Folates in human serum

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SYNOPSIS Constituent serum folates have been shown to be altered and in some cases raised in diseases in which total folate levels are known to be frequently decreased. Absorption experiments showed that orally administered folate analogues affect the folate pool in different ways. The stability of the 10-formyltetrahydrofolate level in normal subjects is demonstrated.

The association of low serum folate levels with adult coeliac disease (Cooke, Fone, Cox, Meynell, and Gaddie, 1963; Dormandy, Waters, and Mollin, 1963) and leukaemia (Rama Rao, Lagerlof, Einhorn, and Reizenstein, 1965) is established although the pathogenesis of the relationship is less well understood. The fate of orally administered pteroyl-L-monoglutamic acid is well documented (Dormandy *et al.*, 1963; Butterworth, Nadel, Perez-Santiago, Santini, Rafael, and Gardner, 1957; Chanarin, Anderson, and Mollin, 1958; Melikian, Paton, Leeming, and Portman-Graham, 1971; Leeming, Portman-Graham, and Blair, 1972). There is information on the absorption of the folate analogues (Baker, Frank, Feingold, Ziffer, Gellene, Leevy, and Sobotka, 1965; Perry and Chanarin, 1970; Nixon and Bertino, 1972; Brown, Scott, Foster, and Weir, 1973) but less on their impact on the components of the total folate pool.

This study describes relative levels of 5-methyltetrahydrofolic acid (5Me-THF), 10-formyltetrahydrofolic acid (10-CHO-THF), and 10-formylfolic acid (10-CHO-FA) in normal subjects and in patients with adult coeliac disease and leukaemia.

The effects of orally administered folates on individual serum constituents are demonstrated.

Materials and Methods

Pteroyl-L-monoglutamic acid was a commercial product. The calcium salt of 5-formyltetrahydrofolic acid (5-CHO-THF) was a gift from Lederle Laboratories Ltd; 5-Me-THF-Ca salt was prepared according to Blair and Saunders (1970), 10-CHO-FA according to Blakley (1959), and 5,10-methenyl-THF by the method of Roth, Hultqvist, Fahrenbach,

Cosulich, Broquist, Brockman, Smith, Parker, Stokstad, and Jukes (1952). All products were checked for purity and identity by (a) microbiological assay with *L. casei*, *S. faecalis*, and *P. cerevisiae*, (b) uv spectra in water at pH 1, 7, and 13, and (c) thin-layer chromatography in three solvent systems, namely, 0.1 M phosphate buffer pH 7.0, butanol:acetic acid:water (4:1:5 v/v, upper phase), and propanol:1% ammonia (2:1 v/v).

Five mg doses of biologically active materials were prepared for absorption experiments. The subjects fasted overnight and during the collection of blood samples. Blood specimens were taken aseptically immediately before the administration of folates and then at 30, 60, 90, 120, 180 and, in some instances, at 240, 300, and 360 minutes. They were immediately decanted into glass specimen containers and stood at room temperature for half to one hour. After centrifugation the sera were stored at -20°C with the addition of 5 mg/ml ascorbic acid and thawed immediately before adding to assay tubes. Assays were carried out as soon as possible after the collection of specimens and always within seven days.

Single samples were taken after a normal lunch.

L. casei assays were carried out using an aseptic technique (Herbert, 1966). *Streptococcus faecalis* and *P. cerevisiae* assays were also performed aseptically. Bioautography (Leeming, Portman-Graham, Swan, and Blair, 1970) with the three test organisms was used to confirm the identity of the serum folates after absorption.

The normal subjects were hospital staff and medical students.

Results

The individual serum folates in normal subjects and in the diseased states are given in table I.

