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The Metabolic Fate of Purified Glucoraphanin in F344 Rats

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Dietary broccoli is commonly eaten cooked, exposing individuals to intact glucoraphanin rather than to its hydrolysis product, the anticarcinogenic isothiocyanate sulforaphane, since cooking destroys the hydrolyzing enzyme myrosinase. There is little information on the absorption and metabolism of glucoraphanin, due partly to the lack of purified compound. In this study, glucoraphanin was purified from broccoli seed and 150 μmol/kg was administered to male F344 rats. Glucoraphanin (5% of an oral dose) was recovered intact in urine, showing that it is absorbed intact, and no glucoraphanin or metabolites were found in feces. Total urinary products accounted for 20 and 45% of oral and intraperitonneal doses, respectively, including sulforaphane *N*-acetyl cysteine conjugate (12.5 and 2%), free sulforaphane (0.65 and 0.77%), sulforaphane nitrile (2 and 1.4%), and erucin (0.1 and 0.1%), respectively. Both glucoraphanin and its reduced form glucoerucin were identified in bile following intravenous glucoraphanin administration. We conclude that orally administered glucoraphanin is absorbed intact, undergoes enterohepatic circulation, and is hydrolyzed in the gut in F344 rats.

KEYWORDS: Glucoraphanin; absorption; metabolism; excretion; broccoli; sulforaphane

INTRODUCTION

Interest in consuming broccoli has increased in the past decade, in part due to potential health benefits associated with the presence of the glucosinolate glucoraphanin and its hydrolysis product, sulforaphane (**Figure 1**; *1*, *2*). Sulforaphane is a potent inducer of detoxification enzymes, thus aiding in the clearance of carcinogens and other xenobiotics (*3*). Purified sulforaphane is available commercially and has been used to conduct a number of studies both in vitro and in animals. However, in the diet, we are more commonly exposed to unhydrolyzed glucoraphanin, since the hydrolyzing enzyme myrosinase is destroyed by cooking.

Several studies have evaluated aspects of glucosinolate metabolism in humans. In an early study, human subjects were given the purified glucosinolate, progoitrin, orally and its metabolite, goitrin, was identified in blood and urine (*4*). On the basis of these results, the authors postulated that gut bacterial enzymes are responsible for the hydrolysis of progoitrin in the absence of plant myrosinase. In support of this mechanism, isolated human fecal bacteria have been shown to hydrolyze progoitrin, glucotropaeolin, and other glucosinolates (*5*, *6*). Furthermore, mechanical cleansing of the bowel, in conjunction with antibiotic treatment of human subjects, strongly decreased urinary excretion of isothiocyanate metabolites, further supporting the concept that gut microbiota are responsible for metabolism of glucosinolates (*7*). One study provided subjects fresh or steamed broccoli to estimate the extent of isothiocyanate bioavailability with and without the endogenous plant myrosinase, and the results indicated a three-fold lower urinary excretion of the *N*-acetyl cysteine conjugate of sulforaphane in individuals ingesting cooked broccoli as compared to those ingesting fresh broccoli (*8*). Although these data are consistent with a role for microbiota in hydrolysis, they may also indicate that microbiota do not support complete hydrolysis or that isothiocyanates are not the major product of hydrolysis by microbiota or even that much of the glucoraphanin is absorbed whole and does not reach the microbiota of the lower gut.

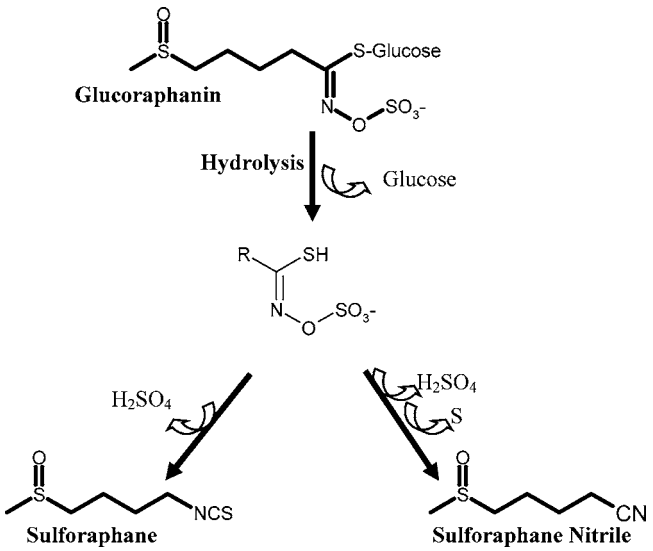

Figure 1. Glucoraphanin hydrolysis to sulforaphane and sulforaphane nitrile.

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Animal studies also support a role for gut bacteria in glucosinolate hydrolysis. When sinigrin was given to germ-free rats inoculated with a human fecal bacterial strain, *Bacteroides thetaiotaomicron*, the metabolites allylisothiocyanate and allylamine were isolated from the gastrointestinal contents (*9*). In an in vitro fermentation study mimicking digestive tract action, 10−30% of a dose of sinigrin was converted to allylisothiocyananate; the authors did not identify any other metabolites (*10*). In a follow-up study, sinigrin metabolism was compared between germ-free rats untreated and those inoculated with a human fecal suspension, and again, only a fraction was converted to allylisothiocyanate (*11*). A more recent study reported that when sinigrin and glucotropaeolin were subjected to anaerobic fermentation mimicking the lower gut, the corresponding nitrile was found as the major hydrolysis product (*6*).

