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Biochemical Studies on 1,1′-Ethylenebis(1-nitrosourea) (EBNU). I. Degradation of EBNU in Mild Conditions

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1,1′-Ethylenebis(1-nitrosourea)(EBNU) possesses antitumor activity against L-1210. The kinetics of EBNU degradation in mild conditions was studied. Aqueous solution of EBNU is unstable in alkaline and neutral sides at 37°. The half-lives of EBNU are 0.9 min in alkaline solution (pH 9.0), 13.3 min in neutral solution (pH 7.0), and 45 hr in acid solution (pH 1.0). The dependence of the observed rate constant on hydroxyl ion concentration is expressed in the following equation.

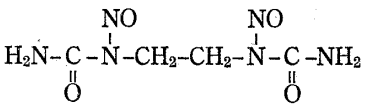
$$k_{obs}\ (min^{-1}) = 1050[OH^-]^{0.65}$$

The effects of various amino acids and thiol compounds on EBNU degradation were investigated. The rate of degradation is accelerated by cysteine and 2-mercaptoethanol, but not by glutathione. However these three thiol compounds seem to react with EBNU through unstable S-nitroso derivatives which have the absorption maximum at 335 nm.

Keywords——1,1′-ethylenebis(1-nitrosourea); antitumor agent; kinetic study; degradation in mild conditions; half-life; pH-rate profiles; effect of cysteine; spectral change; S-nitrosocysteine

N-Alkyl-N-nitrosoureas are active as carcinogens[2] and mutagens,[3] but they also offer a relatively new and promising class of antineoplastic agents. 1,3-Bis(2-chloroethyl)-1-nitrosourea (BCNU), 1-(2-chloroethyl)-3-cyclohexyl-1-nitrosourea (CCNU), and related compounds are useful agents for the treatment of lymphomas and other malignant disease.[4]

1,1′-Ethylenebis(1-nitrosourea) (EBNU) and 1,1′-hexamethylenebis(1-nitrosourea) (HxBNU) have been found to be carcinostatic in primary screening system using a rat ascites hepatoma, AH-13, and a mouse leukemia, L-1210.[5] EBNU is the simplest bisnitrosoureido alkane which has two N-nitrosoureido groups symmetrically, and is a different type of compound compared with BCNU or CCNU which is a typical alkyl nitrosourea with 2-chloroethyl group (Chart 1).



1,1′-ethylenebis(1-nitrosourea)

Chart 1

It has been observed that N-alkyl-N-nitrosoureas are decomposed by nucleophilic attack in physiological conditions, yielding reactive intermediates which serve as alkylating and

1) Location: *18-1 Kamiyoga 1-Chome, Setagaya-ku, Tokyo, 158, Japan.*
2) H. Druckrey, R. Preussmann, S. Ivankovic, and D. Schmäl, *Z. Krebsforsch.*, **69**, 103 (1967); S. Odashima, *Gann*, **61**, 245 (1970); S. Odashima, T. Ogiu, A. Maekawa, and M. Nakadate, *Gann Monograph on Cancer Research*, **17**, 405 (1975).
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carbamoylating agents.[6–10] The alkylation of nucleic acids and proteins by CCNU,[11] and that of polycytidylic acid by BCNU[12] have been demonstrated. It has been also known that the degrees of instability of N-alkyl-N-nitrosoureas are quite important in determining their alkylating activities. Wheeler, *et al.*[13] indicated that there was a good inverse relation between the half-lives of CCNU derivatives and their alkylating activities.

N-Nitroso compounds are known to react with nucleophiles. For examples, CCNU binds to proteins through cyclohexylcarbamoylation of ε-amino group of lysine residues.[14] N-Methyl-N′-nitro-N-nitrosoguanidine (MNNG),[15] N-methyl-N-nitroso-*p*-toluenesulfonamide (MNTS),[15] N-alkyl-N-nitrosourethanes,[16] and 1-methyl-1-nitrosourea (MNU)[17] react with cysteine, but kinetic studies on the reactions have not yet been presented except for a brief report.[17]

The main purpose of this study is to investigate the interaction of EBNU with biopolymers *in vitro* and *in vivo* in order to understand the carcinostatic effect of this agent from the biochemical standpoint. In the present paper, the kinetic studies of EBNU degradation in mild conditions including the effects of pH, amino acids, and thiol compounds were performed.

