Chapter 3

Metabolism, kinetics and genetic variation

Observations in humans

Isothiocyanates

Metabolism and disposition

The –N=C=S group of most isothiocyanates is electrophilic and can react readily with various nucleophiles, including thiols. Studies in both humans and experimental animals have established that isothiocyanates are metabolized in vivo to various dithiocarbamates principally by the mercapturic acid pathway. An initial conjugation with glutathione (GSH, the most abundant cellular thiol), which takes place spontaneously but is further promoted by glutathione *S*-transferase (GST), gives rise to the corresponding conjugates.

In the spontaneous reaction, isothiocyanates react reversibly with cysteinyl thiols, forming dithiocarbamates:

The equilibrium position and the chemical relaxation time for equilibrium have been estimated for phenethylisothiocyanate (ITC) reacting with cysteinyl residues in blood plasma (cysteinyl thiol concentration, about 500 µmol/l) and cells (cysteinyl thiol concentration, 5 mmol/l). The equilibrium constant, *K*, was 730 per mol. At equilibrium, about 27% of phenethyl-ITC is bound to plasma thiols in blood, and about 88% is bound to thiols in cells. For a plasma concentration of 5 µmol/l of phenethyl-ITC, the equilibrium relaxation time for formation of adducts with protein thiols is about 17 min in plasma and about 3 min in cells (Xu & Thornalley, 2000a).

The GSH conjugates undergo further enzymatic modification, in the GSH portion, to give rise sequentially to the cysteinylglycine, cysteine and *N*-acetylcysteine conjugates, which are excreted in urine (Brüsewitz *et al.*, 1977), as shown in Figure 10. Urinary excretion of *N*-acetylcysteine conjugates is the principal route of disposition of ingested isothiocyanates. For example, when benzyl-ITC was administered orally to six men, 54% of a dose of 14.4 mg was recovered in the urine as *N*-acetyl-S-(*N*-benzylthiocarbamoyl)-L-cysteine (benzyl-ITC–*N*-acetylcysteine). This compound was excreted rapidly: maximum excretion occurred within 2–6 h, and the compound was essentially undetectable 10–12 h after dosing (Mennicke *et al.*, 1988). Watercress is rich in gluconasturtiin (constituting more than 30% of total glucosinolates), the precursor of phenethyl-ITC. When four volunteers ate 30 g of watercress containing 21.6 mg of gluconasturtiin, an average of 47% of the amount was recovered as the *N*-acetylcysteine conjugate in 24-h urine (Chung *et al.*, 1992). The amount of phenethyl-ITC–*N*-acetylcysteine in urine is probably greater when phenethyl-ITC is administered, because the myrosinase-catalysed conversion of gluconasturtiin to phenethyl-ITC may be incomplete after ingestion of watercress. In these studies, the amount of urinary *N*-acetylcysteine –ITC might have been underestimated, because these conjugates are unstable and readily dissociate to isothiocyanates (Conaway *et al.*, 2001). The metabolic disposition of other isothiocyanates, including allyl-ITC and sulforaphane, are similar in humans, as discussed below.

Measurement of isothiocyanates and isothiocyanate metabolites

The cyclocondensation assay is a highly sensitive quantitative method for measuring isothiocyanates and their metabolites (referred to collectively here as isothiocyanate equivalent) (Zhang *et al.*, 1996) and is a valuable tool for studying dietary consumption of isothiocyanates and their metabolism. The assay is based on the almost universal ability of isothiocyanates to react quantitatively with 1,2-benzenedithiol, a vicinal dithiol, to give rise to a five-membered cyclic product and a free amine (Zhang *et al.*, 1992a), as shown in Figure 11. Reactive isothiocyanates are converted to the same cyclic product, 1,3-benzodithiole-2-thione, and conversion is complete in the presence of excess 1,2-benzenedithiol. The resultant 1,3-benzodithiole-2-thione can be measured accurately in amounts as low as a few picomoles, with a simple high-performance liquid chromatography (HPLC) procedure. Conjugation products of isothiocyanates with thiols (dithiocarbamates), including *N*-acetyl-cysteine–isothiocyanate, also react quantitatively with 1,2-benzenedithiol, giving rise to the same cyclic product. Thus, this assay allows detection of the total amount of isothiocyanates and their

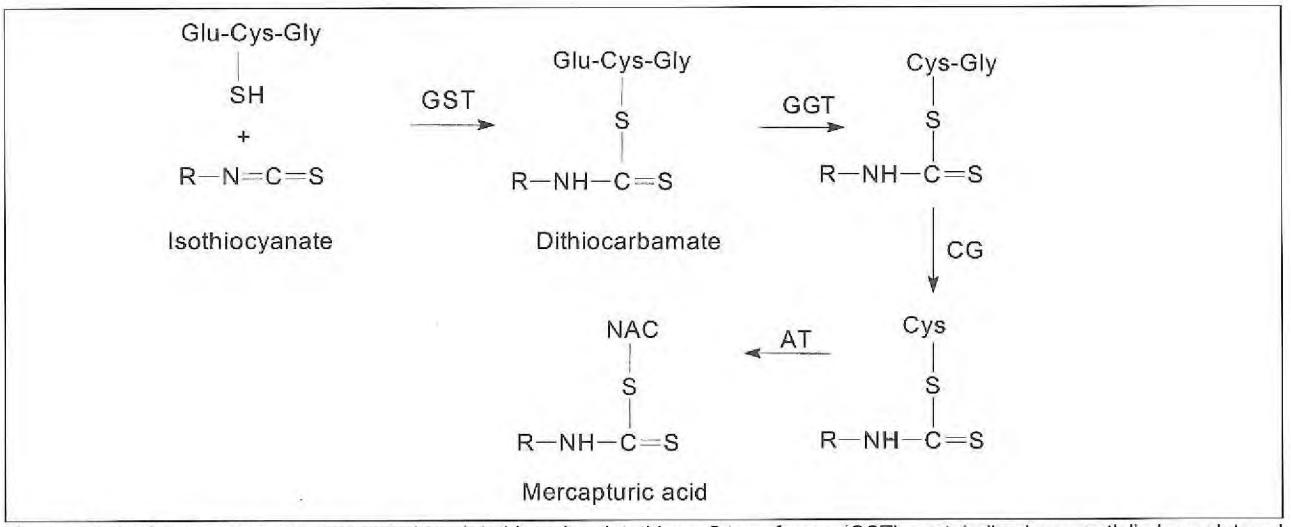

Figure 10 Isothiocyanates are conjugated to glutathione by glutathione *S*-transferase (GST), metabolized sequentially by γ-glutamyl transpeptidase (GGT), cysteinylglycinase (CG) and *N*-acetyltransferase (AT) to form, ultimately, mercapturic acid.
NAC, *N*-acetylcysteine

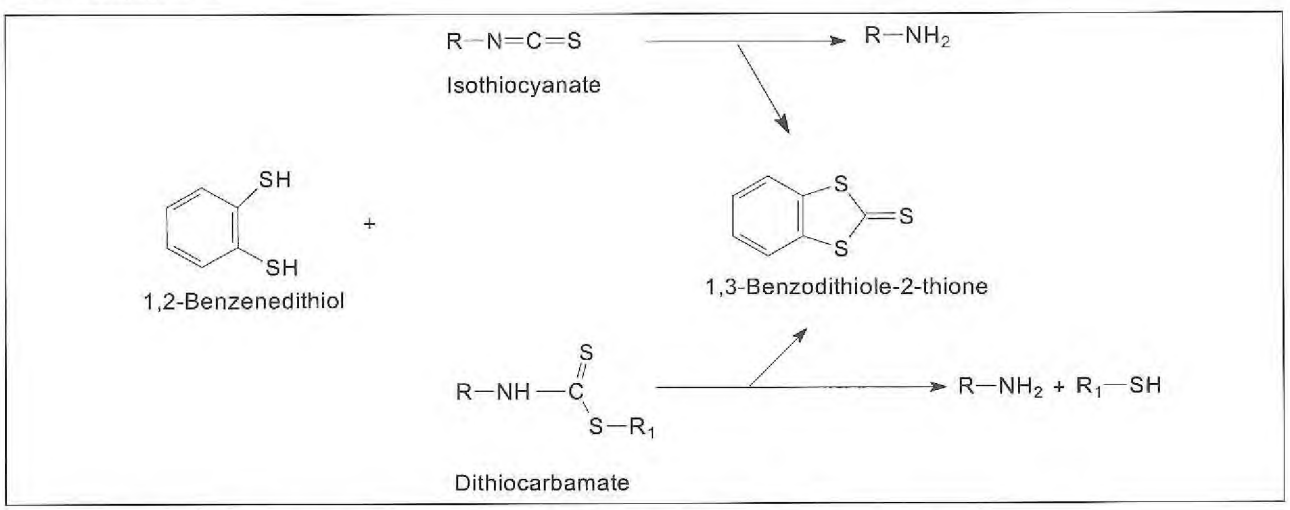

Figure 11 Cyclocondensation reaction of isothiocyanate and dithiocarbamate with 1,2-benzenedithiol

thiol conjugates. The assay has allowed quantitative assessment of total isothiocyanates in cruciferous vegetables and isothiocyanate equivalents in human fluids, including blood and urine. Because it reveals both isothiocyanates and their thiol metabolites, the total urinary concentrations of these compounds are not affected by dissociation of thiol conjugates to isothiocyanates. Even though individual isothiocyanates and isothiocyanate metabolites can be quantified sensitively by other methods, including HPLC–mass spectrometry (Ji & Morris, 2003; Vermeulen *et al.*, 2003), such analyses might not provide accurate values, since the metabolites are unstable and can dissociate to isothiocyanates.

Use of the cyclocondensation assay is nevertheless subject to some caveats. Although there are no endogenous sources of urinary isothiocyanates or dithiocarbamates in humans (Shapiro *et al.*, 1998), non-dietary sources may exist. The contributions of cigarette smoke (possibly due to the presence of carbon disulfide, which is also detected in the assay), agricul-

tural chemicals, chemicals used in the rubber industry and medical compounds such as disulfiram and antiseptics, should be considered (Zhang *et al.*, 1996; Shapiro *et al.*, 1998; Ye *et al.*, 2002). Shapiro *et al.* (1998) found that when urine samples were stored at –20 °C, the cyclocondensation reactivity remained stable, but 35% of the dithiocarbamate content was lost in samples stored for 18 months at the same temperature.

Measuring metabolic disposition of isothiocyanates

Studies on dietary intake of isothiocyanates are still extremely limited, and the information below was obtained from studies of a small number of volunteers. Ye *et al.* (2002) determined the metabolic disposition of isothiocyanates from broccoli sprouts by giving four volunteers a single serving of a myrosinase-treated extract of 3-day-old broccoli sprouts containing 200 µmol total isothiocyanate. The isothiocyanate composition of the preparation was 77.2% sulforaphane or iberin and 22.8% iberin (aliphatic isothiocyanates with closely related chemical structures). The isothiocyanates were absorbed rapidly and reached peak plasma concentrations of 0.94–2.27 µmol/l 1 h after ingestion, which declined according to first-order kinetics (half-life, 1.77 ± 0.13 h). The cumulative urinary excretion of isothiocyanate equivalent at 8 h was 58.3 ± 2.8% of the dose. In a similar experiment, the cumulative urinary excretion of isothiocyanate equivalent at 72 h was 88.9 ± 5.5% (Shapiro *et al.*, 2001). Similar results were obtained in volunteers given horseradish isothiocyanates (Shapiro *et al.*, 1998), most of which are allyl- or phenylethyl-ITC (Liebes *et al.*, 2001; Ji & Morris, 2003). For example, after a single serving of 20 ml of horseradish juice containing 74 µmol of isothiocyanates to 10 volunteers, 42 ± 5% of isothiocyanate equivalent was recovered in urine within 10 h (Shapiro *et al.*, 1998). Thus, isothiocyanates are rapidly and efficiently absorbed, rapidly cleared from blood and eliminated almost exclusively in urine.

