JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY



# pH-Dependent Radical Scavenging Activity of Folates

ANNA GLISZCZYŃSKA-ŚWIGŁO* AND MAŁGORZATA MUZOLF

Faculty of Commodity Science, The Poznań University of Economics, al. Niepodległości 10, 60-967 Poznań, Poland

Folic acid (FA) is used, in many countries, in nutritional supplements or for the fortification of cereals and their products. It is also used in vitamin pills. Recently, it was reported that folates may act as antioxidants; therefore, in the present study, the effect of pH of the surrounding medium on the radical-scavenging activity of FA and its reduced forms [dihydrofolic acid (DHF), tetrahydrofolic acid (THF), 5-methyltetrahydrofolic acid (5-MTHF), and 5-formyltetrahydrofolic acid (5-FTHF)] was investigated. It was found that radical-scavenging activities of folates, measured in the trolox equivalent antioxidant capacity (TEAC) assay, are strongly pH-dependent. FA is a better radical scavenger at acid and basic pH than at neutral pH. Reduced forms of FA are better radical scavengers at acidic pH values than at neutral and basic pH values, with exception of 5-FTHF for which, at a pH higher than 5.0, an increase of the radical-scavenging activity with an increasing pH of the medium is observed. The results of the present study indicate that possible health effects of folates associated with their radical-scavenging activity will vary depending upon the pH of body fluid or tissue considered.

**KEYWORDS: Folic acid; folates; water-soluble vitamins; radical-scavenging activity; TEAC value**

## INTRODUCTION

There is evidence that excessive free radicals in cells and tissues are the causative agents in some chronic diseases, such as cardiovascular disease, some forms of cancer, and aging. Free radicals are normal species produced during metabolic processes of the body, but if they get out of balance, highly reactive forms of free radicals can cause oxidative damage to biological systems. Conditions of oxidative stress may be prevented or delayed by the consumption of dietary antioxidants, such as polyphenols, and antioxidant vitamins C and E. Other micronutrients that are involved include some carotenoids, selenium, zinc, and folate (*1*).

Folates are a class of compounds having chemical structure and nutritional activity similar to that of folic acid (vitamin B9; **Figure 1**). Folates are found in both vegetable and animal foods. Citrus fruits, grains, yeasts, mushrooms, liver, pork meat, eggs, and leafy green vegetables, such as spinach, lettuce, and asparagus, are especially rich in folates. Folic acid (FA) is in vivo reduced to 7,8-dihydrofolate (DHF), which is subsequently reduced to 5,6,7,8-tertahydrofolate (THF) and then enzymatically converted into 5-methyltetrahydrofolate (5-MTHF); in both latter structures, two double bonds of the pterin ring system are reduced (**Figure 1**). Reduced forms of FA are cofactors in the transfer and use of one-carbon groups; they donate a one-carbon group in the biosynthesis of purine, pyrimidine, and DNA and play a key role in the regeneration of methionine (*2*). FA is used, in many countries, in nutritional supplements or for the fortification of cereals and their products. It is also used in vitamin pills. 5-Formyltetrahydrofolic acid (5-FTHF), known also as folinic acid, citrovorum factor, or leucovorin, is one of the coenzyme forms of FA, which is produced commercially. It is used in combination with other chemotherapy drugs to enhance the anticancer effects of fluorouracil or to help prevent or lessen the toxic effects of methotrexate (*3*).

Proper metabolism and sufficient intake of dietary FA before conception and during early pregnancy decreases the risk of a baby developing neural tube defects, which include spina bifida (*4, 5*). Moreover, FA deficiency has been associated with neurological and neuropsychiatric disorders (*6, 7*) and a megaloblastic anemia. FA is considered as potentially protective against cardiovascular disease because of its homocysteine-lowering potential (*8*). It was suggested that folates may enhance endothelial function via the mechanisms independent of homocysteine lowering (*9*). Plausible mechanisms include the role of 5-MTHF in the redox cycling of the inactive quinoid dihydrobiopterin ($BH_2$) or in the chemical stabilization of tetrahydrobiopterin ($BH_4$) (*9, 10*), which is an essential cofactor of endothelial NO synthase (eNOS). The depletion of $BH_4$ results in the uncoupling of eNOS activity and a switch from the production of NO to the generation of superoxide (*11*). Folates may also act as direct antioxidants against superoxide. The last mechanism suggested is the direct effect of folates on eNOS, in which 5-MTHF reduces superoxide generation and increases NO synthesis in a $BH_4$-dependent manner (*9, 10*).

* To whom correspondence should be addressed: Faculty of Commodity Science, The Poznań University of Economics, al. Niepodległości 10, 60-967 Poznań, Poland. Telephone: +48-61-8569368. Fax: +48-61-8543993. E-mail: a.gliszczynska-swiglo@ae.poznan.pl.