Experimental

Materials——EBNU was synthesized according to the usual method for the preparation of N-alkyl-N-nitrosoureas using ethylenediamine dihydrochloride, potassium cyanate, sodium nitrite, and 25% sulfuric acid. The yield of EBNU was approximatey 15% and its melting point was 129° (decomp.) (Reported, 170°[18], 182.9—183.9°[19])[20]. *Anal.* Calcd. for $C_4H_8O_4N_6$: C, 23.53; H, 3.95; N, 41.17. Found: C, 23.81; H, 4.01; N, 40.49. IR (Nujol) cm^{-1}: 3340, 3260, 1715, 1640, 1607, 1510, 1430, 1208, 1012, 856. NMR (DMSO-d_6) ppm: 3.68 (s, $-CH_2CH_2-$), 7.70 (s, NH). Mass Spectrum *m/e*: 102 ($M^+/2$). EBNU was insoluble in water and its solubility in DMSO was approximately 10% at 37°.

Other materials used were special grades.

Kinetic Studies——In the experiment of pH effect the following buffers, 0.05M, were used: KCl–HCl at pH 1.0; citric acid–sodium citrate at pH 3.0—5.0; cacodylate-HCl at pH 5.0—7.5; boric acid–borax at pH 7.5—9.0. For the general investigations cacodylate buffer, pH 7.0, was used. However, the effects of thiol compounds were investigated in Tris-HCl buffer, pH 7.0, because white precipitates were immediately formed when cysteine was added to cacodylate buffer. EBNU was dissolved in DMSO at a concentration of 0.05M. In each test solution, 0.1 ml of the EBNU solution was added in a total volume of 3 ml. Therefore, the aqueous solutions of EBNU in this study contained 3.3% (v/v) DMSO, unless otherwise stated. A constant ionic strength of 0.5 was maintained by adding an appropriate amount of KCl. When amino acid or thiol compound was added to the reaction mixture, the pH of the solution of these compounds was previously adjusted to pH 7.0 by the addition of hydrochloric acid or sodium hydroxide. The spectral characteristic of EBNU was $\lambda_{max}^{H_2O}$(pH 7.0) nm (ε): 396 (95.9). To examine the effects of various compounds on the rate of degradation of EBNU, the change of absorbance at 396 nm was followed by Shimadzu Spectrophotometer, QV-50. The

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17) G.P. Wheeler and B.J. Bowdon, *Biochem. Pharmacol.*, **21**, 265 (1972).
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20) The melting point of EBNU is different from each report, but details on the synthesis of this compound will be reported by the authors in near furture. *cf.* M. Miyahara, M. Nakadate, M. Anzai, and I. Suzuki: The 96th Annual Meeting of Pharmaceutical Society of Japan, Nagoya, April, 1976.

reaction temperature was maintained at 37° or 25°. Linear regression analysis was accomplished by means of a computer (SHARP COMPET CS-365P).

Results

EBNU Degradation and Observed Rate Constant

EBNU was very stable in DMSO and the half-life was 23.1 days at 37°, but it was unstable in aqueous solutions.

