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Mutagenic Properties of Nitrosated Spermidine[1] (40145)

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Recent work on the etiology of gastric cancer and its precursor lesions has focused on the hypothesis of intragastric formation of mutagenic carcinogenic *N*-nitroso compounds (1). The hypothesis calls for ultimate (direct-acting) mutagens–carcinogens which may be formed in stomachs with chronic atrophic gastritis. Under such circumstances the gastric juice is consistently less acid than in people with normal mucosa, usually around or above pH 5 (2).

Many experiments of nitrosation of a variety of amines have been carried out to test this hypothesis but most of them have yielded products that require metabolic activation usually carried out with liver microsomes (3). Attempts to produce tumors of the glandular stomach in experimental animals with simultaneous feeding of amines and nitrite have not been successful; the tumors in such experiments appear mostly in the esophagus and the liver (4, 5).

Spermidine is found in corn–germ, fava beans, rice and barley, prominent items of the diet of populations at high risk to gastric cancer in Colombia (6–8). The same high-risk Colombian populations have an excess of nitrite in their drinking water (9). Besides the *N*-nitroso compound of spermidine, spermidine itself has been observed to have a high affinity for nucleic acids (7). These findings prompted us to study the mutagenic properties of nitrosated spermidine.

Materials and methods. Nitrosation reaction. All nitrosation reactions were conducted at 37° for 10 min in Erlenmeyer flasks with shaking. To 125 mg of spermidine trihydrochloride in 5 ml of 0.1 *M* citrate buffer at pH 4.2, 105 mg crystalline sodium nitrate was added except where noted otherwise. This solution, containing 0.1 *M* spermidine and 0.3 *M* sodium nitrite, was sealed with parafilm and tested for mutagenicity at the end of 10 min incubation at 37°. The product mixture was a clear and homogeneous solution with a yellowish color.

The effect of thiocyanate (SCN) as a nitrosation catalyst for spermidine was studied by adding 0.1 ml of a 1.2 *M* solution of potassium thiocyanate to some reaction mixtures.

Mutagenicity. The mutagenicity test as developed and described by Ames *et al.* (10) was employed to study the mutagenic properties of the nitrosated products of spermidine. The reaction products were initially tested on strains TA1535, TA100, TA1530, TA98, and TA1537. Positive results were obtained with strains TA1535 and TA100 only. Strain TA1535 was chosen as the test organism for the remainder of the study because the background was lower (5–30 colonies) than that of TA100 (100–130 colonies). The cells were grown as described by Ames *et al.* (3), except that Brain Heart Infusion (Difco) was used instead of nutrient broth.

A 20 μl aliquot of the reaction mixture was preincubated with bacterial cells in 0.1 *M* phosphate buffer at pH 7.4 for 20 min and then deposited in a soft agar overlay on minimal medium according to the procedure of Sugimura *et al.* (11). Revertant colonies observed on the plates after 48 hr of incubation at 37° were reported as the number of colonies (colony forming units, CFU) per 20 μl of the reaction mixture. The S9 microsomal extract was prepared from the livers of Wistar rats which had been induced with Aroclor 1254, as described by Ames *et al.* (3). S9 extract was added to the preincubtion mixture as described by Sugimura *et al.* (11).

In order to test the viability of the mutagenized cells, an aliquot was diluted appropriately and spread on the surface of Brain Heart Infusion agar (Difco), and the number of colonies was noted after 18 hr incubation. No loss of viability was evident in any of the experiments reported in this paper. The bac-

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terial strains used were a gift of B.N. Ames.

pH Optimum. Citrate buffers (0.1 M) of differing pH values were used to determine the pH at which nitrosation of spermidine yielded a maximum mutagenic response. The range of pH values (2.2–6.8) used in the experiment corresponds to the effective range of Sorenson's citrate buffer (12). In all cases, after addition of the sample aliquot to the cell suspension in pH 7.4 phosphate buffer, the pH remained above seven and no loss of viability occurred.