The molecular basis for the rapid metabolic disposition of isothiocyanates in humans was elucidated by studying cultured human cells with the cyclocondensation assay (Zhang & Talalay, 1998; Zhang, 2000, 2001; Zhang & Callaway, 2002). Exposure of cells to an isothiocyanate led to rapid accumulation of the compound through conjugation with cellular thiols. GSH, the most abundant intracellular thiol, is the major driving force for isothiocyanate accumulation, and cellular GST enhances isothiocyanate accumulation by promoting conjugation reactions. Peak intracellular isothiocyanate accumulation was achieved within 0.5–3 h after the beginning of exposure to isothiocyanate, reaching 100–200 times the extracellular isothiocyanate concentration. Intracellularly accumulated isothiocyanate and conjugates were also rapidly exported by membrane transporters, including the multidrug resistance-associated protein (MRP)-1 and P-glycoprotein-1: the half-life of the accumulated isothiocyanate and conjugates in human prostate cancer LNCaP cells was only about 1 h.

These studies have begun to reveal the pharmacokinetics of ingested isothiocyanates in humans; however, the question remains as to when urine should be collected for assessment of dietary isothiocyanate intake. As dithiocarbamates are excreted rapidly in urine after ingestion of isothiocyanates, continuous urine collection over a sufficient period (e.g. 8 or 24 h) after isothiocyanate ingestion might be important for comparing urinary total isothiocyanate equivalents among individuals. Analysis of random spot urine samples might lead to incorrect conclusions about long-term dietary intake of isothiocyanates.

Cellular uptake of isothiocyanates is enhanced by GST isozymes (Zhang, 2001); however, export of intracellular isothiocyanate–GSH conjugates is also highly efficient without GST involvement (Zhang & Calloway, 2002). While GSTs are likely to be important in isothiocyanate metabolism, current understanding does not allow a prediction of the consequences of GST polymorphism on exposure of tissues or the rates and extent of urinary excretion of isothiocyanates and isothiocyanate conjugates.

Bioavailability of isothiocyanates from cruciferous vegetables

In a study designed to compare the bioavailability of isothiocyanate in fresh and steamed broccoli, 12 men were asked to eat 200 g of fresh or steamed broccoli, and urine samples were collected during the subsequent 24 h. The total content of isothiocyanate in fresh and steamed broccoli after treatment with myrosinase was virtually identical (1.1 and 1.0 µmol/g wet weight); however, the average 24-h urinary excretion of isothiocyanate equivalent was 32.3 ± 12.7% and 10.2 ± 5.9% of the amount ingested in fresh and steamed broccoli, respectively. Thus, the bioavailability of isothiocyanates from fresh broccoli was approximately three times greater than that from steamed broccoli, in which myrosinase was inactivated by heat (Conaway *et al.*, 2000). In another study (Getahun & Chung, 1999), 350 g of watercress (containing 475 µmol total glucosinolates) was cooked in boiling water for 3 min to inactivate myrosinase and then eaten by nine volunteers. The 24-h urine samples showed a total urinary excretion of isothiocyanate equivalent ranging from 5.6 to 34.8 µmol, corresponding to 1.2–7.3% of the total glucosinolates ingested. In contrast, ingestion of

150 g of uncooked watercress resulted in excretion of 17.2–77.7% of the total ingested glucosinolates in 24-h urine. Clearly, myrosinase in the original vegetable contributed to the release of isothiocyanates from glucosinolates. It can also be inferred from these results that human myrosinase, known to exist in the intestinal flora (Shapiro *et al.*, 1998; Getahun & Chung, 1999; Krul *et al.*, 2002), might hydrolyse only a fraction of the glucosinolates ingested and might also vary widely in activity among individuals. Nevertheless, when enteric flora were reduced by a combination of mechanical cleansing and oral antibiotics after ingestion of 100 μmol of a broccoli glucosinolate preparation in which myrosinase had been inactivated, a dramatic reduction was seen in 72-h urinary excretion of isothiocyanate metabolites, falling from 11.3 ± 3.1% of the dose before treatment to 1.3 ± 1.3% after treatment (Shapiro *et al.*, 1998). Not surprisingly, the extent of glucosinolate conversion to isothiocyanates by the myrosinase in vegetables is also affected by the length of time the vegetable is chewed. In a study in which four volunteers were each given 12 g of fresh broccoli sprouts containing 109 μmol total glucosinolates and were asked to either swallow without chewing or chew thoroughly before swallowing, thorough chewing resulted in significantly greater excretion of isothiocyanate equivalent (42.4 ± 7.5 μmol and 28.8 ± 2.6 μmol, respectively) in 24-h urine (Shapiro *et al.*, 2001).

Assessing consumption of cruciferous vegetables by measuring isothiocyanate equivalents in urine

Controlled metabolic studies have shown that urinary isothiocyanate equivalent assayed by the cyclocondensation reaction reflects the amount of cruciferous vegetables eaten. Shapiro and colleagues (1998) found that the disposition of ingested isothiocyanates was consistent between volunteers in different studies and that the urinary concentration of dithiocarbamates was proportional to an escalating dose regimen ($r^2 = 0.976$). The urinary concentrations peaked within < 8 h of consumption, but complete excretion took 24–72 h. In studies in which volunteers were given glucosinolates and isothiocyanates from broccoli sprouts, measurements of urinary isothiocyanate equivalent with this assay were highly reproducible (coefficient of variation, ≤ 10%), and their concentrations accurately reflected the absorption, metabolism and excretion of the isothiocyanate consumed. A strict linear relationship was observed between isothiocyanate dose and isothiocyanate equivalent in 72-h urine over an eightfold range of isothiocyanate dose (25–200 μmol) (Shapiro *et al.*, 2001), with no evidence of a threshold. Fowke and colleagues (2001) reported that an acceptable dose–response relationship between intake of cruciferous vegetables and urinary dithiocarbamate was found only at intake levels of 100–200 g/day. The intake levels were achieved in a planned dietary intervention and were much higher than the 0.2 serving per day (one serving = one cup of raw leafy or one-half cup of cooked or chopped raw) estimated to be the average intake of cruciferous vegetables by residents of the USA in 1994–96 (Johnston *et al.*, 2000).

In an Asian population (n = 246) who frequently ate cruciferous vegetables (mean daily intake of cooked, 40.6 g; mean daily isothiocyanate intake, 9.1 μmol), concentrations of total isothiocyanate in spot urine samples were significantly associated with cruciferous vegetable intake and isothiocyanate intake, estimated from a semiquantitative food-frequency questionnaire (Seow *et al.*, 1998). In 34 postmenopausal women who provided 24-h dietary recalls, responded to a questionnaire on vegetable and fruit intake and collected a 24-h urine sample (Fowke *et al.*, 2001), the urinary isothiocyanate correlated well with the 24-h consumption of cruciferous vegetables (Pearson correlation coefficient, 0.57; $p < 0.01$) and with an 'unknown but true intake' calculated from a structural equation (Ocké & Kaaks, 1997). Single-void urine samples are clearly less reliable markers than urine collected over 8 or 24 h (Seow *et al.*, 1998; London *et al.*, 2000). The former are useful mainly for assigning participants to reasonable categories of intake (e.g. negative, positive, low, high), while the latter, especially repeated 8- or 24-h urine collection, allow realistic assessments of consumption of cruciferous vegetables or isothiocyanates.

Indoles

Metabolism and disposition

The mildly acid environment of the stomach induces chemical modification of indole-3-carbinol, which is dehydrated in acidic solutions and is converted to its active derivates (Grose & Bjeldanes, 1992). Thus, indole-3-carbinol administered intraperitoneally does not exert metabolic change in the host (Bradfield & Bjeldanes, 1987a). The main products of this chemical reaction are 3,3′-diindolylmethane, triindolylmethane and indolo[3,2-*b*]carbazole (Figure 12). Many other indole-3-carbinol-derived compounds were detected in experimental animals exposed to this compound (Stresser *et al.*, 1995a; Anderton *et al.*, 2003).

Most of the information derives from studies in vitro in which condensation products were collected without ascorbic acid. When glucobrassicin and neoglucobrassicin are degraded in the presence of ascorbic acid, various ascorbigens are formed. It has been estimated that 20–60% of indolyl glucosinolates are converted to

