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INFLUENCE OF INTACT AND MYROSINASE-TREATED INDOLYL GLUCOSINOLATES ON THE METABOLISM *IN VIVO* OF METRONIDAZOLE AND ANTIPYRINE IN THE RAT

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Abstract—Induction of the cytochrome *P*-450 enzymes is a mechanism whereby cruciferous vegetables and their glucosinolates could influence the risk of cancer. The cytochrome *P*-450-inducing capacity of isolated intact broccoli glucosinolates and their degradation products, resulting from myrosinase-catalysed hydrolysis, has been assessed in studies of the metabolism of antipyrine (AP) and metronidazole (MZ) in the rat. The intact glucosinolates had no effect on the metabolism of MZ and AP as measured by the clearance and metabolite formation rates; however, the myrosinase-treated glucosinolates significantly increased the clearance of AP by two-thirds and the formation rates of the three major AP metabolites by 87–100%, and doubled the rate of oxidative metabolism of MZ to its hydroxy and acetic acid metabolites. Active myrosinase was thus essential for the capacity of glucosinolates from broccoli (mainly indolyl glucosinolates) to induce the activity of several cytochrome *P*-450 isoenzymes involved in the metabolism of AP and MZ. The data indicated that hydrolysis products of indolyl glucosinolates had an inducing effect on the activity, but not the total amount, of hepatic cytochrome *P*-450 isoenzymes. The effect of these products on the oxidative metabolism of AP and MZ was similar to that of phenobarbital. The significance of this induction pattern in relation to cancer risk depends primarily on the activation inactivation mechanism of the relevant carcinogen.

INTRODUCTION

Plants belonging to Capparales, including Brassicaceae, are characterized by their content of glucosinolates. These compounds and their transformation products have various physiological effects (Bjerg *et al.*, 1989). In cruciferous vegetables of the Brassica genus, glucosinolates with an indolylic structure are quantitatively predominant (Jensen *et al.*, 1991). The indolylmethylglucosinolate glucobrassicin and a range of indolylic compounds produced by myrosinase (β-thioglucosid glucohydrolase, EC 3.2.3.1.) catalysed hydrolysis have been shown to inhibit chemical carcinogenesis induced by polycyclic aromatic hydrocarbons (Wattenberg *et al.*, 1986; Wattenberg and Loub, 1978). Cruciferous vegetables and their constituents, furthermore, have been shown to inhibit carcinogenesis induced by other chemicals (Boyd *et al.*, 1982; Dashwood *et al.*, 1989; Stoewsand *et al.*, 1988; Wattenberg and Loub, 1978) and to be associated with decreased risk of cancer in humans (Graham *et al.*, 1978; Hu *et al.*, 1988; Marchand *et al.*, 1989; Modan *et al.*, 1975; Young and Wolf, 1988).

The majority of chemical carcinogens are metabolically activated and/or detoxified by the cytochrome *P*-450 enzymes and other enzymes (Guengerich, 1988; Wright, 1980). Alteration of the activity of these enzymes in various organs might be one of the mechanisms whereby cruciferous vegetables and their glucosinolates exert a protective effect against a range of carcinogenic chemicals (Godlewski *et al.*, 1985; Goeger *et al.*, 1986; Ramsdell and Eaton, 1988; Wattenberg, 1980; Whitty and Bjeldanes, 1987).

The capacity of cabbage and indolylic compounds to induce the activity of the cytochrome *P*-450 enzymes has been assessed *in vitro* using specific substrates of the isoenzymes (Bradfield and Bjeldanes, 1984 and 1987; McDanell *et al.*, 1987; Pantuck *et al.*, 1976; Wortelboer, 1991) and by measuring gene expression at the mRNA and/or protein level (Vang *et al.*, 1990 and 1991; Wortelboer, 1991). Induction of cytochrome *P*-450 activities by Brassica species has also been demonstrated *in vivo* in humans using antipyrine (AP), phenacetine and caffeine as probes (Pantuck *et al.*, 1979; Vistisen *et al.*, 1991). However, it is not known to what extent the inducing capacity of the vegetables is due to the intact indolyl glucosinolates or to the transformation products. Little information is available on the hydroxy- and methoxy-substituted indolyl glucosinolates, which occur in appreciable amounts in several cruciferous vegetables; however, it is known that the degradation products of 4-hydroxyglucobrassicin have much more pronounced

Abbreviations: AP = antipyrine; MZ = metronidazole; PBS = phosphate buffered saline.

physiological effects than the intact glucosinolate (Jensen *et al.*, 1991).

