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Studies on Chemical Carcinogens and Mutagens. XXXVII.[1] A Simple Method to Predict Ultimate Structures of Chemical Mutagens, and Probably Carcinogens

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A simple and reliable method is proposed to determine if a mutagen requires any metabolic activation for its genotoxicity. It consists of a comparison of the mutation frequencies induced in tester bacteria treated with the mutagen in the presence and absence of butyl isothiocyanate (Bu-NCS), a non-specific enzyme inhibitor. A mutagen requiring metabolic activation mutates the cells to a lesser extent in the presence of Bu-NCS, whereas direct-acting mutagens are capable of mutating the cells to the same extent in the presence or absence of this inhibitor. Bu-NCS seems to more or less inhibit the metabolizing ability of the enzymes present in the tester cells without any subsequent effects on the cells' viability or mutability after the inhibitor is removed. The tester bacteria to be used should preferably be deficient in the excision repair mechanism for induced deoxyribonucleic acid damage. When a carcinogen is mutagenic in such a tester bacterial system, this method would predict the ultimate, *i.e.*, direct-acting, structure of the carcinogen because a common ultimate reactive species should be responsible for both mutagenicity and carcinogenicity. The validity of this method was tested by using 17 models of mutagenic carcinogens and the resuls supported the reliability of the prediction. Some limitations to the applicability of this method are discussed.

Keywords——ultimate carcinogen; ultimate mutagen; direct-acting; metabolic activation; isothiocyanate

As previously reported,[2,3] the mutation frequency induced in an *E. coli* B tester strain, H/r30R (wild type), by ultrabiolet (UV) irradiation was remarkably decreased by co-treatment with an appropriate acylating agent. When Hs30R (*uvrA*), an isogenic strain deficient in the excision repair system for desoxyribonucleic acid (DNA) damage, was used as the tester bacteria, the frequency was not changed at all by co-treatement with the same acylating agent. Thus, these acylating agents more or less retard DNA synthesis of the cells, giving the cells a greater chance to repair the UV-induced DNA damage by means of the excision mechanism, *i.e.*, by prolonging the lag period before the cell enters the mutable S-phase for DNA synthesis.[2,3] We found that the mutation frequency induced with a chemical mutagen, 4-nitroquinoline 1-oxide (4NQO), was decreased even in Hs30R, an excision repair-deficient strain.[3] These results seemed to suggest that the decrease thus observed was brought about by inhibition of the metabolic acitivation of 4NQO, as some biosyntheses including DNA synthesis were more or less inhibited. More generally, it was expected that such acylating agents might non-specifically inhibit the metabolizing ability of the enzymes endogenously present in the tester cells. It is worth mentioning here that this inhibitory effect was only temporary, *i.e.*, no after-effects were observed on the cells' viability or mutability after the inhibitors were removed.[2,3]

If this is the case, the mutagens which require metabolic activation for their genotoxicity would become less mutagenic by the co-treatment with such an enzyme inhibitor. Mutagenicity of direct-acting mutagens, on the other hand, would only be unchanged or

increased; an increase might be observed when the inhibitor reacts with SH-components and/or enzymes which would have quenched the direct-acting mutagen.[4]

Thus, it may be possible to distinguish indirect-acting mutagens, *i.e.*, promutagens, from the direct-acting mutagens by comparing the mutation frequencies induced in the presence and absence of such non-specific enzyme inhibitors. It is not always straightforward to predict the ultimate structures of mutagens even if they are directly mutagenic toward tester bacterias in the absence of exogenous metabolizing enzymes such as the S-9 mixture[5]; the tester bacteria themselves are usually capable of metabolizing the xenobiotica through endogenous hydrolysis, acylation, reduction, and so on.

The present paper describes the modification of the mutation frequency by co-treatment with a less lethal and non-specific enzyme inhibitor in several experimental mutageneses. The results are discussed in relation to identification of the ultimate structures in chemical mutageneses.

Butyl isothiocyanate was chosen as the inhibitor for the present purpose among many alkyl isocyanates, isothiocyanates, and some other acylating agents previously examined.[3] Butyl isothiocyanate is effective in inhibiting biosyntheses, less toxic to bacteria, less reactive with various types of mutagens, and in addition, non-volatile and water-soluble enough for the bioassay, as compared with other candidates.