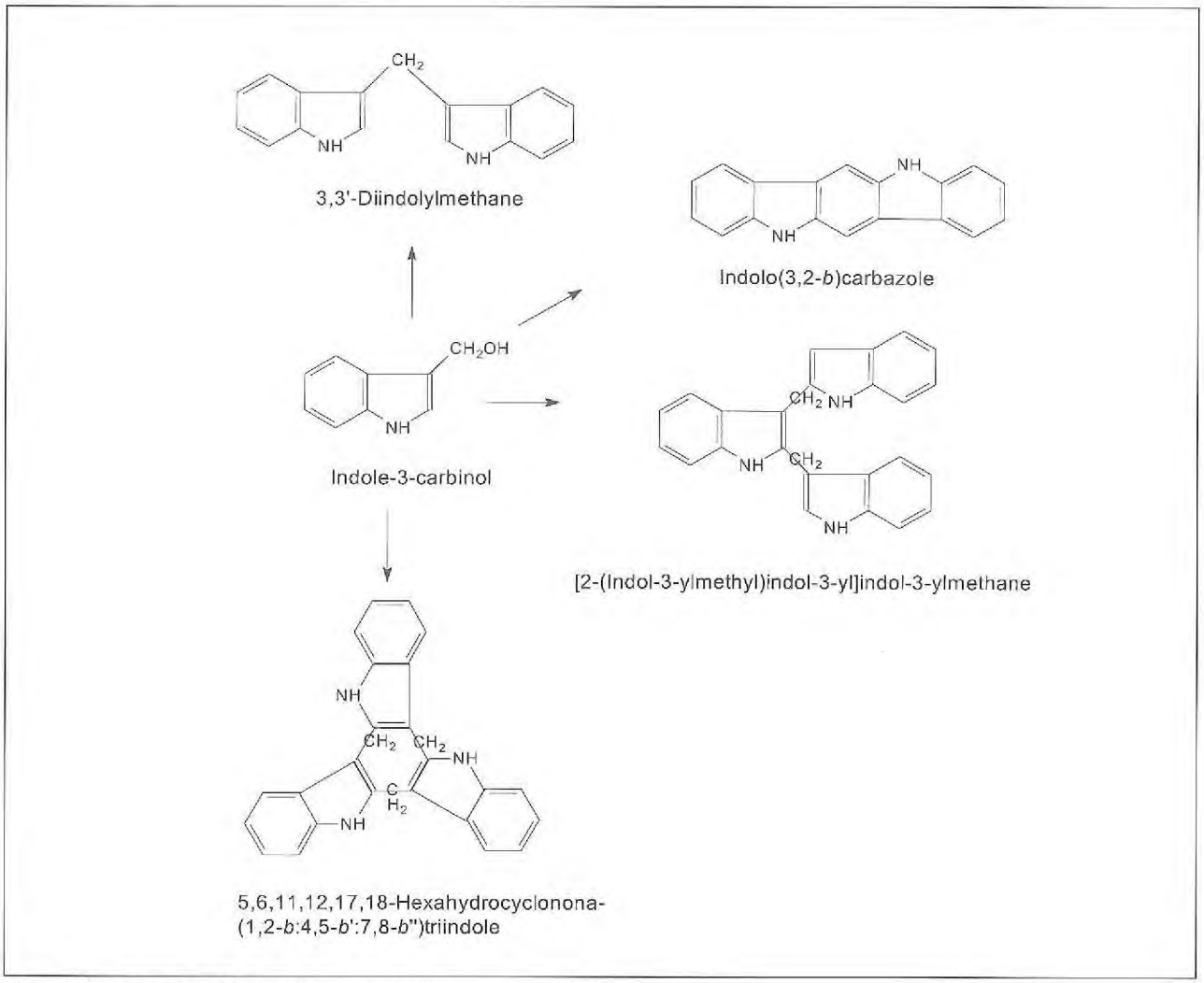

Figure 12 Indole-3-carbinol and its by-products

ascorbigens, depending on the pH (Buskov *et al.*, 2000; Hrncirik *et al.*, 2001).

Ascorbigen is unstable in acidic media (as in the stomach), giving rise to indolo[3,2-*b*]carbazole. About 20 times more indolo[3,2-b]carbazole was formed in vitro after incubation with ascorbigen than after incubation with indole-3-carbinol under identical conditions (Preobrazhenskaya *et al.*, 1993a).

3,3´-Diindolylmethane is the most common of derivative of indole-3-carbinol, and it is the most stable, with the longest biological life (Broadbent & Broadbent, 1998a,b). pH affects the oligomerization process: only 5% of indole-3-carbinol was converted to 3,3´-diindolylmethane at neutral pH (Amat-Guerri *et al.*, 1984), but 80% was transformed at pH 4.5 (de Kruif *et al.*, 1991). As the pH falls, the production of indolo[3,2-*b*]carbazole and larger oligomers increases over that of 3,3´-diindolylmethane. Dilution of indole-3-carbinol favours the formation of higher-order oligomers (Grose & Bjeldanes, 1992). In vitro at physiological pH, only 3,3´-diindolylmethane was detected after 48 h (Niwa *et al.*, 1994). In contrast, Staub *et al.* (2002) found that indole-3-carbinol was stable in cell-free medium or in cultured MCF-7 cells, with a half-life of about 40 h.

3,3´-Diindolylmethane can be extracted from the urine of volunteers given indole-3-carbinol. This assay has been used to estimate indole-3-carbinol intake, in order to correlate it

with the severity of cervical dysplasia. In one volunteer given 150 mg of the indole, from whom urine was collected over the next 18 h, peak urinary excretion was observed after 7 h. In the same study, 10 women were treated with indole-3-carbinol for 4 weeks. In five treated with 200 mg/day, the mean urinary concentration of 3,3′-diindolylmethane was 12.1 ± 2.5 µg/mg creatinine; in the remaining five women, treated with 400 mg/day, the concentration was 15.6 ± 22.2 µg/mg creatinine (Sepkovic *et al.*, 2001). 3,3′-Diindolylmethane was also measured in plasma and blood of four women treated with 400 mg of indole-3-carbinol orally. The concentrations in plasma and serum were 0.1–0.4 µg/ml. No indole-3-carbinol was recovered in blood, confirming that it undergoes oligomerization in the stomach (Arneson *et al.*, 1999).

When indolo[3,2-*b*]carbazole was measured in two samples of faeces of volunteers eating controlled diets, both samples showed a chromatographic peak in the range 2–20 µg/kg (dry weight, w/w) (Kwon *et al.*, 1994).

The presence of other metabolites in human blood, urine or faeces has not been tested.

Induction and inhibition of metabolizing enzymes

Indoles modulate the activity of genes involved in the metabolism of both endogenous and exogenous compounds (Nho & Jeffery, 2001). Differences in dietary habits, in conjunction with metabolic gene polymorphisms, may partially explain ethnic differences in the ratio of 2-hydroxy-: 16-hydroxyestradiol in women's urine (Taioli *et al.*, 1996).

The metabolism of tobacco can also be influenced by dietary indole intake (Morse *et al.*, 1990a; Taioli *et al.*, 1997). Thirteeen healthy smokers treated with 400 mg of indole-3-carbinol for 5 days showed a change in the urinary levels of two metabolites of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK): 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol and its glucuronide. These changes suggest that indole-3-carbinol increases NNK metabolism in smokers. It may also affect the metabolism of several other substances, including aflatoxins, alcohol and certain drugs (Fong *et al.*, 1990; Chung *et al.*, 1993; Manson *et al.*, 1997; Oganesian *et al.*, 1999).

In a study of structure–activity relationships of 3,3′-diindolylmethane analogues in human cells, substitutions at the 5 and 5′ positions induced the activity of both aromatase and ethoxyresorufin *O*-deethylase (EROD) activity, whereas analogues with a substitution in the bridging methylene carbon induced neither enzyme (Sanderson *et al.*, 2001)

Dimethyl 3,3′-diindolylmethane and tetramethyl 3,3′-diindolylmethane derivatives did not induce bacterial chloramphenicol acetyltransferase (CAT) reporter gene activity in T47D cell lines, and no aryl hydrocarbon (Ah) receptor antagonist activity was observed; however, no comparison was made with the induction ability of 3,3′-diindolylmethane under these conditions (McDougal *et al.*, 2001).

Experimental studies

Cruciferous vegetables

Metabolism and disposition

Few studies have been reported on the metabolism of isothiocyanates and indoles in experimental animals fed cruciferous vegetables.

Modulation of phase I and II enzymes[1]

The effects of cruciferous vegetables on phase I and II enzymes have been studied extensively (see also the section on intermediary biomarkers). Some of the studies addressed the effects of the vegetables themselves, while others focused on the effects of specific compounds in vegetables (e.g., glucosinolates, precursors of isothiocyanates and indoles). The vegetables studied were usually Brussels sprouts, cabbage, broccoli and cauliflower. The diets were often prepared by mixing lyophilized vegetables into the regular diet at concentrations of 2.5% up to 50% (w/w). In some studies, vegetables extracts were mixed into the regular diet after evaporation of the solvent, which was usually water, methanol or ethanol. Regardless of the study protocol and diet preparation, most of these studies showed that feeding cruciferous vegetables to animals generally induces phases I and II enzymes in the liver and intestinal tract.

Many biologically active compounds in cruciferous vegetables are likely to contribute to the effects on phase I and II activities, and the increased enzyme activities reflect a combined effect of these active compounds. Isothiocyanates and indoles are likely to have opposite effects on phase I enzymes, as indoles are strong inducers of cytochrome P450 enzymes, whereas isothiocyanates usually inhibit these enzymes (see below). Indoles also inhibit cytochrome P450 activity, however (Stresser *et al.*, 1995b; Takahashi *et al.*, 1995a). The induction of phase II enzymes such as GST by cruciferous vegetables is probably due to the concerted action of

[1]Usually, xenobiotic metabolizing enzymes are subdivided by toxicologists into phase I and phase II enzymes on the basis of whether they catalyse oxidation–reduction or conjugation reactions. Thus, NAD(P)H:quinone oxidoreductase 1 is sometimes classified as a phase I enzyme. In this Handbook, however, it is referred to as a phase II enzyme becuase it is coordinately regulated with xenobiotic conjugating enzymes through an antioxidant response element.

isothiocyanates and indoles. In all these studies, the contribution of isothiocyanates was difficult to assess quantitatively because of the presence of other active compounds in the vegetables (Loub *et al.*, 1975; McDanell *et al.*, 1989). Nevertheless, there is little doubt that the observed effects are due in part to isothiocyanates, as shown in studies with isothiocyanate as the only agent (see below).

Some reports have emphasized both the complexity of the phase I response to *Brassica* vegetables in rat models and the fact that it depends strongly on differences in the chemical composition of vegetables. Vang *et al.* (1991) studied the effects of broccoli on CYP1A1 and CYPIIB mRNA and proteins in rats fed freeze-dried vegetables for 7 days. In the colon, both CYP1A1 mRNA and protein were induced by the broccoli diet. CYPIA2 protein was present in the colon, but it was not altered by the diet, and the mRNA was not detectable. Paradoxically, CYPIIB mRNA was reduced, but the protein was increased. In the liver, CYPIIB and CYPIIE1 proteins were increased by the broccoli diet, but CYPIIB mRNA was not affected. In a later study in which different varieties of broccoli were fed to rats, it was also shown that modulation of cytochrome P450 and other phase I enzymes is critically dependent on the concentrations of glucosinolates and other biologically active phytochemicals in plant tissue (Vang *et al.*, 2001). Various strategies have been used to overcome the problems due to the complexity of *Brassica* vegetables. Sorensen *et al.* (2001) fed rats a Brussels sprout extract containing a complex, incompletely characterized mixture of glucosinolates and their breakdown products for 4 days. No significant modulation of phase I enzymes but significant up-regulation of phase II enzymes was observed.

Bradfield and Bjeldanes (1984) fed rats a diet containing 25% (w/w) Brussels sprouts for 10 days and compared the effects on intestinal and hepatic GST activity with those of a diet containing indole-3-carbinol at 50–500 mg/kg. The Brussels sprouts diet increased GST activity by 1.9-fold in the gut and by 1.6-fold in the liver, but neither activity was significantly affected by purified indole-3-carbinol, even at the highest dose. The diet containing sprouts significantly increased the activities of Ah hydroxylase (by 3.6-fold) and ethoxycoumarin *O*-deethylase (by 3.2-fold). The same group (Bradfield *et al.*, 1985) included hepatic GST in a study of the effects of 12 vegetables on phase I and phase II activity in mice. Diets containing 20% freeze-dried, powdered Brussels sprouts or cauliflower significantly increased the activities of GST (by 2.0- and 1.2-fold, respectively) and epoxide hydratase (both by 1.6-fold): the activity of ethoxycoumarin *O*-deethylase was increased significantly (by 2.2-fold) by cauliflower but that of Ah hydroxylase was not affected. The effects on enzyme activities were not confined to *Brassica* vegetables, although the high doses used make these results difficult to interpret.