10.1021/jf070986x CCC: $37.00 
Published on Web 09/12/2007

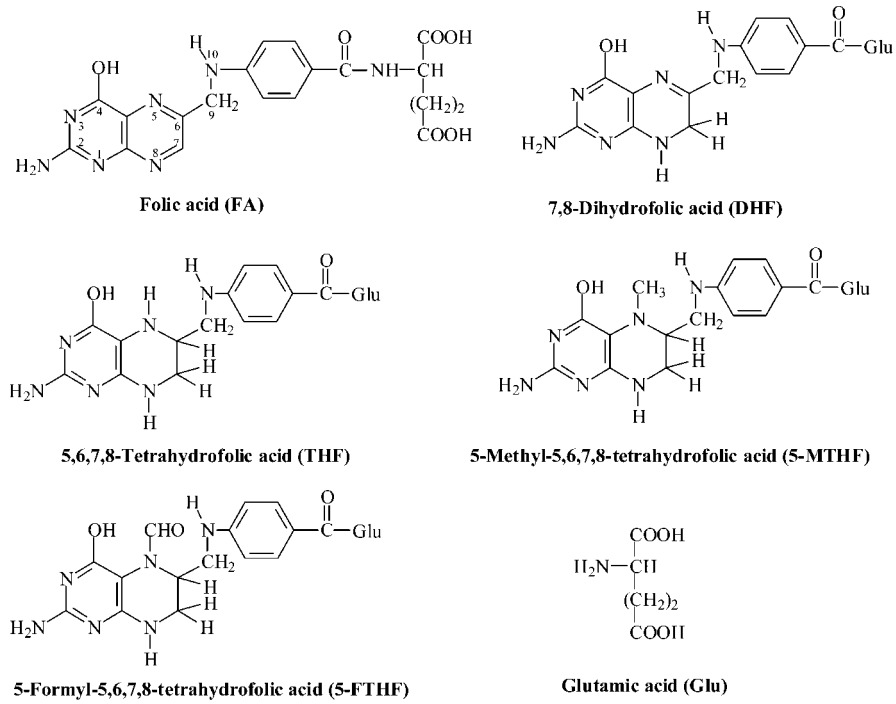


**Figure 1.** Chemical structures and atom-numbering system of FA and its reduced forms.

There is also evidence that the deficiency of folates can cause damage to DNA that may lead to cancer (*12*). It was proposed that presumed protective effects of folates in the pathogenesis of different diseases, such as neural tube defects, megaloblastic anemia, cardiovascular disease, and certain forms of cancer, could be associated, at least in part, with its antioxidant activity (*13*).

In the previous studies (*14–18*), the antioxidant activity of folates was reported but the influence of pH of the surrounding medium on their activity was not taken into account. Thus, the objective of the present study was to investigate the effect of pH on the radical-scavenging activity, measured in the TEAC assay, of FA and its reduced forms.

## MATERIALS AND METHODS

**Chemicals.** FA (approximately 98%), 5-methyltetrahydrofolic acid disodium salt (approximately 90%), dihydrofolic acid (≥90%), microperoxidase-8 (MP8), and folinic acid calcium salt (≥90%) were purchased from Sigma (St. Louis, MO). 2,2′-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), tetrahydrofolic acid (approximately 70%), and hexanesulfonic acid were from Fluka (Buchs, Switzerland). Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) was purchased from Sigma-Aldrich (Steinheim, Germany). Ascorbic acid was from Merck (Darmstadt, Germany). Folates were of the highest purity available, and they were used as received.

**Stability of Folates.** Stock solutions of folates were prepared in dimethyl sulfoxide (DMSO) (FA, DHF, and THF), methanol (THF and 5-MTHF), or demineralized water (5-FTHF), and their stability was immediately analyzed at room temperature using high-performance liquid chromatography (HPLC). The stability of folates was also measured in 0.1 M potassium phosphate buffers at pH 4.0 and 7.4. A Waters 600 high-performance liquid chromatograph (Waters, Millford, MA) equipped with a Nova-Pak $C_{18}$ column (150 × 3.9 mm, 5 $\mu$m) fitted with a $\mu$Bondapak $C_{18}$ cartridge guard column (Waters, Millford, MA) was used. A gradient of the mobile phase, methanol (solvent A) and 0.05 M $NaH_2PO_4$ containing 0.005 M hexanesulfonic acid at pH 3.0 (solvent B) was developed and used according to the following gradient: linear increment from 10 to 40% solvent A during 20 min and the return to the initial conditions within the next 10 min. The flow rate was 1 mL/min. The injection volume was 20 $\mu$L. The eluate was detected at 280 nm using a Waters 996 photodiode-array detector. Degradation of folates is expressed as a percentage of the appropriate folate peak area at $t = 0$ min.

