Disposition of Glucosinolates and Sulforaphane in Humans After Ingestion of Steamed and Fresh Broccoli

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Abstract: The cancer-chemopreventive effects of broccoli may be attributed, in part, to isothiocyanates (ITCs), hydrolysis products of glucosinolates. Glucosinolates are hydrolyzed to their respective ITCs by the enzyme myrosinase, which is inactivated by heat. In this study, the metabolic fate of glucosinolates after ingestion of steamed and fresh broccoli was compared in 12 male subjects in a crossover design. During each 48-hour baseline period, no foods containing glucosinolates or ITCs were allowed. The subjects then consumed 200 g of fresh or steamed broccoli; all other dietary sources of ITCs were excluded. Blood and urine samples were collected during the 24-hour period after broccoli consumption. Total ITC equivalents in broccoli and total ITC equivalents in plasma and urine were assayed by high-performance liquid chromatography as the cyclocondensation products of glucoraphanin and the 3-glucosinolate of sulforaphane (SFN). Total ITC metabolites in plasma peaked between 0 and 8 hours, whereas urinary excretion of total ITC equivalents and SFN-NAC occurred primarily between 2 and 12 hours. Results of this study indicate that the bioavailability of ITCs from fresh broccoli is approximately three times greater than that from cooked broccoli, in which myrosinase is inactivated. Considering the cancer-chemopreventive potential of ITCs, cooking broccoli may markedly reduce its beneficial effects on health.

Introduction

Glucosinolates, thioglycoside conjugates of isothiocyanates (ITCs), are widely distributed in Brassica vegetables, e.g., broccoli, cabbage, cauliflower, radish, mustard, turnip, and rutabaga (1). When plant cells are chewed or damaged, glucosinolates are hydrolyzed by the enzymatic action of myrosinase (thioglycoside glucohydrolase, EC 3.2.3.1) to yield ITCs (Figure 1, top). It has long been recognized that ITCs have diverse biological effects due to their chemical reactivity (1,2), and more recently, ITCs have been shown to be potent inhibitors of tumorigenesis in various animal models (3–5). The cancer-protective effect of ITCs in animals has been attributed to their inhibitory action on phase I enzymes responsible for bioactivation of carcinogens and their activity as inducers of phase II detoxification enzymes (3–9). Similar effects on phase I and phase II enzymes have been observed in humans given large quantities of cruciferous vegetables in the diet (10–12). The exact role of dietary ITCs or glucosinolates in human cancer prevention remains to be defined, however. Epidemiological data indicate that consumption of Brassica vegetables reduces the incidence of cancers at various sites (13–15); in particular, a recent cohort study showed that uptake of ITCs from crucifers is associated with a reduced risk of lung cancer (16).

Reports indicate that 36–60% of glucosinolates in broccoli (Brassica oleracea var. italica), appear as glucoraphanin, the glucosinate of sulforaphane (SFN, 4-methylsulfinylbutyl ITC), an exceptionally active inducer of phase II enzymes (8,17,18). SFN is metabolized in rats by the mercapturic acid pathway, in which SFN initially reacts with glutathione (GSH) but ultimately appears in the urine (60% of dose) as the N-acetyl-L-cysteine conjugate of sulforaphane (SFN-NAC) (19). Other ITCs, e.g., benzyl ITC, allyl ITC (AITC), and phenethyl ITC, are also metabolized in rodents and humans by the mercapturic acid pathway (20–23). Despite extensive knowledge regarding the formation of ITC-NAC conjugates, the human metabolism of broccoli and its glucosinolates is rather poorly defined. Because humans usually ingest cooked broccoli, an important question is whether there are major differences in the metabolism and disposition of glucosinolates and ITCs after ingestion of cooked vs.
This study focuses on the metabolism of glucosinolates, in particular glucoraphanin, in humans after ingestion of steamed broccoli vs. fresh broccoli using a two-period crossover experimental design.

Materials and Methods

Human Subjects

Twelve healthy men of normal body weight, ages 24–51 years, were recruited by clinics at the Bellevue Hospital Center of the New York University (NYU) Medical Center complex and by the employee health clinics of Tisch University Hospital of NYU Medical Center and Bellevue Hospital Center (New York, NY). All subjects completed self-administered baseline questionnaires regarding diet and tobacco use habits and signed informed consent documents before the study. A complete medical history was taken from each subject and was followed by a physical examination, hematology (complete blood count), and liver function tests that included serum glutamic-pyruvic transaminase and bilirubin. Subjects were entered into the crossover design, which was approved by the NYU Medical Center Internal Review Board, and were assigned to four blocks of four, two blocks of three, and one block of two, with balanced randomization within blocks. The experiments were conducted during the period November 13–October 11, 1998. The trials were administered according to International Commission on Healthcare Professions guidelines and the Declaration of Helsinki.