Bogaards *et al.* (1990) studied the effects of dietary supplements of Brussels sprouts (2.5–30%), the sinigrin breakdown product allyl-ITC (0.03 and 0.1%) and goitrin (0.02%) on the GST subunit pattern in the liver and small intestinal mucosa of male rats. A statistically significant linear relationship was found between the amount of Brussels sprouts in the diet and induction of GST, with similar increases in the total amounts of GST subunits. When the average concentrations of sinigrin and progoitrin in the sprouts were 1835 µmol/kg and 415 µmol/kg, respectively, the subunit induction patterns in the liver and the small intestinal mucosa were similar to those observed after feeding allyl-ITC, which caused stronger enhancement of subunit 2. When the average sinigrin concentration in the sprouts was as low as that of progoitrin (about 540 µmol/kg), however, a goitrin-like induction pattern was observed. The authors concluded that at least two compounds (probably allyl-ITC and goitrin) are responsible for the induction of GSTs in rat liver and small intestine by Brussels sprouts.

Thus, much of the induction of phase I and phase II enzymes by vegetables may be due to indoles. The vegetables studied, Brussels sprouts, cabbage, broccoli and cauliflower, have high concentrations of indole glucosinolates (glucobrassicin) and relatively little isothiocyanate glucosinolates (van Etten & Tookey, 1979; Fenwick & Heaney, 1983). Isothiocyanate-rich vegetables with a small amount of indoles might reduce phase I enzyme activity, as was observed in studies with pure isothiocyanate compounds (see below). The preparation of the vegetable diets for this type of experiment is an important consideration, as it might considerably alter the bioavailability of the hydrolysed products. The free isothiocyanates in freeze-dried broccoli samples are less bioavailable, as estimated from the mercapturic metabolite excreted in urine, and less effective in inducing quinone reductase activity in colon than freeze-dried broccoli samples containing intact glucosinolates (Keck *et al.*, 2003). Equally important is the method of storage, which could have significant effects on the stability of compounds such as isothiocyanates.

A question of practical importance is whether cooked vegetables affect phase I and II enzymes. Cooked vegetables are devoid of myrosinase, the enzyme responsible for hydrolysing glucosinolates to release bioactive aglucones. In a study in which Wistar rats were fed a diet supplemented with

cooked Brussels sprouts at 2.5–20% (w/w), various phase I and II enzymes were measured, including CYP1A1, CYP1A2 and CYP2B, GST, UDP-glucuronosyl transferases (UGTs) and NAD(P)H–quinone reductase (Wortelboer *et al.*, 1992a). Almost all the enzymes were induced by the diet containing Brussels sprouts, and effects were seen as soon as 2 days after feeding. Although no direct comparison was made of the effects on these enzymes of cooked and uncooked vegetables, they appeared to have similar effects. This suggests that glucosinolates are hydrolysed to aglycones after ingestion of Brussels sprouts by a myrosinase-like microfloral activity in the rat intestinal tract. In a study of the influence of the intestinal microflora and dietary glucosinolates on hepatic cytochrome P450 enzymes (Nugon-Baudon *et al.*, 1998), conventional rats and germ-free rats were fed a diet containing myrosinase-free rapeseed. While the effects of the glucosinolate-rich diet on these enzymes appeared to be complex, the rapeseed meal decreased total cytochrome P450 activity in conventional rats but not in germ-free rats, suggesting that the microflora present in the gut play a role.

The ability of nitriles to induce phase II enzymes was investigated in two studies. In a comparison of the effects of the major broccoli isothiocyanates and nitrile, groups of five 4-week-old male Fischer 344 rats were given 5-(methylsulfinyl)pentane nitrile in saline at 200, 500 or 1000 µmol/kg bw, sulforaphane in saline at 500 µmol/kg bw or saline, by gavage for 5 days. Controls and test animals given nitrile were pair-fed with semi-synthetic food throughout the study. The animals were killed 24 h after the last dose, and the activities of quinone reductase and GST were determined in cytosolic fractions from the liver, pancreas and colon mucosa. The nitrile had no effect, while sulforaphane statistically significantly reduced both enzyme activities (Matusheski & Jeffery, 2001). In a subsequent study, groups of three male Fischer 344 rats [age not specified] were given the rapide seed nitrile crambene or sulforaphane at a dose of 50 mg/kg bw by gavage in corn oil for 7 days (515 µmol/kg bw for crambene, 282 µmol/kg bw for sulforaphane) (Keck *et al.*, 2002). Controls were given corn oil only. Hepatic cytosolic quinone reductase activity was induced 1.5-fold by crambene and 1.7-fold by sulforaphane.

Isothiocyanates

Metabolism and disposition

The metabolism and tissue disposition of several isothiocyanates have been investigated extensively in rodents. These studies showed that the mercapturic acid pathway is the main route of metabolism of isothiocyanates, involving initial conjugation with GSH mediated by GSTs, followed by enzymatic degradation to its *N*-acetylcysteine conjugate (Meyer *et al.*, 1995; Whalen & Boyer, 1998). Other, minor metabolites of isothiocyanates with an alkyl or aryl moiety have also been identified in animals. This section summarizes the data from these studies (Table 8).

Allyl isothiocyanate

In Wistar rats, the main urinary metabolite of allyl-ITC was the mercapturic acid *N*-acetyl-*S*-(*N*-allylthiocarbamoyl)-L-cysteine (allyl-ITC–*N*-acetylcysteine) (Mennicke *et al.*, 1983). Studies of pharmacokinetics and metabolism were conducted after oral administration of [^{14}C]allyl-ITC labelled in the isothiocyanate moiety to Fischer 344 rats and B6C3F$_1$ mice (Bollard *et al.*, 1997). Within 96 h, male and female mice had excreted ~80% of the ^{14}C in urine, and the rats had excreted 50–55%. The rats had retained 20–25% of the dose in the carcass after 96 h. Faecal ^{14}C accounted for 6–12% and expired CO^2 for 4–7% of the dose. In this study, 67–85% of the ^{14}C in rat urine samples was identified as allyl-ITC–*N*-acetylcysteine by HPLC and positive-ion electrospray mass spectroscopy. Three metabolites were found in mice: 48–85% of the urinary ^{14}C was on thiocyanate, 7–52% on allyl-ITC–cysteine (Cys) conjugate and 8–12% on allyl-ITC–*N*-acetylcysteine (males only). In B6C3F$_1$ mice, allyl-ITC–*N*-acetylcysteine represented less than 20% of total urinary radioactivity; three other major and two minor unidentified urinary metabolites were also detected (Ioannou *et al.*, 1984). In both rats and mice, 70–85% of the administered dose was collected in the urine by 72 h, while 13–15% was trapped as CO_2 (in rats only), and 3–6% of the ^{14}C, consisting of a single unidentified metabolite, was found in faeces. The urinary bladders of animals given [^{14}C]allyl-ITC showed sex and species differences, more ^{14}C being found in the bladder tissue of male rats. The amount of ^{14}C detected in bile from cannulated rats was greater than that in faeces, indicating possible enterohepatic circulation of metabolites and eventual urinary excretion (Borghoff & Birnbaum, 1986; Bollard *et al.*, 1997). Biliary metabolites of allyl-ITC from Fischer 344 rats given [U-^{14}C]allyl-ITC by gavage were similar to urinary metabolites, as identified by HPLC, but the relative proportions differed considerably (Ioannou *et al.*, 1984).

Benzyl isothiocyanate

Brüsewitz *et al.* (1977) identified the mercapturic acid of benzyl-ITC (benzyl-ITC–*N*-acetylcysteine) as its dicyclohexylamine salt after administration of benzyl-ITC and various thiol conjugates orally or by intraperitoneal or intravenous injection to rats. The

Table 8. Urinary metabolites in experimental animals after administration of isothiocyanates (ITCs)

Isothiocyanate	Route	Main metabolite(s)	Administered dose recovered (%)	Strain and species	Reference
Allyl-ITC	Oral	Allyl-ITC–NAC	40–50	Wistar rat	Mennicke *et al.* (1983)
		Allyl-ITC–NAC	75–82	Fischer 344 rat	Ioannou *et al.* (1984)
		Allyl-ITC–NAC	8–20	B6C3F$_1$ mouse	Ioannou *et al.* (1984)
		Allyl-ITC–NAC	67–85	Fischer 344 rat	Bollard *et al.* (1997)
		Allyl-ITC–NAC	8–12	B6C3F$_1$ mouse	Bollard *et al.* (1997)
		Allyl-ITC–Cys	7–52	B6C3F$_1$ mouse	Bollard *et al.* (1997)
		–SCN–	15–33	Fischer 344 rat	Bollard *et al.* (1997)
		–SCN–	48–85	B6C3F$_1$ mouse	Bollard *et al.* (1997)
		Allyl-ITC	0.3–0.4	Fischer 344 rat	Ioannou *et al.* (1984)
		Allyl-ITC	1.9	B6C3F$_1$ mouse	Ioannou *et al.* (1984)
Butyl-ITC	Oral	Butyl-ITC–NAC	10–20	Wistar rat	Mennicke *et al.* (1983)
Benzyl-ITC	Oral	Benzyl-ITC–NAC	Only metabolite	Wistar rat	Brüsewitz *et al.* (1977)
		Benzyl-ITC–NAC	Trace	Guinea-pig	Görler *et al.* (1982)
		4H4CBTT	23 ± 3	Guinea-pig	Görler *et al.* (1982)
Benzyl-ITC–Cys	Oral	Benzyl-ITC–NAC	62	Wistar rat	Brüsewitz *et al.* (1977)
		Benzyl-ITC	< 1	Wistar rat	Brüsewitz *et al.* (1977)
		Benzyl-ITC–Cys	< 1	Wistar rat	Brüsewitz *et al.* (1977)
		Hippuric acid	40	Beagle dog	Brüsewitz *et al.* (1977)
		4H4CBTT	33 ± 4	Guinea-pig	Görler *et al.* (1982)
Benzyl-ITC–NAC	Oral	Benzyl-ITC–NAC	Trace	Guinea-pig	Görler *et al.* (1982)
		4H4CBTT	4–9	Guinea-pig	Görler *et al.* (1982)
Phenethyl-ITC	Oral	Phenethyl-ITC–NAC	9–10	A/J mouse	Eklind *et al.* (1990)
		4H4CPETT	25	A/J mouse	Eklind *et al.* (1990)
		Phenethyl-ITC–NAC	> 90	Fischer 344 rat	Conaway *et al.* (1999)
		Phenethyl-ITC	< 1	Fischer 344 rat	Conaway *et al.* (1999)
Gluconasturtiin	Diet	Phenethyl-ITC–NAC	22± 8	A/J mouse	Chung *et al.* (1992)
6-Phenylhexyl-ITC	Oral	Unidentified	7 ± 1	Fischer 344 rat	Conaway *et al.* (1999)
		6-Phenylhexyl-ITC–NAC	Trace[a]	Fischer 344 rat	Conaway *et al.* (1999)
Sulforaphane	Intraperitoneal	Sulforaphane–NAC	~ 60	Sprague-Dawley rat	Kassahun *et al.* (1997)
		Erucin–NAC	~ 12	Sprague-Dawley rat	Kassahun *et al.* (1997)
Erucin	Intraperitoneal	Sulforaphane–NAC	~ 67	Sprague-Dawley rat	Kassahun *et al.* (1997)
		Erucin–NAC	~ 29	Sprague-Dawley rat	Kassahun *et al.* (1997)

NAC, *N*-acetylcysteine; Cys, cysteine; 4H4CBTT, 4-hydroxy-4-carboxy-3-benzylthiazolidine-2-thione; 4H4CPETT, 4-hydroxy-4-carboxy-3-phenylethylthiazolidine-2-thione

