Effects of Preparation and Cooking of Folic Acid-Fortified Foods on the Availability of Folic Acid in a Folate Depletion/Repletion Rat Model

K. O'Leary* and P. J. A. Sheehy

Nutritional Sciences, Department of Food Science, Food Technology, and Nutrition, University College, Cork, Republic of Ireland

The practice of food fortification with folic acid offers the potential to increase the folate intake of the general population. To fully exploit the potential of fortification for raising folate nutriture, appropriate food vehicles need to be selected. Selection should involve determination of the availability of folic acid as affected by characteristics of the carrier food, food matrix, food preparation, and cooking. The present study investigated the effects of preparation and cooking of a range of folic acid-fortified foods on the folate status of folate-deficient rats. Fifty-six weanling male rats (Wistar strain) were fed a folate-deficient diet containing 1% succinyl sulfathiazole for 28 days. Following depletion, six rats were randomly assigned to each of eight repletion diets containing cooked or uncooked meringue mix, quick bread mix, brownie mix, or pizza base mix. The test foods were fortified with 1400 μg of folic acid/kg of food and incorporated as 19% of the repletion diets. Each of the first four groups was pair-fed a diet containing a cooked fortified food with another group fed the corresponding uncooked fortified food. After a further 28 days, plasma, liver, and kidney folate concentrations were determined by microbiological assay. Mean plasma and liver folate concentrations of rats fed diets containing cooked fortified foods were similar to those of rats fed uncooked fortified foods. Preparation and cooking did not affect the availability of folic acid from the selected cereal-based convenience foods in this rat model system, suggesting that these foods are appropriate vehicles for fortification with folic acid.

Keywords: *Folic acid; fortified foods; rat folate status*

INTRODUCTION

Folic acid supplementation can reduce by a factor of two-thirds the incidence of neural tube defects (NTD) (*1*). Additionally, folic acid can lower plasma homocysteine concentrations (*2*−*6*), a known independent risk factor for cardiovascular disease (*2*), which suggests a possible role for folate in the prevention of heart disease. These findings have heightened interest in folic acid supplementation and adequate folate nutriture for the maintenance of optimal health, as well as the prevention of disease. Three approaches to increasing folate intake have been proposed, namely, increasing dietary folate intake by promoting the consumption of foods rich in folate; fortifying staple foods with folic acid; and advocating the use of folic acid supplements. The critical time for the development of NTD such as spina bifida and anencephaly is before closure of the neural tube, an event that occurs during the fourth week of gestation. It is essential, therefore, that folate be taken periconceptionally to adequately protect against such defects. Because many pregnancies are unplanned (*7*) and many sexually active women of childbearing age do not use effective contraception (*8*), the most effective strategy for increasing folate status is probably one that does not require behavior modification. Consequently, fortification of staple foods with folic acid is more likely to reach a greater proportion of the target population than either of the other two proposed strategies. In response to this, the U.S. government decided to mandate the fortification of enriched cereal grain products with 1.4 μg of folic acid/g of grain. On the basis of the assumption that folic acid added to foods is as bioavailable as that from supplements, this level of fortification would theoretically provide the average woman of reproductive age with an additional 100 μg of folic acid each day. In many other countries, meanwhile, regulations regarding folic acid fortification have yet to be put in place and in these countries an ever-increasing number of foods fortified with various levels of folic acid are appearing in the marketplace. Naturally occurring food folates consist of a mixture of reduced folate polyglutamates, which are chemically extremely unstable (*9*). Several studies have indicated that there are considerable losses of folates during the storage, cooking, and processing of foodstuffs (*10*−*15*). Leichter (*16*) investigated the effect of cooking on the folate content of vegetables and concluded that losses during boiling were primarily as a result of leaching of the folate into the cooking water rather than as a result of actual destruction of the vitamin. Other investigators have also reported losses of food folates as a result of heat processing. Lin et al. (*17*) observed folate losses of 30% following heat treatment of canned garbanzo beans at 118 °C for 53 min. Augustin (*18*) reported total losses similar to those of Lin et al. (*17*) during the boiling of a variety of legume species for up to 1 h. Malin (*19*) examined the effects of cooking, processing, and storage on the total folate activity of Brussels sprouts and found relatively small

* Author to whom correspondence should be addressed (e-mail karencrotty@hotmail.com; telephone 353-21-4903126; fax 353-21-4270244).

