Chem. Pharm. Bull. 29(9)2631—2638(1981)

Studies on Chemical Carcinogens. XX.[1] Inhibitory Effect of Alkyl Isocyanates and Isothiocyanates on Mutagenesis in *E. coli* by Ultraviolet Radiation

Yutaka Kawazoe,* Masanari Kato, and Kazuhiko Takahashi

Faculty of Pharmaceutical Sciences, Nagoya City University, 3-1 Tanabedori, Mizuho-ku, Nagoya 467, Japan

(Received February 7, 1981)

The treatment of *E. coli* B H/r 30R (wild-type) cells with alkyl isocyanates or isothiocyanates decreased the mutability of the cells under ultraviolet irradiation to an appreciable extent, whereas Hs30R (uvrA$^-$) cells were not affected by treatment with these compounds. It is speculated that these compounds delayed the cell division, thus giving the irradiated cells a greater chance to carry out excision repair by prolonging the lag period before entering the S-phase for deoxyribonucleic acid synthesis.

Keywords——isocyanates; isothiocyanates; UV mutagenesis; DNA repair; mutation frequency

Alkyl isocyanates are of interest to chemists in the field of genetic toxicology for the following two reasons. (1) They are acylating agents, which constitute a representative group of electrophiles, ranking with the alkylating agents. Electrophiles are expected to react with cell constituents having nucleophilic reactive centers, and may induce toxic effects, including genetic damage, through chemical modifications of nucleic acids and/or proteins.[2] However, no acylating agents, including alkyl isocyanates, have been reported to be mutagenic or carcinogenic in ordinary bioassay systems, whereas most alkylating agents are well known to be more or less mutagenic and/or carcinogenic.[2,3] (2) The other reason is connected with the carcinogenic and carcinostatic activities of alkyl nitrosoureas, which are degraded under physiological conditions into two components: an alkylating carbonium intermediate and a carbamoylating isocyanate intermediate;[4] the latter has been suggested to have an inhibitory effect on deoxyribonucleic acid (DNA) repair.[5]

In the course of our studies on the genetic toxicity of acylating agents, it was found that some alkyl isocyanates and isothiocyanates decreased the mutation frequency of *E. coli* irradiated with ultraviolet (UV) light, although none of them showed any appreciable mutagenic effect on *E. coli* by themselves under the experimental conditions employed in this study. This paperdescribes the general profile of the inhibitory action of these compounds on UV mutagenesis.

Experimental

Materials——The compounds used here were purchased from Tokyo Kasei Co., Tokyo, except for hexamethylene 1,6-diisothiocyanate, which was a gift from Dr. Mitsuaki Maeda of National Cancer Center Research Institute, Tokyo.

Bacterial Tester Strains——The tester strains of *E. coli* B (argF$^-$) used were a gift from Dr. Sohei Kondo of Osaka University Medical School: H/r30R [wild-type (Exc$^+$ Rec$^+$)], Hs30R [uvrA$^-$ (Exc$^-$ Rec$^+$)], and NG30 [recA$^-$ (Exc$^+$ Rec$^-$)].[6,7]

UV Irradiation——Irradiations were carried out with a 10 watt National GL-10 germicidal lamp (Matsushita Electric Co., Tokyo) on cells plated on agar plates. The applied energies were evaluated by reference to a correlation curve of mutagenicity *versus* UV dose applied, which was calibrated with these strains of mutants.[6] The UV dose rates used were roughly estimated to be 27 erg/mm^2/s for H/r30R and 1.0 erg/mm^2/s for Hs30R cells.

Mutagenic and Cytocidal Effects on *E. coli* Tester Strains——The assay was carried out as described in our previous paper[8] with a slight modification. The cells were grown to the stationary phase in liquid nutrient broth (0.8% Difco nutrient broth containing 0.4% NaCl), then starved in 1/15 M phosphate buffer (pH 6.8) at 37°C for 2 h. To 1 ml of this culture containing about 2.5×10^9 cells, 4 ml of 1/15 M phosphate buffer (pH 6.8) and 0.05 ml of ethanol containing an appropriate amount of isocyanates or isothiocyanates were

added and the mixture was shaken at 37°C for 30 min. The cells were washed twice with the phosphate buffer and suspended in 0.5 ml of the same buffer. This suspension was added to 2 ml of molten top agar (1.2% Difco agar) and layered on semi-enriched plating medium, SEM,[9] in an 86 mm Petri dish. This was then incubated at 37°C for 2 days and the revertant colonies were counted. For the measurement of the surviving cells, colonies obtained by incubation of an appropriately diluted cell suspension at 37°C for 1 d were counted on the SEM plate.

