Nitrosation of dietary precursors

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Summary

The diet contains a large number of constituents which can be nitrosated in the gastrointestinal tract (especially in the stomach) to potentially carcinogenic nitroso compounds (NOC). The nitrosation of food mixtures has been investigated with a number of assays, such as chemical analysis or detection of alkylating potential, mutagenicity and carcinogenicity. Relatively good information is available on the formation of stable nitrosamines using high nitrite concentrations. Little is known, however, about the formation of chemically unstable NOC at low nitrite concentration and their genotoxicity in target cells. A comparison of the precursor classes, alkylamines, aromatic amines, amino acids, amides and peptides, ureas and guanidines, reveals a vast range, both with respect to daily intake (10^5-fold) and nitrosation rate (10^4-fold both for 1st and 2nd order nitrite dependence). A total span of 10^8 results for the relative yield of NOC in the stomach. The endogenous NOC burden from dietary ureas and aromatic amines may represent as large a hazard as the intake of preformed NOC. Recent evidence also indicates that heterocyclic amines and phenols must be considered and that the half-life of nitrosated α-amino acids can be much longer than that of nitrosated primary alkylamines. In these classes, more information should be collected on dietary concentrations, on the nitrosation under realistic conditions and on the genotoxicity in stomach lining cells. Within a chemical precursor class, a wide range is seen with respect to alkylating potency. It cannot, therefore, be excluded that individual precursors within the top ranking classes might become more important than single preformed NOC. Not considered in the above analysis but probably just as important for a risk evaluation in a population is the knowledge of the nitrosation conditions and target cell susceptibility in individuals.

1 Introduction

Research on the nitrosation of food precursors to produce nitroso compounds (NOC) has focused on two complementary fields. Work with food mixtures has aimed to identify foods with large amounts of nitrosatable precursors (Section II), while work with isolated precursors has examined the chemistry of the various types of nitrosatable compounds (Section III). In our laboratory the various precursor classes were compared on the basis of three main variables, and short-term assays were developed to investigate and compare individual precursors within a class (Section IV).

II Nitrosation of food mixtures

Food mixtures were nitrosated in vitro under 'realistic' conditions (micromolar nitrite) or 'extreme' conditions (millimolar nitrite) and then analysed by chemical or biological methods which are reviewed here.

1 Chemical analysis

In a study that examined a large cross-section of the Western diet, food samples were nitrosated under 'realistic' conditions and the products analysed with GC-TEA (TEA = Thermal Energy Analyser) (Groenen *et al*, 1982). Of the volatile NOC, only nitrosodimethylamine (NDMA) was regularly found after nitrosating the food samples, and its presence was restricted almost exclusively to fish. Amounts ranged from <0.1-44 μg/portion, with an average of 0.65 μg NDMA/250 g portion. This is approximately equal to the daily exogenous load of volatile NOC (0.5-1.0 μg; Preussman and Eisenbrand, 1984). Whereas 90% of the fish samples contained no NDMA before nitrite treatment (detection limit 0.1 μg/portion), 97% were positive following nitrite treatment. No other food samples regularly produced detectable amounts of volatile NOC. The non-volatile nitrosamino acids nitrosoproline (NPRO), nitrosohydroxyproline (NHPRO) and nitrososarcosine (NSARC) were found in both higher quantities and in a wider variety of foods. Fried foods were particularly likely to contain nitrosamino acids following nitrosation (average 28 μg/250 g portion ≈ 1 μmol/kg). The occurrence of other non-volatile NOC after nitrosation was not studied. From these results, it appears that the formation of non-volatile NOC could be about 10–100 times greater than the formation of volatile NOC.

Walters *et al* (1974) nitrosated milk and cheese under 'realistic' and 'extreme' conditions, with or without catalysis by thiocyanate. The products were separated into volatile and non-volatile dichloromethane extracts, and a water soluble residue, referred to as the 'NO-peptide fraction'. The three fractions were analysed by a group-specific procedure, but the individual NOC were not identified. After nitrosation for 3 h at pH 3 with 145 μM nitrite, milk contained no measurable volatile NOC, 1 μmol non-volatile NOC and about 8 μmol NO-peptides per litre. Cheese contained 2 μmol volatile, 12 μmol non-volatile and 17 μmol NO-peptides per kilogram.

2 Alkylating potential

In this method, alkylating agents formed by nitrosation of precursors are trapped with the nucleophile 4-(p-nitrobenzyl)pyridine (NBP; introduced to trap alkylating agents by Epstein *et al*, 1955). The resulting blue derivative can be quantified colorimetrically (see also Section IV and Appendix). Detection is therefore not restricted to chemically identified and analysable NOC, but also encompasses hitherto unsuspected precursors and NOC for which analytical methods are lacking.

Foods from a cross-section of the typical Western diet were treated with millimolar nitrite concentrations; the products were worked up under acidic conditions and then allowed to react with NBP. The method was optimized for alkylating agents of the *N*-nitrosourea type (stability at acidic pH). Up to ≈5 μmol alkylating agent per portion was found in sauerkraut, fermented milk products (yoghurt, quark, etc), wine and smoked fish (Groenen and Businк, 1988).

