Chem. Pharm. Bull.
34(2) 775—780 (1986)

Studies on Chemical Carcinogens and Mutagens. XXXV.[1] Standardization of Mutagenic Capacities of Several Common Alkylating Agents Based on the Concentration–Time Integrated Dose

ATSUSHI HAKURA, YASUNARI MIZUNO, MASAFUMI GOTO and YUTAKA KAWAZOE*

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

(Received June 13, 1985)

The mutagenicities of methyl, ethyl, isopropyl, and butyl methanesulfonates and the corresponding alkyl derivatives of N-nitrosoureas were examined using *E. coli* B Hs30R tester strain deficient in the excision deoxyribonucleic acid-repair system. Their mutagenic capacities were standardized by converting the experimentally obtained mutation frequencies into those per unit of the concentration-time integrated dose with various dimensions, μM·h, mM·h, and M·h. The mutation frequencies under various conceptual exposure conditions were computed by using those at unit integrated dose, on the assumption that the dose–response relation is linear on a log–log scale. The results indicate that the relative mutagenic capacities of these alkylating agents were strongly dependent on the measure (dimensions) of the mutagenic capacities computed in the present study. Consequently, care is necessary in the quantitative evaluation of mutagenicity, especially when the dose–response relation is not linear but exponential.

Keywords——integrated dose; unit dose; alkylating agent; methanesulfonate; nitrosourea

Much attention has been paid to the quantitative evaluation of mutagenic capacities of chemicals so as to correlate them with the chemical and physico-chemical properties and also to assess the genotoxic risk to man.[2-12] The present study was undertaken to evaluate the most appropriate measure of mutagenic capacity to carry out standardization for the purpose of quantitative structure-activity relationship analysis. In this paper, an *E. coli* tester strain deficient in the excision deoxyribonucleic acid (DNA)-repair system was used as the assay organism and 8 direct-acting alkylating agents were examined as the test mutagens. The mutation frequencies of these chemicals were evaluated as those per unit of the concentration–time integrated dose. These values were converted, as a tentative measure of mutagenic capacity, to the mutation frequencies at various conceptual doses and durations of exposure on the assumption that the log/log plot of the dose–response relation is linear.[13]

Materials and Methods

Materials——Methyl and ethyl methanesulfonates (MMS and EMS) were purchased from Tokyo Kasei Kogyo Co. (Tokyo) and isopropyl and butyl methanesulfonates[14] (iso-PMS and BMS) and methyl, ethyl, isopropyl and butyl nitrosoureas[15] (MNU, ENU, iso-PNU and BNU) were synthesized according to the cited methods. No impurities in the mutagens examined were detected on the thin-layer and high performance liquid chromatograms or in the nuclear magnetic resonance spectra.

Rate of Hydrolysis of Nitrosoureas——The cells were grown to an early stationary phase in liquid nutrient broth (0.8% Difco nutrient broth containing 0.4% NaCl) in an L-tube at 37 °C for 10—12 h, washed with 1/15 M phosphate buffer (pH 6.8) and starved at 37 °C for 1—1.5 h. The cell suspension (6.4 ml; *ca.* 2×10^9 cells/ml) was added to a mixture of 24 ml of the phosphate buffer and 1.6 ml of dimethyl sulfoxide (DMSO) containing an appropriate amount of the test compound. This reaction mixture was incubated at 37 °C. After the temperature of the mixture had reached 37 °C, 3.5 ml aliquots of the mixture were taken out successively at appropriate intervals, chilled in an ice-bath, and

NII-Electronic Library Service

centrifuged at 3000 rpm (1900 g) for 20 min. The optical density (OD) of the supernatant was measured at 390 nm with a Shimadzu UV-210A spectrophotometer. As iso-PNU was hydrolyzed too rapidly, the hydrolysis rate constant in the medium (without the cells) was used as its disappearance rate in the reaction mixture.

