

[Chem. Pharm. Bull.]  
[34(9)3866—3872(1986)]

## Identification of Mutagenic Nitrosation Products of Antipyrine and Evaluation of Their Formation under Model Stomach Conditions

TAKAFUMI OHTA,\* YOSHIHIRO ASABE and SHOJI TAKITANI

*Faculty of Pharmaceutical Sciences, Science University of Tokyo, 12, Ichigaya  
Funagawara-machi, Shinjuku-ku, Tokyo 162, Japan*

(Received February 20, 1986)

Nitrosation products of antipyrine treated with nitrite under the conditions recommended by the WHO were examined. An unknown product was confirmed to be formed besides 4-nitrosoantipyrine (a known nitrosation product), and was isolated and identified as 1-(2-hydroxyimino-3-oxobutyl)-2-methyl-2-nitroso-1-phenylhydrazine.

This new nitrosation product showed mutagenicity to *Salmonella typhimurium* TA 98 and TA 100 either with or without S9 mix. Model experiments indicated that antipyrine is probably easily nitrosated in the stomach after administration to give these nitrosation products.

**Keywords**—antipyrine; nitrite; nitrosation product; mutagenicity; 1-(2-hydroxyimino-3-oxobutyl)-2-methyl-2-nitroso-1-phenylhydrazine; 4-nitrosoantipyrine; 4-nitroantipyrine

Many drugs administered orally to humans have been shown to react with nitrite in acidic solution to give carcinogenic and/or mutagenic compounds. Rao and Krishna<sup>1)</sup> reported that antipyrine (ANP), a pyrazolone analgesic, reacted with nitrite to give 4-nitrosoantipyrine (ANP-NO) or 4-nitroantipyrine (ANP-NO<sub>2</sub>). Arisawa *et al.*<sup>2)</sup> confirmed the mutagenicity of ANP-NO, and suggested that an unknown product besides ANP-NO was present in the reaction mixture of ANP and nitrite. Although the reaction was carried out under conditions similar to those used by Rao and Krishna,<sup>1)</sup> they did not mention the formation of ANP-NO<sub>2</sub>. There thus seems to be uncertainty regarding the nitrosation products of ANP.

In the present study, ANP was allowed to react with nitrite under the conditions recommended by the WHO, and the reaction products were examined. A new mutagenic compound (X) besides ANP-NO was found and identified. The formation of these compounds was also examined under conditions presumed to resemble those in the stomach.

### Materials and Methods

**Chemicals**—ANP used was of J. P. grade. ANP-NO and ANP-NO<sub>2</sub> were synthesized by the methods of Eisenstaedt<sup>3)</sup> and Knorr,<sup>4)</sup> respectively. 3-Hydroxyimino pentanedione was synthesized by the method of Yamada *et al.*<sup>5)</sup> All other chemicals used were of analytical grade.

**Spectroscopic Measurements**—Proton nuclear magnetic resonance (<sup>1</sup>H-NMR) and carbon-13 nuclear magnetic resonance (<sup>13</sup>C-NMR) spectra were measured with a JEOL JNM FX-100 spectrometer equipped with a dual probe for <sup>1</sup>H and <sup>13</sup>C nuclei operating at 99.6 and 25.0 MHz, respectively; chemical shifts are given on the ppm scale with tetramethylsilane as an internal standard (s, singlet; d, doublet; q, quartet). Mass spectra (MS) were measured with a Hitachi M-80 double-focusing spectrometer equipped with an electron impact (EI) ion source with a direct inlet, operating at 70 eV.

**Chromatographic Analysis**—High-performance liquid chromatography (HPLC) was carried out with a Shimadzu LC-5A equipped with a Zorbax ODS column (4 × 250 mm) and a ultraviolet (UV) detector. The mobile phase used was methanol–0.01M phosphate buffer (pH 7.5) (1 : 1, v/v). The flow rate was maintained at 1.0 ml/min, and the effluent was monitored at 290 nm.

Thin-layer chromatography (TLC) was carried out on pre-coated silica-gel plates (GF<sub>254</sub>, E. Merck) with

chloroform–dioxane (4:1, v/v) (I) and ethyl acetate–acetone (4:1, v/v) (II) as solvents.

