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Studies on Chemical Carcinogens and Mutagens. XXXVI.[1] Apparent Activation Energy for Mutagenic Modification Induced in *E. coli* by Alkylating Agents. Estimation from Mutation Frequency

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An attempt was made to establish a procedure for kinetic formulation of the mutagenic chemical modification of the mutational target(s), even though the mutational initiation processes are unclear. Using the proposed kinetic formulation, a quantitative analysis was made of the temperature dependence of mutation frequency in terms of the activation energies for mutagenic modification induced in the cell by mutagens. *E. coli* B Hs30R (*uvrA*) was treated with alkyl methanesulfonates and *N*-alkyl-*N*-nitrosoureas at 37, 25 and 15 °C for 1 h and the induced mutation frequencies were normalized in terms of the concentration–time integrated dose. The plot of the normalized mutation frequency *versus* the reciprocal of the reaction temperature gave a straight line which corresponds to the Arrhenius plot, enabling us to estimate the apparent activation energy of the initial chemical event leading to mutation. It is worth emphasizing that the activation energy evaluated from a certain biological end-point, the mutagenicity in the present study, may cast light on the chemical characteristics of the induced chemical event leading to the end-point concerned.

Keywords——activation energy; methanesulfonate; nitrosourea; temperature dependence; mutation; integrated dose; chemical modification; mutagenic

It is probable that mutation induction is related substantially to two major events that occur in the cell treated with the mutagen: one is the induction of the mutagenic injury, *i.e.*, the mutagenic chemical modification of the target(s), and the other is the subsequent biological responses of the cell leading to mutation.[2-10] The former is a series of chemical and physico-chemical processes; the incorporation of a given mutagen into the cell from the medium, the metabolic activation of the mutagen, if required, and the chemical modifications of the target site(s) of the informational biopolymer with the (activated) mutagen.

It is expected that, in a given bioassay system, the mutation frequency induced by a chemical mutagen, *i.e.*, the probability of the induction of the mutational event in a cell, may be quantitatively described as a function of the chemical characteristics of the mutagenic modification of the target(s), provided that no repair mechanisms operate during the treatment of the cell. On these assumptions, an attempt was made, in the present study, to establish a procedure for kinetic formulation of the mutagenic chemical modification of mutational target(s), even though the mutational initiation processes are unclear. Using the proposed kinetic formulation, the activation energies were evaluated for the mutagenic alkylations produced by alkyl methanesulfonates and *N*-alkyl-*N*-nitrosoureas, which are all direct-acting mutagens. All the data support the pertinence of the proposed kinetic formulation. The activation energy thus obtained is expected to cast light on the chemical characteristics of the induced chemical event leading to the biological end-point of mutation.

Materials and Methods

Materials——Methyl and ethyl methanesulfonates were purchased from Tokyo Kasei Kogyo Co. (Tokyo) and isopropyl and butyl methanesulfonates[11] and *N*-methyl, *N*-ethyl, *N*-isopropyl, and *N*-butyl-*N*-nitrosoureas[12] were synthesized according to the reported methods. All the mutagens examined were checked for purity by thin-layer and high-performance liquid chromatographies and by nuclear magnetic resonance (NMR) spectroscopy.

Assay for Mutagenicity on *E. coli* B Tester Strain Hs30R——A tester strain of *E. coli* B (Arg^-), Hs30R (*uvrA*), was kindly supplied by Dr. Sohei Kondo, Medical School of Osaka University.[13-15] The tester cells were grown to the 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. The 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. To 0.2 ml of the starved cell suspension containing about 2×10^9 cells/ml, 0.75 ml of the phosphate buffer and 0.05 ml of dimethyl sulfoxide (DMSO) containing an appropriate amount of a chemical to be tested were added under ice-cooling. Then, the "reaction mixture" was shaken at the desired reaction temperature (37, 25, or 15 °C) for 1 h. The reaction was stopped by ice-cooling. The measurements of surviving and reverted cells were made as previously described.[1]

Mutation frequency (*MF*) was calculated as $(M - M_0)/N$, where M and M_0 are the numbers of revertants per 1 ml of the "reaction mixtures" of the test compound and of the control DMSO, respectively, and N is the number of surviving cells per 1 ml of the "reaction mixture" containing the test compound. The values shown in Table I are averages of at least three separate measurements.

