The folate in human milk[1, 2]

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ABSTRACT In previous studies of the folate content of human milk, samples were prepared for assay by a method that resulted in a turbid solution that was then assayed by a turbidimetric microbiological method. We have used an improved microbiological assay in which the milks were treated with rennin to precipitate casein and heated in a buffered ascorbate to coagulate lactalbumin and lactoglobulin. Milks were obtained serially from nursing mothers for periods ranging from 1 day to 6 months postpartum. The results showed that the folate in human milk has few glutamate residues since treatment with a purified folate conjugase preparation released no additional folate activity for *Lactobacillus casei*. Colostrum is relatively low in folate, but milk folate increases as lactation proceeds. During each stage of lactation there was great variation in milk folate content among the women. In the case of a folate-deficient woman, supplementation with folic acid resulted in a prompt increase in milk folate level. *Am J Clin Nutr* 1982:36:576–580.

KEY WORDS Folate, human milk, improved microbiological assay

Introduction

There is a great variation in the values reported for the folate content of human milk. Tamura et al (1) discussed this problem in a recent paper and the range for free folate was 0.3 to 62 ng/ml and for total folate 52 to 280 ng/ml. Part of this variation may be accounted for by the folate nutritional status of the nursing mothers (2–4) and stage of lactation (5–7). In addition, the method used to assay the milks may be an important factor. This is owing to the fact that the technique used in the studies to prepare the milks for assay was based on that for serum and blood. For bloods, this results in a clear, protein-free solution which then can be used in a microbiological assay where the endpoint is a turbidimetric reading of the bacterial growth (8). This is less satisfactory for the assay of milk since casein, unlike the blood proteins, is not coagulated during the process. The resulting opaque solution is not suitable for a turbidimetric assay.

An assay has been devised that overcomes this problem by using a rennin precipitation of the milk casein resulting in a clear, protein-free solution that retains the folate activity (9). We have used this method to determine the amount and nature of the folate in human milks obtained from nursing mothers at various stages of lactation.

Materials and methods

Subjects

Milk samples were obtained from 15 women who gave birth to infants in a large municipal hospital. Samples were obtained daily during the first 10 postpartum days and then once or twice a week for the duration of the nursing period. Milks were expressed manually usually before the first feeding. Casual samples were also obtained from nursing women who made office visits to one of the participating physicians. In each case, the nature of this study was explained and informed consent was obtained. None of the women received folate supplementation during the study or the last trimester of

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pregnancy. Samples were processed for assay (9) immediately and the finished samples stored at −20°C until assayed. Milks were classified according to the system of Macy and Kelly (10). Colostrum is milk obtained 1 to 5 days postpartum, transitional milk 6 to 15 days postpartum, and samples obtained after this period are mature milk.

Folate assay

The method used to prepare the milks using rennin precipitation of the casein is given in detail elsewhere (9). Folate levels were determined by a turbidimetric microbiological assay utilizing *Lactobacillus casei* (ATCC 7469) as the test organism (11). Each specimen was assayed before and after conjugase treatment. During each assay procedure, a sample of a standard yeast extract powder was treated similarly to the milks with a purified folate conjugase preparation (12) to ascertain the activity of the enzyme. Only 5% of the activity of the yeast extract was assayable by the *L casei* assay before treatment with the conjugase preparation.

In one case, folate deficiency was determined from urinary excretion of formiminoglutamic acid serum folate levels, and from megaloblastic changes in red and white blood cells by methods previously described (13).

Results

A total of 220 samples was analyzed for folate content before and after treatment with a folate conjugase preparation. These were classified as colostrum, transitional, and mature milk according to the scheme discussed previously. The results are presented in **Table 1**. In order to insure that these results were independent of sample-to-sample variations, the statistical significance was determined by analysis of variance of differences (14). In this method, the variation in the differences between the values obtained before and after enzyme treatment of the samples was tested for statistical significance and none was found for colostrum, transitional, and mature milks.

The data were plotted to show the variation of folate content with stage of lactation (**Fig 1**). Two observations are apparent. First, the folate content increases as lactation continues. Second, there is a great variation in folate content within each time group. For colostrum the mean was 15.2 μg/l (range 4 to 33.2); for transitional 16.6 (range 7.7 to 41.3); for 16 to 21 days, 28.2 (range 12.0 to 56.8); for 4 wk, 27.9 (range 8.8 to 46.5); for 5 wk, 41.0 (range 21.6 to 84.0); for 6 months, 68.6 (range 62.0 to 84.6).