10.1021/jf010204e CCC: $20.00
Published on Web 08/24/2001

losses after water blanching. In contrast to the chemically unstable food folates, synthetic folic acid (the form used in supplements and also to fortify foods) is almost completely stable for months or even years (*9*, *20*). However, the possible influence of food components, food preparation, and cooking may be important in determining the bioavailability of folic acid from fortified foods. Early human studies concerning the bioavailability of folic acid-fortified foods suggested that there was considerably lower bioavailability of folic acid from fortified cereal grain products than from folic acid in its free form. Colman et al. (*21*) and Margo et al. (*22*) found that the absorption of folic acid added to maize, rice, and bread was only 30−60% of the absorption of folic acid in solution. More recently, evidence from human and animal studies suggests that folic acid-fortified foods are as effective in increasing folate status as are supplements (*3*, *23*−*25*). Nevertheless, to fully exploit the potential of fortification for raising folate nutriture, appropriate food groups suitable for fortification with folic acid need to be selected. The selection process should involve, among other things, determining the availability of folic acid as affected by characteristics of the carrier food, food matrix, food preparation, and cooking.

The objective of the present study was to investigate the effects of preparation and cooking of a range of folic acid-fortified foods on the folate status of folate-deficient rats. Rats were depleted of folate for 28 days and subsequently repleted with a range of cooked and uncooked folic acid-fortified foods incorporated into a basal folate-free diet for a further 28 days. Folate status was determined by measuring plasma, liver, and kidney folate concentrations.

MATERIALS AND METHODS

Depletion Phase. This experiment was carried out under license from the Department of Health and Children, Dublin, Ireland. Sixty-one weanling male rats (Wistar strain) were obtained from the Biological Services Unit, University College, Cork, Ireland. Throughout the experiment, rats were housed individually in wire-bottom stainless steel cages in an environmentally controlled room with a 12 h light/12 h dark cycle at 21 ± 1 °C. Rats were randomly divided into two groups of equal mean weight. One group of 56 rats was depleted of folate for 28 days by feeding a folate-free diet containing 1% succinylsulfathiazole (*26*). The other group of 5 rats was fed the same diet (also containing 1% succinylsulfathiazole), supplemented with 200 μg of folic acid/kg of diet. Rats were given free access to food and water, weighed weekly, and examined daily for general condition and symptoms associated with folate deficiency. At the end of this 28 day depletion phase, the group of 5 folate-supplemented rats and 5 of the group of 56 folate-depleted rats were selected at random, fasted overnight, and their body weights recorded. Rats were anesthetized with diethyl ether and bled to death by cardiac puncture. The blood was transferred into heparinized tubes and the plasma separated by centrifugation (1000*g*). The plasma was transferred to 1.5 mL polypropylene tubes and stored at −20 °C. The liver and kidneys were excised, immediately frozen in liquid nitrogen to preserve labile folates, and subsequently stored at −20 °C.

Diet Preparation. Eight repletion diets were prepared. Four test foods (meringue mix, quick bread mix, brownie mix, and pizza base mix) were purchased and subsequently fortified with 1.4 μg of folic acid/g of food (i.e., 1400 μg/kg). Following fortification, each food was divided in two and half was prepared and cooked according to the instructions on the product label (Table 1). The cooked portions were then weighed, and cellulose was added to the uncooked portions to adjust for changes in weight as a result of the various preparation procedures. The amounts of cellulose added to the uncooked brownie mix, pizza base mix, quick bread mix, and meringue mix were 36, 53, 57, and 3 g/kg, respectively. The repletion diets were then prepared by mixing 10 g/kg of succinylsulfathiazole, 190 g/kg of the test foods, and 800 g/kg of the basal folate-free diet. The folic acid contents of the repletion diets are shown in Table 3. An inclusion level of 19% was considered sufficient to supply the rats with folate without unduly distorting the basal diet. The folate content of the depletion diet was determined by trienzyme extraction (*27*), followed by microbiological assay (*28*, *29*).

Table 1. Cooking Instructions for Test Foods

food type	cooking method	temp, °C	time, min
meringue mix	oven	110	120
quick bread mix	oven	200	40
pizza base mix	oven	220	20
brownie mix	microwave	800 W (full power)	7

Repletion Phase. Following depletion, 48 of the folate-depleted rats were systematically divided into 8 groups of 6. Each of the first four groups was pair-fed a diet containing a cooked fortified food with another group fed the corresponding uncooked fortified food. After 28 days of repletion, rats were killed and blood and tissue samples were taken as previously described.

Extraction of Folate from Tissues. Frozen liver/kidney (∼0.5 g) was added to 9 volumes of 50 mM phosphate buffer (pH 6.1), homogenized for 1 min, autoclaved for 10 min at 121 °C, mixed, and cooled in an ice bath. Chilled homogenates were centrifuged for 15 min at 2000*g*. The clear supernatant was transferred to plastic vials and stored at −20 °C.

