

The Enrichment of Eggs with Folic Acid through Supplementation of the Laying Hen Diet

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ABSTRACT In light of evidence supporting a need for humans to increase their dietary folate intakes, experiments were conducted to evaluate the extent to which egg folate levels could be increased. In Study 1, Hyline W36 hens ($n = 6/\text{diet}$) received a barley-based diet, containing 0 or 10 mg/kg of crystalline folic acid, to establish the potential for folate incorporation into table eggs. In Study 2, 70 hens were divided into seven treatment groups ($n = 10 \text{ hens/diet}$) and received diets supplemented with 0, 1, 2, 4, 8, 16, or 32 mg folic acid/kg diet. In Study 3, 64 hens received the barley-based diet with or without 4 mg folic acid/kg diet. Eggs were collected and stored for 0, 7, 14, 21, or 28 d, prior to folate determinations. The folate content of eggs was determined by

HPLC for 5-methyltetrahydrofolate (the sole form of folate in egg yolk). Results from Study 1 showed that a 10 mg/kg inclusion of folic acid increased folate incorporation into egg yolk ($41.0 \pm 0.7 \mu\text{g/egg}$) over that of an unsupplemented diet ($17.5 \pm 0.7 \mu\text{g/egg}$; $P = 0.0001$). In Study 2, the response of egg folate to dietary folic acid supplementation was saturable, with 90% of maximal egg folate levels established at approximately 4 mg folic acid/kg diet. Results from Study 3 showed that folate levels are stable, in control and fortified eggs, during 28 d of storage at 4 C. In terms of its nutritional value, one large egg collected from a folic acid-supplemented hen provided approximately 12.5% of the recommended dietary allowance (RDA) for adult humans (RDA = 400 mg/d).

(Key words: folate, egg, fortification, laying hen)

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INTRODUCTION

There has been growing awareness of the need for increased consumption of folate by humans. Increased periconceptional intake of this vitamin by women has been shown to reduce the occurrence (Czeizel and Dudas, 1992) and recurrence (Medical Research Council, 1991) of neural tube defects, such as spina bifida, in children. As well, poor to marginal folate status is linked to increased serum levels of the sulfur amino acid homocysteine, due to the role that folate plays as a cofactor in the remethylation of homocysteine to form methionine (House et al., 1999). An increase in the serum level of homocysteine has been shown to be an independent risk factor for the development of cardiovascular disease (Boushey et al., 1995). Therefore, it is critical that efforts be made to ensure adequate intake of this vitamin by humans.

The term folate encompasses a number of different water-soluble compounds; each based on the structure of folic acid, or pteroylmonoglutamate, but differing in oxidation state and number of additional glutamate resi-

dues (Selhub and Rosenberg, 1996). Folic acid does not occur naturally, in appreciable amounts, in foods. However, due to its stability and commercial availability, it is the form that is used in vitamin supplements, fortified foods, and vitamin premixes. In 1998, the US and Canadian governments enacted legislation requiring that cereal products be fortified with folic acid at 140 $\mu\text{g}/100 \text{ g}$. The effectiveness of this strategy to increase serum folate levels in the general population has yet to be fully assessed, although preliminary evidence does support an improvement of folate status as a result of this policy change (Selhub et al., 2000). However, additional strategies may be necessary in order to ensure that all segments of the population are consuming adequate folate, including the use of supplements in specific target groups (women of child-bearing age) as well as educating consumers to eat foods that are rich in folate. Eggs naturally contain folate at approximately 22 μg folate per large egg (USDA, 2001), which is equivalent to 6% of the newly established adult daily requirements for folate (Institute of Medicine, 1998). Increasing the folate content of eggs may position the egg as an important source of dietary folate and lead to an improvement in consumer acceptance of this commodity as a healthful product.

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Abbreviation Key: DFE = dietary folate equivalent; RDA = recommended dietary allowance.