[a] Most metabolites found in faeces

thiol conjugates studied were GSH (benzyl-ITC–GSH), cysteinylglycine (benzyl-ITC–Cys–Gly) and cysteine (benzyl-ITC–Cys). The tissue disposition of benzyl-ITC was studied with ^{14}C-labelled compound synthesized by radiolabelling the carbon adjacent to the isothiocyanate group. When [^{14}C]benzyl-ITC–Cys was administered orally to fasted male and female rats, the mean peak plasma concentration of ^{14}C occurred

within 45 min. The plasma radioactivity declined rapidly, with a half-time of 1–2 h. A total of 92.4% of the ^{14}C was collected in urine, while 5.6% appeared in faeces, and 0.4% was detected in expired air over 3 days. Metabolites in urine were analysed by mass spectrometry after isolation by thin-layer chromatography, and most of the recovered ^{14}C in the urine was identified as the mercapturic acid (see Figure 13A). Free [^{14}C]benzyl-ITC or [^{14}C]benzyl-ITC–Cys accoun-ted for less than 1% of the administered dose in urine. No other metabolite was identified in rat urine after oral administration of benzyl-ITC or its thiol conjugates. Small amounts of free benzyl-ITC were recovered in urine after administration of the conjugates by the other routes, these presumably having been formed by dissociation of the conjugates in vivo. When the [^{35}S]Cys conjugate of benzyl-ITC was administered orally to the rats or unlabelled benzyl-ITC–Cys was given to rats pretreated with [^{35}S]Cys to label the body sulfur pool, [^{35}S]mercapturic acid was recovered in the urine. Excretion of benzyl-ITC–[^{35}S]*N*-acetylcysteine after dosing with benzyl-ITC–[^{35}S]Cys indicates that the conjugate was at least partially absorbed unchanged, subsequently acetylated and then excreted. The excretion of benzyl-ITC–[^{35}S]*N*-acetylcysteine after administration of unlabelled benzyl-ITC–Cys and –[^{35}S]Cys suggests that the benzyl-ITC–Cys was deconjugated and then reconjugated with [^{35}S]Cys or GSH.

The metabolism of benzyl-ITC in other species differs from that in the rat. When male guinea-pigs were given a single dose of benzyl-ITC or benzyl-ITC–Cys by gavage, 23.3 ± 2.8% and 33.1 ± 4.0 %, respectively, of the dose excreted in 24-h urine was identified as the cyclic 4-hydroxy-4-carboxy-3-benzylthiazolidine-2-thione, a mercaptopyruvic acid conjugate of benzyl-ITC (Görler *et al.*, 1982; see Figure 13B). The mechanism for its formation is thought to involve the cysteine conjugate, which is transaminated to the S-substituted mercaptopyruvate, followed by enolysis and cyclization to form the cyclic metabolite instead of the expected *N*-acetylated product. Benzyl-ITC–*N*-acetylcysteine was, however, also identified as a minor metabolite. Similar results were observed after administration of benzyl-ITC or benzyl-ITC–Cys to rabbits. Thus, guinea-pigs and rabbits excrete little mercapturic acid after isothiocyanate metabolism and apparently do not readily form mercapturic acids after receiving other types of xenobiotic.

In beagle dogs given a single dose of [^{14}C]benzyl-ITC–Cys by gavage, absorption occurred more slowly than in rats, with a peak mean plasma concentration of ^{14}C within 1.5–6 h (Brüsewitz *et al.*, 1977). After 3 days, 86.3% of the dose had been excreted in urine and 13.2% in faeces. Rather than the mercapturic acid metabolite, [^{14}C]hippuric acid (40% of the dose) was identified (see Figure 13C). Moreover, when benzyl-ITC–[^{35}S]Cys was administered, the radiolabelled metabolite was not recovered in urine, indicating that the conjugate had lost the cysteine moiety by dissociation. Components in dog urine with retention times on thin-layer chromatography corresponding to the unchanged cysteine conjugate, free benzyl-ITC or other metabolites represented less than 5% of the administered dose. The fate of the free [^{35}S]Cys formed was not reported.

In summary, rats metabolized benzyl-ITC via the mercapturic acid pathway, and the major urinary metabolite formed was benzyl-ITC–*N*-acetylcysteine. Guinea-pigs and rabbits excreted primarily the cyclic mercaptopyruvate conjugate 4-hydroxy-4-car-

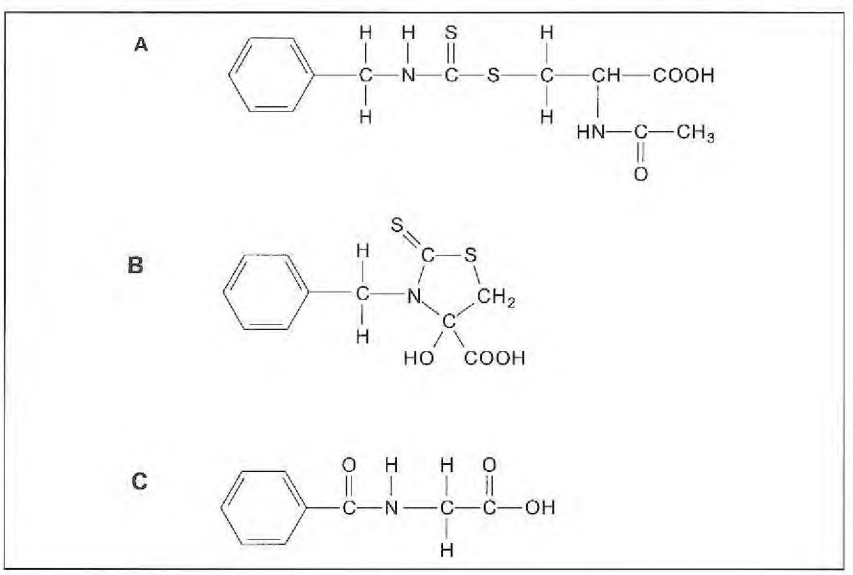

Figure 13 Variations in urinary metabolites of benzyl-isothiocyanate (ITC) and benzyl-ITC–cysteine
A) *N*-Acetyl-*S*-(*N*-benzylthiocarbamoyl)-L-cysteine (benzyl-ITC–*N*-acetylcysteine), the mercapturic acid of benzyl-ITC
B) 4-Hydroxy-4-carboxy-3-benzothiazolidine-2-thione, major benzyl-ITC–cysteine metabolite in urine of guinea-pigs and rabbits (Görler *et al.*, 1982)
C) Hippuric acid, major metabolite in urine of beagle dogs (Brüsewitz *et al.*, 1977)

boxy-3-benzythiozolidine-2-thione. In contrast, dogs excreted the glycine conjugate, hippuric acid, possibly because they readily hydroxylate the benzylic moiety of benzyl-ITC, which is subsequently oxidized to benzoic acid and conjugated.

Phenethyl isothiocyanate

The metabolism and tissue disposition of phenethyl-ITC was first investigated in A/J mice, a model of lung carcinogenesis (Shimkin & Stoner, 1975) widely used used to study the efficacy and mechanisms of action of chemopreventive agents (Eklind *et al.*, 1990; Chung, 2001). When [^{14}C]phenethyl-ITC, synthesized from 2-phenyl[1-^{14}C]ethylamine hydrochloride, was administered by gavage (Eklind *et al.*, 1990), 55.2% of the dose was excreted in urine within 72 h, and 23.3% appeared in faeces. Two major urinary metabolites were isolated in urine and identified by ^{1}H- and ^{13}C-nuclear magnetic resonance spectrometry and by HPLC co-chromatography with ultraviolet standards, as the cyclic mercaptopyruvic acid conjugate 4-hydroxy-4-carboxy-3-phenethylthiazolidine-2-thione (25% of the dose) and the mercapturic acid of phenethyl-ITC, *N*-acetyl-*S*-(*N*-phenethylthiocarbamoyl)-L-cysteine (phenethyl-ITC–*N*-acetylcysteine) (10% of dose) (see Figure 14). Radioactivity in major organs was determined up to 72 h after dosing: the maximum ^{14}C activity in liver and lung occurred within 2–8 h and 4–8 h, respectively. When gluconasturtiin, the glucosinolate precursor of phenethyl-ITC, was mixed into AIN-76 diet with myrosinase and fed to A/J mice, the same two metabolites were identified in urine, with only 22% recovery (Chung *et al.*, 1992). These results indicate that gluconasturtiin is hydrolysed to phenethyl-ITC endogenously, presumably mediated by micro-flora in the gut, as described above.

The pharmacokinetics and metabolism of [^{14}C]phenethyl-ITC were investigated in Fischer rats. After receiving 10 μmol in corn oil by gavage, three rats were killed at various times over 48 h, and ^{14}C was evaluated in whole blood and in 13 major organs at each time, while ^{14}C in expired CO_2, urine and faeces was assayed at 8, 24 and 48 h. The ^{14}C in whole blood peaked at 2.9 h, with two compartments (α and β) with half-times of 2.4 and 21.7 h. The maximum activity of ^{14}C appeared in the liver at 2.5 h, the lungs at 4.5 h and brain at 6.5 h. The time course of absorption and elimination in selected tissues is presented in Figure 15. By 48 h, 88.7% of the ^{14}C was detected in urine, 9.9% in faeces and 0.1% trapped as CO_2. More than 90% of the urinary ^{14}C was identified by HPLC as phenethyl-ITC–*N*-acetylcysteine on the basis of co-chromatography with authentic standards, but less than 1% occurred as phenethyl-ITC in the urine. Measurement of radioactivity in homogenates of liver and lung by HPLC showed the presence of an additional major unidentified metabolite (other than phenethyl-ITC–GSH an phenethyl-ITC–Cys). A considerable amount of the

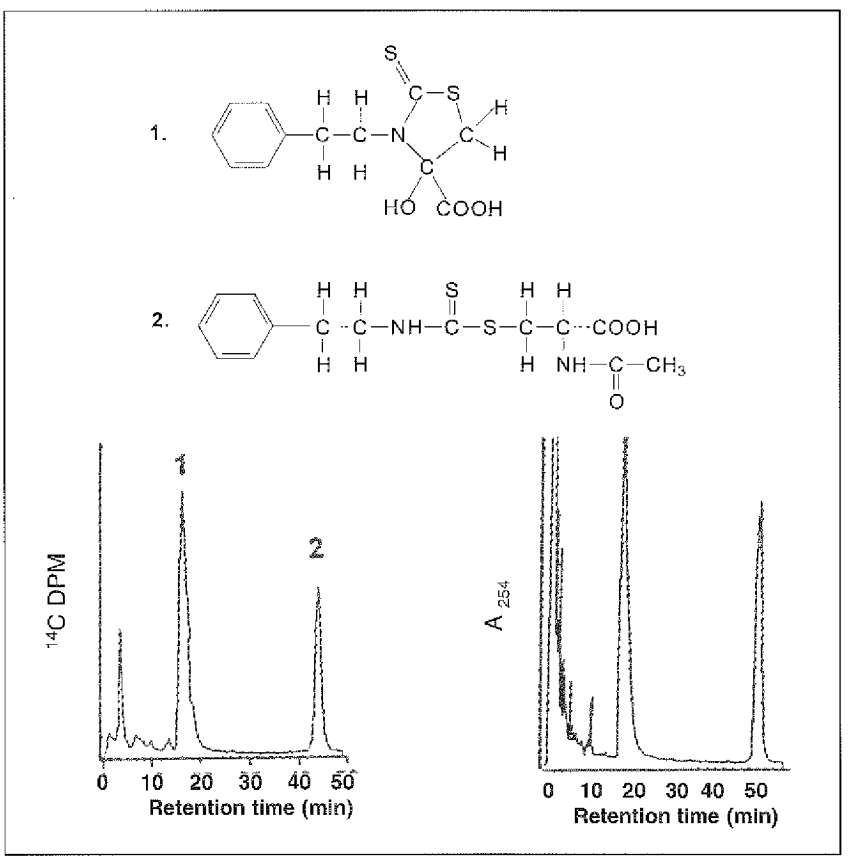

Figure 14 Detection of urinary metabolites of phenethyl-isothiocyanate (ITC) in *A/J* mice by high-performance liquid chromatography with radioflow and confirmation of the identities of metabolites by nuclear magnetic resonance and mass spectroscopy

