J. Agric. Food Chem. **1997**, *45*, 135−139

Food Folate Assay with Protease, α-Amylase, and Folate Conjugase Treatments

Tsunenobu Tamura,* Yasuharu Mizuno, Kelley E. Johnston, and Robert A. Jacob†

Department of Nutrition Sciences, University of Alabama at Birmingham, Birmingham, Alabama 35294, and Western Human Nutrition Research Center, USDA, ARS, San Francisco, California

We evaluated the effect of protease, α-amylase, and folate conjugase treatments on apparent folate content in four food composites designed to be low in folate (31.2 μg/day calculated according to previously published food folate tables) for the determination of folate requirements in humans. Protease and α-amylase treatments were performed for 16 and 4 h, respectively, at pH 4.1 and chicken pancreas folate conjugase (FC) for 3 h at 37 °C. Folate was determined by microbiological assay using the *Lactobacillus casei* method. Mean folate content in these menus was 56 μg/day after treatment with FC alone. However, means were 90 μg/day with protease and FC, 126 μg/day with α-amylase and FC, and 152 μg/day with α-amylase, protease, and FC treatments. Tri-enzyme treatment appears to be essential to determine food folate content accurately. Food folate tables should be revised using these enzyme treatments to accurately establish the dietary folate requirements in humans.

Keywords: *Food folate; folate conjugase; protease; α-amylase*

INTRODUCTION

The important role of folate nutriture in the possible prevention of arteriosclerosis due to increased plasma homocysteine concentrations or neural-tube defect pregnancies has been well recognized in recent years (Clarke et al., 1991; Selhub et al., 1995; MRC Vitamin Study Research Group, 1991). Although these findings have drawn much attention to improve folate nutriture by increasing dietary intakes of the vitamin, it has been known that the calculated dietary folate intakes are notoriously inaccurate due to the lack of dependable data on food folate contents (Bailey, 1995). Food folate concentrations are generally determined by microbiological assay, and two procedures have been traditionally involved prior to the assay (Gregory, 1989; Tamura, 1990). One is the heat treatment of food samples to extract folates by releasing them from folate-binding protein or food matrix (Gregory et al., 1990), and the other, the treatment of food folates with folate conjugase (pteroylpolygammaglutamyl hydrolase, EC 3.4.22.12), which has been used to obtain the "total folate" contents of foods (Tamura, 1990; Engelhardt et al., 1990). Most of the food items are known to contain polyglutamyl forms of folate which must be hydrolyzed to mono- or diglutamyl forms in order to be utilized by the assay organisms (Tamura et al., 1972).

Yamada (1979) reported that protease (EC 3.4.24.31) treatment of samples significantly increases folate contents in breast milk, hog liver, and cod. Cerna and Kas (1983) determined folate contents in food items rich in starch and glycogen and reported that α-amylase (EC 3.2.1.1) treatment of samples is essential for obtaining proper values of folate in certain foods. Furthermore, Pedersen (1988) reported that folate contents in starch-containing food items after a simultaneous treatment with α-amylase and chicken pancreas folate conjugase were 9.3% higher than those treated with folate conjugase alone. Martin et al. (1990) and De Souza and Eitenmiller (1990) reported an important observation indicating that a tri-enzyme treatment is necessary to accurately determine folate contents in certain foods. The method includes the digestion of food extracts with α-amylase and protease in combination with the traditional folate conjugase treatment. However, to our knowledge, there has not been a follow-up study to confirm their observation, and little attention has been given to the methodology of food folate assay in recent years. We undertook this study to evaluate the effect of α-amylase and protease digestions in addition to folate conjugase treatment on folate content in complete food composites prepared for a 4-day rotation menu used to estimate folate requirements in adult females. We intended to establish a method of folate analysis that provides us with the *maximum folate contents* in these food composites.