In the few studies discussed above, glucoraphanin metabolism was investigated using either fresh or cooked broccoli, rather than purified glucoraphanin. In the present study, glucoraphanin was purified from broccoli seed, and its physiological fate in the body was investigated using Fischer 344 rats.

MATERIALS AND METHODS

Materials. Broccoli seed (Marathon cultivar) was obtained from Sakata Seed America Inc. (Morgan Hill, CA). Solvents used for gas chromatography (GC) or high-performance liquid chromatography (HPLC) analysis were of HPLC grade; all others were of reagent grade. All solvents were obtained from Fisher Scientific (Fair Lawn, NJ). Anion exchange resin, Dowex Marathon WBA2, was purchased from Supelco (Bellefonte, PA). Benzylglucosinolate and glucoraphanin standards were purchased from The Royal Veterinary and Agriculture University (Copenhagen, Denmark), and sulforaphane and sulforaphane nitrile standards were purified from broccoli seed as previously described (*12*). Erucin and benzyl isothiocanate were obtained from LKT Laboratories (St. Paul, MN). Sulforaphane, erucin, and erysolin *N*-acetyl cysteine conjugate standards were synthesized from the pure isothiocyanates as previously described (*13*). All other chemicals were obtained from Sigma (St. Louis, MO).

Glucoraphanin Purification. *Seed Extraction.* Rathon seed was oven-dried at 100 °C for 24 h to inactivate myrosinase. Dried seed (250 g) was ground twice for 15 s in a coffee grinder, to form a fine powder. Ground seed was defatted three times with excess hexane (1:4, w/v) and allowed to dry in the fume hood overnight. The defatted seed meal was extracted three times with boiling 70% methanol−water (1:4, v/v) for 2 h (70−75 °C), using a water-chilled condenser. The combined methanol−water extract was concentrated to 300 mL using a rotary evaporator and made up to 500 mL with imidazole formate buffer (final pH 4.15, 50 mM). The extract was deproteinated with 10 mL of freshly mixed $Pb(CH_3COO)_2$/$Ba(CH_3COO)_2$ (1:1 v/v, 1 M) and then centrifuged at 34000*g* for 1 h. The pellet was discarded, and the supernatant was filtered through Whatman filter paper #1, and the pH was adjusted to 4.15, using 1 N HCl.

Ion Exchange Column Chromatography. An anion exchange column (15 cm × 2.5 cm i.d.) was prepared using Dowex Marathon WBA2 resin. This was first washed with distilled, deionized water (1:5 v/v; 15−20 min), then with 1 N NaOH (10−15 min), and then rewashed with distilled, deionized water repeatedly until the rinse water pH dropped to ~7 (about 5−10 washes). Then, the resin was conditioned with an imidazole formate buffer (50 mM; pH 4.15) by repeated washes to a constant pH of 4.15.

Sample (1:2.5 v/v, resin:sample) was loaded onto the packed resin column, and the column was washed with 5 volumes of water followed by 3 volumes of a mix of formic acid:2-propanol:water (2:3:5). The column was rewashed with water to remove any trace of the solvent mix, and glucosinolates were eluted with 3 volumes of 0.5 M K_2SO_4 solution (1:3 v/v, resin:K_2SO_4 solution). The eluate was lyophilized, dissolved in boiling methanol, and filtered through Whatman filter paper #1. Methanol was removed by rotary evaporation, and the residue was dissolved in deionized, distilled water for HPLC purification.

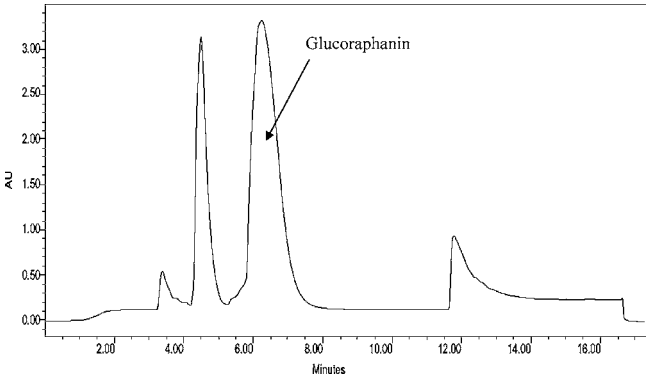

Figure 2. HPLC separation of glucoraphanin from broccoli seed.

HPLC Purification. The water extract was injected onto a 300 mm × 19 mm i.d., 6 μm Novapak C-18 preparative HPLC column (Waters, Milford, MA) previously equilibrated with 98% solvent A (0.1 M ammonium acetate and 0.25% acetic acid in water) and 2% solvent B (0.1 M ammonium acetate and 0.25% acetic acid in methanol). A flow rate of 35 mL/min was used with a gradient mode starting with 98% solvent A for the first 4 min, changing to 100% solvent B over the next 2 min, then maintaining 100% solvent B for 3 min, before changing back to 98% solvent A over 2 min, and maintaining 98% solvent A for 4 min in preparation for the next injection. Glucosinolate elution was monitored at 227 nm using a Waters 2487 dual wavelength absorbance detector.