1. 4-Hydroxy-4-carboxy-3-phenethylthiazolidine-2-thione, a cyclic mercaptopyruvic acid conjugate
2. *N*-Acetyl-*S*-(*N*-phenethylthiocarbamoyl)-L-cysteine (phenethyl-ITC–*N*-acetylcysteine, a mercapturic acid) (Eklind *et al.*, 1990)

radioactivity was not extractable, suggesting covalent binding with proteins. Significant amounts of [^{14}C]phenethyl-ITC occurred in ethyl acetate extracts of faeces, indicating incomplete absorption from the intestinal tract (Conaway *et al.*, 1999).

In beagle dogs given phenethyl-ITC, phenethylamine was identified in plasma by gas chromatography with mass spectrometry and was proposed to be a degradation product of phenethyl-ITC formed at the low pH of the stomach (Negrusz *et al.*, 1998). The urinary metabolites of phenethyl-ITC were not reported, however. Therefore, as in humans, phenethyl-ITC is metabolized mainly via the mercapturic acid pathway in rats and appears in the urine as phenethyl-ITC–*N*-acetylcysteine; other pathways appear to predominate in mice and dogs.

Sulforaphane

Only one large study has been reported on the metabolism of sulforaphane in rodents (Kassahun *et al.*, 1997). After intraperitoneal administration of sulforaphane to male Sprague-Dawley rats, its metabolites were studied in urine and bile. Those identified in 24-h urine were the *N*-acetylcysteine conjugates of sulforaphane and erucin, its reduction sulfur analogue, and accounted for ~60% and ~12% of the dose, respectively. When erucin was administered, the metabolites in 24-h urine consisted of ~67% sulforaphane–*N*-acetylcysteine and ~29% erucin–*N*-acetylcysteine, indicating that oxidative metabolism of erucin was favoured over reductive metabolism of sulforaphane. Sulforaphane appeared to be metabolized by the phase I reactions of S-oxide reduction and dehydrogenation; the mercapturic acid pathway via GSH conjugation was the main route by which sulforaphane and its phase I metabolite erucin were eliminated. In another group of animals, bile was collected 0–4 h after administration of sulforaphane or [1,1-$^{2}H_{2}$]sulforaphane. Biological fluids were analysed by liquid chromatogra-phy–mass spectroscopy. Five meta-bolites were detected in bile, two of which were identified as GSH conjugates of sulforaphane and erucin on the basis of synthesized standards. Two other metabolites were identified as the *N*-acetylcysteine conjugates of these compounds. A fifth biliary metabolite was tentatively identified as the GSH conjugate of a desaturated derivative of sulforaphane, Δ^{1}-sulforaphane, on the basis of studies with deuterated [1,1-$^{2}H_{2}$]sulforaphane.

Modulation of phase I and phase II enzymes

Although the focus of this section is studies in experimental animals in vivo, the following section also briefly discusses some in-vitro studies which should contribute to the interpretation of the in-vivo studies. Modulation of drug metabolizing enzymes by isothiocyanates has been investigated extensively to determine the mechanism of their chemopreventive activity in animal tumour bioassays (Zhang & Talalay, 1994; van Poppel *et al.*, 1999;

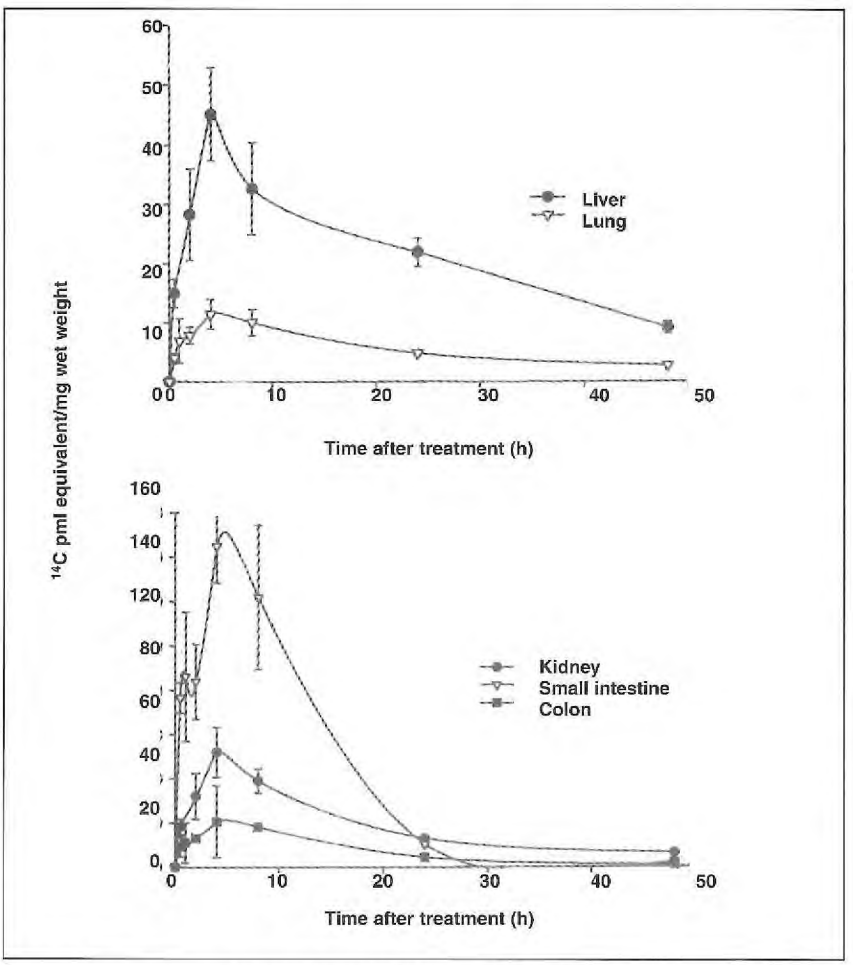

Figure 15 Time course of absorption and elimination of ^{14}C after oral administration of a single dose of 10 µmol of [^{14}C]phenethyl-isothiocyanate

Adapted from Conaway *et al.* (1999)

Hecht, 2000; Smith & Yang, 2000; Chung, 2001).

Phase I enzymes

Studies of structure–activity relationships in vivo and in vitro show that the length of the alkyl chain in arylalkyl isothiocyanates is critical for inhibition of cytochrome P450 enzymes, and increasing the alkyl chain length up to six carbons increases their chemopreventive efficacy (Morse *et al.*, 1991; Guo *et al.*, 1993; Jiao *et al.*, 1994; Conaway *et al.*, 1996). Considerable variability in the selectivity and potency of inhibition of cytochrome P450 enzymes by isothiocyanates has been found in vitro (Smith *et al.*, 1993; Conaway *et al.*, 1996; Jiao *et al.*, 1996; Smith *et al.*, 1996). Isothiocyanates are particularly strong inhibitors of CYP2B1, as determined by assays for pentoxyresorufin *O*-dealkylase (PROD). The inhibitory potency appeared to be positively correlated with the lipophilicity of the isothiocyanates. The most potent inhibitor of PROD was the synthetic 6-phenylhexyl-ITC. Morse *et al.* (1991) showed that it is also a remarkably effective inhibitor of lung tumorigenesis induced by NNK.

Oral administration of phenethyl-ITC to male Swiss-Webster mice inhibited the metabolism of acetaminophen by CYP2E1. Pretreatment with this isothiocyanate significantly reduced the amount of metabolites of acetaminophen in plasma and urine, prevented depletion of hepatic GSH and significantly reduced liver damage (Li *et al.*, 1997).

Typically, administration of isothiocyanates to mice shortly before treatment with a carcinogen inhibits its metabolic activation (Chung *et al.*, 1984a,b; Morse *et al.*, 1989a, 1991). Nevertheless, isothiocyanates given 24 h before sacrifice as a single dose or in the diet sometimes enhanced cytochrome P450 activity measured at sacrifice (Guo *et al.*, 1992, 1993; Smith *et al.*, 1996). For example, an oral dose of phenethyl-ITC to Fischer rats 24 h before death selectively inhibited CYP2E1-mediated *N*-nitrosodimethylamine demethylation by its demethylase, but the activity of PROD and the amount of CYP2B1 (measured by immunoblot analysis) were both markedly induced (Ishizaki *et al.*, 1990). These results illustrate a complex mechanism in which regulation of cytochrome P450 enzymes by isothiocyanates is highly specific. In contrast to Fischer rats, mice given phenethyl-ITC in the diet showed induction of CYP2E1 activity in liver microsomes by a factor of 1.2 and of CYP2E1 activity in lung microsomes by a factor of 1.6 (Smith *et al.*, 1993).

Phase II enzymes

Isothiocyanates induce phase II detoxification enzymes, including GSTs, quinone reductase, sulfatase and UGTs (Zhang & Talalay, 1994). GSTs catalyse the otherwise relatively slow conjugation reaction of GSH with electrophiles, including genotoxic chemicals and isothiocyanates. In vertebrates, GSTs are dimeric enzymes consisting of seven families: alpha, mu, kappa, pi, sigma, theta and zeta (Coles & Ketterer, 1990; Whalen & Boyer, 1998). Some isothiocyanates are readily conjugated by human GSTs M-1 (mu-1) and P1-1 (pi 1-1) but are slowly conjugated by other forms (Kolm *et al.*, 1995; Meyer *et al.*, 1995; Zhang *et al.*, 1995). The rates of catalytic conjugation of isothiocyanates by various forms of GST apparently depend on the structure of the isothiocyanates. It is conceivable that isothiocyanate, as an inducer of GST, facilitates its own excretion by increasing its rate of conjugation with GSH.

