Role of Glucosinolates in the Formation of *N*-Nitroso Compounds

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The hydrolysis of the glucosinolates, sinigrin, gluconapin, glucobrassicanapin, progoitrin, glucotropaeolin, sinalbin, gluconasturtiin, glucobrassicin, and 4-hydroxyglucobrassicin, by myrosinase from white mustard (*Sinapis alba*) or acid was examined. While all glucosinolates were hydrolyzed by myrosinase, only 4-hydroxyglucobrassicin, glucosinalbin, gluconasturtiin, glucobrassicin, and progoitrin were partially hydrolyzed by acid (pH 2). When intact glucosinolates or myrosinase-treated glucosinolate products were treated with nitrite, only glucobrassicin and 4-hydroxyglucobrassicin formed *N*-nitroso compounds. The nitrosated products of myrosinase-treated glucobrassicin alone were mutagenic and induced about 400 *Salmonella typhimurium* TA100 revertants/μmol. The enzymic breakdown products of the alkyl and aryl glucosinolates were cytotoxic, but this activity was not affected by subsequent nitrite treatment. Given the levels at which indole glucosinolates occur in brassica vegetables, these findings suggest that their contribution to the observed mutagenic potential of these vegetables after nitrite treatment will be marginal. Further work is, however, needed to identify the exact chemical natures of both the *N*-nitroso compounds formed in nitrite-treated brassicas and their naturally occurring precursors.

INTRODUCTION

Glucosinolates are a group of sulfur-containing glycosides, which occur in all members of the Cruciferae, including brassica vegetables. Brassicas are frequently consumed by humans as well as by animals. The mean consumption of fresh vegetables in the Netherlands in 1987–1988 is estimated to be 144 g per person per day (Anonymous, 1988); the contribution of brassicas to this figure is not known exactly but will be considerable, since the amount of brassica available per person per day in 1983 exceeded 36 g (Godeschalk, 1987). In the United Kingdom the consumption of green vegetables, mainly brassicas (cabbage and Brussel sprouts), exceeds 40 g per person per day (Lewis and Fenwick, 1988).

Glucosinolates contain a common structure (Figure 1) with different substituents at the side chain, R; currently, more than 100 different glucosinolates have been characterized. Glucosinolates can be hydrolyzed by an enzyme, myrosinase (thioglucoside glucohydrolase, EC 3.2.3.1), which is present in members of the Cruciferae. Such hydrolysis occurs when the cells are damaged, for example, by cutting. Enzymic hydrolysis most usually yields volatile compounds, such as isothiocyanates, nitriles, and thiocyanates. Glucosinolates that contain a hydroxy group in the β-position of the side chain (for example, progoitrin in Figure 1) will form less volatile oxazolidine-2-thiones and epithionitriles. Indolyl glucosinolates will yield nonvolatile indole compounds; thus, indole-3-carbinol, indole-3-acetonitrile, diindolylmethane, and ascorbigen are formed from glucobrassicin (Figure 1). Such compounds have been shown to inhibit the neoplastic effects of carcinogens [see McDanell et al. (1988) and references cited therein; McDanell et al., 1989]. In addition to this enzymic breakdown, glucosinolates may also be hydrolyzed chemically (Fenwick et al., 1983).

Besides the anticarcinogenic effects of indole-3-acetonitrile, it is also known to be a precursor of *N*-nitroso compounds (NOC) which have been isolated from nitrite-treated Chinese cabbage and which exhibit direct mutagenic activity (Wakabayashi et al., 1985). Other workers have also shown indole compounds to be directly mutagenic to bacteria after nitrite treatment (Gatehouse and Wedd, 1983; Yang et al., 1984; Büchi et al., 1986; Ochiai et al., 1986; Tiedink et al., 1989). Approximately 75% of the nitrate ingested by humans is derived from vegetables (Ellen and Schuller, 1983), and part of this load will be converted to nitrite by bacteria in the oral cavity (Mirvish, 1983). Since indole compounds react rapidly with nitrite even at physiological feasible nitrite concentrations (Yang et al., 1984; Wakabayashi et al., 1986), it is possible that endogenous nitrosation of indole compounds may occur. While it has been suggested that nitrosated indole-3-acetonitrile has tumor-initiating and tumor-promoting activity in rats (Furihata et al., 1987; Yamashita et al., 1988), the effects of such compounds in man are unknown.