Rate of Hydrolysis of Sulfonates——A starved cell suspension (4 ml) was added to a mixture consisting of 15 ml of 1/15 M phosphate buffer (pH 6.8) and 1.0 ml of DMSO containing an appropriate amount of the test compound and the whole was incubated at 37 °C. After the temperature of the reaction mixture had reached 37 °C, 2.0 ml aliquots of the mixture were taken out successively at appropriate intervals, chilled in an ice-bath, and centrifuged at 3000 rpm (1900 g) for 20 min. Then, 0.8 ml of the supernatant was combined with 0.8 ml of ether containing an appropriate amount of the internal standard for quantitative analysis by gas chromatography. The internal standard used for this study was either mesitylene, cymene or tetralin.[16] The ether extract thus obtained was dried over anhydrous $MgSO_4$. Quantitative analysis was done with a Shimadzu GC-8APF gas chromatography equipped with an FID detector and a 3.2 mm (i.d.) × 2.0 m 5% SE30 column (column temperature between 60 and 120 °C). The quantification was carried out by measuring the peak area relative to that of the internal standard with the help of working curves previously prepared. All the data shown in Results were the averages from duplicate separate experiments; the deviations fell within ±5% in all cases.

Assay for Mutagenicity on *E. coli* B Tester Strain Hs30R——The tester strain of *E. coli* B (Arg^-) Hs30R ($uvrA^-$) was kindly supplied by Dr. Sohei Kondo, Medical School of Osaka University.[17,18] The tester cells were grown to an early stationary phase in liquid nutrient broth (0.8% Difco nutrient broth containing 0.4% NaCl) in an L-tube at 37 °C for 10—12 h. This cell culture (4.0 ml) was centrifuged at 3000 rpm (1900 g) for 20 min and the collected cells were washed with 4 ml of 1/15 M phosphate buffer (pH 6.8). Then, they were resuspended in 4.0 ml of the buffer and starved at 37 °C for 1.0—1.5 h. The starved cell suspension (0.2 ml) containing about 2×10^9 cells/ml was added to 0.75 ml of 1/15 M phosphate buffer (pH 6.8), to which was added 0.05 ml of dimethyl sulfoxide containing an appropriate amount of a chemical to be tested under ice-cooling. This "reaction mixture" was shaken at 37 °C for a desired period (0.5, 1.0 and 2.0 h), then the reaction was stopped by ice-cooling.

For the measurement of surviving cells, 0.04 ml of the "reaction mixture" was diluted with 4.0 ml of the phosphate buffer, and 0.04 ml of this diluted suspension was further diluted with 4.0 ml of the buffer. Finally, 0.1 ml of this cell suspension was added to 2.5 ml of molten top agar (0.7% Bacto-Agar) maintained at 45 °C, and this was immediately layered on a semi-enriched medium (SEM) agar plate in an 86 mm disposable plastic Petri dish. The SEM consisted of 750 ml of deionized water containing 12 g of Bacto-Agar and 16 ml of the 50-fold concentrated medium E,[19] supplemented with 40 ml of liquid nutrient broth and 8 ml of 40% glucose. The colonies obtained by incubation at 37 °C for 1 d were counted.

For the measurement of revertants, the residual fraction of the "reaction mixture" was centrifuged at 3000 rpm for 20 min. The supernatant was decanted off and the cells were resuspended in 2 ml of the phosphate buffer. This cell suspension was again centrifuged and the supernatant was decanted off. The cells were resuspended in 0.5 ml of the phosphate buffer and mixed with 2.0 ml of 0.7% Bacto-Agar maintained at 45 °C. This was immediately layered on a SEM agar plate. The plate was incubated at 37 °C for 2 d and the number of revertant colonies formed was counted.

Mutation frequency (MF) was calculated as $(M - M_o)/N$, where M and M_o are the numbers of revertants per 1 ml of the "reaction mixture" of the test compound and DMSO, respectively, and N is the number of surviving cells per 1 ml of the "reaction mixture."

Standardization of Mutation Frequency at Unit Integrated Dose——Since the mutation is initiated by chemical damage at mutational site(s) of the target biomolecule (probably DNA), the concentration and the duration of the exposure are determinants of the mutation induction.[4,7,20] Thus, provided that the mutagen in question is "direct-acting," *i.e.*, it does not require any metabolic activation for its mutagenicity, the concentration–time integrated dose (D)[21] required for the induction of mutagenic damage can be formulated as follows.

$$D = \int_{t=0}^{t} fC dt$$

where C is the averaged concentration of the mutagen and f is a factor due to the partition of the mutagen between the inside and the outside of the cell. The partition coefficient is termed as P, and f is formulated as follows.

$$f = P(V_c + V_m)/(PV_c + V_m)$$

where V_c and V_m are the volumes of the cells and the medium, respectively. This may be valid when the partition equilibrium of the mutagen between the inside and outside of the cell is reached quickly enough compared with either the duration of the mutagen treatment of the cell or the half-life of the mutagen in the medium.[22]

Since the dose–response relationship in mutagenesis shows exponential proportionality, in general, over a certain range of mutagen concentration,[13] the induced mutation frequency can be quantitatively described as a function of the integrated dose, as follows.

