Folate Coenzymes of Lactobacillus casei and Streptococcus faecalis

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SUMMARY

During growth in the presence of 10 ng of [H]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 PteGlu3 (~24%), of PteGlu4 (~61%), and PteGlu5 (~4%). After treatment of cell extracts with hog kidney conjugase ~39% of folate compounds in L. casei were converted to tetrahydrofolic acid (H4PteGlu), ~26% to 5-methyl-[H]PteGlu and ~10% to 5- and 10-formyl-[H]PteGlu; in the case of S. faecalis ~25% were converted to H2PteGlu and ~45% to 5- and 10-formyl-H3PteGlu. No 5-methyl-[H]PteGlu derivatives could be found in S. faecalis. Simple methods for the preparation and separation of the biologically active forms of 5-methyl-[H]H4PteGlu and 5-formyl-[H]H3PteGlu, (~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 PteGlu6 (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 purpose of our study was to develop a simple method for the preparation of folate compounds, as developed by Baugh et al. (2), which 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—[H]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-14C]methyl-H4PteGlu (specific activity: 50 mCi per mmole), were obtained from American Biochemical Corp. Both compounds were found to be grossly contaminated after storage of the unopened container at -20° for several weeks and were purified by chromatography on QAE-Sephadex A-25 (Fig. 1, A and B). The radiochemical purity of PteGlu was ~75%; of 5-methyl-H4PteGlu, ~84%. The major impurities of [H]PteGlu are p-amino-[H]benzoic acid (~4%) and p-amino-[H]benzoylglutamic acid (~9%) as determined by co-chromatography with the authentic compounds on Sephadex G-15 and DEAE-cellulose columns. The major impurity of (+)-5-[Me-14C]methyl-H4PteGlu (~13%) did not support the growth of L. casei and was not identified.

10-Formyl-H4PteGlu was prepared from 5-formyl-H4PteGlu (Leucovorin) (5), which was generously supplied by Dr. James M. Smith, Jr. (Lederle Laboratories). 5-Methyl-H4PteGlu was obtained by reduction of 5,10-methenyl-H4PteGlu with sodium borohydride (6), and H4PteGlu by reduction of PteGlu in glacial acetic acid with sodium dithionite (7). Folic acid polyglutamates PteGlu3 to PteGlu9, as well as PteGlu-[U-14C]Glu-Glu and PteGlu3-[U-14C]Glu-Glu, with a specific activity of 0.4 mCi per 

The abbreviations used are: PteGlu, pteroylglutamic acid; folinic acid; PteGlu3, pteroylmono- to nonaglutamic acid; n indicates the number of glutamic acid residues; H4PteGlu, 5,6,7,8-tetrahydopteroylglutamic acid.
Separation of Folate Compounds—QAE-Sephadex columns were eluted with exponential gradients of triethylammonium bicarbonate buffer (0.1 to 0.8 M), pH 7.5 (4). How-

erver, formyl derivatives are adsorbed less than PteGlu, H$_4$PteGlu, and 5-methyl-H$_4$PteGlu. This is valid also for polyglutamates of folate acid and for 10-formyl-H$_4$PteGlu, which elutes approximately at the position of PteGlu$^{+1}$. The chromatographic behavior of folate derivatives is described in detail by Shih 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 folate acid derivatives with these microorganisms. The inoculum used for 100 ml of medium contained not more than 1 ng of unlabeled folate acid derivatives.

For experiments with washed cells, L. casei was grown in the presence of 1 ng of folate acid per ml of medium at 37° for 18 hours. The cells were separated from the medium by centrifugation (2000 x g, 4°) and washed several times (at 4°) with a solution containing 20 g of glucose, 20 g of sodium acetate, 1 g of K$_3$HPO$_4$, 3 H$_2$O, 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 radioisotopic 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°C. 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$_4$PteGlu and 5-formyl-H$_4$PteGlu were treated with the respective conjugase preparation under the conditions used to digest polyglutamate derivatives (6 hours at 37°C in the presence of 0.1% ascorbic acid, ~200 ng of folic acid derivative per ml of assay preparation). 5-Methyl-H$_4$PteGlu could be recovered unchanged. 5-Formyl-H$_4$PteGlu was partially converted to 5,10-methenyl-H$_4$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$_4$PteGlu to 5-formyl-H$_4$PteGlu. 5-Formyl-H$_4$PteGlu was recovered unchanged after treatment with chicken pancreas or cabbage conjugase (pH 7.8).

