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Measurable human milk folate is increased by treatment with α-amylase and protease in addition to folate conjugase

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The application of trienzyme treatment prior to microbiological assay using *Lactobacillus casei* for the measurement of folate in human milk was evaluated. Trienzyme treatment consisted of α-amylase, protease, and folate conjugase. Human milk samples ($n = 84$) were heat treated for 5 min at 100°C in 0.1 M potassium phosphate buffer containing 0.05 mol liter^{-1} ascorbic acid, pH 4.1, then incubated with the enzymes by stepwise addition. Following trienzyme treatment, folate was measured microbiologically using a 96-well microplate method. The results show that treatment at 37°C with α-amylase for 4 h followed by protease for 8 h, and finally by folate conjugase for 3 h significantly increased the measurable folate in human milk by an average of 85% compared with values after folate conjugase treatment alone ($p < 0.001$). Neither the pH of the extraction buffer, the source of folate conjugase, nor the method/length of heat treatment of the samples were significant variables influencing the values of human milk folate. Data indicate that folate in human milk can be underestimated unless samples are treated with α-amylase, protease, and folate conjugase prior to microbiological assay using *L. casei*.

INTRODUCTION

A firm knowledge of the folate content of human milk is essential for estimating requirements of this vitamin for both lactating women and their infants. The quantity of human milk folate serves as the basis for estimating the folate requirement for lactating women for milk secretion. Accordingly, folate intake of exclusively breast-fed infants thriving normally also serves as the basis for evaluating infant requirement. Although human milk folate content has been examined by many investigators, there exist considerable discrepancies in the literature concerning the actual amount present. Reported mean values range from 26 to 141 μg liter^{-1} (Yamada, 1979; Tamura *et al.*, 1980; Thomas *et al.*, 1980; Sneed *et al.*, 1981; Butte and Calloway, 1981; Cooperman *et al.*, 1982; Eitenmiller *et al.*, 1984; Bank *et al.*, 1985; Smith *et al.*, 1985; Ek, 1985; Udipi *et al.*, 1985, 1987; O'Connor *et al.*, 1991; Keizer *et al.*, 1995). The extent to which this wide range reflects methodological difficulties and/or true biological variation is uncertain.

The term 'folate' is a generic descriptor for a family of compounds with chemical structures similar to synthetic pteroylglutamic acid, commonly known as folic acid. Folic acid, the oxidized and stable form of folate, does not occur in nature, but is found in vitamin supplements and fortified food products. Folate in biological samples varies in the nature of the single carbon unit (e.g. formyl, methyl, methylene, etc.) and the reduction status of the pteridine ring. In addition, most naturally occurring folate has glutamate residues (ranging from one to more than 10) attached to the *para*-amino-benzoic acid moiety of the molecule by a series of γ-glutamyl peptide bonds. Given the possibilities of variation in the folate molecule, it is not surprising that over 150 forms of folate are reported to exist (Krumdieck *et al.*, 1983).

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Radiobinding, high-performance liquid chromatography, and microbiological assays are used to determine concentrations and/or identify the forms of folate in biological samples. However, since folate is present in biological samples in nanogram quantities, microbiological assays are commonly employed, because they provide the needed sensitivity and can detect the variety of folate molecules present. Among assay organisms, *Lactobacillus casei* (ATCC 7469) has become the microorganism of choice, because it responds to the broadest spectrum of folate forms and is the only one that responds to 5-methyltetrahydrofolate, the principal derivative found in human milk.

Regardless of the analytical procedure used, the accuracy of the analysis is highly dependent on sample handling and preparative methods employed for analyses. Folate in biological samples is extremely labile; therefore, samples must be protected from oxidation. When the folate content is measured using *L. casei*, two procedures are traditionally involved prior to the folate assay (Gregory, 1989; Tamura, 1990). One involves heat treatment of samples, which is necessary to release folate from binding proteins to which it is quantitatively bound, for example, in human milk (Selhub *et al.*, 1984). The other is treatment with folate conjugase (pteroylpolygammaglutamyl hydrolase: EC 3.4.19.9), to cleave glutamyl residues from polyglutamyl folate. Previous findings from our laboratory showed greater than a 190% increase in measurable milk folate after heat treatment and a 23–47% increase following treatment with folate conjugase (O'Connor *et al.*, 1991).

