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Analytical Methods Section

Physicochemical and biological factors influencing methylfolate stability: use of dithiothreitol for HPLC analysis with electrochemical detection

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Temperature rather than light is the predominant factor influencing 5-methyltetrahydropteroylmonoglutamate loss. This loss is pH dependent. At pH 9·0 in the absence of antioxidant at 25°C it is very unstable, whilst at pH 7·3 and 3·5 the stability is much greater, with the latter two pH values producing very similar rates of loss. In the presence of dithiothreitol, at 25°C, 5-methyltetrahydropteroylmonoglutamate is stable at pH 7·3 and 9·0, although it has no protective effect at pH 3·5 (a problem which can be overcome by storage at +4°C). The relative stability of four commercially available reduced folylmonoglutamates was found to follow the order 5-formyltetrahydropteroylmonoglutamate > dihydropteroylmonoglutamate > 5-methyltetrahydropteroylmonoglutamate > tetrahydropteroylmonoglutamate. In tissue culture experiments the stability of exogenous, extracellular 5-methyltetrahydropteroylmonoglutamate was found to be greater in the presence of human peripheral blood lymphocytes than in the absence of these cells.

INTRODUCTION

High performance liquid chromatography (HPLC) has emerged as a powerful tool in the biological sciences, its application to folate analysis avoids the lack of specificity associated with classical microbiological and radiometric binding assays (Lucock *et al.*, 1989*a*). Earlier reports apply HPLC to the quantitation of authentic materials (Reed & Archer, 1976; Branfman & McComish, 1978; Allen & Newman, 1980; Reingold & Picciano, 1980; Horne *et al.*, 1981) pharmaceutical products (Chapman *et al.*, 1978; Payet *et al.*, 1987) or to in-vivo metabolic studies employing artificially high folate concentrations (Wegner *et al.*, 1986; Payet *et al.*, 1987) where the low sensitivity of UV detection proves adequate. When the complexity of biological matrices complicates selective folate analysis, analytical separation has been hyphenated with off-line microbiological assay (Wilson & Horne, 1986) or more sensitive analytical forms of measurement such as fluorometric (Day & Gregory, 1981; Gregory *et al.*, 1984; Leeming *et al.*, 1990) or electrochemical detection (Lankelma *et al.*, 1980; Birmingham & Green, 1983; Kohashi *et al.*, 1986; Lucock *et al.*, 1989*a*. With such a rapid increase in the use of HPLC for folylmonoglutamate analysis, the criteria for determining the stability characteristics of these labile trace nutrients need re-evaluation since nutritional stability (that is a general growth response determined by *Lactobacillus casei* bioassay (O'Broin *et al.*, 1975)) and molecular integrity determined by a specific analytical technique like HPLC are not necessarily the same phenomenon.

Although vitamin C is well proven in the protection of folate compounds, and is considerably less noxious than β-mercaptoethanol, its use with electrochemical detection (ECD) results in a high background current which eventually prevents routine sample analysis (Lucock *et al.*, 1989*a*). We have therefore evaluated dithiothreitol (DTT) as an antioxidant (AO) for protecting 5-methyltetrahydropteroylmonoglutamate ($5CH_3$—H_4PteGlu) prior to analysis by HPLC as it is a highly water soluble solid with little odour and little tendency to be oxidised directly by air. Its low redox potential (−0·33 V at pH 7·0) makes it ideal for maintaining favourable equilibria for the reduction of many compounds, including vitamin C (Sanderson & Schorah, 1987) and molecules containing sulphydryl groups (Cleland, 1963).

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As $5CH_3$—H_4PteGlu is the main extracellular folylmonoglutamate found in nature, an important dietary nutrient usually existing in its polyglutamate form in food and the main plasma form of the vitamin, the present study uses a rapid HPLC technique to investigate the effect of freeze thawing on $5CH_3$—H_4PteGlu stability, as well as the effects of temperature and light under varying conditions of pH in the presence and absence of DTT. It also investigates the properties of $5CH_3$—H_4PteGlu in the presence of human peripheral blood lymphocytes, and the differential stability of various commercially available reduced folylmonoglutamates.