Effect of Pretreatment of *E. coli* with Isocyanates or Isothiocyanates on their UV Mutagenesis and UV Sensitivity——As described above, 1/15 M phosphate buffer and ethanol containing an appropriate amount of isocyanates or isothiocyanates were added to the starved cell culture. The mixture was shaken at 37°C for 30 min. The cells were washed twice with the phosphate buffer and suspended in 5 ml of the same buffer. For UV mutagenesis, 1 ml of this suspension (about 5×10^8 cells) was added to 1.5 ml of 1.2% molten top agar and layered on an SEM plate. The layered cells were immediately irradiated with various doses of UV light, then incubated at 37°C for 2 d, and the revertant colonies were counted. For UV sensitivity testing, UV irradiation was carried out on an SEM plate on which an appropriately diluted cell suspension had been layered with the molten agar, and the colonies obtained by incubation at 37°C for 1 d were counted as the number of surviving cells. Mutation frequency was calculated as $[M/N-M_0/N_0]$, where M and M_0 are the numbers of revertant colonies per ml on the UV irradiated and unirradiated plates, respectively, and N and N_0 are the numbers of the surviving colonies per ml on the UV irradiated and unirradiated plates, respectively.

Effect of Isocyanates on Cell Growth——1/15 M phosphate buffer and ethanol containing an appropriate amount of isocyanates were added to a starved cell culture and the mixture was shaken at 37°C for 30 min. The cells were washed with the phosphate buffer and suspended in the same buffer, and the cell suspension was appropriately diluted with the buffer. The diluted cell suspension (0.05 ml) was added to 5 ml of liquid nutrient broth in an L-tube (about 2×10^4 cells/ml). The tube was gently shaken at 37°C for various periods of time (0—240 min), then 0.1 ml of this liquid nutrient broth was added to the molten top agar and layered on an SEM plate. Colonies obtained by incubation at 37°C for 1 d were counted as the number of cells present in the medium.

Effects of Liquid-Holding on Recovery from UV Damage——To 3 ml of the starved cell culture was added 12 ml of 1/15 M phosphate buffer. The mixture was irradiated with various doses of UV light in an 86 mm Petri dish. The irradiated cells were incubated at 37°C for various periods of time (holding time; 0—120 min) in the phosphate buffer supplemented with glucose (0.4% final concentration) to allow recovery to occur. The cells were resuspended in the phosphate buffer, and 1 ml of this suspension was added to the molten top agar and layered on an SEM plate, which was then incubated at 37°C for 2 d. The revertant colonies were counted. For measurement of the surviving cells, colonies obtained by incubation of an appropriately diluted cell suspension at 37°C for 1 d were counted on an SEM plate.

Results

Mutagenic and Cytocidal Effects of Butyl Isocyanate on *E. coli*

No appreciable increase in sensitivity toward butyl isocyanate was found with the tester strains deficient in DNA repair enzymes, Hs30R (uvrA$^-$) and NG30 (recA$^-$), compared with the sensitivity of the wild-type strain, H/r30R, as can be seen in Fig. 1. The sensitivity seems to be independent of the presence or absence of either the excision or the recombination repair system of the cell. Mutagenicity of this compound was not observed in any tester strain examined in this study (data not shown). From these results, it seems probable that this compound has not ability to induce genetic lesions in spite of its potent electrophilicity.

Effect of Pretreatment of Cells with Butyl Isocyanate on UV Mutagenesis

The pretreatment of H/r30R cells with butyl isocyanate decreased the mutation frequency induced by UV irradiation, whereas the same treatment of Hs30R cells did not have any effect (Fig. 2).[11] The decrease in mutation frequency observed in H/r30R cells was dependent on the concentration of the isocyanate within a certain concentration range, and the mutation frequency reached a constant level, 6×10^{-7}, at 800 erg/mm^2 UV dose, at concentrations over 100 μM (Fig. 3). The doses of isocyanate used in these experiments were much less than those producing an appreciable killing effect on the cells.

