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Mutagenicity of *N*-Methyl-*N'*-aryl-*N*-nitrosoureas and *N*-Methyl-*N'*-aryl-*N'*-methyl-*N*-nitrosoureas in Relation to Their Alkylating Activity

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ABSTRACT

Mutagenic activities of the series of *N*-methyl-*N'*-aryl-*N*-nitrosoureas (I-X) and *N*-methyl-*N'*-aryl-*N'*-methyl-*N*-nitrosoureas (II-Y) on *Salmonella typhimurium* TA 1535 have been investigated. All I-X compounds had strong mutagenic potency without metabolic activation, and their effectiveness was even greater than that of *N*-methyl-*N*-nitrosourea. The mutagenicity at a given concentration of the compounds (3.35×10^{-2} μmol) was compared with the chemical alkylating activity on 4-(*p*-nitrobenzyl)pyridine. A positive parallelism between them was observed in the cases of *N*-methyl-*N'*-(*p*-methoxyphenyl)-*N*-nitrosourea, *N*-methyl-*N'*-(*p*-methylphenyl)-*N*-nitrosourea, *N*-methyl-*N'*-phenyl-*N*-nitrosourea, and *N*-methyl-*N'*-(*p*-chlorophenyl)-*N*-nitrosourea, whereas this correlation broke down with *N*-methyl-*N'*-(*p*-acetylphenyl)-*N*-nitrosourea and *N*-methyl-*N'*-(*p*-nitrophenyl)-*N*-nitrosourea. These observations were discussed in terms of both the inductive effect and hydrogen-bonding nature of the substituents (X) and the difference in the chemical and biological processes. On the other hand, the II-Y compounds which are the methyl-substituted derivatives on the second nitrogen (N') had no significant or weaker mutagenicity when compared to the corresponding I-X compounds. This was also in agreement with the results of the chemical alkylation. The dose-response curves of the I-X and II-Y compounds showed that all mutagenicities with the exception of *N*-methyl-*N'*-phenyl-*N'*-methyl-*N*-nitrosourea increased similarly in accordance with an increase in their concentrations, indicating that the lethal effect might not influence the observed mutagenicity. Mechanistically, it has been suggested that removal of the hydrogen on N' may be involved in the transition state of both the chemical and mutagenic actions of the I-X compounds. With the II-Y compounds, however, further investigations are needed to elucidate the observed results since other factors such as inductive and steric effects by the methyl group on N' may also be of importance.

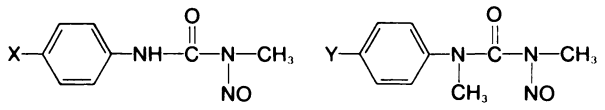
INTRODUCTION

Although *N*-nitrosoureas have been extensively investigated from the viewpoints of mutagenicity (14, 19), carcinogenicity (5, 13, 15, 21), and anticarcinogenicity (3, 7, 9, 18), their mechanism of action has not yet been established (10, 15). There is, however, some evidence that they generally produce active alkylating species without enzymatic activation and that these species react with cellular nucleophiles (4, 17) leading to the alkylated products which may cause the various activities. Thus, relationships between the chemical alkylating and biological action of *N*-nitrosoureas have been discussed from different points of view (13).

In order to gain insight into the factors that influence chemical and biological activity, however, systematic investigations on substituted *N*-nitrosoureas must be carried out (18, 22). Therefore, we synthesized the I-X[2] and II-Y compounds and investigated their alkylating activity toward 4-(*p*-nitrobenzyl)pyridine (6, 25) as a model compound of the cellular nucleophiles.

In this paper, we would like to present the results of the mutagenicity of the I-X and II-Y compounds on *Salmonella typhimurium* TA 1535 (1) and to compare them with their alkylating activity.[3]

MATERIALS AND METHODS



I-X II-Y

X = —OCH_3, —CH_3, —H, —Cl, —$COCH_3$, —NO_2; Y = —H, —NO_2.