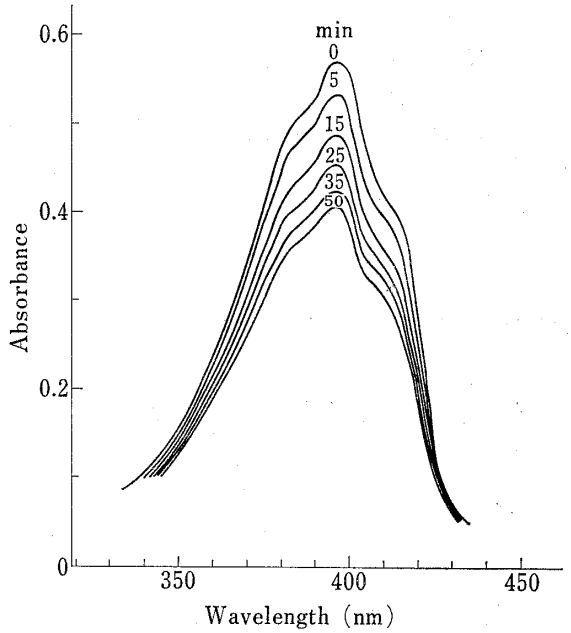

Fig. 1. Change of Absorption Spectrum of EBNU in Aqueous Solution

These studies were performed in 0.05M Tris–HCl buffer, pH 7.0 (μ=0.50) at 25°. Final concentration of EBNU was 0.0047M.

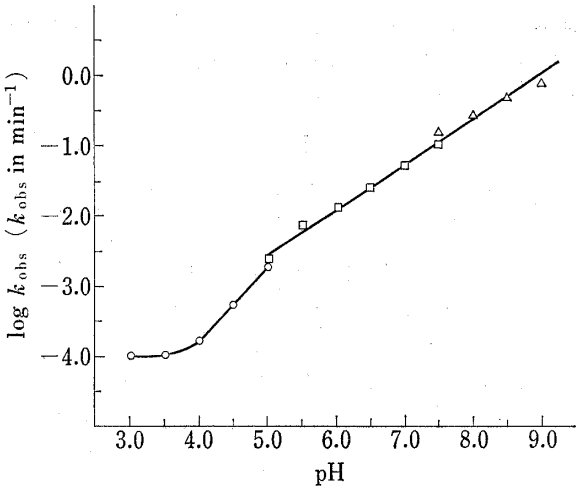

Fig. 2. pH-Rate Profiles of EBNU Degradation at 37° and μ=0.50

△: borate buffer,
□: cacodylate buffer,
○: citrate buffer

Table I. Observed Pseudo First-order Rate Constants of EBNU Degradation at Different pHs[a)]

pH	Buffer species	Rate constant $k_{obs} \times 10^2$(min^{-1})	Half-life (min)
9.0	Borate	76.9	0.90
8.5	Borate	48.8	1.42
8.0	Borate	27.3	2.54
7.5	Borate	16.5	4.20
7.5	Cacodylate	8.89	7.80
7.0	Cacodylate	5.18	13.3
6.5	Cacodylate	2.47	27.7
6.0	Cacodylate	1.35	51.7
5.5	Cacodylate	0.756	91.2
5.0	Cacodylate	0.246	277
5.0	Citrate	0.172	403
4.5	Citrate	0.0509	1360
4.0	Citrate	0.0164	4230
3.5	Citrate	0.0085	8150
3.0	Citrate	0.0088	7880
1.0	HCl	0.0256	2710

a) These studies were performed at 37° and μ=0.50.

When the absorption spectrum of EBNU in Tris-HCl buffer, pH 7.0, was periodically measured at 25°, the absorbance at 396 nm gradually decreased without any alteration in the spectral curve (Fig. 1). More rapid decrease was observed at 37°.

Table I shows the kinetic results of EBNU degradation in the buffer solutions adjusted to various pHs at constant ionic strength (μ=0.5) and temperature (37°). The reactions were followed pseudo first-order kinetics with respect to EBNU. The half-life of EBNU was 0.9 min in alkaline solution (pH 9.0), 13.3 min in neutral solution (pH 7.0), and 45 hr in acid solution (pH 1.0).

pH-Rate Profiles

The pH-log k_{obs} profiles for the degradation of EBNU based on the data of Table I are given in Fig. 2. The observed rate in this experiment is actually a summation of a series of catalytic reaction rates induced by buffer species, hydrogen ion, and hydroxyl ion. The degradation of EBNU was accelerated by hydroxyl ion at above pH 4.0. The contribution of hydroxyl ion to the degradation is shown by the following equation which is calculated from the straight line at pH 5.0—9.0 in Fig. 2.

$$k_{obs} = 1050[OH^-]^{0.65} \quad (r=0.998) \qquad \text{(Eq. 1)}$$

r, correlation coefficient; $[OH^-]=10^{-pOH}=10^{-(pK_w-pH)}$; $pk_w=13.59$ at 37°[21]

From the equation 1, it is indicated that EBNU is very susceptible to hydroxyl ion.