Measurements of Rates of Disappearance of Mutagens from the Assay System——*N*-Alkyl-*N*-nitrosoureas: The cells were grown to the 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, then 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 consisting of 24 ml of the phosphate buffer and 1.6 ml of DMSO containing an appropriate amount of the test mutagen. The reaction mixture was incubated at an appropriate temperature (37, 25, or 15 °C). Samples of 3.5 ml each were taken from the mixture successively at appropriate intervals and chilled in an ice-bath. After being centrifuged at 3000 rpm for 20 min, the optical density of the supernatant was measured at 390 nm with a Shimadzu UV-210A spectrophotometer. As the isopropyl derivative was hydrolyzed very rapidly, the rate constant of hydrolysis in the medium (without the cells) was used as its disappearance rate in the reaction mixture. All the hydrolyses were experimentally confirmed to proceed according to pseudo-first order kinetics.

Alkyl Methanesulfonates: The starved cells (4 ml; *ca.* 2×10^9 cells/ml) were added to the 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 mutagen, and incubated at an appropriate temperature (37, 25, or 15 °C). Samples of 2.0 ml each of the mixture were taken successively at appropriate intervals, chilled in an ice-bath, and centrifuged at 3000 rpm 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 ether extract thus obtained was analyzed as previously reported.[1] All the data are the averages of duplicate measurements; the deviations fell within ±5% in all cases.

Procedure for Evaluation of Apparent Activation Energy——The following assumptions were made in developing a kinetic formulation of the mutation frequency.

(1) The mutagens to be considered are "direct-acting", *i.e.*, they do not require any metabolic activation for their mutagenicity.

(2) Mutation is induced by an appropriate chemical modification of a certain target(s) in the cell, probably deoxyribonucleic acid (DNA), produced by the mutagen.[3-10]

(3) The rate-determining step of such modification, in addition to that of the disappearance of the mutagen from the medium, proceeds with the first-order reaction kinetics with respect to the concentration of mutagen.

(4) No repair of the modification takes place either enzymatically or spontaneously during the treatment of the cells with the mutagen. This may be valid for a short duration of treatment (0.5 to 1 h, in general) of excision repair-deficient tester bacteria.[7,13-15,16]

(5) Mutation frequency is exponentially proportional to the concentration of mutagen. This is generally the case over a certain range of mutagen concentration.[6,17-19]

(6) The mutated fraction, *i.e.*, mutation frequency, is very small, usually less than 10^{-5}, under the experimental conditions chosen. Mutation and killing are mutually independent events, *i.e.*, there is no difference in lethality between mutated and non-mutated cells on the exposure to the mutagen.[19]

(7) Partition equilibrium of the mutagen between the inside and outside of the cell is reached rapidly as compared with either the duration of the mutagen treatment of the cell or the half-life of the mutagen in the medium.[8,20,21]

Under the above conditions, the *MF* can be expressed as a function of the rate constant of mutagenic modification of the target, as illustrated below. Here, it is assumed that the overall process of the modification can be treated as an elementary reaction.

$$dP/dt=\tilde{k}fC$$

where dP/dt is the differential of the probability at which the mutagenic modification occurs on the target, $\tilde{k}$ is the rate constant (the proportionality constant) of the modification reaction, C is the averaged or apparent concentration of mutagen at an arbitrary time, and f is the factor due to the partitioning of the mutagen between the inside and outside of the cell. When the partition coefficient is termed P, then f can be 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. The probability (P) of the induction of mutagenic modification during a period from $t=0$ to t can be written as follows.

$$P=\tilde{k}\int_{t=0}^{t} fCdt$$

Here, $\int_{t=0}^{t} fCdt$ is defined as the integrated dose, D.

$$D=fC_0(1-e^{-kt})/k$$

where C_0 is the apparent initial concentration of mutagen in the medium, k is the pseudo-first order rate constant for the disappearance of the mutagen from the assay system, and t is the time of treatment of the cell with the mutagen. Therefore,

$$P=\tilde{k}D$$

On the assumption that the mutation frequency is exponentially proportional to the probability of mutagenic modification in the target, MF can be written as follows.

$$MF=mP^n$$
$$=m(\tilde{k}D)^n$$

where m is the proportional constant which is related to the mutational efficiency of the cell injured by mutagenic modification.

$$\ln MF=\ln m+n\cdot\ln\tilde{k}+n\cdot\ln D$$
$$n\cdot\ln\tilde{k}=\ln MF-\ln m-n\cdot\ln D \quad (1)$$

Provided that the temperature-dependence of the modification reaction is governed by the Arrhenius relation, then

$$\ln\tilde{k}=-\frac{E_a}{RT}+\ln A \quad (2)$$

where E_a and A are the activation energy and the frequency factor of the Arrhenius equation, respectively, $\boldsymbol{R}$ is the gas constant, and T is absolute temperature. Provided that m, n, and A are regarded as independent of the reaction temperature in a narrow range of temperature, Eqs. 1 and 2 give the following.