Materials and Methods

Materials——Butyl isothiocyanate (Bu-NCS), *N*,*N*-dimethylcarbamoyl chloride (DMCC), *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG), methyl methanesulfonate (MMS), furylfuramide (AF2), and iodoacetamide were purchased from Tokyo Kasei Kogyo Co. Ltd. (Tokyo). The carcinogens used were prepared according to the reported methods: *N*-methyl-*N*-nitrosourea (MNU),[6] *N*-butyl-*N*-nitrosourea (BNU),[7] *N*-pentyl-*N*-nitrosourea (*N*-amyl-*N*-nitrosourea, ANU),[7] 4-nitroquinoline 1-oxide (4NQO),[8] 4-nitrosoquinoline 1-oxide (4NOQO),[9] 4-hydroxyaminoquinoline 1-oxide (4HAQO),[10] 4-(*N*-methylhydroxyamino)-quinoline 1-oxide,[11] 2-naphthohydroxamic acid (2NHA),[12] *O*-propionyl-2-naphthohydroxamic acid (Pr2NHA),[13] *N*-acetoxymethyl-*N*-ethylnitrosamine (AMENA),[14] and *p*-phenylstyrene oxide (PhSTO).[15]

Bacterial Tester Strains——The bacterial strains used were as follows. *Escherichia coli* B Hs30R (Arg^-, *uvrA*)[16] was gift from Prof. S. Kondo, Osaka University Medical School. *Salmonella typhimurium* TA100[5] was the stock strain of Prof. Kondo's laboratory, which was originally a gift from Prof. B. Ames, University of California.

Metabolic Rates of 4NQO in the Reaction Media in the Presence of Bu-NCS——*E. coli* B Hs30R cells were grown to the stationary phase in liquid nutrient broth (0.8% Difco nutrient broth containing 0.4% NaCl) by incubation at 37 °C for about 10 h. The cells were collected by centrifugation and washed twice with 1/15 M phosphate buffer (pH 6.8). The cells were resuspended in the phosphate buffer at 2.5×10^9 cells/ml and starved at 37 °C for 2 h. To 8.0 ml of the starved cell culture were added 32 ml of 1/15 M phosphate buffer (pH 6.8), 0.2 ml of a DMSO solution of 4NQO (final concentration, 1 μM), and 0.2 ml of a DMSO solution of Bu-NCS (final concentration, 0.00, 200, 400, or 800 μM). The reaction mixture thus prepared was shaken at 37 °C for 30 min and immediately chilled in an ice bath. The unreacted 4NQO was extracted from the chilled reaction mixture with 2.0 ml of benzene and analyzed quantitatively by measuring the UV absorbance at 393 nm. The inhibition ratio (%) was calculated with reference to the absorbance of the benzene extract from the blank reaction mixture without the cells (100% inhibition) and to the absorbance of that obtained from the reaction mixture without Bu-NCS (0% inhibition). The data shown in Fig. 3 are the means of duplicate experiments.

Mutagenicity in *E. coli* Hs30R (*uvrA*) in the Presence and Absence of Bu-NCS——First, 4.0 ml of 1/15 M phosphate buffer (pH 6.8), 0.05 ml of DMSO containing an appropriate amount of a mutagen, and 0.05 ml DMSO containing an appropriate amount of Bu-NCS were added to 1.0 ml of the starved cell culture containing about 2.5×10^9 cells, and then the "reaction mixture" was shaken at 37 °C for 30 min. For the assay in the absence of Bu-NCS, 0.05 ml of the solvent DMSO was added. The cells thus treated were washed twice with the phosphate buffer. Mutation assay was carried out as previously described.[3] Mutation frequency was calculated as $[(M-M_0)/N]$, where M and M_0 are the numbers of revertant colonies per ml of the "reaction mixture" of the test compound and those of the blank reaction mixture, respectively, and N is the number of surviving cells per ml of the "reaction mixture".

Mutagenicity in *Salmonella typhimurium* TA100 in the Presence and Absence of Bu-NCS——The cells were grown to the stationary phase in liquid nutrient broth (0.8% Difco nutrient broth plus 0.6% NaCl), collected by centrifugation, and washed twice plus 0.6% NaCl), collected by centrifugation, and washed twice with 2 ml of 0.25 M sodium phosphate buffer (pH 7.4). The cells were resuspended in the same buffer at about 2×10^9 cells/ml and starved

at 37 °C for 2 h. Then, 4.0 ml of the phosphate buffer, 0.05 ml of DMSO containing an appropriate amount of a test compound and 0.05 ml of DMSO containing an appropriate amount of Bu-NCS were added to 1.0 ml of the cell culture. For the assay in the absence of Bu-NCS, 0.05 ml of the solvent DMSO was added. The "reaction mixture" was gently shaken at 37 °C for 30 min. The cells thus treated were washed twice with the phosphate buffer. Mutation assay was carried out as previously reported.[17] The mutation frequency was calculated as $[(M-M_0)/N]$, where M and M_0 are the numbers of revertant colonies per ml of the "reaction mixture" of the test compound and those of the blank reaction mixture, respectively, and N is the number of surviving cells per ml of the "reaction mixture".

