Micro-organisms commonly present in human saliva and three DSM strains (Helicobacter pylori, Campylobacter jejuni and Neisseria cinerea), which can be isolated from the human gastro-intestinal tract, were assayed in vitro for their capacity to catalyse N-nitrosation of a series of medicinal drugs and other compounds. Following incubation at pH 7.2 in the presence of nitrate (or nitrite) for up to 24 (48) h, the yield of N-nitroso compounds (NOC) was quantified by HPLC equipped with a post-column derivatization device, allowing the sensitive detection of acid-labile and acid-stable NOC. Eleven out of the 23 test compounds underwent bacteria-catalysed nitrosation by strains, Campylobacter jejuni and Helicobacter pylori, were shown to catalyse nitrosation in the presence of nitrate at pH 7.2. As compared to Neisseria cinerea used as a nitrosation-proficient control strain, H. pylori was 30–100 times less effective, whilst C.jejuni had intermediary activity. The results of our sensitive nitrosation assay further confirm that bacteria isolated from human sources, possessing nitrate reductase and/or nitrosating enzymes such as cytochrome cd$_1$-nitrite reductase (Calmels et al., Carcinogenesis, 17, 533–536, 1996), can contribute to intragastric nitrosamine formation in the anacidic stomach when nitrosatable precursors from exogenous and endogenous sources are present.

Introduction
Chronic bacterial infections are recognized or suspected risk factors associated with several malignancies including cancer of the stomach, urinary bladder and, possibly, uterine cervix. Bacterial formation of carcinogenic nitrosamines in situ is one of the possible mechanisms thought to play an aetiological role in these cancers (1,2). A number of studies have now unequivocally shown that bacteria-mediated N-nitroso compound (NOC*) formation can occur in vivo and in vitro when using animal models and human studies. A relationship between NOC formation catalysed by bacteria, chronic urinary tract infections, e.g., those accompanying schistosomiasis, and an increased risk for squamous cell carcinoma of the bladder (3,4) is now supported by studies using analytical methods and storage conditions that exclude artifactual NOC formation. When European patients with urinary tract infections (5) or those infested with Schistosoma (6) were compared with healthy controls, the levels of N-nitrosodimethylamine, other volatile and non-volatile NOC and nitrite in urine were greatly increased, particularly in subjects with bacteriuria. Several bacterial strains that were isolated from urine of infected patients catalysed the nitrosation of morpholine in vitro (7). Bacterial catalysis of NOC formation in the stomach and urinary bladder has now been demonstrated in vivo in rat models (8). Thus, formation of NOC from nitrite and secondary amines by bacteria isolated from various human sources is enzyme-mediated (7–12). One of the nitrosating enzymes (pH optimum: 7.25) was purified from Helicobacter pylori (14,15). The results indicate that bacteria possessing nitrate reductase and nitrosating enzymes can contribute to NOC formation in vivo, when precursors are available. Nitrosatable precursors are present in normal Western diet (16), but larger amounts of nitrosatable drugs are often taken orally for medication several times a day after meals. Thus, after reaching the stomach these drugs could undergo chemical or bacteria-catalysed nitrosation, depending on (a) the stomach pH, (b) the degree of colonization of the (achlorhydric) stomach by bacteria and (c) the nitrate content of the diet. Nitrosation in vivo could be shown in a healthy human volunteer living on Western diet, whereby upon ingestion of metamizole the unmetabolized nitrosated product N.mucosa, was also assayed. Our results are discussed in relation to long-term bacterial infections, intragastric nitrosamine formation and gastric cancer risk.

Materials and methods

Chemicals
The following N-containing medicinal drugs or compounds (CAS numbers in parentheses; for chemical formulae see Figure 2) were investigated for their nitrosatability in the presence of bacteria: ambroxol (18683–91–5), aminophenazone (58–15–1), benzathine (140–28–3), cimetidine (51481–61–9), diclofenac (15307–86–5), di-isopropylamine (108–18–9), dimethylamine (124–40–3), etambutol (74–55–5), hydrochlorothiazide (58–93–5), metamizole

*Abbreviations: NOC, N-nitroso compounds; HPLC, high performance liquid chromatography; NO, nitric oxide.
in a tightly sealed glass tube of ground glass for 10 min. Thereafter, 

N-(1-naphthyl)ethylenediamine dihydrochloride, 60 ml of acetonitrile/water (30/70 by vol.) and 6 ml 47% hydrobromic acid were made up to 200 ml with distilled water. Activated charcoal was added to the solution which was then kept at 0–4°C in the dark. Before use the reagent was filtered through a glass fibre filter. Acid-labile N-nitrosocompounds were hydrolysed at 95°C in the reaction coil. 

