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Formation of the Thiocyanate Conjugate of Chlorogenic Acid in Coffee under Acidic Conditions in the Presence of Thiocyanate and Nitrite: Possible Occurrence in the Stomach

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The objective of the present study was to elucidate how chlorogenic acid in coffee was transformed under acidic conditions simulating the mixture of saliva and gastric juice. When coffee was incubated in acidified saliva that contained nitrite and SCN^-, in addition to nitric oxide (NO), four major components were detected. Two of the four components (components 3 and 4) were generated when chlorogenic acid was incubated in acidified saliva and when incubated in an acidic buffer solution in the presence of both nitrite and SCN^-. By the incubation of chlorogenic acid in acidic nitrite in the absence of SCN^-, components 3 and 4 were not formed but the quinone of chlorogenic acid and nitrated chlorogenic acid were formed. The result indicates that SCN^- was indispensable for nitrous acid induced formation of components 3 and 4. Component 4 was isolated and its structure was determined to be (*E*)-5′-(3-(7-hydroxy-2-oxobenzo[*d*] [1,3]oxathiol-4-yl)acryloyloxy)quinic acid. Component 3, which was suggested to be 2-thiocyanatochlorogenic acid, seemed to be formed by the reaction between SCN^- and the quinone of chlorogenic acid. As it has been reported that the quinone of chlorogenic acid can react with thiols and can decompose producing H_2O_2, the formation of component 4 can reduce the toxic effects of the quinone of chlorogenic acid.

KEYWORDS: Coffee; chlorogenic acid; nitrous acid; saliva; thiocyanate

INTRODUCTION

Seeds of coffee plants (*Coffea arabica* L. and *Coffea canephora* Pierre ex Frohner) contain chlorogenic acid (5′-caffeoylquinic acid) (**Figure 1**) and its isomers (3′-caffeoylquinic acid and 4′-caffeoylquinic acid). After roasting the seeds, they are used to make coffee. Many effects of coffee drinking on the human body have been reported (*1*). Immediately after coffee drinking, almost all of the components in coffee are mixed with gastric juice in the stomach. Small amounts of the components may bind to epithelial cells of the oral cavity. The binding is deduced from reports that flavonoids bind to oral epithelial cells after ingesting flavonoid-rich foods (*2*, *3*). The epithelial cells, which bind components in coffee like chlorogenic acid, are easily detached and are washed away from the oral cavity by saliva to be mixed with gastric juice. As saliva normally contains about 0.2 mM nitrite that is transformed to nitrous acid ($pK_a = 3.3$) under acidic conditions, components in coffee, which bind to epithelial cells, can react with nitrous acid in the stomach. The concentration of nitrite in saliva increases to 1∼2 mM after nitrate-rich foods have been ingested (*4*). In addition to nitrite, thiocyanate ($pK_a = 0.9$) is also present in saliva at a concentration of about 1 mM (*5*).

It has been reported that some dietary phenolics with antioxidative activity can reduce nitrite to nitric oxide (NO) under acidic conditions (*6*−*8*), among which are quercetin, caffeic acid, gallic acid, and chlorogenic acid. The *o*-dihydroxyl group of the phenolics may participate in the reduction of nitrous acid to NO. As coffee contains chlorogenic acid, it is possible that this reduces nitrous acid to NO when saliva is mixed with gastric juice after ingestion of coffee. On the other hand, nitration of chlorogenic acid in coffee by nitrogen oxides derived from NO and nitrous acid is also possible (*8*, *9*). At present, there seem to be no reports on the reactions of chlorogenic acid in coffee with nitrous acid in acidified saliva. As saliva contains SCN^-, its effects on the reaction between nitrous acid and chlorogenic acid in coffee should be taken into consideration. The effects of SCN^- on the reduction of nitrous acid by ascorbic acid (*10*) and the nitrous acid induced nitrosation of amines (*11*) and nitration of phenolics (*12*, *13*) have been reported.

One of the objectives of the present study was to characterize the reactions between nitrous acid and chlorogenic acid in coffee in acidified saliva simulating the mixture of saliva and gastric juice. The reaction between nitrous acid and chlorogenic acid

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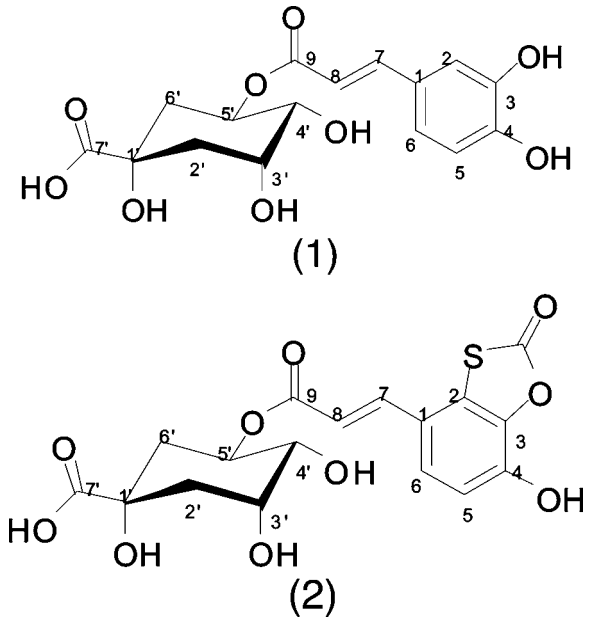

Figure 1. Chemical structure of chlorogenic acid (**1**) and (*E*)-5′-(3-(7-hydroxy-2-oxobenzo[*d*] [1,3] oxathiol-4-yl)acryloyloxy)quinic acid (**2**).

was also studied in an acidic buffer solution. The other objective was to elucidate how the salivary component SCN^- affects the reaction between nitrous acid and chlorogenic acid.

MATERIALS AND METHODS

Reagents. Instant coffee (freeze-dried) was obtained from a local market. Chlorogenic acid and Griess-Romjin reagent for nitrite were from Wako Pure Chem. Ltd. (Osaka, Japan). *N*-(Dithiocarboxy)-sarcosine was from Dojin (Kumamoto, Japan).