Mutagenicity in *E. coli* B Hs30R after Pretreatment with Iodoacetamide or Bu-NCS——Mutagenicity was assayed after the cells had been treated with iodoacetamide (200—1400 μM) or Bu-NCS (800 μM) at 37 °C for 30 min as described above for mutation assay. The cells thus treated were immediately used for mutation assay in the same way as described above.

Results

Toxicity of Bu-NCS on Bacterial Tester Strains

Cytotoxicity of Bu-NCS was tested on the excision repair-deficient tester strains, *E. coli* B Hs30R and *S. typhimurium* TA100. Figure 1 shows the dose–response curves after the treatment of the cells suspended in phosphate buffer at 37 °C for 30 min. More than 80% survival rate was obtained below 800 μM Bu-NCS in Hs30R and below 600 μM in TA100. It is worth noting that no appreciable increase in the number of mutants over the background level was observed in the whole range of concentrations examined (400 to 2000 μM) in either tester strain of bacteria.

Inhibitory Effect of Bu-NCS on Mutagenesis by 4NQO and on the Metabolic Activation Rate

When the cells were treated with 1 μM 4NQO in the presence of Bu-NCS for 30 min, a dose-dependent decrease was observed in the mutation frequency, as shown in Fig. 2. The degree of inhibition of 4NQO metabolic activation thereby observed was also dose-dependent as shown in Fig. 3. Taking account of the more than 80% survival rate below 800 μM Bu-NCS, it is suggested that the decrease in mutation frequency may be caused mainly by a retardation of the metabolic activation of 4NQO.

Comparison between Mutation Frequencies Induced in *E. coli* by Chemical Mutagens in the Presence and Absence of 800 μM Bu-NCS

As already reported,[3] no difference in the mutation frequency was observed in UV-mutagenesis or in X-ray-mutagenesis, regardless of the presence or absence of alkyl

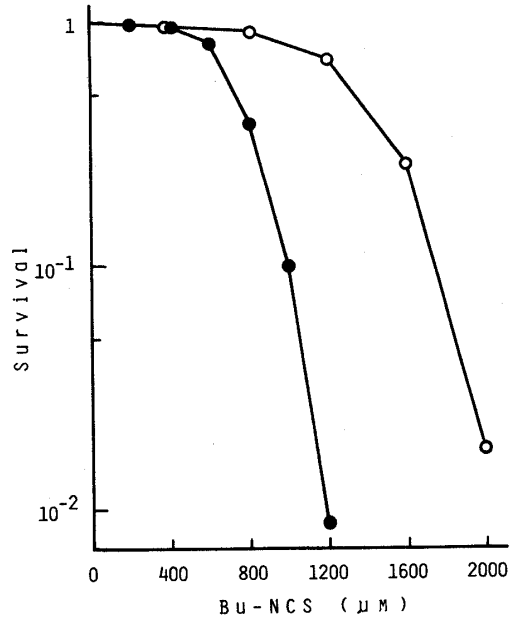

Fig. 1. Survival Rates of *E. coli* B Hs30R (—○—) and *S. typhimurium* TA100 (—●—) after Treatment with Bu-NCS in Phosphate Buffer (pH 6.8) at 37 °C for 30 min

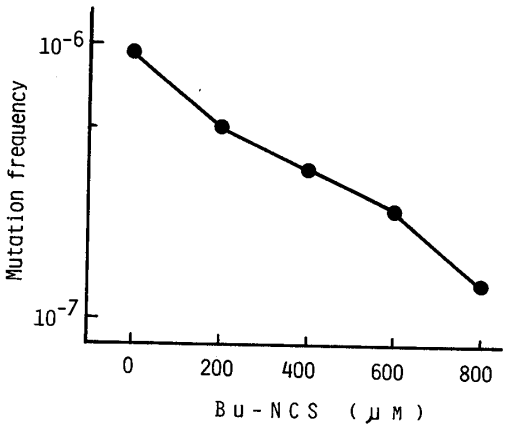

Fig. 2. Mutation Frequency of *E. coli* B Hs30R after Treatment with 1 μM 4NQO in the Presence of Bu-NCS at 37 °C for 30 min

