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pH-Dependent Degradation of Nitrosocimetidine and Its Mechanisms

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The degradation of nitrosocimetidine (NC) and its mechanism were found to be strongly dependent upon pH by monitoring NC and its degradation products by high-performance liquid chromatography (HPLC). NC was relatively stable at neutral pH, but it degraded rapidly under both acidic and alkaline conditions. In a strongly acidic solution, the degradation was shown to be entirely by denitrosation, but under alkaline conditions scarcely any denitrosation was observed and various other degradation products were found. At neutral pH, both these degradation mechanisms were observed. In neutral solution, the presence of thiol compounds greatly shortened the half life of NC, and enhanced its denitrosation. The high degradation rate in acidic solution, the strong influence of thiol groups, and the preference of denitrosation at pH 0.3–5 can explain the discrepancy between the *in vitro* genotoxicity and the lack of carcinogenicity of NC.

Key words: Nitrosocimetidine — Denitrosation — pH-Dependent degradation — Thiol effect

Cimetidine (CIM) is a drug that is widely used for therapy of gastric[1] and duodenal[2] ulcres. As a histamine H_2-receptor antagonist, it inhibits gastric acid secretion,[3] and consequently causes an increase in the pH of the stomach. This results in bacterial growth and higher activity of nitrate reductase[4] which in turn may increase the gastric content of nitrite. Thus the possibility of intragastric nitrosation of CIM has been pointed out. The structure of nitrosocimetidine (NC) is related to that of N-methyl-N′-nitro-N-nitrosoguanidine (MNNG), a potent stomach carcinogen in experimental animals and both are classified as nitrosamidines. Therefore two questions arose shortly after the introduction of this drug: 1) Is NC actually formed *in vivo* from CIM?, and 2) is NC carcinogenic? NC can be formed *in vitro* by the reaction of CIM with nitrite and its yield is 80–300 times higher at pH 1 than at pH 4.[5] Genotoxicity of NC was clearly shown in various *in vitro* assys[6–12] but NC exhibited very low acute toxicity in F344 rats.[13] Moreover, it was found not to be carcinogenic in several animal tests. Its administration in the drinking water to F344 rats,[14] by gavage to Sprague-Dawley rats,[15] or in the drinking water to (C57BL/6×BALB/c)F_1 mice from pre-conception to adult life[16] all failed to induce tumors. Denitrosation of NC catalyzed by a thiol group has been proposed to explain the discrepancy between the biological activities of this compound under *in vitro* and *in vivo* conditions.[17, 18]

This paper reports the pH-dependent degradation of NC and the effect of various sulfur compounds on its degradation rate. The results supported the proposed interpretation of the discrepancy between the *in vitro* genotoxicity and the lack of carcinogenicity of NC.

Materials and Methods

Chemicals NC was synthesized by nitrosating CIM (Sigma Chemical Co., St. Louis, MO) by the protocol of Foster *et al.*,[19] using sodium nitrite in 2*N* HCl. Analytical data of the synthesized NC (m.p., and UV, mass and NMR spectra) were consistent with data reported in the literature.[5, 19] The material gave only one spot on a thin layer chromatogram (Silica gel; $CHCl_3$/methanol=7/3) and its purity was estimated by HPLC to be more than 98%. All other chemicals used were obtained commercially (Wako Pure Chemical Industries, Osaka; Tokyo Kasei Industries, Tokyo; Cica-Merck, Tokyo/Darmstadt; Sigma Chemical Co.) and were of the highest purity available.

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HPLC HPLC was performed using a Shimadzu LC-6A pump, equipped with a Rheodyne 7125 injector, a Toyo Soda UV-8000 detector and a Shimadzu C-R1B Chromatopac integrator. An RP18 ODS $80T_M$ column (250×4.6 mm) from Toyo Soda was used and the eluent was a mixture of 0.1*M* phosphate buffer (pH 7.2) and acetonitrile (4:1, v/v). The injection volume was usually 5 μl, the flow rate 1.0 ml/min, and the detection wavelength 220 nm.

Procedures A solution of 2.8 mg (10 μmol) of NC in 1.0 ml of methanol was diluted to 10.0 ml (1m*M* NC) with various buffer solutions and kept at room temperature (25°). At intervals, aliquots of 5 μl were subjected to HPLC to monitor the rates of disappearance of NC and formation of CIM. The peak area of CIM was corrected by the factor 0.65, since the molar extinction at $\lambda=220$ nm ($\varepsilon=21500$) differ from that of NC ($\varepsilon=14000$). From the logarithms of the areas, linear regression curves were calculated and the half lives of NC under various conditions were estimated.

