Calibration Curve

Conformity to Beer's law over a range of 0.1—0.9 μg/ml of gold(III) was obtained by using the procedure recommended. The coefficient of variation was 1.34% ($n=6$) for 0.5 μg/ml of gold (III). The molar extinction coefficient of the complex at 622 nm was estimated to be $1.1\times10^5\cdot1\cdot mol^{-1}\cdot cm^{-1}$.

Composition of the Gold(III)-DACTB Complex

This was examined by the molar ratio and the continuous variation methods. The ratio of gold(III) to DACTB in the complex was found to be 1: 2.

Effect of Other Ions

As shown in Table III, Fe(II), Mn(VII), Ce(IV), and Pd(II) interfered considerably with the determination. Therefore, these ions should be removed before this method is practically applied. This may be carried out by selective extraction of gold (III) with ethyl acetate or ethyl ether.[3d,4]

Sensitivity of the Method

The sensitivity of this method is higher than those of the *p*-dimethylaminobenzylidene-rhodanine,[3] *o*-tolidine,[5] bromoaurate,[6] tin(IV)-chloride,[7] tetron,[8] and 2-pyridyl-2-thienyl-Z-ketoxime[9] methods. It is comparable to that of rhodamine B method[10] and lower than that of the thio-Michler's ketone method.[11] This method may be applicable for the micro-determination of gold(III) in biological materials from subjects given auric preparations.

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Mutagenicity of Amide Type and Carbamate Type Diazoalkane-generating Agents in *Salmonella*/Microsome Assay

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The mutagenicity of several N-nitroso compounds, $RCONHCH_2N(NO)R'$, which were reported as new diazoalkane-generating agents, was assayed by the *Salmonella*/microsome

test. Among 6 amide type N-nitroso compounds which had an alkyl group at position R, only two which had a benzyl group at position R′ were weakly mutagenic to *Salmonella typhimurium* TA100, a base-pair substitution strain, with metabolic activation by S-9 mix. On the contrary, 6 carbamate type N-nitroso compounds which had an alkoxy or benzyloxy group at position R showed considerable mutagenic activity for TA100 with metabolic activation. Two of the carbamate type compounds which had a benzyl group at position R′ were also mutagenic in *Salmonella typhimurium* TA98, a frameshift strain, even without metabolic activation.

Keywords——amide type N-nitroso compound; carbamate type N-nitroso compound; diazoalkane-generating agent; mutagenicity; *Salmonella typhimurium*; S-9 mix

N-[(N-Nitrosoalkylamino)methyl]amides or -carbamates and N-[(N-nitrosobenzylamino)-methyl]amides or -carbamates, all of which have a common structure, $RCONHCH_2N(NO)R'$, have been reported to be excellent diazoalkane-generating agents by Sekiya *et al.*[1] Among many diazoalkane-generating agents (mostly for diazomethane), N-methyl-N-nitroso-*p*-toluenesulfonamide and N-methyl-N′-nitro-N-nitrosoguanidine are widely favored at present, but these two compounds are not entirely stable and, moreover, the latter agent is a strong carcinogen. Though $RCONHCH_2N(NO)R'$ are stable and generate diazoalkane in comparable yield only on treatment with alkaline solution *in vitro* (Chart 1),[1] it is considered that they may have mutagenic and/or carcinogenic effects *in vivo* like other N-nitroso compounds. Regarding the biological actions of these N-nitroso compounds, it has been reported by Ishidate *et al.*[2] that chromosomal aberrations were induced in cultured Chinese hamster cells. Among the series of chemical agents checked, compounds **1**, **2** and **6** in Table I were included. Compound **1** and **6** induced chromosomal aberrations such as gaps, breaks and translocations of chromatids.

