

Chemoprotective Glucosinolates and Isothiocyanates of Broccoli Sprouts: Metabolism and Excretion in Humans¹

Theresa A. Shapiro,² Jed W. Fahey, Kristina L. Wade, Katherine K. Stephenson, and Paul Talalay

Department of Pharmacology and Molecular Sciences [T. A. S., J. W. F., K. L. W., K. K. S., P. T.] and the Division of Clinical Pharmacology, Department of Medicine [T. A. S.], The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract

Broccoli sprouts are a rich source of glucosinolates and isothiocyanates that induce phase 2 detoxication enzymes, boost antioxidant status, and protect animals against chemically induced cancer. Glucosinolates are hydrolyzed by myrosinase (an enzyme found in plants and bowel microflora) to form isothiocyanates. *In vivo*, isothiocyanates are conjugated with glutathione and then sequentially metabolized to mercapturic acids. These metabolites are collectively designated dithiocarbamates. We studied the disposition of broccoli sprout glucosinolates and isothiocyanates in healthy volunteers. Broccoli sprouts were grown, processed, and analyzed for (a) inducer potency; (b) glucosinolate and isothiocyanate concentrations; (c) glucosinolate profiles; and (d) myrosinase activity. Dosing preparations included uncooked fresh sprouts (with active myrosinase) as well as homogenates of boiled sprouts that were devoid of myrosinase activity and contained either glucosinolates only or isothiocyanates only. In a crossover study, urinary dithiocarbamate excretion increased sharply after administration of broccoli sprout glucosinolates or isothiocyanates. Cumulative excretion of dithiocarbamates following 111- μ mol doses of isothiocyanates was greater than that after glucosinolates (88.9 ± 5.5 and $13.1 \pm 1.9 \mu\text{mol}$, respectively; $P < 0.0003$). In subjects fed four repeated 50- μ mol doses of isothiocyanates, the intra- and intersubject variation in dithiocarbamate excretion was

very small (coefficient of variation, 9%), and after escalating doses, excretion was linear over a 25- to 200- μ mol dose range. Dithiocarbamate excretion was higher when intact sprouts were chewed thoroughly rather than swallowed whole (42.4 ± 7.5 and $28.8 \pm 2.6 \mu\text{mol}$; $P = 0.049$). These studies indicate that isothiocyanates are about six times more bioavailable than glucosinolates, which must first be hydrolyzed. Thorough chewing of fresh sprouts exposes the glucosinolates to plant myrosinase and significantly increases dithiocarbamate excretion. These findings will assist in the design of dosing regimens for clinical studies of broccoli sprout efficacy.

Introduction

Numerous epidemiological studies indicate that consumption of large quantities of fruits and vegetables, particularly cruciferous vegetables (e.g., broccoli, cabbage, kale, and Brussels sprouts), is associated with a reduced incidence of cancer (1–6). In a series of laboratory studies, we found that broccoli is rich in phytochemicals that induce phase 2 detoxication enzymes and bolster antioxidant activities in mammalian cells (7–10). Nearly all of this inducer activity is attributable to sulforaphane, which is an isothiocyanate. In intact plants, sulforaphane and other isothiocyanates are stored as inert precursors, termed glucosinolates. Isothiocyanates are released when glucosinolates undergo hydrolysis by myrosinase (E. C. 3.2.3.1), an enzyme that coexists with glucosinolates in crucifers (Fig. 1A). Young broccoli plants are an especially good source of chemoprotective glucosinolates, with levels 20–50 times those found in mature market-stage plants (9). Dietary broccoli sprouts and their component glucosinolates and isothiocyanates induce phase 2 enzymes and afford protection against chemically induced tumors in rodents (8, 9).

The observation that dietary glucosinolates are not detected in urine suggests they are not absorbed (11). Furthermore, with the exception of an early report (12), all available evidence suggests that mammalian tissues cannot convert glucosinolates to isothiocyanates (reviewed in Ref. 13). However, we and others have shown that dietary glucosinolates are indeed converted to isothiocyanates in animals and humans, and that this conversion is mediated by the myrosinase activity of enteric microflora (11, 14, 15). Once generated, isothiocyanates are absorbed and metabolized by sequential enzymatic reactions, the first of which is conjugation with glutathione (Refs. 16 and 17; Fig. 1B). In this report the glutathione adduct and subsequent metabolites are collectively termed dithiocarbamates.

Isothiocyanates and their dithiocarbamate metabolites react quantitatively with 1,2-benzenedithiol to form 1,3-benzodithiole-2-thione, a cyclic product that is readily separated by

Received 9/8/00; revised 1/3/01; accepted 2/11/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

¹ Supported by Program Project Grant P01 CA44530 and National Center for Research Resources Grant RR00052 from the Department of Health and Human Services, the American Institute for Cancer Research, the Cancer Research Foundation of America, and the Burroughs Wellcome Fund. The Johns Hopkins University, P. T. and J. F. are equity holders in Brassica Protection Products LLC, which is involved in the commercialization of broccoli sprouts. P. T. and J. F. are scientific consultants to this company. The University is managing this arrangement in accordance with its conflict of interest policies. No funding for the reported work was provided by Brassica.

² To whom requests for reprints should be addressed, at Department of Pharmacology and Molecular Sciences, The Johns Hopkins University School of Medicine, 725 North Wolfe Street, Baltimore, MD 21205. Phone: (410) 955-1888; Fax: (410) 955-2634; E-mail: tshapiro@jhmi.edu.