Sample Administration and Collection

All study participants were asked to avoid the foods known to contain glucosinolates or ITCs listed on a dietary questionnaire. Before admission to the Clinical Research Center, a 12-hour urine sample was collected from each participant, beginning at 8 PM in the evening before consumption of broccoli until 8 AM the following morning. Each urine sample was collected in a container containing 0.5 g of ascorbic acid. The study subjects were admitted to the Clinical Research Center of Bellevue Hospital before 8 AM. Each subject was directed to ingest 200 g of steamed or fresh broccoli according to the crossover design. Blood (5 ml) was drawn in heparinized Vacutainer tubes (Becton-Dickinson, Franklin Lakes, NJ) just before ingestion of broccoli (0 h) and 2, 4, 6, 8, 10, 12, and 24 hours after consumption. Plasma, immediately prepared by centrifugation, was stored at −20°C until analyzed. Urine samples were collected at 0–2, 2–4, 4–8, 8–12, and 12–24 hours after broccoli consumption and stored in a refrigerator until each collection period was completed; samples were then frozen (−20°C) until analyzed. Individual dietary records were taken for the 24-hour period before admission and during the feeding trial days. Milk, tea, alcoholic beverages, more than two cups of coffee, food supplements, and vitamins were not allowed during these days. Participants were instructed to drink ≥1 liter of liquids during each 24-hour experimental period.

Preparation of Steamed and Fresh Broccoli

Broccoli of the Emperor cultivar was purchased from Cherry Lane Farms (Bridgeton, NJ) <24 hours before sample preparation. Throughout the entire experimental period, all samples, recently harvested, were provided by the same source. Broccoli prepared for the subjects was washed and cut into bite-sized pieces (florets, ~1.5 in.). For steamed broccoli experiments, 200-g portions were weighed out. Each portion was then cooked in a food steamer (model 4710, Sunbeam Household Products, Schaumburg, IL) for 15 minutes. Under these conditions, myrosinase is completely inactivated (see below). After all portions had been steamed, they were combined to ensure that all participants received homogeneous samples; the steamed broccoli was then reapportioned into 200-g servings. For fresh broc-
coli experiments, prewashed, cut broccoli was divided into 200-g portions immediately before ingestion. Steamed or fresh broccoli was served with lemon or butter; bagels, bread, and fruit juice (lemonade, apple, cranberry, and orange) were also provided.

The optimal time for steaming to deactivate myrosinase in broccoli was determined as follows: fresh broccoli florets were steamed for various time periods and then homogenized in water (60 g/100 ml). An extract was prepared by filtration of the homogenate, washing the retained broccoli puree with water twice, and repeated filtration, and final filtration of the homogenate, washing the retained broccoli puree with water twice, and repeated filtration, and final filtration of the homogenate, washing the retained broccoli puree with water twice. Myrosinase activity was assayed by determining the amount of total ITCs released in 100 μl of the extract using the cyclocondensation assay described below. It was determined that broccoli steamed for 15 minutes was devoid of hydrolytic activity when incubated at 37°C with 1 ml of 0.2 mM sinigrin (Sigma Chemical, St. Louis, MO), the glucosinolate of AITC, for two hours.

Samples of all broccoli lots prepared for each experiment, residual drippings from the steamer, and extracts (see below) were stored at −20°C until treated with myrosinase and analyzed for ITC content.

Analysis of Total Glucosinolates in Broccoli Samples as ITCs

The analysis of total ITCs in steamed broccoli was performed after treatment of the samples with myrosinase. The experiments were performed on the day of broccoli ingestion or, in some instances, after overnight frozen storage at −20°C. Steamed broccoli (15 g) was blended for two minutes in deionized water (75 ml) using a blender (model DLC-1TX Mini-Prep, Cuisinart, East Windsor, NJ) until it had been converted into a fine puree. The puree was poured through six layers of cheesecloth, and the liquid obtained was collected in a 500-ml beaker. The retentate was washed two additional times with 75 ml of deionized water, and the filtrates were pooled and brought to a final volume of 250 ml with deionized water. A 10-ml aliquot of the broccoli extract was then filtered through filter paper (Whatman no. 1) in a Büchner funnel to obtain a clear solution. One milliliter of myrosinase (Sigma Chemical), 0.35 U/ml in 0.1 M potassium phosphate buffer, pH 6.6, was added to 1 ml of filtrate; the mixture was then incubated with shaking at 37°C for two hours. The amount of myrosinase added was determined on the basis of preliminary experiments using 0.1–8 mg (0.02–1.4 U) in the incubation. The amount chosen, 0.35 U, was calculated to provide ~700-fold excess activity to ensure complete hydrolysis. For the analysis of total ITCs released in the incubation mixture, 100 μl of the myrosinase-treated broccoli extract were utilized in the cyclocondensation assay described below (17,24–27).

