Reaction of Sodium Nitrite with Dimethylglycine Produces Nitrososarcosine

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That sodium nitrite reacts with secondary amines under slightly acidic conditions to form potentially carcinogenic nitrosamines is a well-established fact (4,5,12,13,16,17,19). Due to the wide distribution of nitrite salts and secondary amines and the constant human exposure to these compounds, the practical relevance of this observation with reference to human cancer is readily apparent. Recently, the possibility that tertiary and quaternary amines might also serve as nitrosatable substrates has come under intensive investigation (1, 6,11,14).

Dimethylnitrosamine(DMN) is a potent mammalian mutagen and carcinogen (10,15) which has been identified in several nitrite-preserved foodstuffs (18,19). DMN has been assayed and identified as the potential reaction product of a wide range of amines, including trimethylamine, choline chloride, dimethylformamide and many others (6,14).

Nitrososarcosine, on the other hand, is only weakly carcinogenic. In the rat, nitrososarcosine induces cancer of the esophagus and oral pharengeal cavity (3). However, due to the very high concentrations of precursors of nitrososarcosine in the environment, the potential biological significance of this nitrosamine is currently being studied (2). Therefore, it is important to know whether nitrososarcosine, a weak carcinogen, or dimethylnitrosamine, a potent carcinogen, is produced as a result of the nitrosation of sarcosine derivatives such as dimethylglycine (N-methylsarcosine). It has been suggested previously that dimethylnitrosamine is the nitrosation product of dimethylglycine (6). This communication details the ratio of nitrosation products of dimethylglycine.

Materials and Methods

In Vitro Nitrosations: An aqueous solution of 2M dimethylglycine hydrochloride was prepared and the pH was adjusted with 10N NaOH to 1.0, 1.5, 2.0, 2.52, 3.0, 3.55, and 4.0. To test tubes containing 1.5 ml of dimethylglycine solution at each pH was added 0.5 ml of 2M $NaNO_2$. The tubes were then incubated for 45 minutes at 37°C. At the end of this interval, groups of tubes were handled in one of two ways. Total nitrosamines and nitrosamino acids were quantitated following addition of 3 ml 25% HCl containing 3M urea (8). The solutions were diluted 1:50 with distilled water and the absorbency at 230 millimicrons determined. There was no residual $NaNO_2$ as shown by absorbences in undiluted blanks less than 0.005. Dimethylnitrosamine was quantitated by adding 4 ml of 3N NaOH, 4 ml ether or methylene chloride and an excess of NaCl to each tube. Prior to addition of NaOH, aliquots containing 95,652 cpm of DMN-C^{14} were added to each tube. The tubes were shaken for 15 minutes followed by centrifugation. Two ml of ether or

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methylene chloride faction were removed and shaken for 15 minutes with 3 ml distilled water. The ether was aspirated and absorbency of the water fraction at 230 millimicrons was determined. 0.5 ml of the water was pipetted into a scintillation vial and radioactivity determined with toluene-Triton-X-100 (3.1) solution. Efficiency of extraction of DMN was calculated as CPM water fraction divided by CPM when added to original solution. DMN concentration in mM was calculated as (OD230 x 2000) (Efficiency of extraction x 6210)$^{-1}$ where 6210 is the molar extinction coefficient for DMN at 230 millimicrons. Nitrososarcosine concentration was calculated by determining the absorbence of the acid fraction minus the ether extract corrected for efficiency and dilutions. Concentration was calculated using an extinction coefficient for nitrososarcosine of 5,177.

Preparation of NMR Samples: Dimethylglycine hydrochloride (1M, pH 2.0) and $NaNO_2$ (2M) were incubated 45 minutes at 37°C. The reaction was stopped by addition of equal volume of 25% HCl containing 3M urea in order to assure this preparation was the same as that studies in the in vitro experiments. The water was evaporated and the residue was extracted with acetone for 15 minutes (13). The acetone fraction was removed, and the acetone evaporated. The subsequent residue was extracted with ether for 20 minutes, the ether fraction removed, and the ether evaporated. NMR spectrum was determined on the remaining material dissolved on both D_2O and $CDCl_3$.

