FOELATE COENZYMES OF LACTOBACILLUS CASEI AND STREPTOCOCCUS FAECALIS

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SUMMARY

During growth in the presence of 10 ng of [3H]pteroylglutamic acid (PteGlu) per ml of medium, Lactobacillus casei (ATCC 7469) incorporated 80 to 90% and Streptococcus faecalis (ATCC 8043) 40 to 60% of the added radioactivity. Folate compounds in L. casei were identified as reduced derivatives of PteGlu3 (~19%), of PteGlu4 (~42%), of PteGlu5 (~14%), and PteGlu6 (~8%); in S. faecalis as reduced derivatives of PteGlu5 (~24%), of PteGlu4 (~61%), and PteGlu3 (~4%). After treatment of cell extracts with hog kidney conjugase ~39% of folate compounds in Z. casei were converted to tetrahydrofolic acid (HtPteGlu), ~26% to 5-methyl-HIPteGlu, and ~10% to 5- and 10-formyl-HPteGlu; in the case of S. faecalis ~25% were converted to HtPteGlu and ~45% to 5- and 10-formyl-HPteGlu. No 5-methyl-HPteGlu derivatives could be found in S. faecalis. Simple methods for the preparation and separation of the biologically active forms of 5-methyl-[3H]HtPteGlu and 5-formyl-[3H]HtPteGlu (n = 1 to 9 glutamic acid residues) with a high specific radioactivity are described.

Growth of L. casei in the presence of 100 ng of PteGlu-[U-14C]Glu-Glu or 100 ng of PteGlu3-[U-14C]Glu-Glu per ml of medium was the same as in the presence of 100 ng of PteGlu per ml. PteGlu3 (100 ng per ml of medium) did not inhibit the uptake of [3H]PteGlu (0.3 ng per ml of medium), in contrast to PteGlu3 (100 ng per ml) and PteGlu (100 ng per ml). L. casei converted PteGlu-[U-14C]Glu and PteGlu3-[U-14C]Glu-Glu to the same metabolites as [3H]PteGlu without loss of the 14C label.

The Merrifield synthesis for the preparation of folic acid polyglutamates, as developed by Baugh et al. (2), is relatively time-consuming and expensive for preparation of compounds with high specific radioactivity. Furthermore, folate derivatives obtained by this method are pteroyl compounds rather than the tetrahydro derivatives, and reduction of the pteroyl compounds by chemical methods forms two diastereoisomers, of which only one is biologically active.

Consequently, we considered the possibility of a biological synthesis, using Lactobacillus casei (ATCC 7469) and Streptococcus faecalis (ATCC 8043), commonly used for the microbiological assay of folic acid. Both microorganisms lack the ability to synthesize folic acid and therefore require folic acid or its reduced derivatives in the growth medium. Both convert folic acid intracellularly to unidentified reduced polyglutamates (3, 4). The methods developed and identification of the products are described.

MATERIALS AND METHODS

Materials—[3H]PteGlu1 labeled in the 9, 3', and 5' positions (specific activity: 15 to 40 Ci per mmole, depending on the batch received from the supplier) and (±)-5-{Me-i4C}methyl-HPteGlu (specific activity: 50 mCi per mmole), were obtained from Amer-

The abbreviations used are: PteGlu, pteroylglutamic acid; folinic acid: PteGlu3, pteroylmonoo- to nonaglutamic acid: n indicates the number of glutamic acid residues; H4PteGlu, 5,6,7,8-tetra-

The purpose of our study was to develop a simple method for the preparation of biologically active, reduced folate compounds and polyglutamate derivatives, which were labeled with tritium and had the same high specific activity as commercially available [3H]folinic acid (~40 Ci per mmole). Such derivatives should be useful as reference compounds for the identification of folates in natural materials and for studies concerning uptake and metabolism of natural occurring folate derivatives.

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mmole, were synthesized according to the method of Baugh et al. (2). All folic acid derivatives were purified by chromatography on QAE-Sephadex A-25 before use.

Separation of Folate Compounds—QAE-Sephadex columns were eluted with exponential gradients of triethylammonium bicarbonate buffer (0.1 to 1.0 M), pH 7.0 (8). DEAE-cellulose columns with exponential gradients of potassium phosphate buffer (0.01 to 0.5 M), pH 6.0 (9), and Sephadex G-15 and G-25, fine, columns were eluted with 0.1 M potassium phosphate buffer, pH 7.0 (4). The columns were run in the dark at room temperature and all buffers used were 0.2 m in 2-mercaptoethanol.