UV Sensitivity of H/r30R Cells treated with Butyl Isocyanate

Since butyl isocyanate reduced the mutability of H/r30R cells under UV irradiation, the UV sensitivity of H/r30R cells treated and not treated with butyl isocyanate was compared.

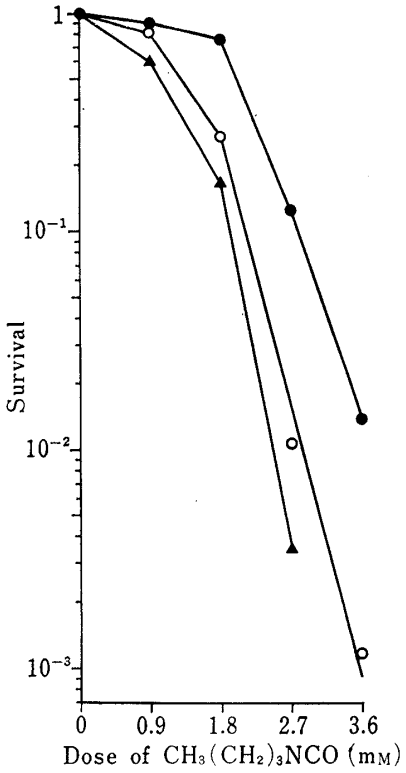

Fig. 1. Killing Effect of Butyl Isocyanate on *E. coli* B strains with different DNA Repair Capacities; -○- H/r30R, -●- Hs30R, -▲- NG30.

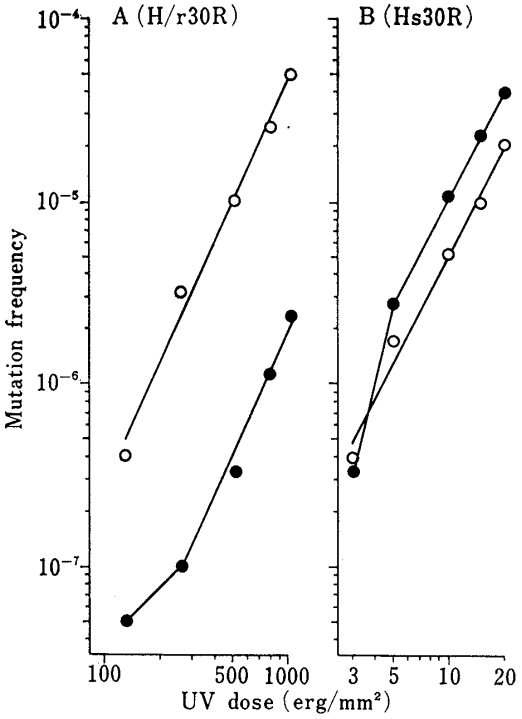

Fig. 2. Inhibitory Effect of Pretreatment with Butyl Isocyanate at Concentrations of 89.9 μM (-●-) and 0 μM (-○-) on UV Mutagenesis of H/r30R (A) and Hs30R (B) strains

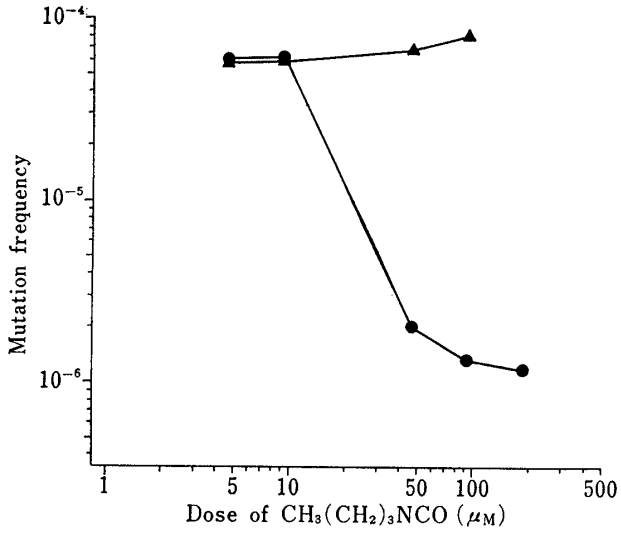

Fig. 3. Dependence of the Mutation Frequency on the Concentration of Butyl Isocyanate used for the Pretreatment; -●- H/r30R treated with 800 erg/mm² UV dose; -▲- Hs30R treated with 20 erg/mm²