Three investigative teams (Martin *et al.*, 1990; Tamura *et al.*, 1997; Pfeiffer *et al.*, 1997) recently reported that treatments with α-amylase (EC 3.2.1.1) and protease (EC 3.4.24.31) in addition to folate conjugase (trienzyme treatment) may be necessary for an accurate determination of folate in biological samples. These additional enzyme treatments are believed to release bound folate from complex protein and/or oligosaccharide matrices. The application of trienzyme treatment prior to quantification is reported to increase measurable folate by 13–71% in commercially available infant formula (De Souza and Eitenmiller, 1990; Martin *et al.*, 1990) and by 4–300% in a variety of foods (De Souza and Ertenmiller, 1990; Martin *et al.*, 1990; Tamura *et al.*, 1997; Pfeiffer *et al.*, 1997).

Mature human milk contains approximately 12 g liter^{-1} of undefined oligosaccharides (Newburg and Neubauer, 1995) and 7 g liter^{-1} of proteins (Lonnerdal and Atkinson, 1995), and a multitude of glycoproteins, glycolipids, and mucins, creating a complex biological matrix in which folate may be trapped. Therefore, the relative merit of the trienzyme treatment prior to microbiological assay for the measurement of folate in human milk was evaluated. Other possible sources of methodological variation in the measurement of human milk folate were also evaluated, including the pH of the buffer, the method and length of heat treatment, and the sources of folate conjugase.

MATERIALS AND METHODS

Samples

A total of 84 human milk samples were collected at 3 and 6 months postpartum from 42 apparently healthy women participating in a study designed to examine folate nutritional status during extended lactation. The use of human subjects in this investigation was annually reviewed and approved by the Institutional Review Board of the Pennsylvania State University. Informed written consent was obtained from each woman. Approximately 30 ml of human milk were collected, either by manual expression or with the assistance of commercially available mechanical pumps (Marshall Baby Care Products, Lincolnshire, IL, USA; Ross Laboratories, Columbus, OH, USA; Evenflo Products, Ravenna, OH, USA). Samples were held on ice and mixed with ascorbate to achieve a final concentration of 0.05 mol liter^{-1} within 6 h of collection. They were stored at −70°C until analyzed.

Enzymes

Folate conjugase

The rat serum folate conjugase used in this study was obtained from Harlan Bioproducts For Science (Indianapolis, IN, USA) and was treated with acid-washed charcoal to remove endogenous folates. Folate conjugase from chicken pancreas was partially purified by the method of Leichter *et al.* (1977), and that from hog kidney by the method of Bird *et al.* (1969) with slight modifications. The activities of these folate conjugases were evaluated using pteroyldiglutamyl-[^{14}C]glutamate (PteGlu$_2$[^{14}C]-Glu) as substrate (Krumdieck and Baugh, 1970). Folate conjugase purifed from hog kidney had the lowest activity with over 500 ng of (PteGlu$_2$[^{14}C]-Glu) per 200 μl per 60 min, which was far more than sufficient to hydrolyse folates in the incubation system. Approximately 5 ml of the clear filtrate obtained at the final stage of preparation of hog kidney folate conjugase was applied on to a Sephadex C-25 column (2.5 × 25.0 cm), and eluted with 0.1 M sodium acetate buffer (pH 4.8). A fraction (15 ml) at the position of void volume was collected, and used as the enzyme source. This fraction did not contain measurable folate by *L. casei* assay.

α-Amylase and protease

α-Amylase (Type X-A) and protease (Type XIV) were obtained from Sigma Chemical Co. (St Louis, MO, USA), and were dissolved in distilled deionized water at concentrations of 20 mg ml^{-1} and 10 mg ml^{-1}, respectively. α-Amylase and protease preparations were filter-sterilized (0.22 μm) prior to use. No endogenous folate

was detected in the protease preparation, but α-amylase contained approximately 1.1 ng of folate per mg solid via microbiological assay following treatment with folate conjugase. The folate added during analyses from the α-amylase preparation was subtracted in the final calculation of total folate.