TABLE 1. Composition of the basal barley-based laying hen ration

Ingredient	%
Barley (10 % CP)	53.1
Soybean meal (46.1 % CP)	16.8
Canola meal (33.8% CP)	10
Fish meal (72% CP)	2
Tallow	7.1
Limestone	8.7
Biophos (monocalcium phosphate)	0.8
Vitamin premix ¹	1
Mineral premix ²	0.5
Calculated nutrient composition	
Crude protein	18.0
Metabolizable energy, kcal/kg	2,700
Calcium	3.75
Phosphorus (total)	0.4
Folate, mg/kg	0.49

¹Provided (per kg of diet): vitamin A, 8225 IU; vitamin B₁₂, 11.2 µg; calcium panthothenate, 4.4 mg; choline chloride, 110 mg; vitamin D₃, 1000 IU; vitamin E, 5.46 IU; ethoxyquin, 125 mg; dl-methionine, 500 mg; niacin, 7.6 mg; riboflavin, 2.2 mg.

²Provided (per kg of diet): MnO, 165 mg; ZnO, 55 mg; salt (iodized), 4.78 g.

The objectives of the current studies included determination of the potential for folate-fortification of eggs from hens receiving barley-based diets. With the first objective realized, subsequent experiments were conducted to determine the optimal level of dietary folate supplementation for maximal egg folate content and to determine the stability of egg folate during storage.

MATERIALS AND METHODS

General

Hyline W36 laying hens, 25 to 28 wk old at peak levels of production, were used in all experiments. Hens were kept in confinement housing under semicontrolled environmental conditions and exposed to a 16-h photoperiod. When birds were housed individually (Experiments 1 and 2), the cage dimensions were 25.4 cm by 40.64 cm, providing 1,032 cm² per bird. When birds were housed in groups of four (Experiment 3), the cage dimensions were 40.64 cm by 40.64 cm, providing 413 cm² per bird. Feed and water were available ad libitum. Animal care approval was received from our institute's Senate Committee on Animal Care, in accordance with recommendations established by the Canadian Council on Animal Care (1984).

Diets

In all experiments, the basal diet was a barley-based ration, formulated to meet the requirements of laying hens consuming 100 g of feed/d (NRC, 1994). The composition of the basal diet is presented in Table 1. The basal diet included no crystalline folic acid, a practice consistent with industry standards (BASF, 2000)

Experiment 1

In order to examine the potential for eggs to be fortified with folic acid, 12 hens were divided equally between two treatment groups: 1) basal diet (n = 6) with no supplemental folic acid and 2) basal diet + 10 mg/kg crystalline folic acid (n = 6). Birds were individually housed and were fed the diet for 14 d before the period of egg collection began. Feed intakes were recorded throughout the study. Eggs were collected twice daily (morning and afternoon) for 7 d, weighed, and immediately processed for determination of egg folate content.

Experiment 2

In order to establish a dose-response relationship between dietary and egg folate levels, 70 hens (n = 10 per level) were assigned to receive one of seven dietary folic acid levels. The dietary treatments consisted of the basal diet plus 0, 1, 2, 4, 8, 16, or 32 mg crystalline folic acid/kg diet. For 2 wk before the start of the study, 90 healthy hens of uniform age and production were monitored for egg production, and the 70 highest-producing hens were selected for the experiment. Hens were placed individually into battery cages at 26 wk of age. Feeding of the treatment diets began 14 d before the egg collection period started and continued during the 5-d collection period. Feed consumption and productivity were recorded throughout the study. After collection, eggs were weighed and processed for egg folate determinations.