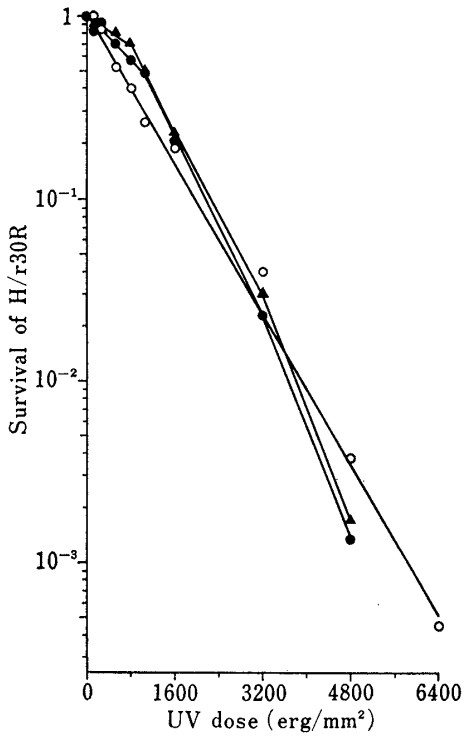

Fig. 4. UV Sensitivity of H/r30R Cells pretreated with Butyl Isocyanate at Concentrations of 180 μM (-▲-), 89.9 μM (-●-), and 0 μM (-○-)

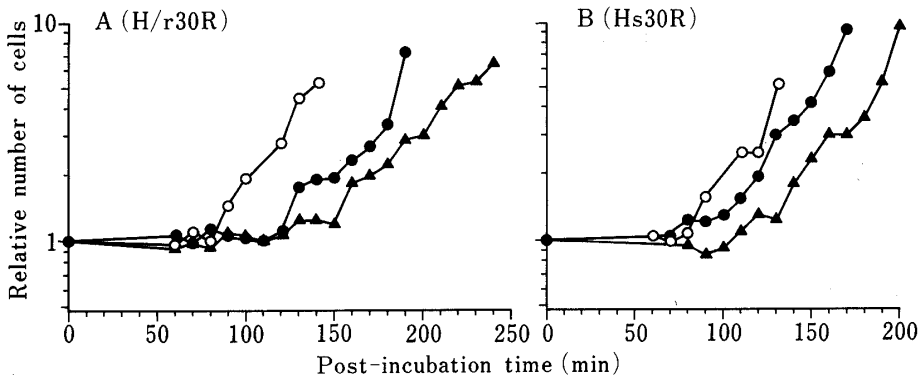

Fig. 5. Rates for Cell Growth of H/r30R (A) and Hs30R (B) after Pretreatment with Butyl Isocyanate at Concentrations of 89.9 μM (-▲-), 45.0 μM (-●-) and 0 μM (-○-)

As shown in Fig. 4, no appreciable difference in UV sensitivity was observed between the treated and untreated cells. The doses of the isocyanate employed were large enough for induction of the maximum level of inhibitory effect on UV mutagenesis.

Effect of Butyl Isocyanate on Cell Growth

As shown in Fig. 5, the first cell-division of H/r30R or Hs30R strain was similarly delayed to some extent by pretreatment with butyl isocyanate. There was no marked difference between H/r30R and Hs30R in this respect.

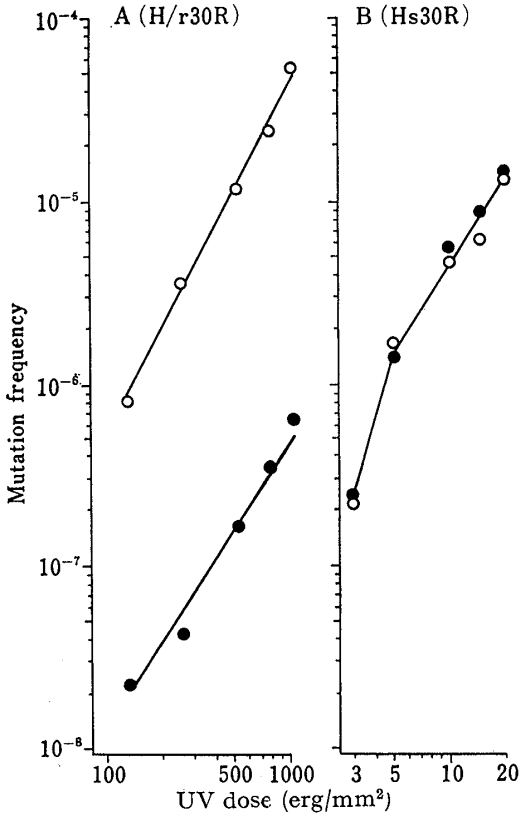

Fig. 6. Inhibitory Effect of Pretreatment with Butyl Isothiocyanate at Concentrations of 827 μM (-●-) and 0 μM (-○-) on UV Mutagenesis of H/r30R (A) and Hs30R (B) Strains

