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Determination of food folate

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Trienzyme treatment appears to be essential for obtaining the maximum possible value of folate in food items. Trienzyme treatment is performed using protease and α-amylase treatment for the extraction of food folate trapped or bound to protein or carbohydrate matrices, and using traditional folate conjugase for the hydrolysis of polyglutamyl folates that are regularly present in foods. Because the conditions (pH and incubation time) of protease and α-amylase treatments vary among individual foods, it would be labor intensive and time consuming to put together reliable and accurate food folate tables. However, considering the importance of folate nutriture for the maintenance of health and the prevention of certain diseases, it is important to establish dependable food folate tables to accurately estimate dietary intake of folate and to recommend adequate dietary folate intake in the general population. (J. Nutr. Biochem. 9:285–293, 1998) © *Elsevier Science Inc. 1998*

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Introduction

During the last decade, interest in the role of adequate folate nutriture has been intensified because of the possible connection between folate intake and reduction of pregnancies with neural tube defects (NTDs) and of occlusive vascular diseases in relation to increased concentrations of homocysteine in the circulation.[1,2] For example, in order to reduce NTD pregnancies, the U.S. Public Health Service recommended in 1992 that daily folate intake by women of childbearing age should be at least 400 μg.[3] In response to this recommendation, the Food and Drug Administration of the U.S. Department of Health and Human Services[4] mandated that, effective in 1998, cereal-grain products should be fortified with folic acid (pteroylglutamic acid) at 140 μg/100 g of cereal-grain products. This fortification program is expected to yield a reduction in the incidence of NTD pregnancies in the United States. It remains to be seen, however, whether this amount of folic acid fortification also reduces the risk of developing occlusive vascular diseases by lowering plasma homocysteine concentrations in certain segments of the population.[5]

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Although the fortification of staple foods with folic acid may be the most effective and economical means to raise the intake of the vitamin in the general population, it would be ideal to increase the intake of this vitamin by the consumption of food items that not only provide a sufficient amount of folate but also are consistent with the dietary guidelines set by the U.S. Department of Agriculture.[6] However, it has been well recognized that the calculated dietary folate intakes based on the existing food folate tables have been inaccurate and unreliable;[7,8] therefore, it has been extremely difficult to correctly estimate the dietary intake of this vitamin. This has resulted in uncertainty in establishing dietary folate requirement or the recommendation of dietary folate intake for the maintenance of health and the prevention of certain diseases. A certain segment of the population is in a state of inadequate folate nutriture;[9] therefore, there is an urgent need to establish accurate and reliable food folate tables. In this article the methods currently available for food folate analysis are reviewed, with particular emphasis on trienzyme treatment; in addition, a brief historical aspect of the analysis of food folate content is reviewed.

Historical aspect of food folate analysis

During the last four decades, numerous reports have been published on food folate content and the various methods of folate determination. These methods include detection using microbiological assay;[10–13] radiobinding[14] or radiometric

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assay;[15] and fluorometric,[16–18] electrochemical,[19] or spectrophotometric methods,[18,20–23] some of which are carried out in combination with gel or high-pressure-liquid chromatography (HPLC). Recently, an extensive review of the interlaboratory variation of food folate analysis using various methods has been published.[24] Among these determinations, the microbiological assay appears to be most commonly used. The majority of investigators reported values of food folate content obtained by microbiological assay using *Lactobacillus casei* (*L. casei*, ATCC 7469) after both heat extraction in the presence of a reducing agent(s) as well as the treatment with pteroyl-γ-glutamyl carboxypeptidase (folate conjugase, EC 3.4.19.9), which hydrolyzes folate polyglutamates to folate with shorter glutamyl residues such as mono- or diglutamates, which can be utilized by the assay organisms. These values have been the basis of the calculations of dietary folate intake and have been used to establish the U.S. Recommended Dietary Allowances.[8,25,26]

Microbiological assay

Microbiological assay has been considered to be one of the best and most versatile methods for the determination of food folate for the past half century. Three organisms have been identified to be useful for this purpose, including *L. casei. Enterococcus hirae* (previously called *Streptococcus lactis R, Streptococcus faecalis*, or *Streptococcus faecium*, ATCC 8043) and *Pediococcus cerevisae* (formerly called *Leuconostoc citrovorum*, ATCC 8081).[27] Among these three microorganisms, *L. casei* has been most widely used for the determination of food folate because this microorganism responds almost equally to the widest variety of folate derivatives. Extensive reviews on microbiological assay are available elsewhere.[27–30]

For many years, despite its popular use, investigators had considered that microbiological assay was extremely laborious and time consuming, and it was difficult to establish this method as a dependable routine in each laboratory. Although numerous attempts have been made to improve the method over the years, it may be safe to say that the two most significant contributions that were made during the last 15 years changed the image of the method to less laborious, less time consuming, and more reproducible. These contributions include the use of cryoprotected *L. casei*[31,32] and the use of a 96-well plate and a microplate reader with a computer for data reduction.[27,33–35]

Extraction of food folate

Prior to the assay of folate content, two sample-treatment procedures have been traditionally involved. These include the heat extraction of folate and treatment with folate conjugase.[6,27] The former is used to extract folates by releasing them from folate-binding protein or food matrix[36]; the latter is used to obtain the "total folate" contents of foods.[37,38]

It was not until the early 1960s that the importance of the use of a reducing agent, such as ascorbic acid, to protect some of the relatively labile reduced folates against oxidation during the extraction and assay procedures, thereby increasing the accuracy of the estimation of food folate content, was recognized.[39] Until then, many investigators reported values of food folate content that were obtained without the use of a reducing agent. For example, in one of the most extensive food folate tables published in the early 1950s by Toepfer and his colleagues,[40] ascorbic acid was not used as a reducing agent during either the extraction or assay procedures. However, it is now well established that the presence of a reducing agent(s) is essential throughout the assay procedure.[41,42]

