Analysis for and Intestinal Metabolism of Precursor Nitroso-Compounds in Normal Subjects and Patients with Chronic Renal Failure

M. L. Simenhoff, S. R. Dunn, P. S. Lele

Department of Medicine
Division of Nephrology
Jefferson Medical College
Thomas Jefferson University
Philadelphia, PA 19107 USA

Running Title - Intestinal metabolism and analysis of nitroso precursors

Key Words - Nitrosodimethylamine, dimethylamine, trimethylamine, intestine, duodenum, humans, chronic renal failure, cancer, gas chromatography

INTRODUCTION

A human model exhibiting increased generation of dimethylamine (DMA), a precursor of nitrosodimethylamine (NDMA) was investigated in order to identify possible NDMA formation therefrom. Patients with chronic renal failure (CRF) fulfill the criteria for such a model (Simenhoff et al., 1963, 1976) but, because of rapid degradation of NDMA in the liver, possible NDMA formation would not be accurately reflected by the blood NDMA levels (Simenhoff et al., 1982). Seeking NDMA formation in uremic patients in particular, is additionally desirable because of the reported increased incidence of cancer in patients with chronic renal failure. The presence of upper intestinal bacterial overgrowth adds further to the possibility of in situ NDMA production (Simenhoff et al., 1978). The probable metabolic steps that occur in the intestine are illustrated in Figure 1.

MATERIALS AND METHODS

Fifteen patients with chronic renal failure (CRF) (12 of whom were on thrice weekly hemodialysis) and eight control subjects were studied by sampling blood and duodenal aspirate. The latter was obtained by intubation under sterile conditions (Simenhoff et al., 1978). From a pool of 56 CRF patients, 39 were solicited randomly for the study but only 21 consented. Of these, only 15 were successfully intubated.

Methylamines and volatile nitrosamines were measured in blood. Bacterial culture, pH, methylamines and volatile nitrosamines were measured in the duodenal aspirate.

Patients blood and duodenal specimens were always distilled at the same time. With every group of specimens a water blank was also run. The water blank was "subtracted" from the corresponding specimens. All glassware was rinsed with DCM prior to use. The dichloromethane that was used was checked for TEA positive peaks by evaporating 300 ml to 1.0 ml and assaying for TEA positive peaks (Pensabene, J.W., 1980 personal communication). The 6N HCl, 5N NaOH and 1N NaOH and water were extracted with DCM to remove any TEA positive peaks. Only polypropylene, glass or teflon stoppers were used to close reagent bottles.

Conditions for Analysis by Gas Chromotography (GC) - Thermal Energy Analyzer (TEA)

Conditions for GC-TEA analysis were the same as reported by Fiddler et al., 1981.

Specimens that gave positive nitrosamine peaks were confirmed by the photolytic technique described by Doerr and Fiddler (1977) and when enough nitrosamines was present, by mass spectrometry according to the technique described by Kimoto and Fiddler (1982).

Aliphatic amines: The aliphatic amines, DMA and TMA, were assayed for in serum and duodenal aspirate using gas-liquid chromatography. Frozen serum or duodenal aspirate was thawed, mixed and centrifuged. Amines were separated from biological fluids by adopting a modification of the procedure described by Conway (1950). A sealable porcelain micro diffusion cell (Arthur H. Thomas, Phila, PA USA) was set up in duplicate (Figure 2). The center well (#1) contained 250 µl of 0.10 N H_2SO_4. Well #2 contained 500 µl of "amine-free" deionized water and 1000 or 500 µl of either serum or duodenal aspirate.

The outermost well (#3) contained 2.0 ml of a mixture of 50% V/V of saturated K_2CO_3 and 12N KOH (called the alkaline solution). This well acted as the sealing well. To the #2 well 1.00 ml of the alkaline solution was carefully added and the cover immediately placed on dish. The dish was gently rocked for about 10-15 seconds and allowed to set overnight (18 hours). Each specimen was run in duplicate. Standards, usually four, were run with each set of specimens. After 18 hours the covers were carefully removed. The center wells (#1) were separately transferred to tared polypropylene microcentrifuge tubes. The volume of each center well was determined by weighing, and tested to assure it was still acidic. This solution containing the liberated volatile aliphatic amines from the specimens (now as acid salts of the amines) was assayed for DMA and TMA. A portion (50 µl) of the acidic solution from the center well was added to glass micro test tubes that contained the dry alkaline residue from 50 µl of 1N KOH that had been previously put into these tubes and allowed to evaporate in an over at 90-100°C. One microliter of resulting alkaline solution was immediately injected a gas chromatograph using conditions similar to those employed by Dunn et al. (1976). The column has changed slightly. It is now 12% Amine 220 and 8% KOH on 100/120 Chromosorb WAW. The column was run at 75° isothermal with N_2 at 17 ml/min.