The present study was designed to investigate the toxicological effects of broccoli glucosinolates, which contain a high amount of *N*-methoxyglucobrassicin (neoglucobrassicin) in addition to smaller amounts of other substituted indolylmethyl glucosinolates. We investigated whether isolated broccoli glucosinolates *per se* or hydrolysed by myrosinase influenced the cytochrome *P*-450 enzymes. It has been shown that the activity of several cytochrome *P*-450-dependent isoenzymes can be assayed *in vivo* by measuring the total and fractional clearances of probes that do not interfere with the activity of these enzymes (Loft, 1990; Loft *et al.*, 1991).

MATERIALS AND METHODS

Isolation of glucosinolates and myrosinase. Glucosinolates were extracted from portions (150–200 g) of 2.6 kg freeze-dried broccoli (*Brassica oleracea* L. cv. *botrytis* (L.) Alef. var. *cymosa* Duch., cultivar 'Skiff') and isolated by means of flash chromatography as described by Jensen *et al.* (1991). The yield was 13.3 g glucosinolate powder quantitatively dominated by indolylmethyl glucosinolates (mainly glucobrassicin and neoglucobrassicin) (Table 1). Active myrosinase (*β*-thioglucosid glucohydrolase, EC 3.2.3.1.) was extracted from rapeseed (*Brassica napus* L. cv. *Optima*) and purified by affinity chromatography as described by Michaelsen *et al.* (1991).

Preparation of test solutions. 2.4 g isolated glucosinolates were dissolved in 88.0 ml phosphate buffered saline (PBS) (0.05 M-NaH_2PO_4 and 0.154 M-NaCl, pH 7.3) to which was added 1.5 ml ascorbic acid (20 mM). A portion of this solution (45.5 ml) was incubated at 30°C for 3 days with myrosinase (29 U) dissolved in 4.5 ml α-D-methyl mannoside (0.5 M). Another 2.4 g glucosinolates were dissolved in 88.0 ml PBS containing ascorbic acid and was incubated with 4.5 ml α-D-methyl mannoside without myrosinase. PBS with ascorbic acid and α-D-methyl mannoside in concentrations as in the test solutions served as the control solution.

Animals and treatment. Male Wistar rats weighing 210–240 g were housed three to a cage with free access to water and feed (Altromin). After a 5-day pretreatment period, three groups each of 9–10 rats were treated with 2 ml intact glucosinolates (215 mg glucosinolate powder/kg body weight), myrosinase-treated glucosinolates (215 mg glucosinolate powder/kg body weight) or control solution. Both treatment groups received a dose of glucosinolate/glucosinolate products corresponding to 35% (w/w) freeze-dried broccoli in the diet. The test and control solutions were administered by gastric intubation twice daily for 3.5 days. Two rats treated with myrosinase-treated glucosinolates died; this may have been caused by maladministration. After the last dose of glucosinolates, the animals were put into individual metabolic cages and dosed ip with a cocktail of antipyrine (AP) and metronidazole (MZ) (14 and 10 mg/kg body weight, respectively). The clearance of each probe was determined from a pilocarpin-stimulated saliva sample collected 4 hr later (Loft *et al.*, 1991). Urine was collected for 20 hr for the determination of fractional clearances (Loft *et al.*, 1991). After completion of urine collection, the rats were anaesthetized and their livers were immediately removed and homogenized for the determination of protein, cytochrome *P*-450 and glutathione contents.

Chemical analysis. Paper chromatography and HPLC of intact glucosinolates were performed as described by Olsen and Sørensen (1981) and Sørensen (1990), respectively. The content of MZ and AP in saliva and urine samples was determined by HPLC according to Loft *et al.* (1991). Urinary concentrations of MZ and AP metabolites were determined before and after incubation of urine samples with glucuronidase/arylsulphatase (Loft *et al.*, 1991). Total cytochrome *P*-450 content was measured by the method of Omura and Sato (1967), and glutathione (reduced and oxidized) was determined by a modification of the method of Tietze (1969) as described by Poulsen *et al.* (1981). Protein was measured according to the method described by Lowry *et al.* (1955).

Statistical analysis. A comparison of the effects of the three treatments on the clearances of AP and MZ was made by analysis of variance. Mean clearances were compared by Duncan's multiple-range test.