EXPERIMENTAL PROCEDURES

Chemicals

DL-$5CH_3$—H_4PteGlu, barium salt; 5-formyltetrahydropteroylmonoglutamate (5CHO—H_4PteGlu), calcium salt; dihydropteroylmonoglutamate (H_2PteGlu); tetrahydropteroylmonoglutamate (H_4PteGlu); β-mercaptoethanol; boric acid; bis-tris and DL-dithiothreitol were purchased from Sigma Chemical Company Ltd (Poole, Dorset, UK). AnalaR grade potassium chloride L-ascorbic acid and EDTA; Aristar grade sodium hydroxide, potassium dihydrogen orthophosphate and orthophosphoric acid were obtained from BDH Chemicals Ltd (Poole, Dorset, UK). Methanol (HPLC grade) was purchased from Rathburn Chemicals (Walkerburn, UK). All aqueous solutions were prepared from filtered deionised and double distilled water obtained from an Aquatron WD1-W4D water deioniser/still supplied by J. Bibby Science Products Ltd (Stone, Staffs, UK).

Effect of freeze-thawing on $5CH_3$—H_4PteGlu

Standard solutions

$5CH_3$—H_4PteGlu (50 μg ml^{-1}) was made up in (i) water containing 0·1% vitamin C and (ii) water containing 30% (v/v) methanol and protected by 0·1% vitamin C. Both solutions were stored at −20°C in the dark, solution (i) being stored in plastic.

Equipment

The high performance liquid chromatograph consisted of a model 6000A pump, U6K injector, Lambda-max 481 variable wavelength LC spectrophotometer (Waters Associates) and a Linseis series LS4, 2 channel, flat bed potentiometric recorder.

Chromatographic conditions

As in Lucock *et al.* (1989*a*). (LC spectrophotometer sensitivity was 0·05 AU full scale.)

Method

Two solutions of $5CH_3$—H_4PteGlu were made up, both were stored at −20°C. However, one solution was stored frozen because it was made up in water, and one was stored as a liquid due to its 30% alcohol content. The study was carried out over a period of 2 months and the effects of freeze-thawing ascertained by comparing the absorbance response of $5CH_3$—H_4PteGlu stored frozen with that of $5CH_3$—H_4PteGlu stored as a liquid after successive freeze thaw cycles. Three × 10 μl of each solution were injected onto the chromatograph and the mean values obtained.

Effect of pH, temperature and light on the stability of $5CH_3$—H_4PteGlu in the presence and absence of DTT

Standard solutions

A stock solution of $5CH_3$—H_4PteGlu in water (25 μg ml^{-1}) was prepared. This stock solution (200 μl) was diluted to 10 ml with either 50 mM phosphate buffer, pH 3·5; 50 mM bis-tris buffer, pH 7·3 or 100 mM borate buffer, pH 9·0 (Clark & Lubs, 1917) to provide a final concentration of 500 ng ml^{-1} $5CH_3$—H_4PteGlu.

Two of each of the above 500 ng ml^{-1} solutions were prepared, one contained 133 μg ml^{-1} DTT (10 μl of 133 mg ml^{-1} DTT in water per 10 ml solution), the other contained no DTT.

Equipment

As for the freeze-thawing experiment above, except an EG and G Model 400 electrochemical detector was used in combination with a glassy carbon oxidative flow cell containing a 20 μm gasket (Type TG-2M). The electrode potential was held at 450 mV versus Ag/AgCl and the current was measured at 2·0 nA full scale.

Chromatographic conditions

As in Lucock *et al.* (1989*a*) except a Waters Associates Nova-Pak phenyl stainless steel column (7·5 cm × 3·9 mm i.d., 4 μm spherical bonded silica) was used at a flow rate of 1·3 ml min^{-1}.

Method

Solutions of $5CH_3$—H_4PteGlu (500 ng ml^{-1}) made up in pH 3·5, 7·3 and 9·0 buffer, and protected by DTT, were placed at 25°C in either total darkness or constant ambient laboratory lighting. Identical solutions containing no DTT were subjected to the same physical conditions. In addition, 500 ng ml^{-1} $5CH_3$—H_4PteGlu made up at pH 3·5 was placed in total darkness at +4°C. Aliquots (10 μl) of each solution under investigation were injected onto the chromatograph at regular intervals over several hours, this being representative of a typical working day. Decay rates were calculated from the diminishing current response peak determined under the described chromatographic conditions. Regression analysis of the log-linear decay rates using the method of least squares has been used to define the line of best fit from which both K (rate constant) and $t_{1/2}$ (half-life in hours) have been calculated (K = slope value × 2·303; 95% confidence interval = $K \pm t$ value corresponding to n-2 degrees of freedom × estimated standard deviation of K; $t_{1/2}(\text{min}) = 0{\cdot}693/K$; only P values >0·05 are shown, indicating where K is not significantly different from 0).

Differential stability of various reduced folylmonoglutamates

Standard solutions
A solution (100 ng ml^{-1}) of $5CH_3$—H_4PteGlu, 5CHO—H_4PteGlu, H_4PteGlu (added from powder immediately prior to use) and H_2PteGlu was made up in water.