3 Mutagenicity

A number of authors, particularly in Japan, have examined the mutagenicity of food items and food mixtures after nitrite treatment. Nitrosation was carried out typically for one to three hours at 40 mM nitrite. The major advantage of this experimental method is that the test screens not only for the amounts but also for the genotoxic activity of NOC produced. A disadvantage is that the NOC must have sufficient stability to survive the extraction procedure.

Tomita *et al* (1984) carried out a study on a cross-section of the Japanese diet. Food mixtures were dried, extracted with methanol and nitrosated. Following a further extraction, the products were tested for mutagenicity with *Salmonella typhimurium* TA100 (preincubation, with and without rodent liver activation system, S-9). Between 3 and 1000 revertants were induced per gram food mixture. Rice and milk products induced the fewest, and beans/soya beans and root vegetables/pickles/seaweed the most revertants. Addition of S-9 (microsomal enzymes) reduced the mutagenicity in every case. Fish and meat were mutagenic even without nitrite treatment.

Extracts of pickled vegetables, sun-dried seafood and soya sauce from various countries have also been tested for mutagenicity in *Salmonella typhimurium* TA100 (preincubation, no S-9 mix) following nitrite treatment. The pickled vegetables induced 1900–18 000 revertants/g; the sun-dried seafood 1400–5500 revertants/g, and the soya sauce 2000-26 000 revertants/ml undiluted sauce (Wakabayashi *et al*, 1985).

An American group compared the mutagenicity of a Japanese fish product (*sanma hiraki*) with hot dogs and beef after nitrite treatment (no preincubation, TA100, no S-9 mix). Whereas the hot dog and beef were not mutagenic after nitrosation, the Japanese fish produced 600 revertants/g (Marquardt *et al*, 1977).

Finally, an extract of fava beans, a staple food in Latin America and the Mediterranean, was nitrosated under drastic conditions. An activation-independent mutagen was produced that was more potent than MNNG (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) (Piacek-Llanes and Tannenbaum, 1982).

It is interesting that in all of these studies the mutagenicity of the nitrosated food mixtures was *reduced* by the addition of rat liver microsomal enzymes (S-9 mix). This activation-independent mutagenicity shows that the NOC formed are not simple secondary nitrosamines, but rather one of the nitrosamide types or an 'activated nitrosamine', such as an α-hydroxynitrosamine or primary nitrosamine, which do not require enzymatic activation to form an electrophile.

4 Carcinogenicity

The nitrosated Japanese fish product mentioned above was tested for carcinogenicity in rats. The fish was nitrosated under drastic conditions (70 mM nitrite, pH 3, 3 h, 25°C) and the products were extracted into ether, dried and redissolved in water. This extract was given orally three times weekly to rats over a period of six months. The amount of extract given weekly was

calculated to have equal mutagenic activity (by Ames assay) to the weekly oral dose of MNNG necessary to induce adenocarcinomas of the glandular stomach in rats. After a further 18 months, eight of the 12 rats developed tumours in the forestomach and glandular stomach. Thus, the nitrosated compound that was an activation-independent mutagen in the bacterial system was a local carcinogen in rats (Weisburger *et al*, 1980).

5 Other assays and comparison

The most relevant assays (for carcinogenicity and mutagenicity) are also the most complicated. Chemical analysis and simple screening tests for alkylating potential do not provide information on the effects in a biological system but might be valuable in establishing priorities for more detailed further studies. In some cases the NOC derivatives might be too unstable to be isolated, but could be carcinogenic at the site of formation in vivo. Such compounds cannot be detected in tests requiring extensive extraction and workup of the sample. They could be investigated in animal experiments by concomitant administration of the precursors in the diet and of nitrite in the drinking water.

DNA binding studies in vivo have not been discussed above because this method called for the use of a defined radiolabelled precursor (see Section IV. 3). With the recent advances in the analysis of DNA adducts by ^{32}P-postlabelling the investigation of food mixtures will become feasible.

The problem of extrapolation also deserves special mention. Most of the methods require large concentrations of nitrite, about 1000x greater than those occurring in the stomach, in order to produce a measurable effect. Extrapolation is therefore necessary; unfortunately, it is difficult to extrapolate the NOC yields down to gastric conditions without knowing the structures of the precursors. This is because the nitrosation rate is linearly proportional to the nitrite concentration for some precursors (amide group, ureas, guanidines), and proportional to the *square* of the nitrite concentration for others (amino group). Thus, depending on the proportions of each precursor type in a particular food item, the ranking order will be partially inverted under gastric conditions. The reaction order for nitrite could be even higher than two for those precursors which require two reactions with nitrite. Thus, swamping the foods with nitrite could lead to artefactual reaction sequences and to products that would not form in detectable amounts under gastric conditions where food precursors are greatly in excess. Unfortunately, the test systems that allow a rapid analysis of the nitrite concentration dependence are the ones with little direct biological importance. It therefore seems that more than one assay has to be performed to answer the most important questions.

