Nitrosation of Sarcosine: Chemical Kinetics and Gastric Assay

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Carcinogenic nitrosamines are formed chemically from the interaction of nitrite salts with secondary amines under acidic conditions (1). Such reactions have been documented in vitro in the presence of enteric bacteria at a near neutral pH (2), in vitro in gastric juice from various species including man (3), and in vivo in the stomach of laboratory animals and man (4,5,6). Furthermore, combined oral administration of sodium nitrite with dimethylamine or methylbenzylamine produced acute toxicity similar to that of the corresponding nitrosamine (7,8,9). Additionally, 7-methylguanine-C^{14} was isolated from liver RNA following treatment of mice with sodium nitrite and dimethylamine-C^{14} (8).

The relevance of nitrosation and nitrosamines to human cancer has been suggested by induction of tumors in rats following long term administration of nitrite and secondary amines. Combined feeding of rats with sodium nitrite and morpholine or methylbenzylamine resulted in liver or esophageal cancer, respectively (10). Lung cancer was observed subsequent to feeding rats with sodium nitrite together with secondary amines (11).

Sarcosine (methylglycine) is one of the most commonly occurring nitrosatable secondary amines. Involved in transmethylation reactions, sarcosine has been quantitated in the cat ranging from 1.3 mg/100 gms bladder to 4.3 mg/100 gms liver (12). Its corresponding nitrosamine is a weak esophageal carcinogen in the rat (13). Due to the concentrations of this amine in the environment and the potential carcinogenicity of the corresponding nitrosamine, the biological significance of sarcosine nitrosation is being studied. This communication presents evidence for sarcosine nitrosation in experimental animals and the kinetic constants for sarcosine nitrosation.

Materials and Methods

PREPARATION OF NITROSO-SARCOSINE STANDARD -- Sarcosine (0.2M) and sodium nitrite (0.6M) were incubated at pH 3.5 for 48 hours at room temperature. The mixture was taken to dryness in a rotary evaporator and dissolved in methanol. Unreacted sodium nitrite and sodium chloride were precipitated by addition of 3 volumes of acetone. Nitroso-sarcosine sodium salt was isolated by addition of an equal volume of ether. Identity and purity of this compound were established by NMR spectroscopy and thin layer chromatography. The molar extinction coefficient was determined as 2.44×10^3 at 250 mμ.

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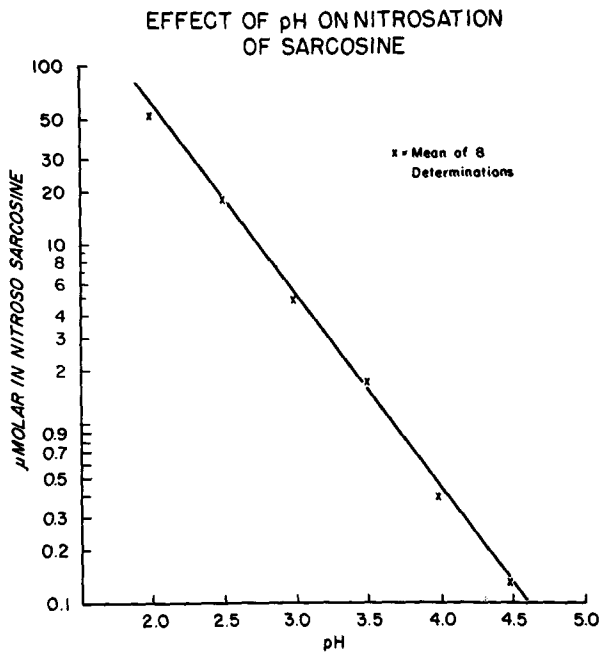

Figure 1 - Sarcosine (50 µM) and sodium nitrite (50 µM) were incubated at 25°C for 21 minutes at the pH indicated above. The reaction was stopped by addition of 0.5 ml of 3.5 M urea and 4 ml of 1N HCL. Nitroso-sarcosine concentration was calculated by using a molar extinction coefficient of $2{,}44 \times 10^3$ at 250 mµ.

