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Studies on Chemical Carcinogens. XXIV.[1] Mutagenic Potency of Alkylated 4-Nitroquinoline 1-Oxides: Its Dependence on the Rate of Metabolic Activation

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Twelve kinds of alkylated derivatives of 4-nitroquinoline 1-oxide (4NQO) were tested for their mutagenicity on *S. typhimurium* TA100. The assay method was slightly modified from the original procedure of Ames in order to obtain a better correlation with intrinsic chemical properties of the compounds. It was found that mutagenic potencies of these derivatives were linearly correlated with the metabolic rates of 4NQO's to the corresponding 4-hydroxyaminoquinoline 1-oxides (4HAQO's), the correlation coefficient being 0.930. The steric requirements for enzymic reduction of the nitro group seem to be the major determinant of the mutagenic activity of the derivatives, and surprisingly, the substituents do not have any appreciable effect either on the subsequent metabolic activation step (aminoacylation of 4HAQO's) or on the ultimate nonenzymatic modification step.

Keywords——4-nitroquinoline 1-oxide; mutagenicity; metabolism; capacity factor; metabolic rate

It is well known that 4-nitroquinoline 1-oxide (4NQO) is metabolically activated to 4-hydroxyaminoquinoline 1-oxide (4HAQO), which is then enzymatically acylated with an amino acid to form the ultimate carcinogen, 4-aminoacyloxyaminoquinoline 1-oxide.[2] It has been assumed that this ultimate metabolite attacks nucleic acid bases through the following two processes: electrophilic aminations through a nitrenium (or nitrene) intermediate (A) and electrophilic arylations through a carbenium intermediate (B), as shown in Chart 1. Steps I and II shown in the chart are enzymic, whereas step III is non-enzymic. A substitution at position 5 should retard the reaction rates at steps I and III (probably also at step II) and that at position 2 should sterically hinder all the reactions of the carbenium intermediate (B) at step III. In addition, 3-substitution should seriously affect all the reaction steps electronically and sterically. In fact, carcinogenicity of 3-methyl-4NQO has not yet been demonstrated.[3] McCoy *et al.*[4] argued that mutagenicity of this non-carcinogenic derivative was due to its electrophilic reactivity only through the nitrenium intermediate, whereas that of non-substi-

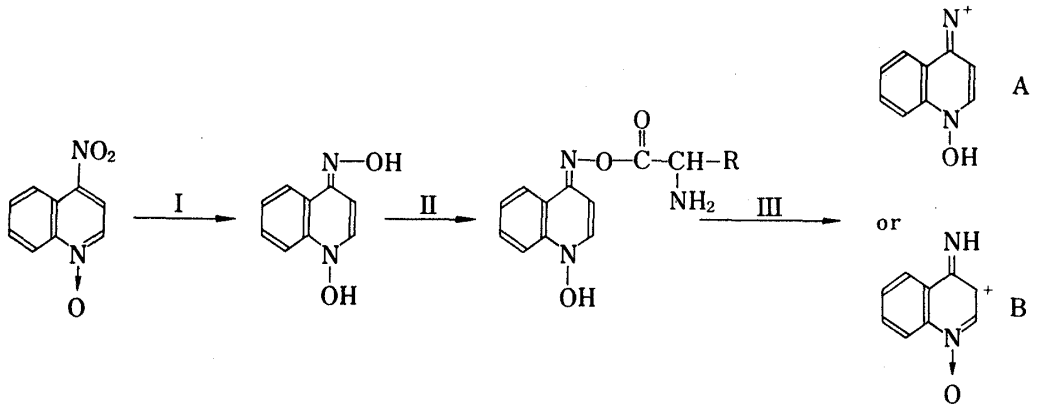

Chart 1. Proposed Molecular Mechanism for Mutagenesis by 4-Nitroquinoline 1-Oxide

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tuted 4NQO was due to reactivities through both intermediates, A and B; *i.e.*, a different molecular process might be operating in the mutagenesis by 3-methyl-4NQO from that by 4NQO.