III Nitrosation of individual precursors

1 Alkylamines

i *Secondary amines*

As attention was first given to in vivo nitrosation because of the extremely potent carcinogenicity of the secondary nitrosamines, it was natural that the

environmental secondary amines were first examined as potential precursors for endogenous nitrosation (Lijinsky and Epstein, 1970; Walker, 1981). With this in mind, a survey of the occurrence and amounts of short-chain volatile secondary amines in foods was carried out (Neurath, 1977). In vivo and in vitro nitrosation studies have shown, however, that these simple, basic dialkylamines nitrosate with very low yield (Mirvish, 1975; Meier-Bratschi *et al*, 1983). This coincides with the fact that diethylamine and piperidine could not cause tumours when fed concurrently with nitrite (Sander, 1971). As yet, there have been no comprehensive studies on the occurrence of less basic, more readily nitrosatable amines in foods. Their role as NOC precursors is unknown.

Glycosylamines and Amadori or Heyns compounds, formed from sugars and amino acids during cooking (Maillard browning reaction) have also been examined as possible precursors of secondary nitrosamines. Model *N*-nitrosoglycosylamines have been synthesized and tested for mutagenicity; they were activation-independent mutagens with a potency in some cases equal to ethylnitrosourea (ENU; Pignatelli *et al*, 1987). Nitrosated Amadori compounds have likewise been synthesized. Most were non-mutagenic or only weakly mutagenic in the Ames test (Röper *et al*, 1984). Neither the amounts of glycosylamines and Amadori compounds in cooked foods nor their nitrosation rates are known at present.

Tertiary amines and quaternary ammonium compounds have also been tested for their ability to form dialkylnitrosamines. Nitrosation yields have in all cases been lower (typically 1000x lower) than with the corresponding secondary amines (Fiddler *et al*, 1972; Obiedzinski *et al*, 1980).

ii *Primary amines*

The possibility of the endogenous formation of *primary* nitrosamines or diazo compounds from primary amines has received scant attention. Stomach DNA became methylated following oral administration of methylamine and nitrite to rats, presumably via the formation of the primary nitrosamine (Huber and Lutz, 1984). Knowing that only a very small fraction of the primary amine had been nitrosated under the given experimental conditions the nitrosomethylamine formed must have had a surprisingly high genotoxic potency in the stomach.

2 Aromatic amines

Aromatic amino groups have a much higher rate of nitrosation than alkylamines (Table 1). Aniline, the most simple aromatic amine, formed an activation-independent mutagen when allowed to react with a large excess of nitrite in gastric juice. The measurable effect was weak, however, because of cytotoxicity even at low doses (Boido *et al*, 1980).

3 Amino acids

Interest has also been growing in the possible nitrosation of amino acids, natural amides, peptides and proteins because of their high gastric concentrations (Piacek-Llanes *et al*, 1982; Walters *et al*, 1982; Challis *et al*, 1984;

Table 1. Evaluation of dietary precursor classes for the yield of nitroso compounds (NOC) in the stomach at 10 μM nitrite

Precursor class	*Daily intake (g)*	*Nitrosation rate*[a]	*Relative yield of NOC*[b]
Second order for reaction with nitrite:			
Alkylamines			
Primary	0.1	0.01	0.001
Secondary	0.01	0.01	0.0001
Arylamines	0.001	100	0.1
Amino acids			
Primary	0.1	0.1	0.01
Secondary	0.01	0.1	0.001
Span	**100-fold**	**10 000-fold**	**1000-fold**
First order for reaction with nitrite:			
Amides			
Peptides	100	0.001	10 000
Carnosine	1	0.001	100
Ureas	0.001	10	1000
Guanidines	1	0.001	100
Span	**100 000-fold**	**10 000-fold**	**100-fold**
Total span	**10^5-fold**	**Not applicable**	**10^8-fold**

[a] k_2 at optimal pH for amines, k_4 at pH 2.5 for amide-type compounds (Mirvish, 1975)
[b] Column 1 × column 2 × nitrite conc (10^{-5}M for reaction order 1; 10^{-10} M^2 for reaction order 2) × 10^{10}

Preussman, 1984; Pollock, 1985). The paper by Challis and coworkers in this issue covers the subject so that a summary is appropriate here.

It has been known for a century that the terminal amine of a protein can be easily nitrosated; the reaction is exploited to estimate the molecular weight of a protein (Van Slyke estimation). The importance of this reaction under stomach conditions has generally been considered to be negligible because of the instability of the resulting diazo compound (half-life at acidic pH is estimated at < 1 second; Challis *et al*, 1987a). However, the conversion rate of the nitrosated amino groups to the diazopeptide might be slower and become rate-limiting, and, secondly, several diazopeptides with glycine at the N-terminus have been synthesized and purified. They are strongly mutagenic; two have been identified as carcinogens in rats (Brambilla *et al*, 1979).

When the N-terminus of a protein is PRO, HPRO or SARC, nitrosation leads not to diazotization and decomposition, but to the production of a stable secondary nitrosamine. For this reason, work on amino acids as NOC precursors has mainly concentrated on these three compounds (Lijinksky *et al*, 1970). Kinetics of nitrosation have been determined: the amino acids react more quickly than simple amines (Mirvish, 1972). NPRO is neither mutagenic nor carcinogenic; NSARC is a weak carcinogen (Druckrey *et al*, 1967). In

theory, it is possible that NPRO could be decarboxylated in vivo to form carcinogenic *N*-nitrosopyrrolidine (NPYR): this reaction occurs readily in frying bacon (Thompson, 1978). However, at 37°C, the reaction is so slow as to have negligible yields (10^{-6}% after 4 hours; Shephard, 1987).