Folate conjugase treatment

In the 1950s and 1960s, most of the publications concerning the analyses of food folate contained folate values as "free folate" and "total folate." *Free folate* content was used to express the value of folate content determined by *L. casei* microbiological assay without prior treatment of folate conjugase, and *total folate*, with the treatment. Until the early 1970s it was not known that *L. casei* responds to a certain extent to polyglutamyl folates with more than three glutamyl residues and that the values of "free folate" do not represent only folates with shorter glutamyl residues.[37] Partially purified hog kidney or chicken pancreas folate conjugase has generally been used for folate analysis.[43–45] Recently, the use of rat serum became common because it is readily available from commercial venders and the removal of endogenous folate can be done with relative ease. The majority of the end product of the reaction using rat serum folate conjugase is monoglutamyl folate.[46]

Food folate analysis using affinity column and HPLC in combination with diode array detection

Many investigators have used HPLC methods to separate, identify, and quantitate various derivatives of folate present in foods.[13,16–24] In 1980 Selhub et al.[47] reported a revolutionary, efficient method for the purification and concentration of naturally occurring folates as intact molecules using affinity chromatography utilizing immobilized milk folate-binding protein. Subsequently, Selhub and his coworkers[20–22,48] used this affinity-column chromatography in combination with an HPLC-diode array ultraviolet (UV) detection system for the isolation and quantification of folates in biological materials, including foods.

The method has various advantages over those developed previously, including traditional ion-exchange or gel chromatography,[29,49] as well as other methods previously reviewed.[50] The most significant advantage of this method is that the distribution of folate coenzymes can be identified and quantified intact, including oxidation states, one-carbon substitutions, and glutamyl-chain lengths of the complex mixture of naturally occurring folates. To date, this appears to be the most advanced method for the identification of various folate coenzymes in food. In the future this method may provide a powerful means to estimate the bioavailability of folates in various food items by providing a complete picture of folate coenzyme distribution, which may have different degrees of bioavailability in humans.[51]

Table 1 Folate contents (pmol/g, mean ± SD) in food composites (n = 4) and dietary folate intake (nmol/day) after four types of enzyme treatments[57]

	Calculated values[6]	Folate conjugase only	Protease and folate conjugase	α-Amylase and folate conjugase	α-Amylase, protease, and folate conjugase
Food content		112 ± 40	180 ± 46	254 ± 78	306 ± 101
Increase over folate conjugase only (%)			61	126	172
Folate intake	71	127	204	286	345

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.

Food folate analysis using trienzyme treatment

In 1990 Eitenmiller and his colleagues[14,52] published a method for the determination of food folate. In addition to the traditional treatment with folate conjugase, the method included treatments with protease (EC 3.4.24.31) and α-amylase (EC 3.2.1.1). They coined this *trienzyme treatment.* It was intended to accomplish a more complete extraction of folates that may be trapped in or bound to the matrices of protein and polysaccharides by using protease and α-amylase in addition to heat treatment rather than the conventional method of heat treatment alone. Using the trienzyme treatment, they observed a substantial increase, in some cases over 100%, in folate content of certain food items compared with the traditional method using folate conjugase treatment alone.

Trienzyme treatment appears to be the most effective means of food folate extraction to date. Many investigators made an effort for complete extraction of food folate prior to the work by Eitenmiller and his colleagues;[14,52] however, no investigator had used the combination of protease and α-amylase treatments. In 1978 the use of the combination of protease and folate conjugase treatments was first performed by Yamada,[53] who reported that these treatments increased folate content in human milk and a few other food items. Eitenmiller et al.[54] used a similar method to determine folate content in human milk. In 1983 Cerna and Kas[55] combined α-amylase and folate conjugase treatments. They reported that the combination of the treatments is essential for obtaining maximum values of folate contents in certain food items rich in starch and glycogen. Similarly, Pedersen[56] reported that food folate contents after a simultaneous treatment with α-amylase and chicken pancreas folate conjugase were significantly higher than those treated with folate conjugase alone. It should be mentioned that in 1951 Toepfer et al.[40] reported the effect of a 24-hr incubation of five food homogenates with Takadiastase (a trade name for amylase) at pH 4.5. Although this treatment was not combined with folate conjugase treatment, there was a small increase in folate values in all five items compared with the values obtained without any enzyme treatment. They concluded that "the Takadiastase preparation, under the conditions and amounts employed, was of little value for increasing the amount of folic acid over that found in the free form." Retrospectively, if they had tried the combination of treatments with α-amylase and folate conjugase, the world of food folate analysis might have been quite different for the following 40 years.

The effectiveness of trienzyme treatment to obtain higher food folate values did not draw much attention until 1997, when two groups of investigators reported that trienzyme treatment is necessary to obtain proper folate content in certain foods.[57,58] Stimulated by a presentation by Dr. Eitenmiller at a workshop titled "Measurement of folates in foods and biological specimens" (organized by Drs. Beecher, G.R., and Bowman, B.A.), which proceeded Experimental Biology '95, Tamura et al.[57] undertook a study to evaluate the effect of trienzyme treatment on folate content in food composites. These diets were prepared as a 4-day-rotation menu that was intended to be a "low-folate diet" to estimate folate requirements in healthy adult females. The food composites were treated by (1) folate conjugase only, (2) protease followed by folate conjugase, (3) α-amylase followed by folate conjugase, or (4) α-amylase followed by protease, and then folate conjugase. Folate content in each food composite was determined by *L. casei* microbiological assay using a 96-well-microplate reader.[27]