Chromatography on QAE-Sephadex A-25 has the advantage that the eluant buffer is a volatile compound which can be removed easily by a flash evaporator at reduced pressure (14 mm Hg, 30°). Furthermore, the high capacity of QAE-Sephadex (～3.0 meq per g compared with 0.7 meq per g of DEAE-cellulose) allows application of samples containing up to 100 mg of folate derivatives on a gel bed of ～0.9 × 50 cm. Since folate compounds are eluted at higher buffer concentrations from QAE-Sephadex (0.3 to 0.8 M) than from DEAE-cellulose (0.1 to 0.6 M), columns with relatively small dimensions could be used to separate or desalt (or both) samples containing relatively large amounts of salt. However, triethylammonium bicarbonate is inhibitory to L. casei and interferes with the microbiological assay at higher concentrations. Also, it was observed that DEAE-cellulose gives better resolution of polyglutamate derivatives than QAE-Sephadex under the conditions employed. QAE-Sephadex was therefore used to desalt fractions obtained from DEAE-cellulose columns, to purify synthetic folate compounds, and for the separation of extracts of L. casei and S. faecalis containing relative large amounts of salt.

Sephadex G-15 and G-25 column chromatography were used to determine the relative number of glutamic acid residues present in folate derivatives from L. casei and S. faecalis and to separate folate acid monoglutamate derivatives. HPteGlu, H2PteGlu, and PteGlu elute in similar positions, but 5-formyl-HPteGlu and 10-formyl-HPteGlu are less absorbed by the gel and well separated. Sephadex column chromatography also separates (see Fig. 5) 5-methyl-HPteGlu and 5-formyl-HPteGlu, which have similar elution positions, from DEAE-cellulose.

Aminobenzoylglutamic acid, a major degradation product of folate acid derivatives, elutes from DEAE-cellulose at a position close to 10-formyl-HPteGlu, but elutes before 5-formyl-HPteGlu with the Sephadex column. Aminobenzoic acid, which elutes from Sephadex at a position similar to 5-formyl-HPteGlu but elutes before 10-formyl-HPteGlu from DEAE-cellulose columns, was not observed as a degradation product of folate derivatives when purified [3H]PteGlu was used as substrate for L. casei and S. faecalis.

For the interpretation of chromatograms from Sephadex columns, it should be noted that folate derivatives are considerably adsorbed to the gel, when buffers with a high ionic strength are used as eluant (for instance, PteGlu is eluted later than glutamic acid, when 0.1 M phosphate buffer is used as eluant) (4). However, formyl derivatives are adsorbed less than PteGlu, HpPteGlu, and 5-methyl-HpPteGlu. This is valid also for polyglutamates of folic acid and for 10-formyl-HpPteGlu, which elutes approximately at the position of PteGlu,+. The chromatographic behavior of folate derivatives is described in detail by Shin et al. (4).

Microbiological Methods—The microbiological assay procedure with L. casei (ATCC 7469), S. faecalis (ATCC 8043), and Pediococcus cerevisiae (ATCC 8081) was carried out according to Bird et al. (10), with slight modifications employed in this laboratory (11). Growth was measured turbidimetrically at 640 nm.

Growth medium and growth conditions for L. casei and S. faecalis were the same as those used for the microbiological determination of folic acid derivatives with these microorganisms. The inoculum used for 100 ml of medium contained not more than 1 ng of unlabeled folic acid derivatives.

For experiments with washed cells, L. casei was grown in the presence of 1 ng of folic acid per ml of medium at 37° for 18 hours. The cells were separated from the medium by centrifugation (2000 × g, 4°) and washed several times (at 4°) with a solution containing 20 g of glucose, 20 g of sodium acetate, 1 g of K2HPO4·3 H2O, 1 g of ascorbic acid, and 150 mg of L-glutamic acid in 1 liter. This solution was adjusted to pH 6.7 with 1 N NaOH. After washing, the cells were used directly as a suspension in the same buffer solution; 1 ml of the final assay mixture contained ~11 mg of cell dry weight corresponding to an optical density of 0.37 after a dilution of 1:40. The cells were incubated in a shaker at 37°, and aliquots were removed at convenient time intervals. Cells were separated from the buffer by centrifugation, washed once with the same volume of buffer, and resuspended in 1.5 ml of buffer. Folate compounds were released by heating the sample for 10 min at 100°. Cell debris was removed by centrifugation and the sample was applied directly to the Sephadex columns.

Preparation of hog kidney conjugase and chicken pancreas conjugase and the conjugase treatment of folate compounds were made according to Bird et al. (10). Cabbage conjugase was prepared as described by Tamura et al. (12). The approximate
activity of hog kidney conjugase was determined by the isotopic assay, as developed by Baugh et al. (2), with PteGlu-[U-14C]Glu-Glu (specific activity: 0.4 mCi per mmole) as substrate. Assays with hog kidney conjugase were composed of (final volume, 5 ml) 3.5 ml of sodium acetate buffer (0.1 M, pH 4.8) containing 0.1% ascorbic acid, 100 to 500 ng of folic acid derivatives, and 0.5 ml of a hog kidney conjugase preparation; 1 ml of this preparation converted ~1 µg of PteGlu to PteGlu in 1 hour. When necessary, the assay preparation was adjusted to pH 4.8 with 0.1 N NaOH. The incubation time was 6 hours at 37°. For short incubations, the amount of folic acid derivatives was 100 to 200 ng, and 0.1 ml of hog kidney conjugase preparation in a total volume of 5 ml was used.