MATERIALS AND METHODS

A 4-day rotation menu was developed for a low-folate diet to estimate the requirement of folate in healthy adult female volunteers at the metabolic unit of the Western Human Nutrition Research Center in San Francisco, CA. The menu consisted of four independent composites of foods. Each food composite (I−IV) was given to the subjects in numerical order every 4 days for 13 weeks, and the compositions of these composites at 1900 kcal are presented in Table 1. To reduce folate contents, five items (green beans, carrots, chicken, turkey, and ham) were heated in boiling water three times for 5 min each and the cooking water discarded each time. Folate content of each composite was calculated on the basis of the information in food folate tables (U.S. Department of Agriculture, 1976−89). However, folate values for 13 of the 40 food items in the compositions were missing from the database and were not included in the calculation. Among these, six were low-protein pasta, bread, crackers, and cookies

* Address correspondence to T. Tamura, M.D., Department of Nutrition Sciences, University of Alabama at Birmingham, 218 Webb Bldg., UAB Station, Birmingham, AL 35294-3360. Phone: (205) 934-7478. FAX: (205) 934-7049. E-mail: NUSC024@UABDPO.DPO.UAB.EDU.

† USDA.

S0021-8561(96)00497-9 CCC: $14.00 © 1997 American Chemical Society

Table 1. Composition of Food Composites I–IV (g = grams of food item)

composite I		composite II		composite III		composite IV	
item	g	item	g	item	g	item	g
				Breakfast			
hash browns	90.5	jelly	13.6	hash browns	90.5	jelly	13.6
applesauce	110.8	low-protein bread	78.7	applesauce	124.9	low-protein bread	78.7
cinnamon	0.2	applesauce	108.6	cinnamon	0.1	applesauce	108.6
		cinnamon	0.1			cinnamon	0.1
				Lunch			
fruit cocktail	108.6	cranberry juice	217.1	pears	158.3	peaches	90.5
casserole	288.6	low-protein cookies[a]	38.9	low-protein bread[b]	83.2	casserole	410.8
somen noodles		white rice	76.9	roast chicken	39.8	low-protein fusilli[c]	
roast chicken		onion powder	0.3	mayonnaise	13.6	zucchini	
stewed tomatoes		deli ham	65.1	dill	0.1	stewed tomatoes	
zucchini		green beans	90.5	onion powder	0.2	roast chicken	
garlic powder	0.1			marinated beans	58.8	chicken broth	
alphacel[c]	4.6			green beans		garlic powder	0.1
salt	0.5			Italian dressing		alphacel	4.6
				Dinner			
casserole	200.8	casserole	281.9	casserole	460.5	low-protein cookies	28.0
low-protein fusilli		whole wheat spaghetti		low-protein rigatini[c]		casserole	134.8
roast turkey		low-protein tagliatelle[c]		deli ham		white rice	
carrots		oregano		zucchini		roast turkey	
Italian dressing		salt		stewed tomatoes		mushrooms	
celery seed	0.2	zucchini		beef broth		basil	0.1
onion powder	0.2	roast turkey		oregano	0.1	applesauce	138.4
applesauce	108.6	stewed tomatoes		applesauce	108.6	cinnamon	0.1
cinnamon	0.1	applesauce and cinnamon	108.7	alphacel	4.6		
		alpacel	4.6	cinnamon	0.1		
				Snack			
carrots	28.0	dried apricots	27.1	fruit jello	150.2	dried prunes	36.2
cinnamon	0.1	low-protein cracker	5.4	peaches		gelatin	117.6
				gelatin			

[a] Wel-Plan (low-protein cookies, Dietary Specialities, Rochester, NY). [b] Wheat starch bread (low-protein bread, Dietary Specialities, Rochester, NY). [c] Aproten (low-protein pasta or cracker, Dietary Specialities, Rochester, NY). [d] Polysaccharide fiber supplement (ICN Biochemicals, Cleveland, OH).

of which folate contents are unknown at the present time. The folate contribution from the other items may be small because six were spices and one a fiber supplement. The study was approved by the Human Subjects Committee of the University of California, Davis, and the Human Studies Review Committee of the Agricultural Research Service, U.S. Department of Agriculture, and signed informed consent was obtained from each subject.