The results of this experiment are presented in *Table 1.*[57] The data indicate that the method of trienzyme treatment was the correct choice of folate analysis in these food composites because this method gave a much higher folate content than the traditional method. Folate content in the composites after folate conjugase treatment alone was the lowest among the four treatments. The mean folate content found in the composites after trienzyme treatment was approximately five times higher than that calculated using the food folate tables.[6] It was also 2.7 times higher (170% increase) than the value obtained after folate conjugase treatment alone. The average dietary folate provided by the 4-day-rotation menu, which was intended to be low in folate, was found to be 152 μg/d using trienzyme treatment. This amount is approximately 84% of the currently recommended dietary folate intake (180 μg/d for adult females) in the U.S. Recommended Dietary Allowances.[26]

This increase in folate values of the food composites obtained after trienzyme treatment were much higher than those based on the information provided by Martin et al.[52] It may be necessary to mention that the procedures used in the study by Tamura et al.[57] were different in the order of enzyme treatments as described by Martin et al.,[52] who first heated the food samples, then treated them with simultaneous folate conjugase and α-amylase, and then digested these treated samples with protease. However, it may be reasonable to assume that if food folate is trapped in or bound to protein and carbohydrate matrices that are digested by protease and α-amylase the enzyme treatment to release folates from the matrices should be performed prior to the

Table 2 Effect of pH of trienzyme treatment on the folate content (pmol/g) of a food composite[57]

pH	Folate conjugase treatment only	Trienzyme treatment	% increase by trienzyme compared with conjugase treatment only
4.1[1]	45 ± 5	123 ± 9	168
6.3[2]	78 ± 5	96 ± 18	24
7.85[3]	83 ± 5	103 ± 6	23

[1]0.1 mol/L potassium phosphate buffer and 5.7 mmol/L of ascorbic acid with a final pH of 4.1.
[2]0.1 mol/L potassium phosphate buffer and 5.7 mmol/L of ascorbic acid with a final pH of 6.3.
[3]50 mmol/L Hepes, 50 mmol/L Ches, 11.4 mmol/L of ascorbic acid, and 0.2 mol/L of 2-mercaptoethanol at a final pH of 7.85.[42]

heat treatment and centrifugation of food homogenates. Furthermore, the treatment with folate conjugase should be done after all folates are released from the food matrices in order to expose the maximum amount of polyglutamyl folates to this enzyme. It may be safe to conclude that these sequential alterations in the treatments produce a greater increase in folate contents in samples.[57] The data of these studies also suggest that the increment in folate contents after trienzyme treatment compared with those after folate conjugase alone is influenced by the types of food.[52,57]

Shortly after the appearance of the publication by Tamura et al.,[57] Pfeiffer et al.[58] reported the results of folate content obtained after trienzyme treatment in cereal-grain products with and without folic acid fortification. Compared with the values with folate conjugase treatment alone, trienzyme treatment resulted in 4%, 34%, and 33% increases in measurable folate content of bread, spaghetti, and rice, respectively. These investigators purified and concentrated folates after trienzyme treatment using affinity chromatography.[47] These purified folates were subjected to an HPLC method with both diode array UV detection and microbiological assay detection method using *L. casei*.[16] The results obtained by both methods showed remarkably good agreement, indicating that the compounds detected by *L. casei* microbiological assay are indeed folates. It should be noted that the identification of folate derivatives using UV detection in their study has extremely important findings. The data reported by Pfeiffer et al.[58] clarified two important questions concerning folate analysis using trienzyme treatment: specifically, the increase in folate content after trienzyme treatment is not due to an artifact (the release of unknown growth factors for the assay organism by the extra enzyme treatments) or to the degradation of unknown growth inhibitor of *L. casei*, which could have resulted in falsely low folate content without trienzyme treatment.

It is known that folate conjugase inhibitors are present in some foods.[27] It is possible that there were folate conjugase inhibitors in food samples that limited the hydrolysis of polyglutamyl forms of folate; hence, folate content after folate conjugase treatment is lower than the actual value. This question has not yet been answered. However, as noted by Tamura et al.,[57] a "positive drift" phenomenon of *L. casei* in folate assay of samples treated with folate conjugase alone was not observed, although a total of six serial twofold dilutions were made for each sample. The positive drift phenomenon is that apparently higher concentrations of folate are observed when a larger amount of sample containing polyglutamyl folates is placed in the assay system.[37] Therefore, it is unlikely that inhibition of folate conjugase is significant among samples used in the study by Tamura et al.[57] It is important to keep in mind that there is always a possibility of the presence of folate conjugase inhibitor(s) in foods, as pointed out by Butterworth and his colleagues.[59]

Is it possible to achieve the degree of folate extraction from food samples by methods other than the labor-intensive and time-consuming trienzyme treatment? In all the studies using trienzyme treatment, the heating procedure was performed for no more than 20 min. It is natural to question whether the temperature and the duration of heating was sufficient for adequate folate extraction. In order to answer this question the effect of long-term heating was performed using a mixed diet. The food homogenate was heated for 30 min at 121°C in 0.1 mol/L Bis-Tris buffer (pH 7.8) containing 0.1 mol/L sodium ascorbate and 10 mmol/L 2-mercaptoethanol.[22] Folate content in a mixed diet after trienzyme treatment increased approximately 2.1 times (110% increase) the value with folate conjugase treatment alone following heat treatment (Aiso and Tamura, unpublished observation). This preliminary result suggests that this 30-min-heating procedure is not sufficient to achieve the same degree of folate extraction in the mixed diet.

Recently, Lim et al.[60] observed an average of 85% increase in folate content in a total of 84 human milk samples after trienzyme treatment (including two 10-min-heating procedures) compared with that obtained by folate conjugase alone (with two 10-min heatings). In this study the optimal incubation time of 4 hr was suggested for α-amylase and 8 hr for the subsequent protease treatment in addition to folate conjugase treatment for the measurement of human milk folate content.

