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Methylfolate exhibits a negative in-vitro interaction with important dietary metal cations

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Methylfolate is an important dietary micronutrient essential for cell maturation and replication. Using HPLC with electrochemical detection we have shown that, at approaching neutral pH, anionic methylfolate complexes with metal cations leading to either oxidative degradation or possibly precipitation, the order of the effect being $Zn^{2+} > Ca^{2+} \simeq K^{+} > Mg^{2+} \simeq Na^{+}$. Equimolar Zn^{2+} and Na^{+} enhance methylfolate decay 33.3- and 7.2-fold, respectively, when compared to the decay in water alone. This effect does not occur in the presence of reduced glutathione or at mildly acid pH. These results may have important implications *in vivo*, particularly with respect to the bioavailability of dietary and pharmacological forms of the vitamin.

INTRODUCTION

Methylfolate ($5CH_3$-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. In a previous report (Lucock *et al.*, 1993) we showed how this labile molecule was influenced by physicochemical and biological factors such as light, temperature, pH, antioxidant and the presence of an unidentified factor associated with human peripheral blood lymphocytes. The present report uses a similar approach to describe a negative micronutrient interrelationship between $5CH_3$-H_4PteGlu and selected metal cations. The implications of this in-vitro study with respect to folate bioavailability are discussed.

MATERIALS AND METHODS

Chemicals

DL $5CH_3$-H_4PteGlu barium salt, dithiothreitol and reduced glutathione were purchased from Sigma Chemical Co. Ltd (Poole, Dorset, UK). AnalaR grade hydrochloric acid, ethylenediaminetetra-acetic acid disodium salt, potassium chloride, sodium chloride, magnesium chloride, calcium chloride and zinc chloride; and Aristar grade potassium dihydrogen orthophosphate and orthophosphoric acid were purchased from BDH chemicals Ltd (Poole, Dorset, UK). Methanol (HPLC grade) was purchased from Rathburn Chemicals (Walkerburn, UK).

Solutions

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). Stock aliquots of 200 μg/ml $5CH_3$-H_4PteGlu were made up in water containing no antioxidant. These were stored at –70°C until used. Working solutions at 250 ng/ml were made up on the day by dilution in water or various aqueous metal cation solutions at equimolar (0.1 M) concentrations. The pH was lowered with HCl or 1 μg/ml reduced glutathione added as indicated. To investigate effects near physiological pH, and in order to avoid any influence from additional ions, the aqueous cation solutions were not buffered to pH 7.4, but left at their original pH which was essentially the same as water (pH 6.42±0.11).

Equipment and chromatographic conditions

An HPLC system coupled to an electrochemical detector (HPLC–EC) was used as described in Lucock *et al.* (1989, 1993).

Method

Solutions of $5CH_3$-H_4PteGlu (250 ng/ml) made up in water, 0.1 M NaCl (containing 1 μg/ml reduced glutathione) or equimolar (0.1 M) aqueous solutions of NaCl, KCl, $MgCl_2$, $CaCl_2$ and $ZnCl_2$ at pH 6.4 and pH 3.5 were placed at 25°C in the dark. Aliquots (30 μl) of each solution under investigation were injected onto the chromatograph at regular intervals and decay rates

calculated from the diminishing current response peak determined under the described conditions. Chromatographic stability was ascertained by periodic injection of 30 μl $5CH_3$-H_4PteGlu (250 ng/ml) in water containing 133 μg/ml dithiothreitol. This standard was stored at 4°C prior to its injection.

Since $5CH_3$-H_4PteGlu stability was so profoundly influenced by zinc, an important dietary nutrient, and given recent reports of zinc's negative interaction with dietary folates *in vivo* (Simmer *et al.*, 1987), decay was followed at 0.05, 0.025, 0.01, 10^{-3} and 10^{-4} M $ZnCl_2$ in order to describe the stoichiometry involved. 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 minutes) have been calculated (K=gradient value × 2.303; 95% confidence interval=K±t value corresponding to n–2 degrees of freedom × estimated standard deviation of K; $t_{1/2}$ (min) = 0.693/ K.

