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The Reaction of Ethylenethiourea with Nitrite and Transnitrosation by *N*-Nitrosoethylenethiourea

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Ethylenethiourea (ETU), a degradation and metabolic product of ethylenebisdithiocarbamate fungicides, was nitrosated by $NaNO_2$ to form *N*-nitrosoethylenethiourea (NETU). ETU was nitrosated much more rapidly than morpholine and methylurea, which are easily nitrosatable compounds, and NETU formation increased with lowering of the pH. NETU was most unstable around pH 10 and was rather stable in strongly alkaline media above pH 12. The main decomposition reaction of NETU in acidic media was denitrosation. Transnitrosation by NETU to secondary amines gave high yields of nitrosamines at pH 3 and even at pH 5.

Keywords——ethylenethiourea; *N*-nitrosoethylenethiourea; nitrosourea; nitrite; nitrosation; transnitrosation; decomposition of nitrosourea

It is well known that nitrite reacts with secondary or tertiary amines, alkylureas or guanides to form carcinogenic *N*-nitroso compounds under acidic conditions. There are many reports on the presence and the formation of *N*-nitroso compounds in foods,[1)] cosmetics,[2)] air,[3)] drugs,[4)] pesticides,[5)] industrial products[6,7)] and cutting fluid.[8)] The estimation of the exposure of the human body to *N*-nitroso compounds is therefore an important problem.

Nitrite is used as a food additive and is also formed by the reduction of nitrate by microorganisms in human saliva.[9)] Ethylenethiourea (ETU) is used as a vulcanization accelerator of neoprene rubbers. It is also a degradation and metabolic product of the very widely used ethylenebisdithiocarbamate fungicides, and is frequently detected in various vegetables and fruits.[10)] Its amount increases during cooking.[11)] ETU was reported to be a mutagen,[12)] a carcinogen[13)] and a teratogen,[14)] and *N*-nitrosated ETU also showed mutagenicity[15)] and teratogenicity.[16)]

In this paper, kinetic studies on *N*-nitrosoethylenethiourea (NETU) formation from the reaction of ETU and nitrite, on the stability of NETU at various pH values, and on transnitrosation from NETU to other secondary amines are described.

Experimental

Materials——ETU was purchased from Tokyo Kasei Kogyo, Co., Ltd., Tokyo and was recrystallized from methanol before use. Methylurea (MU), morpholine (MOR), dimethylamine-HCl (DMA-HCl) and *N*-nitrosodimethylamine (NDMA) were obtained from Wako Pure Chemical Industries, Ltd.

Apparatus——Ultraviolet spectra were measured with a model UV-240 spectrometer (Shimadzu Seisakusho Ltd., Kyoto, Japan). Infrared (IR) spectra were taken on a JASCO A-102 machine. Nuclear magnetic resonance (NMR) spectra were measured on a JEOL JNM-FX 200 NMR spectrometer with tetramethylsilane as an internal standard. The chemical shifts are given in δ values (ppm). A gas chromatograph (GC-6AM, Shimadzu Seisakusho Ltd.) equipped with a thermal energy analyzer Model 502 (TEA) detector (Thermo Electron Corp., Waltham, MA. USA) and a glass column packed with 10% Carbowax 20M on Gaschrom P, 60—80 mesh, 3 mm i.d. × 1.5 m, was used. A high-pressure liquid chromatograph (HPLC, model LC-3A, Shimadzu Seisakusho Ltd.) equipped with spectrophotometric detector SPD-1 (Shimadzu Seisakusho Ltd.) and a stainless steel column packed with Zorbax ODS, 4.6 mm i.d. × 25 cm, was used; the eluent was acetonitrile–water (1 : 10) at a flow rate of 1.0 ml/min.

