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A Technique for Assessing the Biological Availability of Folate in Foods

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ABSTRACT

A method is described for the assessment of folate availability from human foods using the the folate-depleted rat. Animals were maintained on a semisynthetic diet for periods of 4 weeks or more. At the end of this period tissue folate levels were measured using a radioimmunoassay. Folate intakes were assessed by monitoring food intake on a daily basis. The folate content of the diet was determined by microbiological assay. The animals continued to grow and maintain health throughout the depletion period, but levels of folate in liver, serum and intestinal mucosa were significantly reduced in comparison to rats fed a folate supplemented diet. Refeeding depleted rats with crystalline folic acid, or with freeze-dried cabbage incorporated into the semi-synthetic diet led to a measurable increase in tissue folate stores within 24 h. The serum-folate level appeared to be the most consistent and conveniently measurable index of folate status. The method provides a relatively inexpensive and rapid method of measuring the physiological response to the most important sources of folate in the human diet.

INTRODUCTION

The nutritional value of vitamins in foods depends both upon the total concentration of biologically active forms present, and upon their availability for absorption and utilisation by normal metabolic pathways. In the case of folates the multiplicity of naturally occurring substituted and conjugated forms leads to uncertainties in both areas, and there is little conclusive information on the bioavailability of folates from the major

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sources in the human diet. A few indirect studies have been undertaken in man (Tamura & Stokstad, 1973), but there is a need to develop a practical and reliable technique using experimental animals. Several groups have described methods which utilise the growth and folate status of the chick, or the liver folate stores of the rat as experimental indices (Graham *et al.*, 1980; Keagy & Oace, 1982). However, both methods require large numbers of experimental animals, and utilise time-consuming microbiological assay techniques to measure changes in tissue folate levels. In this report we describe a simple method for assessing folate availability to rats, in which the rise in serum folate of previously depleted animals is measured by radioimmunoassay. The application of the technique is illustrated by a comparison of the serum folate response to raw cabbage and pteroylglutamic acid incorporated into semi-synthetic feeds.

METHODS

Preparation of diets

The composition of the folate-free semi-synthetic diet (FFS) fed to all animals during the folate depletion period is shown in Table 1. Experimental diets containing synthetic folic acid were identical, except for the addition of crystalline pteroylglutamic acid (PGA) (Sigma Chemical Co., Poole, Dorset) at the appropriate level. For the preparation of the experimental diets containing a natural source of food folate, green cabbage was purchased locally. The chopped raw cabbage was freeze-dried in bulk, finely ground and incorporated into semi-synthetic diets at levels of 125 and 250 g/kg. The other constituents were adjusted to maintain total energy values to within 5% of the control diet. The folate contents of the diets were determined by microbiological assay. All diets were stored in black polythene bags at −40°C prior to use.

Although stable at −40°C, the folate content of experimental diets declines under animal house conditions. In order to estimate and allow for this, the PGA-supplemented diets were analysed at the beginning and end of the 7 day feeding period (Table 2), and the folate intake of the animals (Table 5) was calculated from the average of these values.

Microbiological assay of folate

The procedure was essentially that of Phillips & Wright (1983), with minor modifications. Samples of diet (1 g) were homogenised in 25 ml of ascorbic

TABLE 1
Composition of the Folate-Deficient Semi-Synthetic Diet

Constituent	*g/kg diet*
Sucrose[a]	655
Casein[b]	200
Maize-oil[c]	80
Mineral-Mix[d]	40
Vitamin-Mix (in starch)[e]	20
DL-Methionine[f]	5

[a] BDH Chemicals, Dagenham, Essex.
[b] Edible casein; Glaxo Farley Foods, Plymouth.
[c] Mazola corn-oil.
[d] Mineral-mix provided (per kilogram of diet): $CaHPO_4$, 13 g; Na_2HPO_4, 7·4 g; $CaCO_3$, 8·2 g; KCl, 7·03 g; $MgSO_4$, 4·0 g; $ZnCO_3$, 0·1 g; $FeSO_4.7H_2O$, 0·144 g; $CuSO_4.5H_2O$, 0·023 g; KIO_3, 0·001 g; $MnSO_4.H_2O$, 0·18 g.
[e] Vitamin-mix provided (per kilogram of diet): Nicotinic acid, 60 mg; cyanocobalamin (B12) in mannitol, 50 mg; Calcium D-Pantothenate, 40 mg; Thiamine HCl (B1), 10 mg; Riboflavin (B2), 10 mg; Pyridoxine, 10 mg; D-Biotin, 1 mg; Menadione (K), 1 mg; Rovimix E-50, 150 mg; Rovimix A-500, 25 mg; Rovimix A-500/D3, 15 mg; Choline bitartrate, 0·9 g.
[f] Sigma Chemical Co, Poole, Dorset.

