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Reaction of Folic Acid with Reducing Sugars and Sugar Degradation Products

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The reaction of folic acid with reducing sugars (nonenzymatic glycation) under conditions that can occur during food processing and preparation was studied by high-performance liquid chromatography with diode array detection. *N*-(*p*-Aminobenzoyl)-L-glutamic acid, a well-established oxidation product, was detected in the reaction mixtures. Furthermore, a new product was isolated and identified as N^2-[1-(carboxyethyl)]folic acid (CEF). CEF was the main product that was formed by the nonenzymatic glycation of folic acid. For preparation, N^2-[1-(carboxyethyl)]folic acid was obtained in high yields when folic acid and dihydroxyacetone (DHA), a sugar degradation product, were heated at 100 °C in phosphate buffer. Mixtures of folic acid and different sugars or DHA were heated under variation of reaction time and temperature, and CEF was quantified. Up to 50% of the vitamin was converted to CEF, with highest yields formed from maltose (49%) and lactose (43%).

KEYWORDS: Folic acid; nonenzymatic glycation; Maillard reaction; N^2-[1-(carboxyethyl)]folic acid; folate degradation; HPLC

INTRODUCTION

Folate is an essential component of our nutrition. During heat treatment and storage of food products, a loss of folate can be observed. Whereas its degradation during cooking of vegetables or meat seems to be rather slow, a considerable decrease of folate concentration was detected, for example, in parenteral nutrition (*1*) and beer (*2*) during storage or in heated milk. Storage of milk powder under adverse conditions can lead to an almost complete loss of folate (*3*), but even pasteurization results in up to 16% reduction (*4*).

It is assumed that the main degradation pathways include thermal degradation by oxidation (*5*) and subsequent C9−N10 bond cleavage, yielding pterin-6-carboxylic acid and *N*-(*p*-aminobenzoyl)-L-glutamate. The same oxidation products were also found after photodegradation of folate in the presence of UV light (*6*).

During food processing, reducing sugars react with amines, for example, proteins, leading to a great variety of products. This process is commonly referred to as nonenzymatic glycation or the Maillard reaction (*7*). The reaction products have a high impact on the quality of food, for example, by browning, flavor formation, or antioxidative activity. On the other hand, lysine modification by Maillard products leads to a decrease of nutritional value.

As a pterin derivative, folate contains an exocyclic amino group linked to a pyrimidine ring. It was shown earlier that the amino groups of guanine derivatives, which possess a similar moiety, react readily with sugars and sugar degradation products. At 100 °C, glucose leads to the formation of a N^2-(glucosylamino)guanine derivative (*8*), whereas at 37 °C 2-(N^2-guanosyl)-4,5,6-trihydroxyhexanoate (*9*) and N^2-(carboxyethyl)guanine (*10*) are formed as the main products. Under oxidative conditions, the modification rate is greatly enhanced and N^2-(carboxymethyl)guanine is formed as the main product (*11*). Other precursors such as D/L-glyceraldehyde, dihydroxyacetone (*12*), or dehydroascorbic acid cause even higher modification rates than glucose itself (*13*).

Therefore, we investigated in this study folate degradation in the presence of reducing carbohydrates and sugar degradation products by nonenzymatic glycation reaction.

MATERIALS AND METHODS

Apparatus. ^{1}H NMR (360.1 MHz) and ^{13}C NMR (90.6 MHz) spectra were recorded with a Brucker AM 360 FT-NMR spectrometer with tetramethylsilane as internal standard in DMSO-d_6 and acetone in D_2O. Chemical shifts are reported in parts per million. Negative FAB mass spectral analysis was performed with an HP 5989 A MS engine and a Kretos MS 80 RFA spectrometer and high-resolution MS with a Finnigan/MAT-MAT 95Q. Analytical HPLC was performed with a Jasco PU-1580 Intelligent HPLC pump, Jasco DG-980-50 three-line degasser, Jasco LG-980-02S tenery gradient unit, Jasco AS-1555 Intelligent sampler, and Jasco MD-1510 multiwavelength diode array detector that included Jasco Borwin software. UV spectra were directly taken from this system and are given in nanometers. For preparative HPLC, the same instruments were used with the exception that the analytic photocell was replaced by a preparative cell.