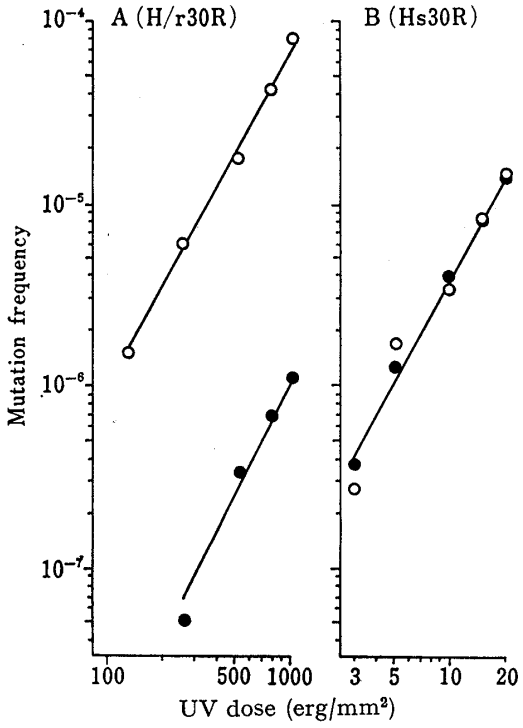

Fig. 7. Inhibitory Effect of Pretreatment with Hexamethylene, 1,6-Diisocyanate at Concentrations of 39.1 μM (-●-) and 0 μM (-○-) on UV Mutagenesis of H/r 30R (A) and Hs 30R (B) Strains

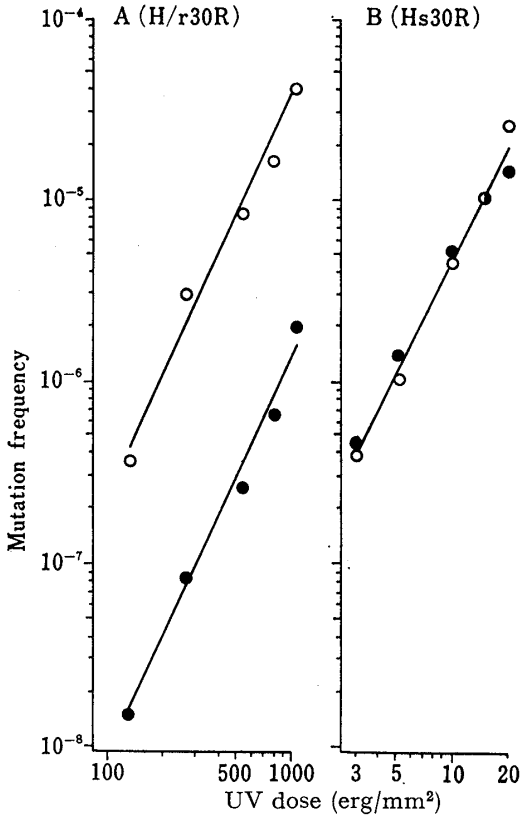

Fig. 8. Inhibitory Effect of Pretreatment with Hexamethylene 1,6-Diisothiocyanate at Concentrations of 5.50 μM (-●-) and 0 μM (-○-) on UV Mutagenesis of H/r30R (A) and Hs30R (B) Strains

