Folate Distribution in Cultured Human Cells

STUDIES ON 5,10-CH_2-H_4PTEGLU REDUCTASE DEFICIENCY

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ABSTRACT We have studied the distribution of folate coenzyme forms in cultured human fibroblasts from control lines and from lines derived from nine patients representing all of the published reports of 5,10-CH_2-H_4PteGlu reductase deficiency. Based on mobility on DEAE-Sephadex and differential microbiological assay the major folate fractions in extracts of human fibroblasts were 5-CH_3-H_4PteGlu, 10-CHO-H_4PteGlu, and 5-CHO-H_4PteGlu with smaller fractions, which included 5-CH_3-H_2PteGlu, 10-CHO-PteGlu, and H_4PteGlu. Evidence that the 5-CHO-H_4PteGlu may have been derived from 5,10-CH=H_4PteGlu during extraction is presented. In most of the mutant fibroblasts the absolute concentration of 5-CH_3-H_4PteGlu was lower than in control cells but the proportion of intracellular folate which was 5-CH_3-H_4PteGlu was strikingly lower in mutant cells when determined by chromatography or differential microbiological assay. In both control and mutant cells most of the 5-CH_3-H_4-PteGlu was polyglutamate. The proportion of intracellular folate which was polyglutamate was similar in control and mutant cells. A direct relationship was observed between the proportion of cellular folate which was 5-CH_3-H_4PteGlu, and both the clinical severity of this disorder and the residual enzyme activity indicating that the distribution of different folates may be an important control of intracellular folate metabolism. These studies indicate that 5,10-CH_2-H_4PteGlu reductase is the only significant intracellular pathway for the generation of 5-CH_3-H_4PteGlu, that the activity of this enzyme regulates the level of this folate in control and mutant cells under conditions of culture used here, that the majority of intracellular folate is in the polyglutamate form, and that the relative distribution of folates may control folate metabolism by interaction in the various folate reactions.

INTRODUCTION

Deficiency of 5,10-CH_2-H_4PteGlu reductase, (EC.1.1.-1.68) an autosomal recessive inborn error of folate metabolism, is characterized by moderate degrees of homocystinemia and homocystinuria accompanied by normal or low plasma methionine levels. The clinical findings in the twelve patients described to date (1–8) has ranged from seizures, apnea, coma, and death in infancy (4) to mild mental retardation and neurological impairment in adolescence (5). The deficiency of 5,10-CH_2-H_4PteGlu reductase was first described in fibroblasts (3, 5, 9) but has now been confirmed in liver (4, 6), kidney (4), brain (4), and leukocytes (3, 4). Preliminary studies showed a decreased ratio of 5-CH_3-H_4PteGlu to total folates in the fibroblasts of several deficient patients (10) and an absence of 5-CH_3-H_4PteGlu in the

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serum and erythrocytes of one patient (4) has been described.

This study describes the distribution of folate monoglutamates and folate polyglutamates and the composition of each in fibroblasts from nine patients representing all of the published reports of 5,10-CH_2-H_4PteGlu reductase deficiency.

METHODS

Materials. Tissue culture medium was purchased from Grand Island Biological Co. (Grand Island, N. Y.). Fetal calf serum was purchased from Flow Laboratories, Inc. (Rockville, Md.). All other chemicals were of reagent grade and obtained commercially.

Cell culture. Fibroblasts were obtained from nine patients representing all known publications of 5,10-CH_2-H_4-PteGlu reductase deficiency (Table I) and from controls.

On arrival in our laboratory all lines were routinely tested for mycoplasma contamination (11). Four such lines (549, 550, 583, and 654) were found to be contaminated with mycoplasma. Because the patients had either died or were not available, repeat biopsy was not possible. These contaminated cells were grown in a separate laboratory. No difference in the parameters involved in these studies could be found between the five mycoplasma-free and the four mycoplasma-contaminated cells except that insufficient mycoplasma-contaminated cells could be obtained for all studies. All cells were grown to confluence in roller bottles (Bellco Glass Inc., Vineland, N. J.) of 690 cm^2 surface area containing 100 ml medium in an atmosphere of 5% CO_2 and 95% air. The standard medium consisted of Eagle's minimum essential medium, which contains PteGlu 2.3 μM (1 mg/liter) plus nonessential amino acids. All media contained 10% fetal calf serum dialyzed where indicated against a 10-fold vol of 0.9% NaCl with two changes. Cells were released from the surface of the roller bottles by exposure to 0.25% trypsin for 10 min at 37°C. The cells were resuspended in Dulbecco's phosphate-buffered saline solution (pH 7.4), and an aliquot was removed for enumeration in a Coulter