Preparation of NETU——A solution containing 1.5 g of ETU and 3.5 g of $NaNO_2$ (300 ml) was adjusted to pH 3 with dil. hydrochloric acid and allowed to stand for 20 min at room temperature. After checking by HPLC that ETU had reacted quantitatively with nitrite, sulfamic acid was added to the solution to decompose excess nitrite and the yellow reaction mixture was extracted five times with 150 ml of dichloromethane. The combined extracts were dried over anhydrous sodium sulfate and concentrated to about 100 ml; the concentrate was kept in a refrigerator. Yellow crystals were collected by filtration, washed with dichloromethane and dried over P_2O_5 *in vacuo*. The melting point was 104.5—105.5 °C. On a Silica-gel HF_{254} thin-layer chromatogram developed with dichloromethane–methanol (9 : 1), only one spot (*Rf* value; 0.50) was visualized under an ultraviolet lamp (253.6 nm) and this spot was colored red by spraying Griess reagent after irradiation by ultraviolet light. Griess reagent was prepared by mixing equal volumes of 1% sulfanilamide in 30% acetic acid and 0.1% *N*-1-naphthylethylenediamine in 30% acetic acid just before use. A solution of the yellow crystals gave one peak (retention time; 7.2 min) on HPLC. The results of instrumental analysis were as follows. NMR spectrum in d_6-acetone; δ; 2.94 (1H, br s, SH), 3.87—4.05 (4H, m, $-CH_2CH_2-$). The IR spectrum showed the presence of a nitrosyl group at 1540 cm^{-1}. The mass spectrum gave two strong peaks, *m/e* 131 for NETU and *m/e* 102 for ETU, in agreement with the result of Khera and Iverson.[16b] Absorption spectra; $\lambda_{max}^{H_2O}$ nm (ε); 220 (10000), 261 (10000), 406.5 (90), $\lambda_{shoulder}^{H_2O}$ nm (ε): 310 (3400), 425 (66), $\lambda_{max}^{CH_2Cl_2}$ nm (ε); 250 (12400), 308 (3000), 414 (70), 432 (60). (Absorption spectrum of ETU: $\lambda_{max}^{H_2O}$ 233.0 nm ($\varepsilon = 15000$).)

The Kinetics of Nitrosation of ETU, MOR and MU——In the reaction of ETU and $NaNO_2$ at various pH values, 0.5 mM ETU was allowed to react with 1 mM $NaNO_2$ in 0.25 M AcONa–HCl buffer (pH 1.8—3.5) and 0.25 M AcONa–AcOH buffer (pH 4 and 5) at 37 °C for 10 min, then a 5 ml aliquot was removed from the reaction mixture, and mixed with 1 ml of 5% sulfamic acid to decompose excess nitrite. The absorbance at 300 nm was measured and the amount of NETU formed was calculated using ε_{300} nm (4000).

In the nitrosation reactions of ETU, MOR and MU at pH 2.5 or 3.4, 5 mM ETU, MOR or MU was treated with 5 mM $NaNO_2$ in the buffer at 37 °C. After a defined period, a 5 ml aliquot was removed from the reaction mixture and mixed with 1 ml of 5% sulfamic acid. *N*-Nitroso compounds formed were determined by measuring the absorbance at 406.5 nm ($\varepsilon = 90$) for NETU, 392 nm ($\varepsilon = 92$) for *N*-nitrosomethylurea (NMU), and 260 nm ($\varepsilon = 3540$) for *N*-nitrosomorpholine (NMOR).[17]

No interfering absorbance was present at the wavelength of measurement because only one peak of the corresponding *N*-nitroso compound was found when the reaction mixture was subjected to HPLC with monitoring at the given wavelength of measurement.

The Stability of NETU——The kinetic measurements of the decomposition of NETU at various pH values were made spectrophotometrically at 24 °C by following the decrease of NETU absorption at 300 nm. When the above reaction was also followed by HPLC with monitoring at 300 nm, no peak other than that of NETU was found and the peak height of NETU decreased with the passage of time. Thus, no interfering substances having absorption at 300 nm are present.

The test solution was prepared as follows. One milliliter of freshly prepared aqueous solution of NETU (2 mM) was mixed with 9 ml of the buffer just before the measurement. In the experiments without the buffer, 1 ml of 2 mM NETU was mixed with 9 ml of water previously adjusted to the desired pH with dil. NaOH or dil. HCl. Buffers employed were 0.05 M borate buffer (pH 8.0, 10.0 and 12.0), phosphate buffer (pH 8.0 and 12.0), carbonate buffer (pH 10.0) and 0.25 M AcONa–HCl buffer (pH 3.0).