In a previous study (Tiedink et al., 1988), we have screened vegetables for their potential to form directly mutagenic NOC. While all the vegetables tested formed NOC following nitrite treatment, brassicas exhibited the highest levels. Whereas no correlation was found between the amounts of NOC formed in nitrite-treated vegetables and the mutagenicity of these vegetables upon nitrosation, an association ($p < 0.01$) was observed between the amounts of NOC formed in extracts of cruciferous vegetables and their glucosinolate (both aryl/alkyl and indolyl) content.

In the present study we have examined whether this association reflects a causal relationship between the two variables. The glucosinolates sinigrin (SIN), gluconapin (GNP), glucobrassicanapin (BNP), progoitrin (PG), glucotropaeolin (GTP), sinalbin (SAL), gluconasturtiin (NAS), glucobrassicin (GB), and 4-hydroxyglucobrassicin (4-hy-

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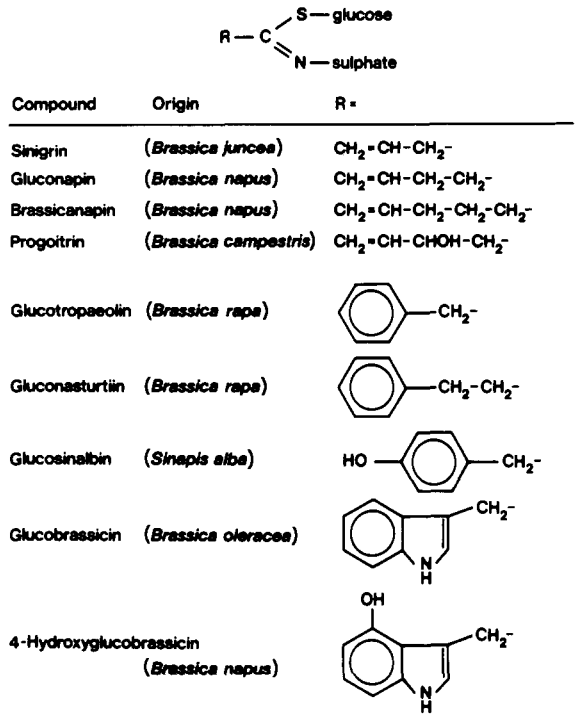

Figure 1. Chemical structures and origins of the glucosinolates tested.

droxyGB) (Figure 1) were screened for their potential to form NOC following nitrosation. Intact and myrosinase-treated glucosinolates were also tested for direct mutagenic activity (cytotoxicity) following nitrosation. Since thiocyanate ion (a known myrosinase-induced hydrolysis product of indole glucosinolates) is able to catalyze the nitrosation reaction (Boyland et al., 1971), the breakdown products of glucosinolates were further tested for their ability to catalyze the nitrosation rate of proline. Because the low pH of the stomach may result in the chemical breakdown of glucosinolates, the chemical hydrolysis of glucosinolates was also examined.

MATERIALS AND METHODS

Chemicals. SGN, GNP, BNP, PGT, GTP, SAL, NST, GB, and 4-hydroxyGB were isolated from the sources shown in Figure 1 according to the methods of Hanley et al. (1983) and Peterka and Fenwick (1988). With the exception of 4-hydroxyGB, all glucosinolates were obtained in >95% purity (checked by HPLC and glucose release). The sample of 4-hydroxyGB was a crude isolate from rapeseed (*Brassica napus*), was prone to oxidation, and was not amenable to purification by the methods described. For this reason the crude material (40% pure) was used directly. Proline was purchased from Sigma (St. Louis MO); myrosinase (200 units mg^{-1}) was obtained from Biocatalysts (Pontypridd, Wales). All other chemicals were at least of analytical grade.