To determine the amount of ITCs and glucosinolates in fresh broccoli samples, a method modified from that of Fahey and associates (28) was utilized. Fresh broccoli (15 g) was chopped and mixed for uniformity; a 1-g sample was homogenized for one minute in 12.5 ml of 0.1 M potassium phosphate buffer, pH 6.6, with 0.35 U of myrosinase, using an Ultra-Turrax T25 Tissue-Mizer (IKA-Works, Cincinnati, OH). The homogenate was then incubated in a water bath shaker for two hours at 37°C to ensure that all glucosinolates had been hydrolyzed. The sample was centrifuged at 3,500 rpm for 10 minutes to pellet the suspended solids; the supernatant was placed in another vial and kept on ice until analyzed by the cyclocondensation reaction. The sediment was extracted twice with 10 ml of methanol (65°C). The myrosinase-treated broccoli juice and the combined methanol extracts were separately analyzed using the cyclocondensation reaction. Total ITCs were calculated as the sum of the amounts in broccoli juice and the combined methanol extract.

Cyclocondensation Assay for Total ITCs

The cyclocondensation reaction mixture consisted of 600 μl of a degassed 2-propanol solution of 10 mM 1,2-benzenedithiol (Lancaster Synthesis, Windham, NH), 500 μl of degassed 0.1 M potassium buffer, pH 8.5, and 100 μl of broccoli extract in 2-ml autosampler vials with screw caps (Chromacol, Trumbull, CT), which was incubated for two hours in a water bath shaker at 65°C (25,26). The product 1,3-benzodithiol-2-thione (Figure 1, bottom) was analyzed by reverse-phase high-performance liquid chromatography (HPLC) using a μBondapak C18 (150 × 3.9 mm) column with a C18 guard column (Waters, Milford, MA) at a detection wavelength of 365 nm. The mobile phase consisted of 7:3 methanol-H2O (vol/vol) with a flow rate of 1.75 ml/min. An HPLC system consisting of a system controller (model SCL-10A), dual pumps (model LC-10AS), an autoinjector (model SIL-10A), and a spectrophotometer (model SPD-10A UV/vis, Shimadzu Scientific Instruments, Columbia, MD) was utilized. Data were collected using an AxxiChrom 727 chromatography data station (Axxiom Chromatography, Moorpark, CA). For quantification of the reaction product, a standard curve was constructed using a series of 1,3-benzodithiol-2-thione solutions in 2-propanol-water (1:1) at concentrations ranging from 0.01 to 20 μM (27).

Analysis of Total ITCs in Urine and Plasma Samples

The amount of ITC equivalents (ITC equivalents = ITCs + thiol conjugates of ITCs) in the urine samples was analyzed by the method described above. For analysis, a 7-ml aliquot of thawed urine was centrifuged at 3,500 rpm for 20 minutes to remove particulates; a 100-μl sample was routinely analyzed in triplicate (25,26). Analytic data for urine were normalized on the basis of the creatinine content, since excretion of creatinine is relatively constant over time, while the urinary void volume is subject to more variation.

Total ITCs in plasma samples were analyzed by using a modified version of the cyclocondensation method, in which 500 μl of plasma, 500 μl of degassed 0.1 M potassium phosphate, pH 8.5, and 800 μl of degassed 10 mM benzene 1,2-benzenedithiol in 2-propanol were used as the reaction mixture. The reaction mixture was incubated with agitation in
were performed on a GC with electronic pressure control chromatograph (GC)/mass spectrometer (MS). Analyses of the extract was then diluted to 1 ml for injection into the gas
terminal range examined (0.001–2.5 mM). The limit of detec-
tration range of 10–200 μg/ml.

Data Analysis and Statistics

Data are expressed as the mean of two determinations in cases of two injections from the same vial; otherwise the means ± SD of triplicate injections are presented. Urine recovery data were evaluated statistically by using the Student’s t-test.

Results

Study Subjects

Twelve healthy male medical students, interns, and hospital workers, with mean age of 34 ± 8.1 years, were recruited for the clinical portion of the study. None of the subjects used tobacco. The subject compliance with the protocol was excellent. Results of a complete analysis of blood count and liver function showed normal ranges for all subjects, indicating no toxicity of the treatment. Nevertheless, two subjects, FM-11 and MF-12, demonstrated high baseline values for urinary ITCs. Subject MF-12 consumed two hamburgers from a fast-food restaurant on the second baseline day of the steamed broccoli experiment. The facts that mustard and mayonnaise contain AITC and that both are common components of fast-food hamburgers may account for the relatively high baseline value observed (see Figure 3). The results for Subject FM-11 are inexplicable.

Glucosinolate and Glucoraphanin Content of Broccoli Samples

Two separate study periods, one with 200 g of steamed broccoli and the other with 200 g of fresh broccoli, were conducted to investigate the metabolic disposition of glucosinolates and to determine the time course of appearance of their metabolites (ITCs) in blood and urine samples after ingestion. The range of total glucosinolates in steamed broccoli measured as ITCs released by myrosinase was 0.9–1.1 μmol ITC equivalents/g for seven experiments (mean 1.0 ± 0.1 μmol/g) and 0.9–1.3 μmol ITC equivalents/g (mean 1.1 ± 0.2 μmol/g) in the fresh broccoli (Table 1). The amount of free ITCs in steamed broccoli was ~5.3% of the total ITCs released by myrosinase. The mean amounts of total glucosinolates determined in fresh or steamed broccoli did not differ significantly (p > 0.05). Therefore, the cooking conditions

