In Vitro Production of N-Nitrosodimethylamine and Other Amines by *Proteus* Species

L. THACKER AND J. B. BROOKS

*Center for Disease Control, Atlanta, Georgia 30333*

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*N*-nitrosodimethylamine, a potent carcinogen, was produced by three strains each of *Proteus mirabilis*, *P. morganii*, and *P. rettgeri*, but not by three strains of *P. vulgaris* grown under the same conditions. Many of the alkaline-extractable volatile metabolites elaborated by these organisms are the same, but there are some qualitative and quantitative differences among species. Representative gas-liquid chromatographic profiles of the four species are presented, and the significance of the differences is discussed. Primary emphasis, however, is given to the importance of the production of *N*-nitrosodimethylamine by these microorganisms and the conditions under which it is produced.

Members of several genera of bacteria have the ability to catalyze the formation of nitrosamines from secondary amines and nitrate or nitrite (3, 5, 12, 15, 21). Until recently most of the work on in vivo formation of nitrosamines has been directed toward their non-enzymatic synthesis in the stomach, where the pH and availability of the two reactive components are conducive to this reaction. That this synthesis does in fact occur has been demonstrated (22-25).

Now, however, with the knowledge that some bacterial species have the ability to nitrosate secondary amines under neutral or even alkaline conditions, it is recognized that any part of the gastrointestinal tract or the urinary tract during infection may be the site of synthesis (5, 12, 15).

Secondary amines are normal constituents of urine. Asatoor and Simenhoff (1) suggested that they may be, in part, derived from the degradation of basic food substances by intestinal bacteria. Dimethylamine, which is the principal secondary amine found in urine, is present in a normal concentration of about 0.5 mM (12). Nitrate is also found in normal urine in varying concentrations, dependent upon the diet and the nitrate content of the drinking water (12, 29).

Since both substrates are normally present in urine, *N*-nitrosodimethylamine (N-NDA) formation could be predicted in cases of bacteriuria caused by an organism that produces nitrate reductase and a nitrosating enzyme (12). In a previous publication (5) we reported the tentative identification of N-NDA in urine from two individuals with bacteriuria due to *Proteus mirabilis* and demonstrated the in vitro production of this same compound in normal urine and in cooked meat medium cultures of one of these organisms. It was decided on the basis of this previous work to test other species of *Proteus* for N-NDA production and to study some of the factors affecting its formation.

**MATERIALS AND METHODS**

*Reagents.* Heptafluorobutyric anhydride (HFBA) was obtained from Pierce Chemical Co., Rockford, Ill.; chloroform (nanograde) was from Mallinckrodt, St. Louis, Mo.; diethyl ether (anhydrous) and sodium nitrite were from Fisher Scientific Co., Pittsburgh, Pa.; and dimethylamine (DMA) was from Eastman Chemical Co., Rochester, N.Y. The 3% OV-1-coated Chromosorb W 80/100 mesh (acid washed, treated with dimethyl dichlorosilane, high performance) column packing material was obtained from Applied Science Laboratories, State College, Pa., and the TA33 Tabsorb packing material was from Regis Chemical Co., Morton Grove, Ill. N-NDA and amines used as standards were obtained from the same sources as previously reported (4, 5).

*Cultures.* Three strains of *P. mirabilis* (CDC no. 2064-70, 2065-70, 313-66), three strains of *P. vulgaris* (CDC no. 5272-68, 1787-64, Pr 1), three strains of *P. rettgeri* (CDC no. 5626-71, 3185-70, 952-68), and three strains of *P. morganii* (CDC no. 4581-69, 1340-69, 542-69) were obtained from the Enterobacteriology Section of the Center for Disease Control, Atlanta, Ga. All cultures were of intestinal origin and were identified by established cultural and biochemical procedures (10).

*Culture medium.* The culture medium employed was a pool of urine from a normal male collected over a period of several days. Each specimen was boiled for 1 min, cooled, and then incubated to test for sterility.
Samples (20 ml) of the urine pool were used to grow the 12 strains of *Proteus*. Inoculation was made with a platinum loop, and the cultures were incubated aerobically for 4 days at 37 C. Two additional pools of urine were collected from the same individual. These were used to determine the consistency of the background in uninoculated controls and to determine the differences in the gas-liquid chromatography (GLC) profiles of organisms grown on various lots of urine.

**Supplemented medium.** Samples (20 ml) of the urine pool were supplemented with 0.2% sodium nitrite and 0.05% DMA. After 4 days of incubation at 37 C, these uninoculated samples were extracted and analyzed in the same way as all others.

**pH monitoring.** All cultures and controls were checked daily for changes in pH with a Corning model 7 pH meter.

**Extraction and derivatization of amines and nitrosamines.** All standards, cultures, and controls were extracted, and HFBA derivatives were prepared by the method of Brooks et al. (4, 5). Of each derivatized sample, 0.8 ml was then injected onto the Tabsorb column, and 1.0 ml was injected onto the 3% OV-1 column.

