

Influence of plant and bacterial myrosinase activity on the metabolic fate of glucosinolates in gnotobiotic rats

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The breakdown of glucosinolates, a group of thioglucoside compounds found in cruciferous plants, is catalysed by dietary or microbial myrosinase. This hydrolysis releases a range of breakdown products among which are the isothiocyanates, which have been implicated in the cancer-protective effects of cruciferous vegetables. The respective involvement of plant myrosinase and gut bacterial myrosinase in the conversion, *in vivo*, of glucosinolates into isothiocyanates was investigated in sixteen Fischer 344 rats. Glucosinolate hydrolysis in gnotobiotic rats harbouring a whole human faecal flora (Flora+) was compared with that in germ-free rats (Flora-). Rats were offered a diet where plant myrosinase was either active (Myro+) or inactive (Myro-). The conversion of prop-2-enyl glucosinolate and benzyl glucosinolate to their related isothiocyanates, allyl isothiocyanate and benzyl isothiocyanate, was estimated using urinary mercapturic acids, which are endproducts of isothiocyanate metabolism. The highest excretion of urinary mercapturic acids was found when only plant myrosinase was active (Flora-, Myro+ treatment). Lower excretion was observed when both plant and microbial myrosinases were active (Flora+, Myro+ treatment). Excretion of urinary mercapturic acids when only microbial myrosinase was active (Flora+, Myro- treatment) was low and comparable with the levels in the absence of myrosinase (Flora-, Myro- treatment). No intact glucosinolates were detected in the faeces of rats from the Flora+ treatments confirming the strong capacity of the microflora to break down glucosinolates. The results confirm that plant myrosinase can catalyse substantial release of isothiocyanates *in vivo*. The results also suggest that the human microflora may, in some circumstances, reduce the proportion of isothiocyanates available for intestinal absorption.

Glucosinolates: Isothiocyanates: Excretion: Myrosinase: Human colonic microflora

The consumption of cruciferous vegetables, such as cabbage and Brussels sprouts, by human subjects has been shown in epidemiological studies to be associated with a reduced incidence of cancer, particularly of the digestive and respiratory tracts (Steinmetz & Potter, 1991; Watson & Kohlmeier, 1999). Cruciferous vegetables contain several antioxidants and phytochemicals, such as carotenoids, vitamin C, flavonoids and glucosinolates (GSL), which have been implicated in the protective effects of such vegetables against cancer (Beecher, 1994; Johnson *et al.* 1994). Whilst most of these phytochemicals are also found in a wide variety of non-cruciferous fruit and vegetables, the essential source of GSL in the human diet is limited to the cruciferous vegetables, a few condiments, such as mustard and horseradish, and one fruit, the papaya (Fenwick *et al.* 1983). The GSL have, therefore, aroused particular interest as potential mediators of the cancer-protective effects of brassica vegetables. It is,

however, difficult to establish accurate estimates of target tissue exposure to GSL derivatives since information on the metabolic fate of GSL *in vivo* is limited.

The breakdown of GSL is thought to occur at two stages following their consumption by mammals. The first stage occurs when plant cells are disrupted during ingestion of plant material. This process exposes GSL to a plant enzyme, myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1) and hydrolysis occurs to yield several metabolites among which the isothiocyanates (ITC) have been implicated as cancer-protective compounds (Zhang & Talalay, 1994; Hecht, 1999). This hydrolysis may occur *in vivo* under the action of plant myrosinase when vegetables are eaten raw. In this situation, the release of the ITC from the GSL precursor *in vivo* may primarily occur in the upper digestive tract. In these circumstances, it is hypothesized that ITC may be absorbed before reaching the colon, although no direct evidence for this phenomenon

Abbreviations: Flora+, presence of microflora; Flora-, absence of microflora; GSL, glucosinolate; ITC, isothiocyanate; Myro+, presence of dietary myrosinase activity; Myro-, absence of dietary myrosinase activity.

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is available. ITC taken up by the intestinal mucosa are subsequently metabolized by the liver before being released into the systemic circulation as their N-acetyl cysteine conjugates and excreted in urine as their respective mercapturic acids (N-acetyl-S-(N-alkylthiocarbamoyl)-L-cysteines) (Conaway *et al.* 2002).

On the other hand, when vegetables are processed and cooked, plant myrosinase is inactivated (Ludikhuyze *et al.* 1999), which may limit the release of breakdown products. Intact GSL may reach the lower intestinal tract and be available as a substrate for the colonic microflora. The existence of microbial myrosinase activity in the microflora of the human bowel may catalyse the hydrolysis of GSL (Elfoul, 1999; Shapiro *et al.* 2001). The amount and site of release of GSL breakdown products probably, therefore, varies according to the activity of plant myrosinase, which will influence the amount of intact GSL reaching the large intestine. The different sites of hydrolysis following consumption of cooked and raw brassica vegetables may also influence the relative exposure of different tissues to ITC.

In the experiment reported here, the respective influence of dietary myrosinase and microbial myrosinase activity, and their interaction, on the release of ITC was measured *in vivo* in rats. The mode of administration of GSL, as a single dose or as part of a dietary regimen, may alter the fate of GSL; two modes of administration of GSL were therefore tested in the present experiment. The fate of dietary prop-2-enyl glucosinolate (sinigrin) contained in Brussels sprouts (*Brassica oleracea* var. *gemmifera*) and the fate of benzyl glucosinolate administered artificially were studied. The release of their related ITC was measured using mercapturic acids (N-acetyl S-(N-alkylthiocarbamoyl)-L-cysteine) as markers of their appearance *in vivo* following GSL hydrolysis (Duncan *et al.* 1997).