The Reactions of NETU, NMU or $NaNO_2$ with Secondary Amines——DMA or MOR in the buffer solution was mixed with freshly prepared NETU or NMU solution (and NaSCN solution in the reaction containing NaSCN) and the mixture was incubated at 37 °C. A 5 ml aliquot was removed after defined periods and mixed with 5 ml of 0.1 M carbonate buffer (pH 10) to stop the reaction by decomposing NETU. NETU reacted with secondary amines in dichloromethane so readily that it was necessary to decompose NETU completely before the reaction mixture was extracted with dichloromethane. After checking by HPLC that NETU was completely decomposed, the reaction mixture was extracted three times with 10 ml of dichloromethane. The combined extracts were dried over anhydrous sodium sulfate and concentrated to 5.0 ml. *N*-Nitrosamines were determined by gas chromatography. The recoveries were 90% for NDMA and 98% for NMOR.

In the reaction of MOR with $NaNO_2$ in the presence and absence of NaSCN, the reaction was terminated by the addition of 2 ml of sulfamic acid (2 g/100 ml) to 5 ml of the reaction mixture after the appropriate periods. Other procedures were the same as described above.

All experiments were done in duplicate at least, and reproducible values were obtained.

Results and Discussion

The Kinetics of Nitrosation of ETU, MOR and MU

The amounts of NETU formed in the reaction of 0.5 mM ETU and 1 mM $NaNO_2$ at various pH values at 37 °C for 10 min are shown in Fig. 1. Generally, the rates of nitrosation

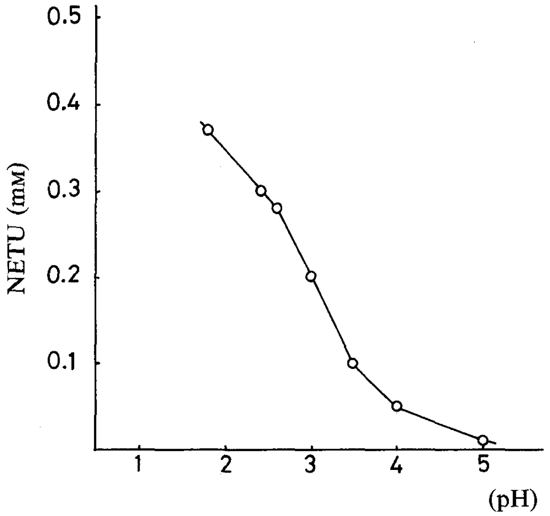

Fig. 1. NETU Formation from ETU and $NaNO_2$ at Various pH Values

The reaction of 0.5 mM ETU with 1 mM $NaNO_2$ was carried out for 10 min at 37 °C.

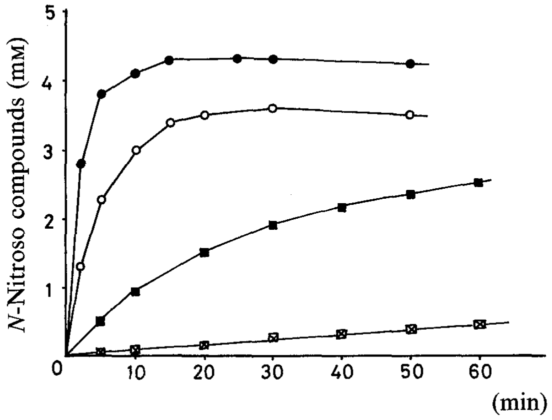

Fig. 2. Time Courses of Nitrosation of ETU, MOR and MU at 37 °C

At pH 2.5 or 3.4, 5 mM ETU, MOR and MU were treated with 5 mM $NaNO_2$. (—●—), ETU (pH 2.5); (—○—), ETU (pH 3.4); (—■—), MU (pH 2.5); (—□—), MU (pH 3.4); (—×—), MOR (pH 3.4).

Table I. The First-Order Rate Constants for Decomposition of NETU

pH	Buffer	k_0/s^{-1}
3.0[a]	AcONa–HCl	1.38×10^{-4}
8.0	Borate	3.81×10^{-3}
	Phosphate	3.51×10^{-3}
10.0	Borate	1.90×10^{-2}
	Carbonate	1.98×10^{-2}
12.0	Borate	7.56×10^{-4}
	Phosphate	6.77×10^{-4}
	None[b]	7.06×10^{-4}
13.0	None[b]	7.09×10^{-5}

[a] The decomposition at pH 3.0 was determined in the presence of 0.2% ammonium sulfamate.
[b] The pH of the reaction mixture was adjusted to the desired value with dil. NaOH.

of secondary amines have the optimum pH near pH 3, while the rates of nitrosation of ureas and guanides increase with decreasing pH.[18] In agreement with this, NETU formation from ETU and $NaNO_2$ increased with decreasing pH, as shown in Fig. 1.

