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Formation of Nitrosamines in Organic Solvents and Aqueous Systems Containing Organic Solvents

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Formation of *N*-nitrosodimethylamine and *N*-nitrosodiethylamine in organic solvents such as chloroform, benzene, ethyl acetate, *n*-hexane, acetonitrile and dioxane was much faster than that in the control citrate buffer at pH 3. This effect of the organic solvents may be due to their ability to suppress ionization of nitrous acid. Formation of the nitrosamines in acetone was faster, but the yield was less than in other organic solvents, probably due to reaction of the solvent with nitrous acid. Ethyl alcohol was not useful as the control buffer, since it consumed nitrous acid to produce nitrite ester. The use of pH-controlled aqueous systems containing acetonitrile and dioxane accelerated the formation of the nitrosamines by increasing the concentration of nitrous acid; the 25% acetonitrile-containing system produced 3 times as much *N*-nitrosodimethylamine and 6 times as much *N*-nitrosodiethylamine as compared to the control citrate buffer at pH 4. Formation of the nitrosamines in citrate was accelerated or inhibited by addition of acetone and ethyl alcohol depending upon the pH conditions. It is concluded that most organic solvents suppressed the ionization of nitrous acid and accelerated the nitrosation of the secondary amines.

Keywords——organic solvent; *N*-nitrosodimethylamine; *N*-nitrosodiethylamine; nitrous acid; nitrite

It has been documented that nitrite reacts readily with secondary amines to produce carcinogenic nitrosamines.[1] Formation of nitrosamines in aqueous solution has been well established,[2] but formation in an organic solvent has not been well characterized. Organic solvents are frequently used in processing foods, cosmetics and drugs, and it is therefore important to elucidate the profiles of formation of the nitrosamines in organic solvent systems. Although several studies have been done on the effect of organic solvents,[3-6] the data are not comparable and the information is limited. It has been demonstrated that an oil phase in an emulsion and acetone in an aqueous solution enhanced the formation of nitrosamines.[3,4] We demonstrated that alcohols enhanced the formation of the nitrosamines at pH 5 and inhibited it at pH 3.[5] The formation of *N*-nitrosodimethylamine (NDMA) in chloroform containing a saturated fatty acid ester was faster than that in the control citrate buffer.[6] This paper deals with systematic investigations of the effect of organic solvents on the formation of nitrosamines. The organic solvents examined in the present experiments were chloroform, benzene, ethyl acetate, *n*-hexane, acetonitrile, dioxane, acetone and ethyl alcohol.

Materials and Methods

Materials——NDMA and *N*-nitrosodiethylamine (NDEA) standards for gas chromatography were obtained from Wako Pure Chemical Industries, Ltd. *n*-Hexane, acetonitrile, dioxane and acetone were special grade reagents. Chloroform, benzene, ethyl acetate and ethyl alcohol were purified by distillation before use. A solution of nitrous acid in chloroform was prepared by shaking a solution of 50 mM sodium nitrite in 1 N hydrochloric acid with 100 ml of chloroform, and the content of nitrous acid was determined as described.[5]

Analytical Methods——A Yanaco G80 gas chromatograph, equipped with a glass column (3 mm i.d. × 3 m) packed with polyethylene glycol 6000 (25%) on 80—100 mesh Chromosorb W AW, was used to determine the nitrosamines. The chromatograph was operated isothermally at 125 °C (column temperature) and at 140 °C (injector and detector temperature) with a nitrogen carrier gas flow of 25 ml/min. The amount of the nitrosamine was determined by comparing the peak area of the sample with that of authentic NDMA or NDEA in chloroform. Ultraviolet absorption spectra were measured with a Shimadzu UV-200S double-beam spectrophotometer.

Formation of the Nitrosamines——In Organic Solvents: A 5.0 ml aliquot of 20 mM nitrous acid in chloroform and 5.0 ml of 50 mM dimethyl(or diethyl)amine hydrochloride in chloroform were mixed in 35 ml of the organic solvent, and the mixture was made up to 50 ml with the same organic solvent. The mixture was incubated at 37 °C in a stoppered flask. In order to remove nitrous acid, each reaction mixture in chloroform, benzene, ethyl acetate and *n*-hexane was shaken with 1/10 volume of 10% sulfamic acid (or ammonium sulfamate) in 3% glacial acetic acid in the presence of 0.5 g of NaCl. This treatment removed more than 99% of nitrous acid in the organic solvent as estimated from the absorbance at 360 nm, and less than 3% of NDMA in the solvent was lost. The organic solvent layer was dried over anhydrous sodium sulfate and subjected to gas liquid chromatographic (GLC) analysis. The reaction mixtures in acetonitrile, acetone and ethyl alcohol were mixed with 1/10 volume of 10% sulfamic acid (or ammonium sulfamate) in 3% glacial acetic acid, and dried over anhydrous sodium sulfate before GLC analysis. Recovery of the authentic standards was quantitative.