All conjugase enzymes used are relatively crude preparations, and the presence of enzymes converting folic acid cannot be excluded. To examine this possibility, 5-methyl-H$_2$PteGlu and 5-formyl-H$_2$PteGlu were treated with the respective conjugase preparation under the conditions used to digest polyglutamate derivatives (6 hours at 37° in the presence of 0.1% ascorbic acid, ~200 ng of folic acid derivative per ml of assay preparation). 5-Methyl-H$_2$PteGlu could be recovered unchanged. 5-Formyl-H$_2$PteGlu was partially converted to 5,10-methenyl-H$_2$PteGlu during treatment with hog kidney conjugase (pH 4.8) and adjusting the sample to pH 7 resulted in the conversion of 5,10-methenyl-H$_2$PteGlu to 10-formyl-H$_2$PteGlu. 5-Formyl-H$_2$PteGlu was recovered unchanged after treatment with chicken pancreas or cabbage conjugase (pH 7.8).

**Extraction of Folate Compounds**—After incubation at 37° for 18 hours the cells were harvested by centrifugation (4°, 20,000 × g). Cells obtained from 100 ml of medium were suspended in 30 ml of acetone (0.2 M 2-mercaptoethanol) and kept at 40° under nitrogen for 1 hour. After centrifugation, the cell residues were washed once more with 30 ml of acetone (0.2 M 2-mercaptoethanol) and resuspended in 30 ml of 0.01 M potassium phosphate buffer, pH 7.0 (0.2 M 2-mercaptoethanol). The acetone washings contained less than 0.1% of the radioactivity incorporated into the cells and were discarded. After centrifugation of the suspension in phosphate buffer, 95% of the radioactivity was found in the supernatant. The washing was repeated once and cell residues were discarded. This phosphate buffer extract is subsequently called "water extract."

To convert oxygen-sensitive derivatives of folic acid such as H$_2$PteGlu and 10-formyl-H$_2$PteGlu to more stable 5,10-methenyl-H$_2$PteGlu, the cells, after treatment with acetone, were suspended in 50 ml of formic acid (97 to 100%, Matheson, Colman & Bell), containing 0.1% ascorbic acid. The mixture was kept at 60° for 1 hour under nitrogen, formic acid was removed by a flash evaporator, and the remaining material was suspended in 30 ml of distilled water (0.2 M 2-mercaptoethanol). Cell residues were removed by centrifugation and the extract (called "formic acid extract") was stored at 4° after adjusting to pH 2.

5- and 10-Formyl-H$_2$PteGlu form 5,10-methenyl-H$_2$PteGlu at pH 2. H$_2$PteGlu is formylated by formic acid in the N-10 position (13) and subsequently converted to 5,10-methenyl-H$_2$PteGlu.

5-Methyl-H$_2$PteGlu is converted by formic acid to a compound with properties consistent with a structure such as 10-formyl-5-methyl-H$_2$PteGlu. This compound elutes at the same position as 10-formyl-H$_2$PteGlu from Sephadex G-20 and slightly before from DEAE-cellulose columns. Treatment with 0.5 N NaOH at 37° for 1 hour results in the quantitative conversion of this compound to 5-methyl-H$_2$PteGlu. This is in agreement with the observation that the formyl group of 10-formyl-H$_2$PteGlu can be removed easily under alkaline conditions (13). After treatment of a mixture of 5-[Me-14C]methyl-H$_2$PteGlu and 5-methyl-[H]H$_2$PteGlu with formic acid, both 14C and 1H activity was found to co-chromatograph on Sephadex and DEAE-cellulose, i.e., formic acid does not remove the methyl group. The compound supports growth of L. casei to the same extent as 5-methyl-H$_2$PteGlu.

When the purification and isolation of formyl derivatives of H$_2$PteGlu was desired, 5,10-methenyl-H$_2$PteGlu was converted to 5-formyl-H$_2$PteGlu according to the method of Beaven and Blair (14). In contrast to 10-formyl-H$_2$PteGlu, 5-formyl-H$_2$PteGlu is quite stable in the presence of oxygen, and column purification could be performed without significant losses.

10-Formyl-5-methyl-H$_2$PteGlu, the reductase product of 5-methyl-H$_2$PteGlu with formic acid, is not converted to 5-methyl-H$_2$PteGlu during reduction with NaBH$_4$ and can be recovered unchanged from the reaction mixture. In order to remove the formyl group, the "formic acid extract" after reduction with NaBH$_4$ was adjusted to pH 7 with 10 N NaOH and was kept at 37° for 1 hour. Since at this pH polyglutamate forms of folic acid are very sensitive to oxygen (15), care was taken to exclude any oxygen during this treatment. Adjustment was made to pH 7, and the solution stored at 4° until further use.