The present study was undertaken to obtain more quantitative information on the mutagenicity of substituted 4NQO's, with the aim of clarifying the molecular mechanism involved in 4NQO mutagenesis and carcinogenesis in more detail.

Experimental

Materials——The compounds examined were prepared by the methods cited in Table I:[5] 4NQO, 2-methyl-4NQO, 2-ethyl-4NQO, 2-propyl-4NQO, 2-isopropyl-4NQO, 3-methyl-4NQO, 5-methyl-4NQO, 6-methyl-4NQO, 6-ethyl-4NQO, 6-isopropyl-4NQO, 7-methyl-4NQO, and 8-methyl-4NQO.

Capacity Factors of 4NQO Derivatives——High-performance liquid chromatography (HPLC) was carried out as described in our previous paper with slight modifications,[6] with a JASCO TWINCLE type HPLC apparatus equipped with a JASCO UV-100 II ultraviolet detector and a 4.5×250 mm JASCO Finepack C_{18} column. The sample was dissolved in MeOH at a concentration of 0.03 mg/ml. The solution thus prepared was injected under full flow using a microsyringe. Elution was done with methanol–water (70: 30 v/v) and the flow rate was 1 ml/min. The eluted compounds were detected by measuring ultraviolet (UV) absorption at 393 and/or 255 nm. The capacity factor was calculated as $[(t_R - t_0)/t_0]$, where t_R and t_0 are the retention times of the compound to be tested and the solvent, respectively.[7]

Mutagenicity on *Salmonella typhimurium* TA100——The assay was carried out as described in our previous paper with a slight modification.[8] The bacterial cells were grown to an early stationary phase in liquid nutrient broth (0.8% Difco nutrient broth plus 0.5% NaCl) in an L-tube at 37°C for 10—12 h. Then, 0.8 ml of 0.25 M sodium phosphate buffer (pH 7.4) and 0.1 ml of dimethyl sulfoxide containing an appropriate amount of the test compound were added to 0.1 ml of the culture containing about 2×10^9 cells/ml. The reaction mixture was gently shaken at 37°C for 30 min, then diluted with 3 ml of the phosphate buffer. It was centrifuged at $1900 \times g$ for 20 min. The cells were suspended in 0.5 ml of the buffer and layered on minimal glucose agar medium (MM plate; 1.5% Difco-bacto-agar in Vogel-Bonner medium E[9] with 0.4% glucose), together with 2.0 ml of 0.8% molten top agar containing NaCl (0.6%), and 1/10 volumes of 0.5 mM histidine and 0.5 mM biotin, in an 86 mm disposable plastic Petri dish. The histidine prototroph revertant colonies were counted after incubation at 37°C for 2 d.

Measurement of Metabolic Rates——The bacterial cells were grown to an early stationary phase in liquid nutrient broth (0.8% Difco nutrient broth plus 0.5% NaCl) in an L-tube after incubation at 37°C for about 10 h. The cells were collected by centrifugation at $1900 \times g$ for 20 min, and resuspended in 0.25 M sodium phosphate buffer (pH 7.4) at 5-fold dilution with respect to the original cell concentration. To this cell suspension, a small about of dimethyl sulfoxide containing an appropriate amount of the test compound was added. The final concentration of dimethyl sulfoxide was 0.1 to 1.0%. At various intervals during incubation at 37°C, the test compound in the reaction mixture was extracted with 1 ml of benzene. The starting nitro derivatives were extractable with benzene but most of their metabolites were not. Then, the benzene

Table I. Metabolic Rates, Capacity Factors, and Melting Points of Alkyl Derivatives of 4-Nitroquinoline 1-Oxide