NII-Electronic Library Service

$$\log MF = n \cdot \log D + \log(\text{constant})$$
$$= n \cdot \log \int_{t=0}^{t} fCdt + \log(\text{constant}) \quad (1)$$

It turns out on these assumptions that the constant in Eq. 1 is the mutation frequency at unit integrated dose, *i.e.*, $D=1$, which will be termed as $MF(D=1)$ here. Since it was experimentally proved that the mutagen disappeared according to pseudo-first order reaction kinetics, Eq. 1 can be solved as follows.

$$\log MF = n \cdot \log\{C_o(1/k)(1-e^{-kt})\} + \log MF(D=1) \quad (2)$$

where

C_o(mM): the averaged initial concentration of the mutagen,
$k(\mathrm{h}^{-1})$: the pseudo-first order rate constant for disappearance of the mutagen from the reaction medium,
n: the slope of the logarithmic dose-response line, and
$MF(D=1)$: the mutation frequency at unit integrated dose with the dimensions of "mM·h."

The dimensions used in the present study were tentatively chosen as described above. Although the concentration inside the cell (fC_o) should be considered for a kinetical study of gene damage, the averaged or net concentration is regarded as appropriate for comparison of mutagenic potency of chemicals, hence the partition equilibrium between the inside and outside of the cell is neglected in Eq. 2.

Results

The mutagenicities of 4 alkyl methanesulfonates, MMS, EMS, iso-PMS and, BMS and the 4 corresponding alkyl derivatives of N-nitrosoureas, MNU, ENU, iso-PNU, and BNU, were examined toward *E. coli* Hs30R (*uvrA*$^-$) at 37 °C. Prior to the mutagenicity assay, the rates of decay of the mutagens under the bioassay condition were determined. The pseudo-first order rate constants are shown below.

MMS	1.67×10^{-1}/h	MNU	1.39/h
EMS	8.67×10^{-2}/h	ENU	1.44/h
iso-PMS	3.46/h	iso-PNU	4.66×10/h
BMS	6.45×10^{-2}/h	BNU	1.49/h

The exposure durations (reaction times) for mutagenesis were 0.5, 1.0, and 2.0 h. The dose–

TABLE I. Mutation Frequency at Unit Integrated Dose with Dimensions of mM·h, Obtained from Assays Involving Various Durations of Exposure to 8 Alkylating Agents at 37 °C

Chemical	$n^{a)}$	Mutation frequency at unit integrated dose (mM·h) computed from the observed data at an exposure time of$^{b)}$			
		0.5 h	1.0 h	2.0 h	Average$^{c)}$
MMS	1.52 (±0.19)	5.53×10^{-8}	5.25×10^{-8}	4.98×10^{-8}	$5.25\ (\pm 0.28) \times 10^{-8}$
EMS	2.96 (±0.37)	6.98×10^{-10}	7.63×10^{-10}	7.84×10^{-10}	$7.49\ (\pm 0.45) \times 10^{-10}$
iso-PMS	1.11 (±0.07)	1.98×10^{-5}	2.14×10^{-5}	2.07×10^{-5}	$2.06\ (\pm 0.08) \times 10^{-5}$
BMS	0.96 (±0.06)	1.43×10^{-7}	1.40×10^{-7}	1.44×10^{-7}	$1.42\ (\pm 0.02) \times 10^{-7}$
MNU	1.55 (±0.15)	5.62×10^{-6}	4.55×10^{-6}	5.33×10^{-6}	$5.17\ (\pm 0.55) \times 10^{-6}$
ENU	1.85 (±0.17)	4.28×10^{-5}	2.86×10^{-5}	2.06×10^{-5}	$3.07\ (\pm 1.12) \times 10^{-5}$
iso-PNU	1.38 (±0.06)	1.56×10^{-4}	1.41×10^{-4}	1.41×10^{-4}	$1.46\ (\pm 0.09) \times 10^{-4}$
BNU	1.26 (±0.15)	7.90×10^{-6}	6.84×10^{-6}	6.26×10^{-6}	$7.00\ (\pm 0.83) \times 10^{-6}$

a) The average value of the slopes of the dose–response plots obtained from all the experiments on each mutagen, the standard deviations being given in parentheses. *b)* Each value is the average obtained from more than 4 separate experiments. *c)* The standard deviations are given in parentheses.

