Degradation of N-Nitrosamines by Intestinal Bacteria

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A major proportion of bacterial types, common in the gastrointestinal tract of many animals and man, were active in degrading diphenylnitrosamine and dimethylnitrosamine, the former being degraded more rapidly than the latter. At low nitrosamine concentrations (<0.05 μmol/ml), approximately 55% of added diphenylnitrosamine, 30% of N-nitrosopyrrolidine, and 4% of dimethylnitrosamine were degraded. The route of nitrosamine metabolism by bacteria appears to be different from that proposed for breakdown by mammalian enzyme systems in that carbon dioxide and formate were not produced. In bacteria, the nitrosamines were converted to the parent amine and nitrite ion and, in addition, certain unidentified volatile metabolites were produced from dimethylnitrosamine by bacteria. The importance of bacteria in reducing the potential hazard to man of nitrosamines is discussed.

Attempts to assess the hazard to human health from nitrosamines have led to the identification of several potential sources of exposure of man to this group of potent carcinogens (18). Certain foods intended for human consumption have been shown to contain nitrosamines (7, 23, 25, 28) which were thought to be derived from the interaction of nitrite with secondary amines in the food, either spontaneously or by the agency of bacteria (6, 13).

Recently the possibility that nitrosamines may be formed from secondary amines and nitrite in the gastrointestinal tract has been explored. The acid conditions which prevail in the human stomach favor the nitrosation of dimethylamine (DMA; 19), and the formation of dimethylnitrosamine (DMN) and other nitrosamines has been demonstrated in human and animal gastric juices (1, 22, 24). In addition, at neutral pH, many secondary amines can be nitrosated by nitrite or nitrate in the presence of intestinal bacteria, or the cecal contents of the rat (14, 17, 21), and soluble enzymes which catalyze the N-nitrosation of several secondary amines have been extracted from two microorganisms (3). The rate of nitrosamine formation depends greatly on the basicity of the amine; the less basic amines such as diphenylamine and pyrrolidine are nitrosated far more readily than the strongly basic DMA and diethylamine (14, 21).

In contrast to the extensive information on bacterial synthesis of nitrosamines, data on their breakdown by bacteria are scarce. In the present paper, an attempt has been made to determine which of the bacteria commonly found in the alimentary tracts of man and animals are capable of degrading diphenylnitrosamine (DPN) and DMN, and the importance of these bacteria in reducing nitrosamine levels in man is discussed.

MATERIALS AND METHODS

Chemicals. The Eastman Kodak Co., New York, supplied DPN, DMN and N-nitrosopyrrolidine, and Nuclear Enterprises Ltd., Edinburgh, Scotland, supplied 2,5-diphenyloxazole and N-bis[2-(5-phenyloxazoyl)]benzene. [14C]dimethylamine hydrochloride was purchased from the Radiochemical Centre, Amersham, England, and converted to the nitroso derivative by the method of Dutton and Heath (12). All bacteriological media were purchased from Oxoid Ltd., London, England, and Silica Gel G was bought from Anderman & Co., East Molesey, England. All other chemicals were purchased from BDH, Poole, Dorset.

Bacteria. Klebsiella aerogenes (NCTC 9644) and Pseudomonas aeruginosa (NCTC 6750) were obtained from the National Collection of Type Cultures, London. All other bacteria were isolated from the intestinal tract of male Wistar rats (20).

Preparation of bacterial suspensions. P. aeruginosa, K. aerogenes, and strains of Proteus, Escherichia coli, and Streptococcus faecalis were grown to mid-exponential phase in nutrient broth no. 2, centrifuged at 10,000 × g for 10 min at 4 C, and then suspended in 0.2 M potassium phosphate buffer (pH 7.0) at a final cell density of approximately 2 × 10^9 viable cells per ml. Suspensions of Lactobacillus strains in the same buffer were derived from mid-exponential phase cultures in MRS broth. Strains of bacteroides and bifidobacteria were grown in reinforced clostridial medium for 2 to 3 days under an atmosphere of 90% H2-10% CO2 in an anaerobic jar (Gaspak, Becton-Dickinson Ltd., Wembley, England).
Degradation of DPN. The bacterial suspensions (5 ml) were shaken at 37 C for 20 h in 50-ml Erlenmeyer flasks containing 0.1 ml of DPN (prepared as a solution of 125 μmol/ml in dimethylsulfoxide). The incubations were performed either aerobically, anaerobically (oxygen-free nitrogen), or, in the case of lactobacilli, under carbon dioxide. In early experiments, the flasks were covered with aluminium foil to minimize photolysis of the nitrosamines, but it was subsequently found that photolytic breakdown was negligible during the incubation times used. In some experiments, the incubation mixture was supplemented with d-glucose (1% wt/vol).