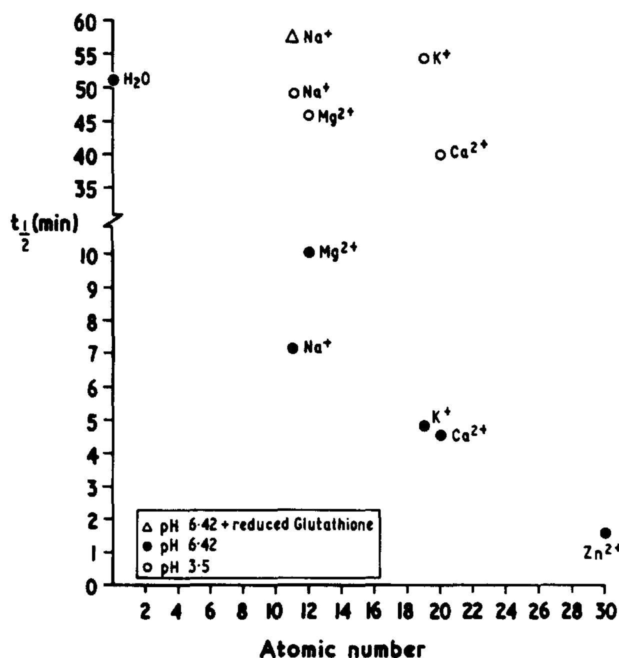

Fig. 1. Half life of the carboxylate anionic (pH 6.4) and protonated (pH 3.5) forms of $5CH_3$-H_4PteGlu in the presence of some biologically important cations (aqueous 0.1 M solutions).

RESULTS AND DISCUSSION

HPLC–EC can evaluate $5CH_3$-H_4PteGlu stability without being influenced by its degradation product (probably 5-methyldihydrofolate (Donaldson & Keresztesy, 1962)). However, *Lactobacillus casei* bioassay, the classical method for folate estimation, is likely to show a growth response to the oxidised form(s) of the vitamin (O'Broin *et al.*, 1975; Lucock *et al.*, 1993). The advantage of HPLC–EC for measuring $5CH_3$-H_4PteGlu stability therefore clearly resides in the assay's specificity.

Table 1 shows how $5CH_3$-H_4PteGlu decay, under approaching neutral and mildly acid conditions, is influenced by a variety of metal chlorides at equimolar concentrations (0.1 M). These rates of decay expressed as a rate constant (K±95% confidence interval) and half-life ($t_{1/2}$ in minutes) are compared to those in water alone and 0.1 M NaCl containing 1 μg/ml reduced glutathione. Each experiment was carried out at 25°C in the dark and the K and $t_{1/2}$ values obtained from a single time *versus* current response curve. Clearly these biologically important cations influence $5CH_3$-H_4PteGlu stability with degradation profoundly enhanced in their presence compared to water alone. Furthermore, a trend is apparent in which $t_{1/2}$ decreases with increasing atomic mass (see Fig. 1). As this phenomenon occurs near neutral pH, and not pH 3.5, and since the effect can be negated in the presence of reduced glutathione,

Table 1. Summary of the half lives ($t_{1/2}$) and rate constants (K ± 95% confidence interval) for $5CH_3$-H_4PteGlu in the presence of various biologically important cations. Tests were carried out at approaching neutral (pH 6·4) and mildly acid (pH 3·5) conditions

