Absence of Volatile Nitrosamines in Human Feces

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ABSTRACT

Using a method for nitrosamine analysis that gives high recovery values and that is free from artificial synthesis of nitrosamines, we have shown that human feces do not contain volatile nitrosamines (detection limit, 0.1 to 0.5 μg/kg). We also showed that nitration reactions are not catalyzed by fecal organisms. Following a 2-day anaerobic incubation of feces with either a secondary amine (dimethylyamine, dipropylamine, or morpholine) or nitrite, no nitrosamine was formed. When the amine and nitrite were added together, nitrosamine was formed, but at a level of 2 to 20% of that formed in autoclaved feces under the same conditions. Nitrosamines were stable following anaerobic incubation with feces for up to 4 days. These results suggest that fecal organisms inhibit the chemical formation of nitrosamines instead of catalyzing it. When morpholine and nitrate were added together, nitrosomorpholine was formed. Morpholine nitrosates so rapidly that it intercepts nitrite formed by the action of nitrate reductase before the nitrite can be further reduced. However, very high concentrations of morpholine and nitrate, which are far from the conditions in normal feces, were required to form measurable nitrosomorpholine. We may conclude that N-nitroso compounds are unlikely to be formed in any significant amounts in the human colon.

INTRODUCTION

N-Nitrosamines may be formed from secondary amines and nitrite at neutral pH in the presence of bacteria including those found in the intestinal tract (3, 4, 9, 12, 15, 17, 19, 24). It was therefore perhaps not surprising when Wang et al. (23) reported the presence of nitrosamines in human feces, for it was known that amines, such as DMA, could arise there by bacterial metabolism (2) or degradation of amino acids (10) and that nitrite could be present in the lower gastrointestinal tract (21).

However, it has become clear that the concentration of nitrate and nitrite in feces is less than initially supposed and that conditions in feces favor bacterial reduction of both nitrate and nitrite (1, 20). It has also become clear that the assay of nitrosamines at low concentrations poses a number of methodological difficulties (13). We have therefore developed a robust method of assaying nitrosamines in fecal material and have used it to determine the magnitude of formation of these compounds in feces under a range of conditions. The results have led us to the conclusion that N-nitroso compounds are unlikely to be formed in any significant amounts at this site.

MATERIALS AND METHODS

NMPA and nitroso-2,6-dimethylmorpholine were prepared according to the method of Druckrey et al. (5). The other secondary amines and volatile nitrosamines used in this study were obtained from Eastman Organic Chemicals, Rochester, N. Y., and other reagents were of the best available quality.

Fecal samples were donated by healthy adults who were eating a free-choice western-style diet.

Our analytical procedures for volatile nitrosamines were based on the methods of Fine et al. (6) for determination of nitrosamines in foods. Fifteen to 30 g of samples were weighed into 500-ml round-bottomed flasks, the internal standard (NMPA, 5 μg/kg), the control amine (2,6-dimethylmorpholine, 10 μg/kg), and 40 μl of mineral oil were added, and the nitrosamines were distilled under vacuum into a liquid nitrogen trap. The temperature was increased from 24° to 120° over approximately 60 min, and the vacuum was maintained for a further 15 min after the heat was removed. The contents of the trap were rinsed into a separatory funnel with 20 ml water and were extracted twice with 20 ml dichloromethane. The extract was then dried with magnesium sulfate, filtered with a sintered glass filter that was prewashed with dichloromethane, concentrated to 10 ml in a Kuderna-Danish evaporator, and further concentrated to 1.0 ml with a slow stream of nitrogen. Three μl of the concentrate were analyzed by gas chromatography-thermal energy analyzer under conditions similar to those described by Fine et al. (6). In this case, the inlet of the furnace of the TEA-502 (Thermo Electron, Waltham, Mass.) was placed directly in the gas chromatograph (Varian 3700; Varian Associates, Inc., Palo Alto, Calif.) to avoid cold spots in any connecting tubing. The column used was 10-ft x 0.125-inch stainless steel column packed with 3% Carbobox 20M TPA-2% KOH on 100 to 120 mesh Chromosorb G-Hp (Chromatographic Specialties, Ltd., Brockville, Ontario, Canada). The carrier gas was argon at a flow rate of 30 ml/min, and the column oven temperature remained at 130° for 9 min and then increased at 5°/min to 180°. The output was recorded on a Hewlett-Packard integrator (Model 3396A, Avondale, Pa.). With this analytical method, the recoveries of NDMA, nitrosodibutylamine, nitrosomethylpropylamine, NDPA, nitrosobutylamine, nitrosopiperidine, and NMOR added to the feces at a concentration of 5 μg/kg were found to exceed 75%, and the detection limits for each nitrosamine were in the range of 0.1 to 0.5 μg/kg.