While the possibility of forming a primary nitrosamine from amino acids has been dismissed (analogous to the situation with primary amines), the reactions of amino acid *side groups* with nitrite have been examined. Tryptophan, tyrosine, cysteine, creatine and citrulline all form nitroso-derivatives that are stable enough to be purified and characterized. Of particular interest are Trp and Tyr; their side chains nitrosate very rapidly, and the products are known to be mutagenic (Bonnett and Holleyhead, 1974; Bonnett and Nicolaidou, 1977).

In summary, the peptides and amino acids have several possible roles as NOC precursors. The relative importance of each pathway has not been fully clarified.

4 Indoles, β-carbolines, phenols

Indoles, β-carbolines and phenols have been quite recently identified as precursors of mutagens in soya sauce, Chinese cabbage and fava beans (cf Section II.3). As they are discussed at length in another paper in this issue (Wakabayashi *et al*) they are discussed only briefly here. Tyramine (a phenol derivative) and derivatives of β-carboline are found as mutagen precursors in soya sauce (Wakabayashi *et al*, 1984). The product of C-nitrosation of tyramine, 3-diazotyramine, is a local carcinogen (Fujita *et al*, 1987). A diazo phenol is also suspected to be the precursor in *sanma hiraki*, the Japanese fish product mentioned in Sections II.3 & 4 (JH Weisburger, personal communication). Indole derivatives have been isolated from Chinese cabbage and fava beans as mutagen precursors: 4-methoxyindole-3-aldehyde is the major precursor identified to date in Chinese cabbage. In the fava bean, 4-chloro-6-methoxy indole was isolated. Note that the C-nitrosation of tyramine, and the nitrosation of the fava bean indole require two successive reactions with nitrite; this raises the difficulty of extrapolations to gastric conditions.

While the rate of nitrosation was proportional to the square of the nitrite concentration for the precursors discussed above, the precursor classes 5, 6 and 7 (discussed below) have a reaction order of one for nitrous acid and the rate of nitrosation is proportional to the concentration of H_3O^+.

5 Amides, peptides

Reaction at the peptide bond in a protein can produce a nitrosamide; however, peptides other than glycine derivatives are resistant to nitrosation, probably for steric reasons. Reaction of *N*-acetylglycylglycine proceeds about 20x slower than nitrosation of a terminal amino group, but the product is relatively stable at gastric pH; it has a half-life of 2 hours at pH 2, 37°C. Two model nitroso-dipeptides have been synthesized under drastic conditions and tested for mutagenicity in the Ames test (strain TA100, no preincubation). *N*-(*N*-acetylprolyl)-*N*-nitrosoglycine and *N*-(*N*-acetylvalyl)-*N*-nitrosoglycine

were both activation-independent mutagens, about 20–30 times less powerful than methylnitro sourea (Bonnett and Nicolaidou, 1977; Challis *et al*, 1984, 1985, 1987b).

6 Ureas

Sander and Mirvish have long maintained that in vivo nitrosation would only be a problem for rapidly nitrosatable compounds, and have thus emphasized the role of ureas as precursors (Mirvish, 1971, 1972, 1983; Sander, 1971). Methylurea and citrulline nitrosated more rapidly than all other amide-type precursors at pH 2.5 in vitro (Mirvish, 1975). Ureides (ureas containing further functional groups) are found in nature, particularly in plant foodstuffs (Mirvish, 1971), but simple alkylureas have not yet been detected in foods (Kawabata *et al*, 1980).

In vivo nitrosation studies have been performed on several ureides: citrulline, allantoin or hydantoin plus nitrite in the diet of rats were non-toxic and did not induce tumours (in 20–40 animals). Allantoin was also non-mutagenic following nitrite treatment. In contrast, methylurea or ethylurea plus nitrite were extremely toxic and resulted in high tumour incidences (Sander, 1971; Lijinsky, 1984).

It seems, then, that the ureas which have been positively identified in foods are not particularly important precursors, while those which remain *suspect* could pose an appreciable health risk.

In the chemically related class of the carbamates, goitrin, a thiocarbamate occurring in cruciferous vegetables, forms an activation-independent mutagen upon nitrosation. Its potency is similar to that of MNNG (Lüthy *et al*, 1984).

7 Guanidines

The potent gastric carcinogenicity of MNNG, a synthetic nitrosated methylguanidine derivative, led to the consideration of dietary guanidines as potential NOC precursors. The sources of guanidines in the diet were reviewed by Mirvish (1971). Sources and amounts of methylguanidine and agmatine in Japanese foods have also been catalogued (Kawabata *et al*, 1978).

The kinetics and products of methylguanidine nitrosation have been reviewed (Mirvish, 1972). Interestingly, simple nitrosoguanidines have never been isolated. The nitrosation reaction, which is slow, appears to go on to make nitrosocyanamides and nitrosoureas. The presence of thiols in the reaction mixture speeds up the process. The kinetics of nitrosation of other natural guanidines is unknown.