A second column, six foot coiled glass packed with 4% Carbowax 20 M and 0.8% KOH on 60/80 Carbopack B (Supleco Inc. Bellefont, PA USA) operated at 75° isothermal was used to further confirm the presence of DMA and TMA.

All specimens were corrected for recoveries of TMA and DMA since the diffusion of each in the Conway cells is temperature dependent. Appropriate

Matas et al. (1975) have described an increase in the incidence of cancer in patients with CRF. This has been subsequently reported by others (Lindner et al., 1981). There is no direct evidence that these findings of cancer are related to the increased concentration of NDMA in the duodenum. It is clear however, there is a spectrum of NDMA duodenal levels, such that some patients who have duodenal NDMA concentrations within the normal range would probably be at less potential risk than patients with increased concentrations, if these single measurements are representative of the daily levels (Figure 6). We do not have information on the diurnal variation or month to month variation.

Two CRF patients agreed to ingest six grams of Vitamin C per day for two weeks and be reintubated. Vitamin C should be capable of blocking nitrosation via interaction with available nitrite thus decreasing NDMA formation. In one patient we were able to demonstrate a decrease in duodenal NDMA levels from 1.22 to 0.20 μg/kg, but in the other, NDMA levels were not significantly different (0.23 to 0.25 μg/kg). A larger series of patients will be necessary to demonstrate if an antioxidant effect is present, and if it is sufficient to reduce NDMA formation in the duodenum.

NDMA formation might be anticipated in the presence of DMA, nitrite, and an appropriate pH (3.4 is optimal for NDMA formation (Mirvish, 1970). In normal man these conditions are not usually met simultaneously. There is much discussion and disagreement on these optimal conditions.

Our results demonstrate significant increases in duodenal DMA in chronic renal failure, the level always being higher than that seen for blood. This suggests that DMA reaches the lumen by ingestion or is produced in situ. In

view of the fact that these were fasting subjects, the latter is more likely. Quantitatively salivary DMA would not account for this rise. Normally, non-absorbed choline in the large gut is degraded in part to trimethylamine by bacterial activity (Zeisel, 1981). TMA is then demethylated, probably in the liver to DMA, which is an end metabolite. However, the potential enzymatic activities of the bacteria found in these patients have the capacity to demethylate TMA sequentially and has been shown to do so in vitro (Colby & Zatmus, 1973)

With large increases of DMA in the intestine are the conditions optimal for NDMA formation? Measured nitrite levels have been variable and have not correlated with the measured NDMA levels. The pH of the duodenal aspirate is usually alkaline (although contamination of the acid contents of the stomach have sometimes occured). Thus the conditions are not optimal for NDMA formation in the duodenum and speculation is that the metabolic bacterial activity is an essential condition for NDMA formation in this location (Klubes & Jondorf, 1971; Mills & Alexander, 1976). We have not yet had the opportunity to repeat the measurements following the administration of non-absorbable antibiotics.

The suggestion from animal experiments (Asatoor & Simenhoff, 1965) is that DMA has both an endogenous and an exogenous source. This paper addresses only the exogenous formation of NDMA in the gut. We have no data on endogenous DMA formation.