Identification and Quantification of Glucoraphanin. The HPLC eluate was collected in fractions, which were hydrolyzed by incubation with excess myrosinase (5−7 mg myrosinase/mL eluate) for 15 min at room temperature. Products were extracted into methylene chloride (1:1 v/v) and analyzed by GC as previously described (*14*). When hydrolyzed, the HPLC fraction eluting at 6.4 ± 1 min contained both sulforaphane and sulforaphane nitrile, indicating that it was glucoraphanin (**Figure 2**). Fractions were combined from multiple injections, concentrated by rotary evaporation, and analyzed by mass spectrometry. Briefly, a sample of the concentrated fraction was dissolved in methanol and subjected to electrospray ionization using a Micromass 70-VSE mass spectrometer. The instrument was operated in the negative ion mode, with a source temperature of 200 °C, probe temperature of 25 °C, electron energy of 70 eV, and a resolution of 1000 for nominal spectra. The sample was further analyzed for glucosinolates by the desulfoglucosinolate method, as previously described (*15*), using benzylglucosinolate as an internal standard and a response factor established by a collaborative study across six countries that compares readings to that of a reference material (*16*). Quantification of pure sinigrin (Sigma) was used as an additional validation. In addition, glucoraphanin was reanalyzed by hydrolysis and quantification of the hydrolysis products by GC, using benzylisothiocyanate as an internal standard (*14*). The concentrations of sulforaphane and sulforaphane nitrile were calculated using standard curves (0.39−25 mg/mL) as previously described (*14*), and the glucoraphanin concentration was estimated as sulforaphane + sulforaphane nitrile.

Animals and Housing. Animal experiments were carried out in accordance with NIH regulations and approved by the Animal Care and Use Committee of the University of Illinois, which is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, International. Adult male F344 rats (Harlan, Indianapolis, IN) were housed under controlled temperature and humidity with a 12 h light−dark cycle throughout the study period and were given food (AIN 93G) and water ad libitum. The AIN 93G diet was formulated in the laboratory (*17*).

Glucoraphanin Administration. Eighteen 9 week old male F344 rats, weighing 170−190 g, were randomly distributed into one of three treatment groups and acclimated for 5 days in individual hanging wire metabolism cages. On the last 3 days of acclimation, all rats received saline solution by gavage, daily. After acclimatization, all rats received both gavage (saline for groups 1 and 3, 150 μmol glucoraphanin/kg for group 2) and an ip injection (saline for groups 1 and 2, 150 μmol glucoraphanin/kg for group 3). A dose of 150 μmol glucoraphanin/kg

(65.4 mg/kg) was approximately equivalent to that present in a 20% dry weight broccoli diet, commonly used in rat feeding studies (*18*). Urine and feces were collected every 4 h for the first 12 h and at 24, 36, 48, 60, and 70 h posttreatment. Urine was stored at −80 °C until analysis. Feces were freeze-dried and stored at −20 °C until analyzed.

At the end of the 72 h collection period, rats were anaesthetized with ketamine/xylazine (87:13, w/w; 10 mg in 0.1 mL/100 g body wt, ip) and killed by cervical dislocation without recovery from anesthesia.

Biliary Excretion Study. Six male F344 rats, 8−9 weeks old, were anesthetized with a mix of urethane (0.8 g/kg) and α-chloralose (65 mg/kg), and the bile duct was cannulated as previously described (*19*). Bile was collected into preweighed vials kept on ice, and body temperature was maintained at 34−36 °C using a heating pad. Gauze soaked in saline was used to maintain hydration. Baseline bile was collected for 20 min immediately prior to glucoraphanin (75 or 150 μmol/kg in saline) injection via the femoral vein.

Urinary, Fecal, and Biliary Analysis. Urine was analyzed for mercapturic acid conjugates of glucorophanin metabolites by HPLC, as previously described (*13*). A standard curve was constructed in pooled control rat urine for each conjugate (sulforaphane conjugate, 0.9−59.5 μmol, $R^2 = 0.986$; erysolyn conjugate, 0.4−28.4 μmol, $R^2 = 0.998$; and erucin conjugate, 0.4−31.2 μmol, $R^2 = 0.999$). For free isothiocyanate and glucoraphanin analysis, urine samples (250 μL) were incubated with and without myrosinase (1 U/mL) for 2 h, extracted into methylene chloride (1:2; v/v), and analyzed by GC, using sinigrin and benzyl isothiocyanate as internal standards (*12*). Urinary creatinine was measured according to the manufacturer's instructions for the Diagnostics Creatinine Kit #555 (Sigma).

Lyophilized feces were extracted with water (1:10 w/v), vortexed, and centrifuged at 24000*g* for 20 min. The pellet was discarded, and the supernatant was analyzed as described for the urine samples for glucoraphanin, hydrolysis products, and *N*-acetyl cysteine conjugates of hydrolysis products. Bile samples were used undiluted for analysis of hydrolysis products and diluted five-fold for glucosinolate analysis. The diluted bile was also analyzed by LC/MS.

Statistical Analysis. The Student *t* test was used to compare treatment main effects using analysis of variance by proc GLM with SAS software (SAS Inc., Cary, NC). Where the treatment main effects were significant ($P < 0.05$), means were compared using the posthoc Scheffe test. Where data were obtained over several time periods, data were analyzed individually per time point for treatment effects.

RESULTS AND DISCUSSION

Glucoraphanin Purification. The glucosinolate content of cruciferous vegetables varies across plant parts (*20*). Because the glucoraphanin concentration is greatest in broccoli seed, this was chosen as the starting material. Hexane defatting and hot methanol extraction were similar to previously reported methods for glucosinolate purification (*21*). Because a crude methanol extract from broccoli seed is not readily filtered through columns of many commonly used anion exchange materials, a weak base anion exchange resin was used, which has a larger particle size (550 ± 50) and greater exchange capacity (1.7 equiv/L), allowing rapid processing of large quantities of seed.