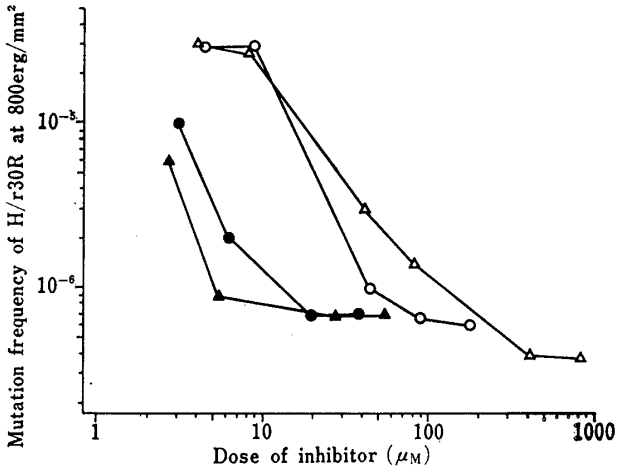

Fig. 9. Dependence of the Mutation Frequency of H/r30R at 800 erg/mm² UV Dose on the Concentrations of Butyl Isocyanate (-○-), Butyl Isothiocyanate (-△-), Hexamethylene 1,6-Diisocyanate (-●-), and Hexamethylene 1,6-Diisothiocyanate (-▲-), used for Pretreatment

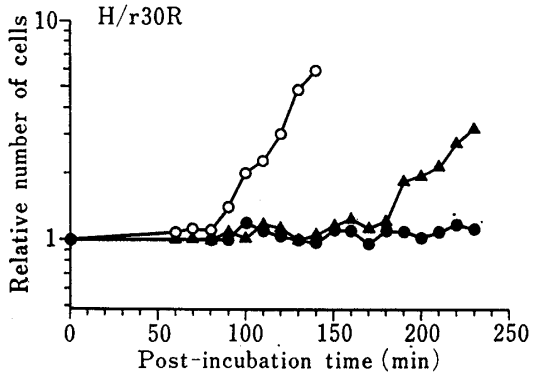

Fig. 10. Rate of Cell Growth of H/r30R after Pretreatment with Hexamethylene 1,6-Diisocyanate at Concentrations of 39.1 μM (-●-), 19.5 μM (-▲-), and 0 μM (-○-)

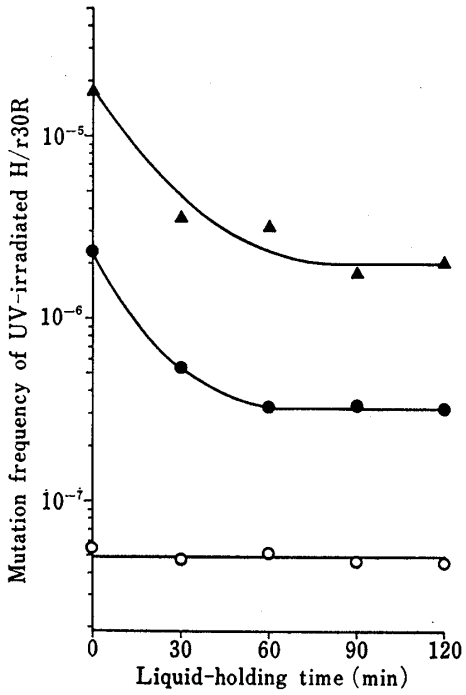

Fig. 11. Effect of Liquid-Holding of H/r30R Cells for Recovery after UV Irradiation at Doses of 600 erg/mm² (-▲-), 200 erg/mm² (-●-), and 0 erg/mm² (-○-) on the Mutation Frequency

Effects of Several Compounds related structurally to Butyl Isocyanate on UV Mutagenesis

Butyl isothiocyanate, hexamethylene 1,6-diisocyanate, and hexamethylene 1,6-diisothiocyanate were examined. All these compounds showed inhibitory effects on UV mutagenesis of H/r30R cells resembling that of butyl isocyanate. Pretreatment of H/r30R cells with these compounds decreased the frequency of mutation induced by UV irradiation, whereas similar treatment of Hs30R did not produce any effects on UV mutagenesis of the cells (Figs. 6—8). The ranges of concentration used in these experiments were less than those inducing an appreciable killing effect on the cells. Furthermore, the mutation frequencies at 800 erg/mm^2 UV dose were reduced to almost the same level, 4×10^{-7}—7×10^{-7}, in all the cases examined, although the effective doses inducing the same degree of decrease in the mutation frequency depended on the compounds used. The results are summarized in Fig. 9. Judging from the effective doses of these compounds, a difunctional structure seems to be more effective than a monofunctional one.