Nitrite released by breakdown of DPN was assayed colorimetrically (2) and compared to the amount of nitrite released by incubation mixtures lacking DPN and mixtures containing heat-killed organisms in place of the viable cell suspensions.

To test the possibility that an inducible enzyme might be involved in DPN breakdown, strains of E. coli were incubated in nutrient broth containing DPN (final concentration, 2.5 μmol/ml) for 44 h, during which time the bacteria were subcultured five times into fresh nitrosamine-containing broth (final DPN concentration, 2.5 μmol/ml). When the final subculture had reached the mid-exponential phase of growth, suspensions in buffer were prepared, and the DPN-degrading ability of the cells was assessed as described above.

Degradation of DMN. The ability of bacteria to degrade DMN was investigated by four methods.

(i) The release of nitrite ion from DMN at various concentrations was measured by using a method essentially the same as that described above for DPN breakdown.

(ii) The formation of [14C]DMA from [14C]DMN was measured. The bacterial suspensions (2 ml) were incubated with shaking at 37 C, under oxygen-free nitrogen, with [14C]DMN (0.01 μCi/μmol; final concentration, 10 μmol/ml) in screw-cap bottles covered with foil. The DMN was prepared as an aqueous solution of 200 μmol/ml. At the end of the incubation period (20 h), saturated barium hydroxide solution (0.1 ml) and 20% (wt/vol) zinc sulfate solution (0.1 ml) were added to precipitate protein. After centrifuging the mixtures (3,000 × g for 15 min), 0.1 ml of each supernatant was applied to plates of Silica Gel G (0.25-mm thickness), and DMN and DMA were separated by benzene (95% [vol/vol] aqueous ethanol [90:10] as solvent) (17). The zones corresponding to the nitrosamine and secondary amine were scraped off into vials containing 10 ml of the scintillant PPQ (5 g/liter)-POPOP (0.3 g/liter) in toluene with 4% Cab-o-sil (Koch-Light Laboratories Ltd., Colnbrook, England), and radioactivity was counted with a Nuclear-Chicago Mark I scintillation counter.

In some experiments, the bacterial suspension in buffer was replaced by nutrient broth which was inoculated with bacteria and incubated at 37 C for 20 h. Extraction of DMN and DMA was performed as described above.

(iii) The conversion of [14C]DMN to [14C]formate was measured. Bacterial suspensions were incubated with [14C]DMN as described above. A 0.1-ml amount of the supernatant, after precipitation with barium hydroxide and zinc sulfate, was applied to a column (8 by 0.5 cm) of Dowex-2-formate resin, and the column was eluted with 0.4 M formic acid to separate DMN from formate (27). The radioactivity in each of the 10 1-ml fractions was counted with a scintillant of PPO (5 g/liter)-POPOP (0.3 g/liter) in a mixture of toluene and ethoxyethanol (1:1).

(iv) The conversion of [14C]DMN to radioactive metabolites soluble in ethanolamine was measured. The bacterial suspension was incubated with [14C]DMN under anaerobic conditions as described above, except that the screw-cap bottle was replaced by a 50-ml Erlenmeyer flask fitted with a Suba-Seal stopper and containing a small tube of ethanolamine (0.5 ml). At the end of the 20-h incubation, the amount of radioactivity in the flask and in the ethanolamine was counted and compared with that in controls containing heat-killed bacteria in place of the suspension of viable bacteria. In some experiments, barium chloride was added to the ethanolamine to precipitate any absorbed carbon dioxide. The precipitate was collected by centrifugation and washed three times with water before being dried in air at 50 C and counted for radioactivity with the scintillant POPOP in toluene with 4% Cab-o-sil, as described above.

In some experiments, the bacterial suspension was replaced by a cell-free extract prepared by disintegration of the cell suspension with an MSE 150-W ultrasonic disintegrator and by centrifugation of the resulting crude extract at 10,000 × g for 10 min at 4 C to remove cell debris.