In pH-Controlled Aqueous Systems Containing Organic Solvents: A 5.0 ml aliquot of 1 M sodium nitrite and 5.0 ml of 4 M dimethyl(or diethyl)amine hydrochloride were added to the mixture of 20 ml of 0.5 M sodium citrate buffer (pH 2—5) and 25 or 50 ml of organic solvent (acetonitrile, acetone or ethyl alcohol), and the mixture was made up to 100 ml with distilled water. It was then incubated at 37 °C for 4 h in a stoppered flask. The pH value of the reaction mixture was kept constant by readjustment at 1 h intervals. A 10 ml portion of the reaction mixture was supplemented with 2 g of NaCl and 1 g of sulfamic acid. The nitrosamine in the mixture was extracted with 40 ml of chloroform and the volume of the extract was measured accurately. The extract was subjected to GLC analysis.

Ultraviolet (UV) Absorption Spectra of Nitrous Acid in Organic Solvents——A mixture of 10 ml of 20 mM nitrous acid in chloroform and 10 ml of the organic solvent was kept at 25 °C for measurement of the absorption spectra.

Determination of pK_a Value of Nitrous Acid in Aqueous Systems Containing 25% Acetonitrile and Dioxane——A mixture of 20 ml of 0.5 M sodium citrate and 25 ml of acetonitrile was adjusted to the required pH value with concentrated hydrochloric acid. Then 10 ml of 0.1 M sodium nitrite was added, and the mixture was made up to 100 ml with distilled water. The UV absorption spectra and pH value of the mixture were measured as soon as possible.

Results and Discussion

Formation of the nitrosamines by the reaction of nitrous acid with dimethylamine and diethylamine in several organic solvents was investigated. A mixture of 2 mM nitrous acid and 5 mM dimethylamine or diethylamine in one of the following organic solvents containing 10% chloroform was incubated at 37 °C for up to 6 h. Organic solvents used were chloroform, benzene, ethyl acetate, *n*-hexane, acetonitrile, dioxane, acetone and ethyl alcohol. The time

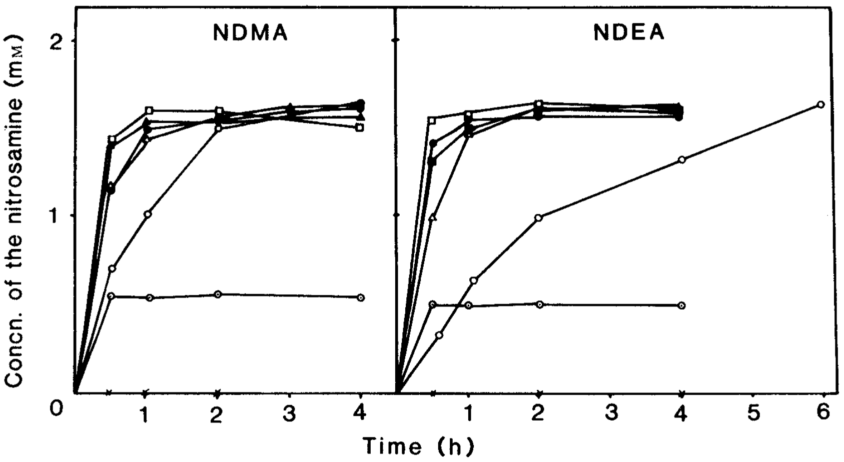

Fig. 1. Formation of NDMA and NDEA in Organic Solvents

A mixture of 2.0 mM nitrous acid and 5.0 mM dimethyl or diethylamine in the following solvents containing 10% chloroform was incubated at 37 °C. Chloroform (○), benzene (●), ethyl acetate (□), *n*-hexane (■), acetonitrile (△), dioxane (▲), acetone (⊙) and ethyl alcohol (×).