Evaluation of assay conditions

Length of incubation with α-amylase and protease
Among investigators who have applied trienzyme treatment to food folate analyses, incubation times range from 2 to 4 h for α-amylase and 1 to 16 h for protease (De Souza and Eitenmiller, 1990; Martin *et al.*, 1990; Tamura *et al.*, 1997; Pfeiffer *et al.*, 1997). Prior to the final application of these enzymes to human milk samples, the effect of incubation time on the contents of folate was assessed. Sample extracts ($n = 20$) treated with α-amylase were incubated for 0, 2, 4, or 6 h at 37°C. Similarly, sample extracts treated with protease were incubated for 0, 4, 8, 12, or 16 h at 37°C. Incubation time for human milk samples with folate conjugase was previously determined to be 3 h.

Length and method of heat treatment of sample
Various investigators have reported that heat treatment of milk samples prior to microbiological assay is necessary to denature binding proteins (Ghitis, 1967; Gregory, 1989; O'Connor *et al.*, 1991). Heat treatment is used to denature these proteins to liberate folate, making it available for detection by the test organism (*L. casei*). To assess various methods of heat treatment, samples ($n = 4$) were mixed with 0.1 M potassium phosphate buffer, pH 4.1, containing 0.05 mol liter^{-1} ascorbate then treated as follows: boiling for 5 min at 100°C, or autoclaving for 5, 15, or 30 min at 121°C.

pH of enzyme treatments
Recently, Tamura *et al.* (1997) reported that the greatest detection of folate in food composites was achieved following α-amylase and protease extraction treatments with 0.1 M potassium phosphate buffer at pH 4.1. The pH optimum for folate conjugase is reported to be pH 6.2–7.5 (Horne *et al.*, 1981). Thus, prior to folate conjugase treatment at pH 7.0, the pH of the buffer during treatments with α-amylase and protease was assessed. Samples ($n = 10$) were extracted in 0.1 M potassium phosphate buffer containing 0.05 mol liter^{-1} ascorbate at pH 2.95, 4.10, or 6.30.

Folate conjugase sources
Sources of folate conjugase commonly employed are chicken pancreas, hog kidney, and rat serum (Tamura, 1990). Possible differences in sources of folate conjugase for the measurement of human milk folate were evaluated. Samples of human milk ($n = 10$) were treated with both α-amylase for 4 h and protease for 8 h then incubated at 37°C for 3 h with one of the three folate conjugases. For rat serum folate conjugase treatment, samples (0.2 ml) were mixed with 0.02 ml of rat serum folate conjugase and 0.38 ml of 0.1 M of potassium phosphate buffer containing 0.05 mol liter^{-1} ascorbate with the final pH of 7.0. For hog kidney folate conjugase treatment, samples (0.2 ml) were mixed with 0.1 ml of conjugase preparation and 0.3 ml of 0.1 M acetate buffer, pH 4.8, containing 1% ascorbate. For chicken pancreas conjugase treatment, samples (0.5 ml) were mixed with 1.0 ml of chicken pancreas conjugase prepared with 0.1 M potassium phosphate buffer, pH 7.0, containing 0.05 mol liter^{-1} ascorbate.

Assay conditions applied to human milk samples

Figure 1 summarizes the stepwise assay conditions applied to human milk samples ($n = 84$) for assessment of the trienzyme treatment prior to microbiological assay. Samples (0.5 ml) were mixed with an equal volume (0.5 ml) of 0.1 M of potassium phosphate buffer, pH 4.1, containing 0.05 mol liter^{-1} ascorbate, and heated at 100°C for 5 min. After cooling on ice, 0.2 ml of the buffered samples were mixed with 0.2 ml of α-amylase and incubated for 4 h at 37°C. Thereafter, 0.2 ml of protease was added to sample mixtures and incubated