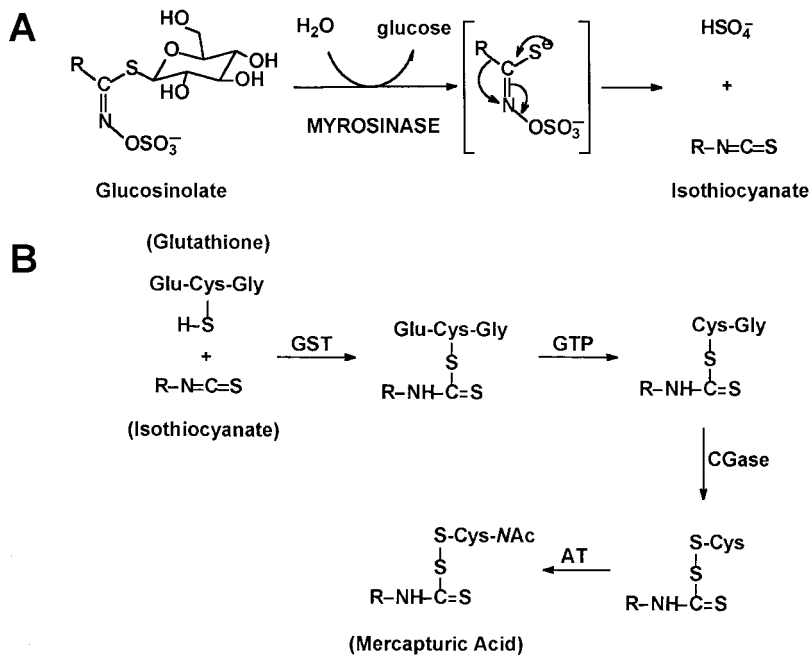


Fig. 1. A, glucosinolates are converted to isothiocyanates by the enzymatic action of myrosinase. For glucoraphanin (a glucosinolate) and sulforaphane (its cognate isothiocyanate), the R group is $\text{CH}_2\text{SO}(\text{CH}_2)_4-$. B, isothiocyanates are conjugated to glutathione by glutathione S-transferase (GST), then metabolized sequentially by γ -glutamyltranspeptidase (GTP), cysteinylglycine (CGase), and acetyltransferase (AT) ultimately to form mercapturic acids.

HPLC³ and detected by UV spectroscopy (11, 18, 19). This cyclocondensation reaction has provided a versatile and highly reproducible method for quantitating glucosinolates and isothiocyanates in plants, as well as their dithiocarbamate metabolites in urine. In previous clinical studies, we found that urinary dithiocarbamates are a sensitive indicator of crucifer consumption, and that dithiocarbamate excretion falls to undetectable levels in the absence of dietary crucifers and cigarette smoking (11). Collective results from dozens of nonsmoking volunteers maintained on diets devoid of glucosinolates or isothiocyanates indicate there are no endogenous sources of urinary dithiocarbamates.⁴

In a previous series of clinical studies, we made extensive use of the cyclocondensation assay: (a) to prepare known doses of dietary glucosinolates and isothiocyanates, administered in the form of various cruciferous vegetables or condiments; and (b) to quantitate their urinary metabolites (11). In this report, we focus exclusively on 3-day-old broccoli sprouts, which are an unusually rich source of glucoraphanin, the glucosinolate precursor of sulforaphane (structures are shown in Fig. 1A). The objective of these clinical trials was to obtain information on the metabolism and excretion of broccoli sprout glucosinolates and isothiocyanates, information essential for the rational design of dietary intervention studies.

Materials and Methods

This report comprises four human studies: two inpatient protocols with broccoli sprout homogenates (termed HS37 and HS43) and two outpatient protocols with uncooked, fresh, intact broccoli sprouts (HS40 and HS42). For all protocols, the first day of the study is designated day 0.

Healthy Volunteers

Subjects were recruited by advertising on local bulletin boards and were eligible to join if they were 18 years of age or older and in good health. Subjects selected for inpatient studies were within 25% of ideal weight for height and build (20), did not use any form of tobacco or regularly take any medications, had not taken any antibiotics for 4 weeks immediately preceding the study, and had no significant abnormalities on medical history, physical examination, or laboratory tests (including routine hematology; chemistry; urine analysis; hepatitis A, B, and C serologies; and HIV testing). Written informed consent was obtained before enrollment. For each of the four studies, subjects were enrolled and studied in a single cohort.

General Study Design

The studies were conducted in the inpatient and outpatient facilities of the NIH-sponsored General Clinical Research Center in the Johns Hopkins Hospital after approval by the Joint Committee on Clinical Investigation. For inpatient studies, a diet free of glucosinolates or isothiocyanates was provided by the Research Nutrition Service. Every component of the diet was assayed (described below) and found to be free of detectable glucosinolates, isothiocyanates, or phase 2 enzyme-inducing activity. Subjects were asked not to eat or drink anything not provided with their meals and not to use any systemic or topical medications, including skin lotions. Urine was collected throughout the entire study in 8-h collection intervals (7 a.m. to 3 p.m.; 3–11 p.m.; and 11 p.m. to 7 a.m.; unless indicated otherwise) and stored at 4°C for ≤ 24 h before analysis. On dosing days, subjects consumed nothing but water after midnight; the sprout preparation was administered with 240 ml water at 7 a.m., and breakfast was withheld until 10 a.m. Outpatient studies were conducted similarly, except that volunteers were asked to abstain from dietary sources of glucosinolates and isothiocyanates and to keep a food diary.

³ The abbreviations used are: HPLC, high-performance liquid chromatography; CV, coefficient of variation.

⁴ T. A. Shapiro, unpublished data.

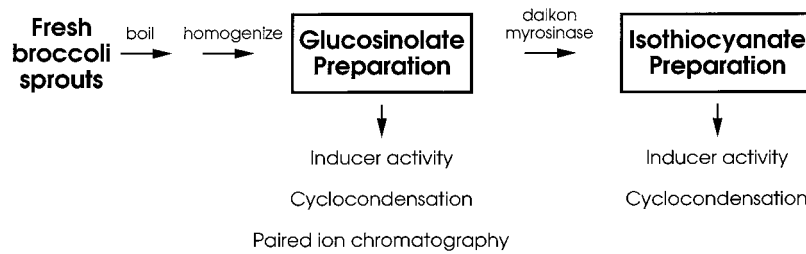


Fig. 2. Scheme for preparing broccoli sprout dosing materials (boxed). As detailed in the text, 3-day-old broccoli plants were harvested quickly and plunged at once into boiling water to destroy endogenous myrosinase activity. The sprouts were homogenized in their cooking water to generate the glucosinolate dosing preparation, and a small aliquot was taken for analysis. A portion of the homogenate was treated with daikons (2% by weight), whose myrosinase activity converted the glucosinolates to isothiocyanates. An aliquot of the isothiocyanate preparation was also analyzed. Each of the indicated analyses was done with and without added myrosinase (Table 1). These preparations were used in the crossover dosing study (HS37). A similar procedure was used to prepare isothiocyanates for the repeated and escalating dose study (HS43).

Preparation and Analysis of Plant Material

Plant preparations were made individually for each of the four studies. All studies were conducted with 3-day-old broccoli sprouts (*Brassica oleracea* var. *italica*; cultivar DeCicco). Seeds untreated with pesticides were obtained from Penn State Seeds, Inc. or from Seeds by Design, surface-sterilized with a 1-min rinse in 70% ethanol, exposed for 15 min to 1.3% sodium hypochlorite containing 0.001% Alconox detergent, then rinsed exhaustively with distilled water. Seeds were sprouted in inclined perforated plastic trays (36 × 42 cm), at 25°C without added nutrients, by watering with four 15-s overhead spray cycles per h and maintaining a 16-h light and 8-h dark photoperiod.