Results. Nitrosation of spermidine with and without thiocyanate was conducted several times under the conditions described. The mutagenic activity of the nitrosated products from spermidine is shown in Table I. Spermidine and SCN produced no mutagenic effect when present alone or in combination. The mutagenic activity of nitrite alone was about 0.02 mutations per n*M*, in reasonable agreement with that observed by McCann *et al.* (13). The combination of nitrite and spermidine was synergistic, producing 6.7 times more mutations than do the separate components, after subtracting the background value.

Dose response curve. When varying amounts of the reaction mixture were incubated with the bacteria, a positive dose-response relationship was found (Fig. 1). The level of mutagenic activity was approximately 0.2 mutations per n*M* of spermidine added to the reaction mixture.

pH dependence. The effect of pH on the production of mutagenic compounds by the reaction of nitrite with spermidine was determined in the presence and absence of thiocyanate. Figure 2 shows the results of one of the experiments and indicates that the extent of formation of mutagenic compounds is dependent upon the pH of the medium, with an

TABLE I. NUMBER OF COLONY FORMING UNITS (CFU) FROM 20 μl OF REACTION MIXTURE.

Added components	CFU	± SD
None	16	± 8
SCN[a]	18	± 3
Spermidine[c]	13	± 2
NO_2[b]	57	± 13
SCN + NO_2	84	± 14
SCN + Spdn.	20	± 9
NO_2 + Spdn.	290	± 100
NO_2 + Spdn. + SCN	338	± 116

[a] SCN = thiocyanate.
[b] NO_2 = nitrite.
[c] Spdn. = spermidine.

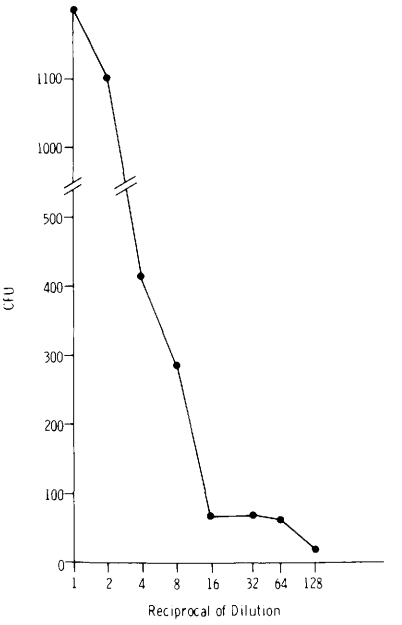

FIG. 1. Dose response curve. A reaction mixture containing 0.4 *M* spermidine and 1.2 *M* sodium nitrite was prepared as described in materials and methods. The reaction mixture was serially diluted two-fold into 0.1 *M* citrate buffer (pH 4.2) and 20 μl of each dilution was tested for mutagenic activity. The number of CFUs is plotted as a function of the reciprocal of dilution.

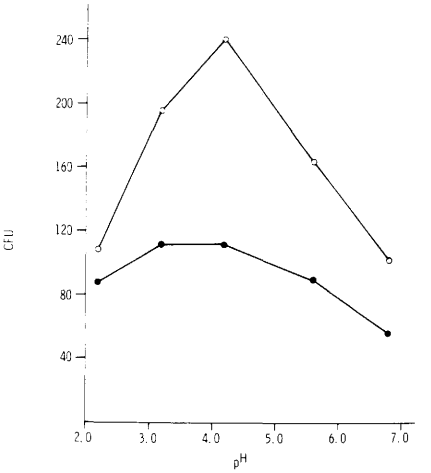

FIG. 2. Effect of thiocyanate on mutagenesis at various pH values. The mutagenic activity obtained at each pH with thiocyanate (○——○) and without thiocyanate (●——●) is plotted as the number of CFUs per 20 μl of reaction mixture.

optimum around four. A significant level of mutations is seen at all pH levels tested with SCN. However, the extent of the catalytic effect of SCN tested by mutagenicity was noted to be variable from one experiment to another. The low mutagenic activity of the experiment in Fig. 2 illustrates this variability. Data from this early experiment was not included in Table I.