**Reaction of ANP with Nitrite and Product Analysis**—(a) The WHO Conditions<sup>6)</sup>: An aqueous solution (100 ml) containing ANP (10 mM) and sodium nitrite (40 mM) was adjusted to pH 3.5 with 3 M HCl, and incubated at 37 °C for 1 or 4 h in the dark. An aliquot of this reaction mixture was subjected to TLC and HPLC.

(b) Conditions Simulating Those in the Stomach: One hundred milliliters of 0.1 M acetate–0.1 M HCl buffer containing 60 mg of ANP (3.2 mM) and 10 or 50 ppm of nitrite (0.145 or 0.725 mM) was incubated in the dark at 37 °C. This ANP concentration was decided on the basis of the usual dose and stomach content as described later. After a defined period, 5  $\mu$ l aliquots of this reaction mixture were subjected to HPLC, and the amounts of ANP–NO formed and ANP remaining were determined. At the same time, 5-ml aliquots withdrawn from the reaction mixture were mixed with 0.5 ml of 0.6% ammonium sulfamate and extracted with ether (2  $\times$  5 ml). The ether layer was evaporated to dryness under reduced pressure, and the residue was dissolved in 0.5 ml of the HPLC mobile phase. An aliquot (5  $\mu$ l) of this solution was subjected to HPLC, and the amount of X formed was determined.

**Isolation and Identification of X**—An aqueous solution (200 ml) containing ANP (50 mM) and sodium nitrite (200 mM) was adjusted to pH 3.5 and incubated at 37 °C for 1 h in the dark. The reaction was stopped by the addition of 5 g of ammonium sulfamate, and the resulting mixture was filtered and extracted with ether (2  $\times$  100 ml). The ether layer was evaporated to dryness under reduced pressure. The residue was dissolved in a small amount of hot benzene, and the solution was allowed to stand to precipitate crude X (430 mg). Recrystallization was done from benzene in the same manner (yield: 340 mg), mp 119 °C. *Anal.* Calcd for  $C_{11}H_{12}N_4O_4$ : C, 50.00; H, 4.58; N, 21.20. Found: C, 50.11; H, 4.51; N, 21.61. MS: the parent ion was not detected; fragments at  $m/z$ : 234 ( $M^+ - NO$ ), 121 ( $C_6H_5NH - NCH_3$ ), 77 ( $C_6H_5$ ), 43 ( $COCH_3$ ). IR (KBr): 3170 (hydrogen-bonded OH), 1700 and 1660 (C=O), 1490 (C=N), 1470 (NO)  $cm^{-1}$ . IR ( $CHCl_3$ ): 3540 (free OH), 3200 (hydrogen-bonded OH)  $cm^{-1}$  (IR ( $CHCl_3$ ) of 3-hydroxyiminopentanedione: 3560 (free OH), 3200 (hydrogen-bonded OH)  $cm^{-1}$ ). UV  $\lambda_{max}^{CHCl_3}$  nm ( $\epsilon$ ): 365 (140). UV  $\lambda_{max}^{methanol-0.01\ M\ phosphate\ buffer\ (pH\ 7.5)\ (1:1,\ v/v)}$  nm ( $\epsilon$ ): 288 (11600), 371 (1060).  $^1H$ -NMR ( $CDCl_3$ ) $\delta$ : 2.10, 2.14, 2.33 and 2.44 (total 3H, each s, C–CH<sub>3</sub>), 3.11, 3.42, 3.80 and 4.07 (total 3H, each s, N–CH<sub>3</sub>), 7.30 and 7.40 (5H, aromatic H), 10.33 and 10.50 (total 1H, each s, exchangeable with D<sub>2</sub>O, =N–OH) (Fig. 3).  $^{13}C$ -NMR ( $CDCl_3$ ) $\delta$ : 25.1 and 25.4 (each q, C–CH<sub>3</sub>), 35.1 and 39.1 (each q, N–CH<sub>3</sub>); 125.2 and 127.2 (each d, phenyl C-2, 6), 127.7 and 129.1 (each d, phenyl C-4), 129.6 and 130.7 (each d, phenyl C-3, 5), 135.7 and 137.2 (each s, phenyl C-1), 151.1 and 151.4 (each s, HO–N=C); 160.1 and 162.1 (each s,  $C_6H_5-N-C=O$ ), 194.3 and 194.4 (each s,  $CH_3-C=O$ ).

**Mutation Test**—X was dissolved in dimethyl sulfoxide, and its mutagenicity was tested by the method of Maron and Ames,<sup>7)</sup> including preincubation (37 °C, 20 min) with *Salmonella typhimurium* TA 98 and TA 100. Three replicate plates were used for each dose.