The I-X compounds were synthesized by nitrosation of the corresponding *N*-methylureas which were made by the reactions of the *p*-substituted anilines with methylisocyanate, and the II-Y compounds were done from the corresponding *p*-substituted *N*-methylanilines. MNU was prepared by the same method developed by Arndt (2) and recrystallized from ether. Dimethyl sulfoxide (Dojindo Laboratories, Kumamoto, Japan) was used without purification.

Mutagenic Activity of *N*-Nitrosoureas. The mutation tests were carried out by the Ames test modified by Yahagi (23). A typical run was as follows. To a test tube containing 0.10 ml of a culture of *S. typhimurium* TA 1535 (about 1×10^8 cells) was added an *N*-nitrosourea (1.0 to 6.0×10^{-2} μmol) in 0.15 ml of 5% dimethyl sulfoxide in water. This mixture was incubated first at 20° for 1 hr and then at 37° for 1 hr. To it were added 2 ml of soft agar (45°) which consists of 50 ml of 0.6% NaCl solution containing 0.35 g of Difco agar and of 5 ml of water containing a limited amount of L-histidine (0.5 mM)-biotin (0.5 mM). The resulting mixture was poured onto a minimal-glucose agar plate. After incubation for 2 days at 37°, the colonies on the plate were counted.

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[2] The abbreviations used are: I-X, *N*-methyl-*N'*-aryl-*N*-nitrosoureas; II-Y, *N*-methyl-*N'*-aryl-*N'*-methyl-*N*-nitrosoureas; MNU, *N*-methyl-*N*-nitrosourea; I-OCH_3, *N*-methyl-*N'*-(*p*-methoxyphenyl)-*N*-nitrosourea; I-CH_3, *N*-methyl-*N'*-(*p*-methylphenyl)-*N*-nitrosourea; I-H, *N*-methyl-*N'*-phenyl-*N*-nitrosourea; I-Cl, *N*-methyl-*N'*-(*p*-chlorophenyl)-*N*-nitrosourea; I-$COCH_3$, *N*-methyl-*N'*-(*p*-acetylphenyl)-*N*-nitrosourea; I-NO_2, *N*-methyl-*N'*-(*p*-nitrophenyl)-*N*-nitrosourea; II-H, *N*-methyl-*N'*-phenyl-*N'*-methyl-*N*-nitrosourea; II-NO_2, *N*-methyl-*N'*-(*p*-nitrophenyl)-*N'*-methyl-*N*-nitrosourea.

[3] K. Yano and K. Yoshida, manuscript in preparation.

RESULTS AND DISCUSSION

Since an approximately linear relationship is known between mutagenicity and carcinogenicity, we investigated the mutagenic potency of the I-X and II-Y compounds on *S. typhimurium* TA 1535, which is a specific strain for base substitutions (1), by the modified Ames test (23) without using the S-9 mixture. The numbers of revertants of TA 1535 at a concentration of the nitrosoureas (3.35×10^{-2} μmol each) were summarized in Table 1 in which their chemical alkylating activities[3] were also presented to discuss the relationships between the chemical and biological activity of *N*-nitrosoureas.

It may be seen from Table 1 that all I-X compounds have strong mutagenic potency, even greater than that of MNU which is known as one of supermutagens (20), without metabolic activation, although their effectiveness is quite variable with change of the substituents (X). Considering the compounds themselves, this variability might be mainly related to an electrostatic (inductive) effect (12) rather than a steric effect (24) of the X groups because their gross structural differences are only in the *p* position of the phenyl groups where the reaction center to produce alkylating species seems far away (16). Thus, the mutagenic activities of the I-X compounds were compared with their alkylating activities, which were linearly influenced by the inductive effect of the X groups.[3] The comparison is shown in Chart 1 using the data presented in Table 1.