Intact Sprouts. Fresh intact sprouts for direct dosing were grown, harvested as described, and stored in the dark at 4°C for no more than 2 days before feeding. Triple solvent (equal volumes of DMSO, dimethylformamide, and acetonitrile) extracts were prepared immediately after harvest (9, 11), evaporated to dryness, then redissolved in distilled water for cyclocondensation, paired-ion chromatography, or assay of inducer activity (described below). Myrosinase activity was assayed in aqueous homogenates, also described below.

Homogenates. An overall scheme for preparing broccoli sprout homogenates suitable for dosing is depicted in Fig. 2. Sprouts were rapidly and gently harvested from the trays, plunged at once into boiling water (1:3.5, w:w) in a 40-liter Groen Model D40 steam-jacketed kettle, and boiled for 5 min. This process effectively destroys the plant myrosinase activity (11). The entire mixture was then homogenized in 2-liter aliquots in a 4-liter stainless steel Waring Blender. The pooled homogenate (termed the glucosinolate preparation) was cooled and divided three ways: a small portion for analysis, a portion for glucosinolate dosing (when required for the study), and a portion to generate the isothiocyanate preparation. The latter was achieved by treating the glucosinolate preparation with daikon (*Raphanus sativus*) sprout myrosinase as detailed previously (11). Briefly, 7-day-old daikon plantlets (2% of the broccoli sprouts, by fresh weight) were homogenized with the glucosinolate preparation and the mixture was incubated at 37°C for 2 h. Samples were retained for analysis before freezing. A dosing preparation to control for the slight contribution daikons make to the broccoli sprout isothiocyanates was prepared by homogenizing 7-day-old daikon sprouts in cold water, then incubating at 37°C for 2 h, and taking portions for analysis. All homogenates were stored at -20°C. Before dosing, homogenates were thawed at 4°C and apportioned to provide the desired

dose of glucosinolates or isothiocyanates. All dosing preparations were analyzed by cyclocondensation reaction and by paired-ion chromatography; some were also analyzed for inducer activity (Table 1).

Preparation and Analysis of Control Diet

For the inpatient studies, volunteers were maintained on a control diet prepared by the General Clinical Research Center Research Nutrition staff, in a research kitchen located on the ward. The diet was devoid of any spices, herbs, known preservatives, condiments (e.g., mustard, horseradish, wasabi, soy sauce, ketchup, or mayonnaise), crucifers (e.g., broccoli, kale, Brussels sprouts, cabbage, radish, daikon, turnip, kohlrabi, or rutabaga), or vegetables in the onion family (e.g., onions, leeks, garlic, or chives). Salt and sugar were permitted. Breads were prepared from flour free of butylated hydroxyanisole (BHA) or butylated hydroxytoluene (BHT), and meats were baked rather than fried or broiled. Coffee, tea, or caffeinated sodas were not served. Three meals and one snack were served each day, and the menu was repeated every 6 days. At each feeding, the subjects received identical portions, and for HS43, an extra meal or snack was prepared for analytical purposes. Shortly after preparation, the entire meal or snack was homogenized in an equal volume of water and a portion was stored at -20°C until analysis. Beverages and thawed food homogenates were added directly to the assay for quinone reductase-inducer activity (highest concentration of sample, 10% v/v in the assay). Bacterial contamination was successfully avoided by including penicillin and streptomycin in the assays. Limit of detection was <70 units of inducer activity/g of food.⁵

Analytical Methods

Quinone Reductase Inducer Activity. Induction of quinone reductase catalytic activity was measured in Hepa 1c1c7 murine hepatoma cells grown in microtiter plate wells each containing 150 μ l of medium (9, 21, 22). Plant homogenates were centrifuged (5600 × g for 3 min at 25°C), and the supernatant was assayed. The highest concentrations of plant

⁵ The inducer activity of sulforaphane is 33,333 units/ μ mol; closely related methylthioalkyl isothiocyanates such as isoberin and erucin have about 10% of the potency of sulforaphane; indole isothiocyanates have only about 1% the potency of sulforaphane (7, 25). One unit of inducer activity is defined as the amount of material that doubles quinone reductase specific activity in 48 h (see "Materials and Methods").

Table 1 Characterization of broccoli sprout dosing preparations^aBroccoli sprouts were grown, processed, and analyzed before each of the four clinical studies. Doses (in μmol) were apportioned based on cyclocondensation results.

Study and dosing preparation	Inducer activity ^b (units/g fresh weight)		Cyclocondensation ^b ($\mu\text{mol/g}$ fresh weight)		Glucosinolates by paired-ion chromatography ^c			Myrosinase (units/g fresh weight)	
	Total	ITC	Total	ITC	Total ($\mu\text{mol/g}$ fresh weight)	Methylthioalkyl			Indole
						GR + GI ^d	GE (percent of total)		
HS37									
Glucosinolate	405,000	20,500	8.55	0.33	8.90	78.9	18.1	2.9	-
Isothiocyanate	389,000	332,000	7.49	7.66	-	-	-	-	-
Daikon control	178,000	163,000	4.22	4.40	-	-	-	-	-
HS40									
Intact sprouts	518,000	<1,000	8.46	ND	10.9	74.7	18.2	7.1	3.03
HS42									
Intact sprouts	-	-	9.08	0.05	8.80	75.0	21.6	3.4	-
HS43									
Isothiocyanate	-	-	8.43	0.18	11.0	77.6	20.5	1.9	-

^a ITC, isothiocyanates; GR, glucoraphanin; GI, glucoiberin; GE, glucoerucin; ND, not detectable; -, not determined. See Fig. 3 for glucosinolate structures.^b Myrosinase was added to the assay to determine total activity (glucosinolates plus isothiocyanates); mock reactions without myrosinase were performed to obtain the contribution of isothiocyanates alone.^c Estimation of total glucosinolates excludes minor unidentified peaks, which constitute <5% of the known glucosinolates.^d Glucoraphanin:glucoiberin ratio is 2:1 or more.

homogenate supernatant and urine were equivalent to 0.75 and 15 μl , respectively. Eight or more replicates of eight 1:1 serial dilutions in medium were assayed. To measure total inducer activity in plant samples, excess purified myrosinase (23) and 500 μM ascorbate to activate the myrosinase were added directly to the microtiter plates. One unit of inducer activity is the amount that doubles quinone reductase specific activity in 48 h in a well containing 150 μl of medium.