Microsomal extract. The enzymatic activation was tested by the addition of varying quantities of S9 microsomal extract. The effect of the microsomal extract on mutagenic activity was dependent on the amount of extract employed, but no more than a 20% stimulation of activity was noted with any concentration of extract.

Inhibition of ascorbic acid. The mutagenic activity of the reaction mixture was inhibited by the addition of L-ascorbic acid in crystalline form to the reaction mixture prior to the addition of nitrite (14). The results of this experiment are shown in Fig. 3. The mutagenic activity was plotted as a per cent of activity detected without ascorbic acid. It declines with increasing amounts of ascorbic acid up to approximately 100 mg.

Ascorbic acid is an effective inhibitor of the mutagenic activity produced in the reaction mixture, whether added before or after the other reagents. The average data from three experiments shows 77% inhibition (SD ± 7) when 100 mg of ascorbic acid was added before the other reagents and 67% inhibition (SD ± 15) when added afterwards.

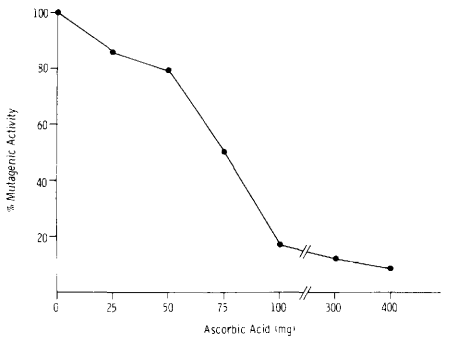

FIG. 3. Effect of ascorbic acid on mutagenesis by nitrosated spermidine. 125 mg spermidine trihydrochloride and 105 mg sodium nitrite incubated in water bath shaker at 37° for 10 min in 5 ml 0.1 *M* citrate buffer of pH 4.2 with varying amounts of sodium ascorbate. Per cent of mutagenic activity plotted as per cent activity detected without ascorbic acid.

Discussion. Our experiments suggest that the nitrosated spermidine model fits many of the requirements of the hypothesis on the causation of gastric cancer: (a) it yields a direct-acting mutagen; (b) it has an optimum pH range very similar to what is found in gastric juice of human populations at high risk to gastric cancer, as a result of chronic atrophic gastritis; (c) its mutagenicity may be enhanced by thiocyanate ions, present in human gastric juice (15); (d) spermidine is present in items prominent in the diet of populations at high gastric cancer risk. High nitrite levels have been found in gastric juice of people with atrophic gastritis and elevated pH in Colombian high-risk populations (15).

The inhibition of mutagenesis by ascorbic acid in our spermidine model further fits the human situation, given the ascorbic acid deficiency reported from Colombian high-risk population (16). Further investigation of this inhibitory role of ascorbate and other compounds is indicated since they offer hope for prevention of the disease.

Our original experiments were based on the assumption that the role of ascorbate would be to prevent the formation of mutagens through its interaction with the nitrite molecules. We then become aware of the work of Guttenplan (17) and the possibility that ascorbate may interfere with the activity of preformed mutagens. We then proceeded to add ascorbate to our reaction mixtures after spermidine, thiocyanate and nitrite had had time to interact. We found that 100 mg of ascorbate added to the reaction mixture 10 min after the addition of nitrite, spermidine and thiocyanate, resulted in a similar degree of inhibition of the mutagenesis observed when the ascorbate was added before the other components.

The optimum pH range found by us in the spermidine model agrees with the hypothesis of Thomas *et al.* (18) to the effect that the mutagen may be a triazene which becomes unstable when there is an increase in the H+ ions.

Summary. The nitrosation of spermidine results in direct-acting mutagens as shown by the Ames assay utilizing *S. typhimurium* TA1535. The mutagenic effect is catalyzed by thiocyanate and inhibited by ascorbic acid.

The reaction takes place at pH ranges 3.0–6.0. The possibility that this reaction may play a role in human gastric cancer etiology is discussed.

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