Preparation of Saliva. After their informed consent had been obtained, mixed whole saliva (5 mL) was collected at 9:00−10:00 a.m. from healthy volunteers (male, 56 years old; female, 28 and 59 years old) by chewing Parafilm. The collected saliva was centrifuged at 20000*g* for 5 min, and the supernatant was used as saliva. The concentrations of nitrite and SCN^- were measured using Griess-Romjin reagent and acidic Fe(III), respectively, as reported previously (*14*, *15*).

Binding of Chlorogenic Acid to Epithelial Cells. Whole saliva contained detached epithelial cells of the oral cavity. To study whether chlorogenic acid in coffee binds to the cells, various amounts of coffee (10 mg/mL) were added to 0.5 mL of mixed whole saliva. After incubation for 10∼15 s at about 25 °C, the saliva was centrifuged at 6500*g* for 5 min. The pH of the supernatant was decreased to 3∼3.5 by adding 1 M HCl to precipitate proteins, and then the supernatant was filtered with a cellulose acetate filter (0.45 μm) (Advantec, Tokyo, Japan). The sediment was suspended in 0.5 mL of methanol and then was centrifuged at 6500*g* for 5 min. An aliquot (0.05 mL) of the supernatant was also applied to a high-performance liquid chromatography (HPLC) column to determine the concentration of chlorogenic acid. The concentrations of chlorogenic acid in the precipitate were calculated using the volume of the precipitate.

Measurement of Electron Spin Resonance (ESR) Signals. ESR spectra were measured using a JE1XG spectrometer (JEOL, Tokyo, Japan) at about 25 °C using a quartz flat cell (0.05 mL). The conditions for the measurement were as follows: microwave power, 10 mW; line width, 0.5 mT; amplification, 1000-fold; scanning speed, 2.5 mT/min (*6*). The reaction mixture (0.5 mL) contained 0.2 mM $NaNO_2$ and 0.5 mg of coffee/mL or 0.1 mM chlorogenic acid in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2.0). When saliva was used, the reaction mixture (0.5 mL) contained 0.5 mg of coffee/mL or 0.1 mM chlorogenic acid in a mixture of 0.25 mL of saliva and 0.25 mL of 50 mM KH_2PO_4−50 mM KCl−HCl (pH 1.4). The final pH was 1.8∼1.9. After incubation of the mixtures for 30 s, 0.5 mL of solution, which contained 5 mM *N*-(dithiocarboxy)sarcosine and 1.5 mM $FeCl_3$ in 0.1 M sodium phosphate (pH 7.6), was added to the mixture. The pH after the addition of the Fe-[*N*-(dithiocarboxy)sarcosine]$_2$ solution was between 6.5∼7. Immediately after the addition of the Fe-[*N*-(dithiocarboxy)sarcosine]$_2$ solution, an aliquot (0.05 mL) was withdrawn into the flat cell and ESR spectra were measured. It is known that Fe-[*N*-(dithiocarboxy)-sarcosine]$_2$ complex reacts with NO producing a stable radical NO−Fe-[*N*-(dithiocarboxy)sarcosine]$_2$ complex around neutral pH (*16*−*18*).

Spectrophotometric Measurements. Spectrophotometric measurements were performed using model 557 (Hitachi, Tokyo, Japan) and UV-260 (Shimadzu, Kyoto, Japan) spectrophotometers at room temperature (20−25 °C). Path length of the measuring beam was 4 mm. Changes in absorption spectra of coffee and chlorogenic acid, which were dissolved in acidified saliva, were measured using a model 557 spectrophotometer as the reaction mixture was turbid. The reaction mixture (1 mL) contained 0.3 mg of coffee/mL or 0.1 mM chlorogenic acid in the mixture of 0.5 mL of saliva and 0.5 mL of 50 mM KH_2PO_4−50 mM KCl−HCl (pH 1.4). The pH of the reaction mixture was 1.8−1.9. Reactions were started by adding coffee or chlorogenic acid. When changes in absorption spectra were measured in an acidic buffer solution, a UV-260 spectrophotometer was used. The reaction mixture (1 mL) contained 0.1 mM $NaNO_2$ and 0.3 mg of coffee/mL or 0.05 mM chlorogenic acid in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2.0). Reactions were started by the addition of nitrite.

HPLC. HPLC for the quantification of chlorogenic acid was performed at room temperature. A column used was a 150 × 6.0 mm i.d. Shim-Pack CLC-ODS column (Shimadzu), and components separated were detected with an SPD-M10Avp spectrophotometric detector with a photodiode array (Shimadzu). Mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (1:2, v/v), the pH of which was adjusted to 3.0 by 1 M H_3PO_4. The flow rate was 1 mL/min. After incubation of acidified saliva which contained coffee (0.3 mg/mL) or chlorogenic acid (0.1 mM) in a mixture of 0.5 mL of saliva and 0.5 mL of 50 mM KH_2PO_4−50 mM KCl−HCl (pH 1.3) for defined periods at about 25 °C, the mixture was filtered using a cellulose acetate filter (0.45 μm) (Advantec, Tokyo, Japan) and 25 μL of the filtrate was applied to the HPLC column. When coffee (0.3 mg/mL) or chlorogenic acid (0.1 mM) was incubated in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2.0) in the presence of 0.2 mM $NaNO_2$, 25 μL of the mixture was directly applied to the column after defined periods of incubation. Anaerobic experiments were performed under argon gas.

Changes in concentration of SCN^- were also measured by HPLC. SCN^- could be separated from nitrite and from chlorogenic acid and its products using a 150 × 6 mm i.d. Shim-Pack CLC-C_8 column (Shimadzu). The mobile phase used was a mixture of methanol and 25 mM KH_2PO_4 (1:4, v/v), the pH of which was adjusted to 3 by 1 M H_3PO_4 and the flow rate was 1 mL/min. SCN^- separated was detected at 210 nm and the retention time was 3.6 min. The concentration of SCN^- was calculated from area under the peak.

Liquid chromatography/mass spectrum (LC/MS) of the products of chlorogenic acid was performed using an Agilent 1100 series LC/MSD combined with a photodiode array detector. A column used for HPLC was a Shim-Pack CLC-ODS column, and mobile phases were mixtures of acetonitrile and 10 mM ammonium acetate [mobile phase-1, 1:10 (v/v); mobile phase-2, 1:6 (v/v)], the pH of which was adjusted to 4.5 by HCl and the flow rate was 1 mL/min. Ionization mode was API-ES. After incubation of the reaction mixture (1 mL) for defined periods, which contained 0.1 mM chlorogenic acid and 0.2 mM $NaNO_2$ in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2.0), 25 μL of the mixture was applied to the HPLC column.