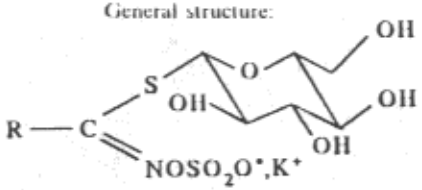
RESULTS

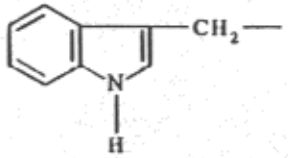
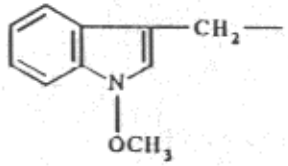
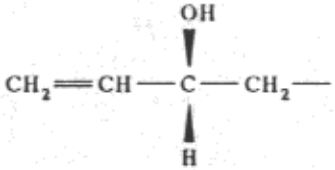
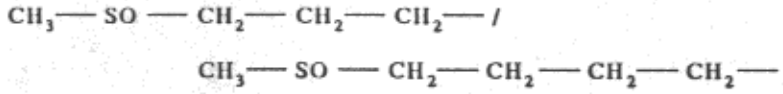
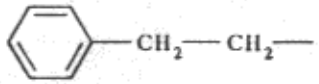
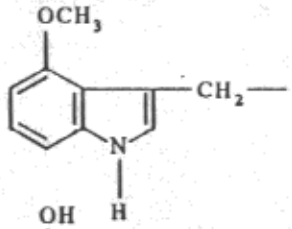
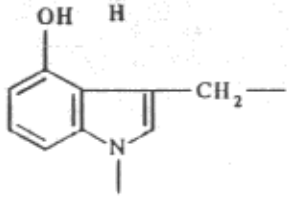
Analyses of different cultivars of broccoli have shown that the glucosinolate content varies from 5 to 35 μmol/g dry weight, with indolyl glucosinolates constituting up to 60% of total glucosinolates. In the isolation procedure used in the present study, advantage was taken of the adsorptive properties of indolylic compounds to produce a glucosinolate powder composed of nearly 70% indolyl glucosinolates (Table 1). Paper chromatography and HPLC revealed that the intact glucosinolates in the test solution were stable throughout the duration of the experiment. Likewise, it was shown that no intact glucosinolates remained in the solutions incubated with myrosinase (Fig. 1); they were broken down to various indolylic compounds including indolyl-3-methanol.

The clearances of AP and MZ were 4.6 ± 0.6 and 5.3 ± 0.6 ml/min/kg body weight (mean ± SD), respectively, in rats treated with the control solution. Intact glucosinolates had no effect on the metabolism of MZ and AP. However, myrosinase-treated glucosinolates significantly ($P < 0.05$) increased the clearance of AP by almost two-thirds to 7.5 ± 2.2 ml/min/kg body weight (Fig. 2B). On average 61% of the administered dose of AP was recovered in the urine, either unchanged (renal) or as the metabolites

Table 1. Structures, contents and daily doses administered to rats of individual glucosinolates in the powder isolated from broccoli

Glucosinolate
General structure:



	Structure of side chain (R)	Concentration (μmol/g powder)	Dose administered (μmol/kg body weight/day)
Glucobrassicin		525	219
Neoglucobrassicin		365	159
Progoitrin		180	75
Glucoiberin/glucoraphanin		155	65
Phenethylglucosinolate		26	11
4-Methoxyglucobrassicin		25	10
4-Hydroxyglucobrassicin		25	10