For the anaerobic incubation studies, freshly passed feces were homogenized in a VirTis homogenizer (VirTis Co., Gardiner, N. Y.) for 2 min with Dulbecco’s phosphate-buffered saline to make a 60% suspension of feces. Thirty g of this sample (representing 18 g of original feces) were weighed into 60-ml screw-topped bottles and autoclaved at this stage if required. Appropriate amounts of nitrosamines or amines were added to the bottle (after cooling if the bottle had been autoclaved) and mixed well. Appropriate amounts of nitrate or nitrite were added, and the mixtures were incubated at 37° under an atmosphere of humidified nitrogen for 24 to 96 hr. Reactions were terminated by chilling the closed bottle and adding 2 ml of 15% sodium azide solution to the incubation mixture. Twenty-five μl of incubation mixture (representing 15 μg of original feces) were used for the analysis of nitrosamines.

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4 The abbreviations used are: DMA, dimethylamine; NMPA, nitrosomethylpropylamine; NDMA, nitrosodimethylamine; NDPA, nitrosodipropylamine; NMOR, nitrosomorpholine; DPA, dipropylamine; MOR, morpholine.

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RESULTS

Nitrosamines in Feces. The analysis of nitrosamines in complex mixtures such as feces poses a number of problems including the possibility of loss or destruction of nitrosamines during storage or analysis of the sample; the possibility for synthesis of nitrosamines from precursors in the sample during the analysis (our early studies showed that artifactual formation of nitrosamines could occur when feces were stored in the frozen state in the presence of precursors); the possibility for contamination of glassware, especially sintered glass; and the possibility of obtaining false positives or false negatives during chromatographic analysis. The analytical procedure described here measured volatile nitrosamines with good recovery, without evidence of artifactual formation and with a detection limit in the range of 0.1 to 0.5 μg/kg. Analytical data for 12 feces samples gave an average recovery of the internal standard NMPA of 78% (range, 75 to 81%), no evidence of artifactual formation of nitroso-2,6-dimethylmorpholine, and no measurable levels of nitrosamines except the internal standard. Furthermore, no loss of NDMA, NDPA, and NMOR added to fresh feces samples at a concentration of 17 μg/kg was observed during anaerobic incubation at 37°C for periods of up to 4 days.

Formation of Nitrosamines in Feces Incubated with Precursors. Since the levels of nitrosamines in feces were not measurable although these compounds were stable in feces, we examined conditions under which nitrosamines might be formed. First, incubations were carried out over 2 days with either secondary amines (DMA, DPA, or MOR) added at a concentration of 200 mg/kg, nitrite, or a combination of amines with nitrite. Nitrosamines were not detected when the amines and nitrite were present alone. However, nitrosamines were formed when amines were present with nitrite (Table 1). In this case, the levels were substantially lower when the feces were fresh than when they were autoclaved.

Next, formation of nitrosamines was studied with 24-hr incubations in which amines were added at 200 mg/kg and nitrite was added at concentrations up to 500 mg/kg (Chart 1). All 3 of the amines were nitrosated in autoclaved feces at a rate that depended on the basicity of the amine (18). With fresh feces, nitrosation was not seen with dimethylamine or dipropylamine at a nitrite concentration below 200 mg/kg, although the pH was the same (within 0.2 unit) as that of the autoclaved feces. During incubations with fresh feces, in which nitrite concentration was 500 mg/kg and amine concentration was varied, NMOR was formed linearly reaching 40 μg/kg at a MOR concentration of 200 mg/kg. Only 2 to 3 μg/kg levels of NDMA and NDPA were seen at 200-mg/kg levels of DMA or DPA.