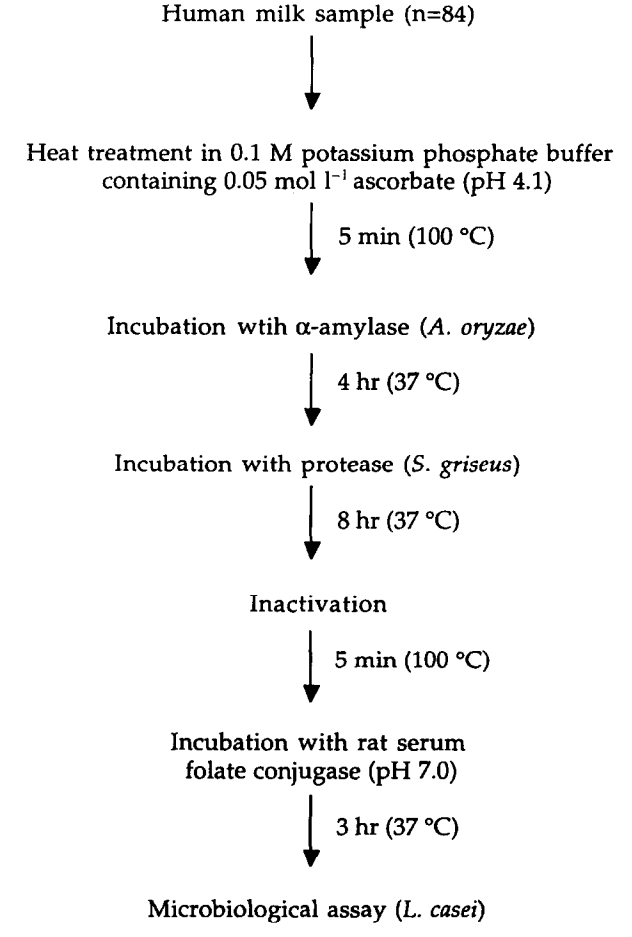

Fig. 1. Flow diagram of trienzyme treatments of human milk samples prior to folate measurement by microbiological assay.

for 8 h at 37°C. Before folate conjugase treatment, samples treated with α-amylase and protease were heated at 100°C for 5 min to inactivate the protease, then cooled to room temperature. Folate conjugase treatment was carried out by incubating 0.2 ml of each enzyme-treated sample with 0.02 ml of rat serum folate conjugase and 0.38 ml of 0.1 M of potassium phosphate buffer, pH 7.0, containing 0.05 mol liter^{-1} ascorbate for 3 h at 37°C.

Microbiological assay

Folate in the samples treated with α-amylase, protease, and folate conjugase were determined by microbiological assay using 0.1 M potassium phosphate buffer, pH 6.3, containing 0.05 mol liter^{-1} ascorbate and *L. casei* as the test organism. The assay was carried out using a 96-well microplate and a microplate reader (Tamura, 1990). The folate standard was 5-formyl-tetrahydrofolate ([6*RS*]-5-HCO-H_4PteGlu, calcium salt; Sigma), a reduced and stable form of folate. The standard concentration was checked using a molar extinction coefficient of 28 000 at 282 nm (pH 13.0). Since the compound is a reacemic mixture, one half of the calculated value was used for the concentration of folate standard (Tamura, 1990). The coefficient of interassay variation for folate analysis is approximately 10–12% in our laboratory.

Statistical analyses

Since data obtained in this study were not normally distributed, data were logarithmically transformed prior to statistical analyses. Analysis of variance statistics were applied to data using the Statistical Analysis System (SAS) for Windows version 6.12 (1996, Cary, NC, USA). Comparisons among treatment means were performed using Tukey's honestly significant difference test. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

Length of incubation with α-amylase and protease

The length of the incubation time with α-amylase had a significant effect ($p < 0.05$) on measurable folates (Fig. 2). Mean values for folate in human milk samples ($n = 20$) treated with α-amylase (μg liter^{-1} ± standard error of the mean) were 71.9 ± 5.0 at 0 h, 81.6 ± 6.0 at 2 h (14% increase, compared with 0 h incubation), 92.1 ± 6.0 at 4 h (28% increase), and 80.5 ± 5.3 at 6 h (12% increase). Since samples treated with α-amylase for 4 h yielded the highest values, the 4 h incubation time was chosen for the assay of human milk folate.