As shown in *Table 2*, folate content in one of the food composites after folate conjugase treatment alone is significantly lower at pH 4.1 than at pH 6.3 or 7.85, whereas the value after trienzyme treatment is highest at pH 4.1.[57] This finding indicates that heat extraction of folates at pH 4.1 was less than at pHs 6.3 and 7.85; however, pH 4.1 was more suitable for trienzyme treatment than the other pHs. It is unknown at the present time whether this pH is optimal for protease and α-amylase treatments using other food items, and it is likely that optimal pHs for enzyme treatments are different for each type of food. In fact, Aiso and Tamura[61] investigated the effect of pH for the treatment using α-amylase and protease independently on folate values in four individual food items and found that there

was a distinctive pH optimum for the treatment with α-amylase of fresh beef and white bread but not for cow's milk and spinach. It is known that the pH optima are different depending on the protease(s) present in a crude enzyme preparation being used for trienzyme treatment because the affinity of the enzymes to substrates may be different depending on the food items.[62] Furthermore, pH optimum of α-amylase isolated from the same source as the one used for trienzyme treatment (*Aspergillus oryzae*) is different depending on the preparations.[63] Therefore, further investigations may be needed to establish the best pH condition for each enzyme treatment for individual food to obtain the maximum folate content.

Based on all findings mentioned above, it may be reasonable to conclude that accurate food folate content must be obtained after trienzyme treatment.[14,52,57,58,60] Without trienzyme treatment, previously obtained values of food folate content or dietary folate intake may be grossly underestimated.[10–12,24,25,64–67] However, it is evident that food folate determination using trienzyme treatment is at a very early stage. A monumental task confronts us in the form of establishing accurate food folate tables. At completion, however, these tables will have a powerful impact, not only nutritionally but also socially and politically; for example, on the estimation and recommendation of dietary folate intake, which has most likely been underestimated.[26,67] Furthermore, the previously estimated bioavailability of food folate in relation to synthetic folic acid may be an overestimation.[65–67] At present, however, it is unknown how much of folate in food is digested and absorbed *in vivo* from the gastrointestinal tract of humans in relation to the values of folate obtained using *in vitro* trienzyme treatment. It is important to note that the apparent minimum dietary folate requirement in humans becomes numerically large. Thus, it may be necessary for us to become accustomed to new folate values. It is acknowledged that this process will take time, and, for some, it may be difficult. However, we must get used to the idea. Needless to say, it is obvious the absolute minimum folate requirement (equivalent to pteroylglutamic acid) remains unchanged in humans.

One may question whether trienzyme treatment is necessary to determine folates in human tissues, such as plasma or red blood cells, as well as in various tissues of experimental animals. The author has tried a long-term incubation (total of 23 hr) of trienzyme treatment on human plasma and red blood cells and rat liver. There was no significant increase after trienzyme treatment in these samples. However, further studies are warranted to clarify this question because, as stated above, there was a noticeable increase in folate content of uncooked beef after a short-term treatment of α-amylase in addition to folate conjugase.[61] Furthermore, it may be of interest to investigate the physiological role of folates trapped in matrices, that can be extracted by α-amylase and protease treatments in plant and animal tissues.

Recommended procedures for food folate determination using trienzyme treatment

For the convenience of readers, an example of a flow chart of the experimental procedure is presented in *Figure 1.*

Food homogenization and heat treatment

To determine folate content in a single food item or mixed diet, an appropriate amount of material is homogenized using a Waring blender in a buffer containing 50 mmol/L Hepes and 50 mmol/L Ches with 11.4 mmol/L of ascorbic acid and 0.2 mmol/L of 2-mercaptoethanol with a final pH of 7.85.[41] The amount of buffer can vary from an equal volume to 10 to 20 times the amount in relation to the food to be homogenized, but this depends on the content of folate in each item. Based on our experience, an additional homogenization using a Polytron did not make any difference in folate content of a diet composite as long as trienzyme treatment was performed.[57] This homogenate can be separated into several aliquots and frozen at −70°C until further processing. One aliquot is heated in boiling water for 10 min before proceeding to the following steps.

Protease, α-amylase, and folate conjugase treatments

In order to obtain the maximum possible folate content in each sample, four types of treatment can be arranged. These treatments include (1) folate conjugase only, (2) protease followed by folate conjugase, (3) α-amylase followed by folate conjugase, and (4) α-amylase followed by protease, and then folate conjugase. It may be necessary to note that certain food items do not require protease treatment. For example, in fresh beef the maximum folate value was obtained with the combination of α-amylase and folate conjugase treatment, and the value declined with an additional protease treatment.[61] In such a case, it is not necessary to do all four treatments.

Protease and α-amylase can be obtained from Sigma Chemical (St. Louis, MO) and are the same as the enzyme preparations recommended by Martin et al.,[52] which are prepared from *Streptomyces griseus* (Type XIV) and *A. oryzae* (Type X-A), respectively. The concentration of protease and α-amylase to be used for the treatment of food mixtures are 20 and 20 mg, respectively, per mL of distilled-deionized water or a buffer depending on the pH that is most suitable for each food homogenate. These enzyme solutions should be filtered using a microfilter (0.22 μm) immediately before use in order to prevent bacterial contamination. The amount of enzyme solution for a unit of heated food homogenate may vary depending on the nature of the food item. In general 1 volume of each enzyme solution is mixed with 1 volume of the heated homogenate; however, this ratio can be flexible.