Folate content in each food composite was determined by microbiological assay using *Lactobacillus casei* (ATCC 7469) as an assay organism. The assay was carried out using a 96-well microplate with a microplate reader (Tamura, 1990). The mean folate values were used for the final calculation based on at least two duplicates which were within the linear portion of the standard response curve. 5-Formyltetrahydrofolate (5-HCO-H_4PteGlu, calcium salt, Sigma Chemical, St. Louis, MO) was used as a standard. The coefficient of interassay variation for folate analysis is approximately 10% in our laboratory (Tamura, 1990).

To determine folate content in each food composite, dietary composites were homogenized using a Waring blender in an equal volume of 0.1 M potassium phosphate buffer (pH 6.3) containing 57 mM of ascorbic acid (Tamura, 1990). Aliquots (50 mL) were frozen at −70 °C and shipped from San Francisco to Birmingham on dry ice. Each of the thawed homogenates was mixed with two volumes of 0.1 M of potassium phosphate buffer containing 57 mM of ascorbic acid at a final pH of 4.1, and this food mixture was subjected to the four types of enzyme treatments, as described below. The effect of additional homogenization using a Polytron on folate content in each food composite was also evaluated.

The food mixtures were treated by (1) folate conjugase only, (2) protease followed by folate conjugase, (3) α-amylase followed by folate conjugase, and (4) α-amylase followed by protease, then folate conjugase. These treatments were independently repeated three times. Protease and α-amylase used in this study were obtained from Sigma Chemical and were the same as the enzyme preparations recommended by Martin et al. (1990) which were prepared from *Streptomyces griseus* (Type XIV) and *Aspergillus oryzae* (Type X-A), respectively. The concentration of protease or α-amylase used for the treatment of food mixtures was 10 mg/mL of distilled-deionized water, and these enzyme solutions were filtered using a microfilter (0.22 μm) immediately before use. A 1 mL sample of each enzyme solution was mixed with 1 mL of the food mixture, which was equivalent to 10 mg of each solid enzyme for approximately 167 mg of the original food composites. The food mixtures were incubated 16 h for protease treatment and 4 h for α-amylase treatment at 37 °C. We observed that the food mixtures after the treatments of both protease and α-amylase appeared more homogeneous and much clearer than the original mixtures. Endogenous folate in α-amylase (approximately 1.1 ng of folate/mg of solid enzyme as *L. casei* activity after protease and folate conjugase treatments) was subtracted for the final calculation of folate content in each food composite. Only a minute amount of endogenous folate was detected in protease; therefore, such a subtraction was not necessary for the calculation after protease treatment.

In addition, the effect of two different pHs on the content of folate in food composite I after folate conjugase treatment with and without protease and α-amylase treatments was evaluated. The buffers included (1) potassium phosphate buffer (0.1 M, pH 6.3) containing 57 mM of ascorbic acid (after the addition of ascorbic acid, the final pH of the buffer was re-adjusted to 6.3 by adding 0.1 M potassium phosphate monobasic solution (Tamura, 1990)) and (2) a buffer containing 50 mM Hepes, 50 mM Ches, 101 mM sodium ascorbate and 0.2 M 2-mercaptoethanol with a final pH of 7.85 (Wilson and Horne, 1984). The pH of these buffers was consistent throughout the extraction and protease and α-amylase treatments. All folate conjugase treatment was performed at pH 7.0 as stated below.