The time courses of nitrosation of ETU, MOR and MU at the same concentrations are compared in Fig. 2. MOR is one of the most easily nitrosatable secondary amines, and the rate constant for the nitrosation of MOR at pH 3.4 is about 250 times that of DMA.[18] ETU was much more rapidly nitrosated than MOR even at pH 3.4, the optimum pH for MOR. ETU was also more rapidly nitrosated than MU, which is a very easily nitrosatable compound.[18] At these concentrations, the nitrosation of ETU attained equilibrium in a short time.

The Stability of NETU

Nitrosoureas are known to be easily decomposed by the addition of hydroxide ion to the carbonyl group in basic media to form an alkyl diazonium ion which is an alkylating agent.

In the kinetic measurements of the decomposition of NETU under alkaline conditions, good first-order behavior was found. The rate constant for decomposition of NETU, k_0, was calculated from the equation $k_0 = 1/t \cdot \ln (A_0 - A_\infty)/(A - A_\infty)$, where A_0 is the initial absorbance of NETU, A is the absorbance at time t and A_∞ is the absorbance after over 10 halflives. In Table I, k_0s at various pHs are shown. They were somewhat influenced by the buffer. The ionic strengths of the reaction solutions were in the range of 0.15—0.22. It was reported, however, that the decomposition rate of NMU was not sensitive to the ionic strength of the reaction solution or to the buffer cation (Na^+ and K^+) at pH 9.75.[19] The influence of the buffer may be due to the buffer anion. NETU was most unstable at pH 10 and was rather stable under strongly alkaline conditions above pH 12. Nitrosoureas usually show characteristic absorption at 390—430 nm[20] NETU also had similar absorption under acidic and neutral conditions. The characteristic absorption of NETU in aqueous solution was lost when the solution was made strongly alkaline, but it recovered on making the solution acidic again.

The decomposition reactions were followed not only by ultraviolet absorption measurement but also by HPLC with monitoring of the absorbance at 250 nm. Above pH 12, the peak of NETU (retention time; 7.2 min) decreased very slowly with the passage of time, while no other new peak appeared. At pH 8—10, an unknown product with λ_{max} at 238 nm was formed as the decomposition product of NETU and was eluted at the retention time of 4.4 min by HPLC. The product was stable, though its chemical structure has not yet been elucidated.

At pH 3, the rates of NETU decomposition were almost the same in the buffer and in aqueous solution whose pH was adjusted with dil. HCl. The decomposition of NETU at pH 3 did not follow first-order kinetics. NETU decomposed to the extent of about 40% in the first 3 h, but thereafter the amount of NETU remained apparently unchanged. When the decomposition products of NETU were analyzed by HPLC, ETU was found as a major product. When the decomposition of NETU was determined in the presence of ammonium sulfamate as a nitrite trap at pH 3, it followed first-order kinetics. It is thus considered that released nitrite reacted with ETU again to form NETU, and the decomposition reaction attained equilibrium in the absence of the nitrite trap.

Transnitrosation to Secondary Amines with NETU

NETU is easily denitrosated under acidic conditions as described above. It is thus possible that the released nitrite from NETU nitrosates other amines, if present. NETU was therefore reacted with secondary amines.

There are some reports[21] on transnitrosation by alicyclic nitrosamines and nitrosoureas in acidic media. Nitrosamides are much more effective transnitrosating agents than alicyclic nitrosamines,[21g] which require nucleophilic catalysts at the step of denitrosation.[21e,f] Protonated alicyclic nitrosamines are attacked by nucleophiles such as SCN^- to form NOSCN, which is a potent nitrosating agent.[22] On the other hand, the denitrosation of nitrosamides

TABLE II. NDMA Formation from the Reaction of 5 mM DMA with 5 mM NETU or NMU in the Presence and Absence of 5 mM NaSCN at pH 3.0 and 37 °C

Time (h)	NDMA formation, % yield[a]			
	DMA+NETU NaSCN		DMA+NMU NaSCN	
	None	5 mM	None	5 mM
1	0.1	0.2		
3	0.3	0.5	ND	ND
24	1.8	2.4	ND	0.2
48	2.3	3.7	ND	0.3

a) Percentage with respect to the concentration of NETU or NMU.

