Human Metabolism and Excretion of Cancer Chemoprotective Glucosinolates and Isothiocyanates of Cruciferous Vegetables

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Abstract
Isothiocyanates and their naturally occurring glucosinolate precursors are widely consumed as part of a diet rich in cruciferous vegetables. When plant cells are damaged, glucosinolates are released and converted to isothiocyanates by the enzyme myrosinase. Many isothiocyanates inhibit the neoplastic effects of various carcinogens at a number of organ sites. Consequently, these agents are attracting attention as potential chemoprotectors against cancer. As a prerequisite to understanding the mechanism of the protective effects of these compounds, which is thought to involve the modulation of carcinogen metabolism by the induction of phase 2 detoxication enzymes and the inhibition of phase 1 carcinogen-activating enzymes, we examined the fate of ingested isothiocyanates and glucosinolates in humans. Recently developed novel methods for quantifying isothiocyanates (and glucosinolates after their quantitative conversion to isothiocyanates by purified myrosinase) and their urinary metabolites (largely dithiocarbamates) have made possible a detailed examination of the fates of isothiocyanates and glucosinolates of dietary crucifers. In a series of studies in normal volunteers, we made these findings. First, in nonsmokers, urinary dithiocarbamates were detected only after the consumption of cruciferous vegetables and condiments rich in isothiocyanates and/or glucosinolates. In sharp contrast, the consumption of noncrucifers (corn, tomatoes, green beans, and carrots) did not lead to the excretion of dithiocarbamates. Moreover, the quantities of dithiocarbamates excreted were related to the glucosinolate/isothiocyanate profiles of the cruciferous vegetables administered (kale, broccoli, green cabbage, and turnip roots). Second, eating prepared horseradish containing graded doses of isothiocyanates (12.3–74 μmol; mostly allyl isothiocyanate) led to a rapid excretion of proportionate amounts (42–44%) of urinary dithiocarbamates with first-order kinetics. The ingestion of broccoli in which myrosinase had been heat-inactivated also led to proportionate but low (10–20%) recoveries of urinary dithiocarbamates. Finally, when bowel microflora were reduced by mechanical cleansing and antibiotics, the conversion of glucosinolates became negligible. These results establish that humans convert substantial amounts of isothiocyanates and glucosinolates to urinary dithiocarbamates that can be easily quantified, thus paving the way for meaningful studies of phase 2 enzyme induction in humans.

Introduction
Numerous epidemiological studies from many parts of the world report strikingly lower cancer rates among individuals who consume large quantities of fruit and vegetables (reviewed in Refs. 1–4). High levels of yellow and green vegetables in the diet, particularly those of the family Cruciferae (mustards) and the genus Brassica (broccoli, cauliflower, Brussels sprouts, and cabbage), reduce susceptibility to cancer at a variety of (mostly epithelial) organ sites (5–7). The mechanisms responsible for these protective effects are multiple, probably involve complex interactions, and are incompletely understood (8). In recent years, however, much evidence has accumulated suggesting that the elevation (induction) of phase 2 detoxication enzymes (e.g., glutathione transferases, glucuronosyltransferases, NAD(P)H:quione reductase, and epoxide hydrolase) is a major strategy for achieving protection against the toxic and neoplastic effects of mutagens and carcinogens (9, 10). A simple cell culture system designed to detect and quantify phase 2 enzyme inducer activity (11, 12) led to the identification of sulforaphane as the principal and exceedingly potent phase 2 enzyme inducer in broccoli (13). Studies in animals have demonstrated that sulforaphane induces phase 2 enzymes in vivo (13) and blocks chemically induced mammary tumor formation in rats (14, 15), confirming the hypothesis that induction is associated with chemoprotection.

Sulforaphane and other isothiocyanates are synthesized and stored in plants in the form of precursors known as glucosinolates, which are not inducers. The structures of representative glucosinolates are shown in Fig. 1. However, glucosinolates are converted to isothiocyanates by the action of myrosinase (Fig. 2A), an enzyme that is physically segregated from glucosinolates in plant cells but is released when these cells are damaged, e.g., by microbial attack, insect predation, mechanical food processing, or chewing (16). Notwithstanding

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Fig. 1. Structures of glucosinolates that are commonly found in crucifers.

\[
\begin{align*}
\text{Glucoraphanin} & \quad (4\text{-methylsulfinylbutyl glucosinolate}) \\
\text{Glucobrassicin} & \quad (indole-3\text{-methyl glucosinolate}) \\
\text{Glucosinolate} & \quad \text{Isothiocyanate} \\
\text{Myrosinase} & \quad \text{Myrosinase}
\end{align*}
\]

Fig. 2. Enzymatic conversions of glucosinolates and isothiocyanates. A, glucosinolates are hydrolyzed by myrosinase to form isothiocyanates. B, isothiocyanates are eventually converted to mercapturic acids by the sequential action of glutathione transferase (GST), \(\gamma\)-glutamyltranspeptidase (GGTP), cysteimylglycine (CG), and N-acetyltransferase (NAT). We refer to the glutathione adduct and its subsequent degradation products collectively as dithiocarbamates.