TABLE 2
Folate Content of PGA Supplemented Diets

Diet	*Folate content (mg/kg diet)*	
	Initial	*Final*
Folate deficient	0·28 (0·7)	ND
PGA-supplemented	10·6 (0·6)	6·0 (0·8)
PGA-supplemented	23·1 (5·3)	12·2 (3·9)

Results are mean and range of microbiological assays performed in triplicate (initial values) or duplicate (final values) at the beginning and end of 7 day feeding period.
ND: None detected.

acid buffer (57 mM, pH 6), boiled for 5 min, cooled and centrifuged (40 000 *g*) for 30 min at 5°C. Supernatants were stored at −20°C prior to assay. To cleave the folate polyglutamates, the extracts were treated with hog kidney deconjugase enzyme, prepared according to the procedure of Gregory *et al.* (1984). The total folate content of the diet extracts was assayed by measuring the growth response of *L. casei* (ATCC, 7469), using the aseptic addition method of Herbert (1966).

The folic acid casein medium (Difco Laboratories, West Molesey, UK) was prepared at single strength with added ascorbic acid (5·7 mM). The pH was adjusted to 6·2 before dispensing into assay tubes (10 ml) which were incubated for 22 h at 37°C. A calibration curve was prepared simultaneously using tubes containing PGA. Growth of *L. casei* was measured using an EEL Nephelometer.

Feeding trials

Male Wistar rats (50–80 g) were purchased from a commercial supplier and housed in pairs in wire-bottomed cages to prevent coprophagy. A depletion of folate stores was then induced by maintaining the animals on the FFS diet, fed *ad libitum*, for a period of 6–7 weeks: water was available *ad libitum*. The folate depleted animals were then randomly divided among three experimental groups, weighed, housed singly and given either a folate-supplemented or folate-deficient diet for a further period of 1, 5 or 7 days *ad libitum*. Food intakes and body weights were recorded daily.

At the end of the refeeding period, the rats were weighed, anaesthetised by intraperitoneal injection (1 ml/kg body weight) of sodium barbiturate (Sagatal; May and Baker, Dagenham, Essex) and killed by cervical dislocation. The abdomen was opened by a mid-ventral incision, and a blood sample (*ca* 2·5 ml) taken from the vena cava by syringe. The liver was flushed *in situ* with ice-cold physiological saline (20 ml), excised and cut into small pieces. The small intestine was removed, flushed with saline, slit open and the mucosa scraped from the jejunal portion (10–50% of the length) using microscope slides. Samples of liver and intestinal mucosa were immediately stored under liquid nitrogen. After storage for 1 h at 4°C, samples of serum were obtained by centrifugation and stored under liquid nitrogen prior to analysis. The availability of folate from food sources was assessed by calculating the intake of folate by each group of rats and comparing the rise in serum folates in response to the foods and to crystalline PGA.

Extraction of tissue sample

Portions (5 g fresh weight) of liver or jejunal mucosa were homogenised in an equal volume of ascorbic acid solution (2% w/w, pH 6), extracted by placing the homogenate in a boiling water bath for 10 min then cooled and centrifuged (1500 *g*, 30 min, 4°C). The supernatant was removed and the pellet re-extracted. Combined supernatants were diluted as necessary prior to radioimmunoassay.

Radioimmunoassay of serum and tissue folate

Folate levels in rat serum and tissue extracts were determined using a commercial radioimmunoassay kit based on the principle of competitive protein binding (Beckton-Dickinson Immunodiagnostics, Cowley, Oxford). Serum samples were diluted with 1% ascorbic acid solution prior to assay, and heated at 100°C for 15 min in the dark to destroy endogenous folate-binding protein. After cooling, known quantities of [^{125}I]-labelled folate-binding protein were added to both serum samples and folic acid standards, and the solutions incubated in buffer (pH 9·3) in the dark for 30 min at room temperature. Free and bound folate were separated by treatment with dextran-coated charcoal, followed by centrifugation. The supernatants were decanted and counted in a Philips PW4750 gamma scintillation counter (Philips, Cambridge).

Statistical analysis

The significance of differences between means was estimated using a one-way analysis of variance, followed by calculation of the least significant difference ($p < 0{\cdot}05$) in cases where the F-test indicated significant inequality of means (Cochran & Cox, 1964).

RESULTS

Folate levels in tissues of depleted rats

Rats fed the FFS-diet for up to 7 weeks continued to gain weight and showed no overt signs of ill-health, in comparison to animals maintained throughout the feeding period on a diet containing 5 mg/kg added PGA. Levels of folate in the serum, liver and small intestinal mucosa of animals fed folate-depleted and control diets for 38–42 days are given in Table 3.