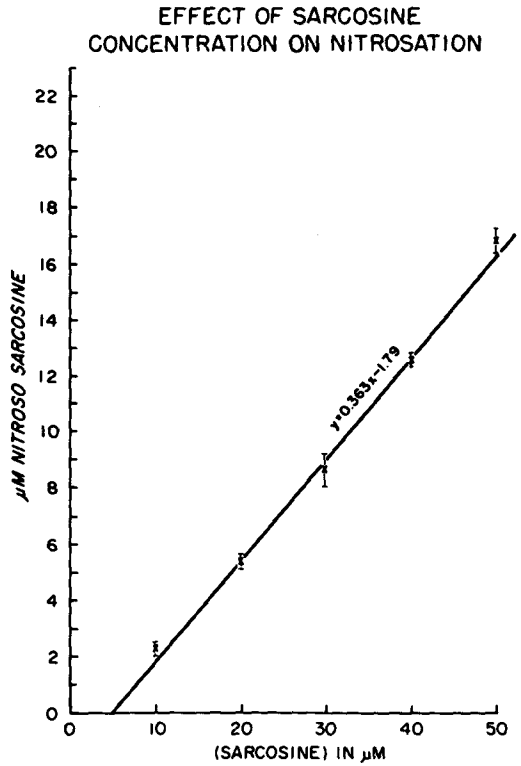

Figure 2 - Buffered sodium nitrite (50 µM pH 2.0) was incubated together with sarcosine at concentrations of 10,20,30,40, or 50 µM for 21 minutes. Nitroso-sarcosine was measured by absorbency at 250 mµ.

IN VITRO NITROSATION -- In vitro reactions were assayed in a final volume of 3 ml. Sarcosine concentration was 50 mM, when held constant, or 10, 20, 30, 40 or 50 mM in experiments where sarcosine concentration was varied. Sodium nitrite concentration was 50 mM when held constant or 10, 20, 30, 40 or 50 mM in experiments where nitrite concentrations were varied. When indicated, 0.01N HCl was used as buffer. The reaction was stopped after 21 minutes by addition of 0.5 ml of 3.5 M urea followed immediately by 4 ml of 1N HCl. Urea reacts with nitrite to produce nitric oxide and carbon dioxide and prevents further nitrosation in vitro. The tubes were shaken for 10 minutes on an Omni shaker to remove dissolved gases prior to assay. In each case, a blank was assayed where urea and 1N HCl were added prior to sarcosine and nitrite. Absorbency at 250 mμ was determined and concentration calculated from the extinction coefficient given above. In another series of experiments, dimethylamine buffered with 0.01N HCl at a concentration of 100 mM was studied in place of sarcosine. Sodium nitrite concentrations in these experiments were 20, 40, 44.8, 60, 63.3, 77.5, 80, 89.4 and 100 mM. Incubation was stopped after 30 minutes and absorbency at 250mμ determined.

SARCOSINE NITROSATION IN MOUSE STOMACH -- Groups of 5 male Swiss mice were killed and their stomachs removed. Following ligation of the gastro-duodenal and gastroesophageal junctions, 0.1 ml of 1.0 M sodium nitrite or 1.0 M sarcosine either alone or in combination were injected intraluminally and incubated at 37°C for 45 minutes. Following addition of 50 ml of 1M urea the stomachs were minced and activated charcoal and 0.5 ml of 1.04 M zinc sulfate were added (14). Following filtration the presence of nitrosarcosine was determined quantitatively by ultraviolet absorbtion. Thin layer chromatography was performed on Silicar with 100% ethanol as the mobile phase.

STATISTICS -- Kinetic determinations represent the results of at least 2 separate experiments, each of which contain at least 4 replicates per point. Regression analyses were performed on an Olivetti computer and regression coefficients always exceeded 0.99.

RESULTS

EFFECT OF pH ON SARCOSINE NITROSATION -- The rate of sarcosine nitrosation as a function of pH is shown in figure 1. The reaction is linearly related to hydrogen ion concentration over a pH range from 2.0 to 4.5.

KINETICS OF SARCOSINE NITROSATION -- The relationship between sarcosine concentration and nitrosation rate is first order (figure 2). With the sodium nitrite concentration constant at 50 mM and in the presence of 0.01N HCl buffer, the rate constant at 25°C was calculated as 3.24×10^6 $hr^{-1} \times mM^{-1}$. Although not shown in figure 2, when similar experiments were performed without the 0.01N HCl buffer the reaction was second order with respect to buffered sarcosine. The other degree of dependence arose because sarcosine was also the source of hydrogen ion.

In similar experiments, with the concentration of sarcosine constant at 50 mM, the reaction rate was first order with respect to sodium nitrite over a range of 10 to 50 mM (fig. 3). The rate constant at pH 2.0 was computed as 2.52×10^6 $hr^{-1} \times mM^{-1}$.