Since in the reductive environment of the feces nitrate is reduced to nitrite which could, before further reduction, react

<table>
<thead>
<tr>
<th>Feces</th>
<th>NDMA (μg/kg)</th>
<th>NDPA (μg/kg)</th>
<th>NMOR (μg/kg)</th>
<th>Others (μg/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh</td>
<td>1.3 (0.7–2.3)</td>
<td>4.2 (1.9–5.1)</td>
<td>43 (30–55)</td>
<td>N.D.</td>
</tr>
<tr>
<td>Autoclaved</td>
<td>54 (35–66)</td>
<td>35 (22–70)</td>
<td>220 (180–295)</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

* Values represent the means of 3 experiments.
  * Numbers in parentheses, ranges.
  * N.D., not detected.

with amines to form nitrosamines, incubations were carried out with nitrate and morpholine (Chart 2). As expected, NMOR was not produced in autoclaved feces but was produced in small amounts in fresh feces, readily measurable amounts being formed only when nitrate and MOR were at high concentrations (μg/kg).

DISCUSSION

The results reported here differ in several important respects from the results of earlier studies. First, we have observed with the improved analytic methodology that the concentration of volatile nitrosamines in feces is undetectable, less than 0.1 to 0.5 μg/kg. This result may be compared with that of Wang et
al. (23), who reported that most normal human feces contained measurable levels of NDMA and nitrosodiethylamine. We have repeated the analytical method used by Wang et al. and have shown it to be subject to artifactual formation of nitrosamines although the exact problem has not been identified.

A second somewhat unexpected result was the stability of nitrosamines in feces. Nitrosamines in the body proper are rapidly metabolized and are excreted or lead to reactive alkylating species (16). The metabolic processes are oxidative. Nitrosamines appear to be relatively inert in the reductive environment of the colon.

A third finding was that the nitrosation reaction is not catalyzed by the bacteria of feces. Following a 2-day anaerobic incubation of fresh feces with secondary amine (DMA, DPA, or MOR) and nitrite, nitrosamines were formed but at a level of 2 to 20% of that formed in autoclaved feces under the same conditions. This result suggests that fecal organisms inhibit the chemical formation of nitrosamines instead of catalyzing it. The opposite conclusion had been reached by Klubes and Jondorf (12) and Klubes et al. (11). They used a 10% suspension of rat cecal, small intestinal, and large intestinal contents incubated with sodium nitrite and DMA in the presence of glucose and observed the formation of NDMA. The conditions of their experiment were far from physiological, and it seems possible that they observed chemical nitrosation, accelerated by the decrease in pH due to the microbial degradation of glucose during the incubation. Although many bacteria isolated from human feces have been reported to be able to form nitrosamines (7–9, 14, 15, 17, 22), it appears that feces form an ecosystem which is not appropriate in this regard.

The data of Charts 1 and 2 indicate that no volatile nitrosamines are formed at concentrations of nitrite much below 200 mg/kg or of nitrate below 500 mg/kg even at high concentrations of amines. These concentrations are probably not seen under physiological conditions. The recent reports of Archer et al. (1) and Saul et al. (20) show that the concentration of nitrite and nitrate found in the feces is generally <1 mg/kg. While Asatoor and Simenhoff (2) and Johnson (10) have reported that amines can be formed in the intestine, the concentration of these amines must be low, since no nitrosamines could be detected in our experiments even after adding high concentrations of exogenous nitrite. It is possible that nitrosamines could be made at low levels close to the intestinal wall where the microenvironment may be aerobic. It is also possible that nitrosamines might be formed in the upper gastrointestinal tract and transported unchanged into the colon. However, in view of the stability of the nitrosamines in feces and their complete absence from fresh feces, these possibilities seem most unlikely.

REFERENCES

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