In addition to the similarity in the decrease in mutation frequency, the other isocyanates examined caused some delay of the first division of the treated cells, just as butyl isocyanate did. The delay seems to be prolonged without reaching a plateau level, as the concentration of the isocyanate increases. The cell-growth curves of H/r30R cells treated with 19.5 and 39.1 μM hexamethylene 1,6-diisocyanate are shown in Fig. 10. This phenomenon is in contrast with the finding that the decrease in the mutation frequency induced by this diisocyanate reached a constant level, 7×10^{-7} at 800 erg/mm^2 UV dose, at concentrations over about 10 μM.

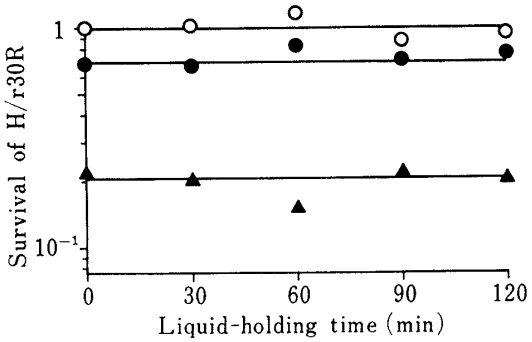

Fig. 12. Effect of Liquid-Holding of H/r30R Cells for Recovery after UV Irradiation at Doses of 600 erg/mm^2 (-▲-), 200 erg/mm^2 (-●-), and 0 erg/mm^2 (-○-) on the Survival

Decrease in Mutation Frequency caused by Liquid-Holding of Irradiated Cells for Various Periods for Recovery

After irradiation of H/r30R cells,[12] the cells were further incubated in a buffer solution supplemented with glucose (0.4% final concentration) for DNA recovery through the excision repair mechanism. As expected, the mutation frequency of H/r30R cells decreased, but that of Hs30R, which lack the excision repair system, was not affected.[13] The extent of the maximum frequency decrease observed with H/r30R was only about 50% of that produced by pretreatment with the isocyanates or isothiocyanates as shown in Fig. 11. No increase in survival of H/r30R was observed in these experiments, as shown in Fig. 12.

Discussion

It was found that the treatment of H/r30R cells with alkyl isocyanates or isothiocyanates altered the mutability of the cells under UV irradiation, whereas Hs30R cells were not affected by treatment with these compounds. If the difference between H/r30R and Hs30R strains is simply the presence or absence of the excision repair system, it may be concluded that the treatment with these compounds enhanced the ability to repair UV-induced damage through the excision mechanism. These compounds may either enhance the enzyme activity for the excision repair or, alternatively, give the irradiated cells a greater chance to carry out excision repair by prolonging the lag period before entering the S-phase for DNA synthesis. Since butyl isocyanate and the other compounds examined produced a marked delay of the first cell division, the latter possibility may be more likely. However, a discrepancy was observed

between the mutability and sensitivity of H/r30R cells with respect to UV irradiation; the former is appreciably reduced but the latter is not affected at all by pretreatment with the chemicals.[14] If the prolonged lag period before entering the S-phase enables the cells to repair DNA damage to a greater extent, the survivor fraction should have been increased compared with that of the cells not treated with these chemicals. However, this was not the case. The same discrepancy was observed in the liquid-holding recovery of the irradiated cells (Fig. 12). More data are required for a better understanding of these phenomena.

The mutation frequency was reduced to a level of 4×10^{-7}—7×10^{-7} at a UV dose of 800 erg/mm^2 by treatment with any of the compounds used, and the frequency never fell below this level, even if higher concentrations of the compounds were employed (the background level was *ca.* 5×10^{-8}). It is speculated from these results that most of the DNA damage induced by UV irradiation may be repaired by error-free excision repair but that a minor part is left unrepaired, being available for subsequent error-prone repairs accompanying the DNA replication process. Recovery caused by post-incubation of the irradiated cells brought about a decrease in mutation frequency to half compared with those produced by the treatment with isocyanates and isothiocyanates, although the experimental conditions were slightly different as indicated in "Experimental." It may be speculated that this discrepancy is a result of differences of experimental conditions for the repair of DNA damage. Details of the mechanism require further investigation. Apart from mechanistic considerations, it appears that the inhibitory effect of alkyl isocyanates and isothiocyanates, produced during the degradation of mutagenic and carcinogenic nitrosoureas, should be taken into consideration whenever the frequency of mutation, and probably carcinogenesis, inducedby nitrosoureas is quantitatively discussed. The inhibitory effects of benzyl isothiocyanate,[15] naphthyl isothiocyanate,[16] and some other classes of compounds [17]on chemical carcinogenesis may be related to the phenomena described in this paper.[18]

Acknowledgement The authors are greatly indebted to Professors Sohei Kondo and Mitsuo Ikenaga of Osaka University for valuable discussions and the kind gift of *E. coli* tester strains. A part of this work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

Added in Proof It was recently reported that some alkyl isocyanates and isothiocyanates were mutagenic in *Salmonella typhimurium* TA 100 although they were very weak. (T. Yamaguchi, *Agric. Biol. Chem.*, **44**, 3017 (1980)).