The original food mixtures or enzyme-treated food mixtures were heated in boiling water for 10 min to denature protease, cooled to room temperature, and centrifuged (900*g*, 10 min) before folate conjugase treatment. The effect of heating (10 min in boiling water) before any enzyme treatment on folate

Table 2. Folate Contents in Food Composites I–IV and Daily Intake after Four Types of Enzyme Treatments[a]

	calcd values (μg/day)	folate conjugase only (ng/g)	protease and folate conjugase (ng/g)	α-amylase and folate conjugase (ng/g)	α-amylase, protease, and folate conjugase (ng/g)
food composite I	31.1	49.8 ± 13.6	70.8 ± 7.0	109.8 ± 24.8	125.2 ± 50.6
food composite II	32.4	63.8 ± 22.8	94.4 ± 29.2	124.0 ± 36.4	167.2 ± 39.4
food composite III	33.5	51.0 ± 16.0	79.4 ± 19.4	128.2 ± 40.8	132.0 ± 51.6
food composite IV	27.8	33.8 ± 10.8	70.6 ± 21.6	87.0 ± 35.8	115.2 ± 43.6
mean ± SD	31.2 ± 2.5	49.6 ± 17.8	79.8 ± 20.2	112.2 ± 34.2	135.0 ± 44.6
mean daily content (μg/day)	31	56	90	126	152

[a] The values represent mean ± SD. All enzyme digestions and folate assays were independently repeated three times. Heat extraction and protease and α-amylase treatments were carried out at pH 4.3 and folate conjugase treatment at pH 7.0

content in each food composite was also evaluated. Chicken pancreas folate conjugase was prepared according to the method described by Leichter et al. (1977), and all folate conjugase treatments were carried out by incubating 0.25 mL of either the original or enzyme-digested food mixtures with 0.1 mL of chicken pancreas folate conjugase at pH 7.0 for 3 h at 37 °C. The amount of folate conjugase used in this investigation was sufficient to hydrolyze over 120 μg of pteroyldiglutamyl-[^{14}C]glutamic acid per h at 37 °C (Krumdieck and Baugh, 1970). This was far in excess to completely hydrolyze polyglutamyl folates in all food mixtures.

In addition, the stability of synthetic folates (5-HCO-H_4-PteGlu and pteroylglutamic acid, PteGlu) was tested by incubating the solutions of these folates with the enzymes for a total of 23 h (α-amylase, 4 h; protease, 16 h; folate conjugase, 3 h) at 37 °C. The statistical analysis was performed using Wilcoxon-rank-sum test to evaluate the differences between various enzyme treatments. The P value of less than 0.05 was considered to be significant.

RESULTS

The dietary folate contents in each food composite determined by the four types of enzyme treatments are shown in Table 2. Folate contents in the composites after the traditional folate conjugase treatment alone was the lowest among the four treatments. However, it was approximately 1.8 times higher than that calculated on the basis of the information available in food folate tables (U.S. Department of Agriculture, 1976–89). The yield of measurable folate was increased by an average of 60% in these food composites with protease treatment ($P < 0.005$) and approximately 125% by treatment with α-amylase and folate conjugase as compared to the values obtained with folate conjugase treatment alone ($P < 0.001$). When the food samples were treated with all three enzymes, the mean folate content in the four composites increased to approximately 271% of the value obtained after folate conjugase treatment alone ($P < 0.001$). This represented approximately 152 μg per day of folate intake using the 4-day rotation menu designed to be low in folate.

As shown in Table 3, folate content in food composite I after folate conjugase treatment was significantly lower at pH 4.1 than those at pH 6.3 or 7.85. On the other hand, the value after protease, α-amylase, and folate conjugase treatments was highest at pH 4.1, and this was significantly higher than that at pH 7.85 ($P = 0.05$). Probably due to the small number of assays, the differences between the values at pHs 4.1 and 6.3 were not significant. It should be noted that the discrepancy among the values of folate in food composite I shown in Table 2 and those in Table 3 is due to the degradation of folate (approximately 60%) during the storage at −70 °C for over a few months. This degradation was unexpected; however, it did occur despite the presence of 57 mM of ascorbic acid at pH 6.3. This particular batch of composite I used for the experiment to evaluate the effect of pH on the enzyme treatment was thawed only once during the storage.