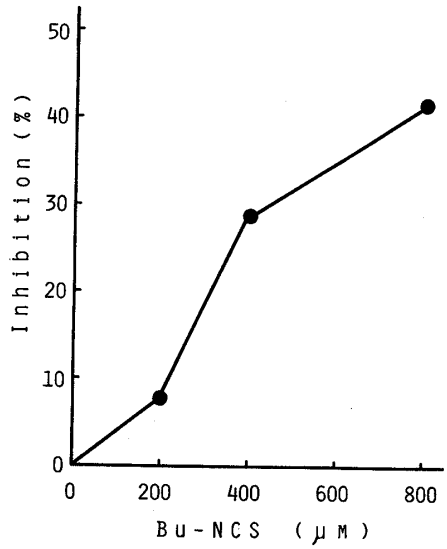

Fig. 3. Inhibitory Effect of Bu-NCS on the Rate of Metabolism of 4NQO under the Same Experimental Conditions as Used for Mutation Assay

See the legend to Fig. 2.

isocyanates and isothiocyanates, when an excision repair-deficient tester strain was used. In the present study, 17 chemicals were assayed for mutagenicity in *E. coli* B Hs30R (*uvrA*) in the presence and absence of 800 μM Bu-NCS. The dose–response curves are shown in Figs. 4 and 5. The mutagens shown in Fig. 4 are those which were subject to a more or less inhibitory effect of Bu-NCS, and those in Fig. 5 are the others, not included in this category. The mutagens included in the former category require metabolic activation for their mutagenicity. Thus, 4-nitro- and 4-nitrosoquinoline 1-oxides are metabolically reduced to 4-hydroxyaminoquinoline 1-oxide, which is then aminoacylated by an aminoacyl-transfer ribonucleic acid synthetase[17]; *N*-methyl-4-hydroxyaminoquinoline 1-oxide is probably metabolized to its acylated derivative,[11] although this has not been experimentally confirmed; *N*-acetoxymethyl-*N*-ethylnitrosamine is enzymically hydrolyzed to its *N*-hydroxymethyl derivative, which is converted to alkyldiazohydroxide[13]; 2-naphthohydroxamic acid is activated to its *O*-acyl derivative, which is thought to convert spontaneously to a certain type of reactive species, possibly naphthyl isocyanate[12]; furylfuramide is known to require reductive metabolism for its mutagenicity, although the ultimate form has not yet been identified.[19]

In contrast, the compounds shown in Fig. 5, *i.e.*, methyl methanesulfonate, three *N*-alkyl-*N*-nitrosoureas, *p*-phenylstyrene oxide and *N*,*N*-dimethylcarbamoyl chloride are all considered to modify cellular DNA without any metabolic activation. It is worth noting that *O*-propionyl-2-naphthohydroxamic acid may also be included in this category.[13]

Inhibitory Effect of Bu-NCS on Mutagenicity of MNNG: An Example of the Limitation of the Present Method

It is known that MNNG does not require any enzymic activation for its genotoxicity; however, an appreciable decrease in mutation frequency was observed upon co-treatment with Bu-NCS, as shown in Fig. 6. Here, it is necessary to recall that the hydrolytic activation of MNNG to methyldiazohydroxide is stimulated by the SH-functions of cell constituents,[20] and that Bu-NCS reacts quickly with the potently nucleophilic SH group. Therefore, the mechanism for this exceptional decrease may involve quenching by Bu-NCS of the SH-functions which would have activated MNNG. This mechanism is supported by the finding