**Trolox Equivalent Antioxidant Capacity (TEAC) Assay.** The TEAC assay is based on the ability of the antioxidant to scavenge the blue–green-colored $ABTS^{\bullet+}$ radical cation relative to the $ABTS^{\bullet+}$ scavenging ability of the water-soluble vitamin E analogue, Trolox (*19, 20*). Radical-scavenging activities of folates were measured by the modified TEAC assay performed essentially as described previously (*19, 20*), with some modifications (*21*). In the present study, microperoxidase-8 (MP8) instead of metmyoglobin was used to generate the $ABTS^{\bullet+}$ in PBS (0.01 M phosphate buffer, 0.14 M NaCl, and 0.002 M KCl) at pH 7.4. MP8 (final concentration of 0.2 $\mu$M) and ABTS (final concentration of 3.0 mM) in PBS were mixed, and the reaction was initiated by the addition of hydrogen peroxide (final concentration of 0.1 mM). The incubation of ABTS with MP8/$H_2O_2$ was carried out for 1 h at 30 °C. The major advantage of the modified TEAC assay is that it permits studies of the radical scavenging activity over a wide pH range (2–10) (*21*) in contrast to many other assays, such as ORAC, FRAP, TRAP, DPPH, and TEAC/myoglobin (*22*).

$ABTS^{\bullet+}$ solution thus obtained was diluted 1:1 (v/v) using 0.2 M potassium phosphate buffers of various pH values to give $ABTS^{\bullet+}$ solutions at pH values varying between 2 and 10 (an absorption was about 0.6 at 734 nm). The $ABTS^{\bullet+}$ buffer solutions, which have a stable absorbance at 734 nm for at least 2 h were used for the determination of the TEAC values. Folates, ascorbic acid, and Trolox were added as 1% (v/v) of 100 times concentrated stock solutions in DMSO (FA and DHF), methanol (THF, 5-MTHF, and Trolox), or water (5-FTHF and ascorbic acid) to give the final concentration required (2–20 $\mu$M for FA and 2–10 $\mu$M for other compounds). Control incubation with the solvent was carried out for each determination. The decrease in absorption caused by the compounds tested, measured after 6 min, is reflecting the $ABTS^{\bullet+}$ radical-scavenging capacity and was plotted against the concentration of the compound. The TEAC value was calculated as the ratio of the slope of the plot for scavenging of the $ABTS^{\bullet+}$ radical cation by the folate under investigation to the slope of the plot for $ABTS^{\bullet+}$ scavenging by Trolox. The radical-scavenging activity of Trolox was previously shown to be unaffected over the whole pH range tested (*21*).

To limit degradation of THF, all TEAC measurements were done using degas organic solvents and buffers. The linear correlation obtained for the plot of the increasing concentrations of folates to the absorbance at 734 nm, measured after 6 min of incubation, allows for the

assumption that the decrease in absorbance especially reflects the reaction between the $ABTS^{\bullet+}$ radical cation and folates, and this reaction is not significantly affected by possible side reactions.

**Absorption Spectra.** All UV absorption spectra were recorded using a UV–vis–NIR Varian Cary 5E spectrophotometer.

## RESULTS AND DISCUSSION

Recently, it was demonstrated that FA can efficiently scavenge such free radicals as $CCl_3O_2^{\bullet}$, $N_3^{\bullet}$, $SO_4^{\bullet-}$, $Br_2^{\bullet-}$, $^{\bullet}OH$, and $O^{\bullet-}$. Moreover, FA can also scavenge and repair thiyl radicals at physiological pH (*14*). The activity of FA against the radical-mediated oxidative damage in human whole blood was also reported (*16*). Its physiological reduced forms (DHF, THF, and 5-MTHF) are peroxynitrite scavengers and inhibitors of lipid peroxidation (*13*, *15*). Antioxidant activities of folates were also observed in the TEAC, DPPH (2,2-diphenyl-1-picrylhydrazyl), and FRAP (ferric reducing antioxidant power) assays (*18*). In the present study, the effect of pH of the surrounding medium on the radical-scavenging activities of folates, measured in the TEAC assay, was investigated.

The first aspect which should be taken into account is sensitivity of THF toward oxidation. It was reported that the stability of THF is dependent upon oxygen and light access and the pH of the solution. It can be easily cleaved to dihydroxanthopterin or to other pterins and to 4-aminobenzoylglutamic acid; the later compound is the main product of the THF air oxidation at pH 4.7 and 10.0 (*3*). After the evaluation of currently available data on the antioxidant activity of THF (*15*, *18*), it appears that the sensitivity of THF toward oxidation resulting in its poor stability in organic solvents, which are used to prepare solutions of THF, is not taken into account. It implies that these literature data may have to be ascribed to lower concentrations of THF than expected. **Figure 2A** shows the time course of THF degradation in dimethyl sulfoxide (DMSO) and methanol. For a comparison, the stability of other folates used in the present study is presented. From this figure, it follows that the stability of THF in DMSO is much lower (a half-time of degradation was estimated to be about 30 min) than in methanol (half-time degradation is about 90 min). Other folates are stable in organic solvents or demineralized water used for the preparation of their solutions. Moreover, parts **B** and **C** of **Figure 2** present the stability of folates in phosphate buffer at pH 4.0, at which the highest radical scavenging activity of folates is observed (**Figure 3**) and pH 7.4. THF in aqueous solutions is the least stable folate followed by DHF. The stability of other folates for at least 4 h is relatively high. From these figures, it can be concluded that more than 90% of folates, in their parent forms, is present at 6 min of incubation with the $ABTS^{\bullet+}$ radical cation. The oxidation of THF could be prevented by the addition of ascorbic acid or 2-mercaptoethanol (*3*), but these compounds could not be used in the present study because of the fact that they also scavenge the $ABTS^{\bullet+}$ radical cation. To limit the oxidation of folates, especially THF, all measurements were done using degas organic solvents and buffers.