250 ng/ml $5CH_3$–H_4PteGlu solution containing	$t_{1/2}$(min) for $5CH_3$–H_4PteGlu at pH 6·4			$t_{1/2}$(min) for $5CH_3$–H_4PteGlu at pH 3·5		
	$t_{1/2}$	K	95% CI[a] for K	$t_{1/2}$	K	95% CI[a] for K
Water (to pH 3·5 with HCl)				175·6	−0·004	−0·005 to −0·003
Water (no pH adjustment)	50·9	−0·014	−0·019 to −0·008			
NaCl (0·1 M)	7·1	−0·097	−0·149 to −0·046	49·4	−0·014	−0·021 to −0·007
$MgCl_2$ (0·1 M)	10·0	−0·069	−0·092 to −0·046	45·9	−0·015	−0·023 to −0·007
KCl (0·1 M)	4·8	−0·145	−0·185 to −0·105	54·8	−0·013	−0·029 to 0·003
$CaCl_2$ (0·1 M)	4·5	−0·154	−0·282 to −0·025	39·6	−0·018	−0·022 to −0·013
$ZnCl_2$ (0·1 M)	1·5	−0·452	−1·177 to 0·273			
$ZnCl_2$ (0·05 M)	1·8	−0·378	−0·792 to 0·716			
$ZnCl_2$ (0·025 M)	5·8	−0·119	−0·380 to 0·143			
$ZnCl_2$ (0·01 M)	2·2	−0·311	−2·160 to 1·538			
$ZnCl_2$ (0·001 M)	15·2	−0·046	−0·052 to −0·040			
$ZnCl_2$ (0·0001 M)	40·6	−0·017	−0·024 to −0·011			
NaCl (0·1 M) + reduced glutathione	57·1	0·012	0·011 to 0·013			

[a] CI = confidence interval.

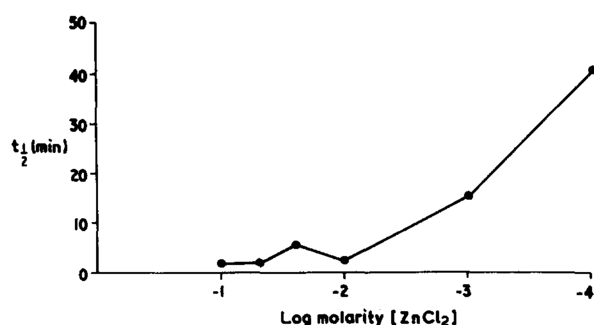

Fig. 2. Effect of $ZnCl_2$ concentration on $5CH_3$-H_4PteGlu half life in aqueous solution at pH 6.4.

an oxidative process dependent upon the ionic state of $5CH_3$-H_4PteGlu seems a likely cause, although precipitation of the $5CH_3$-H_4PteGlu cation complex should not be excluded. At pH 6.4 $5CH_3$-H_4PteGlu exists in its anionic form (2 × COO—, the phenolic—OH remaining in its protonated form); in this state complex formation with metal cations could render it more labile. Since the stability of $5CH_3$-H_4PteGlu increased at pH 3.5 in the presence of the same cations, the protonated free acid is probably less available for complex formation and consequently more stable; from information generated using reversed phase and weak anion-exchange chromatography (unpublished results) we believe that, like pteroylmonoglutamate and 5-formyltetrahydrofolate (Merck Index; Elsborg, 1974), ionisation of the $5CH_3$-H_4PteGlu carboxylic acid groups would be minimal at pH 3.5. However, the exact mechanism remains unclear. Zn^{2+} could associate with the glutamyl carboxylate ions or induce degradation via polarization of the carbonyl group associated with the peptide linkage between *p*-aminobenzoic acid and glutamic acid. Furthermore, although the differential effect of cations on $5CH_3$-H_4PteGlu is removed at pH 3.5 (decay rates being similar to that in water alone at pH 6.4), the rates are dramatically reduced compared to those in water alone at pH 3.5 ($t_{1/2}$=175.6 min). Therefore, even at pH 3.5 some general effect of the cations is present.

$5CH_3$-H_4PteGlu was profoundly unstable in the presence of 0.1 M $ZnCl_2$. Table 1 shows the concentration-dependent effect of Zn^{2+} on $5CH_3$-H_4PteGlu degradation. By referring to Fig. 2, a maximal effect can be seen to occur at approximately 0.01 M $ZnCl_2$ (the antilog of -2).

The implications of these findings may have significance *in vitro* where maintenance of isotonicity is important, for instance in tissue-culture medium containing $5CH_3$-H_4PteGlu. However, more importantly, under certain circumstances the negative interaction of $5CH_3$-H_4PteGlu with zinc could be of crucial biological importance since reduced zinc absorption with iron-folate supplements in pregnant women has been reported (Simmer *et al.*, 1987). Other studies suggest that zinc-folate (Pteroylmonoglutamate) complex formation at pH 2.0 could interfere with intestinal transport of both micronutrients and account for their reduced absorption (Gishan *et al.*, 1986). Results of the present study indicate a similar complex formation with $5CH_3$-H_4PteGlu but at a higher pH (6.4). Therefore, although the present study has been carried out on an artificial system, the results may have important implications when considering the bioavailability of folyl micronutrients under conditions where zinc and folate supplements are co-administered such as in early pregnancy.