TABLE I

Features of 5,10-CH_2-H_4PteGlu Reductase Deficient Cell Lines

Patient and line designation	Rank of clinical severity*	Sex	Age	Cell type in literature	CH_2-H_4PteGlu reductase activity‡	Reference No.
			yr			
CP (488)	1	M	16	Fibroblasts	1.39	5
					1.7	9
LM (451)	3	F	15	Fibroblasts	0.77	5
					1.6	9
BM (452)	3	F	17	Fibroblasts	0.82	5
					1.2	9
WM (548)	3	M	11	Fibroblasts	1.1	1
TC (549)	5 1/2	F	9	Fibroblasts	0.53	2
MEC (550)	5 1/2	F	7	Fibroblasts	0.97	2
					1.3	§
GS (654)	7 1/2	M	2 4/12	Leukocytes	0.94	3
GP (583)	7 1/2	M	2	Fibroblasts	0.16	‖
SS (670)	9	F	5/12	Leukocytes	0	4
				Fibroblasts	0.1	§

Reference	Control 5,10-CH_2-H_4PteGlu reductase activity‡	Cell type
Rosenblatt and Erbe (9)	8.6±1.4	Fibroblasts
Mudd et al. (5)	5.04±1.36	Fibroblasts
Narisawa et al. (4)	2.68±0.78	Leukocytes
Wong and Berlow (3)	9.38±1.22	Leukocytes
Wong et al. (2)	7.88	Fibroblasts
Baumgartner and Wong‖	4.72	Fibroblasts

* Least severe (1), most severe (9).

‡ Nanomoles of formaldehyde per milligrams protein per hour.

§ Rosenblatt, D. S., and B. A. Cooper. Unpublished observation.

‖ Baumgartner, R., and P. W. K. Wong. Unpublished observations.

counter (Coulter Electronics, Inc., Hialeah, Fla.). The cell pellet was washed twice in phosphate-buffered saline and collected by centrifugation (12). 5,10-CH_2-H_4PteGlu reductase activity in cell extracts were measured as previously described (13).

Folate determinations. Folate was assayed microbiologically with *Lactobacillus casei* ATCC 7469, *Pediococcus cerevisiae* ATCC 8081, and *Streptococcus fecalis* ATCC 8083, using aseptic addition of samples to sterile medium already inoculated with assay organisms as described previously (14). This technique represented a slight variation of standard techniques. Because *S. fecalis* required a higher folate concentration than the other organisms for logarithmic growth, folate concentration in fibroblasts could not always be reliably determined with this organism.

For fractionation of folates on Sephadex G-25, washed fibroblasts were extracted in 0.05 M sodium phosphate pH 9 containing 8.5 mM ascorbate, protein precipitated with heat, and the clear supernate obtained after centrifugation was analyzed. For assay of folate content by microbiological methods and by DEAE-Sephadex, fibroblasts were extracted in 0.1 M sodium acetate pH 4.5 containing 8.5 mM ascorbate, and the extract was treated with 10% (vol/vol) of folate-depleted normal human serum for 90 min at 37°C followed by heating and analysis of the supernate. The pH of the mixture of extract serum and buffer was 5.2–5.3.

Fractionation of folate on DEAE-Sephadex was as described previously (15, 16) using a linear gradient (0.1–0.8 M) of potassium phosphate pH 6.1. Folate polyglutamates were differentiated from short-chain folates in extracts untreated with conjugase by filtering through Sephadex G-25 as described previously (17). To allow assay of the relatively small amount of *Pediococcus*-active folate from specimens containing 10^6 cells, filtration utilized a column of 0.9 × 30 cm, and 1-ml fractions were collected.

Folate polyglutamates were deconjugated to monoglutamates with 10% folate-depleted serum within the assay tubes at pH 4.5, after which the pH was adjusted to 6.9 and 0.8 ml of each fraction was mixed with concentrated assay medium containing the assay organism, and incubated without precipitation of protein. Standards and controls were assayed in a similar fashion. These samples were assayed only with *L. casei* and *P. cerevisiae*. This technique allowed assay of as little as 500 pg/sample. When fibroblast folate excluded from the gel was refiltered, it was again excluded from the gel. When the extract was treated with conjugase before filtration, all the folate filtered within the included volume of the gel, indicating that the technique differentiated between polyglutamyl and short-chain folates.

