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Folate Metabolism in *Streptococcus faecalis*

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The possibility that the inability of *Streptococcus faecalis* to utilize 5-methyltetrahydropteroylglutamate or pteroyltriglutamate might be due to permeability was investigated. Whereas the former was taken up by *S. faecalis* cells growing on pteroylglutamic acid, the latter was not. No subsequent conversion of the 5-methyltetrahydropteroylglutamate took place and accumulation, which was against a considerable concentration gradient, was inhibited by fluoride. It would thus appear to be an active process.

It has been known for some time that *Streptococcus faecalis* is unable to use either 5-CH_3-H_4PteGlu* or $PteGlu_{3-7}$. *Lactobacillus casei*, however, can grow on both 5-CH_3-H_4PteGlu and any folate having less than four glutamic acid residues (Stokstad, 1954; Johns & Bertino, 1965; Baugh *et al.*, 1970). These differences in growth response between the two organisms have been used frequently to estimate these forms of the vitamin in the presence of other folate derivatives (Herbert, *et al.*, 1962; Luhby & Cooperman, 1964; Perry & Chanarin, 1970).

The inability of these forms of folate to support growth of *S. faecalis* is presumably due to inability of the organism either to transport them or, having concentrated them within a cell, to metabolize them further.

Evidence is presented in this paper that 5-CH_3-H_4PteGlu is taken up by *S. faecalis*. This uptake by the cells takes place against a considerable concentration gradient and, together with the demonstration that there is no subsequent conversion of the vitamin into other forms of folate, it would appear that active transport has taken place. However, $PteGlu_3$ was not taken up by growing *S. faecalis* cells. Both forms of folate were taken up by *L. casei*.

Materials and Methods

Folates

PteGlu was supplied by Lederle Laboratories American Cyanamid Co., Pearl River, N.Y., U.S.A. Radioactive (±)-5-$^{14}CH_3$-H_4PteGlu with a specific radioactivity of 91 μCi/mg was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Non-radioactive 5-CH_3-H_4PteGlu was prepared by the method of Blair & Saunders (1970). $PteGlu_3$ was prepared by the solid-phase peptide synthesis of Krumdieck & Baugh (1969) by using pteroic acid prepared by the method of Houlihan & Scott (1972). Stock solutions of 5-CH_3-H_4PteGlu and $PteGlu_3$ were assayed spectrophotometrically on a Unicam SP.800, by using the molar extinction coefficient of 31.7×10^3 litre·mol^{-1}·cm^{-1} at 290nm (0.1M-sodium phosphate buffer, pH7.0) for 5-CH_3-H_4PteGlu and 26.3×10^3 litre·mol^{-1}·cm^{-1} at 255nm (0.1M-NaOH) for $PteGlu_3$, and by microbiological assay using *L. casei* in conjunction with a standard curve based on PteGlu. $PteGlu_3$ was found to have 62.5% of the growth-promoting activity of PteGlu for *L. casei*. In all microbiological estimations it was taken into account that chemically prepared 5-CH_3-H_4PteGlu is a racemate with only 50% biological activity.

Whatman chromatography paper no. 1 and Whatman DE52 cellulose were supplied by W. and R. Balston Ltd., London, U.K. Cellulose powder MN300 UV254 was obtained from Macherey, Nagel and Co., Düren, West Germany. Filter membranes were supplied by Millipore Ltd., Wembley, U.K.

Micro-organisms

S. faecalis (N.C.I.B. 6459, A.T.C.C. 8043) and the chloramphenicol-resistant strain of *L. casei* (N.C.I.B. 10463, A.T.C.C. 7469) isolated by Davis *et al.* (1970) were both supplied by the Torry Research Station, Aberdeen, U.K.