S9 was prepared from polychlorinated biphenyl (PCB)-treated male rats, and 50  $\mu$ l of S9 was used per plate.

## Results

### Analysis and Identification of Nitrosation Products

TLC and HPLC of the mixture obtained by the reaction of ANP with nitrite under the WHO conditions showed that ANP–NO ( $R_f$ =0.06 with I, 0.10 with II;  $t_R$ =3.4 min) and an unknown product (X:  $R_f$ =0.58 with I, 0.81 with II;  $t_R$ =3.0 min) were formed in this reaction, and that the latter is clearly different from ANP–NO<sub>2</sub> ( $R_f$ =0.12 with I, 0.32 with II;  $t_R$ =3.6 min). Since the concentration of nitrite is four times that of the drug under the WHO conditions, the reaction of ANP with equimolar or 1/10 molar concentration of nitrite was also examined by TLC. ANP–NO was detected under both conditions, while X was detected only with the equimolar concentration of nitrite. ANP–NO<sub>2</sub>, however, was not formed under these conditions. A similar experiment at pH 2.0 also gave no ANP–NO<sub>2</sub>.

The product X was isolated from the reaction mixture. Elemental analysis of X gave a molecular formula of  $C_{11}H_{12}N_4O_4$ , indicating that one molecule of HNO<sub>2</sub> was incorporated into an ANP–NO molecule. In fact, X was ascertained to be formed from ANP–NO treated with nitrite in acidic solution. The molecular ion was not detected by mass spectrometry, while a fragment of  $C_{11}H_{12}N_3O_3$  (234.0882 Calcd 234.0876), which could arise from loss of the NO group, was detected.

The infrared (IR) spectrum showed two absorption bands at 1700 and 1660  $cm^{-1}$  due to two carbonyl groups, and a broad absorption band at near 3200  $cm^{-1}$ , which was in good agreement with that of 3-hydroxyiminopentanedione measured at the same time, suggesting the presence of an intramolecularly hydrogen-bonded hydroxyl group.<sup>8)</sup>

The  $^1\text{H}$ -NMR spectrum (Fig. 1) showed one hydroxyl proton, five aromatic protons and six protons due to two methyl groups. Each of these two methyl groups gave signals that consisted of four singlets, indicating that at least four conformers and/or tautomers should be present in this solution.

The  $^{13}\text{C}$ -NMR spectrum also showed two signals for each of *C*-methyl carbon, *N*-methyl carbon, aromatic carbons, oxime carbon and two carbonyl carbons.

The above-mentioned results suggested that **X** was formed from ANP-NO by nitrosation and hydrolytic cleavage of the pyrazolone ring between the 2 and 3 positions, and that its structure is 1-(2-hydroxyimino-3-oxobutyl)-2-methyl-2-nitroso-1-phenylhydrazine.