Isolation of a Component Formed in HNO_2/SCN^-/Chlorogenic Acid Systems. Chlorogenic acid (0.1 g) was incubated in the reaction mixture (300 mL) that contained 10 mM NaSCN and 1 mM $NaNO_2$ in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2) for 60 min at about 25 °C, and then the mixture was extracted with 250 mL of ethyl acetate three times. The ethyl acetate extracts were combined and dried with anhydrous sodium sulfate. After evaporating ethyl acetate with a rotary evaporator, the residue was dissolved in 50 mL of ethyl acetate and the solvent was evaporated with a rotary evaporator again. Methanol (1 mL) was added to the residue, and then 3 mL of water was added. The solution of the mixture of methanol and water was kept at 4 °C for one night. A white precipitate was formed, which was collected by

centrifugation and was dried in vacuo. The yield was about 30 mg. This procedure was repeated twice. The dried materials were combined (about 60 mg in total) and dissolved in 2 mL of methanol to apply to a 35 × 2.2 cm i.d. column of Sephadex LH-20 equilibrated with methanol. The column was eluted with methanol (150 mL), and 3 mL fractions were collected. The component isolated was found in fractions between 30 and 38 with absorption peaks at 230 and 309 nm. The fractions were combined, and methanol was evaporated with a rotary evaporator. The residue was dissolved in 1 mL of methanol. When the methanol solution was kept at 4 °C for one night, a white precipitate was formed. The precipitate was collected by centrifugation and was dried in vacuo. The compound obtained (about 40 mg) was used for analysis.

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with an ECA-500 FT-NMR spectrometer (JEOL) with CD_3OD as the solvent and tetramethylsilane as the internal standard. ^{1}H NMR was performed at 500.16 MHz, and the ^{1}H−^{1}H chemical shift correlated (COSY) technique was employed to assign ^{1}H shifts and couplings. ^{13}C NMR was performed at 125.77 MHz with proton decoupling. Heteronuclear multiple-bond correlation (HMBC) and heteronuclear multiple-quantum coherence (HMQC) techniques were used to assign correlations between ^{1}H and ^{13}C signals. Electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) mass spectra of the isolated compound were measured with an LCMS QP8000α quadrupole mass spectrometer (Shimadzu). Sample was delivered into the ion source using methanol/water/formic acid (50:50:0.2, v/v/v) at 0.2 mL/min. Elemental analysis was performed by a CHN Corder MT-6 (Yanako CO., Kyoto, Japan).

The isolated compound had the following characteristics: UV (methanol) λ max, 225, 309 nm; ESI-MS (negative mode), *m*/*z* 411 ([M − H]$^-$), 219, 191, 163; APCI-MS (positive mode), *m*/*z* 413 ([M + H]$^+$), 221; ^{1}H NMR (CD_3OD) δ 2.05 (m, 1H, H-6′a), 2.08 (m, 1H, H-2′a), 2.18 (m, 1H, H-2′b), 2.24 (m, 1H, H-6′b), 3.74 (dd, J = 2.9, 8.6 Hz, 1H, H-4′), 4.17 (m, 1H, H-5′), 5.36 (ddd, J = 4.6, 9.2, 9.2 Hz, 1H, H-3′), 6.27 (d, J = 16.0 Hz, 1H, H-8), 6.90 (d, J = 8.6 Hz, 1H, H-5), 7.43 (d, J = 8.6 Hz, 1H, H-6), 7.54 (d, J = 16.0 Hz, 1H, H-7); ^{13}C NMR (CD_3OD) δ 36.95 (C-2′), 37.53 (C-6′), 70.10 (C-5′), 71.18 (C-3′), 72.22 (C-4′), 74.92 (C-1′), 115.20 (C-5), 117.73 (C-8), 120.11 (C-1), 123.89 (C-2), 126.87 (C-6), 136.61 (C-3), 140.99 (C-7), 144.47 (C-4), 166.29 (C-9), 167.56 (C═O), 175.77 (C-7′). Anal. calcd for $C_{17}H_{16}O_{10}S\cdot0.6H_2O$: C, 48.24; H, 4.10. Found: C, 48.35; H, 4.16.

RESULTS AND DISCUSSION

Binding of Chlorogenic Acid to Epithelial Cells. The concentration of chlorogenic acid in the precipitate of whole saliva filtrate was about 1.5-fold of that in the supernatant independent of the concentration of coffee added (10−400 μg/mL) to the saliva. Isomers of chlorogenic acid also bound to the filtrate. This result indicates that chlorogenic acid can bind to epithelial cells of oral tissues. If chlorogenic acid remains in the oral cavity, it is washed away slowly from oral cavity by saliva. The chlorogenic acid washed away from the oral cavity can also mix with gastric juice to react with salivary nitrite and SCN^- as described below.

NO Formation. The concentrations of nitrite and SCN^- in saliva used in this study were 0.18 ± 0.02 and 0.82 ± 0.14 mM (means with standard deviations (SDs)). **Figure 2** shows typical ESR spectra of the NO−Fe-[*N*-(dithiocarboxy)sarcosine]$_2$ complex formed in acidified saliva simulating the mixture of saliva and gastric juice. Coffee (0.5 mg/mL) enhanced the formation of NO by about 3.5-fold and chlorogenic acid (0.1 mM) by about 2-fold. The enhancement was also observed in other saliva preparations. NO−Fe-[*N*-(dithiocarboxy)sarcosine]$_2$ complex was also formed in an acidic buffer solution that contained 0.2 mM $NaNO_2$ in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2.0). Coffee and chlorogenic acid enhanced the formation of NO, and the degree of the enhancement by 0.5 mg of coffee/mL (about 3.5-fold) was similar to that of the enhancement by 0.1 mM chlorogenic acid. The addition of 1 mM SCN^- did not significantly affect the intensity of ESR signal independent of the presence of coffee or chlorogenic acid. It has been reported that SCN^- does not affect the formation of NO by nitrite/chlorogenic acid and nitrite/chlorogenic acid in apple extract systems under acidic conditions (*8*).