Extraction of Folate Compounds—After incubation at 37°C for 18 hours the cells were harvested by centrifugation (4,000,000 × g). Cells obtained from 100 ml of medium were suspended in 30 ml of acetone (0.2 M 2-mercaptoethanol) and kept at 40°C 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$_4$PteGlu and 10-formyl-H$_4$PteGlu to more stable 5,10-methenyl-H$_4$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°C 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°C after adjusting to pH 2.

5- and 10-Formyl-H$_4$PteGlu form 5,10-methenyl-H$_4$PteGlu at acid pH. H$_4$PteGlu is formylated by formic acid in the N-10 position (13) and subsequently converted to 5,10-methenyl-H$_4$PteGlu.

5-Methyl-H$_4$PteGlu is converted by formic acid to a compound with properties consistent with a structure such as 10-formyl-5-methyl-H$_4$PteGlu. This compound elutes at the same position as 10-formyl-H$_4$PteGlu from Sephadex G-20 and slightly before from DEAE-cellulose columns. Treatment with 0.5 N NaOH at 37°C for 1 hour results in the quantitative conversion of this compound to 5-methyl-H$_4$PteGlu. This is in agreement with the observation that the formyl group of 10-formyl-H$_4$PteGlu can be removed easily under alkaline conditions (13). After treatment of a mixture of 5-[14C]methyl-H$_4$PteGlu and 5-methyl-[1H]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$_4$PteGlu.

When the purification and isolation of formyl derivatives of H$_4$PteGlu was desired, 5,10-methenyl-H$_4$PteGlu, was converted to 5-formyl-H$_4$PteGlu according to the method of Beavon and Blair (14). In contrast to 10-formyl-H$_4$PteGlu, 5-formyl-H$_4$PteGlu is quite stable in the presence of oxygen, and column purification could be performed without significant losses.

5,10-Methenyl-H$_4$PteGlu was converted to 5-methyl-H$_4$PteGlu by the method of Chanarin and Perry (6). One gram of sodium borohydride was dissolved in the necessary amount of 0.1 M K$_2$PO$_4$ solution to bring the “formic acid extract” to pH 7. This solution was added slowly to the “formic acid extract,” which contained 1 drop of 1-octanol to prevent excessive foaming and the mixture was kept for 30 min at room temperature under nitrogen.

10-Formyl-5-methyl-H$_4$PteGlu, the reaction product of 5-methyl-H$_4$PteGlu with formic acid, is not converted to 5-methyl-H$_4$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°C 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°C until further use.

Since S. faecalis does not contain 5-methyl derivatives of folic acid, i.e., no 10-formyl-5-methyl-H$_4$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 30 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 [14C]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 folic 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 folic acid derivatives or breakdown products (or both) into the medium. A level of 10 ng of folic 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 folic compounds in S. faecalis.

Fig. 3A shows the chromatographic pattern of folic 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 folic 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 folic acid, polyglutamates with fewer glutamic acid residues are formed (Fig. 3A). At levels of 1 and 10 ng per ml, folic 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 folic 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 folic 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 folic 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 folic derivative, it is convenient to deal with only one form of folic acid (i.e. 5-formyl- or 5-methyl-H4PteGlu, etc.), preferably one which is relatively stable against oxidation. This was accomplished by converting 5-formyl- to 5-formyltetrahydro, and H4PteGlu, to 5,10-methenyl-H4PteGlu, and consecutive reduction of the latter to 5-methyltetrahydro, PteGlu, and H2PteGlu, 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 H2PteGlu, if present at all, was too small to be determined. 5-Methyl-H4PteGlu, is converted by formic acid to 5-methyl-10-formyl-H4PteGlu, 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

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**Fig. 2.** Growth curve and decrease of the radioactivity in the medium during growth of *Lactobacillus casei* in the presence of various amounts of PteGlu. Growth curves: ◆, 1 ng per ml; ●, 10 ng per ml; ▲, 100 ng per ml; Δ, 1000 ng per ml. Decrease of the radioactivity in the medium: ◆, 1 ng per ml; ●, 10 ng per ml; ▲, 100 ng per ml; Δ, 1000 ng per ml.