N2O3 released by hydrolysis reacted with sulphanilamide to give the respective diazo compound that coupled with N-

= 540 nm. 

Method B1: analysis of acid-stable N-nitrosocompounds. Aliquots of 100 µl of the dichloromethane extract dissolved in solvent (acetonitrile/water, 30/70 by vol.) were injected and chromatographed on the HPLC (Figure 1) that contained a photoreactor. In the photoreactor the dichloromethane extract was dissolved in 1000 µl solvent (acetonitrile/water, 30/70 by vol.). Aliquots of 100 µl were injected and chromatographed on the HPLC (Figure 1) without photoreactor at a flow rate of 0.5 ml/min. Colorimetric reagent IA (2 g sulphanilic acid and 75 ml glacial acetic acid made up to 150 ml with distilled water) was added and after 10 min, colorimetric reagent II to make up to 5 ml. After an additional 10 min the absorbance was measured at \(\lambda = 540 \text{ nm} \). 

Quantitative determination of N-nitrosocompounds 

Method A: determination of total N-nitrosocompounds. One millilitre of the concentrated dichloromethane extract was mixed with 2 ml of 0.2 M hydrogen bromide in glacial acetic acid (18). After 15 min, 2 ml colorimetric reagent IA (2 g sulphanilic acid and 75 ml glacial acetic acid made up to 150 ml with distilled water) was added and after 10 min, colorimetric reagent II to make up to 5 ml. After an additional 10 min the absorbance was measured at \(\lambda = 540 \text{ nm} \). 

Method B: analysis of acid-labile N-nitrosocompounds. The dry dichloromethane extract was dissolved in 1000 µl solvent (acetonitrile/water, 30/70 by vol.). Aliquots of 100 µl were injected and chromatographed on the HPLC (Figure 1) without photoreactor at a flow rate of 0.5 ml/min. Colorimetric reagent III was added behind the column at a flow rate of 0.1 ml/min. The colorimetric reagent III was prepared as follows: 700 mg sulphanilamide, 100 mg N-(1-naphthyl)ethylenediamine dihydrochloride, 60 ml of acetonitrile/water (30/70 by vol.) and 6 ml 47% hydrobromic acid were made up to 200 ml with distilled water. Activated charcoal was added to the solution which was then kept at 0–4°C in the dark. Before use the reagent was filtered through a glass fibre filter. Acid-labile N-nitrosocompounds were hydrolysed at 95°C in the reaction coil. 

N2O3 released by hydrolysis reacted with sulphanilamide to give the respective diazo compound that coupled with N-

The chemical formulae of the drugs and chemicals tested in the bacterial nitrosation assay are shown in Figure 2. These compounds were selected because all are known from the

\(\lambda = 540 \text{ nm} \). 

The purpose of the Extrelut/ascorbic acid layer is to remove amines from the eluate. 

Extrolut ® 20 column was packed with 2 g of a mixture of equal weight parts of Extrelut/sodium sulphate (anhydrous, finely powered, high-purity grade) and 10–14 g (depending on the volume of the sample) of Extrelut. 

5, 10 or 20 ml of the incubation medium were applied to the packed column and extracted three times with 20 ml dichloromethane each. The combined extracts were concentrated to 1 ml or to dryness under vacuum. The extraction procedure was validated using the polar compounds proline and thioproline as substrates in the nitrosation assay. Artificial nitrosation is inhibited by borate addition to stop nitrosation. The purpose of the Extrelut/ascorbic acid layer is to remove amines from the eluate. 

Quantitative determination of N-nitrosocompounds 

Method A: determination of total N-nitrosocompounds. One millilitre of the concentrated dichloromethane extract was mixed with 2 ml of 0.2 M hydrogen bromide in glacial acetic acid (18). After 15 min, 2 ml colorimetric reagent IA (2 g sulphanilic acid and 75 ml glacial acetic acid made up to 150 ml with distilled water) was added and after 10 min, colorimetric reagent II to make up to 5 ml. After an additional 10 min the absorbance was measured at \(\lambda = 540 \text{ nm} \). 