For the microbiological assays, care was taken to ensure that the samples were diluted into the range where growth of the microorganism was directly proportional to the folate concentration (18). In all circumstances at least two dilutions within this assay range were achieved and concentrations calculated from these different dilutions were within 5% of each other.

Statistical analysis employed the Student *t* test, using paired or unpaired observations, and the Mann-Whitney analysis for nonparametric statistics. When not otherwise qualified, *P* values indicate the probability that values are different based on unpaired *t* test analysis.

The distribution of folate forms at confluence was determined by chromatography of deconjugated extracts on DEAE-Sephadex. Typical chromatograms of extracts of normal and mutant cells are shown in Fig. 1. Folate fractions eluted from the column were identified by comparison of the conductivity of the eluting buffer with that of standard folate coenzymes, including DL-10-CHO-H_4PteGlu, DL-10-CHO-PteGlu, DL-5,10-CH=H_4PteGlu, DL-5-CHO-H_4PteGlu, DL-5-CH_3-H_4PteGlu, and PteGlu, with data published previously and by their support of growth of the assay organisms (15, 16). Because of the known lability of 5,10-CH=H_4PteGlu and 5,10-CH_2-H_4PteGlu under the conditions of extraction of the cells (19), these folates would not have been found in these cell extracts; the former would have been converted to a mixture of 10-CHO-H_4PteGlu and 5-CHO-H_4PteGlu, and the latter to H_4PteGlu. The folates eluting from the column were therefore identified as: fraction I, 10-CHO-H_4PteGlu; fraction II, 10-CHO-PteGlu and 5-CH_3-H_2PteGlu; fraction III, 5-CHO-H_4-PteGlu; fraction IV, 5-CH_3-H_4PteGlu; and fraction V, H_4PteGlu. Note that the folates in fraction II are oxidation products of those in fractions I and IV, and always represented a minority of folates eluting from the columns. Fractions II and V were small and may also have contained traces of other folates.

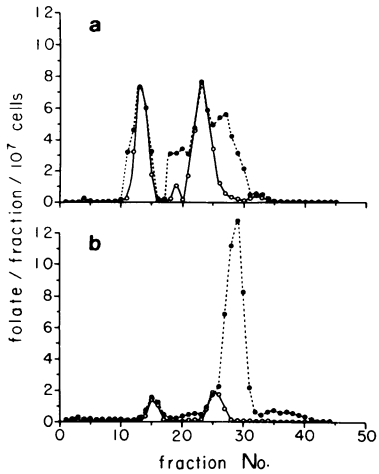

FIGURE 1 DEAE-Sephadex elution pattern of folate forms in a deconjugated extract of 5,10-CH_2-H_4PteGlu reductase deficient fibroblast line 451 (*a*) and a normal line (316) (*b*). 7.8 × 10^7 cells of line 451, and 5.1 × 10^7 cells of line 316 were suspended in 12 ml of 0.1 M sodium acetate pH 4.5 containing 8.5 mM ascorbate and the extract was treated with 10% (vol/vol) of folate-depleted human serum for 90 min at 37°C followed by heating. The extract was then fractionated on DEAE-Sephadex using a linear gradient (0.1–0.8 M) potassium phosphate pH 6.1. The folate forms were identified on the basis of mobility and support of growth of test organisms *L. casei* (●) and *P. cerevisiae* (○). The ordinate indicates total folate in nanograms per 9-ml fraction per 10^7 cells.

RESULTS

The distribution of folate coenzyme forms determined from DEAE-Sephadex chromatography of extracts of control cell lines and of eight of the nine mutant cells is summarized in Table II. In six of the eight mutant lines analyzed by this method, the absolute concentration of 5-CH_3-H_4PteGlu (fraction IV) was lower than in the control lines, but the groups were not statistically different ($P = 0.31$ [Student]; $P = 0.30$ [Mann-Whitney]). The concentration of total folate coenzymes and of other folate fractions was also not different in normal