Since, at pH 6.4, equimolar Zn^{2+} and Na^+ enhance $5CH_3$-H_4PteGlu decay 33.3- and 7.2-fold, respectively, when compared to the decay in water alone (a process inhibited by the antioxidant glutathione or mildly acid conditions), further biologically specific interactions might be anticipated. For instance, the digestive enzyme γ-glutamyl hydrolase (EC 3.4.22.12)(folate deconjugase), which acts at γ- rather than α-carboxyl glutamate residues on dietary folylpolyglutamate molecules, exhibits exo- or carboxypeptidase activity alongside endopeptidase activity (McGuire & Coward, 1984). Since Zn^{2+} can stimulate γ-glutamyl hydrolase activity from some sources—the bovine hepatic enzyme being a zinc metalloprotein (Silink *et al.*, 1975), a parallel might exist with carboxypeptidase-A which is a similar proteolytic enzyme containing a tightly bound zinc ion near its catalytic cleft and which is essential for enzyme activity. A complex of dietary folylpolyglutamates and oral Zn^{2+} supplements might therefore hinder polyglutamate hydrolysis and consequently reduce dietary folate absorption. Such speculation is not inconsistent with workers who have shown that Zn^{2+} can actually inhibit γ-glutamyl hydrolase activity (McGuire & Coward, 1984).

We conclude by drawing attention to the need for further detailed investigations of the potentially negative in-vivo interactions of $5CH_3$-H_4PteGlu with Zn^{2+} and similar micronutrients such as iron and copper. This is particularly important in the periconceptional period where the role of folate in preventing congenital malformations such a neural tube defects is well established (MRC Vitamin Study Research Group, 1991).

REFERENCES

Donaldson, K. O. & Keresztesy, J. C. (1962). Naturally occurring forms of folic acid. *J. Biol. Chem.*, **237**, 3815.

Elsborg, L. (1974). Folic acid: A new approach to the mechanism of its intestinal absorption. *Danish. Med. Bull.*, **21**, 1–11.

Gishan, F. K., Said, H. M., Wilson, P. C., Murrell, J. E. & Greene, H. L. (1986). Intestinal transport of zinc and folic acid: A mutual inhibitory effect. *Am. J. Clin. Nutr.*, **43**, 258–62.

Lucock, M. D., Hartley, R. & Smithells, R. W. (1989). A rapid and specific HPLC–electrochemical method for the determination of endogenous 5-methyltetrahydrofolic acid in plasma using solid phase sample preparation with internal standardization. *Biomed. Chromatogr.*, **3**, 58–63.

Lucock, M. D., Green, M., Hartley, R. & Levene, M. I. (1993). Physicochemical and biological factors influencing methylfolate stability: Use of dithriothreitol for hplc analysis with electrochemical detection. *Food Chem.*, **47**, 79–86.

McGuire, J. J. & Coward, J. K. (1984). Pteroylpolyglutamates: Biosynthesis, degradation, and function. In *Folates and Pterins*, ed. R. L. Blakley & S. J. Benkovic, John Wiley and Sons, New York, pp 135–78.

MRC Vitamin Study Research Group (1991). Prevention of neural tube defects: Results of the Medical Research Council Vitamin Study. *Lancet*, **338**, 131–7.

O'Broin, J. D., Temperley, I. J., Brown, J. P. & Scott, J. M. (1975). Nutritional stability of various naturally occurring monoglutamate derivatives of folic acid. *Am. J. Clin. Nutr.*, **28**, 438–44.

Silink, M, Reddel, R., Bethel, M. & Rowe, P. (1975). γ-glutamyl hydrolase (conjugase) purification and properties of the bovine hepatic enzyme. *J. Biol. Chem.*, **250**, 5982–93.

Simmer, K., James, C. & Thompson, R. P. H. (1987). Are iron folate supplements harmful? *Am. J. Clin. Nutr.*, **45**, 122–5.