Hydrolysis of Glucosinolates. To study the hydrolysis of glucosinolates by acid, glucosinolate solutions (25 μM) were adjusted to pH 2 and incubated at 40 °C. Enzymic hydrolyses were carried out at pH 6 by addition of 1 unit of myrosinase μmol^{-1} of glucosinolate, followed by incubation at 40 °C for 1 h. Samples were taken at various times during this incubation and the intact glucosinolates analyzed by HPLC (Tiedink et al., 1990). The stationary phase was Hypersil APS (3 × 200 mm; Chrompack B.V., Middelburg, The Netherlands) with 0.01 M H_3PO_4, pH 4.5 (flow of 0.4 mL min^{-1}) as mobile phase. Glucosinolates were detected by UV absorption at 220–227 nm.

Nitrite–Glucosinolate Reaction As Detected by HPLC–Photohydrolysis. Solutions of intact and myrosinase-treated glucosinolates (1 unit of enzyme μmol^{-1} of glucosinolate, 1-h incubation at pH 6 and 40 °C) were adjusted to pH 2. A solution of $NaNO_2$ was added (final concentration 40 mM) and the pH again adjusted to pH 2. The resulting solutions were incubated in the dark for 15 min at 37 °C, nitrosation reactions stopped by addition of ammonium sulfamate ($NH_2SO_3NH_4$, final concentration 48 mM), and the NOC analyzed by HPLC with photohydrolysis detection (PHD). The HPLC conditions were the same as used in the preceding section. The HPLC–PHD system, described elsewhere (Shuker and Tannenbaum, 1983), was modified according to the procedure of Tiedink et al. (1989). NOC are cleaved by light (400–420 nm) to nitrosyl radicals, which form nitrite in the aqueous medium. The nitrite subsequently reacts with Griess reagent to yield an azocompound which is measured spectrophotometrically at 546 nm. This systems does not allow quantitative measurements, since the percentage of breakdown by the high-intensity discharge lamp varies according to the nature of the *N*-nitroso compound, but it is suitable for screening solutions for the presence of nonvolatile NOC.

Table I. Percentage of Breakdown of Glucosinolates 2 h after the Addition of Acid (37 °C, Final pH 2) and 30 min after the Addition of Myrosinase (1 Unit μmol^{-1} Glucosinolate, 40 °C, Final pH 6)

compound	treatment with acid	treatment with myrosinase
glucosinalbin	25	100
brassicanapin	0	100
gluconasturtiin	5	100
glucobrassicin	17	100
4-hydroxyglucobrassicin	45	56
glucotropaeolin	0	100
gluconapin	0	100
progoitrin	15	100
sinigrin	1	100

Determination of Total *N*-Nitroso Content. Glucosinolate samples containing NOC after nitrosation were also subjected to thermal energy analysis [Thermal Energy Analyzer (TEA, Thermo Electron Corp., Waltham, MA)] to determine total NOC content (Walters et al., 1978). The method is based on the chemical denitrosation of NOC by HBr/HAc in refluxing ethyl acetate (EtAc); nitrosyl radicals thus formed are carried in a nitrogen atmosphere to the TEA reaction chamber. Pollutants are prevented from entering the reaction chamber by cold traps. A correction for responses of compounds other than NOC, for example, *C*-nitroso compounds and nitrite (Pignatelli et al., 1987), was made by carrying out parallel analyses in boiling EtAc containing HCl/HAc.

To study the possible catalytic effects of glucosinolate breakdown products on the rate of formation of NOC, proline was nitrosated in the absence/presence of the appropriate products. Aqueous solutions of myrosinase-treated glucosinolates (final concentration 413–494 μM), or distilled water, were added to proline solutions (437 μM) and treated with nitrite (0–60 min) as described under Nitrite–Glucosinolate Reaction As Detected by HPLC–Photohydrolysis. Samples were removed at various times and analyzed for their total NOC content.

