OCCURRENCE OF INDOLE COMPOUNDS IN SOME VEGETABLES; TOXICOLOGICAL IMPLICATIONS OF NITROSATION WITH EMPHASIS ON MUTAGENICITY

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Proefschrift

ter verkrijging van de graad van
doctor in de landbouw- en milieuwetenschappen,
op gezag van de rector magnificus,
dr. H.C. van der Plas,
in het openbaar te verdedigen
op vrijdag 4 oktober 1991
des namiddags te vier uur in de aula
van de Landbouwuniversiteit te Wageningen.

STELLINGEN

1. Indolen zijn niet primair verantwoordelijk voor de mutageniteit van brassica groenten na nitrosering.
   -Dit proefschrift.

2. Het is onwaarschijnlijk dat afzonderlijke componenten effecten op de gezondheid kunnen veroorzaken zonder dat hierbij interferentie optreedt met andere componenten, van nature aanwezig in de matrix. Toxicologisch onderzoek aan individuele stoffen kan derhalve leiden tot irreële risico-voorspellingen.
   -Dit proefschrift.

3. Gezien de lage recovery van 4-chloro-6-methoxyindool bij isolatie uit tuinbonen via een methode beschreven in de literatuur, zullen de werkelijke gehalten van deze stof in Colombiaanse tuinbonen hoger zijn dan gepubliceerd.
   -Yang, D., *et al.* (1984), Carcinogenesis 5, 1219-1224.
   -Dit proefschrift.

4. Nederlandse groenten bevatten, afgezien van nitraat en nitriet, precursors van direct mutagene N-nitroso verbindingen.
   -Dit proefschrift.

5. In het kader van de wereldvoedselvoorziening wordt de verbouw van *Leguminosae* gepropageerd. Gezien de aanwezigheid van 4-chloro-6-methoxyindool in tuinbonen, een verbinding die na nitrosering potentiële genotoxische en tumor-bevorderende eigenschappen bezit en de positieve correlatie, die in Colombia is aangetoond tussen de consumptie van tuinbonen en het voorkomen van maagkanker, is nader onderzoek naar de aanwezigheid van 4-chloro-6-methoxyindool in vlinderbloemigen geboden.

6. Detectie van N-nitroso verbindingen met behulp van HPLC in combinatie met een post-kolom reactie, gevolgd door chemiluminescentie, kan een nieuwe dimensie aan N-nitroso onderzoek geven.
   -Harvery, D.C. (1990), J. Anal. Toxicol. 14, 181-185.

7. Ames *et al.* (1990) suggereren ten onrechte dat natuurlijke toxinen categorisch als pesticiden moeten worden beschouwd.
   -Ames, B.N. *et al.* (1990), Proc. Natl. Acad. Sci. USA 87, 7777-7781.

8. De vaststelling van een ADI op basis van een NEL gedeeld door een veiligheidsfactor moet bij voorkeur gereserveerd blijven voor de evaluatie van niet van nature voorkomende verbindingen; bij van nature voorkomende stoffen dient men in eerste instantie rekening te houden met achtergrondwaarden.

9. Hogere N-giften aan aardappelen leiden tot een betere sortering, maar zullen ook leiden tot een hoger nitraat gehalte in de knol. Het verdient derhalve aanbeveling om bij de prijsbepaling van aardappelen naast sortering ook rekening te houden met het nitraat gehalte.

10. Bij bezuinigingsoperaties worden vaak ondoordachte beslissingen genomen. Het feit dat N-nitroso onderzoek in Nederland vrijwel stil is komen te liggen, terwijl door de nitraatproblematiek de vraag naar dergelijk onderzoek nog steeds urgent is, is hier een goed voorbeeld van.

11. Universiteiten zouden bijscholing en refreshment-onderwijs moeten aanbieden, om de kansen van herintredende vrouwelijke afgestudeerden en promovendi op de arbeidsmarkt te vergroten.

12. Gezien het feit dat geen dierexperimenten zijn uitgevoerd in het toxicologische onderzoek beschreven in dit proefschrift, kan de verdediging zonder gewetenswroeging plaatsvinden op Wereld Dierendag.

H.G.M. Tiedink
Occurrence of indole compounds in some vegetables; Toxicological implications of nitrosation with emphasis on mutagenicity
Wageningen, 4 oktober 1991

Voor Matthijs
Voor mijn ouders

VOORWOORD

Het in dit proefschrift beschreven onderzoek is tot stand gekomen mede dankzij de inzet en het enthousiasme van een groot aantal mensen die ik hierbij graag wil bedanken.

Allereerst wil ik hier noemen dr. Leo van Broekhoven en dr. Wim Jongen, die de aanzet hebben gegeven tot het in dit proefschrift beschreven onderzoek. Beste Leo en Wim jullie begeleiding, al was dat soms vanaf (zeer grote) afstand, was onmisbaar bij de uitvoer van dit onderzoek. Door onze discussies en jullie correcties van vele (concept) manuscripten konden publicaties en dit proefschrift "vlot" tot stand komen.

Het onderzoek is destijds gestart als een project voor 9 maanden bij het Centrum voor Agrobiologisch Onderzoek (CABO). Dankzij prof. dr. J.H. Koeman kreeg het onderzoek een vervolg in de vorm van een promotie-onderzoek, waarbij een samenwerkingsverband werd opgezet tussen de vakgroep Toxicologie en het CABO. Beste Jan, bedankt voor het in mij gestelde vertrouwen en voor de leerzame begeleiding met name tijdens de laatste fase van het onderzoek. De directie Voedings- en Kwaliteitsaangelegenheden van het Ministerie van Landbouw, Natuurbeheer en Visserij heeft het promotie-onderzoek voor het overgrote deel gefinancieerd.

Bij de start van het onderzoek heeft Jacques Davies (CABO) mij ingewerkt in het analytische werk ten aanzien van N-nitroso verbindingen, waardoor al snel waardevolle resultaten konden worden verkregen, die de basis hebben gevormd voor het promotie-onderzoek. In de loop van het onderzoek hebben de hulp en/of adviezen van de volgende personen bijgedragen aan het verder welslagen van het onderzoek: Mijn collega's Bert Spenkelink en Irene Bruggeman; dr. Maarten Posthumus, Cees Teunis en Gerrit Lelyveld (vakgroep Organische Chemie); Henk van der Kamp en Diny Venema (Rikilt); dr. Roger Fenwick en Jenny Lewis (AFRC, Norwich, UK); Wim Verhagen en Jacques Withagen (CABO). Ook de vermelding van de enthousiaste medewerking van de studenten: Nanette Visser, Joke Herremans, Marianne Verbruggen, Erna Hissink, Menthe Malingré, Monique Lodema, Hanneke Kayser en Mariska Wubben is hier op zijn plaats. In het bijzonder noem ik hier nog Laura de Haan, zonder haar inbreng was "deel 2" nooit geworden zoals het nu is.

Voor de uitvoer van het onderzoek waren de aanwezigheid van groenten en van diverse chemicaliën een wezenlijk voorwaarde, daarom ben de volgende personen/firma's/instellingen zeer erkentelijk: Het Centraal Bureau Tuinbouwveilingen (begeleiding bij het verkrijgen van diverse bekende rassen Brassica's); ir. G. van Bentum van Nickerson-Zwaan B.V. (gift diverse tuinboonrassen); dr. Roger Fenwick van het AFRC (gift diverse glucosinolaten en myrosinase); dr. P. Martin van Ciba-Geigy, Bazel, Zwitserland (gift 4-chloro-6-methoxyindool); dr. ir. Tiny van Boekel van de vakgroep Zuivel (gift caseïne).

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Last but not least wil ik ook Matthijs in dit voorwoord betrekken. Lieve Matthijs, jij was de beste steun en toeverlaat, die ik mij kon wensen.

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

N-nitroso compounds (NOC) are a structurally diverse family of chemicals bearing a common functional >N-N=O group, to which man is exposed through a variety of routes, e.g. environment, food, occupation and through the endogenous reaction of nitrite with nitrosatable substances naturally occurring in foods. The nitrosation of naturally occurring substances in food has been studied least and forms the main subject of this study.

NOC are probably the most investigated group of chemicals with carcinogenic potential; of the circa 300 different NOC tested for carcinogenicity, over 90% were able to induce tumours in experimental animals (Preussmann & Stewart 1984). Paradoxically, despite of the wealth of information on the carcinogenicity of NOC for different animal species, there is only circumstantial evidence for their role in the etiology of human cancer (see Chapter 8).

N-nitroso formation

NOC are formed when oxides of nitrogen (NO_x), with nitrogen in oxidation state +3 and +4, react with nucleophilic amines, amides, alcohols or anions (Challis 1981; Figure 1.1). While only NO_x in these oxidation states are capable of nitrosation, nitrogen in other oxidation states can be converted to the +3 or +4 states by several biological and chemical mechanisms (Figure 1.2).

$$2\,HNO_2 \rightleftharpoons N_2O_3 + H_2O$$

$$R_2NH + N_2O_3 \longrightarrow R_2NNO + HNO_2$$

Figure 1.1: The classical nitrosation reaction; under acidic conditions two moles of HNO_2 react with one mole of a nucleophilic nitrogen containing compound.

Characteristics of NOC

NOC can be divided into two classes (Figure 1.3): N-nitrosamines and N-nitrosamides (and related compounds). N-nitrosamines are N-nitroso derivatives of secondary amines, while N-nitrosamides are those of substituted ureas, amides, carbamates, guanidines and similar compounds (Mirvish 1975). The difference between the two classes is not merely based on the chemical structure, there are also biological and environmental differences:

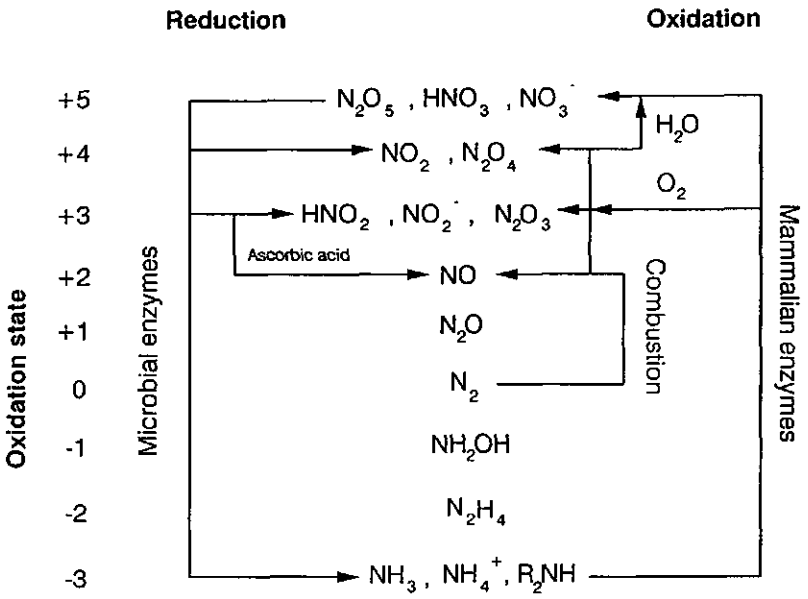
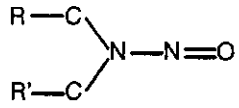
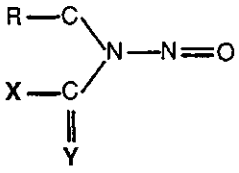


Figure 1.2: Oxidation states of nitrogen; Nitrosating agents are those in oxidation states +3 and +4. (Adapted from Hotchkiss 1989).

N-nitrosamines



N-nitrosamides



Compounds	X	Y
N-Nitrosamides	alkyl, aryl	O
N-Nitrosoureas	NH_2, NHR, NR_2	O
N-Nitrosocarbamates	RO	O
N-Nitrosoguanidines	NH_2, NHR, NR_2	NH

Figure 1.3: Chemical structures of N-nitrosamines and N-nitrosamides and related compounds.

1. N-nitrosamines are quite stable compounds, while many nitrosamides have half-lives in the order of minutes.

2. The maximum rate of N-nitrosamine formation is in the range of pH 2.5-3.3 (Mirvish 1975, Challis 1985) and is proportional to the concentration of the unprotonated amine so that weak bases are more rapidly nitrosated than strong bases (Archer 1982). No optimal pH can be given for the nitrosation of amides and related compounds; nitrosation rates increase with decreasing pH (Mirvish 1975).

3. In general N-nitrosamines are volatile compounds, which are stable enough for gas-chromatographic analysis. Recently also non-volatile N-nitrosamines and N-nitrosamides can be analyzed by high performance liquid chromatography coupled to a photohydrolysis detector (see Chapter 2).

4. N-nitrosamines are only biologically active after enzymatic activation by mono-oxygenases (Figure 1.4). The resulting α-hydroxylnitrosamines are unstable and spontaneously split off an aldehyde group to leave primary N-nitrosamines. Primary N-nitrosamines rearrange to form highly electrophilic diazonium ions, which react with cellular nucleophiles as water, proteins and nucleic acids. N-nitrosamides do not require oxidation in the body. They hydrolyse without the aid of enzymes and likewise form primary N-nitrosamines. The carcinogenicity of NOC is thought to be the result primarily of a reaction of the diazonium ions with various nucleophilic sites of DNA bases. DNA adducts thus formed can cause mispairing of the bases during replication, which in turn can result in the formation of mutations (Druckrey *et al.* 1967). Mutations in critical genes can initiate the process of tumour formation.

Exposure to preformed N-nitroso compounds

The vast majority of preformed NOC to which humans are exposed will be N-nitrosamines, because of their stability.

From a quantitative point of view, the most important source of human exposure to NOC is through the occupational environment. NO_x are ubiquitous in polluted air and many NOC precursors are valuable intermediates in industrial processes. The occurrence of volatile NOC in workplaces of rubber, leather tanning, metal working and chemical industries has recently been reviewed by Ecetoc (1990). However, no attention was paid to endogenous formation of NOC resulting from the uptake of amines by inhalation or dermal absorption together with NO_x exposure (Preussmann & Eisenbrand 1984).

In rubber factories the concentrations of N-nitrosodimethylamine (NDMA) and N-nitrosomorpholine (NMOR) in air mostly ranged between 0.05-20 $\mu g/m^3$, while in rubber chemicals concentrations of 4-3500 $\mu g/kg$ were found. Due to improving factory conditions in the leather tanning and chemical industries total N-nitrosamine levels were reduced to $\leq$ 1

$\mu g/m^3$, whereas before levels up to respectively 47 and 43 $\mu g/m^3$ of NDMA were detected. In metal working industries concentrations of N-nitrosodiethanolamine (NDELA) in synthetic cutting fluids ranged between 0.5-250 mg/kg with extreme values up to 750 mg/kg.

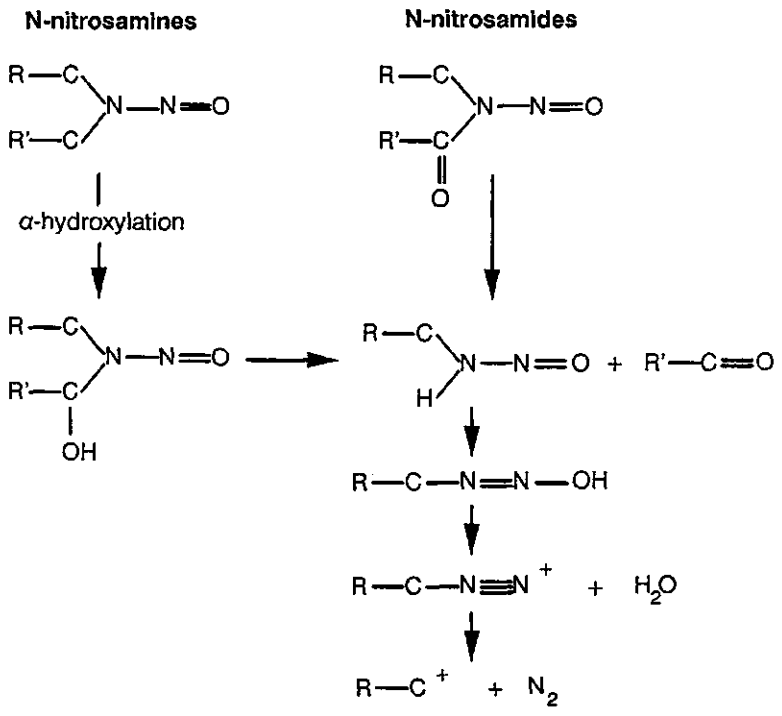


Figure 1.4: Formation of alkylating products by spontaneous or enzymatic degradation of N-nitroso compounds.

Tobacco use is a major source of "lifestyle" NOC exposure. During smoking and processing (e.g. curing, ageing and fermentation) of tobacco N-nitrosamines are formed, with levels and composition varying per type of tobacco used (Hoffmann *et al.* 1982). More than 20 different NOC have been detected in tobacco smoke (main and side stream) (Preussmann 1989), which can be divided in three categories: volatile N-nitrosamines, non-volatile NOC (mainly N-nitroso amino acids) and tobacco specific N-nitrosamines (TSNA; Hill 1988). Snuff-dipping is a rage in the USA and Scandinavia, by which tobacco is used orally. Hoffmann *et al.*(1987) detected 10-289 mg/kg of total NOC in snuff.

Volatile NOC commonly occur in different foodstuffs. In the last decade many investigations of the volatile NOC content of foodstuffs have been carried out. Probably the study conducted in Germany between 1978 and 1981, in which about 3000 samples of food items were screened for volatile NOC (reviewed by Spiegelhalder 1983), is the most extensive one. NOC were consistently found in beer and meat products in concentrations >1 μg/kg, while extremely high concentrations could be found in dark beer, beer made from smoked malt and cooked bacon (respectively up to 47, 68 μg/l and 57 μg/kg). NOC are formed in beer during direct-fired drying (kilning) of wet malt, while in many meat products (bacon, ham and various types of sausages) the formation of NOC is due to addition of nitrite or nitrate containing mixtures before curing. Since beer brewing processes and processes of curing meat have been improved, the concentrations of volatile NOC have dropped drastically in these products (Institute of Food Technologists' Expert Panel on Food Safety and Nutrition 1987). Occasionally, volatile NOC have also been found in milk and milk products, cheese, spices and spiced products (Gough *et al.* 1977, Massey *et al.* 1989, Spiegelhalder 1983).

The American Chemical Society calculated the daily uptake of volatile NOC through the diet in various countries. The results vary between 0.3 μg/person/day for Sweden and 1.8 μg/person/day for Japan (in: Ecetoc 1990).

Endogenous formation of N-nitroso compounds

The chemistry of nitrosation suggests that NOC may be formed in the acidic environment of the stomach. The first demonstration that *in vivo* formation of NOC could give rise to tumours in animals was that of Sander (1971). In this study rats were fed with methylbenzylamine and morpholine together with nitrite and tumours were formed in the oesophagus and liver. Subsequently, the endogenous nitrosation has been demonstrated in humans using different techniques. Groenen *et al.* (1984) could detect NDMA (up to 30 μg/kg) in the gastric juice of healthy persons a few hours after the consumption of a nitrate rich meal containing fish. The endogenous formation in humans has also been shown by the detection of N-nitroso derivatives of proline, aminopyrine and piperazine in the urine after the intake of their precursors and a nitrite/nitrate source (Ohshima & Bartsch 1981, Spiegelhalder & Preussmann 1985, Bellander *et al.* 1984). Nowadays, the N-nitrosoproline test, in which urinary N-nitrosoproline is measured after ingestion of a standard dose of proline and nitrate, is being widely used as an experimental tool to measure and compare yields of endogenous nitrosation amongst human populations (discussed by Tannenbaum 1987).

The formation of NOC in the stomach depends on several factors, the most important being: the concentration of nitrite and precursors (in case of amines also the pK_a of the amine), the gastric pH, the temperature and the amount of food in the stomach, the rate of stomach emptying, the rate of removal of nitrite (Mirvish 1975). The presence of inhibitors and/or catalysts of the nitrosation reaction also influences the amounts of endogenously formed NOC (reviewed by Lijinski 1984). Ascorbic acid, α-tocopherol, glutathione, some

phenols and tannins are known to inhibit the nitrosation reaction, by competition for nitrous acid. Oppositely, nucleophilic ions (as thiocyanate, which is found in enlarged concentrations in the saliva of smokers), some phenols, metal ions, certain carbonyl compounds including formaldehyde and chloral are known to accelerate the nitrosation reaction.

Although the stomach will be the cradle of the vast majority of the endogenously formed NOC, small amounts can be formed elsewhere in the body. In human saliva NOC are formed (Hart & Walters 1983), but the unfavourable pH and the short residence time limit the amounts formed. Although bacteria taken from the large bowel of rats and human faeces catalyse the formation of N-nitrosamines from nitrite, nitrate and amines at neutral pH *in vitro* (Klubes *et al.* 1972, Hill & Hawksworths 1971), subsequent studies failed to detect volatile NOC in faeces of healthy persons (Archer *et al.* 1981, Eisenbrand *et al.* 1981, Lee *et al.* 1981). In infected urinary tracts and vaginas nitrate reducing bacteria were found, and NOC could be detected in infected urine (Ohshima *et al.* 1987) and in vaginal exudate from some patients (Allsbrooke *et al.* 1974).

Already in 1978 the US National Academy of Sciences Panel (National Academy of Sciences 1978) concluded that the major source of human exposure to NOC will be through nitrosation of precursors in the stomach. Consequently the intake of nitrate, nitrite and nitrosatable compounds is of concern.

Nitrate as a precursor

Many toxicity studies have been performed with nitrate and the World Health Organisation (WHO 1974, 1980) consider 500 mg $NaNO_3$ per kg of body weight the highest daily dose over lifetime without adverse effects in rats. By application of a safety factor of 100, the "Acceptable Daily Intake" (ADI) is given as 3.65 mg NO_3^-/kg body weight, equivalent to 219 mg NO_3^-/day for a 60 kg person. In The Netherlands the average daily intake of nitrate was estimated to be 110 mg (Stephany & Schuller 1980), although recently van Loon & van Klaveren (1990) reported that the ADI is frequently exceeded, especially by children (0-10 year).

About 25% of ingested nitrate is absorbed from the gastrointestinal tract and will be excreted in the saliva. In the oral cavity bacteria are present which can reduce nitrate to nitrite and ultimately about 5% of ingested nitrate will enter the stomach as nitrite (Spiegelhalder *et al.* 1976). The nitrate which is not reduced to nitrite can be reabsorbed and thus can recirculate, so a single high intake of nitrate will have long term effects on the nitrite levels in the stomach (Spiegelhalder *et al.* 1976).

Ellen & Schuller (1983) reported that about 75% of the daily nitrate intake originates from vegetables. Other nitrate sources are meat products and drinking water, especially in the case of contaminated ground water. The amounts of nitrate in vegetables will depend on several factors including variety, culture and harvesting practices, fertilizer type, application rate and time in relation to harvest. In general, green leafy vegetables contain high concentrations of nitrate. In The Netherlands for these vegetables maximally allowed levels of nitrate are regulated by law. In drinking water the WHO (1985) maximally allows 50 mg

NO_3^-/l, while the European Community (EC) guideline is 25 mg NO_3^-/l (EC 1980). In 1986, 71% of the samples of drinking water, obtained from 225 pumping stations in The Netherlands, contained less than 5 mg NO_3^-/l, while in one case the level exceeded 50 mg/l. The nitrate levels in Dutch drinking water (and vegetables as well as) probably will further increase in the coming years, as a result of intensive breeding of domestic animals and fertilizer usage and it is predicted that before the turn of the century 4% of all drinking water plants will extract ground water with nitrate levels exceeding 50 mg/ml (Basisdocument nitraat 1987).

In many epidemiological studies a positive correlation was shown between the ingestion of nitrate and the development of gastric cancer (reviewed by Hartman 1983), while in some studies this relationship was lacking or even reversed (Forman *et al.* 1985). In the former studies, the endogenous formation of NOC was hypothesized to be a causative factor (Mirvish 1983), while the negative outcome was supposed to be the result of differences in source of the ingested nitrate. Since vegetables contain nitrate and inhibitors of the nitrosation reaction, Mirvish (1985) supposed that the ratio of nitrosation inhibitors to nitrate would be of concern in relation to gastric cancer rather than nitrate alone. The equivocal results of the epidemiological studies can probably also be explained by differences in the ratio of secondary amines/secondary amides in the diets.

Endogenously also small amounts of nitrate can be formed by macrophages (Stuehr & Marletta 1985).

Nitrite as a precursor

In the seventies and early eighties in Europe and North America the average daily intake of nitrite was about 4 mg, and meat and meat products were the major sources (reviewed in Ellen & Schuller 1983). After the usage of nitrite as a preservative has been restricted, a recent study showed that the maximal daily intake of nitrite in The Netherlands is about 0.7 mg (in: Groenen 1990), while the levels of nitrite deriving from nitrate, based on a 5% conversion of 110 mg ingested nitrate, will be about 5.5 mg. Thus human exposure to nitrite mainly comes from the reduction of ingested nitrate by bacteria in the oral cavity.

Ingested nitrite is rapidly absorbed and in the blood it will convert haemoglobin (Hb) to methaemoglobine (MetHb), by which the oxygen transport is inhibited (Jaffé 1981). At low levels of nitrite, MetHb formation is reversible. Infants are extremely susceptible to nitrite induced MetHb formation, since fetal Hb is more easily converted to MetHb and neonatal erythrocytes lack the enzyme which catalyse MetHb reduction to Hb. On the basis of MetHb formation, rather than on the basis of NOC formation, an ADI of 0.2 mg $NaNO_2$/kg body weight, equivalent to 8.04 mg NO_2^-/day for a 60 kg person was given by the WHO (1974). This ADI is obtained by application of a safety factor of 50 to 10 mg $NaNO_2$/kg body weight, which is considered the highest daily dose over lifetime without adverse effects in rats. However, recently 10 mg of KNO_2/kg body weight administered to rats over a 3 months period was found to induce hypertrophy of the zona glomerulosa of

the adrenal gland (Falke & Til 1988). Therefore it is likely that the ADI of nitrite will be adjusted.

Dietary nitrosatable compounds as precursors

The nitrosation of dietary substrates is reviewed by Shephard & Lutz (1989). Primary and secondary amines, aromatic amines, amino acids, indoles, ß-carbolines, phenols, amides, peptides, ureas and guanidines were indicated as potential precursors of endogenously formed NOC.

In many *in vitro* studies the presence of large amounts of nitrosatable compounds in foods was investigated by nitrosation of extracts of these foods followed by total NOC measurements or by determination of alkylating- or mutagenic-activity. In most of these studies the experimental conditions during nitrosation were modifications of the so called "Nitrosation Assay Procedure" (NAP), which is a test described by the WHO (1978) to investigate the occurrence of nitrosatable compounds in drugs. After nitrite treatment fish, milk and cheese contained volatile NOC (Groenen *et al.* 1982, Walters *et al.* 1974), sauerkraut, fermented milk products, wine and smoked fish possessed direct alkylating activity (Groenen & Bussink 1988) and meat, fish, soy sauce, pickled vegetables, Chinese cabbage and fava beans possessed direct mutagenic activity (Marquardt *et al.* 1977, Yano *et al.* 1988, Tahira *et al.* 1986, Wakabayashi *et al.* 1983, 1984, 1985, Piacek-Llanes & Tannenbaum 1982, van der Hoeven *et al.* 1984, Jongen *et al.* 1987). Although no chemical analyses were performed when alkylating and mutagenic activity were studied as endpoints, it can be deduced from the direct acting activity that the majority of the NOC formed were non-volatiles. This is in agreement with a study of van Broekhoven *et al.* (1987), in which the majority of the biologically active NOC formed in nitrite treated extracts of lettuce and fish were determined as non-volatiles. Moreover, only small portions of preformed NOC found in Japanese foods appeared to be volatiles (Kawabata *et al.* 1984) and also in beer only 6 nM of NDMA was found, while the concentrations of "apparent total NOC" exceeded 450 nM (Preussmann & Eisenbrand 1984, Massey *et al.* 1987). These studies indicate that the endogenous formation of non-volatile NOC form precursors occurring in foods would be of more importance than the exposure to volatiles (both preformed and endogenously formed).

Scope of the present study

From the preceding information it can be concluded that human exposure to NOC mainly occurs through endogenous nitrosation of dietary precursors. Vegetables are the main source of nitrosating compounds, while also drinking water can contribute significantly. It is most likely that as a consequence of the present practices of fertilizer use and intensive animal husbandry the nitrate concentrations in both vegetables and drinking water will further increase, which will result in an enhanced risk of endogenous NOC formation. Some vegetables also appeared to contain nitrosatable compounds, from which directly mutagenic NOC can be formed. Therefore the first objective of the present study was to

screen Dutch vegetables on their potential to form directly mutagenic NOC and secondly to study the identity and mutagenic properties of the NOC formed. Soon after the start of this study it became apparent that regarding the second objective it was not feasible to cover all vegetables. Therefore the efforts were concentrated on two types of vegetables, brassicas and fava beans, which were both known to contain precursors of directly mutagenic NOC (Wakabayashi *et al.* 1985, Yang *et al.* 1984). Consequently this study consists of two parts, the first dealing with brassicas, the second with fava beans.

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CHAPTER 2

PRINCIPLES OF THE TEST METHODS

N-NITROSO ANALYSIS

So far the chemical analysis of N-nitroso compounds (NOC) in food products has mainly been restricted to volatile ones, because they can easily be distilled from their matrix and because of their relative stability. Volatile NOC in foods can be detected and identified by chemiluminescence, while in this way also total amounts of NOC (volatile and non-volatile) in foods can be quantitatively measured. Recently also non-volatile NOC can be detected by a photohydrolysis detector (PHD), although only qualitative measurements can be made. In the present study the emphasis was on non-volatile and heterocyclic NOC, which are almost non-volatile. Therefore, in Chapters 4 and 6 of this thesis, in which the amounts of NOC formed in vegetable extracts were related to induced mutagenicity, total amounts (volatile and non-volatile) of NOC were measured, while in Chapter 5 and 6, non-volatile NOC formed from model compounds were determined using high performance liquid chromatography (HPLC) in combination with PHD.

Total N-nitroso measurements

The thermal energy analyzer (TEA), developed by Fine *et al.* (1975) for the detection of volatile NOC is based on the catalytic cleavage of the N-nitroso binding and subsequent chemiluminescence detection of formed nitrosyl radicals. In the reaction chamber of the TEA, nitric oxides react with ozone to form excited nitrodioxides. These nitrodioxides will return to the ground state by emission of light in the 600 nm region (near infrared), which is detected by a very sensitive photomultiplier. For the quantitative detection of total NOC (both volatile and non-volatile) Walters *et al.* (1978) used the chemiluminescence detection of nitrosyl radicals after chemical cleavage of the N-nitroso binding. Samples to be tested for total NOC are injected in boiling ethylacetate containing HBr/HAc, by which the N-nitroso binding is cleaved and nitrosyl radicals formed are carried to the reaction chamber of the TEA in a nitrogen atmosphere. This method is schematically depicted in Figure 2.1.

Pollutants are prevented from entering the reaction chamber by 6 M NaOH traps, a water/ice (0°C) and an isopentane/liquid nitrogen (-110°C) trap. The whole system is made out of glass and teflon (Figure 2.2).

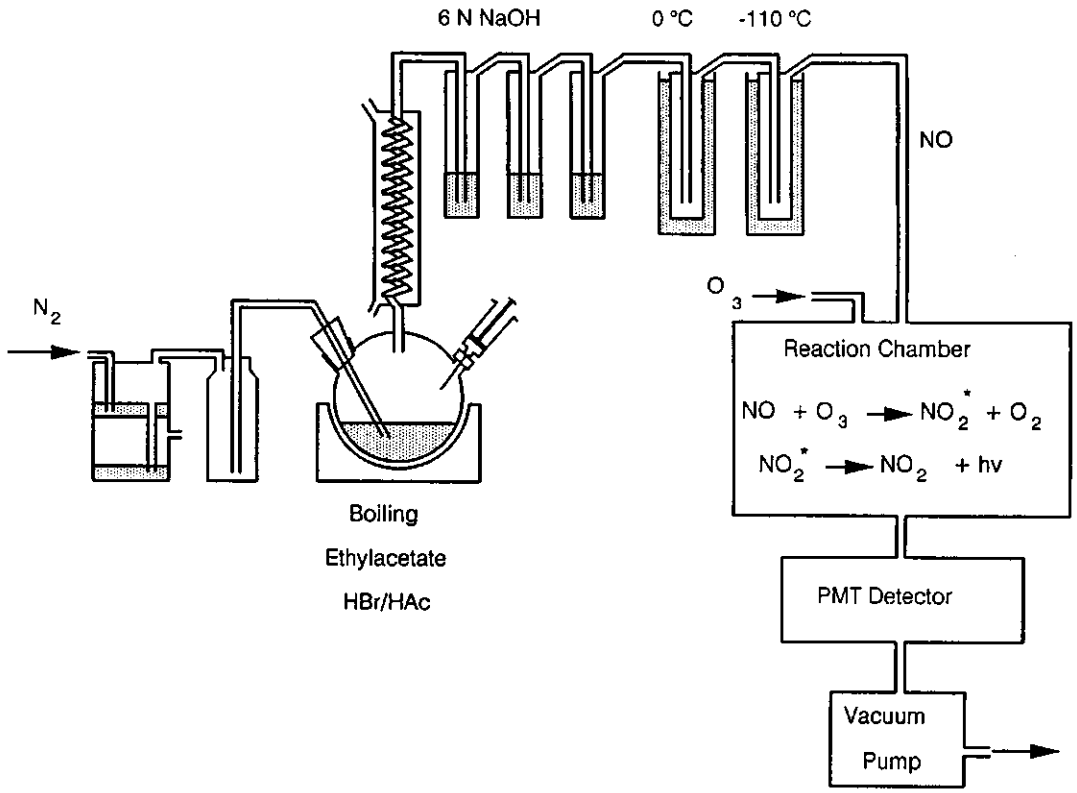


Figure 2.1: Schematic representation of the quantitative detection of total N-nitroso compounds (volatile and non-volatile) by a Thermal Energy Analyzer.

Using the method to determine total NOC by TEA as described above, besides NOC also other nitrogenous compounds like nitrite, alkylnitrites, pseudonitrosites, nitrosothiols and organic nitroso compounds (C-NO, O-NO), can form nitric oxides upon breakdown by HBr/HAc. Therefore also measurements were performed in which HBr/HAc is replaced by HCl/HAc (Pignatelli *et al.* 1987). NOC (and some aliphatic C-nitroso compounds, nitrolic acids and nitrothiols) are not cleaved by HCl/HAc and therefore will not give a response in these measurements. So by subtracting responses obtained in HBr measurements by those in HCl measurements, the analysis of NOC is corrected for interference by other nitrogenous compounds. The resulting responses are expressed in terms of nitric oxide produced *in situ* by comparing peak areas with a calibration curve made with nitrite standards. In the operating system the detection limit was 5 μmol/l.

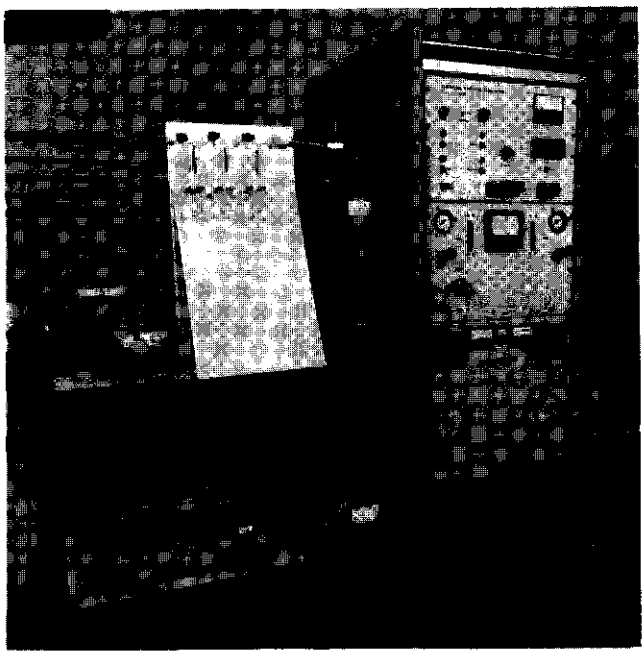


Figure 2.2: The Thermal Energy Analyzer.

Non-volatile NOC measurements

The analytical technique to measure non-volatile NOC by HPLC in combination with a PHD was developed in the early eighty's by Professor Tannenbaum's laboratory at the MIT in Boston (Shuker & Tannenbaum 1983). The method is schematically depicted in Figure 2.3.