Equipment
As in previous experiment above. Sample concentration was achieved by using a UniVap centrifugal evaporator in combination with a refrigerated solvent trap (Model RST 551) and Javac double stage high vacuum pump (Model JD60). The equipment was supplied by Uniscience Ltd (London).

Chromatographic conditions
A modification of the analytical separation method in Lucock *et al.* (1989*a*) was used, viz. twin Waters Associates Nova-Pak phenyl columns (7·5 cm × 3·9 mm i.d., 4 μm spherical bonded silica) were used in series and were protected by a LiChro-CART, LiChrospher 5 μm, C_{18} guard column (E. Merck, Darmstadt, Germany). The mobile phase consisted of 1% (v/v) methanol in 0·05 M KH_2PO_4; 0·001 M EDTA (pH adjusted to 5·5 with orthophosphoric acid) and was filtered through a 0·22 μm Millipore filter (type GVWP) before being degassed in an ultrasonic bath. Analysis was performed at ambient temperature using a flow rate of 0·8 ml min^{-1} which produced a back pressure of 13·64 mPa (1500 psi). These modifications to the method permit resolution of a wider range of folylmonoglutamates.

Method
Commercial preparations of H_4PteGlu contain β-mercaptoethanol as the solvent of crystallisation; this acts as an effective antioxidant. To discriminate between β-mercaptoethanol and H_4PteGlu following separation on an HPLC column, β-mercaptoethanol has been injected separate from H_4PteGlu/β-mercaptoethanol. The only peak not common to β-mercaptoethanol and H_4PteGlu containing β-mercaptoethanol, is assumed to be H_4PteGlu.

The resolution, general stability under ambient conditions and relative susceptibility to elevated temperature (35°C) of H_4PteGlu, 5CHO—H_4PteGlu, $5CH_3$—H_4PteGlu and H_2PteGlu have been ascertained by timed HPLC injections and by subjecting the different folylmonoglutamates (each at 100 ng ml^{-1}) to a constant temperature *in vacuo*, of 35°C for 3·75 h.

$5CH_3$—H_4PteGlu stability in the presence of human peripheral blood lymphocytes

Standard solutions
$5CH_3$—H_4PteGlu (394 ng ml^{-1}) was made up in medium containing either 129 or 64 μg ml^{-1} DTT. Each concentration of DTT was further divided into medium containing human peripheral blood lymphocytes and medium without lymphocytes.

Medium
RPMI-1640 (Gibco, Paisley, UK) without folate and *p*-aminobenzoic acid, but containing:

438 μg ml^{-1} glutamine (Gibco)
1% Nutridoma HU (Boehringer Mannheim, Horsham, UK)
5% foetal calf serum (Gibco)
2% phytohaemagglutinin—M form (Gibco)
1·25 μg ml^{-1} amphotericin B (Gibco)
50 μg ml^{-1} penicillin G (Gibco)
50 μg ml^{-1} streptomycin (Gibco).

Cells
Human peripheral blood lymphocytes were isolated on a density gradient medium (Lymphocyte separation medium (Gibco)). Monocytes were removed following overnight incubation in the medium on a tissue culture treated substrate (Nunc, Paisley, UK), and the cells activated with phytohaemagglutinin during culture in the medium for 4 days. The effect on $5CH_3$—H_4PteGlu stability was then investigated over 46 h.

Incubation conditions
pH 7·4; 37°C; 5% CO_2–17% O_2, balance air.

Equipment and chromatographic conditions
As in second experiment above.

Method
The protective effect of two concentrations of DTT (64 and 129 μg ml^{-1}) on $5CH_3$—H_4PteGlu (394 ng ml^{-1}) in the presence and absence of human peripheral blood lymphocytes has been investigated after varying periods of incubation at 37°C using the HPLC system of Lucock *et al.* (1989) as modified in second experiment above.