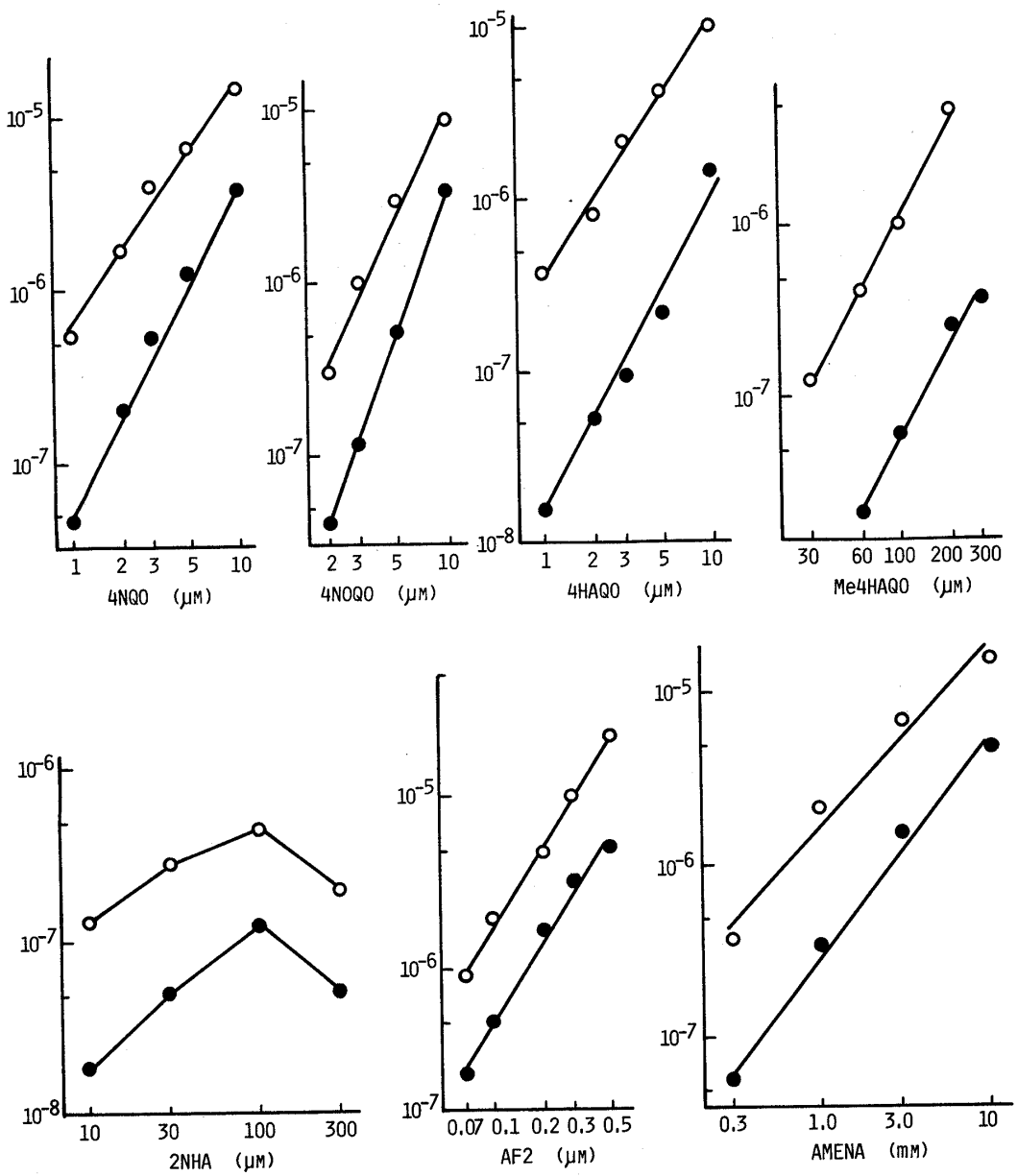

Fig. 4. Mutation Frequency of *E. coli* B Hs30R after Treatment with Mutagens at 37 °C for 30 min in the Presence (—●—) and Absence (—○—) of 800 μM Bu-NCS

This figure includes plots of mutation frequency (on the ordinate) *versus* dose (on the abscissa) for mutagens which showed an appreciable decrease in mutation frequency in the presence of Bu-NCS, *i.e.*, indirect-acting mutagens.

that the "frequency-decrease" in MNNG mutagenesis was also caused by pretreatment of the cells with iodoacetamide, which is known to be an effective quencher of the SH-group,[21] as shown in Fig. 7. Mutagenicity of 4NQO was not affected at all by the pretreatment with iodoacetamide, as shown in Fig. 7.

Assay with *S. typhimurium* TA100 as the Tester Strain

It was confirmed that TA100 (a *uvrA* strain)[5] could be used, instead of Hs30R, as the tester bacteria for the present purpose. Thus, mutagenicity of 4NQO was assayed in the presence and absence of Bu-NCS. TA100 is slightly more sensitive to this isothiocyanate, as shown in Fig. 1, so that 600 μM Bu-NCS was used as the metabolizing enzyme inhibitor. As