Cyclocondensation Reaction. This assay detects, with equal efficiency, isothiocyanates and their dithiocarbamate metabolites. Glucosinolates (which do not react in the cyclocondensation reaction) were determined after quantitative conversion to isothiocyanates by treating with purified myrosinase (23) before cyclocondensation. All plant dosing materials and all urine samples were analyzed by cyclocondensation as described in detail previously (11, 18, 19). Just before cyclocondensation, plant homogenates and urine specimens were centrifuged to remove particulate matter (2000 $\times g$ for 10 min at 4°C or 200 $\times g$ for 5 min at 4°C, respectively). Plant supernatants were maintained for 2 h at 37°C, with or without myrosinase, before cyclocondensation. Because urine samples contain no glucosinolates (11), they were not treated with myrosinase. Cyclocondensation reactions [2 ml; 50% methanol (v/v); 125 mM sodium borate (pH 9.25); 20 mM 1,2-benzenedithiol; and 5% to 25% (v/v) sample] were performed under N_2 in 4-ml glass vials (2 h; 65°C). The vials were loaded into a Waters WISP Autosampler; aliquots of each reaction mixture were injected onto a Whatman Partisil 10 octadecylsilane-2 reverse-phase HPLC column and eluted isocratically with 80% methanol and 20% water (v/v). The area of the 1,3-benzodithiole-2-thione peak was integrated at 365 nm by means of a Waters Photodiode Array detector and Waters Millennium software (version 2.15.01). All samples were assayed at three dilutions, and each analytical run included a water blank; a 1,3-benzodithiole-2-thione standard curve; and three dilutions of the *N*-acetylcysteine derivative of allyl isothiocyanate as a positive control. The lower limits of detection are 80 pmol/g fresh weight broccoli sprouts or 0.2 nmol/ml of urine.

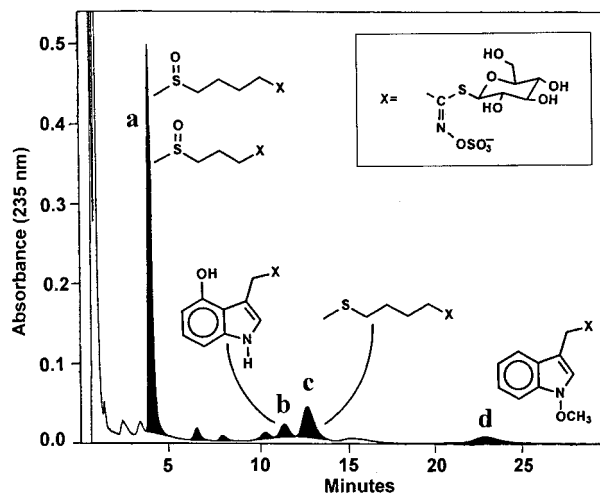
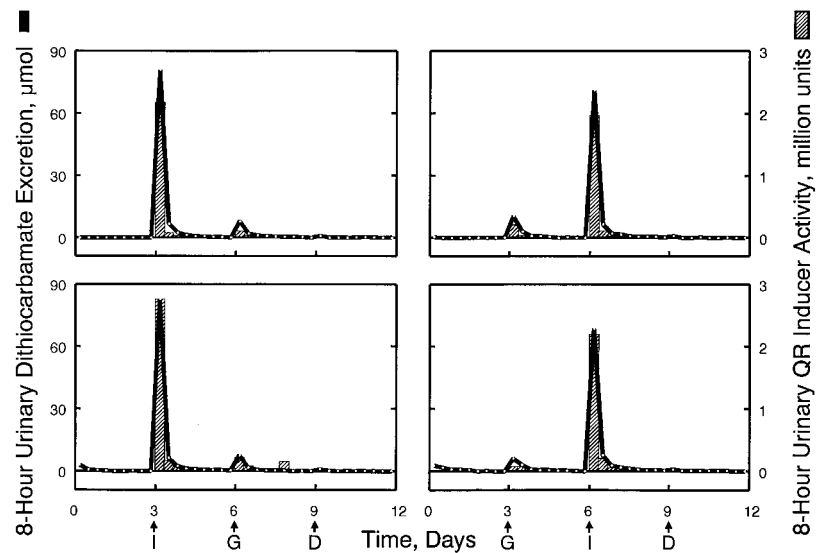


Fig. 3. Paired-ion chromatography of broccoli sprout glucosinolates. Plant extracts were separated by HPLC in the presence of tetradecylammonium bromide. Glucosinolates (shaded black peaks) were identified by their absence from a repeat chromatogram after treatment of the extract with myrosinase. In order of increasing retention times, the known glucosinolates are glucoraphanin and glucoiberin (a), 4-hydroxyglucobrassicin (b), glucoerucin (c), and neoglucobrassicin (d). Unlabeled minor peaks are tentatively identified as alkyl glucosinolates. Under different experimental conditions, peak a can be resolved into its components; in dozens of such analyses, the glucoraphanin content of broccoli sprouts is reproducibly more than twice that of glucoiberin.⁶

Paired-Ion Chromatography of Glucosinolates. Plant extracts were chromatographed in acetonitrile:water (1:1, by volume) containing 5 mM tetradecylammonium bromide on a Whatman Partisil 10 octadecylsilane-2 column (11, 24). This provides a qualitative analysis of the glucosinolate content. To

⁶ J. W. Fahey, unpublished data.

Fig. 4. Time course of dithiocarbamate excretion and quinone reductase-inducer activity in urine of volunteers fed broccoli sprout preparations in a crossover dosing study (HS37). Each panel represent the results from a single volunteer. After a 3-day period on control diet, the subjects were fed 111- μmol portions of broccoli sprout isothiocyanates (I) or glucosinolates (G), as indicated; 3 days later, the doses were crossed. On day 9, all subjects were fed 0.92 μmol daikon sprout isothiocyanates (D). Urine was collected throughout the entire 12-day study in 4- or 8-h intervals and assayed for dithiocarbamate content (connected \circ) and for quinone reductase inducer activity (bars). The small peak of inducer activity on day 7 for one of the subjects may be an artifact or may reflect a dietary indiscretion.



estimate the quantity of each glucosinolate, an assumption was made that the relative integrated absorbance areas at 235 nm for alkylthioglucosinolates (*e.g.*, glucoraphanin, glucoiberin, and glucoerucin) and indole glucosinolates (*e.g.*, neoglucobrassicin and 4-hydroxyglucobrassicin) are 1.0 and 2.7 times, respectively, those of an equimolar quantity of allyl glucosinolate (9). A representative tracing and glucosinolate structures are presented in Fig. 3.