Table 3. Effect of pHs on the Extraction and Tri-enzyme Treatments for Food Composite I

pH (no. of assays)	folate conjugase treatment only (ng/g)	tri-enzyme treatment (ng/g)	% increase by tri-enzyme treatment as compared to conjugase treatment only
4.1[a] (3)	19.9 ± 2.4	54.1 ± 4.1	168
6.3[b] (2)	34.6 ± 2.2	42.5 ± 7.8	24
7.85[c] (3)	36.7 ± 2.2	45.3 ± 2.8	23

[a] 0.1 mol/L potassium phosphate buffer containing 57 mmol/L ascorbic acid with a final pH of 4.1. [b] 0.1 mol/L potassium phosphate buffer containing 57 mmol/L ascorbic acid with a final pH of 6.3, which was adjusted by the addition of 0.1 mol/L potassium phosphate monobasic solution (Tamura, 1990). [c] 50 mmol/L Hepes and 50 mmol/L Ches containing 101 mmol/L sodium ascorbate and 0.2 mol/L 2-mercaptoethanol at a final pH of 7.85 (Wilson and Horne, 1984).

Folate content in the food composite determined after additional Polytron homogenization was similar to that without this additional homogenization, indicating that the increase in folate content after tri-enzyme treatment was not due to incomplete homogenization of food samples by the Waring blender. Furthermore, folate contents in the food composites after the heat treatment of the food mixtures in the presence of 57 mM of ascorbic acid before protease and α-amylase treatments were similar to those with the heat treatment only before folate conjugase treatment. There was approximately a 23% increase in *L. casei* activity of 5-HCO-H_4PteGlu solution during the 23 h incubation for tri-enzyme treatment, although the reason for this increase is unknown at the present time. On the other hand, approximately 16% of *L. casei* activity of PteGlu was lost during the incubation as compared to that of PteGlu without incubation.

DISCUSSION

Our data indicate that the content of folate in food mixtures must be determined after the tri-enzyme treatment as suggested by Martin et al. (1990) and De Souza and Eitenmiller (1990). The mean folate contents found in the composites after the tri-enzyme treatments were approximately 5 times higher than those calculated on the basis of the food folate tables currently available (U.S. Department of Agriculture, 1976–89). It was also 2.7 times higher than the folate value obtained after traditional folate conjugase treatment alone. The amount of dietary folate in the 4-day rotation menu designed to be low in folate was 152 μg of folate/day (Table 2). This amount is approximately 84% of the recommended folate intake (180 μg/d for females over 15 years old) in the U.S. Recommended Dietary Allowances (National Research Council, 1989).

The data of the comparison between various pHs for the content of folate in composite I indicate that tri-enzyme treatment gave the highest value at pH 4.1 as compared to pH 6.3 and 7.85 (Table 3). Although we are not certain whether or not this is the best pH for protease and α-amylase treatments for other food items, it was best for the food composites prepared for this study. In fact, folate contents in other food items were often higher at pH 7.85 than at pH 4.1 after tri-enzyme treatment (Mizuno et al., unpublished). The value after folate conjugase treatment alone of which folate was extracted at pH 4.1 was significantly lower than that at pHs 6.3 and 7.85 (Table 3). This finding indicates that the extraction of folates at pH 4.1 was less complete than at pHs 6.3 and 7.85, and this is consistent with that observed by Gregory et al. (1990). It is known that the pH optima are different depending on protease present in such crude enzyme preparation as used in this study and the affinity of the enzymes to substrates may be different depending on food items (Birktoft and Breddam, 1994). Therefore, the optimal pHs for enzyme treatment might be different for each type of food. Further investigations may be needed to establish the best method to obtain *the maximum folate contents in individual food items*.

There were no marked changes in *L. casei* response to synthetic folates after a total of 23 h of incubation as compared to that before the incubation, suggesting that food folates might be quite stable during the incubation. However, more studies may be needed to ensure the stability of other forms of folate during such a long-term incubation (Gregory, 1989). Furthermore, we observed a marked decrease in folate content in our food composites within a few months under the storage condition of −70 °C. This was unexpected, since ascorbic acid was added in the buffer for the preparation of the homogenates. Careful evaluation of folate stability in certain foods may be warranted.