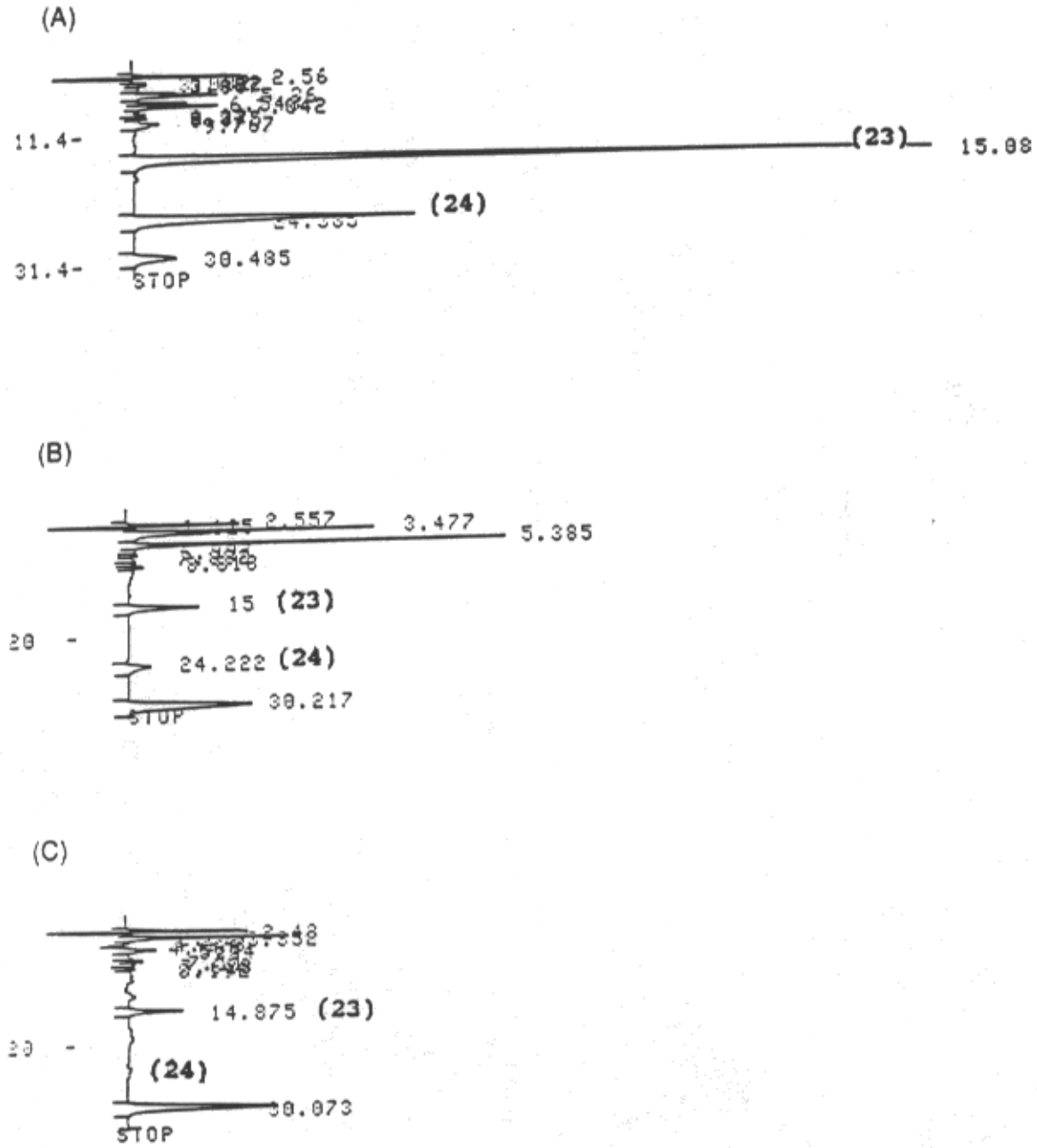


Fig. 1. HPLC chromatograms of the glucosinolate solutions administered to rats: A, incubation without myrosinase for 3 days; B, immediately after incubation with myrosinase and C, incubation with myrosinase for 3 days. Peaks 23 and 24 are glucobrassicin and neoglucobrassicin, respectively. The degradation products of 23 and 24 appear in front and at 30 min of chromatograms B and C. (detection 280 nm), and the peak at retention time 5.385 corresponds to indolyl-3-methanol.

hydroxymethylantipyrine, hydroxyantipyrine and norantipyrine (Fig. 3). Treatment with degradation products of glucosinolates increased the formation rates of these three metabolites by 87–100%, whereas the renal clearance of unchanged AP was not altered (Fig. 2B). The urinary recovery of MZ was on average 40% of the administered dose. The major part was excreted unchanged or conjugated as metronidazole glucuronide (Fig. 3). Neither of the glucosinolate treatments significantly altered the renal clearance to the unchanged compound or that of the glucuronide conjugate. However, the fractional clearances to the oxidative metabolites of MZ, metronidazole acetic acid and hydroxymetronidazole were almost doubled in rats administered with myrosinase-treated glucosinolates ($P < 0.05$) (Fig. 2A).

Weight gain and liver weight were not significantly altered by intact or myrosinase-treated glucosinolates. Variations between animals have been seen and discussed in other experiments, where individual glucosinolates (±myrosinase) were fed to rats in balance trials (Bjerg *et al.*, 1989; Jensen *et al.*, 1991). The relative liver weight (nourished rats) was 4.6 ± 0.4 g/100 g body weight in all groups.

The contents of hepatic protein and cytochrome *P*-450 were not significantly affected by any of the treatments (Table 2). The reduced-CO absorption maximum of liver homogenates was 450 nm in all groups. No shift in absorption maximum towards 448 nm was observed with liver homogenates from any of the glucosinolate-treated rats. A small and not significant increase in the average hepatic glutathione