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Chemicals and Reagents. Deionized water for HPLC was distilled before use. HPLC grade methanol was used without further purification. All chemicals were of analytical reagent grade quality. Dihydroxyacetone, D-fructose, and D-maltose were obtained from Merck (Darmstadt, Germany). Folic acid, D-glucose, D-glucose-6-phosphate, ammonium formiate, D-lactose, and d-ribose were purchased from Fluka-Sigma-Aldrich (Deisenhofen, Germany).

High-Performance Liquid Chromatography. Analytical HPLC was performed on a column packed with Nucleosil (RP 18, 125 × 3 mm i.d., 5 μm particle size) from Macherey and Nagel (Düren, Germany) and protected with a guard cartridge (8 × 3 mm) packed with the same material as the column. The eluents used were ammonium formate buffer (20 mmol, pH 3.65, solvent A), methanol (solvent B), and water (solvent C). For elution, a gradient was used of 10% solvent A for the whole run time, 10−40% solvent B from 0 to 12 min, 40−90% solvent B from 12 to 15 min, and then continuing with 90% B from 15 to 22.5 min at a flow rate of 0.8 mL/min. The substances were detected with a diode array detector from 190 to 600 nm.

Preparative HPLC was performed on a column packed with Nucleosil (RP-18, 250 × 21 mm i.d., 10 μm particle size) from Macherey and Nagel. The column was protected with a guard column packed with Nucleosil (RP-18, 30 × 16 mm i.d., 7 μm particle size). As eluents, ammonium formate buffer (50 mmol, pH 4.5, solvent A), methanol (solvent B), and water (solvent C) were used. A gradient of 10% solvent A for the whole run time and 10−50% solvent B from 0 to 25 min and 90% solvent B from 30 to 35 min at a flow rate of 6 mL/min was used. The detection wavelength was 283 nm.

Preparation of N^2-[(1-Carboxy)ethyl]folic Acid (CEF). Folic acid (pteroylglutamic acid) (441.5 mg, 1 mmol) and dihydroxyacetone (90.1 mg, 4 mmol) were incubated in a closed vessel in phosphate buffer (1 M, pH 7.4) at 37 °C for 30 days in a shaking water bath. The isolation of CEF was achieved by preparative HPLC as described above. The diastereomers could not be separated by HPLC; therefore, one peak with the retention time of 14.7 min was collected. Methanol was removed under reduced pressure, and the aqueous solution was lyophilized. N^2-Carboxyethylfolic acid was obtained as a bright yellow solid (yield after purification = 22.8%): ^{1}H NMR (DMSO-d_6 COSY) δ 1.37 (d, 3H, ^3J = 7.4 Hz, H-3′), 1.85−2.05 (m, 2H, ^3J = 6.9, 7.8 Hz, H-21a,b), 2.60 (dd, 2H, ^3J = 7.8, 7.4 Hz, H-22), 4.25−4.35 [m(ddd, 1H, ^3J = 6.9, 7.8 Hz, H-20/q, 1H, ^3J = 7.4 Hz, H-2′)], 4.50 ("s", 2H, H-9), 6.65 (d, 2H, ^3J = 8.7 Hz, H-12,14), 6.93 (t, 1H, ^3J = 4.6, 6.0 Hz, H-10), 7.65 (d, 2H, ^3J = 8.7 Hz, H-13,15), 7.95 (d, 1H, ^3J = 6.9 Hz, H-18), 8.64 (s, 1H, H-7) (quotation marks around a signal assignment indicate the apparent signal that represents obviously a doublet signal); ^{13}C NMR (DMSO-d_6, C,H-COSY) δ 18.56 (CH_3-3′), 27.08 (CH_2-21), 31.23 (CH_2-22), 45.93 (CH_2-9), 50.55 (CH-2′), 52.45 (CH-20), 111.23 (CH-12, CH-14), 121.69 (C-16), 128.14 (C-4a), 128.76 (CH-13, CH-15), 148.31 (CH-7), 150.66 (C-11), 151.65 (C-8a), 151.83 (C-2), 156.26 (C-6), 161.02 (C-4), 164.73 (C-17), 174.23 (C-19), 174.51 (C-23), 174.82 (C-1′); FAB-MS (Cs, 20 kV, m-NBA) (negative ion mode), m/z 512.7 [M − H] ; exact mass for $C_{22}H_{23}N_7O_8$ = 512.1530; found = 512.1532 by ESI-MS [M − H] (negative ion mode); UV λ_{max} 283, 355 nm.