NII-Electronic Library Service

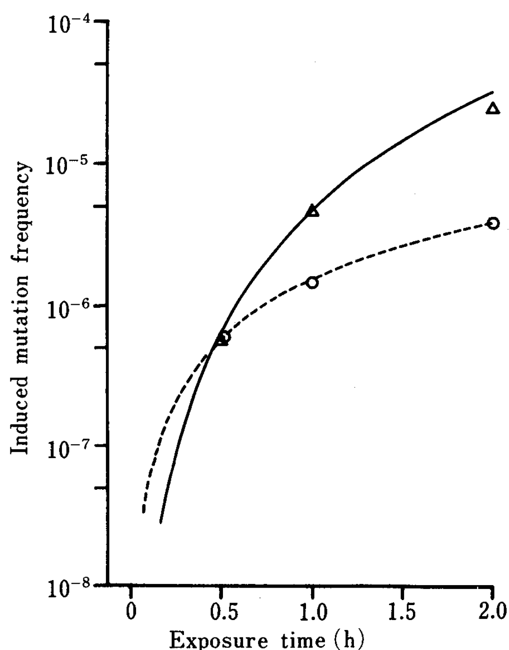

Fig. 1. Observed and Computed Mutation Frequencies Plotted *versus* Exposure Time (on Hs30R at 37 °C)

○, observed frequency induced by exposure to MMS at the initial concentration of 10 mM; △, observed frequency induced by exposure to EMS at the initial concentration of 20 mM. The dotted (MMS) and solid (EMS) lines are the computed frequencies under the conditions indicated.

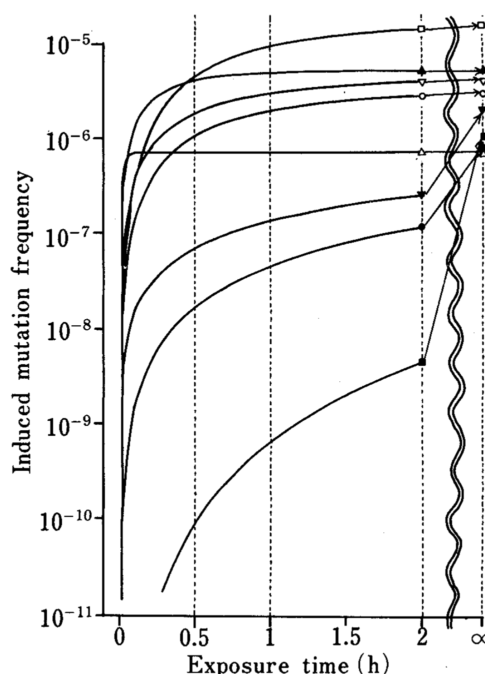

Fig. 2. Computed Time-Course of Mutation Frequency Induced by Exposure to 1 mM (Initial Concentration) of Alkylating Agents (on Hs30R at 37 °C)

—□—, ENU; —▲—, iso-PMS; —▽—, BNU; —○—, MNU; —△—, iso-PNU; —▼—, BMS; —●—, MMS; —■—, EMS.

Table II. Comparison of Mutation Frequencies at Unit Integrated Doses with Different Dimensions, μM·h, mM·h, and M·h

Chemical	Mutation frequency at unit integrated dose with the dimensions of[a] μM·h	mM·h[b]	M·h
MMS	1.45×10^{-12} (1.00)	5.25×10^{-8} (1.00)	1.91×10^{-3} (1.00)
EMS	9.87×10^{-19} (6.8×10^{-7})	7.49×10^{-10} (0.014)	5.68×10^{-1} (300.)
iso-PMS	9.66×10^{-9} (6700.)	2.06×10^{-5} (390.)	4.41×10^{-2} (23.)
BMS	1.86×10^{-10} (130.)	1.42×10^{-7} (2.7)	1.09×10^{-4} (0.057)
MNU	1.16×10^{-10} (80.)	5.17×10^{-6} (98.)	2.31×10^{-1} (120.)
ENU	8.64×10^{-11} (60.)	3.07×10^{-5} (580.)	1.09×10^{1} (5700.)
iso-PNU	1.06×10^{-8} (7300.)	1.46×10^{-4} (2800.)	2.02 (1100.)
BNU	1.16×10^{-9} (800.)	7.00×10^{-6} (130.)	4.22×10^{-2} (22.)