Substituent	Compound number	mp (°C)	Rate constant[a] (min^{-1})	Half-life	Capacity[b] factor
(Nil)	(1)	153	0.1591	4.4	0.796
2-Methyl	(2)	157	0.0604	11.5	1.076
2-Ethyl	(3)	128	0.0277	25.0	1.537
2-Propyl	(4)	86	0.0285	24.3	2.042
2-Isopropyl	(5)	89	0.0141	49.1	2.058
3-Methyl	(6)	180	0.0000		1.065
5-Methyl	(7)	175	0.00224	309.5	0.969
6-Methyl	(8)	186	0.1159	6.0	1.196
6-Ethyl	(9)	126	0.0185	37.4	1.708
6-Isopropyl	(10)	100	0.00634	109.0	2.262
7-Methyl	(11)	165	0.1951	3.3	1.181
8-Methyl	(12)	154	0.1091	6.4	1.176

a) Pseudo-first order rate constants at 37°C in the culture medium for mutation assay.
b) Calculated from the retention times in high performance liquid chromatography.

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extracts were analyzed by UV spectroscopy, directly or after separation by HPLC. Thus, the starting nitro compound (compound **1**—**4**, **6**—**8**, **11**, or **12**) was quantitatively analyzed by measurement of UV absorption at around 390 nm of the benzene extract. In the cases of compounds **5**, **9**, and **10**, the benzene extract was subjected to HPLC analysis under the same conditions as in the procedure for capacity factor measurements, except for the eluting solvent; methanol–water (80: 20 or 70: 30 v/v). The benzene extracts from the reaction mixtures of the latter group of nitro compounds seemed to be contaminated with certain metabolites. The metabolic rates thus obtained are summarized in Table I.

Results and Discussion

Metabolic Rate of Alkylated 4NQO's

The time-course of decrease in the concentration of nitro compound was found to be a pseudo-first order process for each compound; the values of k_{obs} and $t_{1/2}$ are shown in Table I. The 3-methyl derivative was so resistant to metabolic change that no appreciable decrease in its concentration was observed under the experimental conditions employed.

The metabolic rates seem to be strongly influenced by the presence and position of the substituent. The metabolic rates increased in the following orders, depending on the position of substitution and the kind of substituent.

(1) Dependence on the position of alkyl substituents

3-Me<5-Me≪2-Me<8-Me≈6-Me<(H)<7-Me

6-Et<2-Et

6-iso-Pr<2-iso-Pr

(2) Dependence on the kind of alkyl substituent

at position-6

isopropyl<ethyl<methyl<(H)

at position-2

isopropyl<propyl≈ethyl<methyl<(H)

As far as the methyl substituents are concerned, substitutions at position-3 and position-5, which are close to the nitro group to be reduced, more effectively reduce the metabolic rate than those at any other position. However, as can be seen with a series of 6-substituted derivatives, the bulkiness of the alkyl group also influences the rate, although the 6-position is distant from the nitro group concerned. Since similar sizes of retarding effect were produced by the presence of ethyl and propyl groups, the retarding effect may be dependent primarily on the number of alkyl group on the α-carbon; *i.e.*, primary carbon ($-\underline{C}H_3$), secondary carbon ($-\underline{C}H_2CH_3$ and $-\underline{C}H_2CH_2CH_3$), or tertiary carbon ($-\underline{C}H(CH_3)_2$). Thus, bulkiness, which might be related to deviations of the molecule from a planar structure, may affect the formation of enzyme-substrate complexes and result in retardation of the metabolic rate.