The increases in folate values in the food composites obtained after tri-enzyme treatment were much higher than the values which we expected on the basis of the information reported by Martin et al. (1990). As stated above, incomplete extraction of folates from the food composites carried out at pH 4.1 is likely to be one reason. However, it may be necessary to note that our procedures used in this study were slightly different from those described by Martin et al. (1990). We speculated that, if folates in food items are trapped in protein or carbohydrate matrix which can be digested by the use of both protease and α-amylase, the enzyme treatment to release folates from the matrix should be carried out before the heat treatment and centrifugation of food mixtures, not after the removal of undigested food residues where folates might still be trapped. However, Martin et al. (1990) first heated the food samples followed by simultaneous folate conjugase and α-amylase treatment, and these treated samples were then digested with protease. We thought that folate conjugase treatment should be carried out after all folates are released from the food matrix and that this step allows the possible maximum hydrolysis of folate polyglutamates. The changes in the order of these treatments might have made for a greater increase in folate contents in our samples as compared to the increments ranging from 2.3 to 50.7% depending on food items as reported by Martin et al. (1990). De Souza and Eitenmiller (1991) reported, however, over a 500% increase in folate in rye bread and approximately a 340% increase in beef franks after the tri-enzyme treatment as compared to the values after folate conjugase treatment alone. The data reported by Martin et al. (1990) and De Souza and Eitenmiller (1990) may indicate that the increment in folate contents after the tri-enzyme treatment over folate conjugase alone is influenced by the type of foods. Nevertheless, our data suggest that it is essential to reevaluate food folate contents.

It might be important to note that there is always a possibility of bacterial growth during a total of more than 20 h of incubation at 37 °C as pointed out by Pedersen (1988). The bacteria which synthesize folates may grow during the incubation and give falsely high folate values. We encountered suspected bacterial growth in approximately 3% of assays particularly for protease treatment during this investigation. We used toluene (20 μL/tube) to overcome this problem; however, it inhibited *L. casei* growth, even after the samples were heated for 10 min at 100 °C before the treatment with folate conjugase. For this reason, we used presterilized plasticware and microfilter-sterilized reagents and enzymes for all assays.

It is known that there are folate conjugase inhibitors present in some foods (Tamura, 1990). Therefore, one may wonder whether there were folate conjugase inhibitors in our samples which limited the complete hydrolysis of polyglutamyl forms of folate; hence our folate contents after folate conjugase treatment were lower than they actually were. However, we did not observe a "positive drift" phenomenon in folate assays using *L. casei*, where a total of six serial dilutions (two times each) were made for each sample. This phenomenon is the observation of apparently higher concentrations of folate when a larger amount of sample containing polyglutamyl folates is placed in the assay well (Tamura, 1972). Therefore, it is likely that insignificant inhibition of folate conjugase may have taken place during the treatment, if it occurred, among our samples used in the study presented here. Furthermore, as indicated above, the amount of chicken pancreas folate conjugase used for each treatment was approximately 2000−6000 times of the activity needed for the complete hydrolysis of polyglutamyl folates present in our samples.

Our findings together with those by others (Yamada, 1979; Cerna and Kas, 1983; Pedersen, 1988; Martin et al., 1990; De souza and Eitenmiller, 1990) indicate that food folate contents must be determined after the treatments with α-amylase and protease under these conditions in addition to the traditional folate conjugase. Therefore, it is now evident that (1) the previously obtained food folate values may be underestimated (Perloff and Butrum, 1977; Hoppner et al., 1977; Chanarin, 1979; Subar et al., 1989; Tamura and Stokstad, 1973; Tamura et al., 1976; Babu and Srikantia, 1976) (therefore, food tables worldwide including the handbook used for the study presented here (U.S. Department of Agriculture, 1976−89) may require reevaluation); (2) the requirements of dietary folate in humans may also be underestimated (National Research Council, 1989; Sauberlich et al., 1987); and (3) the bioavailability of food folate previously estimated by comparing to synthetic folic acid may be an overestimation (Tamura and Stokstad, 1973; Tamura et al., 1976; Babu and Srikantia, 1976). However, it is not clear, at the present time, how completely each food item is digested in the gastrointestinal tract in vivo in humans as compared to the in vitro tri-enzyme digestion. In addition, it may be necessary to investigate whether there are other vitamins which are trapped in the food matrix.