Effects of Ionic Strength, DMSO, Various Amino Acids, and Thiol Compounds

As EBNU was rapidly degraded at 37°, subsequent studies were performed at 25°.

Table II shows the effect of ionic strength on EBNU degradation at pH 7.0. When the ionic strength was varied in the range of 0.1 to 1.0 by the addition of KCl, no significant difference in rate constant was observed.

Table II. Effect of Ionic Strength on Degradation of EBNU[a]

Ionic strength	Rate constant $k_{obs}\times10^3$ (min^{-1})
0.10	17.3
0.25	17.3
0.50	18.0
0.75	18.0
1.00	17.7

a) These studies were performed in 0.05M cacodylate buffer, pH 7.0 at 25°. Ionic strength was adjusted by the addition of KCl.

Table III. Effect of Dimethyl Sulfoxide on Degradation of EBNU[a]

Dimethyl sulfoxide % (v/v)	Rate constant $k_{obs}\times10^3$ (min^{-1})
2.0	18.2
3.8	17.4
5.0	17.9
7.5	17.8
10.0	18.5

a) These studies were performed in 0.05M cacodylate buffer, pH 7.0 (μ=0.50) at 25°.

As shown in Table III, DMSO had no effect on EBNU degradation in the range of 2.0 to 10.0% (v/v).

Fig. 3 presents the comparison of relative degradation rates of EBNU in the presence of a definite concentration of various amino acids and other compounds. Any amino acids tested had no or little effect except cysteine which accelerated the degradation of EBNU. Such an acceleration was also observed in 2-mercaptoethanol, but not in glutathione.

Kinetic Analysis of EBNU Degradation in the Presence of Thiol Compounds

Table IV shows the effects of various concentration of cysteine and 2-mercaptoethanol on degradation rate of EBNU. The accelerating effect of these thiol compounds increased with increasing the concentration.

21) A.N. Martin, "Physical Pharmacy," 2nd ed., Lea and Febiger, Inc., Philadelphia, 1969, p. 196.

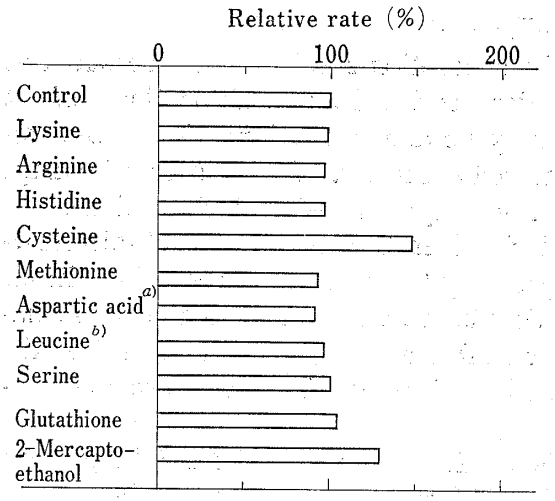

Fig. 3. Effects of L-Amino Acids and Other Compounds on EBNU Degradation

These studies were performed at pH 7.0 (μ=0.50) and 25°. Final concentration of each compound was 0.033M except for *a*) 0.010M and *b*) 0.023M.

TABLE IV. Effects of Cysteine and 2-Mercaptoethanol on Degradation of EBNU[a)]

Concentration (10^{-3}M)	Rate constant $k_{obs}\times10^{3}$ (min^{-1})	
	Cysteine	2-Mercaptoethanol
0.0	18.6	19.1
16.7	24.3	22.6
33.3	27.6	24.6
50.0	30.5	26.5
66.7	32.5	28.9

a) These studies were performed in 0.05M Tris-HCl buffer, pH 7.0 (μ=0.50) at 25°.