Lindner, A.M., Farewell, V.T. & Sherrard, D.J. (1981) High incidence of neoplasia in uremic patients receiving long-term dialysis. Nephron., 27, 292-295

Matas, A.J., Simmonds, R.L., Kjellstrand, C.M., Buselmeier, T.J. & Najarian, J.S. (1975) Increased incidence of malignancy during chronic renal failure. Lancet, i, 883-886

Mills, A.L. & Alexander, M. (1976) N-Nitrosamine formation of cultures of several microorganisms. Appl. Environ. Microbiol., 31, 892-895

Mirvish, S.S. (1970) Kinetics of dimethyl nitrosation in relation to nitrosamine carcinogenesis. J. Natl. Cancer Inst., 44, 633-639

Simenhoff, M.L., Dunn, S.R., Fiddler, W. and Pensabene, J.W. (1982) Presence of nitrosamines in blood of normal and diseased human subjects. Banbury Report 12, Nitrosamines and Human Cancer, P.N. Magee, ed, Cold Spring Harbor Laboratory, NY, pp 283-293

Simenhoff, M.L., Milne, M.D. & Asatoor, A.M. (1963) Retention of aliphatic amines in uremia. Clin. Sci., 25, 65-77

Simenhoff, M.L., Saukkonen, J.J., Burke, J.F. Wesson, L.G. & Schaedler, R.W. (1976) Amine metabolism and the small bowel in uremia. Lancet, II, 818-821

Simenhoff, M.L., Saukkonen, J.J., Burke, J.F., Wesson, L.G., Schaedler, R.W. & Gordon, S. (1978) Bacterial populations of the small bowel in uremia. Nephron., 22, 460-464

Zeisel, S.H. (1981) Dietary Cholines: Biochemistry, physiology and pharmacology. Ann. Reviews Nutrition, 1, 95-121

LEGENDS FOR FIGURES

Figure 1 Proposed metabolic steps leading to the formation of NDMA in the intestine

Figure 2 Conway micro diffusion cell. Well #1 (collection well) contains 250 µl of 0.1N H_2SO_4. Well #2 (reaction well) contains specimen (100-500 µl), water (500 µl) and KOH/K_2CO_3 (1000 µl). Well #3 (sealing well) contains 2000 µl of KOH/K_2CO_3

Figure 3 Mean DMA concentration is expressed in micrograms per liter. For blood, control n = 8, CRF = 15; for duodenal aspirate, control n = 7, CRF = 14

Figure 4 Mean TMA concentration is expressed in micrograms per liter. For blood, control n = 8, CRF = 15; for duodenal aspirate, control n = 7, CRF = 14

Figure 5 Mean NDMA concentration is expressed in micrograms per kilogram. For blood, control n = 8, CRF = 15; for duodenal aspirate, control n = 7, CRF = 14

Figure 6 Individual NDMA levels in blood and duodenal aspirates expressed in micrograms per kilogram.

Figure 7 Numerator shows the number of patients with the respective nitrosamine. Denominator shows the total number surveyed.

Figure 8 Aerobic and anaerobic bacteria counts are expressed as log of colonies per ml.