**Analysis by GLC.** HFBA derivatives were analyzed with a Varian Model 3700 gas chromograph equipped with a tritium 300-mCi EC detector. Operating parameters of the instrument were: injector and detector temperatures, 220 C; column temperature, 90 C for 5 min (this was then programmed for a linear increase of 5 C per min to 220 C and maintained at this temperature for 30 min).

Approximately 1 h was required for each analysis. The electrometer settings were: attenuation, 32; range, 10; and balance, 10. Prepurified nitrogen was used as the carrier gas at a flow rate of 36 ml/min. The recorder was operated at a speed of 30 inches (76.2 cm)/h and an input signal of 1 nV. Two U-shaped glass columns (7.3 m long, 0.6 cm inside diameter) were used in this study. One column was packed with Chromosorb W 80/100 mesh (AW-DMCS H.P.) coated with 3% OV-1 (nonpolar) and the other was coated with TA33 Tabsorb (polar).

Tentative identification of N-NDA and other amines was made by a comparison of retention times of the unknown compounds on both polar and nonpolar columns with the retention times of known standards.


**RESULTS**

Of the four species of *Proteus* tested, three (*P. mirabilis*, *P. morganii*, and *P. retti*eri) were able to produce N-NDA when incubated aerobically in normal urine. GLC profiles were also distinctly unique and allowed species differentiation when all organisms were grown on the same lot of urine but not when grown on different lots. It was observed that variations in the composition of urine substrate resulted in some differences in the GLC profiles of these organisms. However, the production or nonproduction of N-NDA was consistent for a given organism regardless of the lot of urine on which it was grown.

Although detection of N-NDA production was the primary purpose of this study, the fact that the *Proteus* genus can be speculated under controlled laboratory conditions by GLC is of taxonomic interest. As can be seen from Fig. 1 and 2, all four species of *Proteus* produced many of the same amines; note particularly isobutyramine, isoamylamine, pyrrolidine, di-n-butyamine, 1,2-phenylethylamine, putrescine, cadavrine, and unidentified peaks 1, 2, 3, 4, and 5. Peaks 6 through 12 showed considerable quantitative variation but were relatively consistent for a given species. With the exception of *P. vulgaris* (Fig. 1, curve B), which did not produce N-NDA, the other three species were distinguished on the basis of the relative quantities of the various amines produced. Previous work in this laboratory with flame ionization detectors (6, 7) indicated that GLC analysis for other volatile products of in vitro metabolism such as fatty acids or hydroxy acids may also be of value in taxonomic studies of this genus. In contrast to the controlled laboratory conditions described above, actual clinical conditions where the composition of each urine specimen may differ would possibly make speciation on the basis of volatile amines impractical.

Studies were conducted to determine whether the N-NDA detected after growth of *Proteus* species was produced by acid catalysis or by bacterial-mediated nitrosation. The initial pH of the urine substrate was 6.1, but within 24 h after inoculation, all cultures had reached a pH of 9.0 to 9.2, which was maintained for the remainder of the 4-day incubation period. Thus, it is unlikely that any appreciable amount of N-NDA could have been produced by acid catalysis. This was verified by the fact that urine cultures extracted and analyzed after 24 h of growth contained only minute amounts of N-NDA, and in some cases none was detected. Therefore, the major portion of N-NDA was produced while the pH of the cultures was 9.0 to 9.2.

The possibility that the *Proteus* organism's only function was to produce nitrite ions and that the actual nitrosation was a simple non-enzymatic combination of nitrite and DMA was investigated. Normal urine at pH 6.1 was supplemented with 0.2% sodium nitrite and 0.05% DMA and incubated for 4 days at 37 C. No
N-NDA was detected upon analysis.

In addition, reports that higher nitrate concentrations enhance the non-enzymatic formation of nitrosamines prompted us to determine the level of nitrate present in our urine substrate. The urinary nitrate concentration, however, was less than 1 μmol/ml, which is well below the threshold value considered by some to be necessary, even for the enzymatic formation of nitrosamines (12).

**DISCUSSION**

The results of this study indicate that the urine of patients with bacteriuria due to strains of *P. morganii*, *P. rettgeri*, or *P. mirabilis* may contain N-NDA. The previously reported in vivo and in vitro production of N-NDA in urine by a strain of *P. mirabilis* (5) serves to demonstrate the feasibility of in vitro testing of these organisms for N-NDA production. Furthermore, although *P. vulgaris* formed no detectable N-NDA under these conditions, no attempt was made to determine the presence of other nitrosamines that may have been formed. Sander (21) demonstrated the formation of nitrosamines from aromatic secondary amines and sodium nitrate by several bacterial species, including *P. vulgaris*.