Since S. faecalis does not contain 5-methyl derivatives of folic acid, i.e., no 10-formyl-5-methyl-H$_2$PteGlu is present in the "formic acid extract," the last step was omitted, and the solution after reduction with NaBH$_4$ was directly adjusted to pH 7 to destroy excessive NaBH$_4$. Before application to a QAE-Sephadex column, the extract was diluted to ~300 ml to reduce the salt concentration.

In the case of S. faecalis, a large percentage (~40%) of the added radioactivity remained in the medium, mainly as polyglutamates. The radioactive material was recovered as follows. The medium (adjusted to pH 7.5) was applied to a QAE-Sephadex column (1.5 x 50 cm) previously washed with ~200 ml of 0.1 M triethylammonium bicarbonate buffer. After application of the sample, the column was washed with 300 ml of 0.2 M buffer, and folate compounds eluted with 200 ml of 1.0 M buffer. The buffer was removed by a flash evaporator, with the last traces of triethylamine and water removed by drying the material over P$_2$O$_5$ in a vacuum (0.1 mm Hg). The residue was dissolved in formic acid (97 to 100%) and treated as described above.

Radioactivity counting was carried out in a Beckman CPM-100 liquid scintillation counter using Aquasol as scintillation liquid.

**RESULTS AND DISCUSSION**

**Identification of Folic Acid Coenzymes in L. casei and S. faecalis** —To determine the optimal conditions for [H]folic acid uptake, L. casei and S. faecalis were grown in the presence of various amounts of folic acid. The growth response of L. casei to 10, 100, and 1000 ng of folic acid per ml of medium is the same (Fig. 2), indicating that all of these levels of folic acid support maximum growth for L. casei under the conditions employed.
In the presence of 1 and 10 ng of PteGlu per ml of medium, 80 to 90% of the added radioactivity was incorporated into the cells after 15 hours of incubation; in the presence of 100 ng per ml, only 40 to 60% was incorporated. At the end of the logarithmic growth phase an increase of the radioactivity in the medium was observed at lower folate acid levels (1 and 10 ng per ml). We did not investigate whether the increase was due either to cell lysis or to excretion of folate acid derivatives or breakdown products (or both) into the medium. A level of 10 ng of folate acid per ml of medium and an incubation time of 18 hours were chosen for the further experiments.

In the case of S. faecalis, 65 to 75% of the added radioactivity was incorporated into the cells in the presence of 1 ng of PteGlu per ml of medium; 40 to 60% in the presence of 10 ng per ml; and less than 30% at a level of 100 ng per ml. A level of 10 ng per ml and an incubation time of 18 hours were chosen for study of folate compounds in S. faecalis.

Fig. 3A shows the chromatographic pattern of folate derivatives in cells on Sephadex G-25 columns after incubation of L. casei in the presence of 10 and 100 ng of [3H]PteGlu per ml of medium for 24 hours. The chromatographic pattern of folate acid derivatives in the medium after incubation of L. casei in the presence of 1, 10, and 100 ng of [3H]PteGlu per ml of medium for 24 hours is shown in Fig. 3B. At a level of 1 and 10 ng per ml, the elution pattern of the cell extract is the same. However, at a level of 100 ng per ml, the maximum radioactivity eluted later, an indication that, in the presence of large amounts of folate acid, polyglutamates with fewer glutamic acid residues are formed (Fig. 3A). At levels of 1 and 10 ng per ml, folate acid derivatives in the medium are almost exclusively polyglutamate derivatives, but at a level of 100 ng per ml a large proportion of PteGlu remained in the medium (Fig. 3B). Folate compounds in the medium elute slightly later (maximum elution of radioactivity at Fractions 35 and 36 in the presence of 10 ng of folate acid per ml of medium) than folate derivatives extracted from the cells (maximum elution at Fractions 34 and 35, in the presence of 10 ng of folate acid per ml of medium). This suggests that folates with fewer glutamic acid residues may be preferentially released by the cell. The identity of folates in the medium was not further determined in the case of L. casei. However, the values for folate derivatives (determined as folic acid by the L. casei assay) in the medium after treatment with hog kidney conjugase were 80 to 90% as high as values calculated from the radioactivity. Thus, less than 20% of the radioactivity remaining in the medium can be attributed to breakdown products of folic acid, which cannot support growth for L. casei.