Degradation of N-nitrosopyrrolidine. The breakdown of N-nitrosopyrrolidine was determined by measuring the release of nitrite ion at various nitrosamine concentrations by a method identical to that used to study DPN breakdown.

RESULTS

Five main groups of bacteria, isolated from the gastrointestinal tract of the rat, were investigated for their ability to degrade DPN and DMN. The proportion of strains capable of degrading the nitrosamines in each of the five groups is shown in Table 1. The experiments were performed at pH 7.0 (which was found to be the pH optimum for the reaction) and in the absence of glucose, since strains of lactobacilli and E. coli degraded DPN at similar rates in the presence or absence of the sugar (Table 2). To simulate conditions in the alimentary tract, experiments were conducted under anaerobic conditions. However, for E. coli strains and Proteus sp., the release of nitrite from DPN was measured aerobically, since these organisms possessed nitrate reductase which was active only under anaerobic conditions. Other types of bacteria tested (lactobacilli, streptococci, and
TABLE 1. Degradation of DPN and DMN by intestinal bacteria

| Bacterium* | No. tested | No. positive | Rangea | DMN breakdown |
|------------|------------|--------------|---------|---------------|
|            |            |              |         | To DMA       | To formate   | To volatile metabolite |
|            |            |              |         | No. tested   | No. positive | Rangeb       | No. tested   | No. positive | Rangeb       | No. tested   | No. positive | Rangeb       |
| E. coli    | 25         | 19           | 0.08–0.20 | 8            | 3            | 0.04–0.09   | 4            | 0           | 8            | 0.10–0.56    |
| Bacteroides| 7          | 2            | 0.05–0.15 | 5            | 4            | 0.03–0.06   | 4            | 0           | 4            | 0.35         |
| Bifidobacterium | 6       | 2            | 0.04–0.09 | 9            | 6            | 0.01–0.04   | 2            | 0           | 2            | 0            |
| Lactobacillus | 15      | 11           | 0.12–0.35 | 11           | 7            | 0.02–0.09   | 8            | 0           | 5            | 0.82–1.75    |
| S. faecalis | 14         | 13           | 0.06–0.25 | 10           | 0            | 5           | 0           | 5           | 1            | 0.20         |

* Bacteria (2 × 10⁸ viable cells/ml) and nitrosamine were incubated for 20 h at 37 C in 0.2 M potassium phosphate buffer, pH 7.0.

bacterial suspensions in 0.2 M buffer (pH 7.0) were incubated for 20 h at 37 C in the presence of 2.5 µmol of DPN per ml.

The results of two independent experiments are shown.

a One percent (wt/vol).

b D. E. coli was incubated in nutrient broth containing 2.5 µmol of DPN per ml for 44 h, during which time the bacteria was subcultured five times into fresh DPN-containing broth. A bacterial suspension in buffer was prepared from the final subculture and incubated with DPN as described above.

bifidobacteria) did not appear to possess the reductase under anaerobic or aerobic conditions.

Although many strains of bacteria could release nitrite from DPN, the rate of breakdown varied considerably; for example, bacteroides and bifidobacteria strains released 0.04 to 0.15 µmol of nitrite ion per ml of incubation mixture per 20 h from 2.5 µmol of DPN per ml, whereas many strains of lactobacilli released approximately 0.35 µmol of nitrite ion per ml per 20 h at the same initial concentration of DPN. Several pathogenic or potentially pathogenic organisms, K. aerogenes, Proteus sp., and, to a lesser extent, P. aeruginosa, were also active in degrading DPN (Table 3).

Suspensions of E. coli strains derived from cultures grown in nutrient broth containing DPN (final concentration, 25 µmol/ml) degraded the nitrosamine at similar rates to control cultures having no prior exposure to DPN (Table 2), which suggests that an inducible enzyme is not involved in DPN breakdown.

The rate of breakdown of DMN to DMA by bacteria was considerably slower than the rate of DPN breakdown (Table 1), and although over 90% of S. faecalis strains degraded DMN alone were able to convert DMN to DMA. Cultures of E. coli strains growing in nutrient broth were unable to form DMA from DMN (I. Rowland, unpublished data), though this may reflect the smaller number of bacteria in the growing cultures compared with that in the bacterial suspensions used in other experiments.