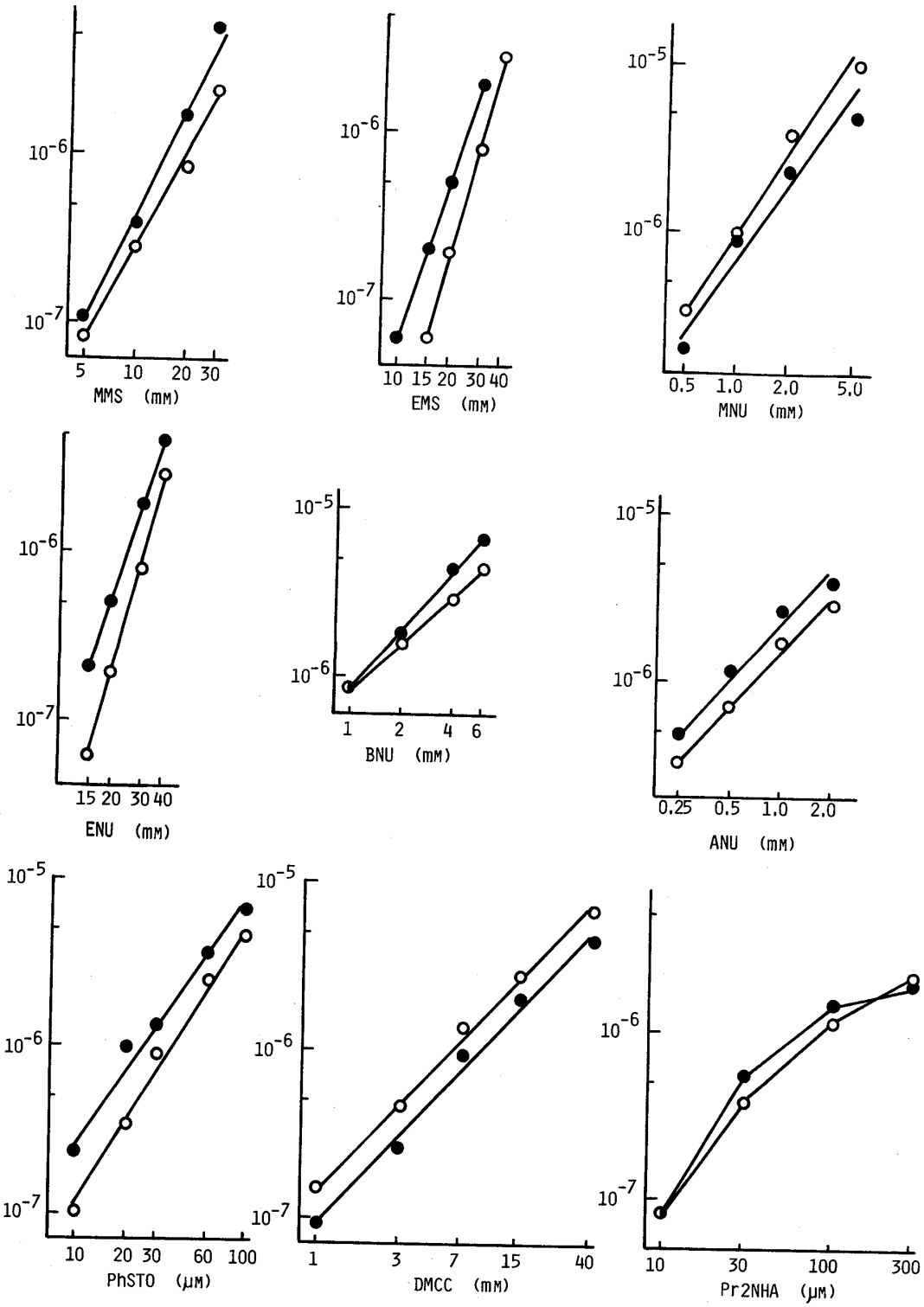

Fig. 5. Mutation Frequency of *E. coli* B Hs30R after Treatment with Mutagens at 37 °C for 30 min in the Presence (—●—) and Absence (—○—) of 800 μM Bu-NCS

This figure includes plots of mutation frequency (on the ordinate) *versus* dose (on the abscissa) for mutagens which did not show any appreciable decrease in mutation frequency in the presence of Bu-NCS, *i.e.*, direct-acting mutagens.

shown in Fig. 8, an appreciable decrease of mutagenicity was observed, as also seen in Hs30R mutagenesis.

