Initial and Final Products, Nitriles, and Ascorbigens Produced in Myrosinase-Catalyzed Hydrolysis of Indole Glucosinolates

Niels Agerbirk,† Carl Erik Olsen, and Hilmer Sørensen*

Chemistry Department, Royal Veterinary and Agricultural University, Thorvaldsensvej 40, DK-1871 Frederiksberg C, Denmark

Micellar electrokinetic capillary chromatography (MECC) was used to follow the myrosinase (*β*-thioglucoside glucohydrolase EC 3.2.3.1)-catalyzed transformation of glucobrassicin (indol-3-ylmethylglucosinolate, **1a**) and neoglucobrassicin (*N*-methoxyglucobrassicin, **1b**) into nitriles, ascorbigens, and other products. The influence of pH, ascorbic acid, and Fe(II) ions was investigated. In the presence of ascorbic acid, (5 mM), thiocyanate ion and ascorbigens were the dominating products from **1a** and **1b**. In the presence of Fe(II) ions (2.5 mM), nitriles were the dominating products between pH 4 and 6−7. During hydrolysis of **1b** in neutral or weakly basic solution, an unstable intermediate was detected by MECC. Comparisons of the rate of ascorbigen formation from **1a**, **1b**, and indol-3-ylcarbinol showed that ascorbigens were formed directly from ascorbate and unstable products of the hydrolysis of indole glucosinolates and that indol-3-ylcarbinols were not important intermediates. Structures of **1a**, **1b**, and products of **1b** were confirmed by ^{1}H NMR, MS, and UV spectroscopy.

Keywords: *Indole glucosinolate; myrosinase; ascorbigen; isothiocyanate; nitrile*

INTRODUCTION

The products of the myrosinase (*β*-thioglucoside glucohydrolase, EC 3.2.3.1)-catalyzed hydrolysis of indole glucosinolates (indol-3-ylmethylglucosinolates) have attracted attention for a number of reasons: as precursors of ascorbigens and thiocyanate ion in crushed *Brassica* vegetables (Kutácek et al., 1960; Gmelin and Virtanen, 1961, 1962; Kiss and Neukom, 1966); as precursors of phytoalexins (Monde et al., 1994) or plant hormones (Rausch et al., 1983) in infected plants; and as possible antinutritional and anticarcinogenic constituents of oilseed rape and *Brassica* vegetables (Bradfield and Bjeldanes, 1987a,b; McDanell et al., 1987, 1988; Sørensen, 1990; Jensen et al., 1991; Loft et al., 1992). Glucobrassicin (indol-3-ylmethylglucosinolate, **1a**) and neoglucobrassicin (*N*-methoxyglucobrassicin, **1b**) are common constituents of rape and various cabbage and kale species (McDanell et al., 1988).

There is conflicting evidence of the relative abundance of the several types of hydrolysis products of indole glucosinolates (Bjergegaard et al., 1994) (Figure 1). While some authors found that indol-3-ylcarbinols were the dominant products of indole glucosinolates from autolysis of crushed *Brassica* vegetables (Bradfield and Bjeldanes, 1987a; Slominski and Campbell, 1989), other authors found that ascorbigens were the dominant products (McDanell et al., 1987; Preobrazhenskaya et al., 1993). The poor stability of indol-3-ylcarbinol (**5a**) in acidic solution could be one explanation of the conflicting evidence (Bradfield and Bjeldanes, 1987a), and use of extraction methods unsuitable for ascorbigens could be another (Preobrazhenskaya et al., 1993). As ascorbigen (**4a**) may be formed from reaction between **5a** and ascorbic acid in vitro (Kiss and Neukom, 1966), indol-3-ylcarbinols could possibly be the initial products of the hydrolysis of indole glucosinolates, forming ascorbigens and indole oligomers (Grose and Bjeldanes, 1992, Agerbirk et al., 1996) by further reactions. However, intermediacy of **5a** in the formation of **4a** during autolysis has not been proved, and it has been suggested that indol-3-ylmethyl isothiocyanate (**3a**) is the reactive intermediate during autolysis (Preobrazhenskaya et al., 1993; Bjergegaard et al., 1994).

It is well established that indol-3-ylacetonitriles are important products of the nonenzymatic degradation of indole glucosinolates during cooking (Slominski and Campbell, 1989), but their importance as products of myrosinase-catalyzed hydrolysis has been debated. In vitro myrosinase-catalyzed hydrolysis of **1a** gave a high molar yield of indol-3-ylacetonitrile (**2a**) near pH 3 (Gmelin and Virtanen, 1961; Latxague et al., 1991), but the molar yield of **2a** at pH values relevant for plant tissue (pH 5−6) was 1% or below (Schraudolf and Weber, 1969; Searle et al., 1982). While nitriles were the dominant glucosinolate products from autolysis (pH 5−6) of aliphatic glucosinolates in cabbage (Daxenbichler et al., 1977), only low molar yields of indol-3-ylacetonitriles were reported from autolysis of several *Brassica* vegetables (Slominski and Campbell, 1989). The high yield of nitriles from autolysis of aliphatic glucosinolates in fresh vegetables was presumably due to the effect of endogenous Fe(II) ion (Van Etten and Tookey, 1979). A stimulation of nitrile formation from myrosinase-catalyzed hydrolysis of **1a** was reported, but the stimulation was surprisingly weak, as the presence of Fe(II) ions increased the molar yield of **2a** to only 3% at pH 4−6 (Searle et al., 1982).

The myrosinase-catalyzed hydrolysis of the substituted indole glucosinolate **1b** seems to follow the same pattern as known for **1a**, including formation of ascorbigens (Gmelin and Virtanen, 1962; Hanley et al., 1990),

* Corresponding author (tel., +45 35 28 24 32; fax, +45 35 28 20 89).

† Current adress: Danisco Biotechnology, Langebrogade 1, DK-1001 Copenhagen K, Denmark.