Several factors indicate that the formation of N-NDA by *Proteus* is an enzymatic reaction. (i) The major portion of N-NDA was produced when the pH of the cultures was from 9.0 to 9.2, a pH range that is not conducive to the non-enzymatic formation of N-NDA. Mirvish (17) has shown that the rate of nitrosation of DMA is maximal at about pH 3.4, and that for each unit increase in the pH range 5 to 9 the nitrosation rate decreases by a factor of 10. (ii) Normal urine at pH 6.1 was supplemented with 0.2%...
sodium nitrite and 0.05% DMA and incubated for 4 days at 37 C. Although these conditions are considerably more favorable for the formation of N-NDA via acid catalysis than those that existed in the cultures, no N-NDA was detected upon analysis. (iii) Finally, had N-NDA production in the presence of these organisms been merely a chemical reaction occurring as a function of the pH of the medium, the P. vulgaris cultures should also have formed the nitrosamine since the pH of all cultures was the same.

Secondary amines that are normally found in urine may be derived from amine precursors such as proteins, amino acids, phospholipids, quaternary ammonium compounds, and other basic substances present in many foods (1, 11, 29). Degradation of these substances by intestinal flora results in the production of secondary amines that are readily absorbed into the body and subsequently excreted in the urine. Nitrates are also present in urine, in varying amounts, dependent upon the diet and the nitrate content of the drinking water. Many vegetables contain very high concentrations of nitrates (2), and either sodium nitrate or potassium nitrate is commonly added to meat products in this country at levels of up to 3.50 oz (108.86 g) per 100 lb (37.32 kg) of meat (20). Thus, both substrates (amines and nitrate) are present in urine at the same time. Nitrates, however, are a hazard only under conditions that favor their reduction to nitrite. Proteus, which is frequently involved in urinary tract infections, characteristically possesses nitrate reductase, as do most other Enterobacteriaceae. Some (but apparently not all) of these organisms also have the ability to nitrosate secondary amines. Hawksworth and Hill (12) reported that 5 of 10 strains of Escherichia coli tested were able to form nitrosamines when incubated with nitrate and secondary amines. They also suggested that a relatively high urinary nitrate concentration may be necessary for significant nitrosamine formation by infecting coliform bacteria.

Although relatively high levels of nitrate may be necessary before some microorganisms form nitrosamines, the results of this study demonstrate that most species of Proteus are able to form N-NDA in urine that contains less than 1.0 μmol of nitrate per ml. Klubes et al. (14) have demonstrated the formation of N-NDA by rat intestinal bacteria from 14C-labeled DMA and

Fig. 2. Gas chromatographic profiles of the HFBA derivatives of basically extracted compounds from representative urine cultures of two Proteus species on 3% OV-1. (A) P. morganii 4561-69; (B) P. rettgeri 5626-71.
sodium nitrite, incubated in various molecular proportions. From 0.05 M of DMA, they obtained the highest net yield of enzymatically formed N-NDA when the nitrite concentration was between 0.05 and 0.2 M (a ratio of 1:1 to 1:4).

The normal concentration of DMA in urine is 0.5 mM, and the mean urinary nitrate level of persons living in an area where the nitrate content of the drinking water was less than 4 ppm (nitrate nitrogen) was 1.0 mM (12). This is a ratio of 1 part DMA to 2 parts nitrate. Thus, it would appear that even in areas of so-called low nitrate consumption there is adequate nitrate in the urine of most individuals for the nitrosation of DMA by some microorganisms.

Microorganisms, even within the same genus, vary greatly in their ability to reduce nitrate to nitrite. Thus, the nitrate-reducing capacity of the infecting organism may be the factor determining whether sufficient nitrite is present in the urine for nitrosamine formation. Regardless of the mechanism of formation, the presence of a nitrate-reducing organism such as Proteus in the urinary environment may present more of a danger than is currently recognized. It has been shown that single doses of very short periods of exposure to N-NDA may produce tumors in a number of laboratory animals (8, 16, 26, 27). N-NDA administered orally to rats has been shown to cause tumors of the liver, kidney, and lung. It is thought that the site of tumor formation is linked to the organ or tissue that most actively metabolizes the nitrosamine rather than the point of entry or synthesis in the body (9, 16). Even though no direct evidence exists that man is sensitive to the carcinogenic action of N-NDA, the number of species of animals that are susceptible and the results of in vitro studies that demonstrated that the rate of N-NDA metabolism by human and rat liver slices is comparable (19) indicate that man is probably no exception.

It is estimated that, at any given time, 8 × 10⁴ Americans have a urinary tract infection, and the concentration of organisms in the urine usually exceeds 100,000 per ml (28). It is fairly common for pregnant women to have asymptomatic bacteriuria. This may be of particular concern in view of the teratogenic action of some nitrosamines as demonstrated in laboratory animals (13, 18). Another large group of patients, many of whom are elderly, have chronic bacteriuria with recurrent symptomatic episodes. Klebsiella-Enterobacter, Proteus, Pseudomonas and some strains of E. coli are the usual pathogens involved. For this group conventional antimicrobial therapy is relatively ineffective, and the recurrence rate in these patients exceeds 80% (28).

Since microorganisms with the ability to reduce nitrate to nitrite are widely distributed in our environment, and since some are able to catalyze the formation of nitrosamines, it may become essential in the near future to identify and catalog those organisms capable of nitrosamine production and to define the in vivo and in vitro conditions under which they do so. This information may be of great value in future studies of cancer etiology.

LITERATURE CITED