A 29-yr-old patient of a physician participating in this study was a food faddist who consumed a diet consisting of cereal grains, vegetables, occasional chicken and milk, but no red meat or vitamin supplements during her pregnancy. Studies done a day before delivery revealed that the subject excreted 212 mg formiminoglutamic acid/24 h (normal <35) after a histidine load, had a serum folate level of 3.8 ng/ml (normal >7) and megaloblastic changes in both the red and white blood cells of the peripheral blood. She nursed her infant, and her milk contained between 7 and 9 μg of folate per liter during the first 7 days postpartum (**Fig 2**). She left the hospital 3 days after delivery and was convinced to take 5 mg folic acid orally on days 8, 9, and 10. One day after initiation of folic acid therapy, her milk folate rose to 15 μg/l and to 40 μg/l during the next day. These levels were maintained during the next 3 wk without further folic acid supplementation.

Discussion

During the early studies of the folate content of milks, it was noted that the turbidity of the milks prepared for assay made the use of a turbidimetric microbiological assay unfeasible so that an acidimetric method was used (15, 16). The low values obtained by these workers probably reflected the fact that ascorbic acid was not used to protect the heat labile folates. In one study (15) an attempt to

TABLE 1
Mean values for folate content of human milks collected at various stages of lactation and determined by microbiological assay before and after folate conjugase treatment*

Milk	n	Mean folate content		Difference of mean ± SEM	p value
		Before conjugase	After conjugase		
		ng/ml	*ng/ml*		
Colostrum	70	15.2	15.1	0.1 ± 1.1	> 0.10
Transitional	70	16.3	16.6	0.3 ± 1.0	> 0.10
Mature	80	33.4	26.2	7.2 ± 3.8	> 0.05

* The difference between the mean value for colostrum and transitional milks was not statistically significant ($p > 0.10$). The difference between the mean for transitional and mature milk was statistically significant ($p < 0.05$).

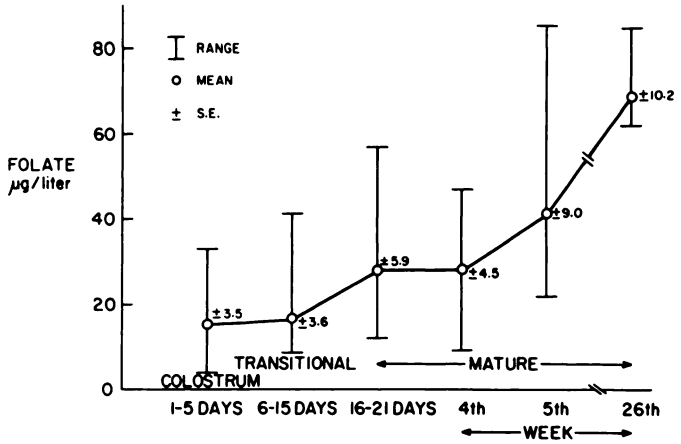

FIG. 1. Folate content of human milks at various stages of lactation showing mean, range, and SEM.

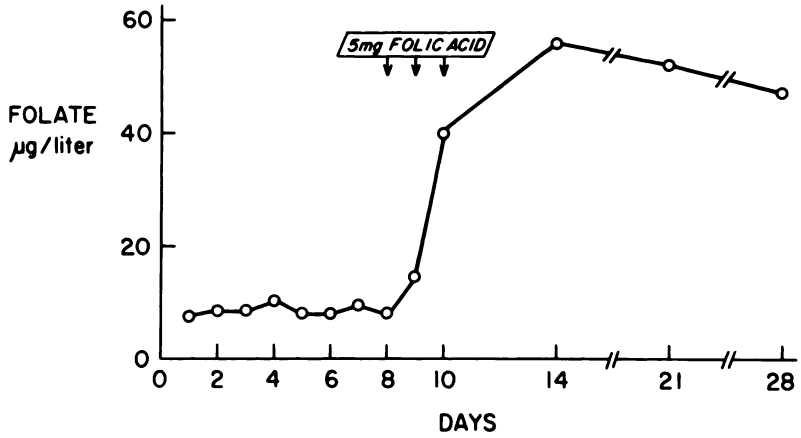

FIG. 2. The effect of folic acid supplementation on the milk folate level of a folic acid-deficient nursing mother.

precipitate the milk proteins at pH 4.5 resulted in considerable loss of added synthetic pteroylglutamic acid which would not be effected by the lack of inclusion of ascorbic acid.