a) The values in parentheses are the values relative to the frequency of MMS in the same column. *b*) The unit dose taken in the present study.

response plots were recorded on a log–log scale in all cases. The data obtained from each experiment (the mutation frequency at an arbitrary initial concentration and the slope of the dose–response plot) were computationally converted, by using Eq. 2, to a constant "$MF(D=1)$" value, which is the mutation frequency at unit integrated dose, the dimensions of which, in the present study, are taken as "mM·h," as already stated. As shown in Table I,

NII-Electronic Library Service

Table III. Mutation Frequencies Expected for a 1-h Exposure at $C_o = 1$ mM and for Infinite Exposures at $C_o = 1$ mM and 1 μM

Chemical	Mutation frequency computed by Eq. 2[a] MF_1 ($C_o = 1$ mM)	MF_∞ ($C_o = 1$ mM)	MF_∞ ($C_o = 1$ μM)
MMS	4.63×10^{-8} (1.00)	8.00×10^{-7} (1.00)	2.21×10^{-11} (1.00)
EMS	6.59×10^{-10} (0.014)	1.04×10^{-6} (1.30)	1.37×10^{-15} (6.20×10^{-5})
iso-PMS	5.01×10^{-6} (108.)	5.20×10^{-6} (6.50)	2.44×10^{-9} (110.)
BMS	1.38×10^{-7} (2.98)	1.98×10^{-6} (2.48)	2.59×10^{-9} (117.)
MNU	2.00×10^{-6} (43.20)	3.12×10^{-6} (3.90)	6.98×10^{-11} (3.16)
ENU	9.50×10^{-6} (205.)	1.57×10^{-5} (19.6)	4.41×10^{-11} (2.00)
iso-PNU	7.27×10^{-7} (15.7)	7.27×10^{-7} (0.91)	5.28×10^{-11} (2.39)
BNU	3.07×10^{-6} (66.3)	4.24×10^{-6} (5.30)	7.04×10^{-10} (31.9)

a) MF_1 ($C_o = 1$ mM): mutation frequency for a 1-h exposure at an initial concentration of 1 mM. MF_∞ ($C_o = 1$ mM): mutation frequency for infinite exposure at an initial concentration of 1 mM. MF_∞ ($C_o = 1$ μM): mutation frequency for infinite exposure at an initial concentration of 1 μM.

the "$MF(D=1)$" values obtained by the treatments for 0.5, 1.0, and 2.0 h all coincided in all the experiments with each mutagen. Each mutation frequency listed in Table I is the average of the data obtained from more than 4 separate experiments, and the slope "n" used for the calculation is the average of the data from all the exposure times. Using the averaged "$MF(D=1)$" values of MMS and EMS listed in the last column of Table I, the time-course changes in the mutation frequency induced by MMS ($C_o = 10$ mM) and EMS ($C_o = 20$ mM) are plotted in Fig. 1; the dotted and solid lines are the curves computed by using Eq. 2 and the circles and triangles are the observed values.

The dimension of the concentration was next converted from mM to μM or M. The mutation frequencies at the converted dimensions of the concentration are shown in Table II. It is evident that the relative mutagenic capacity of these chemicals depends on the dimension of the concentration chosen.

The values relative to the mutation frequency at unit integrated dose of MMS are shown in parentheses in Table II for each dimension of the unit dose.

Some other notations of mutagenic capacities are shown in Table III; mutation frequency induced by a 1-h exposure at an initial concentration (C_o) of 1 mM ($MF_1(C_o = 1$ mM)), that by infinite exposure at $C_o = 1$ mM ($MF_\infty(C_o = 1$ mM)), and that by infinite exposure at $C_o = 1$ μM ($MF_\infty(C_o = 1$ μM)). The values in parentheses in Table III are the relative values of the mutation frequency with respect to those of MMS in these notations. Figure 2 shows the computed time-course changes in the mutation frequencies of these chemicals when the exposure is initiated at a concentration of 1 mM of each chemical.