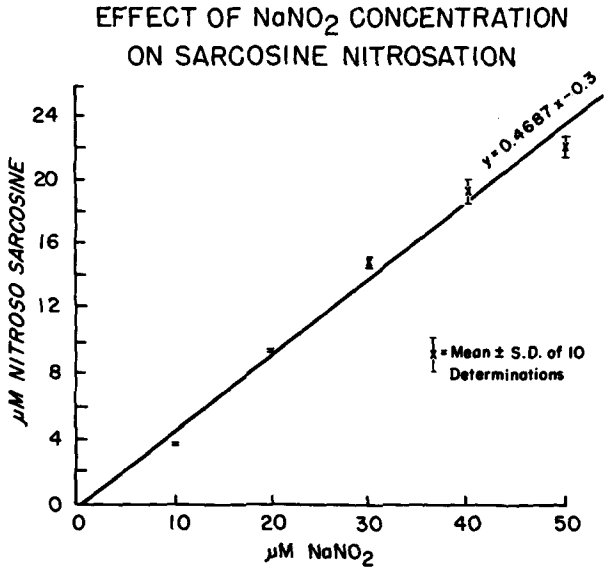

Figure 3 - Sarcosine (50 µM, pH 2.0) was incubated at 25°C with sodium nitrite at 10,20,30,40,or 50 µM. After 21 minutes, the nitroso-sar-osine concentration was determined by absorbency at 250 mµ.

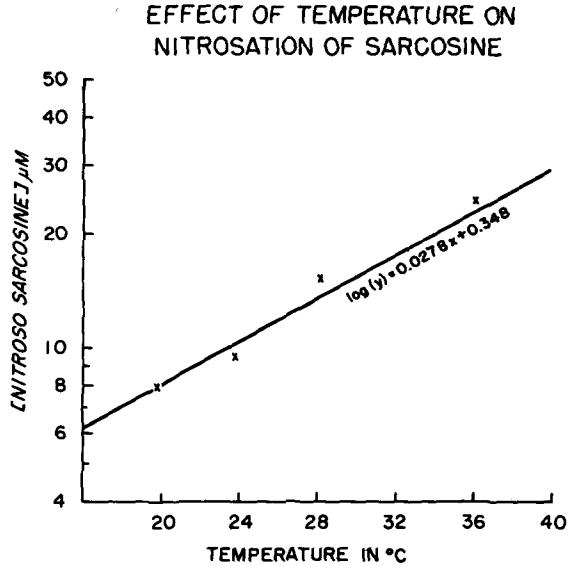

Figure 4 - Sarcosine and sodium nitrite (50 µM) at pH 2.0 were incubated for 21 minutes at 20, 24, 28, and 36°C. Nitroso-sarcosine was determined by absorbency at 250 mµ.

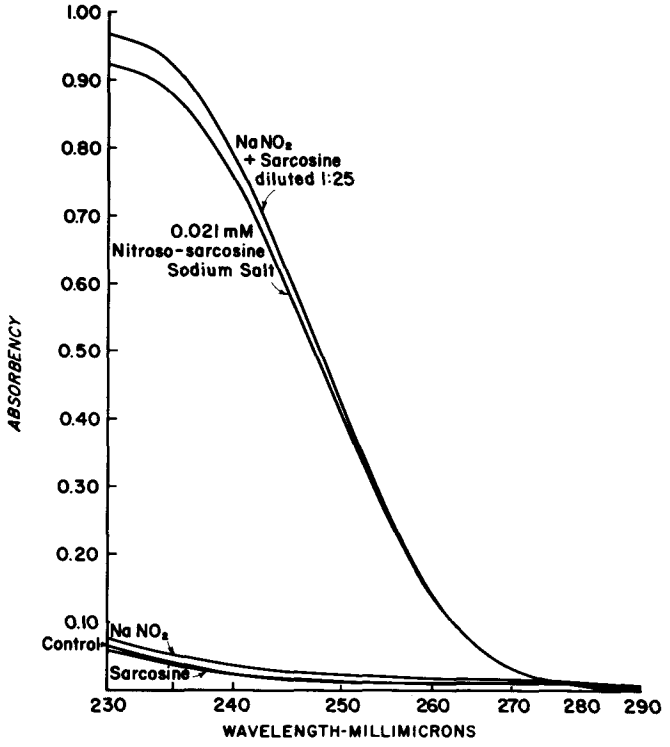

Figure 5 - Ultraviolet spectra of stomach extract from stomachs injected with sodium nitrite or sarcosine, either alone or in combination, or nitroso-sarcosine. Stomach contents extracted with activated charcoal and zinc sulfate.