Preparative HPLC of this partially purified glucosinolate preparation obtained from anion exchange chromatography gave a distinct absorbance peak (227 nm) at 6.4 min (**Figure 2**). The mass spectra of both this fraction and a purchased glucoraphanin standard gave a *m*/*z* of the $[M - H]^-$ ion of 436.2. Rotary evaporation of this fraction gave white crystals that became liquid upon standing at room temperature for 30 min or longer. Upon freeze drying, the rotary evaporated fraction lost ∼95% (95.4 ± 0.8) weight, weighing ∼1.5 g/kg original broccoli seed. Although much of the excess weight prior to lyophilization was no doubt water, discrepancies in weight could also be due to the presence of residues of sulfate or potassium ions from the anion exchange chromatography or ammonium acetate or acetic acid from the HPLC solvent (*22*, *23*). Methanol, being a poor solvent for salts, was used to remove the excess K_2SO_4, but the sharp odor of acetic acid remained with the product. Dissolution into deionized distilled water and glucorinolate analysis revealed that the product was 96% pure by weight, yielding 3.5 μmol/g original broccoli seed. To avoid weight changes due to the hygroscopic nature of glucoraphanin, a stock solution in deionized, distilled water was made up and stored at −80 °C for use in future experiments. The concentration of glucoraphanin in the stock solution was estimated using two methods, HPLC of the desulfogulcosinolate and GC analysis of hydrolysis products following incubation with excess myrosinase (*14*, *15*). Glucorapahnin anlysis by HPLC gave two peaks corresponding to glucoraphanin and the internal standard benzylglucosinolate (**Figure 3**). Hydrolysis and GC analysis revealed that sulforaphane and sulforaphane nitrile (94.5:5.5) together accounted for 96% of the value obtained by glucosinolate analysis; 3.4 μmol/g original broccoli seed. GC analysis of hydrolysis products was used for quantification in all further studies. Analysis of unprocessed Marathon seed showed that it contains ∼19 μmol glucoraphanin/g DW, suggesting an extraction and purification efficiency of ∼18%. Thus, there is considerable scope for improving the extraction efficiency, even though recovery was almost three-fold improved over high-speed countercurrent chromatography (*24*) and very similar to that using any of three recently reported preparative HPLC systems (*25*).

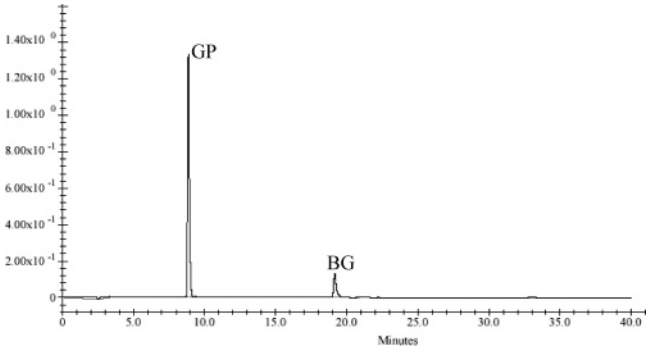

Figure 3. Glucosinolate analysis. Glucoraphanin (GP) purity and analysis by the desulfoglucosinolate method; internal standard, benzyl glucosinolate (BG).

Administration of Purified Glucoraphanin to Rats. Rats were administered purified glucoraphanin orally, in the absence of endogenous plant myrosinase, to model ingestion of cooked broccoli. Rats were also dosed ip as a "positive control" to aid in viewing metabolic pathways that occur within the rat. At this dose, rats tolerated both oral and ip administration of glucoraphanin. Following an overnight fast immediately prior to providing glucoraphanin, feed intake tended to recover more slowly in the treatment groups as compared to control and was slightly but significantly ($p < 0.05$) lower in rats administered glucoraphanin ip as compared to the control group on days 1 and 2; food intake had recovered by day 3 (data not shown). Body weights did not vary significantly among groups (data not shown). Previous studies have shown that long-term feeding of rapeseed meal results in growth retardation, loss of appetite, and liver enlargement (*26*). However, no such effects were seen with this single 150 μmol/kg dose of glucoraphanin, equivalent to a 20% dry weight broccoli diet, which has been used previously with no adverse consequences (*27*). A recent study administered 240 mg (550 μmol) glucoraphanin/ kg by gavage, a dose approximately 3.5-fold greater than the present study, daily for 4 days. However, they did not report food intake, body weight at termination, or glucoraphanin metabolism (*28*). To

Table 1. Urinary Analysis of Glucoraphanin and Metabolites[a]

metabolite/product	oral	ip
urine volume (mL)	11.75 ± 2.74	13.16 ± 2.31
sulforaphane	0.16 ± 0.11	0.17 ± 0.06
sulforaphane nitrile	0.51 ± 0.17	0.34 ± 0.23
erucin	0.03 ± 0.01	0.07 ± 0.04
erucin nitrile	detected/not quantified	detected/not quantified
erucin conjugate	0.03 ± 0.00	not detected
erysolin conjugate	0.01 ± 0.00	not detected
sulforaphane conjugate	3.80 ± 0.20	0.67 ± 0.06
glucoraphanin	1.20 ± 0.5	9.70 ± 2.70
glucoerucin	0.25 ± 0.1	1.40 ± 0.10
total	5.99	12.35
% dose	~20	~45

[a] μmol per 36 h urine.

our knowledge, the present study is the first to investigate the effect of glucoraphanin given by ip injection.