The identification of natural folates is difficult due to the variety and instability of reduced folate acid derivatives. In preliminary experiments we observed considerable amounts of degradation products even in the presence of 0.1% ascorbic acid in the extraction buffer, when the folates were released by autoclaving (120°, 5 min). The extraction procedure involving acetone treatment of the cells avoids high temperature and fewer breakdown products are obtained. It has the further advantage of yielding cell residues essentially free of water, which can be treated directly with formic acid. A temperature of 40° was maintained during the acetone treatment to destroy enzymatic activities.

To determine the number of glutamyl residues in folate derivatives, it is convenient to deal with only one form of folate acid (i.e. 5-formyl-, 5-methyl-[H]PteGlu, etc.), preferably one which is relatively stable against oxidation. This was accomplished by converting 5-formyl-, 10-formyl-, and H1PteGlu to 5,10-methenyl-[H]PteGlu, and consecutive reduction of the latter to 5-methyl-[H]PteGlu. PteGlu and H1PteGlu are formylated by formic acid in the N 10 position (13); the product of reduction with NaBH4 was not examined. However, the amount of PteGlu and H1PteGlu, if present at all, was too small to be determined. 5-Methyl-[H]PteGlu, was converted by formic acid to 5-methyl-10-formyl-[H]PteGlu. After reduction of the “formic acid extract” with NaBH4, the formyl group was cleaved with NaOH. The formylation and reduction steps gave yields of between 90 and 90% as determined by co-chromatography of the reaction product after treatment with hog kidney conjugase, with authentic compounds on DEAE-cellulose and Sephadex G-15 columns.

To determine the relative proportion of formyltetrahydro, 5-methyltetrahydro, and tetrahydro forms in the bacterial cells, the “water extract” was treated with hog kidney conjugase and applied to a DEAE-cellulose column (Fig. 4, A and B) and to various amounts of folic acid under comparable conditions.
FIG. 4. DEAE-cellulose column chromatography of the "water extract" of (A) Lactobacillus casei and (B) Streptococcus faecalis after treatment with hog kidney conjugase. Column size, 0.9 x 40 cm; gradient, 100 ml of 0.01 M potassium phosphate buffer (pH 6.0) in the mixing chamber and 0.8 M potassium phosphate buffer (pH 6.0) in the reservoir; sample size, 10 ml, containing ~1 mg of 10-formyl-H₃PteGlu and ~10,000 dpm 5-[Me⁻¹⁴C]methyl-H₃PteGlu. (A) indicates the elution position of H₃PteGlu and PteGlu, (B) of 5-formyl-H₃PteGlu, and (C) of 10-formyl-PteGlu under comparable conditions.

Sephadex G-15 columns (Fig. 5, A and B). The exact proportions of 10-formyl-, 5-formyl-, and 5,10-methenyl-H₃PteGlu could not be determined, since the hog kidney conjugase treatment is made at pH 4.8 and considerable interconversion of these derivatives occurs under this condition. The value obtained for H₃PteGlu represents the sum of H₃PteGlu and 5-methyl-H₃PteGlu. 5,10-Methylene-H₃PteGlu is stable only in solution and is hydrolyzed easily to H₃PteGlu at acid pH (16).

The radioactive material (eluted as Peak II in Fig. 4, A and B) co-chromatographed with H₃PteGlu on DEAE-cellulose and Sephadex G-15, and it was found to support the growth of L. casei, S. faecalis, and P. cerevisiae. H₃PteGlu represents ~39% of the total radioactivity applied to the column in the case of L. casei and ~25% in the case of S. faecalis. Peak II in Fig. 4A (~33%) consists mainly of 5-methyl-H₃PteGlu (~80%) of the radioactivity, eluted as Peak I) according to its elution pattern on DEAE-cellulose and Sephadex G-15 and its microbiological behavior. 5-Formyl-H₃PteGlu elutes slightly before 5-methyl-H₃PteGlu on DEAE-cellulose (Fig. 4B). The proportion of 5-formyl-H₃PteGlu in Fig. 4A was determined by differential microbiological assay or Sephadex G-15 column chromatography, where 5-formyl-H₃PteGlu is well separated from 5-methyl-H₃PteGlu (Fig. 5A). According to Fig. 5A, ~7% of the radioactivity applied to the Sephadex column consists of 5-formyl-H₃PteGlu.