S0021-8561(97)00849-2 CCC: $15.00
Published on Web 04/03/1998

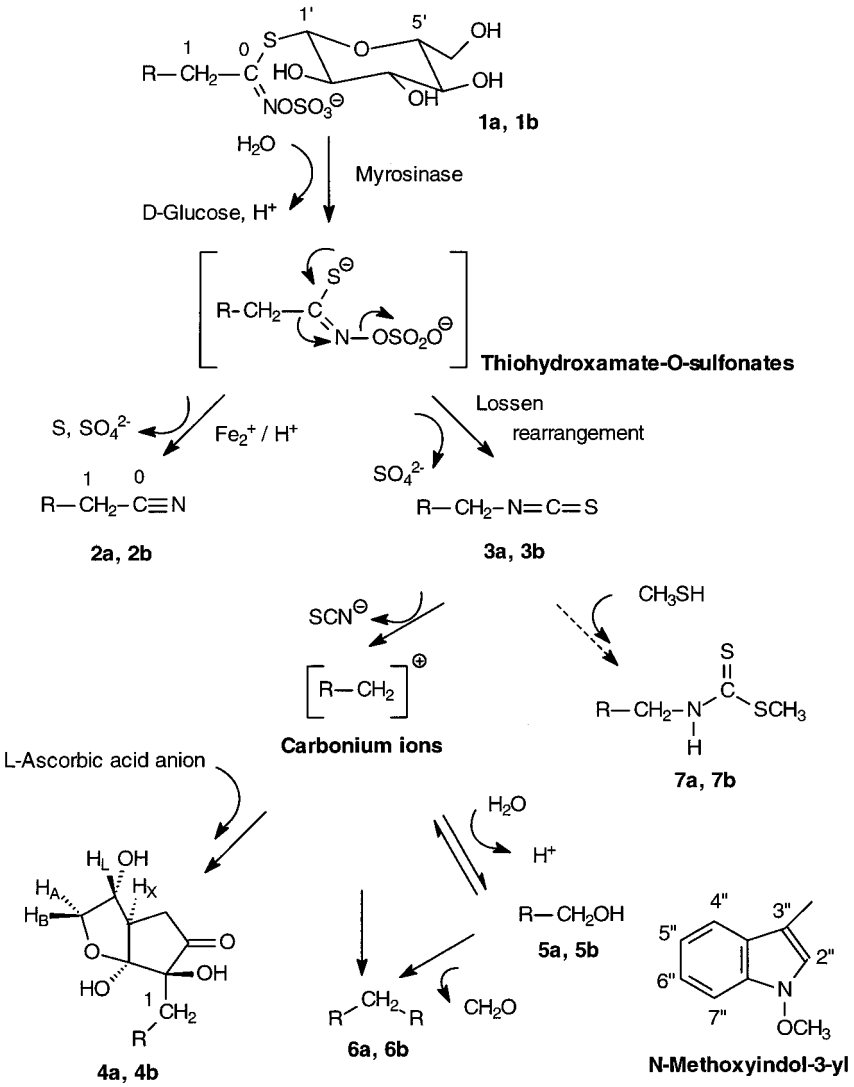

Figure 1. Products of the myrosinase-catalyzed hydrolysis of glucobrassicin (**1a**) and neoglucobrassicin (**1b**). The numbering system in reported NMR spectra is indicated. Key to numbers: **2a**, indol-3-ylacetonitrile; **3a**, indol-3-ylmethyl isothiocyanate; **4a**, ascorbigen (A-stereoisomer is shown); **5a**, indol-3-ylcarbinol; **6a**, di(indol-3-yl)methane; **7a**, brassinin; **2b**, *N*-methoxy-**2a**, etc.; **6b**, di(*N*-methoxyindol-3-yl)methane; **7b**, *N*-methoxybrassinin. The formation of 2,3-bis(indol-3-ylmethyl)indole from **5a** and **6a** is not shown (Agerbirk et al., 1996).

but there are no reports of detailed variation of the reaction conditions.

We have previously developed analytical techniques for the determination of intact indole glucosinolates (Michaelsen et al., 1992; Feldl et al., 1994a), as well as both their monomeric and oligomeric hydrolysis products (Feldl et al., 1994a; Agerbirk et al., 1996) and the thiocyanate ion (Bjergegaard et al., 1995b).

In the present study, the stimulation of nitrile formation by Fe(II) ions was reinvestigated. The stimulation was found to be significantly higher than previously reported by Searle et al. (1982).

In addition, the yields of indol-3-carbinols, oligomers, and ascorbigens from myrosinase-catalyzed hydrolysis of **1a** and **1b** were determined in the presence or absence of ascorbic acid. Ascorbigens were found to be the dominant products. It was shown that indol-3-ylcarbinols do not transiently accumulate during the formation of ascorbigens from indole glucosinolates, and that indol-3-ylcarbinols are not important intermediates in the formation of ascorbigens at the pH values of crushed plant tissue (pH 5−6) (Schraudolf and Weber, 1969; Daxenbichler et al., 1977).

MATERIALS AND METHODS

Reference Compounds and Reagents. Potassium thiocyanate (photographic grade), ammonium iron(II)sulfate, *N*-acetylcysteine, **5a**, and **2a** were obtained from Sigma (St. Louis, MO). Allyl isothiocyanate was from Aldrich-Chemie (Steinham, Germany). L-Ascorbic acid was from Bie og Berntsen (Rødovre, Denmark). Allylglucosinolate (sinigrin) and but-3-enylglucosinolate (gluconapin) were from the collection of reference compounds of this laboratory (Sørensen, 1990). **6a** was synthesized as previously described (Agerbirk et al., 1996). All other reagents were from the laboratory collection of pro analysi grade chemicals.

Potassium thiocyanate was dried in a vacuum before weighing. UV: 193 nm (max), 215 nm (shoulder), $\epsilon_{220} = 3.1 \times 10^3$ M^{-1} cm^{-1}.

4a was synthesized by reaction of **5a** with L-ascorbic acid. L-Ascorbic acid (1.2 g) and **5a** (1.0 g) were dissolved in 250

mL of water and left for 2 h at room temperature. Soluble **4a** was separated from precipitated **6a** by filtration. The aqueous phase was neutralized with NaOH and extracted with 3 × 75 mL of ethyl acetate. After evaporation of the solvent, the product was dried in a vacuum. Yield: 0.61 g (29%) of **4a** (red, amorphous solid) with a trace of ascorbic acid. Structure confirmed by ^{1}H NMR, UV, and MS (vide infra). MECC showed that only one dominant stereoisomeric ascorbigen was formed (Feldl et al., 1994b).

Spectroscopy. UV spectra were measured in quartz cuvettes on a Shimadzu MPS-2000 UV−visible light spectrophotometer. UV data are reported in the following way: λ_{max} (relative intensity). ^{1}H NMR spectra were recorded at 250 MHz on a Bruker AC250P NMR spectrometer. In deuterated chloroform and methanol, chemical shifts (δ) are relative to internal TMS; in water, chemical shifts are relative to dioxane (3.75 ppm) unless otherwise stated. NMR data are reported in the following way: chemical shift δ_H/ppm (relative intensity, multiplicity, coupling constant *J*, assignment).

Mass spectra were recorded on a JEOL AX505W mass spectrometer. EI spectra were measured at 70 eV and at a source temperature of 240 °C. MS data are reported in the following way: *m*/*z* value, assignment (relative abundance). The direct inlet mass spectra of **4a** and **4b** and to a lesser degree **2b** revealed the presence of major impurities.

MECC. MECC of glucosinolates, thiocyanate ion, and indolylic monomeric hydrolysis products was performed as described by Feldl et al. (1994a) but with UV detection at 220 nm. The pH of the buffer was adjusted to 7.0 before the addition of 1-propanol, at 80% of the final volume. In some experiments the pH of the buffer was adjusted to 8.0 instead of 7.0, to slow the hydrolysis of **3b** during the electrophoresis time. To separate **1a** and **2a**, the temperature was in some cases increased to 60 °C. MECC of indolyl oligomers was performed as described by Agerbirk et al. (1996), with UV detection at 220 nm. Satisfactory peak shape of **4a** and **4b** was only accomplished at one set of conditions: DTAB 30 °C.

Response Factors. The response factor of thiocyanate ion was determined by MECC (DTAB, 30 °C) of solutions of pure KSCN. Molar response factors of indole derivatives at 220 nm in MECC (DTAB, 30 °C) relative to **2a** were measured in three different ways: (1) from the relative magnitude of ϵ_{220}, assuming identical ϵ_{280} of monomeric indole compounds (Feldl et al., 1994a); (2) from UV spectroscopy (280 nm) and MECC of pure compounds; and (3) from the normalized areas of substrate and product peaks in MECC, using an argument of stoichiometry. Standards of **5a** and **5b** were dissolved in phosphate buffer pH 8, and the compounds were stable at this pH. All reported yields of monomeric indole derivatives obtained with the DTAB method were calculated using the response factor found for **2a**. With the cholate method, yields of **5b** and **6b** were calculated from response factors obtained with standards of **5a** and **6a** (Agerbirk et al., 1996).

Determination of SCN^- in the presence of Fe(II) and Fe(III) ions. The influence of the reaction conditions on the quantitative determination of thiocyanate ion by MECC was tested. Neither Fe(II) ions, Fe(III) ions, ascorbic acid, the various buffer solutions used for glucosinolate hydrolysis (pH 3.5, 4, 5.6, or 7), nor strongly acidic or strongly basic conditions influenced the determination.

Glucosinolates. **1a** and **1b** were purified from frozen broccoli (*Brassica oleracea* L. var. *botrytis* L. subvar. *cymosa* Lam.) bought in a food store, by a procedure modified from Thies (1988) and Visentin et al. (1992). While nitrate, formiate, and the aliphatic glucosinolate 4-(methylsulfinyl)butylglucosinolate were eluted from the ion exchange column with the salt front, **1b**, 4-methoxyglucobrassicin and **1a** adsorbed more strongly to the column material and were eluted in the order mentioned. Fractions pure in **1b** and in **1a** were obtained and desalted, and they contained 22 μmol (34% yield) of **1a** and 10 μmol (20% yield) of **1b**, as potassium salts (Agerbirk, 1997). The purity was tested by ^{1}H NMR and quantitative UV spectroscopy, by HPLC and MECC of desulfoglucosinolates (EU, 1990; Bjergegaard et al., 1995a), and by MECC of intact glucosinolates (Michaelsen et al., 1992).

Table 1. Influence of Neoglucobrassicin (1b) Concentration and Ascorbic Acid on the Products of Myrosinase-Catalyzed Hydrolysis of 1b at pH 5.6[a]

conditions		products (% of initial [**1b**])		
[**1b**] (mM)	asc. ac	**5b**	**6b**	sum of indolyl groups in **5b** and **6b**
2.1		17	12	41
0.60		42	26	94
0.43	+	4	0.6	5
0.35	+	4	2	8
0.33		73	7	87
0.13		67	4	75

[a] The analysis method included indole oligomers, but ascorbigens could not be determined quantitatively. Organic solvent (1-propanol and acetonitrile, see text) was added after the complete hydrolysis of **1b**, and the mixture was analyzed by the MECC−cholate method. In some experiments, 5 mM ascorbic acid was present during the hydrolysis, as indicated with a "+" in the column "asc. ac.".