**Figure 3** presents the pH-dependent TEAC values for FA and its reduced forms. For a comparison, the TEAC profile of ascorbic acid is also presented. The TEAC profiles of α-tocopherol and Trolox are the same as measured for ascorbic acid (results not shown). From this figure, it follows that radical-scavenging activities of ascorbic acid or α-tocopherol are not significantly changing over the whole pH range, whereas the radical-scavenging activities of folates are strongly pH-dependent. The TEAC value of DHF and THF at pH 2.5–5.0 is about 1.3–1.6-fold higher than those of vitamin C or E. Above the

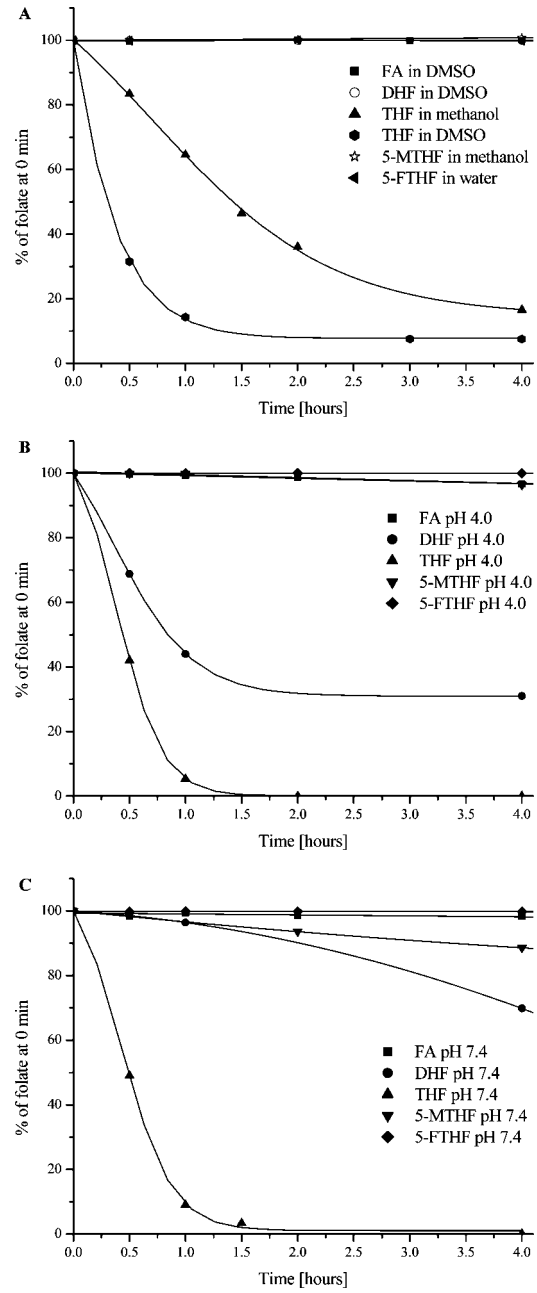


**Figure 2.** Stability of 20 μM soutions of folates (A) in organic solvents or in water, (B) in 0.1 M phosphate buffer at pH 4.0 and (C) pH 7.4. Quantification of folates is based on the HPLC peak area expressed as a percentage of samples at $t = 0$ min. Each point is a mean of two replicates.

pH value 6.0, only the TEAC value of THF is higher than the TEAC values of ascorbic acid and α-tocopherol. From the plots presented, the TEAC values for folates at pH 7.4 were derived and it was found that, only for THF, the TEAC value was significantly higher than already published (*18*). The order of activity of folates in the TEAC assay at pH 7.4 was verified, and it was as follows: THF ($1.24 \pm 0.11$ mM) > DHF ($0.94 \pm 0.02$ mM) > 5-MTHF ($0.77 \pm 0.03$ mM) > 5-FTHF ($0.55 \pm 0.02$ mM) > FA ($0.08 \pm 0.02$ mM). The TEAC values of DHF, 5-MTHF, and THF at pH 7.4 are comparable or even higher than those of vitamins C and E and other natural antioxidants, such as some carotenoids and extensively studied polyphenols (*23*–*25*).