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Results 

The chemical formulae of the drugs and chemicals tested in the bacterial nitrosation assay are shown in Figure 2. These compounds were selected because all are known from the

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Fig. 2. Drugs and chemicals tested for bacterial nitrosation (presented in the order as listed in Table I); all compounds are known from the literature to be nitrosatable by acidified nitrite. A typical example for the time course of nitrosation by salivary bacteria in the presence of nitrate and 4-(methylamino)antipyrine is shown in Figure 3.

In a typical experiment, bacteria started to proliferate exponentially after a short initial lag phase and then absorbance at \( \lambda = 680 \), a measure of bacterial density increased steadily. The pH only changed from 7.4 to 7.2, but never fell to an acidic range during a 24-h incubation period. Nitrate was reduced to nitrite by bacterial enzymes most actively during the exponential growth phase. After 6 h, a peak nitrite concentration was reached which decreased thereafter. In proportion to a decrease in nitrite concentration, increasing amounts of \( N\)-nitroso-4-(methylamino)antipyrine were formed.

Using this sensitive assay for simulating \textit{in vivo} nitrosation conditions at pH 7.2 (molar ratio of drug:nitrate = 1:4), 11 out of 23 test compounds underwent bacteria-catalysed nitrosation to yield the respective acid-stable or acid-labile \( N\)-nitroso compound (Table I, nos 1–11). Nitrosation rates, i.e. yield of NOC after 24 h of incubation, varied 800-fold with 4-(methylamino)antipyrine being most rapidly nitrosated and \( L\)-thioproline having the lowest rate.

Upon incubation of compounds nos 12–19 (Table I) in the presence of \( NO_3^- \) and salivary bacteria, the micro-organisms proliferated, allowing transitory formation of nitrite. NOC were not formed in any case, although these precursor compounds are shown in Figure 3.

Bacterial strains from the human gastro-intestinal tract, \textit{Helicobacter pylori} and \textit{Campylobacter jejuni} (obtained from Deutsche Sammlung von Mikroorganismen, Braunschweig, Germany) were compared to \textit{Neisseria cinerea} (DSM 4630). Neisseria strains present in human saliva were previously shown to possess nitrosating activity (13). Nitrosation assays were carried out according to procedure B (Materials and methods), using a molar ratio of nitrite:metamizole = 1:1 and 48 h of incubation under micro-aerobic conditions. Taking the absorbance of the incubation media at \( \lambda = 720 \) nm (1-cm cuvette) as an indicator of bacterial proliferation, the
same value of 0.800 was recorded in all the experiments involving the above strains. The pH values always ranged between 7.2 and 7.8. All three strains yielded detectable amounts of N-nitroso-4-(methylamino)antipyrine, whereby the yield increased as a function of precursor concentration (Figure 4).

Under our assay conditions the yield of NOC was highest in the presence of Neisseria cinerea, followed by Campylobacter jejuni (strain DSM 4688), but was 30–100 times lower with Helicobacter pylori (strain DSM 4867). The weak nitrosation activity of this Helicobacter pylori strain was confirmed in a series of 72-h incubation assays with different amounts of nitrite, using metamizole, a rapidly nitrosatable precursor. The yield of N-nitroso-4-(methylamino)antipyrine was up to 200 times higher than in the control assays where bacteria had been omitted (Figure 5), confirming that bacteria-catalysed nitrosation reaction has occurred under these conditions.