The mutagenicity of some naturally occurring guanidines following nitrite treatment has been compared in several *Salmonella* strains. Note that this type of experiment measures the *sum* of the nitrosation yield + mutagenicity yield of each precursor, rather than the mutagenicity of the purified *N*-nitroso derivative. Methylguanidine was the most potent precursor, agmatine, and guanidinobutyric acid were moderately mutagenic and arginine showed only a weak mutagenicity. In this test, *N*-nitro-*N'*-methylguanidine, the precursor of

MNNG, was as potent a precursor as methylguanidine, and both were more effective than methylurea (Endo *et al*, 1974). On the other hand, methylguanidine and nitrite, given in high doses to rats over two years, did not produce tumours (Sander, 1971). The importance of guanidines as NOC precursors remains uncertain.

IV Comparative studies

1 Model class analysis

The food mixture and individual precursor approaches have provided important information in determining what kinds of compounds can be nitrosated, and what their properties are. However, the diversity of experimental approaches and substances tested makes it somewhat difficult to compare the results of different investigators and assess which precursors are most likely to pose a health risk to humans. The problem is compounded by the fact that the risk associated with the intake of *N*-nitroso-precursors is a function of three variables: (1) the amount of precursor and nitrite ingested, (2) the rate of in vivo nitrosation and (3) the carcinogenic potency of the resulting *N*-nitroso compound. In order to establish priorities for further research in this area, it is necessary to make a systematic analysis of each of the precursor types with respect to each of these variables.

One attempt has been made (Shephard *et al*, 1987a). From a review of the literature, an average daily intake of each precursor was calculated from the amounts found in various foods. It was found that the daily intakes of various *N*-nitroso-precursor classes in a typical European diet spanned five orders of magnitude (Table 1). Amides in the form of protein and guanidines in the form of creatine and creatinine are the nitrosatable groups found most abundantly in the diet, approaching levels of 100 g/day and 1 g/day, respectively. Approximately 100 mg of primary amines and amino acids are consumed daily, whereas arylamines, secondary amines and ureas appear to lie in the 1–10 mg range.

The ease of nitrosation of each precursor was estimated, the reactivities being found to span five orders of magnitude, with ureas and arylamines at the top and guanidines and alkylamines at the bottom of the scale, for the two types of reaction kinetics, respectively. These nitrosation rate constants were used to estimate in vivo nitrosation yields. Assuming a nitrite concentration of 10 μM, the relative yields of nitroso compounds spanned eight orders of magnitude, with nitrosamide formation from peptides and nitrosoureas ranking top, and secondary nitrosamines ranking at the bottom (Table 1).

The third variable that was taken into account was the carcinogenicity of the various NOC. Nitrosoureas and nitrosamines were given high potency values, whereas secondary nitrosamino acids and nitrosamides ranked low. Then, the *combined* effects of these risk variables were analysed using a simple mathematical model:

$$\text{Risk} = [\text{daily intake of precursor}] \times [\text{gastric concentration of nitrite}]^n \times [\text{nitrosatability rate constant}] \times [\text{carcinogenicity of derivative}]$$

(The exponent 'n' equals 2 for amine nitrosation and 1 for amide-type nitrosation.)

The risk estimates for the various dietary components spanned nine orders of magnitude. Dietary ureas and aromatic amines combined with a high nitrite burden could pose as great a risk as the intake of preformed dimethylnitrosamine in the diet. In contrast, the risk posed by the in vivo nitrosation of primary and secondary amines is probably negligible. The risk contribution by amides (including protein), guanidines and primary amino acids is intermediate between these two extremes (Shephard *et al*, 1987a).

Thus, three priorities arising out of this review were the need for a comprehensive study of the sources and levels of arylamines and ureas in the diet (indoles would now also be included), determination of the carcinogenic potencies of key nitrosated products to replace the necessarily vague categories used so far, and the development of short-term in situ tests for studying the nitrosation speed and alkylating power or genotoxicity of *N*-nitroso compounds too unstable for inclusion in long-term tests.

2 Alkylating potential

i *One-step NBP test*

The colorimetric method with the nucleophile 4-(p-nitrobenzyl)pyridine (introduced in Section II.2) was modified to detect unstable alkylating species formed during the nitrosation reaction (see Appendix). In the one-step test system, precursor, nitrite and NBP are mixed together at acidic pH, and the amount of blue NBP derivative is determined after various times. The test is suitable for comparing the overall nitrosation/alkylation speeds of precursors of highly unstable NOC (Shephard *et al*, 1987b).

Reactivities of a series of primary amines, amino acids and dipeptides covered four orders of magnitude! As all compounds were reacting at the primary amine group, such a large reactivity range was quite unexpected. Several factors could be identified as promoting a high rate of NBP-adduct formation: (a) Weak basicity of the precursor. This leads to an increased nitrosation speed, clearly seen in the series alkylamines $<$ amino acids $<$ arylamines. (b) Presence of an aromatic side chain. This may have stabilized the resulting diazonium ion and/or carbonium ion, thus preventing it from reacting so quickly with water and allowing it to be more selective for NBP alkylation. (c) Presence of an ester or amide group alpha to the amine. This removes the negative charge of the carboxyl group of $\alpha-$ = amino acids, but the precise mechanism of rate enhancement is unknown.