the early suggestion by Goodman et al. (17), there is no convincing evidence for the presence of significant myrosinase activity in mammalian cells. Numerous studies have suggested that in fact this activity derives from the gut microflora, as demonstrated by the use of antibiotic treatment and cecectomy (18). Some of the most convincing evidence was reported by Rabot et al. (19), who introduced whole human fecal flora or single strains of Escherichia coli or Bacteroides vulgatus into gnotobiotic rats, resulting in the hydrolysis of dietary glucosinolates. Even more recently, Chung et al. (20) have demonstrated that the direct incubation of human feces with glucosinolate-containing watercress in which myrosinase has been inactivated results in the formation of isothiocyanates.

Measurement of isothiocyanates and glucosinolates has been greatly facilitated by the development of two analytical methods: (a) paired-ion chromatography to separate and identify glucosinolates (21, 22); and (b) a cyclocondensation reaction of isothiocyanates with 1,2-benzenedithiol to yield a product (1,3-benzodithiole-2-thione) with highly favorable properties for spectroscopic quantitation (23, 24). The cyclocondensation reaction may be used to quantify glucosinolates after their complete hydrolysis to isothiocyanates by the addition of an excess of highly purified myrosinase (15). These methods have revealed that little, if any, free isothiocyanate is present in most fresh intact plants (15).

Isothiocyanates are not only inducers of phase 2 enzymes but are also substrates for glutathione transferases, which are phase 2 enzymes (Fig. 2B; Refs. 25 and 26). The enzyme-catalyzed nucleophilic attack of the sulfhydryl group of glutathione on the central carbon of the isothiocyanate group results in the formation of glutathione dithiocarbamates that are modified by a sequence of enzymatic reactions, leading ultimately to the formation of N-acetylcyctisteine dithiocarbamates (15). Multiple lines of evidence suggest that the protective effects of sulforaphane against cancer are attributable to phase 2 enzyme induction (13, 32) and probably also to phase 1 enzyme inhibition (33, 34). The powerful new analytical tools developed in our laboratory to quantify isothiocyanates and glucosinolates and their dithiocarbamate metabolites have made it possible to characterize and quantify the metabolism of dietary glucosinolates and isothiocyanates in humans, as reported in this study. The value of these methods has been pointed out by

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Chung et al. (35), who have very recently shown that the cyclocondensation reaction (23, 24) can be used to monitor the excretion of the N-acetylcysteine conjugates of phenethylisothiocyanate and allylisothiocyanate from watercress and brown mustard, respectively, and have also reported preliminary results on the levels of dithiocarbamates in urine samples obtained from consumers of green vegetables (35).

The clinical studies reported here were undertaken to explore the quantitative relationship between the dietary intake of cruciferous vegetables and the urinary excretion of dithiocarbamates and to ascertain the role of plant myrosinase in the metabolism of dietary glucosinolates.

**Materials and Methods**

**Healthy Volunteers.** Clinical studies were approved by the Joint Committee for Clinical Investigation of the Johns Hopkins Medical Institutions. Volunteers were recruited by advertising on local bulletin boards and were eligible to join if they were 18 years or older and in good health. Subjects selected for inpatient studies were within 25% of ideal weight for height and build (36), did not use any form of tobacco (unless indicated otherwise) 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, and HIV testing). Written informed consent was obtained before enrollment.

**Inpatient Studies.** Volunteers were admitted to the General Clinical Research Center of the Johns Hopkins Hospital. To eliminate dietary sources of glucosinolates or isothiocyanates, they were provided (for the indicated interval) with a diet (referred to hereafter as the "control diet") that contained no beans (America’s Choice), carrots (America’s Choice), and corn (Birds Eye); canned tomatoes ( Hunt’s); locally prepared horseradish; and fresh kale, turnip roots, green cabbage, and broccoli were purchased from area markets. Endogenous myrosinase was inactivated by boiling the fresh plants into boiling water and returning to a boil for at least 3 min or by microwaving them with a minimal amount of water. Heated vegetables were homogenized in a Brinkmann Polytron Homogenizer or Waring Blender, depending on the sample size (usually with less than 3 volumes of distilled water), and then stored at -80°C until use. Immediately before administration, individual doses of the homogenate were warmed in a microwave oven to render them more palatable. For analysis, a portion of the vegetables was extracted at -50°C (dry ice-ethanol bath) with 10 volumes of a triple solvent mixture (equal volumes of DMSO, dimethylformamide, and acetonitrile), as described previously (15). Small aliquots of aqueous or triple solvent homogenates were centrifuged (2000 × g for 10 min at 4°C), and the supernatant fraction was stored at -20°C until analyzed. Homogenates were assayed for residual myrosinase activity and total glucosinolate and isothiocyanate content, and individual glucosinolates were identified.