From MECC and NMR, **1b** contained 7% **1a** and 2% 4-methoxyglucobrassicin, and **1a** contained 5% **1b** and 4% 4-methoxyglucobrassicin. The apparent ϵ_{280} around 5000 M^{-1} cm^{-1} of the indole glucosinolate preparations indicated a low content of inorganic salts etc. (Feldl et al., 1994a).

Myrosinase was purified, characterized, and assayed by use of previously described procedures (Michaelsen et al., 1991) from seeds of yellow mustard (*Sinapis alba* L. cv. Albatros), obtained from Trifolium A/S (Roskilde, Denmark). The myrosinase for use in glucosinolate degradation experiments was dissolved in the con A elution buffer and stored until use at 4 °C.

Glucosinolate Hydrolysis. Purified glucosinolate (10−100 nmol) was dissolved in 200 μL of the appropriate buffer, and 2−4 μL of myrosinase solution (6 units/mL) was added. The mixture was analyzed by MECC before the addition of myrosinase and by repeated injections after the addition of myrosinase. The reported yields of the thiocyanate ion were corrected for a trace of thiocyanate ion in the glucosinolate preparation.

Glucosinolate hydrolysis at all the reported conditions was followed by repeated MECC analyses. The temperature of the hydrolysis mixture was 30 °C, which was the temperature of the autosampler. Before the measurements reported in Table 1, but after the complete hydrolysis of **1b**, 1-propanol−acetonitrile (3:2) was added to 16%, in an attempt to keep the oligomers in solution. In Table 1, the analysis result of the first measurement (10 min after the addition of myrosinase) is reported, and it was confirmed by additional measurements that the concentrations of both **5b** and **6b** were unchanged during longer incubation. Formation of the *N*-methoxyindol-3-ylmethylpropyl ether (neoIPE; Agerbirk et al., 1996) was insignificant when 1-propanol was added after the complete hydrolysis of **1b**.

The analysis result after 30−60 min, or after the complete disappearance of the unstable intermediate **3b**, is reported in Table 2. Some experiments were repeated, and representative results are reported. For the analysis of indole oligomers, addition of organic solvent was necessary to keep the hydrophobic oligomers in solution (Agerbirk et al., 1996).

The buffers for glucosinolate hydrolysis were either 200 mM Tris-HCl (pH 7.2), 200 mM Na_2HPO_4−NaH_2PO_4 (pH 7 (100 mM) or pH 8 (200 mM)), 50 mM maleic acid−sodium maleate (pH 6), 100 or 200 mM acetic acid−sodium acetate (pH 5.6 or 4), or 100 mM formic acid−sodium formiate (pH 3.5). The Fe(II) ion was added as $FeSO_4$ or $(NH_4)_2Fe(SO_4)_2$. Phosphate buffer was avoided whenever iron salts were added. Tris buffer was avoided whenever reaction with isothiocyanates could occur. Cysteine was added as the hydrochloride. Buffers containing reducing compounds were prepared fresh. The final pH of the hydrolysis buffers with added reagents was measured and adjusted by titration with NaOH.

Purification of Hydrolysis Products: 2b. **1b** (1 μmol) was hydrolyzed by myrosinase in aqueous 5 mM cysteine, 2.5

Table 2. Yields of Thiocyanate Ion and Monomeric Indolyl Compounds from Myrosinase-Catalyzed Hydrolysis of 1a and 1b in the Presence of 5 mM Ascorbic Acid[a]

conditions		products (% of initial [glucosinolate])			
pH	glucosinolate (concn/mM)	SCN^-	**2a**	**4a**	**5a**
7.0	**1a** (0.67)	61	tr.	27	2.5
5.6	**1a** (0.33)	73	tr.	37	3.0
4.0	**1a** (0.63)	59	2.7	36	2.7
			2b	**4b**	**5b**
7.0	**1b** (1.30)	80	<1.5	34	13
5.6	**1b** (1.30)	94	<1.2	38	8
4.0	**1b** (0.58)	84	<4.3	36	9

[a] The pH was varied as indicated. At pH 7, phosphate buffer was used, at pH 5.6 and 4, acetate buffer was used. MECC conditions: DTAB (30 °C).

mM $FeSO_4$, 100 mM AcOH−NaOH (pH 4), yielding SCN^-, **5b**, and 0.7 μmol of **2b**. Upon incubation overnight, **5b** disappeared from the solution. After centrifugation, the supernatant was neutralized with Na_2HPO_4, and iron phosphate removed by centrifugation. **2b** was extracted with diethyl ether, yielding 0.3 μmol of **2b**. The product was pure by MECC, NMR, and MS, except for some aliphatic hydrocarbons detectable in MS and NMR, and a small impurity giving rise to an ion at 530 *m/z* in the EI mass spectrum.

4b. 1b (0.7 μmol) was hydrolyzed by myrosinase in a pH 4 acetate buffer with 5 mM L-ascorbic acid. Extraction with ethyl acetate gave 0.2 μmol of **4b**, which was characterized by NMR and MS.

Unknown Products 1 and 2. The unknown reaction products of *N*-acetylcysteine and hydrolysis products of **1b** were prepared as described in Results and loaded on a Sephadex DEAE A-25 column (acetate). The unknowns were eluted with 0.2 M potassium sulfate, and individual fractions were analyzed by MECC and UV spectroscopy.

RESULTS

Figure 1 illustrates the myrosinase-catalyzed hydrolysis of the indole glucosinolates, at the various conditions used in the present work. The mechanisms of formation of ascorbigens (**4a** and **4b**) and of **6b**, which were deduced from the time course experiments (vide infra), are shown.

Response Factors of Glucosinolates and Their Products (Conditions: DTAB, 30 °C). The molar response factor of thiocyanate ion was 0.10 relative to the molar response factor of IAN.

From their nearly similar ϵ_{220}, the molar response factors of most monomeric indole derivatives were assumed to be similar. When the response factors of the glucosinolates were calculated from the amount of thiocyanate ion produced, the indole glucosinolates seemed to have the same response factors as the other investigated indole derivatives. When the response factors were measured by MECC and UV spectroscopy of purified compounds, **4a** and an impure preparation of **5b** had significantly lower molar response factors than **2a**, while pure **5a** had exactly the same response factor as **2a**. A lower response factor for **4a** than for other indole derivatives was possibly due to poor chromatographic peak shape (Figure 2). Thus, the data as a whole suggested that the response factors of monomeric indole derivatives, with the possible exception of **4a** and **4b**, were roughly similar. All reported yields in Table 2 were calculated using the response factor found for **2a**, meaning that reported yields of ascorbigens may be moderately underestimated.

Characterization of Myrosinase. Myrosinase used in this study was isolated from seeds of *Sinapis alba*. The native myrosinase behaved like the dimeric myrosinase isolated from seeds of *Brassica napus* during gel filtration. IEF indicated that the myrosinase preparation consisted of a number of isoenzymes, with p*I* values from 5.6 to 6.2. The specific activity (Michaelsen et al., 1991) of the purified enzyme was 5 units/mg. The molecular weight of the subunits was 73 kDa as determined by reducing SDS−PAGE. The lower pH limit of activity of the enzyme was near pH 3.5. It hydrolyzed both allyl- and but-3-enylglucosinolate to the corresponding isothiocyanates at neutral pH, as shown by conversion of the products of hydrolysis into the corresponding substituted thiourea compounds in 70−80% of the theoretical yields (results not shown). In the experiments with **1a** and **1b** reported below, total hydrolysis of the glucosinolates was always observed at the end of each incubation (e.g. Figure 2A).

Initial Products from Indole Glucosinolate Hydrolysis. In the presence of Fe(II) ions at 2.5 mM, which were protected from oxidation by 5 mM cysteine added to the buffers, the indol-3-ylacetonitriles were the dominant products formed at pH 4 and 5.6, and were formed to a considerable extent even in neutral solution (Figures 2 and 3). The yield of thiocyanate ion was lowered quantitatively in response to nitrile formation, except for a few deviating observations (Figure 3). The formation of the indol-3-ylacetonitriles was dependent on both myrosinase and Fe(II) ions, as no degradation of the glucosinolate was detectable during incubations of the glucosinolates in the reaction buffer with cysteine and Fe(II) ions, but without myrosinase.