Mutagenicity Assay. The *Salmonella typhimurium* (*S. typhimurium*) assay was performed with tester strain TA100 without S9 mix as reported by Ames et al. (1975), with minor modifications as described by van der Hoeven et al. (1983). Solutions of either intact or hydrolyzed glucosinolates both before and after nitrosation (0.1–1.0 mL), bacterial suspension (0.1 mL, OD 0.4 at 700 nm), and Vogel–Bonner solution (0.5 mL) were added to an agar solution (1%, 2.5 mL). The volume was adjusted with distilled water to 4.1 mL and the mixture poured on Petri dishes containing minimum (histidine-free) medium; acidic samples were observed to have no significant effect on the pH of the overlayer agars. After incubation of the plates at 37 °C (48–72 h), the number of revertants was counted automatically by using an Autocount (Artek Systems Corp., Framingdale, NY). The assay was performed at least twice (independent experiments) for each glucosinolate, triplicate plates being used for each sample. The data presented in Figure 3 represent the average values of one representative experiment. A sample was considered to be mutagenic when the number of revertants induced following nitrite treatment was at least twice the number of revertants in the blank (which had not been treated with nitrite).

RESULTS AND DISCUSSION

In Table I the hydrolyses of glucosinolates both by acid and by myrosinase are shown. While almost half of the