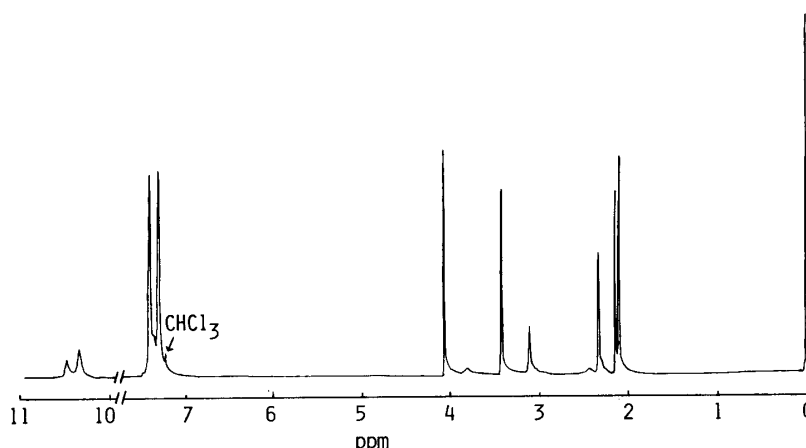


Fig. 1.  $^1\text{H}$ -NMR Spectrum of **X**

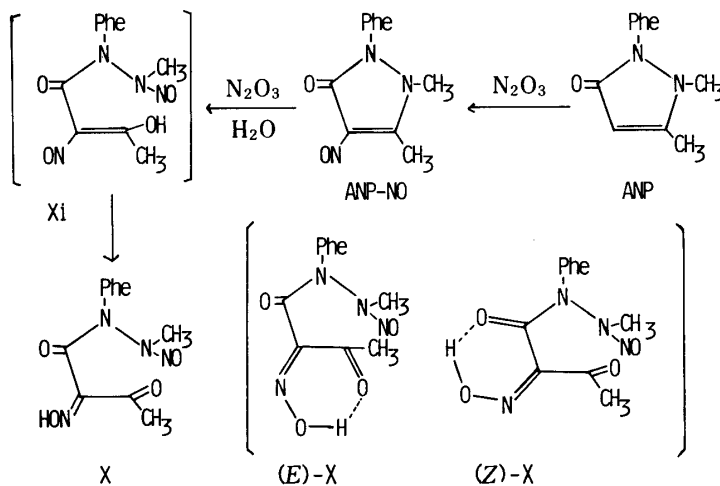


Chart 1

TABLE I. Solvent Effect on Chemical Shifts of *N*-Methyl Signals of **X**

| Solvent and $\Delta$ ppm |              |                        |              | Shift <sup>a)</sup> |
|--------------------------|--------------|------------------------|--------------|---------------------|
| $\text{CDCl}_3$          | $\Delta$ ppm | $\text{C}_6\text{D}_6$ | $\Delta$ ppm |                     |
| 3.11                     | 0.69         | 2.66                   | 0.55         | 0.45                |
| 3.80                     |              | 3.24                   |              | 0.56                |
| 3.42                     | 0.65         | 3.01                   | 0.51         | 0.41                |
| 4.07                     |              | 3.52                   |              | 0.55                |

a)  $(\text{CDCl}_3) - (\text{C}_6\text{D}_6)$ .

This compound has been synthesized by the reaction of ANP with nitrous acid and converted to 4-aminoantipyrine by reduction with  $\text{SnCl}_2\text{-HCl}$  by ring closure.<sup>9)</sup> The formation of 4-aminoantipyrine from X by such reduction was confirmed by TLC and UV spectral measurement.

Compound X may be present in nitroso-enol form, such as Xi, because the enol group is stabilized by forming a hydrogen bond with the carbonyl-oxygen or C-nitroso-oxygen. However, the UV spectrum of X showed no absorption band at around 700 nm, characteristic of the C-nitroso group,<sup>10)</sup> but showed an absorption band at 290 nm in alkaline methanol solution, which is characteristic of an ionized oxime.<sup>11)</sup>

The conformation of the N-NO moiety was assigned as follows. In the  $^1\text{H-NMR}$  spectra of dialkyl nitrosamines, the *syn* N-methyl protons with respect to the nitroso oxygen resonate at higher field than the *anti* N-methyl protons.<sup>12)</sup> In addition,  $\Delta$  ppm (*anti-syn*) values of the N-methyl protons range from 0.56 (benzene) to 0.80 ( $\text{CCl}_4$ ) depending on the solvent, and a larger upfield shift of the *anti* N-methyl protons than the *syn* N-methyl protons is observed when the solvent is changed from  $\text{CCl}_4$  to benzene.<sup>12)</sup> Table I shows the solvent effect on the chemical shifts of N-methyl protons of X. Larger upfield shifts were observed for the two signals at lower fields, and the  $\Delta$  ppm values of the two sets of signals shown in Table I coincided with the previously reported values. Consequently, the N-methyl proton signals at  $\delta$  3.11 and 3.42 of X should be due to the *syn* conformers and those at  $\delta$  3.80 and 4.07, due to the *anti* conformers. The signals at  $\delta$  3.11 and 3.80 thus should arise from one oxime conformer, and those at 3.42 and 4.07 from another oxime conformer ((*E*)-X, (*Z*)-X).

