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Photodegradation of 5-methyltetrahydrofolate: Biophysical Aspects

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ABSTRACT

5-methyltetrahydrofolate (5MTHF) absorbs UV radiation and has an absorption coefficient of 24 250 ± 1170 M^{-1} cm^{-1} at 290 nm. It has a weak fluorescence emission in the wavelength region around 360 nm. Our data demonstrated induction of 5-methyldihydrofolate by exposure to UVB and, after continues irradiation, *p*-aminobenzoyl-L-glutamic acid was found. The photodegradation of 5MTHF follows a first order kinetic with a degradation rate constant of 9.2×10^{-3} min^{-1} under our conditions (fluence rate of 2.15 mW cm^{-2}, exposure wavelengths from 280 to 350 nm). Our results indicate that a direct degradation of 5MTHF by UV exposure in humans *in vivo* is rather unlikely. 5MTHF mainly absorbs, and is degraded by, UVB and UVC, radiation that does not penetrate the earth's atmosphere and the human skin well.

INTRODUCTION

Folate is an important vitamin for human health (1). Folate deficiency, or impairment of the folate metabolism in an organism, leads to several diseases, including megaloblastic anemia (1) and complications arising in pregnancy, such as neural tube defects (2). Folate deficiency may also increase the risk of developing cardiovascular diseases (3) and cancer (4).

It has been suggested that exposure to large doses of solar radiation may lead to folate deficiency, and that sun-induced folate degradation may play a key role in evolution of human skin color. This hypothesis was first proposed by Branda and Eaton (5) and further developed by Jablonski and Chaplin (6). No definite conclusion about the possibility of folate photodegradation *in vivo* has been drawn yet. As a first step in the elucidation of this problem, investigations of the photophysics and photochemistry of folate in simple model systems would be of great value.

The photodegradation of folic acid (FA), a synthetic form of the folate, has been thoroughly studied (7,8). 5-methyltetrahydrofolate (5MTHF) belongs to the naturally occurring folates. In the present work we have investigated the photodegradation kinetic of 5MTHF by absorption and fluoresence measurements. The chemical structure of 5MTHF is shown in Fig. 1. It consists of three groups: a pteridine residue, *p*-aminobenzolate and glutamic acid.

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MATERIALS AND METHODS

Chemicals. (6R,S)-5-methyl-5,6,7,8-tetrahydrofolate calcium salt and (6R,S)-5-methyl-5,6-dihydrofolate ammonium salt was purchased from Schircks Laboratories (Jona, Switzerland), while *p*-aminobenzoyl-L-glutamic acid was purchased from Sigma Chemical Co. (St. Louis, MO). Dulbecco's phosphate buffered saline (PBS) was purchased from PAA Laboratories GmbH (Pasching, Austria). The solutions were freshly made in PBS before each experiment. Because of the instability of the compounds, the solutions were always kept on ice before use.

UV/Vis absorption and fluorescence measurements. Absorption spectra were registered with a Perkin-Elmer Lambda 40 UV/Vis spectrometer (Shelton, CT). Fluorescence emission and excitation spectra were recorded by means of a Perkin-Elmer LS50B luminescence spectrometer equipped with a Hamamatsu R-928 photomultiplier (Iwata, Japan). All measurements were performed in standard 10 mm quartz cuvettes.

Photolysis. Solutions of 5MTHF were exposed to UV radiation in closed quartz cuvettes (1 mL). The radiation source was a broad-band UVB lamp with five Philips TL 20W/12 RS UVB tubes (Amsterdam). The fluence rate of the lamp was measured after each experiment with an UVB detector (PMA2106) connected to a photometer (PMA2200), both from Solar Light Co. (Philadelphia, PA). The intensity at the sample position was 2.15 mW cm^{-2}, and the radiation was emitted mainly in the wavelength region 280–350 nm with a maximum at 312 nm. The temperatures of the samples were approximately 25 to 30°C. All experiments were performed in constant dim light in order to avoid external, uncontrolled light exposure.

One point that must be stressed is the fact that our UV source contains radiation that is not present in the solar radiation (9). In the starting phase of our project we used a narrow-band UVB lamp (Phillips TL 20W/01 RS, 312 nm peak). The results were the same as for the broad-band UVB lamp, but the process was slower because 5MTHF absorbs less at 312 nm than at shorter wavelengths. 5MTHF is rather unstable *in vitro*, so we decided to use the broad-band UVB lamp.