Experiment 3

In order to determine the stability of folate in eggs during storage, 64 hens (n = 32 per treatment) were assigned to receive one of two dietary folic acid levels: the basal diet (0 mg folic acid/kg) or 4 mg crystalline folic acid/kg diet, the latter level being set at a level yielding 90% maximal egg folate concentrations from the regression equation generated in Experiment 2. Hens were housed four per cage, with feed and water available ad libitum, as described above. Feeding of the treatment diets began 14 d before the egg collection period started. Fifteen eggs were collected per treatment per day during the 5-d collection period. On each collection day, three eggs per treatment were processed immediately for egg folate determinations (0 storage) or were stored at 4 C for 7, 14, 21, or 28 d. After the designed storage period, eggs were weighed and processed for egg folate determinations. Eggs collected over and above the requirement for the storage study were pooled and processed for future animal feeding trials.

Extraction of Egg Yolk Folate

All chemicals used in the extraction and analysis of folates were purchased from Sigma Chemical Co.² Eggs were weighed and then hard-boiled for 10 min. Immediately after boiling, eggs were immersed in chilled water.

²Oakville, ON, Canada.

Once cooled, the yolks were removed, weighed, and lyophilized, and the dry weights were noted. Dried yolks were stored at -20°C until analyzed. With respect to egg folate content, previous studies have clearly shown that virtually all of the folate found in eggs is 1) limited to the yolk fraction (Sherwood et al., 1993), 2) present as 5-methyltetrahydrofolate (Seyoum and Selhub, 1998), and 3) present as the monoglutamate (Seyoum and Selhub, 1998). Our initial investigations support these findings. As a result, additional steps were not required to deconjugate glutamate residues or to determine multiple species forms for folate. Approximately 0.5 g of dried yolk were weighed into glass tubes with lids. Ten milliliters of an extraction buffer (20 g/L sodium ascorbate; 12.1 g/L Trizma base; pH 7.8) was added to each tube, and the tubes were topped with N_2 gas, vortexed, and placed in a boiling water bath for 60 min. After boiling, the tubes were centrifuged at $4,000 \times g$ for 30 min. The supernatant from each tube was decanted and retained. An additional 10 mL of extraction buffer was added to each tube, and the tubes were vortexed and centrifuged as before. The supernatants were pooled, and the final volume brought to 25 mL. A sample from each flask was placed into microcentrifuge tubes and frozen at -20°C until analyzed.

Analysis of Egg Yolk Folate Content

The concentration of 5-methyltetrahydrofolate in egg yolk extracts was determined by reverse-phase HPLC with fluorescence detection,³ as previously described (Vahteristo et al., 1997). An external standard curve with purified 5-methyltetrahydrofolate was used to quantify egg folate concentrations. The inter- and intra-assay CV for determinations was $<3\%$, and recovery of 5-methyltetrahydrofolate added to dried egg yolk was 98.9%. The folate content was expressed as micrograms of folic acid per egg.

Statistical Analysis

The experiments consisted of completely randomized designs with main effects partitioned between treatments and collection day. Data were subjected to ANOVA, using the PROC GLM function of SAS software (SAS Institute Inc., 1988). Differences between means were assessed using the protected-LSD method. Level of significance was set at an α -level of $P < 0.05$. Data for the dose-response relationship (Experiment 2) were fitted to a three-parameter, single rectangular hyperbola, with the following equation: $y = y_0 + (ax/b+x)$ (SPSS, Inc., 2000).

RESULTS

Experiment 1

In this preliminary trial, the supplementation of barley-based diets with 10 mg crystalline folic acid resulted in