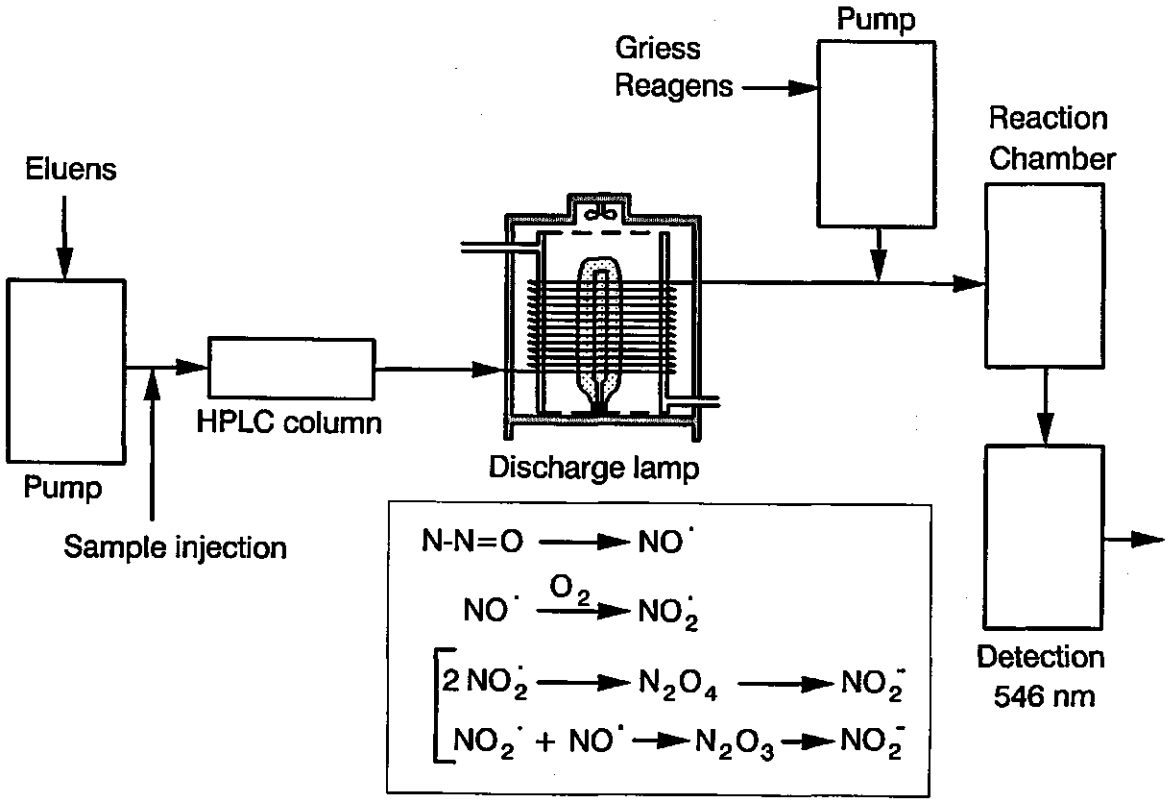


Figure 2.3: Schematic representation of the detection of non-volatile N-nitroso compounds by high performance liquid chromatography in combination with photohydrolysis.

After separation by HPLC, NOC are transferred through a teflon tubing (15.25 m x 0.25 mm) to a high intensity discharge lamp (type HP/AB, 400 W, 290 x 53 mm, Philips, The Netherlands), emitting 400-420 nm light. To prevent overheating the lamp is surrounded by a water jacket which is cooled with tap water and the teflon tubing is wound around this jacket. The lamp is housed in a reflecting, light tight box, which is cooled by a fan. NOC are cleaved by the lamp and the formed nitrosyl radicals are oxidized to NO_2 radicals, which further react to N_2O_3 and N_2O_4. N_2O_3 and N_2O_4 both are unstable in aqueous surroundings and will convert to nitrite. The effluent from the photohydrolysis coil is mixed with Griess reagent in a low dead volume mixing tee. The resulting solution is pumped to a stainless steel column packed with glass beads (0.1-0.11 mm) kept at 65°C by a water jacket and then to a second stainless steel column cooled with tap water. Finally, the azo-compound formed by reaction of nitrite with Griess reagent is measured spectrofotometrically at 546 nm.

Turning off the high intensity discharge lamp results in the detection of only free nitrite and other non-NOC, which react with Griess reagent. HPLC runs in which the lamp is turned off thus serve as control measurements.

IN VITRO MUTAGENICITY TESTS

In order to detect chemical mutagens and potential carcinogens, a large number of short term assays has been developed (in: IARC 1986). In these assays prokaryotic cells (bacteria) can be used to detect point mutations and small deletions, while in eukaryotic cells (fungi, yeast, mammalian cells) also chromosomal abberations can be detected. In the present study an assay with *Salmonella* (*S.*) *typhimurium* bacteria was extensively used for screening chemicals and vegetable extracts for mutagenicity, while for some model compounds also assays with mammalian cells were used: the sister chromatid exchange (SCE) test and the forward mutation assay using the hypoxanthine-guanine-phophoribosyltransferase (HGPRT) locus as an endpoint.

S. typhimurium assay

The *Salmonella*/microsome (the so called "Ames") test (Ames *et al.* 1975, Maron & Ames 1983) is the most popular of the bacterial assays and has been validated thoroughly (Kier *et al.* 1986).

The principle of the assay is to measure reverse mutations from histidine auxotrophy to prototrophy in several specially constructed *S. typhimurium* strains. In the present study strain TA100 was used, which is designed to detect base pair substitutions (AT→GC) in the "His" operon and is widely used for testing the direct mutagenic activity of vegetable extracts (van der Hoeven *et al.* 1983, 1984, Wakabayashi *et al.* 1984, 1985, Yang *et al.* 1984, van Broekhoven *et al.* 1987, Jongen *et al.* 1987).

Strain TA100 contains several mutations which increase its sensitivity to mutagens: The "gal" and "rfa" mutations enhance permeability to large molecules by elimination of the lipopolysaccharide barrier that normally coats the surface of the bacteria (Maron & Ames 1983) and by which the bacteria are no longer pathogenic. By a "uvrb" mutation the bacteria are DNA repair deficient, as a consequence of a deletion in a gene coding for the biotin operon. The involvement of the biotin operon causes that addition of biotin to the medium is a prerequisite for growth. The plasmid "pkM101" carrying a mutated His-gene is multi-copied by addition of a "R-factor" (resistance-factor), by which the bacteria are more sensitive to backward mutations towards prototrophy. The number of spontaneous and chemically induced revertants is further increased by stimulation of an error-prone repair system, which is normally present in these bacteria (McCann *et al.* 1975, Shanabruch & Walker 1980). The presence of this plasmid can be checked because it also contains a tetracycline-resistance gene which confers resistance to the presence of ampicillin in the growth medium (Ames *et al.* 1975, Maron & Ames 1983). In the absence of mutagens, spontaneous reversion will occur and for strain TA100 these should be within the range of 80-200 per plate, otherwise an alteration in the genetics of the strain or a contamination can not be excluded.

Stock cultures of *S. typhimurium* TA100 were maintained on master plates, which contain minimal agar medium (25 ml) enriched with 1.5 mg of L-histidine (Merck, Darmstadt, Germany) per plate, and were stored for no longer than 1 month at 4°C. In each experiment fresh cultures were prepared from an inoculum of one single colony isolated from master plates in 10 ml of nutrient broth (no. 2, Oxoid Ltd., Basingstoke, Hants, UK) enriched with L-histidine (10 μg/ml). The cultures were incubated in a shaking water bath at 37°C for 6-8 h until a density of 1 x 10^9 cells/ml was reached, as measured by optical density at 700 nm.

Samples to be tested for mutagenicity were added to a 1% agar solution (2.5 ml), followed by addition of the bacterial suspension (0.1 ml; about 10^8 cells) and a Vogel-Bonner solution (0.5 ml). The volume was adjusted with distilled water to 4.1 ml and the mixture poured on Petri dishes containing minimum glucose agar medium with biotin (0.5 μg/ml) and a trace of histidine. Histidine is required to allow initial growth (auxotrophy), necessary for expression of mutagenesis and which results in the formation of the so called background lawn (Ames 1971). The background lawn can be examined by phase contrast microscopy and can be used as an indicator for cytotoxicity (Ames 1971, Ames *et al.* 1975, Maron & Ames 1983). Mutations induced by tested samples result in the growth of hundreds to thousands colonies (prototrophy), which are thick and white after 2 days of incubation at 37°C and which are visible by eye. Counting the number of prototrophic colonies (revertants) allows quantification of the mutagenic activity. In all experiments triplicate plates were used per sample and appropriate positive and negative controls were included. Samples were considered to be mutagenic when the number of revertants induced following nitrite treatment were at least twice as much as those induced by untreated samples. Moreover, a dose-response curve should be obtained.

SCE test

A SCE represents a symmetrical exchange of chromatid segments within a single chromosome. In 1958, this phenomenon in plant chromosomes was described by Taylor, by using autoradiographic methods. In this study SCE's were measured in Chinese hamster V79 cells, using visible staining techniques. The points of exchange in two sister chromatids appear to occur cytologically at the same locus, but the precision of exchange at the molecular level is unknown. At this moment there is no evidence that SCE's per se are cytotoxic or lethal events, but they are extensively used as an endpoint in cytogenic and mutagenicity research (reviewed by Sandberg 1982, Wolff 1982, Tice & Holaender 1984) and the SCE test has been generally accepted as a standard method for testing genotoxicity (in: IARC 1986).

In this study SCE's were detected by a method using 5-bromodeoxy-uridine, a thymidine analogue. Incorporation of this compound for two rounds of replication in V79 Chinese hamster cells results in chromosomes consisting of one unfiliairly substituted chromatid and one bifillairly substituted chromatid. These chromatids stain differentially with Giemsa or fluorescent dyes. The best results are obtained with a combination of these (Zakharov & Egolina 1974, Latt 1973, Korenberg & Freedlender 1974, Perry & Wolff 1974) (Figure 2.4).

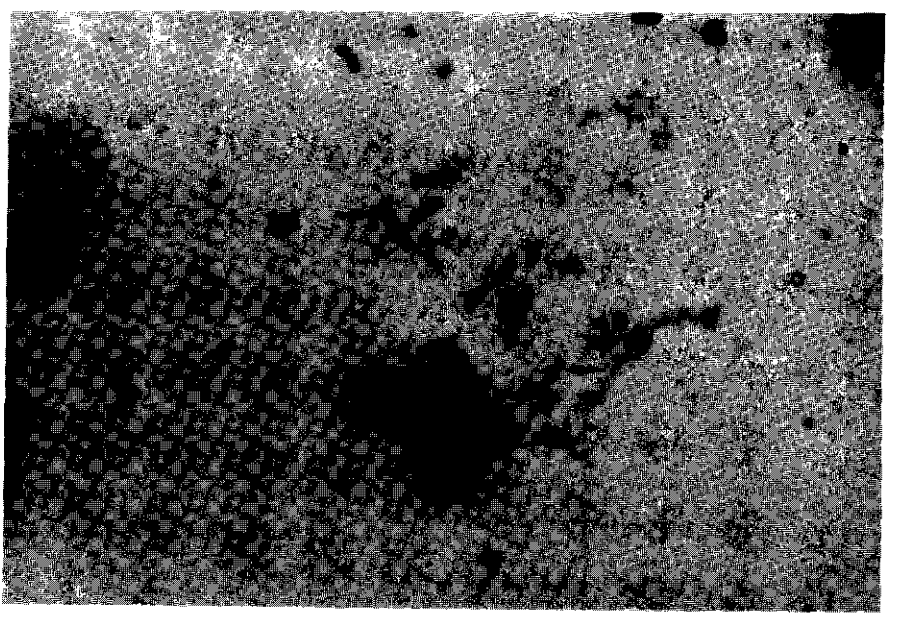


Figure 2.4: Metaphase of V79 Chinese hamster cell containing differential stained chromatids. Arrows indicate reciprocal exchanges between homologous sister chromatids induced by nitrosated 4-chloro-6-methoxyindole (see Chapter 9).

a

b

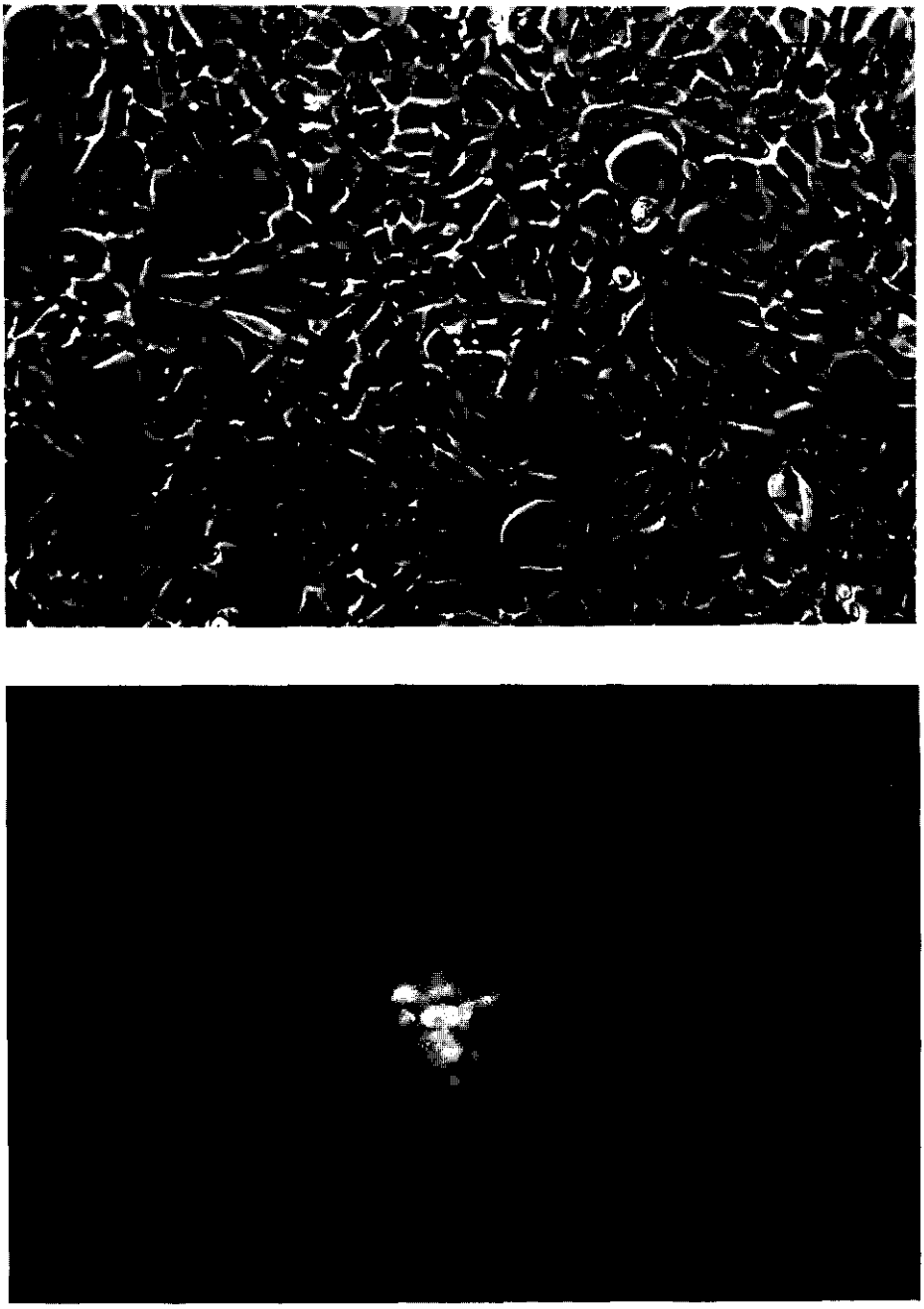
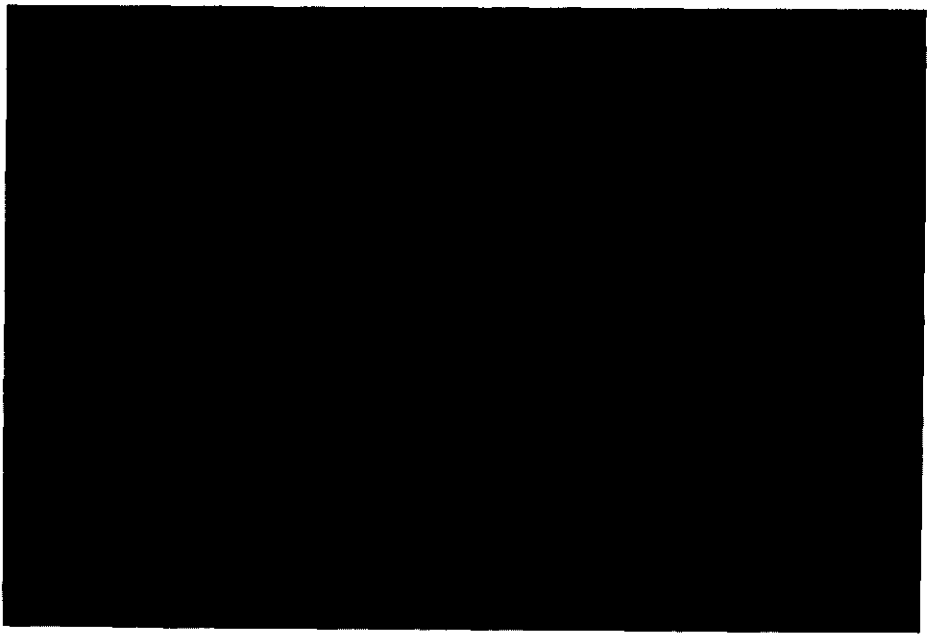


Figure 2.5: Photomicrographs depicting lucifer yellow transfer in cultures of V79 Chinese hamster cells. Cells injected with lucifer yellow dye are indicated by arrows. Bar = 12.5 μm.

c

d



(a) Phase contrast micrograph of untreated cells. (b) Lucifer yellow fluorescence in the same field. (c) Phase contrast micrograph of cells after exposure to nitrosated 4-chloro-6-methoxyindole (see Chapter 9). (d) Lucifer yellow fluorescence in the same field.

Forward mutation assay on the HGPRT locus

Cultured mammalian cells need purines and pyrimidines for their growth. Besides *de novo* synthesis of these bases from simple precursors, cells are able to recycle them from degraded DNA and RNA. Hypoxanthine and guanine are salvaged by the dual purpose hypoxanthine guanine phosphoribosyl transferase (HGPRT), an enzyme coded for by a gene on the X chromosome (Polani *et al.* 1981). When poisonous base analogues 6-thioguanine or 8-azaguanine are added to the culture medium of cells, these compounds will be incorporated into nucleic acids, which will result in cell death. Only mutated cells which lack the HGPRT activity will survive in this selective medium, because these cells will not salvage purine and pyrimidine bases from the medium, but will synthesize them *de novo*.

In the present study V79 Chinese hamster cells were exposed to nitrosated indole compounds followed by an expression time of 7 days in a non-selective medium. Then the medium was replaced by a selective medium (containing 6-thioguanine), in which only the mutants will grow (van Zeeland and Simmons 1975, 1976a+b). After an incubation of 9 days colonies were fixed, stained and counted.

Forward mutation assays are extensively used in mutagenicity research (reviewed by Bradley *et al.* 1981, Howard-Flanders 1981, Hsie *et al.* 1981, Cox 1982, Chu 1983, Clive *et al.* 1983, Morrow 1983, Chu *et al.* 1984), since these assays will detect a broader spectrum of genetic lesions in comparison with reverse mutation assays.

IN VITRO TEST SYSTEM FOR TUMOUR PROMOTION

Potential tumour promoting activity of chemicals can be tested by morphological changes or by the inhibition of intercellular communication. The latter method was used in the present study.

Inhibition of intercellular communication

At cellular level, growth is controlled by various forms of cell-cell interaction and communication (Loewenstein 1979, Yamasaki & Mesnil 1987). A characteristic of cancer cells is their anarchic growth, in which control mechanisms of cell proliferation and differentiation are ignored. From this it was concluded that intercellular communication could be the basis for control of the cellular society. Murray & Fitzgerald (1979) and Yotti *et al.* (1979) were the first to show that tumour promoters inhibit gap junctional intercellular communication (GJIC). Since then a considerable body of data has been collected showing that the blockage of GJIC might be an important determinant in the promotion stage of carcinogenesis (reviewed by Yamasaki 1987). The amount of inhibition of GJIC by several compounds correlates well with their *in vivo* promoter activity (Yamasaki 1984, Enomoto *et al.* 1984a+b).

The inhibition of GJIC in the present study was measured using a technique, in which

a fluorescent dye is injected into single V79 Chinese hamster cells by a microinjector. Since the plasma membrane is impermeable for the dye, it will only be transferred to neighbouring cells by means of gap junctions. Ten minutes after the injection of the dye the transfer to surrounding cells is measured by counting fluorescent cells (Figure 2.5).

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PART 1

BRASSICAS

CHAPTER 3

INTRODUCTION BRASSICAS; MAIN EMPHASIS ON GLUCOSINOLATES AND THEIR BREAKDOWN PRODUCTS

Brassica vegetables comprise all kinds of cabbages, Brussels sprouts, broccoli, cauliflower, kohlrabi, turnip and swede. They belong to the family of Cruciferae, and are botanically known as *Brassica* species. Brassicas are frequently consumed by humans from western and eastern cultures (Fenwick & Heaney 1983, Hill *et al.*, 1987), as well as by animals. In The Netherlands, the amounts consumed by humans are not exactly known, but more than 36 g of brassica are available per person per day (Godeschalk 1987).

The typical flavour of brassicas is largely due to glucosinolate-derived volatiles. The occurrence of glucosinolates in plants for human consumption appears to be limited to cruciferous plants. The chemistry and occurrence of glucosinolates and their breakdown products has been reviewed extensively by van Etten & Wolff (1973) and by Fenwick *et al.* (1983). Glucosinolates contain a common structure (Figure 3.1), with different substituents (alkyl, alkenyl, alkylthioalkyl, aryl, ß-hydroxyalkyl or indolylmethyl groups) at the side chain R. Currently more than 100 different glucosinolates have been characterised. The levels of total glucosinolates in plants may depend upon variety, cultivation conditions, climate and agronomic practice, while the levels in a particular plant vary between the parts of the plant. In plants glucosinolates and their hydrolysis products act as allelochemicals, which are compounds affecting behaviour, health and growth of other species. For instances the white butterfly *Pieris rapae* is attracted to brassicas, and probably needs glucosinolates for essential functions, while in general glucosinolates and their hydrolysis products also have insecticidal and fungicidal properties. In higher plants the hydrolysis product of indolylglucosinolates, indole-3-acetonitrile (I_3A) and indole-3-acetic acid, formed from I_3A (Gmelin & Virtanen 1961), also act as hormones (family name auxins).

Glucosinolates can be hydrolyzed by myrosinase (thioglucoside glucohydrolase EC 3.2.3.1.), an enzyme also present in cruciferae, which becomes active when cells are damaged, e.g. by cutting or chewing. Myrosinase hydrolyses glucosinolates by splitting off the glucose, the unstable aglucone thereafter eliminates sulphate by a Lossen rearrangement (Figure 3.1). The structure of the resulting products depend on a variety of factors, but in general isothiocyanates, nitriles and thiocyanates are formed. Less volatile compounds such as epithionitriles and oxazolidine-2-thiones are formed from glucosinolates with an hydroxyl group at the ß-position of the side chain. Myrosinase can be inactivated by heating, although equivocal data exist about the levels of inactivation. Bradfield and Bjeldanes (1987) only found a 20% reduction in myrosinase activity after cooking cauliflower for 10 min., while McMillan *et al.* (1986) detected about 0-2.5% of its original activity in Brussels

sprouts after cooking for 9 min. Slominski & Campbell (1987) found a complete inactivation of myrosinase, when rape meal was heated to 95°C for 10 min., although substantial decomposition of indolylglucosinolates was observed during heating. Glucosinolates are lost during cooking of cruciferous vegetables due to leaching into cooking liquor (Srisangham *et al.* 1980, Fenwick *et al.* 1983). The effects of processing on glucosinolates levels in vegetables have been reviewed by de Vos & Blijleven (1988).

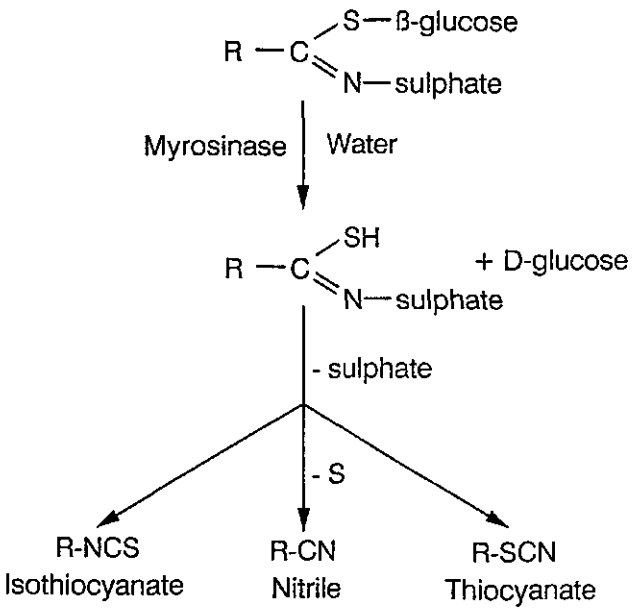

Figure 3.1: Common structure of glucosinolates and their breakdown products generally formed upon myrosinase hydrolysis (adapted from van Etten & Wolff 1983).

From a toxicological point of view all the volatile hydrolysis products of glucosinolates are of interest (reviewed by van Etten 1969, Fenwick *et al.* 1983). Among the problems associated with these compounds, those effecting the thyroid have been studied most extensively. Thiocyanate ions and oxazolidine-2-thiones both have shown to be goitrogenic in animals, although the mechanism involved seemed to be different. Thiocyanate ions are considered to behave as iodine competitors and therefore will only cause goitrogenicity in

case of iodine deficiency, while oxazolidine-2-thiones interfere with thyroxine synthesis and therefore will be goitrogenic irrespective of the iodine status. Additionally, some isothiocyanates and nitriles may also lead to goitrogenic symptoms resulting from the thiocyanate-mediated detoxication process. In most animal studies performed in the past, goitrogenicity induced by glucosinolate breakdown products appeared to be limited to situations of iodine deficiency. In a study of McMillan *et al.* (1986), also no effects could be found on the human thyroid function, as measured by the levels of thyroid stimulating hormone, triiodothyronine and thyroxine after a daily consumption of 150 g of Brussels sprouts over a period of 9 weeks.

Besides the effects of glucosinolate breakdown products on the thyroid also other effects have been observed. Hydroxynitriles and glucosinolates themselves were suggested to be responsible for enlargement of adrenal glands, kidneys and liver found in rats after longterm feeding of rapeseed and for liver haemorrhage observed in poultry. Several isothiocyanates have been shown to be embryotoxic in rat, while in *in vitro* studies a number of them appeared to be cytotoxic and mutagenic. Moreover, allylisothiocyanate has been found to be able to induce tumours in rats (NTP 1981).

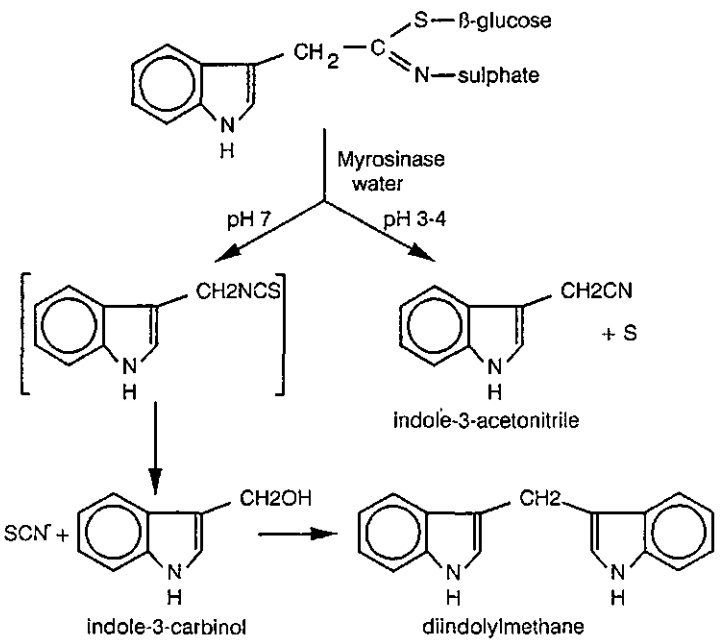

Figure 3.2: The breakdown products of indolylglucosinolates upon myrosinase hydrolysis (adapted from van Etten & Wolff 1973).

Upon hydrolysis of indolylglucosinolates (Figure 3.2) unstable isothiocyanates are formed which degrade to indole-3-carbinol (I_3C), while at more acidic conditions I_3A and elemental sulphur are formed. I_3C may condense to diindolylmethane (DIM) or react with ascorbic acid to form ascorbigen (ASC). I_3C, I_3A, DIM and ASC have shown to inhibit neoplastic effects of carcinogens (reviewed by McDanell *et al.* 1988). Initially these effects were thought to be the result of induction of enzymes of the "Mixed Function Oxidase" (MFO) system (Loub *et al.* 1975), which can be induced by intact glucobrassicin as well (McDanell *et al.* 1989). Later I_3C was also found to be an inducer of glutathione-S-transferase, glucoronyl transferase and epoxide hydroxylase, enzymes involved in the detoxification of xenobiotics (Sparnins *et al.* 1982, Cha *et al.* 1985). Studies performed with cell cultures revealed that I_3C and I_3A treatment prior to carcinogen exposure led to a shift in the equilibrium of enzymes involved in the biotransformation of these compounds (Jongen *et al.* 1989). This shift towards increased detoxification capacity resulted in decreased DNA damage (Jongen *et al.* 1987). Contrary, indole compounds can also enhance the formation of tumours in trouts and rats, when administered after a carcinogen (Bailey *et al.* 1987, Pence *et al.* 1986).

As can be concluded from the above the toxicological implications of the consumption of brassicas is not yet clear. Epidemiological studies showed only beneficial effects of their consumption. In studies of Graham *et al.* (1978) and Graham & Mettlin (1979) it was shown that diets rich in Brussels sprouts, broccoli or turnip lowered the risk for developing colon and rectum cancer.

The main reason to concentrate on indole compounds in the present study was the previous finding that I_3A, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-aldehyde were identified as precursors of N-nitroso compounds (NOC) in Chinese cabbage (Wakabayashi *et al.* 1985, 1986). Since these indole compounds are hydrolysis products of glucosinolates, in this study special attention was paid to the role of indole compounds and glucosinolates in the formation of NOC in vegetables.

In the study described in Chapter 4, 31 vegetables, commonly consumed in The Netherlands were screened for their potential to form directly mutagenic NOC upon nitrite treatment. For brassicas this potential was compared with their glucosinolate levels. In Chapter 5, the formation of nitrosated products of I_3A, I_3C and indole (I) was studied and special attention was paid to the stability of these products. After investigation of the hydrolysis of glucosinolates by myrosinase and acid, the contribution of intact and hydrolysed glucosinolates, as well as several known indole compounds to the total mutagenicity of nitrite treated brassica extracts was studied (Chapter 6).

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CHAPTER 4

FORMATION OF DIRECTLY MUTAGENIC N-NITROSO COMPOUNDS IN VEGETABLE EXTRACTS UPON NITRITE TREATMENT

INTRODUCTION

In the present study 31 Dutch vegetables (including 9 brassicas) that are commonly consumed in The Netherlands were tested for their potential to form directly mutagenic NOC, irrespective of their nitrate content. Extracts of the vegetables were incubated with nitrite under acidic conditions. After incubation the direct mutagenic activity to *Salmonella* (*S.*) *typhimurium* was measured as well as the amounts of total NOC (volatile and non-volatile) formed. Levels of total NOC were measured rather than that of non-volatiles (the directly mutagenic ones), since at this moment no methods are available for the quantitative detection of non-volatiles. However, since several studies have shown that the majority of the NOC formed in different foods are non-volatiles (Kawabata *et al.* 1984, van Broekhoven *et al.* 1987, Preussmann & Eisenbrand 1984, Massey *et al.* 1987) and since in the present study the pH during nitrosation was 2, at which N-nitrosamides and related compounds will be much faster formed than volatile N-nitrosamines, it can be suggested that the NOC formed in the vegetable extracts will predominantly be non-volatiles.

Since indole-3-acetonitrile, 4-methoxyindole-3-acetonitrile and 4-methoxyindole-3-carboxaldehyde were identified as precursors of directly mutagenic NOC in Chinese cabbage (Wakabayashi *et al.* 1985, 1986) and since these indole compounds are hydrolysis products of indolylglucosinolates, in this study special attention was paid to the occurrence of glucosinolates (both aryl/alkyl and indolyl) in the vegetables (Figure 4.1).

This chapter is based on:
Tiedink, H.G.M., J.A.R. Davies, L.W.van Broekhoven, H.J. van der Kamp & W.M.F. Jongen (1988) Formation of mutagenic N-nitroso compounds in vegetable extracts upon nitrite treatment; A comparison with the glucosinolate content. Fd. Chem. Toxicol. 26, 947-954.

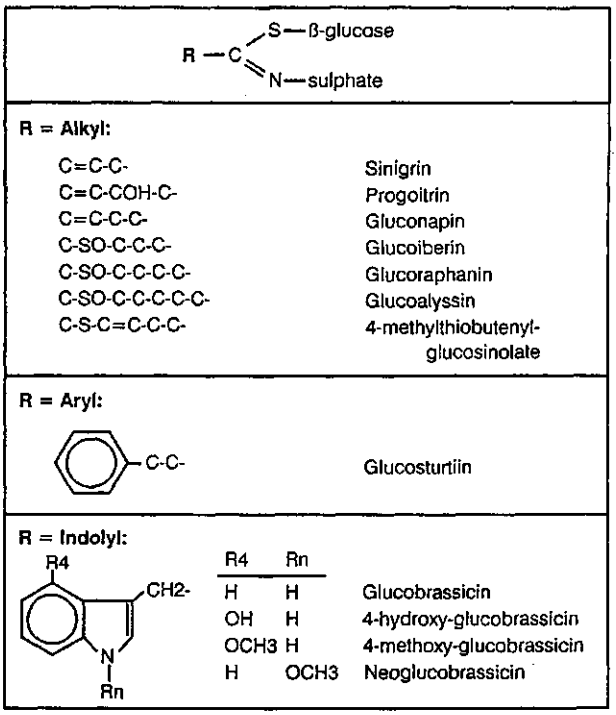

Figure 4.1: Chemical structures of the most commonly occurring glucosinolates in cruciferous vegetables.

MATERIALS AND METHODS

Treatment of vegetables

Fresh vegetables were purchased at a local greengrocery. Inedible parts, sand, dirt and water were removed from the vegetables. The remaining parts were shredded into pieces of about 1.5 cm x 1.5 cm, and weighed before freeze-drying (Virtis, Gardiner, New York, USA). Lyophilized vegetables were used instead of fresh, because of seasonal availability.

The lyophilized vegetables were ground, homogenized and stored at -20°C. All experiments were performed within 10 weeks after lyophilization.

Nitrite incubation

An amount of lyophilized vegetable, corresponding to 60 g of fresh material was made up to 80 g with distilled water. After shaking for 15 min. in a satellite flask-shaker (Griffin & George Ltd, Alperton, Wembley, UK), the homogenate was centrifuged for 20 min. at

16,300 x g, 0-10°C. The supernatant was filtered over a Büchner funnel and the weight was filled up again to 80 g with distilled water. The vegetable extract thus obtained was divided in two equal parts of 40 g, and adjusted to pH 2 with a 6 M HCl solution. To one part a $NaNO_2$ solution was added (final concentration 40 Mm), and to the other an equal volume of distilled water was added. Both extracts were incubated for 1 h at 37°C in the dark. A 40 mM $NaNO_2$ solution and distilled water alone were also incubated as blanks. The nitrosation reaction was stopped by addition of a ammoniumsulphamate solution ($NH_4SO_3NH_2$, final concentration 48 mM). Samples (1 ml) were taken from each incubation mixture for determination of the total N-nitroso content. The rest of the incubation mixture was adjusted to pH 8 with NaOH solutions, because for some vegetables a greater stability of the NOC was found at pH 8 than at pH 2.

The determination of total N-nitroso content and the mutagenicity assay were both performed no longer than 3 h after termination of the incubation.

Total N-nitroso determination

The total N-nitroso content of all incubated extracts was measured using a thermal energy analyzer (TEA; Thermedics Inc., Woburn MA, USA), according to a modified method of Walters *et al.* (1978; see Chapter 2).

Samples (2.5 μl) of the vegetable extracts were injected in the refluxing ethylacetate containing HBr/HAc. The obtained TEA responses were corrected for possible responses of blanks and for responses of the samples measured in ethylacetate containing HCl/HAc (Pignatelli *et al.* 1987).