The two diastereomers of CEF are indistinguishable in UV absorption and ^{1}H NMR/^{13}C NMR in DMSO-d_6, but the ^{1}H NMR and ^{13}C NMR in D_2O show a peak splitting of the benzoylic protons and carbons, which is analytical evidence of the coincidence of the two diastereomers.

Reaction of Folic Acid with Dihydroxyacetone in Phosphate Buffer at Different Temperatures. Folic acid (441.5 mg, 1 mmol) and dihydroxyacetone (90.1 mg, 4 mmol) were dissolved in 10 mL of phosphate buffer (1 M, pH 7.4) and incubated in a closed vessel at 100 °C. Samples were taken after 0, 0.5, 1, 1.5, 2, 3, and 4 h. Additionally, folic acid and DHA (as described above) were incubated at 37 and 70 °C. Samples incubated at 70 °C were taken after 0, 3, 6.5, 9.5, 21, 24, 27, and 45 h, whereas samples incubate at 37 °C were taken after 25, 58, and 86 days.

Reaction of Folic Acid with D-Glucose, D-Glucose-6-phosphate, D-Fructose, D-Ribose, D-Maltose, and D-Lactose in Phosphate Buffer at 37, 70, and 100 °C. Folic acid (441.5 mg, 1 mmol) and D-glucose (720.64 mg, 4 mmol), D-glucose-6-phosphate (1360.52 mg, 4 mmol), d-fructose (720.64 mg, 4 mmol), D-ribose (600.52 mg, 4 mmol), D-maltose (1441.28 mg, 4 mmol), or D-lactose (1369.2 mg, 4 mmol) were dissolved in 10 mL of 1 M phosphate buffer (pH 7.4) and incubated in a closed vessel at 37, 70, and 100 °C. Samples were taken after 0, 1, 2, 3, 4, 5, 8, and 24 h at 70 and 100 °C. The mixtures that stood at 37 °C were analyzed after 25, 58, and 86 days.

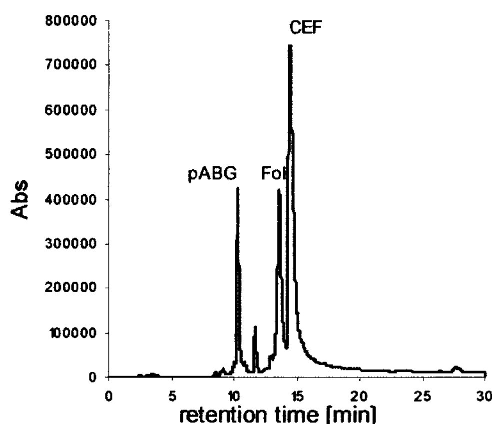

Figure 1. HPLC chromatogram of a mixture of folic acid and dihydroxyacetone that was heated at 100 °C for 24 h in phosphate buffer (pH 7.4) (UV detection at 283 nm). Fol, folic acid. For chromatographic conditions see Materials and Methods.