At pH 0.3 (1*N* HCl solution) the degradation of NC was too fast to measure by this method, and so samples (100 μl) of the acidic NC solution were taken at intervals, mixed with 1 ml of phosphate buffer (pH 7) in order to stop the rapid degradation and then subjected to HPLC analysis. For the study of the influence of sulfur compounds on the degradation of NC, the methanolic NC solution (1 ml) was mixed with an 11m*M* solution of the test sulfur compound (9 ml) in buffer. All experiments were performed in duplicate or triplicate.

Results

As shown in Table I, the rate of degradation of NC varied greatly with the pH value. At room temperature (25°), NC was stable around pH 7 but decomposed rapidly under both acidic and alkaline conditions. However, its degradation processes under acidic and alkaline conditions were quite different. The HPLC chromatograms in Fig. 1 show that under acidic conditions, CIM was produced in high yield, whereas under alkaline conditions some other degradation products were

Table I. Degradation of Nitrosocimetidine[a] at Room Temperature (25°) at Different pH Values

pH	$t_{1/2}$
0.3[b]	7.2±1.1 min
1	2.7±1.0 hr
3	3.3±1.1 d
5	12.4±0.4 d
7	9.3±2.3 d
9	12.7±2.5 hr

a) The initial concentration of NC was 1m*M*.
b) 1*N* HCl solution.

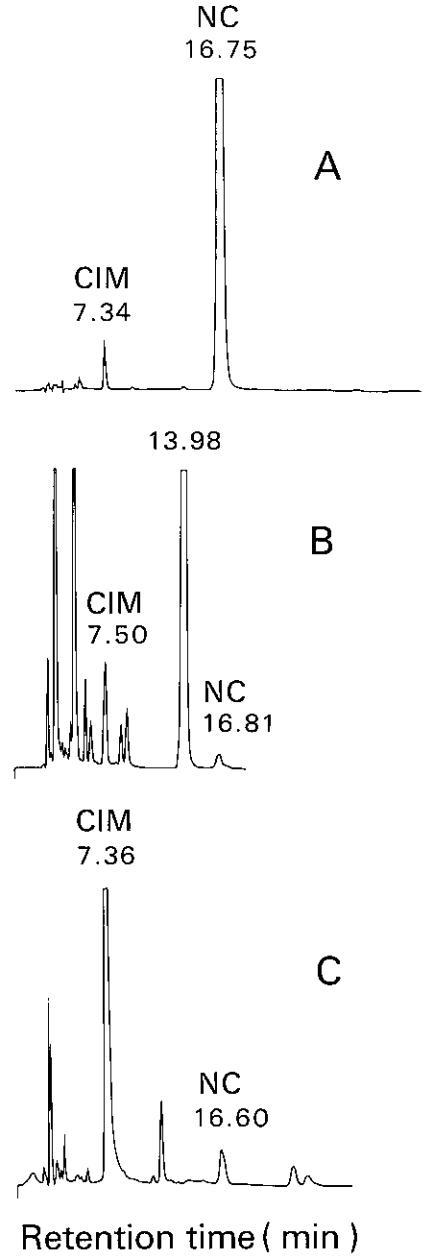

Fig. 1. HPLC chromatograms of NC (1m*M*) at pH 9 initially (A) and after 1 week (B), and at pH 1 after 24 hr (C). HPLC conditions are described in "Materials and Methods."

formed with scarcely any CIM. At pH 5 and pH 7, CIM was found together with other degradation products (for pH 7, see A in Fig. 2). For examination of the quantitative relationship between the rate of degradation of NC and denitrosation at different pH values, the normalized amounts of NC and CIM were plotted against time. Figure 3 shows the fast degradations of NC at pH 1 and pH 9. At pH 1, the increase of CIM corresponded quantitatively to the decrease of NC, whereas at pH 9 only a marginal amount of CIM was found. The time courses for pHs 3, 5 and 7 are plotted in Fig. 4. With decrease in the amount

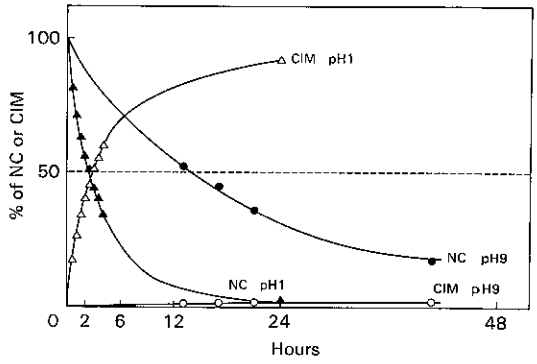

Fig. 3. Time courses of NC degradation (closed symbols) and CIM formation (open symbols) at pH 1 (triangles) and pH 9 (circles).