The treatment of samples with protease similarly increased detection of the measurable folate in human milk. Incubation with protease for 8 h yielded significantly ($p < 0.001$) higher detectable folate than when treated for 0, 4, 12, or 16 h (Fig. 3). Mean values (μg liter^{-1}) of folate in human milk samples ($n = 20$) treated with protease were 31.8 ± 3.7 at 0 h, 37.0 ± 4.8 at 4 h (16% increase), 65.4 ± 5.8 at 8 h (110% increase), 62.8 ± 5.9 at 12 h (97% increase), and 42.6 ± 5.6 at 16 h (34% increase). For the purpose of assaying human milk folate, the 8 h incubation was chosen as the length of time for subsequent protease treatments.

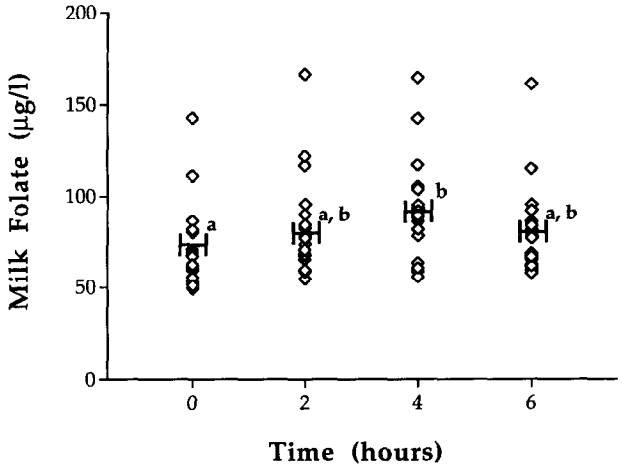

Fig. 2. Effect of length of incubation with α-amylase on the measurement of human milk folate. The mean at each time point is indicated by (|—|). Means with unlike letters differ at $p < 0.05$. Samples were subsequently treated with protease and conjugase for 8 and 3 h, respectively, at 37°C. Folates were measured by microbiological assay using *Lactobacillus casei*.

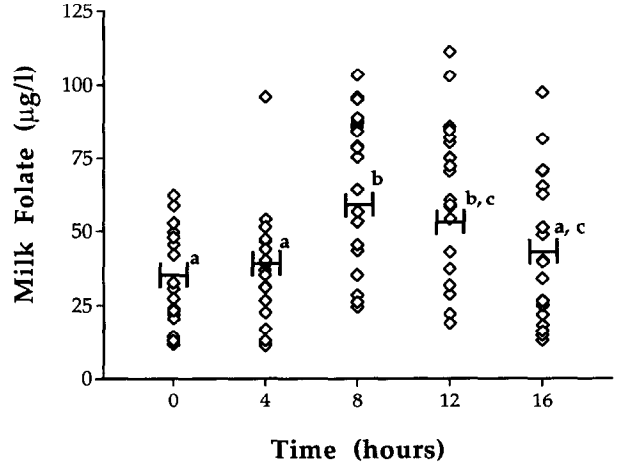

Fig. 3. Effect of length of incubation with protease on the measurement of human milk folate. The mean at each time point is indicated by (|—|). Means with unlike letters differ at $p < 0.001$. Samples were treated with α-amylase for 4 h at 37°C before protease treatment, followed by folate conjugase for 3 h at 37°C. Folates were measured by microbiological assay using *Lactobacillus casei*.

Length and method of heat treatments, pH of buffers, and sources of folate conjugase

Mean values for human milk folate in samples heated at 100°C for 5 min were 99.5 ± 11.7, and in samples heated at 121°C for 5, 10, 15, or 30 min were 96.2 ± 12.3, 99.1 ± 12.7, 88.0 ± 9.7, and 86.0 ± 10.6 μg liter^{-1}, respectively. Even though heating to 121°C for 15–30 min appeared to decrease measurable folate, the apparent decline was not statistically significant. Heating to 100°C for 5 min in a boiling water bath was chosen for subsequent human milk analyses, because this procedure was the most rapid and required minimal equipment.

