Studies on the Formation of Nitrosamines I.

Kinetical Studies on the Nitrosation of Piperidine and Morpholine

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Rates of formation of nitrosopiperidine (NP) and nitrosomorpholine (NM) were studied. Initial rates of formation of NP from piperidine and sodium nitrite were calculated from the absorbance at 260 nm after decomposition of nitrite with sulfamic acid. They were proportional to [piperidine] $[\text{NaNO}_2]^2$ both at pH 4.2 and 3.0. Initial rates of formation of NM from morpholine and sodium nitrite at pH 4.2 and 3.0 were also proportional to [morpholine] $[\text{NaNO}_2]^2$.

The effect of SCN^- on the formation of NP at various pH values was also studied. SCN^- in concentration of 15 mM accelerated the initial rate of formation of NP by three times compared with one in the absence of SCN^- at pH 1.25, but accelerated only by 1.2 times at pH 3.5. According to Boyland *et al.*, the content of SCN^- in human saliva was estimated to be 2~5.7 mM. Therefore, SCN^- in human saliva does not seem so much effective to the rate of formation of NP.

It is well known that nitrosamines could be formed in foods or in the stomach of rats or rabbits by the action of nitrite on the secondary amines present in foods during storage, cooking, or digestion. Many of them are shown to be carcinogenic^{1),2)}, and dimethylnitrosamine is one of the most potential carcinogenic nitrosamine among them. Besides, dimethylamine, which is one of the precursor of dimethylnitrosamine, is contained in many kinds of foodstuffs, especially in marine fish and fish products^{3),4)}. Although dimethylamine is nitrosated relatively slowly^{5),6)}, more rapidly nitrosatable secondary amines might be more potently hazard to human health. So, it is important to estimate the rates of nitrosation of various secondary amines.

Piperidine is detected in fish^{7),8)}, soy bean⁹⁾, and tobacco smoke¹⁰⁾, and it could be nitrosated to nitrosopiperidine (NP) which is carcinogenic to rats^{11)~13)} and hamsters¹⁴⁾. Alam *et al.*, reported *in vitro* formation of NP from piperidine and nitrite¹⁵⁾ or nitrate¹⁶⁾ in the stomach or the small intestine of rats. Mirvish¹⁷⁾ described in his paper that the rates of nitrosation of secondary amines depend on the pK_a

values of the amines and he also described that the rates of the formation of nitrosamines from secondary amines and nitrite are proportional to the concentration of secondary amines and the square of the nitrite concentration. There are some papers showing that the rates of nitrosation of dimethylamine and other secondary amines are proportional to the square of the nitrite concentration^{18),19)}. On the other hand, Friedman reported that the rates of nitrosation of dimethylamine and sarcosine are proportional to nitrite concentration²⁰⁾.

In this paper, the effects of the concentration of nitrite and piperidine, and the effect of thiocyanate on the formation of NP are described. The effect of concentration of nitrite and morpholine, whose pK_a value is greatly different from that of piperidine, on the formation of *N*-nitrosomorpholine (NM) is also described.

Methods

1. Identification of the products

Sodium nitrite (200 mM) and piperidine (20

mm) in 1M sodium acetate-hydrochloric acid buffer solution, pH 3.0, were incubated at 37°C for 1 hr. The reaction was performed in a final volume of 20ml. Four gram of potassium carbonate were added to the reaction mixture and extracted three times with each 10 ml of dichloromethane. The combined extract was concentrated to about 1 ml and used for further analyses.

Thin layer chromatography was performed on silicagel spotfilm (Tokyo Kasei Kogyo Co. Ltd.). The developing solvents used were hexane-ether-dichloromethane, (2:3:10) and chloroform-methanol-water, (65:25:4). The spots were visualized with Griess reagent or ninhydrin reagent after spraying 30% acetic acid and exposing to ultraviolet light (253.6 nm) for 10 min. When Wakogel UA plates (Wako Pure Chemical Ind., Ltd.) containing inorganic fluorescent indicator were used, the spots could be seen directly with ultraviolet light.

The authentic NP was purchased from Eastman Organic Chemicals.

In the case of NM formation, 3 mM of morpholine and 15 mM of nitrite were used and the other procedures were the same as described above.