Material and methods

Experimental design

The experiment was a 2 × 2 factorial design with factors consisting of presence or absence of dietary myrosinase activity (Myro+ and Myro- respectively) and presence or absence of microflora (Flora+ and Flora- respectively). Two diets differing in myrosinase activity were offered to rats. One diet contained intact plant myrosinase (Myro+ diet) while the other diet contained the same cruciferous material heat-treated to inactivate the plant myrosinase (Myro- diet). To study the role of microbial myrosinase activity, two sets of animals differing in microbial status were used. The Flora+ group consisted of eight rats harbouring a whole human faecal flora while the Flora- group consisted of eight germ-free rats. Study of the interaction between dietary and microbial myrosinase was achieved by offering the Myro+ or Myro- diets to rats from the Flora+ or Flora- groups. Within each group of rats differing in microflora status, the order of offering the two diets was balanced, with four animals receiving the Myro+ diet first followed by the Myro- diet and vice versa. The total duration

of the experiment was 44 d. Each period of feeding lasted 22 d. In each feeding period, the first 8 d acted as an adaptation phase to allow rats to habituate to the food. The following 14 d were split into two dosing periods (see later; p. 397) lasting 7 d each.

Animals

Sixteen male, adult Fischer 344 rats were used. They were born germ-free and bred in germ-free conditions according to established methods (Coates, 1968) (INRA breeding unit, Jouy en Josas, France). Rats were aged 6–9 weeks at the start of the experiment (mean weight 163 (SD 11) g).

Animals were randomly allocated to Flora+ and Flora- groups. To maintain their bacterial status, rats were kept in groups of four animals in four sterile isolators (La Calhène, Vélizy, France). Within each isolator, rats were individually housed in metabolism cages (Iffa-Credo, Saint Germain sur l'Arbresle, France). Sterilized diet and autoclaved ultra-filtered water were given *ad libitum* to rats. On day 1, rats from the Flora+ group were orally administered with 1.0 ml 10⁻² dilution of a fresh faecal suspension. The faecal suspension was made from the stools of a healthy human male subject. Fresh stools (2 g) were dispersed in 20 ml Difco Brain Heart Infusion medium (Becton Dickinson, Le Pont de Claix, France). The microbiological diversity of the inoculum was assessed by microscopic examination.

Diet composition and preparation

The Myro+ and Myro- diets were semi-synthetic diets simulating a human-type diet (Table 1). They contained 150 g freeze-dried Brussels sprouts (*Brassica oleracea* variety *cyrus*)/kg DM (Novartis Seeds, Enkhuizen, The Netherlands). Analysis of GSL concentrations in the Brussels sprouts showed that sinigrin (prop-2-enyl glucosinolate) accounted for 54 % of the total amount of identified GSL and that benzyl glucosinolate was absent (Table 2). Diets were pelleted and packed in double-vacuum bags, which were sterilized by γ irradiation at 45 kGy (U.A.R., Villemoisson, France). In the Myro- diet, freeze-dried Brussels sprouts were treated to denature myrosinase before inclusion in the diet. Inactivation of myrosinase was achieved by soaking 100 g ground, freeze-dried Brussels sprouts in 1 litre 70 % (v/v) boiling ethanol for 10 min at 75°C. Ethanol was subsequently evaporated at 60°C using a rotary evaporator and aqueous residues were removed by oven-drying at 70°C. The success of the inactivation process was verified by adding 0.2 g freeze-dried Brussels sprouts treated for myrosinase inactivation to 2 ml distilled water. Duplicate samples were made and incubated at 37°C overnight. GSL concentrations were subsequently measured in the freeze-dried material and in the aqueous suspensions using the method of Minchinton *et al.* (1982). The inactivation process was considered successful if the difference between the concentration of sinigrin in the aqueous suspensions and in the freeze-dried Brussels sprouts was less than 10 % (Table 2).

Table 1. Composition of the diets (g/kg dry matter) given to germ-free rats and rats harbouring a human faecal microflora

Ingredients	Diets	
	Myro+	Myro–
Casein	50.00	50.00
Soya isolate*	90.00	90.00
Brussels sprouts with native myrosinase	150.00	–
Brussels sprouts with denatured myrosinase†	–	150.00
Saccharose	50.00	50.00
Mashed potato	230.00	230.00
Maize starch	229.85	229.85
Lard	30.00	30.00
Maize oil	30.00	30.00
Cholesterol	0.15	0.15
Cellulose	60.00	60.00
Mineral additive‡	70.00	70.00
Vitamin additive§	10.00	10.00
Protein content	15.70	16.07
Energy (kJ/kg DM)	16.83	17.08

Myro+, diet with presence of myrosinase activity; Myro–, diet with absence of myrosinase activity.

* Soya protein isolate PP500E; Protein Technologies International (St Louis, MO, USA).

† Myrosinase was denatured by heat treatment.

‡ The mineral additive included (g/kg DM of diet): CaHPO₄ 30.1, KCl 7.0, NaCl 7.0, MgO 0.735, MgSO₄ 3.5, Fe₂O₃ 0.21, FeSO₄·7H₂O 0.35, MnSO₄·H₂O 0.17, CuSO₄·5H₂O 0.035, ZnSO₄·7H₂O 0.141, CoSO₄·7H₂O 2.8 × 10⁻⁴, KI 5.6 × 10⁻⁴.