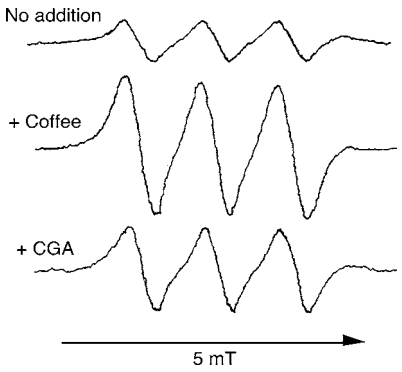

Figure 2. ESR spectroscopic measurement of formation of NO in acidified saliva. Coffee, 0.5 mg/mL; chlorogenic acid, 0.1 mM.

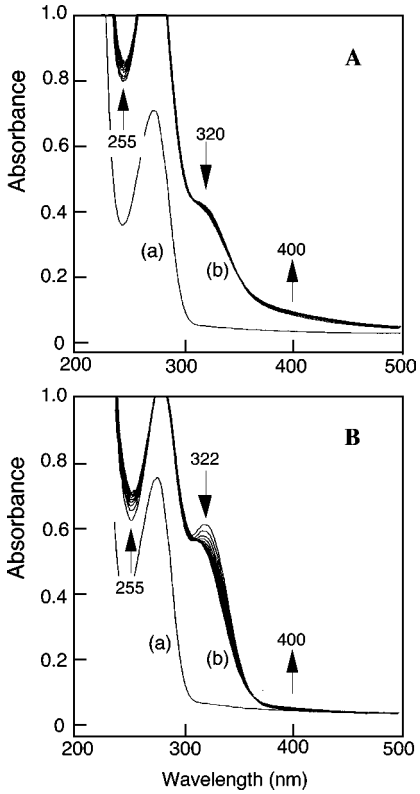

Figure 3. Changes in absorbance spectra of coffee and chlorogenic acid in acidified saliva. **A**, coffee; **B**, chlorogenic acid. Curve a, no addition; curve b, 0.3 mg of coffee/mL or 0.1 mM chlorogenic acid. Scanning (curve b) was repeated every 2.5 min from 500 to 240 nm.

Transformation of Components in Coffee and Chlorogenic Acid in Acidified Saliva. Figure 3A shows typical changes in absorption spectrum of coffee that was dissolved in acidified saliva. A decrease (around 320 nm) and increase (around 255 and 400 nm) in absorbance were observed during the incubation. When chlorogenic acid was incubated in the acidified saliva, similar changes in the absorption spectrum were also observed (**Figure 3B**).

To study which components in coffee were transformed during the incubation, HPLC was performed before and after incubation of coffee in acidified saliva (**Figure 4A**). Before

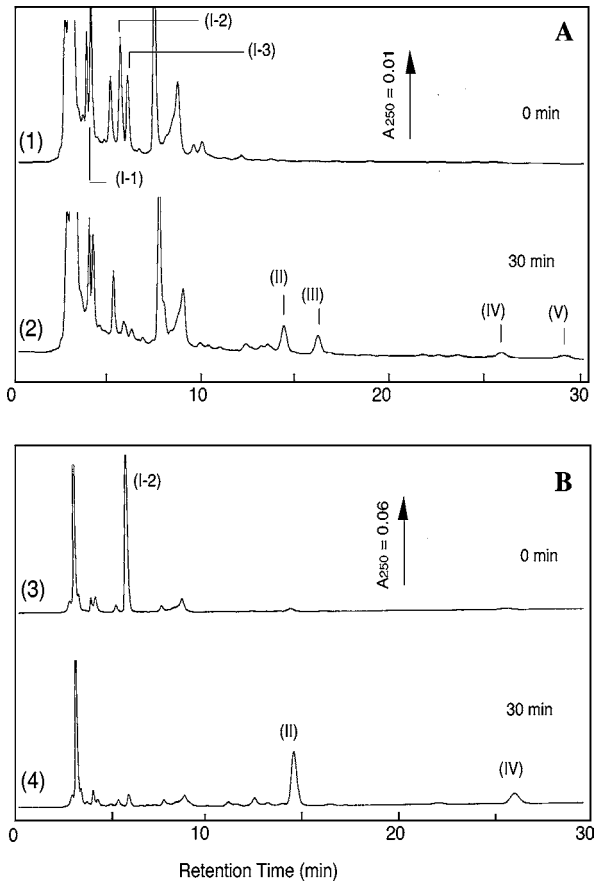

Figure 4. HPLC analysis of products formed during incubation of coffee and chlorogenic acid in acidified saliva. **A**, coffee (0.3 mg/mL); **B**, chlorogenic acid (0.1 mM). Traces 1 and 3, immediately after the preparation of the mixtures; traces 2 and 4, 30 min after incubation of the mixtures.

incubation, at least nine components were separated within a retention time of 10 min or less (trace 1). Component I-2 was identified as chlorogenic acid by comparing the retention time (5.2 min) and the absorption spectrum with standard chlorogenic acid (trace 3). The absorption spectrum of peak I-3 was identical with that of chlorogenic acid suggesting that the peak component was an isomer of chlorogenic acid. Peak I-1 consisted of at least two components. This was confirmed by HPLC using another mobile phase; one component, the absorption spectrum of which was identical with that of chlorogenic acid, was separated from the other component. The result indicates that an isomer of chlorogenic acid was included in peak I-1. The presence of chlorogenic acid and its two isomers (3′-caffeoylquinic and 4′-caffeoylquinic acid) in coffee has been reported (*19*, *20*). A peak at retention time of about 8.5 min was nitrous acid/nitrite ion. This was confirmed from the result that the peak was observed when HPLC was performed using nitrite dissolved in water (data not shown), and the absorption spectrum of standard nitrite (peak, 210 nm) was identical with that of the peak at a retention time of 8.5 min. A peak at retention time of about 7.5 min remained to be identified.