Analysis of SFN-NAC in Urine Samples

One-milliliter urine samples were acidified with 10 μl of 2 N HCl; the samples were then frozen on dry ice, allowed to thaw to room temperature, and centrifuged at 3,500 rpm for 10 minutes to remove matter precipitated by acid treatment. The supernatant urine was subsequently filtered through a 0.45-μm nylon syringe filter before use. Analysis of 10 μl of filtered urine was performed using the same reverse-phase HPLC system described above, except the mobile phase was 0.1% trifluoroacetic acid in 9:1 H₂O-acetonitrile, and the mobile phase flow rate was 1 ml/min. A photodiode array detector (model SPD-10A, Shimadzu Scientific Instruments) was used for detection and integration of peaks and for spectral scans (see Figure 4). The standard NAC (Aldrich, Milwaukee, WI) conjugate of SFN (LKT Laboratories, St. Paul, MN) was synthesized (19) in our laboratory. The standard curve for urinary SFN-NAC was constructed using synthetic SFN-NAC dissolved in urine; it was linear over the concentration range examined (0.001–2.5 nM). The limit of detection using this method was ~0.1 μM in urine.

Analysis of SFN in Broccoli Samples

The SFN content of fresh and steamed broccoli was determined using a modification of a published method (30). Briefly, frozen broccoli samples (1 g) were thawed and homogenized in 10 ml of water; samples were treated with myrosinase as described above to release SFN and other ITCs. The homogenate was filtered to remove debris and extracted twice with 25-ml portions of methylene chloride. The pooled methylene chloride fraction was dried over sodium sulfate and filtered through a 0.45-μm Teflon filter. The filtrate was concentrated using a rotary evaporator, and the extract was then diluted to 1 ml for injection into the gas chromatograph (GC)/mass spectrometer (MS). Analyses were performed on a GC with electronic pressure control (5890 Series II Plus, Hewlett-Packard, Palo Alto, CA) equipped with a flame ionization detector and connected to a mass selective detector (model 5972, Hewlett-Packard). The GC was operated in the splitless mode using a fused capillary column (model 5MS Hewlett-Packard; 30 m, 0.25 mm ID, cross-linked to 5% phenyl methyl siloxane stationary phase). Mass spectra were obtained by electron ionization over a range of 50–550 mass units; ion source temperature was 177°C, and electron multiplier voltage was 1,753 eV. SFN was quantified by flame ionization detection using a five-point calibration curve that was linear over the concentration range of 10–200 μg/ml.

Glucosinolate and Glucoraphanin Content of Broccoli Samples

Two separate study periods, one with 200 g of steamed broccoli and the other with 200 g of fresh broccoli, were conducted to investigate the metabolic disposition of glucosinolates and to determine the time course of appearance of their metabolites (ITCs) in blood and urine samples after ingestion. The range of total glucosinolates in steamed broccoli measured as ITCs released by myrosinase was 0.9–1.1 μmol ITC equivalents/g for seven experiments (mean 1.0 ± 0.1 μmol/g) and 0.9–1.3 μmol ITC equivalents/g (mean 1.1 ± 0.2 μmol/g) in the fresh broccoli (Table 1). The amount of free ITCs in steamed broccoli was ~5.3% of the total ITCs released by myrosinase. The mean amounts of total glucosinolates determined in fresh or steamed broccoli did not differ significantly (p > 0.05). Therefore, the cooking conditions...
used in the study did not significantly reduce the glucosinolate content of the broccoli.

The glucoraphanin content of the broccoli samples was determined by analysis of broccoli extracts for SFN after treatment with myrosinase using a published GC/MS method (30). The SFN content was expected to equal the glucoraphanin content, inasmuch as myrosinase releases \( \frac{1}{109} \) mol of SFN for each \( \frac{1}{109} \) mol of glucoraphanin hydrolyzed. The GC/MS analysis showed that the mean glucoraphanin content of steamed broccoli (0.46 ± 0.12 \( \mu \text{mol/g} \)) did not differ significantly \((p > 0.05)\) from that of fresh broccoli (0.48 ± 0.23 \( \mu \text{mol/g} \)). The glucoraphanin content was calculated to be 46.1% and 45.5% of total glucosinolates in steamed and fresh broccoli, respectively, in good agreement with data reported previously (17).

### Urinary Excretion of Total ITC Equivalents

The amounts of total ITC equivalents excreted in the urine samples collected at different time points within the first 24 hours after ingestion are illustrated in Figures 2 and 3. The maximum excretion of urinary ITC equivalents was observed within two to eight hours in both studies, but the peak excretion of ITC equivalents after ingestion of steamed broccoli appeared to be somewhat delayed compared with excretion after ingestion of fresh broccoli. The average cumulative excretion of ITC equivalents in urine during the 24 hours after consumption of steamed or fresh broccoli was 20.6 ± 12.8 and 68.1 ± 22.6 \( \mu \text{mol} \), respectively. The average excretion rate of glucosinolates to ITC equivalents was determined to be 10.2 ± 5.9% and 32.3 ± 12.7% after consumption of steamed and fresh broccoli.