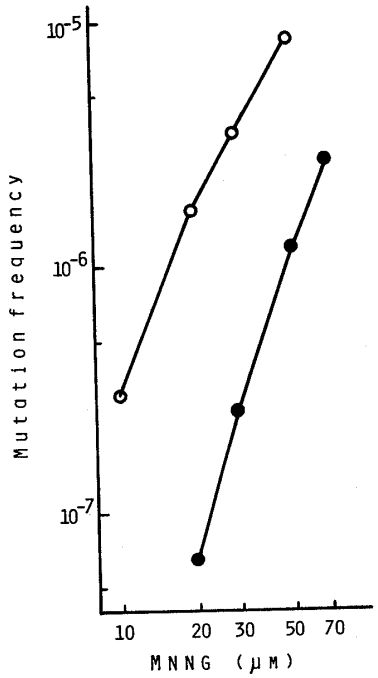

Fig. 6. Mutation Frequency of *E. coli* B Hs30R after Treatment with MNNG at 37 °C for 30 min in the Presence (—●—) and Absence (—○—) of 800 μM Bu-NCS

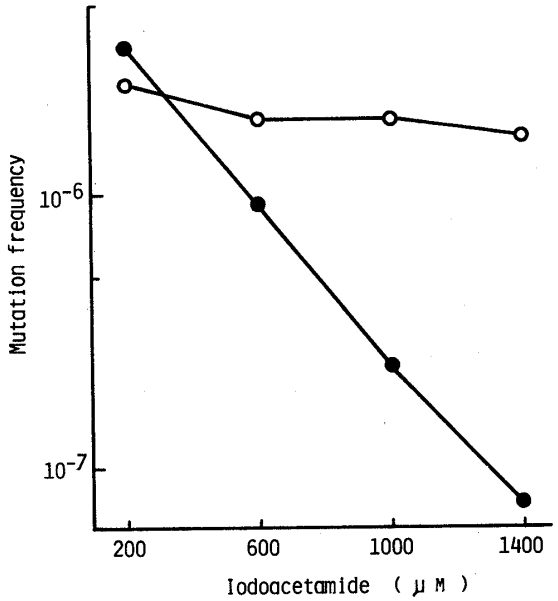

Fig. 7. Mutation Frequencies of *E. coli* B Hs30R after Pretreatment with Iodoacetamide, Followed by Treatment with 20 μM MNNG (—●—) or 1 μM 4NQO (—○—)

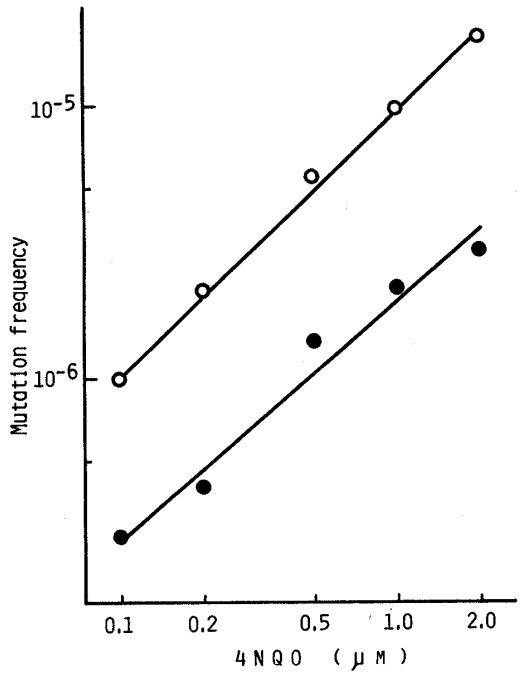

Fig. 8. Mutation Frequency in *S. typhimurium* TA100 after Treatment with 4NQO in the Presence (—●—) and Absence (—○—) of 600 μM Bu-NCS

Discussion

As summarized in Table I, all of the 7 promutagens showed a decreased mutation frequency in the case of co-treatment with Bu-NCS, whereas 9 direct-acting mutagens induced almost the same degrees of mutation regardless of the presence or absence of Bu-NCS. As a numerical expression of the frequency decrease, the ratio of mutation frequency obtained by the treatment without Bu-NCS *versus* mutation frequency in the case of co-treatment with

TABLE I. Mutation Frequencies Modified by Co-treatment with Butyl Isothiocyanate in Mutageneses by Several Direct-Acting and Indirect Chemical Mutagens