For inoculation, *S. faecalis* was grown overnight in single strength Lactobacilli Broth AOAC (B901-15) supplied by Difco Laboratories, Detroit, Mich., U.S.A. The cells were washed five times in ice-cold 0.9% (w/v) saline to remove folates. Three flasks each containing 50 ml of Difco Folic Assay Medium (B318-15) and 0.25% (w/v) ascorbic acid (Bakerman, 1961; Herbert, 1961) were used for growth. The flasks

* Abbreviations: 5-CH_3-H_4PteGlu, 5-methyl-5,6,7,8-tetrahydropteroylglutamic acid; PteGlu, pteroylglutamic acid; $PteGlu_{3-7}$, polyglutamyl forms of folate with three or more glutamic acid residues.

contained, respectively, 0.4ng of PteGlu/ml, 0.4ng of PteGlu/ml plus a microbiologically equivalent amount of 5-CH_3-H_4PteGlu (0.4ng/ml), and 0.4ng of PteGlu/ml plus a microbiologically equivalent amount of $PteGlu_3$ (0.63ng/ml). It was always ensured that the medium was capable of supporting at least twice the final growth obtained. Three drops of the washed inoculum, which had a turbidimetric extinction of 0.05 at 640nm, were used to inoculate 50ml of the media. In order to measure folate uptake during growth, samples (5ml) of the growing cells were withdrawn at various time-intervals. Their extinction at 640nm was measured and after centrifugation at 10000*g* for 15min at 4°C the supernatants were assayed for folate content by using *L. casei* (Freed, 1966; Davis *et al.*, 1970). Column chromatography on DE52 DEAE-cellulose (phosphate form) was used to identify the folates present in the medium after growth by *S. faecalis*. Separation of PteGlu and $PteGlu_3$ was carried out on a 0.5cm × 20cm column with a linear gradient formed from 500ml of 0.005M-sodium phosphate buffer, pH7.0, in the mixing vessel and 500ml of 0.5M-NaCl in 0.005M-sodium phosphate buffer, pH7.0, in the second vessel. The column was calibrated by applying 1ml of a 1mg/ml solution of PteGlu or $PteGlu_3$ in the starting buffer. Samples (1ml) of the growth media were adjusted with 0.1M-NaOH to pH7.0 and similarly applied. The flow rate was 0.165ml/min. Fractions (3.3ml) were collected every 20min and assayed for folate with *L. casei*. A standard curve of turbidimetric extinction against mg dry wt. of bacterial cells was plotted for *S. faecalis* and *L. casei* by using a Unicam SP. 600 series II spectrophotometer (Koch, 1970).

Uptake of 5-$^{14}CH_3$-H_4PteGlu

L. casei was grown in 50ml of Folic Acid Assay Broth (11267) supplied by BBL Division of Bioquest, Cockeysville, Md., U.S.A., and *S. faecalis* was grown in 50ml of Difco Folic Acid Assay Medium. The media contained (per ml) 40ng of biologically active (by *L. casei* assay) 5-$^{14}CH_3$-H_4PteGlu, which is equivalent to 74ng of (±)-5-CH_3-H_4PteGlu (15000 c.p.m.), and 0.4ng of PteGlu. Cells were harvested, after growth for 24h, by centrifugation at 10000*g* for 15 min and were washed three times on membrane filters (pore size 0.2μm) with ice-cold 0.9% NaCl to remove any extracellular radioactive material. The cells were lysed by autoclaving at 103.5kN/m^2 (15lb/in^2) for 5min in 1ml of water. Lysate (1ml) plus 0.4ml of 1.0M-HCl was counted for radioactivity in 10ml of toluene–Triton X-100 (2:1, v/v) scintillation fluid, by using a Packard model 3375 liquid-scintillation counter (Turner, 1967). This procedure gave the same efficiency as counting the extracted radioactive material in the absence of cells. Efficiency was determined by using the channels-ratio technique and also by means of an external standard.