Subjects	No. of Subjects	5 Me-THF (ng/cm ³)			10 CHO-THF (ng/cm ³)			10 CHO-FA (ng/cm ³)			<i>S. faecalis</i> (ng/cm ³)		
		Mean	± SEM	t Test (%)	Mean	± SEM	t Test (%)	Mean	± SEM	t Test (%)	Mean	± SEM	t Test (%)
Normal	51	4.80	± 0.28	—	0.57	± 0.03	—	0.21	± 0.03	—	0.78	± 0.03	—
Leukaemia	41	3.70	± 0.37	<1	0.50	± 0.03	NS	0.70	± 0.08	<0.05	1.23	± 0.07	<0.05
Adult coeliac disease	56	4.59	± 0.46	NS	0.56	± 0.03	NS	0.54	± 0.07	<0.05	1.1	± 0.06	<0.05

Table I Mean levels of folate analogues in normal controls and patients with leukaemia and adult coeliac disease

Folate Analogues	Rf Values	<i>L. casei</i>	<i>S. faecalis</i>	<i>P. cerevisiae</i>
Folic acid	0.05	+	+	—
10 CHO-FA	0.60	+	+	—
10 CHO-THF	0.45 (t) ¹	+	+	+
5 CHO-THF	0.50	+	+	+
5 Me-THF	0.65	+	—	—

Table II Bioautographic Rf values and microbiological activity of folates on cellulose TLC developed in 3% ammonium chloride

¹(t) = tail

The Rf values of the folates are given in table II, together with their activity for the three test organisms.

L. casei is active for all folates in serum whilst *Strep. faecalis* is active for all except 5-methyltetrahydrofolic acid (Johns and Bertino, 1965); therefore, the difference in activity of these two test organisms is a measure of 5-methyltetrahydrofolic acid. *P. cerevisiae* is active for 10-formyl-tetrahydrofolic acid 5-formyltetrahydrofolic acid but not for 10-formylfolic acid or folic acid whereas *Streptococcus faecalis* is active for all four compounds. Therefore the difference between the *S. faecalis* and *P. cerevisiae* assays measures the amount of folic acid and 10-formylfolic acid present.

Bioautography of serum and urine samples showed that except after folic acid administration the folate responsible for the *S. faecalis*-*P. cerevisiae* difference is 10-formylfolic acid. Of the folates active for *P. cerevisiae*, only 10-CHOTHF is found in serum (Nixon and Bertino, 1972). Individual folates were further identified by bioautography in 3% aqueous ammonium chloride, a solvent system in which all commonly occurring folate monoglutamates are clearly separated (Leeming *et al.*, 1970; Brown, Davidson, and Scott, 1973). Additional precision in identification was obtained by carrying out bioautography with *L. casei*, *S. faecalis*, and *P. cerevisiae* as developing microorganisms.

The correlation of total folate and 5-Me-THF is shown in figs 1 and figs 2-6 show serum folate constituents after oral administration of folate analogues. In 10 patients with adult coeliac disease on long-term treatment with folic acid, the mean values of the total folate, *Streptococcus faecalis*, and *P. cerevisiae* (10-CHOTHF) levels were > 20.0, > 3.0, and 0.56 ng/cm³ respectively.

Discussion

Three folates can be assayed in normal human blood serum. The means of our values were 5-methyltetrahydrofolic acid, 4.80 ng/cm³, 10-formyltetrahydrofolic acid, 0.57 ng/cm³, and 10-formylfolic acid 0.21 ng/cm³ (table I). As 10-formyltetrahydrofolic acid is very readily oxidized to 10-formylfolic acid (similar

Fig 1 Correlation of total folate and 5-methyltetrahydrofolate levels in 51 normal subjects.

Symbols for figs 2-6 together with standard errors of means.

- *L. casei* (total folate)
- 5-Me THF
- 10-CHO FA
- 10-CHO THF
- *—* Pteroyl-L-monoglutamic acid

Fig 2 The effect of orally administered 5-Me-THF (10 mg) on the mean levels of folate analogues in six subjects.

to tetrahydrofolic acid (Blair and Pearson, 1974), the 10-formylfolic acid is very likely an analytical artifact. When serum samples were taken and analysed under carefully controlled conditions no 10-formylfolic acid could be identified. The 10-formyltetrahydrofolic acid level in serum is thus best represented by the *S. faecalis* level. Normal serum folates are therefore 5-methyltetrahydrofolic acid 4.80 ng/cm³ and 10-formyltetrahydrofolic acid 0.78 ng/cm³.