Another set of four singlets due to C-methyl protons was assigned as follows. The intensity of each C-methyl signal at  $\delta$  2.10, 2.14, 2.33 and 2.44 nearly coincided with that of each N-methyl signal at  $\delta$  4.07, 3.42, 3.11 and 3.80, respectively. This relationship was also observed when benzene and dimethyl sulfoxide were used as solvents for measurement. Therefore, the signals at  $\delta$  2.10 and 2.44 are likely to arise from the conformers having the *anti* nitroso group, and those at  $\delta$  2.14 and 2.33 from the conformers having the *syn* nitroso group. The two signals at higher fields thus should arise from one oxime conformer, and those at lower fields from another conformer, the former being present predominantly in every solvents employed (67–70%). The ratio of intensity of the two hydroxyl signals at  $\delta$  10.33 (69%) and 10.50 (31%) was in good agreement with that of these two oxime conformers. It is reasonable to assume that (*E*)-X is predominant because of the larger steric hindrance of (*Z*)-X due to hindered rotation between the 2 and 3 positions of the butyryl moiety. We accordingly concluded that the C-methyl signals at  $\delta$  2.10 and 2.14 arise from (*E*)-X and those at  $\delta$  2.33 and 2.44 from (*Z*)-X. It was also concluded that the N-methyl signals at  $\delta$  3.11 and 3.80, and at  $\delta$  3.42 and 4.07 were attributable to *syn* and *anti* nitroso conformers of (*Z*)-X and (*E*)-X, respectively.

### Mutagenicity of X

The mutagenic activity of X to TA 98 and TA 100 is shown in Fig. 2. The mutagenicity to TA 98 was higher than that to TA 100, and was reduced to one-half by S9 mix. On the other hand, the mutagenicity to TA 100 was not altered significantly in the presence of S9 mix. The mutagenic activity of an analogous compound, 1-dioxobutyryl-2-methyl-2-nitroso-1-phenylhydrazine is known to be enhanced with cysteine and glutathione.<sup>13)</sup> The mutagenicity of X, however, was not altered by these SH compounds (data not shown).

### Evaluation of X and ANP-NO Formation under Conditions Simulating Those in the Stomach

The formation of X and ANP-NO was examined under conditions presumed to resemble those in the stomach after administration of ANP, as described under method (b) in Materials and Methods. Figure 3 shows the time courses of formation of ANP-NO and X in the presence of 10 or 50 ppm of nitrite at pH 3.0. These compounds were formed relatively rapidly

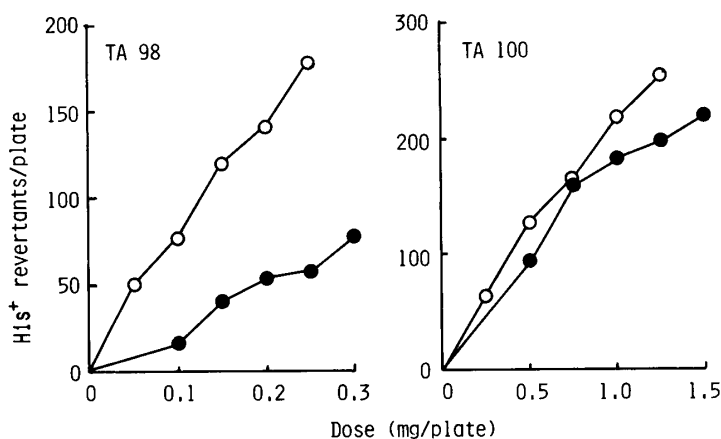


Fig. 2. Mutagenic Activity of X

○, without S9 mix; ●, with S9 mix.

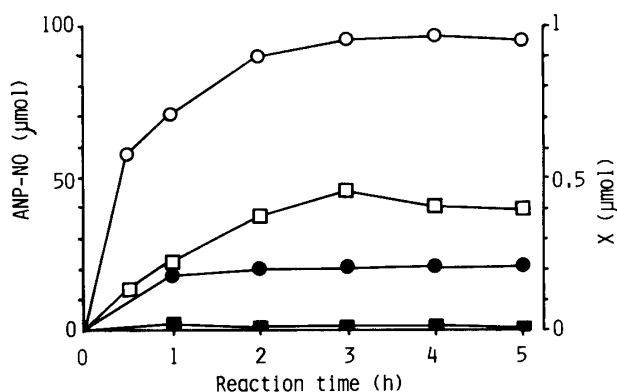


Fig. 3. Time Courses of Formation of ANP-NO and X during the Reaction of ANP with Nitrite

The reaction was carried out as described in Materials and Methods (b). 50 ppm NO<sub>2</sub><sup>-</sup>: ○, ANP-NO; □, X. 10 ppm NO<sub>2</sub><sup>-</sup>: ●, ANP-NO; ■, X.