**Measurement of Isothiocyanates and Glucosinolates.** Isothiocyanate concentrations in plant extracts were determined spectroscopically by cyclocondensation with 1,2-benzenedithiol to produce 1,3-benzodithiole-2-thione (23, 24). Aqueous extracts were used directly; triple solvent extracts were evaporated to dryness and then redissolved in water. To quantify glucosinolates, extracts were treated with excess purified daikon myrosinase (2 h, 37°C) before cyclocondensation. In-dole glucosinolates cannot be measured in this fashion because their hydrolysis products decompose spontaneously. Furthermore, the hydrolysis of β-hydroxyalkenyl glucosinolates yields oxazolidine-2-thiones, which do not react in the cyclocondensation reaction (24).

Samples (5 ml) of each urine collection were centrifuged (200 × g for 5 min at 4°C) to remove particulate matter. The cyclocondensation reaction with urine was carried out in 4-mL screw-top glass vials in a final volume of 2.0 ml containing 100, 200, or 500 μl of urine and enough water to equal 500 μl, 1.5 ml of a mixture containing 0.5 ml of 500 mM sodium borate buffer (pH 9.25), and 1.0 ml of 40 mM 1,2-benzenedithiol in methanol. The vials were flushed with nitrogen gas and sealed with screw caps equipped with Teflon-lined septa, and the contents were mixed with a Vortex mixer and incubated for 2 h at 65°C. The samples were cooled to room temperature and briefly centrifuged (350 × g for 5 min) before loading into a Waters WISP Autosampler. Aliquots (200 μl) of each reaction mixture were injected onto a reverse-phase HPLC (Partisil 10 μm ODS-2, 4.5 × 250 mm; Whatman, Clifton, NJ) and eluted isocratically with 80% methanol and 20% water (v/v) at a flow rate of 2 ml/min. The cyclocondensation product peak, 1,3-benzodithiole-2-thione, was eluted at ~5.0 min, and its area was integrated at 365 nm by means of a Waters Photodiode Array detector (Waters Millennium software, version 2.15.01).

Three sets of controls were included with each analytical run: (a) purified cyclocondensation product (200 μl of 2.5, 5, and 10 μM solutions) was injected to assess the validity of the standard curve; (b) a reaction mixture containing only 1,2-

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1 The abbreviation used is: HPLC, high-performance liquid chromatography.
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The indicated quantities of allyl-NAC and urine were included in the 2.0-mL cyclocondensation reaction. 100 μL of which were injected onto the HPLC column, and the 1.22, and 2.70 times those of an equimolar quantity of sinigrin, respectively (Ref. 15).

**Myrosinase Purification.** The enzyme was purified from 8-day-old daikon (Raphanus sativus) seedlings by sequential ion exchange and lectin binding chromatography procedures. The dimeric Mf 120,000 subunit ascorbic acid-requiring myrosinase was purified approximately 230-fold over the initial homogenates to a specific activity of 194 μmol sinigrin hydrolyzed/min/mg protein at 25°C.

**Statistical Analyses.** Analytical values are expressed as means ± SDs.

**Results**

**Validation of Spectroscopic Quantitation of the Cyclocondensation Product in Urine.** Under specified conditions, the reaction of isothiocyanates and diithiocarbamates with 1,2-benzenedithiol is quantitative, producing a single cyclocondensation product, 1,3-benzodithiole-2-thione, which can be sensitively and precisely quantified spectroscopically by area integration after simple, reverse-phase, isocratic HPLC separation (23, 24). Chung et al. (35) have recently demonstrated the utility of this analytical method in studies of humans ingesting isothiocyanates. The details of the method, the linearity of the standard curve, the reproducibility of measurements, the proportionality to analytical sample size, and the limits of detection have been described in “Materials and Methods.”

To validate the quantitative nature and the suitability of the method for measuring diithiocarbamates in human urine, we measured the analytical recovery of a precisely determined quantity (0.571 nmol) of the N-acetylcyesteine derivative of allyl isothiocyanate when added as an internal standard to 10, 50, or 100 μL of a urine sample obtained from a subject who was eating crucifers and excreted 166 nmol/mL diithiocarbamate/isothiocyanate (Table 1). For the three urine samples (10, 50, or 100 μL; each of which was carried through a separate cyclocondensation reaction), the deviations from the expected values were less than 2%. This establishes that an internal standard is recovered with high accuracy and precision.