The amount of chlorogenic acid in coffee was calculated after separating it by HPLC as about 9.2 μg/mg of coffee. Under the assumption that molar extinction coefficients of isomers of chlorogenic acid at 320 nm were the same as that of chlorogenic acid, the amounts of two isomers of chlorogenic acid were 6.8 and 5.9 μg/mg of coffee. The values were similar to those reported in the literature (*20*). The concentration of chlorogenic acid plus its isomers in the reaction mixture, which contained 1 mg of coffee/mL, was estimated to be equivalent to 62 μM chlorogenic acid.

After incubation of coffee in acidified saliva for 30 min, the peaks of chlorogenic acid (peak I-2) and its isomer (peak I-3) decreased (**Figure 4A**). Peak I-1 also decreased although the decrease was smaller than those of peaks I-2 and I-3. The decrease in concentrations of chlorogenic acid and its isomers resulted in the formation of new major peaks II∼V. When coffee (0.3 mg/mL) was incubated in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2) in the presence of 0.2 mM nitrite and 1 mM SCN^-, peaks corresponding to II∼V were also formed (data not shown). No changes in concentration of chlorogenic acid and no formation of components, which gave peaks II∼V, were observed in the absence of nitrite.

Figure 4B shows HPLC profiles before (0 min) and after (30 min) incubation of chlorogenic acid with acidified saliva. In trace 3, chlorogenic acid (peak I-2) was detected as a major component before incubation. Two components were formed after incubation for 30 min. Retention times of the two components corresponded to peaks II and IV in **Figure 4A** and absorption spectra were also identical. Peaks II and IV were also detected when chlorogenic acid was incubated in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2) in the presence of both nitrite and SCN^-. No formation of components, which gave peaks II and IV, was observed when only SCN^- was contained in the acidic buffer solution. The above results indicate that nitrous acid participated in the transformation of chlorogenic acid and that the manner of transformation of chlorogenic acid in coffee was the same as that of reagent chlorogenic acid. As SCN^-, which is contained in saliva, may affect the reaction between nitrous acid and chlorogenic acid, we studied effects of SCN^- on nitrous acid induced transformation of chlorogenic acid.

Effects of SCN^- on Nitrous Acid Induced Change in Absorption Spectrum of Chlorogenic Acid. Figure 5A shows changes in the absorption spectrum during incubation of chlorogenic acid in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2) in the presence of nitrite but in the absence of SCN^-. Absorption around 320 nm decreased and absorption around 250 and 400 nm increased. Isosbestic points were detected at 274 and 364 nm. The changes in absorption spectrum were significantly affected by SCN^- (**Figure 5B**). Absorption increased around 250 and 306 nm during the decrease in absorption around 320 nm, and an isosbestic point was observed at 317 nm. The absorbance increase around 400 nm was small. The changes in absorption spectrum of chlorogenic acid in the presence of SCN^- were similar to those when the reagent was incubated in acidified saliva (absorption increase, 255 and 400 nm; isosbestic point, 316 nm) (**Figure 3**).

HPLC Analysis of Reaction Products of Chlorogenic Acid in the Absence of SCN^-. Products of chlorogenic acid, which were formed by nitrous acid in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2) in the absence of SCN^-, were analyzed by HPLC. In addition to chlorogenic acid, three components (CGA-Q and components 1 and 2) were detected (**Figure 6A**). CGA-Q (retention time, 4 min) had absorptions peaks at about 250, 310, and 400 nm (**Figure 6C**). The peak of CGA-Q disappeared by adding $NaBH_4$ to the reaction mixture, and accompanying the $NaBH_4$ induced disappearance of the peak, the peak due to chlorogenic acid increased. The molecular ion obtained by LC/MS (retention time in the mobile phase-1 for LC/MS, 5.2 min) was *m*/*z* 351 ($[M - H]^-$). The molecular ion of chlorogenic acid (retention time in the mobile phase-1 for LC/MS, 6.7 min) was *m*/*z* 353 ($[M - H]^-$). The results indicate that CGA-Q was

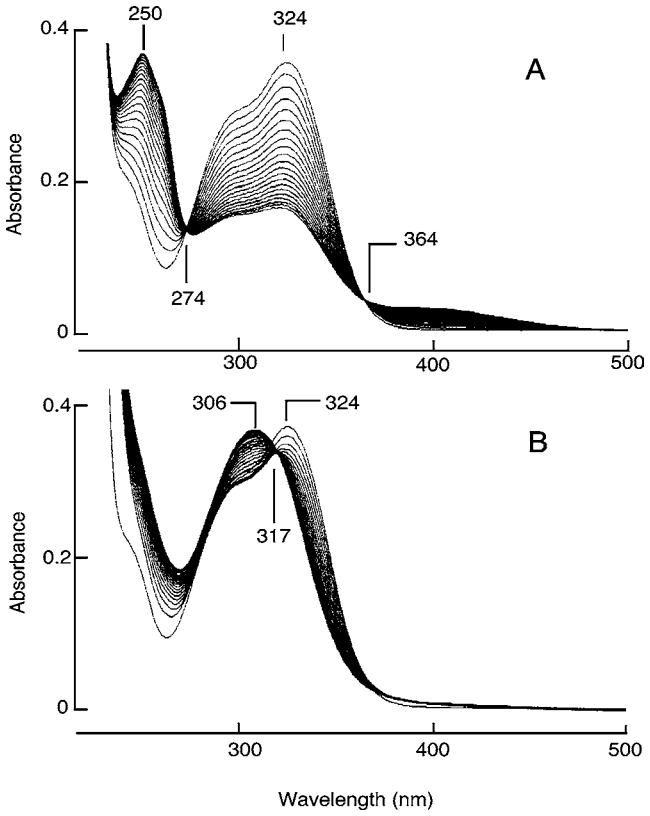

Figure 5. Nitrous acid induced changes in absorbance spectrum of chlorogenic acid. **A**, no addition; **B**, 1 mM NaSCN. Scanning was repeated every 1.5 min from 500 to 220 nm.