In summary, we suggest that the appropriate method of tri-enzyme treatment should be established to obtain

the maximum content of folate in each food item. This may be an urgent matter because of recent developments showing the prevention of important diseases by adequate folate nutriture (Clarke et al., 1991; Selhub et al., 1995; MRC Vitamin Study Research Group, 1991). Better understanding of appropriate dietary folate intake is needed more than ever, since a significant segment of a certain population appears to have inadequate folate nutriture (Selhub et al., 1993).

ACKNOWLEDGMENT

We dedicate this paper to the memory of Dr. E. L. Robert Stokstad. We thank Ms. Lynn Oehler of Healthcare Management Composite for help with planning the diet and Ms. Vickie Jen of Bionetics Corp. for preparing the food composites.

LITERATURE CITED

Babu, S.; Srikantia, S. G. Availability of folates from some foods. *Am. J. Clin. Nutr.* **1976**, *29*, 376–379.

Bailey, L. B. Folate requirements and dietary recommendations. In *Folate in health and diseases*; Bailey, L. B., Ed.; Marcel Dekker: New York, 1995; pp 123–151.

Birktoft, J. J.; Breddam, K. Glutamyl endopeptidases. *Methods Enzymol.* **1994**, *244*, 114–126.

Cerna, L.; Kas, J. New conception of folacin assay in starch or glycogen containing food samples. *Nahrung* **1983**, *10*, 957–964.

Chanarin, I. *The megaloblastic anaemias*; Blackwell: Oxford, U.K., 1977.

Clarke, R.; Daly, L.; Robinson, K.; Naughten, E.; Cahalane , S.; Fowler, B.; Graham, I. Hyperhomocysteinemia: an independent risk factor for vascular disease. *New Engl. J. Med.* **1991**, *324*, 1149–1155.

De Souza, S.; Eitenmiller, R. Effects of different enzyme treatments on extraction of total folate from various foods prior to microbiological assay and radioassay. *J. Micronutr. Anal.* **1990**, *7*, 37–57.

Engelhardt, R.; Gregory, J. F., III. Adequacy of Enzymatic Deconjugation in Quantification of Folate in Foods. *J. Agric. Food Chem.* **1990**, *38*, 154–158.

Gregory, J. F., III. Chemical and nutritional aspects of folate research: analytical procedures, methods of folate synthesis, stability and bioavailability of dietary folates. *Adv. Food Nutr. Res.* **1989**, *33*, 1–101.

Gregory, J. F., III; Engelhardt, R.; Bhandari, S. D.; Sartain, D. B.; Gustafson, S. K. Adequacy of extraction techniques for determination of folate in food and other biological materials. *J. Food Comput. Anal.* **1990**, *3*, 134–144.

Hoppner, K.; Lampi, B.; Smith, D. C. Data on folacin activity in foods: availability, applications, and limitations. In *Folic acid. Biochemistry and physiology in relation to the human nutrition requirement*; National Academy of Sciences: Washington, DC, 1977; pp 69–81.

Krumdieck, C. L.; Baugh, C. M. Radioactive assay of folic acid polyglutamate conjugase(s). *Anal. Biochem.* **1970**, *35*, 123–129

Leichter, J.; Butterworth, C. E., Jr.; Krumdieck, C. L. Partial purification and some properties of pteroylpolyglutamate hydrolase (conjugase) from chicken pancreas. *Proc. Soc. Exp. Biol. Med.* **1977**, *154*, 98–101.