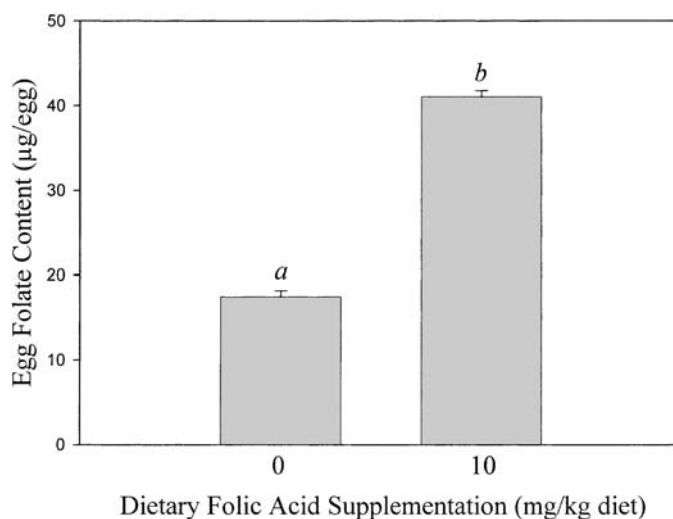


FIGURE 1. Effects of dietary folic acid supplementation to barley-based laying hen rations on the content of folate in eggs. Data ($n = 6$ hens/diet) are expressed as means plus standard errors. ^{a,b}Values with different letters are significantly different ($P < 0.05$) by protected least squares difference.

an increased folate content of eggs by a factor of 2.4 ($P < 0.05$; Figure 1). Examination of the main effects of the ANOVA revealed a significant effect of both treatment and day. When averaged across treatments, egg folate content exhibited significant variation due to collection day; however, there was no significant interaction.

Experiment 2

Percentage egg production averaged across treatments was 94%. The dose-response relationship between dietary folic acid levels and egg folate content is shown in Figure 2. Consistent with the observations from Experiment 1,

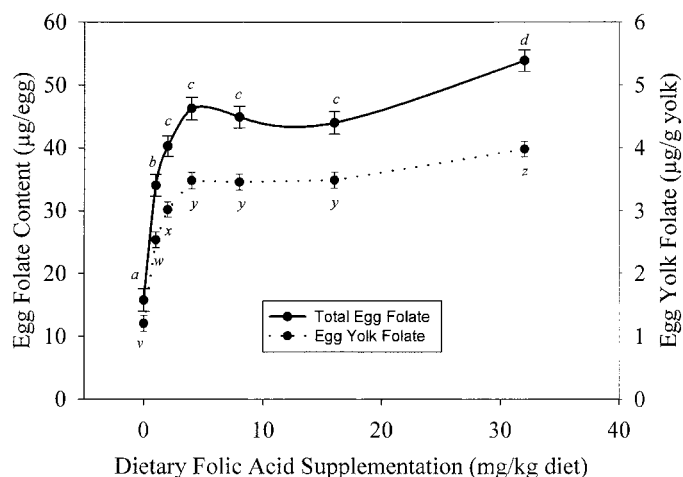


FIGURE 2. Dose-response relationship between dietary folic acid supplementation and the folate content of eggs, total or per gram of egg yolk, from hens receiving barley-based diets. Data ($n = 10$ hens/diet) are expressed as least squares means and standard errors. ^{a-d; v-}Values within a line with different letters are significantly different ($P < 0.05$) by protected least squares difference.

³Shimadzu, Mantech, Guelph, ON, Canada.

the supplementation of crystalline folic acid to barley-based laying hen diets increased the folate content of eggs, with the most sensitive responses observed between 0 and 2 mg crystalline folic acid/kg diet. With diet supplementation between 2 and 16 mg folic acid/kg, egg folate levels were not significantly different, but supplementation of diets with 32 mg folic acid/kg led to a further increase in egg folate content ($P < 0.05$). Average daily feed consumption was significantly higher for birds consuming the diets fortified with 32 mg/kg folic acid (106 ± 1.6 g/d) when compared to those consuming diets containing folic acid at 4 mg/kg (98 ± 1.6 g/d), 8 mg/kg (97 ± 1.6 g/d) or 16 mg/kg (93 ± 1.6 g/d), but not when compared to birds eating folic acid at 0 mg/kg (102 ± 1.6 g/d), 1 mg/kg (103 ± 1.6 g/d) or 2 mg/kg (102 ± 1.6 g/d) mg of diet). Consistent with the observed reductions in feed intake, birds consuming diets containing 8 and 16 mg folic acid/kg produced eggs weighing 53.3 ± 0.5 and 52.6 ± 0.5 g. These values were significantly less ($P < 0.05$) than those observed for birds consuming the diets containing 0 to 4, and 32 mg folic acid/kg (55.0, 55.8, 55.4, 55.5, & 55.7 g; SEM = 0.5 g). Expression of egg folate concentrations on a per gram of egg yolk basis led to a similar pattern as that observed to the values expressed on a per egg basis (Figure 2), with folate concentrations being highest ($P < 0.05$) at dietary folate levels of 32 mg/kg diet.