The pH of the buffer for treatments with α-amylase and protease had no statistically significant impact on the measurement of human milk folate by microbiological assay with *L. casei*. Mean values of folate in human milk samples treated with α-amylase and protease in 0.1 M potassium phosphate buffer containing 0.05 mol liter^{-1} ascorbate at pH 6.30, 4.10, and 2.95 were 34.6 ± 6.1, 40.7 ± 6.7, and 38.0 ± 5.9 μg liter^{-1}, respectively. A pH of 4.10 was selected for the buffer during α-amylase and protease treatments.

The source of folate conjugase was not found to be an important variable in the measurement of human milk folate. Mean values of folate in samples treated with rat serum, hog kidney, or chicken pancreas folate conjugase were 37.61 ± 5.59, 28.80 ± 2.53, and 34.09 ± 4.62 μg liter^{-1}, respectively.

Assay of human milk folates

Applying the assay conditions presented in Fig. 1 to a total of 84 samples, an average increase of 85% was found in the measurable folate in human milk (Table 1). Using the most commonly employed method of folate detection (i.e. folate conjugase treatment alone prior to *L. casei* analysis), the mean value for human milk folate was 46.5 ± 2.3 μg liter^{-1}. In comparison, the detection of human milk folate in samples following treatment with α-amylase, protease, and folate conjugase was increased to 86.0 ± 2.5 μg liter^{-1} ($p < 0.001$). The measurable increase in detectable folate following trienzyme treatment was similar for samples collected at 3 months (84% increase) and at 6 months (86% increase). Of the samples ($n = 84$) treated with the three enzymes prior to microbiological assay, only two showed no increase in measured folate compared with treatment with folate conjugase only. The increase in measurable folate in human milk samples with trienzyme treatment ranged from 8 to 375% for individual samples.

DISCUSSION

Data from the present study show that human milk samples treated with α-amylase, protease, and folate conjugase prior to the microbiological assay using *L. casei*, resulted in a marked increase in measurable folate as compared with samples treated with folate conjugase alone. The average increase in measured folate in human milk samples (85%) is within the ranges reported by other investigators who applied trienzyme treatment prior to folate measurement of different foods (Martin *et al.*, 1990; De Souza and Eitenmiller, 1990; Tamura *et al.*, 1997; Pfeiffer *et al.*, 1997). The magnitude of the increase in detectable folate in samples following trienzyme treatment varies considerably among individual foods. Previous investigators who have applied this methodology suggest that the food matrix may be responsible for the variable responses. An important finding of this study is that the conditions of the assay must be established for individual biological samples prior to trienzyme treatment.

Although a 16 h incubation with protease yielded the highest folate values for food composites (Tamura *et al.*, 1997), labile folates in human milk were degraded with this lengthy incubation time. Previously, Yamada (1979) reported enhanced detection following protease treatment for 6 h. In this study, an 8 h incubation time yielded the greatest measurable folates in human milk samples. Incubation times exceeding 8 h resulted in a decrease in measurable folates. This observation is similar to that of Pfeiffer *et al.* (1997), who reported a 20% decrease in the measurable folates in cereal grains following a 16 h incubation with protease.

Samples treated with α-amylase for 4 h yielded the highest measurable folates in human milk. This was a surprising finding, since human milk does not contain any polysaccharides. However, the oligosaccharide

Table 1. Mean folate content (μg liter^{-1} ± standard error of the mean) of human milk samples collected at 3 and 6 months postpartum measured after treatment with folate conjugase alone or with α-amylase, protease, and folate conjugase

	No. of samples	Folate conjugase alone[a]	α-Amylase, protease, and folate conjugase[b]	% Increase
Human milk at 3 months	42	49.3 ± 3.8[3a]	90.6 ± 3.5[b]	84
Human milk at 6 months	42	43.7 ± 2.9[a]	81.5 ± 3.5[b]	86
Total	84	46.5 ± 2.3[a]	86.0 ± 2.5[b]	85

[a]Samples were treated with folate conjugase at 37°C for 3 h prior to microbiological assay with *Lactobacillus casei*.
[b]Samples were treated with α-amylase, protease, and folate conjugase at 37°C for 4, 8, and 3 h, respectively, prior to microbiological assay with *L. casei*.
Means with unlike superscript letters differ at $p < 0.001$.

fraction in human milk is reported to be approximately 12 g liter^{-1}, making it one of the largest solid components in human milk, just behind fat and lactose (Newburg and Neubauer, 1995). Apparently the total polysaccharide content of a food is not predictative of the impact that α-amylase treatment will have during the extraction procedure. Other investigators reported that foods rich in starch and glycogen show a 10–80% increase in measurable folates, following treatment with α-amylase (Pedersen, 1988; Cerna and Kas, 1983).