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>( \mu \text{mol SFN/g} )</th>
<th>( \mu \text{mol glucosinolates/g} )</th>
<th>Glucoraphanin, % of total glucosinolates</th>
<th>Calculated glucosinolates consumed by each subject, ( \mu \text{mol} )</th>
<th>( \mu \text{mol SFN/g} )</th>
<th>( \mu \text{mol glucosinolates/g} )</th>
<th>Glucoraphanin, % of total glucosinolates</th>
<th>Calculated glucosinolates consumed by each subject, ( \mu \text{mol} )</th>
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<tbody>
<tr>
<td>I</td>
<td>0.47</td>
<td>0.9 ± 0.0</td>
<td>52.2</td>
<td>180</td>
<td>0.68</td>
<td>1.1 ± 0.0</td>
<td>61.8</td>
<td>220</td>
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<td>1.0 ± 0.0</td>
<td>58.0</td>
<td>200</td>
<td>0.30</td>
<td>1.2 ± 0.0</td>
<td>25.0</td>
<td>240</td>
</tr>
<tr>
<td>III</td>
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<td>1.1 ± 0.0</td>
<td>49.1</td>
<td>220</td>
<td>0.50</td>
<td>1.0 ± 0.0</td>
<td>50.0</td>
<td>200</td>
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<tr>
<td>IV</td>
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<td>0.9 ± 0.0</td>
<td>46.7</td>
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<td>1.0 ± 0.0</td>
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<td>200</td>
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<td>0.9 ± 0.0</td>
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<td>180</td>
</tr>
<tr>
<td>VI</td>
<td>0.51</td>
<td>1.1 ± 0.0</td>
<td>46.3</td>
<td>220</td>
<td>0.31</td>
<td>1.3 ± 0.0</td>
<td>23.8</td>
<td>260</td>
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</table>

Mean ± SD 0.46 ± 0.12 1.0 ± 0.1 46.1 ± 11.6 200 ± 17.8 0.48 ± 0.2 1.1 ± 0.1 45.5 ± 21.2 216 ± 29.4

### Table 1. Glucosinolate Content of Steamed and Fresh Broccoli

<table>
<thead>
<tr>
<th>Expt No.</th>
<th>SFN Assayed</th>
<th>glucosinolates</th>
<th>Glucoraphanin, % of total glucosinolates</th>
<th>Calculated glucosinolates consumed by each subject, ( \mu \text{mol} )</th>
</tr>
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<tr>
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<td>0.9 ± 0.0</td>
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<tr>
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<td>0.54</td>
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<td>49.1</td>
<td>220</td>
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<td>IV</td>
<td>0.42</td>
<td>0.9 ± 0.0</td>
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<td>180</td>
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<td>0.51</td>
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<td>46.3</td>
<td>220</td>
</tr>
</tbody>
</table>

Mean ± SD 0.46 ± 0.12 1.0 ± 0.1 46.1 ± 11.6 200 ± 17.8

\(a\): Each subject consumed 200 g of steamed broccoli and 200 g of fresh broccoli in separate experiments. SFN, sulforaphane.

\(b\): Assayed as isothiocyanate (ITC) equivalents.

\(c\): Values are means ± SD.

\(d\): Assayed as \( \mu \text{mol SFN/g broccoli, equivalent to } \mu \text{mol glucoraphanin/g broccoli.} \)

**Figure 2.** Time course of urinary excretion of ITC equivalents after ingestion of steamed broccoli by individual subjects.
fresh broccoli, respectively, within a 24-hour period. The total excretion of ITC equivalents in the urine after consumption of fresh broccoli was thus three- to fourfold higher (statistically significant, \( p < 0.001 \)) than the total urinary excretion of ITC equivalents after ingestion of steamed broccoli. Although most ITCs were excreted within 24 hours, the appearance of ITCs in urine was not complete within the 24-hour period of urine collection. Of the total amount of ITCs excreted after ingestion of steamed broccoli, 21.8 ± 8.3% of the amount excreted was detected in urine collected between 12 and 24 hours; during the same period, 12.2 ± 5.3% of excreted ITC equivalents from fresh broccoli appeared in the urine (data not shown). These data suggest that additional amounts of ITCs would probably have been collected at later time periods. Because creatinine excretion is rather constant for each individual during 24 hours, while excretion of urine is highly variable and dependent on fluid consumption or other factors, for graphing purposes the urinary ITC excretion data were normalized on the basis of creatinine levels.