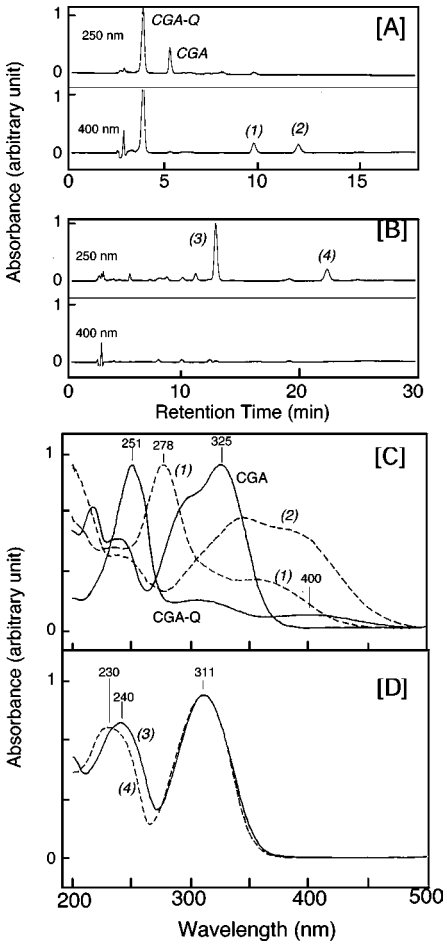

Figure 6. HPLC analysis of products formed during nitrous acid dependent decomposition of chlorogenic acid. **A**, no addition (30 min of incubation); **B**, 1 mM NaSCN (10 min of incubation); **C** and **D**, absorption spectra of components separated in the mobile phase. Each number in **C** and **D** corresponds to the peak numbers in **A** and **B**.

a quinone form of chlorogenic acid. It has been reported that the quinone of chlorogenic acid had peaks at about 305−310 and 390−400 nm and that its retention time was shorter than that of chlorogenic acid when they were separated by an ODS column (*21*). Coffee (0.3 mg/mL) was incubated in the presence of 0.2 mM nitrite but in the absence of SCN^- at pH 2 and was analyzed by HPLC. Components corresponding to quinones of chlorogenic acid and its isomers were detected at retention times between 3 and 5 min. The formation of quinones was supported by the result that these peaks disappeared and that the peaks corresponding to chlorogenic acid and its isomers increased after treating the incubated reaction mixture with $NaBH_4$ (data not shown). Furthermore, formation of quinone of chlorogenic acid was also studied under anaerobic conditions in the presence of 0.2 mM $NaNO_2$ and 0.1 mM chlorogenic acid in 50 mM sodium phosphate−50 mM KCl−HCl (pH 2.0). The formation of the quinone during 30 min of incubation under anaerobic conditions was 42 ± 13% (mean ± SD, $n = 3$) of that under aerobic conditions.

Component 1 (**Figure 6A**) had a retention time of 9.6 min and absorption maximum at 278 nm with a shoulder at about 360 nm (**Figure 6C**). Component 2 (**Figure 6A**) had a retention time of 11.8 min and absorption maximum at about 347 nm with a shoulder at about 380 nm (**Figure 6C**). The two components were also formed when coffee was incubated in an acidic buffer in the presence of nitrite but not in the presence of SCN^- (data not shown). The absorption spectrum of component 1 was similar to that of nitrotyrosine and 3-nitro-4-hydroxyphenylacetic acid (peaks around 360 nm) (*14*, *22*) suggesting that the component was nitrated chlorogenic acid. It has been reported that reagent chlorogenic acid and chlorogenic acid in apple extract are nitrated by nitrite under acidic conditions (*8*, *9*). Component 2 may also be a nitrated form of chlorogenic acid according to its absorption spectrum.

HPLC Analysis of Reaction Products of Chlorogenic Acid in the Presence of SCN^-. Incubation of chlorogenic acid in the presence of both nitrite and SCN^- resulted in the formation of components 3 and 4 inhibiting the formation of CGA-Q and components 1 and 2 (**Figure 6B**). Component 3 had a retention time of 13.2 min and absorption maxima at 240 and 312 nm (**Figure 6D**). The values corresponded to those of peak II in **Figure 4**. Component 4 had a retention time of 22.6 min and absorption maxima at 230 and 312 nm (**Figure 6D**). The values corresponded to those of peak IV in **Figure 4**. The formation of component 3 plus component 4 under anaerobic conditions was 74 ± 13% (mean ± SD, $n = 3$) of that under aerobic conditions when incubated for 30 min in the presence of 0.2 mM $NaNO_2$ and 0.1 mM chlorogenic acid at pH 2 for 30 min. Molecular ions of components 3 and 4 were *m*/*z* 410 ($[M - H]^-$) and *m*/*z* 411 ($[M - H]^-$), respectively, and retention times for components 3 and 4 were 6.4 and 12.7 min, respectively, in the mobile phase-2 for LC/MS.

To understand the relation between components 3 and 4, time courses of changes in concentrations of chlorogenic acid, SCN^-, and components 3 and 4 were studied as a function time (**Figure 7A**). Accompanying the consumption of chlorogenic acid, SCN^- was also consumed. No detectable consumption of SCN^- was