Mutagenicity test

The *S. typhimurium* assay was performed with strain TA100 without S9 mix as described by Ames *et al.* (1975) with minor modifications (van der Hoeven *et al.* 1983).

Because the incubations were performed in an aseptic way, none of the extracts was filter-sterilized before testing. At least two independent experiments were carried out with 4 doses (0.2, 0.5, 0.8 and 1.0 ml vegetable extracts filled up to 1 ml with distilled water), using triplicate plates for each sample. The numbers of revertants that occurred in tests of extracts without nitrite treatment were subtracted from those that occurred with nitrite-treated extracts. From the resulting values dose-response curves were drawn and the mutagenic activity of the extracts after nitrite treatment was calculated from the linear part of these curves. For all vegetable extracts the dose corresponding with 25 mg of dry weight was within this range.

In each experiment a blank (an incubated sodium nitrite solution) and a positive control (1 μg 1-methyl-1-nitroso-3-nitroguanidine (MNNG) per plate) were also tested, which induced an average of 110 ± 25 and 775 ± 187 revertants respectively.

Glucosinolate determination

Glucosinolates were determined in the dry matter of the vegetables according to a

method reported by Muuse & van der Kamp (1987). This method is based on extraction of the glucosinolates with boiling water followed by desulphatation with aryl sulphatase on a micro column with 20 mg DEAE Sephadex A-25 (Pharmacia, S-75182 Uppsala, Sweden). The desulphoglucosinolates were eluted with water and separated by high performance liquid chromatography (HPLC) using a Lichrosorb 5RP18 column (150 x 4.6 mm; Chrompack International, Middelburg, The Netherlands). The solvent programme consisted of water/acetonitrile 98/2 (v/v) for 1 min. and a linear gradient over 18 min. to water/acetonitrile 75/25 (flow 1.5 ml/min.). The desulphoglucosinolates were monitored by UV-absorption at 229 nm, quantified against the internal standard glucotropaeolin and expressed as μmol/g dry matter. The sequence of elution of the individual desulphoglucosinolates was: glucoiberin (1.13), progoitrin (1.15), sinigrin (1.05), glucoalyssin (1.13), glucoraphanin (1.13), gluconapin (1.17), 4-hydroxyglucobrassicin (0.29), glucotropaeolin, 4-methylthiobutenylglucosinolate (0.52), glucobrassicin (0.31), glucosturtiin (1.00), 4-methoxyglucobrassicin (0.26), neoglucobrassicin (0.21). The relative response factor of the compounds in comparison with glucotropaeolin at 229 nm is indicated in brackets.

Statistics

Linear regression was calculated by the least square analysis. Calculated correlation coefficients (r) were two-sided tested for significance.

The mean N-nitroso concentration was calculated from duplicate measurements of 2 independent experiments. The standard deviation (SD) was calculated from the total variance of the 4 observations.

RESULTS

N-nitroso content

The concentrations of NOC formed in the vegetable extracts after nitrite treatment were calculated from TEA measurements (Table 4.1). In contrast to untreated extracts, in all vegetable extracts treated with nitrite, NOC could be detected. From this it can be concluded that precursors of NOC are present in vegetable extracts.

Brassicas, with the exception of swede, showed high levels of NOC, when expressed per 25 mg dry matter. When expressed per 25 mg fresh weight most of the *Brassica oleracea* varieties had higher levels than most other vegetables.

Mutagenic activity

The mutagenic activity of the vegetable extracts after nitrite treatment is shown in Table 4.2. About half of the vegetables were found to be mutagenic upon nitrite treatment (induced at least twice as much revertants after nitrite treatment compared to before). The number of revertants induced by extracts of brassica species were high in comparison with those induced by extracts of other vegetables, although Brussels sprouts, oxheart cabbage

Table 4.1: Levels of N-nitroso compounds formed in vegetable extracts upon treatment with nitrite under acidic conditions.

Vegetable		*N*-Nitroso compounds (nmol)* expressed	
Common name	Scientific name	per 25 mg dry matter	per 25 mg fresh weight
Brassica	*Brassica oleracea*		
Cauliflower	var. *botrytis* L. subvar. cauliflora DC.	263 ± 9	23 ± 1
Brussels sprouts	var. *gemmifera* DC.	789 ± 57	147 ± 8
Savoy cabbage	var. *sabauda* L.	628 ± 65	86 ± 9
Broccoli	var. *botrytis* L. subvar. *cymosa* Lam.	492 ± 53	73 ± 8
Red cabbage	var. *capitata* L.	492 ± 90	50 ± 9
Green cabbage	var. *sabauda* L.	697 ± 134	92 ± 18
Oxheart cabbage	var. *alba* DC.	277 ± 46	28 ± 5
White cabbage	var. *capitata* L.	370 ± 59	32 ± 5
Kohlrabi	var. *gongylodes* L.	186 ± 12	14 ± 1
Chinese cabbage	*Brassica pekinensis* (Lour.) Rupr.	247 ± 31	15 ± 2
Swede	*Brassica napus* L. var. *napobrassica* (L.) Rehb.	76 ± 5	6 ± 0
French beans	*Phaseolus vulgaris* L.	188 ± 15	20 ± 2
Slicing beans	*Phaseolus vulgaris* L.	172 ± 24	17 ± 2
Fava beans	*Vicia faba* L.	42 ± 3	8 ± 1
Peas	*Pisum sativa* L.	23 ± 4	8 ± 1
Marrowfat	*Pisum arvense* L.	104 ± 18	25 ± 5
Endive	*Chicorium endivia* L.	103 ± 11	6 ± 1
Chicory	*Chicorium intybus* L.	311 ± 10	18 ± 1
Spinach	*Spinacia oleracea* L.	274 ± 122	17 ± 8
Lettuce	*Lactuca sativa* L.	158 ± 57	9 ± 3
Onion	*Allium cepa* L.	187 ± 11	25 ± 1
Leek	*Allium porrum* L.	213 ± 23	22 ± 2
Red beet	*Beta vulgaris* L.	69 ± 23	10 ± 3
Carrots	*Daucus carota* L.	25 ± 13	4 ± 1
Radish	*Raphanus sativa* L.	143 ± 117	6 ± 5
Horseradish	*Amoracia lapathifdia* Gilib	190 ± 16	7 ± 1
Green pepper	*Capsicum annuum* L.	79 ± 22	5 ± 1
Red pepper	*Capsicum annuum* L.	237 ± 9	25 ± 1
Tomato	*Lycopersicon lycopersicum* L.	58 ± 4	4 ± 0
Cucumber	*Cucumis sativus* L.	287 ± 17	17 ± 1
Mushroom	*Agaricus campestris* L.	241 ± 25	19 ± 2

*Values are means ± SD for four samples.

Table 4.2: Mutagenic activity present in vegetable extracts after treatment with nitrite under acidic conditions.

Vegetable	No. of revertants induced† per 25 mg dry matter	No. of revertants induced† per 25 mg fresh weight	Mutagenicity ratio‡
Cauliflower	176 ± 16	15 ± 1	5.76*
Brussels sprouts	61 ± 2	12 ± 0	2.15*
Savoy cabbage	101 ± 3	14 ± 0	2.35*
Broccoli	148 ± 18	22 ± 3	5.23*
Red cabbage	124 ± 39	13 ± 4	4.76*
Green cabbage	203 ± 55	27 ± 7	3.62*
Oxheart cabbage	84 ± 21	8 ± 2	1.84
White cabbage	203 ± 18	17 ± 2	3.05*
Kohlrabi	134 ± 50	10 ± 4	2.61*
Chinese cabbage	326 ± 30	20 ± 2	3.71*
Swede	307 ± 13	26 ± 1	4.27*
French beans	30 ± 10	3 ± 1	1.67
Slicing beans	40 ± 10	4 ± 1	1.41
Fava beans	62 ± 5	11 ± 1	1.47
Peas	20 ± 8	7 ± 3	1.69
Marrowfat	39 ± 2	9 ± 0	1.51
Endive	81 ± 42	5 ± 2	5.50*
Chicory	59 ± 8	3 ± 0	1.64
Spinach	85 ± 58	5 ± 4	3.00*
Lettuce	55 ± 37	3 ± 2	3.08*
Onion	8 ± 4	1 ± 1	1.16
Leek	12 ± 5	1 ± 0	1.48
Red beet	81 ± 17	12 ± 2	8.36*
Carrots	57 ± 18	6 ± 2	29.50*
Radish	233 ± 63	9 ± 3	7.82*
Horseradish	55 ± 5	2 ± 0	1.73
Green pepper	7 ± 0	0 ± 0	1.17
Red pepper	13 ± 8	1 ± 1	1.27
Tomato	45 ± 6	3 ± 0	2.47*
Cucumber	17 ± 9	1 ± 1	1.16
Mushroom	155 ± 1	12 ± 0	1.78

†No. of revertants (mean ± SD) induced by nitrite-treated extracts in *Salmonella typhimurium* TA100 without microsomal activation, as calculated from dose–response curves of two independent experiments.

‡No. of revertants induced by nitrite-treated extracts/no. of revertants induced by untreated extracts (both corrected for no. of spontaneous revertants). Where ratios are marked with asterisks, the nitrite-treated extracts were defined as mutagenic.

and kohlrabi did not show more revertants (per 25 mg fresh) than some non-brassica vegetables. Of the 11 brassica species tested 10 were found to be mutagenic.

Glucosinolate content

The concentrations of glucosinolates in cruciferous vegetables are shown in Table 4.3. The other vegetables gave no peaks or only minor peaks with deviating retention times, and these minor peaks could not be identified as desulphoglucosinolates.

Correlations

Although, in general, nitrite treated extracts of brassica species contained more NOC and induced higher numbers of revertants upon nitrite treatment than did other vegetables, no significant correlation was found between the amount of NOC formed and the number of revertants induced ($r=0.20$ based on dry matter and $r=0.42$ based on fresh weight). Neither a significant correlation was found between the number of revertants induced by nitrite-treated extracts of cruciferous vegetables and the levels of glucosinolates in these vegetables ($r=0.31$ based on dry matter and $r=0.21$ based on fresh weight). However, the amounts of NOC formed upon nitrite treatment in extracts of cruciferous vegetables were significantly correlated ($p<0.01$) with the levels of glucosinolates in these vegetables. When the glucosinolates were subdivided in aryl/alkyl- and indolylglucosinolates this significant correlation ($p<0.01$) was maintained for both subgroups (Figure 4.2). When expressed per fresh weight the amounts of NOC formed correlated more closely with the levels of glucosinolates than when expressed per dry weight.

Based on dry weight, only the levels of glucoiberin, sinigrin, gluconapin and glucobrassicin were significantly ($p\leq0.05$) correlated with NOC formed upon nitrite treatment, while based on fresh weight the levels of all individual glucosinolate compounds, with the exception of glucosturtiin and 4-methylthiobutenylglucosinolate, were significantly ($p\leq0.05$) correlated.

DISCUSSION

The results of this study show that all vegetable extracts tested contained NOC after nitrite treatment. This indicates that precursors of NOC are present in the vegetables. In mutagenicity tests the number of revertants induced by the extracts was increased when the extracts were treated with nitrite. There was no correlation between the number of induced revertants and the amount of NOC formed. This can be explained by a variation in the pattern of NOC formed in each vegetable and by the variation in the mutagenic activity of different NOC.

After nitrite treatment about half of the vegetables tested were mutagenic. Apart from the brassica species many other vegetables were defined mutagenic. Endive, beet and carrots had low levels of NOC formed, but were mutagenic (Tables 4.1 & 4.2). This sug-

Table 4.3: Glucosinolate concentrations in the lyophilized cruciferous vegetables.

	Glucosinolates (μmol/g dry weight vegetable)															
	Aryl/alkyl-glucosinolates*										Indolyl-glucosinolates*					
Vegetables	1	2	3	4	5	6	7	8	9	Total	10	11	12	13	Total	Total (1–13)
Cauliflower	0.2	—	0.2	—	0.3	—	—	—	—	0.7	0.1	0.7	0.2	0.1	1.1	1.8
Brussels sprouts	3.1	7.6	8.2	2.1	0.3	7.0	0.3	—	—	28.3	1.2	4.5	0.9	—	6.6	34.9
Savoy cabbage	4.3	0.3	4.3	0.4	0.3	0.4	—	—	—	10.0	0.4	2.4	1.8	0.1	4.7	14.7
Broccoli	0.5	6.2	—	5.9	0.3	0.6	—	—	—	13.5	—	2.1	0.2	0.9	3.2	16.7
Red cabbage	1.6	1.2	2.7	0.3	0.1	0.4	—	—	—	6.2	0.1	3.8	0.3	—	4.2	10.4
Green cabbage	7.3	0.2	10.2	—	0.2	—	—	—	—	17.9	—	6.8	1.3	—	8.1	26.0
Oxheart cabbage	0.7	0.3	0.1	0.9	0.3	—	—	—	—	2.3	0.1	1.1	0.1	—	1.3	3.6
White cabbage	6.8	0.2	4.2	0.1	0.2	—	—	—	—	11.5	—	3.4	0.4	0.1	3.9	15.4
Kohlrabi	0.2	—	—	0.2	0.1	—	—	—	—	0.5	0.1	1.3	0.1	0.5	2.0	2.5
Chinese cabbage	—	—	—	—	—	—	0.5	—	—	0.5	—	1.3	1.5	0.1	2.9	3.4
Swede	—	2.3	—	—	0.7	—	2.9	—	0.5	6.4	0.2	0.9	0.3	0.9	2.3	8.7
Radish	—	0.2	—	—	0.2	—	—	4.9	0.7	6.0	0.2	0.3	0.5	—	1.0	7.0
Horseradish	—	—	—	—	0.2	—	—	9.6	0.4	10.2	0.3	0.3	0.1	—	0.7	10.9

*The compounds were as follows: 1 = glucoiberin; 2 = progroitrin; 3 = sinigrin; 4 = glucoalyssin; 5 = glucoraphanin, 6 = gluconapin; 7 = glucosturtiin; 8 = 4-methylthiobutenylglucosinolate; 9 = rest; 10 = 4-hydroxyglucobrassicin; 11 = glucobrassicin; 12 = 4-methoxyglucobrassicin; 13 = neoglucobrassicin.

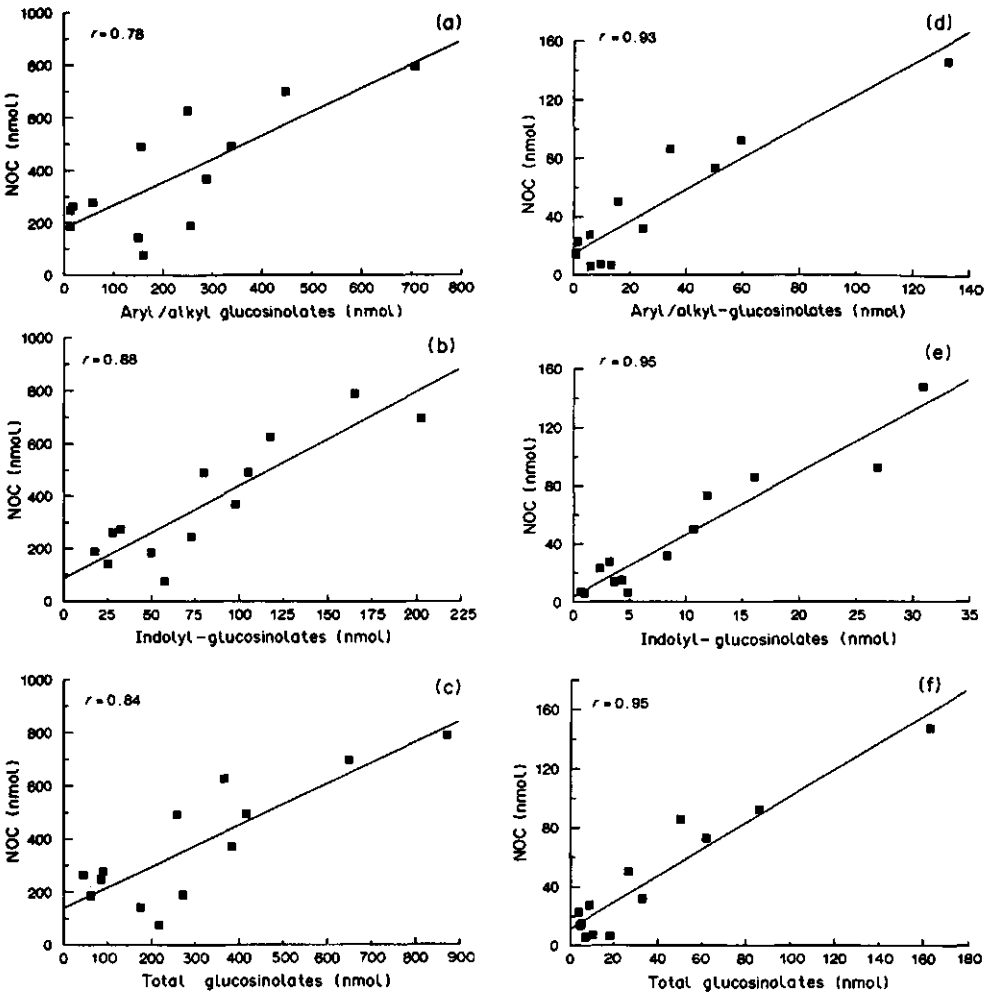

Figure 4.2: Relationship between the content of aryl/alkyl-, indolyl- and total-glucosinolates in the dry matter of cruciferous vegetables and the amount of N-nitroso compounds formed upon nitrite treatment in extracts of these vegetables: (a,b,c) calculated per 25 mg dry matter; (d,e,f) calculated per 25 mg fresh weight.

gests the presence of precursors which become highly mutagenic after nitrite treatment. Further investigations are needed to identify these precursors.

Fava beans were not mutagenic upon nitrite treatment. This result contrasts with those of Piacek-Llanes & Tannenbaum (1984) and van der Hoeven *et al.* (1984), and this may be due to differences in the methods of incubation. In the present study the vegetable extracts were filtered before the incubation with nitrite, whereas in the other studies the incubations were done in the presence of the solid matter of the fava beans, and the incubates were filtered just before the mutagenicity assay. Moreover, van der Hoeven *et al.* (1984) showed a more than 15-fold difference in the mutagenic response of various cultivars of the fava beans. It is possible that our non-specified cultivar was a low responder. A wide variation in mutagenic activity between cultivars of various vegetables was also shown in another study (van der Hoeven *et al.* 1983). Therefore it is necessary to extrapolate the results of this study with care, because only one cultivar per vegetable was investigated.

In the present study glucosinolates could only be detected in cruciferous vegetables. This is in agreement with previous reports (Fenwick *et al.* 1983). The glucosinolates were determined in the dry matter of the vegetables, because in the vegetable extracts no glucosinolates could be detected. Even glucotropaeolin, added to the vegetable extracts as an internal standard for HPLC analysis could not be detected in (non-incubated) vegetable extracts. This indicates that myrosinase was activated by adding water to lyophilized vegetables.

The levels of glucosinolates in the fresh intact starting material probably were not much higher than those determined in the lyophilized vegetables, because only minimal losses of glucosinolates would be expected as a result of chopping. Disruption of cells is a prerequisite for the myrosinase-glucosinolate interaction leading to the formation of biologically reactive metabolites. In the case of chopping the disruption is relatively minor (Fenwick *et al.* 1983). Moreover grinding of the lyophilized material probably did not lead to losses of glucosinolates, because myrosinase is inactive in dry material. In some vegetables glucosinolate levels were analyzed after storage at -20°C for 3 months; no significant decreases were observed.

The levels of glucosinolates were better correlated with NOC formed if expressed per fresh weight than per dry matter (Figure 4.2). On a molar basis, the amount of total glucosinolates was similar to that of the NOC formed. Moreover, both the aryl/alkyl- and the indolylglucosinolates were significantly correlated with the NOC formed. This suggests that all glucosinolates are somehow involved in the formation of NOC. A role of indolylglucosinolates in the formation of NOC can be explained. Indoles are the breakdown products of the indolylglucosinolates and can be easily nitrosated. However, the aryl/alkyl glucosinolates, which make up 15-94% (mean 64%) of the total level of glucosinolates, give in general thiocyanates, isothiocyanates and nitriles upon hydrolysis (Fenwick *et al.* 1983). These products are not considered to be nitrosatable. Thiocyanates, however, are known catalysts of the nitrosation reaction (Fan & Tannenbaum 1973). Only hydrolysis of hydroxy-alkyl-glucosinolates (e.g. progoitrin) can give oxazolidine-2-thiones (Fenwick *et al.*

1983) and 2-oxazolidones (Lüthy *et al.* 1984), which are nitrosatable products. Therefore it is not clear in what way the aryl/alkyl-glucosinolates are involved in the formation of NOC, but from the similarity of the concentrations of the total glucosinolates and the NOC it seems likely that the aryl/alkyl-glucosinolates serve as precursors of NOC.

In conclusion, all vegetables tested, but especially the brassica species, contain precursors of NOC. The identity of the precursors is unknown, but it is likely that the glucosinolates are involved in the formation of NOC. It must be kept in mind that in this study no attention was paid to factors that can inhibit or catalyze the nitrosation reaction or to factors that can influence the mutagenic activity of NOC.

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CHAPTER 5

THE STABILITY OF THE NITROSATED PRODUCTS OF INDOLE, INDOLE-3-ACETONITRILE, INDOLE-3-CARBINOL AND 4-CHLOROINDOLE

INTRODUCTION

In the study described in Chapter 4, a striking correlation was found between the levels of glucosinolates (both alkyl- and indolylglucosinolates) in brassica vegetables and the amount of N-nitroso compounds (NOC) formed in extracts of these vegetables after treatment with nitrite. Upon hydrolysis by myrosinase, an enzyme also present in brassicas, indolylglucosinolates form indole derivatives (Fenwick *et al.* 1983). Many indole compounds have shown to be directly mutagenic to different tester strains of *Salmonella* (*S.*) *typhimurium* after nitrosation. Ochiai *et al.* (1986) tested 31 indole derivatives and found that upon nitrite treatment 22 of them had direct mutagenic activity to bacteria. Tryptophan, an essential amino acid for humans, tryptamine and 5-hydroxytryptamine also showed direct mutagenic activity to *S. typhimurium* TA1537 and other tester strains upon nitrite treatment (Gatehouse & Wedd 1983). Moreover, naturally occurring indole compounds were identified as precursors of directly mutagenic NOC in Chinese cabbage and fava beans (Wakabayashi *et al.* 1985a, 1986, Yang *et al.* 1984).

The nitrate (NO_3^-) levels of most brassicas usually do not exceed 2500 mg/kg fresh product, but higher concentrations have been observed (Corré & Breimer 1979). About 5% of nitrate ingested by humans enters the stomach as nitrite (Spiegelhalder *et al.* 1976) and therefore theoretically it is possible that the indole compounds present in brassicas are converted to NOC endogenously. Indole-3-acetonitrile (I_3A), indole-3-carbinol (I_3C) and indole (I) are the breakdown products of glucobrassicin, the most commonly occurring glucosinolate in brassica vegetables (Figure 5.1). In order to obtain information about the possible endogenous nitrosation of these indole compounds, their nitrosation rates were investigated. 4-Chloroindole (4Cl), which has a structure and properties that resemble those of 4-chloro-6-methoxyindole, the precursor of the mutagenic N-nitroso compound in fava beans (Yang *et al.* 1984), was also investigated.

This chapter is based on:
Tiedink, H.G.M., J.A.R. Davies, N.A. Visser, W.M.F. Jongen & L.W. van Broekhoven (1989) The stability of the nitrosated products of indole, indole-3-acetonitrile, indole-3-carbinol and 4-chloroindole. Fd. Chem. Toxicol. 27, 723-730.

Jongen *et al.* (1987) showed that NOC formed in fava beans can interact with proteins. From this it can be assumed that proteins might be able to serve as carriers for NOC *in vivo*, thereby transporting the NOC from the place of formation, the stomach, into the intestines. Since the binding may be reversible, NOC could be released from the proteins in the intestines. This implies that both the stomach and the intestines may be target organs for directly mutagenic NOC. In the study described in Chapter 4, it was noticed that for some vegetables the stability of NOC in the extracts was pH dependent. Regarding the fact that in the gastro-intestinal tract the pH varies from 1.5 to 8, this could be of great importance. Therefore in this study the stability of the nitrosated indole compounds at pH 2 and 8 was investigated.

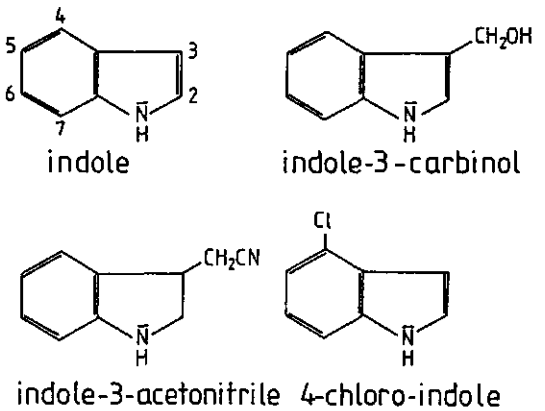


Figure 5.1: Chemical structures of indole, indole-3-carbinol, indole-3-acetonitrile and 4-chloroindole.

MATERIALS AND METHODS

Chemicals

I3A, I3C, I and 4Cl were purchased from Sigma, St. Louis MO, USA. All chemicals used were at least of analytical grade.

Nitrite incubation

The indole compounds were dissolved in DMSO and then diluted in distilled water (final concentration DMSO 1% at most). The nitrosation reaction was started by addition of a $NaNO_2$ solution (final concentration 40 mM) to the indole compounds solutions of pH 2. After re-adjustment of the pH to 2, the indole compounds were incubated in the dark at 37°C. Unless stated otherwise the nitrosation reaction was stopped after 15 min. by addi-

tion of ammoniumsulphamate or ascorbic acid (final concentration 80 mM or as indicated).

Mutagenicity assay

The *S. typhimurium* assay was performed with tester strain TA100 without S9 mix as described by Ames *et al.* (1975) with minor modifications (van der Hoeven *et al.* 1983). Although the pH of the samples (volume of 0.1-1.0 ml) varied (pH 2 and 8), they hardly had any effect on the pH of the overlayered agars. At least two independent experiments were performed with triplicate plates for each sample and the data presented are the average values of one representative experiment. In each experiment a water blank and a positive control (1 μg/plate 1-methyl-1-nitroso-3-nitroguanidine; MNNG) were included. MNNG induced >2000 revertants.

High performance liquid chromatography-photohydrolysis detection

Non-volatile NOC formed upon nitrosation of the indole compounds were determined by high performance liquid chromatography (HPLC) using a photohydrolysis detector. The analysis of NOC by photohydrolysis has been described by Shuker & Tannenbaum (1983) (see Chapter 2).

When a Hypersil APS column (100 x 3 mm; Chrompack International BV, Middelburg, The Netherlands) was used, the mobile phase consisted of 0.01 M H_3PO_4 (pH 4.5) at a flow rate of 0.4 ml/min.. In the case of a Chromsphere C18 column (100 x 3 mm; Chrompack International BV, Middelburg, The Netherlands), 20 mM ammoniumacetate (pH 6.5)/acetonitrile 85/15 (v/v) was used as mobile phase. Griess reagent consisted of 1.92 mM N-naphtylethyleendiamine-diHCl), 29.04 mM sulphanilamide and 3% (v/v) H_3PO_4, and was pumped at a flow rate of 0.4 ml/min..

Total N-nitroso determination

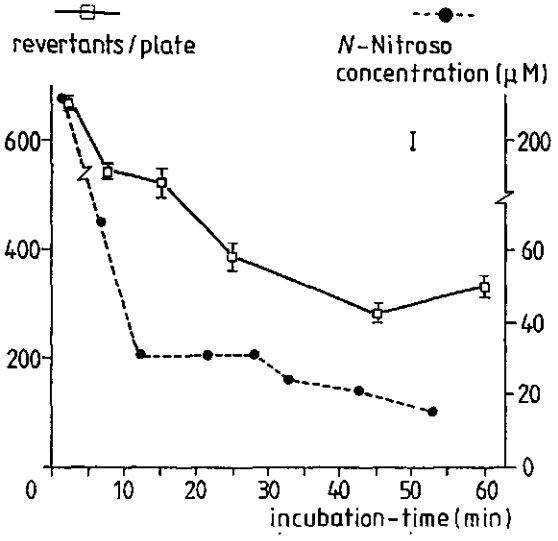
The total N-nitroso levels formed upon nitrosation of the indole compounds were determined by a thermal energy analyzer (TEA; Thermedics Inc., Woburn MA, USA), according to a modified method of Walters *et al.* (1978; see Chapter 2).

Samples were injected in the refluxing ethylacetate containing HBr/HAc and the TEA responses were corrected for those obtained in HCl/HAc measurements (Pignatelli *et al.* 1987).

HPLC-UV detection

Nitrosated indole compounds were separated from indole compounds by HPLC, using a Lichrosorb 5RP18 column (150 x 4.6 mm; Chrompack International BV). The solvent programme consisted of a linear gradient over 15 min. from 75/25 to 35/65 (v/v) 20 mM ammoniumacetate (pH 6.5)/acetonitrile (flow rate 1 ml/min.). The compounds were monitored by UV-absorption at 280 nm.

a



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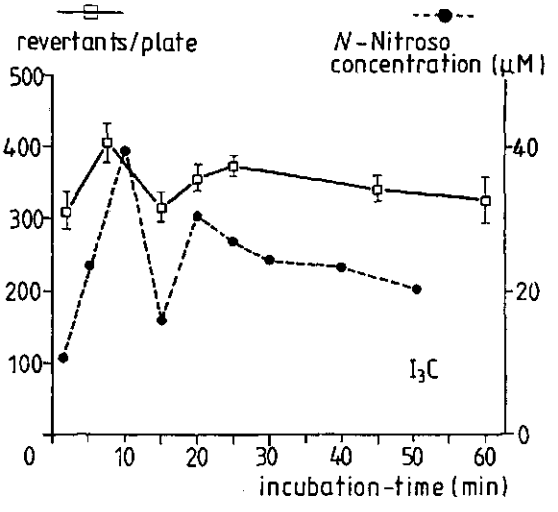
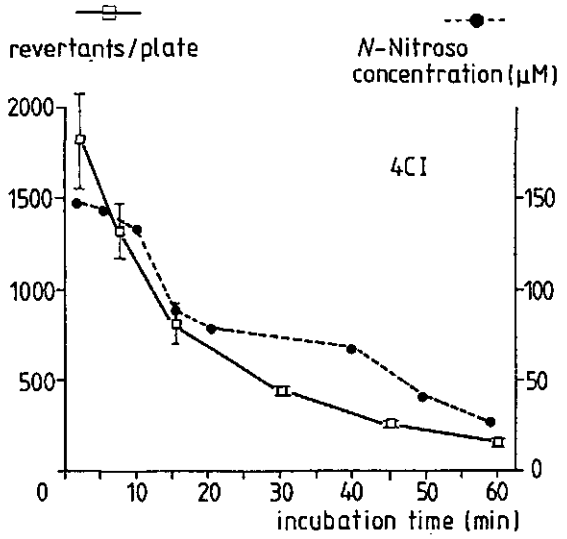


Figure 5.2: Effects of incubation time of (a) indole (I), (b) indole-3-carbinol (I_3C), (c) 4-chloroindole (4Cl) and (d) indole-3-acetonitrile (I_3A) with nitrite on the nitrosation level and on mutagenicity in S. typhimurium TA100, -S9 mix.*
Initial concentrations of the indole solutions, in experiments to determine the N-nitroso levels (NOC) were:

c



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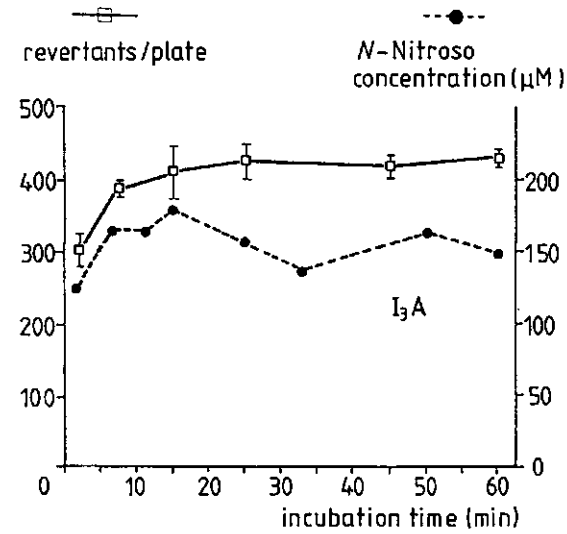


728 μM I; 588 μM I_3C; 266 μM 4CI; 1280 μM I_3A and in mutagenicity assays: 154 nmol I/plate; 170 nmol I_3C/plate; 6.1 nmol 4CI/plate; 154 nmol I_3A/plate

**Number of revertants shown are without correction for that induced by blanks (I_3A, I_3C, I, 156 ± 8; 4CI, 168 ± 20).*

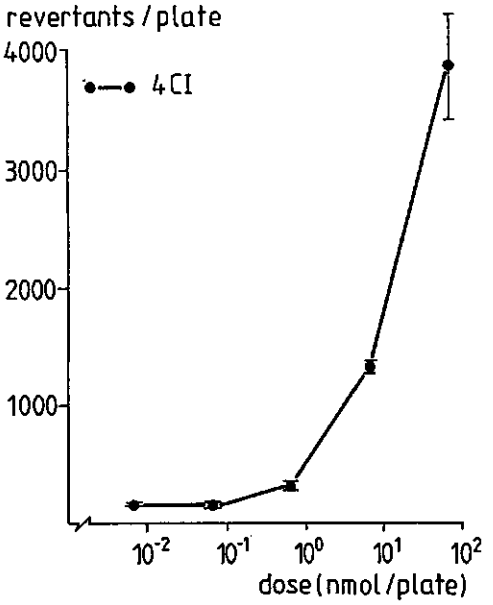
RESULTS

The effects of the length of incubation of the indole compounds with nitrite on the amounts of NOC formed and on the mutagenic activity to *S. typhimurium* are shown in Figure 5.2. For I and 4CI the amounts of NOC formed decreased sharply from the first point of measurement indicating that: 1. the formation of the NOC was almost instantaneous and 2. the NOC formed were highly unstable under the experimental conditions. In contrast, the nitrosation rates of I_3A and I_3C were slower and the amount of NOC formed increased up to about 5 (I_3A) and 10 (I_3C) min. of incubation. When the amounts of nitrosated products were expressed as the percentage of the initial concentration of the indole compound, the maximal percentages were about 55, 29, 14 and 7 for 4CI, I, I_3A and I_3C, respectively. Since 4CI and I will be double nitrosated (a nitroso group at the N1 and an oxime group at the C3 positions) the percentages of the nitrosated product would have been 27.5 for 4CI and 14.5 for I. In Figure 5.2 also the mutagenicity of the nitrite treated indole compounds to *S. typhimurium* is shown. With the exception of I, the profiles of the mutagenic activities parallelled to those of the formed NOC. The decrease in mutagenicity over time was never due to cytotoxicity. Although the responses in the *S. typhimurium* assay and the amounts of NOC were not at maximum at an incubation time of 15 min., in the rest of the experiments this incubation time was used for reasons of comparison.

In Figure 5.3 the dose-response curves in the *S. typhimurium* test of the indole compounds after nitrite treatment are shown. Upon nitrosation about 0.5 nmol 4CI/plate induced twice as much revertants as the negative control, while for I, I_3C, and I_3A this was only observed at respectively 100 nmol, 100 nmol and 200 nmol/plate. From this it can be concluded that 4CI is the most mutagenic indole compound after nitrite treatment, with mutagenic activities 200-400 times those of I_3C, I and I_3A. The activities of nitrosated I_3C and I were similar, but I_3C became cytotoxic at much lower concentrations than I. Without nitrite treatment all indole compounds gave about the same number of revertants as the distilled water blank.