§ The vitamin additive included (mg/kg DM of diet): thiamin 20, riboflavin 15, pantothenic acid 70, pyridoxine 10, myoinositol 150, cyanocobalamin 0.05, ascorbic acid 800, α-tocopherol 170, menadione 40, niacin 100, choline 1360, folic acid 5, p-aminobenzoic acid 50, biotin 0.3, retinol 5.9 and cholecalciferol 0.0625.

Administration of glucosinolates and isothiocyanates

The proportion of intact sinigrin and benzyl glucosinolate that were absorbed as allyl isothiocyanate and benzyl isothiocyanate from the gut was estimated in a similar fashion to a previous study (Duncan *et al.* 1997). Estimation of ITC release required the measurement of urinary output of mercapturic acids following two separate dosing events within each feeding period. On one occasion, urinary output of mercapturic acids arising from GSL precursors was measured (GSL dosing period). On another occasion urinary output of mercapturic acids derived directly from orally

delivered ITC was quantified (ITC dosing period). Urinary mercapturic acid concentrations were then used to estimate ITC release as explained later (p. 398) and in Fig. 1. At the beginning of the GSL dosing period, a dose of benzyl glucosinolate was administered orally to rats. Sinigrin was ingested as part of the diet and its intake was measured by recording the daily food intake of individual rats over the GSL dosing period. At the beginning of the ITC dosing period, an oral dose of allyl and benzyl isothiocyanate was administered to rats. Butyl isothiocyanate was also given to all animals on both dosing occasions as a recovery standard. Doses were administered either on day 9 or 16 of each feeding period. The sequence of administration of the dosing mixtures was balanced within each sub-group of four rats. When the GSL dose was administered, animals received 25 µmol benzyl glucosinolate (Merck, Darmstadt, Germany) diluted in 0.25 ml water followed by 25 µmol butyl isothiocyanate (Sigma, Saint-Quentin Fallavier, France) diluted in 0.25 ml maize oil. The ITC dose contained 25 µmol each of allyl, benzyl and butyl isothiocyanates (Sigma) diluted in 0.25 ml maize oil. The ITC dose was preceded by the administration of 0.25 ml water so that all rats received equivalent amounts of the dosing vehicles. On each occasion, the doses were flushed with 0.25 ml pure maize oil. The GSL dosing mixtures were sterilized by filtration (0.22 µm Millex-GS filters; Millipore, Saint Quentin-en-Yvelines, France) and the ITC mixtures by autoclaving, before being administered. Preliminary tests showed that sterilization procedures did not influence concentrations of GSL or ITC. Dosing mixtures were orally administered using a sterile stainless-steel stomach tube to rats under light diethyl ether anaesthesia.

Sample collection

The day before each dosing, metabolism cages were cleaned. Urine and faeces were subsequently collected at 0, 6, 24, 48, 72, 120 and 144 h after administration of dosing mixtures. To avoid bacterial degradation of urinary markers, sodium azide (final concentration 0.02% (v/v); Sigma) was added to the urine collection receptacles of

Table 2. Concentrations of glucosinolates in the Brussels sprouts (*Brassica oleracea* variety *cyrus*) and in the diets given to rats (µmol/g dry matter)* (Mean values and standard deviations)

Glucosinolates	Brussels sprouts		Myrosinase-inactivated Brussels sprouts		Myro+ diet	Myro– diet
	Mean	SD	Mean	SD		
Glucoiberin	3.31	0.08	4.64	0.42	0.50	0.70
Progoitrin	2.96	0.18	3.71	0.35	0.44	0.56
Sinigrin	12.2	0.04	11.7	0.76	1.83	1.76
Gluconapin	2.46	0.15	2.19	0.19	0.37	0.33
4-OH glucobrassicin	2.91	0.15	ND	ND	0.43	ND
Glucoibassicin	0.32	0.04	0.92	0.25	0.05	0.14
4-Methoxy glucobrassicin	0.58	0.08	ND	ND	0.09	ND
Total	24.74	0.72	23.24	1.53	3.71	3.49

Myro+, presence of myrosinase activity; Myro–, absence of myrosinase activity; ND, not detected.

* For details of diets and procedures, see Table 1 and p. 396.