Myrosinase Activity. Uncooked sprouts were homogenized in 33 mM potassium phosphate buffer (pH 7.0) with a Brinkman Polytron homogenizer (0.5 \times full speed for 15 s at 4°C) and centrifuged (5600 \times g for 10 s at 4°C). Myrosinase activity in supernatants was determined by measuring the rate of decrease in absorbance at 235 nm resulting from hydrolysis of allyl glucosinolate, as described previously (23) and with the following modifications. The 1.0-ml reaction mixture contained 33 mM potassium phosphate buffer (pH 7.0), 50 μM allyl glucosinolate, 500 μM ascorbic acid, and 1 mM EDTA. The reaction was initiated by the addition of 50 μl of supernatant. One unit of myrosinase activity hydrolyzes 1 μmol of allyl glucosinolate/min.

Statistical Methods

Results are expressed as means \pm SDs. CV is the (SD/mean) \times 100%. Student's *t* tests are paired and two-tailed.

Results

Dosing Materials and Control Diet. We made new plant preparations for each of the four studies and analyzed them for (a) inducer activity; (b) glucosinolate and isothiocyanate content by cyclocondensation; (c) glucosinolate profile by paired-ion chromatography; and, in some cases, (d) myrosinase catalytic activity (Fig. 2, Table 1). As expected, the broccoli sprouts contained high levels of phase 2 enzyme-inducer activity, comparable with those we reported previously (9). By far the greatest contribution to inducer activity is provided by methylthioalkyl glucosinolates, which constitute >93% of the mass of glucosinolates (Fig. 3, Table 1) and whose cognate isothiocyanates have the greatest inducer potency.⁵ Indole glucosinolates were present at <7% of total mass (Table 1) and afforded only weak inducer potency (25).

Goitrogenic β -hydroxyalkenyl glucosinolates were not detected (Fig. 3). Multiple independent assays of activity and composition provided consistent results of ~ 9 μmol glucosinolates/g fresh weight broccoli sprouts (Table 1). The inducer potency of broccoli plants depends on numerous factors, including genotype, time of harvest, and conditions of growth (26, 27). The four batches of broccoli sprouts produced over time for these studies were of the same genotype and were grown under controlled environmental conditions, which contributed to the uniformity of the dosing preparations.

During HS43, the control diet was extensively tested for evidence of phase 2 enzyme inducer activity (quinone reductase assay). For all 18 meals and six snacks, inducer activity was $53,000 \pm 37,000$ units/meal, which is equivalent to <2 μmol of sulforaphane. (By comparison, the glucosinolate/isothiocyanate doses in the four studies reported here ranged from 25 to 200 μmol).

Glucosinolate and Isothiocyanate Crossover Study (HS37).

In a 12-day inpatient study conducted with four healthy male volunteers (age range, 43–53 years; 3 blacks and 1 white) we compared the urinary dithiocarbamate excretion after feeding equimolar doses of broccoli sprout glucosinolates or isothiocyanates. Glucosinolate and isothiocyanate dosing preparations (Fig. 2) were characterized by bioassay, cyclocondensation reaction, and paired-ion chromatography (Table 1). The glucosinolate preparation contained <4% isothiocyanate; conversely, the isothiocyanate preparation contained no detectable glucosinolates. To make the isothiocyanate preparation, we added daikon sprouts (as a source of myrosinase), which themselves contain glucosinolates/isothiocyanates (Table 1). However, based on the fact that daikons were added at just 2% by weight, this resulted in a contribution of <1% to the isothiocyanate dosing preparation. After a 3-day washout period, subjects were dosed at 7 a.m. with 111 μmol glucosinolate preparation (two subjects) or isothiocyanate preparation (two subjects). Three days later, the doses were crossed; and on day 9 all four subjects were given a volume of daikon homogenate (containing 0.92 μmol isothiocyanates) equivalent to that used to prepare the broccoli sprout isothiocyanate dose. After each dose, urine was collected in 4-h periods, from 7 a.m. to 11 a.m. and 11 a.m. to 3 p.m., and thereafter in 8-h collection periods.

For all four subjects, urinary dithiocarbamate excretion and urinary inducer activity fell below the level of detection during the baseline period (Fig. 4). Thereafter there was a brisk rise in both of these end points in response to dosing with isothiocyanate or glucosinolate preparations. The time course and magnitude of dithiocarbamate excretion closely parallel inducer activity throughout. In all but one case, dithiocarbamate excretion peaked in the 0–4 h postdose collection; for one subject dosed with glucosinolates it peaked in the 4–8 h collection (data not shown). Cumulative 72 h urinary dithiocarbamate excretion after isothiocyanates (corrected for daikon contribution) was significantly greater than that after glucosinolates ($88.9 \pm 5.5 \mu\text{mol}$ and $13.1 \pm 1.9 \mu\text{mol}$, respectively; $P < 0.0003$). Overall, the variation among subjects in excretion after isothiocyanate dosing (CV, 8.2%) was smaller than that after glucosinolate dosing (CV, 17.7%).

Repeated and Escalating Doses of Isothiocyanates (HS43).

The reproducibility of dithiocarbamate excretion and the relationship of dithiocarbamate excretion to isothiocyanate dose were evaluated in four male subjects (age range, 21–53 years; two blacks and two whites) who were enrolled in a 20-day inpatient study. A broccoli sprout isothiocyanate preparation was made and characterized as described above (Fig. 2, Table 1), and individual aliquot doses were frozen until use. After a 2-day washout period, subjects were fed $50 \mu\text{mol}$ of isothiocyanate on days 2, 4, 6, and 8; then $25 \mu\text{mol}$ on day 11, $100 \mu\text{mol}$ on day 14, and $200 \mu\text{mol}$ on day 17. Urine was collected throughout the entire 20-day study, in 8-h intervals.

Three subjects completed the entire study; one withdrew on day 6 just before the third dose. For all subjects, urinary dithiocarbamate excretion fell below detectable levels during the initial control period. After each dose of broccoli sprout isothiocyanates, there was a brisk increase in urinary dithiocarbamate excretion, which peaked (in every case) within the first 8 h after dosing and then decreased in a non-first-order fashion (Fig. 5A). There is remarkably little variation between subjects in the time course of excretion, or in the extent of excretion after (a) repeated doses of $50 \mu\text{mol}$ (CV $\leq 5.5\%$; 0–48 h collections); or (b) escalating doses of 25–200 μmol (CV $\leq 9.0\%$; 0–72 h collections). The intrasubject variability with repeated doses of $50 \mu\text{mol}$ was similarly low (CV $\leq 7.9\%$). The dose-response curve is linear over an 8-fold range of doses and extrapolates through the origin (Fig. 5B).