It can be also seen that THF and DHF are better radical scavengers than other folates. The TEAC value of FA increases at pH from 2.0 to 3.5, and then, it decreases to pH of about

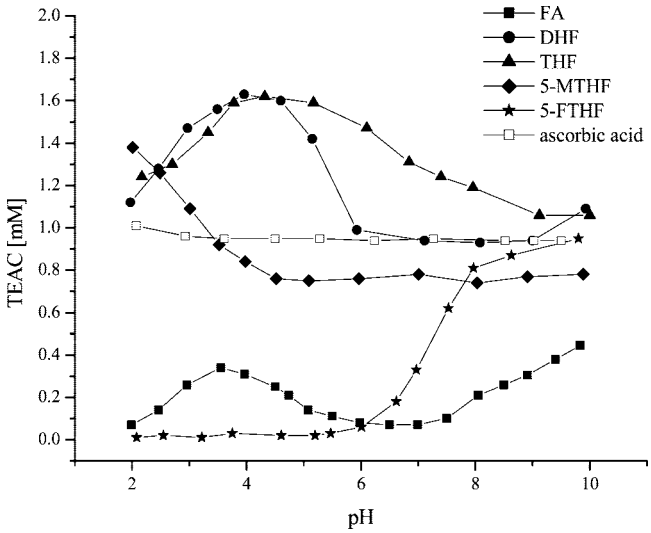


**Figure 3.** Effect of pH on the TEAC values of ascorbic acid, FA, and its physiological reduced forms.

6.5; at pH higher than 6.5, it increases again. For DHF, the increase of the TEAC value is observed up to pH of about 4.0 and the decrease is observed to pH 6.5; at pH higher than 9.0, the TEAC value increases again. For THF, the increase in the TEAC value is observed up to pH 5.0 and then the decrease is observed to pH 10.0. On the basis of the data presented, it can be concluded that FA is a better free radical scavenger at acidic and basic pH than at neutral pH, whereas DHF and THF are better free radical scavengers at acidic pH than at neutral and basic pH. The radical-scavenging activity of FA, over the whole pH range tested, is much lower than that of DHF, THF, and 5-MTHF but, at pH 2.5–5, is higher than observed for 5-FTHF. Because ot the relatively low purity and stability of THF, it can be expected that its TEAC values at different pH could be even higher than those reported in this study. The influence of the decomposition products of THF on the course of its TEAC profile, especially at acidic pH, can be rather excluded because the TEAC profile of THF is similar to those of FA and DHF, which are stable in the time course of the experiment. Moreover, the changes in the absorption spectra (i.e., the appearance or disappearance of an absorption band at the wavelength of about 350 and 380 nm for FA and DFA, respectively, and the appearance of several isosbestic points) indicate rather on protonation/deprotonation of the compounds than on their degradation at pH 2–6 (**Figure 4**). Finally, the decrease in the absorbance at 734 nm after 6 min of incubation of folates with the $ABTS^{\bullet+}$ radical cation is proportional to the concentration of folates. It allows for the assumption that it especially reflects the reaction between the $ABTS^{\bullet+}$ radical cation and folates in their parent forms.

The pH-dependent TEAC profiles of 5-MTHF and 5-FMTH are quite different than those of other folates. For 5-MTHF, the TEAC value decreases from pH 2.0 to 4.5 and is unaffected by the pH at the values higher than 4.5. Interesting is that the TEAC profile for 5-FTHF, which has a CHO instead of $CH_3$ group at position N5, is quite different than that of 5-MTHF; the TEAC value for 5-FTHF increases with an increasing pH of the medium above pH 5.0.

This effect of pH on radical-scavenging activities of folates may be of biological relevance because the pH range of different human body fluids is known to vary widely from pH 1 in the stomach, pH 5.3 in the small intestine, pH 6.8 in mouth saliva, pH 7.4 in blood and tissue fluid, pH 8 in the large intestine, pH 7–8.7 in the pancreas, to pH 8.3–9.3 in the duodenum (*26*).

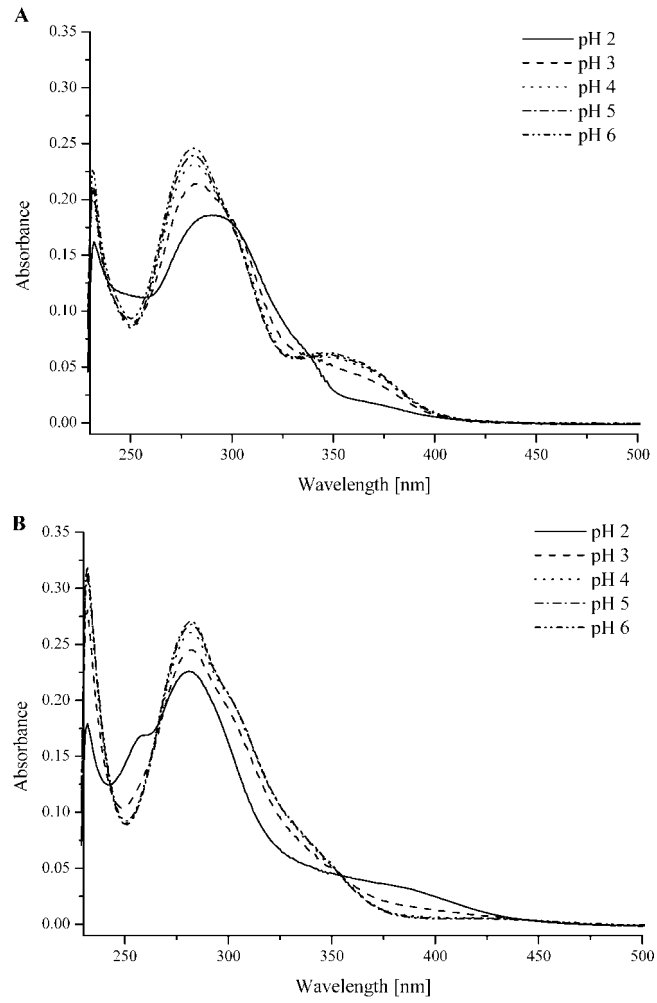


**Figure 4.** UV absorption spectra of 10 $\mu$M phosphate buffer solutions of (A) FA and (B) DHF at the pH range of 2–6.