In the study described in Chapter 4, it was mentioned that the NOC formed in some nitrite treated vegetable extracts (mainly of brassicas) were more stable at pH 8 than at pH 2. In the present study the stability of nitrosated I_3A, I_3C and I in the *S. typhimurium* assay just after termination of the nitrosation reaction, without changing the pH (about pH 2) as well as after raising the pH to 8, was investigated. This was done by measuring the mutagenic activity as a function of time (Figure 5.4). All three nitrosated indole compounds showed higher mutagenic activity at pH 8 than at pH 2, indicating that they were more stable at pH 8 and thus respond to pH in the same way as the vegetable extracts. Nitrosated 4CI showed no mutagenic activity at all at pH 8, and it was tested further: after termination of the nitrosation reaction, the pH of the nitrosated 4CI solution was either left unchanged (about pH 2) or was raised to pH 3, 4, 5, 6 and 7, and then after 15 min. the mutagenicity assay was performed (Table 5.1). In contrast to nitrosated I_3A, I_3C and I, nitrosated 4CI appeared to be more stable at low pHs (2-4). It was not mutagenic at pH≥6,

a



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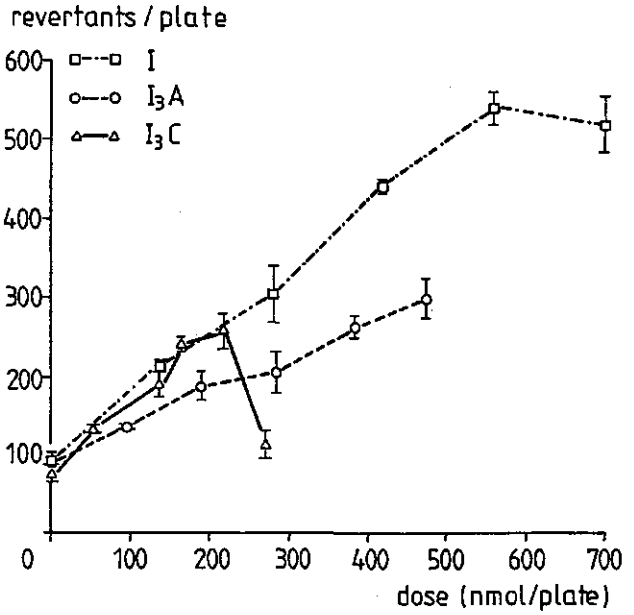

Figure 5.3: Dose-response curves of: (a) 4-chloroindole (4CI) and (b) indole-3-acetonitrile (I_3A), indole-3-carbinol (I_3C) and indole (I) after nitrite treatment in a mutagenicity assay using S. typhimurium TA100, -S9 mix. The indole dose is expressed as the initial concentration of the indole solutions.

Table 5.1: Effect of pH on the mutagenic activity of nitrosated 4-chloroindole (4Cl) determined 15 min. after termination of the nitrosation reaction. Initial concentration: 16.5 nmol 4Cl/plate.*

pH	# revertants/plate
2	690 ± 100
3	696 ± 151
4	808 ± 31
5	426 ± 25
6	124 ± 12
7	52 ± 20

**to S. typhimurium TA100, -S9 mix; number of revertants shown are without correction for that induced by blank (80 ± 37).*

which indicates a rapid breakdown at these pHs.

The stability of nitrosated I_3A and 4Cl at pH 2 and 8 was also investigated by HPLC in combination with a photohydrolysis detector (PHD; Figure 5.5 & 5.6). Figure 5.5 shows the HPLC-PHD chromatograms of nitrosated I_3A and 4Cl. The samples were kept for 2.5 min. after termination of the nitrosation reaction, at pH 2 and 8, and then injected. Nitrosated 4Cl gave two peaks: peak 1, the larger at pH 2, was very small at pH 8. At pH 8, the chromatograms show not only the peaks of the nitrosated products but also a peak (4) with the retention time of nitrite. HPLC carried out with the high intensity discharge lamp turned off resulted in the detection of only nitrite and non-NOC, which interfere with Griess reagent, and indicated that the identity of peak 4 was indeed nitrite. The minor peak (2) for 4Cl that was observed with HPLC-PHD was also detected with the high intensity discharge light turned off. The nitrite probably originated from the breakdown, just before the injection, of nitrosated products, which could not be scavenged by sulphamate because sulphamate is inactive at high pHs. Since sulphamate was added in excess in comparison with nitrite, in the indole solutions at pH 2 no nitrite could be present. However, in the pH 2 chromatograms small nitrite peaks can be seen. This is probably due to the continuous breakdown of nitrosated products, which results in the continuous presence of small amounts of nitrite. Although the experimental method does not allow a quantitative comparison between different NOC, different measurements for the same compound can be compared.

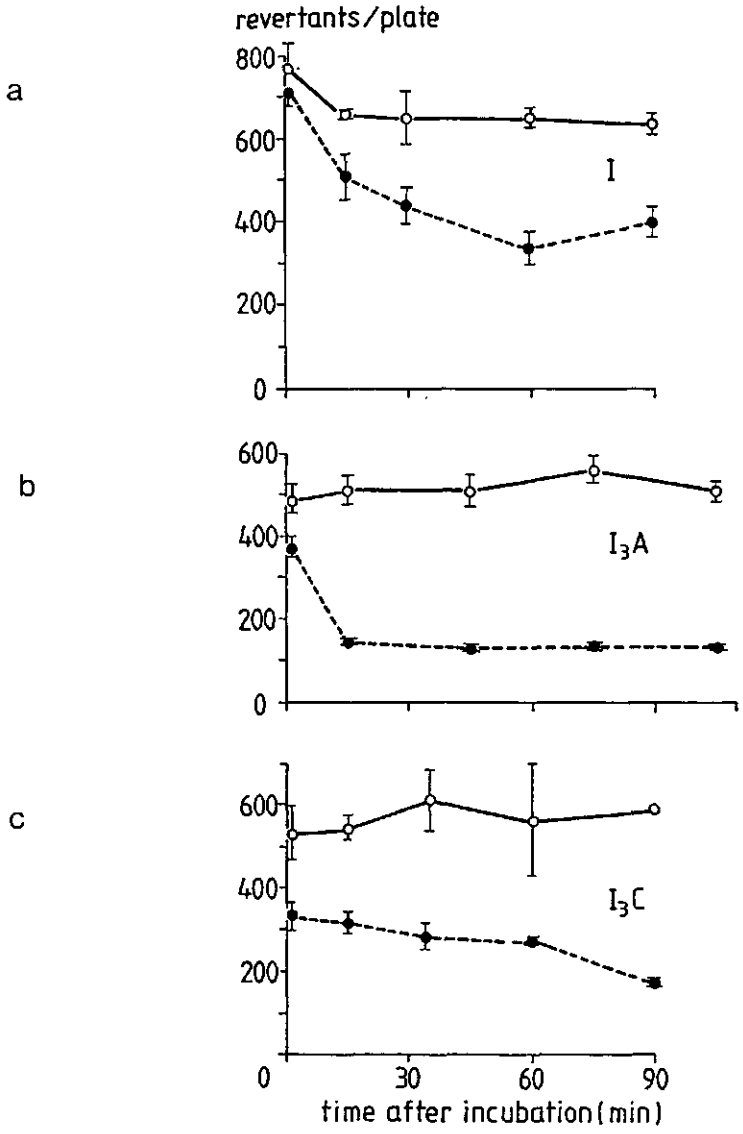

Figure 5.4: Mutagenic activity of nitrosated (a) indole (I), (b) indole-3-acetonitrile (I_3A) and (c) indole-3-carbinol (I_3C) at pH 2 and pH 8 as a function of time after the incubation. Initial concentrations: 855 nmol I/plate, 640 nmol I_3A/plate, 680 nmol I_3C/plate.*

**to S. typhimurium TA100, -S9 mix; number of revertants shown are without correction for that induced by blanks (I_3A, I_3C, I, 119 ± 15).*

The peak areas of nitrosated I_3A at pH 8 and of nitrosated 4Cl at pH 2 in Figure 5.5 were set at 100%. The amounts of the nitrosated products left over time, expressed as a percentage of the peak areas, are shown in Figure 5.6: again nitrosated I_3A was more stable at pH 8 than at pH 2, while for nitrosated 4Cl it was the other way around. However, the minor peak of nitrosated 4Cl was more stable than the major peak at pH 2 and 8. The small nitrite peaks seen in the pH 2 chromatograms of Figure 5.5 decreased as a function of time and eventually disappeared. Since the amounts of nitrosated products left in the pH 2 samples decreased with time, the amounts of nitrosated products that break down progressively decreased and so do the amounts of released nitrite.

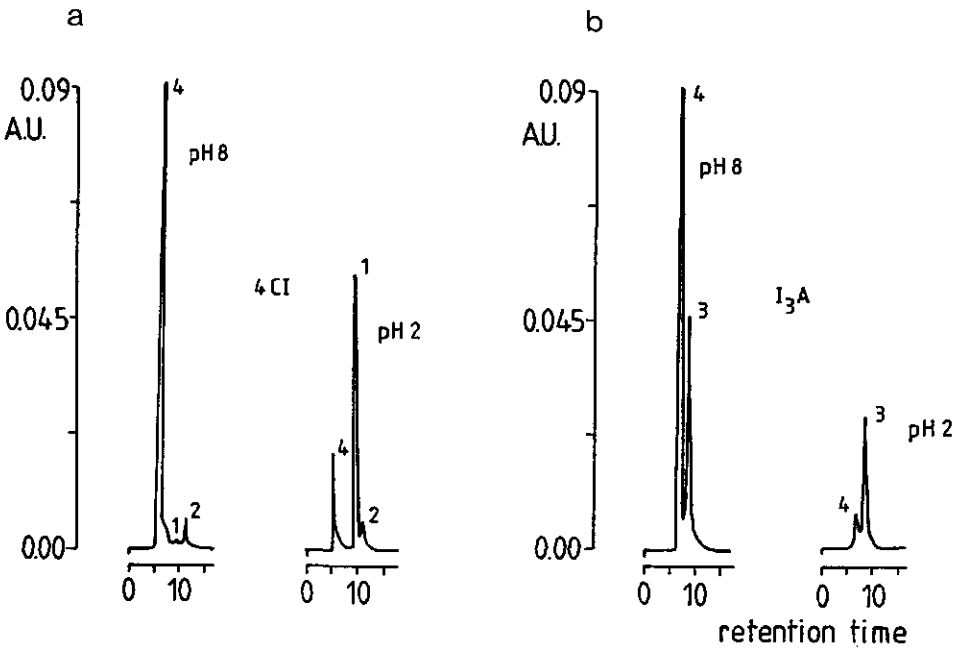

Figure 5.5: HPLC profile of (a) nitrosated 4-chloroindole (4Cl) and (b) indole-3-acetonitrile (I_3A) at pH 2 and 8, 2.5 min. after termination of the nitrosation reaction. Peaks 1 and 2: nitrosated products of 4Cl, peak 3: nitrosated product of I_3A, peak 4: nitrite. The HPLC was fitted to the photohydrolysis detector.

From the HPLC-PHD results it was hypothesized that the stability of nitrosated I_3A, I_3C and I at pH 8 was due to the presence of nitrite, and so an equilibrium would exist between the nitrosated indole compound and the free indole compound plus nitrite. This hypothesis was examined in two experiments. In all experiments described so far the nitrosation reac-

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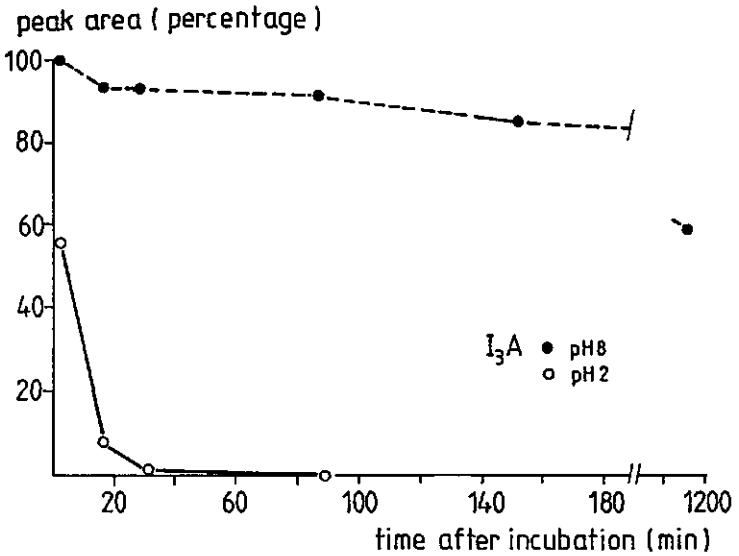

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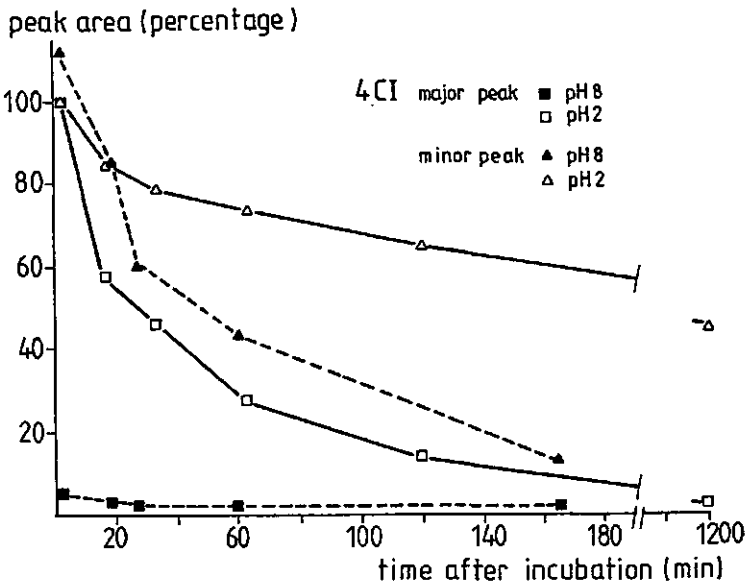

Figure 5.6: The percentage decrease of the peak areas of the nitrosated products of (a) indole-3-acetonitrile (I_3A) and (b) 4-chloroindole (4Cl) at pH 2 and 8 as a function of time. The peak area of nitrosated I_3A in the pH 8 chromatogram and of nitrosated 4Cl in the pH 2 chromatogram of Figure 5.5 were set at 100%.

tion was stopped by addition of an excess of sulphamate over nitrite. Now, experiments were performed in which sulphamate was added in less than equimolar amounts in comparison with nitrite, so that nitrite could be present even in pH 2 solutions. This should result in the maintenance of the equilibrium, manifested by the stability of the nitrosated indole compound. The results of these experiments, which were carried out using the *S. typhimurium* assay and I_3A as test compound, show that nitrosated I_3A was stable at pH 2 in the presence of nitrite (Figure 5.7).

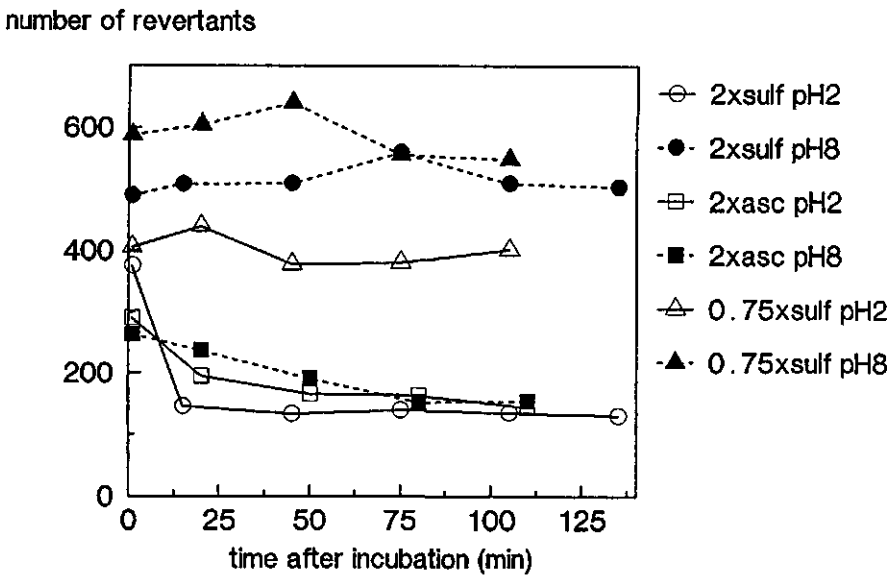

Figure 5.7: Mutagenic activity of nitrosated indole-3-acetonitrile (I_3A) at pH 8 and 2, as a function of time after the incubation, when sulphamate 2x and 0.75x the nitrite concentration and ascorbic acid 2x the nitrite concentration were used to stop the nitrosation reaction. Initial concentration: 640 nmol I_3A/plate.*

**to S. typhimurium TA100, -S9 mix; number of revertants shown are without correction for that induced by blank (107 ± 5).*

Secondly, instead of sulphamate to stop the nitrosation reaction, ascorbic acid was added in amounts that were twice equimolar in comparison with nitrite. After the addition of ascorbic acid the pH was raised to 8. For unknown reasons, in all experiments in which ascorbic acid was used the pH dropped to pH 5-6 in about 20 min.. At these pHs ascorbate scavenges nitrite effectively (Mirvish 1981), which should result in a

shift of the equilibrium to the free indole compound at the cost of the nitrosated indole compound. This should be manifested by the instability of the nitrosated indole compound, and indeed Figure 5.7 shows that nitrosated I_3A was no longer stable at higher pH when no nitrite is present. Therefore both experiments confirmed the hypothesis that the presence of nitrite is a prerequisite for the stability of the nitrosated indole compounds. It should be noted that stopping the nitrosation reaction of I_3C with ascorbic acid resulted in cytotoxicity, probably because of the formation of ascorbigen, a product consisting of an ascorbic acid molecule and an I_3C molecule (Fenwick *et al.* 1983).

To check that the results of the experiments at high pHs were not influenced by the nucleophilic properties of the nitrite scavengers, the experiments were repeated, with modifications, and samples were analyzed by HPLC using UV detection. For comparison, experiments at pH 2 were also repeated. Table 5.2 shows the results, using I_3A as the test compound, in which no sulphamate or ascorbic acid was used at all, or in which twice the equimolar amount of sulphamate or 1.2 times (not twice) the equimolar amount of ascorbic acid (in comparison with nitrite) was used to stop the reaction.

Table 5.2: Effects of using sulphamate or ascorbic acid to stop the nitrosation reaction on the relative peak areas of indole-3-acetonitrile (I_3A) and nitrosated I_3A at pH 2 and 8 as a function of time.

			peak areas*	
	sulphamate or ascorbic acid	min. after incubation	I_3A	nitrosated I_3A
pH 2	none**	60	96%	89%
	sulphamate 2x	20	170%	<1%
	ascorbic acid 1.2x	60	165%	<1%
pH 8	none	60	103%	90%
	sulphamate 2x	45	101%	103%
	ascorbic acid 1.2x	60[+]	155%	11%

**The peak areas in chromatograms obtained just after the nitrosation reaction were set at 100%.*

***none, no ammonium sulphamate or ascorbic acid; sulphamate 2x, ammonium sulphamate at twice the concentration of nitrite; ascorbic acid 1.2x, ascorbic acid 1.2 times the concentration of nitrite was used to stop the nitrosation reaction.*

[+]pH dropped to 5.3.

The peak areas in the chromatograms obtained just after termination of the nitrosation reaction (and raising of the pH) were set at 100% and compared with the peak areas in chromatograms obtained from later injections. Under all conditions that a decrease in mutagenic activity was found with time (Figure 5.7), a decrease in the nitrosated I_3A peak was found while the I_3A peak was increased (Table 5.2). This again confirmed the hypothesis of the existence of an equilibrium between the nitrosated indole compound and the free indole compound plus nitrite. Moreover, these experiments proved that the nucleophilic properties of neither ascorbic acid nor sulphamate influenced the results. If there would have been a nucleophilic reaction of the nitrite scavengers with nitrosated I_3A, a decrease in the mutagenic activity would not have been mirrored by a decrease in the nitrosated I_3A peak and an increase in the I_3A peak.

DISCUSSION

The results of the experiments in which the effects of the incubation time on the amounts of NOC formed were studied show that each indole compound almost immediately forms NOC upon nitrite treatment (Figure 5.2). It is striking that the indole compounds with an unsubstituted C3 atom (I and 4Cl) have higher nitrosation rates than the other indole compounds. Challis & Lawson (1973) have also reported that the C3 nitrosation of indole compounds is very rapid. According to Bonnett & Nicolaidou (1979), indole compounds with unsubstituted C3 atoms will be nitrosated first at this atom. If the C3 atom is substituted, in general the N1 atom will be nitrosated, unless the C3 substituent is a strong electron donor, in which case the C2 atom is nitrosated preferably. For I_3A the chemical structure of the nitrosated product was indeed found to be 1-nitroso-3-acetonitrile (Wakabayashi *et al.* 1985b).

Except for I at a very short incubation time, the profiles of the amounts of NOC formed and the responses in the mutagenicity assay were very similar (Figure 5.2). It is probable that I formed very high amounts of NOC just after addition of nitrite, but that since it took more time to perform the *S. typhimurium* assay than the total N-nitroso measurements, and since nitrosated I is not very stable at pH 2 in the absence of nitrite (Figure 5.4), the nitrosated I had already broken down before the mutagenicity assay was performed.

In the HPLC-PHD experiments nitrosated 4Cl gave two peaks. Büchi *et al.* (1986) also found two products of 4Cl upon nitrosation. The minor, more polar, compound was identified as 3-formyl-4-chloroindazole and the major peak as 4-chloro-2-hydroxy-N1-nitroso-3H-indolin-3-one-oxime (Yang *et al.* 1984). This indazole is not mutagenic to *S. typhimurium* TM677 (Büchi *et al.* 1986), while 4-chloro-2-hydroxy-N1-nitroso-3H-indolin-3-one-oxime shows strong mutagenic activity in various *S. typhimurium* tester strains (Yang *et al.* 1984). In the HPLC-PHD chromatograms for nitrosated 4Cl at pH 8 the minor peak (2) persisted (Figure 5.5) and this peak was also seen in HPLC

measurements with the high intensity discharge lamp turned off. As can be concluded from Table 5.1, this peak would not have been responsible for mutagenic activity, and therefore it is likely that the minor peak in the chromatograms is 4-chloro-2-hydroxy-N1-nitroso-3H-indolin-3-one-oxime. However, mass spectrometric or nuclear magnetic resonance measurements are needed to confirm this.

4CI differed from the other indole test compounds in mutagenicity and stability after nitrite treatment. From the HPLC-PHD results, it was hypothesized that for I_3A, I_3C and I an equilibrium exists between the nitrosated compound and the free indole compound plus nitrite. Therefore experiments were done in which nitrite was present in a nitrosated I_3A solution of pH 2 and in which nitrite was absent in a nitrosated I_3A solution of pH 8. The first experiments resulted in a stability of nitrosated I3A even at pH 2, while the second experiments resulted in the decay of nitrosated I3A. Therefore both types of experiments confirmed the hypothesis. Moreover, it was found that the responses of nitrosated I_3A, I_3C and I in the *S. typhimurium* assay and the amounts of NOC formed tended to be constant from incubation times of about 15 min. (I_3A, I_3C) or 20-25 min. (I) and up (Figure 5.2), which indicates that a steady state had been reached.

Experiments with HPLC-UV detection indicated that nucleophilic reactions are not responsible for the observed results. For experiments in which sulphamate was used, this can also be deduced from the fact that the mutagenicity of nitrosated I_3A, I_3C and I in pH 8 solutions in the presence of sulphamate does not decrease with time (Figure 5.4). If sulphamate would have reacted with the nitrosated products then the amounts to which the bacteria were exposed would have been decreased, with corresponding reductions in mutagenic activities. Although no chemical characterizations were done, effects of pH changes can probably be excluded. This because of: (1) HPLC chromatograms of solutions at pH 2 and 8 were always identical, even when different columns and eluents were used (data not shown). (2) When an I_3A solution was nitrosated with 10 mM $NaNO_2$ without stopping the nitrosation reaction and then divided into two parts, one of which was kept at pH 2 and the other adjusted to pH 8, both parts showed identical mutagenic responses (data not shown).

Mellet *et al.* (1986) also found evidence for the existence of an equilibrium between tryptophan and its nitrosated form by comparing experimental values of optical densities with theoretical ones, while using different ratios of the N-acetyl derivative of L-tryptophan to nitrite. These data suggest that tryptophan behaves the same as I_3A, I_3C and I did in the present study.

It was quite difficult to obtain a linear dose-response curve for nitrosated I_3C and the lowest concentration at which cytotoxic effects were observed varied from experiment to experiment. In many cases I_3C solutions became flocculent when adding acid. This has also been reported by Bradfield & Bjeldanes (1987). They found that I_3C will form a series of acid condensation products such as linear and cyclic methylene indole trimers and tetramers as well as di-indolylmethane. This implies that it is not

sure that the effects of nitrosated I_3C as such have been measured in the present study; possibly the effects of nitrosated condensation products of I_3C were measured.

Since it has been reported that nitrosated I_3A has potential genotoxic and tumour promoting activity (Yamashita *et al.* 1988, Furihata *et al.* 1987) and many other indole compounds also show mutagenic activity (Yang *et al.* 1984, Wakabayashi *et al.* 1985, 1986a, Ochiai *et al.* 1986, Gatehouse & Wedd 1983) and since the formation of nitrosated products is rapid even at physiological feasible nitrite concentrations (Yang *et al.* 1984, Wakabayashi *et al.* 1986), it is important to investigate the risk of nitrosated indole compounds to humans. The results of the present study indicate that nitrosated I_3A, I_3C and I when formed endogenously, will be stable in the presence of nitrite, while 4CI will also be stable without nitrite.

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CHAPTER 6

THE ROLE OF GLUCOSINOLATES AND SEVERAL KNOWN INDOLE COMPOUNDS IN THE FORMATION OF N-NITROSO COMPOUNDS IN BRASSICAS

INTRODUCTION

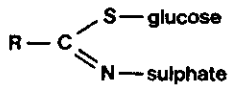
In the study described in Chapter 4, vegetables were screened for their potential to form directly mutagenic N-nitroso compounds (NOC). While all the vegetables tested formed NOC following nitrite treatment, brassicas exhibited the highest levels. Whereas no correlation was found between the amounts 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 it was examined whether this association reflects a causal relationship between the two variables. The glucosinolates, sinigrin (SGN), gluconapin (GNP), glucobrassicanapin (BNP), progoitrin (PGT), glucotropaeolin (GTP), sinalbin (SAL), gluconasturtiin (NST), glucobrassicin (GB) and 4-hydroxyglucobrassicin (4-OH-GB) (Figure 6.1), 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 indolylglucosinolates) is able to catalyze the nitrosation reaction (Boyland *et al.* 1971), the breakdown products of glucosinolates were further tested for their ability to catalyse 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.

This chapter is based on:

Tiedink, H.G.M., A.M. Hissink, S.M. Lodema, L.W. van Broekhoven & W.M.F. Jongen (1990) Several known indole compounds are not important precursors of direct mutagenic N-nitroso compounds in green cabbage. Mutation Res. 232, 199-207.

Tiedink, H.G.M., C.E. Malingré, L.W. van Broekhoven, W.M.F. Jongen, J. Lewis & G.R. Fenwick (1991) The role of glucosinolates in the formation of N-nitroso compounds. J. Agric. Food Chem. 39, 922-926.

Since indole (I), indole-3-acetonitrile (I_3A) and indole-3-carbinol (I_3C), the hydrolysis products of glucobrassicin were found to be mutagenic to *Salmonella* (*S.*) *typhimurium* upon nitrite treatment (Chapter 5), their role in the formation of directly mutagenic NOC in extracts of brassicas was further investigated in the study described in this chapter. Green cabbage (*Brassica oleracea* var. *sabauda* L.) was used as model vegetable, because it generally contains high levels of glucosinolates and it induced high numbers of revertants upon nitrite treatment (Chapter 4). Besides I_3A, I_3C and I, tryptophan (Trp) was also tested (Figure 6.2) for its role in the formation of directly mutagenic NOC. Additionally, experiments were performed to detect other indole compounds then I_3A, I_3C, I and Trp by analyzing green cabbage extracts with high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS).



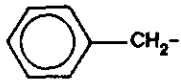
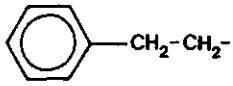
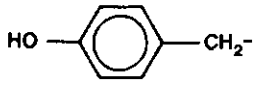
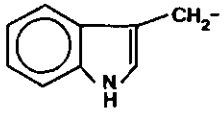
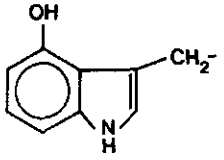
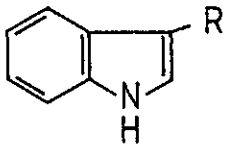
Compound	Origin	R =
Sinigrin	(*Brassica juncea*)	CH_2=CH-CH_2-
Gluconapin	(*Brassica napus*)	CH_2=CH-CH_2-CH_2-
Brassicanapin	(*Brassica napus*)	CH_2=CH-CH_2-CH_2-CH_2-
Progoitrin	(*Brassica campestris*)	CH_2=CH-CHOH-CH_2-
Glucotropaeolin	(*Brassica rapa*)	
Gluconasturtiin	(*Brassica rapa*)	
Glucosinalbin	(*Sinapis alba*)	
Glucobrassicin	(*Brassica oleracea*)	
4-Hydroxyglucobrassicin	(*Brassica napus*)	

Figure 6.1: Chemical structures and origins of the glucosinolates tested.



R =

CH_2CN	INDOLE-3-ACETONITRILE
CH_2OH	INDOLE-3-CARBINOL
H	INDOLE
$CH_2CH(NH_2)COOH$	TRYPTOPHAN
CHO	INDOLE-3-CARBOXALDEHYDE
C_2H_4OH	INDOLE-3-ETHANOL
CH_2COOH	INDOLE-3-ACETIC ACID

Figure 6.2: Chemical structures of some indole compounds.

MATERIALS AND METHODS

Chemicals and green cabbage

In the laboratory of Dr. G.R. Fenwick (AFRC, Norwich, UK), SGN, GNP, BNP, PGT, GTP, SAL, NST, GB and 4-OH-GB were isolated from the sources shown in Figure 6.1, according to the methods of Hanley *et al.* (1983) and Peterka and Fenwick (1988). With the exception of 4-OH-GB, all glucosinolates were obtained in >95% purity (checked by HPLC and glucose release). The sample of 4-OH-GB was a crude isolate from rapeseed (*Brassica napus*), was prone to oxidation and was not amenable to purification using the methods described. For this reason the crude material (40% pure) was used directly. Myrosinase (200 U per mg) from white mustard (*Sinapis alba*) was obtained from Biocatalysts, Pontypridd, Wales, UK.

Proline, Trp and all indole compounds were purchased from Sigma, St. Louis Mo, USA. All other chemicals were at least of analytical grade.

A specified cultivar of green cabbage was obtained from the Central Hortical Auctions in The Netherlands and was treated as described in the study of Chapter 4. This cultivar contained high levels of indolylglucosinolates (11.82 μmol 4-OH-GB, 1300.2 μmol GB, 130.0 μmol 4-methoxyglucobrassicin and 47.3 μmol neoglucobrassicin per kg fresh material) and induced about 2000 (16600) *S. typhimurium* TA100 revertants per gram fresh (dry) material upon nitrite treatment.

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. Enzymatic hydrolyses were carried out at pH 6 by addition of 1 U myrosinase per μmol of glucosinolate, followed by incubation at 40°C for 1 h. Samples were taken at various times during this incubation and analyzed for intact glucosinolates by HPLC using a Hypersil APS column (3 x 200 mm; Chrompack International BV, Middelburg, The Netherlands) with 0.01 M H_3PO_4 (pH 4.5; flow of 0.4 ml/min.) as mobile phase. Glucosinolates were detected by UV-absorption at 220-227 nm.

Determination of nitrite-glucosinolate reaction by HPLC-photohydrolysis

Solutions of intact and myrosinase-treated glucosinolates (1 U enzyme per μmol glucosinolate for 1 h at pH 6 and 40°C) were adjusted to pH 2. After addition of a $NaNO_2$ solution (final concentration 40 mM) the pH was again adjusted to pH 2, and the resulting solutions were incubated in the dark for 15 min. at 37°C. The nitrosation reaction was stopped by addition of ammonium sulphamate ($NH_4SO_3NH_2$, final concentration 48 mM) and the formation of NOC was determined by HPLC coupled to a photohydrolysis detector (PHD). The HPLC-PHD system has been described by Shuker & Tannenbaum (1983; see Chapter 2). The HPLC conditions were the same as used in the preceding section.

Extraction of green cabbage

Extracts of green cabbage used in GPC experiments were obtained by a stepwise extraction with distilled water and methanol (MeOH). After addition of distilled water (7.5 ml per g of lyophilized green cabbage), the cabbage mixture was shaken for 15 min. in a satellite flask-shaker (Griffin & George Ltd., Wembley, Middx, UK) followed by centrifugation (30 min.; 16,300 x g). Then MeOH (7.5 ml per gram of lyophilized cabbage) was added to the pellet and the extraction procedure was repeated. The two supernatants were combined, centrifugated (30 min.; 16,300 x g), and the obtained supernatant was evaporated by vacuum. The residue was resuspended in distilled water (about 1.3 ml per gram cabbage), centrifuged (15 min.; 950 x g) and filtered (1.2 μm, Schleicher & Schuell, D-3354 Dassel, Germany). During this procedure over 90% of the glucosinolates was hydrolyzed by the action of myrosinase, present in the lyophilized vegetables. The glucosinolate analysis has been described in Chapter 4.

Extracts of green cabbage used in HPLC/GC-MS experiments were obtained by extraction with distilled water/MeOH 1/1 (v/v) (11 ml per g of lyophilized green cabbage). After mixing (15 min. on a Vortex), and centrifugation (30 min.; 11,700 x g), the obtained supernatant was evaporated by vacuum and resuspended in 5 ml of distilled water and was ready for HPLC analysis. In case of GC or GC-MS analysis it was applied to a Seppak C18 cartridge (Millipore, Waters Associates, Milford MA, USA), which was eluted with MeOH.

Gel permeation chromatography (GPC)

GPC was performed using a 1.6 x 30 cm column, containing Sephadex G15 (Pharmacia, S-75182 Uppsala, Sweden) as stationary phase and 0.1 M $(NH_4)_2HPO_4$ (pH 6.5)/acetonitrile, 85/15 (v/v) as mobile phase (flow 1 ml/min.). A maximum of 2.25 ml of

green cabbage extract was brought onto the column. Fractions of about 10 or 20 ml were collected and concentrated by vacuum to about 4 ml. In this way all acetonitrile was removed from the buffer (checked by HPLC with refractive index detection). The concentrated fractions were adjusted to the desired volume with distilled water and nitrosated as described before.

Indole compounds used to spike green cabbage extracts were dissolved in dimethyl sulphoxide (DMSO) from which 50 μl was mixed with 2.20 ml of a green cabbage extract.

Total N-Nitroso determination

The total levels of NOC formed in nitrite treated glucosinolate solutions as well as in nitrite treated GPC fractions of green cabbage extracts were determined by thermal energy analysis (Thermal Energy Analyzer (TEA; Thermedics Inc., Woburn MA, USA) as described by Walters *et al.* (1978; see Chapter 2).

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 to 494 μM), or distilled water, were added to proline solutions (437 μM) and treated with nitrite (0 to 60 min.) as described before. Samples were removed at various times and analyzed for their total NOC content.

Mutagenicity Assay

The *S. typhimurium* assay was performed as described in Chapter 4 and 5. At least two independent experiments were carried out with 5 doses of either intact or hydrolysed glucosinolates (0.2, 0.4, 0.6, 0.8 and 1.0 ml, filled up to 1 ml with distilled water) and 2 doses (0.4/0.5 ml and 0.8/1.0 ml) of each GPC fraction of green cabbage extracts. In each experiment a positive control (1 μg 1-methyl-1-nitroso-3-nitroguanidine per plate) was include, which induced >2000 revertants.

The number of revertants induced by untreated glucosinolates/GPC fractions were subtracted from those induced after nitrite treatment and the data presented represent the average values of one representative experiment.

HPLC

Green cabbage extracts were analyzed for the presence of several known indole compounds using a hypersil APS column (100 x 3 mm; Chrompack International BV) with 0.01 M H_3PO_4 (pH 4.5)/acetonitrile 80/20 (v/v) (flow 0.4 ml/min.) as mobile phase. A Waters 490 multiwave UV detector (Millipore, Waters Associates) was used for monitoring.

GPC fractions were analyzed for the presence of I, I_3A, I_3C and Trp by HPLC, using a lichrosorb 5RP18 column (150 x 4.6 mm; Chrompack International BV). The solvent programme consisted of a linear gradient over 20 min. from 80/20 to 20/80 (v/v) 0.02 M CH_3COONH_4 (pH 6.5)/acetonitrile and subsequently in 2 min. to 100% acetonitrile (flow 1 ml/min.). The compounds were monitored by UV-absorption at 280 nm.

GC-MS

The identity of indole compounds found in HPLC fractions of green cabbage extracts were analyzed by GC using a capillary CP-Sil 19CB column (25 x 0.25 mm; Chrompack International BV) coupled to a VG MM7070F mass spectrometer, electron impact ionization at 70 eV (VG-isogas ltd, Middlewich, Cheshire, UK). After 4 min. at 120°C the temperature programme of the GC consisted of a linear gradient over 40 min. to 280°C. In case of using "probe-MS" the tested compounds were directly applied to the mass spectrometer.