RESULTS AND DISCUSSION

Absorption measurements

The absorption spectra of different concentrations of 5MTHF are shown in Fig. 2. 5MTHF absorbs radiation in the UV region and has an absorption peak at 290 nm. At wavelengths longer than 340 nm, the absorption is weak. The insert shows a linear plot of the absorbance at 290 nm as a function of concentration. The linear relationship between absorbance and concentration indicates that the absorbance of 5MTHF follows the Beer–Lambert law, thus aggregation plays no major role.

The absorption coefficient, ε, for 5MTHF was found to be 24 250 ± 1170 M^{-1} cm^{-1} at 290 nm. This is lower than 30 800 M^{-1} cm^{-1}, found by Donaldson and Keresztesy (10) and 29 000 M^{-1} cm^{-1} found by Larrabee *et al*. (11), and the reason for this can be that 5MTHF is a hygroscopic substance. The powder of 5MTHF may absorb water from the air and increase its weight. This may lead to

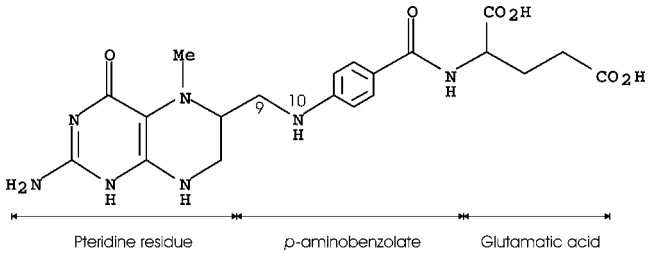

Figure 1. Chemical structure of 5-methyltetrahydrofolate (5MTHF).

a lower concentration in our experiments than calculated by the weight of the substance.

FA has a peak at 280 nm (7), so the absorption spectrum of 5MTHF is red shifted compared with that of FA. The main difference between the spectrum of FA and that of 5MTHF is that FA absorbs much more UVA than 5MTHF does. This difference can be explained by considering the absorption spectra of *p*-aminobenzoyl-L-glutamic acid (PGA), 6-formyl pterin (FPT) and pterin-6-carboxylic acid (PCA), the photoproducts of FA photodegradation (see Off *et al.* [7]). FA consists of a fully oxidized pterin group. FPT and PCA contain the pterin group and the absorption spectra show UVA absorption (7). PGA (without pterin) does not absorb UVA. Therefore, the high UVA absorption of FA is probably due to the pterin moiety.

The reason why 5MTHF absorbs less UVA may be that the pterin residue of 5MTHF is in a reduced form. Another reason could have been that the methyl group is attached to atom N-5 but the effect of this is probably small. Besides, tetrahydrofolate, which is identical to 5MTHF except for the methyl group, only absorbs UVA to a small degree (12). PGA has a weaker absorption than 5MTHF. The reason for this may be that the pterin residue of 5MTHF absorbs UVB radiation or, most likely, that 5MTHF absorbs more radiation being a larger molecule.

When 5MTHF is exposed to UVB radiation, its spectral characteristics are changed. This indicates that it is transformed to other compounds. Figure 3a shows the absorption spectra of 5 μM 5MTHF after 0, 60, 120, 180 and 240 min of UVB exposure, while in Fig. 3b the difference spectra are presented. The peak at 288 nm

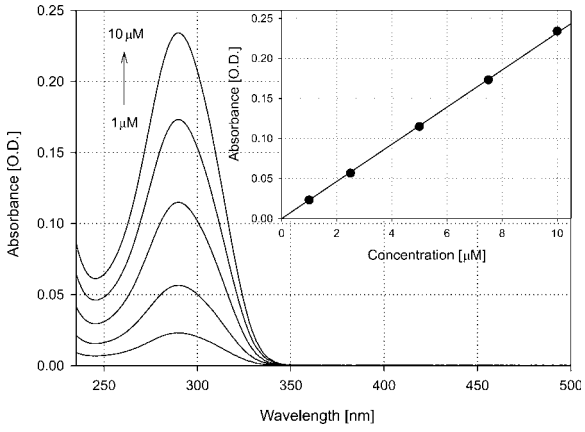

Figure 2. Absorption spectra of different concentrations of 5MTHF. The concentrations are 1, 2.5, 5.0, 7.5 and 10.0 μ*M*, pH 7.4. Path length of cuvette is 1.0 cm. Insert: The points show the absorbance at 290 nm as function of concentration. The regression line in the insert follows the equation $y = 0.0232x$, ($R^2 = 0.9997$).