Peak II in Fig. 4B (~38%) represents exclusively 5-formyl-H₃PteGlu according to its elution pattern (Figs. 4B and 5B) and microbiological assay. The absence of 5-methyl-H₃PteGlu derivatives in S. faecalis is in agreement with the observation that this compound does not support the growth of S. faecalis (3) and is not metabolized by this organism (10). Peak I in Fig. 4A and B, which is eluted at a position similar to the position of 10-formyl-H₃PteGlu, gave only a partial response to P. cerevisiae (~11% of Peak I in Fig. 4A, ~15% in Fig. 4B) and S. faecalis (~20% in Fig. 4A, ~36% in Fig. 4B) compared to the value calculated from the radioactivity. The higher growth response of S. faecalis than of P. cerevisiae indicates that part of the material eluted as Peak I in Fig. 4, A and B, consists of 10-formyl-PteGlu, the oxidation product of 10-formyl-H₃PteGlu, since P. cerevisiae responds only to 10-formyl-H₃PteGlu, whereas S. faecalis shows growth response to 10-formyl-H₃PteGlu and 10-formyl-PteGlu. Peak I in Fig. 4A and B elutes between 10-formyl-H₃PteGlu and 5-formyl-H₃PteGlu from DEAE-cellulose and approximately at the same position as 10-formyl-H₃PteGlu from the Sephadex column. Of the total radioactivity applied to the column 17% eluted as Peak I in Fig. 4A, ~19% in Fig. 4B. The sum of 10-formyl- forms can therefore be estimated as ~3% in the case of L. casei and ~7% in S. faecalis. The remaining material eluted in Peak I (Fig. 4, A and B) and radioactive material eluted before Peak I (Fig. 4, A and B) represents...
degradation products of folic acid derivatives. Their identities were not further determined. Less than 3\% of the radioactivity applied to the DEAE-cellulose column in Fig. 4A eluted later than H2PteGlu (Peak III) at an elution position similar to the elution position of H2PteGlu. However, the amount of this material was too small for the identification.

Fig. 6A shows that more than 95\% of the radioactivity in the "water extract" from L. casei eluted before PteGlu from the Sephadex column and more than 80\% before PteGlu7. No reduced derivatives of PteGlu or PteGlu itself were present in either L. casei or S. faecalis (Fig. 6A and B). Folate compounds in S. faecalis eluted between PteGlu4 and PteGlu6, indicating that folate derivatives in S. faecalis are mainly tetra- and pentaglutamates (Fig. 6B).

For the further identification of folate compounds isolated from L. casei and S. faecalis, the folates were converted to the respective 5-methyl derivatives and subjected to QAE-Sephadex A-25 column chromatography (Fig. 7, A and B). The material in Peak III in Fig. 7A (~42\% of the applied radioactivity) was treated with hog kidney conjugase, and the reaction product was identified as 5-methyl-H4PteGlu by co-chromatography with authentic compounds. Short time incubation of this material with hog kidney conjugase results in the formation of polyglutamates with intermediate chain length as shown in Fig. 8.

The reaction products, obtained after incubation for 0, 1, 5, and 30 min, were combined and applied to a DEAE-cellulose column (Fig. 9). Nine peaks were observed, which co-chromatographed with 5-methyl-H4PteGlu after complete digestion with hog kidney conjugase. When chicken pancreas conjugase was used instead of hog kidney conjugase, the compounds eluted as Peaks II to IX in Fig. 9 co-chromatographed with Peak II (Fig. 9), and, when cabbage conjugase was used, the compounds in Peaks III to IX (Fig. 9) eluted at the same position as Peak

Fig. 6. Sephadex G-25 column chromatography of the "water extract" of (A) Lactobacillus casei and (B) Streptococcus faecalis. Column size, 0.8 × 175 cm; sample size, 1.5 ml, containing ~2200 dpm PteGlu3-[U-14C]Glu-Glu (specific activity: 0.4 mCi per mmole); flow rate, 13 ml per hour; V0: elution position of blue dextran; arrows indicate the elution position of reference compounds under comparable conditions.

Fig. 7. QAE-Sephadex A-25 column chromatography of the reduced "formic acid extract" of (A) Lactobacillus casei and (B) Streptococcus faecalis. Column size, 0.9 × 50 cm; gradient, 500 ml in A (300 ml in B) 0.1 M triethylamine bicarbonate buffer (pH 7.5) in the mixing chamber and 1.0 M triethylamine bicarbonate buffer (pH 7.0) in the reservoir; sample size, 100 ml (fractions were collected after absorption of the sample to the gel bed); flow rate, 30 ml per hour.

Fig. 8. Sephadex G-25 column separation of mixtures of 5-methyl-[3H]tetrahydrofolic acid polyglutamate derivatives obtained by treatment of Peak III of Fig. 7A with hog kidney conjugase for various time intervals. Column size, 0.8 × 190 cm; sample size, 1.5 ml; flow rate, 13 ml per hour; V0: elution volume of blue dextran.
flow rate, III (Fig. 9). Chicken pancreas conjugase and cabbage con-
ml, containing -100,000 dpm (&)-5-[Me-14C]methyl-H4PteGlu; 
obtained by treatment of Peak ZZZ of Fig. 7A with hog kidney 
conjugase. Column size, 0.9 X 50 cm; gradient, 300 ml, 0.01
of 5-methyl-[3H]tetrahydrofolic acid polyglutamate derivatives 
methyl-HIPteGluS, respectively, and eluted at the same position 
glutamyl residues the elution position is almost identical. In 
the position of PteGlu, Peak VI at the position of PteGlus, etc. 
before the respective PteGlu, and HJ'teGlu, derivatives (4). 
elution position of every peak in Fig. 9 was determined on 
Sephadex G-25 columns. Peak VII eluted approximately at 
to folic acid di- and triglutamates, respectively (10, 12). The 
chromatographed with synthetii: 5.methyl-HJ'teGlu3 and 5-
as the reference compound.
the case of Peaks III and V (Fig. 9), the material was co-
isolated and converted to the respective 5-methyl derivatives.
After treatment with hog kidney conjugase -70% of the ma-
lified as 5-methyl-H4PteGlu3. According to this, -61% of 
folate derivatives in S. faecalis is unable to synthesize 5-methyl-H4PteGlu.