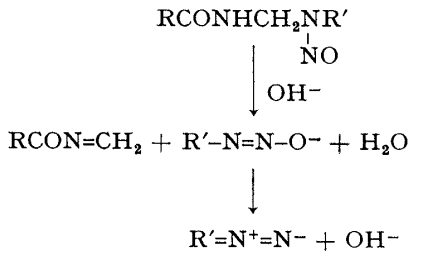

Chart 1. Generation of Diazoalkane from $RCONHCH_2N(NO)R'$ in Alkaline Solution[1]

The present paper describes the mutagenicity of the above amide and carbamate types of N-nitroso compounds in the *Salmonella*/microsome assay. The differences in mutagenic activity between the compounds are also discussed.

Experimental

Chemicals——All N-nitroso compounds tested (Table I) were kindly prepared by Professor Sekiya, Shizuoka College of Pharmacy, Shizuoka. Glucose-6-phosphate (G6P) and G6P-dehydrogenase (EC 1.1.1.49) were purchased from Sigma Chemical Co. (St. Louis). NADPH was from Kyowa Hakko Kogyo Co. (Tokyo).

Bacterial Strains——*Salmonella typhimurium* TA100 (base-pair substitution strain) and TA98 (frameshift strain) were kindly supplied by Professor Ames,[3] University of California, Berkeley. Eighteen-hr cultures of these strains in Nutrient Broth (Difco), which contained 8 to 10×10^8 cells/ml, were used for the assay.

Assay of Mutagenic Activity——The assay was carried out as described by Ames *et al.*[4] with some modifications.[5] A 0.1 ml aliquot of test compound dissolved in distilled water was preincubated with 0.5 ml of S-9 mix and 0.1 ml of bacterial culture at 37° for 60 min. After 2 ml of soft agar (0.7% agar, 0.6% NaCl, 0.05 mM *l*-histidine, 0.05 mM biotin) had been added, the preincubation mixture was poured onto a minimal glucose agar plate containing 0.1 μmol of *l*-histidine. The histidine prototrophic colonies on the plate were counted after incubation for 2 days at 37°.

For each compound, 2 to 3 independent experiments with 5 plates were carried out. The final concentrations of compounds in the preincubation mixture were adjusted to between 0.027 μmol (4×10^{-5} M) and 27 μmol (4×10^{-2} M). Mutagenic activity was assessed from the slope of a linear dose-response curve and expressed as revertants/plate/μmol of compound.

Preparation of S-9 Mix——S-9 fractions were obtained from male Wistar strain rats (average 150 g) treated with 500 mg/kg of polychlorinated biphenyl (Kanechlor 500) as described by Ames *et al.*[4] S-9 mix was prepared as described by Yahagi *et al.*,[5] and contained per ml: 0.3 ml of S-9 fraction, 8 μmol of $MgCl_2$, 33 μmol of KCl, 5 μmol of G6P, 0.5 unit G6P-dehydrogenase and 4 μmol of NADPH in 100 μmol of sodium

phosphate buffer (pH 7.4).

Results

Mutagenicity of Amide Type N-Nitroso Compounds

Spontaneous histidine prototrophic revertants per plate amounted to 110 to 150 in TA100 and 17 to 45 in TA98 throughout the experiments. The number of spontaneous revertants was subtracted from the appropriate experimental values before calculating the mutagenic activity of each compound.

The results obtained with 6 amide type N-nitroso compounds (**1** to **6**) are given in Table I. Without metabolic activation by S-9 mix, none of the amide type compounds showed any mutagenicity in TA100 and TA98. When S-9 mix was added to the preincubation mixture, compounds **5** and **6**, which have a benzyl group at position R′, were weakly mutagenic to TA100. The mutagenic activity of both compounds was lower than that of dimethylnitrosamine (DMN), a positive control. DMN induced 167 revertants/plate/μmol (Table I).