Deconjugation of Folate Extracts. The frozen extracts of livers and kidneys were thawed, and 250 μL aliquots were transferred to tubes containing 4.33 mL of phosphate buffer (50 mM, pH 6.1), 200 μL of hog kidney conjugase preparation (*30*), and 100 μL of mercaptoethanol solution (1 mL of 2-mercaptoethanol commercial preparation obtained from Merck, Munich, Germany/10 mL of phosphate buffer) and mixed. The mixture was incubated in a shaking water bath for 6 h to allow folyl polyglutamates to be converted to monoglutamates and stored at −20 °C until required for folate analysis.

Folate Analysis of Plasma and Tissues. The folate contents of plasma and conjugase-treated liver and kidney extracts were analyzed by microbiological assay (*28*, *29*).

Statistical Analysis. Plasma, liver, and kidney folate concentrations were analyzed by analysis of variance, and when significant differences were found ($P < 0.05$) within the data, multiple comparisons between means were carried out using Duncan's new multiple-range test (SPSS 8.0).

RESULTS AND DISCUSSION

The first phase of this experiment involved the induction of moderate folate deficiency by feeding rats a folate-free diet containing 1% succinylsulfathiazole (*26*) for 28 days. During this time food consumption of rats fed the folate-deficient diet was similar to that of rats fed the control diet. Both groups continued to gain weight, and there was no significant difference in body weights at the end of the depletion phase. Following depletion and in agreement with previous investigators (*30*−*35*), plasma and liver folate were found to be sensitive indicators of folate status. Rats fed the folate-deficient diet had significantly lower ($P < 0.05$) plasma and liver folate concentrations at the end of the depletion phase than did controls, with mean plasma and liver folate concentrations of the folate-depleted rats being only 50 and 59%, respectively, of those of rats fed

Table 2. Weight Gain and Plasma, Liver, and Kidney Folate Concentrations of Rats Fed either a Folate-Deficient or Control Diet during a 28 Day Depletion Period[a]

measure	folate-deficient diet[b]	control diet[c]
wt gain, g/week	39.3 ± 1.95	37.9 ± 1.40
final wt, g	200 ± 9.13	188 ± 4.09
plasma folate, ng/mL	16.3 ± 1.41[d]	32.6 ± 4.17
liver folate, μg/g	3.74 ± 0.11[d]	6.37 ± 0.39
kidney folate, μg/g	6.63 ± 0.41	6.12 ± 0.22

[a] Values are means ± SEM for five rats. [b] <5 μg of folate/kg of diet. [c] 200 μg of folate/kg of diet. [d] Statistically significant differences between dietary treatment groups at the end of the 28 day period ($P < 0.05$) (ANOVA).

the control diet supplemented with 200 μg of folic acid/kg of diet (Table 2). The mean plasma folate concentrations of depleted rats in this study (16.3 ± 1.41 ng/mL) were much higher than those observed in previous studies by other authors (*36*, *37*). Liver folate concentrations of depleted rats (3.74 ± 0.11 μg/g) were in good agreement with those of Martinez and Roe (*36*) (4.1 μg/g) and Swiatlo et al. (*37*) (5.7 μg/g) but were considerably higher than those reported by Clifford et al. (*38*) (0.73 μg/g), Miller et al. (*35*) (0.23 μg/g), Martin (*30*) (0.14 μg/g), and O'Leary and Sheehy (*25*) (0.45 μg/g). Initially we thought the higher plasma and liver folate concentrations were a result of endogenous folate present in the basal folate-free diet. However, trienzyme extraction (*27*), followed by microbiological assay (*28*, *29*), revealed that the folate content of the depletion diet was <5 μg/kg. It is possible that the folate status of these rats on arrival at our laboratory may have been higher than was the case with the rats used in the other studies cited. Despite this, it is clear that both plasma and liver folate concentrations successfully distinguished between folate-depleted and folate-replete rats, and these were therefore considered appropriate response variables for measuring changes in folate status during the repletion phase.

Surprisingly, kidney folate concentrations were unable to distinguish between folate-depleted and -replete rats (Table 2). We previously found kidney folate to be a useful indicator of folate status in rats (*25*) as did other investigators (*30*, *37*). However, both groups noted that kidney folate was less sensitive than serum folate as an indicator of folate status. Consequently, we considered kidney folate to be a poorer measure of folate status for the purpose of this experiment.

The objective of the repletion phase was to investigate the effects of preparation and cooking of selected folic acid-fortified foods incorporated into a basal folate-free diet on plasma, liver, and kidney folate concentrations of folate-depleted rats. The results are given in Table 3. Mean plasma and liver folate concentrations of rats fed diets containing the cooked fortified foods (meringue, quick bread, brownies, and pizza base) were similar to those of rats given the uncooked fortified products. Mean kidney folate concentrations of rats fed the cooked brownie-containing diet were also similar to those of rats fed the uncooked counterpart. However, kidney folate concentrations of rats fed the other cooked products were significantly higher ($P < 0.05$) than those of rats fed the uncooked products.