This preparation of α-amylase contains a large amount of folate ranging from 1.1 to 1.8 ng/mg of the solid as *L. casei* activity after folate conjugase treatment. This was a significant amount for the final calculation of folate content; therefore, it was essential to subtract the amount of folate in the enzyme solution. Recently, we successfully removed endogenous folate from the α-amylase preparation. One volume of the α-amylase solution (50 mg/mL) was mixed with one-tenth volume of activated charcoal and gently vortexed on ice for 20 min. After centrifugation (900 × g for 10 min), the supernatant was filtered using a 0.22 μm filter, and this filtrate was used as the enzyme. After this

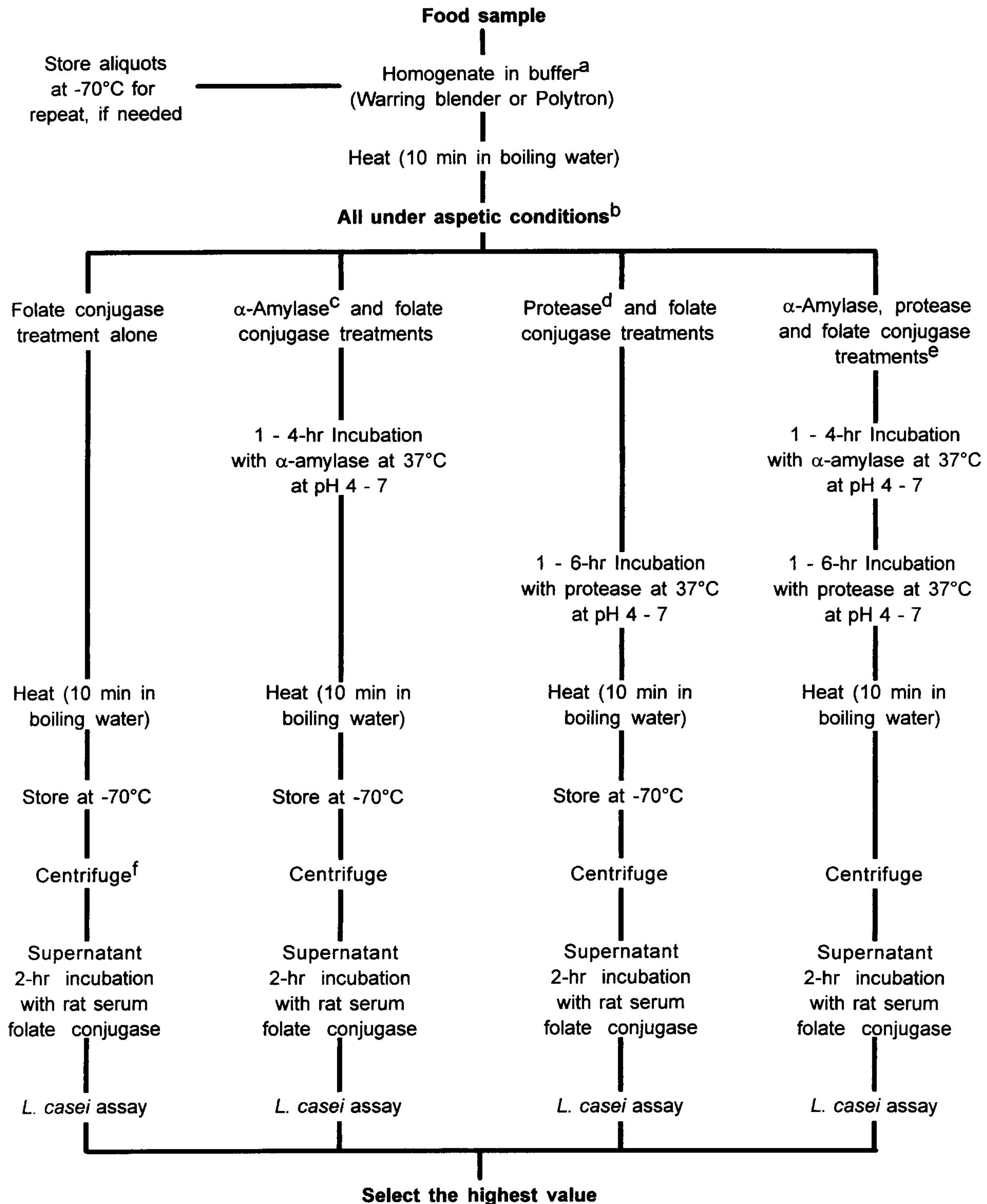

Figure 1 Flow chart of food folate analysis using trienzyme treatment. [a]50 mmol/L Hepes, 50 mmol/L Ches buffer (pH 7.85) containing 11.4 mmol/L of ascorbic acid, and 0.2 mol/L of 2-mercaptoethanol.[41] [b]All procedures after the first heating should be performed under aseptic conditions to avoid any bacterial contamination. [c]α-Amylase (Sigma Chemical, cat. #A 0273) at 20 mg/mL of water or buffer. This should be treated with charcoal before the treatment (see text). [d]Protease (Sigma Chemical, cat. #P 5147) at 20 mg/mL. [e]This trienzyme treatment can be done after the optimal conditions are identified by the remainder of treatments. Even without this procedure, the total number of test tubes to perform one sample (food mixture or a single food item) may exceed 30. [f]Centrifuge at 5,000 × g for 10 min using a microfuge.

treatment, no folate was detected by *L. casei* assay after conjugase treatment, and less than 15% of enzyme activity was lost.[68] If we use this preparation, it is no longer necessary to subtract α-amylase folate blank for the calculation. Furthermore, it is possible to increase the amount of enzyme used to treat food homogenate without disturbing high folate blank by the enzyme. The elimination of endogenous folate provides the possibility of using a shorter incubation to reduce degradation of food folate during the treatment by α-amylase. This filtrate can be stored at −70°C for at least 1 week.