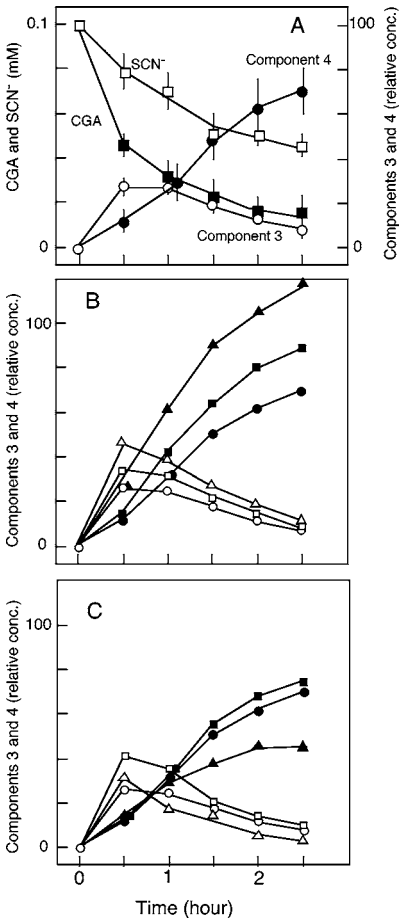

Figure 7. Consumption of chlorogenic acid and SCN^- and relation between components 3 and 4. **A**: Time courses of consumption of chlorogenic acid and SCN^- and formation of components 3 and 4. The reaction mixture contained 0.1 mM chlorogenic acid, 0.1 mM NaSCN, and 0.1 mM $NaNO_2$ in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2.0). **B**: Effects of NaSCN concentration on the formation of components 3 and 4. The reaction mixture contained 0.1 mM chlorogenic acid and 0.1 mM $NaNO_2$ in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2.0). Open symbols, component 3; closed symbols, component 4. ○ and ●, 0.1 mM NaSCN; □ and ■, 0.2 mM NaSCN; △ and ▲, 1 mM NaSCN. **C**: Effects of $NaNO_2$ concentration on the formation of components 3 and 4. The reaction mixture contained 0.1 mM chlorogenic acid and 0.1 mM NaSCN in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2.0). Open symbols, component 3; closed symbols, component 4. ○ and ●, 0.1 mM $NaNO_2$; □ and ■, 0.4 mM $NaNO_2$; △ and ▲, 1 mM $NaNO_2$.

observed when 0.1 mM NaSCN and 0.1 mM $NaNO_2$ were incubated for 30 min in 50 mM KH_2PO_4−50 mM KCl−HCl (pH 2) in the absence of chlorogenic acid (data not shown). This result indicates that chlorogenic acid/HNO_2 systems participated in the consumption of SCN^-. During the consumption of chlorogenic acid and SCN^-, component 3 was formed; the concentration increased and then decreased during incubation. The concentration of component 4 increased as a function of time. This result suggests that component 4 was formed from component 3.

When the concentration of SCN^- was increased in the presence of 0.1 mM $NaNO_2$ and 0.1 mM chlorogenic acid, not only the rate of the formation of component 3 but also the rate of the formation of component 4 increased as a function of concentration of SCN^- (**Figure 7B**), supporting SCN^- participation in the formation of component 4 as well as component 3 in HNO_2/chlorogenic acid systems. SCN^- did not significantly

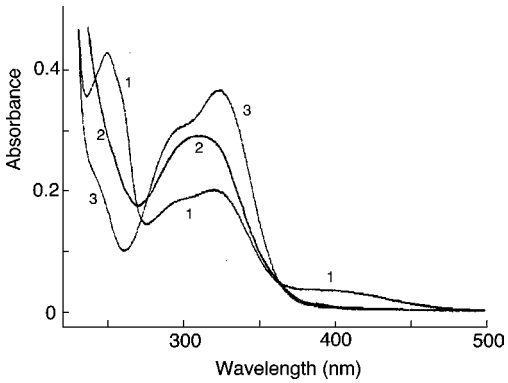

Figure 8. SCN^--induced changes in absorption spectrum of quinone of chlorogenic acid. The reaction mixture (1 mL) contained 0.05 mM chlorogenic acid and 0.1 mM $NaNO_2$ in 50 mM KH_2PO_4–50 mM KCl–HCl (pH 2). After incubation for 30 min, 1 mM NaSCN or a grain of $NaBH_4$ was added. Spectrum 1, before the addition of reagents; spectrum 2, 4.5 min after the addition of NaSCN; spectrum 3, 4.5 min after the addition of $NaBH_4$.

affect the decrease in concentration of chlorogenic acid (data not shown). When the concentration of $NaNO_2$ was increased to 0.4 mM in the presence of 0.1 mM chlorogenic acid and 0.1 mM NaSCN, the rate of the formation of component 3 increased (**Figure 7C**). When the concentration of nitrite was increased above 0.4 mM, the rate of the formation of component 3 was inhibited and the decrease in the concentration of component 3 was enhanced. The amount of component 4 formed decreased as a function of concentration of $NaNO_2$. The rate of consumption of chlorogenic acid increased as a function of concentration of nitrite (data not shown). The result in **Figure 7B,C** indicates that concentration of SCN^- relative to that of nitrite was important for the formation of components 3 and 4.

To study how components 3 and 4 were formed, SCN^- was added to the reaction mixtures, in which quinone of chlorogenic acid had been formed (**Figure 8**). By the addition of SCN^-, absorption due to quinone of chlorogenic acid (at about 250 and 400 nm) (spectrum 1) disappeared and absorption around 310 nm (spectrum 2) increased. When $NaBH_4$ was added, absorbance around 325 nm increased indicating the formation of chlorogenic acid (spectrum 3). Components, which gave spectrum 2, were analyzed by HPLC. Both components 3 and 4 were detected. This result suggests that quinone of chlorogenic acid participated in the formation of components 3 and 4. The formation of components 3 and 4 was also observed when SCN^- was added to acidic solution (pH 2), in which chlorogenic acid had been oxidized to the quinone by sodium hypochlorite.

Identification of Component 4. The ^1H MNR spectrum of component 4 showed two doublets in the aromatic region (J = 8.6 Hz) ascribed to *ortho*-coupled H-5 and H-6 protons. The *E* configuration at the double bond was maintained (J = 16.0 Hz). The ^{13}C NMR spectrum showed 17 carbon signals, including 16 carbon atoms of the chlorogenic acid moiety. The signal at δ 167.6 was derived from the carbon atom of a carbonyl moiety. The $[M - H]^-$ peak was observed at *m*/*z* 411 in the negative ESI-MS and the $[M + H]^+$ peak was observed at *m*/*z* 413 in the positive APCI-MS by LC/MS and by fast bombardment mass spectrometry (FABMS). No nitrogen atoms were found in the component and carbon (48.35%) and hydrogen (4.16%) atoms were found by elemental analysis. If sulfur is contained in the molecule, the data of NMR, mass spectra, and elemental analysis suggest the molecular formula of component 4 to be $C_{17}H_{16}O_{10}S$. Thus, the structure of component 4 was determined

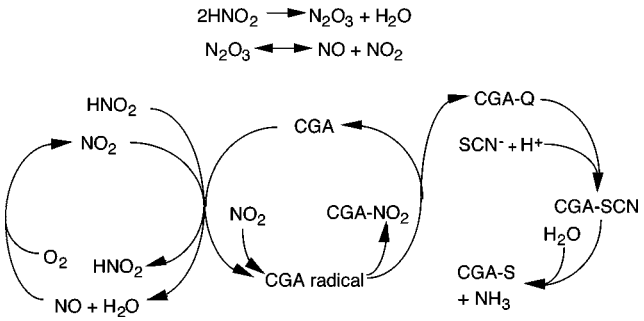

Figure 9. Possible reactions among chlorogenic acid, SCN^-, and HNO_2. CGA, chlorogenic acid; CGA-Q, quinone of chlorogenic aicd; CGA-SCN, 2-thiocyanatochlorogenic acid; CGA-S, (*E*)-5′-(3-(7-hydroxy-2-oxobenzo-[*d*] [1,3] oxathiol-4-yl)acryloyloxy)quinic acid.

to be (*E*)-5′-(3-(7-hydroxy-2-oxobenzo[*d*] [1,3]oxathiol-4-yl)-acryloyloxy)quinic acid (**Figure 1**).