Quantification. The contents of CEF in the reaction mixtures of folic acid and DHA or D-glucose, d-glucose-6-phosphate, D-fructose, D-ribose, D-maltose, or D-lactose were determined. Identification of the product in the reaction mixtures was achieved by comparison of the retention time and UV spectra with those of the isolated reference compound. An external calibration curve was calculated from injections of solutions of 28.75, 57.5, 61, 115, 122, 172.5, 230, 290, 345, 414, 460, and 690 μg/mL CEF. Quantification was achieved using DAD-System-Manager software Jasco Borwin version 1.5 (Jasco) with manual baseline correction.

The yields are given as a percentage relative to the starting concentration of folic acid and are means of triplicates.

Determination of the Time-Dependent Degradation of Folic Acid at 100 °C. Folic acid (441.5 mg, 1 mmol) was dissolved in 10 mL of phosphate buffer (1 M, pH 7.4) and incubated in a closed vessel at 100 °C. Samples were taken after 0, 1, 2, 3, 4, 5, 6, 7, and 8 h. The loss of folic acid was determined by quantification of the non-degraded folic acid using an external calibration curve.

RESULTS AND DISCUSSION

Isolation and Identification of N^2-[(1-Carboxy)ethyl]folic Acid. When folic acid and carbohydrates are heated in neutral aqueous solutions or allowed to stand for a longer period at 37 °C, a reaction product can be detected by HPLC/DAD that is characterized by an absorption maximum at 283 nm (**Figure 1**). The highest yield of this new product was observed when folic acid was heated in phosphate buffer under neutral conditions with DHA. DHA is a sugar degradation product that is formed in food, for example, by retro-aldol cleavage of fructose and that reacts readily in nonenzymatic glycation reactions.

The described product was then isolated from the mixture and identified by spectral analyses as CEF. The ^{1}H NMR spectrum of the product in DMSO-d_6 features, in addition to the signals of folic acid, a characteristic doublet of a methyl group at 1.37 ppm, which couples with the methine group at 4.25−4.35 ppm. This methine group causes a signal in the HMBC NMR spectrum that correlates with the C^2 of the pteridinyl ring. The exchangeable protons at N^{10} and N^{18} give rise to obvious one-proton resonances at 6.93 and 7.95 ppm, respectively. Chemical shifts of ^{13}C NMR indicate the presence of a carboxyethyl group as well. In both ^{1}H NMR and ^{13}C NMR

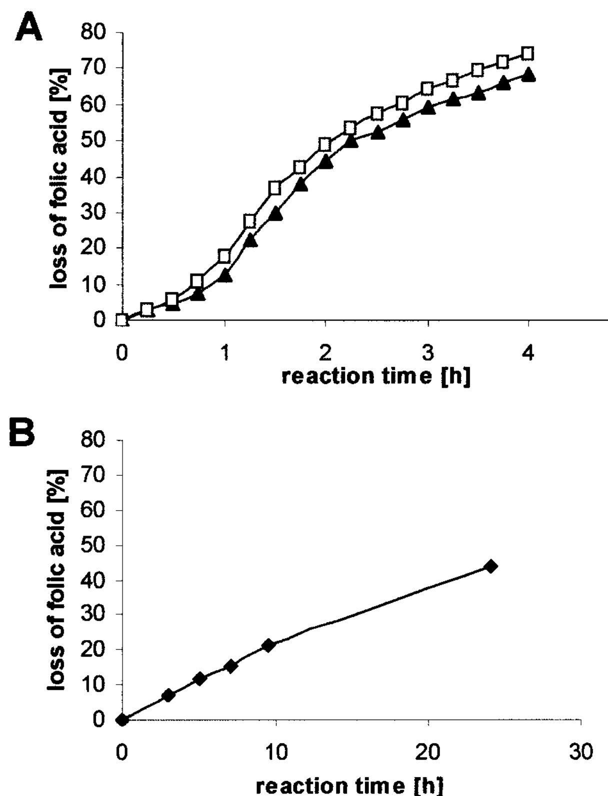

Figure 2. Time-dependent loss of folic acid during the heating of 0.1 mM folic acid in neutral phosphate buffer at 100 °C (A) in the presence of 0.4 mM dihydroxyacetone (□, total loss of folic acid; ▲, loss of folic acid due to CEF formation) or (B) in the absence of carbohydrates.