Our results show that L. casei and S. faecalis are useful for a 
relatively simple preparation of poly- and monoglutamate 
derivatives of folic acid with a high specific radioactivity. 5-
Formyl derivatives with 4 glutamic acid residues or less are more 
conveniently prepared with S. faecalis, since this organism does 
not form 5-methyl-H4PteGlu4. 5-Formyltetrahydro derivatives 
can be easily converted into the respective 10-formyltetrahyr 
derivatives by the method of May et al. (5). Mono, di, , and 
triglutamate derivatives were obtained in yields of ~50% of 
the initial amount of folic acid added to the medium by digestion 
of the extracts with the suitable conjugase enzymes. 5-Formyl-
[3H]H4PteGlu as well as 5-methyl-[3H]H4PteGlus, isolated from 
L. casei or S. faecalis supported growth of L. casei as effectively 
as folic acid, i.e., values determined with the microbiological 
assay agreed with values calculated from the radioactivity in the 
range of experimental error (~10%). For the preparation of 
derivatives of PteGlus, S. faecalis gives the best yield. For 
higher polyglutamates L. casei should be used. The mixture of 
polyglutamates, obtained after digestion with hog kidney 
conjugase for suitable periods, can be separated on DEAE-
cellulose columns.

It is obvious that 14C-labeled folic acid derivatives can be ob-
tained by this same method, using as starting material commerci-
ally available 14C-labeled folic acid.

Because of the high specific activity possible (~40 Ci per 
nmole or 91 mCi per mg), these compounds can be used as refer-
cence compounds in amounts of a few nanograms for the iden-
tification of folate derivatives in biological materials. Folate 
compounds in rat red blood cells have been found to be 
mainly 5-methyl-H4PteGlus and 5-methyl-H4PteGlus,2 mainly 
reduced derivatives of PteGlus and PteGlus, in cabbage (19), 
mainly reduced derivatives of PteGlus and PteGlus, in romaine 
lettuce (20), and in orange juice.

Properties of Conjugase Enzymes—With respect to the differ-
ent conjugase preparation used in this study, our results indi-
icate that hog kidney conjugase removes the glutamyl residues 
one after the other (Fig. 8), i.e., the enzyme can be considered
2 Y. S. Shin, K. U. Buehring, and E. L. R. Stokstad, un-
published results.
3 T. Tamura, K. U. Buehring, Y. S. Shin, and E. L. R. Stokstad, 
unpublished results.
as an exopeptidase. In this respect, hog kidney conjugase shows the same properties as a conjugase enzyme isolated from human liver (21), which has also a similar pH optimum (3.5 to 4.5 in acetate buffer).

During the treatment with chicken pancreas conjugase (Fig. 10) or cabbage conjugase, negligible amounts of intermediates were formed. Thus, these enzymes either hydrolyze polyglutamates with fewer glutamyl residues faster than the original compound (or the substrate is released from the enzyme only after complete hydrolysis) or these enzymes are endopeptidases which convert folate polyglutamates specifically to diglutamates in the case of chicken pancreas conjugase, and to triglutamates in the case of cabbage conjugase. In agreement with the latter possibility, Rosenberg and Neumann (22) isolated an enzyme from chicken intestine, which converted PteGlu to PteGlu2 and Glu3.

Metabolism of Folic Acid in Washed Cells of L. casei—Fig. 11 shows the removal of [3H]PteGlu from the buffer at various levels of PteGlu by “washed” cells of L. casei (11 mg of cell dry weight per ml of buffer) at 37°. In the presence of 11, 110, and 1100 ng of PteGlu per ml of medium, the incorporation of radioactivity into the cells is rapid; more than 90% of the radioactivity is removed from the buffer after 10 min of incubation at levels of 11 and 110 ng of PteGlu per ml, ~85% at a level of 1100 ng of PteGlu per ml of buffer. In the presence of 1 ng of PteGlu per ml of saturation of the cells with folic acid is reached and only ~20% of the radioactivity is incorporated into the cells after 30 min. At longer incubation periods an increase of radioactivity in the buffer was observed in the presence of 1.1 and 11 μg of PteGlu per ml of buffer. Whether this was due to lysis of the cells or to an “overshoot” of the folic acid transport system was not determined.