The identity of **2b** was confirmed by various spectroscopic methods (Table 3), while the identities of **2a** and thiocyanate ion were confirmed by spiking with reference compounds.

In the absence of Fe(II) ions, the yield of nitriles was low at pH higher than 4. Extraction of the isolated preparation of **1a** with ethyl acetate prior to the hydrolysis resulted in an apparent lowering of the yield of **2a** between pH 4 and 7, suggesting that the majority of the yield of **2a** and **2b** reported here (Figure 3), in the absence of Fe(II) ions, was due to contamination of the glucosinolate preparations with small amounts of the corresponding nitrile.

Thiocyanate ion, **5a**, **5b**, or indolyl oligomers were in general detected immediately following the hydrolysis of the indole glucosinolates in the absence of ascorbic acid (Figure 4B; Table 1). **6b** was formed even at pH 5.6, where **5b** was stable. The identities of **5a** and **6a** were confirmed by spiking with reference compounds, while the identity of **5b** and **6b** were tentatively confirmed by MS, UV spectroscopy, and instability of **5b** in dilute acid (results not shown). Taken together, the yields of **5b** and **6b** accounted for the majority of the hydrolyzed glucosinolate (except for the highest concentration, where precipitation probably occurred), but the relative amounts depended on the initial concentration of glucosinolate (Table 1).

An Unstable Intermediate from Hydrolysis of Neoglucobrassicin (1b). Under certain conditions, we saw exceptions to the immediate formation of thiocyanate ion and **5b** from **1b** in the absence of ascorbic acid. In phosphate buffer at pH 7, a trace of an unstable intermediate was detected by MECC (DTAB 30 °C). The intermediate was not detected at lower pH. In phosphate buffer pH 8, the intermediate was more promi-

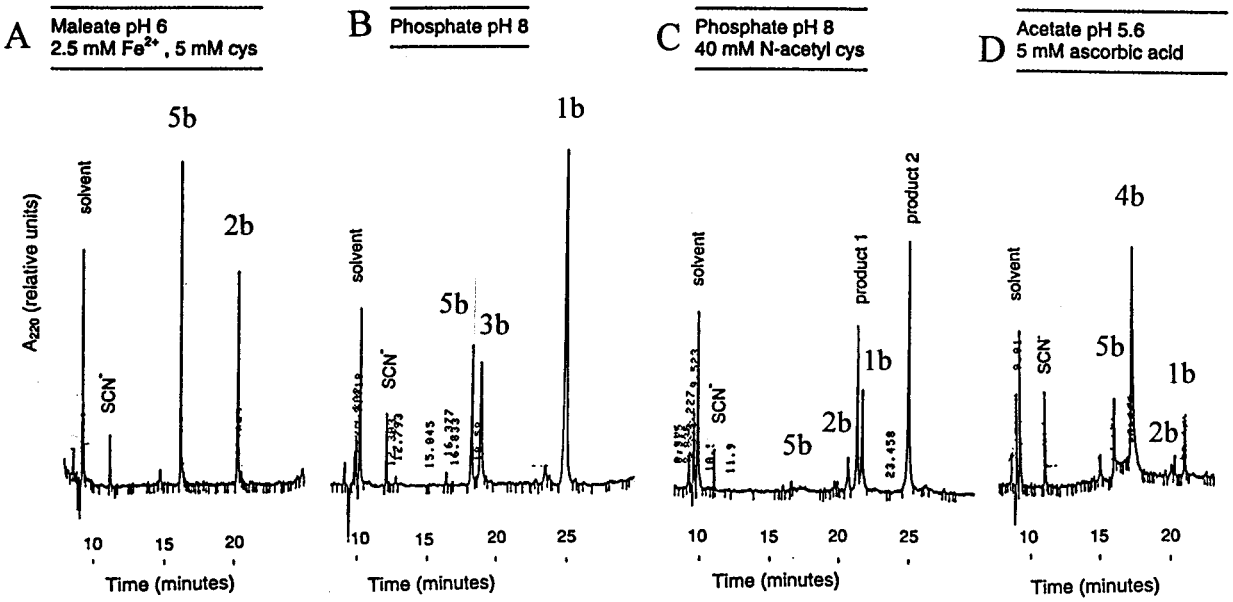

Figure 2. MECC chromatograms of neoglucobrassicin (**1b**) and its hydrolysis products, showing the effect of the reaction conditions on the products formed. Small, unlabeled peaks represent the corresponding products from hydrolysis of contaminating **1a**. (A–D) Reaction conditions as indicated.

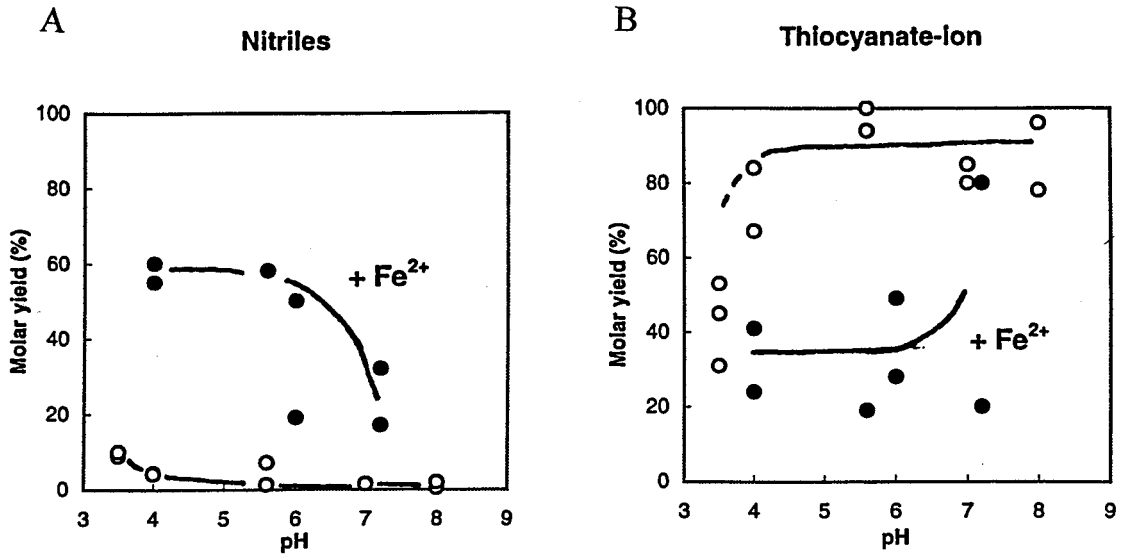

Figure 3. Influence of pH and Fe(II) ions on molar yields of (A) nitriles A and (B) thiocyanate ion from hydrolysis of glucobrassicin (**1a**) and neoglucobrassicin (**1b**). Data from both **1a** and **1b** hydrolyses were used.

nent. If the pH of the MECC buffer was likewise changed to pH 8, the peak representing the unstable intermediate was further enlarged (Figure 2B). A study of the time course of the formation of the carbinol, the thiocyanate ion, and the unstable intermediate (Figure 4A) suggested that the intermediate could be *N*-methoxyindol-3-ylmethyl isothiocyanate (**3b**). **1a** was also degraded at pH 8 to investigate whether an unstable intermediate would appear during the degradation of this glucosinolate, but in this case the carbinol was the first product detected (Figure 4B).

In Tris buffer (400 mM, pH 8), the intermediate was not observed, the molar yield of **5b** was reduced from 31 to 11%, while the yield of thiocyanate ion was unaltered. This result indicated a reaction between the intermediate and the amine Tris. Similarly, in the presence of the thiol *N*-acetylcysteine (at 10, 40, or 200 mM), two new products were formed instead of **5b** (Figure 2C). After purification, the two unknown products were characterized by UV spectroscopy. The UV spectrum of the unknown product **2** was similar to the reported UV spectrum of **7b** (Takasugi et al., 1988).