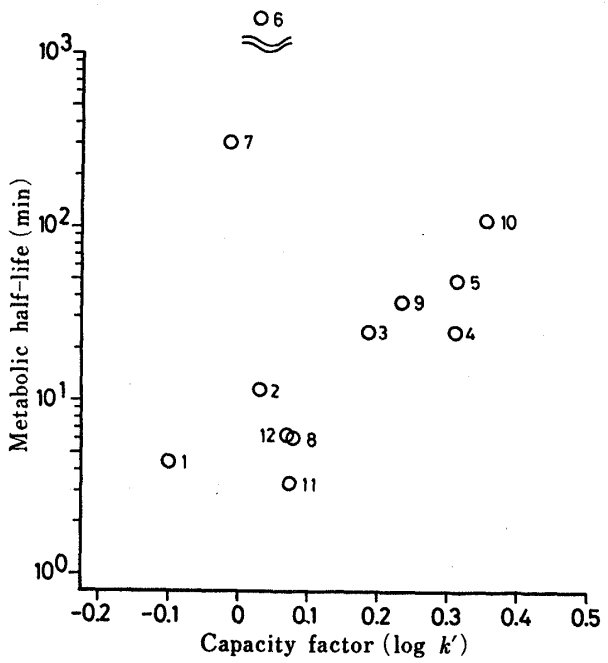

Fig. 1. log-log Plots of Metabolic Half-Lives *versus* Capacity Factors of alkylated 4NQO's

The numbers listed in the figure are the compound numbers tabulated in Table I.

Partition Properties of Alkylated 4NQO's

The capacity factors of alkylated 4NQO's were measured by liquid chroma-

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tography as a measure of their partition properties,[9] which might be related to the stability of the enzyme-substrate complex, the ability to permeate into the cell, *etc.* The data shown in Table II indicate that the order of capacity factor seems to parallel that of the number of carbon involved in the alkyl substituent. Thus, an increasing order of capacity factor is shown, depending on the size of substituent, as follows.

$$(H)(0.79)<CH_3\ (0.96—1.20)<C_2H_5\ (1.53—1.71)<C_3H_7\ (2.05—2.26)$$

These data are plotted *versus* metabolic half-life on log-log scales in Fig. 1. It seems that the contribution of the partition properties of the compound is not significant in determining of the enzymic reduction rate. The steric requirements for the enzymic reduction of the nitro group seem to be related to the crowdedness around the nitro group and probably to the planarity of the molecule, which might affect the formation of enzyme-substrate complex.

Mutagenicity on *S. typhimurium* TA100

These derivatives were tested for mutagenicity by means of a modified Ames' test,[10,11] which includes an additional process of washing the cells treated with the mutagen in order to eliminate the residual mutagen before the plating. This modified method is much more effective to obtain quantitative correlations of the mutagenicity of a series of compounds with their chemical and physicochemical properties,[12] compared with the method widely used as the original Ames' test.[11] The data are shown in Table II. max. Rev/μM is the maximum value of the number of revertants/plate divided by the concentration under these experimental conditions. R_{max} is the maximum number of revertants/plate when the dose–response curve showed a maximum. C_{max} is the concentration required for giving R_{max}. C_{max} is a measure of the cytotoxic potency of the compounds, giving an appreciable extent of cell killing, *e.g.*, roughly correspondingto D_0 (37% survival doses) in certain cases.[13] The values of max. Rev/μM are usually obtained from the linear portion of the dose–response curves. Therefore, they represent a measure of the relative mutation frequency among the compounds examined, since no appreciable cell killing is induced under such experimental conditions.

Table II. Mutagenic Parameters of Alkyl Derivatives of 4-Nitroquinoline 1-Oxide

Substituent	max. Rev/μM[a]	C_{max}[b] (μM)	R_{max}[c]
(Nil)	870.	8.0	4700
2-Methyl	410.	20.0	4850
2-Ethyl	193.	80.0	7600
2-Propyl	115.	80.0	4067
2-Isopropyl	31.5	160.0	3500
3-Methyl	1.65		
5-Methyl	14.5	800.0	5350
6-Methyl	1120.	8.0	6000
6-Ethyl	130.	150.0	9300
6-Isopropyl	6.85	500.0	2000
7-Methyl	4014.	1.5	3600
8-Methyl	261.	40.0	3690

a) Maximum value of Rev/μM.
b) Concentration giving maximum number of revertants/plate.
c) Maximum number of revertants/plate at the maximum of the dose-response curve.