Excreted Products following Glucoraphanin Administration. Total urine voided in 70 h was not significantly different among treatment groups (**Table 1**). Fecal form and weight over the 70 h treatment were not significantly different among groups (data not shown). Analysis of urine from both groups of glucoraphanin-treated rats identified intact glucoraphanin, the reduced analogue glucoerucin, and several metabolites. Rats dosed orally excreted 3.8 ± 0.20 μmol of *N*-acetyl cysteine conjugate of sulforaphane during 36 h posttreatment (**Table 1**), accounting for approximately 12% of the dose in the orally treated rats. This is comparable to the 7.5% reported previously in a human study of urinary dithiocarbamates following ingestion of steamed broccoli (*8*). Peak excretion (6.7 ± 2.1 μmol/mg of creatinine) was achieved by 8 h, and detectable amounts were seen in the urine up to 36 h postdosing (**Figure 4A**,**B**). In contrast, the total amount of sulforaphane conjugate excreted in 36 h by the ip-treated rats was significantly less (0.67 ± 0.06 μmol) and accounted for ~2% of the glucoraphanin dose (**Table 1**). In addition to the sulforaphane conjugate, two minor peaks not detected in control urine were collected and identified as erucin and erysolin conjugates by LC/MS (**Table 1**). Previous studies using the dithiocarbamate assay, which accurately reflect total isothiocyanates plus conjugates, were not able to distinguish among individual conjugates or the free isothiocyanates (*7*). Using both HPLC analysis of derivatized conjugates and GC analysis of free hydrolysis products, we were able to identify not only the sulforaphane conjugate but also individual conjugates of the reduced (erucin) and oxidized (erysolyn) analogues of sulforaphane, in addition to free sulforaphane and erucin. These data support a previous finding that sulforaphane can be reduced to erucin in vivo (*29*). There was a substantially larger fraction of the dose excreted as *N*-acetyl conjugates of hydrolysis products by the orally dosed animals than by the ip-dosed animals. Yet, the ip-dosed animals had far greater systemic levels of glucoraphainin. One explanation consistent with the literature is that glucoraphanin is not hydrolyzed systemically. We considered the possibility that glucoraphanin administered systemically was excreted in the bile. It could then be hydrolyzed in the gut, absorbed, and metabolized to conjugates systemically, as others have suggested (*6*, *7*, *11*).

Urine samples contained small amounts of free, unconjugated sulforaphane, sulforaphane nitrile, and erucin, totaling 2.7 and 2.1% of the glucoraphanin dose in oral and ip-treated rats, respectively (**Table 1**). Trace amounts of erucin nitrile were also identified but not quantified. In contrast, myrosinase treatment of urine samples resulted in much higher concentrations of these same hydrolysis products, reflecting the presence

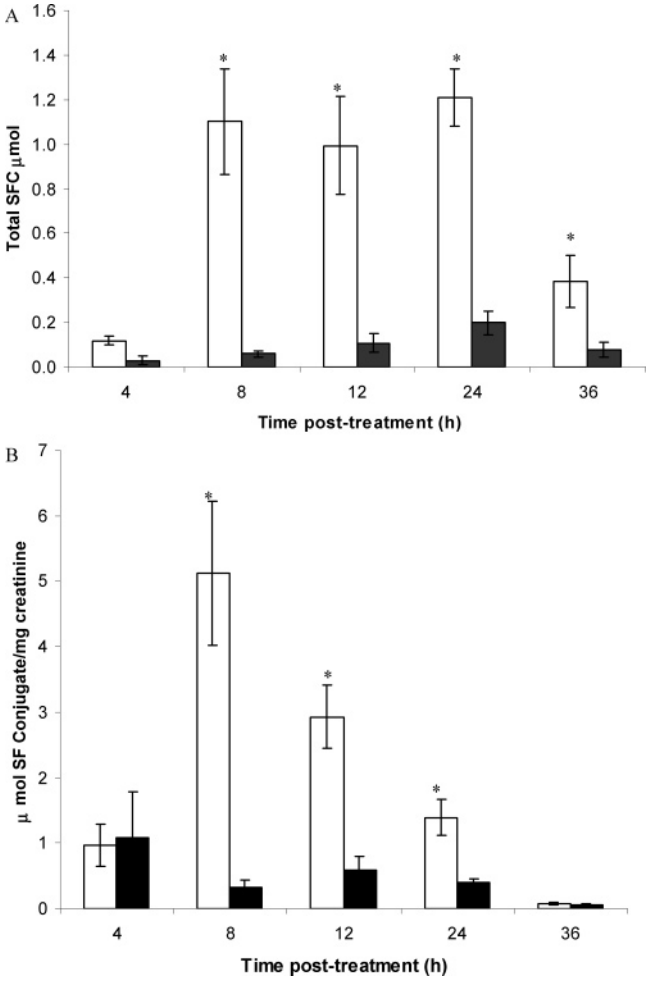

Figure 4. (**A**) Sulforaphane *N*-acetyl cysteine conjugate in urine following adminstration of glucoraphanin (150 μmol/kg BW) orally (open bars) or by ip injection (closed bars). Mean ± SE, $n = 6$. *Significantly different from ip-treated rats ($P < 0.05$). (**B**) Excretion of sulforaphane N-acetyl cysteine conjugate per mg creatinine, following administration of glucoraphanin (150 μmol/kg BW), orally (open bars) or by ip injection (closed bars). Mean ± SE; $n = 6$. *Significantly different from ip-treated rats ($P < 0.05$).