Discussion

The mutation frequency at unit integrated dose may be a measure of how much mutational damage would be produced by the mutagen in question for the exposure duration indicated in an imaginary experiment where the cells are exposed to an constant concentration of the mutagen without any decay of the mutagen. This measure of the mutagenic capacity may tentatively be regarded as appropriate to a chemical approach to the structure–activity relationship. Attention should, however, be paid to the fact that the mutation frequency strongly depends on the dimension chosen for the mutagen concentration, especially in cases where the slope "n" is larger than unity.

As can be seen in Tables II and III, it seems very difficult not only to determine unambiguously which compound is more potently mutagenic than another, but also to select

NII-Electronic Library Service

the most appropriate measure (dimensions) of the mutagenic capacity to elucidate the structure–activity relationship. These difficulties are definitely due to deviations in the slope "n" from unity, *i.e.*, the exponential dose–response relationship.

Therefore, care should be exercised in the quantitative evaluation of mutagenic capacity when the dose–response relation is not linear but exponential.

Acknowledgements This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References

1) Part XXXIV: T. Kaiya, N. Shirai and Y. Kawazoe, *Chem. Pharm. Bull.*, **34**, 881 (1986).
2) P. M. Calabro-Jones, J. E. Byfield, J. A. Ward and T. R. Sharp, *Cancer Res.*, **42**, 4413 (1982).
3) A. J. de Kok, P. de Knijff and G. R. Mohn, *Mutat. Res.*, **120**, 81 (1983).
4) L. Ehrenberg, E. Moustacchi, S. Osterman-Golkar and G. Ekman, *Mutat. Res.*, **123**, 121 (1983).
5) S. Osterman-Golkar, L. Ehrenberg and C. A. Wachtmeister, *Radiat. Bot.*, **10**, 303 (1970).
6) B. W. Penman, C. L. Crespi, E. A. Komives, H. L. Liber and W. G. Thilly, *Mutat. Res.*, **108**, 417 (1983).
7) R. J. Weinkam and D. F. Deen, *Cancer Res.*, **42**, 1008 (1982).
8) M. F. Lyon, I. D. Alder, B. A. Bridges, L. Ehrenberg, L. Golberg, D. J. Kilian, S. Kondo, E. Moustacchi, A. Putrament, K. Sankaranarayanan, F. H. Sobels, R. J. Sram, G. Streisinger and K. Sundaram, *Mutat. Res.*, **115**, 255 (1983).
9) H. Bartsch, B. Terracini, C. Malaveille, L. Tomatis, J. Wahrendorf, G. Brun and B. Dodet, *Mutat. Res.*, **110**, 181 (1983).
10) G. Streisinger, *Mutat. Res.*, **114**, 93 (1983).
11) I. Turtóczky and L. Ehrenberg, *Mutat. Res.*, **8**, 229 (1969).
12) M. S. S. Murthy, *Mutat. Res.*, **94**, 189 (1982).
13) M. H. L. Green and W. J. Muriel, *Mutat. Res.*, **38**, 13 (1976); S. Hussain and S. Osterman-Golkar, *Hereditas*, **101**, 57 (1984); R. H. Haynes, F. Eckerdt and B. A. Kurz, *Mutat. Res.*, **150**, 51 (1985).
14) A. M. Kolodziejczyk and M. Manning, *J. Org. Chem.*, **46**, 1944 (1981).
15) E. R. Garrett, S. Goto and J. F. Stubbins, *J. Pharm. Sci.*, **54**, 119 (1965).
16) A. Hakura, S. Ninomiya, K. Kohda and Y. Kawazoe, *Chem. Pharm. Bull.*, **32**, 3626 (1984).
17) Y. Ishii and S. Kondo, *Mutat. Res.*, **16**, 13 (1972).
18) Y. Ishii and S. Kondo, *Mutat. Res.*, **27**, 27 (1975).
19) H. J. Vogel and D. M. Bonner, *J. Biol. Chem.*, **218**, 97 (1956).
20) A. A. van Zeeland, G. R. Mohn, C. S. Aaron, G. W. Glickman, M. Brendel, F. J. de Serres, C. Y. Hung and H. E. Brockman, *Mutat. Res.*, **119**, 45 (1983).
21) L. Ehrenberg, K. D. Hiesche, S. Osterman-Golkar and I. Wennberg, *Mutat. Res.*, **24**, 83 (1974).
22) R. J. Weinkam and D. F. Deen, *Cancer Res.*, **42**, 1008 (1982); V. A. Levin, *J. Med. Chem.*, **23**, 682 (1980).

NII-Electronic Library Service