Formation of Nitrososarcosine - C^{14} in isolated mouse stomachs: Stomachs were removed from six male mice and the gastro-esophageal and gastroduodenal junctions ligated (9). All stomachs were injected intraluminally with 0.1 ml of dimethylglycine-1-C^{14} (10 microcuries per ml). Three stomachs were simultaneously injected with 0.1 ml of 8M sodium nitrite. Stomachs were incubated for 45 minutes at 37°C after which the reaction was stopped by mincing each stomach with 25 ml of 25% HCl containing 3M urea. Following saturation with NaCl, stomachs were extracted three times with ether. The ether fraction was removed and evaporated in a scintillation vial. To the residue was added 1 ml water and 8 ml scintillation solution, toluene-Triton-X-100 3:1 (7).

Thin Layer Chromatography: Ether extracts of mouse stomach were prepared and evaporated as described above. The residue was dissolved in 1 ml ether and spotted on TLC plates (Silicar: TLC-G6F). Aliquots of nitrososarcosine-C^{14} and dimethylglycine-1-C^{14} were also spotted. The plates were eluted with 95% ethanol or chloroform-methanol (1:1). At increments of 0.75 inches for the ethanol and 0.5 inches for the chloroform-methanol, the surface of the plates was scraped into scintillation vials. To each vial was then added 1 ml of ether and 8 ml scintillation solution.

Results

In vitro Nitrosation: Dimethylglycine reacted with sodium nitrite to form both nitrososarcosine and dimethylnitrosamine. The relative proportion of these products is shown in Figure 1. Formation of both dimethylnitrosamine and nitrosarcosine were pH dependent and increased markedly with decreasing pH. At all pH's there was considerably more nitrososarcosine than dimethylnitrosamine produced with the ratio generally inversely related to the pH. Identical results were obtained subsequent to extraction with ether or methylene chloride. Extraction efficiencies in ether were 58.9 $\pm$ 0.8% and in methylene chloride 40.0 $\pm$ 1.2%. There was less than 1% decarboxylation of nitrososarcosine to dimethylnitrosamine under these experimental conditions on studies on the extraction of pure nitrososarcosine into ether or methylene chloride.

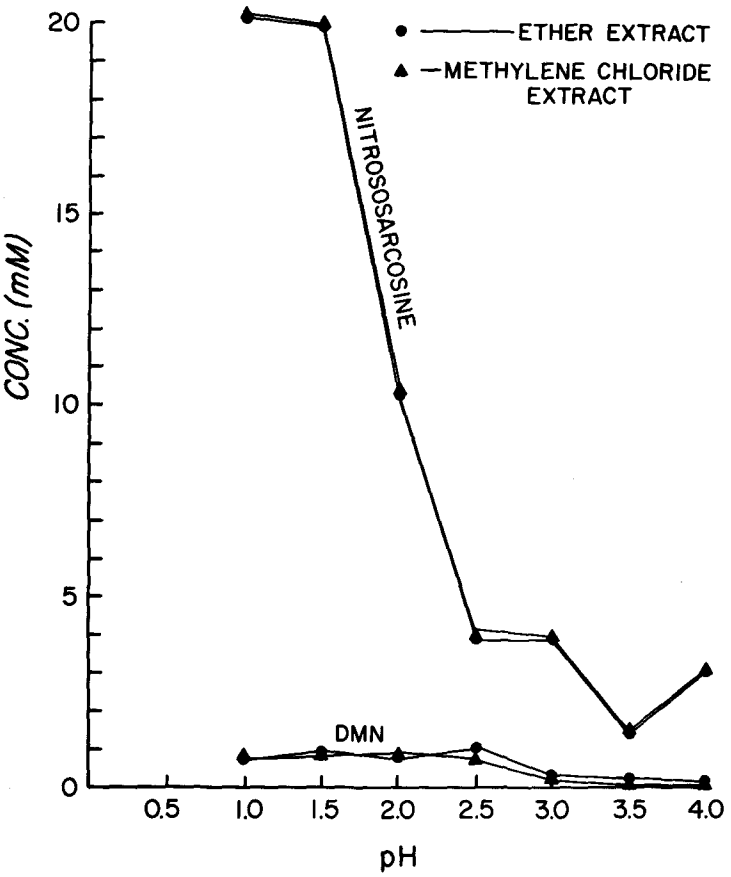

Figure 1. Effect of pH of formation of dimethylglycine and sodium nitrite.

