriglutamate derivatives, namely $\text{H}_4\text{PteGlu}_3$ and 10-CHO- $\text{H}_4\text{PteGlu}_3$, have been obtained enzymically from the unlabelled 5,10-CH=H₄PteGlu₃ of *Clostridium acidurici* (Curthoys *et al.* 1972). At present, enzymic synthesis (either *in vitro* or *in vivo*) of formyl substituted or unsubstituted tetrahydropteroylglutamates is the only method of providing biologically active diastereoisomers of these compounds (Nixon & Bertino, 1971; Matthew & Huennekens, 1960). Chemically-synthesized racemic methenyltetrahydropteroyl-L-glutamate (5,10-CH₂-H₄PteGlu) can be resolved chromatographically into its biologically active and inactive diastereoisomers (Yeh & Greenberg, 1965; Kaufman,

Donaldson & Keresztesy, 1963) and these may in turn be chemically reduced to the corresponding diastereoisomers of 5-methyltetrahydropteroyl-L-glutamate ($5\text{-CH}_3\text{-H}_4\text{PteGlu}$) (Rudiger, 1970; Weir, Brown, Freedman & Scott, 1973).

Chromatographic separation of pteroylpoly- γ -L-glutamates having differing numbers of glutamate residues, degrees of reduction with respect to the pterin nucleus and a variety of substituents on the N5 and/or N10 position is probably impossible to achieve by existing techniques (Shin *et al.* 1972). However, if the variation is confined to either the degree of conjugation or to the type of substitution and reduction, chromatographic separation becomes possible as evidenced by the present study employing either chemical oxidation of the C9-N10 bond or enzymic hydrolysis of γ -peptide bonds.

It appears that useful approaches to the synthesis of substituted reduced pteroylpolyglutamates could be based on the synthetic properties of the bacteria described in this paper. High specific activity ^3H -labelled or lower specific activity ^{14}C -formyl derivatives with four to seven glutamate residues could be synthesized by *E. coli* or *S. faecalis* and separated chromatographically after conversion to 10-formyltetrahydropteroylglutamates by acidification and neutralization (Houlihan, Davidson, Scott & Brown, 1973). The advantage of using the latter organism is its apparent inability to synthesize or use methylated folates (McElwee & Scott, 1972). Methylated derivatives could be obtained by chemical reduction (Chanarin & Perry, 1967) of the formyl derivatives after isolation or, in the case of mixtures of formyl and methyl derivatives (e.g. *L. casei*), before separation.

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