Folic acid is rapidly converted to polyglutamate derivatives at a level of 11 ng of PteGlu per ml, 46% of the radioactivity incorporated after 5 min elutes at the position of PteGlu on the Sephadex column, ~18% after 10 min of incubation (Fig. 12A). After 5 min, polyglutamate derivatives elute mainly between PteGlu2 and PteGlu3; after 30 min, they elute before PteGlu5. This is an indication that polyglutamate derivatives are not synthesized by the use of preformed polyglutamic acid chains, but that glutamyl residues are attached one after the other to the folic acid moiety. This is in agreement with...
Although Griffin and Brown (23) in *E. coli* and Sakami et al. (24) in *Neurospora crassa*. In the presence of 1.1 μg of folic acid per ml of buffer, considerable amounts of PteGlu or H₂PteGlu (H₂PteGlu elutes at the same position as PteGlu from the Sephadex column and its proportion was not determined) remained inside the cell even after 1 hour of incubation and the major conversion product eluted at the position of PteGlu (Fig. 12B). These results show that *L. casei* can concentrate folic acid monoglutamates from the medium to a considerable degree—about 100-fold, assuming the intracellular water volume is ~4 μl per ng of cell dry weight (25)—without converting them to polyglutamate derivatives.

**Uptake and Metabolism of PteGlu**

Tamura et al. (11) showed that PteGlu is ~20% as active as folic acid in supporting growth of *L. casei*. PteGlu was found to support the growth of *L. casei* to the same degree as PteGlu. We were interested therefore to determine whether higher polyglutamates of folic acid enter the bacteria cell intact or are hydrolyzed before uptake by a conjugase enzyme.

When *L. casei* was incubated in the presence of 100 ng of [H]PteGlu, 100 ng of PteGlu-[U-¹³C]Glu-Glu, or 100 ng of PteGlu-[U-¹³C]Glu-Glu per ml of medium, the decrease in the radioactivity in the medium was the same in all three cases (Fig. 13) as was the growth response. Growth roughly paralleled the decrease of radioactivity in the medium under these conditions. After incubation for 24 hours the cells were extracted as described and the extract from incubations with PteGlu and PteGlu were co-chromatographed with the extract of cells incubated in the presence of 100 ng of [H]PteGlu per ml of medium. Both ¹⁴C and ¹³C radioactivity co-chromatographed on DEAE-cellulose and Sephadex G-25 columns, indicating that PteGlu and PteGlu enter the cell as polyglutamates and are converted to the same compounds as folic acid itself. These results also suggest that *L. casei* is not able to hydrolyze polyglutamates. Conjugase activity was not detectable in the medium or in sonically treated cell extracts. This is particularly surprising, since it is known that resting cells of *L. casei* can convert methotrexate to 4-amino-4-deoxy-10-methylpterioic acid (26). However, even in the presence of methotrexate at various levels we did not observe any conversion of PteGlu to Pte.

The initial rate of uptake of [H]PteGlu (3 ng per ml of medium) was not affected by PteGlu-[U-¹³C]Glu-Glu (100 ng per ml of medium) (Fig. 13), i.e. it was the same in the absence or presence of PteGlu. After longer incubation times, an increase of ³⁴Cl radioactivity in the medium is observed. This might be due to dilution of ³⁴Cl-labeled folic acid derivatives by derivatives derived from PteGlu and excretion of ³⁴Cl-labeled polyglutamates, which are taken up subsequently at the same slower rate as PteGlu itself.

PteGlu-[U-¹³C]Glu-Glu (100 ng per ml of medium) inhibited considerably the uptake of [H]PteGlu (0.3 ng per ml of medium), but to a lesser extent than did the addition of 100 ng of unlabeled PteGlu per ml of medium. On the basis of these results, one can speculate that PteGlu and PteGlu, but not PteGlu, share the same mechanism of uptake. Also the uptake of PteGlu is slower than that of PteGlu. Studies are in progress with ³⁴Cl and ¹³C labeled polyglutamate derivatives with a high specific radioactivity to determine the relative rates of uptake of these compounds in comparison with folic acid and transport characteristics of folic acid derivatives.

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**REFERENCES**


Fig. 13. Decrease of radioactivity during growth of *Lactobacillus casei* in medium containing, per ml, 100 ng of PteGlu plus: 1, 0.3 ng of [H]PteGlu (specific activity: 30 Ci per mmole); 1, 100 ng of PteGlu-[U-¹³C]Glu-Glu (specific activity: 0.4 mCi per mmole); 1, 0.3 ng of [H]PteGlu; 1, 100 ng of PteGlu-[U-¹³C]Glu-Glu; O, 0.3 ng of [H]PteGlu.
Folate Coenzymes of *Lactobacillus casei* and *Streptococcus faecalis*

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