The gas-liquid chromatograph used was a Yanaco Model G 80 equipped with an FID detector and 2.25 m glass column packed with 10% PEG plus 3% KOH on Gas-chrom P. Column temperature was 148°C.

2. Determination of nitrosamines

The concentrations of NP and NM were measured spectrophotometrically at 260 nm using $\epsilon_{260} = 1450$ for NP and $\epsilon_{260} = 3540$ for NM²³⁾, respectively. Although, λ_{max} for NP is 234~235 nm ($\epsilon = 8300$) and for NM is 235 nm, the strong absorption of nitrite that might remain in the reaction mixture makes it simpler to use 260 nm.

3. Measurement of the rate of formation of NP

Sodium nitrite solution was added to piperidine solution in acetate-hydrochloric acid buffer solution kept at 37°C. The pH of the reaction mixture was adjusted to pH 4.2 or 3.0 when reaction was started. The final concentration of piperidine was 10~25 mM and that of sodium nitrite was 100~200 mM. Five ml of the aliquots were withdrawn periodically from the reaction mixture and stop the reaction by the addition of a large excess of sulfamic acid. Optical density at 260 nm was measured and the initial rate of the formation of NP was calculated. For the experiments on the effect of thiocyanate, NH_4SCN was used in the concentration of 15 or 2.5 mM.

4. Measurement of the rate of formation of NM

The experimental procedures were very similar to that described in measurement of the rate of formation of NP. The differences were that the final concentration of morpholine used was 1~3 mM and that of sodium nitrite

Table 1. Initial Rates of Nitrosation of Piperidine with Nitrite

Piperidine was dissolved in acetate-hydrochloride buffer and kept at 37°C. Sodium nitrite solution was added to start the reaction. The final volume of the reaction mixture was 20 ml, and the pH values and concentration shown in the table were the final values. At regular time intervals, 5 ml of the sample solution was withdrawn and stop the reaction by the addition of a large excess of sulfamic acid. Then the optical density at 260 nm was measured and the initial rate of formation of NP was calculated using the ϵ value of 1450.

pH 4.2			pH 3.0		
Piperidine (mM)	NaNO ₂ (mM)	Initial rate (mM/hr)	Piperidine (mM)	NaNO ₂ (mM)	Initial rate (mM/hr)
10	100	0.052	10	150	0.760
20	100	0.115	10	200	1.243
25	100	0.137	15	200	1.851
25	150	0.247	25	200	3.002
25	200	0.461			

was 5~15 mM.

Results

1. Identification of the products

The extract of the reaction mixture of piperidine and nitrite gave only one spot coinciding with authentic NP on thin layer chromatograms developed with two different solvent systems and detected by Griess reagent or ultraviolet absorption. The extract gave two spots on thin layer chromatograms treated with ninhydrin reagent, one of which was coincident with NP and the other with piperidine. The extract gave also only one peak whose retention time was coincident with NP (11 min) on the gas chromatogram. Under this condition, the peak of unreacted piperidine was not separated from that of the solvent. The ultraviolet absorption curve of the product was the same as that of NP.

From these results, it is clear that the product is only NP and that the optical density at 260 nm is proportional to the amount of NP. Therefore, the amount of NP was measured with optical density at 260 nm in the following experiments.

The extract from the reaction mixture of morpholine and sodium nitrite gave also one spot on the thin layer chromatograms and gave one peak (12 min 20 sec) on the gas chromatogram.

2. Nitrosation of piperidine

The reaction proceeded linearly for 20 min at pH 3.0 and for 2 hr at pH 4.2, respectively. The amount of NP formed reached to about 4% of initial piperidine. Table 1 shows the initial rates of NP formation varying the stoichiometric concentration of piperidine and nitrite at pH 4.2 and 3.0.

With the constant concentration of sodium nitrite at 100 mM at pH 4.2, the relationship between piperidine concentration and the initial rate of nitrosation was first order (Fig. 1).

In similar experiments, with the constant concentration of piperidine at 25 mM, the initial rate at pH 4.2 was second order with respect to sodium nitrite concentration (Fig. 2).