**Baseline Excretion of Diithiocarbamates.** To obtain an estimate of baseline diithiocarbamate excretion in the absence of known dietary sources of glucosinolates, 18 volunteers (age

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### Table 1 Recovery of allyl-N-acetylcysteine conjugate (allyl-NAC) added to urine as determined by cyclocondensation with 1,2-benzenedithiol

<table>
<thead>
<tr>
<th>Sample</th>
<th>Allyl-NAC (nmol)</th>
<th>Urine (μL)</th>
<th>Area of peak (μV · s)</th>
<th>Amount (nmol)</th>
<th>Recovery of allyl-NAC (nmol % difference)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.571</td>
<td>0</td>
<td>0</td>
<td>378.877</td>
<td>0.571</td>
<td>5.576 (+0.876)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>10</td>
<td>54.281</td>
<td>0.062</td>
<td>6.058</td>
</tr>
<tr>
<td>0.571</td>
<td>0</td>
<td>10</td>
<td>436.498</td>
<td>0.658</td>
<td>5.82 (+1.93)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>50</td>
<td>272.582</td>
<td>0.411</td>
<td></td>
</tr>
<tr>
<td>0.571</td>
<td>0</td>
<td>50</td>
<td>659.145</td>
<td>0.993</td>
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</tr>
<tr>
<td>0</td>
<td>0</td>
<td>100</td>
<td>562.859</td>
<td>0.848</td>
<td>5.72 (+0.175)</td>
</tr>
<tr>
<td>0.571</td>
<td>0</td>
<td>100</td>
<td>942.072</td>
<td>1.420</td>
<td></td>
</tr>
</tbody>
</table>

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*The indicated quantities of allyl-NAC and urine were included in the 2.0-mL cyclocondensation reaction, 100 μL of which were injected onto the HPLC column, and the areas were quantitated.*

benzenedithiol was included to ensure that no peak is given by 1,2-benzenedithiol alone; and (c) three concentrations (2.5, 5, and 10 μM) of the N-acetylcyesteine derivative of allyl isothiocyanate were analyzed with and without 1,2-benzenedithiol to ensure that the cyclocondensation reaction went to completion. A standard curve was developed using purified 1,3-benzodithiole-2-thione in a mixture of equal volumes of methanol and 125 mM sodium borate buffer (pH 9.25), similar to that described by Zhang et al. (24). The calibration curve is linear over at least a 200-fold range of concentrations from 9.6 pmol to 19.2 nmol of 1,3-benzodithiole-2-thione (r² > 0.999) with a slope of 675.455 μV/nmol.

The reproducibility of the measurements was excellent. In hundreds of assays extending over 3 years, the coefficients of variation for the areas for standards, the cyclocondensation product, and the N-acetylcyesteine derivative of allyl isothiocyanate were within ± 3%. Additional evidence for the reproducibility of the cyclocondensation assay and the entire analytical system is provided by comparing the linearity of the response to 100-, 200-, and 500-μL urine aliquots, each of which was subjected to a separate cyclocondensation reaction. For 41 urine samples assayed in this manner, the linear regressions (based on the inclusion of the zero intercept value) gave proportionality to analytical sample size, and the limits of detection for the areas from 6,070 μVs (approximately 6.54 nmol) to 4,414,743 μVs (approximately 9 pmol) with less than a 1% loss in cyclocondensation (r² > 0.999) with a slope of 0.98 ± 0.033 (± SD) spanning integrated areas from 6,070 μVs (approximately 9 pmol) to 4,414,743 μVs (approximately 6.54 nmol). We place the lower limit of reliable quantitation at 10 pmol of cyclocondensation product in the injected samples.

For each urine sample that could be analyzed with a full standard curve, the reproducibility of measurements, the proportionality to analytical sample size, and the limits of detection have been described in “Materials and Methods.”

Bioassay of Inducer Potency. The induction of quinone reductase was measured in Hepa 1c1c7 murine hepatoma cells grown in 96-well microtiter plates as described previously (11, 12), with minor modifications (15). Excess purified myrosinase (0.0003 unit/mL) and 500 μM ascorbate were added to each well to achieve complete glucosinolate hydrolysis.

Inducer potencies of vegetable extracts are expressed in terms of units. One unit of inducer activity is defined as the amount required to double the quinone reductase activity in a microtiter well containing 150 μL of medium (12). These potencies are related to the fresh weights of vegetables used to prepare the extracts, i.e., units/g fresh weight.

**Paired-Ion Chromatography of Isothiocyanates and Glucosinolates.** Plant extracts (75–200 μL) were separated by HPLC as described previously (22). The relative integrated absorbance areas at 235 nm for glucoraphanin, glucobrassicin, and neoglucobrassicin (for structures, see Fig. 1) were 1.00, 4 W. D. Holtzclaw, personal communication.

range, 20–45 years; 12 blacks, 5 whites, and 1 Asian; 9 males) were enrolled in a simple outpatient study. Subjects were instructed to maintain a control diet (devoid of crucifers or condiments) for 2 days, to keep a food diary, and, on the second day, to collect all urine in 8-h intervals. By the time of the third collection, dithiocarbamate excretion had fallen from a mean of 0.23 ± 0.22 μmol/8 h (range, 0.01–0.91 μmol/8 h) to a mean of 0.12 ± 0.08 μmol/8 h (range, 0.01–0.34 μmol/8 h). Based on the food diaries, some of the higher values were attributable to dietary indiscretions, but in one subject, the only identifiable contributing factor was cigarette smoking. In a subsequent small study, nine cigarette smokers on a diet devoid of crucifers and condiments had baseline excretion values ranging from 0–7.6 μmol/8 h. To avoid the possible contribution of active tobacco use to urinary dithiocarbamates, tobacco users were excluded from further studies. Because the cyclocondensation reaction detects carbon disulfide (24), which is a component of tobacco smoke, it is possible that this substance contributes to the elevated values.