As shown in Fig 4(a), four plots obtained by plotting ($k_{obs}-k'_{obs}$) against cysteine concentration, [CySH], in log scale were found to fall on a linear positive slope. k_{obs} and k'_{obs} are rate constants of EBNU degradation with and without cysteine, respectively. Thus the dependence of the observed rate constant on cysteine concentration can be expressed by Eq. 2.

$$k_{obs} = 0.081[CySH]^{0.64} + 0.019 \quad (r=0.999) \qquad \text{(Eq. 2)}$$

From the equation 1 and 2, it is indicated that the effectiveness of hydroxyl ion on EBNU degradation is far higher than that of cysteine.

The similar result was obtained in the case of 2-mercaptoethanol, as shown in Fig. 4(b). The dependence of the observed rate constants on 2-mercaptoethanol concentration, [ME], can be expressed by Eq. 3.

$$k_{obs} = 0.055[ME]^{0.67} + 0.019 \quad (r=0.999) \qquad \text{(Eq. 3)}$$

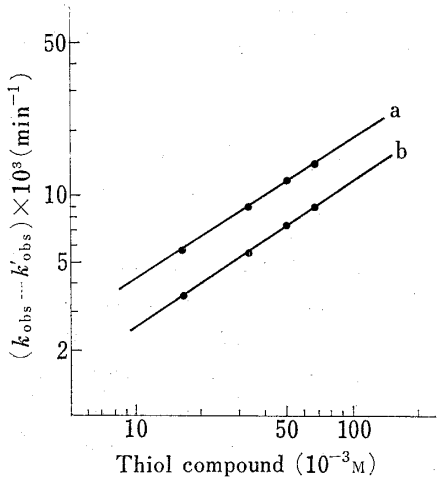

Fig. 4. Plots of EBNU Degradation Rate Constants *versus* Concentration of Thiol Compounds

These studies were performed in 0.05M Tris-HCl buffer, pH 7.0 (μ=0.50) at 25°. k_{obs} and k'_{obs} are rate constants of EBNU degradation with and without thiol compounds. (a) cysteine, (b) 2-mercaptoethanol.

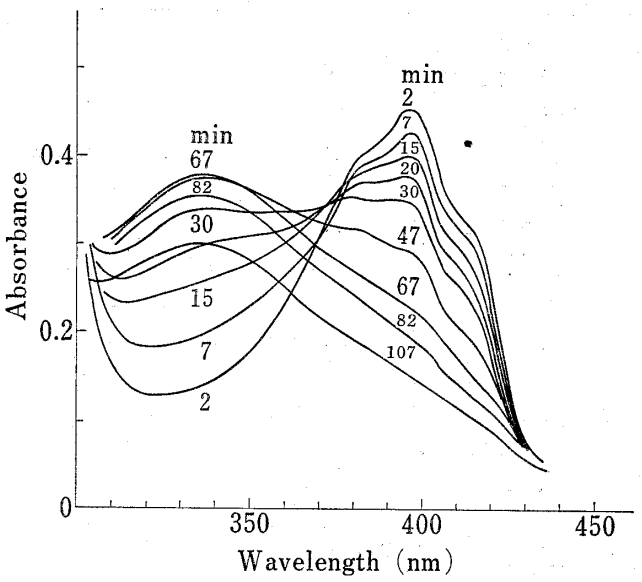

Fig. 5. Effect of Cysteine on Spectral Change of EBNU in Aqueous Solution

These studies were performed in 0.05M Tris-HCl buffer, pH 7.0 (μ=0.50) containing of 0.0033M EBNU and 0.033M cysteine at 25°.

Spectral Change of EBNU in the Presence of Thiol Compounds

When EBNU was reacted with cysteine and the absorption spectrum was measured at various intervals, the absorbance at 396 nm rapidly decreased and a new absorption peak at 335 nm appeared (Fig. 5). The latter peak which may be due to the formation of S-nitrosocysteine increased up to about 1 hr, but then decreased gradually. Such a spectral change was also observed in the reaction of EBNU with 2-mercaptoethanol and glutathione.