Table I. Mutagenic Activities of $RCONHCH_2N(NO)R'$

No.	$RCONHCH_2N(NO)R'$ Substituents R	R′	Mutagenic dose range to TA100 (μmol)[a]	Revertants/plate/μmol compound[b] TA100 −S-9	TA100 +S-9[c]	TA98 −S-9	TA98 +S-9
Amide type							
1	CH_3	CH_3		0	0	0	0
2	CH_3	C_4H_9		0	0	0	0
3	CH_3	$CH(CH_3)COOH$		0	0	0	0
4	CH_3	$CH(COOH)CH(CH_3)_2$		0	0	0	0
5	CH_3	$CH_2C_6H_5$	2.7—27	0	79	0	0
6	C_2H_5	$CH_2C_6H_5$	2.7—27	0	114	0	0
Carbamate type							
7	CH_3O	CH_3	2.7—27	0	559	0	0
8	C_2H_5O	CH_3	1.4—27	0	610	0	0
9	$(CH_3)_2CHO$	CH_3	2.7—27	0	517	0	0
10	$C_6H_5CH_2O$	CH_3	0.27—2.7	0	2860	0	0
11	$(CH_3)_2CHO$	$CH_2C_6H_5$	1.4—27	0	293	0	99
12	C_2H_5O	$CH_2C_6H_5$	0.14—2.7	0	550	84	173
Positive control							
	Dimethylnitrosamine		0.27—270	0	167	0	0
	2-Acetylaminofluorene		50μg[d]	0	23200	0	35000
	Benzo(*a*)pyrene		10μg[d]	0	18700	0	7500

a) The dose range of each compound in the preincubation mixture was 0.027 μmol (4×10^{-5} M) to 27 μmol (4×10^{-2} M). Compound **11** and **12** were mutagenic to TA98 at 27 μmol.
b) Average of 2 to 3 independent experiments with 5 plates.
c) Metabolic activation with polychlorinated biphenyl-induced rat liver homogenate (S-9) was included.
d) Used for checking S-9 activity.

Mutagenicity of Carbamate Type N-Nitroso Compounds

The results obtained with 6 carbamate type N-nitroso compounds (**7** to **12**) are also presented in Table I. All of them showed mutagenicity to TA100 in the presence of S-9 mix. The mutagenic activities of these carbamate type compounds were higher than those of DMN and varied substantially. There was about a 10-fold difference in mutagenic activity between compounds **11** and **10**, which induced 293 and 2860 revertants/plate/μmol, respectively.

Compounds **11** and **12**, which have a benzyl group at position R′ also showed mutagenic effects on TA98. Compound **12** gave a positive response with or without S-9 activation.

The mutagenic activities of 2-acetylaminofluorene and benzo(*a*)pyrene, which were used

as positive controls for checking the activity of S-9 fraction, are also shown in Table I.

Discussion

A clear structure–mutagenicity relationship was observed for amide type and carbamate type N-nitroso compounds. Chemical generation of diazoalkane or phenyldiazomethane from the test compounds in alkaline solution (see Chart 1) proceeds faster and gives higher yields in the case of carbamate type compounds.[1b,c] The yield of phenyldiazomethane from compound **5** in alkaline solution was 35%, whereas that from compounds **7** to **12** was 36% (compound **11**) to 81% (compound **9**). However, this difference is probably due to higher solubility of carbamate type compounds in the reaction mixture compared with that of amide type compounds.[1b] Though we have no direct evidence regarding the pathway of metabolic activation of these N-nitroso compounds, it is considered that biological activation under physiological conditions (pH 7.4) may proceed other than by chemical reaction. As demonstrated by Czygan *et al.*[6] and Druckrey *et al.*,[7] enzymatic α-hydroxylation of N-nitroso compounds may occur first. The α-hydroxylated derivative is then converted nonenzymatically into R′ (alkyl or benzyl)-diazonium *via* R′-diazohydroxide to generate the alkylcarbonium or phenylcarbonium ion (Chart 2). Differences of R substituents, methyl (compound **1**) or methoxy (compound **7**) and ethyl (compound **6**) or ethoxy (compound **12**), resulted in large differences in mutagenic activity. Replacement of an alkyl group at position R with an alkoxy or benzyloxy group may increase the rate of α-hydroxylation in some way. A detailed study of the differences in metabolic activation of these compounds is in progress.