Discussion

The composition of the microflora present in the oral cavity shows great inter-individual variations but seems to be constant in a human subject over a longer time period (19). Using a sensitive in vitro assay for the nitrosation capacity of bacteria (pH 7.2) in the presence of nitrate or nitrite, HPLC quantification combined with a post-column derivatization method allowed the detection of acid-labile or acid-stable NOC generated. Our results (Table I) confirm that bacteria from human saliva (collected repeatedly from the same volunteer) contain nitrate- and nitrite-reducing species, and can catalyse the formation of NOC from a series of drugs or compounds that contain a nitrosatable amino group. Among the 23 test compounds, 11 underwent bacteria-catalysed nitrosation by salivary bacteria. However, under standard assay conditions the yield of the respective N-nitrosated product varied by two orders of magnitude. 4-(Methylamino)antipyrine exhibited the highest nitrosation rate and l-thioproline the lowest. Thirteen drugs including the H2-blocker cimetidine and nicotine did not yield any NOC under our assay conditions. As all test compounds are known from the literature to be nitrosated by acidified nitrite, our data revealed that the yield of nitrosated products by bacterial catalysis does not parallel that of the chemical nitrosation. Previous investigations on the bacteria-catalysed formation of NOC revealed that the nitrosation activity in non-denitrifying bacterial strains like E.coli was associated with nitrate reductase, while in denitrifying bacteria such as Neisseria mucosa and Pseudomonas aeruginosa (isolated from gastric juice of achlorhydric patients) nitrosation capacity was linked to nitrite reductase (20). Recently, the identification of cytochrome cd1-nitrite reductase (14) as one of the enzymes responsible for catalysis of nitrosation has been reported (15). The production of nitric oxide (NO) or [NO]+-like species from nitrate has been proposed which were also generated from non-denitrifying enteric bacteria like E.coli or Proteus morganii. This confirms earlier observations on the reduction of nitrite to nitric oxide (NO) by enteric bacteria (21). Under aerobic conditions NO is oxidized to form...
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Fig. 4. Nitrosation of metamizole catalysed by the bacterial strains that can be isolated from the gastro-intestinal tract. The incubation time was 48 h; the yield of N-nitroso-4-(methylamino)antipyrine in 50 ml is plotted.

NO₂ that exists in equilibrium with potent nitrosating agents N₂O₃ and N₂O₄, which react at neutral pH with (secondary) amines to form N-nitroso compounds. It is very likely that NO or [NO]⁻–like species are also produced under our assay conditions from various bacterial strains present in human saliva. Here, the situation is more complex in that a mixture of nitrate-reducing strains devoid of nitrosation activity together with strains that have both enzymatic properties may coexist. This may account for the observed differences; concerning the nitrosation reaction and substrate specificity. E.coli strains nitrosated secondary amines with a rate that was inversely related to their pKₐ value; morpholine and L-thioproline were readily nitrosated and dimethylamine less fast (10). Although the incubation conditions in our assay with salivary bacteria were not identical, dimethylamine was not found to be nitrosated, while the nitrosation yield for thioproline was 800 times lower than for the fastest nitrosatable precursor 4-(methylamino)antipyrine (Table I).

The bacterially catalysed nitrosation of drugs that we have demonstrated to occur in vitro yielding in some instances carcinogenic nitroso compounds (reviewed in 22), may probably not occur in substantial amounts in healthy subjects. Even when saliva is swallowed, bacteria are immediately killed by hydrochloric acid in the stomach. However, in patients with chronic gastric achlorhydria causing bacteria colonization, nitrosation of precursors arising from the diet, of amines produced by physiological metabolism or of ingested drugs could be substantial. Indeed, recent results confirm that both acid-catalysed and bacteria-catalysed N-nitrosation reactions occur in the human stomach (23). Studies including several hundred untreated patients categorized by diagnosis to be at greater risk of developing gastric cancer, including patients with chronic atrophic gastritis, partial gastrectomy and pernicious anaemia, showed significantly higher nitrite and total nitroso compound concentrations in the gastric juice. As in these patients the second peak concentration of nitroso compounds was found at pH 7 of the gastric juice, it strongly suggests that bacteria-catalysed nitrosation is the origin of these nitrosated products. As found earlier, 25 out of 38 concerning the nitrosation reaction and substrate specificity.

As in these patients the second peak concentration of nitroso compounds was found at pH 7 of the gastric juice, it strongly suggests that bacteria-catalysed nitrosation is the origin of these nitrosated products. As found earlier, 25 out of 38 organisms isolated from diverse human sources including gastric juice from achlorhydric patients and subjects with urinary tract infections were able to catalyse nitrosation in vitro at pH 7.2, including most of the E.coli strains as well as other species (7).

Taking advantage of the sensitivity of our in vitro nitrosation assay using metamizole as a most readily nitrosatable substrate, we investigated the nitrosation catalysis by strains that can be isolated from the human gastro-intestinal tract, i.e. Campylobacter jejuni and Helicobacter pylori. Both strains in the presence of nitrite were compared for their nitrosation capacity with Neisseria cinerea, a species known to contain a high nitrosation capacity—likely due to the presence of cytochrome c₁-nitrite reductase—as previously shown for Neisseria mucosa (15). When comparing these three strains, Campylobacter jejuni showed intermediate nitrosation capacity, while the Helicobacter strain DSM 4867 gave only 30–100 times lower nitrosation yield as found for Neisseria. Because of the very similar incubation conditions
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Fig. 5. Nitrosation of metamizole by Helicobacter pylori DSM strain.