Therefore, the pH-dependent changes in the radical-scavenging activity of FA and its metabolites suggest that possible health effects of folates, associated with their radical-scavenging activity, will vary with the tissue under investigation.

Physiological concentrations of folates are much lower than the level of vitamin C, the main biological water-soluble antioxidant. On the other hand, blood concentrations of folates as well as some polyphenols and carotenoids can reach a similar level in humans following their habitual or supplemented diets (*27–30*). The role of folates as biological antioxidants is still uncertain; however, the observed reactions of folates with the stable $ABTS^{\bullet+}$ radical cation used as a model radical in our study may also indicate that, under the conditions of excessive oxidative stress, the depletion of folates may occur. This conclusion may be supported by the result that reduced folates are very active as free radical scavengers as shown in **Figure 3**.

All folates contain an amide-like structure involving N3 and C4 atoms that resonates between lactam and lactim tautomeric forms (**Figure 5**). These forms exhibit identical behavior (*31*). In the lactam form, the hydrogen atom at N3 on the purine-type ring is shifted to the oxygen atom at C4, giving C4–OH moiety. It was postulated that this group is responsible for the antiradical activity of FA (*14*). In another study, it was suggested that the antioxidant activity of the fully reduced forms of folate (THF and 5-MTHF) resides in the pterin core and an electron-donating effect is probably important for their antioxidant

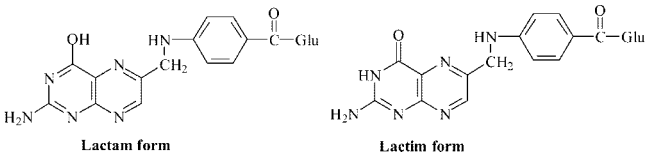


**Figure 5.** Lactam and lactim forms of folates, taking FA as an example.

**Table 1.** Dissociation Constants of Some Folates

| | p$K_a$ of | | | | | |
|---|---|---|---|---|---|---|
| compound | amide | N(1) | N(5) | N(10) | α-carboxyl | γ-carboxyl |
| FA | 8.38[a] | 2.35[a] | < −1.5[a] | 0.20[a] | ND[a] | ND[a] |
| | 8.30[b] | 1.60[b] | | | | |
| DHF | 9.54[a] | 1.38[a] | 3.84[a] | 0.28[a] | ND[a] | ND[a] |
| THF | 10.50[c] | 1.24[c] | 4.82[c] | −1.25[c] | 3.5[c] | 4.8[c] |
| 5-MTHF | | | | 5.20[a] | | |

[a] From ref *35*. [b] From ref *36*. [c] From ref *37*. ND = not determined because of insufficient solubility. It is assumed that the p$K_a$ values of these carboxyl groups are similar for all folates.

activity (*15*). Up to now, there is no clear answer as to which part of folates is in fact responsible for their antioxidant activity.

The pH dependence of the TEAC value previously observed for polyphenols was attributed to an effect of the pH on the deprotonation of their OH moieties (*32*–*34*). A comparison of the p$K_a$ values to the pH-dependent TEAC profiles of polyphenols led the authors to the conclusion that a significant increase in the TEAC value of these compounds is related to deprotonation of their most acidic hydroxyl moiety. Upon deprotonation of this OH group, phenolic compounds become better free radical scavengers (*32*–*34*). For folates, the p$K_a$ of the amide group, which refers to the dissociation of the N3–C4 amide-like site (OH group in the lactam form; **Figure 5**) is in the range of basic pH values (**Table 1**) (*35*–*37*). Therefore, on the basis of a comparison of the p$K_a$ values for the amide group to the pH-dependent TEAC profiles of folates, it could be concluded that the increase in the TEAC value at basic pH could be related to the deprotonation of this group. In **Figure 3**, the significant increase in the TEAC value at pH higher than 7.5 can be seen for FA and at pH higher than 9.0 for DHF, for which the p$K_a$ values of the amide-like group are 8.38 and 9.54, respectively. For other folates, e.g., THF, such an increase is not observed because the dissociation of the N4–C4 amide-like side is outside the range of pH values applied in the study. The p$K_a$ values of other ionisable groups of folates are in the range of acidic pH (**Table 1**). On the basis of a comparison of these values to pH-dependent TEAC profiles, it is difficult to conclude if the changes in the radical-scavenging activity of folates at acidic pH are associated with protonation/deprotonation of ionisable groups of folates. The contribution of carboxyl groups can be rather excluded because the side chain of *N*-(*p*-aminobenzoyl)-L-glutamic acid is present in all compounds tested and the deprotonation of its carboxyl groups would give similar pH-dependent TEAC profiles for all folates.