spectra of the substance recorded in D_2O, a peak splitting of the signals of the benzoylic ring gave analytical evidence of the coincidence of two diastereomers. The negative FAB-MS spectrum of the adduct showed a base peak at *m/z* 512.7 corresponding to the [M − H] ion, and high-resolution ESI-MS had a consistent, exact mass for $C_{22}H_{23}N_7O_8$ [M − H]. Additionally, we noted a bathochromic shift of the UV maxima of the product to λ_{max} 283 and 355 nm, compared to λ_{max} 279 and 347 nm for folic acid. Taken together, these data were consistent with a structure bearing a carboxyethyl moiety attached to the N^2 position of folic acid. The proposed structure is in accordance with previous findings that describe DHA as a very potent glycation agent, which readily and almost exclusively forms carboxyethyl adducts (*12*). Similar to the reaction of DHA with guanosine that forms N^2-[1-(1-carboxy)ethyl]guanosine (CEG), the carboxyethyl moiety of CEF is covalently linked to the NH_2 group at position 2 of the pterin ring.

Kinetic Analysis of CEF Formation and Comparison to Other Degradation Pathways. A representative kinetic analysis of CEF formation at 100 °C from DHA and folic acid is shown in **Figure 2A** and indicates that CEF is formed in a fast, time-dependent manner. CEF was detected after <30 min, and the maximum yield of almost 68% was reached after 4 h of reaction. To compare the loss of the vitamin as a result of nonenzymatic glycation with the thermal degradation, mainly caused by oxidation (*14*), we heated folic acid in the absence and presence of carbohydrates under the same conditions and determined the remaining folic acid by HPLC/DAD (**Figure 2B**). In contrast to the nonenzymatic glycation reaction, the loss due to thermal degradation was slower. Without carbohydrates added, the vitamin content decreased continuously with a maximum loss of almost 45% after 25 h of heating. After 3 h of incubation with DHA at 100 °C, CEF formation leads to a 10-fold higher reduction of the folic acid content (59%) than thermal degradation (7%).

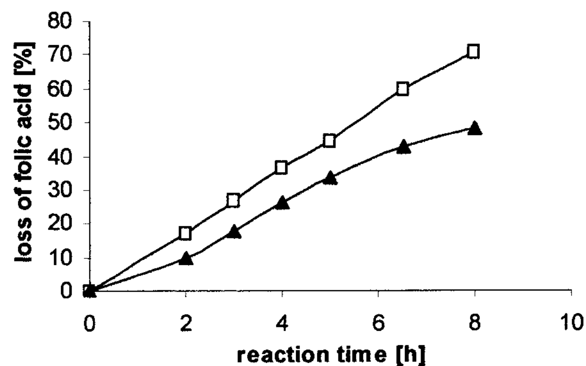

Figure 3. Time-dependent loss of folic acid in a mixture of folic acid (0.1 mM) and maltose (0.4 mM) under neutral conditions at 100 °C (□, total loss of folic acid; ▲, loss of folic acid due to CEF formation). CEF was measured by HPLC/DAD as described under Materials and Methods.

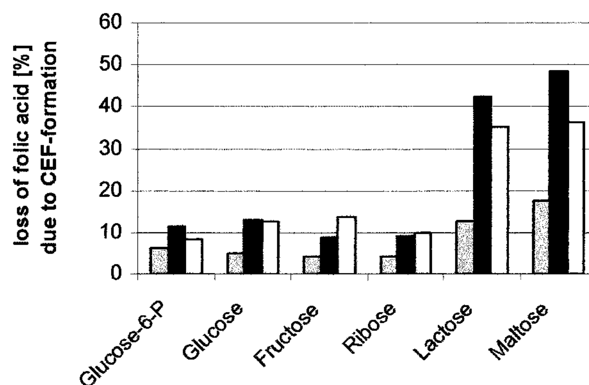

Figure 4. Loss of folic acid (due to CEF formation) in mixtures of folic acid and various sugars that were heated at 100 °C for 3 h (gray bars), 8 h (black bars), and 24 h (white bars) at pH 7.4.