RESULTS AND DISCUSSION

Samples for folate analysis are usually stored frozen and may, especially in research situations, be used on more than one occasion. A full storage trial has already been undertaken for $5CH_3$—H_4PteGlu stored frozen with no AO protection in which no losses were found to occur (see Lucock *et al.*, 1989*a*). By studying the effects of repeat freeze-thawing on $5CH_3$—H_4PteGlu integrity through the comparison of one solution of $5CH_3$—H_4PteGlu (stored at −20°C frozen) with another (stored at −20°C as a liquid), the potential variability in response resulting from fluctuating chromatographic conditions over the period of study are negated. Therefore any degradation observed here probably results from the successive stress of freeze-thawing a single solution of $5CH_3$—H_4PteGlu and not storage loss. Expressed graphically (Fig. 1), the present results clearly demonstrate that successive freeze-thawing does have a detrimental effect on $5CH_3$—H_4PteGlu. However, it seems unlikely that a realistic 1–2 freeze-thaw

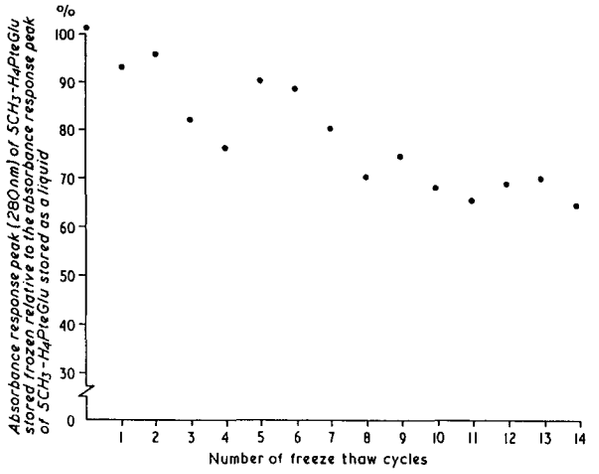

Fig. 1. Relationship between the number of freeze-thaw cycles $5CH_3$—H_4PteGlu is subjected to and the absorbance response peak of $5CH_3$—H_4PteGlu stored frozen relative to the absorbance response peak of $5CH_3$—H_4PteGlu stored as a liquid. Computer generated regression analysis of the degradation of $5CH_3$—H_4PteGlu yields a coefficient (r) of 0·896 and a slope value of 2·407. The chromatographic conditions used are described in the first experiment.

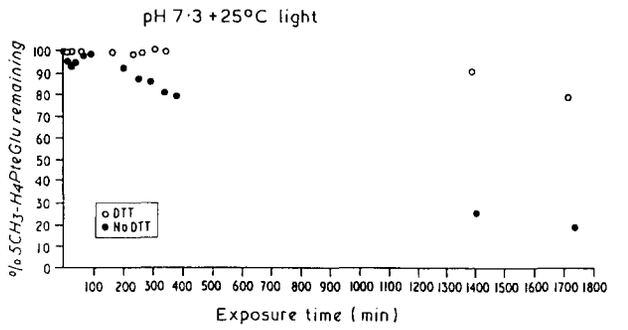

Fig. 2. Percentage of $5CH_3$—H_4PteGlu remaining after exposure to either light or dark at pH 7·3 in the presence and absence of 133 μg ml^{-1} DTT at 25°C. Chromatographic conditions used are described in the second experiment.

cycles will have any real impact on the integrity of $5CH_3$—H_4PteGlu. UV detection as used in this instance, unlike ECD, is unaffected by the presence of vitamin C, the addition of which is a standard precaution adopted by most workers. These results may therefore be of value not only in the analysis of $5CH_3$—H_4PteGlu using HPLC, but also in more traditional bio- and radioassay methods.

It is well documented that pH influences the stability of $5CH_3$—H_4PteGlu, and that the antioxidants β-mercaptoethanol, and especially vitamin C, are useful protective agents. However, no study has thoroughly investigated the effects of light and temperature on $5CH_3$—H_4PteGlu stability at different pH values (3·5, 7·3 and 9·0) in the presence and absence of DTT. All data have been summarised in Table 1, whilst Fig. 2 provides an untransformed example of the typical decay rate for $5CH_3$—H_4PteGlu (Fig. 3 shows representative chromatographic traces). The findings show that:

(i) Overlap in the range of values within which the true K is 95% likely to lie indicates that for a given pH, exposure to light seems to have no obvious effect on the decay rate of $5CH_3$—H_4PteGlu maintained at 25°C, irrespective of whether DTT is present or not.

(ii) The rate of $5CH_3$—H_4PteGlu loss varied dramatically with pH. In the presence of DTT, little loss of $5CH_3$—H_4PteGlu occurred at pH 9·0 or 7·3 as indicated by slope values which are not significantly different from zero ($P > 0{\cdot}05$). However, at pH 3·5, DTT has little or no protective effect as the half-life (h) of $5CH_3$—H_4PteGlu was 19·82 versus 18·93 and 23·32 versus 17·55 for solutions containing DTT versus solutions without DTT maintained in the dark and light, respectively (in all cases $P < 0{\cdot}0001$). Although solutions of $5CH_3$—H_4PteGlu at pH 9·0 and 7·3 were well protected from degradation at 25°C by incorporating DTT, in the absence of this antioxidant a rapid loss of $5CH_3$—H_4PteGlu occurred. This decay was particularly evident at pH 9·0 ($t_{1/2}$ = 5·25 and 5·92 h for solutions maintained in the dark and light, respectively), whilst at pH 7·3 the rate of decay was almost identical to that observed at pH 3·5 ($t_{1/2}$ = 16·22 and 20·68 h for solutions maintained in the dark and light respectively). Indeed overlap of the 95% confidence intervals for K at pH 3·5 and 7·3 in the absence of DTT for solutions