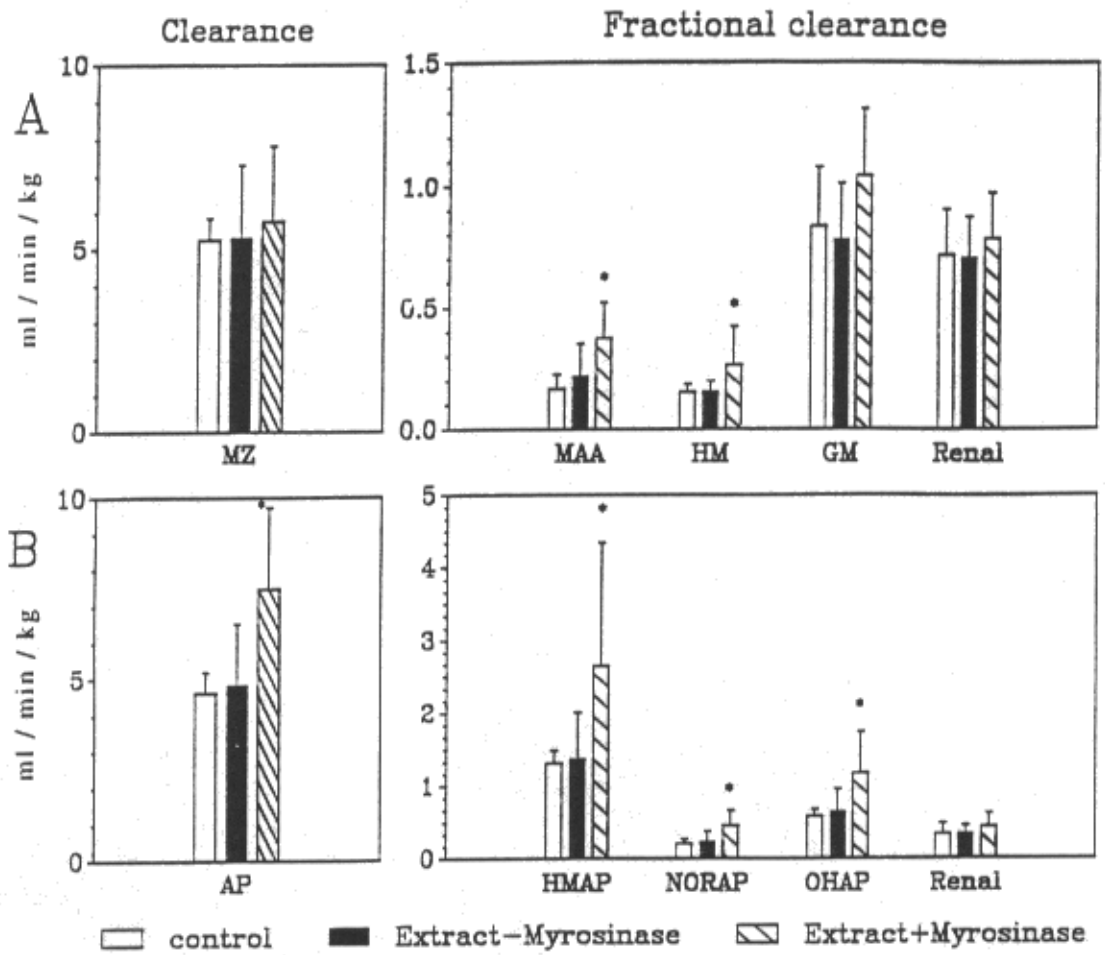


Fig. 2. Clearance and fractional clearance of metronidazole (A) and antipyrine (B) in rats pretreated with intact (Extract – Myrosinase) or myrosinase-treated glucosinolates (Extract + Myrosinase). Asterisk indicates significant difference from controls ($^*P < 0.05$).

content was seen in rats dosed with myrosinase-treated glucosinolates (Table 2).

DISCUSSION

Existing techniques have allowed the isolation of appreciable amounts of intact glucosinolates in the form of potassium salts (Bjerg *et al.*, 1989; Jensen *et al.*, 1991) and highly purified samples of active myrosinases (Michaelsen *et al.*, 1991). Test solutions containing intact glucosinolates isolated from broccoli or their degradation products obtained from myrosinase-catalysed hydrolysis have been investigated for their effects on the metabolism of AP and MZ *in vivo* in rats (Loft *et al.*, 1991). The results from these studies showed that myrosinase-catalysed transformation of glucosinolates was indispensable for the formation of compounds capable of inducing the activity of the cytochrome *P*-450 enzymes involved in the oxidative metabolism of AP and MZ. These results are in accordance with those of McDanell *et al.* (1987), who observed that cooking of cabbage (i.e. inactivation of myrosinase) reduced the capacity to induce cytochrome *P*-450. As myrosinase is specific to glucosinolates (Michaelsen *et al.*, 1991), the cytochrome *P*-450-inducing compounds formed must be the degradation products of glucosinolates, presumably indolylic compounds formed from the indolylmethyl glucosinolates, which were quantitatively predominant among the broccoli glucosinolates isolated (Table 1).