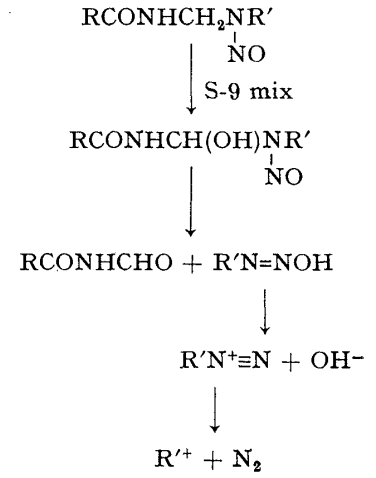

Chart 2. Proposed Pathway of Metabolic Conversion of $RCONHCH_2N(NO)R'$

Though amide type compounds tested in this experiment were not mutagenic or only weakly mutagenic to the bacteria in amounts of up to 27 μmol (4×10^{-2} M), it was reported by Ishidate *et al.*[2] that compounds **1** and **6** induced chromosomal aberrations in cultured Chinese hamster cells at concentrations of 2 mg/ml (1.5×10^{-2} M) and 1 mg/ml (4.5×10^{-3} M), respectively. Since the above data were obtained after incubation for 24 hr or 48 hr with the compounds, there is a posibility that reverse mutation of *Salmonella typhimurium* could be induced by prolonged treatment with higher concentrations of the amide type compounds. In this experiment, the highest dose was 27 μmol (4×10^{-2} M), which was limit of solubility in water of some of the test compounds. To test higher concentrations, dimethylsulfoxide (DMSO) had to be used for solubilizing the compounds. However, the mutagenicity of all the N-nitroso compounds tested in this experiment was inhibited by the addition of DMSO (data not shown). Yahagi *et al.*[8] have also reported an inhibitory effect of DMSO on the mutagenicity of dimethylnitrosamine and diethylnitrosamine.

Mutagenicity to frameshift strain TA98 was observed only in compounds **11** and **12**, which have a benzyl group at position R′. In particular, compound **12** was mutagenic to TA98 without any metabolic activation. It is likely that compound **11** is also mutagenic to TA98 without activation, but this compound had a bactericidal action at a concentration of 27 μmol, which showed positive response to TA98 in the presence of S-9 mix. It is not clear why compounds **5** and **6**, which also have a benzyl group at position R′, were not mutagenic to TA 98 without activation.

Carcinogenicities of these 12 N-nitroso compounds have not been tested, but it seems

very likely in view of their mutagenicity that these compounds, especially the carbamate type compounds, have a carcinogenic effect.

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Stereoisomeric Alanine Peptides as Substrates for Human Spleen Fibrinolytic Proteinase (SFP)[1)]

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Four kinds of stereoisomeric Z–Ala–Ala–OMe and eight kinds of stereoisomeric Z–Ala–Ala–Ala–OMe were tested as substrates for human spleen fibrinolytic proteinase (SFP) in comparison with porcine pancreatic elastase. Both enzymes exhibited esterase activity towards not only Z-L-Ala-L-Ala-L-Ala–OMe (VI) but also Z-D-Ala-L-Ala-L-Ala–OMe (VII). The rate of esterolysis of VI by elastase was only about twice the rate of esterolysis by SFP although the rate of amidolysis of Suc-L-Ala-L-Ala-L-Ala-*p*NA (XVI) by elastase was tenfold faster than that by SFP.

Keywords——synthetic substrate; stereoisomeric alanine peptide; human spleen fibrinolytic proteinase; porcine pancreatic elastase; rate of hydrolysis

Nagamatsu has described the isolation and purification of a neutral proteinase capable of degrading fibrin and fibrinogen from human spleen tissue (SFP).[2)] Later, Okamoto *et al.*[3)] reported that the properties of this enzyme, as far as examined, were similar to those of elastase, especially human leucocyte elastase, except for the substrate specificity. For instance, the activity of SFP to release *p*-nitroaniline from the substrate, Suc–L–Ala–L–Ala–L–Ala–*p*NA, is much poorer than that of porcine pancreatic elastase. Further studies on SFP are under way in our laboratories. In the present paper, we report on the properties of stereoisomeric alanine peptides as substrates for SFP and porcine pancreatic elastase, and compare the esterolytic and amidolytic activities of SFP and porcine pancreatic elastase.