Formation of NO was enhanced by coffee and reagent chlorogenic acid (**Figure 2**). As chlorogenic acid and its isomers in coffee as well as reagent chlorogenic acid were oxidized by nitrite under acidic conditions producing quinone of chlorogenic acid, we deduced that chlorogenic acid and its isomers in coffee could participate in the reduction of nitrous acid to NO (**Figure 9**). It has been reported that antioxidative activities of chlorogenic acid are the same as those of its isomers (*23*). Coffee, which contained chlorogenic acid plus its isomers equivalent to 31 μM chlorogenic acid, enhanced the formation of NO about 3.5-fold, whereas 0.1 mM chlorogenic acid enhanced the NO formation about 2-fold in acidified saliva (**Figure 2**). The result suggests that not only chlorogenic acid and its isomers but also other components in coffee contributed to the reduction of nitrous acid to NO.

Nitrated chlorogenic acid was formed during the incubation of chlorogenic acid with nitrite in an acidic buffer, and SCN^- inhibited the nitration (**Figure 6**). The SCN^--dependent inhibition of nitration of 4-hydroxyphenylacetic acid, which is induced by nitrous acid/H_2O_2 systems, has been reported (*12*). In addition to nitrated chlorogenic acid, quinone of chlorogenic acid was also formed during the incubation of chlorogenic acid with nitrite under acidic conditions (**Figure 6**). SCN^- also inhibited the formation of quinone of chlorogenic acid. The inhibition may be due to reduction of the radical of chlorogenic acid by SCN^- or to reaction between quinone of chlorogenic acid with SCN^-. The inhibition of the formation of quinone of chlorogenic acid under anaerobic conditions suggests that NO_2 formed by autoxidation of NO also participated in the oxidation of chlorogenic acid.

During SCN^--dependent inhibition of the formation of nitrated chlorogenic acid and quinone of chlorogenic acid, component 3 was initially formed and then component 4 was formed accompanying the decreases in concentrations of both chlorogenic acid and SCN^- (**Figure 7**). The rate of the consumption of chlorogenic acid was independent of the concentration of SCN^- and rates of formation of components 3 and 4 increased as a function of concentration of SCN^-. The result suggests that oxidation products of chlorogenic acid were at first formed and that the products reacted with SCN^- producing components 3 and 4. Quinone of chlorogenic acid seemed to have an important role for SCN^--dependent formation of components 3 and 4 because SCN^- could transform quinone of chlorogenic acid to components 3 and 4 (**Figure 8**). This idea is supported by the result that when the catechol group of chlorogenic acid was oxidized by two-electron oxidation, for example, by tyrosinase/O_2 in the presence of gluthathione, one molecule of glutathione binds to the benzene ring of chlorogenic acid (*25*, *26*). Alternatively, if SCN radical, which is formed by the reaction between the radical of chlorogenic acid and SCN^-, is reacted with another molecule of chlorogenic acid radical, formation of components 3 and 4 is possible. The formation of components 3 and 4 was partly inhibited by replacing air with argon. This result suggests that NO_2 formed by autoxidation of NO also contributed to the formation of components 3 and 4. Component 4 was determined as (*E*)-5′-(3-(7-hydroxy-2-oxobenzo[*d*] [1,3]oxathiol-4-yl)acryloyloxy)-quinic acid (**Figure 1**). The molecular ion of component 3 was *m*/*z* 410 [(M− H)$^-$]. It was deduced from the molecular ion that the component 3 was 2-thiocyanatochlorogenic acid (**Figure 9**). The attachment of cysteine and glutathione to the 2-position of chlorogenic acid has been reported (*26*−*29*). If component 3 is 2-thiocyanatochlorogenic acid, it might be hydrolyzed to (*E*)-5′-(3-(7-hydroxy-2-oxobenzo[*d*][1,3]oxathiol-4-yl)acryloyl-oxy)quinic acid producing ammonia (**Figure 9**). It has been reported that thiocyanate can be hydrolyzed producing carbonyl sulfide and ammonia (*30*).

Some functions of SCN^- in saliva have been proposed. One is the formation of an antimicrobial agent $OSCN^-$ by salivary peroxidase/H_2O_2 systems in the oral cavity (*31*). The other function is the inhibition of nitration of salivary phenolics and proteins (*32*). In this study, SCN^- inhibited nitrous acid induced formation of quinone of chlorogenic acid and nitrated chlorogenic acid producing 2-thiocyanatochlorogenic acid. The SCN^--dependent inhibition can be explained by rapid reaction of SCN^- with quinone or radical of chlorogenic acid that was formed during the oxidation of chlorogenic acid by nitrous acid and NO_2. NO_2 can be formed by the decomposition of nitrous acid and the autoxidation of NO (**Figure 9**). In general, quinones are known to be reactive species that can react with a variety of nucleophiles such as thiol-containing compounds and protein thiols (*25*). Such reactivities of quinones may result in their cytotoxicity (*25*). Furthermore, it has been reported that quinone of chlorogenic acid can produce H_2O_2 (*21*). If quinones are stabilized by SCN^-, the toxicity of quinones can be reduced. Taking this into consideration, we can propose an additional function of salivary SCN^- as a stabilizer of quinones formed in the stomach. In this way, the cooperation between dietary chlorogenic acid and salivary nitrite/SCN^- seems to result in the production of a physiological important substance NO scavenging toxic substances like NO_2 and quinone of chlorogenic acid in the stomach.

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