TABLE II

Distribution of Folate Coenzyme Forms in Fibroblasts—DEAE-Sephadex

	Fraction number					
	I	II	III	IV	V	Total
	ng folate/10^6 cells					
Control lines						
33	0.44	0.09	0.36	3.09	0.18	4.16
316	0.46	0.14	1.09	4.33	0.30	6.32
Mean	0.45	0.115	0.725	3.71	0.24	5.24
Mutant lines						
451	2.02	0.19	2.37	2.10	0.20	6.88
452	0.63	0.19	0.8	1.53	0.24	3.40
488	0.52	0.27	2.32	9.42	0.74	13.29
548	2.00	0.92	2.32	4.66	0.77	10.68
549	0.86	0.36	0.35	0.43	0.24	2.24
550	1.21	0.24	0.59	1.25	1.25	4.55
583	3.69	1.59	1.96	0.76	0.31	8.32
670	1.50	0.5	0.86	0.04	0.22	3.29
Mean	1.55	0.53	1.45	2.52	0.496	6.58

Fibroblast extracts were fractionated on DEAE-Sephadex as described in Methods. The presumed identity of the folate fractions eluted sequentially: I, 10-CHO-H_4PteGlu; II, 10-CHO-PteGlu and 5-CH_3-H_2PteGlu; III, 5-CHO-H_4PteGlu; IV, 5-CH_3-H_4PteGlu; V, H_4PteGlu.

TABLE III

Folate Content of Fibroblasts by Differential Microbiological Determination

	L. Casei	*P. Cerevisiae*	5-CH_3-H_4PteGlu	
	ng folate/10^6 cells		*ng/10^6 cells*	*fraction of total folate*
Control cells (n = 10)				
Mean	3.31	0.56	2.75	0.83
SD	1.57	0.51	1.26	0.10
Mutant cells				
451	2.29	1.13	1.17	0.51
452	2.90	1.28	1.62	0.56
488	3.72	0.60	3.12	0.84
548	4.05	1.61	2.44	0.60
549	1.89	0.80	1.09	0.57
550	0.43	0.30	0.13	0.30
583	9.50	7.00	2.50	0.26
654	2.73	1.64	1.08	0.39
670	4.23	3.19	1.03	0.24
Mean	3.53	1.95	1.58	0.48
SD	2.53	2.06	0.94	0.19
P*	0.41	0.027	0.017	0.00004
Heterozygotes (parents of 488)				
394	1.49	0.34	1.15	0.77
395	2.56	0.66	1.90	0.74

Folate levels in fibroblast extracts were determined as described in Methods.

* Probability that mutants and controls are the same (Student t test).

and mutant cells (P = 0.30–0.40 by Student t and Mann-Whitney test).

The concentration of 5-CH_3-H_4PteGlu and of total folates in the cells was also measured by comparing the folate activity in cell extracts for *L. casei* and for *P. cerevisiae*. These data are summarized in Table III. By this method most mutant cells were found to have a lower concentration of 5-CH_3-H_4PteGlu than did normal cells (P = 0.017) although again this was not invariably true. Based both on column chromatography (Table II) and direct assay of extracts (Table III) the proportion of intracellular folate which was 5-CH_3-H_4-PteGlu was significantly lower in mutant cells (P = 0.019 and 0.0004, respectively) and the proportion of fractions I and II are possibly higher in mutant cells (P = 0.09 and 0.14 Student; P = 0.04 and 0.04 Mann-Whitney).

The proportion of intracellular folate which was 5-CH_3-H_4PteGlu determined by differential microbiological assay alone correlates well with that calculated from column chromatography (r = 0.90, P = 0.0008 [Student]), but the results from the former were consistently higher than from those from the column (least-squares analysis of the data in Tables II and III revealed that $y = 0.219 + 0.846x$, where y is the proportion of intracellular 5-CH_3-H_4PteGlu determined by differential microbiological assay, and x, that calculated from column chromatography).

An attempt was made to determine if the distribution of nonmethylated folates observed in the cell extracts reflected the distribution of these within the cells before extraction. To determine if the 5-CHO-H_4PteGlu found in the extracts could have come from 5,10-CH=H_4PteGlu, the latter was prepared by acid incubation (20) from commercial 5-CHO-H_4PteGlu and purified by chromatography. This material, the identity of which was verified spectrophotometrically, was mixed with serum and treated exactly as was a cell extract. All of the 5,10-CH=H_4PteGlu was converted to 10-CHO-H_4PteGlu, 10-CHO-PteGlu, and 5-CHO-H_4PteGlu. The largest fraction formed was 5-CHO-H_4PteGlu, suggesting that this material could have been formed from 5,10-CH=H_4PteGlu during extraction of folates. The quantity of 10-CHO-H_4PteGlu in the extracts could not be explained by such conversion.