**Urinary Excretion of SFN-NAC**

SFN-NAC was quantified by HPLC in the urine samples after ingestion of steamed or fresh broccoli. Figure 4, top, shows a typical chromatogram of urine from an individual ingesting fresh broccoli. Identification of the peak corresponding to SFN-NAC was based on its ultraviolet spectrum (Figure 4, middle) and retention time, which were identical with those of the synthetic standard (Figure 4, bottom). Table 3 provides individual urine sample data for SFN-NAC within 24 hours. Individuals who ingested steamed broccoli excreted a mean of 6.9 ± 2.5 \( \mu \)mol of SFN-NAC, or 41.4 ± 21.9% of total ITCs recovered in urine. After ingestion of fresh broccoli, 25.8 ± 13.9 \( \mu \)mol of SFN-NAC were detected in the urine, which was 40.0 ± 21.0% of total ITC equivalents recovered. As expected, the approximately threefold differences between the amounts of SFN-NAC recovered in the urine of the two treatment groups were highly significant (\( p < 0.001 \)). Approximately 7.5% of glucoraphanin in steamed broccoli was excreted as SFN-NAC in 24 hours, while 24.9% of glucoraphanin in fresh broccoli was excreted in the urine as SFN-NAC during the same period. A small proportion of SFN in fresh and steamed broccoli probably was excreted as other metabolites not quantified (see Discussion).

**Analysis of Plasma for Total ITCs and SFN-NAC**

Total ITC equivalents were quantified in plasma as the cyclocondensation product before and after the ingestion of steamed and fresh broccoli. Mean plasma ITC values for all subjects after ingestion of steamed and fresh broccoli are presented in Figure 5. In the experiments with steamed broccoli, interpretation of the data was hampered by high baseline values of plasma ITC equivalents (range 0.6–1.1 \( \mu \)M) compared with the plasma total ITC values of 0.7–1.1 \( \mu \)M after zero to six hours. The ITC peaks in plasma samples after ingestion of steamed broccoli were small, often irregular, and sometimes not discernable. Definite increases in plasma ITC equivalents were observed 0–10 hours after ingestion of fresh broccoli (range 0.8–1.7 \( \mu \)M) compared with baseline values (range 0.7–1.1 \( \mu \)M).

SFN and SFN-NAC were not detected in plasma samples from individuals consuming steamed or fresh broccoli by the HPLC methods utilized for analysis of urine samples.

**Discussion**

This study was conducted to examine differences in the metabolism of glucosinolates, specifically glucoraphanin, after ingestion of steamed and fresh broccoli in humans. Results of the study provide important quantitative information on the effects of cooking on the absorption and excretion of
glucosinolates and ITCs. Amounts of glucosinolates that appeared in steamed broccoli and fresh broccoli were similar, although ~4% of total glucosinolates were detected in the residual cooking fluids (drippings) as cyclocondensation products after steaming (data not shown). The data indicate that only a minimal amount of thermal degradation or extraction of glucosinolates in broccoli occurs by steaming. In contrast, our previous study showed that the glucosinolate content of watercress was significantly reduced by cooking in boiling water for three minutes (27). The mean proportion of

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**Figure 4.** High-performance liquid chromatography analysis of N-acetyl-L-cysteine conjugate of sulforaphane (SFN-NAC) in urine after ingestion of fresh broccoli. Top: urine sample from Subject JHU collected at 4–8 h, overlaid with synthesized SFN-NAC standard (see Materials and Methods for description of analytical method). Middle: spectrum from Subject JHU of 4- to 8-h peak identified as SFN-NAC on basis of retention time. Bottom: spectrum of SFN-NAC synthesized standard.
glucosinolates identified as glucoraphanin in fresh and steamed broccoli was also relatively constant, although there was considerable variation within the respective treatment groups. Various factors, such as weather, growing location and soil nutrients, cultural factors and plant spacing, date of harvest, position of the inflorescence on the stem of the plant, and storage conditions and length of storage after harvest, alter the content of glucosinolates in broccoli and, in particular, the levels of glucoraphanin (18,31–33). These results show that, for maintaining the glucosinolate content of vegetables such as broccoli, steaming has minimal impact and, therefore, is the cooking method of choice. Another method, microwave cooking of broccoli at full power for eight minutes, caused considerable loss of SFN (33).

This study showed that urinary excretion of total ITCs released from glucosinolates after ingestion of steamed broccoli was approximately one-third the amount excreted in the urine after ingestion of fresh broccoli, even though the glucosinolate content of the broccoli ingested was approximately the same in both sets of experiments. Less than 25% of the total ITCs in steamed broccoli, often considerably less, were accounted for in the urine during the 24-hour testing periods (Table 2). These data indicate that myrosinase plays a critical role in the conversion of glucosinolates to ITCs in humans and that inactivation of myrosinase by cooking considerably reduces the hydrolysis of glucosinolates and the dietary absorption and subsequent excretion of ITCs. Reduction of the absorption of ITCs, including SFN, could reduce the potential health benefits of these components, inasmuch as they have been demonstrated to modulate the metabolism of carcinogens and to effectively reduce their potency in animal experiments (3–5,34).