Other enzyme preparations, such as Type XII-A (*Bacillus licheniforms*) and Type II-A (*Bacillus* species), have been tried in the author's laboratory. However, these are not suitable for the purpose of food folate extraction because the former contains an unacceptably large amount of endogenous folate and the latter does not contain sufficient activity to digest carbohydrates in a mixed diet, probably due to the extremely specific nature of this highly purified enzyme. On the other hand, only a minute amount of endogenous folate has been detected in the preparation of protease. Therefore, a subtraction of endogenous folate value is not necessary for the calculation after protease treatment. However, investigators are encouraged to check the amount of endogenous folate for each batch of enzyme preparations.

As a source of folate conjugase, rat serum appears to be the most suitable.[46] It can be obtained from commercial venders (e.g., Harlan Bioproducts for Science, P.O. Box 29176, Indianapolis, IN 46229). To remove endogenous folate, rat serum is mixed with one-tenth weight of acid-treated charcoal for 1 hr on ice. This step lowers endogenous folate to a level that is undetectable by *L. casei* assay. After centrifugation, the supernatant is filtered using a microfilter (0.22 μm), and aliquots are stored at −70°C. The activity of folate conjugase at pH 7.0 in this preparation has been approximately 1 μmol of [^{14}C]-glutamic acid formed/mL/hr using synthetic pteroyldiglutamyl-[^{14}C]-glutamic acid as substrate.[57] This activity is usually in far excess of what is required to hydrolyze food folates present in the incubation mixture. Folate conjugase treatment is done at 37°C for 1 to 3 hr in the presence of 114 mmol/L of ascorbic acid with a final pH of 7.0.

As stated above, optimal pHs for protease and α-amylase treatment are different from food to food; therefore, it may be important to identify the appropriate pH for each food item. Furthermore, the optimal incubation time for protease and α-amylase treatments also ranges from 1 to 6 hr depending on the food item.[61] Again, studies concerning the identification of the most suitable incubation time for each food item are encouraged. In some foods folates may be labile under the condition of the incubation at 37°C even in the presence of 114 mmol/L of ascorbic acid. Based on the experience of the author, the stability of synthetic folates (5-HCO-H_4PteGlu and pteroylglutamic acid) was within the acceptable range of the assay after a total of 23 hr incubation at 37°C compared with the coefficient of variation (10%) of microbiological assay.[27,57] Further studies may be needed, however, to examine the stability of various forms of folate during the incubation for more than a few hrs. The food homogenates usually appear more homogeneous and much clearer than the original homogenates after incubation with protease and α-amylase. The mixtures of food and enzyme(s) should be heated for 10 min in boiling water to denature protease for the prevention of the inactivation of folate conjugase by this enzyme. After centrifugation, the supernatant is used for folate conjugase treatment.

As cautioned by Pedersen,[56] it is extremely important to avoid contamination with bacteria that can synthesize folates during the incubations with three independent enzymes. The growth of the bacteria possibly provides falsely high folate values. Therefore, the use of presterilized plasticware, microfilter-sterilized reagents, and enzymes as well as heat-treated food samples is essential for all assays. It may be helpful to note that suspected bacterial growth was encountered in less than 3% of assays in the author's laboratory.

Microbiological assay

Detailed techniques of microbiological assay using a 96-well microplate reader have been reviewed.[27,35] Readers are encouraged to refer to these reviews. Usually, 10 to 100 μL of enzyme treated samples, depending on the folate content of the samples, can be placed in the first well before serial dilutions. The mean folate values are used for the final calculation based on at least two duplicates that are within the linear portion of the standard response curve. 5-Formyltetrahydrofolate (5-HCO-H_4PteGlu, calcium salt, Sigma Chemical) is used as a standard.[27]

Conclusion

The procedures for the various methods of food folate determination have been reviewed. It is now apparent that trienzyme treatment prior to folate determination is essential to obtain the true folate content in foods. Trienzyme treatment is performed using α-amylase and protease for the extraction of folate trapped in or bound to protein and carbohydrate matrices, and folate conjugase, for the hydrolysis of polyglutamyl folates. It should be noted that it may not always be necessary to perform all three enzyme treatments for every single food item. The conditions of enzyme treatment, including pH and incubation time, are required to be optimized for each food. The use of trienzyme treatment is currently in its infantile stage, and it should become more refined and specific as more investigators are involved. It may be wise to perform trienzyme treatment on a mixed diet representative of a single meal or one-day menu rather than single food items. Even though these processes are extremely laborious, time consuming, and admittedly scientifically unstimulating, it is essential that food folate tables more reliable than are currently available be established. This information is *fundamentally important* for the estimation of appropriate dietary folate intake.

In summary, we suggest that the method of trienzyme treatment be used to obtain the maximum content of folate in each food item for reliable food folate tables. This is needed more than ever because recent investigations have indicated that certain diseases can be prevented by increasing dietary folate intake, by use of fortifying cereal-grain

products with folic acid or supplementing the vitamin,[1–5,69,70] and that a significant segment of a certain population appears to have inadequate folate nutriture.[9]

References

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

2 Boushey, C.J., Beresford, S.A.A., Omenn, G.S., and Motulsky, A.G. (1995). A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *JAMA* **274,** 1049–1057

3 Public Health Service. (1992). Recommendations for the use of folic acid to reduce the number of cases of spina bifida and other neural tube defects. *Morbid. Mortal. Wkly. Rep.* **41,** 1–7

4 Food and Drug Administration. (1996). Food standards: Amendment of standards of identity for enriched grain products to require addition of folic acid. *Fed. Regist.* **61,** 8781–8797

5 Tucker, K.L., Mahnken, B., Wilson, P.W.F., Jacques, P., and Selhub, J. (1996). Folic acid fortification of the food supply. Potential benefits and risks for the elderly population. *JAMA* **276,** 1879–1885