To determine the proportion of intracellular folates which were present as short-chain and long-chain folates (poly-γ-glutamates), cell extracts were filtered

through Sephadex G-25 and assayed for microbiological activity after deconjugation. The proportion of folate which was present in the excluded volume of the column and thus presumed to be polyglutamates (17) was not different in control and mutant cells (Table IV). The proportion of 5-CH_3-H_4PteGlu within the polyglutamate fraction was decreased in mutant cells. This study indicates that the enzyme deficiency is reflected by a relative lack of 5-CH_3-H_4PteGlu in the folate polyglutamate fraction and in the total folate of the cell.

The relationship between the proportion of cellular folate as 5-CH_3-H_4PteGlu and the residual level of the mutant enzyme in the mutant cells is shown in Fig. 2. The residual level of enzyme was determined by comparing the mutant enzyme activity to the mean control level for the individual laboratory where the patient was studied. There was a direct relationship between the residual activity and the proportion of cellular folate as 5-CH_3-H_4PteGlu ($r = 0.94$, $P = 0.0001$). When patients were ranked as to clinical severity (Table I) there was again a strong correlation between clinical findings and cellular 5-CH_3-H_4PteGlu fraction ($r = -0.95$, $P = 0.00005$). When these were compared with the intracellular concentration of 5-CH_3-H_4PteGlu rather than with the proportion of intracellular folate in this form, correlation was observed, but this was less than with the latter ($r = 0.69$ and 0.71, $P = 0.029$ and 0.013, respectively). A strong relationship was observed also between the proportion of intracellular folate which supported growth of *L. casei* in extracts, but did not support *P. cerevisiae* and both residual enzyme activity and clinical severity of the illness ($r = 0.835$ and -0.088, $P = 0.0025$ and 0.0008, respectively).

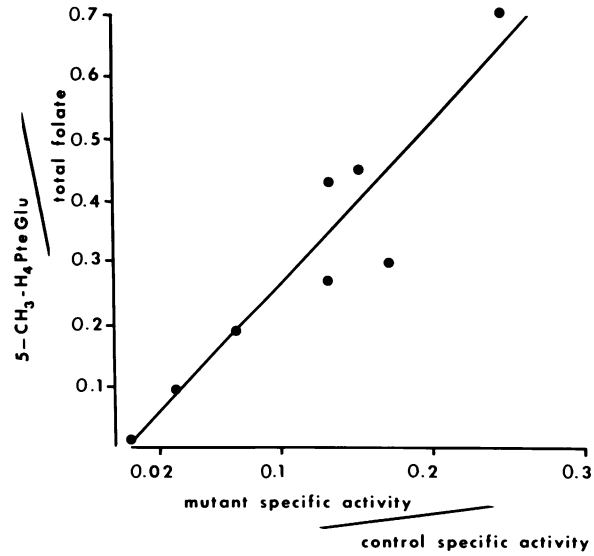

FIGURE 2 The proportion of total cellular folates as 5-CH_3-H_4PteGlu as a function of residual 5,10-CH_2-H_4PteGlu reductase activity in deficient fibroblasts. The residual level of enzyme was determined by comparing published activities to the control values for the individual laboratory where the patient was studied. The proportion of 5-CH_3-H_4PteGlu was determined from the DEAE chromatogram (Table II).

TABLE IV
5-CH_3-H_4PteGlu Distribution in Polyglutamyl Folates

	Fraction of total folate which is polyglutamyl	Fraction of polyglutamyl folates which is 5-CH_3-H_4PteGlu
Controls		
Mean	0.92	0.87*
SD	0.09	0.04
Mutants		
451	0.93	0.28
452	0.86	0.41
488	0.78	0.55
548	0.96	0.29
654	0.93	0.26
670	0.83	0.23
Mean	0.88	0.35*
SD	0.07	0.12

Extracts of fibroblasts were filtered through Sephadex G-25 as described in Methods to distinguish short-chain folates from folate polyglutamates. The fraction of folate as 5-CH_3-H_4PteGlu was determined as the differential activity between *L. casei* and *P. cerevisiae*.

* Means significantly different by Student *t* test ($P \leq 0.003$).