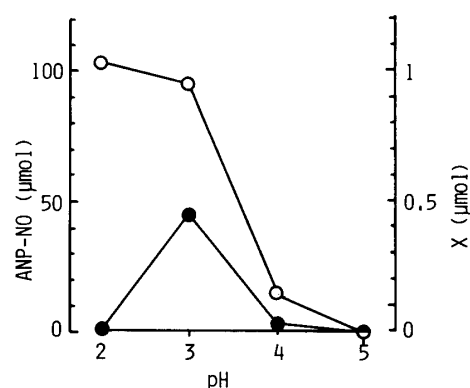


Fig. 4. pH Dependency of Formation of ANP-NO and X

The reaction was carried out for 3 h with 50 ppm of nitrite as described in Materials and Methods (b). ○, ANP-NO; ●, X.

even at the low concentration of nitrite, and the formation reached a plateau within 2–3 h. The amount of X formed under these conditions was very small, and the decrease of ANP during the reaction was almost wholly accounted for by the ANP-NO formed.

Figure 4 shows the pH effect on the formation of X and ANP-NO. The formation of ANP-NO was depressed with increase in pH value. On the other hand, the formation of X was depressed not only at higher pH but also at lower pH than 3.0. In order to clarify the reason for the depression at pH 2.0, several experiments were carried out as follows. First, X (7.6 μM) was incubated at 37°C in pH 2.0 and pH 3.0 buffers containing 50 ppm of nitrite. It was found that X was completely stable in both buffers up to 3 h. ANP-NO (0.93 mM) was incubated similarly in both buffers containing 50 ppm of nitrite, and it was found that the amount of X formed at pH 2.0 was about three times that of X formed at pH 3.0. These results thus do not explain the depression of X formation from ANP at pH 2.0. However, no further study was carried out on the cause of depression.

## Discussion

Rao and Krishna<sup>1)</sup> carried out the reaction of ANP (about 50 mM) with nitrite (about

300 mM) in 10% HCl (for the reaction at pH 1—2) or in 10% acetic acid (for the reaction at pH 3—4), and reported that ANP-NO<sub>2</sub> was readily formed at 37 °C in both pH ranges. ANP-NO, however, was reported to be formed only when the reaction was carried out in 10% acetic acid at room temperature. Arisawa *et al.*<sup>2)</sup> carried out the nitrosation reaction in acidic solution (pH 1.5—3.5, adjusted with hydrochloric acid or acetic acid) using the same concentrations of the drug and nitrite as Rao and Krishna,<sup>1)</sup> and reported the formation of ANP-NO and an unknown compound, not ANP-NO<sub>2</sub>. In the present study, we used the WHO conditions, because these have been used by many investigators in *in vitro* nitrosation assays to evaluate the nitrosatability of drugs and chemicals.<sup>6)</sup> ANP was found to give ANP-NO and 1-(2-hydroxyimino-3-oxobutyl)-2-methyl-2-nitroso-1-phenylhydrazine. The latter compound seems to be identical with the unknown compound reported by Arisawa *et al.*<sup>2)</sup> We then reexamined the experiment carried out by Rao and Krishna<sup>1)</sup> to confirm the formation of ANP-NO<sub>2</sub> only under their reaction conditions. However, we detected X, not ANP-NO<sub>2</sub>, under these conditions. In addition, 10% HCl (used for the reaction at pH 1—2 in their study) was confirmed not to be adequate: the pH of the mixture immediately after the beginning and at the end of that reaction was far lower than 1—2. ANP-NO<sub>2</sub> thus seems not to be the nitrosation product of ANP. Rao and Krishna<sup>1)</sup> may have identified X erroneously as ANP-NO<sub>2</sub>, because they identified ANP-NO<sub>2</sub> only on the basis of the mass spectrum.

1-Dioxobutyl-2-methyl-2-nitroso-1-phenylhydrazine is formed by the reaction of aminopyrine or sulpyrine with nitrite.<sup>14, 15)</sup> The cleavage of the pyrazolone ring between the 2 and 3 positions seems to be common in the reaction of a pyrazolone drug with nitrite.