TABLE 3
Serum, Liver and Small Intestinal Folate Content of Rats Fed Folate Free or Folate Supplemented Semi Synthetic Diets

Diet	*Serum folate* (*μg/ml*)	*Mucosal folate* (*μg/g*)	*Liver folate* (*μg/g*)
Supplemented	$41{\cdot}4 \pm 1{\cdot}6_a$	$1{\cdot}56 \pm 0{\cdot}17_a$	$4{\cdot}75 \pm 0{\cdot}45_a$
Folate deficient	$14{\cdot}6 \pm 4{\cdot}1_b$	$0{\cdot}67 \pm 0{\cdot}12_b$	$3{\cdot}26 \pm 0{\cdot}21_b$

Results are means with standard errors for six animals. Results not sharing the same subscript differed significantly ($p < 0{\cdot}05$).

TABLE 4
Final Liver Mucosa and Serum Folate Levels of Folate-Depleted and Re-fed PGA Supplemented Rats

Diet	*Liver* (*μg/g*) (*fr. wt.*)	*Mucosa* (*μg/g*) (*fr. wt.*)	*Serum* (*ng/ml*)
Depleted = 0d	4·33 (2·24)	0·32 (0·06)	40 (23)
5d	2·69 (0·53)	0·19 (0·10)	15 (13)
PGA-supplemented			
(10 mg/kg) 1d	7·36 (2·45)	1·49 (0·54)	152 (128)
5d	16·93 (6·3)	3·46 (1·15)	328 (272)
PGA-supplemented			
(50 mg/kg) 1d	11·20 (2·55)	3·93 (1·93)	360 (240)
5d	11.41 (0·9)	1·66 (0·20)	305 (70)

Results are means and range of two animals.

TABLE 5
Food Intake, Folate Intake and Final Serum Foiate Levels of Folate-Depleted Rats Re-fed PGA-Supplemented Diets

Diet	*Food intake* (*g/day*)	*Wt. gain* (*g/7 day*)	*PGA intake* (*μg/7 day*)	*Serum folate* (*μg/ml*)
Folate deficient	$19{\cdot}1 \pm 0{\cdot}5_a$	$12{\cdot}5 \pm 1{\cdot}5_a$	$< 8_a$	$28 \pm 0{\cdot}9_a$
PGA-supplemented (10 mg/kg)	$20{\cdot}1 \pm 0{\cdot}7_a$	$16{\cdot}3 \pm 2{\cdot}0_a$	$1\,249 \pm 41_b$	$146 \pm 11_b$
PGA-supplemented (20 mg/kg)	$20{\cdot}3 \pm 0{\cdot}7_a$	$15{\cdot}6 \pm 1{\cdot}5_a$	$2\,655 \pm 92_c$	$242 \pm 25_c$

Results are means with standard errors for ten animals. Results not sharing the same subscript differed significantly ($p < 0{\cdot}5$).

Response to re-feeding with PGA or cabbage supplements

Twelve folate-depleted rats were divided into three groups of four rats, and given diets deficient in or supplemented with 10 or 50 mg/kg PGA for 1 or 5 days, with each treatment in duplicate. The final serum, liver and mucosal folate levels are given in Table 4. The increases in tissue folate concentration reflected the amount of folate in the diet, and showed that increases occur in all the measured folate pools within 24 h of being transferred from a deficient diet.

In a more rigorous experiment 30 rats were fed the FFS-diet for a depletion period of 9 weeks. Two groups of 10 rats were then given diets supplemented with 10 or 20 mg/kg PGA, for a further period of 7 days, whilst the remaining 10 rats continued to receive FFS. The folate contents of these diets are given in Table 2. The mean intakes of feed and folic acid during this period, together with the final serum folate level of both the folate-supplemented and folate-depleted groups, are shown in Table 5. The significant increases in serum folate reflected the higher PGA concentrations in similar amounts of diet consumed.

In a further experiment, folate-depleted rats were re-fed with cabbage at levels of 125 and 250 g/kg. The lower level of cabbage supplementation (1·8 mg folate/kg diet) was well accepted by the animals and there was no adverse effect upon food intake or weight gain. At the higher level of cabbage content (3·3 mg folate/kg diet) the rats showed some aversion to the experimental diet. Table 6 shows the mean feed and folate intake of the three groups, together with their average weight gain during the 7 day feeding period. Both the cabbage-supplemented groups attained serum folate levels which were significantly higher than the folate-depleted controls, the increase being approximately proportional to the estimated folate intake.