Formation of Ascorbigens. When ascorbic acid was present in the hydrolysis mixture at 5 mM, the yields of both **5b** and **6b** were reduced 10-fold or more (Table 1). When the hydrolysis into monomeric indole derivatives was followed by MECC with DTAB as surfactant, the yields of the ascorbigens from both **1a** and **1b** was found to be higher than the yields of **5a** and **5b** (Table 2). Formation of oligomers was not quantitatively detected in this experiment, as organic solvent was not added to the reaction mixture. Yields of oligomers are therefore not reported in Table 2. The identity of **4a** was confirmed by spiking with an authentic standard, while the identity of **4b** was tentatively confirmed by MS, NMR, and UV spectroscopy (Table 3). The molar yields of the ascorbigens were always ca. 3–10-fold higher than the molar yields of indol-3-ylcarbinols, even at pH values where the latter

Table 3. Spectroscopic Data of Indole Glucosinolates and Purified Hydrolysis Products of 1b (Spectra of Authentic Hydrolysis Products of 1a Are Shown in Addition for Comparison)

glucobrassicin (**1a**)
UV (H_2O): 219 nm (100), 270 nm (shoulder, 15), 279 nm (16), 286 nm (shoulder, 13)
^1H NMR (D_2O, int. st. H_2O at 4.75 ppm): 2.94 (1 H, d tr, $J_{5'-4'} = 10$ Hz, $J_{5'-6'} = 4$ Hz, 5′-H), 3.15−3.4 (3 H, m, 2′-H, 3′-H, 4′-H), 3.57 (2H, d, 4 Hz, 6′-H), 4.18 (1H, d, 16 Hz, 1-H_a or 1-H_b), 4.29 (1H, d, 16 Hz, 1-H_a or 1-H_b), 4.81 (1H, d, 10 Hz, 1′-H), 7.20 (1H, tr, 8 Hz, 5″-H), 7.28 (1H, tr, 8 Hz, 6″-H), 7.34 (1H, s, 2″-H), 7.54 (1H, d, 8 Hz, 7″-H), 7.75 (1H, d, 8 Hz, 4″-H)
neoglucobrassicin (**1b**)
UV (H_2O): 221 nm (100), 274 nm (14), 288 nm (14)
^1H NMR (D_2O): 2.99 (1H, d tr, $J_{5'-4'} = 10$ Hz, $J_{5'-6'} = 4$ Hz, 5′-H), 3.15−3.4 (3H, m, 2′-H, 3′-H, 4′-H), 3.55 (2H, irregular doublet, 4 Hz, 6′-H), 4.11 (3H, s, OCH_3), 4.16 (1H, d, 16 Hz, 1-H_a or 1-H_b), 4.25 (1H, d, 16 Hz, 1-H_a or 1-H_b), 4.81 (1H, d, 10 Hz, 1′-H), 7.25 (1H, tr, 8 Hz, 5″-H), 7.37 (1H, tr, 8 Hz, 6″-H), 7.53 (1H, s, 2″-H), 7.59 (1H, d, 8 Hz, 7″-H), 7.77 (1H, d, 8 Hz, 4″-H)
indol-3-ylacetonitrile (**2a**)
UV (H_2O): 216 nm (100), 270 nm (16), 277 nm (17), 286 nm (12)
^1H NMR ($CDCl_3$): 3.83 (1H, d, 1 Hz, 1-H), 7.18 (1H, tr, 8 Hz, 5″-H), 7.25 (1H, s, 2″-H), 7.26 (1H, tr, 8 Hz, 6″-H), 7.40 (1H, d, 7″-H, 8 Hz), 7.59 (1H, d, 4″-H, 8 Hz), 8.2 (1H, broad s, NH)
N-methoxyindol-3-ylacetonitrile (**2b**)
UV (H_2O): 219 nm (100), 270 nm (18), 283 nm (18), 295 (shoulder)
EI-MS *m/z* (%): 186, M^+ (87); 155, $(M - OCH_3)^+$ (100); 128, $(M - OCH_3 - HCN)^+$ (49)
^1H NMR ($CDCl_3$): 3.81 (2H, s, 1-H), 4.09 (3H, s, OCH_3), 7.17 (1H, tr, 8 Hz, 5″-H), 7.31 (1H, tr, 8 Hz, 6″-H), 7.32 (1H, s, 2″-H), 7.45 (1H, d, 8 Hz, 7″-H), 7.56 (1H, d, 8 Hz, 4″-H), and additional long-range couplings between aromatic protons
ascorbigen (**4a**)
EI-MS *m/z* (%): 305, M^+ (10); 130, $(M-C_6O_6H_7)^+$ (55); 116, $(M-C_6O_6H_7\text{-}CH_2)^+$ (100)
^1H NMR (CD_3OD): 3.22 (1H, d, $J_{ab} = 14$ Hz, 1-H_a or 1-H_b), 3.40 (1H, d, $J_{ab} = 14$ Hz, 1-H_a or 1-H_b), 3.77 (1H, s, ($J_{LX} < 1$ Hz), H_X), 3.98 (1H, dd, $J_{AB} = 10$ Hz, $J_{AL} = 3.1$ Hz, H_A), 4.12 (1H, dd, $J_{AB} = 10$ Hz, $J_{BL} = 5.6$ Hz, H_B), 4.20 (1H, dd, $J_{BL} = 5.6$ Hz, $J_{AL} = 3.1$ Hz, ($J_{LX} < 1$ Hz), H_L), 6.97 (1H, tr, 8 Hz, 5″-H), 7.06 (1H, tr, 8 Hz, 6″-H), 7.19 (1H, s, 2″-H), 7.31 (1H, d, 8 Hz, 7″-H), 7.62 (1H, d, 8 Hz, 4″-H), and additional long-range couplings in aromatic system and 2″-H and 1-H_a/H_b
neoascorbigen (**4b**)
EI-MS *m/z* (%): 335, M^+ (13); 160, $(M-C_6O_6H_7)^+$ (100)
^1H NMR (CD_3OD, int. std. TMS): 3.20 (1H, d, 14 Hz, 1-H_a or 1-H_b), 3.89 (1H, s, H_X), 4.05 (3H, s, OCH_3), 7.02 (1H, tr, 8 Hz, 5″-H), 7.17 (1H, tr, 8 Hz, 6″-H), 7.33 (1H, s, 2″-H), 7.36 (1H, d, 8 Hz, 7″-H), 7.61 (1H, d, 8 Hz, 4″-H), some peaks from impurities were also present and additional long-range couplings between aromatic protons were seen; signal at 3.20 ppm (1-H) showed coupling to a proton further downfield, which seemed to be covered by the signal of the solvent at 3.3 ppm
unknown product **1**
UV (H_2O): ca. 220 nm (shoulder, 100), 270 nm (18), 290 nm (shoulder, 14)
unknown product **2**
UV (H_2O): 218 (100), 250 (shoulder, 36), 269 (39)

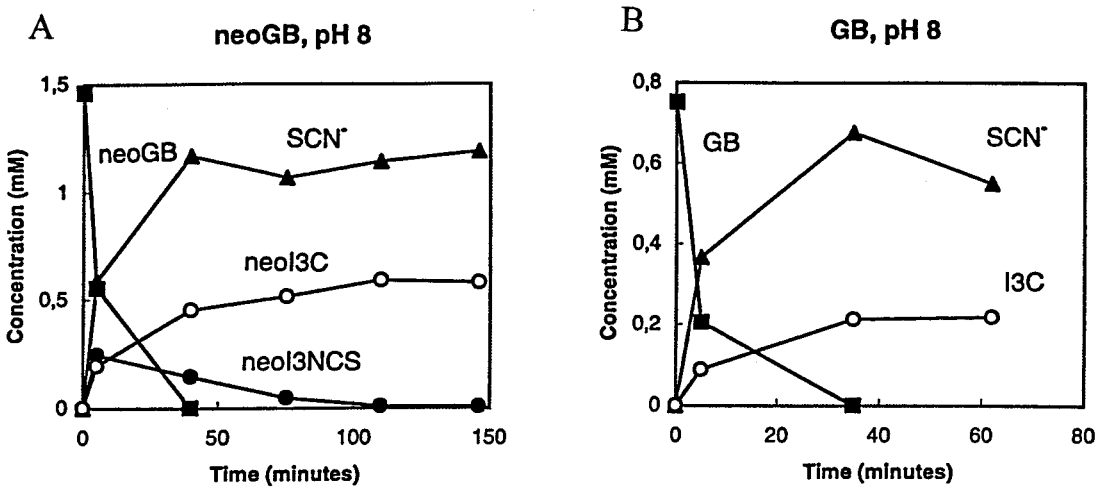

Figure 4. Time course of hydrolysis of (A) neoglucobrassicin (**1b**) and (B) glucobrassicin (**1a**) at pH 8, illustrating the formation and disappearance of an unstable intermediate from **1b** hydrolysis. No such intermediate was detected from hydrolysis of **1a**. MECC conditions: DTAB 30 °C, pH 8.

are perfectly stable (**5a**, pH 7; **5b**: pH 5.6 and 7; Agerbirk et al. (1996) and unpublished results).