None of the bacteria tested could degrade [¹⁴C]DMN to [¹⁴C]formate (Table 1). However, in the presence of many bacterial strains, DMN appeared to be converted to a volatile compound or compounds, since the amount of radioactivity absorbed by a tube of ethanolamine was greater when [¹⁴C]DMN was incubated with suspensions of viable bacteria than when the suspensions were replaced by heat-killed cells or buffer (Table 1). Strains of lactobacilli and E. coli were most active in this respect, some being capable of metabolizing 1.75% of added radioactivity in 20 h. Few of the strains of bacteroides, bifidobacteria, or S.
faecalis appeared to form the volatile compounds from DMN, but *P. aeruginosa* and *K. aerogenes* were able to metabolize DMN to a certain extent by this route (Table 3).  

The identity of the volatile metabolite or metabolites remains obscure. Precipitation with barium chloride of the carbon dioxide absorbed by the ethanolamine in such experiments revealed that less than 0.04% of the added radioactivity could be accounted for as [14C]CO₂.

The ability of a cell-free extract of an *E. coli* strain to degrade DMN was investigated, but no increase in the rate of breakdown (as measured by [14C]DMA formation and by estimation of ethanolamine-soluble metabolites) as compared with the rate in the presence of a suspension of intact cells of the same bacterium was found (I. Rowland, unpublished data).

At low DPN concentrations (less than 1 μmol/ml), the percentage of added nitrosamine degraded increases (Fig. 1 and 2). For a strain of *Lactobacillus*, the maximal rate of nitrosamine breakdown (0.1 μmol/ml per 20 h) was reached at an initial DPN concentration of 0.5 μmol/ml, so that approximately 20% of added nitrosamine was metabolized at this concentration (Fig. 1). For *E. coli*, although the maximal rate of DPN breakdown was slightly lower than that in the presence of *Lactobacillus* sp., this maximal rate was achieved at a lower initial DPN concentration (0.12 μmol/ml); hence the percentage breakdown of DPN was as high as 50% at low concentrations (Fig. 2).

The rates of release of nitrite from DMN and N-nitrosopyrrolidine at low nitrosamine concentrations were slower than from DPN. For example, at a nitrosamine concentration of 0.05 μmol/ml, 55% of added DPN was degraded, whereas only 8 and 5% of added *N*-nitrosopyrrolidine and DMN, respectively, were released as nitrite.

**DISCUSSION**

The ability to degrade DPN and DMN appeared to be a common property of bacteria in most of the bacterial types tested, although the rate of nitrosamine breakdown varied considerably both within and between the groups. Strains of lactobacilli and *E. coli* were the most active in degrading nitrosamines; rates of breakdown of up to 0.35 μmol of DPN per ml per 20 h and 0.09 μmol of DMN per ml per 20 h were recorded. Several strains of bifidobacteria and bacteroides, which constitute more than 99% of

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**Table 3. Degradation of DPN and DMN by bacteria**

| Strain        | Nitrite released from DPN (μmol/ml per 20 h) | DMA formed from DMN (μmol/ml per 20 h) | 14C absorbed by ethanolamine (% of added [14C]DMN) |
|---------------|---------------------------------------------|----------------------------------------|---------------------------------------------------|
| *Proteus sp.* | 0.22                                        | 0.0                                    | NT                                                |
| *P. aeruginosa* | 0.07                                       | 0.0                                    | 0.70, 0.86                                        |
| NCTC 6750     | 0.21                                        | 0.0                                    | 0.63, 0.47                                        |
| *K. aerogenes* | 0.07                                       | 0.0                                    | 0.70, 0.86                                        |
| NCTC 9644     | 0.21                                        | 0.0                                    | 0.63, 0.47                                        |

* Bacteria (2 × 10⁸ viable cells/ml) and nitrosamine were incubated for 20 h at 37 °C in 0.2 M potassium phosphate buffer, pH 7.0.
* Initial DPN concentration, 2.5 μmol/ml.
* Initial DMN concentration, 10.0 μmol/ml.
* After correction for absorption of [14C]DMN by ethanolamine.
* NT, Not tested.