NDMA IN BLOOD AND DUODENAL ASPIRATE

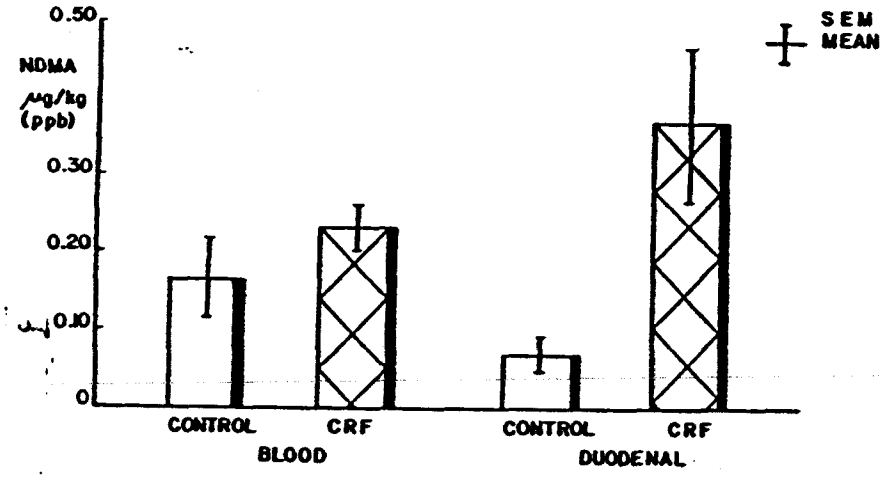

FIG 5

DUODENAL MICROFLORA IN CHRONIC RENAL FAILURE

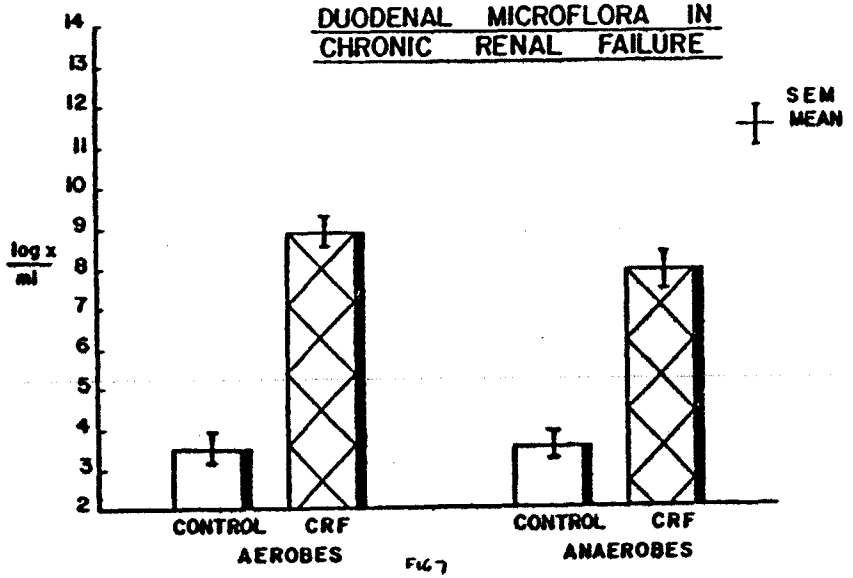

FIG 7

NDMA IN BLOOD AND DUODENAL ASPIRATE INDIVIDUAL VALUES

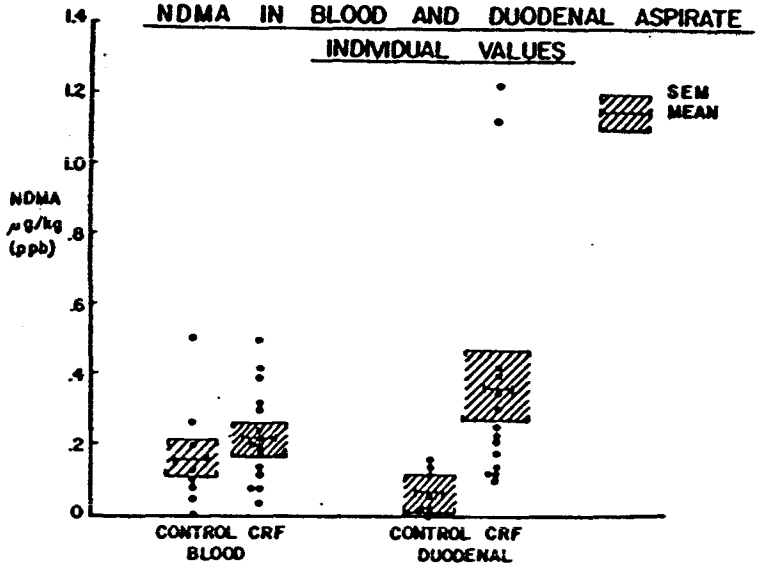

FIG 6

APPEARANCE OF NDMA AND NPIP IN BLOOD AND DUODENAL ASPIRATE FROM CONTROLS AND CRF PATIENTS IN %

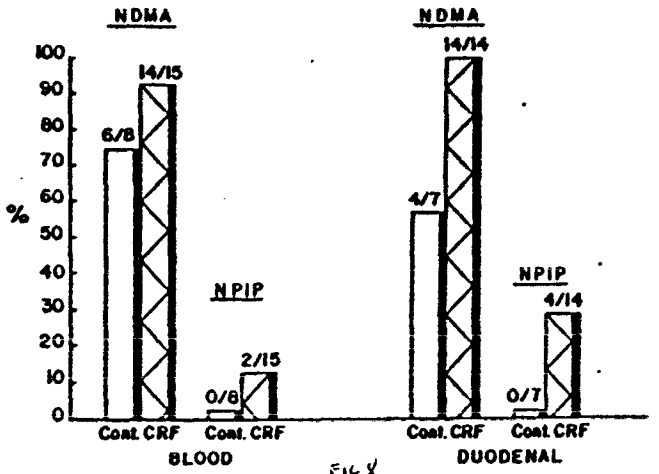

FIG 8