Phenethyl-ITC is a tissue-specific inducer of phase II enzymes. In rats, a single administration of phenethyl-ITC by gavage induced quinone reductase and GST activity by 5.0- and 1.5-fold, respectively, in the liver, but the activities of these enzymes in lung and nasal mucosa were not significantly affected (Guo *et al.*, 1992). Hepatic GST alpha and GST mu activity, protein levels and specific mRNAs were induced in a dose-dependent manner in rats given phenethyl-ITC orally for 3 days, and the hepatic GSH content increased twofold (Seo *et al.*, 2000). In another study, administration of a relatively high concentration of phenethyl-ITC in the diet (1000 mg/kg) for 2 weeks induced both phase I and phase II enzymes in Fischer rats (Manson *et al.*, 1997). The concentration of GST T1-1 in gastric mucosa was significantly increased after dietary administration of phenethyl-ITC at 450 mg/kg to male Wistar rats, but no such effect was observed in oesophagus, colon or liver (van Lieshout *et al.*, 1998a).

Benzyl-ITC induced GST activity in the small intestine and liver of female ICR/Ha mice (Sparnins *et al.*, 1982a). It was also the most potent component of papaya juice in inducing GST P1 in cultured rat liver epithelial cells. Subsequent investigations suggested that reactive oxygen intermediates are also involved (Nakamura *et al.*, 2000a,b).

Sulforaphane was an extremely potent inducer of phase II enzymes in primary cultures of rat and human hepatocytes and in murine hepatoma cells (Prochaska *et al.*, 1992; Zhang *et al.*, 1992b; Mahéo *et al.*, 1997). Related three- to eight-carbon (C3–C8) analogues of sulforaphane, the C3–C5 methyl sulfide isothiocyanates, C3–C5 methyl sulfone isothiocyanates and the methyl dithiocarbamyl analogue of sulforaphane, sulforamate, were also active inducers of a phase II detoxification enzyme (quinone reductase) in murine hepatoma cells (Zhang *et al.*, 1992b; Gerhäuser *et al.*, 1997; Rose *et al.*, 2000). Northern blotting analysis of rat hepatocytes after treatment with sulforaphane showed dose-dependent induction of the mRNA of GST

A1/A2 and P1, but not M1 (Mahéo *et al.*, 1997).

In order to understand better the relationship between structure and inducer activity, the effects of modifying the structural features of sulforaphane (including its chirality, oxidation state of the methylthiol moiety and the number and rigidity of methylene bridging units) were evaluated, with quinone reductase as the marker of induction of phase II enzymes in murine hepatoma Hepa 1c1c7 cells (Zhang *et al.*, 1992b; Posner *et al.*, 1994). Sulforaphane is chiral, the natural compound having the *R* configuration; however, (*R*)-sulforaphane and synthetic (*R,S*)-sulforaphane had identical inducing potency. The chirality of the molecule has also not been found to affect its other anticarcinogenic activities. Changing the oxidation state of the sulfur atom in the methylthiol group from sulfoxide to sulfone, however, reduced the inducing activity only slightly, and the sulfide analogue was three to six times less active. Moreover, when the sulfoxide group was replaced by a methylene group, the inducing activity was reduced 75-fold (Posner *et al.*, 1994). Significantly, the sulfoxide group could be replaced by a carbonyl group with no loss of inducing activity; this is important because the latter compound is more easily synthesized. Changing the number of methylene units from four to five or three did not significantly affect inducing activity, nor did the rigidity of the methylene bridge appear to have much effect, as the norbonyl isothiocyanates were almost equally active. It should be noted, however, that these analyses of structure–activity relationships were based only on induction of quinone reductase in specific murine cells.

Sulforaphane, erucin (C4 methyl sulfide analogue) and erysolin (C4 methyl sulfone analogue) induced GST and quinone reductase in various tissues (including liver, stomach, small intestine and lung) of female CD-1 mice (Zhang *et al.*, 1992b).

When Fischer rats were given sinigrin, the glucosinolate of allyl-ITC, at 24 mg/day for 11 days, total liver GST activity was induced but phase I enzymes were not significantly affected (Manson *et al.*, 1997). The relative potency of nine isothiocyanates in inducing phase II enzymes in several cultured cell lines depended on the rate of cellular uptake of the isothiocyanates (Zhang & Talalay, 1998).

Limited results indicated that thiol conjugates of isothiocyanates also induced GSTs in various organs. Specifically, the cysteine conjugates of benzyl-ITC and 3-phenylpropyl-ITC induced GST in liver, small intestine mucosa, forestomach, lung, colonic mucosa and urinary bladder of female A/J mice (Zheng, G.Q. *et al.*, 1992).

Genetic variation

No studies were available on intraspecies genetic variation in experimental animals. There is, however, strong evidence from studies in several species (see above) that genetic variation influences isothiocyanate metabolism. The evidence derives from detection of the cyclic mercaptopyruvate conjugate in the urine of mice, guinea-pigs and rabbits, but not rats or humans, after ingestion of benzyl-ITC or phenethyl-ITC. Detection of mercapturic acid as the sole urinary product in rats and humans after ingestion of isothiocyanate suggests that these two species process dietary isothiocyanates similarly. The results also indicate that the metabolism of isothiocyanates is species-dependent. Although only limited information is available on genetic variation and isothiocyanate metabolism, the evidence indicates that genetic differences among species can affect isothiocyanate metabolism quantitatively and qualitatively.

Indoles

The indoles ascorbigen and indole-3-carbinol are naturally occurring plant alkaloids formed by the hydrolysis of indole glucosinolate (glucobrassicin) during grinding or chewing of cruciferous vegetables. Ascorbigen is the main indole formed in the presence of L-ascorbic acid, whereas indole-3-carbinol is the main one formed in the absence of ascorbic acid. Both indoles are further converted to several oligomers, including 3,3′-diindolylmethane and indolo[3,2-*b*]carbazole, in the acid environment of the stomach (Grose & Bjeldanes, 1992; Preobrazhenskaya *et al.*, 1993b). Indole-3-carbinol and 3,3′-diindolylmethane are both available as over-the-counter dietary supplements, implying that relatively large amounts of dietary indoles may be ingested from sources other than cruciferous vegetables.

Metabolism and disposition

The metabolism and disposition of dietary indoles in experimental animals in vivo has not been studied extensively. The distribution of indole-3-carbinol has been monitored in rat and trout models, while the fate of ascorbigen has been examined only in the mouse model and in vitro. Under mild acidic conditions with gastric juice in vitro, ascorbigen was converted to indolo[3,2-*b*]carbazole and 3,3′-diindolylmethane, vitamin C and several other oligomers (Preobrazhenskaya *et al.*, 1993a). The oligomerization components of ascorbigen in an acidic environment were similar to those obtained from indole-3-carbinol in acids in vitro, although about 20 times more indolo[3,2-*b*]carbazole was produced from ascorbigen than indole-3-carbinol. After incubation of ascorbigen in bovine serum or with mouse liver microsomes and serum in vitro after oral administration of ascorbigen to mice in vivo, the main products were

similar to those produced in buffer at pH 7, and included 1-deoxy-1-(indol-3-yl)-α-sorbopyrenose and 1-deoxy-1-(indol-3-yl)-α-tagatopyrenose rather than indolo[3,2-*b*]carbazole, 3,3′-diindolylmethane or vitamin C (Reznikova *et al.*, 2000). Within 1 h of oral administration to mice, ascorbigen had almost completely disappeared from the stomach and intestine and the concentrations in blood and liver had reached a maximum. Neither indolo[3,2-*b*]carbazole nor vitamin C was detected.

Acid condensation products were observed in the gastric and intestinal contents of rats and trout after dietary administration of indole-3-carbinol. Similarly, indole-3-carbinol was converted to higher-order oligomers under mild acidic conditions in vitro (Dashwood *et al.*, 1989a; Bjeldanes *et al.*, 1991; de Kruif *et al.*, 1991; Stresser *et al.*, 1995b). When rats were given indole-3-carbinol at 0.2–0.5 mmol/kg bw by gavage, the gastric and small intestinal contents contained primarily 3,3′-diindolylmethane and higher-order oligomers, with small amounts of indolo[3,2-*b*]carbazole and no detec-table indole-3-carbinol (Bjeldanes *et al.*, 1991; de Kruif *et al.*, 1991). Similar results were obtained after 7 days of dietary administration of indole-3-carbinol at 0.88 mmol/kg bw to rats, in which the liver concentration of indole-3-carbinol equivalent (1.15 μmol/l) exceeded that found in lung and blood by 2.6-fold and 3.6-fold, respectively (Stresser *et al.*, 1995b). These results suggest that indole-3-carbinol acid condensation products are absorbed systemically and preferentially target the liver. All studies in rats showed little conversion and distribution of indole-3-carbinol to indolo[3,2-*b*]carbazole. Bjeldanes *et al.* (1991) estimated that gastric conversion to indolo[3,2-*b*]carbazole before absorption represented 0.01% of the administered dose, whereas the amounts measured in the liver after absorption were in the order of 1×10^{-7}% to 1×10^{-5}% (Kwon *et al.*, 1994; Stresser *et al.*, 1995b). In rats, the urine was the main route of elimination during the first 24 h after dietary intake of indole-3-carbinol, but faecal excretion was greater after 40 h and up to three times greater after 110 h of dietary intake (Stresser *et al.*, 1995b).

These findings in rats are consistent with observations on the distribution of indole-3-carbinol in trout after administration in the diet or gavage with indole-3-carbinol at 40 mg/kg bw (Dashwood *et al.*, 1989a). Uptake from the stomach into blood occurred within the first hour after exposure and corresponded to 4–7% of the administered dose. Hepatic indole-3-carbinol-related compounds were detected within the first hour, but the amounts increased significantly between 12 and 72 h. While 3,3′-diindolylmethane was the main acid condensation product in both rat and trout liver, it comprised 40% of the dose in liver in trout and only 1.5% in rats. Furthermore, about 20% of the dose in trout liver was parent indole-3-carbinol, which was not detected in rat liver. These disparities may be due to species differences in the oligomerization, absorption and elimination of indole-3-carbinol in the stomach and and even to differences in hepatic 3,3′-diindolylmethane metabolism, as observed previously (Stresser *et al.*, 1995a; Shilling *et al.*, 2001). Excretion by trout occurred primarily through the urine or gills; however, biliary excretion was also important in this model. Biliary excretion might also contribute to faecal elimination in rats (Stresser *et al.*, 1995b).

In the transplacental rat model, dietary indole-3-carbinol acid condensation products crossed the placenta and differentially induced neonatal hepatic CYP1A1 and CYP1B1 in a sex-specific manner (Larsen-Su & Williams, 2001). Interestingly, only a single peak was detected by HPLC in liver extracts from all male and female pups examined, which corresponded by retention time to a linear trimer of indole-3-carbinol. As the pups were not nursed after birth, it was assumed that this derivative had selectively crossed the placenta. These results indicate that indole-3-carbinol can change the overall metabolic profile of xenobiotics to which the fetus is exposed transplacentally.

Overall, the studies of the disposition of indole-3-carbinol and ascorbigen indicate that acid condensation products are formed in the gastric contents in vivo and are absorbed. The liver appears to be a main target organ after absorption, implying effects on xenobiotic metabolism. Nevertheless, indole-3-carbinol and ascorbigen acid condensation products were also detected in blood, lung, kidney and intestinal tract. The main indole-3-carbinol acid condensation product found in the liver was 3,3′-diindolylmethane, in both rat and trout models; however, other higher-order oligomers were also found, including indolo[3,2-*b*]carbazole, at lower concentrations. These studies indicate that the overall effects of indole-3-carbinol and ascorbigen on metabolizing enzymes in vivo depend on tissue-specific distribution of their acid condensation products.