The reactivities of the various compound types can be grouped as follows: arylamines $\geq$ N-terminal amino acid of peptides $>$ aromatic amino acids $>$

amines with aromatic side chain > aliphatic amino acids > aliphatic amines.

A surprising result of these tests was the extremely high reactivity of the N-terminal residue of peptides. Until now interest has rested primarily on peptides N-terminal in proline, or on nitrosation of the peptide bond. The one-step test result suggested that peptides with a primary N-terminal amino acid, particularly Trp, Met, Tyr, Phe or His, deserve further study.

ii *Two-step NBP test*

In the two-step test, the nitrosation and alkylation reactions are separated from one another, spatially and temporally, as is the case in the human body. The first reaction, nitrosation, takes place under conditions simulated the stomach lumen—acidic, aqueous. Only precursor and nitrite are present. The aklylation reaction takes place under simulated cellular conditions, at neutral pH in a half aqueous solvent mixture to accommodate NBP. With this protocol it is possible to measure both nitrosation and alkylation speeds as well as the stability of the NO-products at various pH values. It is most suited for precursors with a moderate stability in acid and good reactivity at neutral pH.

A series of 13 ureas, 4 guanidines, the common amino acids, 3 biogenic amines, 9 dipeptides with N-terminal Met, Gly or Ala and aspartame were tested for their ability to alkylate NBP following nitrite treatment (Shephard 1987). As expected, the ureas, stable at acidic pH, showed a different pattern of reactivity than did the precursors of primary NOC. The ureas produced increasing amounts of alkylating agent with increasing nitrosation times, reaching a plateau after ~ 5 min nitrosation in the fastest cases (methylurea), and >60 min in the slowest cases (β-carbamoylalanine). The amines, amino acids and peptides showed a definite peak in the production of alkylating agent, producing the most NBP-derivative after 5 minutes' nitrosation in the case of the aromatic amino acids, amines with aromatic side chain and Met peptides, and after 20–30 min in the case of the aliphatic amino acids, Gly and Ala peptides and aspartame. The guanidines produced no measurable alkylation of NBP.

The compounds with the optimum combination of nitrosation speed and stability/alkylating ability were aspartame, the Gly peptides and the ureido amino acids (β-carbamoylalanine, carbamoylaspartate). These were followed by methionine > the Met peptides and methylurea > ureides (citrulline, carbamoylputrescine) > amines/amino acids with aromatic side chains > alkylamino acids and Ala peptides. Below the limit of detection, at levels 10 000 times and less reactive than aspartame, were the guanidines, alkylamines, allantoic acid and analogues, and cyclizing amino acids (Lys, Glu, Gln).

iii *Summary*

The one-step and two-step NBP systems provided both a qualitative and quantitative picture of the nitrosatability and alkylating power of the various precursors and precursor classes. The tests demonstrated that large variations

in nitrosation rate, half-life of NOC derivative and alkylating ability exist not only between classes but also within each precursor class; this leads in turn to large variations in overall reactivity (up to three orders of magnitude within a precursor class). It has thus become clear that a precursor class cannot be evaluated on the basis of one or two arbitrarily chosen examples.

The results shed new light on the evaluations of the precursors presented in Sections II and III. Firstly, primary nitrosamines may be much more stable than has been generally assumed: electrophilic species accumulate in acidic aqueous solution over 5–15 minutes' reaction time, and NBP adducts are still formed even after a 30–60 minutes' nitrosation incubation. This information justifies the inclusion of the primary amino compounds in the model calculations and supports the assumption that the primary NOC are stable enough to reach DNA in human stomach cells. However, it is necessary to distinguish between distinct primary NOC subgroups: those with an alkyl side chain appear to pose a much smaller risk of nitrosation than those with an aryl side chain. The rapid nitrosation/alkylation by indoles (for example Trp) and Tyr, recognized by Wakabayashi *et al* (1984, 1986) and Yang *et al* (1984) and confirmed in these tests, and the rapid nitrosation on the sulphur atom of Met and Cys suggests that these two amino acid subgroups should be treated as classes in their own right.

Peptides also provided some surprising results: here again, the contribution of the primary amino group appears to have been underestimated. In the model calculations, the peptides were treated as a source of amide groups—a re-evaluation as source of primary NOC is necessary, particularly since these primary nitroso derivatives are so stable (half-life 3 minutes and longer). Major questions involve the length of the peptides in the stomach, the gastric concentration of free N-termini, and the ability of nitrosated peptides to penetrate through the cell wall. The bacterial cell membrane is apparently not a barrier for glycine ethyl ester (GEE; see Section III.3 below).

On the other hand, guanidines reacted more *weakly* than expected. In the risk assessment, the guanidines were assigned high carcinogenicity potency values on the basis of the potent carcinogenicity of MNNG. However, these data strongly suggest that MNNG is not representative of the nitrosated guanidines in general; the alkylating ability of MNNG is probably dramatically increased by the presence of the nitro group.

The ureas were correctly evaluated in the risk assessment; they showed, in general, both rapid nitrosation and efficient alkylating abilities in these tests. The detailed kinetic profiles allowed a more precise ranking of this group.