Myrosinase-catalysed degradation of glucobrassicin under conditions similar to those used in the present study initially leads to the formation of indolyl-3-methanol, but this compound is gradually transformed into various other products (Fig. 4), including ascorbigens (H. Sorensen, C. Feldl, L. Michaelson and J. Otte, unpublished data, 1992). In other studies, indolyl-3-methanol in appreciable amounts has been shown to induce several cytochrome *P*-450-dependent enzymes *in vitro* (Bradfield and Bjeldanes, 1984 and 1987; Wortelboer, 1991). If all indolylmethyl glucosinolates administered in the present study were degraded to indolyl-3-methanol derivatives, the daily dose would be 391 μmol/kg body weight (56 mg/kg body weight) corresponding to about 550 ppm. This dose is comparable with the 500 ppm used in the experiments of Babish and Stoewsand (1978), Bradfield and Bjeldanes (1984) and Fong *et al.* (1990), which did not induce hepatic aryl hydrocarbon or ethoxycoumarin-*O*-deethylase activity in microsomal fractions. Therefore, the inducive effect observed in the present experiment might be due to compounds more potent than indolyl-3-methanol. In addition to glucobrassicin, the glucosinolates isolated from broccoli were quantitatively

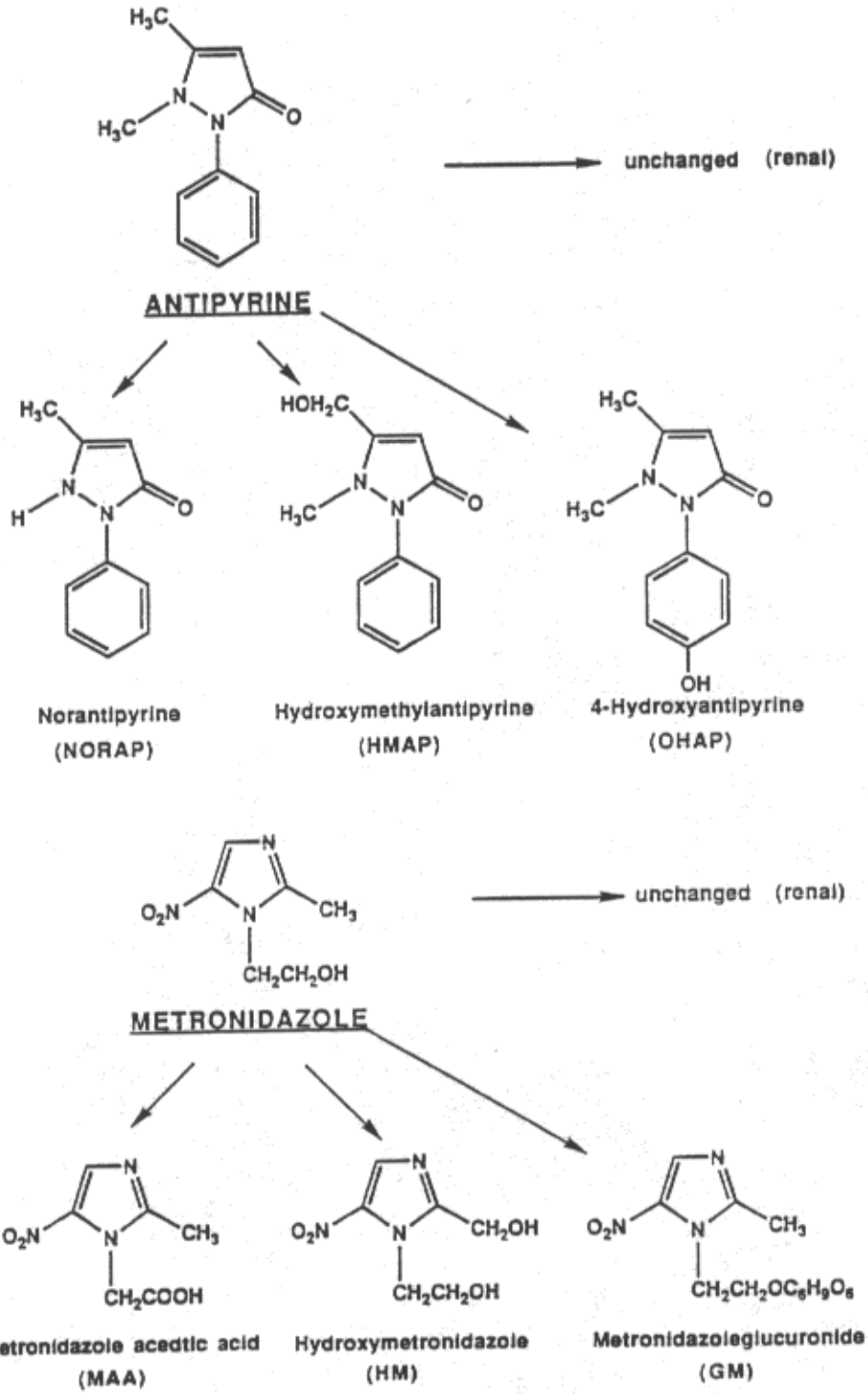


Fig. 3. Metabolism of antipyrine and metronidazole in humans and rats.