In three inpatient studies (described in detail below) in which diets were strictly controlled, dithiocarbamate excretion in 16 subjects fell from 0.90 ± 1.8 to 0.17 ± 0.35 μmol/8 h by 16–24 h after admission and was below the limit of detection (0.2 mmol/ml of urine) in the 40–48 h collection. In one study of four subjects, the levels consistently remained undetectable during the next 3 days. These findings suggest that there are no significant endogenous sources of urinary dithiocarbamates, and that the levels detected in the assay are entirely attributable to dietary intake (or to smoking).

**Dithiocarbamate Excretion after Horseradish Ingestion.** To circumvent potential variability in the conversion of glucosinolates to isothiocyanates among individuals, the initial feeding studies were conducted with horseradish, a commonly consumed condiment rich in allyl isothiocyanate. The allyl isothiocyanate is formed during the manufacturing process from its glucosinolate precursor by the action of myrosinase present in the horseradish root. A pilot study was designed to evaluate excretion after escalating doses of horseradish. All horseradish studies were conducted on an outpatient basis. Locally prepared horseradish was centrifuged (27,000 × g for 20 min at 4°C). The supernatant fluid was analyzed by cyclocondensation (2.5 μmol isothiocyanate/ml) and stored at −20°C. Immediately before dosing, the supernatant fluid was thawed, and the desired volume was applied to a single slice of white bread. A 72-year-old healthy white male volunteer abstained for 7 days from known dietary sources of glucosinolates or isothiocyanates and collected urine from 11 p.m. to 7 a.m. each day. The first 48 h were the control period. Thereafter, at 11 p.m. on each of 5 successive days, he was given an escalating dose of horseradish supernatant preparation containing 12.3–74 μmol of isothiocyanate. Urinary dithiocarbamate excretion was linear over this 6-fold range of doses (Fig. 3A), and excretion that occurred about 6 h after dosing and may arise from the enterohepatic recycling of metabolites.

**Dithiocarbamate Excretion after Vegetable Ingestion.** To further test the hypothesis that urinary dithiocarbamate excretion can be specifically attributed to the ingestion of cruciferous vegetables, an 18-day inpatient study was conducted with four healthy male volunteers (age range, 30–75 years; 2 blacks and 2 whites). There was an initial 48-h period on the control diet only; then, on days 2, 3, 4, and 5, volunteers were fed 250 g of a noncruciferous vegetable (green beans, corn, tomatoes, or carrots). On days 6, 9, 12, and 15, volunteers were given a 250-g portion of cruciferous vegetable (kale, broccoli, green cabbage, or turnips). Each volunteer consumed all eight vegetables, and the vegetables were administered in random order, such that no two volunteers received the same vegetable on any day.

All eight vegetables, except corn, were chopped, heated in a microwave oven to destroy myrosinase activity, and stored at −80°C. Aliquots were analyzed for glucosinolate and isothio-
Quinone reductase induction was assessed by microtiter plate assay after the hydrolysis of glucosinolates by myrosinase. The activity is due almost exclusively to the free isothiocyanates, which only become active upon conversion by myrosinase. For comparison, 1 μmol of sulforaphane is equivalent to 33.333 units of inducer activity.

### Table 2: Glucosinolate (GS) and isothiocyanate (RNCS) content of cruciferous vegetable preparations fed to volunteers

<table>
<thead>
<tr>
<th>Vegetable</th>
<th>Inducer bioassay activity*</th>
<th>RNCS analysis by cyclocondensation</th>
<th>GS analysis by paired-ion chromatography</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>units/g fresh weight</td>
<td>μmol/g fresh weight (%)</td>
<td>μmol/g fresh weight (%)</td>
</tr>
<tr>
<td>Broccoli</td>
<td>33.333</td>
<td>0.775</td>
<td>2.013</td>
</tr>
<tr>
<td>Cabbage</td>
<td>11.111</td>
<td>0.631</td>
<td>1.044</td>
</tr>
<tr>
<td>Kale</td>
<td>10.000</td>
<td>0.317</td>
<td>0.396</td>
</tr>
<tr>
<td>Turnips</td>
<td>2.000</td>
<td>0.536</td>
<td>1.210</td>
</tr>
</tbody>
</table>

* Quinone reductase induction was assessed by microtiter plate assay after the hydrolysis of glucosinolates by myrosinase. The activity is due almost exclusively to the free isothiocyanates, which only become active upon conversion by myrosinase. For comparison, 1 μmol of sulforaphane is equivalent to 33.333 units of inducer activity.