The formation of ascorbigens from **1a** and **1b** was followed by repeated analysis of the reaction mixture during the glucosinolate hydrolysis. The ascorbigens were formed immediately after glucosinolate hydrolysis, without prior accumulation of the indol-3-ylcarbinols as intermediates (Figure 5A−C). To test whether **5a** could be short-lived intermediate in ascorbigen formation, the formation of **4a** from pure **5a** and ascorbic acid was investigated (Figure 5D). **4a** was not formed from **5a** and ascorbic acid at pH 7, but it was formed relatively slowly at pH 5.6, and was formed rapidly at pH 4.

DISCUSSION

Myrosinase. Myrosinase used in the present investigation was isolated from seeds of *S. alba* and tested by standard procedures (Michaelsen et al., 1991). From the subunit molecular weight and extraction method, it seemed to be a member of the so-called MA or Myr 1 family of myrosinases (Xue, 1994; Bones and Rossiter, 1996).

Identification of Glucosinolates and Hydrolysis Products. The identifications of **1a** and **1b** by chromatography and spectroscopy were unambiguous and were in agreement with previously reported NMR spectra of indole glucosinolates (Hanley et al., 1990;

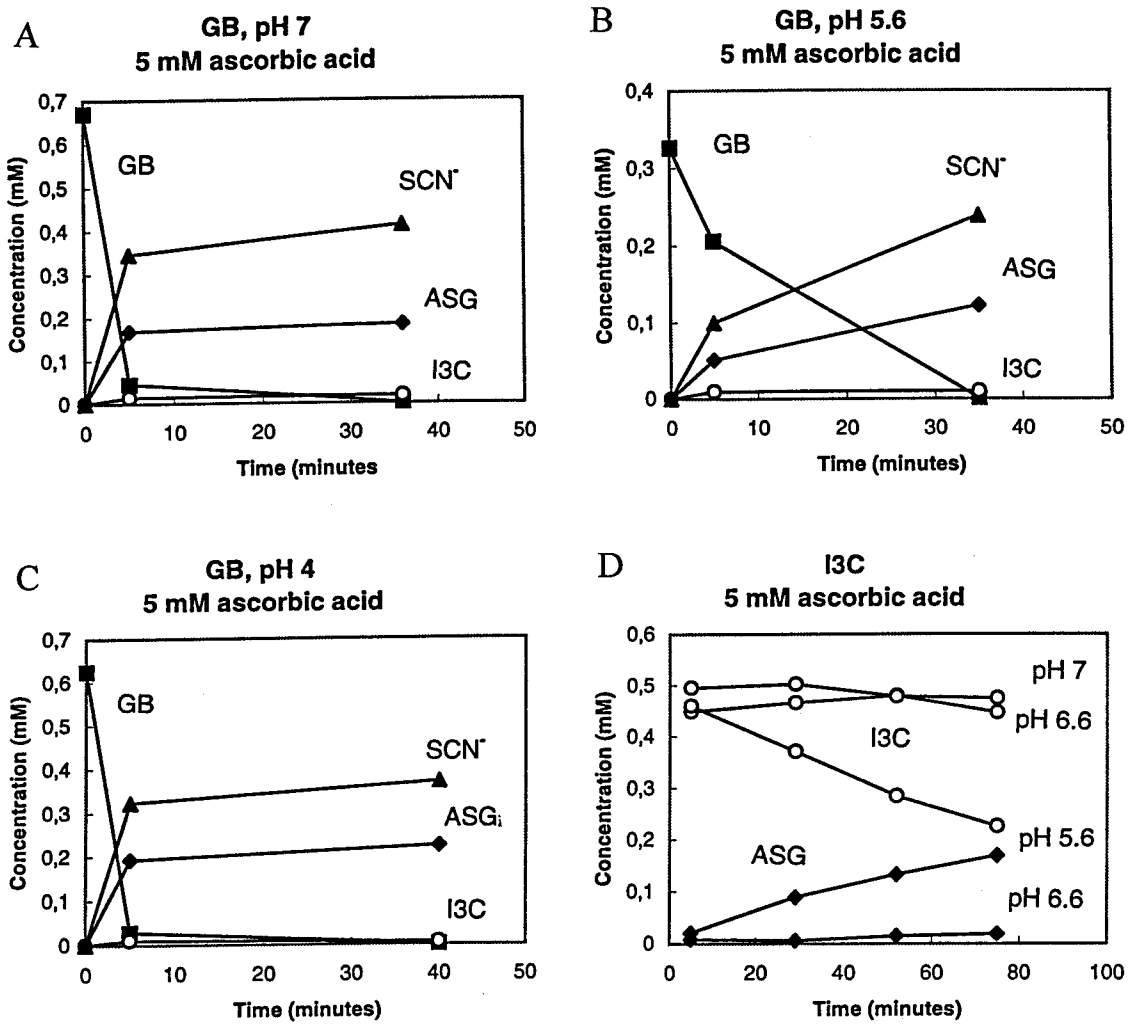

Figure 5. Time course of the formation of ascorbigen (**4a**) from glucobrassicin (**1a**) (A−C) or from indol-3-ylcarbinol (**5a**) (D) at varying pH, showing that **5a** was not an important intermediate in the formation of **4a**.

Viaud et al., 1992). The identification of **2b** was likewise unambiguous. The position of the methoxy group at the heteroatom was supported by the absence of an NH signal in the NMR spectrum of **2b** compared to the spectrum of **2a**.

The identification of **4b** was tentative and was based on the mass spectrum, analogy with the formation of **4a**, and an NMR spectrum showing major impurities. In support of the proposed structure, a doublet representing one of the $C(1)H_2$ protons was detected, while the other seemed to be masked by the solvent signal. The coupling constant of this doublet (14 Hz) was in agreement with the corresponding coupling constant of **4a** and a published NMR spectrum of synthetic **4b** (Muchanov et al., 1994). Signals of the *N*-methoxyindol-3-yl group were unambiguously identified. A singlet representing H_X of the ascorbic acid residue was detected with δ 3.89 (δ 3.79 reported by Muchanov et al. (1994)), while the expected multiplets of H_A, H_B, and H_L of the ascorbic acid residue were not clearly identified, due to the very low amount of **4b** analyzed.

General Reaction Mechanism and Formation of Nitriles. The well-established general mechanism of glucosinolate hydrolysis involves hydrolysis of the β-thioglucoside bond by myrosinase. Nonenzymatic degradation of the aglucone, a thiohydroxamate-*O*-sulfonate or thiohydroxamic acid-*O*-sulfonate depending on pH, yields either an isothiocyanate via Lossen rearrangement, or a nitrile without rearrangement (Ettlinger and Lundeen, 1956, 1957; Ettlinger et al., 1961; Miller, 1965; Saarivirta, 1973; Uda et al., 1986; Iori et al., 1996). The formation of either nitrile or isothiocyanate, depending on pH and the presence or absence of Fe(II) ions, is consistent with this mechanism.

In the case of the indole glucosinolates, rearrangement to the isothiocyanate was proved by isolation of **3b** from myrosinase-catalyzed hydrolysis of **1b** at "low water conditions" (Hanley et al., 1990) and by trapping **3a** by methyl thiolate, forming the phytoalexin **7a** (Monde et al., 1994). The reported increased formation of nitrile at low pH was also in agreement with hydrolysis of indole glucosinolates to the thiohydroxamate-*O*-sulfonate, while the atypical, low stimulation of nitrile formation by Fe(II) ions was unexpected. The strong stimulation of nitrile formation from myrosinase-catalyzed hydrolysis of **1a** and **1b** reported here (Figure 4), at pH 4−7 in the presence of Fe(II) ions, supports that the primary product of the myrosinase-catalyzed hydrolysis of a (substituted) indole glucosinolate is a (substituted) indol-3-ylmethylthiohydroxamate-*O*-sulfonate, analogous to the hydrolysis of aliphatic glucosinolates.