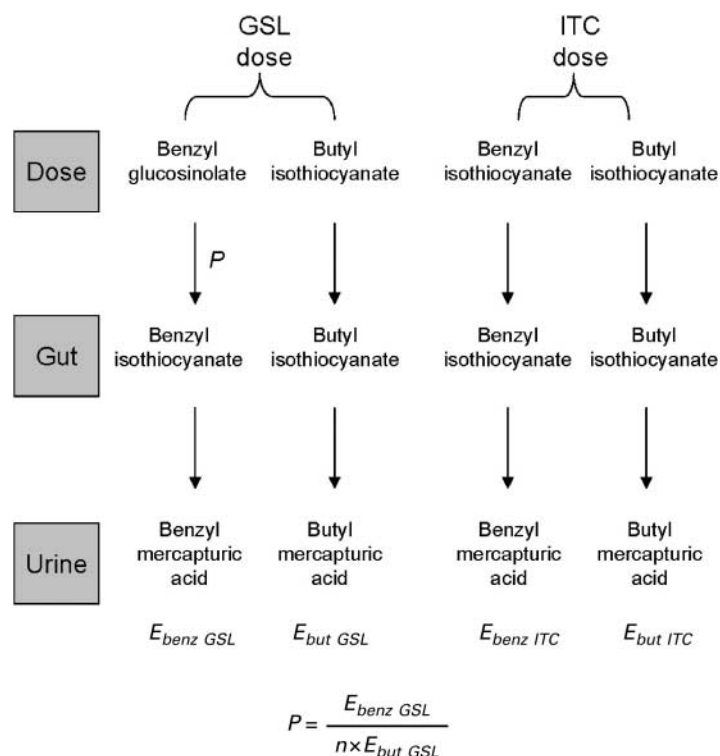


Fig. 1. Calculation of proportional release of benzyl isothiocyanate from benzyl glucosinolate following administration of benzyl glucosinolate to rats. GSL, glucosinolate; ITC, isothiocyanate; *P*, proportion of administered benzyl glucosinolate yielding benzyl isothiocyanate; *benz*, benzyl; *but*, butyl; *E*, molar amount of mercapturic acid excreted in 144 h; *n*, mean $E_{benzITC}:E_{butITC}$ value in all rats.

the Flora+ group of rats. No sodium azide was added to the receptacles of the Flora− group since these animals were in a sterile environment. Preliminary tests showed that sodium azide did not interfere with the analysis.

Analysis

Analysis of urinary endproducts. Mercapturic acids in urine were analysed using a modification of the method of Mennicke *et al.* (1987). The isocratic HPLC analysis was replaced in the present study by a gradient of distilled water and acetonitrile to allow the separation of mercapturic acids of allyl, propyl, butyl and benzyl isothiocyanates. The mobile phase consisted of 30% (v/v) acetonitrile in water changing linearly to 70% over 20 min. Detection was by u.v. absorbance at 240 nm. A solution of 1.5 mM-propyl mercapturic acid (*N*-acetyl-*S*-(*N*-propylthiocarbonyl)-*L*-cysteine) was synthesized (Mennicke *et al.* 1983) and used as an internal standard. Preliminary analysis showed that 95% of mercapturic acids was excreted in 48 h. Consequently, urine samples collected at times 72, 120 and 144 h were bulked in proportion to their volume before analysis.

Analysis of intact glucosinolates in faeces. Residual GSL, recovered intact in the faeces, were measured after desulfatation. Before analysis, faeces samples were freeze-dried and ground. Phenethyl glucosinolate (200 μ l, 1.0 mM; Sigma, Poole, Dorset, UK) was added to 0.2 g ground faeces as an internal standard. Samples were

analysed in duplicate. Extraction of GSL was carried out twice using 15 ml aliquots of 70% (v/v) boiling methanol. Pooled extracts were concentrated by rotary evaporation to obtain a final volume of 5 ml extract. Extracts were subsequently enzymically desulfated (Minchinton *et al.* 1982). Separation of desulfo-glucosinolates was carried out by HPLC (Gilson, Villiers le Bel, France) using a C18 reverse-phase column (Lichrospher 250 \times 4 mm; Merck). The mobile phase consisted of pure distilled water changing to 20% (v/v) acetonitrile in distilled water over 45 min as previously described (Spinks *et al.* 1984). Eluted peaks were detected by u.v. absorbance at 228 nm (u.v./visible detector, model 118; Gilson). Equimolar amounts of 1.0 mM-sinigrin, benzyl glucosinolate and phenethyl glucosinolate, which had been desulfated identically to test samples, were used to determine the detector response factors of measured desulfo-glucosinolates relative to the internal standard.

Calculations and statistical analysis

Calculation of isothiocyanate release from precursor glucosinolates in the intestinal tract of rats. The molar amount of benzyl mercapturic acid recovered in the urine during the GSL dosing period was divided by the predicted amount of benzyl mercapturic acid that would have been excreted assuming complete hydrolysis of benzyl glucosinolate to benzyl isothiocyanate. This prediction was based on the mean benzyl:butyl mercapturic acids value in the urine of individual rats collected during the ITC

dosing period multiplied by the amount of butyl mercapturic acid excreted during the GSL dosing period for each rat. Fig. 1 describes the calculation more fully. The calculation of allyl isothiocyanate released from sinigrin was based on the same principle.

Calculation of glucosinolate intake for individual rats and glucosinolate excretion in faeces. Daily food intake was averaged over the last 16 d of each feeding period. The amount of intact GSL excreted in the faeces of rats was calculated by multiplying faecal GSL concentrations by total faecal output.

Statistics. The experiment was analysed as a split-plot design using ANOVA. Isolator, isolator \times rat, feeding period \times isolator and feeding period \times rat were treated as blocks in the analysis. Terms analysed as treatments in the ANOVA model were diet, microbial status, diet \times microbial status, feeding period and dosing day. The effect of microbial status was analysed in the isolator stratum. The effect of diet and the interaction of diet with microbial status was analysed in the isolator \times period stratum. All calculations were performed using Genstat 5 (Lawes Agricultural Trust, Harpenden, Herts, UK, 1995).