Urinary Dithiocarbamate Excretion after Feeding Intact Sprouts (HS40 and 42). To evaluate the extent to which the hydrolysis of glucosinolates to isothiocyanates may be mediated by the myrosinase activity present in intact uncooked sprouts, in HS40 we monitored urinary dithiocarbamate excretion in a 2-day outpatient study with two male and nine female volunteers (age range, 23–75 years; one Asian, two black, one Hispanic, and seven white). At 7 a.m. on the 2nd day of the study, subjects were fed 11.8 g fresh weight broccoli sprouts (containing $100 \mu\text{mol}$ of glucosinolates; Table 1) and then given 240 ml of water. Urine was collected continuously for 48 h: three 8-h intervals before dosing and a single 24-h collection after dosing. Two subjects were excluded from additional analysis (one spilled a portion of the 24-h postdosing urine collection; and, based on the food diary, one ate condiments that likely contributed to urinary dithiocarbamates within the 24 h after dosing). Urinary dithiocarbamate excretion fell during the control period from $0.38 \pm 0.56 \mu\text{mol}$ (first baseline) to $0.21 \pm 0.34 \mu\text{mol}$ (third baseline) and rose to $55.8 \pm 7.8 \mu\text{mol}$ during the 24 h after dosing.

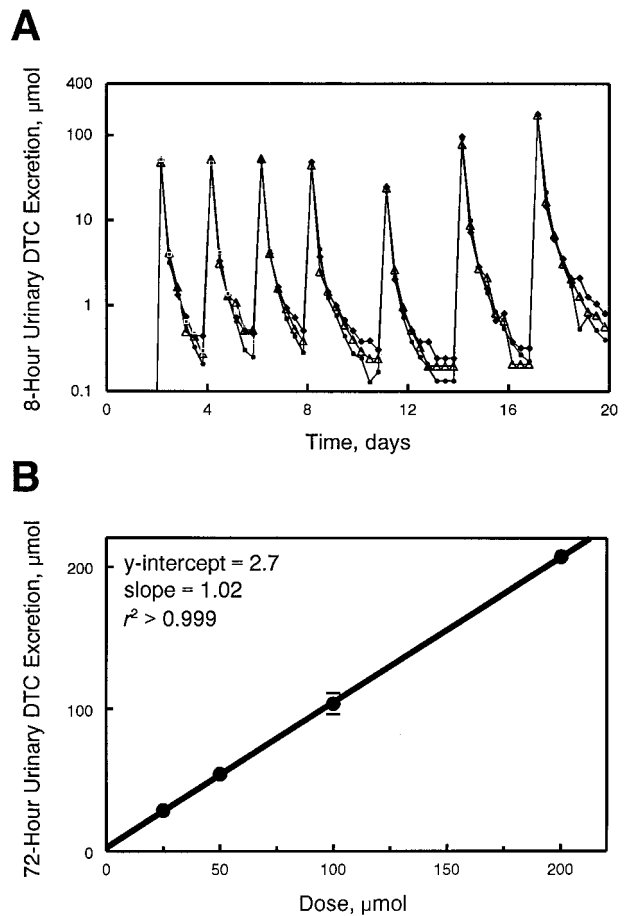


Fig. 5. Urinary dithiocarbamate (DTC) excretion after feeding of broccoli sprout isothiocyanates (HS43). A, time course of dithiocarbamate excretion after repeated, then escalating, doses. After a 2-day period on control diet, four subjects were fed $50 \mu\text{mol}$ of isothiocyanates on days 2, 4, 6, and 8; then $25 \mu\text{mol}$ on day 11; $100 \mu\text{mol}$ on day 14; and $200 \mu\text{mol}$ on day 17. Urine was collected throughout the entire 20-day study in 8-h intervals and assayed for dithiocarbamate content. Each subject is indicated by a different symbol; one subject (small \square) withdrew from the study just before the third dose. Some trough levels fell below the limit of detection; to avoid breaks in the curves, the value immediately preceding these points is repeated in their stead. For all subjects, the dithiocarbamate content of trough samples was $<1\%$ that of succeeding peaks, whether the dosing interval was 48 or 72 h. B, dithiocarbamate excretion as a function of dose. Cumulative 72-h dithiocarbamate excretion after the final four doses depicted in A are plotted as a function of dose. Excretion is proportional to dose, and the line extrapolates through the origin. Bars, one SD; some are too small to extend outside the points. Results are not significantly different if 48-h excretion is plotted for all available dosings, including the repeated administration of $50 \mu\text{mol}$ (first four doses, A).

To determine whether the extent of conversion of glucosinolates to isothiocyanates is affected by chewing intact uncooked broccoli sprouts, two male and one female subject (age range, 44–75 years; three whites) were enrolled in a 4-day outpatient study (HS42). On the 2nd day, subjects swallowed, without chewing, 12 g fresh weight broccoli sprouts containing $109 \mu\text{mol}$ of glucosinolates. On the 4th day, they thoroughly chewed before swallowing a $109\text{-}\mu\text{mol}$ dose. Urine was collected continuously throughout the study in three 8-h intervals before each dose and in a single 24-h interval after dosing. Thorough chewing, as compared with swallowing whole sprouts, resulted in significantly greater dithiocarbamate excretion ($42.4 \pm 7.5 \mu\text{mol}$ and $28.8 \pm 2.6 \mu\text{mol}$, respectively; $P = 0.049$; Fig. 6).

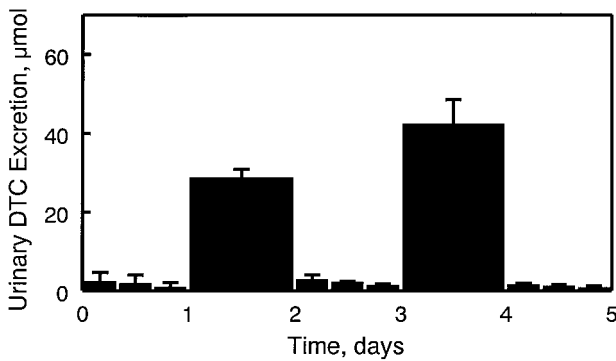


Fig. 6. Urinary dithiocarbamate (DTC) excretion after ingestion of fresh, intact, and uncooked broccoli sprouts (HS42). Urine was collected throughout the entire 5-day study in 8- or 24-h intervals, as indicated by bar widths. Subjects consumed 12 g of fresh broccoli sprouts (containing 109 μmol glucosinolates) either without chewing (day 1) or after chewing thoroughly (day 3). Dithiocarbamate excretion increased significantly if sprouts were chewed before being swallowed ($P = 0.049$).