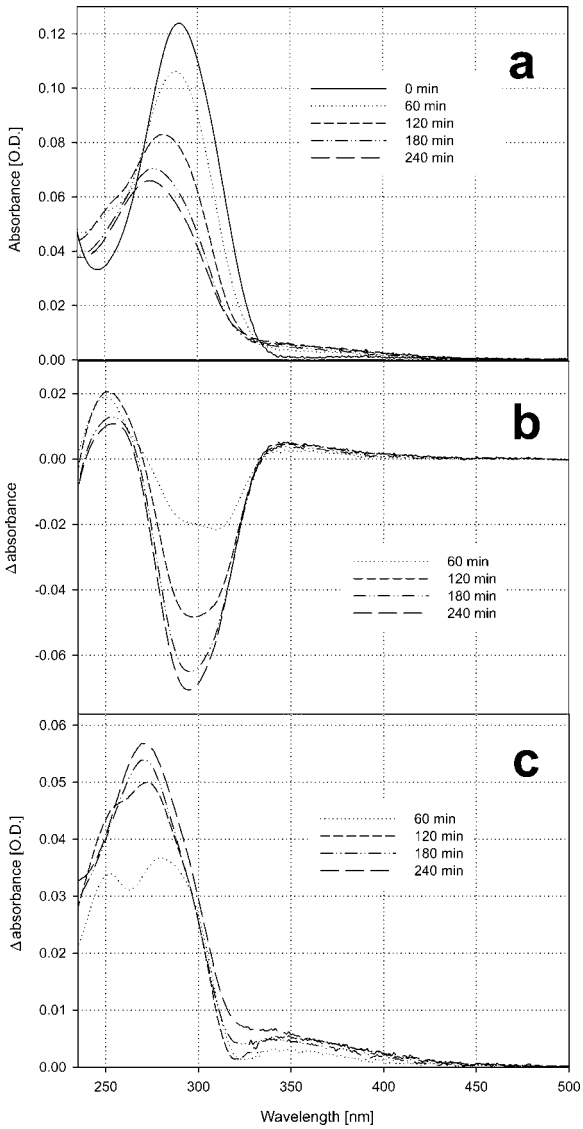

Figure 3. a: Absorption spectra of 5 μ*M* 5MTHF irradiated for 0, 60, 120, 180 and 240 min with the UVB lamp; pH 7.4. b: Difference absorption spectra, obtained by subtracting the spectra of 5MTHF solution from the spectra of solutions after UVB exposure. c: Absorption spectra of the photoproducts of 5MTHF after UVB exposure calculated under the assumption that the degradation is of first order with rate constant of 9.2×10^{-3} min^{-1}.

decreases during irradiation. Subsequently, it is blueshifted. At around 250 nm the absorption increases.

Figure 3c shows the absorption spectra of the photoproducts for different UVB exposures. The spectra were obtained by subtracting a fraction of the initial absorption spectrum from the absorption spectra after irradiation. The fraction of the initial absorption spectrum subtracted was estimated under the assumption that the degradation was of first order, and that the resultant spectrum had to be positive for all wavelengths. The following equation was used to calculate the absorption spectra of the photoproducts:

$$A'_{\text{exp}}(\lambda, t_{\text{exp}}) = A_{\text{exp}}(\lambda, t_{\text{exp}}) - A_0(\lambda) \cdot e^{-k \cdot t_{\text{exp}}},$$

where A'_{exp} is the absorbance at the wavelength λ after exposure time t_{exp}, and k is the degradation rate constant. A_0 and A_{exp} are the