Table 1. Summary of the protective effect of 133 μg ml^{-1} DTT on 500 ng ml^{-1} $5CH_3$—H_4PteGlu under a variety of physical conditions

pH	3·5				7·3				9·0				3·5
	Dark	+25°C	Light	+25°C	Dark	+25°C	Light	+25°C	Dark	+25°C	Light	+25°C	Dark +4°C
	DTT	No DTT	DTT	No DTT	DTT	No DTT	DTT	No DTT	DTT	No DTT	DTT	No DTT	DTT Only
$t_{1/2}$ (h)	19·82	18·93	23·32	17·55	[a]	16·22	571·21	20·68	[a]	5·25	[a]	5·92	259·05
Rate constant (K)	$-5{\cdot}828 \times 10^{-4}$	$-6{\cdot}102 \times 10^{-4}$	$-4{\cdot}953 \times 10^{-4}$	$-6{\cdot}582 \times 10^{-4}$	$5{\cdot}177 \times 10^{-5}$[b]	$-7{\cdot}123 \times 10^{-4}$	$-2{\cdot}022 \times 10^{-5}$[b]	$-5{\cdot}586 \times 10^{-4}$	$5{\cdot}760 \times 10^{-5}$[b]	$-2{\cdot}202 \times 10^{-3}$	$8{\cdot}061 \times 10^{-5}$[b]	$-1{\cdot}949 \times 10^{-3}$	$-4{\cdot}459 \times 10^{-5}$
95% CI	$-6{\cdot}509 \times 10^{-4}$ to $-5{\cdot}147 \times 10^{-4}$	$-7{\cdot}215 \times 10^{-4}$ to $-4{\cdot}989 \times 10^{-4}$	$-6{\cdot}638 \times 10^{-4}$ to $-3{\cdot}267 \times 10^{-4}$	$-8{\cdot}478 \times 10^{-4}$ to $-4{\cdot}684 \times 10^{-4}$	$-6{\cdot}999 \times 10^{-5}$ to $1{\cdot}735 \times 10^{-4}$	$-7{\cdot}916 \times 10^{-4}$ to $-6{\cdot}330 \times 10^{-4}$	$-7{\cdot}178 \times 10^{-5}$ to $3{\cdot}134 \times 10^{-5}$	$-6{\cdot}984 \times 10^{-4}$ to $-4{\cdot}188 \times 10^{-4}$	$-3{\cdot}848 \times 10^{-5}$ to $1{\cdot}537 \times 10^{-4}$	$-2{\cdot}403 \times 10^{-3}$ to $-2{\cdot}001 \times 10^{-3}$	$-9{\cdot}593 \times 10^{-5}$ to $2{\cdot}571 \times 10^{-4}$	$-2{\cdot}092 \times 10^{-3}$ to $-1{\cdot}806 \times 10^{-3}$	$-8{\cdot}824 \times 10^{-5}$ to $-9{\cdot}359 \times 10^{-7}$

[a] No loss measured.
[b] $P > 0{\cdot}05$ (slope is not significantly different from zero).

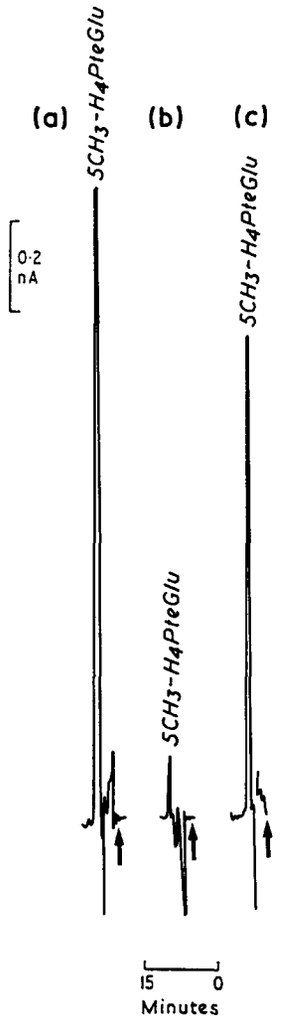

Fig. 3. Three chromatograms of 5 ng $5CH_3$–H_4PteGlu injected under the chromatographic conditions described in the second experiment following exposure to light at 25°C (pH 9·0) for: (a) 0 min; (b) 1399 min; (c) 1375 min. Only (a) and (c) contained DTT (133 μg ml^{-1}). Arrows indicate point of injection.

kept in the dark and light respectively, indicate that at these pH values no significant differences in the decay rates occur.