For all four volunteers, dithiocarbamate excretion fell below the limit of detection during the 48-h control period and remained low throughout the 4 days of noncruciferous vegetable dosing (Fig. 5). In contrast, all four volunteers demonstrated a brisk rise in dithiocarbamate excretion after the ingestion of crucifers. Excretion generally reached a peak in the first 8-h collection, was ≥80% complete in 24 h, and had returned to baseline by 72 h after dosing. Subjects 2 and 4 had double peaks of excretion after broccoli and turnips, respectively. Total dithiocarbamate excretion, as a percentage of the dose, was greatest after the consumption of kale (mean 43%), followed by that of broccoli (21%), cabbage (16%), and turnips (8.6%; Fig. 6). Interestingly, this rank order was consistent for subjects 1–3 and was (just barely) reversed in subject 4 for cabbage and turnips (9.4% as compared to 10%, respectively). This ranking also parallels the percentage of free isothiocyanates (Table 2) and is thus consistent with our observation that the ingestion of isothiocyanates leads to a higher recovery of dithiocarbamates in urine than does dosing with glucosinolates.

Urine from subject 2 was further investigated to determine whether free glucosinolates were present. One control urine (which was negative for dithiocarbamates via cyclocondensation reaction) and a pooled 24-h urine sample obtained after dosing with turnips were analyzed. Turnips were selected because they contained the highest proportion of glucosinolates (91%) and thus afforded the greatest opportunity to detect glucosinolates in the urine. Cyclocondensation with and without myrosinase demonstrated that no glucosinolates were present.

**Dithiocarbamate Excretion after Escalating Doses of Glucosinolates from Broccoli.** The previous study included an array of vegetables containing variable amounts and types of both glucosinolates and isothiocyanates. To reduce the complexity of the dosing material and eliminate the contribution of isothiocyanates, we conducted a pilot study on an outpatient basis in a single volunteer (a 73-year-old white male) and evaluated the kinetics of excretion and the dose-response relationship with a broccoli preparation devoid of isothiocyanates.

### Fig. 4: Paired-ion chromatographic analysis of cruciferous vegetables. All samples were extracted with triple solvent, and the glucosinolates were resolved by paired-ion reverse-phase HPLC in the presence of tetracetylammonium bromide. Vegetable extracts were injected onto the column directly (200-μl volumes equivalent to 15.6, 25.5, 20.6, and 27.3 mg fresh weight for broccoli, cabbage, kale, and turnips, respectively). Glucosinolates (shaded black peaks) were identified by their disappearance from a repeat chromatogram after treatment of the extracts with myrosinase. Chemical identities were confirmed by comparison with authentic standards. In order of increasing retention time, the known glucosinolates are glucoraphanin (a), progoitrin (b), sinigrin (c), 4-hydroxyglucobrassicin (d), glucobrassicin (e), and neoglucobrassicin (f). Several (unlabeled) peaks in the turnip chromatogram have only been tentatively identified. Glucoraphanin accounts for 36, 40, 70, and 0% of the total glucosinolates of broccoli, cabbage, kale, and turnips, respectively.
Frozen Birds Eye Broccoli Cuts (lot 6x126-1, containing cultivars Marathon, Patriot, and Pirate; Dean Foods Vegetable Company, Green Bay, WI) were heated to 100°C in a microwave oven and then homogenized in water. The homogenate contained 0.413 μmol non-indole glucosinolate/g homogenate (0.651 μmol/g fresh weight) with no detectable isothiocyanates. Based on paired-ion chromatography, glucoraphanin constituted 43.9% of the glucosinolates in this preparation (the remainder were indole glucosinolates).

The subject was instructed to avoid all dietary sources of glucosinolates and isothiocyanates, and all urine was collected during the study. After a 2-day control period, he was given a dose of broccoli homogenate (25, 50, 100, or 200 μmol of non-indole glucosinolates) at 3-day intervals, and urine was obtained in 8-h collections. Total dithiocarbamate excretion was proportional to the dose of broccoli glucosinolates, and about 20% of the dose was recovered at each level (Fig. 7A). An elevated baseline at the time of dosing and a documented dietary indiscretion during the collection period led to a spuriously high recovery after the lowest dose. Although the kinetics of excretion were remarkably similar at each dosing level, they did not obey first-order kinetics (the terminal half-life was about 35 h; Fig. 7B). This curvilinear behavior is distinctly different from that seen after allyl isothiocyanate ingestion (Fig. 5B) and may be attributed to multiple factors, including a slow rate of glucosinolate breakdown with ongoing absorption, biliary excretion and reabsorption of metabolites, or overlapping kinetic curves for different glucosinolates and their respective metabolites.