Naiman and Oski (17) determined the folate content of cow's milk and infant forumlas using a turbidimetric *L casei* assay in which the basal medium contained ascorbic acid. They added the milks asceptically to previously sterilized basal medium, and included an uninoculated blank in an attempt to compensate for the turbidity. Subsequent investigators, with few exceptions, also used turbidimetric microbiological assays with buffered ascorbic acid used to protect the heat labile folates.

Matoth et al (2) prepared milks for assay by heating them in a buffered ascorbic acid solution similar to the method for blood folates (8). Highest results were obtained with the *L casei* assay which was labeled "total folates." Ramasastri (5) also treated human milks as blood before assay but added an additional step. After the samples were autoclaved in buffered ascorbic acid, he adjusted the pH to 4.5 to obtain a clear solution. If the solutions were still turbid, he used an acidimetric assay.

Karlin and coworkers (6, 7) added an additional step. After heating cow and human milk samples in a buffered ascorbic acid solution, they treated a portion of the solution with a chick pancreas solution containing folate conjugase and assayed samples before and after treatment. Ford and Scott (18)

heated cow's and human milk in a buffered ascorbic acid solution at pH 7.8 for 2 min in a boiling water bath, and treated the cooled solution with a chick pancreas extract, then acidified to pH 4.8, filtered, and then assayed the solution with a turbidimetric *L casei* assay. Hurdle et al (19) autoclaved cow's milk in a buffered ascorbic acid solution, and then added a chick pancreas extract to an aliquant and all solutions were assayed by a turbidimetric *L casei* assay. Subsequent investigators have used one of the previous methods with slight variations (20–25).

The method described herein overcomes the problem of turbidity by the use of rennin to precipitate casein, and by heating the solution in buffered ascorbic acid solution to precipitate lactalbumin and lactoglobulins. Although human milk has about half the casein content of cow's milk, this is still sufficient to result in turbid solutions if the milks are prepared for assay by the method used for blood.

In this study it was found that treatment of human milk with a folate conjugase preparation resulted in no further release of folate activity for *L casei*. This contrasts with the folate in cow's milk where about one-half the folate activity was released after conjugase treatment indicating a high level of folate polyglutamates (9). This signifies that the folate in human milk is primarily in the monoglutamate form. It is possible that some di- and triglutamates may also occur in human milk since Tamura et al (26) have shown that the latter have equal growth promoting properties for *L casei*. Shin et al (27) have shown by chromatographic separation that cow's milk contains 60% of its folate content as monoglutamate and the rest as polyglutamates.

Previous investigators found appreciable release of *L casei* active folate in human milk after conjugase treatment (1, 6, 7, 19). In view of the problems involved in assaying turbid milk solutions with a turbidimetric microbiological assay, comparisons with results in this study are difficult.

It is possible that the folate monoglutamates are more available for infants than polyglutamate forms. This may explain the fact that megaloblastic anemia owing to folic acid deficiency rarely occurs in breast-fed infants (28). In this regard it has been shown, for example, that synthetic folic acid is more readily available to adults than synthetic folic acid heptaglutamate (29–31).

We have confirmed the previous findings (5–7, 20) that the folate content of human colostrum is considerably lower than that in mature milk. Karlin and coworkers (6, 7) have shown that in the cow, colostrum is richer in folate than mature milk. At about 5 months postpartum the folate content of both cow's and human milk are almost equal and remain so during later periods of lactation.

There has been some question as to whether folate supplementation of nursing mothers can influence the folate content of their milk. In the well-nourished woman, such supplementation did not appear to have effected the folate content of the milk (1, 25). Supplementation of nursing mothers of low socioeconomic status resulted in a significant increase in milk folate levels (4). In the folate-deficient nursing mother we have confirmed the observation that folic acid therapy results in a rapid rise in milk folate levels (3).

The milk folate values at each state of lactation varied greatly among the women. Large variations in folate content were also found in the milk of well-nourished women by Tamura et al (1). We have made no attempt to correlate milk folate levels with folate status of the mothers since the purpose of the study was to apply an improved method to determine the content and nature of human milk folates. Only in the case of the folate-deficient woman, were such studies done.

It is apparent that the values for human milk will depend on the stage of lactation of the nursing mother and her folate nutritional status. It is thus important that listing of the folate content of milk of normal mothers give the stage of lactation of the milk.

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