6 U.S. Department of Agriculture. (1995). *Nutrition and Your Health: Dietary Guidelines for Americans*, 4th ed, U.S. Department of Agriculture, Washington, D.C., USA

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

8 Bailey, L.B. (1995). Folate requirements and dietary recommendations. In *Folate in Health and Diseases* (Bailey, L.B., ed), pp. 123–151, Marcel Dekker, New York, USA

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

10 U.S. Department of Agriculture. (1976–1989). *Agriculture Handbook 8-1-8-21. Composition of Foods.* U.S. Department of Agriculture, Washington, D.C., USA

11 Hoppner, K., Lampi, B., and Smith, D.C. (1977). Data on folacin activity in foods: Availability, applications, and limitations. In *Folic Acid. Biochemistry and Physiology in Relation to the Human Nutrition Requirement*, pp 69–81, National Academy of Sciences, Washington, D.C., USA

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

13 Shin, Y.S., Kim, E.S., Watson, J.E., and Stokstad, E.L.R. (1975). Studies of folic acid compounds in nature. IV. Folic acid compounds in soybeans and cow milk. *Can. J. Biochem.* **53,** 338–343

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

15 Chen, M.F., Hill, J.W., and McIntyre, P.A. (1983). The folacin contents of foods as measured by radiometric microbiological method. *J. Nutr.* **113,** 2192–2196

16 Day, B.P. and Gregory, J.F., III. (1981). Determination of folacin derivatives in selected foods by high-performance liquid chromatography. *J. Agric. Food Chem.* **29,** 374–377

17 Gregory, J.F., III, Sartain, D.B., and Day, B.P.F. (1984). Fluorometric determination of folacin in biological materials using high performance liquid chromatography. *J. Nutr.* **114,** 341–353

18 Vahteristo, L.T., Ollilainen, V., Koivistoinen, P.E., and Varo, P. (1996). Improvements in the analysis of reduced folate monoglutamates and folic acid in food by high-performance liquid chromatography. *J. Agric. Food Chem.* **44,** 477–482

19 White, D.R., Jr., Lee, H.S., and Krüger, R.E. (1991). Reversed-phase HPLC/EC determination of folate in citrus juice by direct injection with column switching. *J. Agric. Food Chem.* **39,** 714–717

20 Selhub, J., Darcy-Vrillon, B., and Fell, D. (1988). Affinity chromatography of naturally occurring folate derivatives. *Analy. Biochem.* **168,** 247–251

21 Selhub, J. (1989). Determination of tissue folate composition by affinity chromatography followed by high-pressure ion pair liquid chromatography. *Analy. Biochem.* **182,** 84–93

22 Seyoum, E. and Selhub, J. (1993). Combined affinity and iron pair column chromatographies for the analysis of food folate. *J. Nutr. Biochem.* **4,** 488–494

23 Bagley, P.J. and Selhub, J. (1997). Analysis of folates using combined affinity and ion-pair chromatography. *Methods Enzymol.* **281,** 16–25

24 Finglas, P.M., Faure, U., and Southgate, D.A.T. (1993). First BCR-intercomparison on the determination of folates in food. *Food Chem.* **46,** 199–213

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

26 National Research Council. (1989). *Recommended Dietary Allowances.* 10th Ed. pp 150–158, National Academy Press, Washington, D.C., USA

27 Tamura, T. (1990). 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., and Gregory, J.F., III, eds.), pp. 121–137, Wiley-Liss, New York, USA

28 Cooperman, J.M. (1971). Microbiological assay of folic acid activity in serum and whole blood. *Methods Enzymol.* **18,** part B, 629–642

29 Bird, O.D. and McGlohon, V.M. (1972). Differential assays of folic acid in animal tissues. In *Analytical Microbiology*, vol. 2, (Kavanagh, F., ed.), pp. 409–437, Academic Press, New York, USA

30 Cossins, E.A. (1984). Folates in biological materials. In *Folates and Pterins, vol. 1, Chemistry and Biochemistry of Folates* (Blakley, R.L., and Bencovic, S.J., eds.), pp. 1–59, John Wiley & Sons, New York, USA

31 Grossowicz, N., Waxman, S., and Schreiber, C. (1981). Cryoprotected *Lactobacillus casei*: An approach to standardization of microbiological assay of folic acid in serum. *Clin. Chem.* **27,** 745–747

32 Wilson, S.D. and Horne, D.W. (1982). Use of glycerol-cryoprotected *Lactobacillus casei* for microbiological assay of folic acid. *Clin. Chem.* **28,** 1198–1200

33 Newman, E.M. and Tsai, J.F. (1986). Microbiological analysis of 5-formyltetrahydrofolic acid and other folates using an automatic 96-well plate reader. *Analy. Biochem.* **154,** 509–515

34 Horne, D.W. and Patterson, D. (1988). *Lactobacillus casei* microbiological assay of folic acid derivatives in 96-well microtiter plates. *Clin. Chem.* **34,** 2357–2359

35 Horne, D.W. (1997). Microbiological assay of folates in 96-well microtiter plates. *Methods Enzymol.* **281,** 38–43

36 Gregory, J.F., III, Engelhardt, R., Bhandari, S.D., Sartain, D.B., and Gustafson, S.K. (1990). Adequacy of extraction techniques for determination of folate in foods and other biological materials. *J. Food Comp. Analy.* **3,** 134–144

37 Tamura, T., Shin, Y.S., Williams, M.A., and Stokstad, E.L.R. (1972). *Lactobacillus casei* response to pteroylpolyglutamates. *Analy. Biochem.* **49,** 517–521

38 Engelhardt, R. and Gregory, J.F., III. (1990). Adequacy of enzymatic deconjugation in quantification of folate in foods. *J. Agri. Food Chem.* **38,** 154–158