Further 5-$^{14}CH_3$-H_4PteGlu-uptake studies were carried out on *S. faecalis* cells that had been grown for 3h in single-strength Difco Folic Acid Assay Medium containing 1.0ng of PteGlu/ml. Washed exponential-phase cells were incubated for 15min in 0.1M-sodium phosphate buffer, pH6.5, and 0.03M-glucose, containing 0.25% (w/v) ascorbate and (±)-5-$^{14}CH_3$-H_4PteGlu to give a radioactivity of 15000c.p.m./ml (74ng/ml). Uptake was studied in the same medium that also contained (per ml) 11.0ng of (±)-5-$^{14}CH_3$-H_4PteGlu (2300c.p.m.), which was found, by using the *L. casei* assay, to contain 5.7ng of biologically active 5-CH_3-H_4PteGlu. The incubation medium contained 5mg dry wt. of cells/ml in a total volume of 2.5ml. Cells were washed as described above and the intracellular folates released by autoclaving. Uptake was determined by counting the radioactivity of the lysate in the Packard scintillation counter.

Identification of transported 5-CH_3-H_4PteGlu

To identify the extracted radioactive material, *S. faecalis* cells were lysed by autoclaving in 1.0ml of

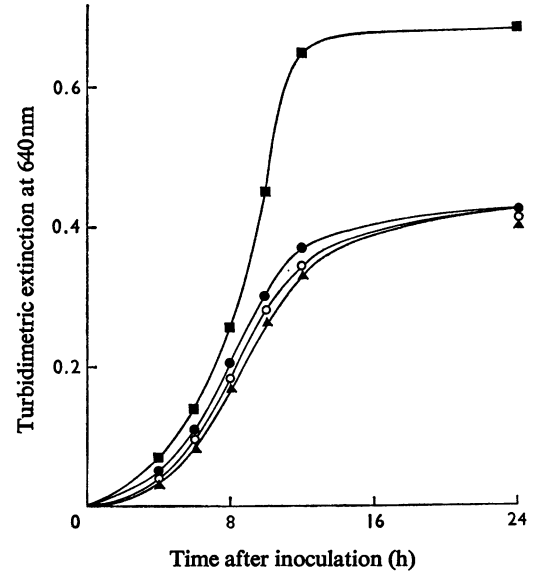

Fig. 1. *Growth of S. faecalis on PteGlu* (●), *PteGlu plus 5-CH_3-H_4PteGlu* (▲) *and PteGlu plus $PteGlu_3$* (○)

The concentration of each form of the vitamin was the microbiological equivalent of 0.4ng of PteGlu/ml. The effect on growth of *S. faecalis* of doubling the concentration of PteGlu (0.8ng/ml) is also shown (■). For experimental details see the Materials and Methods section.

0.1M-sodium phosphate, pH7.0, containing 0.5% (w/v) ascorbic acid, and the cell debris was removed by centrifugation at 10000*g* for 10min at 4°C. The compound was identified as 5-CH_3-H_4PteGlu by ascending chromatography at 4°C on Whatman no. 1 paper with 0.1M-sodium phosphate buffer, pH7.0, containing 0.5% (v/v) β-mercaptoethanol as solvent (Gupta & Huennekens, 1967). The chromatogram was cut into strips 1cm in length, which were counted for radioactivity in 10ml of scintillation fluid. The identity of the substance extracted from *S. faecalis* was confirmed as 5-CH_3-H_4PteGlu by t.l.c. on MN300 UV cellulose powder, with 3.0% (w/v) NH_4Cl containing 0.5% mercaptoethanol as solvent.