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**Fig. 3.** Sephadex G-25 column chromatography of (A) cell extracts ("water extract") and (B) medium of *Lactobacillus casei* grown in the presence of various amounts of folic acid for 24 hours. Column size, 0.8 X 190 cm; sample size, 1.5 ml; flow rate, 13 ml per hour; V15, elution position of blue dextran; arrows indicate the elution position of reference compounds 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, 190 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-H,PteGlu and ~10,000 dpm [4-3H]methyl-H,PteGlu. (A) indicates the elution position of H,PteGlu and PteGlu, (B) of 5-formyl-H,PteGlu, 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-H,PteGlu 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 H,PteGlu represents the sum of H,PteGlu and 5,10-methene-H,PteGlu. 5,10-Methylene-H,PteGlu is stable only in solution and is hydrolyzed easily to H,PteGlu at acid pH (16).

The radioactive material (eluted as Peak III in Fig. 4, A and B) co-chromatographed with H,PteGlu on DEAE-cellulose and Sephadex G-15, and it was found to support the growth of L. casei, S. faecalis, and P. cervevisiae. H,PteGlu represents ~39% of the 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-H,PteGlu (~80%) of the radioactivity, eluted as Peak II) according to its elution pattern (Figs. 4A and 5B) and microbiological assay. The absence of 5-methyl-H,PteGlu 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. 4, A and B, which is eluted at a position similar to the position of 10-formyl-H,PteGlu, 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-H,PteGlu, the oxidation product of 5-formyl-H,PteGlu, since P. cerevisiae responds only to 10-formyl-H,PteGlu, whereas S. faecalis shows growth response to 10-formyl-H,PteGlu and 5,10-dihydro-H,PteGlu. 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 H₃PteGlu. 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 PteGlu₄ 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 PteGlu₅, 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-3H₄PteGlu 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
III (Fig. 9). Chicken pancreas conjugase and cabbage conjugase at pH 7.8 are known to convert folic acid polyglutamates to folate derivatives by the method of May et al. (5). The author used KMnO₄ oxidative degradation of polyglutamates of folic acid to their common oxidation product p-aminobenzoylpolyglutamate (18) and identified these compounds by co-chromatography on ion exchange columns with synthetic reference compounds. The author also observed that 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-H₄PteGlu. 5-Formyltetrahydro derivatives can be easily converted into the respective 10-formyltetrahydro 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-[H]H₄PteGlu as well as 5-methyl-[H]H₄PteGlu₃ 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 (~8%). For the preparation of derivatives of PteGlu₄, 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 obtained by this same method, using as starting material commercially available ¹⁴C-labeled folic acid.

Because of the high specific activity possible (~40 Ci per mmole or 91 mCi per mg), these compounds can be used as reference compounds in amounts of a few nanograms for the identification of folate derivatives in biological materials. Folate compounds in S. faecalis were identified by the same procedure. Peak III in Fig. 7B (~24%) as 5-methyl-H₄PteGlu₄, Peak II (~61%) as 5-methyl-H₄PteGlu₃. approximately 30% of the radioactivity, eluted as Peak I (~12%), was identified as 5-methyl-H₄PteGlu. According to this, ~61% of folate derivatives in S. faecalis are tetraglutamates, ~24% pentaglutamates, and ~4% triglutamates. In the case of S. faecalis, ~50% of the added radioactivity remained in the medium after 18 hours of incubation. Folate compounds were therefore isolated and converted to the respective 5-methyl derivatives. After treatment with hog kidney conjugase ~70% of the material eluted as 5-methyl-H₄PteGlu. Approximately 7% of the radioactive material were identified as 5-methyl-H₄PteGlu₄ (~36%) as 5-methyl-H₄PteGlu₃ (~18%) as 5-methyl-H₄PteGlu₂ and ~5% as 5-methyl-H₄PteGlu. As in the case of L. casei, the percentage of polyglutamates with fewer glutamyl residues was larger in the medium than in the cell.