In order to directly demonstrate nitrososarcosine synthesis (i.e. proof of structure), the material was isolated and NMR spectra determined. The NMR spectra distinctly showed the two sets of peaks characteristic of nitrososarcosine. One set appeared at 3.18δ and 4.41δ while the other set appeared at 3.90δ and 5.11δ. This spectrum is identical to those performed on pure nitrososarcosine and with published spectra(13). The two sets were in the ratio of three to two, respectively. Other minor impurity peaks were also obtained, the most significant of which were attributable to diethylether. Impurities did not include dimethylnitrosamine, dimethylglycine, or sarcosine as shown by the absence of characteristic NMR peaks of these compounds.

TABLE 1

Formation of Nitrososarcosine-C^{14} In Isolated Mouse Stomach From Dimethylglycine-1-C^{14} And Sodium Nitrite

Treatment	No. of Stomachs	CPM/Stomach (mean ± S.E.)
Dimethylglycine-1-C^{14}	3	867 ± 103
Dimethylglycine-1-C^{14} + Sodium Nitrite	3	3606 ± 45

Gastric Formation of Nitrososarcosine: Isolation of nitrososarcosine-C^{14} from mouse stomachs is shown in Table 1. There was 412 times more radioactivity extracted with ether from stomachs injected with both nitrite and dimethylglycine-1-C^{14}. This radioactivity could not have been in the form of dimethylnitrosamine since the C^{14} was on the methylene carbon. However, in order to support the contention that the radioactive material was nitrososarcosine, the ether extracts were chromatographed on thin layer plates. The distribution of radioactivity is shown in Figures 2 and 3. In the case of ethanol elution, the bulk of the radioactivity extracted from stomachs injected with both nitrite and dimethylglycine-1-C^{14} eluted at the fraction 4.5 inches up the plate (Rf = 0.75). Nitrososarcosine standards also eluted in this fraction. In similar fashion, when chromatographed in chloroform-methanol, the radioactivity chromatographed with an Rf of 0.68 as did the nitrososarcosine standard. There was a slight amount of nitrososarcosine isolated from stomachs not treated with nitrite. Chromatography of the starting material revealed no material which chromatographed with nitrososarcosine.

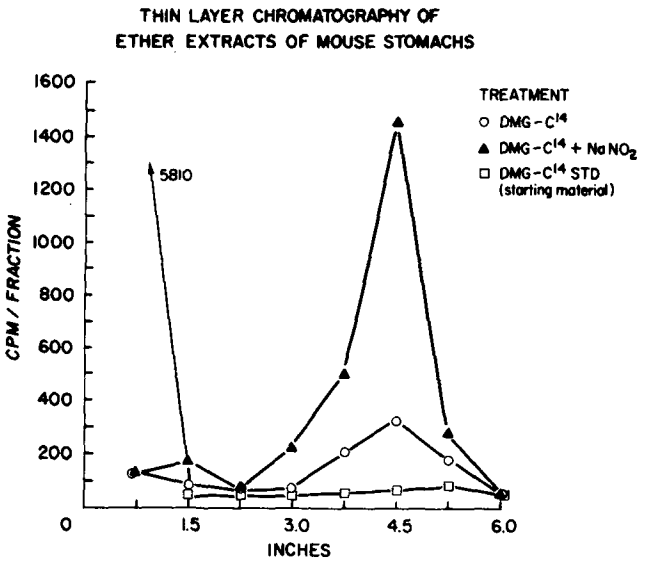

Figure 2. Thin layer chromatography of ether extracts of mouse stomachs eluted with 95% ethanol.