3 Genotoxic potential

i *Mutagenicity*

Some of the same compounds were further tested in a two-step test with a biological endpoint (Shephard *et al*, 1987b). Following a 30 minute nitrosation period, rapid destruction of residual nitrite and pH change from 2.5 to 7, the mixture was tested in the Ames test with TA100 without metabolic

activation system. The order of reactivity of the precursors tested followed the same pattern as was found with the two-step NPB test. The excellent correlation between the two tests suggested that (a) the alkylation of NBP is a good model for the induction of mutagenicity by NOC in bacterial systems; (b) NBP has nucleophilic characteristics similar to the DNA bases; and (c) neither the cell membrane nor the presence of other cellular nucleophiles dramatically hinders the alkylation of DNA by primary NOC, nitrosoureas or nitrosoguanidines.

ii *In vivo covalent DNA binding*

In vivo covalent binding experiments have been carried out on several key precursor compounds. In these experiments, rats are orally administered tritium- or ^{14}C–labelled precursor and a large nitrite dose in rapid succession, killed after 30 to 60 min and the DNA from the digestive tract organs and liver purified to constant specific activity. Nucleotide analysis to investigate the formation of DNA-test compound adducts can be performed on DNA samples if the specific activity is sufficiently high.

Five radiolabelled precursors were investigated. The experiments with methylamine have been reported in detail (Huber and Lutz, 1984); the work with glycine ethyl ester and dicarbamoylputrescine is from Shephard (1987) while the studies with tyramine and aniline have not been published so far.

The results were very surprising. Yields of NOC formation and DNA binding potency were in the expected range only with the urea compound dicarbamoylputrescine. With methylamine, the DNA-binding potency of nitrosomethylamine was much higher than expected, while glycine ethyl ester, aniline, and tyramine appeared much less potent than predicted on the basis of in vitro assays (Meier *et al*, 1990).

The investigation of DNA adduct formation in animals is biologically highly relevant, but two problems require special attention. Firstly, the oral gavage of precursor and nitrite does not reflect the situation of NOC formation during a meal with a continuous flow both of precursor and nitrite. Secondly, the determination of DNA adducts in a homogenized stomach does not allow for a cell-specific determination of the DNA damage. Future investigations might therefore include histochemical analysis so that the DNA damage can be assessed in that population of (stem) cells which can be considered at risk for neoplastic transformation.

V Gaps in present knowledge; future trends

Table 2 gives a summary of achievements and of the most prominent open questions. It is clearly seen that much is known about the chemical aspects of the nitrosation reaction. It remains to be shown, however, to what extent inhibitors and catalysts of the nitrosation reaction alter the rates of reaction in the gastrointestinal tract. All data obtained in humans should be evaluated in this respect. It will also be very important to define and understand inter- and intraindividual variabilities with respect to the extent of NOC formation.

Table 2. Overview of achievements (+) and current open questions (??) in research on endogenous formation of nitroso compounds (NOC) from dietary precursor classes

Precursor class	*Concentration in stomach*	*Nitrosation rate*	*Reaction order for nitrite*	*Genotoxicity of NOC in vivo*
Second order for reaction with nitrite:				
Alkylamines				
Primary	+	+	+	??
Secondary	+	+	+	+
Weakly basic	??	+	+	+
Maillard products	??	??	+	??
Aromatic amines	??	+	+	??
Heterocyclic amines and phenols[a]	??	??	??	??
Amino acids				
Primary amino group	??	+	+	??
Secondary	??	+	+	+
Side chain reaction[a]	??	??	??	??
First order for reaction with nitrite:				
Amides				
Peptides	?? size?	+	+	??
Carnosine	+	+	+	??
Ureas	??	+	+	+
Guanidines	+	+	+	??

[a] including *N*-, *C*- and *S*-nitrosation, where appropriate; reaction order for nitrite may vary

A very important gap is the assessment of the genotoxicity of chemically unstable NOC. The use of intragastric trapping agents, such as the microcapsules described by O'Neill *et al* (1987), could become a useful tool. Another uncertainty is about the abundance of certain precursor classes in the diet. This is most important for the weakly basic amines, the aromatic compounds and the alkylureas. It is possible that ureas have as yet not been identified in 'nitrite-swamped' food studies because they have only a linear dependence on nitrite concentration.

The indoles, phenols and sulphur-containing amino acids are turning up as important precursors in studies using high nitrite concentrations. However, in both cases where the structure of the (mutagenic) NOC derivative has been elucidated, the precursor underwent two sequential reactions with nitrosating agents to produce the product. It is not clear whether such reaction sequences could also take place under stomach conditions where nitrite concentrations are much lower. For this reason, the dependence of the reaction kinetics on nitrite concentration should be examined for these types of compounds.

Further peptides should be screened in vitro. Those with an optimum of reactivity and stability should be tested in vivo. More basic information is also required on the nature and stability of peptide-DNA adducts.