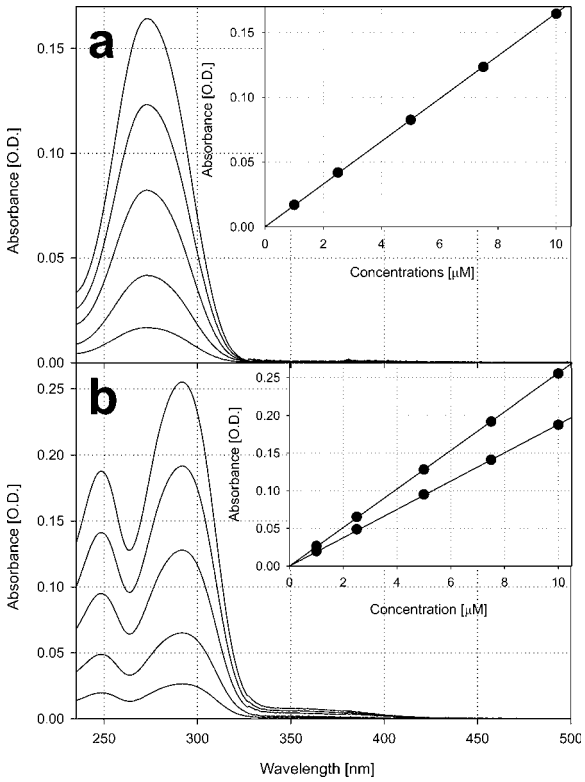

Figure 4. a: Absorption spectra of *p*-aminobenzoyl-L-glutamic acid (PGA) and b: 5-methyldihydrofolate (5MDHF) at different concentrations (1, 2.5, 5, 7.5 and 10 μ*M*). Insert in a: The points show absorbance at 273 nm as function of concentration for PGA and corresponding regression line follows the equation $y = 0.0165x$, ($R^2 = 1$). Insert in b: The points show absorbance at 292 and 248 nm as functions of the concentration of 5MDHF, with regression lines following the equations $y = 0.0256x$ ($R^2 = 0.9999$) and $y = 0.0188x$ ($R^2 = 0.9997$), for 292 and 248 nm, respectively.

measured absorptions before and after exposure, respectively. The degradation rate constant, k, was 9.2×10^{-3} min^{-1}.

These data indicate that two different products are formed. First a substance with two peaks, at 252 nm and 285 nm, is produced. Then, after about 3 h of UVB irradiation, there is only one peak left, with an absorption maximum at 270 nm.

When FA is irradiated with UV the bond between atom 9 and 10 (indicated in Fig. 1) is broken and the photoproducts are PGA and FPT (7). For 5MTHF it is not likely that FPT is a product of the photodegradation. If the bond between atom 9 and 10 is split in 5MTHF, as in FA, one of the products is possibly PGA. To check if this is true, the absorption spectrum of PGA was recorded. The absorption spectra of PGA at different concentrations are shown in Fig. 4a and has a peak at 273 nm. The inserted graph shows the absorption at this wavelength plotted against the concentration. The absorption coefficient, ε, is 16 580 ± 170 M^{-1}cm^{-1} at this wavelength. According to these data, PGA is likely to be a product of the photodegradation of 5MTHF.

The increase in absorption at 250 nm may indicate the formation of 5-methyldihydrofolate (5MDHF). 5MDHF has two absorption peaks in the UV region, one at 248 nm and the second one at 292 nm. The absorption spectra of 5MDHF at different concentrations are shown in Fig. 4b. The peaks at 248 and 292 nm have absorption coefficients of 19 090 ± 450 and 25 870 ± 440 M^{-1}cm^{-1}, respectively. This means that 5MTHF may be photooxidized to

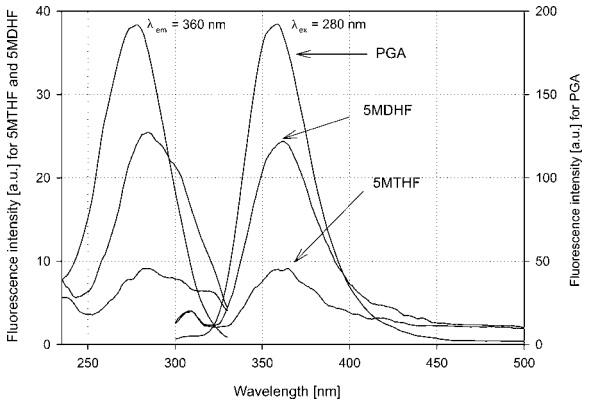

Figure 5. Fluorescence excitation and emission spectra of 5 μ*M* 5MTHF, 5MDHF and PGA, pH 7.4. λ_{em} is 360 nm for the excitation spectra and λ_{ex} is 280 nm for the emission spectra. The x axis to the left is for 5MTHF and 5MDHF, while the axis to the right is for PGA.