dominated by neoglucobrassicin and contained small amounts of other substituted indolylmethyl glucosinolates (Table 1). Neoglucobrassicin gives rise to products that are especially potent inducers of the cytochrome *P*-450-dependent 7-ethoxycoumarin-*O*-deethylase (Bradfield and Bjeldanes, 1987) and inhibitors of human lung-cancer cells *in vitro* (P. Jensen, J. Otte and H. Sorenson, unpublished data, 1992). Furthermore, these *N*-methoxylated products are not substrates for *N*-nitrosoindolyl formation,

Table 2. Effect of intact glucosinolates and degradation products of myrosinase-treated glucosinolates administered to rats by gavage on weight gain and hepatic contents of protein, cytochrome *P*-450 and glutathione

Treatment	No. of rats	Liver weight (g/100 g body weight)	Protein (mg/g liver)	Cytochrome *P*-450 (nmol/g liver)	Glutathione (μmol/g liver)	Weight gain (g/3.5 days)
Control	10	4.63 ± 0.48	85.70 ± 8.83	36.11 ± 4.93	5.02 ± 0.97	12.5 ± 6.6
Intact glucosinolates	9	4.59 ± 0.46	90.04 ± 4.76	30.72 ± 7.22	4.96 ± 0.87	5.2 ± 12.7
Myrosinase-treated glucosinolates	7	4.63 ± 0.41	89.32 ± 14.91	36.88 ± 11.97	5.85 ± 0.98	9.4 ± 15.4

Values are means ± SD.