RESULTS

Hydrolysis of glucosinolates

The hydrolysis of glucosinolates by both acid and myrosinase is shown in Table 6.1. While almost half of 4-OH-GB was hydrolysed by acid within 2 h, only small amounts of SAL, NST, GB and PGT and none of BNP, GTP, GNP and SGN were hydrolysed by acid. With the exception of the 4-hydroxyindole compound, all glucosinolates were hydrolysed by myrosinase within 0.5 h.

Table 6.1: Percentage of breakdown of glucosinolates 2 h after the addition of acid (37°C, pH 2) and 30 min. after the addition of myrosinase (1 U per µmol glucosinolate, 40°C, pH 6).

Compound	Treatment with Acid	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

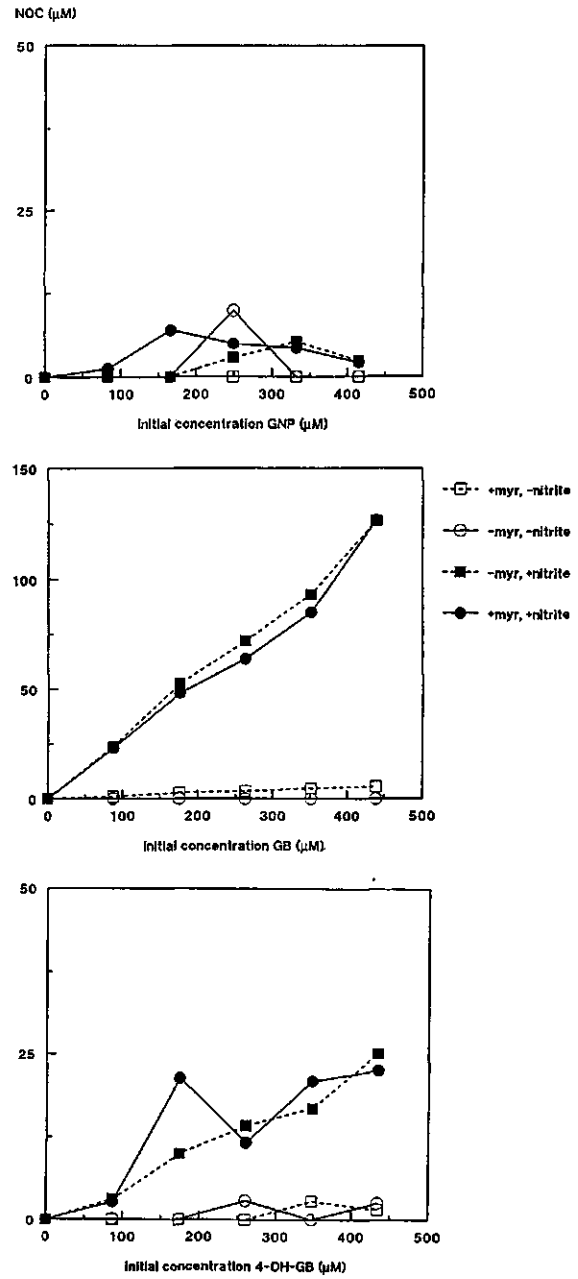


Figure 6.3: Levels of N-nitroso compounds formed in solutions of intact and myrosinase-treated gluconapin (GNP), glucobrassicin (GB) and 4-hydroxy-glucobrassicin (4-OH-GB).

Ability of glucosinolates to form NOC, and to catalyse NOC formation

The ability of intact and hydrolysed glucosinolates to form NOC following nitrite treatment was examined using the HPLC-PHD system. Intact GB, 4-OH-GB, GNP and hydrolysed GB and 4-OH-GB 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 (Figure 6.3). It was noted that GNP did not form significant amounts of NOC; the traces observed may possibly originate from residual nitrite, which is supported by the observation that no dose-response curve was obtained. This result contrasts with those of the HPLC-PHD experiments, in which GNP gave a response after nitrite treatment. Given the structural similarity (Figure 6.1) of GNP to SIN and BNP, both of which did 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-OH-GB 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-OH-GB and 5% of the intact 4-OH-GB were nitrosated.

In order to investigate the ability of glucosinolate breakdown products to catalyse the nitrosation reaction, the nitrosation rate of proline was examined in the presence and absence of myrosinase-treated aryl- and alkyl-glucosinolates. Since the breakdown products of indolylglucosinolates also form NOC, which would have interfered with the N-nitrosoproline measurements, the catalytic effects of the breakdown products of GB and 4-OH-GB could not be determined. No catalytic effect of the breakdown products of the tested alkyl/aryl glucosinolates was apparent.

Mutagenic activity of glucosinolates

The mutagenic activity of nitrosated products of intact and myrosinase-treated glucosinolates to *S. typhimurium* TA100 was examined. None of the alkyl/aryl-glucosinolates showed mutagenic activity. However, all were found to be cytotoxic upon myrosinase treatment irrespective of whether or not they were nitrosated. Of the indolylglucosinolates only myrosinase-treated GB showed direct mutagenic activity upon nitrosation, this being observed from the first point of measurement (Figure 6.4).

GPC fractionation of a green cabbage extract

GPC fractions obtained from a green cabbage extracts were nitrosated and tested for the presence of NOC and for mutagenic activity (Figure 6.5). Fraction 3 was the most mutagenic fraction and also contained high levels of NOC. Fraction 2 also contained high levels of NOC, but the mutagenicity could not be tested as a consequence of the presence of histidine, which disturbs the *S. typhimurium* assay.

In previous experiments it was observed that even after elution with 11 times the total column volume (Vt) compounds eluted, indicating that adsorption of compounds to the column material occurred. To reduce adsorption 15% (v/v) acetonitrile was added to the

buffer, by which all compounds were eluted at 5 x Vt.

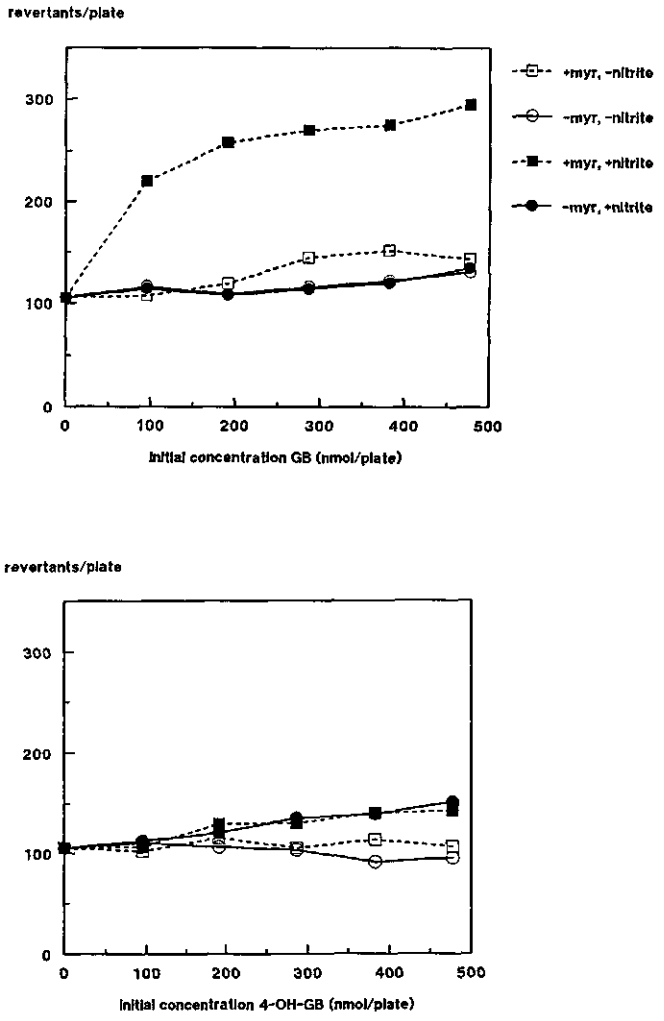


Figure 6.4: Mutagenic activity of intact and myrosinase-treated glucobrassicin (GB) and 4-hydroxyglucobrassicin (4-OH-GB) before and after nitrite treatment.*

** to S. typhimurium TA100, -S9 mix.*

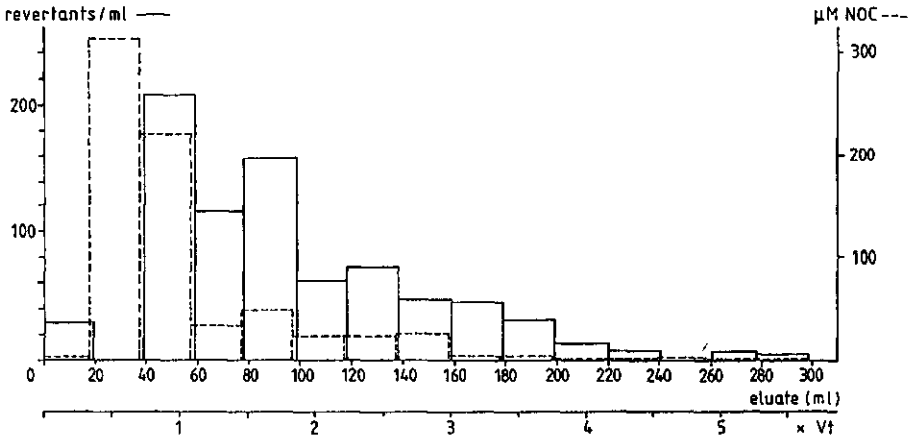


Figure 6.5: Mutagenic activity and total N-nitroso concentration induced by nitrite treatment, expressed per ml of a GPC fraction obtained from an extract of 1.80 g of green cabbage applied to the column.*

** to S. typhimurium TA100, -S9 mix.*

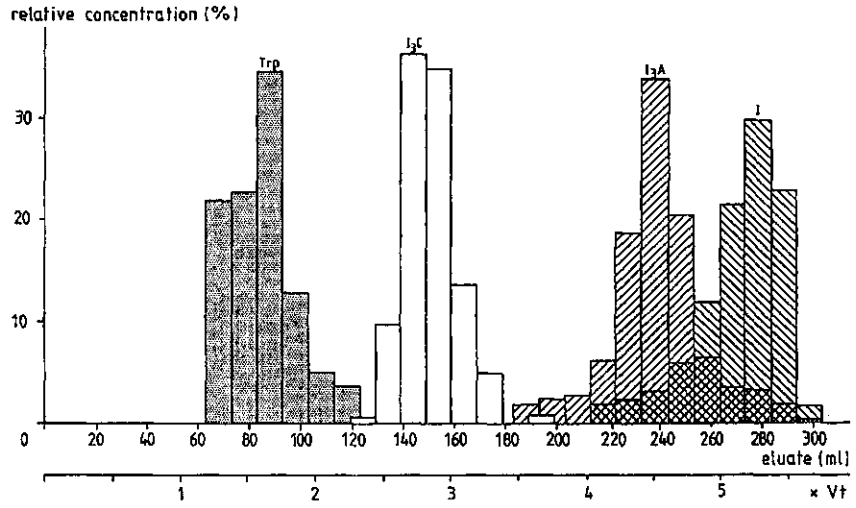


Figure 6.6: Amounts of indole compounds in GPC fractions of an extract of 1.68 g of green cabbage spiked with 200 µg indole-3-acetonitrile (I_3A), 283 µg indole-3-carbinol (I_3C), 150 µg indole (I) and 159 µg tryptophan (Trp), determined by HPLC.*

** expressed as the percentage of the total amounts detected in all fractions.*

In order to determine the fractions in which I_3A, I_3C, I and Trp eluted, a green cabbage extract was spiked with these indole compounds, and the GPC fractions thus obtained were analyzed by HPLC for their presence (Figure 6.6). The amount of an individual indole compound in one particular fraction was expressed as the percentage of the total amounts of this indole compound appearing in all fractions. The recoveries of the spiked indole compounds were ≥100%. Comparing Figure 6.3 to 6.2, shows that the indole compounds with the exception of Trp eluted in fractions with minor mutagenic activity. This indicates that I_3A, I_3C and I are not important precursors of NOC in green cabbage.

Identification of indole compounds in green cabbage extracts

To identify other indole compounds, which could also play a role as precursor of NOC, different analytical methods were used. In HPLC chromatograms of green cabbage extracts peaks were present that had the same retention times as indole-3-acetic acid (IAA), I_3C, indole-3-carboxaldehyde (ICHO), I_3A and indole-3-ethanol (IE) (Figure 6.7). Analysis of UV spectra confirmed the identity of ICHO, I_3A and IE (Figure 6.8), but did not of IAA and I_3C. The peaks with retention times corresponding with those of ICHO, I_3A and IE were collected and analyzed by direct-probe MS. The identity of I_3A and ICHO was confirmed, but not that of IE (Figure 6.9), indicating that I_3A and ICHO are present in the green cabbage extracts and can be precursors of NOC. Neither I_3A nor ICHO were very important precursors of NOC in green cabbage. For I_3A this has already been argued before, while for ICHO it can be concluded from the observation that it did not show mutagenic activity to *S. typhimurium* TA100 after nitrite treatment (Table 6.2).

Table 6.2: Mutagenic activity of indole-3-carboxaldehyde (ICHO) upon nitrite treatment.*

ICHO/plate (nmol)**	Revertants/plate
0	133 ± 8
300	154 ± 3
400	138 ± 13
500	166 ± 7
600	157 ± 8
700	143 ± 13

** to S. typhimurium TA100, -S9 mix.*

*** Initial concentration ICHO.*

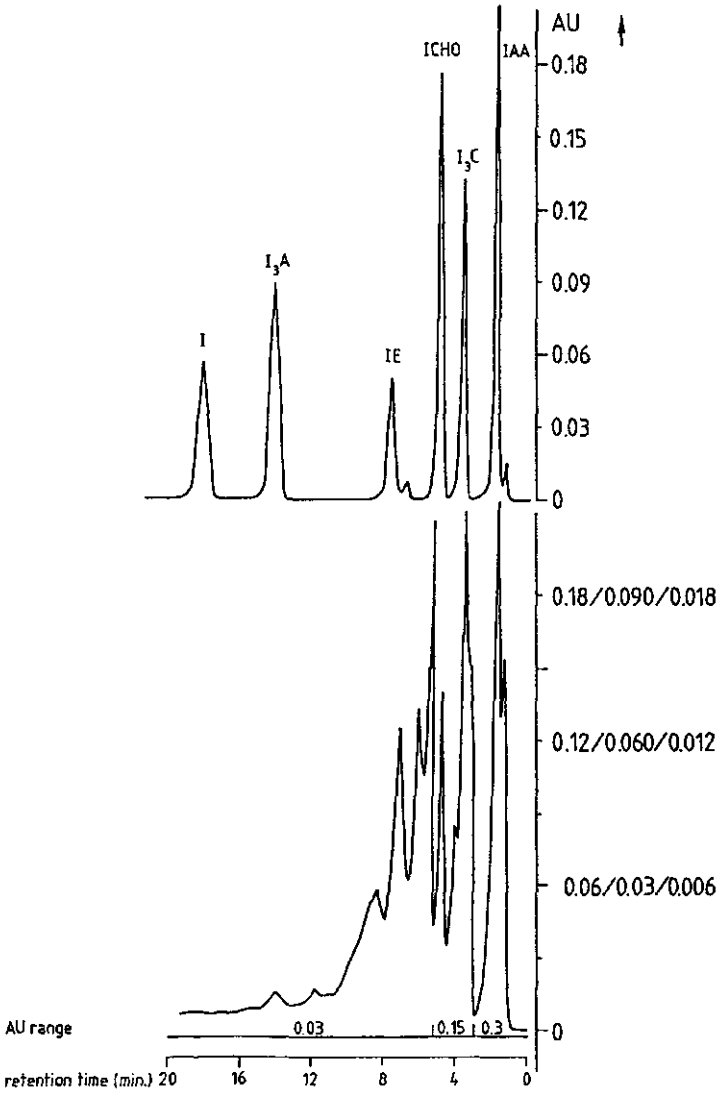


Figure 6.7: HPLC chromatograms of a green cabbage extract (20 µl of a distilled water/methanol extract, about 300 mg/ml green cabbage) and of an indole mixture (20 µl of a standard solution containing 20 µg/ml indole-3-acetic acid (IAA), indole-3-carbinol (I_3C), indole-3-carboxaldehyde (ICHO), indole-3-acetonitrile (I_3A), indole (I) and 32 µg/ml indole-3-ethanol (IE)).

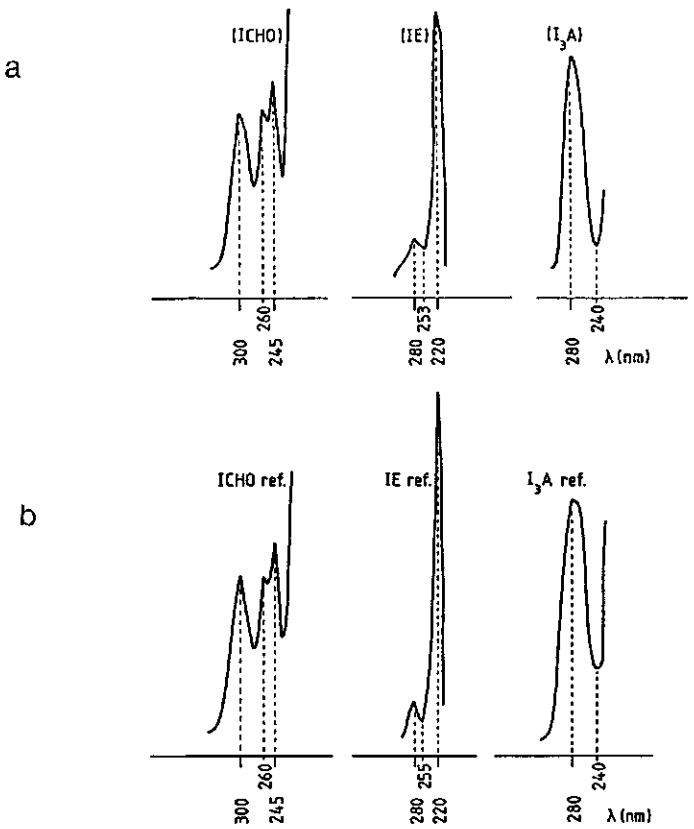


Figure 6.8: UV spectra of (a) compounds that could be I_3A, IE and ICHO, based on their HPLC retention times and of (b) I_3A, IE and ICHO standards.

Finally green cabbage extracts were analyzed for the presence of indole compounds by GC-MS, which resulted in the only detection of I_3A.

Recovery of I_3A in GPC fractions

The concentration of I_3A in green cabbage extracts was estimated by comparing the I_3A peak areas in both HPLC and GC chromatograms of green cabbage extracts with calibration curves made with I_3A standards. The amount of I_3A was estimated to be 0.002% of the dry weight using HPLC and 0.010% using GC.

The amount of I_3A in the combined GPC fractions 10 to 15 from Figure 6.5 was estimated by probe MS to be 83 mg/kg dry green cabbage, which implied a 83% recovery (on basis of GC experiments).

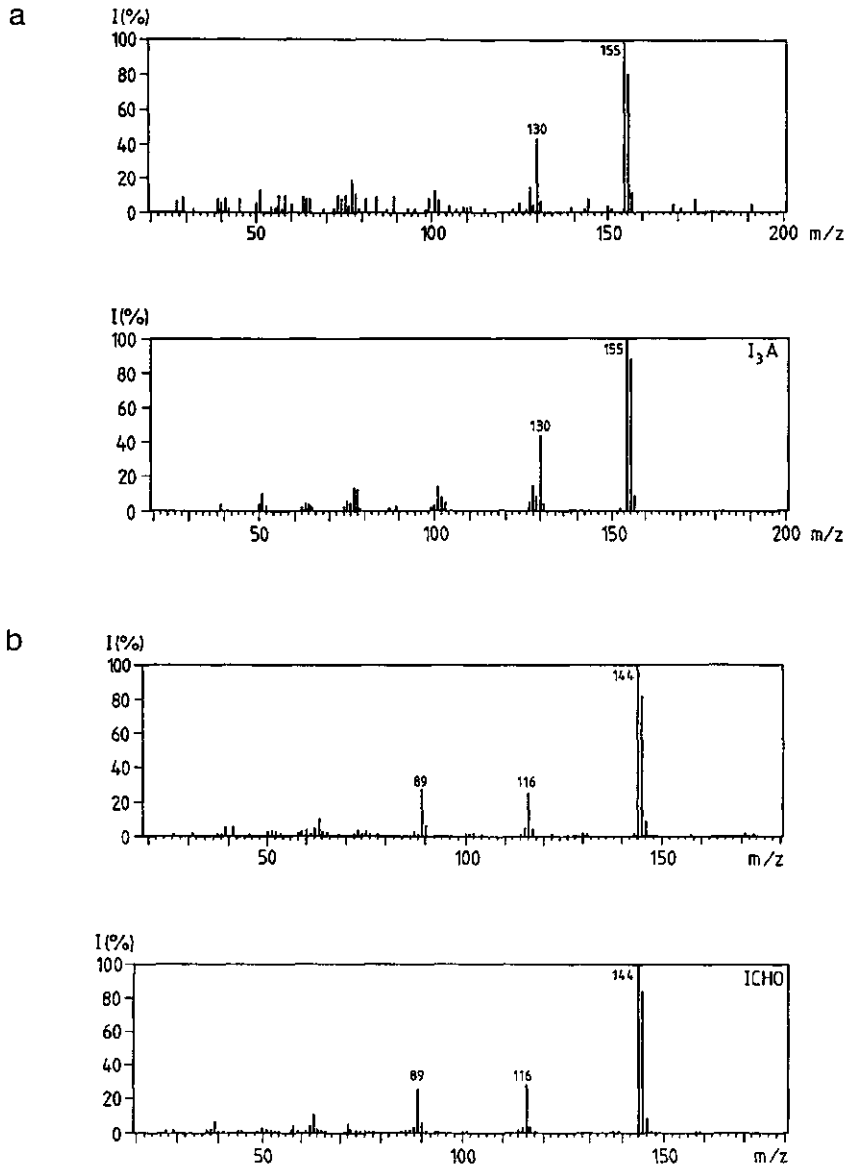


Figure 6.9: Mass spectra of compounds collected from HPLC, derived from a green cabbage extract, which are expected to be (a) indole-3-acetonitrile (I_3A) and (b) indole-3-carboxaldehyde (ICHO) (top) and of (a) I_3A and (b) ICHO standards (bottom).

DISCUSSION

The results of the present study show that of all the glucosinolates tested only indolylglucosinolates are able to form NOC upon nitrite treatment. Although both intact and myrosinase-treated indolylglucosinolates formed NOC upon nitrite treatment, only myrosinase-treated GB was found to be directly mutagenic to *S. typhimurium* after nitrite treatment. Given the similar rates of nitrosation of the intact and myrosinase-treated indolylglucosinolates (24 and 25% respectively), it can be suggested that steric hindrance prevents alkylation of DNA by nitrosated intact GB. The observation that no mutagenicity could be detected upon nitrosation of myrosinase-treated 4-OH-GB might be the result of the incomplete hydrolysis of 4-OH-GB by myrosinase (only 56% in 30 min.), its low nitrosation rate (5%) and the low purity of the sample available (40%).

None of the alkyl/aryl-glucosinolates examined 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 the present study originated from isothiocyanates formation. Apparently, isothiocyanates will not be able to catalyze the nitrosation reaction, since none of the myrosinase-treated aryl/alkyl-glucosinolates were able to catalyse the nitrosation reaction of proline.

From these results it can be concluded that the correlation found in Chapter 4, between the amounts of NOC formed in extracts of brassica vegetables following nitrite treatment, and their aryl/alkyl-glucosinolate content does not appear to reflect a causal relationship. Moreover, it can be concluded that despite the fact that myrosinase-treated GB can form NOC upon nitrite treatment, it is not an important precursor of directly mutagenic NOC in brassicas. This can be deduced by comparing the levels of GB in brassicas (Table 4.3) to the mutagenicity of myrosinase-treated GB after nitrite treatment (Figure 6.4), and is supported by the observation that also I, I_3C and I3A at levels present in extracts of green cabbage were not found to be important precursors of NOC (Figure 6.5 & 6.6). The latter was also observed using Brussels sprouts (data not shown).

From the GPC experiments in which green cabbage extracts were spiked with I_3A, I_3C, I and Trp, it could be deduced that Trp might be a precursor of directly mutagenic NOC, since Trp eluted in fractions, which were mutagenic upon nitrosation. However, the free amino acid Trp was only present in small amounts in the lyophilized green cabbage (64 nmol/g, and in previous experiments Trp could only be detected in GPC fraction 5 (unpublished data). The maximum concentration in this concentrated fraction could have been about 21 μM, which implies a maximum of only 4.29 μg per plate in the *S. typhimurium* test. Since Trp induced 4740 *S. typhimurium* TA100 revertants per mg upon nitrosation (Ochiai *et al.* 1986), Trp would not have been an important precursor of NOC in green cabbage extracts either.

In spite of the fact that we spiked with rather high concentrations of indole com-

pounds and Trp in comparison with the levels naturally occurring in green cabbage, the method was reliable. This was shown by probe MS analysis of a non-spiked green cabbage extract in which I_3A eluted in the same GPC fractions as spiked I_3A with a recovery of 83%. Additionally, it should be noted that the separation of compounds present in green cabbage extracts by means of GPC was not merely based on molecular size. This can be concluded from the fact that histidine, which is a relative small molecule, already eluted in fraction 2, while the indole compounds with comparable molecular sizes eluted in later fractions up to 5 x Vt, even when using acetonitrile in the buffer (Figure 6.5). Nevertheless, the method appeared to be quite suitable for the purpose of the present study.

The most commonly occurring indole compound in green cabbage was found to be ICHO. ICHO, however, did not show mutagenic activity. I_3A was the second most commonly occurring indole compound. No other indole compounds could be detected in green cabbage, although I_3C has been reported to be the main product formed from GB upon hydrolysis at neutral pH (Virtanen 1965). Bradfield & Bjeldanes (1987) indicated why I_3A and ICHO could have been isolated with success relative to I_3C. I_3C is an unstable compound, which can easily be oxidized to ICHO, by which levels of I_3C in vegetable extracts will decrease and those of ICHO will increase as a function of time. Although all experiments were performed with freshly prepared extracts, no I_3C could be detected. However, the ICHO peak in HPLC chromatograms of extracts, increased over time (data not shown). This can imply that I_3C was present in fresh green cabbage extracts but could not be detected for unknown reasons.

Wakabayashi *et al.* (1985) reported that I_3A, occurring in Chinese cabbage in amounts ranging from 0.07-0.33 mg/kg fresh weight (Wakabayashi *et al.* 1985, 1986a, 1986b), is an important precursor of NOC. In the present study the amounts of I_3A in green cabbage were estimated to be much higher (11.82 mg/kg fresh weight). Although the amounts of I_3A will vary per species, other authors found similar levels of I_3A in different brassicas (Wall *et al.* 1988, Bradfield & Bjeldanes 1987) as the ones in the present study. Wakabayashi *et al.* (1985) estimated the contribution of I_3A to the total mutagenicity of nitrosated Chinese cabbage to be less than 10%, while in the present study it was roughly estimated to be 2%. It should be noted that the calculation of the contribution of one compound such as I_3A or of one GPC-fraction to the total mutagenicity of an extract is probably not very accurate, because in an extract or a fraction the presence of many compounds will have an effect (stimulating or inhibiting) on the nitrosation rate of other compounds. For this reason the calculated recoveries of the mutagenic activity (71%) and amounts of NOC (124%) in the GPC-fractions may not be very accurate.

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CHAPTER 7

CONCLUDING REMARKS TO CHAPTERS 3-6

In the first part of this thesis the attention was focused on glucosinolates and their breakdown products, with special emphasis on their role in the formation of N-nitroso compounds (NOC) in brassica vegetables and on the stability of their NOC formed upon nitrosation. After an introduction to brassicas (Chapter 3), the results are presented of the assessment made of the relationship between the levels of glucosinolates in brassicas and the amounts of NOC formed in extracts of these vegetables upon nitrosation (Chapter 4). No causal relationship was found between the two parameters (Chapter 4 & 6). Only indolylglucosinolates and their breakdown products formed NOC upon nitrite treatment. Mutagenicity to *Salmonella* (*S.*) *typhimurium* TA100 was restricted to the nitrosated breakdown products (Chapter 6). Since indole-3-carbinol (I_3C), indole-3-acetonitrile (I_3A) and indole (I), the breakdown products of the most commonly occurring indolylglucosinolate, are known to be precursors of directly mutagenic NOC (Wakabayashi *et al.* 1985, 1986, Ochiai *et al.* 1986), the nitrosation of these indole compounds and the stability of their nitrosated products was further investigated (Chapter 5). Already after a 5-15 min. incubation with nitrite maximal amounts of NOC were formed. Therefore in subsequent experiments a 15 min. incubation period was used instead of that of one h, as described by the WHO (1978) in the "Nitrosation Assay Procedure". The nitrosated products of I_3C, I_3A and I were stable both at pH 2 and 8, but only when nitrite was present.

Chemical analytical experiments to study the occurrence of indole compounds in brassica extracts revealed that indole-3-carboxaldehyde (ICHO) was the most commonly occurring indole compound (Chapter 6). However, ICHO did not form directly mutagenic NOC. I_3A was the second most commonly occurring indole compound; amounts up to 100 mg per kg dried green cabbage were found (Chapter 6). Although, I_3A formed directly mutagenic NOC upon nitrite treatment, its contribution to the total mutagenicity of nitrite treated brassicas was marginal (Chapter 6). From this observation the important conclusion can be drawn that when a compound is tested out of the context of its normal matrix results can be obtained, which differ considerably from those obtained under more realistic conditions. So in general extrapolation of results obtained in model studies will be more valuable when effects of the normal matrix are taken into account.

From the results presented in the Chapters 4-6 it is difficult to predict whether there will be any negative effects of the consumption of brassicas on human health due to the endogenous nitrosation of indolylglucosinolates and their breakdown products. Of particular importance is the observation that the most commonly occurring indole compounds

ICHO and I_3A are not considered as important precursors of NOC in extracts of brassicas. The former did not form directly mutagenic NOC at all, and the latter, although occurring in considerable amounts (11.82 mg/kg fresh green cabbage), only contributed for about 2% to the total mutagenicity of nitrite treated brassicas. Hence it can be concluded, that the rate of nitrosation of I_3A in the presence of vegetable extracts is much lower than nitrosation of the individual compound would suggest. When I_3A is individually treated with 40 mM of nitrite for 15 min., about 18% forms NOC (Tiedink *et al.* 1991) and so this percentage will be less when I_3A is surrounded by its normal matrix. In case I_3A is nitrosated endogenously, the rate of nitrosation will probably be far less than 18%, since the nitrite concentration in the stomach of healthy persons will maximally be 0.3 mM (Walters *et al.* 1979). After the consumption of brassicas the nitrite concentration will probably be less than 0.3 mM, since most brassicas generally do not contain high levels of nitrate (NO_3^-; Corré & Breimer 1979). It is therefore most unlikely that the endogenous nitrosation of I_3A will result in the formation of DNA adducts in the gastric mucosa. Such an effect was found by Yamashita *et al.* (1988). However, they applied unrealistic dose levels up to 100 mg/kg body weight of pure nitrosated I_3A to rats. Also in other animal experiments the doses of individually administered nitrosated I_3A were very high compared to those which might theoretically be formed endogenously. In a study of Furihata *et al.* (1987) nitrosated I_3A was administered individually to rats in amounts up to 300 mg/kg body weight, which resulted in the induction of ornithine decarboxylase activity and DNA synthesis in the pyloric mucosa. Very recently, in 70% of rats administered with 25-50 mg/kg nitrosated I_3A once a week for 2 years, tumorous lesions were found in the forestomach (pers. comm. Dr. K. Wakabayashi). It is probably not very realistic to extrapolate the results of such experiments to the human situation, because of the following reasons:

1. In all animal studies high doses of nitrosated I_3A were used, which were administered in pure form and not in their normal matrix.
2. The endogenous nitrosation of I_3A out of brassica vegetables may be low or even negligible, since under normal conditions in man the gastric nitrite concentration is much lower than under the experimental conditions used in the present study. Moreover, other compounds present in the diet will compete for or scavenge nitrite (discussed in Chapter 11).
3. As concluded from Chapter 5, nitrosated I_3A will only be stable in the presence of nitrite. Because of the low nitrite concentration in the stomach, it can be suggested that nitrosated I_3A will not be very stable when formed endogenously.

Now it is known that several indole compounds and glucosinolates contribute only marginally to the total amounts of directly mutagenic NOC formed in the brassicas investigated so far, one may wonder what the identity of the major precursors of the directly mutagenic NOC will be. In the last decade a lot of studies have been performed with brassicas, however, in most of them the attention was focused on the anti-carcinogenic pro-

perties (reviewed by McDanell *et al.* 1988). Of the few studies, in which the formation of NOC was of concern, none provided information about the identity of the major precursors of the directly mutagenic NOC formed in these vegetables upon nitrosation. Recently, Kumar *et al.* (1990) reported the formation of considerable amounts of the non-volatile NOC, N-nitrosoproline (NPRO; up to 20 μmol/kg dry weight) and N-nitrosopipecolic acid (NPIC; 22 μmol/kg dry weight), in kale (*Brassica oleracea*, var. *acephala*) upon treatment with 210 mM of nitrite. However, both NPRO and NPIC are not mutagenic to bacteria (Stolz & Sen 1977, Rao *et al.* 1977). NPRO was also detected in the nitrosated extracts of green cabbage and Brussels sprouts used in Chapter 6 (Tiedink & Davies 1989). The GPC fractions 1 to 5 of Figure 6.5 were examined for the presence of NPRO by thermal energy analysis (method: van Broekhoven *et al.* 1984). Only in GPC-fraction 2 (18.5-38.5 ml eluate), a significant concentration of NPRO (94 μM) could be detected, which contributed for about 15% to the total amounts of NOC formed in green cabbage extracts. The formation of NPRO is not unexpected, since proline is abundantly present in the brassica vegetables tested in the study described in Chapter 4 (Tiedink & Davies 1989). Proline will accumulate in plants as a response to stress (Stewart 1981). Stress may be caused by a range of environmental conditions, for example temperature and wilting and can occur in excised leaves or in intact plants in light and in darkness. It concerns *de novo* synthesis of proline from glutamic acid. It is possible that proline accumulation will also occur during freeze drying.

Kumar *et al.* (1990) also showed the formation of small amounts of the volatile NOC, N-nitrosodimethylamine, N-nitrosopyrrolidine and N-nitrosopiperidine (respectively <1.9 μmol, <0.6 μmol and <0.3 μmol per kg of dried kale). It might be possible that these NOC were also formed in the brassicas of Chapter 4 and 6, since traces of volatile NOC were found in GPC fraction 2 of Figure 6.5. The contribution of these volatile NOC to the total amounts of NOC formed, however, will be negligible, since in the extracts of brassicas used in Chapter 4 and 6 the total amounts of NOC formed were >10 μmol per g dry weight. Although the nitrite treated brassica extracts used in Chapter 4 and 6 were not tested for mutagenicity in the presence of S9 mix, it can be deduced that no additional mutagenicity would be found after metabolic activation, since even the amounts of volatile NOC found in kale are far below the detection levels of these compounds in a *S. typhimurium* assay. Therefore the conclusion can be drawn that it is most likely that the majority of the NOC formed in brassicas will be non-volatiles. Further studies are needed to elucidate the identity of these directly mutagenic NOC formed in brassicas, as well as to their possible endogenous formation.

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PART 2

FAVA BEANS

CHAPTER 8

INTRODUCTION FAVA BEANS; MAIN EMPHASIS ON THEIR ASSOCIATION WITH GASTRIC CANCER

Fava beans also named faba beans are botanically known as *Vicia faba* L. and belong to the family of Leguminosae. The fava beans can be divided in three varieties namely: the large seeded var. *major*, the intermediate var. *equina* and the small seeded var. *minor*. The last two varieties are usually referred to as field beans and are mainly produced as animal feed. *Major* types are commonly known as broad beans and are almost exclusively grown for human consumption. The background and history of fava bean production has been extensively reviewed by Hawtin & Hebblethwaite (1983) and some of the information described below comes from this review.

The production of fava beans is widespread in temperate and subtropical regions of the world; the crop can even be found at high elevations within the tropics, for instance in Ethiopia and the northern Andean region of Latin America. In Table 8.1, the areas, yields and production figures of fava beans throughout the world are shown. No sub-division is made for the various types of beans. Much of the production of broad beans is on small scale, however, significant areas are grown for canning or quick-freezing in England, Scotland, The Netherlands and Germany and they are considered to be one of the most important "secondary" vegetables. In these countries the fava beans are harvested green, while in the rest of the world the fava beans grown for human consumption are mainly harvested dry and at the present time fava beans are one of the most important grain legume crops.