References and Notes

1) Part XIX: Y. Kawazoe and M. Kato, *Heterocycles*, **15**, 899 (1981).
2) J.A. Miller, *Cancer Res.*, **30**, 559 (1970); D.E. Hathway and G.F. Kolar, *Chem. Soc. Rev.*, **9**, 241 (1980).
3) J. McCann, E. Choi, E. Yamasaki, and B.N. Ames, *Proc. Natl. Acad. Sci. (U.S.A)*, **72**, 5135 (1975); R.M. Peck, T.K. Tan, and E.B. Peck, *Cancer Res.*, **36**, 2423 (1976); H. Hoppe IV, T.R. Skopeck, H.L. Liber, and W.G. Thilly, *Cancer Res.*, **38**, 1595 (1978); A.R. Peterson, H. Peterson, and C. Heidelberger, *Cancer Res.*, **39**, 131 (1979).
4) D.J. Reed, H.E. May, R.B. Boose, K.M. Gregory, and M.A. Beilstein, *Cancer Res.*, **35**, 568 (1975); A. Begleiter, H.P. Lam, and G.J. Goldenberg, *Cancer Res.*, **37**, 1022 (1977); H.E. Kann, Jr., *Cancer Res.*, **38**, 2363 (1978).
5) H.E. Kann, Jr., K.W. Kohn, and J.M. Lyles, *Cancer Res.*, **34**, 398 (1974); A.J. Fornace, Jr., K.W. Kohn, and H.E. Kann, Jr., *Cancer Res.*, **38**, 1064 (1978); L.C. Erickson, M.O. Bradley, and K.W. Kohn, *Cancer Res.*, **38**, 672 (1978); J.M. Heal, P.A. Fox, and P.S. Schein, *Cancer Res.*, **39**, 82 (1979); H.E. Kann, Jr., M.A. Schott, and A. Petkas, *Cancer Res.*, **40**, 50 (1980).
6) S. Kondo, H. Ichikawa, K. Iwo, and T. Kato, *Genetics*, **66**, 187 (1970).
7) Y. Ishii and S. Kondo, *Mutation Res.*, **16**, 13 (1972); Y. Ishii and S. Kondo, *Mutation Res.*, **27**, 27 (1975).
8) K. Takahashi, Y. Kawazoe, Mi. Tada (the late), Ma. Tada N. Ito, and M. Okada, *Gann*, **69**, 499 (1978).
9) SEM consists of 750 ml of deionized water containing 12 g of Difco agar and 16 ml of the medium E,[10] supplemented with 8 ml of 40% glucose and 40 ml of liquid nutrient broth consisting of 0.32 g Difco NB and 0.16 g of NaCl.
10) H.J. Vogel and D.M. Bonner, *J. Biol. Chem.*, **218**, 97 (1956).
11) It is known that mutation is not induced in NG30 strain (recA$^-$) by UV irradiation.

12) In this experiment, the cell suspension was irradiated in 1/15 M phosphate buffer.
13) A.K. Ganesan and K.C. Smith, *J. Bacteriol.*, **97**, 1129 (1969).
14) A.K. Ganesan and K.C. Smith, *J. Bacteriol.*, **96**, 365 (1968).
15) L.W. Wattenberg, *J. Natl. Cancer Inst.*, **58**, 395 (1977).
16) H. Sidransky, N. Ito, and E. Verney, *J. Natl. Cancer Inst.*, **37**, 677 (1966).
17) J.L. Speier and L.W. Wattenberg, *J. Natl. Cancer Inst.*, **55**, 469 (1975); L.W. Wattenberg, *J. Natl. Cancer Inst.*, **52**, 1583 (1974).
18) L.W. Wattenberg, *Adv. Cancer Res.*, **26**, 197 (1978).