(iii) Since a low pH is desirable for maintaining hydrophobic interactions during the reversed phase chromatography of folylmonoglutamates (Lucock *et al.*, 1989*a*), and as a low pH would be necessary for certain extraction techniques, the stability of a solution of 500 ng ml^{-1} $5CH_3$—H_4PteGlu in pH 3·5 buffer stored at +4°C with DTT in the dark has been investigated. Although the decay rate showed a significant deviation from zero ($P < 0{\cdot}05$), little loss was detected over two working days ($t_{1/2}$ = 259·05 h). Therefore, given the long half-life of $5CH_3$—H_4PteGlu at +4°C in the dark and in the presence of DTT, it seems likely that the rate of loss at 25°C under otherwise similar conditions ($t_{1/2}$ = 19·82 h) was entirely due to a temperature dependent oxidative degradation.

It is interesting to compare the present values obtained using DTT and HPLC with those of Gyorgy & Pearson (1967) who describe the use of *L. casei* to measure the protective effect of vitamin C on

Table 2. Time (h) for 50% loss of $5CH_3$—H_4PteGlu

Previous study[a]	Present study
39 pH 3·0	17·55 pH 3·5
119/98[b] pH 7·0	20·68 pH 7·3
329 pH 9·0	5·92 pH 9·0

[a] Data from O'Broin *et al.* (1975).
[b] Results using different buffers.

$5CH_3$—H_4PteGlu. On the basis of this it would appear that $5CH_3$—H_4PteGlu is better protected by 133 μg ml^{-1} DTT in pH 7·3 buffer than it is by 5 mg ml^{-1} vitamin C in serum (10% loss in 23 h using DTT versus 23% loss in 24 h using vitamin C (storage in both cases was at 25°C)). However, in the absence of any antioxidant, our results indicate only 24·3% of $5CH_3$—H_4PteGlu is left after 23 h at pH 7·3 whilst 70·9% is left after 24 h in the serum (again storage in both cases was at 25°C). Two hypotheses exist which might explain this disparity: either (i) serum contains intrinsic factors which afford endogenous $5CH_3$—H_4PteGlu protection against degradation, or (ii) the endogenous $5CH_3$—H_4PteGlu is degraded but yields a product which is *L. casei* active (such a product may elicit a bacterial growth response which is greater or less than with the native molecule).

The latter hypothesis could explain why the present results are diametrically opposed to those reported by O'Broin *et al.* (1975) (see Table 2). Their finding, that $5CH_3$—H_4PteGlu is considerably more stable than observed in the present study at all pH values, but especially at an alkaline pH where we found the molecule to be least stable, might be a consequence of $5CH_3$—H_4PteGlu forming a more stable, *L. casei* active degradation product, and that the stability of this degradation product at various pH values is significantly different from that of $5CH_3$—H_4PteGlu itself. If this hypothesis is correct, it is unlikely that the degradation product will produce a growth response equal to that of $5CH_3$—H_4PteGlu, although this in itself, if the response is greater, may contribute to the apparent stability or nutritional usefulness to *L. casei* of $5CH_3$—H_4PteGlu in O'Broin's study. In further support of the present findings, it is known that $5CH_3$—H_4PteGlu is reasonably acid-stable, since this phenomenon has been used in the cleavage and conversion to azo dye derivatives of three distinct pools of folates using the Bratton-Marshall reaction (Eto & Krumdieck, 1980). In this determination the three pools, (1) 5,10-methylene-tetrahydropteroylmonoglutamate, H_4PteGlu, H_2PteGlu, (2) $5CH_3$—H_4PteGlu and (3) 5,10-methenyltetrahydro-pteroylmonoglutamate, 10-formyltetrahydropteroylmono-glutamate, 5CHO—PteGlu and 5-formiminotetrahydro-pteroylmonoglutamate are subject to 0·1 M HCl treatment thereby converting them to (1) *p*-aminobenzoic acid, (2) $5CH_3$—H_4PteGlu and (3) 5,10-methenyltetrahydropter-oylmonoglutamate, respectively. Clearly discrimination between the three pools relies on the ability of $5CH_3$—H_4PteGlu to remain unchanged under acid conditions. H_4PteGlu is