Results

Intake and faecal recovery of glucosinolates

Mean daily intake by individual rats was 17 (SEM 0.3) g DM. Rats harbouring an active microflora (Flora+) ate significantly less than germ-free rats (Flora-) ($P < 0.05$; Table 3), but the amount of sinigrin ingested by rats was not significantly affected by their bacterial status (Table 3). The amount of the Myro+ diet ingested by

rats was marginally, but significantly, higher than the amount of Myro- diet ingested ($P < 0.01$; Table 3) and this variation was also observed in the intake of sinigrin. Rats ingested marginally more sinigrin when they were fed the Myro+ diet than when the Myro- diet was offered ($P < 0.01$; Table 3). In rats harbouring a microflora (Flora+), intact sinigrin and benzyl glucosinolate were not detected in faeces indicating total hydrolysis or absorption. Among germ-free rats (Flora-), a proportion of dosed benzyl glucosinolate and dietary sinigrin was recovered in the faeces and these amounts were higher when dietary myrosinase was inactive (Myro-) (Table 3).

Excretion of mercapturic acids

ITC were recovered as anticipated as mercapturic acids in the urine following their administration during the ITC period. Benzyl isothiocyanate was recovered more fully than butyl isothiocyanate following an equimolar dose (Table 4; mean proportional recoveries of 0.60 (SEM 0.041) and 0.41 (SEM 0.018) respectively). Microbial status and diet had no significant influence on urinary output of mercapturic acids following oral administration of butyl isothiocyanate and benzyl isothiocyanate (Table 4). Urinary excretion of benzyl and butyl isothiocyanates as their respective urinary mercapturic acids was correlated across animals (Fig. 2) confirming that concurrently administered butyl isothiocyanate could be used to reliably predict the post-absorptive recovery of benzyl isothiocyanate as its mercapturic acid when the GSL precursor was administered to animals in the GSL dosing period. Proportional recovery of the mercapturic acid derived

Table 3. Mean intake of experimental diets (g/d) and sinigrin ($\mu\text{mol/d}$) and faecal output of glucosinolates ($\mu\text{mol per 144 h}$) by rats during the glucosinolate dosing period*

(Mean values and standard errors of differences of means)

Precursor	Diet	Bacterial status		Mean	SED	Level of significance (Myro+ v. Myro-)
		Flora+	Flora-			
Intake of pelleted food	Myro+	16.9	18.9	17.9	0.52	$P < 0.01$
	Myro-	15.5	16.4	16.0		
	Mean	16.2	17.7			
	SED	0.50				
Level of significance (Flora)		$P < 0.05$				
Intake of sinigrin	Myro+	30.9	34.6	32.7	1.50	$P < 0.01$
	Myro-	27.3	28.9	28.1		
	Mean	29.1	31.7			
	SED	1.80				
Level of significance (Flora)		NS				
Faecal output of benzyl glucosinolate ($\mu\text{mol over 144 h}$)†	Myro+	<0.001	2.0	1.0	1.85	$P < 0.01$
	Myro-	<0.001	8.5	4.3		
	Mean	<0.001	5.3			
	SED	1.81				
Level of significance (Flora)		$P < 0.01$				
Faecal output of dietary sinigrin ($\mu\text{mol over 144 h}$)†	Myro+	<0.001	0.05	0.028	5.26	$P < 0.01$
	Myro-	<0.001	27.1	12.7		
	Mean	<0.001	12.7			
	SED	4.15				
Level of significance (Flora)		$P < 0.01$				

Flora+, rats harbouring a human faecal microflora; Flora-, germ-free rats; Myro+, presence of myrosinase activity; Myro-, absence of myrosinase activity; NS, not significant at $P < 0.05$.

* For details of diets and procedures, see Tables 1 and 2 and p. 396.

† Amount of mercapturic acids below the threshold of HPLC detection (0.001 μmol) were considered as 0.001 μmol in the statistical analysis.

Table 4. Cumulative excretion of urinary mercapturic acids (μmol) over 144 h after administration of an isomolar solution of allyl, benzyl and butyl isothiocyanates to rats differing in microbial status and diet offered*
(Mean values and standard errors of differences of means)

Isothiocyanate precursor†	Diet	Bacterial status		Mean	SED	Level of significance (Myro+ v. Myro-)
		Flora+	Flora-			
Benzyl isothiocyanate	Myro+	15.6	18.6	17.1	0.68	NS
	Myro-	12.2	14.1	13.2		
	Mean	13.9	16.4			
	SED	3.51				
Level of significance (Flora)		NS				
Butyl isothiocyanate	Myro+	11.0	12.6	11.8	1.20	NS
	Myro-	9.1	10.1	9.3		
	Mean	9.8	11.4			
	SED	2.14				
Level of significance (Flora)		NS				
Allyl isothiocyanate	Myro+	67.2	58.9	63.3	2.61	$P < 0.05$
	Myro-	16.4	13.2	14.7		
	Mean	43.5	34.5			
	SED	5.98				
Level of significance (Flora)		NS				

Flora+, rats harbouring a human faecal microflora; Flora-, germ-free rats; Myro+, presence of myrosinase activity; Myro-, absence of myrosinase activity; NS, not significant at $P < 0.05$.

* For details of diets and procedures, see Tables 1 and 2 and p. 000.

† Isothiocyanate solution contained 25 μmol of allyl, butyl and benzyl isothiocyanate.

from allyl isothiocyanate following the ITC dose was greater than 1.0 due to the presence of sinigrin in the diet, which led to production of allyl isothiocyanate of dietary origin in the gut. Excretion of the mercapturic acid from allyl isothiocyanate was significantly higher when rats were fed the Myro+ diet v. the Myro- diet but excretion was not significantly different in the presence or absence of microbial activity (Table 4).