A positive parallelism is observed in a range from I-OCH_3 to I-Cl, arranged in order of increasing σp values (11), while this correlation breaks down in the case of I-$COCH_3$ and I-NO_2 which are the hydrogen acceptors (8) as well as strong electron-withdrawing groups (11). The above parallelism suggests that the electron-withdrawing nature of the X groups linearly increases both mutagenicity and alkylating activity of the nitrosoureas. With I-$COCH_3$ and I-NO_2, however, in addition to the inductive effect, other factors must be taken into consideration. When the chemical and mutagenic processes are compared, the latter is much more complicated than the former not only since the agents must enter recipient cells effectively for production of biological potency but also because there are many nucleophiles inside and outside the cells for the agents to react with. Thus, the agents with higher reactivity might competitively react with other nucleophiles under the experimental conditions (*e.g.*, —SH, —NH_2) as well as the targets of cellular nucleophiles. Furthermore, compounds with the hydrogen acceptors appear to have more difficulty entering the cells through the hydrophobic cell membranes than do those with the non-hydrogen bonders (8). On the other hand, in the chemical process, the agents can directly react with 4-(*p*-nitrobenzyl)pyridine as a nucleophile. Consequently, the mutagenicity of I-$COCH_3$ and I-NO_2 might decrease in contrast to their increasing alkylating activity.

This explanation is supported by the dose-response curves of the nitrosoureas, which are shown in Chart 2. All mutagenicities increased similarly in accordance with an increase in their concentrations. This indicates that the lethal effect of I-$COCH_3$ or I-NO_2, which is a strong alkylating agent, on *S. typhimurium* TA 1535 might not be an essential factor to reduce its mutagenicity. This may also be applied to explain the weaker mutagenicity and the stronger alkylating activity of MNU compared to those of the I-X compounds except I-$COCH_3$ and I-NO_2.

On the other hand, II-H, which is a methyl-substituted derivative on N′ of I-H, had no significant mutagenic potency up to a concentration of 6.0×10^{-2} μmol; while the mutagenicity of II-NO_2, which has a stronger electron-withdrawing group, increased with the increase in its dose; and its potency was much higher than that expected from Table 1. This discrepancy may be due to the fact that the mutagenicity in Table 1 was measured at low dose probably near its threshold.

When the mutagenicity of II-NO_2 is compared to that of I-NO_2, it will be known that II-NO_2 has at least one-half of the activity of I-NO_2. This observation cannot be explained on the same basis as that for the I-X compounds since inductive and steric effects by the methyl group on N′ may be of importance. Nevertheless, this result in agreement with the alkylating activity indicates that the hydrogen on N′ plays an important role on both chemical and biological action of *N*-nitrosoureas when there is no need of metabolic activation.

Mechanistically, the results presented above suggest that the hydrogen on N′ of the I-X compounds may be removed by

Table 1

Mutagenic activity of N-nitrosoureas on S. typhimurium TA 1535

A mixture of *S. typhimurium* TA 1535 (approximately 1×10^8 cells) and an *N*-nitrosourea (3.35×10^{-2} μmol) in dimethylsulfoxide-water was incubated at 20° for 1 hr and at 37° for 1 hr in the dark and mixed with 2 ml of soft agar (45°). The resulting mixture was poured onto a minimal-glucose agar plate. After incubation for 2 days at 37°, the colonies on the plate were counted.

N-Nitrosourea	His^+ revertants/plate[a]	Relative mutagenicity[b]	Alkylating activity[c]
I-OCH_3	218.8 ± 18.0[d]	1.15	0.372
I-CH_3	266.7 ± 18.2	1.43	0.440
I-H	358.0 ± 35.4	1.97	0.515
I-Cl	497.0 ± 11.5	2.79	0.615
I-$COCH_3$	269.4 ± 53.3	1.45	0.682
I-NO_2	264.4 ± 33.1	1.42	0.762
II-H	19.4 ± 10.4		0.030
II-NO_2	48.8 ± 7.9		0.040
MNU	193.6 ± 28.4	1.00	0.675
Blank	24.8 ± 10.7		0.015

[a] The results represent at least 3 experiments performed in duplicate except those of II-H and II-NO_2 which represent 2 experiments in duplicate.