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**FIG. 1. Extent of DPN breakdown at various concentrations by Lactobacillus sp.** The bacteria (2 × 10⁸ viable cells/ml) and nitrosamine were incubated at 37 °C for 20 h under CO₂, and the amount of nitrite ion released was estimated as described in the text.

**FIG. 2. Extent of nitrosamine breakdown at various concentrations by *E. coli*.** For further details see Fig. 1. Symbols: ⌠, DPN; ▲, DMN; and ○, *N*-nitrosopyrrolidine.
the total number of bacteria in the alimentary tracts of man and many animals (8, 10), were able to degrade DPN and DMN, but the rates of breakdown recorded were considerably lower than those of other types of bacteria tested.

The rate of degradation of DPN by a bacterium was often more than five times that of DMN. This difference correlates with the relative rates of formation of the two nitrosamines, diphenylamine being far more readily nitrated than dimethyamine (14, 22). It seems unlikely that the lower rate for DMN breakdown was due to a slow rate of entry of DMN to the cell, since a cell suspension and a cell-free extract of an E. coli strain metabolized DMN at similar rates. The observation that several of the bacteria tested could degrade DPN, yet were incapable of degrading DMN, suggests that the two processes may be mediated by different mechanisms.

The route of DMN metabolism in bacteria would appear to be different from that proposed for its metabolism by mammalian enzyme systems, where [14C]formaldehyde and [14C]CO₂ have been isolated after [14C]DMN metabolism by liver extracts (5, 16). In bacteria both DPN and DMN appear to be converted to the parent amine and nitrite. [14C]Formate is not formed from [14C]DMN, although a volatile, labeled metabolite would appear to be produced. Although the metabolite was not identified, it is unlikely that it was carbon dioxide since only a very small portion of the radioactivity absorbed by ethanolamine could be precipitated as barium carbonate, and, in addition, the anaerobic conditions under which the incubations were performed and which prevail in the gastrointestinal tract probably preclude the oxidation of DMN to carbon dioxide.

The nitrosamines so far discovered in food — DMN, diethylnitrosamine, N-nitrosopyrrolidine, and N-nitrosopiperidine — are present in concentrations up to 10 μg/kg; consequently only minute quantities of these nitrosamines are likely to be ingested (7). In situations where such low nitrosamine concentrations and intestinal bacteria are present together, a substantial percentage of the nitrosamine could be degraded, for example, at 0.01 μmol/ml (approximately 1 μg/ml), 33% of added N-nitrosopyrrolidine and over 55% of DPN were degraded by E. coli and 17% of added DPN was degraded by Lactobacillus sp. in 20 h.

In the normal human gastrointestinal tract, it is unlikely that the bacterial flora will come into contact with ingested nitrosamines, since studies in rats have demonstrated that DMN is absorbed mainly from the upper part of the small intestine and partly from the stomach (15); in man it is precisely these regions that are very sparsely populated with bacteria for most of the time (8, 10). However, several abnormal conditions exist (e.g., achlorhydria, blind loop syndrome, Crohn's disease) where the stomach and duodenum harbor large numbers of bacteria, including E. coli, bacteroides, and lactobacilli (4, 9, 10, 26). Clearly, under conditions of bacterial colonization of the stomach and duodenum, the bacteria present may reduce to some extent the concentration of any nitrosamine ingested with the food and any nitrosamines formed in the stomach due to bacterial action on ingested secondary amines and nitrite.

Hawksworth and Hill (14) have identified the bladder as the most probable site of bacterial nitrosamine synthesis in man, since both nitrite and secondary amines (DMA, pyrrolidine, and piperidine) are present in the urine and, during urinary tract infections (which are quite common in women), bacteria are present in large numbers. However, under such conditions bacterial degradation of nitrosamines is feasible.

The most common bacterial type associated with urinary tract infection is E. coli, and many strains of this organism tested in the present investigation were active in the degradation of both DPN and DMN; in addition, E. coli released nitrite from N-nitrosopyrrolidine. K. aerogenes, P. aeruginosa, and Proteus are also associated with some urinary tract infections, and, although these organisms could not convert DMN to DMA, they were apparently capable of metabolizing to a certain extent DMN by other routes and were active in degrading DPN (Table 3). Thus, under conditions where bacterial synthesis of nitrosamines is possible, it is feasible that net synthesis of nitrosamines may be reduced by the degradative action of bacterial enzymes.

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