Mercapturic acids were also detected in the urine collected during the GSL dosing period. Mean proportional recovery of benzyl glucosinolate as benzyl mercapturic acid was 0.16 (SEM 0.032). Diet had a significant influence on the excretion of benzyl mercapturic acid following the GSL dose. Excretion was higher when rats were consuming the Myro+ diet than when the Myro- diet was offered

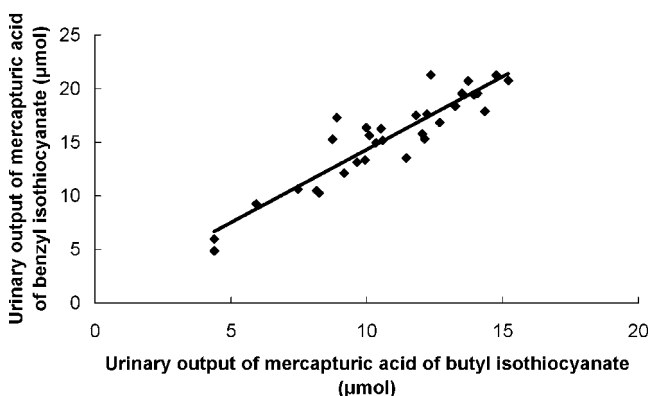


Fig. 2. Relationship between cumulative excretion of mercapturic acids of butyl and benzyl isothiocyanate after administration of isothiocyanate dose: $y = (1.36 \times x) + 0.67$ ($R^2 = 0.847$; $P < 0.001$) where y is the urinary output of mercapturic acid of benzyl isothiocyanate over 144 h and x is the urinary output of mercapturic acid of butyl isothiocyanate over 144 h.

(Table 5) indicating GSL hydrolysis by intact myrosinase present in the Myro+ diet. The urinary output of benzyl mercapturic acid was higher in the Flora- rats than in the Flora+ rats indicating a negative effect of the presence of the microflora on the amount of benzyl isothiocyanate available for intestinal uptake. The highest excretion of benzyl mercapturic acid was found when plant myrosinase only was active (Flora-, Myro+) (Table 5). When both sources of myrosinase were active (Flora+, Myro+), the urinary output of benzyl mercapturic acid was lower than when plant myrosinase only was active. In the absence of myrosinase (Flora-, Myro-) excretion of benzyl mercapturic acid was close to zero. Benzyl mercapturic acid excretion when bacterial myrosinase only was active (Flora+, Myro-) was also close to zero. The sequence of offering the diet had no significant effect on excretion of benzyl mercapturic acid. Excretion of butyl mercapturic acid during the GSL dosing period followed a similar pattern to its excretion during the ITC dosing period. Neither diet nor microbial status significantly influenced urinary output of dosed butyl isothiocyanate as its mercapturic acid and mean proportional recovery was 0.38 (SEM 0.032). Excretion of allyl mercapturic acid during the GSL dosing period was strongly influenced by diet with higher excretion in rats consuming the Myro+ diet than those consuming the Myro- diet (Table 5). Microbial status had no significant influence on allyl mercapturic acid excretion.

Estimation of proportional release of benzyl isothiocyanate

The estimated proportional release of benzyl isothiocyanate from benzyl glucosinolate in the digestive tract was considerably higher in rats consuming a diet containing intact myrosinase than in rats consuming a myrosinase-free diet ($P < 0.05$; Table 6). In rats harbouring a microflora,

Table 5. Cumulative excretion of urinary mercapturic acids (μmol) excreted over 144 h after simultaneous administration of 25 μmol benzyl glucosinolate and butyl isothiocyanate to rats differing in microbial status and diet offered*
(Mean values and standard errors of differences of means)

Precursor	Diet	Bacterial status		Mean	SED	Level of significance (Myro+ v. Myro-)
		Flora+	Flora-			
Benzyl glucosinolate	Myro+	5.1	9.8	7.5	0.54	$P < 0.05$
	Myro-	0.42	0.40	0.41		
	Mean	2.75	5.14			
	SED	0.30				
Level of significance (Flora)		$P < 0.05$				
Butyl isothiocyanate	Myro+	8.6	10.7	9.6	1.02	NS
	Myro-	10.0	9.2	9.6		
	Mean	9.3	10.0			
	SED	2.41				
Level of significance (Flora)		NS				
Dietary sinigrin	Myro+	31.8	42.4	37.1	1.83	$P < 0.05$
	Myro-	4.7	0.84	2.8		
	Mean	18.3	21.6			
	SED	2.86				
Level of significance (Flora)		NS				

Flora+, rats harbouring a human faecal microflora; Flora-, germ-free rats; Myro+, presence of myrosinase activity; Myro-, absence of myrosinase activity; NS, not significant at $P < 0.05$.

* For details of diets and procedures, see Tables 1 and 2 and p. 396.

proportional release of benzyl isothiocyanate was lower than in germ-free rats ($P < 0.05$). The interaction between microbial status and diet was not significant.

Discussion

A fuller understanding of the cancer-protective effects of GSL requires more information about the fate of GSL from their synthesis in plant cells to their absorption from the gut as breakdown products and delivery to tissues. Recent studies on the effect of cooking on brassica vegetables have provided a better appreciation of the fate of GSL during food preparation (Dekker *et al.* 2000). The fate of GSL following ingestion is much less well understood. Recent experiments, demonstrating the release of ITC in the lower gut following microbial hydrolysis (Elfoul *et al.* 2001; Shapiro *et al.* 2001), have aroused interest since ITC may exhibit their cancer-protective effect on the colorectal epithelium (Smith *et al.* 1998) and ingestion of brassica vegetables is particularly associated with inhibition of tumour occurrence at this site.