[b] The value obtained by subtracting the blank from each value was divided by that of MNU.

[c] The alkylating activity was estimated spectrophotometrically at λ_{max} (540 nm) of the reaction mixture of an *N*-nitrosourea and 4-(*p*-nitrobenzyl)pyridine.[3]

[d] Mean ± S.D.

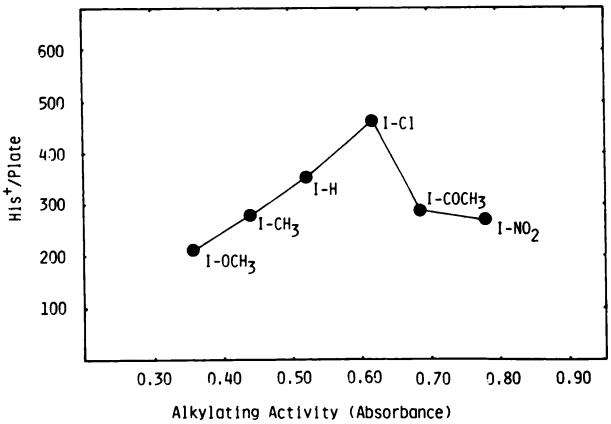


Chart 1. Relationship between mutagenicity and alkylating activity of the I-X compounds. The mutagenic activity was plotted *versus* the alkylating activity by using the data presented in Table 1.

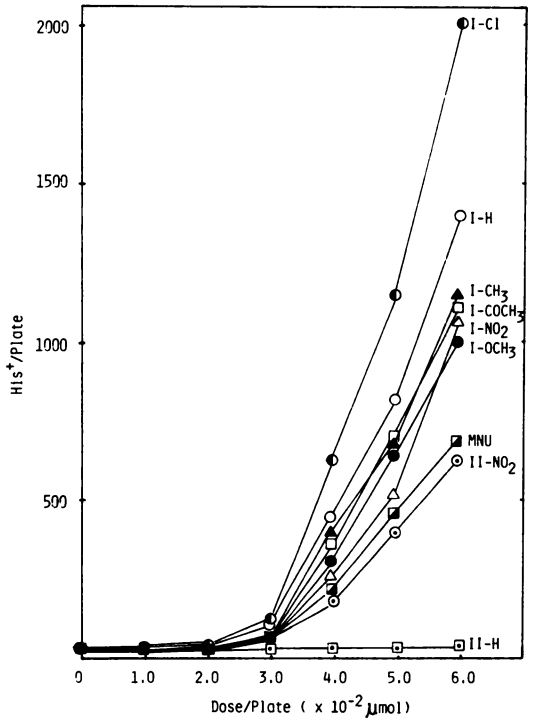
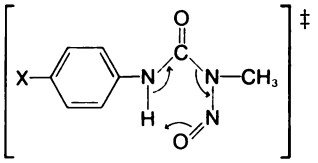


Chart 2. Dose-response curves of mutagenicity of the I-X and II-Y compounds and MNU on *S. typhimurium* TA 1535 without S-9 mix.

a concerted process which leads to methyldiazohydroxide and an isocyanate derivative which give alkylated and other products. The chemical structure of this transition state is:



Accordingly, the compounds with stronger electron-withdrawing groups that easily remove the hydrogen have higher alkylating activities resulting in higher mutagenicities. This mechanistic implication may be related to the concerted mechanism proposed by Druckrey (4) in carcinogenic experiments. With the II-Y compounds, further investigations are needed to elucidate the observed mutagenicity.

Our present results demonstrate that, if one carefully chooses proper experimental designs considering several factors, one will be able to obtain a distinct relationship between specific structures and biological activities of the alkylating agents. Furthermore, the future investigations about correlation of the mutagenicity of the I-X and II-Y compounds with their carcinogenicity will provide some fundamental information for the mechanistic study of carcinogenesis by *N*-nitrosoureas.

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