One of the attractive attributes of all food legumes is their relatively high protein content. The protein concentrations of fava beans range from 22.0 to 37.0% of the dry weight. And since fava beans are rich in lysine, they are supposed to form an excellent supplement to cereal grain diets (Bressani & Elias, 1988). On the other hand fava beans are relatively poor in sulphur containing amino acids and may contain anti-nutritive factors. The following anti-nutritive factors may be of importance:

1. Condensed tannins, which are known to depress appetite, retention of nutrients and growth rates in rats, mice and chicks. It should be noted that there is a considerable difference in the levels of tannins between coloured flowered and white flowered cultivars of fava beans. The former contain condensed tannins up to levels in the order of 8 to 9 g/kg dry weight, while the latter are free or almost free of tannins. Therefore only white flowered cultivars (free of anthocyanin) are acceptable for canning as vegetables, since their seeds do not discolour when cooked (Lawes *et al.* 1983). The colour of these seeds are pale or bright green and in the following chapters the white flowered cultivars are referred to as white cooking cultivars, in contrast to

the brown cooking (the coloured flowered cultivars).

2. Vicine and convicine are glucosides, which are believed to be the main factors responsible for favism, a form of haemolytic anaemia which can occur in people with a genetic deficiency of erythrocyte glucose-6-phosphate dehydrogenase.
3. Phytic acid is a strongly negatively compound, which may sequester trace elements and proteins with positively charged groups, resulting in a lower availability of the latter.
4. Protease inhibitors, which can inhibit trypsin and chymotrypsin in the small intestine of monogastric animals, by which pancreatic hyperplastic lesions can be induced at high levels of exposure.
5. Haemagglutinines (or lectins), which are proteins with specific affinity for certain sugar molecules. They can react with cell membranes that contain a specific carbohydrate moiety, by which the absorption of nutrients is declined.

For a review of the anti-nutritive factors in fava beans see Marquardt & Bell (1988).

Table 8.1: Area, yield and production of fava beans in the period 1979-1981 in different regions. (Source: FAO 1981).

Region	Area 1000 ha	Yield kg/ha	Production 1000 metric tonnes
Africa	734	994	729
North + Central America	68	1119	78
South America	227	490	111
Asia	2325	1200	2789
Europe	355	1345	477
Oceania	11	1167	12
USSR + Eastern Europe	47	1785	84
World	3721	1128	4198

Of the 31 vegetables tested in the study described in Chapter 4, fava beans were the second vegetable to be chosen for further investigation (part 2 of this thesis). The interest for fava beans stems from the fact that this vegetable has already been associated with gastric cancer. Evidence for a correlation between the consumption of fava beans and the prevalence of cancer was obtained from an epidemiological study of Correa *et al.* (1983). This study was conducted in Nariño, a district of Colombia, where one of the world's highest gastric cancer rates is found (age adjusted incidence rate of 150/100,000).

Moreover, treatment of fava beans with nitrite under simulated gastric conditions resulted in the formation of N-nitroso compounds (NOC), highly mutagenic to *Salmonella typhimurium* (Piacek-Llanes & Tannenbaum 1982, van der Hoeven *et al.* 1984, Jongen *et al.* 1987). Already before Correa and colleagues found the correlation between fava bean consumption and the prevalence of gastric cancer, they postulated a model for gastric cancer epidemiology, in which the gastric carcinoma of the "intestinal" type is suggested to be the end result of a series of mutations beginning in the first decade of life (Correa *et al.* 1976): The first step is supposed to be generated by endogenously formed NOC, which can only reach epithelial gastric cells, if the mucous barrier is previously damaged by hard grains, salts, surfactants or other abrasives or irritants. After the first mutation, the glandular gastric epithelium will gradually change to intestinal type epithelium, by which the mucous barrier alters and the pH elevates. Under such conditions, bacteria, which catalyze the conversion of nitrate to nitrite, proliferate and contribute to a high nitrite pool in the stomach. The high nitrite concentrations enhance the bacterially mediated formation of carcinogenic NOC. This process of gastric atrophy and intestinal metaplasia will go on for years, before an ultimate mutation or cell transformation leads to cell autonomy and subsequently to invasion into other tissues. So far the theory.

The possible endogenous formation of fava bean NOC would fit very well in the model for gastric cancer outlined above, and in multidisciplinary studies conducted in Nariño, a number of other factors were found which could also play a role in the onset of gastric cancer, such as:

1. Salt intake. The salt intake of patients with precancerous lesions (chronic atrophic gastritis, intestinal metaplasia and dysplasia) were significantly higher than that of controls (healthy persons and patients with superficial gastritis) (Chen *et al.* 1990a) and the usage of salt as a preservative of meat was correlated with the incidence of gastric cancer (Haenszel *et al.* 1976).
2. Gastric pH. The mean pH of gastric juices of patients with precancerous lesions was significantly higher than that of controls (4.7 versus 3.8; Chen *et al.* 1990b).
3. Nitrate in drinking water. Significantly high levels of nitrate have been found in well water (up to 300 mg/ml; Cuello et la. 1976).
4. Nitrite in gastric juice. Detectable amounts of nitrite in the gastric juice of patients with precancerous lesions were significantly higher than in controls, whereas no nitrite was found when the pH of the gastric juice was below pH 5 (Chen *et al.* 1990b).
5. Gastric biopsies. Gastric biopsies revealed that the relative risk of lesions as hyperplasia, atrophy, metaplasia and dysplasia increased linearly with higher pH, nitrate and nitrite levels in the gastric juice. Moreover, the severity of atrophy correlated with the prevalence of metaplasia and the severity of metaplasia correlates with the prevalence of dysplasia, suggesting a sequential relationship between the stages (Correa *et al.* 1990a). However, comparison of initial with subsequent biopsies revealed that besides a very dynamic flow of progressive also regressive events

occurred (Correa *et al.* 1990b).

A very interesting discovery was the identification of 4-chloro-6-methoxyindole (4C6MI) in fava beans, especially since this compound seemed to be responsible for mutagenicity induced by fava beans after nitrite treatment (Yang *et al.* 1984). In the second part of this thesis special attention was paid to the role of this compound as a precursor of NOC. Since, as far as known the mutagenicity of nitrosated 4C6MI has only been tested for mutagenicity in bacterial assays, in the present study the genotoxicity of nitrosated 4C6MI was evaluated by using assays with bacteria as well as mammalian cells. Moreover, the potential tumour promoting activity of nitrosated 4C6MI was tested (Chapter 9). In Chapter 10, the detection and the levels of 4C6MI in Dutch fava beans is described and the effects of matrix components on the mutagenicity of fava beans were investigated in the study described in Chapter 11.

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CHAPTER 9

POTENTIAL GENOTOXIC AND TUMOUR PROMOTING EFFECTS OF NITROSATED 4-CHLORO-6-METHOXYINDOLE, 4-CHLOROINDOLE AND INDOLE-3-ACETONITRILE

INTRODUCTION

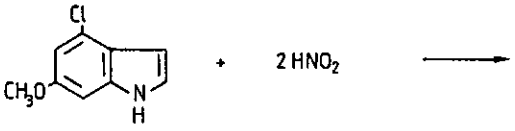
In fava beans treated with nitrite a powerful mutagen is formed with properties similar to an N-nitrosourea (Piacek-Llanes & Tannenbaum 1982). A nutritional survey conducted in Nariño, a district of Colombia, revealed a positive correlation between the consumption of fava beans and the incidence of gastric cancer (Correa *et al.* 1983). This led to the hypothesis that the formation of mutagenic N-nitroso compounds (NOC) in the stomach, from precursors present in fava beans, could be a causative factor in the etiology of gastric cancer.

Yang *et al.* (1984) isolated the compound occurring in fava beans, which was responsible for the mutagenicity after nitrosation. It was identified as 4-chloro-6-methoxyindole (4C6MI), from which 4-chloro-2-hydroxy-N1-nitroso-3H-indolin-3-one-oxime was formed upon nitrosation (Büchi *et al.* 1985) (Figure 9.1). The nitrosated compound was highly mutagenic to *Salmonella* (*S.*) *typhimurium* TA1535, inducing comparable numbers of revertants as aflatoxin B1 (Yang *et al.* 1984). As far as known no other studies on the mutagenicity or carcinogenicity of nitrosated 4C6MI have been reported.

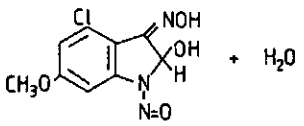
In the present study the genotoxicity of nitrosated 4C6MI was evaluated. Since in the study described in Chapter 5, 4-chloroindole (4CI) was used as a model compound for 4C6MI, nitrosated 4CI was also used, as well as a non-chlorinated indole compound: indole-3-acetonitrile (I_3A), naturally occurring in brassica vegetables (see Part 1). Three different test systems were performed to determine initiator activity: the *S. typhimurium* assay and two assays using mammalian cells; the gene mutation test on the Hypoxanthine Guanine Phosphoribosyl Transferase (HGPRT) locus and the Sister Chromatid Exchange (SCE) test. Tumour promoter activity was determined by inhibition of gap junctional intercellular communication (GJIC) using V79 cells.

This chapter is based on :

H.G.M. Tiedink, L.H.J. de Haan, W.M.F. Jongen & J.H. Koeman (submitted) *In vitro* testing with respect to carcinogenic potential of several nitrosated indole compounds.



4 - chloro - 6 - methoxy indole



4- chloro - 6-methoxy - 2-hydroxy - N^1 - nitroso - 3H - indoline - 3 - one oxim

Figure 9.1: Chemical structure of 4-chloro-6-methoxyindole and its nitrosated form.

Intercellular communication by gap junctions is considered to be an important cellular mechanism for regulating growth and differentiation (Loewenstein 1979, Bennett & Spray 1985). Gapjunctions are hydrophillic channels employed for the exchange of important signal ions and molecules between cells. Blockage of the transfer of regulatory molecules via the gap junctions between normal and initiated cells could lead to abnormal cell proliferation (Yamasaki *et al.* 1988). Inhibition of GJIC is therefore considered to be the key mechanism by which tumour promoters contribute to the process of carcinogenesis (Trosko 1987, Yamasaki *et al.* 1988).

In the study of Chapter 5 it was shown that nitrosated 4Cl was not stable in aqueous solutions of pH ≥8. In mutagenicity assays using mammalian cells, it is necessary to maintain a narrow pH range. Therefore the stability of nitrosated 4-chloroindoles in cell culture media were also studied.

MATERIALS AND METHODS

Chemicals

4C6MI was kindly provided by Dr. P. Martin, Ciba Geigy AG, Basel, Switzerland, (Martin 1989). 4Cl, I_3A, 5-bromo-2-deoxyuridine (BrdU), colchicine, bis-benzimid (Hoechst nr. 33258), lucifer yellow CH and 12-O-tetradecanoylphorbol-13-acetate (TPA) were purchased from Sigma Chemicals CO. (St. Louis MO, USA). Hepes, ethyl methanesulphonate (EMS) and 1-methyl-1-nitroso-3-nitroguanidine (MNNG) were purchased from Merck (Darmstadt, Germany). 4-nitro-quinoline-N-oxide (NQO) and 6-thioguanine (6TG) were obtained from Fluka AG (Buchs SG, Switzerland).

Nitrosation reaction

Indole compounds were first dissolved in dimethyl sulphoxide (DMSO; 4.75-10.00 mg/ml). The nitrosation reaction was accomplished by the addition of the indole compounds (50 μl) to 1.950 ml of a $NaNO_2$ solution at pH 2 (final nitrite concentration 40 mM). This solution was incubated at 37°C in the dark for 15 min.. The reaction was stopped by addition of an ammoniumsulphamate solution (final concentration 48 mM). Then samples were taken, to perform genotoxicity assays and the assay on the inhibition of GJIC. A 40 mM NaNO2 solution treated with ammoniumsulphamate and solutions of the indole compounds in distilled water of pH 2 served as blanks.

BACTERIAL ASSAY

S. typhimurium assay

The *S. typhimurium* assay was performed as described in Chapter 4 and 5. At least two independent experiments were performed carried out with different doses of nitrosated indoles (0.1-1 ml), using triplicate plate plates for each sample. The data presented are the average values of one representative experiment. In each experiment a water blank and a positive control (1 μg MNNG/plate) were tested.

The indole compounds were nitrosated as before, with the exception that the nitrosation reaction was started by the addition of a $NaNO_2$ solution to 10 ml of indole solutions, whereafter the pH was again adjusted to 2. The mutagenic activity, expressed as the number of revertants induced per nmol of an indole compound, was calculated from the slope of the linear part of dose-response curves.

The stability of the nitrosated 4-chloroindoles in different media was determined by measuring the mutagenicity as a function of time. In these experiments the nitrosated indole compounds were applied to a Seppak C18 cartridge (Millipore, Waters Associates, Milford MS, USA), which was eluted with DMSO (4 ml). Then the DMSO was diluted in different media (EBSS, Ham's F10 and Ham's F10 + NCS).

MAMMALIAN CELL ASSAYS

Chinese hamster epithelial (V79) cells

V79 cells were cultured in Ham's F10 medium (Flow, Irvine, Scotland) supplemented with 10% newborn calf serum (Flow), penicillin (200 units/ml; Gist-Brocades, Delft, The Netherlands) and streptomycin (100 μg/ml; Gist-Brocades), indicated as Ham's $F10^{++}$. Monolayers were grown at 37°C in 75-cm^2 flasks (Costar, Badhoevedorp, The Netherlands) in a humified atmosphere containing 5% CO_2; when the cells became confluent they were subcultured by trypsinization with 0.25% trypsin (Difco, Detroit MI, USA) and 0,05% EDTA (Merck).

In both the SCE assay and the forward mutation test on the HGPRT locus 1 ml cell suspensions, containing 10^6 cells/ml, were exposed to the test compounds. After

centrifugation (3 min., 1200 rpm) Ham's $F10^{++}$ was replaced by Earle's balanced salt solution (EBSS) with 10 mM $NaHCO_3$ and 20 mM Hepes of pH 6.4 or by EBSS of pH 7.4, containing 40 mM of Hepes instead of 20 mM. Then the nitrosated indole compounds (10-100 μl) were added, resulting in a pH decrease of maximally 0.4 pH units in EBSS of pH 6.4 and of 0.2 pH units in EBSS of pH 7.4. The final volume was 1.0 ml with final DMSO concentrations of 0.25% v/v at most.

In the assay on the inhibition of GJIC, 10^6 V79 cells were plated in Petri dishes of 2 cm diameter and cultured in Ham's $F10^{++}$ till the cells became confluent. Then Ham $F10^{++}$ medium was removed, cells were washed with Hanks Balanced Salt Solution (HBSS; Flow) and EBSS of pH 6.4 was added. To the EBSS the nitrosated indole compounds were added (50-200 μl). The final volume was 2.0 ml with final DMSO concentrations of 0.5% v/v at most.

In all experiments cells were exposed to the nitrosated compounds for 1 h at 37°C in the dark. Then the cells were washed with HBSS containing 20 mM Hepes and with Ham $F10^{++}$, followed by resuspension in Ham's $F10^{++}$. In the assay on the inhibition of GJIC, however, the cells were only washed with Ham's $F10^{++}$, followed by addition of Ham's $F10^{++}$ containing 20 mM of Hepes to the dishes.

In order to measure cytotoxicity, all assays with mammalian cells were preceded by cloning efficiency experiments. Survival was measured by seeding 200 cells in 6 cm diameter Petri dishes. After a 7 day incubation, colonies were fixed in methanol, stained in Giemsa (Merck) and counted.

Since the percentage of the indole compounds, which is nitrosated is unknown and so the dose of the nitrosated compounds, the concentrations in all Tables and Figures are indicated as the initial dose of the indole compounds.

SCE assay

The SCE experiments were performed according to the method of Perry and Wolff (1974) with slight modifications. After exposure, 10^5 cells were seeded in 1 ml Ham's $F10^{++}$ medium on flame sterilised slides and were allowed to attach for 2.5 to 3 h. Following the addition of BrdU and Ham's $F10^{++}$ medium without thymidine and hypoxanthine (Flow), the incubation was continued in darkness for 22 h. Colchicine (0.2 μg/ml) was then added and after an overnight incubation the cells were swollen in 0.56% KCl solution and fixed in a 3:1 mixture of methanol and acetic acid. The slides were washed in distilled water and immersed for 10 min. in a bis-benzimid solution (1.25 μg/ml). The cells were then irradiated for 5 h with a 100 W mercury lamp and stained with 5% Giemsa for 10 min.. Slides were scored for SCE's (25-50 metaphases per treatment group with 18-22 chromosomes per metaphase). At least two independent experiments were performed. The data presented are the average values of one representative experiment. NQO (final concentration 5 μM) served as positive control.

Gene mutation test on the HGPRT locus

The experiments on forward mutations were slight modifications of those described by van Zeeland & Simmons (1975, 1976a,b). After exposure, 200 cells were plated for survival measurements. Simultaneously 2.5 x 10^5 cells were plated and subcultured every second day to maintain a logarithmically growing population. After an expression time of 7 days 10^5 cells were seeded into 9 cm diameter Petri dishes, to which medium supplemented with 10 μg/ml 6TG was added. For estimation of plating efficiency 200 cells were seeded. Mutant selection dishes were incubated for 9 days and plating efficiency dishes for 7 days before fixation (in methanol), staining (in 10% Giemsa) and counting of colonies. At each dose level, cells were plated into 5 dishes. EMS (final concentration 4.8 μM) served as positive control.

Inhibition of GJIC

The inhibition of GJIC was measured by a dye transfer assay described by Enomoto *et al.* (1984). Immediately after exposure (or 4 h after exposure in recovery experiments), a 10% lucifer yellow CH in 0.33 M lithium chloride solution was brought into single cells close to the nucleus by microinjection. In each culture dish at least 20 individual cells were microinjected using a vertical injection system (Olympus injectoscoop IMT-2-syf) (Yamamoto & Furusawa 1978) with a dye-filled capillary glass tip (Clark, Pangbourne, UK). This capillary glass tip was prepared by an automatic magnetic puller (Narishige, Tokyo, Japan) with a tip diameter of ca. 1 μm. Injected cells were checked with phase-contrast and fluorescence microscopy directly after microinjection. Ten min. after injection the number of fluorescent cells was measured.

Each experiment was performed in triplicate and at least two independent tests were done. TPA (final concentration 162 nM), served as positive control.

RESULTS

S. typhimurium assay

In Table 9.1 the direct mutagenic activity of several nitrosated indole compounds to *S. typhimurium* is shown. The chlorinated compounds were much more mutagenic than the non-chlorinated ones.

In the study of Chapter 5 it was shown that the nitrosated products of I_3A, indole-3-carbinol and indole were stable at pH 8, while the nitrosated products of 4CI were unstable. The aim of this study is to investigate the genotoxicity and tumour promoting potential of nitrosated 4-chloroindoles using V79 cells. Since V79 cells are cultured at pH 7.4, the stability of nitrosated 4C6MI in EBSS, Ham's F10 and Ham's F10 + NCS of pH 7.4 was determined. Nitrosated 4C6MI was most stable in EBSS, although in EBSS it was less mutagenic to *S. typhimurium* than in distilled water. Moreover, the mutagenicity steadily declined as a function of time. Decreasing the pH of EBSS to 6.4, however, resulted in

stability of nitrosated 4C6MI for about 60 min.. Similar results were found for nitrosated 4CI. Unless stated otherwise, in the following experiments EBSS of pH 6.4 was used during exposure.

SCE test

A preceding study revealed that V79 cells could be cultured at pH 6 for several hours (unpublished data). Therefore SCE induction in V79 cells, exposed to 4C6MI and 4CI and their nitrosated products was tested, using EBBS of pH 7.4 and 6.4 during exposure (Table 9.2). Survival of the V79 cells exposed to the indicated doses of nitrosated indoles (24.13 μg/ml 4CI and 13.30 μg/ml 4C6MI) was about 80% at pH 6.4 and 100% at pH 7.4.

Table 9.1: Directly mutagenic activity of nitrosated indole compounds to S. typhimurium TA100.

compound	# revertants/nmol*
indole	0.80
indole-3-carbinol	0.95
indole-3-acetonitrile	0.43
4-chloroindole	179
4-chloro-6-methoxyindole	1348

**concentration before nitrosation.*

From Table 9.2 it can be concluded that only the nitrosated products of 4CI and 4C6MI are inducers of SCE's, while the level of induction depends on the pH during exposure. In Figure 9.2 dose-response curves are shown of cells exposed to nitrosated 4-chloroindoles. From Table 9.2 and Figure 9.2 it can be concluded that both nitrosated 4C6MI and 4CI are potent inducers of SCE's. At equimolar amounts their effects were almost the same, although nitrosated 4C6MI became cytotoxic at lower doses.

To compare SCE induction of nitrosated 4-chloroindoles to that of non-chlorinated indole compounds, SCE's were also measured in cells exposed to nitrosated I_3A (Table 9.3). Nitrosated I_3A induced SCE's, although at much higher concentrations than the nitrosated 4-chloroindoles. (At the maximal dose tested: 0.96 mg/ml, a 100% survival was found).

Forward mutation assay

The induction of HGPRT-deficient mutants in V79 cells exposed to nitrosated 4CI and

4C6MI is shown in Figure 9.3 and 9.4. For both nitrosated 4CI and 4C6MI no clear dose-response curves were obtained. Although the numbers of 6TG resistant mutants induced by 159 μM of nitrosated 4CI and by ≥37 μM of nitrosated 4C6MI were significantly higher than their appropriate controls, they were not considered mutagenic since their responses were less than 3 times as high as their controls (Bradley *et al.* 1981).

Table 9.2: Influence of pH during exposure of V79 cells to 4-chloroindole, 4-chloro-6-methoxyindole and their nitrosated forms on the induction of SCE's.

compound	initial indole concentration	# of SCE's/chromosome[a] pH 6.4*	pH 7.4**
control[b]		0.37 ± 0.08[b]	0.43 ± 0.02
4-chloroindole	159 μM	0.39 ± 0.03	0.43 ± 0.03
4-chloro-6-methoxyindole	73 μM	0.36 ± 0.02	0.44 ± 0.02
nitrosated 4-chloroindole	159 μM	1.74 ± 0.08	0.85 ± 0.03
nitrosated 4-chloro-6-methoxyindole	73 μM	1.37 ± 0.06	0.98 ± 0.04

**final pH=6.0.*

***final pH=7.2.*

[a] *mean of 50 metaphases ± SEM.*

[b] *solution of 40 mM $NaNO_2$ treated with 48 mM ammoniumsulphamate.*

[+] *Positive control (5 μM NQO) induced 4-6 times as much SCE's as control.*

Inhibition of GJIC

The effects of the nitrosated 4-chloroindoles and their precursors on GJIC of V79 cells is shown in Figure 9.5 and 9.6. In these Figures the numbers of cells in which lucifer yellow is found after injection into a single cell of a control culture is set at 100%. The average number of cells ± SEM, which communicated in a control culture was 10.99 ± 0.63 (77 dishes). It should be noted that as a result of exposure in monolayer the maximal concen-

Table 9.3: Number of SCE's/chromosome induced by indole-3-acetonitrile and its nitrosated form in V79 cells.*

compound	initial indole concentration	# of SCE's[a]
control[b]		0.39 ± 0.02
indole-3-acetonitrile	6.2 mM	0.50 ± 0.02
nitrosated indole-3-acetonitrile	3.1 mM	1.03 ± 0.04
	6.2 mM	1.38 ± 0.07

**in EBSS, final pH 7.2-7.4, depending on the volume of the nitrosated sample added.*
[a]mean values of 50 metaphases ± SEM.
[b]a 40 mM $NaNO_2$ solution treated with 48 mM ammoniumsulphamate.

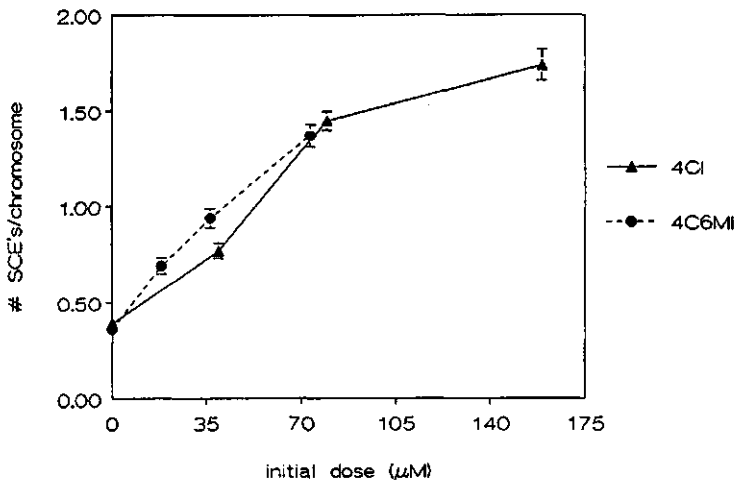


Figure 9.2: Number of SCE's/chromosome induced by nitrosated 4-chloro-indole and nitrosated 4-chloro-6-methoxyindole** in V79 cells.*
**mean of 50 metaphases ± SEM.*
***in EBSS, final pH 6.0-6.2, depending on the volume of nitrosated sample added.*

trations of the indole compounds are higher than in the preceding assays. Both nitrosated 4C6MI (≥21 μM) and 4C6MI (41 μM and 166 μM) significantly inhibited GJIC, while nitrosated 4CI (≥25 μM) significantly inhibited and 4CI (198 μM) significantly stimulated GJIC.

The effects of I_3A and its nitrosated form on GJIC were also measured (Figure 9.7). Nitrosated I_3A significantly inhibited GJIC at much higher doses (≥6.4 mM) than the nitrosated 4-chloroindoles did. I_3A (0.6 mM and 12.8 mM) significantly inhibited GJIC, while at a concentration of 1.3 mM it significantly stimulated GJIC.

The recovery of GJIC by the highest dose of the nitrosated indole compounds was determined 4 h after exposure (Figure 9.8). It appeared that the inhibition generated by nitrosated I_3A was partially restored, while the inhibition generated by the nitrosated 4-chloroindoles did not differ from that observed immediately after exposure.

In all experiments TPA caused a ≥95% inhibition of GJIC.

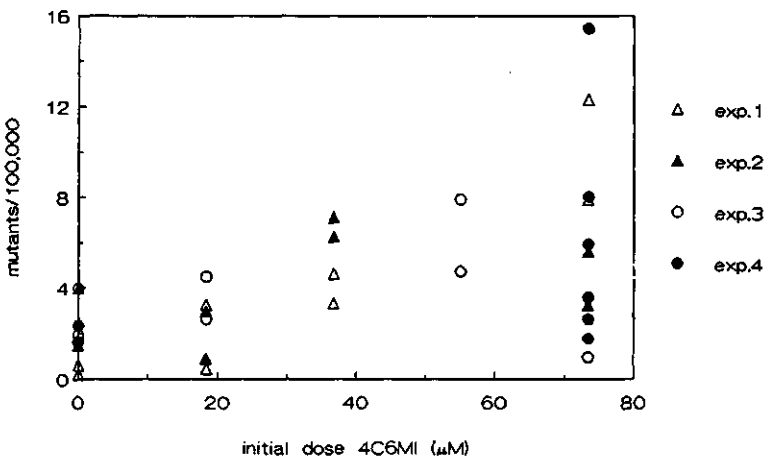


Figure 9.3: Number of HGPRT-deficient mutants induced by nitrosated 4-chloro-6-methoxyindole**.*

**expressed per 10^5 survived V79 cells.*

***in EBSS, final pH 6.0-6.2, depending on the volume of the nitrosated sample added.*

$^+$each point is mean of 5 plates; $^{++}$number of mutants induced by 4-chloro-6-methoxyindole lies within the range of control, EMS (4.8 μM) induced 23.38 mutants; $^{+++}$number of mutants induced by 37, 55 and 73 μM are significantly higher than control (respectively $p=0.01$, $p=0.025$ and $p=0.025$; Wilcoxon test.

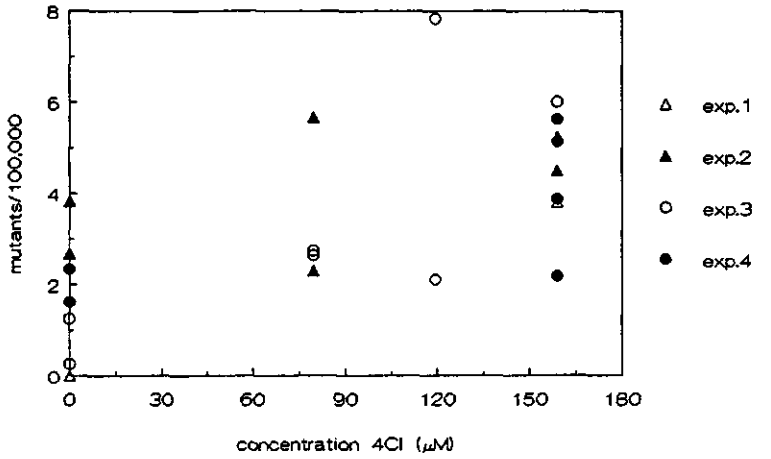


Figure 9.4: Number of HGPRT-deficient mutants induced by nitrosated 4-chloro-indole**.*

**expressed per 10^5 survived V79 cells.*

***in EBSS, final pH 6.0-6.2, depending on the volume of the nitrosated sample added.*

+each point is mean of 5 plates; ++number of mutants induced by 4-chloroindole lies within the range of control, EMS (4.8 μM) induced 20.70 mutants; +++number of mutants induced by 159 μM is significantly higher than control ($p<0.005$; Wilcoxon test).

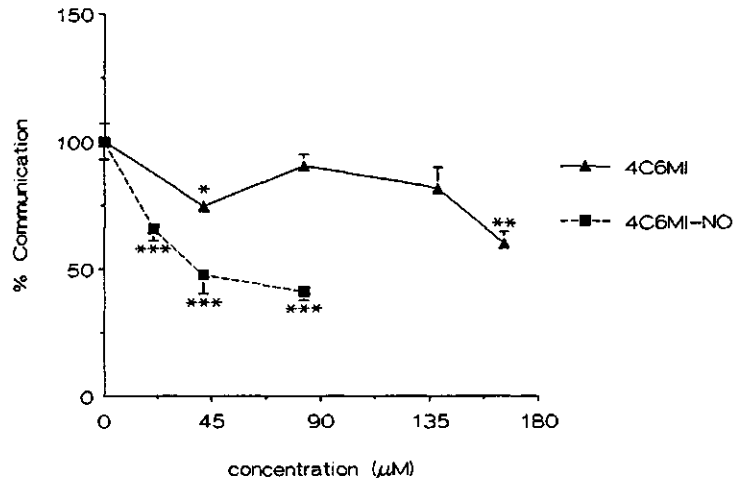


Figure 9.5: Effect of 4-chloro-6-methoxyindole (4C6MI) and its nitrosated form (4C6MI-NO) on gap junctional intercellular communication of V79 cells.

*+mean values ± SEM from 6-12 dishes; ++Significant inhibition, * $p=0.05$, ** $p=0.005$, *** $p=0.001$; Wilcoxon test.*

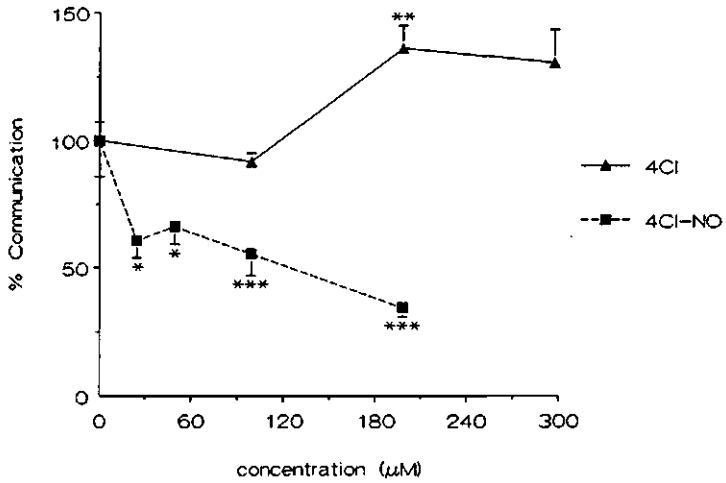


Figure 9.6: Effect of 4-chloroindole (4Cl) and its nitrosated form (4Cl-NO) on gap junctional intercellular communication of V79 cells.

*+mean values ± SEM from 6-12 dishes; ++Significant inhibition/stimulation, * p=0.025, ** p=0.005, *** p=0.001; Wilcoxon test.*

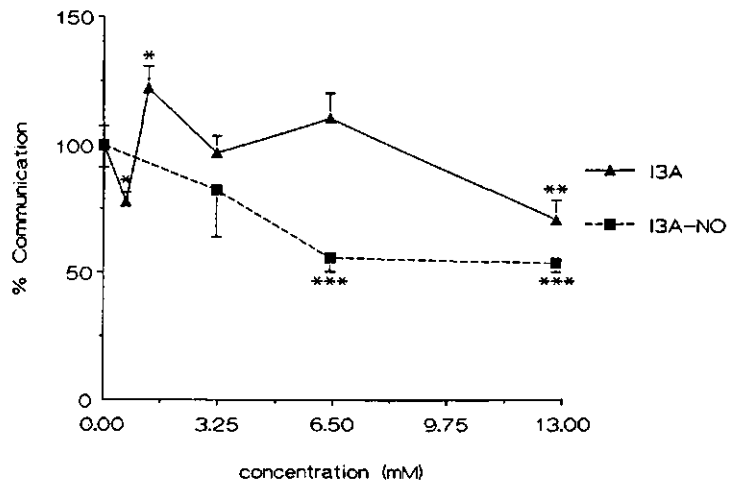


Figure 9.7: Effect of indole-3-acetonitrile (I3A) and its nitrosated form (I3A-NO) on gap junctional intercellular communication of V79 cells.

*+mean values ± SEM from 6-15 dishes; ++Significant inhibition/stimulation, * p=0.05, ** p=0.025, *** p=0.001; Wilcoxon test.*

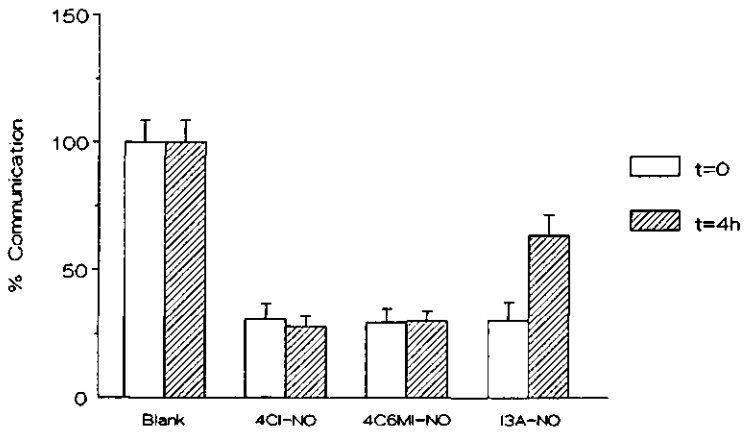


Figure 9.8: Recovery of inhibition of gap junctional intercellular communication of V79 cells 4 h after exposure to nitrosated 4-chloroindole (4Cl-NO), nitrosated 4-chloro-6-methoxyindole (4C6Ml-NO) and nitrosated indole-3-acetonitrile (I3A-NO).

DISCUSSION

In the present study nitrosated 4-chloroindoles appeared to be potent mutagens for *S. typhimurium* TA100 (without metabolic activation). This is in accordance with findings of Yang *et al.* (1984). Nitrosated 4Cl, tested in a forward mutation assay using strain TM677, induced about 13 times less mutations than nitrosated 4C6Ml (Yang *et al.* 1984). In the present study nitrosated 4Cl induced about 7.5 less revertants as nitrosated 4C6Ml using strain TA100.

Nitrosated 4-chloroindoles also appeared to be potent inducers of SCE's in V79 cells, but equivocal results were obtained in a forward mutation assay using the HGPRT locus. No explanations can be given for these equivocal results. However, it can be possible that the SCE assay is more sensitive than the forward mutation assay, which was also demonstrated by Perry and Evans (1975) for a range of chemicals. Moreover, in the present study the sensitivity in the *S. typhimurium* assay was much higher than in the assays using mammalian cells (ngs versus μgs).

Besides genotoxic effects, nitrosated 4-chloroindoles also caused inhibition of GJIC, which is a remarkable observation. The dose related inhibition of GJIC by nitrosated 4-chloroindoles indicates that these compounds might also have tumour promoting properties. Combining this with the results of the genotoxicity assays it can be concluded that nitrosated 4-chloroindoles should be considered as potential genotoxic and potential

tumour promoting agents. Previously, only for a few other genotoxic chemicals or mixture of chemicals like dimethylbenzanthracene (Jongen *et al.* 1987), cigarette smoke condensate (Jongen *et al.* 1985) and airborne particulate matter (Heussen 1991) the ability to inhibit GJIC has been reported.