of intact glucoraphanin and glucoerucin in the urine (**Table 1**). Intact urinary glucosinolates (glucoraphanin plus glucoerucin) accounted for 4.9 (1.42 ± 0.2 μmol) and 38.7% (11.04 ± 3.05 μmol) of the oral and ip doses, respectively. The amount of glucoraphanin and glucoerucin excreted in urine of orally dosed rats (1.2 ± 0.5 and 0.25 ± 0.1 μmol, respectively, was significantly lower than that excreted by ip-treated rats (9.7 ± 2.7 and 1.4 ± 0.9 μmol, respectively; **Table 1**). These data suggest that a fraction of an oral dose of glucosinolates is absorbed intact. The initial 4 h urine collection accounted for 58 and 93% of the total intact glucoraphanin and glucoerucin excreted from oral and ip-dosed rats, respectively (see **Figure 4**). By 8 h, 95 and 99% of urinary excretion of intact glucosinolates were complete for orally and ip-dosed rats, respectively. The larger fraction excreted early following ip administration reflects a higher circulating level occurring following ip administration as compared to oral administration. Only trace amounts were found by 12 and 24 h, and there was none detectable by 36 h. Fecal analysis did not result in detectable levels of any of the above metabolites, and no differences were noted among fecal samples from different treatments.

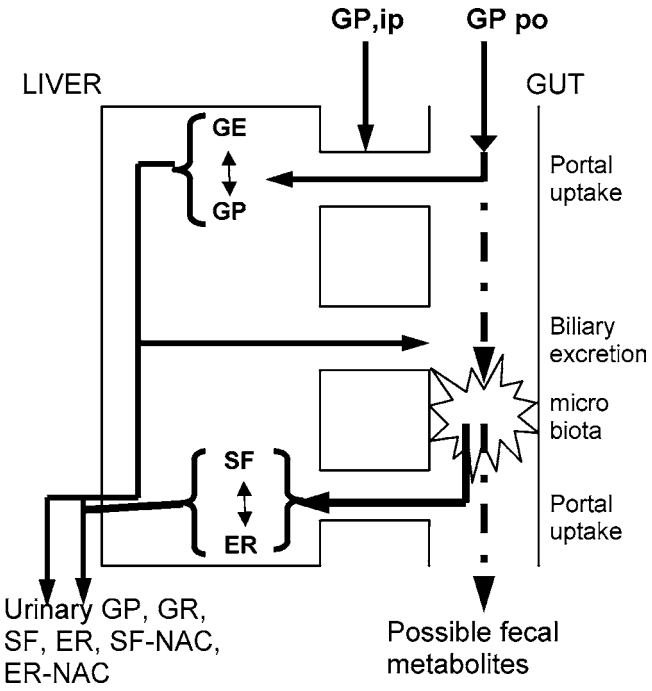

Figure 5. Metabolic scheme for glucoraphanin metabolism. Glucoraphanin (GP) is taken up from the gut to the liver where it is intercovertible with glucoerucin (GE). These glucosinolates may be excreted whole in the urine or secreted via the bile to the gut where microbiota hydrolyze them to sulforaphane (SF) and erucin (ER), which are absorbed, interconvertible in the liver, and excreted unchanged and as *N*-acetyl cysteine conjugates (NAC).

Biliary Excretion of Glucoraphanin. To confirm that the hydrolysis products found in the urine following parenteral administration reflected biliary excretion of glucosinolates, rats were anaesthestised, bile ducts were cannulated, and glucoraphanin was administered intravenously. Bile was collected for 1−2 h, and analysis indicated the presence of glucoraphanin and glucoerucin but no hydrolysis products and no conjugates. Rats given 75 μmol ($n = 3$) excreted 36.96 ± 17.9 nmol of glucoraphanin into bile and rats that were dosed with 150 μmol ($n = 3$) excreted 175.63 ± 67.8 nmol of glucoraphanin. However, these values do not reflect complete biliary efflux, as bile was only collected for 1−2 h, and biliary efflux would have continued for several hours. The role of enterohepatic recycling in formation and availability of glucoraphanin hydrolysis products needs further investigation. Analysis by LC/MS revealed no other metabolites.

Metabolic Scheme. Urine analysis revealed intact glucoraphanin in rats treated orally or ip with glucoraphanin, although there was substantially more in the urine of ip-treated rats. In contrast, there was a greater amount of metabolites present in the urine of the orally treated rats than in the urine of those receiving glucoraphanin ip. **Figure 5** shows a metabolic scheme consistent with these data. Finding intact glucoraphanin in the urine of orally dosed rats shows for the first time that glucoraphanin is absorbed intact. Our results are in agreement with previous studies that reported absorption of intact progoitrin from rapeseed meal fed to rats and transport of glucotropaeolin and sinigrin from the mucosal to the serosal side of the gut wall, using inverted hamster gut sacs from the small intestine and colon (*30*, *31*). However, no absorption of glucosinolates was detected when a human subject was given turnip roots that contained progoitrin, sinigrin, and indole glucosinolates (*8*). This difference could be due to the difference in the dose of glucosinolates in the two studies. The glucosinolate content of the turnip meal (~3−5 μmol/kg BW) may have provided insufficient urinary glucosinates to be above the limit of detection. In the present study, rats were given 150 μmol/kg BW. There was considerably less glucoerucin in the urine of orally dosed rats than ip-dosed rats (**Table 1**), which we interpret to mean that glucoraphanin can be reduced to glucoerucin within rat tissues (**Figure 5**). Conversion of sulforaphane to erucin has previously been shown to occur systemically, following ip administration of sulforaphane (*29*). Finally, the greater amount of metabolites in the orally treated rats as compared to the ip-treated rats suggested to us that glucoraphanin introduced parenterally had to re-enter the gut for hydrolysis (**Figure 5**). Analysis of bile from parenterally treated rats supports this route. Overall, urine analysis could only account for approximately 20 and 45% of the glucoraphanin dose given to rats by oral and ip routes, respectively. Although no unique peaks were identified on HPLC or GC, it is possible that other metabolites are formed, which were not detected in our study.