course of the formation of the nitrosamines is shown in Fig. 1. Formation of the nitrosamines in chloroform gradually increased, and NDMA or NDEA was produced in more than 80% yield after 4 or 6 h, respectively. Formation of the nitrosamines in benzene, ethyl acetate, *n*-hexane, acetonitrile and dioxane, each containing 10% chloroform, was faster than that in chloroform, and the yields of NDMA and NDEA reached maximum (80%) within 2 h. Control mixtures of 2 mM sodium nitrite and 5 mM dimethyl(or diethyl)amine in citrate buffer (pH 3) incubated at 37 °C produced no detectable amount of NDMA or NDEA after 4 h; the pH of the buffer was optimal for nitrosation in an aqueous medium.[2] The results indicate that the formation of the nitrosamines was much faster in organic solvents than in aqueous media.

It has been shown that nitrous acid dissociates into nitrite ion in aqueous media, and the active species for the nitrosation is the undissociated form of nitrous acid.[2] UV absorption spectra of nitrous acid in a 1 : 1 mixture of chloroform and the above solvent revealed characteristic absorption maxima at 345, 357, 370 and 386 (shoulder) nm, which were identical to the reported values for nitrous acid in 1 M hydrochloric acid.[7] The absorption spectra in these solvent mixtures remained unchanged during 1 h at 25 °C, indicating that the acid remained undissociated or undegraded. The high rate of formation of NDMA and NDEA in these organic solvents can be attributed to the high concentration of the undissociated form of nitrous acid.

It is interesting to note that the yields of NDMA and NDEA in acetone containing 10% chloroform did not exceed 25%, a much lower value than in other organic solvents (Fig. 1). UV absorption spectra of nitrous acid stored in a mixture of acetone–chloroform changed progressively (Fig. 2). The characreristic absorption maxima of nitrous acid completely disappeared after 1.5 h at 25 °C, indicating that the acid was lost by reaction with the solvent. The lower yields are presumably due to the loss of available nitrous acid for nitrosation.

When a mixture of nitrous acid and each of the secondary amines in ethyl alcohol containing 10% chloroform was incubated at 37 °C, no detectable amount of NDMA or

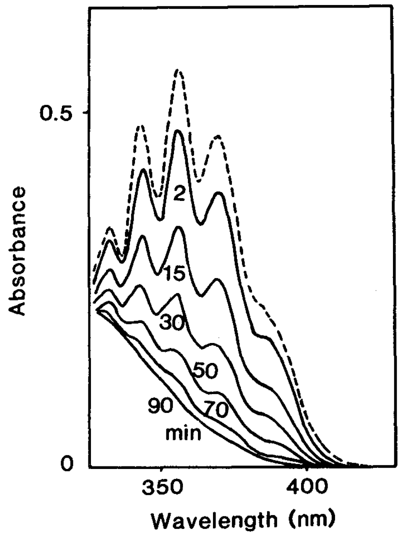

Fig. 2. Changes of the UV Absorption Spectrum of 10 mM Nitrous Acid in Acetone–Chloroform (1 : 1) at 25 °C

The dotted line indicates the spectrum of 10 mM nitrous acid in chloroform.

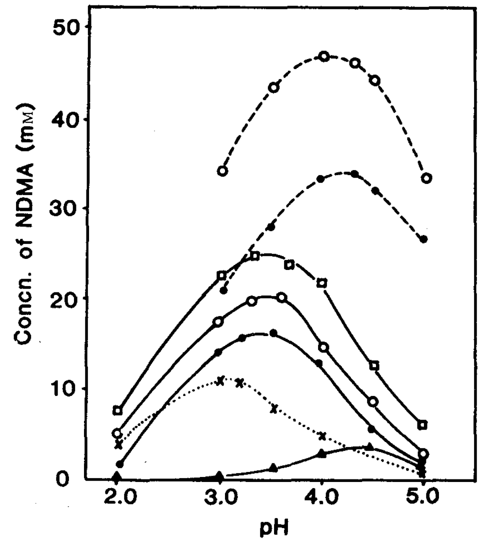

Fig. 3. pH-Dependency of the Formation of NDMA in the pH-Controlled Systems Containing Organic Solvents

A mixture of 50 mM sodium nitrite and 200 mM dimethylamine in citrate buffer containing organic solvent was incubated at 37 °C for 4 h. 25% acetonitrile (—○—), 50% acetonitrile (---○---), 25% dioxane (—□—), 25% acetone (—●—), 50% acetone (---●---), 25% ethyl alcohol (—△—) and the control (citrate buffer) (--- × ---).