In order to aid in the definition of an optimal dietary folate content, the data for egg folate content, for dietary folic acid contents of 0 to 16 mg/kg, were fitted to a three-parameter, single rectangular hyperbola, with the following equation: $y = y_0 + (ax/b+x)$. The resultant parameters were (μg folate/egg; SEM) $y_0 = 16.76 \pm 1.71$; $a = 30.59 \pm 2.20$; $b = 0.64 \pm 0.18$; $r^2 = 0.44$; $P < 0.05$. The data for dietary folate concentrations of 32 mg/kg were excluded from this regression analysis, due to the fact that the resulting egg folate concentrations were significantly higher than preceding values, which were presumed to be at plateau. Based on the regression analysis, the maximum value obtainable for egg folate concentrations was $47.4 \mu\text{g}/\text{egg}$. The level of dietary folic acid addition required to yield a conservative value of 90% maximal egg folate concentrations was $3.6 \mu\text{g}/\text{kg}$, a value close to the tested $4 \mu\text{g}/\text{kg}$ level. This level was chosen for the subsequent storage study.

Experiment 3

The effect of duration of storage at 4 C on egg folate stability is shown in Figure 3. Examination of the main effects of the ANOVA indicated a significant ($P < 0.05$) effect of treatment on egg folate concentrations, consistent with previous experiments; however, the effects of duration time ($P = 0.82$) and treatment by time interaction ($P = 0.17$) were not significant.

DISCUSSION

The present studies provide strong evidence of the sensitivity of egg folate concentrations to dietary folate levels.

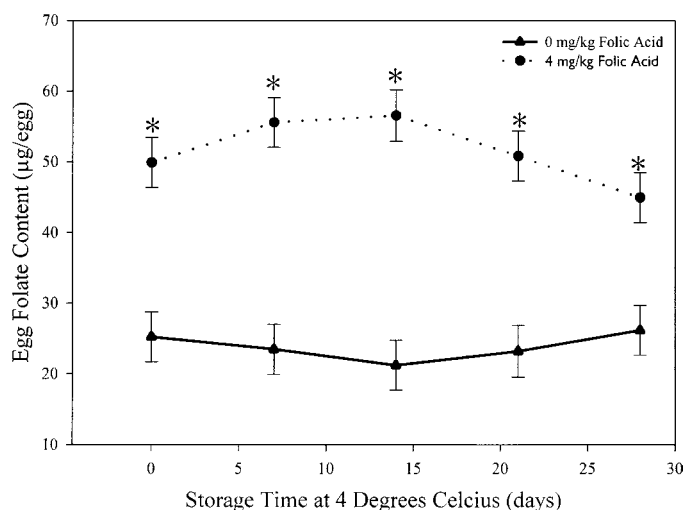


FIGURE 3. The effect of duration of storage at 4 C on the folate content of eggs collected from hens fed a barley-based diet with or without 4 mg crystalline folic acid/kg. Data ($n = 15$ eggs per diet per storage period) are expressed as least squares means and standard errors. Solid and dotted lines represent the 0 and 4 mg crystalline folic acid/kg diet, respectively. An asterisk denotes values that are significantly different ($P < 0.05$), as assessed by protected least squares difference, from corresponding time point in the diet containing no crystalline folic acid.