In the present experiment, urinary mercapturic acids were used as markers of ITC release. Such an approach has been successfully used in other studies (Duncan *et al.* 1997; Shapiro *et al.* 1998; Getahun & Chung, 1999) to estimate ITC production in the digestive tract under different circumstances. Before their excretion in urine as mercapturic acids, ITC undergo a series of biotransformation reactions involving conjugation with glutathione under the catalysis of glutathione-S-transferase (Mennicke *et al.* 1983). The efficiency of the conjugation step as well as the availability of conjugation substrates may vary between individuals and may limit the reliability of mercapturic acids used as markers of metabolism. Recovery of artificially administered ITC as urinary mercapturic acids did show considerable inter-individual variation in the present experiment (Fig. 2). The administration of butyl isothiocyanate, a compound that did not occur in the diet but which follows a similar detoxification pathway to food-borne ITC, allowed variation in the post-absorptive fate of ITC to be accounted for. The use of butyl isothiocyanate as a recovery standard relies on it having a similar metabolic fate to ITC formed following GSL hydrolysis.

Table 6. Estimated proportion of benzyl isothiocyanate released in the digestive tract of germ-free rats (Flora-) and rats harbouring a whole human faecal flora (Flora+) following administration of 25 μmol benzyl glucosinolate*
(Mean values and standard errors of differences of means)

Precursor	Diet	Bacterial status		Mean	SED	Level of significance (Myro+ v. Myro-)
		Flora+	Flora-			
Benzyl glucosinolate	Myro+	0.50	0.80	0.65	0.049	$P < 0.05$
	Myro-	0.05	0.04	0.05		
	Mean	0.27	0.42			
	SED	0.029				
Level of significance (Flora)		$P < 0.05$				

Myro+, presence of myrosinase activity; Myro-, absence of myrosinase activity.

* For details of diets and procedures, see Tables 1 and 2, p. 396.

This assumption may not be fully satisfied since its instantaneous introduction into the digestive tract may influence its recovery relative to the more gradual appearance of GSL-derived ITC. Furthermore, recovery following oral administration into the upper digestive tract may be different from recovery following production in the lower digestive tract under microbial action. The use of a recovery standard does, however, represent an improvement on previous studies in which inter-individual variation in post-absorptive recovery has not been accounted for. Although absolute values for ITC production may not be precise, the use of a recovery standard increases the confidence of estimates of the release of ITC in the digestive tract.

The present study was designed to allow the relative influence of dietary and microbial myrosinase, as well as their interaction, on GSL hydrolysis to be assessed. Diets differed in their activity of plant myrosinase and contained similar concentrations of GSL, ensuring that the observed difference in breakdown product release was not related to a modification of the GSL composition of the diets. The method of treating Brussels sprouts to simulate cooking (by soaking in boiling ethanol) was effective in denaturing myrosinase but may also have had unknown effects on various other characteristics of the vegetable. Cooking of brassica vegetables leads to myrosinase inactivation and this probably is the major effect of cooking on GSL metabolism. GSL hydrolysis is, however, a complex process and it is conceivable that other changes to the vegetable may also have influenced the course of GSL hydrolysis. Little is known of the influence of changes in brassica vegetables following cooking, other than changes to myrosinase activity, on GSL hydrolysis and further research in this area would be useful.

The substantial release of allyl and benzyl isothiocyanate when rats were fed the Myro+ diet, which simulated the intake of raw cruciferous vegetables, confirmed that conditions in the digestive tract are favourable to ITC production from GSL after disruption of plant cells. This supports the findings of previous studies where human subjects have been fed raw cruciferous vegetables and mercapturic acids have been detected in the urine indicating ITC release (Shapiro *et al.* 1998; Getahun & Chung, 1999). Previous estimates of ITC release have, however, been limited by uncertainty about the post-absorptive recovery of ITC.

In the present study, the proportional release of benzyl isothiocyanate by microbial myrosinase, as estimated by the excretion of its mercapturic acid, was relatively low (0.05; Table 6). This conflicts with previous studies, which have indicated more substantial release of ITC under the action of myrosinase of bacterial origin (Duncan *et al.* 1997; Elfoul *et al.* 2001). There are various possible explanations for this finding: GSL hydrolysis by microbial myrosinase may have led to non-ITC metabolites being produced; benzyl isothiocyanate may have been produced but its post-absorptive recovery may have been less efficient than when it arose in the upper tract; finally, benzyl isothiocyanate may have arisen in the colon but been further metabolized by micro-organisms before its absorption and excretion as its mercapturic acid. Contrary to expectations, the effect of plant myrosinase was not enhanced by the myrosinase activity of the microflora.

Indeed, the presence of an active microflora reduced the excretion of the mercapturic acid arising from benzyl isothiocyanate. These observations suggest that the microflora may be able to catalyse both the release of ITC and their further breakdown to other final products resulting in a lower net absorption of ITC by the distal digestive tract in the presence of an active microflora. A recent study, identifying the production of allyl amine and benzyl amine as a result of the microbial hydrolysis of sinigrin and benzyl glucosinolate by human microflora (Combourieu *et al.* 2001), would appear to support these observations.