Cross-Over Dosing with Broccoli Glucosinolate and Isothiocyanate Preparations. To gain better understanding of the contribution of the glucosinolate-to-isothiocyanate conversion to overall metabolism and excretion, we designed an inpatient cross-over study with six volunteers to compare the extent of excretion after equimolar doses of glucosinates on the two vegetable preparations. To overall metabolism and excretion, we designed an inpatient cross-over study with six volunteers to compare the extent of excretion after equimolar doses of glucosinates on the two vegetable preparations.
Disposition of glucosinolates by humans

After a 48-h control period, a single dose of glucosinolate (144 μmol; three subjects) or isothiocyanate (118 μmol; three subjects) was administered (Fig. 8). Three days later, the doses were crossed, and on day 8, the subjects were given 6.84 μmol of daikon extract, an amount equivalent to that used in the isothiocyanate preparation [volunteer 1 declined to participate in the final (daikon) phase of this study]. In all six subjects, the 72-h cumulative excretion after isothiocyanate administration (47 ± 7% of the dose; range, 34–53%; corrected for daikon contribution) was substantially greater than that after glucosinolate feeding (10 ± 5% of the dose; range, 2.5–19%). This difference in response was independent of the order of dosing and is highly statistically significant (P < 0.0001, two-tailed, paired t test). Of note, subjects in this study tended to be consistently either high or low excreters, regardless of whether they received the isothiocyanate or glucosinolate preparation. As observed in the broccoli dose-ranging study (Fig. 7B), kinetics after glucosinolate dosing were curvilinear, with a terminal half-life of about 20 h. In contrast, the terminal kinetics after isothiocyanate dosing were linear, with a half-life of about 12 h (data not shown). This difference supports the notion that the slower elimination and nonlinear kinetics observed after glucosinolate administration are attributable to some aspect of the glucosinolate-to-isothiocyanate conversion.

Effect of Bowel Preparation on Glucosinolate Metabolism. To determine whether enteric microflora contribute to glucosinolate metabolism in humans, we evaluated (in an inpatient study) the effect of a standard preoperative bowel preparation on dithiocarbamate excretion after the ingestion of glucosinolates from broccoli.

To prepare glucosinolates, florets from ice-packed broccoli were cut and immediately boiled in a steam-jacketed kettle (5.164 kg in 8 liters of water, 5 min). Florets were removed, the remaining liquid was reduced to about half of its original volume (vigorous thin film boiling, 5 min) and added back to the cooked florets, and the mixture was homogenized in a Waring Blender. Samples were taken for analysis, and the homogenate was stored at −80°C. Individual doses consisted of 112 g of fresh weight broccoli in 182 ml of fluid, containing

Analysis by cyclocondensation after myrosinase hydrolysis established the presence of 144 μmol non-indole glucosinolates/dose (0.64 μmol/g homogenate; 0.86 μmol/g fresh weight broccoli; 97.3% glucosinolates and 2.7% isothiocyanates). Glucoraphanin accounted for 55.9% of the glucosinolates, as determined by paired-ion chromatography. The remaining half of the homogenate (the isothiocyanate preparation) was mixed with 2% (by weight) of 7-day-old daikon sprouts as an abundant source of myrosinase. This mixture was homogenized and incubated (for 2 h at 37°C and then for 24 h at 4°C) to achieve the quantitative conversion of glucosinolates to isothiocyanates.

To assess the contribution of daikon to the isothiocyanates in the final (daikon) phase of this study, 7-day-old daikon sprouts were homogenized in cold water and allowed to autolyze. Samples were taken for analysis, and the myrosinase-treated homogenates were stored at −80°C until dosing. Each dose contained 118 μmol of total isothiocyanates (0.43 μmol/g homogenate; no detectable glucosinolates), of which 6.84 μmol (5.8%) were contributed by the added daikon preparation. Although it was our intention to administer equimolar quantities of glucosinolates and isothiocyanates to the subjects, the actual dose of glucosinolate (144 μmol) exceeded that of isothiocyanate (118 μmol) by 18%, because some isothiocyanates were lost during storage of the preparations.

Six volunteers (age range, 19–46 years; 4 blacks and 2 whites; 3 males) were enrolled in an 11-day inpatient study.

Fig. 7. Urinary excretion of dithiocarbamates after the ingestion of increasing doses of broccoli glucosinolates. A, escalating doses (equivalent to 25, 50, 100, and 200 μmol of isothiocyanate) gave rise to proportionate urinary dithiocarbamate excretion (r² = 0.976). B, urinary dithiocarbamate excretion is greatest in the first 8 h and decreases in a curvilinear fashion over time, regardless of the dose.