Previously, our laboratory has shown that human milk samples must be heated to denature folate binding proteins for the release of folate, making folate available to test organisms (O'Connor *et al.*, 1991). Human milk is a unique biological fluid with respect to folate concentration. High affinity folate binding proteins in milk (approximately 120 ng folate binding protein per ml milk) are believed to facilitate the secretion of folate. The folate in human milk is quantitatively bound to these glycoproteins (Ghitis, 1967; Ford *et al.*, 1969; Selhub *et al.*, 1984). In biological fluids other than human milk, total folate content far exceeds the folate binding capacity. For example, the folate in human serum (2–23 μg liter^{-1}) is bound to a variety of proteins, including albumin, α-macroglobulin, and a small amount of folate binding proteins (Holm *et al.*, 1980).

The pH of the extraction buffer (prior to folate conjugase treatment) did not appear to have an effect on treatments with α-amylase and protease. Tamura *et al.* (1997) reported the highest measurable folate in food composites following trienzyme treatment at pH 4.1. The source of folate conjugase was also found to have no effect on the measured folate in human milk samples. The enzymatic deconjugation of polyglutamyl folate is an essential preparative techniques in human milk folate analyses, resulting in marked increases in detectable folate (O'Connor *et al.*, 1991). However, in other foods, the source of folate conjugase may be an important variable. Although Tamura and Stokstad (1973) reported that there was no difference in food folate contents with the treatment by folate conjugase from hog kidney and chicken pancreas, Phillips and Wright (1982) suggested that deconjugation of food folate with hog kidney folate conjugase, as compared with chicken pancreas conjugase, may increase measurable folate by approximately 50%. Kirsch and Chen (1984) reported that chicken pancreas folate conjugase was superior for folate analysis in peas and beans, while no advantage was found in potatoes. The presence of a folate conjugase inhibitor in legumes is reported to be responsible for incomplete deconjugation with the use of hog kidney extracts (Butterworth *et al.*, 1974).

The use of an anti-oxidant (ascorbic acid) to preserve labile folate forms, heat treatment of samples for release from binding proteins, protection from destruction by light, and treatment with enzymes are all important procedures in the analytical scheme for measuring folate in human milk. Results from this study suggest that the wide range of values for human milk folate is due in part to methodological issues rather than true biological variation.

A firm knowledge of the folate content of human milk is essential for estimating the nutritional cost for folate during lactation. The folate in human milk also serves as the basis for dietary recommendations for folate during infancy. The current Recommended Dietary Allowance for folate during lactation is 100 μg day^{-1} above the estimated requirement for non-reproducing women (NRC, 1989). This increment is based on the assumption that folate absorption from a mixed diet is 50% and human milk folate content averages 50 μg liter^{-1} (Matoth *et al.*, 1965) with an average daily milk production of 800 ml day^{-1} in established lactation. Based on the average folate measured in these human milk samples (86 μg liter^{-1}) treated with α-amylase, protease, and folate conjugase, and typical milk secretion rate (800 ml day^{-1}), the daily amount of folate needed to cover losses in milk is approximately 70 μg day^{-1}. The amount of dietary folate that needs to be ingested to cover average losses in milk is approximately 140 μg day^{-1}, based on 50% bioavailability. Based on results from this study, the average folate needs of the lactating woman are currently underestimated by at least 40%.

In summary, we evaluated the methods to measure folate content in human milk using the microbiological assay. Our data indicate that treatment of milk samples at 37°C with α-amylase for 4 h, followed by protease for 8 h, and finally with folate conjugase for 3 h appears to give the maximum measurable folate values in human milk.

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