Discussion

In previous clinical studies, we evaluated the metabolism and disposition of glucosinolates and isothiocyanates in a variety of crucifers, including market stage broccoli (11). The current studies focus entirely on broccoli sprouts, which differ from mature broccoli plants in two important aspects (9, 13, 25): (a) on a gram-fresh-weight basis, they contain up to 50 times more glucoraphanin, the glucosinolate precursor of sulforaphane, which is the most potent natural phase 2 enzyme-inducer known. Second, they contain substantially less or no detectable amounts of the indole and β -hydroxyalkenyl glucosinolates that are associated with potential toxicities (Table 1 and Fig. 3).

The principal end point in these studies was urinary dithiocarbamate excretion, as measured by the cyclocondensation reaction. Although this assay detects with equal efficiency isothiocyanates and each of the sequential metabolites (Fig. 1B), the available evidence indicates that urine contains no isothiocyanates, and that dithiocarbamates are the major metabolites that appear in urine (28–30). Urinary dithiocarbamates therefore reflect absorption, sequential metabolic conversions, and then excretion of the dose. In view of this, the remarkable absence of experimental variability in these studies is particularly striking. We consistently find CVs of $\leq 10\%$ for dithiocarbamate excretion values that include error contributions from: (a) the cyclocondensation assay; (b) repeated doses in the same individual over time; (c) different individuals; and (d) experiments conducted at different times and with different dosing preparations. This indicates that our methods are highly precise and also suggests that among different individuals, the overall pathway for disposing of dietary isothiocyanates is the same, and that it is operating well below capacity. The latter notion is supported by the strict linear relationship between isothiocyanate dose (over an 8-fold range) and urinary dithiocarbamate excretion (Fig. 5B). In future studies it will be interesting to see whether more marked differences can be found in a larger sample of the population and to determine whether such differences relate to glutathione *S*-transferase genotype.

The time course and extent of urinary dithiocarbamate excretion differ markedly after administration of broccoli sprout isothiocyanates or glucosinolates. Both preparations result in brisk excretion of urinary dithiocarbamates (Figs. 4 and

5A). However, isothiocyanate preparations yield dithiocarbamate peaks within 4 h after dosing, and $92.9 \pm 1.1\%$ of total dithiocarbamates appear in the first 16 h (total dithiocarbamates are defined as the amount excreted within 72 h after dosing). In contrast, after eating broccoli sprout glucosinolates, urinary dithiocarbamates peak within 8 h and are only $79.5 \pm 5.3\%$ complete by 16 h after dosing. Most notably, the extent of dithiocarbamate excretion after isothiocyanates is more than six times greater than that after an equimolar dose of glucosinolates (Fig. 4). The delayed peak and lower bioavailability after feeding glucosinolates are in keeping with our previous observation that the overall rate and extent of glucosinolate disposition is dictated by the initial conversion of glucosinolates to isothiocyanates (11). In the absence of plant myrosinase, as is the case with the glucosinolate preparations used in these studies, this glucosinolate-to-isothiocyanate conversion is mediated by bowel microflora.

In the glucosinolate/isothiocyanate crossover study (HS37), we found extraordinarily close correspondence in the time course and relative magnitude of urinary dithiocarbamate content and urinary phase 2 enzyme-inducer activity (Fig. 4), which affords additional validation of the analytical methods. The inducer activity in these urine samples is quite potent, at $\sim 24,000$ units/ μmol (as compared with 33,333 units/ μmol for sulforaphane; Ref. 7). This activity may be attributable to the dissociation of dithiocarbamates *in vitro* to yield sulforaphane (31).

Glucosinolates are inert as phase 2 enzyme inducers and must be hydrolyzed to generate the active isothiocyanates. The isothiocyanate and glucosinolate preparations used in this study were designed to represent the upper and lower limits of inducer activity that can be provided by intact broccoli sprouts. The isothiocyanate preparation entirely circumvents the problem of glucosinolate hydrolysis and thus represents a maximum achievable dose of inducer activity, whereas the glucosinolate preparation (which contains no plant myrosinase and depends entirely on gut flora for hydrolysis) represents a minimum achievable dose. We were intrigued to find that total dithiocarbamate excretion after feeding intact uncooked sprouts ($\sim 50\%$ of dose) was intermediate between that seen after feeding the limiting glucosinolate or isothiocyanate preparations ($\sim 15\%$ or 90% of dose, respectively). This suggests that myrosinase activity in intact sprouts contributes significantly to bioavailability by boosting the glucosinolate-to-isothiocyanate conversion. In view of this finding, it is not surprising that dithiocarbamate excretion was enhanced when fresh sprouts were chewed thoroughly, a process that facilitates hydrolysis by releasing myrosinase and glucosinolates from their sequestered sites within the plant cells.

These findings provide important insights into the disposition of broccoli sprout glucosinolates and isothiocyanates, which will be valuable for the design of rational dosing regimens for clinical studies to evaluate the safety of broccoli sprouts and their efficacy as phase 2 enzyme inducers.

Acknowledgments

We thank the staffs of the Clinical Pharmacology Drug Development Unit and the General Clinical Research Centers for their considerable expertise and help in conducting the clinical work. Jerry Collins and Yuesheng Zhang provided valuable advice.

References

- Block, G., Patterson, B., and Subar, A. Fruit, vegetables, and cancer prevention: a review of the epidemiological evidence. *Nutr. Cancer*, 18: 1–29, 1992.