Fig. 8. Urinary excretion of dithiocarbamates after feeding broccoli glucosinolates or isothiocyanates to six volunteers. Each panel represents the excretion pattern in a single volunteer. All subjects were maintained on a control diet for 48 h; at indicated time points (arrows pointing up), they received broccoli glucosinolates (G), isothiocyanates (I), or the equivalent quantity of daikon homogenate (D) used to convert the glucosinolates to isothiocyanates. Urine was obtained in 8-h collections and was analyzed for dithiocarbamate content. Three subjects received the glucosinolate preparation first (left panels), and the other three subjects received the isothiocyanate preparation first (right panels).
100 μmol of non-indole glucosinolates, 0.5 μmol of isothiocyanates, and no residual myrosinase activity; glucoraphanin constituted 76.1% of the glucosinolates. Four healthy volunteers (age range: 27–46 years; 2 whites and 2 blacks; 3 males) were studied. Subject 1 was evaluated in a pilot study, and subjects 2–4 were subsequently enrolled in a second cohort. After an initial 72-h control diet period, glucosinolates were administered on days 3 and 6 (before the bowel preparation) and on days 11 and 14 (after the bowel preparation; Fig. 9). For subject 1, the post-bowel preparation doses were given on days 10 and 13. In all four cases, there was a dramatic reduction in dithiocarbamate excretion after the bowel microflora had been reduced [from 11.3 ± 3.1% of the dose (pretreatment) to 1.3 ± 1.3% of the dose (posttreatment); $P = 0.001$, two-tailed paired $t$ test]. The pattern of recovery of the glucosinolate-to-urinary dithiocarbamate conversion was variable. At 4 days after the intervention, dithiocarbamate excretion ranged from none to at least the level observed before bowel preparation. Five weeks after the intervention, excretion had rebounded to 0.48–1.9 times the pretreatment levels.

### Discussion

New analytical methods have made it possible for us to administer vegetable preparations that are fully characterized with respect to the qualitative and quantitative content of glucosinolates and isothiocyanates. In addition, the cyclocondensation reaction provides highly reliable quantitative information on the content of non-indole isothiocyanates and their metabolites in urine, an observation that has been recently confirmed elsewhere (35). For the first time, the extent of metabolism and the kinetics of excretion of known quantities of dietary glucosinolates in humans can be measured reliably.

The results from 16 volunteers in a number of different studies indicate that there is no endogenous source of urinary dithiocarbamates. In nonsmokers who consume no glucosinolates or isothiocyanates, dithiocarbamates are not detectable in the urine. Although a wider application of the method may identify individuals with some endogenous source of urinary dithiocarbamates, it seems likely this method provides a sensitive indicator of exposure to exogenous materials, most importantly, dietary glucosinolates and isothiocyanates.

Allyl isothiocyanate, an excellent substrate for recombinant human glutathione transferases *in vitro* (26), is rapidly and extensively metabolized when administered to humans in the form of horseradish. The urinary excretion of metabolites is linear with the dose (Fig. 3A), indicating that the metabolic process is not saturated by daily doses up to 74 μmol. In view of its rather brisk 2-h half-life of urinary excretion (Fig. 3B), it is possible that allyl isothiocyanate is metabolized in its first pass through the gut wall and liver and never reaches the systemic circulation. This behavior is distinctly different from that observed with other vegetable preparations. When the glucosinolates in a broccoli homogenate (containing largely glucoraphanin) are treated with myrosinase, the resulting isothiocyanate product is almost exclusively sulforaphane, because indole isothiocyanates decompose immediately upon formation (38). After a dose of sulforaphane (in the form of hydrolyzed broccoli homogenate), the extent of metabolism (47%) is comparable to that of allyl isothiocyanate (44%).

Fig. 9. Effects of the reduction of bowel microflora on dithiocarbamate excretion after the administration of broccoli glucosinolates. Four volunteers on a control diet received 100 μmol of a broccoli glucosinolate preparation in which the myrosinase had been inactivated on two occasions (days 3 and 6; arrows pointing up). Enteric flora were reduced by a regimen of mechanical cleansing and antibiotics (39, 40). After such an intervention, the mean glucosinolate-to-isothiocyanate conversion was reduced only 1.6-fold. There was, however, an excellent correlation between excretion and the amount of the dose contributed by isothiocyanates.

Although the disposition of isothiocyanates is more rapid and complete than that of glucosinolates, it is intriguing that glucosinolates administered in plant preparations devoid of myrosinase activity are indeed metabolized. This was true in every volunteer we studied, with a multitude of protocols and a variety of cruciferous test vegetables. We found compelling evidence that in humans, the first step in glucosinolate metabolism, the conversion of glucosinolates to isothiocyanates, dictates the overall extent and rate of disposition. This notion is further supported by the results of the multiple-vegetable study. In that experiment, there was no correlation between the 72-h excretion of dithiocarbamates and the total dose of glucosinolates plus isothiocyanate consumed (Fig. 6). There was, however, an excellent correlation between excretion and the amount of the dose contributed by isothiocyanates.

These metabolic studies underscore the versatility of newly available analytical methods for the quantitative and qualitative analysis of glucosinolates, isothiocyanates, and their metabolites. They also reveal, for the first time, the quantitative
disposition in humans of dietary glucosinolates and isothiocyanates. Such information will be critically important in determining the role of cruciferous vegetables in phase 2 enzyme induction and in devising cancer chemoprevention strategies based on achieving such inductions in humans.

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