TABLE III. NMOR Formation from the Reaction of 5 mM MOR with 2 mM NMU, NETU or $NaNO_2$ in the Presence and Absence of 5 mM NaSCN at pH 3.0 or 5.0 (37 °C)

Time (h)	MOR+NMU pH 3.0 NaSCN None	MOR+NMU pH 3.0 NaSCN 5 mM	MOR+NETU pH 3.0 NaSCN None	MOR+NETU pH 3.0 NaSCN 5 mM	MOR+NETU pH 5.0 NaSCN None	MOR+NETU pH 5.0 NaSCN 5 mM	MOR+$NaNO_2$ pH 3.0 NaSCN None	MOR+$NaNO_2$ pH 3.0 NaSCN 5 mM	MOR+$NaNO_2$ pH 5.0 NaSCN None	MOR+$NaNO_2$ pH 5.0 NaSCN 5 mM
	NMOR formation, % yield[a]									
1	ND	0.1	7.7	20.2	2.5	2.6	3.5	22.1	0.2	1.2
3	ND	0.6	20.1	48.6	5.6	7.9	7.8	44.8	0.6	3.6
24	0.1	6.1	73.0	96.6	26.7	36.7	17.5	81.0	5.3	23.9
48	0.5	14.4	77.6	91.9	41.5	56.2	19.7	79.7	10.2	42.9

a) Percentage with respect to the concentration of NETU, NMU or $NaNO_2$.

requires no nucleophilic catalysts, though the transnitrosation is somewhat accelerated by thiocyanate ion.[21g] Its rate-limiting step is known to be the proton transfer from the solvent.[23]

In the present study, the reactions of NETU and NMU with secondary amines were carried out in the presence and absence of thiocyanate ion. Table II shows the amounts of NDMA formed in the reaction of 5 mM DMA with 5 mM NETU or NMU at pH 3 and 37 °C. DMA was nitrosated by NETU more effectively than by NMU and the transnitrosation was accelerated in the presence of thiocyanate ion. According to Snyder *et al.*,[19] the main decomposition reaction of NMU in the pH range 2—4 is hydrolysis and not denitrosation. NMU is therefore a less effective nitrosating agent than NETU which easily denitrosates at pH 3. NMU, however, is a more effective nitrosating agent than alicyclic nitrosamines. MOR was more easily nitrosated by NMU and NETU than DMA, as shown in Table III, even though the concentration of nitrosourea was 2 mM. MOR was nitrosated rapidly by NETU even in the absence of thiocyanate ion. As there was a possibility that chloride ion, a weak nucleophile, in the medium catalyzed the reaction, the pH of the reaction mixture was adjusted with $HClO_4$, which is known to be a very weak nucleophile.[24] The amounts of NMOR formed were the same. It is thus likely that NETU itself is an active nitrosating agent and nitrosates MOR directly in the absence of a nucleophile, being different from the transnitrosation by alicyclic nitrosamines which requires a nucleophile. Re-nitrosation of ETU may occur competitively in these reactions, and NOSCN may also be formed in the presence of thiocyanate ion. As NOSCN is an active nitrosating agent for secondary amines but not for nitrosamides, the amounts of NMOR formed were larger in the reactions with SCN^- than in those without SCN^-. MOR was considerably nitrosated by NETU even at pH 5 with or without SCN^-. Comparing the amounts of NMOR formed by NETU and $NaNO_2$ at each pH, NETU was a more active nitrosating agent than $NaNO_2$. MOR was less nitrosated with $NaNO_2$ at pH 5 than at pH 3 because the proportion of HNO_2, which is the active form of $NaNO_2$, was only 2% at pH 5 and 70% at pH 3.

There is a possibility that the formation of nitrosamines could occur by transnitrosation under mild conditions if ETU or NETU exists in the environment, producing a more potent carcinogen such as NDMA or NMOR.

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