Table I. Formation of Nitrosamines in pH-Controlled Aqueous Systems Containing 25% Organic Solvents

Solvent	pH	pH-Specific ratio to the control	
		NDMA	NDEA
Acetonitrile	2	1.32	2.55
	3	1.59	3.17
	4	2.97	6.42
	5	4.54	—
Dioxane	2	2.12	—
	3	2.03	—
	4	4.40	—
	5	11.4	—
Acetone	2	0.44	1.49
	3	1.30	2.22
	4	2.82	4.92
	5	4.22	—
Ethyl alcohol	3	0.03	0.03
	4	0.51	0.75
	5	3.19	—

A mixture of 50 mM sodium nitrite and 200 mM dimethyl (or diethyl)amine in citrate buffer containing 25% organic solvent was incubated at 37 °C for 4 h.

NDEA was formed even after 4 h (Fig. 1). It is not surprising that the production of the nitrosamines in ethyl alcohol occurred in extremely low yields, since the solvent may consume nitrous acid to produce ethyl nitrite.[8] While the UV absorption spectrum of nitrous acid in a mixture of ethyl alcohol–chloroform remained unchanged during 1 h, the spectral data could not be used to check the stability of the acid, since nitrite esters exhibit absorption spectra and molecular extinction coefficients similar to those of nitrous acid.[7,9]

The effects of acetonitrile, dioxane, acetone and ethyl alcohol on formation of the nitrosamines in pH-controlled aqueous media were investigated. These solvents were freely soluble in citrate buffer and homogeneous mixtures are obtained. Figure 3 shows the pH-dependency of the formation of NDMA in the reaction of 50 mM sodium nitrite and 200 mM dimethylamine at 37 °C for 4 h. Formation of the nitrosamine was markedly influenced by the presence of these organic solvents. The optimal pH for the formation of NDMA in the control buffer system was 3.0, which is close to the reported value.[2] The optimal pH was shifted to higher pH values by acetonitrile; 3.5 by 25% acetonitrile and 4.0 by 50% acetonitrile. The ratios of the amount of NDMA produced at the optimal pH to that in the control buffer system at pH 3.0 were 1.9 (in 25% acetonitrile) and 4.2 (in 50% acetonitrile). The optimal pH was similarly shifted to higher values by acetone (3.5 by 25% acetone and 4.2 by 50% acetone), but the ratios of the amount of NDMA at the optimal pH values were lower than those in the systems containing acetonitrile. The relatively low ratios of NDMA formation in these systems may be due to the reaction of nitrous acid with acetone, since the absorption spectrum of nitrous acid in the 25% acetone-containing buffer (pH 2.0) progressively changed, losing the characteristic absorption maxima of nitrous acid. The extent of the shift of the optimal pH by 25% ethyl alcohol was much larger than that by acetonitrile (pH 4.5), but the ratio of the amount of NDMA at the optimal pH was 0.2. Extremely low ratios in the systems containing ethyl alcohol may be due to the loss of nitrous acid by the acid-catalyzed reaction of the solvent with nitrous acid to produce ethyl nitrite.[7]

The pH-specific formation ratios of NDMA and NDEA in the aqueous systems containing 25% organic solvents to that in the control system are given in Table I. Formation

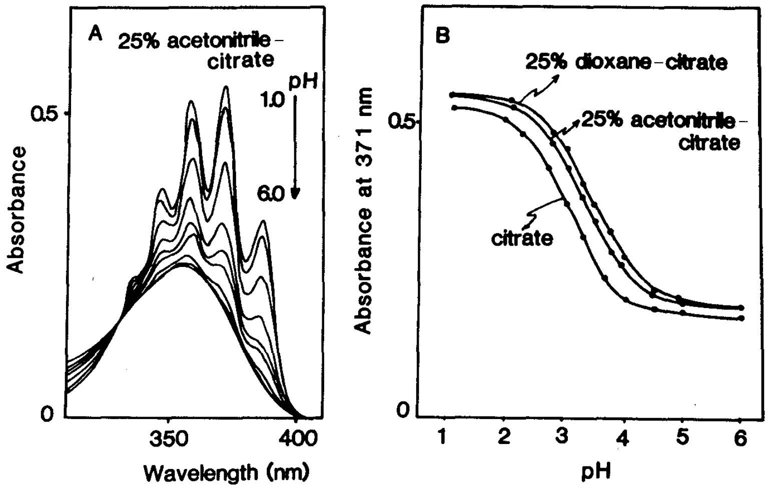

Fig. 4. UV Absorption Spectra of Nitrite (A) and Plots of Absorbance *versus* pH Values (B)