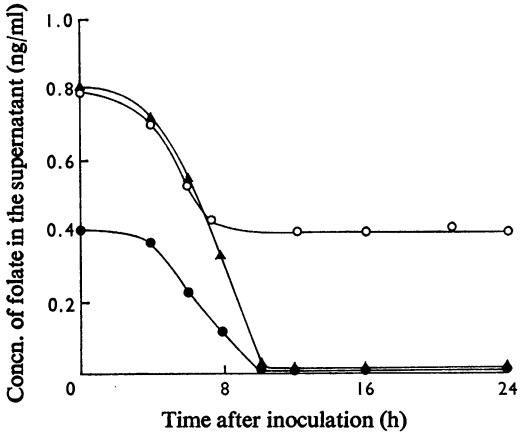

Fig. 2. *Removal of folate activity from a medium containing PteGlu* (●), *PteGlu plus 5-CH_3-H_4PteGlu* (▲) *and PteGlu plus $PteGlu_3$* (○), *during normal growth of S. faecalis for 24h*

The concentration of each folate was the microbiological equivalent of 0.4ng of PteGlu/ml. For experimental details see the Materials and Methods section.

Results

Growth studies on S. faecalis

The growth of *S. faecalis* was followed in folate-free medium supplemented with 0.4ng of PteGlu/ml and a microbiologically equivalent amount of either 5-CH_3-H_4PteGlu or $PteGlu_3$. It was found that when growth in either case was compared to the growth obtained with 0.4ng of PteGlu/ml on its own, no difference was seen (Fig. 1). This confirmed that neither of these compounds can support growth in this organism.

Uptake of folates

During growth under the above conditions the uptake of folate was examined (Fig. 2) by assaying the total folate activity remaining in the medium. Clearly not only was all of the PteGlu removed when growth was complete, but so was the 5-CH_3-H_4-PteGlu. For supplementation with $PteGlu_3$ the amount of folate remaining when growth was complete was exactly equal to the amount of $PteGlu_3$ added. The folate, active for *L. casei*, remaining in the medium was identified as $PteGlu_3$ by column chromatography on DE52 DEAE-cellulose in the phosphate form. Column chromatography also showed that no PteGlu remained in the medium after growth. This indicated that $PteGlu_3$ could not be removed from the medium by the growing cells.

Uptake of 5-$^{14}CH_3$-H_4PteGlu

By using radioactive 5-CH_3-H_4PteGlu and by measuring radioactivity in lysates of washed cells, it was clear that uptake was taking place (Tables 1 and 4). *S. faecalis* and *L. casei* cells grown for 24h on PteGlu and radioactive 5-CH_3-H_4PteGlu were lysed in ascorbate, and the radioactive material was identified by the paper-chromatographic method of Gupta & Huennekens (1967). With *S. faecalis* nearly all of the radioactivity was recovered from a single spot

Table 1. *Uptake of 5-$^{14}CH_3$-H_4PteGlu by S. faecalis and L. casei*

Growth was for 24h in media containing (per ml) 0.4ng of PteGlu and 74ng (15000c.p.m.) of (±)-5-$^{14}CH_3$-H_4PteGlu. Further experimental details are given in the Materials and Methods section.

Micro-organism	Turbidimetric extinction at 640nm after 24h growth	Dry wt. of cells (mg/ml of medium)	Radioactivity of 5-$^{14}CH_3$-H_4PteGlu accumulated (c.p.m./mg dry wt. of cells)	Wt. of 5-$^{14}CH_3$-H_4PteGlu accumulated (ng/mg dry wt. of cells)	Percentage recovery from cell lysates of 5-CH_3-H_4PteGlu taken up from the growth medium
L. casei	1.08	50	24–31	0.12–0.15	96–98
S. faecalis	0.38	4.5	6–16	0.03–0.08	90–91

Table 2. *Paper chromatography of L. casei and S. faecalis extracts*

Cells were grown for 24h in medium containing 5-$^{14}CH_3$-H_4PteGlu and were lysed in 0.1M-sodium phosphate buffer, pH7.0, containing 0.5% ascorbate and the extracts were analysed by paper chromatography on Whatman no. 1 paper. The solvent was 0.1M-sodium phosphate buffer, pH7.0, containing 0.5% mercaptoethanol, at 4°C.