DISCUSSION

These studies demonstrate the accumulation of polyglutamates of 5-CH_3-H_4PteGlu as the major intracellular folate in fibroblasts grown in PteGlu, and the distribution of nonmethylated folates most of which were also in the polyglutamate form. The conditions of extraction and analysis of intracellular folate allowed chemical interconversion of some of these; indeed conversion of 5-10-CH=H_4PteGlu to 10-CHO-H_4PteGlu, 10-CHO-PteGlu, and 5-CHO-H_4PteGlu was demonstrated during the procedure. The major folate fractions in the cell extracts after conversion of polyglutamate to monoglutamate folate were 5-CH_3-H_4PteGlu and 10-CHO-H_4PteGlu, and smaller quantities of 10-CHO-PteGlu, 5-CH_3-H_2PteGlu, and H_4PteGlu were also found. Of the last three, 10-CHO-PteGlu and 5-CH_3-H_2PteGlu could have been formed during extraction and chromatography despite the presence of ascorbate and mercaptoethanol in the solutions, and much of the H_4PteGlu could have been derived from 5-10-CH_2-H_4-PteGlu which would have been converted to H_4PteGlu under the conditions of analysis. The data presented do, however, define the major intracellular folate as 5-CH_3-H_4PteGlu, and 10-CHO-H_4PteGlu as the major nonmethylated folate. 5-CHO-H_4PteGlu may not be present within the unextracted cells.

The mutant cells, deficient in 5,10-CH_2-H_4PteGlu reductase, contained the same intracellular concentration of folate, and the same group of folate coenzymes, although in all cases, the proportion of intracellular folate which was 5-CH_3-H_4PteGlu folate was decreased below that of control cells. The deficiency of intracellular 5-CH_3-H_4PteGlu in mutant cells was not surprising, because the deficient enzyme is considered the major pathway for formation of this folate. It was of interest to note that compensatory metabolic adjustments to correct this imbalance were not observed, and the only defect observed in growth was the previously described dependence of mutant cells on methionine as a growth factor not required by normal cells in the presence of homocysteine (5).

The patients from whom these fibroblasts were obtained had a variety of different clinical syndromes including psychological, neurological, and developmental abnormalities. Clinical ranking of the severity of their illness correlated very well with the residual enzyme activity and with the proportion of intracellular folate which was 5-CH_3-H_4PteGlu, and less well with the absolute concentration of this folate within the cultured cells. The absolute concentration of 5-CH_3-H_4PteGlu was usually, but not always lower in mutant cells than in normal cells, suggesting that this intracellular concentration might not be the critical determinant of cellular metabolism. The close correlation of the proportion of intracellular folate which was 5-CH_3-H_4PteGlu with residual enzyme activity and clinical severity of disease suggests that the relative distribution of different folate coenzymes might be important in controlling folate-related metabolism. Such control might depend on competition by different folates for folate-dependent enzymes, so that excess or deficiency of one folate form might alter the availability of other folates for enzymes. Competition has been reported by 10-CHO-H_4PteGlu, H_2PteGlu, PteGlu, and $PteGlu_5$ for human thymidylate synthetase with its natural coenzyme (21). The least-squares line comparing residual enzyme concentration with proportion of intracellular 5-CH_3-H_4PteGlu passed through the origin of the graph, indicating that the pathway uses the enzyme 5-10-CH_2-H_4PteGlu reductase is the only significant one for producing 5-CH_3-H_4PteGlu in these cells, under these conditions of growth. The total intracellular folate of both mutant and normal cells was equal, indicating that failure to convert intracellular folate to 5-CH_3-H_4-PteGlu does not affect folate accumulation by human fibroblasts in vitro. The intracellular folate concentration in human fibroblasts grown in PteGlu does not exceed that of the PteGlu in the culture medium (22).

Comparison of the proportion of intracellular folate which was 5-CH_3-H_4PteGlu, determined by chromatography with that by differential microbiological assay alone showed poor correlation, but that calculated by the latter was consistently higher when either *S. fecalis* or *P. cerevisiae* was compared with *L. casei*. This suggests that some of the nonmethyl folates were much more effectively used by *L. casei* for growth than by the two other test organisms. Differential microbiological assay alone on extracts of cultured fibroblasts however, does appear to be a good screening test for 5,10-Ch_2-H_4-PteGlu reductase deficiency.

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