Investigations of the Reaction of Folic Acid with Different Sugars. The most relevant carbohydrates in foodstuffs are glucose, fructose, ribose, maltose, lactose, and glucose-6-phosphate. Therefore, we investigated CEF formation from these sugars under conditions that can occur during food processing and preparation. Although the amounts of CEF in the samples incubated at 70 °C for 3, 8, and 24 h were nearly at the detection limit and could not be quantified, CEF could be detected in every sample when the reaction temperature was raised to 100 °C (**Figure 3**, shown for maltose). Samples were taken every hour for 8 h and after 24 h. The yields were determined by HPLC/DAD. Similar to the experiments with DHA, the nonenzymatic glycation of folic acid by maltose was fast and time-dependent. Loss of folic acid by CEF formation was determined after <1 h and was 2-fold higher compared to incubation of the vitamin in the absence of sugar and sugar degradation products. Considerable amounts of CEF were formed from all carbohydrates investigated, most notably in the reaction mixtures of maltose and lactose, with maximum yields of almost 49 and 43%, respectively, after 8 h. Appreciable differences in the formation rate of pABG among the various sugars could not be determined (data not shown). Contrary to our expectations, the monosaccharides glucose, fructose, ribose, and glucose-6-phosphate reacted not as intensely as the disaccharides and led to losses of folic acid from almost 7% for ribose to 13% for glucose after 8 h of heating (**Figure 4**). CEF is not a stable end product so a decrease of product yield was noticed during