By adding crystalline folic acid to cereal-based laying hen diets, it is possible to increase the folate content of eggs by two- to four-fold. A linear increase in egg folate levels was observed when crystalline folic acid was added from 0 to 2 mg/kg to the laying hen diet, after which egg folate levels appeared to reach a plateau. Additions of folic acid above 2 mg/kg diet yielded no further significant increases in egg folate content until dietary concentrations reached 32 mg/kg. The shape of the dose-response curve suggests a saturable process, from 0 to 16 mg folic acid/kg inclusion rate. As a result of the significantly higher egg folate concentration present with 32 mg of dietary folic acid/kg of diet, it is tempting to speculate that a biphasic response pattern is present. Above a critical inclusion level for dietary folate, folate accumulation in the egg yolk may surpass the saturable processes via another mechanism (i.e., noncarrier-mediated transport). However, the current data do not permit the definitive demonstration of a biphasic response, due to a lack of more data points beyond 32 mg/kg. Studies designed with higher folic acid inclusion rates are required to more accurately characterize the response in egg folate concentrations in this region of the curve.

The data from the present studies are in general agreement with the work of Sherwood et al. (1993), who examined the relationship between dietary folate and plasma and egg yolk folate concentrations, using a radioisotope dilution assay. They examined the impact of added dietary folic acid to cereal-based diets and purified diets and established a dose-response relationship using data pooled from several experiments. These authors stated that saturation of egg folate content is not due to limitations in transport processes from plasma to the egg

yolk, as plasma and yolk folates are proportionate across the range of dietary folic acid levels investigated (0 to 7 mg/kg). They concluded that regulatory processes controlling plasma folate levels might be the point of metabolic control. Through the use of brush-border membrane vesicles derived from rat jejunum, folate transport has been shown to be a saturable process (Said et al., 2000). Therefore, intestinal folate uptake may play a role in the regulation of egg yolk folate concentrations.

The folate in the control and fortified eggs was stable during storage at 4 C for 4 wk. This period corresponds with the recommended shelf life of table eggs. The current data are important as the primary form of folate in eggs, 5-methyltetrahydrofolate, is sensitive to oxidation (Luccock et al., 1995). Considerable interest has been raised pertaining to the potential development of oxidation products of cholesterol as a result of storage or processing. Although some researchers have not found cholesterol oxidation products in fresh egg yolk (Tsai and Hudson, 1984)), others have (van de Bovenkamp et al., 1988). The discrepancies, however, may be more reflective of sensitivity of analytical techniques. Yang and Chen (2001) demonstrated the presence of measurable oxidation products in fresh yolk (measured as TBA-reactive substances), but these levels were greatly enhanced by pickling or long-term storage. Despite the potential for oxidation to occur in yolk compounds, the current data suggest that the folate in egg yolk is stable and thus not oxidized during refrigerated storage for 4 wk.

In the present experiments, the addition of supplemental folic acid to laying hen diets did not impact the performance of the birds, as reflected by no significant differences in egg production or egg weights. There was a marginal, but significant, effect of folic acid, especially at higher levels, on feed intake, but this finding did not translate into differences in egg production. The present experiments, however, were not primarily designed with the intent to measure the impact of folic acid on laying hen productivity. The current estimated requirement for folic acid for laying hens, based on experiments conducted in the 1950s using productivity as an endpoint, is between 0.21 to 0.31 mg/kg (NRC, 1994), a level of folic acid, well below the calculated level of folate in the unsupplemented diet (0.49 mg/kg). It is the lack of demonstrable impacts on performance that has generally limited the inclusion of crystalline folic acid in layer diets, a fact supported by recent surveys of commercial feed mills (BASF, 2000). Considerations beyond traditional measures of productivity and egg quality are needed to reflect the growing awareness and concerns that consumers have regarding the nutritional quality of the foods they are selecting.