The role of the Fe(II) ions in the formation of nitriles is not fully understood, but interference with the Lossen rearrangement caused by affinity of Fe(II) ions for the sulfide group of the primary product is one possible

explanation. Control experiments without myrosinase showed that the formation of nitriles was dependent on both Fe(II) ions and myrosinase, excluding Fe(II)-catalyzed hydrolysis of the glucosinolates to be significant at our conditions.

The very low stimulation of **2a** formation by Fe(II) ions reported by Searle et al. (1982), can be attributed to the use of a citrate−phosphate buffer, which will complex-bind Fe(II) ions. Due to a probable contamination of our glucosinolate preparations with trace amounts of the corresponding nitriles, the yields reported here in the absence of Fe(II) ions are maximum estimates but do not contradict much lower yields reported by other workers (Schraudolf and Weber, 1969; Searle et al., 1982).

Formation of so-called abnormal hydrolysis products (Benn, 1977) depends on additional proteins or factors (Hasapis and MacLeod, 1982; Cole, 1980). Formation of organic thiocyanates (known for allyl-, benzyl-, and 4-(methylthio)butylglucosinolates only) seems to be associated with formal ability to form a resonance-stabilized carbonium ion upon formal loss of thiocyanate ion (Lüthy and Benn, 1977). The indole glucosinolates could thus a priori be able to form organic thiocyanates. The fact that allylglucosinolate formed the corresponding isothiocyanate in high yield upon hydrolysis catalyzed by our myrosinase preparation (result not shown) showed that the purified myrosinase did not contain factors with ability to change the product to organic thiocyanates. Involvement of indol-3-ylmethyl thiocyanates in the formation of the various products of indole glucosinolate hydrolysis can thus be excluded in our experiments.

Formation of Ascorbigens. The time course investigations of ascorbigen formation from either **5a** or from myrosinase-catalyzed hydrolysis of **1a** or **1b**, suggested that **5a** and **5b** were not important as intermediates in the formation of **4a** and **4b** (Figure 5). Instead, the ascorbigens may have been formed from reaction between either the isothiocyanate (vide infra) or the carbonium ion, in competition with the formation of indol-3-ylcarbinols from reaction with water (Figure 1). This reaction mechanism was also suggested on a theoretical basis (Preobrazhenskaya et al., 1993; Bjergegaard et al., 1994). At lower pH, where the isothiocyanate is less stable and the carbinol more reactive, it could not be concluded from the time course experiments whether **5a** was important as a reaction intermediate. In any case, neither **5a** nor **5b** accumulated during glucosinolate hydrolysis at low pH (pH 4) prior to ascorbigen formation (Figure 5 C).

Unstable Intermediate from Neoglucobrassicin (1b). On the basis of the previous isolation of **3b** from hydrolysis of **1b** at "low water conditions" (Hanley et al., 1990) and the formation of allyl isothiocyanate, not allyl thiocyanate, from allylglucosinolate by our myrosinase preparation, we suggest that the reaction intermediate observed during hydrolysis of **1b** at pH 8 could be the isothiocyanate (Figures 2 and 5A). The high reactivity of the intermediate with nucleophilic compounds supports this suggestion. The stabilizing influence of the *N*-methoxy group of **3b** (Hanley et al., 1990) explains our inability to detect the corresponding isothiocyanate from **1a** (Figure 4).

The existence of the short-lived isothiocyanate intermediate also at lower pH is suggested from the observed immediate formation of **6b** from **1b** hydrolysis, at conditions were **5b** is stable (Table 1). The isothiocyanates—and not the carbinols—may also be the true precursors of the carbonium ion intermediate during ascorbigen formation, as suggested from the time course of the formation of **4a** and **4b** (vide supra).

ABBREVIATIONS USED

DTAB, dodecyltrimethylammonium bromide; EI, electron impact, MECC, micellar electrokinetic capillary chromatography; Tris, tris(hydroxymethyl)aminomethane.

ACKNOWLEDGMENT

Sandro Palmieri is gratefully acknowledged for a stay of one of us (N.A.) at Istituto Sperimentale per le Colture Industriali, Bologna, Italy. The skilled technical assistance of S. Cinti, K. Mortensen, J. Hallick, and P. Møller is acknowledged.

LITERATURE CITED

Agerbirk, N. Products of the myrosinase catalysed hydrolysis of glucosinolates in *Brassica* vegetables. Thesis, Royal Veterinary and Agricultural University, Frederiksberg, Denmark, 1997; pp 159−174.

Agerbirk, N.; Bjergegaard, C.; Olsen, C. E.; Sørensen, H. Kinetic investigations of the transformations of indol-3-ylcarbinol into oligomeric compounds based on micellar electrokinetic capillary chromatography. *J. Chromatogr. A* **1996**, *745*, 239−248.

Benn, M. Glucosinolates. *Pure Appl. Chem.* **1977**, *49*, 197−210.

Bjergegaard, C.; Li, P. W.; Michaelsen, S.; Møller, P.; Otte, J.; Sørensen, H. Glucosinolates and their transformation products−compounds with a broad biological activity. *Proc. Int. Euro Food Tox IV Conference*; H. Kozlowska, H., Fornal, J., Zdunczyk, Z., Eds.; 1994; Vol. I, pp 1−15 (ISBN 83-901475-0-5).

Bjergegaard, C.; Michaelsen, S.; Møller, P.; Sørensen, H. Separation of desulphoglucosinolates by micellar electrokinetic capillary chromatography based on a bile salt. *J. Chromatogr. A* **1995a**, *717*, 325−333.

Bjergegaard, C.; Møller, P.; Sørensen, H. Determination of thiocyanate, iodide, nitrate and nitrite in biological samples by micellar electrokinetic capillary chromatography. *J. Chromatogr. A* **1995b**, *717*, 409−414.

Bones, A. M.; Rossiter, J. T. The myrosinase−glucosinolate system, its organisation and biochemistry. *Physiol. Plant.* **1996**, *97*, 194−208.

Bradfield, C. A.; Bjeldanes, L. F. High-performance liquid chromatographic analysis of anticarcinogenic indoles in *Brassica oleracea*. *J. Agric. Food Chem.* **1987a**, *35*, 46−49.

Bradfield, C. A.; Bjeldanes, L. F. Dietary modification of xenobiotic metabolism: contribution of indolylic compounds present in *Brassica oleracea*. *J. Agric. Food Chem.* **1987b**, *35*, 896−900.

Cole, R. Volatile components produced during ontogeny of some cultivated crucifers. *J. Sci. Food Agric.* **1980**, *31*, 549−557.

Daxenbichler, M. E.; Van Etten, C. H.; Spencer, G. F. Glucosinolates and derived products in cruciferous vegetables. Identification of organic nitriles from cabbage. *J. Agric. Food Chem.* **1977**, *25*, 121−124.

Ettlinger, M. G.; Lundeen, A. J. The structures of sinigrin and sinalbin; an enzymatic rearrangement. *J. Am. Chem. Soc.* **1956**, *78*, 4172−4173.

Ettlinger, M. G.; Lundeen, A. J. First synthesis of a mustard oil glucoside; the enzymatic Lossen rearrangement. *J. Am. Chem. Soc.* **1957**, *79*, 1764−1765.

Ettlinger, M. G.; Dateo, G. P., Jr.; Harrison, B. W.; Mabry, T. J.; Thompson, C. P. Vitamin C as a coenzyme: The hydrolysis of mustard oil glucosides. *Proc. Natl. Acad. Sci. U.S.A.* **1961**, *47*, 1875−1880.

EU. Determination of glucosinolates in oilseeds by liquid chromatography (HPLC). *J. Eur. Communities* **1990**, *L 170*, (03.07), 27−34.

Feldl, C.; Møller, P.; Otte, J.; Sørensen, H. Micellar electrokinetic capillary chromatography for determination of indolyl glucosinolates and transformation products thereof. *Anal. Biochem.* **1994a**, *217*, 62−69.

Feldl, C.; Møller, P.; Olsen, C. E.; Otte, J.; Sørensen, H. Structure and properties of ascorbigens and other transformation products of indolyl glucosinolates; potential anticancerogens. *GCIRC Bull.* **1994b**, *10*, 128−133.

Gmelin, R.; Virtanen, A. I. Glucobrassicin, der Precursor von SCN^-, 3-Indolylacetonitril und Ascorbigen in *Brassica oleracea* Species. *Ann. Acad. Scientiarum Fennicae, Ser. A, II. Chem.* **1961**, *107*, 1−25.