In the present study nitrosated 4CI reacted comparable as nitrosated 4C6MI, although the magnitude of the response was less in various assays. This suggests that 4CI can be used as a model compound for the compound occurring in fava beans. The much lower activity of I_3A compared to the nitrosated 4-chloroindoles in both the genotoxicity assays and the assay on the inhibition of GJIC suggests that the presence of a chloro atom in the molecule is an important prerequisite for the extreme toxic action of the indole compounds. Moreover, the inhibition of GJIC generated by nitrosated I_3A seemed to be transient, since 4 h after exposure the GJIC was partially restored. This contrasts to the results obtained with the nitrosated 4-chloroindoles. Nevertheless nitrosated I_3A should also be considered as a potential genotoxic and tumour promoting agent. This is in accordance with a study of Yamashita *et al.* (1988), in which the ability of nitrosated I_3A to induce DNA adducts in gastric mucosa of rats was shown both *in vitro* and *in vivo* and with a study of Furihata *et al.* (1987), in which nitrosated I_3A was shown to be an inducer of ornithine decarboxylase activity and DNA synthesis in pyloric mucosa of rat stomach. In both studies high doses of nitrosated I_3A (respectively 100 mg/kg and 300 mg/kg body weight) were used, while in the present study initial doses of 1-2 mg/ml I_3A were used. Assuming that about 18% of I_3A is nitrosated in the presence of 40 mM nitrite (Tiedink *et al.* 1991), the maximal concentrations of nitrosated I_3A were only 180-360 μg/ml in the present study. Very recently, Wakabayashi has shown that nitrosated I_3A can induce tumorous lesions in the forestomach of rats (pers. comm. Dr. K. Wakabayashi; see also Chapter 7).

The hypothesis that the formation of a NOC in the stomach from a precursor present in fava beans can be a causative factor in the etiology of gastric cancer is strengthened by the present study, in which nitrosated 4C6MI appeared to be a potential tumour initiating and tumour promoting agent. The only missing link for confirming the hypothesis is an animal experiment, in which the carcinogenicity of nitrosated 4C6MI can finely be proofed.

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CHAPTER 10

DETECTION AND OCCURRENCE OF 4-CHLORO-6-METHOXYINDOLE IN DUTCH FAVA BEANS

INTRODUCTION

In the study described in Chapter 9, it was concluded that nitrosated 4-chloro-6-methoxyindole (4C6MI) should be considered as a potential genotoxic and tumour promoting agent. Yang *et al.* (1984) isolated this indole compound from Colombian fava beans and considered this compound to be largely responsible for the mutagenicity of the beans after nitrite treatment. Chloro containing compounds are rare in biological systems, but it is known that immature seeds of peas (*Pisum sativa*) also contain a chloro compound. This compound, a methyl ester of 4-chloroindolacetic acid was shown to exert growth regulatory activity (Marumo *et al.* 1968). Harborne *et al.* (1971) suggest that it is a growth hormone, which may occur in all members of *Leguminosae* and from this it can be suggested that 4C6MI found in the Colombian fava beans is a metabolite of this methyl ester.

In previous studies it was found that fava beans cultivated in the Netherlands were also highly mutagenic after nitrosation (van der Hoeven *et al.* 1984, Jongen *et al.* 1987). However, no data were obtained about the presence of 4C6MI. Therefore one of the aims of the present study was to identify 4C6MI in Dutch fava beans.

In one of the previous studies a more than 15-fold difference was found in the mutagenic response of 16 cultivars after nitrite treatment (van der Hoeven *et al.* 1984). It appeared that the brown cooking (coloured breeding) cultivars were low in mutagenic activity in comparison with the white cooking (white coloured breeding) cultivars. Therefore both types of cultivars were taken into consideration in the present study.

This chapter is based on:
H.G.M. Tiedink, C.J. Teunis, L.W. van Broekhoven & W.M.F. Jongen (submitted) Detection and occurrence of 4-chloro-6-methoxyindole in fava beans (*Vicia faba* L.), cultivated in The Netherlands.

MATERIALS AND METHODS

Chemicals

4C6MI was a generous gift of Dr. P. Martin (Ciba Geigy AG, Basel, Switzerland). Methanol (MeOH, 99.5%) was obtained from Janssen Chimica (Beerse, Belgium), while n-hexane (analytical grade) and ethylacetate (EtAc; lichrosolv quality) were purchased from Merck (Darmstadt, Germany).

Fava beans

Fava beans (three white cooking and two brown cooking cultivars) were obtained from a Dutch seed breeding company. The beans were grown under identical conditions at one location. They were harvested in July 1989. The harvest moment was determined according to firmness. After shelling, the beans were frozen at -40°C till freeze-drying. Lyophilized beans were ground and stored in the dark at room temperature under nitrogen in the presence of silicagel.

Purification of fava bean extracts

Extraction A: (Piacek-Llanes & Tannenbaum 1982)

Fava beans were added to hot isopropanol, blended and filtered by suction. The filtrate residue was washed with hot isopropanol and subsequently blended with chloroform:isopropanol (1:1) and chloroform. The combined filtrates were evaporated. The residual lipids were taken up with chloroform and freed from non-lipids by washing with water. The aqueous phase should contain the promutagen.

Extraction B: (Yang *et al.* 1984)

Fava beans were extracted with isopropanol by using a Soxhlet apparatus. The isopropanol was evaporated to dryness and the residue was resuspended in hexane. The hexane was evaporated to dryness and the residue was resuspended in a 2% MeOH in water solution, which should contain the promutagen.

Extraction C: (new developed method)

Portions of 30 g of fava beans were extracted with MeOH for 11 h, using a soxhlet apparatus. The MeOH was evaporated, till only 4 ml remained (= 1/100 of the initial volume). To the flask containing the residue n-hexane (50 ml) and distilled water (25 ml) were added. When the residue was visually dissolved, the mixture was transferred to a separation funnel. Then the flask was rinsed with n-hexane (20 ml) and distilled water (30 ml), which were added to the n-hexane/water mixture in the separation funnel. After stirring the aqueous phase was collected, while the n-hexane phase was 4 times more extracted with distilled water. The combined aqueous phases (150 ml), which would contain 4C6MI were then 6 times extracted with EtAc (final volume 225 ml). The EtAc extracts were evaporated

till about 1.5 ml remained (volumes were estimated by weighing) and centrifuged for 10 min. at 10,000 rpm in an eppendorf centrifuge 5415C (Merck).

The extraction efficiency of the MeOH extraction was checked by re-extraction of the dry matter with MeOH. In order to calculate the recovery of 4C6MI purification, the procedure was also performed using 4C6MI standard solutions and fava beans spiked with 4C6MI.

In all purification methods used, the recovery of the total mutagenicity was checked after each step by measuring the mutagenicity of the obtained fraction after nitrite treatment. The nitrosation reaction was performed as described in Chapter 6.

Detection and quantification of 4C6MI in EtAc

For detection and quantification of 4C6MI in EtAc extracts a gaschromatography-mass spectrometer (GC-MS) combination was used (Hewlett Packard MSD, type 5970 B, electron impact ionisation at 70 eV). Two types of columns were used: a DB 17 Durabond column (30 m x 0.25 mm, film thickness 0.25 μm, J&W Scientific, Folsom CA, USA) with linear temperature programmes over 10 min. from 200°C to 240°C, followed by 10 min. at 240°C and a Sil-19CP WCOT (26 m x 0.22 mm, film thickness 0.18 μm, Chrompack, Middelburg, The Netherlands) with a linear temperature programme over 10 min. from 180°C to 220°C, followed by 5 min. at 220°C. 4C6MI was analyzed by Mass Selective Detection (MSD) on m/e of 181, 166 and 138 with a dwell time of 30 msec.. Only when the relative intensities at the three m/e values were comparable to that of a 4C6MI standard, and the retention time coincided with that of a 4C6MI standard, the 4C6MI concentration in the EtAc extracts was estimated by comparing the intensity of peaks at m/e 166 to that of a calibration curve made with 4C6MI standards.

Mutagenicity assay

After nitrite treatment, 4C6MI and extracts of fava beans were tested for mutagenicity using the *Salmonella* (*S.*) *typhimurium* assay with tester strain TA100 without metabolic activation, as described in Chapter 4 and 5.

4C6MI was first dissolved in DMSO (stock 1.0 mg/ml) and then diluted in distilled water (final DMSO concentration ≤0.25%). MeOH and EtAc extracts of fava beans were evaporated to dryness. The residues were dissolved in distilled water at 37°C and nitrosated as described in Chapter 5 and 6. Samples (0.3, 0.6 and 1.0 ml) were tested in triplicate for mutagenicity. The number of revertants induced by samples treated without nitrite was subtracted from that in treated samples.

Total N-nitroso determination

Total N-nitroso levels in nitrite treated MeOH extracts were determined with a Thermal Energy Analyzer (Thermedics Inc., Woburn MA, USA), according to a modified method of Walters *et al.* (1978; see Chapter 2).

RESULTS

To investigate the presence of 4C6MI in Dutch fava beans, purification methods A and B were used, described by Piacek-Llanes & Tannenbaum (1982) and Yang *et al.* (1984).

Using method A no 4C6MI was detectable. Already after the first step of purification (extraction with isopropanol), the mutagenicity was only one third of that after extraction with MeOH. And after extraction of the lipid fraction, almost no mutagenicity was found in the aqueous phase, which according to published data should contain 4C6MI. Also when method B was used no 4C6MI could be detected. In these experiments the first step of extraction was replaced by extraction with MeOH, because of the poor results of isopropanol extractions using method A. n-Hexane added to the residue of the MeOH, should contain 4C6MI. But almost no mutagenicity was found in the 2% MeOH solution, in which the residue of the evaporated hexane fraction was dissolved. Moreover, it was found that standards of 4C6MI did not dissolve in n-hexane.

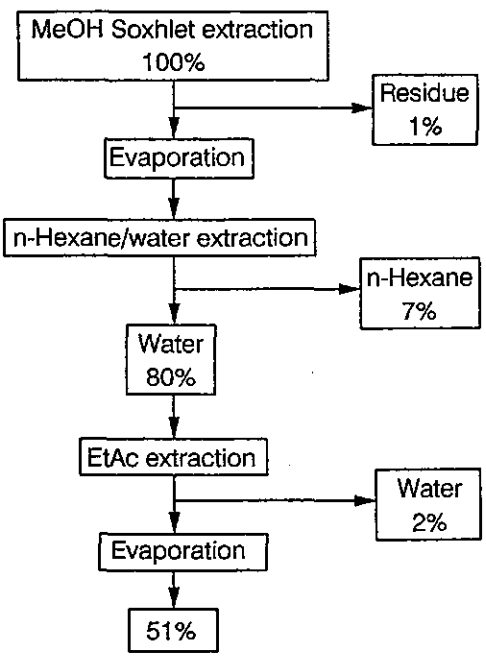

Figure 10.1: Schematic purification procedure of the promutagen in fava beans. The recovery is estimated from mutagenicity data obtained after nitrite treatment, whereas that of a nitrite treated MeOH extract is set at 100%.

Because of the poor recoveries found when using method A & B, a new method was developed. This method is described in detail under Materials and Methods and is depicted schematically in Figure 10.1. In each step the recovery of the mutagenicity is indicated as compared to that found in the MeOH .extract. The mutagenicity in the MeOH extract was set at 100%, since in experiments performed to check the efficiency of the MeOH extraction, no significant mutagenicity was found after re-extraction of the dry matter with MeOH. The EtAc extracts were analyzed by GC-MS and in Figure 10.2 one of the obtained GC-chromatograms is shown. The peak with a retention time of 6.587 min. appeared to have a mass-spectrogram comparable to that of 4C6MI standards (Figure 10.3). To confirm the identity of this peak, an EtAc extract was spiked with a standard of 4C6MI and analyzed by GC. This resulted in an increase of the intensity of the peak at 6.587 min.. So these experiments showed that 4C6MI is present in Dutch fava beans.

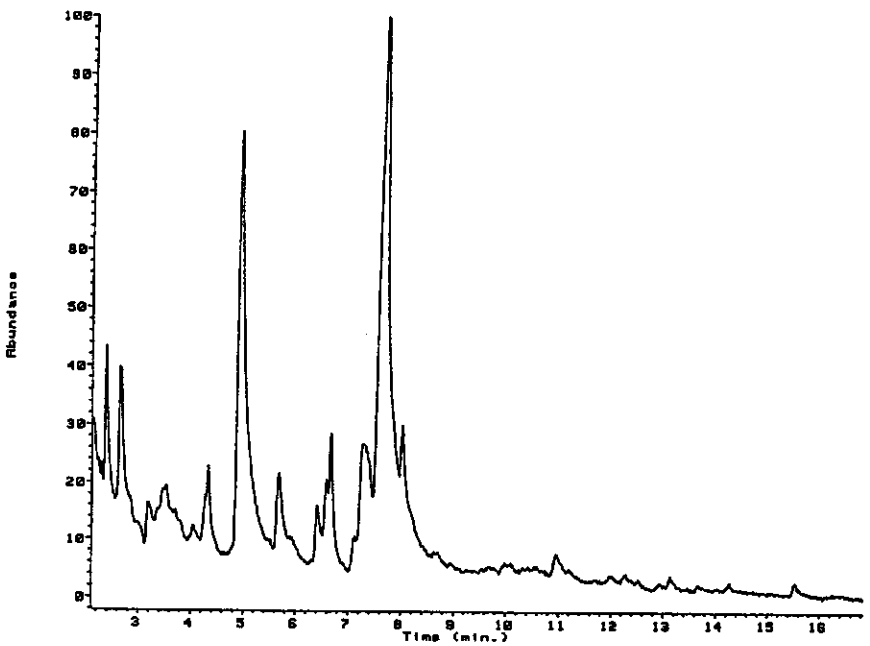

Figure 10.2: Gaschromatogram of an ethylacetate extract of fava beans, obtained after the purification procedure outlined in Figure 10.1.

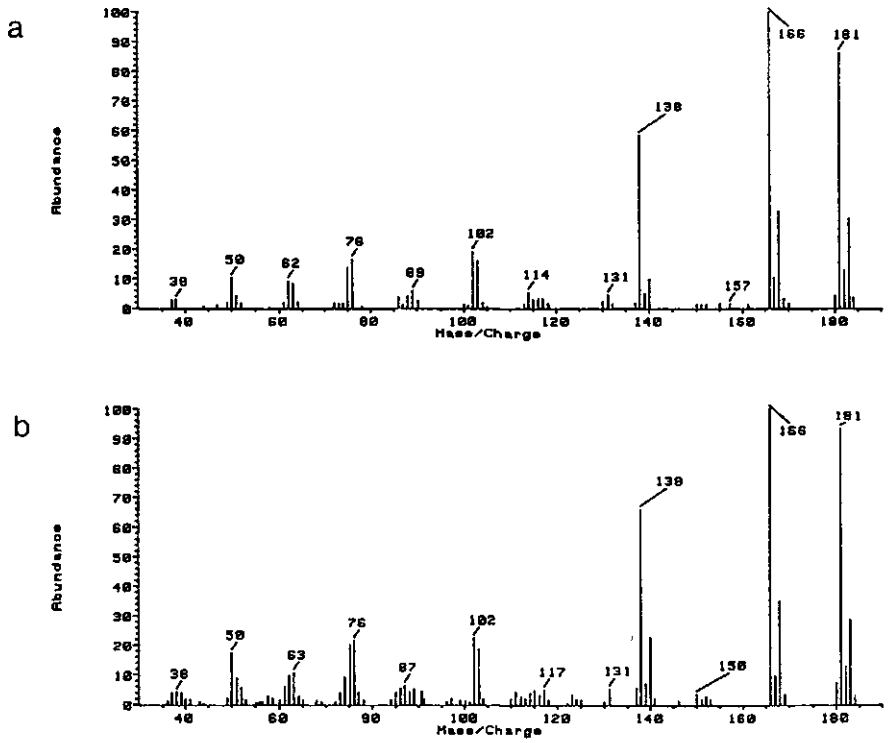

Figure 10.3: Mass spectrogram of (a) a compound, present in ethylacetate extracts of fava beans, expected to be 4-chloro-6-methoxyindole and (b) of a 4-chloro-6-methoxyindole standard.

To determine the amounts of 4C6MI in Dutch fava beans, five cultivars (3 white cooking and 2 brown cooking) were purified according to the new method. In Table 10.1 data are given on: (a) total N-nitroso levels in nitrite treated MeOH extracts (b) the mutagenicity in nitrite treated MeOH and EtAC extracts (c) the amounts of 4C6MI in the EtAc extracts estimated by comparison of the mutagenicity with a dose-response curve of pure 4C6MI. The amounts of 4C6MI in the EtAc extracts were also estimated from GC analyses using MSD (Table 10.2). It should be noted that in EtAc extracts of white cooking cultivars also another compound was present, which gave responses at m/e 138, 166 and 181. The retention time of this peak was longer than that of 4C6MI. The peak intensity was too low to obtain mass spectra.

Table 10.1: Total N-nitroso levels (NOC) in methanol (MeOH) extracts, mutagenicity in MeOH and ethylacetate (EtAc) extracts of fava beans after nitrosation and the amounts of 4-chloro-6-methoxyindole (4C6MI) in the EtAc extracts as estimated from the mutagenicity data.*

	(per 10 mg dry weight)			(mg/kg dry weight)
	NOC**	# revertants		4C6MI
	MeOH	MeOH	EtAc	EtAc
white cooking cultivar				
135	25.8	484	172 (35.54%)[a]	2.49
136	18.4	546	254 (46.52%)	3.98
134	29.5	1208	613 (50.74%)	8.66
brown cooking cultivar				
130	15.2	718	277 (38.58%)	4.01
131	11.7	516	141 (27.33%)	2.11

** to S. typhimurium TA100, -S9 mix.*
*** in nmol.*
[a] *recovery, compared to MeOH extract.*

Experiments using the new purification method, in which 4C6MI was either spiked to fava beans or was used as a standard solution, revealed recoveries of 5% and 34% respectively.

In Figure 10.4 the levels of 4C6MI in fava beans estimated from GC experiments (Table 10.2) are plotted against the levels estimated from the mutagenicity assays (Table 10.1). From this Figure it can be concluded that levels of 4C6MI estimated from mutagenicity data are in good agreement with those determined by GC-MSD. This means that most of the mutagenicity in EtAc extracts can be ascribed to 4C6MI.

Table 10.2: Levels of 4-chloro-6-methoxyindole (4C6MI) in dried fava beans, determined with GC-MSD.

	4C6MI mg/kg ± sd (n*)
white cooking	
cultivar	
135	3.24 ± 0.41 (5)
136	5.61 ± 0.32 (3)
134	7.44 ± 1.44 (3)
brown cooking	
cultivar	
130	5.89 ± 0.30 (4)
131	4.47 ± 0.47 (4)

* *number of GC injections.*

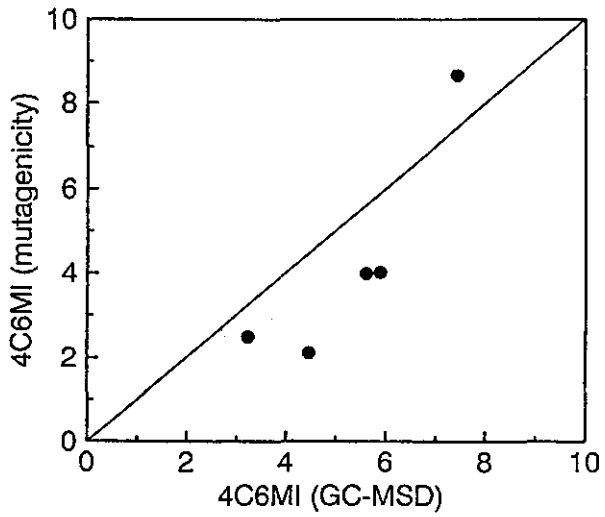

Figure 10.4: Amounts of 4-chloro-6-methoxyindole (4C6MI) in fava beans, as determined by GC-MSD plotted against those estimated from mutagenicity data. Amounts expressed in mg/kg dry weight.

DISCUSSION

In the present study a method was developed to detect 4C6MI in Dutch fava beans. Although, it is possible to estimate the amounts of 4C6MI in fava bean extracts by using this method, data should only be considered as preliminary. Before validated measurements can be made, both the purification method and the GC analysis (Table 10.2) require improvement. The purification method following the MeOH extraction should be revised in order to obtain higher recoveries. Moreover, multiple analysis of one single sample are needed to validate the method.

The differences found between the recoveries of 4C6MI spiked to fava beans and that of 4C6MI standard solutions can probably be attributed to binding of 4C6MI to the dry matter of the fava beans. In a previous study it was shown that mutagens formed in fava beans bind to fava bean proteins (Jongen *et al.* 1987). The 34% recovery of 4C6MI in the absence of dry matter is in the same range as the recoveries of mutagenicity in EtAc extracts (about 40%; Table 10.1). Since almost all mutagenicity found in EtAc extracts can be ascribed to 4C6MI, it can be suggested that the absolute amounts of 4C6MI in the fava beans are those found in the EtAc extracts (Table 10.2) multiplied by a factor 2.5-3.0.

In EtAc extracts of white cooking fava beans besides 4C6MI a compound was present, which contained the characteristic peaks of 4C6MI at m/e 138, 166 and 181, but which was less volatile than 4C6MI. Although no mass spectra of this compound could be obtained, it can be concluded that this compound does not have a chemical structure comparable to 4C6MI. This because of differences in the relative intensities of the peaks at m/e 138, 166 and 181 compared to those of 4C6MI. On the other hand, if it is true that 4C6MI is a metabolite of the methyl ester of 4-chloroindoleacetic acid, then it would have been likely that more compounds with a 4-chloroindole structure were present in the extracts. Yang (1985) supposed that 4C6MI in fava beans is a metabolite of indole alkaloids with a 6-methoxyindole nucleus as found for instance in reserpine and vindoline alkaloids (Geismann & Crout 1969). However, he also raised the possibility that 4C6MI arises from microbial sources, since chloro indoles are a family of tremorigenic mycotoxins called Penitrens isolated from *Penicillium crustesum* (Steyn *et al.* 1983).

The mutagenicity found in nitrite treated MeOH extracts of fava beans (Table 10.1) was not related to the total amount of NOC measured in these extracts. Regarding the amounts of 4C6MI determined in the fava beans (Table 10.2), nitrosated 4C6MI could only have made a small contribution to the total levels of NOC formed in fava bean extracts. This implies that also other NOC are formed. However, their contribution to the mutagenicity will be low, since the mutagenicity found in fava beans can almost entirely be ascribed to nitrosated 4C6MI. This can be deduced from the observation that almost all mutagenicity in EtAc extracts can be ascribed to 4C6MI and the recovery of the mutagenicity in EtAc is in the same range as that of 4C6MI as determined by GC-MSD.

The levels of 4C6MI determined in Dutch fava beans (Table 10.2) appeared to be much higher than those found in Colombian beans. Yang *et al.* (1984) estimated the

amounts of 4C6MI in Colombian fava beans to be 80 μg per kg dried beans, while the levels found in the present study were one to two orders of magnitude higher. Apart from methodological differences, this could be the result of the ripeness of the beans. Fava beans in Colombia are of the *major* type and are harvested dry, while in The Netherlands, most of the beans for human consumption are also of the *major* type, but are harvested green. The difference in the moment of harvesting comes from the fact that in Colombia, at least in the district of Nariño, fava beans are consumed throughout the year as staple food (Correa *et al.* 1983), while in The Netherlands they are seasonally consumed as one out of many available green vegetables. Since it was suggested that 4C6MI is a metabolite of a methyl ester of 4-chloroindoleacetic acid, as found in immature seeds of peas and which exerts growth hormone (auxin) activity (Marumo *et al.* 1968), the levels of 4C6MI may be related to the harvest time. In order to confirm this hypothesis, studies on the levels of 4C6MI in fava beans grown and harvested under different conditions are recommended.

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CHAPTER 11

THE INFLUENCE OF EXPERIMENTAL CONDITIONS AND FOOD MATRIX COMPONENTS ON THE MUTAGENICITY OF NITRITE TREATED FAVA BEANS

INTRODUCTION

In the study described in Chapter 10 the mutagenicity of nitrite treated fava beans was much higher than that found in previous studies (5000-12000 revertants versus 120-2000 revertants induced per 100 mg of dry weight; van der Hoeven *et al.* 1984, Jongen *et al.* 1987). In the latter studies some experimental conditions were different, the most important being: (1) the method of extraction (water versus methanol (MeOH) extraction) (2) the condition of the fava beans (cooked versus uncooked before freeze drying) (3) the conditions during the nitrosation reaction (nitrite concentration, duration of incubation). The main aim of the present study was to investigate the relative importance of these factors with regard to the mutagenicity of nitrite treated fava bean extracts. It is very probable that this mutagenicity is mainly due to nitrosated 4-chloro-6-methoxyindole (4C6MI; Chapter 10).

Van der Hoeven *et al.* (1984) found a significant difference in the mutagenicity of white and brown cooking cultivars of fava beans after nitrosation. White cooking maximally induced about 2000 revertants per 100 mg dry weight, while brown cooking maximally did 200. However, in the study described in Chapter 10 no obvious differences were found between the mutagenicity of the two types of cultivars. Therefore this aspect was also considered in the present study.

Finally the inhibition of the mutagenicity of nitrite treated fava bean extracts by food matrix components was investigated. In a previous study it was found that the mutagenicity of nitrosated fava beans could be inhibited by casein, pectin, gelatin, fava bean proteins and to a lesser extent by whey protein and starch (Jongen *et al.* 1987). In the study described in this chapter the binding of mutagens formed in nitrite treated fava beans to casein was further investigated, while also the binding to cellulose and wheat bran was studied.

MATERIALS AND METHODS

Chemicals

Casein was obtained from Dr. M.A.J.S. van Boekel, Department of Food Science, Agricultural University, Wageningen. Cellulose powder cF1 was obtained from Whatman (W

& R Balston Ltd, UK). Wheat bran (biodynamic cultivated wheat) was obtained from a local supermarket.

Fava beans

All fava beans used were obtained from a Dutch seed breeding company. In experiments, in which binding to casein, cellulose and wheat bran was investigated, the beans were harvested, cooked and canned in a commercial food processing plant in 1986. After 1-6 months of storage at room temperature the beans were freeze dried and ground, followed by storage at room temperature under nitrogen in the presence of silica gel in the dark for about 2 years. Storage had no effect on the mutagenicity of the beans after nitrosation. In experiments, in which the influence of incubation time with nitrite was investigated, the same beans as in Chapter 10 (1989 harvest) were used. In all other experiments, two brown cooking (cultivar 130 and 131) and two white cooking (cultivar 135 and 137) cultivars were used, which were cultivated under identical conditions (1990 harvest). The latter beans were divided in two portions. One of them was cooked for 7 min. at 98°C (checked by thermocouple) before freeze drying, while the other was not. The beans were stored as described before.

Extraction method

Portions of 3 g of cooked/uncooked fava beans were extracted with methanol (MeOH) for 3 h, using a soxhlet apparatus. Aliquots of MeOH extracts were evaporated to dryness and the residues dissolved in distilled water under heating to 37°C.
Cooked/uncooked fava beans were also extracted with water. To 100-125 mg fava beans 10 ml distilled water was added. After soaking for 0.5 h and stirring for 15 min., these "extracts" (actually suspensions, but referred to as water extracts) and MeOH extracts were tested for mutagenicity after nitrosation. When necessary the water extracts were centrifugated before the performance of the mutagenicity assay.

Nitrosation reaction & mutagenicity assay

The aqueous extracts of fava beans were nitrosated as described before (Chapter 6) and tested for mutagenicity using the *S. typhimurium* assay, as described in Chapter 4 and 5.

Inhibition of mutagenicity by casein, cellulose and wheat bran

In these experiments and those described in the "Reversibility of fava bean protein binding" section, water extracts of fava beans (1986 harvest) were used. Water extracts were nitrosated in the presence of the dry matter, because removing of the dry matter before nitrosation resulted in a decrease of the mutagenicity by a factor 6. This indicates that the promutagens present in the fava beans have a greater affinity for the dry matter than for the aqueous phase.

After termination of the nitrosation reaction, the extracts were centrifugated for 20 min.

at 10,000 rpm. Then either casein, cellulose or wheat bran were added to the supernatants, followed by a 15 min. incubation at 37°C (in the dark) and centrifugation. The obtained supernatants were tested for mutagenicity. In order to investigate the reversibility of the binding to casein, the pellet obtained after the first centrifugation was resuspended in distilled water of different pHs.

Reversibility of fava bean protein binding

To investigate the reversibility of the binding of mutagens to the dry matter of fava beans, the following experiments were performed: Water extracts were nitrosated in presence of the dry matter. After centrifugation (20 min. at 10,000 rpm), the supernatant was tested for mutagenicity. The pellet was resuspended in distilled water of pH 2. Then the procedure from centrifugation on was repeated 3 times.

RESULTS

Influence of extraction method, cultivar and cooking

Fava beans, both cooked and uncooked before freeze drying, were extracted by water and MeOH. In Table 11.1 the mutagenicity of nitrite treated water and MeOH extracts of two brown and two white cooking cultivars is shown. In general the method of extraction did not affect the mutagenicity. Only the brown cooking cultivar 130 induced about twice as much revertants when MeOH extracts were used instead of water extracts. Cooking before freeze drying had almost no effect on the mutagenicity of white cooking cultivars. However, it decreased the mutagenicity of brown cooking cultivars by a factor 2.5 (and by a factor 3.5 in the water extract of cultivar 130). All brown cooking cultivars, which were not cooked before freeze drying, were much more mutagenic after nitrosation than white ones.

Influence of incubation time and nitrite concentration

The influence of the length of the incubation of MeOH extracts on the mutagenicity is shown in Table 11.2. After an incubation time of 1 h the mutagenicity was only 40% of that found after 15 min. of incubation, irrespective of the cultivar used.

In Figure 11.1 the influence of the nitrite concentration on the mutagenicity of a white cooking cultivar is shown. The mutagenicity already reached a plateau when 5 mM of nitrite was used.

Inhibition of mutagenicity by proteins and other complex molecules

Previously it was shown that mutagens formed in fava beans bind to proteins, including those occurring in fava beans (Jongen *et al.* 1987). In the present study the reversibility of this binding was investigated (Table 11.3). Fava bean mutagens reversibly bound to proteins present in the dry matter. This being observed in the pH range of 2-6. From Table 11.3 it can be concluded that the extracts generally tested (supernatant I) contain

Table 11.1: Mutagenicity of nitrite treated methanol (MeOH) and water extracts of fava beans, whether or not cooked before freeze drying.*

	# of revertants in			
	MeOH extracts		water extracts	
	cooked	uncooked	cooked	uncooked
white cooking cultivar				
135	465	481	610	604
137	467	528	321	428
brown cooking cultivar				
130	953	2317	440	1520
131	533	1369	540	1394

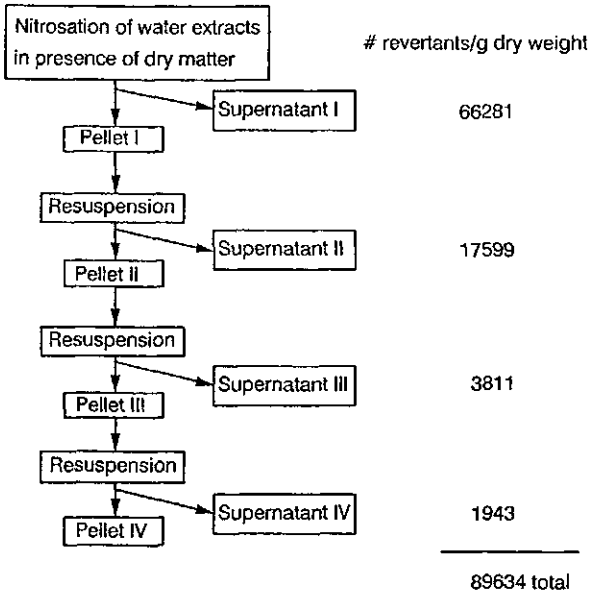
**S. typhimurium TA100, -S9 mix.*
+ 10 mg dry matter per plate.

Table 11.2: Number of revertants induced by fava beans (1989 harvest) after varying incubation periods with nitrite. In brackets the relative mutagenicity compared to that found after an 15 min. incubation.

incubation time (min.)	white cooking+ cultivar	brown cooking++ cultivar
15	1693 (100%)	973 (100%)
30	1080 (64%)	637 (65%)
45	1009 (60%)	602 (62%)
60	740 (44%)	414 (43%)

**S. typhimurium TA100, -S9 mix.*
+ 10 mg dry weight/plate; ++ 20 mg dry weight/plate.

Table 11.3: Reversibility of binding of fava bean mutagens to proteins occurring in fava beans, as measured by mutagenicity after repeated resuspension of dry matter of nitrosated fava beans in distilled water.*



**S. typhimurium TA100, -S9 mix.*

Table 11.4: Inhibition of the mutagenicity of nitrosated fava beans by casein, cellulose and wheat bran, when added after the nitrosation reaction.*

Addition per g of fava beans	Inhibition of mutagenicity
1.0 g casein	81.8%
1.5 g cellulose	2.4%
2.0 g wheat bran	28.8%

**S. typhimurium TA100, -S9 mix; Mutagenicity of a nitrite treated extract of fava beans without addition of matrix components was set at 100%.*

about 75% of the total mutagens formed in fava beans, while the other 25% reversibly bind to proteins present in the dry matter.

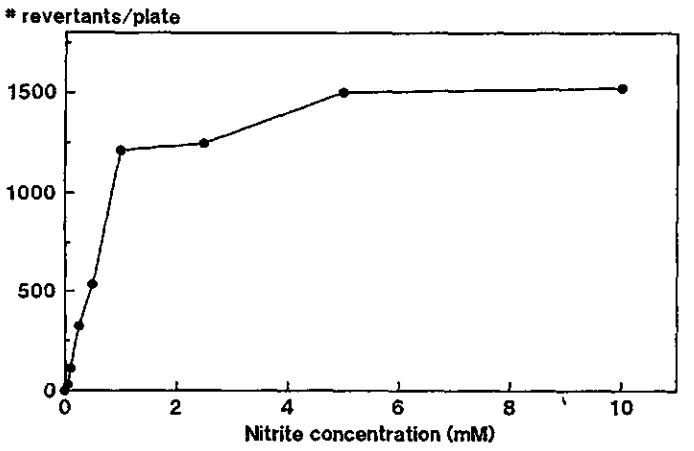


Figure 11.1: The mutagenic activity of fava beans** after treatment with varying concentrations of nitrite.*

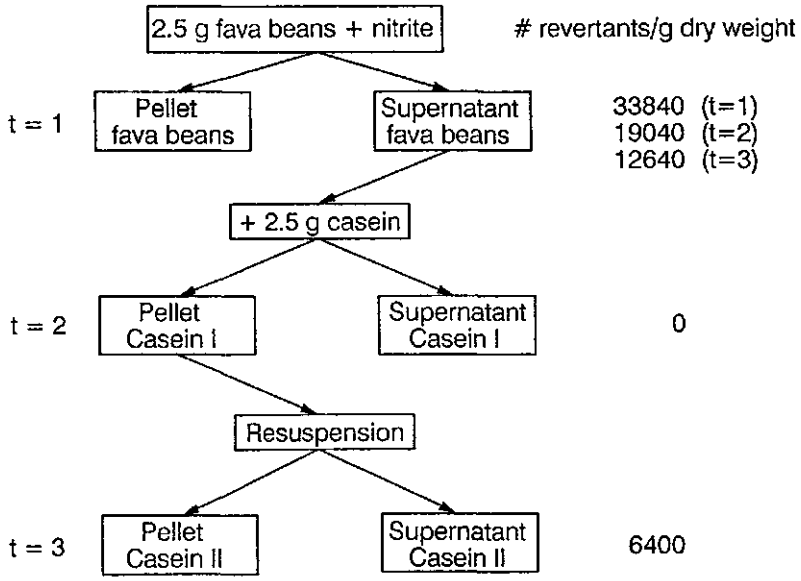
** to S. typhimurium TA100, -S9 mix.*

*** white cooking cultivar 135; 20 mg of dried weight/plate.*

In Table 11.4 the inhibition of fava bean mutagenicity by casein, cellulose and wheat bran is shown, as measured by addition of these components to supernatants of nitrosated fava bean extracts. Casein inhibited the mutagenicity of fava beans effectively. However, wheat bran and cellulose did not affect or weakly affected the mutagenicity. This indicates that wheat bran can bind small amounts of fava bean mutagens, whereas cellulose can not. The binding of the mutagens to casein was independent of the pH (tested pHs: 2.0, 4.5 and 6.0).