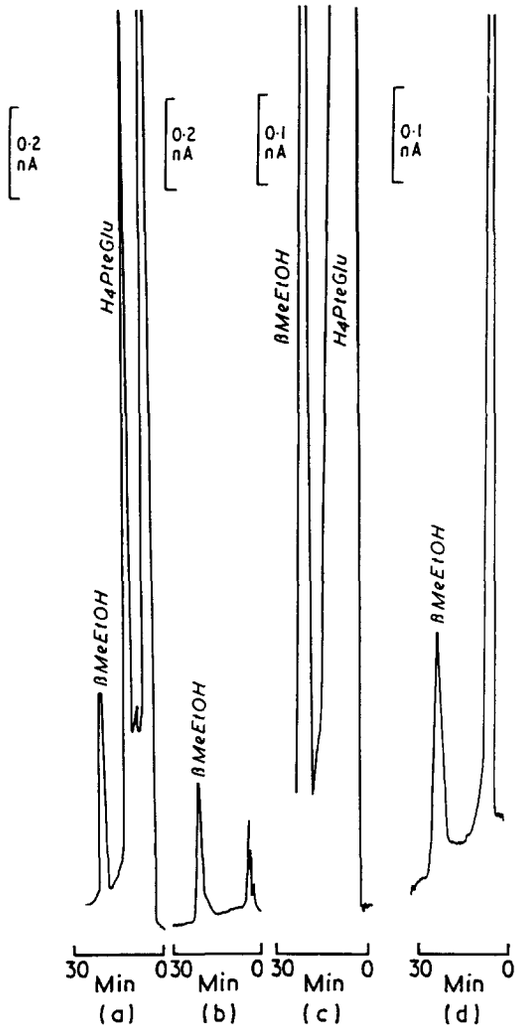

Fig. 4. Chromatographic separation of H_4PteGlu from its solvent of crystallization, β-mercaptoethanol. Traces (a) and (b) were achieved at 450 mV, traces (c) and (d) at 800 mV. Chromatographic conditions are as described in third experiment.

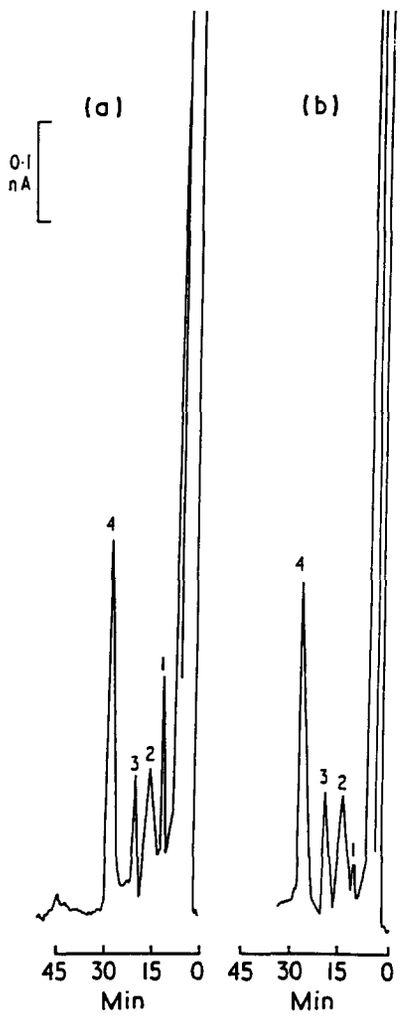

Fig. 5. Chromatographic separation of (1) H_4PteGlu, (2) 5CHO—H_4PteGlu, (3) H_2PteGlu/β-mercaptoethanol and (4) $5CH_3$—H_4PteGlu. Chromatographic conditions as described in the third experiment except 800 mV. Whilst the 5-methyl and formyl coenzymes show no deterioration, the tetrahydro form is highly unstable as evidenced by consecutive chromatograms (a) and (b) which show 50% loss in 29·54 min.