Correlation of Mutagenicity with Metabolic Rate

The values of max. Rev/μM obtained here were correlated with the metabolic half-lives, the plots being shown in Fig. 2. As can be seen in the figure, there is a linear correlation on the log-log scales. The correlation coefficient was 0.9301.

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This indicates that the mutagenic potency of this class of 4NQO derivatives, presented in terms of max. Rev/μM, is primarily determined by the rate of metabolic reduction of the nitro group into a hydroxyamino group, *i.e.*, by step I in Chart 1. This result suggests that step II and III are not significantly related to the determination of the mutagenic potency of the derivatives. It is rather surprising that neither the rate at step II nor that at step III is influenced by the presence of a methyl substituent at position 3 or 5, when one takes account of the molecular mechanism proposed in Chart 1.

Correlation of C_{max} with Metabolic Rate

It is worth noting that C_{max} is linearly correlated with the metabolic half-life on the log-log scales and that the slope is almost unity, as shown in Fig. 3. The correlation coefficiency was 0.9453. Therefore, it can be said that the potency for cell killing is mainly dependent on the ease of metabolic reduction of the nitro group, regardless of the substituent effects on further metabolic activation to acylamino derivative and on the reactivity of the ultimate structure, just as seen in connection with the mutagenicity of this class of compounds.

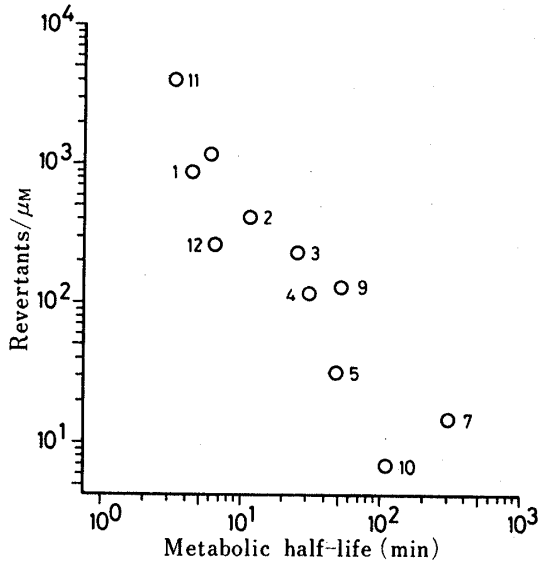

Fig. 2. log-log Plot of Maximum Value of Revertants/μM *versus* Metabolic Half-Lives of Alkylated 4NQO's

The numbers listed in the figure are the compound numbers tabulated in Table I. The correlation equation is log (max. Rev/μM) = −1.251 log ($t_{1/2}$) + 3.870 (r=0.9301; n=11).

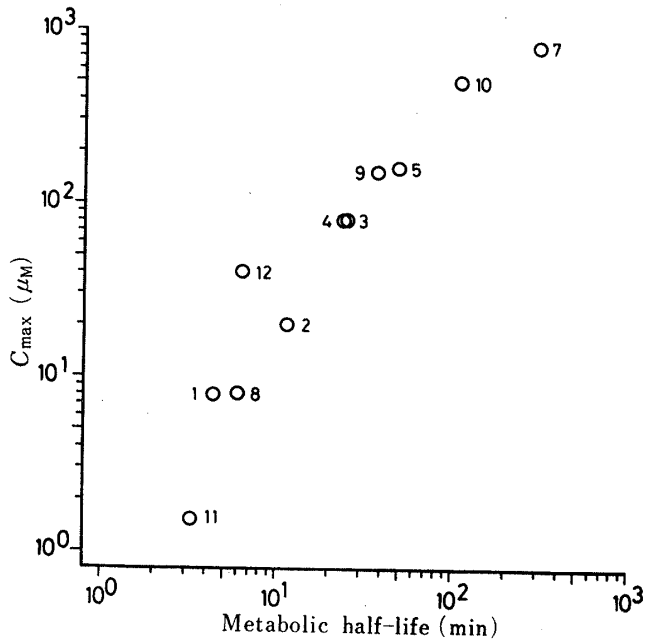

Fig. 3. log-log Plots of C_{max} Values *versus* Metabolic Half-Lives of Alkylated 4NQO's

The numbers listed in the figure are the compound numbers tabulated in Table I. The correlation equation is log (C_{max}) = 1.258 log ($t_{1/2}$) + 0.052 (r=0.9453; n=11).