Compound	R_F	Radioactivity recovered (%) *L. casei* extract	Radioactivity recovered (%) *S. faecalis* extract
5-$^{14}CH_3$-H_4PteGlu	0.65	44	95
Unknown	0.20	6	5
Unknown	0.48	10	—
Unknown	>0.81	40	—

with an R_F value corresponding to 5-CH_3-H_4PteGlu, whereas with *L. casei* several radioactive areas were found (Table 2). A similar result was obtained when the *S. faecalis* extract was chromatographed on thin-layer cellulose powder with 3% (w/v) NH_4Cl and 0.5% mercaptoethanol as solvent (Table 3). Nearly all (95%) of the extracted radioactive material was identified as 5-CH_3-H_4PteGlu. However, of the 74ng of (±)-5-$^{14}CH_3$-H_4PteGlu/ml of medium available to *S. faecalis* growing for 24h (Table 1), only 0.36ng/ml (4.5mg dry wt. of cells/ml) was found to be present in the washed cells after harvesting. The remainder of the radioactivity remained in the medium after growth and was identified as 5-$^{14}CH_3$-H_4PteGlu by chromatography with the above procedures. By using washed exponential-phase cells and incubation for 15min with 74ng of (±)-5-$^{14}CH_3$-H_4PteGlu/ml (15000c.p.m./ml), 4.6ng of radioactive folate/ml was taken up and stored by the cells (5mg dry wt. of cells/ml). Since quantitative recoveries of 5-$^{14}CH_3$-H_4PteGlu were possible from cells grown for either 24h or incubated for only 15min (Tables 1 and 4), it was concluded that no metabolism of 5-$^{14}CH_3$-H_4PteGlu had taken place.

Uptake did not take place when exponential-phase cells were incubated with 0.1M-KF. Uptake was impaired when cells were incubated in the absence of glucose. Also, the quantity of 5-$^{14}CH_3$-H_4PteGlu taken up by the cells was found to vary depending on the concentration of PteGlu present in the medium. High concentrations of PteGlu effectively inhibited uptake of 5-$^{14}CH_3$-H_4PteGlu. These experiments suggest that PteGlu and 5-CH_3-H_4PteGlu share the same transport mechanism, probably an active one, in *S. faecalis*.

Table 3. *Chromatography of S. faecalis extract*

S. faecalis cells were grown for 24h in a medium containing 5-$^{14}CH_3$-H_4PteGlu and were lysed in 0.1M-sodium phosphate buffer, pH7.0, containing 0.5% ascorbate and the extracts were analysed by t.l.c. on MN300 UV cellulose powder. The solvent was 3.0% (w/v) NH_4Cl containing 0.5% mercaptoethanol at room temperature.

Compound	R_F	*S. faecalis* extract (% of radioactivity recovered)
5-$^{14}CH_3$-H_4PteGlu	0.81	95
PteGlu	0.22	—
H_4PteGlu	0.65	—
Unknown	0.91	4

Discussion

The observation that folates that are reduced and substituted with a methyl group in the 5-position or that have three glutamic acid residues support growth for *L. casei* but not for *S. faecalis* has been used to assay these compounds in the presence of other folates, although the reason for this lack of response was unknown.

It seemed likely either that these folates could not be taken up by *S. faecalis* cells or that this micro-organism has some alteration in folate metabolism making it impossible to convert accumulated folates into the folate pool. Inability to transport $PteGlu_3$ would seem to be the reason that this form of the vitamin does not support growth in *S. faecalis* (Fig. 2). The presence of three glutamic acid residues makes the molecule too large to enter the cell or ensures that it is not recognized by the cell transport mechanisms.