It should be emphasized that the proportion of various folic acid polyglutamate derivatives depends on the amount of folic acid supplied in the medium. When L. casei was incubated in the presence of 100 ng of folic acid per ml of medium, the percentage of derivatives of PteGlu_4 was ~40 to 50%, compared with 12% in the presence of 10 ng per ml. The same conclusion holds for S. faecalis. In the presence of 100 ng of folic acid per ml of medium, roughly equal amounts of derivatives of PteGlu₄ and PteGlu₃ were found inside the cell, in contrast to only 3% of derivatives of PteGlu₄ and 61% of PteGlu₃ in the presence of 10 ng of PteGlu per ml of medium.

Similar results concerning the identification of folate acid conjugates in L. casei and S. faecalis were obtained recently by Dobbs (17). The author used KMnO₄ oxidative degradation of polyglutamates of folic acid to their common oxidation product p-aminobenzoylpolyglutamate (18) and identified these compounds by co-chromatography on ion exchange columns with synthetic reference compounds. The author also observed that 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-H₄PteGlu. 5-Formyltetrahydro derivatives can be easily converted into the respective 10-formyltetrahydro 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-[H]H₄PteGlu as well as 5-methyl-[H]H₄PteGlu₃ 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 (~8%). For the preparation of derivatives of PteGlu₄, 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 obtained by this same method, using as starting material commercially available ¹⁴C-labeled folic acid.

Because of the high specific activity possible (~40 Ci per mmole or 91 mCi per mg), these compounds can be used as reference compounds in amounts of a few nanograms for the identification of folate derivatives in biological materials. Folate compounds in rat red blood cells have been found to be mainly 5-methyl-H₄PteGlu₃ and 5-methyl-H₄PteGlu₂, mainly reduced derivatives of PteGlu₃ and PteGlu₄ in cabbage (19), mainly reduced derivatives of PteGlu₄ and PteGlu₃ in romaine lettuce (20), and in orange juice.³

Properties of Conjugase Enzymes—With respect to the different conjugate preparation used in this study, our results indicate that hog kidney conjugase removes the glutamyl residues one after the other (Fig. 8), i.e., the enzyme can be considered


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 PteGlu; and Glus.

**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 11 μg 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, ~13% after 10 min of incubation (Fig. 12A). After 5 min, polyglutamate derivatives elute mainly between PteGlu; and PteGlu; after 30 min, they elute before PteGlu; 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
FIG. 13. Decrease of radioactivity during growth of Lactobacillus casei in medium containing, per ml, 100 ng of PteGlu plus: △, 0.3 ng of [H]PteGlu (specific activity: 30 Ci per mmole); ●, 100 ng of PteGlu-[U-14C]GluGlu (specific activity: 0.4 mCi per mmole); □, 0.3 ng of [H]PteGlu; ○, 100 ng of PteGlu-[U-14C]GluGlu; O, 0.3 ng of [H]PteGlu.

studies of 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 H₃PteGlu (H₃PteGlu 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 and PteGlu₂. Tamura et al. (11) showed that PteGlu is ~20% as active as folic acid in supporting growth of L. casei. PteGlu₂ 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-14C]GluGlu, or 100 ng of PteGlu-[U-14C]GluGlu 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 PteGlu₂ 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 ³²P radioactivity co-chromatographed on DEAE-cellulose and Sephadex G-25 columns, indicating that PteGlu and PteGlu₂ 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 PteGlu₂-[U-¹⁴C]GluGlu (100 ng per ml of medium) (Fig. 13), i.e. it was the same in the absence or presence of PteGlu₂. After longer incubation times, an increase of ³²P radioactivity in the medium is observed. This might be due to dilution of ³²P-labeled folic acid derivatives by derivatives derived from PteGlu₂ and excretion of ³²P-labeled polyglutamates, which are taken up subsequently at the same slower rate as PteGlu₂.

PteGlu-[U-¹⁴C]GluGlu (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 PteGlu₂, but not PteGlu, share the same mechanism of uptake. Also the uptake of PteGlu₂ is slower than that of PteGlu. Studies are in progress with ³H- and ¹⁴C-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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