Correlation of R_{max} with Metabolic Rate

R_{max} is a measure of the relative efficiency of mutagenesis to killing activity of compounds. This term corresponds roughly to relative mutagenic potency at D. dose (37% survival dose).[14] Fig. 4 shows a plot of R_{max} *versus* metabolic half-life. The deviations of R_{max}'s among all the derivatives examined do not seem to be substantial. In addition, there is no apparent correlation of R_{max} with the chemical structure of the derivatives. These results suggest that all the derivatives may have a common relative efficiency (mutagenic/killing activities); in other words, a common molecular mechanism may be operating in the mutagenic and killing processes.

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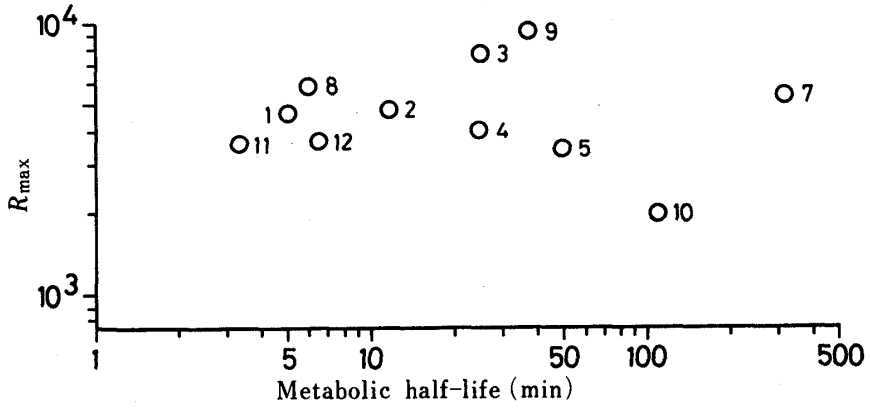

Fig. 4. log-log Plots of R_{max} Values *versus* Metabolic Half-Lives of Alkylated 4NQO's

The numbers listed in the figure are the compound numbers tabulated in Table I. The correlation equation, log $(R_{max})=0.020$ log $(t_{1/2})+3.689$ ($r=0.0682$; $n=11$), indicates that there is no significant correlation.

Conclusion

The metabolic rate of alkylated 4NQO derivatives in bacteria is strongly dependent on both the size of the alkyl group and the position of substitution. Vicinity of the alkyl group to the nitro group is a crucial rate-determining factor, but bulkiness of the alkyl group also makes a remarkable contribution in retarding the metabolic rate, even if the position of substitution is distant from the nitro group. These results indicate that the steric requirement for the enzymic reduction of the nitro group is a crucial structural requirement for the mutagenic activity of this class of compounds. The electronic status of the nitro group seems to have a lesser contribution, although correlations have been found between the reduction potentials of nitro groups and some biological activities of some classes of compounds.[15] It is also worth noting that the mutagenic potency, expressed as max. Rev/μM, was linearly correlated with the metabolic rate. This is an unexpected result, because both the second metabolic activation process (step II) and the ultimate chemical modification process (step III) had been thought to have a substantial contribution to the mutagenic potency.

In conclusion, it is worth mentioning that a much worse correlation was obtained when we used mutagenicity data obtained by the method widely used as the Ames' test, reported by Yahagi *et al.*,[11] although higher concentrations were required under the present experimental conditions for obtaining the same numbers of revertants, as compared with the experiments by the original method.

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