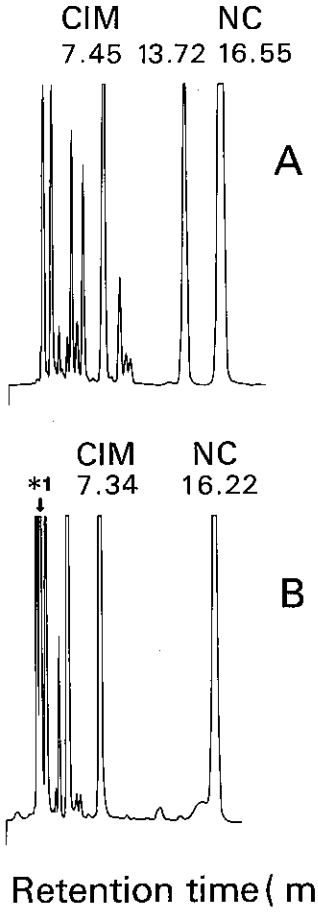

Fig. 2. HPLC chromatograms of NC (initially 1 m*M*) at pH 7. After 9 days in the absence of NAC (A) and after 2.5 hr in the presence of 10m*M* NAC (B). *1 Peak of NAC.

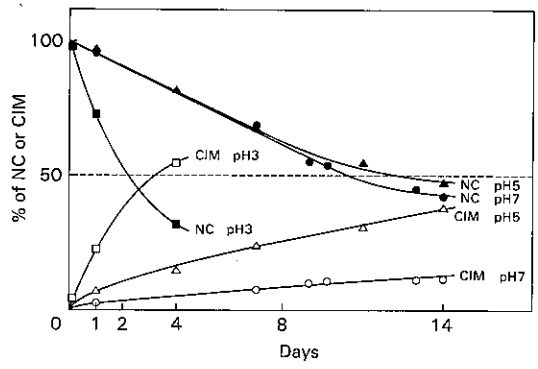

Fig. 4. Time courses of NC degradation (closed symbols) and CIM formation (open symbols) at pH 3 (squares), pH 5 (triangles) and pH 7 (circles).

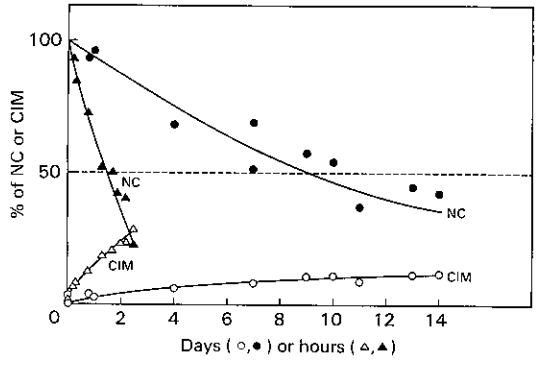

Fig. 5. NC degradation (closed symbols) and CIM formation (open symbols) at pH 7 in the absence of NAC (circles; time scale in days) and in the presence of 10m*M* NAC (triangles; time scale in hours). Points are averages of values in at least two experiments.

Table II. Effects of Various Sulfur Compounds on the Degradation of Nitrosocimetidine[a] at pH 7

Sulfur compound[b]	$t_{1/2}$
None	9.3±2.3 d
Cysteine	17.7±3.1 min
N-Acetylcysteine	95.5±5.7 min
Glutathione	34.0±1.4 min
Thioproline	1.9±1.0 d

a) The initial concentration of NC was 1m*M*.
b) The initial concentration of the sulfur compounds was 10m*M*.

of NC, a concomitant increase in the amount of CIM was observed. At pH 3, only small amounts of degradation products other than CIM were detected. At pH 5, the rate of CIM production was less than that of degradation of NC due to the formation of other minor degradation products, and at pH 7 degradation products other than CIM were mainly formed.

Figure 2 shows a comparison of the HPLC chromatograms of NC in phosphate buffer solution (pH 7) after 9 days (Fig. 2A) and the same buffer solution containing N-acetylcysteine (NAC) after 2.5 hr (Fig. 2B). In the absence of NAC, CIM as well as other degradation products were observed (Fig. 2A). In the presence of NAC (Fig. 2B), CIM formation was predominant. The effects of NAC on the time courses of NC disappearance and CIM formation are shown in Fig. 5. At pH 7, CIM formation was only 12% at 14 days in the absence of NAC, whereas it was 23% at 2 hr and 38% at 26 hr in the presence of NAC. Thus, thiol compounds also markedly stimulated denitrosation of NC.