5MDHF. It is known that 5MTHF is easily oxidized; oxidation may even take place without any irradiation (13). Therefore, absorption spectra of samples of 5MTHF, covered with a UV filter, were recorded (data not shown). These experiments show that the increase at 250 nm is not caused by irradiation alone, or stated differently, some increase at 250 nm takes place even without UV exposure. The spectra of the control samples also decreased at 288 nm, but did not shift towards shorter wavelengths. Therefore, 5MTHF is oxidized to 5MDHF in the absence of UV radiation but slower, and the bond between atom 9 and 10 will not be broken. The degradation rate constant for sample covered with UV filter was 1.8×10^{-3} min^{-1}.

Fluorescence measurements

The fluorescence emission spectrum of 5 μ*M* 5MTHF in PBS has a peak at 360 nm, and the fluorescence excitation spectrum has a peak at 284 nm (Fig. 5). In further work we used an excitation wavelength of 280 nm, since previous work with FA was performed with this wavelength (7). There is also a small peak at 308 nm in the fluorescence emission spectrum, but this peak is also seen for pure PBS and water (data not shown) and is most probably the Raman line of water (14).

The fluorescence emission peak at 360 nm resembles that of PGA. The fluorescence emission and excitation spectra of PGA are also shown in Fig. 5. Thus, it is probably the aminobenzolate part of the 5MTHF molecule that is fluorescent. The pterin moiety, which is known to have a fluorescence peak at around 450 nm when it is fully oxidized (Thomas *et al.* [15] and Off *et al.* [7]), is probably not fluorescing when it is reduced. The lack of fluorescence in the region around 450 nm might also be due to the methyl group attached to the pterin ring. This group may act as an internal quencher.

Lucock *et al.* (16) found that the tetrahydro forms of folate have a stronger fluorescence intensity than the dihydro forms. In Fig. 5 the fluorescence emission and excitation spectra of 5MDHF is shown together with the spectra of 5MTHF and PGA. The relative higher fluorescence of 5MDHF, compared with 5MTHF, may be due to impurities of 5MDHF solutions with the presence of some PGA. After storage of 5MTHF, and, therefore, oxidation of 5MTHF to 5MDHF, the fluorescence is reduced, which indicates that 5MDHF has a lower fluorescence than 5MTHF.

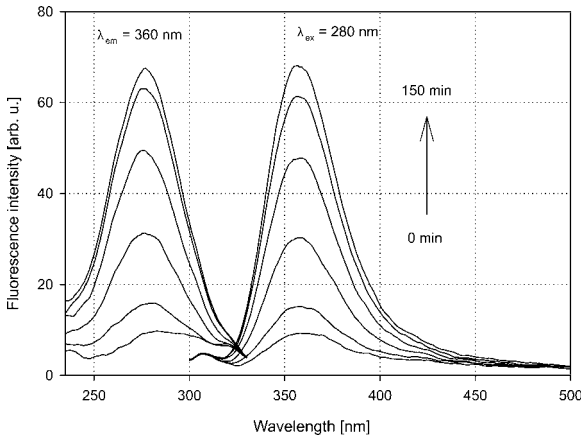

Figure 6. Fluorescence excitation and fluorescence emission spectra of 5 μ*M* 5MTHF exposed to UVB irradiation for 0, 30, 60, 90, 120 and 150 min. λ_{em} is 360 nm for the excitation spectra and λ_{ex} is 280 nm for the emission spectra; pH 7.4.

Figure 6 shows the evolution of the fluorescence excitation and emission spectra after 30, 60, 120 and 150 min of UVB exposure. When solutions of 5MTHF are exposed to UVB radiation, the fluorescence of the solutions increases. This means that at least one of the photoproducts of 5MTHF fluoresces stronger than 5MTHF itself. The peak at 284 nm is blueshifted to 277 nm.

The fluorescence yield for PGA is around 20 times higher than that of 5MTHF (*cf.* Fig. 5). A two-step process may explain the observed kinetics: 5MTHF is first oxidized to 5MDHF and then the bond between atom 9 and 10 is broken.