The above discussion assumes that in vivo nitrosation of at least *some*

precursors is of toxicological importance. However, estimates of risk have all been rather low: averaged over all DNA isolated from an organ, the damage appears to be minute. Two aspects should, however, be kept in mind. Firstly, individuals within a population can exhibit large differences with respect to nitrate and nitrite concentration in saliva, the concentration of catalysts and inhibitors of the nitrosation reaction and the pH in the gastrointestinal tract. Therefore, stomach lesions that lead to small erosions and regenerative cell division might form the basis for a higher level of DNA damage and a subsequently higher probability of tumour formation. Thus biological investigations of the process of stomach cancer formation must complement the research on the endogenous formation of genotoxic nitroso compounds from dietary precursors.

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(The authors are responsible for the accuracy of the references.)

Appendix. Method of NBP assays (from Shephard, 1987)

One-step NPB assay

pH calibration. The following solutions were prepared: (a) 0.2 mmol precursor dissolved in 1 ml 250 mM phosphoric acid, and (b) 0.2 mmol sodium nitrite (puriss, Fluka, Buchs, Switzerland) in 1 ml 250 mM sodium phosphate buffer, pH 2.5. The two solutions were mixed and the pH measured. If the pH did not lie at the desired value of 2.5 ± 0.1, it was corrected by the dropwise addition of 1 M hydrochloric acid or sodium hydroxide, and the required volume recorded. This same volume of acid or base was added to the appropriate incubation before the start of each run.

Incubation. The following three solutions were prepared: (a) 0.2 mmol precursor dissolved in 1 ml 250 mM phosphoric acid; (b) 0.2 mmol sodium nitrite (puriss, Fluka) in 1 ml. 250 mM phosphate buffer, pH 2.5; and (c) 50 mg (0.23 mmol) 4-(p-nitrobenzyl)pyridine (zur Synthese, Merck) dissolved in 0.5 ml acetone. 2.5 ml ethylene glycol was added to the NBP solution.

The precursor solution (a) was added to the NBP solution (c) with stirring. The pH of the solution was corrected, if necessary, at this point. The nitrite solution (b) was added to start the reaction. Final concentrations of the components were precursor 40 mM, nitrite 40 mM, NBP 47 mM. The mixture was incubated at 37°C.

For compounds with extremely high or low reactivity, or with solubility problems, appropriately concentrated or dilute runs were made. Maximum concentrations: precursor 80 mM, nitrite 80 mM, NBP 97 mM. Minimum 5 mM, 40 mM, 47 mM, respectively. The pH was recalibrated to 2.5 ± 0.1 for each change in concentration.

Colour measurement. At intervals of 5–30 min, 0.6 ml reaction mixture was removed and added to 0.2 ml acetone, mixing vigorously with a vortex mixer. 0.2 ml triethylamine (zur Synthese, Merck) was added and again vigorously mixed. Some NBP adducts had a short half-life at pH 10, approximately one minute. Absorption of the mixture at 580 nm was measured exactly 15 and 60 seconds after addition of the base. By calculating the half-life of the unstable blue colour, the absorbance at the moment of base addition was determined.

Two-step NBP assay

pH calibration. Precursor and nitrite were mixed to a final concentration of 40 mM in 250 mM sodium phosphate buffer, pH 2.5, and the resulting pH measured. The pH was calibrated as described in the one-step test.

First incubation. 40 mM precursor and 40 mM nitrite (and 1 M hydrochloric acid, if necessary, to adjust the pH) were incubated together at 37°C in 250 mM sodium phosphate buffer, pH 2.5 ± 0.1. (a) Procedure with ureas and guanidines: a 1 ml aliquot was removed after 10, 30 and 60 minutes, and nitrite destroyed by the addition of 1.1 equivalents amidosulphonic acid (as 1 M solution). (b) Procedure with primary amines and amino acids: a 1 ml aliquot was removed after 2, 5, 10, 15, 20 and 25 minutes. Nitrite was not destroyed. (c) Procedure with peptides and glycine derivatives: a 1 ml aliquot was removed every 5 or 10 minutes until peak of NBP-adduct concentration was past. Nitrite was not destroyed.

Second incubation. A mixture of 50 mg NBP in 0.5 ml acetone, 2 ml ethylene glycol, 0.5 ml 1 M sodium phosphate buffer, pH 8.1, was prepared and added to the aliquot. Final concentration of NBP 58 mM. Maximal concentration of nitroso-derivative 10 mM. pH was neutral. Mixture was incubated at 37°C.

For compounds with extremely high or low reactivity, or with solubility problems, appropriately concentrated or dilute runs were made. Maximum concentrations: precursor 80 mM, nitrite 80 mM, NBP 116 mM. Minimum 0.5 mM, 40 mM, 58 mM, respectively. For the concentrated runs, the pH of the phosphate buffer was roughly adjusted to 2.5 by mixing together 2 ml 250 mM H_3PO_4 and 3 ml 250 mM phosphate buffer, pH 2.5.

Colour measurement. An aliquot of 0.8 ml was removed at the times indicated. 0.2 ml triethylamine was added. The position of the peak maximum, the maximum absorbance and the absorbance at 580 nm were recorded. The solution was pink-violet or blue and had a half-life of 2–5 min. (a) Procedure with ureas and guanidines: aliquots were removed after 10, 30, 60 and 90 minutes. (b & c) Procedure with primary amino compounds: aliquots were removed after 2, 5 and 10 minutes.