In conclusion, most published research on glucosinolate metabolism has utilized whole cruciferous vegetables, such as broccoli. Disadvantages of using broccoli as the source of glucoraphanin for such studies are that it is difficult to separate effects of glucoraphanin from those of other glucosinolates, glucosinolate hydrolysis products, and even other nonglucosinolate broccoli components. Furthermore, because glucoraphanin is in very low concentration in the whole vegetable, this limits, by bulk, the amount that one can provide in the diet (*15*). Thus, we developed a method to purify gram quantities of glucoraphanin from broccoli seed so that we could use the purified product to study its metabolism, its physiological effects, and the factors that affect its conversion to bioactive sulforaphane. Once effects of glucoraphanin have been evaluated in preclinical studies in rats, this can clarify interpretation of clinical studies, using whole broccoli or even semipurified glucoraphanin. Using purified glucoraphanin, we have been able to show for the first time that it is absorbed intact and undergoes enterohepatic circulation. We also showed for the first time that it is converted to the reduced analog glucoerucin in the body. Our data showing more urinary hydrolysis products from an oral dose of glucoraphanin than from an ip dose are consistent with the growing body of knowledge showing that in the absence of myrosinase, such as when ingesting cooked broccoli, glucosinolates are hydrolyzed by the gut microbiota and not in animal tissues (*11*).

LITERATURE CITED

(1) USDA-NASS Agricultural statistics—vegetables and melons; http://www.nass.usda.gov/Publications/Ag_Statistics/agr05/05_ch4.PDF, accessed 2-09-2007.

(2) Holst, B.; Williamson, G. A critical review of the bioavailability of glucosinolates and related compounds. *Nat. Prod. Rep.* **2004**, *21* (3), 425−447.

(3) Talalay, P.; Fahey, J. W. Phytochemicals from cruciferous plants protect against cancer by modulating carcinogen metabolism. *J. Nutr.* **2001**, *131* (11), 3027S−3033S.

(4) Greer, M. A.; Deeney, J. M. Antithyroid activity elicited by the ingestion of pure progoitrin, a naturally occurring thioglycoside of the turnip family. *J. Clin. Invest.* **1959**, *38*, 1465−1474.

(5) Oginsky, E. L.; Stein, A. E.; Greer, M. A. Myrosinase activity in bacteria as demonstrated by the conversion of progoitrin to goitrin. *Proc. Soc. Exp. Biol. Med.* **1965**, *119*, 360−364.

(6) Cheng, D. L.; Hashimoto, K.; Uda, Y. In vitro digestion of sinigrin and glucotropaeolin by single strains of Bifidobacterium and identification of the digestive products. *Food Chem. Toxicol.* **2004**, *42* (3), 351−357.5.

(7) Shapiro, T. A.; Fahey, J. W.; Wade, K. L.; Stephenson, K. K.; Talalay, P. Human metabolism and excretion of cancer chemoprotective glucosinolates and isothiocyanates of cruciferous vegetables. *Cancer Epidemiol. Biomarkers Prev.* **1998**, *7* (12), 1091−1100.6.

(8) Conaway, C. C.; Getahun, S. M.; Liebes, L. L.; Pusateri, D. J.; Topham, D. K.; Botero-Omary, M.; Chung, F. L. Disposition of glucosinolates and sulforaphane in humans after ingestion of steamed and fresh broccoli. *Nutr. Cancer* **2000**, *38* (2), 168−178.

(9) Elfoul, L.; Rabot, S.; Khelifa, N.; Quinsac, A.; Duguay, A.; Rimbault, A. Formation of allyl isothiocyanate from sinigrin in the digestive tract of rats monoassociated with a human colonic strain of *Bacteroides thetaiotaomicron*. *FEMS Microbiol. Lett.* **2001**, *197* (1), 99−103.

(10) Krul, C.; Humblot, C.; Philippe, C.; Vermeulen, M.; van Nuenen, M.; Havenaar, R.; Rabot, S. Metabolism of sinigrin (2-propenyl glucosinolate) by the human colonic microflora in a dynamic in vitro large-intestinal model. *Carcinogenesis* **2002**, *23* (6), 1009−1016.

(11) Rouzaud, G.; Rabot, S.; Ratcliffe, B.; Duncan, A. J. Influence of plant and bacterial myrosinase activity on the metabolic fate of glucosinolates in gnotobiotic rats. *Br. J. Nutr.* **2003**, *90* (2), 395−404.

(12) Matusheski, N. V.; Jeffery, E. H. Comparison of the bioactivity of two glucoraphanin hydrolysis products found in broccoli, sulforaphane and sulforaphane nitrile. *J. Agric. Food Chem.* **2001**, *49* (12), 5743−5749.