39 Bakerman, H.A. (1961). A method for measuring the microbiological activity of tetrahydrofolic acid and other labile reduced folic acid derivatives. *Analy. Biochem.* **2,** 558–567

40 Toepfer, E.W., Zook, E.G., Orr, M.L., and Richardson, L.R. (1951). Folic acid content of foods. Microbiological assay by standardized methods and complication of data from literature. In *Agriculture Handbook* No. 29, United States Department of Agriculture, Washington, D.C., USA

41 Wilson, S.D. and Horne, D.W. (1983). Evaluation of ascorbic acid in protecting labile folic acid derivatives. *Proc. Natl. Acad. Sci.* **80,** 6500–6504

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

43 Bird, O.D., Binkley, S.B., Bloom, E.S., Emmitt, A.D., and Pfeffner, J.J. (1945). On the enzymatic formation of vitamin B_c from its conjugate. *J. Biol. Chem.* **157,** 413–414

44 Mims, V. and Laskowski, M. (1945). Studies on vitamin B_c conjugase from chicken pancreas. *J. Biol. Chem.* **160,** 493–503

45 Leichter, J., Butterworth, C.E., Jr., and Krumdieck, C.L. (1977).

Partial purification and some properties of pteroylpolyglutamate hydrolase (conjugase) from chicken pancreas. *Proc. Soc. Exp. Biol. Med.* **154,** 98–101

46 Horne, D.W., Krumdieck, C.L., and Wagner, C. (1981). Properties of folic acid γ-glutamyl hydrolase (conjugase) in rat bile and plasma. *J. Nutr.* **111,** 442–449

47 Selhub, J., Ahmad, O., and Rosenberg, I.H. (1980). Preparation and use of affinity columns with bovine milk folate-binding protein (FBP) covalently linked to Sepharose 4B. *Methods Enzymol.* **66,** 686–690

48 Varela-Moreiras, G., Seyoum, E., and Selhub, J. (1991). Combined affinity and ion pair liquid chromatographies for the analysis of folate distribution in tissues. *J. Nutr. Biochem.* **2,** 44–53

49 Shin, Y.S., Buehring, K.U., and Stokstad, E.L.R. (1972). Separation of folic acid compounds by gel chromatography on Sephadex G-15 and G-25. *J. Biol. Chem.* **247,** 7266–7269

50 Krumdieck, C.L., Tamura, T., and Eto, I. (1983). Synthesis and analysis of the pteroylpolyglutamates. *Vit. Horm.* **40,** 45–104

51 Gregory, J.F., III. (1997). Bioavailability of folate. *Eur. J. Clin. Nutr.* **51,** S54–S59

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

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

54 Eitenmiller, R.R., Bryan, W.D., Khalsa, I.K., Feeley, R.M., and Barnhart, H.M. (1984). Folate content of human milk during early lactational stages. *Nutr. Res.* **4,** 391–397

55 Cerna, L. and Kas, J. (1983). New conception of folacin assay in starch or glycogen containing food samples. *Nahrung* **27,** 957–964

56 Pedersen, J.C. (1988). 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.* **59,** 261–271

57 Tamura, T., Mizuno, Y., Johnston, K.E., and Jacob, R.A. (1997). Food folate assay with protease, α-amylase, and folate conjugase treatments. *J. Agri. Food. Chem.* **45,** 135–139

58 Pfeiffer, C.M., Rogers, L.M., and Gregory, J.F., III. (1997). Determination of folate in cereal-grain food products using trienzyme extraction and combined affinity and reversed-phase liquid chromatography. *J. Agri. Food. Chem.* **45,** 407–413

59 Butterworth, C.E., Jr., Newman, A.J., and Krumdieck, C.L. (1974). Tropical sprue: A consideration of possible etiologic mechanisms with emphasis on pteroylpolyglutamate metabolism. *Trans. Am. Clin. Clinatol. Assoc.* **86,** 11–22

60 Lim, H.-S., Mackey, A.D., Tamura, T., Wong, S.C., and Picciano, M.F. (1998). Measurable human milk folate increased by treatment with α-amylase and protease in addition to folate conjugase. *Food Chem.* (in press)

61 Aiso, K. and Tamura, T. (1998). Trienzyme treatment for food folate analysis: Optimal pH and incubation time for α-amylase and protease treatments. *J. Nutr. Sci. Vitaminol.* (in press)

62 Birktoft, J.J. and Breddam, K. (1994). Glutamyl endopeptidases. *Methods Enzymol.* **244,** 114–126

63 Vihinen, M. and Mäntsälä, P. (1989). Microbial amylolytic enzymes. *Crit. Rev. Biochem. Mol. Biol.* **24,** 329–418

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

65 Tamura, T., Shin, Y.S., Bueħing, K.U., and Stokstad, E.L.R. (1976). The availability of folates in man: Effect of orange juice supplement on intestinal conjugase. *Br. J. Haematol.* **32,** 123–133

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

67 Sauberlich, H.E., Kretsch, M.J., Skala, J.H., Johnson, H.L., and Taylor, P.C. (1987). Folate requirement and metabolism in nonpregnant women. *Am. J. Clin. Nutr.* **46,** 1016–1028

68 Bernfeld, P. (1955). Amylase, α and β. *Methods Enzymol.* **1,** 149–158

69 Pfeiffer, C.M., Rogers, L.M., Bailey, L.B., and Gregory, J.F., III. (1997). Absorption of folate from fortified cereal-grain products and of supplemental folate consumed with or without food determined by using a dual-label stable-isotope protocol. *Am. J. Clin. Nutr.* **66,** 1388–1397

70 Tamura, T. (1997). Bioavailability of folic acid in fortified food. *Am. J. Clin. Nutr.* **66,** 1299–1300