Mutagen	Modification of DNA[a]	Decrease in[b] mutation freq. ($MF_{without}/MF_{with}$)	Metabolic[a] activation
Not requiring metabolic activation			
(UV irradiation)	Pyridine–pyridine dimers	−	None
(X-Ray irradiation)	Oxidative damages	−	None
Methyl methanesulfonate	Methylation	−(0.59±0.14)	None
Ethyl methanesulfonate	Ethylation	−(0.43±0.13)	None
N-Methyl-*N*-nitrosourea	Methylation	−(1.61±0.32)	None
N-Ethyl-*N*-nitrosourea	Ethylation	−(0.43±0.12)	None
N-Butyl-*N*-nitrosourea	Butylation	−(0.83±0.11)	None
N-Pentyl-*N*-nitrosourea	Pentylation	−(0.66±0.04)	None
p-Phenylstyrene oxide	Aralkylation	−(0.55±0.14)	None
N,*N*-Dimethylcarbamoyl chloride	Carbamoylation	−(1.52±0.14)	None
12-Naphthohydroxamic acid *O*-propionate	Carbamoylation?	−(0.89±0.18)	None
N-Methyl-*N'*-nitro-*N*-nitrosoguanidine	Methylation	+(15.9±7.8)	(SH-stimulated)
Requiring metabolic activations			
4-Nitroquinoline 1-oxide	Adduct formation	+(7.5±2.7)	Reduction, acylation
4-Nitrosoquinoline 1-oxide	Adduct formation	+(6.2±2.3)	Reduction, acylation
4-Hydroxyaminoquinoline 1-oxide	Adduct formation	+(18.2±6.3)	Acylation
N-Methyl-4-hydroxyamino-quinoline 1-oxide	Adduct formation?	+(19.9±2.9)	Acylation?
2-Naphthohydroxamic acid	Carbamoylation?	+(5.1±1.4)	Acylation?
Furylfuramide	Adduct formation?	+(3.6±0.6)	Reduction
N-Acetoxymethyl-*N*-ethylnitrosamine	Ethylation	+(5.2±1.4)	Hydrolysis

a) Assumed from the chemical characteristics of the mutagen. *b*) $MF_{without}$ and MF_{with} are mutation frequencies obtained by the treatments without and with 800 μM Bu-NCS, respectively; ±value is S.D. The sign − is used when the ratio is below 2.

Bu-NCS is given in Table I; those of the promutagens range from 19.9 to 3.6 and those of the suspected direct-acting mutagens range from 1.61 to 0.43. The exceptional decrease shown by direct-acting MNNG may be accounted for by the fact that Bu-NCS scavenges the cellular SH-functions, which stimulate the activation of MNNG. In conclusion, the method presented here can effectively test whether the mutagen in question requires metabolic activation by enzymes endogenously present in the test organism, although some limitations exist. Thus, it is possible to predict the ultimate, *i.e.*, direct-acting structures of mutagens, provided that they are inert to Bu-NCS. The ultimate structures of genotoxic mutagens are mostly electrophilic,[22] so that they are generally inert to electrophilic Bu-NCS. Further, the prediction thus obtained may also be valid in chemical carcinogenesis, when the carcinogen in question is mutagenic in the bacterial tester system, because a common ultimate reactive species should be responsible for both mutagenesis and carcinogenesis.

With regard to the tester bacteria for the present purpose, an excision repair-proficient strain should be avoided, because Bu-NCS may cause a delay of DNA synthesis, giving the cell more chance to repair the induced DNA damage through the excision repair mechanism. This might cause a similar decrease in the mutation frequency. In this study, *E. coli* B Hs30R was used in most cases and *S. typhimurium* TA100 in several cases.

In the present study, the cells were treated simultaneously with the mutagen and Bu-NCS because the co-treatment was more effective in inducing a decrease in mutation frequency than a serial treatment, *i.e.*, treatment with Bu-NCS prior to that with the mutagen (pretreatment). The co-treatment method, however, cannot be applied to mutagens which react with Bu-NCS. For example, azide ion (N_3^-) is so potently nucleophilic that it was quickly quenched by the added Bu-NCS (data not shown). This is a limitation of this method.

Finally, we wish to comment on the mutagenic mechanism of a hydroxamic acid. As already shown in Fig. 4, the dose–response curve of naphthohydroxamic acid is characterized by a declining trend at high doses, regardless of the presence or absence of Bu-NCS. This unusual deviation from linearity can probably be understood in terms of a series of processes. A hydroxamic acid is metabolically activated to the *O*-acylated derivative, followed by Lossen rearrangement[23] to naphthyl isocyanate, which is suspected to be the ultimate structure.[13] Since it is expected that naphthyl isocyanate may be a non-specific enzyme inhibitor like Bu-NCS, this ultimate mutagen may block the enzymic acylation of the parent hydroxamic acid when a sufficient concentration of naphthyl isocyanate is supplied. This speculation will be tested in the near future.

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