2. Steinmetz, K. A., and Potter, J. D. Vegetables, fruit, and cancer prevention: a review. *J. Am. Diet. Assoc.*, *96*: 1027–1039, 1996.
3. World Cancer Research Fund. Food, Nutrition, and the Prevention of Cancer: A Global Perspective. Washington, DC: American Institute for Cancer Research, 1997.
4. van Poppel, G., Verhoeven, D. T., Verhagen, H., and Goldbohm, R. A. Brassica vegetables and cancer prevention. Epidemiology and mechanisms. *Adv. Exp. Med. Biol.*, *472*: 159–168, 1999.
5. Beecher, C. W. Cancer preventive properties of varieties of *Brassica oleracea*: a review. *Am. J. Clin. Nutr.*, *59*: 1166S–1170S, 1994.
6. Michaud, D. S., Spiegelman, D., Clinton, S. K., Rimm, E. B., Willett, W. C., and Giovannucci, E. L. Fruit and vegetable intake and incidence of bladder cancer in a male prospective cohort. *J. Natl. Cancer Inst.*, *91*: 605–613, 1999.
7. Zhang, Y., Talalay, P., Cho, C-G., and Posner, G. H. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. *Proc. Natl. Acad. Sci. USA*, *89*: 2399–2403, 1994.
8. Zhang, Y., Kensler, T. W., Cho, C-G., Posner, G. H., and Talalay, P. Anticarcinogenic activities of sulforaphane and structurally related synthetic norbornyl isothiocyanates. *Proc. Natl. Acad. Sci. USA*, *91*: 3147–3150, 1994.
9. Fahey, J. W., Zhang, Y., and Talalay, P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. *Proc. Natl. Acad. Sci. USA*, *94*: 10367–10372, 1997.
10. Fahey, J. W., and Talalay, P. Antioxidant functions of sulforaphane: a potent inducer of Phase II detoxication enzymes. *Food Chem. Toxicol.*, *37*: 973–979, 1999.
11. Shapiro, T. A., Fahey, J. W., Wade, K. L., Stephenson, K. K., and Talalay, P. Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. *Cancer Epidemiol. Biomarkers Prev.*, *7*: 1091–1100, 1998.
12. Goodman, I., Fouts, J. R., Bresnick, E., Menegas, R., and Hitchings, G. H. A mammalian thioglucosidase. *Science (Wash. DC)*, *130*: 450–451, 1959.
13. Fahey, J. W., Zalcman, A. T., and Talalay, P. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Photochemistry*, *56*: 5–51, 2001.
14. Rabot, S., Nugon-Baudon, L., Raibaud, P., and Szyliet, O. Rape-seed meal toxicity in gnotobiotic rats: influence of a whole human faecal flora or single human strains of *Escherichia coli* and *Bacteroides vulgatus*. *Br. J. Nutr.*, *70*: 323–331, 1993.
15. Getahun, S. M., and Chung, F-L. Conversion of glucosinolates to isothiocyanates in humans after ingestion of cooked watercress. *Cancer Epidemiol. Biomarkers Prev.*, *8*: 447–451, 1999.
16. Zhang, Y., Kolm, R. H., Mannervik, B., and Talalay, P. Reversible conjugation of isothiocyanates with glutathione catalyzed by human glutathione transferases. *Biochem. Biophys. Res. Commun.*, *206*: 748–755, 1995.
17. Kolm, R. H., Danielson, U. H., Zhang, Y., Talalay, P., and Mannervik, B. Isothiocyanates as substrates for human glutathione transferases: structure-activity studies. *Biochem. J.*, *311*: 453–459, 1995.
18. Zhang, Y., Cho, C-G., Posner, G. H., and Talalay, P. Spectroscopic quantitation of organic isothiocyanates by cyclocondensation with vicinal dithiols. *Anal. Biochem.*, *205*: 100–107, 1992.
19. Zhang, Y., Wade, K. L., Prestera, T., and Talalay, P. Quantitative determination of isothiocyanates, dithiocarbamates, carbon disulfide, and related thiocarbonyl compounds by cyclocondensation with 1,2-benzenedithiol. *Anal. Biochem.*, *239*: 160–167, 1996.
20. Anonymous. 1983 Metropolitan height and weight tables. *Stat. Bull. Metrop. Life Found.*, *64*: 2–9, 1983.
21. Prochaska, H. J., and Santamaria, A. B. Direct measurement of NAD(P)H: quinone reductase from cells cultured in microtiter wells: a screening assay for anticarcinogenic enzyme inducers. *Anal. Biochem.*, *169*: 328–336, 1988.
22. Prochaska, H. J., Santamaria, A. B., and Talalay, P. Rapid detection of inducers of enzymes that protect against cancer. *Proc. Natl. Acad. Sci. USA*, *89*: 2394–2398, 1992.
23. Shikita, M., Fahey, J. W., Golden, T. R., Holtzclaw, W. D., and Talalay, P. An unusual case of “uncompetitive activation” by ascorbic acid: purification and kinetic properties of a myrosinase from *Raphanus sativus* seedlings. *Biochem. J.*, *341*: 725–732, 1999.
24. Prestera, T., Fahey, J. W., Holtzclaw, W. D., Abeygunawardana, C., Kachinski, J. L., and Talalay, P. Comprehensive chromatographic and spectroscopic methods for the separation and identification of intact glucosinolates. *Anal. Biochem.*, *239*: 168–179, 1996.
25. Fahey, J. W., and Stephenson, K. K. Cancer chemoprotective effects of cruciferous vegetables. *Hortscience*, *34*: 1159–1163, 1999.
26. Kushad, M. M., Brown, A. F., Kurilich, A. C., Juvik, J. A., Klein, B. P., Wallig, M. A., and Jeffery, E. H. Variation of glucosinolates in vegetable crops of *Brassica oleracea*. *J. Agric. Food Chem.*, *47*: 1541–1548, 1999.
27. Farnham, M. W., Stephenson, K. K., and Fahey, J. W. Capacity of broccoli to induce a mammalian chemoprotective enzyme varies among inbred lines. *J. Am. Soc. Hort. Sci.*, *125*: 482–488, 2000.
28. Mennicke, W. H., Görler, K., Krumbiegel, G., Lorenz, D., and Rittman, N. Studies on the metabolism and excretion of benzyl isothiocyanate in man. *Xenobiotica*, *4*: 441–447, 1988.
29. Jiao, D., Ho, C-T., Foiles, P., and Chung, F-L. Identification and quantitation of the *N*-acetylcysteine conjugate of allyl isothiocyanate in human urine after ingestion of mustard. *Cancer Epidemiol. Biomarkers Prev.*, *3*: 487–492, 1994.
30. Chung, F-L., Morse, M. A., Eklind, K. I., and Lewis, J. Quantitation of human uptake of the anticarcinogen phenethyl isothiocyanate after a watercress meal. *Cancer Epidemiol. Biomarkers Prev.*, *1*: 383–388, 1992.
31. Zhang, Y. Role of glutathione in the accumulation of anticarcinogenic isothiocyanates and their glutathione conjugates by murine hepatoma cells. *Carcinogenesis (Lond.)*, *21*: 1175–1182, 2000.

Chemoprotective Glucosinolates and Isothiocyanates of Broccoli Sprouts: Metabolism and Excretion in Humans

Theresa A. Shapiro, Jed W. Fahey, Kristina L. Wade, et al.

Cancer Epidemiol Biomarkers Prev 2001;10:501-508.

Updated version Access the most recent version of this article at:
<http://cebp.aacrjournals.org/content/10/5/501>

Cited articles This article cites 25 articles, 12 of which you can access for free at:
<http://cebp.aacrjournals.org/content/10/5/501.full#ref-list-1>

Citing articles This article has been cited by 31 HighWire-hosted articles. Access the articles at:
<http://cebp.aacrjournals.org/content/10/5/501.full#related-urls>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, use this link
<http://cebp.aacrjournals.org/content/10/5/501>.
Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.