The explanation of the pH-dependent radical-scavenging activities of folates, especially at acidic pH, is far from straightforward and is currently under investigation. Quantum mechanical calculations are performed and radical-scavenging activities of related model compounds are measured to get more insight into the mechanism of pH-dependent radical-scavenging activities of folates. Nevertheless, the results of the present study indicate that possible health effects of folates associated with their radical-scavenging activity will vary depending on the pH of body fluid or tissue considered.

## ABBREVIATIONS USED

5-FTHF, 5-formyltetrahydrofolic acid; 5-MTHF, 5-methyltetrahydrofolic acid; ABTS, 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt; $ABTS^{\bullet+}$, 2,2′-azinobis(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt radical cation; $BH_2$, dihydrobiopterin; $BH_4$, tetrahydrobiopterin; DHF, dihydrofolic acid; DMSO, dimethyl sulfoxide; FA, folic acid; MP-8, microperoxidase-8; PBS, phosphate-buffered saline; TEAC, trolox equivalent antioxidant capacity; THF, tetrahydrofolic acid; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

## LITERATURE CITED

(1) Godfrey, D.; Richardson, D. Vitamins and minerals for health. *Brit. Food J.* **2002**, *104*, 913–933.
(2) Stanger, O. Physiology of folic acid in health and disease. *Curr. Drug Metab.* **2002**, *3*, 211–223.
(3) Friedrich, W. Folic acid and unconjugated pteridins. In *Vitamins*; Walter de Gruyter: Berlin, Germany, 1988; pp 631–634.
(4) Daly, L. E.; Kirke, P. N.; Molloy, A.; Weir, D. G.; Scott, J. M. Folate levels and neural tube defects. Implications for prevention. *J. Am. Med. Assoc.* **1995**, *274*, 1698–702.
(5) Olney, R. S.; Mulinare, J. Trends in neural tube defect prevalence, folic acid fortification, and vitamin supplement use. *Semin. Perinat.* **2002**, *26*, 277–285.
(6) Alpert, J. E.; Fava, M. Nutrition and depression: The role of folate. *Nutr. Rev.* **2003**, *55*, 145–149.
(7) Manzoor, M.; Runcie, J. Folate-responsive neuropathy: Report of 10 cases. *Brit. Med. J.* **1976**, *1*, 1176–1178.
(8) Boushey, C. J.; Beresford, S. A.; Omenn, G. S.; Motulsky, A. G. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. Probable benefits of increasing folic acid intakes. *J. Am. Med. Assoc.* **1995**, *274*, 1049–1057.
(9) Doshi, S. N.; McDowell, I. F. W.; Moat, S. J.; Payne, N.; Durrant, H. J.; Lewis, M. J.; Goodfellow, J. Folic acid improves endothelial function in coronary artery disease via mechanism largely independent of homocysteine lowering. *Circulation* **2002**, *105*, 22–26.
(10) Verhaar, M. C.; Stroes, E.; Rabelink, T. J. Folates and cardiovascular disease. *Arterioscler., Thromb., Vasc. Biol.* **2002**, *22*, 6–13.
(11) Vásquez-Vivar, J.; Kalyanaraman, B.; Martásek, P.; Hogg, N.; Masters, B. S.; Karoui, H.; Tordo, P.; Pritchard, K. A. Superoxide generation by endothelial nitric oxide synthase: The influence of cofactors. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9220–9225.
(12) Duthie, S. J.; Narayanan, S.; Brand, G. M.; Pirie, L.; Grant, G. Impact of folate deficiency on DNA stability. *J. Nutr.* **2002**, *132*, 2444S–2449S.
(13) Nakano, E.; Higgins, J. A.; Powers, H. J. Folate protects against oxidative modification of human LDL. *Brit. J. Nutr.* **2001**, *86*, 637–639.
(14) Joshi, R.; Adhikari, S.; Patro, B. S.; Chattopadhyay, S.; Mukherjee, T. Free radical scavenging behavior of folic acid: Evidence for possible antioxidant activity. *Free Radical Biol. Med.* **2001**, *30*, 1390–1399.
(15) Rezk, B. M.; Haenen, G. R. M. M.; van der Vijgh, W. J. F.; Bast, A. Tetrahydrofolate and 5-methyltetrahydrofolate are folates with high antioxidant activity. Identification of the antioxidant pharmacophore. *FEBS Lett.* **2003**, *555*, 601–605.
(16) Stocker, P.; Lesgards, J.-F.; Vidal, N.; Chalier, F.; Prost, M. ESR study of a biological assay on whole blood: Antioxidant efficiency of various vitamins. *Biochem. Biophys. Acta* **2003**, *1621*, 1–8.
(17) Gliszczyńska-Świgło, A. Antioxidant activity of water soluble vitamins in the TEAC (trolox equivalent antioxidant capacity) and the ferric reducing antioxidant power (FRAP) assays. *Food Chem.* **2005**, *96*, 131–136.
(18) Gliszczyńska-Świgło, A. Folates as antioxidants. *Food Chem.* **2007**, *101*, 1497–1500.