Table I. Yield of nitrosated products when drugs and compounds were incubated in the presence of human salivary bacteria and nitrate at pH 7.2 for 24 h; total acid-labile and acid-stable NOC were determined as described in ‘Materials and methods’ by procedures A, B or B1, respectively.

<table>
<thead>
<tr>
<th>No.</th>
<th>Nitrosatable drug/compound</th>
<th>µmol (N-nitroso compounds per assay)</th>
<th>Method used</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>4-(Methylamino)antipyrine</td>
<td>39.80</td>
<td>B</td>
</tr>
<tr>
<td>2</td>
<td>Diclofenac</td>
<td>12.12</td>
<td>B1</td>
</tr>
<tr>
<td>3</td>
<td>Metamizole</td>
<td>6.12</td>
<td>B</td>
</tr>
<tr>
<td>4</td>
<td>Piperazine</td>
<td>3.22</td>
<td>A</td>
</tr>
<tr>
<td>5</td>
<td>Etambutol</td>
<td>2.57</td>
<td>A</td>
</tr>
<tr>
<td>6</td>
<td>Phenmetrazine</td>
<td>1.30</td>
<td>B1</td>
</tr>
<tr>
<td>7</td>
<td>N’-Methylsalicyline</td>
<td>1.19</td>
<td>A</td>
</tr>
<tr>
<td>8</td>
<td>Methylaminoacetanilide</td>
<td>0.66</td>
<td>A</td>
</tr>
<tr>
<td>9</td>
<td>Morphinol</td>
<td>0.20</td>
<td>B1</td>
</tr>
<tr>
<td>10</td>
<td>L-proline</td>
<td>0.08</td>
<td>A</td>
</tr>
<tr>
<td>11</td>
<td>L-thioproline</td>
<td>0.05</td>
<td>A</td>
</tr>
<tr>
<td>12</td>
<td>Aminophenazone</td>
<td>ND</td>
<td>A</td>
</tr>
<tr>
<td>13</td>
<td>Benzathine</td>
<td>ND</td>
<td>A</td>
</tr>
<tr>
<td>14</td>
<td>Cimetidine</td>
<td>ND</td>
<td>A</td>
</tr>
<tr>
<td>15</td>
<td>Disopropylamine</td>
<td>ND</td>
<td>A</td>
</tr>
<tr>
<td>16</td>
<td>Dimethylamine</td>
<td>ND</td>
<td>A</td>
</tr>
<tr>
<td>17</td>
<td>Nicotine</td>
<td>ND</td>
<td>A</td>
</tr>
<tr>
<td>18</td>
<td>Propranolol</td>
<td>ND</td>
<td>B1</td>
</tr>
<tr>
<td>19</td>
<td>Pyrrole-2-carboxylic acid</td>
<td>ND</td>
<td>A</td>
</tr>
<tr>
<td>20</td>
<td>Sarcosine</td>
<td>ND</td>
<td>A</td>
</tr>
<tr>
<td>21</td>
<td>Ambroxol</td>
<td>ND</td>
<td>B1</td>
</tr>
<tr>
<td>22</td>
<td>Hydrochlorothiazide</td>
<td>ND</td>
<td>A</td>
</tr>
<tr>
<td>23</td>
<td>Trimepramine</td>
<td>ND</td>
<td>A</td>
</tr>
</tbody>
</table>

ND = not detected (detection limit: 0.01 µmol NOC/assay).

for all three strains, the difference in yield of N-nitroso-4-(nitrosamino)antipyrine must be attributed to their different nitrosation capacity. As for logistic reasons only this one strain H.pylori was assayed, more research is needed to investigate whether different H.pylori strains vary in their nitrosation capacity. A recent report indicates that intact Helicobacter pylori cells can stimulate the murine macrophage system by the t-arginine/nitric oxide (NO) pathway (24). As a consequence chronic H.pylori infection of the human stomach may increase the endogenous formation of NO, yielding after oxidation the nitrosating agents N2O3 and N2O4 that could produce nitrosamines or cause other types of DNA damage (25,26). Thus, elevated NO formation via the nitric oxide synthase pathway in macrophages together with the generation of NO or [NO]+-like compounds enzymatically derived from colonized bacteria (14, 15) may be contributing aetologic factors explaining the association between bacterial colonization, long-term H.pylori infection and gastric cancer (27–29).

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References

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