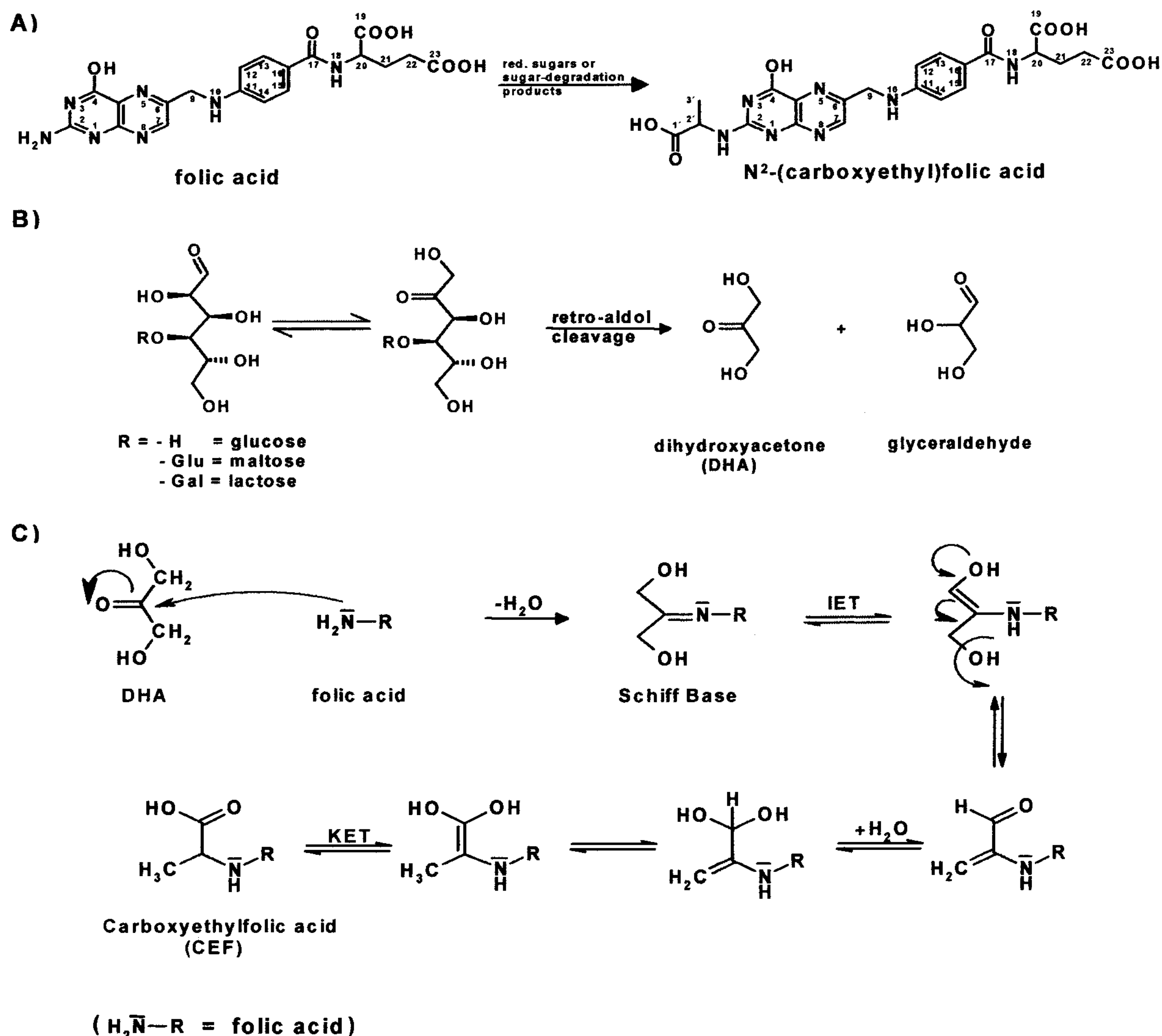

Figure 5. (A) Nonenzymatic glycation of folic acid by reducing carbohydrates resulting in the formation of carboxyethylfolic acid. (B) Formation of dihydroxyacetone by retro-aldol cleavage of sugars. (C) Proposed mechanism for the formation of CEF from dihydroxyacetone and folic acid (IET = imine–enamine tautomery; KET = keto–enol tautomery).

prolonged heating (24 h), which could result, for example, from oxidative degradation.

CEF was also detected in long-term incubations (12 weeks) of folic acid and carbohydrates, which were kept at 37 °C and pH 7.4 (data not shown).

A reaction mechanism for the formation of CEF from DHA and folic acid is proposed in **Figure 5**. It is likely that DHA reacts directly with folate to form the carboxyethyl moiety and is not converted to methylglyoxal. The latter leads mainly to cyclic reaction products (*15*).

Conclusion. These results indicate that under certain conditions nonenzymatic glycation may represent an important pathway of folic acid degradation in addition to the well established thermal degradation by oxidation. Therefore, CEF, which is a nonenzymatic glycation product of folic acid, deserves particular attention with regard to the loss of folate's nutritional value in foodstuff during heating. It can be expected that particularly products that contain an excess of lactose such as heated milk, milk powder, or infant formula are prone to CEF formation. Furthermore, CEF formation can be expected in heat-treated cereal-derived products such as biscuits and breakfast cereals, which are rich in folates and maltose, and in folate-enriched, pasteurized fruit juices. Besides investigations of CEF formation in foodstuffs, the bioavailability and the vivo activity of CEF should be determined. Additionally, pathophysiological and toxicological effects on human metabolism of this newly identified product formed by nonenzymatic glycation of folic acid cannot be ruled out.

ABBREVIATIONS USED

CEF, N^2-[1-(carboxy)ethyl]folic acid; DHA, dihydroxyacetone; pABG, *N*-(*p*-aminobenzoyl)-L-glutamate; Glc, glucose; Glc-6-P, glucose-6-phosphate; DAD, diode array detection; HPLC, high-performance liquid chromatography; HMBC NMR, heteronuclear multiple bond correlation NMR; Fol, folic acid.

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