There may be a number of reasons for the wide variability in amounts of ITC-NAC and SFN-NAC excretion among subjects in this study (Table 3). For example, intake of other foods at the time of ingestion of broccoli would tend to dilute myrosinase activity and impede release of ITCs. Also, differences in individual patterns of mastication, which release myrosinase from broccoli cells, may have been important. Such variables may have been particularly notable on ingestion of fresh broccoli, which potentially has much higher myrosinase activity but is less easily macerated during the processes of mastication and digestion. In the mercapturic acid pathway of metabolism of ITCs, the initial conjugation reaction with GSH is catalyzed by glutathione S-transferases. The human glutathione S-transferases are subject to considerable polymorphism in individuals and could possibly account for some of the differences observed in plasma ITC levels and in rates of ITC excretion (16, 35,36).

In the instance of steamed broccoli, hydrolysis of glucosinolates by intestinal microflora more than likely contributed to the release of ITCs and their subsequent absorption from the intestine (17,27,34). In this study, the urinary excretion of ITCs and their conjugates from steamed broccoli was somewhat delayed compared with that of fresh broccoli (Figures 2 and 3). These data suggest that, after ingestion of steamed broccoli, absorption of ITCs from the intestinal tract was delayed. Differences in the microflora activity of the intestinal tract in the experimental subjects may have contributed to the wide variations in rates of absorption of ITCs from steamed broccoli (27,34).

Approximately 40% of ITC equivalents in urine samples were identified as SFN-NAC (Table 3). This value is comparable to the percentage of glucosinolates in broccoli identified as glucoraphanin on the basis of the SFN content of broccoli samples (Table 1). In studies with rats, other urinary metabolites of SFN have been identified; besides SFN-NAC...
erucin-NAC, the mercapturic acid of the sulfide analog of SFN (12% of dose), and a desaturated derivative were excreted during a 24-hour period (19). No attempt was made to identify minor metabolites of SFN possibly present in the human urine samples. Minor metabolites of SFN formed after human consumption could account for the small discrepancy between the fraction of total glucosinolates identified as glucoraphanin in broccoli samples and the fraction of total ITCs measured in urine as SFN-NAC. The failure to identify SFN-NAC in plasma can most likely be attributed to the relative insensitivity of the method used.

The measurement of total ITC equivalents in plasma samples using the cyclocondensation method is subject to several confounding difficulties. ITCs absorbed from the gastrointestinal tract are initially distributed by circulation of blood throughout the body (37,38), considerably reducing the plasma concentration of ITCs. Furthermore, ITCs readily undergo reactions by binding with sulfhydryl, hydroxyl, and amino groups; ITCs are quickly bound to some degree to GSH, plasma proteins, and cellular sulfhydryls and proteins. The covalently bound ITCs, including dithiocarbamates, are in equilibrium with ITCs. The reaction of 1,2-benzenedithiol with ITCs removes all ITCs in the aqueous phase, thus favoring the complete dissociation of ITCs bound to protein or conjugated with thiols. The background ITC levels in plasma or urine of individuals who have not

Table 2. Cumulative Amounts of ITC Equivalents in 24-Hour Urine Samples After Ingestion of Steamed and Fresh Broccoli

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>ITC equivalents excreted within 24 h, ( \mu )mol</th>
<th>Cumulative % of dose excreted( c )</th>
<th>ITC equivalents excreted within 24 h, ( \mu )mol</th>
<th>Cumulative % of dose excreted( c )</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB-1</td>
<td>25.0</td>
<td>13.8</td>
<td>90.1</td>
<td>37.5</td>
</tr>
<tr>
<td>LK-2</td>
<td>26.2</td>
<td>11.9</td>
<td>86.0</td>
<td>39.1</td>
</tr>
<tr>
<td>NR-3</td>
<td>54.3</td>
<td>24.6</td>
<td>67.0</td>
<td>30.4</td>
</tr>
<tr>
<td>MV-4</td>
<td>17.3</td>
<td>9.6</td>
<td>77.0</td>
<td>32.1</td>
</tr>
<tr>
<td>AL-5</td>
<td>23.0</td>
<td>11.5</td>
<td>46.8</td>
<td>23.4</td>
</tr>
<tr>
<td>HI-6</td>
<td>15.0</td>
<td>7.5</td>
<td>44.3</td>
<td>22.1</td>
</tr>
<tr>
<td>PU-7</td>
<td>7.3</td>
<td>4.0</td>
<td>44.0</td>
<td>24.4</td>
</tr>
<tr>
<td>CB-8</td>
<td>15.0</td>
<td>8.3</td>
<td>73.0</td>
<td>40.5</td>
</tr>
<tr>
<td>AS-9</td>
<td>28.8</td>
<td>16.0</td>
<td>111.3</td>
<td>61.8</td>
</tr>
<tr>
<td>JH-10</td>
<td>17.4</td>
<td>7.9</td>
<td>82.0</td>
<td>41.0</td>
</tr>
<tr>
<td>FM-11</td>
<td>10.5</td>
<td>4.0</td>
<td>36.8</td>
<td>14.2</td>
</tr>
<tr>
<td>MF-12</td>
<td>7.6</td>
<td>3.8</td>
<td>59.1</td>
<td>21.2</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>20.6 ± 13*</td>
<td>10.2 ± 5.9*</td>
<td>68.1 ± 22.6</td>
<td>32.3 ± 12.7</td>
</tr>
</tbody>
</table>

\( a \): Statistical significance is as follows: *, significantly different from fresh broccoli (\( p < 0.001 \)).
\( b \): Value obtained after subtracting the baseline; values not normalized using urinary creatinine levels.
\( c \): Percentage on the basis of analyzed amount of ITC equivalents (\( \mu \)mol) in broccoli.