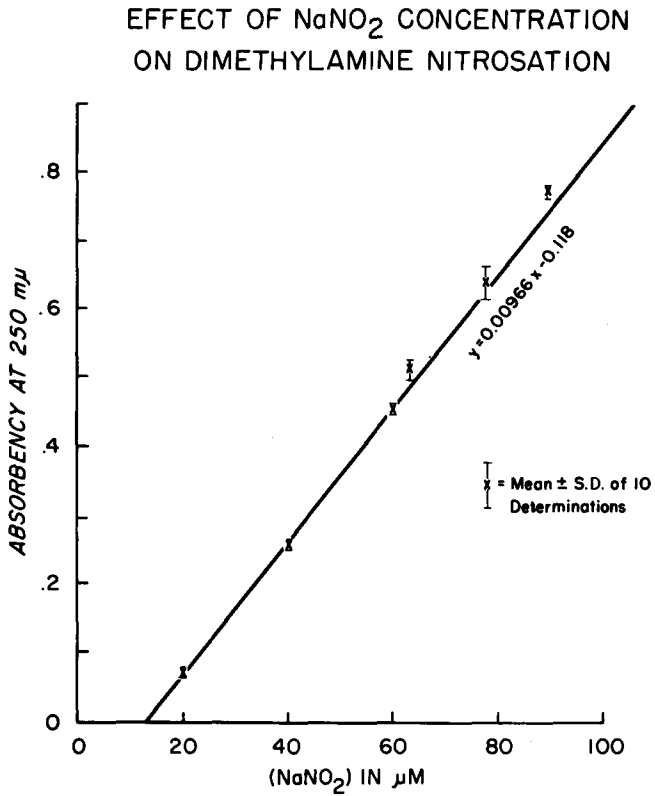

Figure 6 - Dimethylamine (100 μM, pH 2.0) was incubated for 30 minutes with 20,40,60,80, or 100 μM sodium nitrite. Following addition of urea and HCl absorbency at 250 mμ was determined.

The thermal response of sarcosine nitrosation is shown in figure 4. The reaction rate increased exponentially as a function of temperature. The reaction proceeded 2.6 times faster at 37°C than 24°C.

NITROSATION IN MOUSE STOMACH -- Stomachs from mice were injected intraluminally with 0.1 ml of 1 M sodium nitrite or sarcosine, either alone or in combination. Following extraction, the supernatant isolated from stomachs injected with both sodium nitrite and sarcosine contained material with a UV spectrum identical to nitroso-sarcosine (figure 5). No UV absorbing material was isolated from stomachs treated with sarcosine or sodium nitrite alone. The UV absorbing material also co-chromatographed on thin layer chromatography with nitroso-sarcosine. Following extraction, 109 μmol of nitroso-sarcosine were isolated from stomachs of 5 mice.

NITROSATION OF DIMETHYLAMINE -- As with sarcosine, the nitrosation of dimethylamine is linear with respect to sodium nitrite concentration (figure 6). Buffered dimethylamine (100 mM) was incubated with sodium nitrite for 30 minutes, prior to addition of urea. The absorbance increases linearly with sodium nitrite concentration over a range of 20 to 100 mM sodium nitrite.

DISCUSSION

The physical constants characterizing the nitrosation of sarcosine have been determined. The reaction is first order with respect to both sarcosine and sodium nitrite and may be represented mathematically as follows:

$$\frac{d[\text{nitroso-sarcosine}]}{dt} = k\,[\text{sarcosine}]\,[\text{sodium nitrite}]$$

At pH 2.0 and 25°C, k has been determined as 2.52×10^6 hr^{-1} x mM^{-1} and 3.24×10^6 hr^{-1} x mM^{-1} by varying sodium nitrite and sarcosine concentrations, respectively. Increasing the temperature to 37°C increases k by 2.6 fold. Additionally, k is linearly related to hydrogen ion concentration implying a 10 fold change for each pH unit.

Sarcosine nitrosation proceeds rapidly in mouse stomach. There was 22% conversion of sarcosine and sodium nitrite to nitroso-sarcosine in isolated mouse stomachs within 45 minutes.

Previous reports have indicated that dimethylamine nitrosation proceeds via second order kinetics with respect to sodium nitrite (15). Since sarcosine nitrosation proceeds via first order kinetics with respect to nitrite concentration, the response of dimethylamine nitrosation to sodium nitrite concentration was assayed. Under experimental conditions described here, this reaction, likewise, proceeds via first order kinetics with respect to nitrite concentration. In fact if the original data in reference 10 are plotted as rate vs. nitrite concentration, first order kinetics are observed under those conditions also.

The practical relevance of establishing first order kinetics with respect to each individual reactant is readily apparent. Laboratory data on nitrosation of secondary amines can be linearly extrapolated to humans. Synergistic toxicity and carcinogenicity data may likewise be extrapolated to humans.

The significance of nitroso-sarcosine as a human carcinogen is under active investigation. Nitroso-sarcosine produces esophageal tumors in rats at elevated levels (3). Esophageal cancer in man has been correlated with molybdenum deficiency in soil (16). Since molybdate is a cofactor in nitrite reductase, nitrate levels in plants in areas deficient in molybdenum are markedly elevated (16). Esophageal cancer has also been related to Norwegian populations who consume large quantities of salted fish (17). Additionally, nitroso-sarcosine may be formed from reaction of sodium nitrite with creatine (18). There is no estimate of dietary sarcosine intake. Average daily intake of sodium nitrite has been estimated at 1.4 mg per day. With these considerations the importance of assessing the potential hazard posed by nitroso-sarcosine must be underscored.

Acknowledgements

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