Gmelin, R.; Virtanen, A. I. Neoglucobrassicin, ein zweiter SCN^--Precursor vom Indoltyp in *Brassica*-Arten. *Acta Chem. Scand.* **1962**, *16*, 1378−1384.

Grose, K. R.; Bjeldanes, L. F. Oligomerisation of indole-3-carbinol in aqueous acid. *Chem. Res. Toxicol.* **1992**, *5*, 188−193.

Hanley, A. B.; Parsley, K. R.; Lewis, J. A.; Fenwick, G. R. Chemistry of indole glucosinolates: Intermediacy of indol-3-ylmethyl isothiocyanates in the enzymic hydrolysis of indole glucosinolates. *J. Chem. Soc., Perkin Trans. 1*, **1990**, 2273−2276.

Hasapis, X.; MacLeod, A. J. Benzyl glucosinolate degradation in heat treated *Lepidium sativum* seeds and detection of a thiocyanate forming factor. *Phytochemistry* **1982**, *21*, 1009−1013.

Iori, R.; Rollin, P.; Streicher, H.; Thiem, J.; Palmieri, S. The myrosinase−glucosinolate interaction mechanism studied using some synthetic competitive inhibitors. *FEBS Lett.* **1996**, *385*, 87−90.

Jensen, S. K.; Michaelsen, S.; Kachlicki, P.; Sørensen, H. 4-Hydroxyglucobrassicin and degradation products of glucosinolates in relation to unsolved problems with the quality of double low oilseed rape. *Proc. 8th. Int. Rapeseed Congr.* **1991**, 1359−1364.

Kiss, G.; Neukom, H. Über die Struktur des Ascorbigens. *Helv. Chim. Acta* **1966**, *49*, 989−992.

Kutácek, M.; Procházka, Z.; Grünberger, D. Biogenesis of ascorbigen, 3-indolyl acetonitril and indole-3-carboxylic acid from D,L-tryptophan-3-^{14}C in *Brassica oleraceae* L. *Nature* **1960**, *187*, 61−62.

Latxague, L.; Gardrat, C.; Coustille, J. L.; Viaud, M. C.; Rollin, P. Identification of enzymatic degradation products from synthesised glucobrassicin by gas chromatography−mass spectrometry. *J. Chromatogr.* **1991**, *585*, 166−170.

Loft, S.; Otte, J.; Poulsen, H. E.; Sørensen, H. Influence of intact and myrosinase-treated indolyl glucosinolates on the metabolism *in vivo* of metronidazole and antipyrine in the rat. *Food Chem. Toxicol.* **1992**, *30*, 927−935.

Lüthy, J.; Benn, M. H. Thiocyanate formation from glucosinolates: a study of the autolysis of allylglucosinolate in *Thlaspi arvense* L. seed flour extracts. *Can. J. Biochem.* **1977**, *55*, 1028−1031.

McDanell, R.; McLean, A. E. M.; Hanley, A. B.; Heaney, R. K.; Fenwick, G. R Differential induction of mixed-function oxidase (MFO) activity in rat liver and intestine by diets containing processed cabbage: Correlation with cabbage levels of glucosinolates and glucosinolate hydrolysis products. *Food Chem. Toxicol.* **1987**, *25*, 5, 363−368.

McDanell, R.; McLean, A. E. M.; Hanley, A. B.; Heaney, R. K.; Fenwick, G. R Chemical and biological properties of indole glucosinolates (glucobrassicins): A review. *Food Chem. Toxicol.* **1988**, *26*, 59−70.

Michaelsen, S.; Mortensen, K.; Sørensen, H. Myrosinases in *Brassica*: Characterisation and properties. *Proc. 8th Int. Rapeseed Congr.* **1991**, 905−910.

Michaelsen, S.; Møller, P.; Sørensen, H. Factors influencing the separation and quantitation of intact glucosinolates and desulphoglucosinolates by micellar electrokinetic capillary chromatography. *J. Chromatogr.* **1992**, *608*, 363−374.

Miller, H. E. The aglucone of sinigrin. Thesis, Rice University, Houston, Texas, 1965.

Monde, K.; Takasugi, M.; Ohnishi, T. Biosynthesis of cruciferous phytoalexins. *J. Am. Chem. Soc.* **1994**, *116*, 6650−6657.

Muchanov, V. I.; Kaganski, M. M.; Sorokin, A. A.; Antonian, S. G.; Tananova, G. V.; Mikhailevskaya, L. L.; Kinsirski, A. S.; Preobrazhenskaya, M. N. Neoascorbigen and its analogues: Synthesis and study. *Khim.-Farm. Zh.* **1994**, *28* (7), 6−10.

Preobrazhenskaya, M. N.; Bukhman, V. M.; Korolev, A. M.; Efimov, S. A. Ascorbigen and other indole-derived compounds from Brassica vegetables and their analogues as anticarcinogenic and immunomodulating agents. *Pharmac. Ther.* **1993**, *60*, 301−313.

Rausch, T.; Butcher, D. N.; Hildenberg, W. Indol-3-methylglucosinolate biosynthesis and metabolism in clubroot diseased plants. *Physiol. Plant.* **1983**, *58*, 93−100.

Saaravirta, M. The formation of benzylcyanide, benzylthiocyanate, benzylisothiocyanate and benzylamine from benzylglucosinolate in *Lepidium*. *Planta Med.* **1973**, 24, 112−119.

Schraudolf, H.; Weber, H. IAN-Bildung aus Glucobrassicin: pH Abhängigkeit und wachstumsphysiologische Bedeutung. *Planta* **1969**, *88*, 136−143.

Searle, L. M.; Chamberlain, K.; Rausch, T.; Butcher, D. N. The conversion of 3-indolylmethylglucosinolate to 3-indolylacetonitrile by myrosinase, and its relevance to the clubroot disease of the Cruciferae. *J. Exp. Bot.* **1982**, *33*, 935−942.

Slominski, B. A.; Campbell, L. D. Formation of indole glucosinolate breakdown products in autolyzed, steamed and cooked *Brassica* vegetables. *J. Agric. Food Chem.* **1989**, *37*, 1297−1302.

Sørensen, H. Glucosinolates: Structure−Properties−Function. In *Canola and Rapeseed: Production, Chemistry, Nutrition and Processing Technology*; Shahidi, F., Ed.; Van Nostrand Reinhold: New York, 1990; ISBN 0-442-00295-5.

Takasugi, M.; Monde, K.; Katsui, N.; Shirata, A. Novel Sulfur-Containing Phytoalexins from the Chinese Cabbage *Brassica campestris* L. ssp. *pekinensis* (Cruciferae). *Bull. Chem. Soc. Jpn.* **1988**, *61*, 285−289.

Thies, W. Isolation of sinigrin and glucotropaeolin from cruciferous seeds. *Fat Sci. Technol.* **1988**, *90*, 311−314.

Uda, Y.; Kurata, T.; Arakawa, N. Effects of pH and ferrous ion on the degradation of glucosinolates by myrosinase. *Agric. Biol. Chem.* **1986**, *50*, 2735−2740.

Van Etten, C. H.; Tookey, H. L. Chemistry and Biological Effects of Glucosinolates. In *Herbivores: Their interaction with secondary plant metabolites*; Rosenthal, G. A., Janzen, D. H., Applebaum, S. W., Eds.; Academic Press: San Diego, CA, 1979.

Viaud, M.-C.; Rollin, P.; Latzague, L.; Gardrat, C. Synthetic studies on indole glucosinolates. Part 1. Synthesis of glucobrassicin and its 4- and 5-methoxy derivatives. *J. Chem. Res. (miniprint)* **1992**, 1664−1672.

Visentin, M.; Tava, A.; Iori, R.; Palmieri, S. Isolation and identification of *trans*-4-(methylthio)-3-butenyl glucosinolate from radish roots (*Raphanus sativus* L.). *J. Agric. Food Chem.* **1992**, *40*, 1687−1691.

Xue, J. Molecular studies of myrosinase in Brassicaceae−from protein to gene and gene to protein. Dissertation, Sveriges Lantbruksuniversitet, Uppsala, Sweden, 1994; ISBN 91-576-4848-1.

Received for review October 1, 1997. Revised manuscript received January 8, 1998. Accepted February 17, 1998. This research work was partially supported from the Commission of the European Communities, Agriculture and Fisheries (FAIR) specific RTD Program CT95-0260, High Quality Oils, Proteins and Bioactive Products for Food and Non-Food Purposes Based on Biorefining of Cruciferous Oilseed Crops.

JF9708498