Table 11.5 shows the reversibility of the binding of fava bean mutagens to casein. This time 100% of the fava bean mutagens bound to casein and about 50% (80/158 x 100%) of the activity could be released by single resuspension in distilled water of pH 2-5, indicating a non-covalent binding. When the casein was resuspended in distilled water of higher pHs less mutagenic activity was found (data not shown). Although this can indicate a lesser reversibility of the casein binding, it is more likely that this is due to the instability of the fava bean mutagens at these pHs.

Table 11.5: Reversibility of the binding of fava bean mutagens to casein; After centrifugation, casein was resuspended in distilled water, from which the mutagenicity was measured. Corrections were made for the decrease in mutagenicity of fava bean mutagens in time.*



DISCUSSION

In the present study the influence of different experimental conditions on the mutagenicity of nitrosated fava beans was investigated. In general none of the parameters examined could solely explain the differences in the mutagenicity of nitrosated fava beans as found between studies by van der Hoeven *et al.* (1984) and Jongen *et al.* (1987) and that of Chapter 10. Only differences in incubation time may partially explain the lower mutagenicity rates found in the former studies. It should be noted that the nitrite/dry matter ratio could probably have affected the mutagenicity (Figure 11.1).

Cooking fava beans before freeze drying only had an effect on brown cooking cultivars. The most important difference between brown and white cooking cultivars is their tannin content. The former cultivars can contain considerable amounts, while the latter are free or almost free of them (Lawes *et al.* 1983). Therefore it is possible that tannins are responsible for the observed effect. However, it would be more likely that the mutagenicity

of tannin containing cultivars increase instead of decrease by cooking. This because during cooking tannins can transfer from the beans into the cooking liquor. Since tannins are known to inhibit the nitrosation reaction (Lijinski 1984), this would result in increases in the mutagenicity. Losses of tannins during cooking were previously observed in common beans (Norton *et al.* 1985).

In the present study brown cooking cultivars, especially those uncooked before freeze drying, were much more mutagenic after nitrosation than white ones. This is in contrast with previous studies (van der Hoeven *et al.* 1984; Chapter 10). None of the experimental conditions could explain this observed effect. Therefore it can not be excluded that environmental conditions during growth of the beans and/or culture practices have an effect on the amounts of 4C6MI, the compound being responsible for almost all mutagenicity in nitrite treated fava beans (Chapter 10).

As stated before, the length of incubation period of fava beans with nitrite had an effect on the mutagenicity. This was expected, since 4C6MI behaves in many ways as 4-chloroindole (4CI; Chapter 9) and it is known that the mutagenicity of nitrosated 4CI decreases with increasing incubation times (Tiedink *et al.* 1989, Chapter 4). Büchi *et al.* (1986) reported that 2 products will be formed when 4 chloroindoles are nitrosated; mutagenic 4-chloro(-6-methoxy)-2-hydroxy-N1-nitroso-3H-indolin-3-one oximes and non-mutagenic formyl-4-chloro(-6-methoxy)-indazoles. When an equilibrium exists between these two compounds, which will shift to the latter at prolonged incubation times, then this can explain the decrease in mutagenic activity after extended incubations. However, Yang (1985) postulated that the methoxy group at the C6 position determines the product ratio, whereas the formation of the α-hydroxy N-nitroso compound is preferred to that of the side products.

In all studies of the preceding chapters the nitrosation reactions were performed with 40 mM of $NaNO_2$. It should be realised that this is a very high nitrite concentration compared to those occurring in the human stomach. However, this concentration is described by the World Health Organization in the so called "Nitrosation Assay Procedure", a test developed to analyze the occurrence of nitrosatable compounds in drugs (WHO, 1978). Consequently in many studies 40 mM or even higher nitrite concentrations are used, which facilitates the detection of N-nitroso compounds formation in the samples under investigation. The nitrite concentration in the human stomach and saliva of healthy persons varies during the day, depending on the intake of nitrate, but different authors do not agree on the absolute concentrations. Walters *et al.* (1979) measured intragastric nitrite concentrations and found levels ranging from 14 μM to 0.3 mM. Gatehouse & Tweats (1982) found 1-5 μM in normal fasting gastric juices, while Correa *et al.* (1979) found levels up to 3 mM in the gastric juice of patients with gastritis. In the present study it was shown that in principle fava beans can form mutagens under physiological feasible nitrite concentrations. So the formation of fava bean mutagens is of relevance in relation to man. However, no definite conclusions can be drawn about the formation of fava bean mutagens *in vivo*, because there is still a number of uncertainties. First of all there are no data about the amounts of

promutagens available for nitrosation, whereas the availability of nitrite will be affected by the presence of scavengers in the food matrix, such as ascorbic acid, diphenols, α-tocopherol (Lathia *et al.* 1989), lignin (Dence 1971), wheat bran and in lesser extent to whole wheat flour (Moller *et al.* 1988). In the present study it was observed that also the fava bean mutagens bind to wheat bran and other food matrix components. Fava bean mutagens bound effectively (80-100%) to casein and in lesser extent to fava bean proteins (>25%; Table 11.3 & 11.4), which is in accordance with a study of Jongen *et al.* (1987). The binding of the mutagens to casein was not affected by pH, in a range of pH 2.0 to 6.0, and was non-covalent, since 50% of the mutagens could be released. However, it seemed that the release was more dependent on pH, since at pH 6.0 less mutagens were freed than at the lower pHs tested. Berg *et al.* (1990) showed that also the carcinogenic N-nitroso compound, 1-methyl-1-nitroso-3-nitroguanidine, binds to casein. Similarly, Stebler & Guentert (1990) reported the binding of benzodiazepine drugs to casein of human milk and of other mammals.

The binding of mutagens to fibres requires further attention, since fibres are not digested and therefore could serve as a vehicle for mutagens to leave the body without harmful effects. Epidemiological studies showed that the intake of dietary fibres is negatively associated with the incidence of colon (Kritchevsky *et al.* 1987) and gastric cancer (La Vecchia *et al.* 1987, Risch *et al.* 1985). Also animal studies revealed that dietary fibres can reduce the incidence of colon tumours, induced by several carcinogens (Barnes *et al.* 1983, Sinkeldam *et al.* 1990). The anti-carcinogenic effects of dietary fibres were previously supposed to be the result of a more rapid transit time for the material to pass the gut (Burkitt 1971, Burkitt *et al.* 1972), by which: (1) The opportunity for bacteria in the bowel to metabolize chemicals to their carcinogenic form is reduced. (2) The opportunity for the carcinogen to work on the gut wall is reduced. (3) The carcinogenic material is diluted, since the total bulk of content is increased. The fact that fibres can serve as vehicles for carcinogenic compounds can be an additional protecting effect.

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CHAPTER 12

CONCLUDING REMARKS TO CHAPTERS 9-11

4-Chloro-6-methoxyindole (4C6MI), the naturally occurring indole compound in fava beans, was evaluated for its genotoxic and tumour promoting potential after nitrite treatment (Chapter 9). Nitrosated 4C6MI was highly mutagenic to bacteria and mammalian cells, which is an indication for tumour initiation. Hence, nitrosation of 4C6MI might be one of the first causative factors in the process of gastric cancer of the intestinal type, as observed in Colombia. Remarkably, nitrosated 4C6MI also inhibited gap junctional intercellular communication, which suggests tumour promoting potency. This indicates that nitrosated 4C6MI could also be active in a later stage of carcinogenesis. 4-Chloroindole (4CI), previously used as a model compound for 4C6MI (Chapter 5) reacted the same as the naturally occurring compound after nitrosation, although the magnitude of the response was lower in various assays.

A method was developed to detect 4C6MI in Dutch fava beans (Chapter 10). Although, a reasonable estimate could be made of the amounts of 4C6MI in fava bean extracts, the results should still be considered as preliminary. Especially, the purification procedure requires further improvement before validated measurements can be made. The results showed that the levels of 4C6MI found in Dutch beans are one to two orders of magnitude higher than those reported for Colombian beans. The conclusion can be drawn that almost all mutagenicity of the nitrosated beans could be ascribed to 4C6MI.

The influence of different experimental conditions (extraction method, cooking of fava beans before freeze drying, nitrite concentration, length of incubation) on the mutagenicity of fava beans is described in Chapter 11. None of the experimental conditions tested could solely explain the differences found in the studies performed so far. However, the length of incubation significantly affected mutagenicity. Fava beans treated with physiologically realistic nitrite concentrations showed mutagenic activity to bacteria, even when relatively low doses of fava beans were used.

The differences between the mutagenicity of nitrite treated brown and white cooking cultivars as found in a previous study (van der Hoeven *et al.* 1984), could not be reproduced (Chapter 11).

Mutagens formed in fava beans appeared to bind to constituents present in the dietary food matrix (Chapter 11). The mutagenicity of fava beans could, for instance, be inhibited for 80-100% by the addition of casein, indicating binding to casein. Binding was independent of pH in the range of pH 2-6. Moreover, it was reversible, since mutagens could be released from the casein. About 25% of the mutagens formed in fava beans bound to proteins, also occurring in the beans. This binding was reversible too. A 30% decrease in mutagenicity of nitrite treated fava beans was found after addition of wheat bran, indicating

that fava bean mutagens also bind to wheat bran.

The finding that nitrosated 4C6MI should be considered as a potential genotoxic and possibly also a tumour promoting agent, indicates that it might be a complete carcinogen (Chapter 9). Hence, the endogenous nitrosation of 4C6MI, fits well into the model of gastric cancer etiology, previously postulated by Correa *et al.* (1976; discussed in Chapter 8). Nitrosated 4C6MI might be one of the main causative factors in the etiology of gastric cancer in the region concerned. So far no studies have been performed on the carcinogenic potential of nitrosated 4C6MI. Therefore no pertinent conclusions can yet be drawn on the possible risks involved for human exposure to this compound. Moreover, it is still uncertain to what extent 4C6MI will be nitrosated *in vivo*, although there are a few data which indicate that 4C6MI will be nitrosated endogenously:

1. Nitrosation of 4-chloroindoles occurred at nitrite concentrations found in human saliva and/or gastric juice (Yang *et al.* 1984).
2. Extracts of fava beans corresponding to only 20 mg of dry weight induced a significant response in the *S. typhimurium* (TA100) assay after treatment with 0.25 mM nitrite (Chapter 11), a concentration actually observed in gastric juice of healthy persons (Walters *et al.* 1979). The positive outcome of this test strongly points to the presence of nitrosated 4C6MI, since almost all mutagenic potential of nitrite treated fava beans could be attributed to 4C6MI (Chapter 10).

The data of the *in vitro* studies outlined above suggest that 4C6MI can in principle react with nitrite in the stomach. However, it is not yet known whether 4C6MI will be available to be nitrosated in the stomach and whether or not other dietary constituents may have an effect on the nitrosation rate. Moreover, in the literature no data are available on the effects of processing of fava beans on the availability of 4C6MI. In the study described in Chapter 11 cooking of fava beans reduced the mutagenicity after nitrosation, however, this was only observed using brown cooking cultivars. This observation can be the result of a higher affinity of 4C6MI to bind to denaturated proteins in comparison with native ones.

An epidemiological study conducted in Colombia revealed a positive correlation between the consumption of fava beans and the incidence of gastric cancer (Correa *et al.* 1983). The results of the study described in Chapter 9 support the hypothesis that a causal relationship exists between consumption of fava beans and the etiology of gastric cancer (Correa *et al.* 1976, 1983). A pertinent question now is whether fava bean consumption in Western countries could also be a risk. For different reasons it seems likely that the risk for the population in The Netherlands is probably much lower than that for the Colombian population, or even negligible. The most important reasons are:

1. In Colombia fava beans are an important source of proteins and form a prominent component of the every day diet (Correa *et al.* 1983). In The Netherlands fava beans are only consumed seasonally as one out of many available green vegetables. The mean consumption of legumes in The Netherlands is 6.3 g per capita per day (Anony-

mous, 1988). The contribution of fava beans to this figure is not exactly known, but will be much lower. In the Colombian villages under investigation, the mean consumption of fava beans ranged from about 50 to >100 g (dry weight) per capita per day (Correa *et al.* 1983), and these consumption figures have hardly been changed during recent years (Correa *et al.* 1990). This means that these people are exposed to about 4-8 μg of 4C6MI per day (based on the levels of 4C6MI reported by Yang *et al.* 1984). However, there is reason to assume that the levels of 4C6MI reported by Yang *et al.* largely underestimate the real levels. By using the purification method developed by Yang *et al.* 4C6MI could not be detected at all in Dutch fava beans and it was shown that in particular the first purification step (soxhlet extraction with isopropanol) had a low yield (about one third of that of a methanol extraction; see Chapter 10). Therefore it seems likely that the daily exposure of the inhabitants of the Colombian villages to 4C6MI will much higher than 4-8 μg. In The Netherlands the average level of exposure will be about 9 μg at most (based on an average daily consumption of 6 g of fresh fava beans, with levels of 4C6MI up to 7.5 mg/kg of dry weight). But again, it should be realised that fava beans are not commonly consumed in The Netherlands. Only people, who frequently consume high amounts of fava beans could be assigned as persons at risk.

2. The levels of nitrate (NO_3^-) in the drinking water in The Netherlands are much lower than in Colombia. In 1986, 75% of the drinking water obtained from 225 pumping stations in The Netherlands contained less than 5 mg NO_3^-/l (Basisdocument nitraat 1987). Although it is most likely that these levels will rise in the future when the present practices of intensive animal husbandry and fertilizer use will continue (Basisdocument nitraat 1987), they will very probably never reach the high levels found in Colombian mineral water (the average levels were reported to be 12.5 and 39 mg NO_3^-/l, and incidentally levels up to 300 mg/l were found (Cuello *et al.* 1976)).
3. In general the consumption of fresh vegetables and dairy products is much higher in The Netherlands than in Colombia. In The Netherlands the mean consumption of fresh vegetables in 1987-1988 was estimated to be 144 g per person per day (Anonymous 1988), while from the results of Correa *et al.* (1983, 1990) it can be calculated that the mean consumption in Colombia is about one third of this. Consequently the intake of compounds negatively correlated with different kind of tumours (for instance: ascorbic acid, ß-carotene and α-tocopherol) are higher in The Netherlands. On the other hand, the higher consumption of vegetables will also lead to higher intakes of nitrate. Previously Mirvish (1985) reported that the ratio nitrate to vitamin C + nitrosation inhibitors in vegetables should be of concern in relation to gastric cancer instead of nitrate alone. From the higher consumption of dairy products it can be assumed that the matrix effects described in Chapter 11 may be more significant in The Netherlands than in the Colombian situation.

   Since in The Netherlands there are people, whose food habits deviate from the average (for instance vegetarians), it can not be excluded that these people have a higher

risk.

Finally, it is recommended that the *in vivo* nitrosation of 4C6MI and the carcinogenicity of nitrosated 4C6MI will be investigated in further detail in an adequate animal study.

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SUMMARY AND CONCLUSIONS

From the introduction to the chemistry, occurrence and formation of N-nitroso compounds (NOC) (Chapter 1), it can be concluded that human exposure to these potent carcinogenic compounds is mainly through the endogenous nitrosation of dietary precursors. Vegetables are the major source of nitrosating compounds, while also nitrosatable substrates can occur in vegetables. Therefore the first aim of the present study was to screen Dutch vegetables for the presence of nitrosatable compounds by measuring their potential to form directly mutagenic NOC upon nitrite treatment. The second aim was to study the identity and mutagenic properties of the NOC formed. Since it was not feasible to cover all vegetables in connection to the second aim of the study, the efforts were concentrated on two of them, brassicas and fava beans. The latter were chosen because they were already known to contain precursors of directly mutagenic NOC and consequently the study was divided into two parts: Part 1 dealing with brassicas and Part 2 with fava beans.

Part 1

In Chapter 4 experiments are described in which extracts of 31 Dutch vegetables were screened for their ability to form directly mutagenic NOC upon nitrite treatment, irrespective of their nitrate content. All vegetables tested formed NOC upon nitrosation and induced *Salmonella* (*S.*) *typhimurium* revertants; Brassica vegetables were high responders on both of these parameters. Moreover, a significant correlation was found between their glucosinolate content (both alkyl/aryl- and indolylglucosinolate) and the amounts of NOC formed in extracts of these vegetables upon nitrosation. This suggests that glucosinolates are involved in the formation of NOC. Therefore purified glucosinolates were tested for their potential to form NOC (Chapter 6). Only indolylglucosinolates and their hydrolysis products formed NOC upon nitrosation. Mutagenicity was restricted to the nitrosated hydrolysis products. Since upon hydrolysis of indolylglucosinolates indole compounds are formed, which are nitrosatable substrates, the kinetics of the formation of NOC from indole compounds were investigated, as well as the stability of the nitrosated products formed (Chapter 5). Indole-3-acetonitrile (I_3A), indole-3-carbinol (I_3C) and indole, the hydrolysis products of the most commonly indolylglucosinolate, glucobrassicin, immediately reacted with nitrite to form directly mutagenic NOC and after an incubation time of about 15 min. maximal amounts of NOC were formed. The formed NOC were stable at both pH 2 and 8, but only when nitrite was present.

In order to determine the contribution of indole compounds to the total mutagenicity of nitrite treated brassicas, the presence of several known indole compounds in green cabbage was investigated (Chapter 6). Only indole-3-carboxaldehyde and I_3A could be detected. Both were not found to be important precursors of directly mutagenic NOC in

green cabbage. The former did not form NOC at all and the second, although it occurred in considerable amounts (12 mg/kg fresh weight), only contributed for 2% to the total mutagenicity of nitrite treated green cabbage.

From these results it can be concluded that:

1. The correlation found between the amounts of glucosinolates in brassicas and the levels of NOC formed in extracts of these vegetables upon nitrite treatment is not based on a causal relationship.
2. In brassicas both glucosinolates and indole compounds should not be considered as important precursors of NOC.
3. By testing compounds out of their normal matrix, results can be obtained which differ considerably from those obtained under more realistic situations; I_3A, when tested pure, was found to be a potent precursor of NOC, while in green cabbage its contribution to the total mutagenicity was marginal.

Although I_3A was not considered an important precursor of NOC and contributed only marginally to the mutagenicity of nitrite treated brassicas, it was found to be a potential genotoxic and tumour promoting agent after nitrosation (Chapter 9). Recently it was shown in a Japanese study that nitrosated I_3A is able to induce tumorous lesions in the forestomach of rats. Therefore the possible risk involved in the consumption of brassicas due to the endogenous formation of nitrosated I_3A was assessed (Chapter 7). For different reasons it seems most unlikely that the endogenous formation of nitrosated I_3A out of brassicas should be considered as a realistic threat to human health: (1) The nitrosation rate of I_3A *in vivo* is expected to be low. (2) In animal studies nitrosated I_3A was administered pure, in very high concentrations. (3) Nitrosated I_3A will only be stable in the presence of nitrite.

Since in brassicas other precursors of directly mutagenic NOC occur, further studies are recommended to elucidate their identity and the implications of their potential endogenous nitrosation.

Part 2

4-Chloro-6-methoxyindole (4C6MI), the naturally occurring indole compound in fava beans, was evaluated for its genotoxic and tumour promoting potential after nitrite treatment (Chapter 9). Remarkably, nitrosated 4C6MI appeared to have both genotoxic and tumour promoting potentials. The initiation effects were measured in bacteria and mammalian cells, the tumour promoting effects were measured by inhibition of gap junctional intercellular communication of V79 Chinese hamster cells. Both effects were observed at concentrations, which were in the same order of magnitude as the estimated daily intake of 4C6MI in a Colombian population. Hence, the results support the model for gastric cancer etiology, proposed by Correa et al. (1976, 1983). In this model the formation of NOC out of fava beans was supposed to be a causative factor of gastric cancer of the intestinal type, endemic in Colombia.

In the study described in Chapter 10 the occurrence of 4C6MI in Dutch fava beans was investigated. An improved purification method was developed because the procedures described in the literature proved to be inadequate. Although the new purification method requires further improvement, a reasonable estimate of the levels of 4C6MI could be made. The levels ranged from about 3 to 7.5 mg/kg dry weight, which is one to two orders of magnitude higher than those reported for Colombian beans. However, in Chapter 12 it was assumed that the levels of 4C6MI in Colombian beans may be much higher than those reported. Further studies are required to investigate this aspect. From the results described in Chapter 10 it was concluded that almost all mutagenicity of nitrite treated Dutch fava beans can be attributed to 4C6MI.

Although in previous studies a difference was observed in the mutagenicity of nitrite treated white and brown cooking cultivars after nitrosation, this was not found in the present study (Chapter 10 & 11). Moreover, all cultivars induced much more *S. typhimurium* revertants after nitrosation than in previous studies. No explanation can be given for these equivocal results and therefore studies are recommended to investigate the influence of environmental factors on the levels of 4C6MI in fava beans (Chapter 10).

The mutagenicity of nitrite treated fava beans could be inhibited for 80-100% by addition of casein, indicating binding of nitrosated 4C6MI to casein (Chapter 11). This binding is independent of pH, in a range of pH 2-6 and appeared to be reversible, since mutagens could be released from the casein. It was estimated that about 25% of nitrosated 4C6MI formed in nitrite treated fava beans binds to proteins present in fava beans. This binding is reversible too. Fava bean mutagens also bind to wheat bran. Although the binding efficiency to wheat bran is less than to casein and the reversibility of this binding has not been studied, the binding of fava bean mutagens to wheat bran can be an important observation. Wheat bran fibres will not be digested and therefore might serve as vehicles for mutagens to leave the body without harmful effects. Further investigation of this is recommended.

The results of the present study are further support for the hypothesis that the consumption of fava beans is causally related to the etiology of gastric cancer in Colombia. But the risks involved in the consumption of fava beans due to the endogenous nitrosation of 4C6MI for the population in The Netherlands is considered to be low, because of: (1) The low consumption of fava beans. (2) The lower levels of nitrate in Dutch drinking water. (3) Differences in food patterns between the Colombian and the Dutch population (the levels of consumption of vegetables and dairy products in particular). However, it can not be excluded that there are groups in The Netherlands with consumption habits deviating from that of the average population, who can in principle be assigned as persons at risk.

It is recommended to investigate the *in vivo* nitrosation of 4C6MI and the carcinogenicity of its nitrosated product in further detail by using an appropriate animal study.

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SAMENVATTING EN CONCLUSIES

In de inleiding van dit proefschrift wordt de chemie en vorming van N-nitroso (NO)-verbindingen, een klasse van potente carcinogenen, beschreven (Hoofdstuk 1). Uit dit overzicht kan worden geconcludeerd, dat humane blootstelling aan deze verbindingen hoofdzakelijk plaatsvindt door de endogene nitrosering van voedingscomponenten. Groenten vormen de belangrijkste bron van nitroserende componenten, terwijl zij ook nitroseerbare verbindingen kunnen bevatten. Daarom was het eerste doel van het onderzoek beschreven in dit proefschrift, het "screenen" van Nederlandse groenten op het voorkomen van nitroseerbare verbindingen. Dit werd gerealiseerd door extracten van groenten onder zure condities te incuberen met nitriet en vervolgens de vorming van direct mutagene NO-verbindingen te bepalen. Het tweede doel van dit onderzoek was het bepalen van de identiteit van de in groenten-extracten gevormde NO-verbindingen en hun mutagene eigenschappen. Ten aanzien van het tweede doel bleek al snel, dat het binnen het tijdsbestek van het onderzoek niet haalbaar zou zijn om alle groenten te onderzoeken. De aandacht werd beperkt tot brassica groenten en tuinbonen, omdat van beiden reeds bekend was, dat zij verbindingen bevatten welke met nitriet kunnen reageren tot direct mutagene NO-verbindingen. Het onderzoek werd overeenkomstig in twee delen opgesplitst. In het eerste deel zijn brassica's onderwerp van onderzoek, in het tweede deel tuinbonen.

Deel 1

In Hoofdstuk 4 zijn experimenten beschreven waarin extracten van 31 Nederlandse groenten, afgezien van hun gehalte aan nitraat, onderzocht werden op de mogelijkheid direct mutagene NO-verbindingen te vormen. In alle groenten werden door behandeling met nitriet NO-verbindingen gevormd en *Salmonella* (*S.*) *typhimurium* revertanten geïnduceerd. Brassica groenten gaven een hoge respons op beide parameters. Bovendien werd een significante correlatie gevonden tussen hun glucosinolaatgehalte (zowel aryl/alkyl- als indolylglucosinolaat) en de hoeveelheid NO-verbindingen gevormd in extracten van deze groenten na behandeling met nitriet. Dit suggereert dat glucosinolaten betrokken zijn bij de vorming van NO-verbindingen. Daarom werden geïsoleerde glucosinolaten onderzocht op hun vermogen NO-verbindingen te vormen (Hoofdstuk 6). Slechts indolylglucosinolaten en hun afbraakprodukten bleken in staat te zijn met nitriet te reageren tot NO-verbindingen, terwijl alleen hun genitroseerde hydrolyseprodukten mutageen werden bevonden. Omdat bij de hydrolyse van indolylglucosinolaten, goed nitroseerbare indoolverbindingen worden gevormd, werd de kinetiek van de vorming van NO-verbindingen uit enkele indolen bepaald. Tevens werd de stabiliteit van de gevormde genitroseerde produkten onderzocht (Hoofdstuk 5). Indool-3-acetonitril (I_3A), indool-3-carbinol (I_3C) en indool (I), de hydrolyseprodukten van het meest voorkomende indolylglucosinolaat, glucobrassicine, werden gelijk na toevoeging van nitriet genitroseerd en na een incubatieduur van ongeveer 15 minuten

werden maximale hoeveelheden aan genitroseerd produkt gevonden. De gevormde NO-verbindingen waren alleen stabiel in aanwezigheid van nitriet en dit gold zowel bij pH 2 als pH 8.

Om de bijdrage van indoolverbindingen aan de mutageniteit van met nitriet behandelde brassica's te bepalen, werd de aanwezigheid van verschillende indoolverbindingen in extracten van groene kool bepaald (Hoofdstuk 6). Alleen indool-3-carboxyaldehyde (ICHO) en I_3A konden worden aangetoond. Echter geen van beide bleek een belangrijke precursor van direct mutagene NO-verbindingen te zijn. ICHO werd niet mutageen bevonden na nitrosering. I_3A droeg, ondanks dat het in behoorlijke hoeveelheden voorkwam (12 mg/kg vers gewicht), slechts voor ongeveer 2% bij aan de totale mutageniteit van genitroseerde groene kool. Uit deze resultaten kan het volgende worden geconcludeerd:

1. De gevonden correlatie tussen de glucosinolaatgehalten in brassica's en de hoeveelheid gevormde NO-verbindingen in extracten van deze groenten na behandeling met nitriet, berust niet op een causaal verband.
2. Zowel glucosinolaten als de daaruit gevormde indoolverbindingen vormen geen belangrijke precursors van NO-verbindingen in brassica's.
3. De onderzochte componenten kunnen in zuivere vorm andere eigenschappen vertonen dan in de aanwezigheid van hun matrix; De modelstof I_3A vormde significante hoeveelheden direct mutagene NO-verbindingen bij behandeling met nitriet, terwijl in een extract van groene kool, slechts een geringe bijdrage aan de mutageniteit werd gevonden.

Ondanks het feit dat I_3A geen belangrijke precursor bleek te zijn van NO-verbindingen in extracten van groene kool en mogelijk andere brassica's, moet genitroseerd I_3A toch worden beschouwd als een potentiële genotoxische stof met tumor bevorderende eigenschappen (Hoofdstuk 9). Recentelijk is in Japan vastgesteld dat genitroseerd I_3A in staat is tumorigene laesies te induceren in de voormaag van ratten. Daarom werd het mogelijke risico verbonden aan de consumptie van brassica's ten gevolge van de endogene nitrosering van I_3A geschat (Hoofdstuk 7). Het lijkt zeer onwaarschijnlijk dat de endogene nitrosering van I_3A uit brassica's beschouwd moet worden als een reëel risico voor de volksgezondheid, om de volgende redenen: (1) De nitroseringssnelheid van I_3A *in vivo* laag wordt verondersteld. (2) In dierproeven zeer hoge concentraties aan genitroseerd I_3A, in zuivere vorm, werden gebruikt. (3) De stabiliteit van genitroseerd I_3A in afwezigheid van nitriet gering is. Echter, gezien het feit dat brassica's naast I_3A nog andere precursors van direct mutagenen NO-verbindingen bevatten, wordt vervolgonderzoek geadviseerd, om de identiteit van deze precursors en de gevolgen van mogelijke endogene nitrosering te bepalen.

Deel 2

De van nature in tuinbonen voorkomende indoolverbinding, 4-chloro-6-methoxyindool (4C6MI), werd geëvalueerd op zijn potentiële genotoxische en tumor bevorderende eigen-

schappen (Hoofdstuk 9). Het is opmerkelijk dat genitroseerd 4C6MI over beide eigenschappen bleek te beschikken. Genotoxische effecten, gemeten in bacteriën en zoogdiercellen, en tumor bevorderende effecten, gemeten aan de hand van remming van intercellulaire communicatie van V79 Chinese hamstercellen, werden waargenomen bij lage concentraties van genitroseerd 4C6MI. Deze concentraties zijn in dezelfde grootte-orde als de geschatte dagelijkse 4C6MI inname door de bevolking van Colombia. De resultaten besproken in Hoofdstuk 9 ondersteunen het model voor maagkanker, opgesteld door Correa et al. (1976, 1983). In dit model wordt de vorming van NO-verbindingen uit tuinbonen gezien als één van de oorzakelijke factoren van maagkanker van het intestinale type, endemisch in Colombia.

In Hoofdstuk 10 is het onderzoek naar het voorkomen van 4C6MI in Nederlandse tuinbonen beschreven. Omdat bij gebruikmaking van methoden zoals beschreven in de literatuur, geen 4C6MI in Nederlandse tuinbonen kon worden aangetoond, werd een verbeterde zuiveringsmethode voor 4C6MI ontwikkeld. Ondanks het feit dat verdere verbeteringen in de zuiveringsprocedure moeten worden aangebracht, kon een redelijke schatting gemaakt worden van de hoeveelheid 4C6MI in Nederlandse tuinbonen. De gehaltes variëerden van ongeveer 3 tot 7.5 mg per kg droog gewicht. Dit is één tot twee grootte-ordes meer dan die gerapporteerd voor Colombiaanse bonen. Echter in Hoofdstuk 12 worden redenen genoemd, waaruit kan worden afgeleid dat de gehaltes in Colombiaanse bonen mogelijk veel hoger zijn dan gerapporteerd. Verder onderzoek hierna wordt aangeraden. De mutageniteit van Nederlandse tuinbonen na nitrosering kan bijna geheel geweten worden aan 4C6MI.

In vorig onderzoek werd een verschil waargenomen in de mutageniteit van bruin en wit kokende tuinbonen na nitrosering. Dit werd echter in het onderzoek beschreven in de hoofdstukken 10 en 11 niet geconstateerd. Ook induceerden alle tuinboonrassen na nitrosering aanzienlijk meer revertanten dan in voorgaand onderzoek. Er konden geen verklaringen worden gegeven voor deze tegenstrijdige resultaten en daarom wordt onderzoek naar de invloed van milieufactoren op het gehalte aan 4C6MI in tuinbonen aanbevolen.

De mutageniteit van genitroseerde tuinbonen kon met 80-100% worden geremd door toevoeging van caseïne, waaruit blijkt dat genitroseerd 4C6MI bindt aan caseïne (Hoofdstuk 11). Deze binding is onafhankelijk van de gebruikte pH, in het pH gebied 2-6, en bleek reversibel, omdat mutagenen vrij konden komen bij resuspendering van de caseïne. Er werd bepaald dat ongeveer 25% van genitroseerd 4C6MI bindt aan eiwitten aanwezig in tuinbonen en dat ook deze binding reversibel is. Tuinboon-mutagenen bleken ook te binden aan zemelen. Hoewel de effectiviteit van de binding aan zemelen minder is dan die aan caseïne en de reversibiliteit van deze binding niet is onderzocht, kan dit een belangrijke waarneming zijn. Vezels aanwezig in zemelen worden in het lichaam niet verteerd en kunnen zodoende als drager voor mutagenen fungeren, waardoor deze zonder schadelijke effecten het lichaam kunnen verlaten.

De resultaten van het onderzoek beschreven in dit proefschrift zijn een verdere ondersteuning van de hypothese dat de consumptie van tuinbonen in Colombia oorzakelijk

gerelateerd is aan maagkanker. Echter de gezondheidsrisico's, verbonden aan de consumptie van tuinbonen ten gevolge van de endogene nitrosering van 4C6MI worden voor de Nederlandse bevolking gering geacht (Hoofdstuk 12). Dit gezien: (1) De geringe consumptie van tuinbonen. (2) Het lagere nitraat gehalte in het Nederlandse drinkwater. (3) Verschillen in voedingsgewoontes tussen de Colombiaanse en Nederlandse bevolking (met name ten aanzien van de consumptie van verse groenten en melkprodukten). Toch kan niet worden uitgesloten dat groepen, met consumptiepatronen afwijkend van de gemiddelde bevolking, een verhoogd risico hebben.

Verdere bestudering van de *in vivo* nitrosering van 4C6MI en de carcinogeniteit van het genitroseerde produkt aan de hand van dierexperimenteel onderzoek wordt aanbevolen.

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ABBREVIATIONS

ASC	ascorbigen
BNP	glucobrassicanapin
BrdU	5-bromo-2-deoxyuridine
4CI	4-chloroindole
4C6MI	4-chloro-6-methoxyindole
DIM	di-indolylmethane
DMSO	dimethyl sulphoxide
EBSS	Earle's balanced salt solution
EtAc	ethylacetate
EMS	ethyl methanesulphonate
GB	glucobrassicin
GC	gas chromatography
GC-MS	gas chromatography-mass spectrometry
GJIC	gap junctional intercellular communication
GNP	gluconapin
GPC	gel permeation chromatography
GTP	glucotropaeolin
HBSS	Hanks balanced salt solution
HGPRT	hypoxanthine guanine phosphoribosyl transferase
HPLC	high performance liquid chromatography
I	indole
IAA	indole-3-acetic acid
I_3A	indole-3-acetonitrile
I_3C	indole-3-carbinol
ICHO	indole-3-carboxaldehyde
IE	indole-3-ethanol
MeOH	methanol
MFO	mixed function oxygenase
MNNG	1-methyl-1-nitroso-3-nitroguanindine
MS	mass spectrometry
NDMA	N-nitrosodimethylamine
NDELA	N-nitrosodiethanolamine
NMOR	N-nitrosomorpholine
NO_x	nitric oxides
NPRO	N-nitrosoproline
NPIC	N-nitrosopipecolic acid
NOC	N-nitroso compounds
NST	gluconasturtiin

NQO	4-nitroquinoline N-oxide
4-OH-GB	4-hydroxyglucobrassicin
PGT	progoitrin
PHD	photohydrolysis detection
SAL	glucosinalbin
SCE	sister chromatid exchange
SD	standard deviation
SEM	standard error of mean
SGN	sinigrin
S. typhimurium	*Salmonella typhimurium*
TEA	thermal energy analyzer
6TG	6-thioguanine
TSNA	tobacco specific N-nitrosamines
Trp	tryptophan
TPA	12-O-tetra-decanoylphorbol-13-acetate
rt	retention time
Vt	total column volume

CURRICULUM VITAE

Erica Tiedink werd geboren op 11 januari 1962 te Utrecht. In 1980 behaalde zij het diploma Atheneum-B aan het Revius Lyceum te Doorn. In datzelfde jaar begon zij haar studie Humane Voeding aan de Landbouwuniversiteit te Wageningen. Tussen de kandidaats- en doctoraalfase werkte zij enkele maanden bij het "Department of (Bio)chemistry", Bucknell University, Lewisburg PA, USA. In de doctoraalfase deed zij de hoofdvakken Toxicologie en Humane Voeding en een extra vak Biochemie. Haar stageperiode bracht ze door bij het Laboratorium voor Carcinogenese en Mutagenese van het Rijksinstituut voor de Volksgezondheid en Milieuhygiëne (RIVM) te Bilthoven. De studie werd in januari 1987 met lof afgerond.

Op 1 april 1987 werd het in dit proefschrift beschreven onderzoek gestart bij het Centrum voor Agrobiologisch Onderzoek (CABO) te Wageningen. Op 1 januari 1988 werd het onderzoek voortgezet bij de vakgroep Toxicologie van de Landbouwuniversiteit in samenwerking met het CABO. Gedurende de periode 1 april 1987 - 1 maart 1991 was Erica achtereenvolgens in dienst van het CABO, de directie Voedings- en Kwaliteitsaangelegenheden van het Ministerie van Landbouw, Natuurbeheer en Visserij en de Landbouwuniversiteit.