In man 5-formyl and 5-methyltetrahydrofolic acid and folic acid are converted into 10-formyltetrahydrofolic acid (Albrecht and Broquist, 1956; Silverman, Ebaugh, and Gardiner, 1956; Nixon and Bertino, 1972) and 10-formyltetrahydrofolic acid adminis-

Fig 3 The effect of orally administered 5-CHO THF (10 mg) on the mean levels of folate analogues in six subjects.

tered as 5,10-methenyltetrahydrofolic acid is converted into 5-methyltetrahydrofolic acid (fig 4). Thus the two serum folates are in dynamic metabolic equilibrium.

When the total serum folates are plotted against the serum 5-methyltetrahydrofolic acid, an excellent straight line fit with an intercept on the total folate axis at 0.8 ng/cm³ is obtained (fig 1). Therefore, normal serum folates consist of a constant level of 10-formyltetrahydrofolic acid maintained by some homeostatic mechanism and a variable level of 5-methyltetrahydrofolic acid acting as storage form. Statistical analysis of the *S. faecalis* and *P. cerevisiae* values show them to be normally distributed about the mean as might be expected for the analysis of a serum component maintained at a constant level. Similar analysis of the *L. casei* values show them to have a skewed distribution about the mean as might be expected from a summation of serum components, one of which varies with dietary intake.

Further confirmation of the homeostatic control of 10-formyltetrahydrofolic acid levels and the role of 5-methyltetrahydrofolic acid as a storage form is shown by the changes in serum folates after oral

Fig 4

Fig 5

Fig 6

Fig 4 The effect of orally administered 5,10 methenyl THF (10 mg) on the mean levels of folate analogues in six subjects.

Fig 5 The effect of orally administered 10-CHO FA (5 mg) on the mean levels of folate analogues in six subjects.

Fig 6 The effect of orally administered pteroyl-L-monoglutamic acid (5 mg) on the mean levels of folate analogues in six subjects.

doses of folates. After oral doses of folic acid, 10-formylfolic acid, 5-formyltetrahydrofolic acid, 5,10-methenyltetrahydrofolic acid, and 5-methyltetrahydrofolic acid total serum folates rise rapidly to high levels while the 10-formyltetrahydrofolic acid remains practically constant (figures 2-6). Long-term administration of folic acid also fails to raise the 10-formyltetrahydrofolic acid level. The 10-formylfolic acid is not converted to 5-methyltetrahydrofolic acid (figure 5). As previously noted (Leeming *et al*, 1972), folic acid is slowly converted to 5-methyltetrahydrofolic acid (figure 6). The 5,10-methenyltetrahydrofolic acid also gives a small amount of serum 10-formylfolic acid probably derived by oxidation of 10-formyltetrahydrofolic acid in the jejunum before absorption (figure 4) (Beavon and Blair, 1972; Blair and Pearson, 1974). The persistence of these small amounts of 10-formylfolic acid in the serum again shows that this compound is not converted to 5-methyltetrahydrofolic acid.

The relative peak serum levels obtained after administration of folic acid and the tetrahydrofolates, particularly the high level after 5,10-methenyl-THF, was similar to that shown by Brown *et al* (1973) although these authors did not use differential microbiological assays or separative techniques to identify individual serum constituents.

Measurement of the individual serum folates in adult coeliac disease and leukaemia shows a significant increase over normals of the 10-formylfolic acid level (table I). However, if as in normal subjects this is an analytical artefact derived from the oxidation of 10-formyltetrahydrofolic acid then in both adult coeliac disease and leukaemia there are significantly increased levels of serum 10-formyltetrahydrofolic acid. In the leukaemic subjects both total folate and 5-methyltetrahydrofolic acid are significantly reduced (table I).

Sotobayashi, Rosen, and Nichol (1965) showed that tissues with high cell replication rates had a much smaller proportion of folate as 5-methyltetrahydrofolic acid and a much higher proportion as 10-formyltetrahydrofolic acid (or derived compounds) than tissues with slow cell replication rates. Thus the increased serum levels of 10-formyltetrahydrofolic acid in adult coeliac disease and leukaemia may be due to the increased cell replication rate in these diseases.

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