We also examined the nitrosation reaction under conditions presumed to resemble those in the stomach after administration of ANP in order to estimate whether ANP-NO and X would be formed *in vivo*. The single dosage of ANP for adults is 0.3—1.0 g, and the estimated volume of the stomach content after a normal meal is about 500 ml.<sup>16)</sup> Tannenbaum and Shinsky<sup>17)</sup> reported that human saliva contained 3—10 ppm of nitrite, and Okabe<sup>18)</sup> reported that the nitrite content of Japanese saliva was 0.1—200 ppm. The nitrite level of human gastric juice is assumed in any event to be less than 10 ppm.<sup>19)</sup> The data presented in this paper, accordingly, indicate that ANP may be easily nitrosated in the stomach after administration to give ANP-NO and X.

ANP-NO is known to be mutagenic to *Salmonella typhimurium* TA 98 and TA 100. X was also found to be mutagenic to these test strains either with or without S9 mix, but its activity was lower than the reported mutagenic activity of ANP-NO.<sup>2)</sup> On the other hand, X was found to enhance dimethylnitrosamine formation in the reaction of dimethylamine with nitrite; this will be described elsewhere. Therefore, these nitrosation products of ANP are likely to be harmful to humans.

The Ministry of Health and Welfare of Japan have decided to restrict the use of pyrazolone drugs in nonproprietary drugs because of these adverse effects.<sup>20)</sup> ANP, however, is also administered to humans as a probe for assessing hepatic mixed function oxidase activity.<sup>21, 22)</sup> A study on *in vivo* nitrosation of ANP seems desirable to elucidate the actual risk.

#### References

- 1) G. S. Rao and G. Krishna, *J. Pharm. Sci.*, **64**, 1579 (1975).
- 2) M. Arisawa, M. Fujiu, Y. Suhara and H. B. Murayama, *Mutat. Res.*, **57**, 287 (1978).
- 3) E. Eisenstaedt, *J. Org. Chem.*, **3**, 153 (1938—1939).
- 4) L. Knorr, *Justus Liebigs Ann. Chem.*, **238**, 214 (1887).
- 5) K. Yamada, Katsuura, H. Kasimura and H. Iida, *Bull. Chem. Soc. Jpn.*, **49**, 2805 (1976).
- 6) G. G. Gibson and C. Ioannides (eds.), "Safety Evaluation of Nitrosatable Drugs and Chemicals," Taylor and Francis, London, 1981, pp. 104—117.

- 7) D. M. Maron and B. N. Ames, *Mutat. Res.*, **113**, 173 (1983).
- 8) N. J. Patel and B. C. Halder, *J. Inorg. Nucl. Chem.*, **29**, 1037 (1967).
- 9) M. Bockmühl, *Med. u. Chem. Abhandl. med.-chem. Forschungstätten I. G. Farbenind.*, **3**, 294 (1936) [*Chem. Abstr.*, **31**, 5796 (1937)].
- 10) H. Feuer (ed.), "The Chemistry of the Nitro and Nitroso Groups," Interscience Publishers, New York, 1969, pp. 137—163.
- 11) A. M. Talati, P. M. Desai and T. I. Patel, *Indian J. Appl. Chem.*, **27**, 159 (1964).
- 12) G. J. Karabatos and R. A. Taller, *J. Am. Chem. Soc.*, **86**, 4373 (1964).
- 13) A. Sakai, K. Yoshikawa, A. Tanimura and I. Tomita, *Mutat. Res.*, **90**, 57 (1981).
- 14) S. S. Mirvish, B. Gold, M. Eagen and S. Arnold, *Z. Krebsforsch.*, **82**, 259 (1974).
- 15) A. Sakai and A. Tanimura, *Chem. Pharm. Bull.*, **28**, 1820 (1980).
- 16) G. Scheunig and D. Ziebarth, "Environmental *N*-Nitroso Compounds Analysis and Formation (IARC Scientific Publications, No. 14)," ed. by International Agency for Research on Cancer, Lyon, 1976, pp. 269—277.
- 17) S. R. Tannenbaum and A. J. Shinsky, *J. Natl. Cancer Inst.*, **53**, 79 (1974).
- 18) S. Okabe, *Hikone-ronso*, **162—163**, 165 (1973).
- 19) Y. Takeda and H. Kanaya, *Cancer Lett.*, **15**, 53 (1982).
- 20) Official Announcement, No. 525 (1st June, 1977) [*Iyakuin Kenkyu*, **8**, 675 (1977)].
- 21) E. S. Vesell, *Clin. Pharmacol. Ther.*, **26**, 275 (1979).
- 22) J. Böttcher, H. Bässmann and R. Schüppel, *J. Pharm. Pharmacol.*, **34**, 168 (1982).