Discussion

In this communication the nitrosation of a naturally occurring tertiary amine was investigated. There were at least two nitrosation products of dimethylglycine, dimethylnitrosamine and nitrososarcosine. From statistical considerations the expected ratio of nitrososarcosine to dimethylnitrosamine should be two to one since there are two methyl groups which could leave to form nitrososarcosine and only one carboxymethyl group which could leave to form dimethylnitrosamine. Experimentally, however, this ratio was generally far greater ranging from 4 to 49 (mean 20.4 $\pm$ 5.8). It has been recently suggested that steric considerations are the most important physical determinant in nitrosation of tertiary and quanternary amines (14). Nitrososarcosine was also produced in mouse stomach from reaction of nitrite with dimethylglycine.

The practical relevance of these observations lies in two areas. First, the potential importance of nitrososarcosine is further emphasized. Dimethylglycine is characteristic of a wide range of environmental amines which may react to produce nitrososarcosine. Nitrososarcosine is a weak esophageal and oral-pharengeal carcinogen in the rat(3). Since nitrososarcosine is structurally similar to dimethylnitrosamine, consideration might be given to the possibilities that these two compounds may not be differentiated is some nitrosamine assays. Thus, these observations underscore the importance of identifying and monitoring human exposure to nitrososarcosine.

Secondly, the simultaneous production of two different nitrosamines from the same amine raises the possibility of interaction of

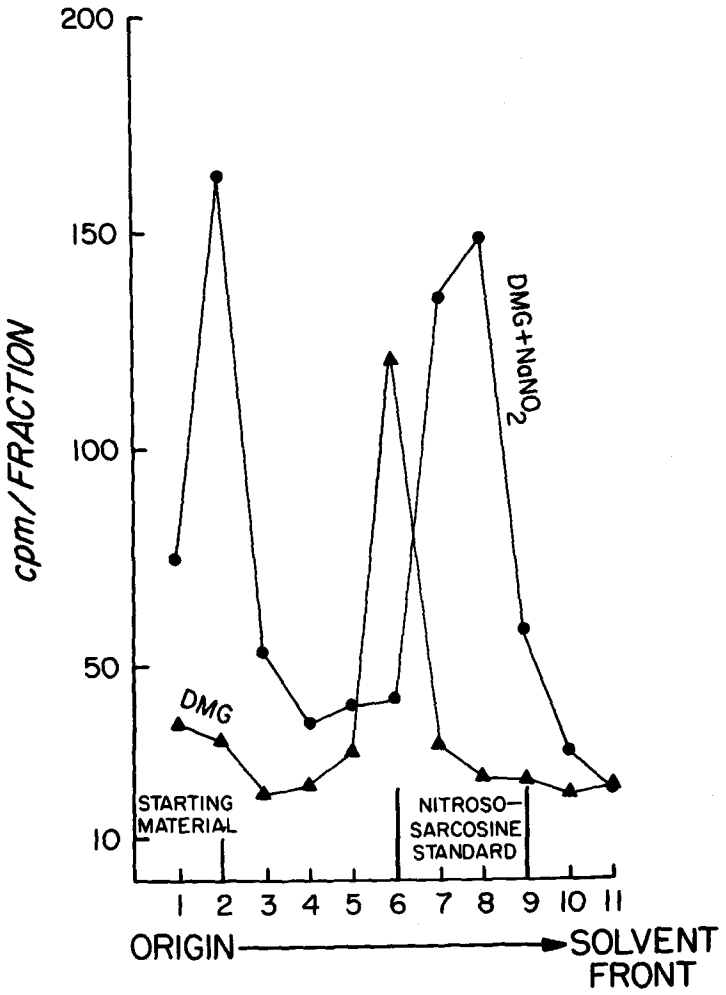

Figure 3. Thin layer chromatography of ether extracts of mouse stomachs eluted with chloroform methanol.

nitrosamines. Since nitrosamines are enzymatically activated and dialkylnitrosamines, by definition, are structurally related, there may be an interaction at the enzyme which activates them. The biological activity of nitrosamines vary with the chemistry of the alkyl groups (15). One of three possibilities (synergistic carcinogenicity, antagonistic carcinogenicity or altered organotropic action) may result from interaction between nitrosamines.

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