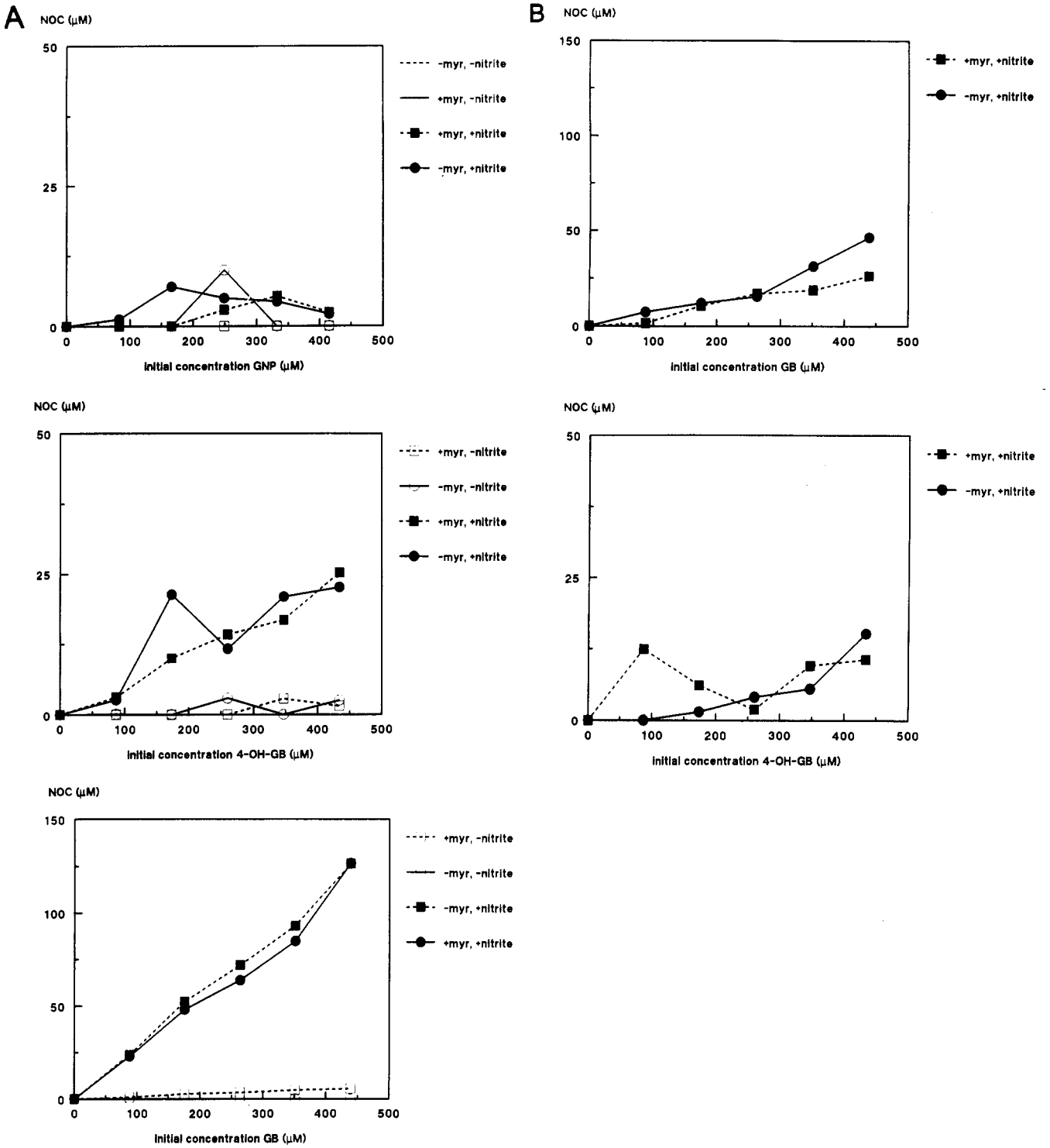

Figure 2. (A) Total *N*-nitroso determination of intact and myrosinase-treated glucosinolates after nitrite treatment. (B) Control measurements in HCl/HAc. GNP, gluconapin; GB, glucobrassicin; 4-OH-GB, 4-hydroxyglucobrassicin.

4-hydroxyGB was hydrolyzed by acid within 2 h, only small amounts of SAL, NST, GB, and PGT and none of BNP, GTP, GNP, and SGN were hydrolyzed by acid. With the exception of the 4-hydroxyindole compound, all glucosinolates were hydrolyzed by myrosinase within 0.5 h. Because of the low purity of the available sample (40%), it is possible that impurities present may have inhibited myrosinase-mediated hydrolysis. This possibility will be examined when sufficiently pure 4-hydroxyGB becomes available.

The ability of intact and hydrolyzed glucosinolates to form NOC following nitrite treatment was examined by using the HPLC–PHD system. Intact GB, 4-hydroxyGB, and GNP and hydrolyzed GB and 4-hydroxyGB exhibited positive responses, indicative of the presence of NOC. To quantify the amounts of NOC formed from these compounds, they were treated with nitrite and the total NOC content was measured by TEA. The results of these experiments with different (initial) glucosinolate concentrations are shown in Figure 2A. It was noted that GNP did not form significant amounts of NOC; the traces observed may possibly originate from residual nitrite. While excess sulfamate was added to scavenge the unreacted nitrite, the presence of trace amounts cannot be excluded. This result contrasts with those of the HPLC–PHD experiment, in which GNP gave a response after nitrite treatment. Given the structural similarity (Figure 1) of GNP to SIN and BNP, both of which do not form NOC, it may be suggested that the observed responses of GNP on the HPLC–PHD system were false positives.

Following nitrite treatment both intact and myrosinase-treated GB and intact and myrosinase-treated 4-hydroxyGB formed significant amounts of NOC ($p < 0.01$). About 25% of the myrosinase-treated GB, 24% of the intact GB, 5% of the myrosinase-treated 4-hydroxyGB, and 5% of the intact 4-hydroxyGB were nitrosated (Figure 2A). The nitrosation rates of the intact and myrosinase-treated glucosinolates were thus equivalent. The nitrosation rate of the myrosinase-treated GB was intermediate between those of indole-3-acetonitrile (29%) and indole-3-carbinol (18%) (Tiedink et al., 1991), two of the breakdown products of GB (Fenwick et al., 1983). It is probable that the actual percentage of nitrosation of 4-hydroxyGB is higher, given the crude nature of the sample employed.