When both sources of myrosinase were active, the proportion of benzyl glucosinolate broken down to benzyl isothiocyanate found in the present studies (0.50) was similar to previous findings on allyl isothiocyanate release from sinigrin in conventional rats (0.41; Duncan *et al.* 1995). The amounts were also broadly similar to previous estimates derived from feeding trials where human volunteers ingested raw cruciferous vegetables (Shapiro *et al.* 1998, 2001).

The proportion of allyl isothiocyanate released from dietary sinigrin could not be confidently estimated in the present study. Due to the background excretion of allyl mercapturic acid, the correlation between the excretion of butyl isothiocyanate and allyl isothiocyanate administered in the ITC dose was poor. Butyl isothiocyanate could not, therefore, be used as a predictor of allyl isothiocyanate recovery since allyl mercapturic acid in urine originated both from dietary sinigrin and dosed allyl isothiocyanate. While precise estimates of allyl isothiocyanate release were not possible, the amounts of allyl mercapturic acid excreted in urine and intact sinigrin excreted in faeces under the various treatments applied were in broad agreement with information derived from benzyl glucosinolate.

Information about the fate of GSL in the digestive tract can also be derived from the extent of their recovery in faeces. When plant myrosinase only was active, the total amount of dosed benzyl glucosinolate accounted for by ITC release and faecal excretion on a molar basis was 0.84 of the amount dosed. This high proportion suggests that other breakdown products of myrosinase hydrolysis, such as nitriles, were only released in minor amounts when GSL were ingested in their intact form. Isothiocyanates therefore appeared to be the major products of GSL hydrolysis in the digestive tract.

The presence of the intestinal microflora presumably led to a total degradation or absorption of GSL since intact GSL were not detected in the faeces of the Flora+ rats. This finding supports the suggestion that the human microflora is able to effectively degrade GSL. Several studies have shown similar effects *in vitro* following incubation of human faeces and using human single strains of bacteria (Rabot *et al.* 1995; Getahun & Chung, 1999; Krul *et al.* 2002).

Faecal recovery of GSL was not complete when both dietary and microbial myrosinase were inactive. Only 0.34 of dosed benzyl glucosinolate was excreted in the faeces of Flora+ rats consuming a Myro- diet. In a parallel experiment the faecal recovery of sinigrin was similar at 0.28 (Elfoul, 1999). These results suggest a possible

metabolism other than enzymic hydrolysis for the proportion of GSL (66–72%) that are not accounted for. It has been shown *in vitro*, using everted intestinal sacs, that GSL may passively cross the intestinal mucosa (Michaelsen *et al.* 1994). No evidence exists, however, for this mechanism occurring *in vivo*. The measurement of intact GSL in tissues and fluids other than faeces would be necessary to clarify the fate of non-digested GSL.

The microflora of rats in the Flora+ treatment were derived from a single healthy human subject. Multiple donors were not used since in previous work it has been found that such an approach leads to unpredictable interactions that result in a reduction of the degradative potential (Krul *et al.* 2002). The composition of the microflora was not determined due to the limitations of conventional culture techniques and since molecular techniques require cDNA probes that are only currently available for a few taxa. Bacterial myrosinase is, anyway, thought to be ubiquitous in man and this idea is supported by findings where the excretion of urinary endproducts dramatically decreased after consumption of GSL when a number of human volunteers were subjected to bowel cleansing and antibiotherapy (Shapiro *et al.* 1998). Although large differences in the composition and xenobiotic metabolizing capacity between human and rat intestinal microflora are well documented (Debure *et al.* 1989; Rumney *et al.* 1993; Meselhy *et al.* 1997), various studies have demonstrated that enzyme activities of the human colonic flora, for example, hydrolysis of glucuronides and hydroxylation of heterocyclic compounds, can be reliably simulated in human-flora-associated rats (Mallett *et al.* 1987; Debure *et al.* 1989; Rumney *et al.* 1993). Therefore, despite uncertainties surrounding the taxonomic and metabolic profiles of the flora of human-flora-associated rats, these animals are probably a more suitable model than conventional rats to investigate xenobiotic metabolism in the human context.

Under the conditions tested in the present experiment, plant myrosinase had a dominant influence on the production of ITC with microbial myrosinase having less marked effects. Myrosinase contained in a diet simulating raw vegetable consumption induced a substantial and rapid release of ITC. In contrast, after ingestion of a diet simulating cooked vegetables, the ITC were released to a lesser extent, if at all. This finding contrasts with previous studies in which the microflora appeared to catalyse ITC release (Elfoul *et al.* 2001, Shapiro *et al.* 2001). In the present study the microflora may have contributed to the release of ITC *in vivo* with a proportion of these newly formed ITC being further metabolized before they could be absorbed from the digestive tract. Alternatively, the microflora may have caused degradation of GSL and metabolism to other breakdown products. Absorption of ITC following consumption of brassica vegetables probably reflects the relative rates of production and further degradation of ITC. Further work is required before drawing general conclusions on the influence of the microflora on GSL hydrolysis. The present experiment has demonstrated the importance of the treatment of brassica material before ingestion (for example, cooking) in influencing the extent and site of production

of ITC from GSL ingested as part of the diet. Further work is required to correlate the extent and site of ITC release from brassica vegetables prepared in different manners with protection against tumour formation at different tissue sites.

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