(19) Miller, N. J.; Rice-Evans, C. A.; Davies, M. J.; Gopinathan, V.; Milner, A. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonate. *Clin. Sci.* **1993**, *84*, 407–412.
(20) Rice-Evans, C.; Miller, N. J. Total antioxidant status in plasma and body fluids. *Methods Enzymol.* **1994**, *234*, 279–283.
(21) Tyrakowska, B.; Soffers, A. E. M. F.; Szymusiak, H.; Boeren, S.; Boersma, M. G.; Lemańska, K.; Vervoort, J.; Rietjens, I. M. C. M. TEAC antioxidant activity of 4-hydroxybenzoates. *Free Radical Biol. Med.* **1999**, *27*, 1427–1436.
(22) Arnao, M. B.; Cano, A.; Acosta, M. Methods to measure the antioxidant activity in plant material. A comparative discussion. *Free. Radical Res.* **1999**, *31*, 89–96.
(23) Rice-Evans, C.; Miller, N. J.; Paganga, G. Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radical Biol. Med.* **1996**, *20*, 933–956.
(24) Miller, N. J.; Sampson, J.; Candeias, L. P.; Bramley, P. M.; Rice-Evans, C. A. Antioxidant activities of carotenes and xanthophylls. *FEBS Lett.* **1996**, *384*, 240–242.
(25) Rice-Evans, C. A.; Miller, N. J.; Bolwell, P. G.; Bramley, P. M.; Pridham, J. B. The relative antioxidant activities of plant-derived polyphenolic flavonoids. *Free Radical Res.* **1995**, *22*, 375–383.
(26) Grzymisławski, M. Physique of systems related to food bioavailability. In *Human Nutrition. Principals of Nutritional Science* (in Polish); Gawędzki, J., Hryniewiecki, L., Eds.; PWN: Warszawa, Poland, 2000; pp 56–72.
(27) Mackey, A. D.; Picciano, M. F. Maternal folate status during extended lactation and the effect of supplemental folic acid. *Am. J. Clin. Nutr.* **1999**, *69*, 285–292.
(28) Erlund, I.; Silaste, M. L.; Alfthan, G.; Rantala, M.; Kesäniemi, Y. A.; Aro, A. Plasma concentrations of the flavonoids hesperetin, naringenin and quercetin in human subjects following their habitual diets, and diets high or low in fruit and vegetables. *Eur. J. Clin. Nutr.* **2002**, *56*, 891–898.
(29) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Rémésy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81*, 230S–242S.
(30) Lamers, Y.; Prinz-Langenohl, R.; Brämswig, S.; Pietrzik, K. Red blood cell folate concentrations increase more after supplementation with [6*S*]-methyltetrahydrofolate than with folic acid in women of childbearing age. *Am. J. Clin. Nutr.* **2006**, *84*, 156–161.
(31) Gregory, J. F., III. Vitamins. In *Food Chemistry*, 3rd ed.; Fennema, O. R., Ed.; Marcel Dekker: New York, 1996; pp 590–591.
(32) Lemańska, K.; Szymusiak, H.; Tyrakowska, B.; Zieliński, R.; Soffers, A. E. M. F.; Rietjens, I. M. C. M. The influence of pH on antioxidant properties and the mechanism of antioxidant action of hydroxyflavones. *Free Radical Biol. Med.* **2001**, *31*, 869–881.
(33) Lemańska, K.; van der Woude, H.; Szymusiak, H.; Boersma, M. G.; Gliszczyńska-Świgło, A.; Rietjens, I. M. C. M.; Tyrakowska, B. The effect of catechol O-methylation on radical scavenging characteristic of quercetin and luteolin—A mechanistic insight. *Free Radical Res.* **2004**, *38*, 639–647.
(34) Borkowski, T.; Szymusiak, H.; Gliszczyńska-Świgło, A.; Rietjens, I. M. C. M.; Tyrakowska, B. Radical scavenging capacity of wine anthocyanins is strongly pH-dependent. *J. Agric. Food Chem.* **2005**, *53*, 5526–5534.
(35) Poe, M. Acidic dissociation constants of folic acid, dihydrofolic acid, and methotrexate. *J. Biol. Chem.* **1977**, *252*, 3724–3728.
(36) Moorthy, P. N.; Hayon, E. One-electron redox reactions of water-soluble vitamins. II. Pterin and folic acid. *J. Org. Chem.* **1976**, *41*, 1607–1613.
(37) Kallen, R. G.; Jencks, W. P. The dissociation constants of tetrahydrofolic acid. *J. Biol. Chem.* **1966**, *241*, 5845–5850.

**Received for review April 4, 2007. Revised manuscript received July 16, 2007. Accepted July 17, 2007.**

JF070986X