TEA measurements were also performed by using HCl/HAc in boiling EtAc in place of HBr/HAc to correct for responses of compounds other than NOC. Both intact and myrosinase-treated GB and 4-hydroxyGB gave significant responses upon nitrosation ($p < 0.01$, $p < 0.01$, $p = 0.03$, and $p = 0.02$, respectively) (Figure 2B). The responses in HCl/HAc ranged between 21% and 44% of the responses in HBr/HAc. These results suggest that part of the amounts of NOC calculated from the HBr/HAc measurements did not originate from NOC and that hence the levels of NOC would need to be corrected. However, it is known that the nitrosated products of indole-3-acetonitrile, indole-3-carbinol, and indole are unstable under acidic conditions (Tiedink et al., 1990). Since GB is the precursor of these indole compounds, it is possible that the nitrosated products of GB and 4-hydroxyGB are labile in EtAc containing HCl/HAc. For this reason the levels of NOC formed from GB and 4-hydroxyGB in Figure 2A are not corrected.

The results presented here demonstrate that only intact and myrosinase-treated indolyl glucosinolates form NOC upon nitrosation. The correlation previously found (Tiedink et al., 1988) between the amounts of NOC formed in extracts of brassica vegetables following nitrite treatment and their aryl/alkyl glucosinolate content does not thus appear to reflect a causal relationship.

Thiocyanate is a product of the myrosinase-catalyzed breakdown of indolyl glucosinolates (Fenwick et al., 1983) and has been reported to be a catalyst for the nitrosation reaction (Boyland et al., 1971). It may also be possible that other breakdown products of glucosinolates catalyze this reaction, and hence the nitrosation rate of proline was examined in the presence and absence of myrosinase-treated aryl and alkyl glucosinolates. Since the breakdown products of indolyl glucosinolates also form NOC, which interfere with the *N*-nitrosoproline measurements, the catalytic effects of the breakdown products of GB and 4-hydroxyGB could not be determined. No obvious catalytic effect of the breakdown products of the tested alkyl/aryl glucosinolates was apparent. The correlation previously found between the amounts of NOC formed in vegetable extracts upon nitrosation and their alkyl/aryl glucosinolate content cannot thus be ascribed to catalytic effects of breakdown products of these glucosinolates.

Finally the mutagenic activity of nitrosated products of intact and myrosinase-treated glucosinolates was examined; Figure 3 shows the mutagenic activity of the indolyl glucosinolates to *S. typhimurium* TA100. Only myrosinase-treated GB showed direct mutagenic activity upon nitrosation, this being observed from the first point of measurement. Given that intact GB forms similar amounts of NOC to that of myrosinase-treated GB, the possibility exists that steric hindrance prevents alkylation of DNA. Because of its low nitrosation rate, the concentrations of 4-hydroxyGB examined may well have been too low to detect mutagenic activity.