In order to confirm the formation of S-nitroso derivatives, S-nitrosocysteine, S-nitroso-2-mercaptoethanol and S-nitrosoglutathione were prepared by the general method using nitrous acid and thiol compounds,[22] and by the method of Schulz, *et al.* using MNTS and thiol compounds.[15] Since these S-nitroso derivatives were unstable and could not be isolated, the absorption spectra were directly measured in the reaction mixtures. They had also a characteristic absorption maximum at 335 nm.

Therefore, it is assumed that the reactions of these thiol compounds with EBNU proceed *via* the attack of thiol group on nitroso nitrogen of EBNU forming the S-nitroso derivatives.

Discussion

EBNU is stable in DMSO, but unstable in aqueous solution especially in alkaline and neutral sides at 37°. The most stable pH is around 3.5, where the half-life is 5.7 days. It was reported that the half-lives of BCNU and CCNU were 101 min and 208 min at pH 7.2, 37°, respectively,[9] and that of MNU was 15 min at pH 7.18, 35°.[7] As the stability of EBNU is comparable to MNU, it is supposed that the alkylating activity of EBNU is also the same order as that of MNU.

The pH-rate profiles of EBNU indicate that EBNU is mainly decomposed by hydroxyl ion attack. The obvious pH dependence of EBNU degradation is also in agreement with that of streptozotocin[6] and other N-alkyl-N-nitrosoureas[7] reported by Garrett, *et al.* Garrett and Goto[8] observed that *n*-butyl alcohol and secondary butyl alcohol were produced through *n*-butyl carbonium ion in alkaline degradation of N-*n*-butyl-N-nitrosourea. In general, it is most probable that the degradation of N-nitrosourea go through a carbonium ion intermediate. The carbonyl carbon of EBNU is extremely susceptible to nucleophilic attack of hydroxyl ion. The alkaline degradation may thus result in the formation of corresponding alcohol. On the other hand, the acid degradation of EBNU may proceed through denitroso reaction as the case of streptozotocin.[6]

It is interested that the rate of EBNU degradation is accelerated in the presence of thiol compounds such as cysteine and 2-mercaptoethanol with the exception of glutathione. The sulfhydryl group of cysteine may attack on the two reactive centers, carbonyl carbon and nitroso nitrogen of EBNU.

When EBNU was reacted with cysteine, the absorption spectrum of the reaction mixture gradually changed with a decrease of the absorbance at 396 nm and an appearance of a new peak at 335 nm of which absorbance decreased afterward. The latter peak seems to be caused by S-nitrosocysteine. Barrett, *et al.*[23] reported that S-nitrosotoluene-α-thiol and other S-nitroso derivatives of thiols had a characteristic absorption maximum around 340 nm in hexane solution. Schulz and McCalla[15] presumed that S-nitrosocysteine was an unstable intermediate in the reaction of cysteine with MNTS or MNNG.

S-Nitrosocysteine was prepared according to the two different methods.[15,22] It had a characteristic absorption maximum at 335 nm in aqueous solution. Thus the spectral change at 335 nm may show the formation of unstable S-nitrosocysteine followed by its degradation.

22) B. Saville, *Analyst*, **83**, 670 (1958).
23) J. Barrett, D.F. Debenham, and J. Glauser, *Chem. Commun.*, **248** (1965).

The similar spectral change was also observed in the reaction of EBNU with 2-mercaptoethanol and glutathione, accompanying the formation of corresponding S-nitroso derivatives.

However, a decrease of the absorbance at 396 nm which was mainly due to the degradation of EBNU by hydroxyl ion was accelerated by cysteine and 2-mercaptoethanol, but not by glutathione.

On the other hand, an increase of the absorbance at 335 nm which was probably due to the formation of S-nitroso derivatives by thiol compounds was brought about by all the thiol compounds tested to the same extent. It was unexpected that glutathione had no effect on EBNU degradation in spite of the formation of S-nitrosoglutathione. The cause of this phenomenon was obscure and remained to be solved.

The effectiveness of thiol group on EBNU degradation is much weaker than that of hydroxyl ion, but thiol compounds such as cysteine may influence the alkylating activity of EBNU in biological systems which is possibly correlated to the carcinostatic process of EBNU.

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