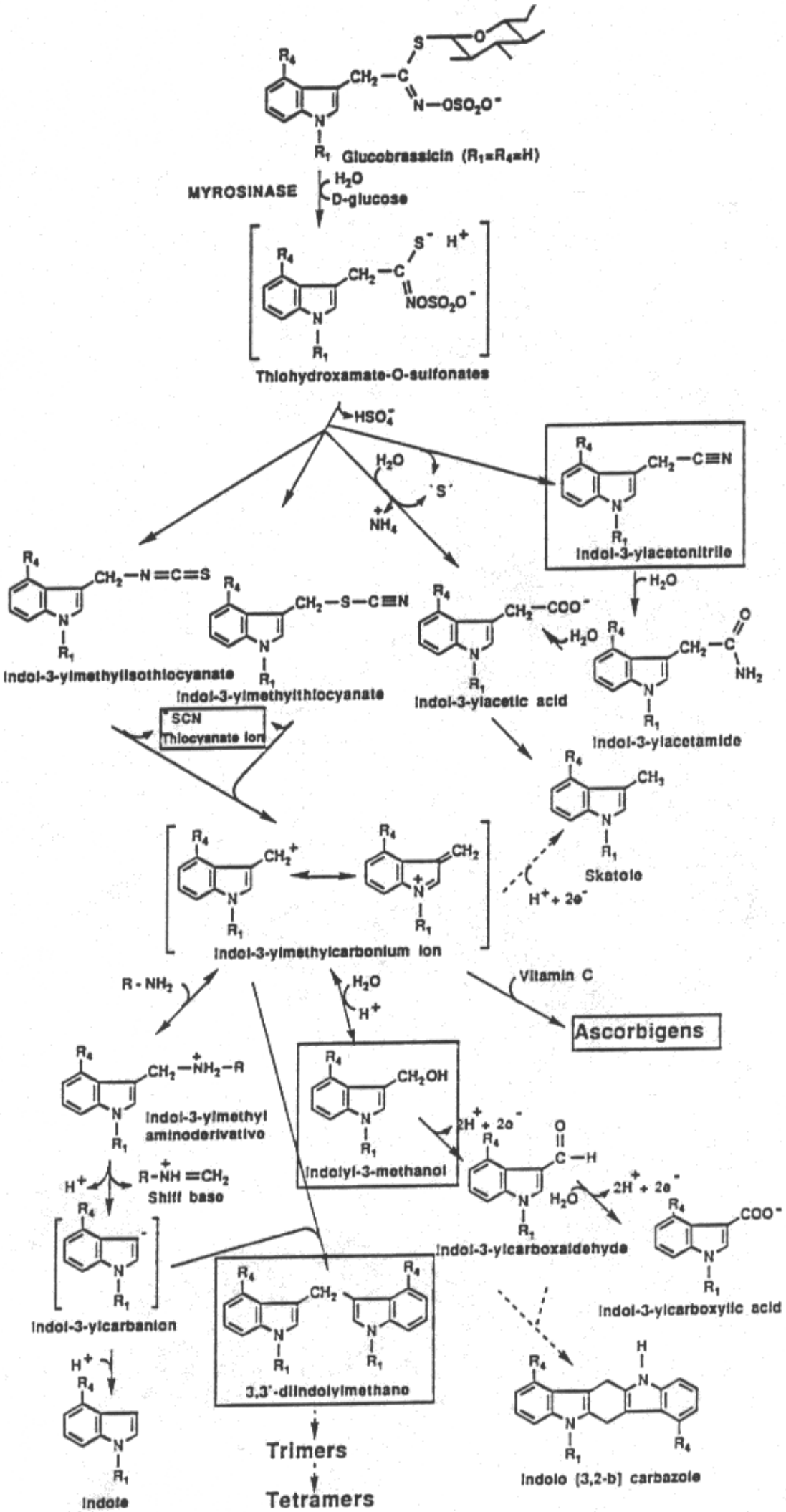


Fig. 4. Enzymatic degradation of indolylmethyl glucosinolates. See Table 1 for structures of neoglucobrassicin, 4-methoxyglucobrassicin and 4-hydroxyglucobrassicin.

a process believed to confer hazardous potential on indolyl compounds (Tiedink *et al.*, 1988; Wakabayashi *et al.*, 1986).

The active compound(s) derived from the glucosinolates in broccoli induced all cytochrome *P*-450 enzymes involved in the formation of the quantifiable oxidative metabolites of AP and MZ (Figs 1 and 3). In this respect, the effect of the glucosinolate degradation products is similar to that of phenobarbital (Loft *et al.*, 1991). An induction of several cytochrome *P*-450 isoenzymes (IA and the phenobarbital-inducible IIB and IIE), measured at the apoprotein level, has also been seen in hepatic microsomes from rats fed broccoli-supplemented diets (Vang *et al.*, 1991). In hepatic microsomes from rats fed a diet supplemented with Brussels sprouts, which mainly contain glucobrassicin, there was a marked induction of IA2 apoprotein only (Wortelboer, 1991). Considering that several cytochrome *P*-450 isoenzymes were induced in the present experiments, it is surprising that no observable increase in the total concentration of cytochrome *P*-450 was measured in the livers of rats dosed with the glucosinolate degradation products. However, the clearance rates of AP and MZ increased about two-fold in these animals as compared with a four-fold increase in β-naphthoflavone- and phenobarbital-treated rats (Loft *et al.*, 1991), and could reflect a shift in enzyme activities, with a decreased activity of some isoenzymes and an increased activity of those studied.

The risk from chemical carcinogens seems to depend on the balance betwen activation and inactivation of these compounds, the type of cytochrome *P*-450 isoenzymes activated, and the activity of the conjugating enzymes and the availability of their substrates (Bock *et al.*, 1987; Parke, 1987). According to Parke (1987), the activation of many carcinogens is catalysed preferably by the cytochrome *P*-450 isoenzymes IA1 and IA2 (former cytochrome *P*-448). In the present investigation, the amount of cytochrome *P*-450 isoenzymes IA1 and IA2 was apparently not increased since no shift from 450 to 448 nm was observed in the absorption maximum. This indicates that the activity of the detoxification enzymes might be increased. Furthermore, the availability of glutathione, the substrate of glutathione *S*-transferases, was slightly increased, although not significantly, in rats treated with the glucosinolate degradation products, suggesting that detoxification by this conjugation pathway was not decreased by the active indolylic compounds. These results lead to the hypothesis that the enzyme-induction pattern observed in the present experiments may have a protective effect against carcinogens normally activated by the *P*450IA isoenzymes.

In the present study, rats were fed 85 μmol indolyl glucosinolates (or degradation products) corresponding to 38 g broccoli/day. This is significantly higher than the average daily human consumption of this vegetable (about 1 g/day); however, during short periods quantities of up to 500 g can be consumed (Vistisen *et al.*, 1991).

In conclusion, the results from the present study reveal that products from the myrosinase-catalysed hydrolysis of broccoli glucosinolates have an inducing capacity towards cytochrome *P*-450 isoenzymes. Further studies are needed concerning the structure of the active compounds as well as the storage and processing conditions that favour their formation. Broccoli has a relatively high content of substituted indolylmethyl glucosinolates compared with other cruciferous vegetables, which should also be taken into account in the evaluation of vegetables in relation to cancer.

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