Modulation of phase I and phase II enzymes

The ability of dietary indoles to modulate xenobiotic-metabolizing enzymes is well documented and was reviewed by Vang & Dragsted (1996).

Phase I enzymes

Phase I enzyme activities, protein levels and mRNA expression were induced in the liver, intestine and colon of mice and rats given indole-3-carbinol or ascorbigen in the diet, indole-3-carbinol having greater hepatic activity (Loub *et al.*, 1975;

Shertzer, 1982; Miller & Stoewsand, 1983; Cha *et al.*, 1985; Bradfield & Bjeldanes, 1987b; McDanell *et al.*, 1987; Vang *et al.*, 1990; Shertzer & Sainsbury, 1991a,b; Vang *et al.*, 1991). In studies in which lower dietary concentrations of indole-3-carbinol were given, cytochrome P450-associated activity was induced only in the intestine and not in liver, indicating that relatively large amounts of indole-3-carbinol are required to alter systemic cytochrome P450 activity (Bradfield & Bjeldanes, 1984; Salbe & Bjeldanes, 1986). Generally, the rainbow trout model appeared to be less sensitive than mammalian species to sustained induction of phase I enzymes by indole-3-carbinol. This disparity might be due to differences in gastric oligomerization or disposition in vivo (Eisele *et al.*, 1983; Fong *et al.*, 1990; Takahashi *et al.*, 1995b; Oganesian *et al.*, 1999). Conversion of indole-3-carbinol to acid condensation products was found to be necessary for effects on metabolizing enzymes to be exerted. Indole-3-carbinol did not alter cytochrome P450 enzymes when given in vivo by routes of administration that bypass the stomach or when enzymatic induction was examined in vitro (Bradfield & Bjeldanes, 1987a; Renwick *et al.*, 1999).

Loft *et al.* (1992) and Vang *et al.* (1999) showed that purified indolyl glucosinolates, individually or as a mixture, efficiently induced phase I enzyme activity and altered carcinogen and drug metabolism after oral administration to rats.

Examples of the effects in animals of indole-3-carbinol, its acid condensation products and purified glucobrassicins on phase I metabolizing enzymes are summarized in Table 9. While many cytochrome P450 enzymes are induced in a tissue-specific manner by dietary indole in animal models, inhibition has also been observed. 3,3´-Diindolylmethane was a potent, non-competitive inhibitor of rat, trout and human liver microsomal EROD and rat liver microsomal PROD (associated with CYP2B1), with inhibition constants in the low or submicromolar range (Stresser *et al.*, 1995a). This range would seem to be relevant for the concentrations of 3,3´-diindolylmethane measured in trout and rat liver after systemic uptake of indole-3-carbinol from the diet (Dashwood *et al.*, 1989a; Stresser *et al.*, 1995b; Takahashi *et al.*, 1995a). 3,3´-Diindolylmethane and indole-3-carbinol also down-regulated flavin-containing mono-oxygenase protein and activity, while upregulating CYP1A1 in rat liver and intestine (Larsen-Su & Williams, 1996; Katchamart *et al.*, 2000). These indoles could alter the metabolism of drugs that are substrates for both cytochrome P450 and flavin-containing monooxygenase families. There-fore, while alterations in phase I enzyme metabolism by dietary indoles is a mechanism for modulation of carcinogen metabolism, these results also show that there might be drug–drug interactions. Furthermore, the fact that dietary indoles have been found to both induce and inhibit phase I enzymes suggests that they are important for both activation and detoxification of carcinogens.

Phase II enzymes

Dietary indoles have been found to function as bifunctional modulators of metabolism, altering both phase II and phase I enzymes. Dietary indole-3-carbinol induced GST, UGT, quinone reductase and epoxide hydrolase activities in the livers of both rats and mice (Cha *et al.*, 1985; Shertzer & Sainsbury, 1991a,b; Wortelboer *et al.*, 1992b; Manson *et al.*, 1997; Staack *et al.*, 1998). A mixture of glucosinolate breakdown products also induced GSH and quinone reductase and GST activity in rat pancreas, while indole-3-carbinol alone had no effect (Wallig *et al.*, 1998). Doses of indole-3-carbinol lower than those required to induce phase I enzymes generally did not alter phase II enzymes in various animal models (Fong *et al.*, 1990; Wong *et al.*, 1995).

The family of GST enzymes, including soluble cytosolic GST and membrane-bound microsomal GST, includes many subclasses, which are regulated differentially by dietary indoles. Dietary indole-3-carbinol (at 250 mg/kg) did not induce total GST activity in the rat model after 2 weeks but did increase GST alpha levels in the liver and GST mu levels in the stomach, without affecting GST-P (van Lieshout *et al.*, 1998b). A number of studies consistently found that GST-A5 (also known as GST Yc2) is up-regulated by indole-3-carbinol in rat liver, males being more sensitive than females (Stresser *et al.*, 1994b; Manson *et al.*, 1997; Hayes *et al.*, 1998; Nho & Jeffery, 2001). GST A5 is important for the scavenging and phase II metabolism of the carcinogenic aflatoxin B_1 intermediate, aflatoxin B_1-8,9-epoxide, produced by cytochrome P450 activation. Another phase II enzyme that is important in metabolizing the aflatoxin B_1 epoxide, by a separate pathway, is aflatoxin B_1 aldehyde reductase, which was also up-regulated by indole-3-carbinol (Manson *et al.*, 1997). The ability of dietary indoles to up-regulate phase II enzymes in most animal models, depending on dose and target organ, suggests that this mechanism is important in their ability to act as chemopreventive agents.

Structure–activity relationships

The potent inducer, 2,3,7,8-tetrachloro-*para*-dibenzodioxin (TCDD) is known to regulate *CYP1A1* gene expression by binding with high affinity to the Ah receptor, a ligand-induced transcription factor that interacts with the dioxin response element or

Table 9. Modulation of phase I enzymes by dietary indoles in experimental animal models

Indole	Species	Dose	Duration	Organ	Effect on phase I enzymes	Reference
Indole-3-carbinol 3,3´-Diindolylmethane	Rat	25 µmol/l	2–28 days	Hepatocytes	– EROD ↑ EROD	Wortelboer *et al.* (1992b)
Indole-3-carbinol	Rat	2000 mg/kg	7 days	Liver	↑ CYP1A1/2 ↑ CYP2B1/2 ↑ CYP3A1/2 ↑ EROD	Stresser *et al.* (1994a)
Indole-3-carbinol	Fingerlings	2000–4000 mg/kg	7 days	Liver	↑ CYP1A1 ↑ EROD (transient only)	Takahashi *et al.* (1995b)
Indole-3-carbinol	Rat	2000 mg/kg	1–28 days	Liver, intestine	↓ FMO1 ↑ CYP1A1	Larsen-Su & Williams (1996)
Indole-3-carbinol	Rat	10–50 mg/kg	12 h	Liver	– MROD, EROD – CYP1A1/2	Xu *et al.* (1997)
		100–1000 mg/kg	12 h	Liver, colon	↑ MROD, EROD ↑ CYP1A1/2	
3,3´-Diindolylmethane	Rat	5 mg/kg	20 days	Mammary gland	– EROD	Chen, I. *et al.* (1998)
Ascorbigen	Mouse	1–1000 µmol/l	24 h	Hepa1c1c7 cells	↑ EROD ↑ CYP1A1	Stephenson *et al.* (1999)
3-3´-Diindoylmethane and indole-3-carbinol	Rat	1000, 2500 mg/kg	4 weeks	Liver	↓ FM01	Katchamart *et al.* (2000)
Indole-3-carbinol	Mouse, rabbit, guinea-pig	2000 mg/kg	4 weeks	Liver	↑CYPA1/2 – FMO1	Katchamart & Williams (2001)
Indole-3-carbinol	Rat	250 mg/kg	4 days	Liver	↑ CYP1A1 ↑ CYP2B1/2 ↑ CYP1B1, total P450 ↑ EROD, MROD, BROD, PROD ↑ NIFOX	Horn *et al.* (2002)
				Mammary gland	↑ CYP1A1 only	
Indolo[3,2-*b*]carbazole	Rat	127 µg/kg	4 days	Liver, stomach, intestine	↑ CYP1A1 ↑ EROD	Pohjanvirta *et al.* (2002)
3,3´-Diindoylmethane	Rat	20 mg/kg	1 year	Liver	↑ CYP1A1/2 ↑ CYP3A2 (females only)	Leibelt *et al.* (2003)

Table 9 (contd)

Indole	Species	Dose	Duration	Organ	Effect on phase I enzymes	Reference
Glucobrassicin, neoglucobrassicin, ascorbic acid	Rat	88 μmol/kg bw	1/day, 3 days	Liver	↑ CYP1A ↑ EROD – CYP1A2 ↑ MROD – CYP2B1/2 – CYP2E1	Bonnesen *et al.* (1999)

Increase (↑), decrease (↓) or no change (–) in phase I enzymes measured as protein, mRNA or activity
EROD, ethoxyresorufin *O*-deethylase; CYP, cytochrome P450; FMO, flavin-related monooxygenase; MROD, methoxyresorufin *O*-deethylase; BROD, benzyloxyresorufin *O*-deethylase; PROD, pentoxyresorufin *O*-deethylase; NIFOX, nifedipine oxidation

enhancer sequences of the *CYP1A1* gene (Swanson & Bradfield, 1993). Structure–activity studies showed a general correlation between binding affinity for the Ah receptor and the CYP1A1-inducing capacity of various polycylic aromatic hydrocarbons (Safe, 1998).

The Ah receptor-binding affinities of indole-3-carbinol, 3,3′-diindolylmethane and indolo[3,2-*b*]carbazole in mouse liver cytosol were 2.6×10^{-7}, 7.8×10^{-5} and 3.7×10^{-2}, respectively, relative to that of TCDD, standardized at 1.0 (Bjeldanes *et al.*, 1991). Therefore, indole-3-carbinol and 3,3′-diindolylmethane are weaker ligands for the Ah receptor than indolo[3,2-*b*]carbazole. These findings are generally consistent with other observations of the binding of indoles to the Ah receptor and CYP1A1 induction. The binding affinity of 3,3′-diindolylmethane, however, is lower than would be expected from its activity in vivo (Gillner *et al.*, 1985; Rannug *et al.*, 1991; Safe, 1998).

Indolo[3,2-*b*]carbazole is an extremely potent agonist for the Ah receptor and competitively inhibited TCDD, with a median inhibitory concentration of 3.6 nmol/l, suggesting that the two ligands bind to the same site on the receptor (Gillner *et al.*, 1985). The factors important for Ah receptor binding are mainly compound size and structural stability or planarity, which may explain why indolo[3,2-*b*]carbazole is a better Ah receptor ligand than either indole-3-carbinol or 3,3′-diindolylmethane. The finding of only low concentrations of indolo[3,2-*b*]carbazole in vivo, however, cast doubt on the significance of this metabolite in the effects of indole-3-carbinol in the whole animal. Although dietary indoles are less persistent than their environmental polycylic aromatic hydrocarbon counterparts, they are eaten in relatively large amounts in the diet and elicit common Ah receptor-mediated responses, which should be considered in their ultimate effects, individually or in combination.