The foods chosen for this experiment are typical of the kinds of prepacked convenience baking foods currently available in the marketplace and come with a variety of preparation and cooking instructions. It should be noted that some endogenous folate may have been present in the foods, but this was not measured because our objective was simply to study the effect of preparation and cooking on folate availability and any endogenous folate would have been equally distributed in the cooked and uncooked portions. The heat stability of folic acid has been studied under various conditions. Colman et al. (*21*) found it to be stable to boiling in aqueous solution for up to 2 h. Keagy et al. (*39*) found that baking caused an average loss of folic acid of only 11% in bread fortified with 5 μg of folic acid/g of flour. Additionally, Cooper et al. (*40*) observed folic acid to be stable when heated at 100 °C for 65 min in a neutral solution, and Ristow et al. (*41*) concluded that thermal processing had little or no effect on the bioavailability of folic acid in liquid model systems. In agreement with these observations, Table 3 clearly demonstrates that the preparation and cooking techniques employed in this study did not affect the availability of folic acid from selected folic acid-fortified foods; rats fed diets containing cooked meringue mix, quick bread mix, brownie mix, and pizza base mix had plasma and liver folate concentrations similar to those of rats given the uncooked products. This suggests that for the fortified foods selected, folic acid remains stable over a broad range of cooking temperatures and times.

The kidney folate concentrations of rats following repletion (Table 3) would give the impression that folic acid from all but one of the fortified foods is more available after preparation and cooking than it is in the uncooked product. However, because kidney folate concentrations were unable to distinguish between folate-depleted and-replete animals at the end of the depletion phase of the experiment, it is likely that this conclusion is unwarranted.

Table 3. Plasma, Liver, and Kidney Folate Concentrations of Folate-Depleted Rats Fed Diets Containing a Variety of Cooked and Uncooked Folic Acid-Fortified Foods[a]

treatment	folic acid content of repletion diets, μg/kg	plasma folate, ng/mL	liver folate, μg/g	kidney folate, μg/g
meringues (uncooked)	265	21.9 ± 2.19	13.0 ± 0.78	20.5 ± 1.21[b]
meringues (cooked)	265	25.3 ± 0.75	14.2 ± 1.21	28.2 ± 3.54
quick bread (uncooked)	252	28.2 ± 1.75	14.5 ± 0.48	18.6 ± 0.73[b]
quick bread (cooked)	252	33.7 ± 4.06	16.3 ± 1.44	24.1 ± 1.99
brownies (uncooked)	257	27.0 ± 1.61	13.7 ± 0.62	19.1 ± 0.78
brownies (cooked)	257	24.8 ± 0.84	13.2 ± 0.48	21.9 ± 1.25
pizza base (uncooked)	253	37.9 ± 1.20	17.4 ± 0.86	23.1 ± 1.16[b]
pizza base (cooked)	253	35.8 ± 0.64	17.5 ± 1.52	28.6 ± 2.07

[a] Values are means ± SEM for six rats. [b] Statistically significant differences between cooked and uncooked dietary treatments ($P < 0.05$) (ANOVA).

Table 3 also shows that rats fed diets containing an uncooked brownie mix had plasma and liver folate concentrations similar to those of rats fed diets containing a brownie mix cooked by microwave heating. Cooper et al. (*40*) also found microwave heating to have little effect on the stability of folic acid. On the other hand, Augustin et al. (*42*) found variable results with regard to folate retention when comparing microwave and conventionally reheated potatoes.

In conclusion, there is increasing evidence of the positive effect that food fortification with folic acid has on folate status. It is not, however, enough to simply recognize the ability of folic acid-fortified foods to raise the folate status of the population. If we are to adequately protect against NTD and cardiovascular disease by increasing folate intake, and at the same time avoid exposing some segments of the population to the possible adverse effects of overconsumption of the vitamin, we need to be careful in our choice of food vehicles for fortification. We also need to be able to accurately quantify the contribution that such fortified foods make to the overall folate supply of the diet. This will require information on the effects of processing, storage, preparation, and cooking methods on folate bioavailability from fortified foods. The present study shows that preparation and cooking did not affect the availability of folic acid from selected cereal-based convenience foods in a male rat model system. Although caution must be exercised in excluding the possibility of sex differences and in extrapolating the results to humans, our data suggest that these foods are appropriate vehicles for fortification with folic acid.

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Received for review February 15, 2001. Revised manuscript received July 3, 2001. Accepted July 3, 2001.

JF010204E