recognised as one of the least stable biologically active folylmonoglutamates. Commercial preparations contain β-mercaptoethanol as the solvent of crystallisation, this acts as an effective antioxidant. In order to discriminate between β-mercaptoethanol and H_4PteGlu following separation on an HPLC column, β-mercaptoethanol must be injected separately from H_4PteGlu/β-mercaptoethanol. Figure 4 (a)–(d) shows separation of β-mercaptoethanol (b) and (d) and H_4PteGlu containing β-mercaptoethanol (a) and (c). An ECD set at 450 mV was used for traces (a) and (b) and 800 mV for traces (c) and (d). The only peak not common to β-mercaptoethanol and H_4PteGlu containing β-mercaptoethanol is assumed to be H_4PteGlu. The resolution of H_4PteGlu from other folylmonoglutamates and its stability is shown in Fig. 5 (a) and (b). Although baseline resolution is not achieved, separation is adequate to illustrate the poor stability of H_4PteGlu relative to the other folylmonoglutamates. Since only 48 min expired between the start of these two chroma-tographic traces, the stability of H_4PteGlu is extremely poor (half of H_4PteGlu has disappeared in 29·54 min, whilst no measurable loss of $5CH_3$—H_4PteGlu and 5CHO—H_4PteGlu has occurred). The minimum detection limit for these compounds is given in Table 3.

Table 3. Minimum detection limit of folylmonoglutamates

	Detection limit on column—450 mV	Detection limit on column—800 mV
H_4PteGlu	1·1 ng	435 pg
5CHO—H_4PteGlu	—	800 pg
H_2PteGlu	1·7 ng	182 pg
$5CH_3$—H_4PteGlu	286 pg	241 pg

Table 4. Differential loss of folylmonoglutamates (100 ng ml^{-1}) subjected to 35°C for 3·75 h

Congener	Recovery after 3·75 h at 35°C
5CHO—H_4PteGlu	100·0%
H_2PteGlu	52·4%
$5CH_3$—H_4PteGlu	7·6%
H_4PteGlu	0·0%

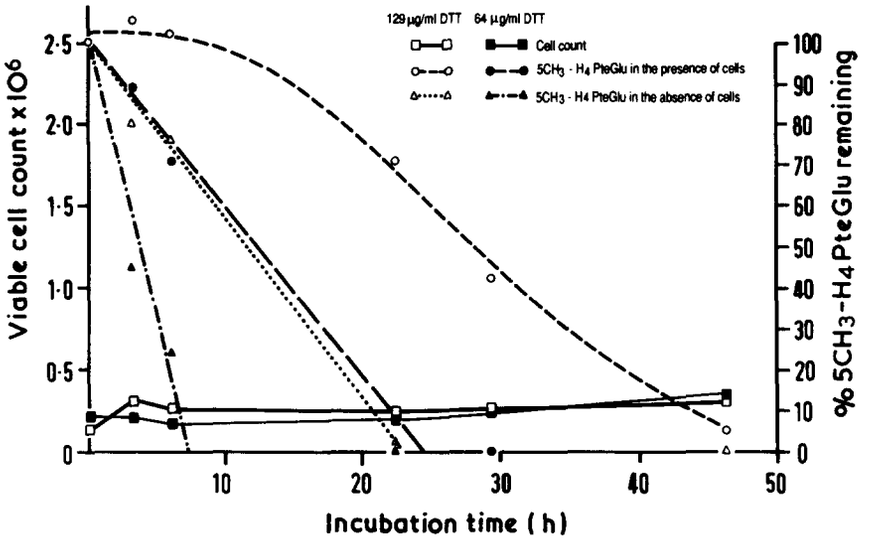

Fig. 6. Protective effect exhibited by human peripheral blood lymphocytes on extracellular $5CH_3$—H_4PteGlu. The study was carried out in the presence of two concentrations of DTT.

By subjecting these reduced folylmonoglutamates (each at 100 ng ml^{-1}) to a constant temperature of 35°C for 3·5 h it has been possible to work out their relative stability (see Table 4). Therefore in order of stability 5CHO—H_4PteGlu > H_2PteGlu > $5CH_3$—H_4PteGlu > H_4PteGlu.

One of the most interesting aspects of this study was the finding that the integrity of exogenous $5CH_3$—H_4PteGlu, extracellular with respect to human peripheral blood lymphocytes, was preserved quite markedly over $5CH_3$—H_4PteGlu integrity in media without cells. The degree of protection, whether in the presence or absence of cells, was directly proportional to the concentration of DTT in the media (64 or 129 µg ml^{-1}) (see Fig. 6). An explanation for this is lacking, although one could speculate that either a cell surface binding protein or transmembrane movement of intracellular antioxidants might elicit a certain degree of protection. Protection could also arise through an intracellular binding protein released as a result of senescence and cell death. However, since media with and without cells were injected directly onto the liquid chromatograph, oxidative protection as a consequence of $5CH_3$—H_4PteGlu entry into the cell seems unlikely.

Combined, these studies provide useful information on both the in-vitro and by inference in-vivo stability of a labile trace nutrient essential for important biochemical processes such as the intracellular synthesis of protein and DNA.

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