Per capita annual egg consumption in Canada declined from a high of 25.0 dozen in 1957 to a low of 14.4 dozen in 1995 (Statistics Canada, 2001). This reduction was due to numerous factors, chief among them was the fact that eggs represent a significant source of dietary cholesterol. The perceived link between eggs, dietary cholesterol, serum cholesterol, and the risk for cardiovascular disease

has been fostered over the last three decades. More recent studies (Hegsted et al., 1993; Clarke et al., 1997) have provided strong evidence that the impact of dietary cholesterol on serum low-density lipoprotein cholesterol is not as strong as originally stated. More specifically for eggs, data from Hu et al. (1999) showed that, in healthy, nondiabetic subjects, the risk for the development of cardiovascular disease was not significantly different between those consuming less than 1 egg/wk versus those consuming greater than 1 egg/d. It is perhaps an increased awareness of the latter information that has led to an increase in egg consumption of 1.3 dozen per person per year for 1995 to 2000 (Statistics Canada, 2001). Additionally, the availability of specialty or designer eggs, such as omega-3 fatty acid-enriched eggs, might have contributed to this surge in consumption (Sim, 2000). Further enhancements to the nutritional image or profile of eggs may be necessary to maintain this surge.

Increasing the folate content of the laying hen diet will increase the egg folate content, thus offering the potential to improve the nutritional image of the egg by positioning it as an important vehicle to increase folate intakes by humans. The increased awareness of the need for women of child-bearing age to increase folate intakes to reduce their risk of having a baby with a neural tube defect, such as spina bifida, presents an opportunity to specifically market folate-enriched eggs to this target population. Furthermore, an increase in folate intake may be beneficial for the general population through reductions in the concentration of homocysteine in plasma, an independent risk factor for cardiovascular disease (Boushey et al., 1995).

One large folate-enriched egg provides approximately 45 to 50 μg of folate, or roughly 25% of the current recommended nutrient intakes for Canadian adults (180 $\mu\text{g}/\text{d}$ males; 220 $\mu\text{g}/\text{d}$ females; Health and Welfare Canada, 1990). Therefore, under current labeling regulations, folate-enriched eggs could be classified as an excellent source of folate. However, the harmonization of Canadian and US nutrient requirements through the dietary reference intake (DRI) process has resulted in a doubling of the adult human recommended dietary allowance (RDA) estimate for folate (Institute of Medicine, 1998). The new adult RDA for folate is 400 dietary folate equivalents, where 1 DFE = 1 μg of food-derived folate, 0.5 μg supplemental crystalline folic acid in an empty stomach, or 0.6 μg folic acid from fortified foods or a supplement with food. Therefore, upon adoption of the new RDA, a folate-enriched egg, produced using the current protocol, will contain 12.5% of the RDA for folate, whereas an unfortified egg will contain 5%. The DFE for folate are based on the assumption that food-derived folates are only 50% bioavailable. Despite this, there is evidence suggesting that the bioavailability of food folates is significantly higher than 50% (Seyoum and Selhub, 1998). The bioavailability of egg folate is likely to be very high due to the fact that the predominant folate form (5-methyltetrahydrofolate) is the metabolically active form, and it is present as a monoglutamate that is readily absorbed (Seyoum

and Selhub, 1998). Studies designed to assess the bioavailability of food folates, using new and sensitive indices of folate status, will define the true DFE content of eggs.

In summary, the current data indicate a potential for fortification of table eggs with folic acid by supplementation of crystalline folic acid to barley-based diets at levels in excess of 2 mg/kg. Furthermore, folate is stable during cold storage for up to 4 wk. One large folate-fortified egg provides approximately 12.5% of the newly established RDA for folate. However, studies designed to assess the true bioavailability of egg folates will more adequately reflect the folate value of fortified eggs. Additional research is needed to not only assess bioavailability but to assess management-related factors that are likely to influence the level of folate in eggs, including stage of production, age of the flock, and dietary factors. This research will help to ensure the development and availability of a folate-enriched egg that has the potential to improve the marketability of eggs by virtue of an enhanced nutritional image.

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