TABLE 6

Food Intake, Folate Intake and Final Serum Folate Levels of Folate-Depleted Rats Re-fed Cabbage-Supplemented Diets

Diet	*Food intake (g/day)*	*Wt. gain (g/7 day)*	*Folate intake (μg/7 day)*	*Serum folate (μg/ml)*
Folate deficient	$19{\cdot}2 \pm 0{\cdot}5_a$	$13{\cdot}8 \pm 1{\cdot}5_a$	$<8_a$	$8{\cdot}6 \pm 1{\cdot}1_a$
Cabbage-supplemented (125 g/kg)	$19{\cdot}8 \pm 1{\cdot}5_a$	$16{\cdot}0 \pm 4{\cdot}0_a$	$249 \pm 20_b$	$21{\cdot}9 \pm 3{\cdot}0_b$
Cabbage-supplemented* (250 g/kg)	$15{\cdot}3 \pm 1{\cdot}4_a$	$7{\cdot}3 \pm 5{\cdot}5_a$	$347 \pm 36_c$	$34{\cdot}0 \pm 2{\cdot}0_c$

Results are means with standard errors for five animals or three animals where indicated by asterisk. Results not sharing the same subscript differed significantly ($p < 0{\cdot}5$).

DISCUSSION

The ideal approach to the investigation of folate availability from human foods would involve direct measurements in man. Several studies with human subjects have been carried out (Tamura & Stokstad, 1973; Babu & Srikantia, 1976), but they are expensive and subject to both technical and ethical difficulties which make them unsuitable for routine work. Graham *et al.* (1980), and other groups have used the chick bioassay to assess folate availability, but a mammalian system is more appropriate for the study of human foods. Folate deficiency is difficult to achieve in the rat, mainly because of coprophagy, but the present study demonstrates that with the use of single wire-bottomed cages it is possible to achieve a significant reduction in tissue folate stores without recourse to tail cups or antibiotics. The response of such stores to re-feeding with defined quantities of folate then provides a means of assessing uptake and utilisation of the vitamin by the rat.

In the present investigation, restriction of folate intake for periods in excess of one month led to a reduction in folate stores in serum, liver and small intestinal mucosa. However, as in the recent study of Abad & Gregory (1987), the broadest response range was obtained with serum folate, which also has the added advantage of being easily measurable using the radio-immunological technique. The assay of solid tissues by this method proved more complex and prone to inaccuracy, and their surgical removal was time-consuming. Moreover, both the liver and gut mucosa may be heavily contaminated with blood, and this may contribute to the lack of consistency of the hepatic folate response to dietary intake noted by Abad & Gregory (1987). One of the major difficulties encountered in vitamin bio-availability assays is the problem of incorporating human foods into animal diets at levels which are both palatable, and high enough to provoke a measurable physiological response. The use of freeze-dried cabbage as a component of an otherwise semi-synthetic diet appears to have overcome this problem, and a significant increase in serum folate level was achieved, even at a relatively low level of incorporation which did not lead to a fall in food consumption.

In spite of the advantages of the radio-immunological procedure for the measurement of folate in blood and other tissues, it does not appear to be a reliable means of measuring folate in a variety of complex foods. Microbiological assay with *L. casei* was used in the present study, although it is possible that alternative methods of food analysis may emerge in the near future. Gregory *et al.* (1982) have described the use of high performance liquid chromatography for this purpose and have suggested that microbiological assay may underestimate the folate content of cabbage

because of the presence of hypothetical growth inhibitors. However, in a subsequent report from this group, significant variations in the results obtained by the two methods were observed, and the *L. casei* method indicated a higher level of total folate in a sample of cabbage than that determined by HPLC (Gregory *et al.*, 1984). The case for the presence of microbiological growth inhibitors in cabbage therefore remains unproven, and it is not yet certain that HPLC provides a reliable method for all foods.

Folates in cabbage are present as polyglutamates which must be hydrolysed prior to absorption. In the rat this is accomplished by a specific PGA hydrolase (Elsenhans *et al.*, 1984) which appears to be located in the cytoplasm of the mucosal cells, whereas in man and the pig the enzyme is localised in the brush border (Wang *et al.*, 1985). The physiological significance of this difference is unclear, but clearly it is a point to be borne in mind when interpreting the results of absorption studies in the rat. Tamura & Stokstad (1973) reported that the availability to human subjects of folate from both raw and cooked cabbage was only 47%, although the range of results for raw cabbage was 0–127%. Abad & Gregory (1987), using a rat bioassay, reported a higher value of 68%. The preliminary results obtained here do not enable us to calculate a precise value for the availability of cabbage folate, but it was evidently well utilised and its uptake and incorporation appeared to be of the same order as that of PGA.

We conclude that the use of a radio-immunoassay to measure the serum folate response of the depleted rat to dietary folate provides a relatively rapid and inexpensive means of investigating the availability of folates in human foods. The sensitivity of the technique is such that it should prove applicable to foods such as potatoes and cereal products which, though relatively low in folate content, are consumed in such quantities as to provide a large proportion of the folates in the average United Kingdom diet.

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