(13) Hwang, E. S.; Jeffery, E. H. Evaluation of urinary N-acetyl cysteinyl allyl isothiocyanate as a biomarker for intake and bioactivity of Brussels sprouts. *Food Chem. Toxicol.* **2003**, *41* (12), 1817−1825.

(14) Matusheski, N. V.; Wallig, M. A.; Juvik, J. A.; Klein, B. P.; Kushad, M. M.; Jeffery, E. H. Preparative HPLC method for the purification of sulforaphane and sulforaphane nitrile from *Brassica oleracea*. *J. Agric. Food Chem.* **2001**, *49* (4), 1867−1872.

(15) Kushad, M. M.; Brown, A. F.; Kurilich, A. C.; Juvik, J. A.; Klein, B. P.; Wallig, M. A.; Jeffery, E. H. Variation of glucosinolates in vegetable crops of *Brassica oleracea*. *J. Agric. Food Chem.* **1999**, *47* (4), 1541−1548.

(16) Heaney, R. K.; Fenwick, G. R. Methods for glucosinolate analysis. *Methods Plant Biochem.* **1993**, *8*, 531−550.

(17) Reeves, P. G. Components of the AIN-93 diets as improvements in the AIN-76A diet. *J. Nutr.* **1997**, *127*, 838S−841S.

(18) Keck, A. S.; Qiao, Q.; Jeffery, E. H. Food matrix effects on bioactivity of broccoli-derived sulforaphane in liver and colon of F344 rats. *J. Agric. Food Chem.* **2003**, *51* (11), 3320−3327.

(19) Jaw, S.; Jeffery, E. H. Role of metallothionein in biliary metal excretion. *J. Toxicol. Environ. Health* **1989**, *28* (1), 39−51.

(20) Carlson, D. G.; Daxenbichler, M. E.; VanEtten, C. H. Glucosinolates in crucifer vegetables: broccoli, Brussels sprouts, cauliflower, collards, kale, mustard greens and kohlrabi. *J. Am. Soc. Hortic. Sci.* **1987**, *112* (1), 173−178.

(21) Vaughn, S. F.; Berhow, M. A. Glucosinolate hydrolysis products from various plant sources: pH effects, isolation, and purification. *Ind. Crops Prod.* **2005**, *21*, 193−202.

(22) Prestera, T.; Fahey, J. W.; Holtzclaw, W. D.; Abeygunawardana, C.; Kachinski, J. L.; Talalay, P. Comprehensive chromatographic and spectroscopic methods for the separation and identification of intact glucosinolates. *Anal. Biochem.* **1996**, *239* (2), 168−179.

(23) Bennett, R. N.; Mellon, F. A.; Botting, N. P.; Eagles, J.; Rosa, E. A.; Williamson, G. Identification of the major glucosinolate (4-mercaptobutyl glucosinolate) in leaves of *Eruca sativa L.* (salad rocket). *Phytochemistry* **2002**, *61* (1), 25−30.

(24) Fahey, J. W.; Wade, K. L.; Stephenson, K. K.; Chou, F. E. Separation and purification of glucosinolates from crude plant homogenates by high-speed counter-current chromatography. *J. Chromatogr. A* **2003**, *996* (1−2), 85−93.

(25) Rochfort, S.; Caridi, D.; Stinton, M.; Trenerry, V. C.; Jones, R. The isolation and purification of glucoraphanin from broccoli seeds by solid phase extraction and preparative high performance liquid chromatography. *J. Chromatogr. A* **2006**, *1120* (1−2), 205−210.

(26) Tookey, H. L.; VanEtten, C. H.; Daxenbichler, M. E. Toxic constituents of plant foodstuffs. In *Glucosinolates*, 2nd ed.; Liener, I. E., Ed.; Academic Press: New York, 1980; pp 103−142.

(27) Staack, R.; Kingston, S.; Wallig, M. A.; Jeffery, E. H. A comparison of the individual and collective effects of four glucosinolate breakdown products from brussels sprouts on induction of detoxification enzymes. *Toxicol. Appl. Pharmacol.* **1998**, *149* (1), 17−23.

(28) Paolini, M.; Perocco, P.; Canistro, D.; Valgimigli, L.; Pedulli, G. F.; Iori, R.; Croce, C. D.; Cantelli-Forti, G.; Legator, M. S.; Abdel-Rahman, S. Z. Induction of cytochrome P450, generation of oxidative stress and in vitro cell-transforming and DNA-damaging activities by glucoraphanin, the bioprecursor of the chemopreventive agent sulforaphane found in broccoli. *Carcinogenesis* **2004**, *25* (1), 61−67.

(29) Kassahun, K.; Davis, M.; Hu, P.; Martin, B.; Baillie, T. Biotransformation of the naturally occurring isothiocyanate sulforaphane in the rat: identification of phase I metabolites and glutathione conjugates. *Chem. Res. Toxicol.* **1997**, *10* (11), 1228−1233.

(30) Lo, M. T.; Hill, D. C. Glucosinolates and their hydrolytic products in intestinal contents, feces, blood, and urine of rats dosed with rapeseed meals. *Can. J. Physiol. Pharmacol.* **1972**, *50* (10), 962−966.

(31) Michaelsen, S.; Otte, J.; Simonsen, L.-O.; Sorensen, H. Absorption and Degradation of individual intact glucosinolates in the digestive tract of rodents. *Acta Agric. Scand. A: Anim. Sci.* **1994**, *44*, 25−37.

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