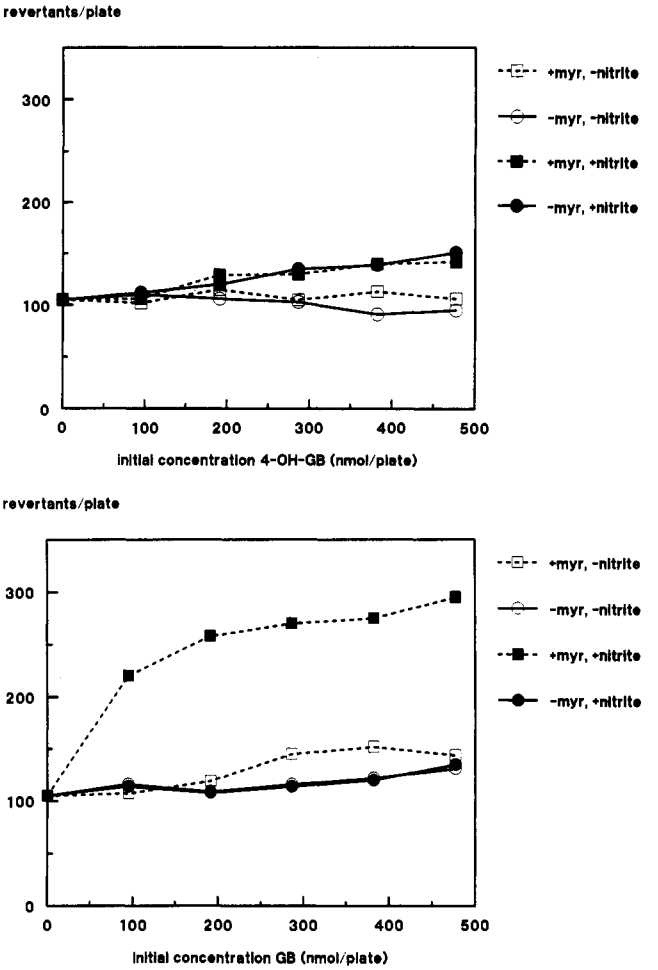

Figure 3. Mutagenicity of intact and myrosinase-treated glucobrassicin and 4-hydroxyglucobrassicin following nitrite treatment. Number of revertants in positive control [1 μg of 1-methyl-1-nitroso-3-nitroguanidine (MNNG)/plate], >2000; SD of triplicate plates, <15 revertants.

Of the brassica vegetables examined in a previous study (Tiedink et al., 1988), Chinese cabbage [*Brassica pekinensis* (Lour.) Rupr.] induced the highest amounts of revertants (326 25 mg^{-1} dry weight). The Chinese cabbage did not contain detectable amounts of 4-hydroxyGB, while the level of GB was 1.3 nmol mg^{-1} dry weight. Wakabayashi et al. (1985) have found indole-3-acetonitrile to be an important precursor of NOC in Chinese cabbage; it can be deduced from Figure 3 that the contribution of nitrosated breakdown products of the low level of GB to the total mutagenicity of Chinese cabbage upon nitrite treatment is negligible. These findings accord with those from a study of the role of several indole compounds in the formation of NOC in green cabbage. It was found that the indole compounds made a negligible contribution to the total mutagenicity of green cabbage following nitrite treatment (Tiedink et al., 1990).

None of the alkyl/aryl glucosinolates examined here showed mutagenic activity upon nitrite treatment. However, all alkyl/aryl glucosinolates were found to be cytotoxic upon myrosinase-treatment irrespective of whether or not they were nitrosated. Fenwick et al. (1983) discussed the cytotoxicity of different isothiocyanates to *S. typhimurium* TA100, and it is possible that the cytotoxicity found in this study originated partly from isothiocyanate formation.

The current study demonstrates that the correlation previously found between the amounts of NOC formed in extracts of cruciferous vegetables and their glucosinolate content (Tiedink et al., 1988) does not reflect a causal

relationship. Thus, the chemical natures both of the precursors of the NOC in cruciferous vegetables and of the biologically active compounds themselves remain unclear. Further investigations are needed to clarify these areas and to determine the significance, if any, of the findings reported here and elsewhere to human health and well-being.

ABBREVIATIONS USED

Sinigrin (SGN); gluconapin (GNP); glucobrassicanapin (BNP), progoitrin (PGT); glucotropaeolin (GTP); glucosinalbin (SAL); gluconasturtiin (NST); glucobrassicin (GB); 4-hydroxyglucobrassicin (4-hydroxyGB); *N*-nitroso compounds (NOC); high-performance liquid chromatography (HPLC); photohydrolysis detection (PHD); thermal energy analyzer (TEA); retention time (rt).

ACKNOWLEDGMENT

This study was financially supported by the Directory for Nutrition and Quality affairs of the Ministry of Agriculture, Nature Management and Fisheries of the Netherlands, and by the U.K. Ministry of Agriculture, Fisheries and Food (MAFF). We thank Mr. J. A. R. Davies (CABO) for technical assistance on *N*-nitroso compound measurements.

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Received for review July 9, 1990. Revised manuscript received November 16, 1990. Accepted December 6, 1990.

Registry No. Glucosinalbin, 19253-84-0; brassicanapin, 132912-08-4; gluconasturtiin, 499-30-9; glucobrassicin, 4356-52-9; 4-hydroxyglucobrassicin, 83327-20-2; glucotropaeolin, 499-26-3; gluconapin, 19041-09-9; progoitrin, 585-95-5; sinigrin, 3952-98-5; nitrite, 14797-65-0.