For 5-CH_3-H_4PteGlu, transport definitely takes place. This derivative of folate at a concentration sufficient to double the growth of an *S. faecalis* culture growing on PteGlu, caused no increase in growth (Fig. 1). However, the 5-CH_3-H_4PteGlu was completely removed from the medium by the cells (Fig. 2). That this loss of folate from the medium was actual uptake and not simply adhesion to the cell walls is indicated by the fact that *S. faecalis* cells, either grown for 24h in a medium containing 5-$^{14}CH_3$-H_4-PteGlu and PteGlu (Table 1) or exponential-phase cells incubated for 15min with 5-$^{14}CH_3$-H_4PteGlu (Table 4), when washed three times in saline and lysed, still contained a considerable concentration of radioactivity (Tables 1 and 4). No destruction of 5-$^{14}CH_3$-H_4PteGlu occurred, as radioactive material removed from the media was recovered quantitatively and identified as 5-$^{14}CH_3$-H_4PteGlu by chromatography (Tables 1 and 4). Incubations of exponential-phase *S. faecalis* cells with 5-$^{14}CH_3$-H_4PteGlu in the presence of KF failed to show any accumulation of

Table 4. *Uptake of 5-$^{14}CH_3$-H_4PteGlu by exponential-phase S. faecalis cells*

Washed exponential-phase cells were incubated for 15min usually in the presence of (per ml) 74ng of (±)-5-$^{14}CH_3$-H_4PteGlu (15000c.p.m.) (equivalent to 40ng of biologically active 5-CH_3-H_4PteGlu). The volume of the incubation mixture was 2.5ml and contained 5mg dry wt. of cells/ml in 0.1M-sodium phosphate buffer, pH6.5, 0.03M-glucose and 0.25% ascorbate.

Conditions of incubation	Radioactivity accumulated (c.p.m./mg dry wt. of cells)	Wt. of 5-CH_3-H_4PteGlu accumulated (ng/mg dry wt. of cells)	Percentage recovery from cell lysates of 5-CH_3-H_4PteGlu taken up from the incubation medium
37°C	185	0.92	100
0°C	18	0.09	92
0.1M-KF added	0	0	—
10ng of PteGlu/ml added	13	0.06	93
1μg of PteGlu/ml added	3	0.015	90
37°C; 11.0ng of (±)-5-$^{14}CH_3$-H_4PteGlu/ml (2300c.p.m./ml) added	79	0.39	98
37°C; no glucose present	35	0.18	96

this radioactive folate. It was also found that at 0°C or in the absence of glucose uptake was impaired.

Increasing concentrations of PteGlu in the incubation medium decreased the amount of 5-$^{14}CH_3$-H_4PteGlu taken up by the cells (Table 4). This accounted for the smaller amount of 5-$^{14}CH_3$-H_4PteGlu found in cells grown for 24h (Table 1) compared with the amount found in exponential-phase cells incubated for 15min (Table 4). *S. faecalis*, grown for 24h in a medium containing 5-$^{14}CH_3$-H_4PteGlu, required an additional form of folate capable of supporting growth (Fig. 1). This was provided in the form of 0.4ng of PteGlu/ml of medium. This concentration of PteGlu partially inhibited uptake of 5-$^{14}CH_3$-H_4PteGlu.

By taking the intracellular water volume to be 4μl/mg dry wt. of cells (Davis *et al.*, 1968), the ability of *S. faecalis* to deplete the medium of 0.4ng of 5-CH_3-H_4PteGlu (Fig. 2) represents a 40-fold concentration of the vitamin. Wood & Hitchings (1959), using a similar uptake system, found that *S. faecalis* does transport and store PteGlu, but degrades the 5-formyltetrahydropteroylglutamate. This may be a consequence of lysing cells after uptake by autoclaving in the absence of a reducing agent. Using a related micro-organism (*Pediococcus cerevisiae*), Mandelbaum-Shavit & Grossowicz (1970) have demonstrated that, whereas this organism cannot utilize 5-CH_3-H_4PteGlu as a source of folate, it does actively transport it. One of the difficulties in investigating the transport of any compound is that frequently it is metabolized, resulting in a constantly changing concentration gradient. The finding that these organisms do not carry out such metabolism makes them suitable for folate-transport studies.

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