Reaction kinetics

Figure 7 shows the fluorescence emission intensity of irradiated 5MTHF (5 μ*M*) at 360 nm as a function of exposure time. The shape of the graph is sigmoidal.

When FA was UV exposed it was found that the degradation rate was concentration dependent (7). The photodegradation rate of 5MTHF is not concentration depended and, therefore, follows first order kinetics. The rate constant was 9.2×10^{-3} min^{-1}. This rate

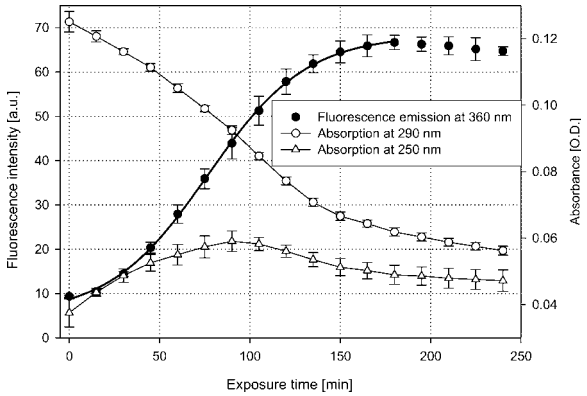

Figure 7. The fluorescence emission intensity at 360 nm (λ_{ex} = 280 nm) and the absorption at 290 and 250 nm as a function of exposure time for 5 μ*M* 5MTHF, pH 7.4. Data are from three different experiments with mean and standard deviation given. The line for fluorescence intensity at 360 nm as a function of exposure time is sigmoidal ($P < 0.0001$ for all parameters) and the fitting was made with SigmaPlot 2001 version 7.101 (SPSS Inc., Chicago).

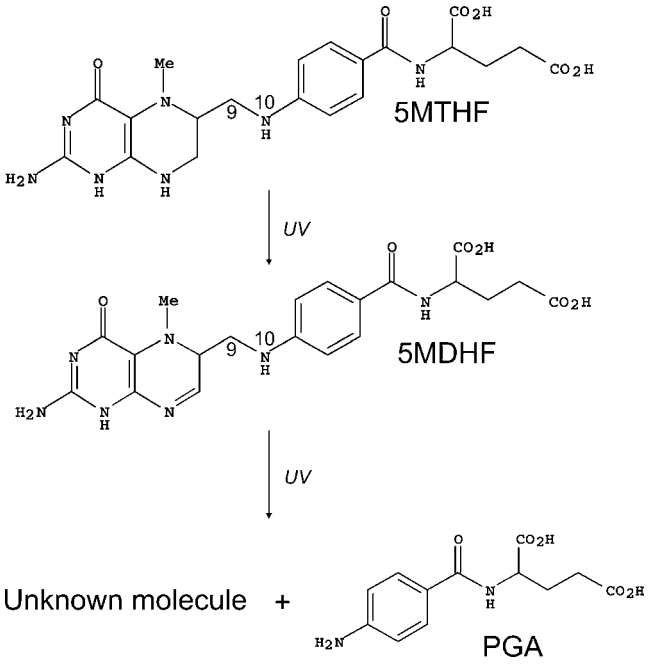

Figure 8. A possible scheme of the photodegradation of 5MTHF.

constant is five times larger than that of an unirradiated solution (1.8×10^{-3} min^{-1}).

In Fig. 8 a scheme of 5MTHF photodegradation is proposed. The unknown molecule is probably a reduced form of a pterin. 5MTHF is first oxidized to 5MDHF, and then the molecule is cleaved into PGA and an unknown pterin.

5MTHF absorbs UV radiation, mostly in the UVB region. This means that the UV penetrating the epidermis and reaching the human blood, mainly UVA (17), probably does not degrade 5MTHF significantly by direct absorption. It is more likely that 5MTHF in the human body is oxidized by radical oxygen species produced by naturally photosensitizers (flavins, porphyrins, bilirubin, *etc.*) after UVA and near-UV exposure. 5MTHF is a strong antioxidant, comparable to ascorbic acid (18,19). The oxidized form of 5MTHF, 5MDHF, is probably not re-entering the folate pool (20), and therefore the first step of folate degradation is sufficient to reduce the folate concentrations in humans. Further studies have to be performed to be able to conclude whether folates are degraded in humans and the implications for human health.

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