Table 3. SFN-NAC Excreted in 24 Hours After Ingestion of Steamed and Fresh Broccoli

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>SFN-NAC within 24 h, ( \mu )mol</th>
<th>Urinary SFN-NAC 24 h after steamed broccoli, %</th>
<th>SFN-NAC within 24 h, ( \mu )mol</th>
<th>Urinary SFN-NAC 24 h after steamed broccoli, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB-1</td>
<td>5.3</td>
<td>21.2</td>
<td>45.6</td>
<td>50.6</td>
</tr>
<tr>
<td>LK-2</td>
<td>7.1</td>
<td>27.1</td>
<td>15.9</td>
<td>18.4</td>
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<tr>
<td>NR-3</td>
<td>11.7</td>
<td>21.5</td>
<td>19.0</td>
<td>28.3</td>
</tr>
<tr>
<td>MV-4</td>
<td>6.4</td>
<td>36.9</td>
<td>43.2</td>
<td>56.1</td>
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<tr>
<td>AL-5</td>
<td>7.1</td>
<td>30.8</td>
<td>20.0</td>
<td>42.7</td>
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<tr>
<td>HI-6</td>
<td>11.3</td>
<td>75.0</td>
<td>11.9</td>
<td>26.8</td>
</tr>
<tr>
<td>PU-7</td>
<td>5.8</td>
<td>79.4</td>
<td>37.4</td>
<td>85.0</td>
</tr>
<tr>
<td>CB-8</td>
<td>6.5</td>
<td>43.3</td>
<td>42.3</td>
<td>57.9</td>
</tr>
<tr>
<td>AS-9</td>
<td>6.7</td>
<td>23.2</td>
<td>15.9</td>
<td>14.2</td>
</tr>
<tr>
<td>JH-10</td>
<td>3.8</td>
<td>21.8</td>
<td>35.7</td>
<td>43.5</td>
</tr>
<tr>
<td>FM-11</td>
<td>7.4</td>
<td>70.4</td>
<td>15.3</td>
<td>41.6</td>
</tr>
<tr>
<td>MF-12</td>
<td>3.5</td>
<td>46.5</td>
<td>8.5</td>
<td>14.4</td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>6.9 ± 2.5*</td>
<td>41.4 ± 21.9</td>
<td>25.8 ± 13.9</td>
<td>40.0 ± 21.0</td>
</tr>
</tbody>
</table>

\( a \): Statistical significance is as follows: *, significantly different from fresh broccoli (\( p < 0.001 \)).
\( b \): Percentage of total ITC equivalents excreted in 24 h; values not normalized using urinary creatinine levels. SFN-NAC, N-acetyl-L-cysteine conjugate of SFN.
eaten food containing free ITCs or glucosinolates recently may be attributed, at least in part, to ITCs released from plasma proteins; the ITCs or their glucosinolates may have been ingested several days or even a week before sample collection. The HPLC-based assay of the product from the cyclocondensation reaction is quite specific for ITCs and their thiol conjugates and is highly accurate and precise. However, products of hydrolysis of indole-containing glucosinolates and glucosinolates containing β-hydroxalkenyl do not react in the cyclocondensation reaction (17,25). In addition, some dithiocarbamates, thiourea, and xanthates will also react weakly with 1,2-benzenedithiol (25) and, when present, could have contributed to the background levels detected in the assays for total ITCs. Our validation studies have confirmed that dithiocarbamates in the rubber stoppers of Vacutainer tubes used in blood collection contribute to the baseline levels of plasma cyclocondensation products, but the extent was ≤10% of measured values (29). Cigarette smoking produces carbon disulfide, which reacts with 1,2-dibenzothiol and contributes somewhat to the amount of cyclocondensation products detected in the urine (17); for that reason, only non-tobacco users were selected for our work. A cursory examination of 24-hour urinary ITC excretion (Table 2) suggests that subjects who excreted relatively high amounts of ITCs after ingestion of fresh broccoli tended to do the same when steamed broccoli was provided. Further investigations are required to explain the variability among individuals and the high baselines for total ITCs in urine and plasma.

In conclusion, these results indicate that cooking broccoli by steaming significantly limits the bioavailability of ITCs to human subjects by inactivation of myrosinase. Therefore, considering the potential chemopreventive activity of ITCs in broccoli, including SFN, cooking may substantially reduce the health benefits of broccoli in the diet. This conclusion is further supported by the epidemiological observation that raw vegetables are more consistently associated with lower cancer risk (15). The information obtained here may serve as a guideline for the evaluation of potential cancer-chemopreventive effects of dietary consumption of broccoli and other cruciferous vegetables by humans.

Acknowledgments and Notes

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References