of the nitrosamines was enhanced by 25% acetonitrile and dioxane at all pH regions between 2 and 5. Formation of the nitrosamines was also enhanced by 25% acetone at every pH value except pH 2 where formation of NDMA was inhibited. Formation of the nitrosamines was effectively inhibited at pH 2—4 and accelerated at pH 5 by 25% ethyl alcohol, and the results were consistent with earlier observations.[6]

In order to obtain information on the acid dissociation of nitrous acid in aqueous systems containing inert organic solvents, the dissociation constants of nitrous acid in 25% acetonitrile and dioxane were determined spectrophotometrically. Nitrous acid in the control buffered solution and the systems containing 25% acetonitrile and dioxane was converted into nitrite with increasing pH values, showing an absorption maximum at 353 nm at pH 6[9] (Fig. 4A). Plots of absorbance at 371 nm against pH value (Fig. 4B) showed that the pK_a values of the acid were 3.06 in the control, 3.22 in 25% acetonitrile and 3.32 in 25% dioxane. Hence, ionization of nitrous acid in the aqueous system was suppressed by the addition of acetonitrile or dioxane.

The results indicate that the level of nitrous acid in aqueous systems containing organic solvents was higher than that in the control at every pH value between 2 and 5. Acceleration of the formation of the nitrosamines in the aqueous systems containing organic solvents may be due to an increase in available nitrous acid level in these pH ranges.

The systems containing organic solvents inert to nitrous acid increased the nitrous acid level by suppressing the ionization of nitrite, and stimulated the formation of the nitrosamines. While acetone and ethyl alcohol readily reacted with nitrous acid, these solvents were stimulatory to nitrosamine formation, depending upon the conditions. It can be concluded that most organic solvents suppress the ionization of nitrous acid and accelerate the nitrosation of the secondary amines. The secondary amines also dissolved readily in organic solvents as the unprotonated forms, which are the reactive forms for nitrosation.[10] In aqueous systems containing organic solvents, the protonation of the amines may be suppressed because of the organic solvent effect demonstrated in the present paper for nitrite. Suppression of protonation of the amines in non-aqueous solvent systems or aqueous systems containing organic solvents may also contribute to the accelerated formation of the nitrosamines. Organic solvents are frequently used in processing foods, cosmetics and drugs, and our results suggest that it is important to take the effect of organic solvents into consideration in the processing of products containing nitrous acid or nitrite.

References

1) H. Druckrey, R. Preussmann, S. Ivankovic, and D. Schmahl, *Z. Krebsforsch.*, **69**, 103 (1967).
2) S. S. Mirvish, *J. Nat. Cancer Inst.*, **44**, 633 (1970).
3) B. L. Kabacoff, R. J. Lechnir, S. F. Vielhuber, and M. L. Douglass, "N-Nitroso Compounds" (R. A. Scanlan and S. R. Tannenbaum eds.), ACS Symposium Series No. 174, American Chemical Society, Washington, D.C., 1981, pp. 149—156.
4) E. A. Walker, B. Pignatelli, and M. Castegnaro, *J. Agric. Food Chem.*, **27**, 393 (1979).
5) T. Kurechi, K. Kikugawa, and T. Kato, *Food Cosmet. Toxicol.*, **18**, 591 (1980).
6) T. Kato and K. Kikugawa, *Food Chem. Toxicol.*, **22**, 419 (1984).
7) S. E. Aldred, D. L. H. Williams, and M. Garley, *J. Chem. Soc., Perkin Trans. 2*, **1982**, 777.
8) R. N. Haszeldine and B. J. H. Mattinson, *J. Chem. Soc.*, **1955**, 4172.
9) G. Y. Markovits, S. E. Schwartz, and L. Newman, *Inorg. Chem.*, **20**, 445 (1981).
10) W. J. Mergens and H. L. Newmark, "N-Nitroso Compounds" (R. A. Scanlan and S. R. Tannenbaum eds.), ACS Symposium Series No. 174, American Chemical Society, Washington, D.C., 1981, pp. 193—205.