At pH 3.0 and 4.2, the rates of nitrosation of piperidine depended upon the piperidine concentration and the square of the nitrite concentration as shown in Fig. 3.

Fig. 1. Effect of piperidine concentration on nitrosation

Fig. 2. Effect of NaNO₂ concentration on nitrosation

Fig. 3. Third-order rate plot for nitrosation of piperidine

Table 2. Initial Rates of Nitrosation of Morpholine with Nitrite

Experimental procedures were the same as described in the legend of Table 1. In this case, the ϵ value of 3450 was used for the estimation of NM from the optical density at 260 nm.

pH 4.2			pH 3.0		
Morpholine (mM)	NaNO ₂ (mM)	Initial rate (mM/hr)	Morpholine (mM)	NaNO ₂ (mM)	Initial rate (mM/hr)
1	10	0.029	1	5	0.059
2	10	0.054	1	10	0.171
2	15	0.127	1	15	0.305
3	10	0.087	2	15	0.593

Fig. 4. Third-order rate plot for nitrosation of morpholine

3. Nitrosation of morpholine

The initial rates of nitrosation of morpholine at pH 3.0 and 4.2 varying the concentrations of morpholine and nitrite were shown in Table 2. The amount of NM formed was proportional to reaction period while the concentration of NM reached about to 10% of initial morpholine.

The rate was also dependent upon the morpholine concentration and the square of the nitrite concentration (Fig. 4).

4. The effect of thiocyanate on nitrosation of piperidine

The effect of thiocyanate on nitrosation of piperidine was studied at a variety of pHs of the incubation mixture, which contained 10 mM of piperidine, 100 mM of sodium nitrite and 15 mM of ammonium thiocyanate. As shown in Fig. 5, compared to the rate of nitrosation of piperidine without thiocyanate ion, the rate of

Fig. 5. Effect of pH on the initial rate of nitrosation of piperidine with and without thiocyanate

nitrosation of piperidine with thiocyanate ion was accelerated with increasing acidity of the incubation mixture. Formation of NP was accelerated by 3.18 times at pH 1.3, 1.97 times at pH 2.2, 1.24 times at pH 3.0 and 1.08 times at pH 3.5. When thiocyanate concentration was 2.5 mM at pH 3.0, NP formation was accelerated only by 1.08 times.

Discussion

Rate constants of the reaction could be calculated from the following equation: Rate = k [total amine] $\times$ [total nitrite]² and from the data listed in Tables 1 and 2.

At pH 4.2, $k_{\text{piperidine}} = 0.46 \text{ L}^2/\text{mole}^2 \cdot \text{hr}$, $k_{\text{morpholine}} = 2.82 \times 10^3 \text{ L}^2/\text{mole}^2 \cdot \text{hr}$, and at pH 3.0, $k_{\text{piperidine}} = 3.17 \text{ L}^2/\text{mole}^2 \cdot \text{hr}$, $k_{\text{morpholine}} = 1.28 \times 10^3 \text{ L}^2/\text{mole}^2 \cdot \text{hr}$. The values at pH 3.0 measured at 37°C are comparable with $k_{\text{piperidine}} = 1.62 \text{ L}^2/\text{mole}^2 \cdot \text{hr}$ and $k_{\text{morpholine}} = 8.88 \times 10^3 \text{ L}^2/\text{mole}^2 \cdot \text{hr}$ that have been shown

by Mirvish¹⁷⁾ that measured at 25°C.

He also reported that the optimum pH for nitrosation of piperidine was pH 3.0, but in Fig. 6, it was a little lower than pH 3.0 and was near pH 2.8. Recently, Lane and Bailey²¹⁾ reported that the optimum pH for nitrosation of dimethylamine in human gastric juice was a little lower than that reported by Mirvish. One of the reasons of these differences might be interpreted as the contribution of nitrosyl chloride present in the buffer used.

There are some papers showing that thiocyanate ion present in human saliva as a natural component is an effective accelerator of nitrosation of *N*-methyl aniline and morpholine^{22), 23)}. Thiocyanate content in human saliva is reported to be 2~5.7 mM. But, from the data described above, thiocyanate in human saliva does not seem to play an important role in nitrosation of piperidine in human stomach.

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