Martin, J. I.; Landen, W. O., Jr.; Soliman, A. G. M.; Eitenmiller, R. R. Application of a tri-enzyme extraction for total folate determination in foods. *J. Assoc. Off. Anal. Chem.* **1990**, *73*, 805–808.

MRC Vitamin Study Research Group. Prevention of neural tube defects: results of the Medical Research Council Vitamin Study. *Lancet* **1991**, *338*, 131–137.

National Research Council. *Recommended dietary allowances*, 10th ed.; National Academy Press: Washington, DC, 1989; pp 150–158.

Pedersen, J. C. Comparison of γ-glutamyl hydrolase (conjugase; EC 3.4.22.12) and amylase treatment procedures in the microbiological assay for food folates. *Br. J. Nutr.* **1988**, *59*, 261–271.

Perloff, B. P.; Butrum, R. R. Folacin in selected foods. *J. Am. Diet. Assoc.* **1977**, *70*, 161–172.

Sauberlich, H. E.; Kretsch, M. J.; Skala, J. H.; Johnson, H. L.; Taylor, P. C. Folate requirement and metabolism in nonpregnant women. *Am. J. Clin. Nutr.* **1987**, *46*, 1061–1028.

Selhub, J.; Jacques, P. F.; Wilson, P. W. F.; Rush, D.; Rosenberg, I. H. Vitamin status and intake as primary determinants of homocysteinemia in an elderly population. *JAMA, J. Am. Med. Assoc.* **1993**, *270*, 2693–2698.

Selhub, J.; Jacques, P. F.; Bostom, A. G. D'Agostino, R. B.; Wilson, P. W. F.; Belenger, A. J.; O'Leary, D. H.; Wolf, P. A.; Schaeffer, E. J.; Rosenberg, I. H. Association between plasma homocysteine concentrations and extracranial carotid-artery stenosis. *New Engl. J. Med.* **1995**, *332*, 286–291.

Subar, A. F.; Block, G.; James, L. D. Folate intake and food sources in the US population. *Am. J. Clin. Nutr.* **1989**, *50*, 508–516.

Tamura, T. Microbiological assay of folates. In *Folic acid metabolism in health and disease. Contemporary Issues in Clinical Nutrition*, Vol. 13; Picciano, M. F., Stokstad, E. L. R., Gregory, J. F., III, Eds.; Wiley-Liss: New York, 1990; pp 121–137.

Tamura, T.; Stokstad, E. L. R. The availability of food folate in man. *Br. J. Haematol.* **1973**, *25*, 513–532.

Tamura, T.; Shin, Y. S.; Williams, M. A.; Stokstad, E. L. R. *Lactobacillus casei* response to pteroylpolyglutamates. *Anal. Biochem.* **1972**, *49*, 517–521.

Tamura, T.; Shin, Y. S.; Buehring, K. U.; Stokstad, E. L. R. The availability of folates in man: effect of orange juice supplement on intestinal conjugase. *Br. J. Haematol.* **1976**, *32*, 123–133.

U.S. Department of Agriculture. *Agriculture Handbook 8-1-8-21. Composition of foods*; U.S. Department of Agriculture: Washington, DC, 1976–1989.

Wilson, S. D.; Horne, D. W. High-performance liquid chromatographic determination of the distribution of naturally occurring folic acid derivatives in rat liver. *Anal. Biochem.* **1984**, *142*, 529–535.

Yamada, M. Folate contents in milk. *Vitamins (Japanese)* **1979**, *53*, 221–227.

Received for review July 9, 1996. Revised manuscript received October 16, 1996. Accepted October 24, 1996.[⊗] The results were in part presented at the minisymposium entitled "B Vitamins: A tribute to E. L. R. Stokstad" at the Experimental Biology '96 meeting in April, 1996, and the abstract was published in *FASEB J.* **1996**, *10*, A464.

JF960497P

[⊗] Abstract published in *Advance ACS Abstracts,* December 15, 1996.