Table II shows the effects of cysteine, NAC, glutathione and thioproline (thiazolidine-4-carboxylic acid) on the rate of degradation of NC at pH 7. With a 10-fold excess of these sulfur compounds, the degradation rate was strongly increased by compounds with a free thiol group, but only slightly by thioproline, which is a thioether and an effective nitrite trapping agent in the human body.[20] On addition of an equimolar amount of cysteine, degradation of NC was initially rapid but then became similar to that in the absence of cysteine, probably because cysteine was oxidized to cystine,[21] which did not increase NC degradation.

Discussion

The values for the degradation rate of NC obtained at various pHs were compatible with those obtained by Jensen and Magee[17] by measurement of the rate of disappearance of the UV-chromophore at $\lambda=390$ nm. Our experimental results indicate that there is a 29-fold difference between the half-life at pH 1 ($t_{1/2}=2.7$ hr) and pH 3 ($t_{1/2}=79.2$ hr) (Table I). In the human stomach, where the pH may be assumed to be 1–3, NC degradation is probably concurrent with formation of NC, because the nitrite level is very low (0.4–60 μM)[22] compared with the initial CIM concentration after intake of the usual amount (200 mg/dose) of this drug. If the stomach pH is increased by cimetidine treatment, the rate of NC degradation should decrease. In addition, the extent of NC formation also decreased in a model experiment using equimolar amounts of CIM and nitrite.[5]

By use of HPLC for monitoring the degradation of NC, we observed different pathways of degradation in acidic and alkaline solutions. Denitrosation occurred not only under strongly acidic conditions, but also to some extent at pH 5 and pH 7. Other degradation products were also formed apparently at alkaline pH and also in smaller amounts at pH 5 and 7. The degradation under alkaline conditions is probably initiated by nucleophilic attack of a hydroxide anion on the proton at the guanidine moiety of NC, which results in liberation of a methyldiazohydroxide.[23]

Tests of mutagenicity and *in vitro* cell damage by NC have all been performed in the pH range of 6.8 to 7.4. Under neutral pH conditions, degradation of NC may produce more of a methylating species than of a denitrosated one. However at pH 1–3, as in the human stomach, degradation of NC — if it is formed — should be almost exclusively by denitrosation and would therefore result in detoxification. Between pH 3 and 5, denitrosation is still the main route of degradation.

Inoue *et al.*[12] showed that induction of sister-chromatid exchanges by NC in cultured human lymphocytes was inhibited in the presence of thiol compounds such as cysteine. In our experiments, thiol compounds markedly increased the rate of NC degradation, causing

a 140- (NAC) to 740- (cysteine) fold decrease in NC half life at pH 7 (Table II). But thioproline, a thioether compound, was much less effective, with only a 5-fold decrease in NC half-life. This thiol-accelerated degradation of NC essentially involves two reaction pathways, denitrosation and formation of a methylating species.[18]

Denitrosation of NC in the presence of NAC occurred much faster and more effectively than in the absence of NAC, as shown in Fig. 5. Schulz and McCalla[21] reported that nucleophilic attack by the thiol group of cysteine on the nitroso group of N-methyl-N-nitroso-*p*-toluenesulfonamide resulted in denitrosation to form S-nitrosocysteine (which converted to cystine via a free radical mechanism) and N-methyl-*p*-toluenesulfonamide almost selectively in 25% ethanolic aqueous solution. Our experimental results revealed that a thiol group, rather than a thioether like thioproline, is important for effective denitrosation catalysis though thioproline has been shown to be an effective nitrite trapping agent in the human body.[20] Jensen has proposed a similiar mechanism for the thiol-accelerated denitrosation of NC and MNNG.[18] However, denitrosation of MNNG in the presence of thiol compounds was shown to be much less effective compared with that of NC in parallel experiments.

Another degradation pathway of NC at neutral pH stimulated by thiol compounds is the formation of a methylating species such as methyldiazohydroxide via nucleophilic attack by the thiol group on the imino carbon of NC.[18] This pathway is related to the genotoxicity of NC via DNA methylation. However, a certain amount of methylating species thus formed must be trapped by excess thiol groups[19, 21] before reacting with DNA and probably resulting in a reduction of genotoxicity.[12] It is also well documented that the DNA methylating capability of MNNG increases in the presence of thiol compounds,[18, 21, 23] whereas it has been reported that the methylating capability of NC is decreased in the thiol-accelerated NC degradation.[17–19] In the stomach of humans and animals, thiol compounds are present in gastric mucosa[24] or supplied as food components. These thiol compounds may stimulate the detoxification of NC genotoxicity.

In conclusion, the almost complete selective denitrosation of NC under stomach pH conditions and the acceleration by thiols of denitrosation in NC degradation provide an explanation for the discrepancy between the *in vitro* genotoxicity and the lack of carcinogenicity of NC.

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