$$\ln(MF/D^n)=-\frac{nE_a}{RT}+\text{constant}$$

where constant $=\ln A^n+\ln m$. Thus, when the mutation frequency observed under appropriate assay conditions is normalized to the frequency to be induced with the nth power of the integrated dose, the temperature dependence of the normalized MF gives the activation energy (E_a) of the chemical modification leading to mutagenesis. It is worth noting that, as we previously reported, $\ln(MF/D^n)$ can be simply written as follows[1)]

$$\{\ln(MF/D^n)\text{ at }D=1\}=\ln MF(D=1)$$
$$=\ln MF_{obs}-n\cdot\ln\{fC_0(1-e^{-kt})/k\} \quad (3)$$

Thus, E_a can be evaluated on the assumption that f is temperature-independent, as are n, m, and A in Eqs. 1 and 2, over a narrow range of temperature.

$$\log\left(\frac{MF_1}{MF_2}\right)-n\cdot\log\left\{\frac{C_0(1)(1-e^{-k_1t})/k_1}{C_0(2)(1-e^{-k_2t})/k_2}\right\}=\frac{nE_a}{2.303R}\left(\frac{1}{T_2}-\frac{1}{T_1}\right)$$

where T_1 and T_2 are the reaction temperatures in absolute temperature unit, MF_1 and MF_2 are the mutation frequencies observed at T_1 and T_2, respectively, k_1 and k_2 are the rate constants for the disappearance observed at T_1

and T_2, respectively, $C_0(1)$ and $C_0(2)$ are the apparent initial concentrations of the mutagen in the experiments at T_1 and T_2, respectively, t is the duration of mutagen treatment of the cell (1 h in all the assays done here), and n is the slope of the dose-response line on log–log coordinates. Alternatively, E_a can be graphically obtained from the slope of the Arrhenius plot, *i.e.*, the plot of $(1/n)\cdot\log[MF_{obs}\{k/C_0(1-e^{-kt})\}^n]$ *versus* the reciprocal of absolute temperature. It is reasonable to neglect f, because f is assumed to be temperature-independent.

Results

All the experiments were designed to meet the above assumptions, as far as possible. Thus, the mutagens used here are all well-characterized "direct-acting" ones, and all were confirmed to disappear with pseudo-first order reaction kinetics in terms of the mutagen concentration under the same conditions as used for the mutation assay. The mutation frequencies were carefully determined by averaging the data from several separate measurements, each of which was confirmed to fit a linear dose-response relation under conditions of low mutation frequency (less than 10^{-5}) and high survival (more than 85% in most cases). The slopes of the log–log dose-response plots, n, are the averages obtained from repeated measurements at each reaction temperature, followed by further averaging of those obtained at three different temperatures. The reaction temperature was regulated within ± 0.1 °C. It does not seem that there are substantial differences of the slope dependent on the reaction temperature. The values used for the calculation of the normalized mutation frequency are

TABLE I. Mutation Frequencies Normalized in Terms of Integrated Dose and Rate Constants of Disappearance of Alkylating Agents at Various Temperatures

	Temp[a] (°C)	k[b] (h^{-1})	$MF\,(D=1)$[c] ($mM^{-1}\cdot h^{-1}$)
Methyl methanesulfonate	37	1.67×10^{-1}	7.38×10^{-8}
	25	3.75×10^{-2}	1.22×10^{-8}
	15	9.86×10^{-3}	3.39×10^{-9}
Ethyl methanesulfonate	37	8.67×10^{-2}	2.87×10^{-10}
	25	2.13×10^{-2}	3.94×10^{-12}
	15	6.04×10^{-3}	2.24×10^{-13}
Isopropyl methanesulfonate	37	3.46	2.04×10^{-5}
	25	7.25×10^{-1}	4.53×10^{-6}
	15	1.74×10^{-1}	1.42×10^{-6}
Butyl methanesulfonate	37	6.45×10^{-2}	1.34×10^{-7}
	25	1.47×10^{-2}	4.67×10^{-8}
	15	3.89×10^{-3}	1.85×10^{-8}
N-Methyl-*N*-nitrosourea	37	1.39	5.35×10^{-6}
	25	2.25×10^{-1}	5.49×10^{-7}
	15	4.25×10^{-2}	2.03×10^{-7}
N-Ethyl-*N*-nitrosourea	37	1.44	2.09×10^{-5}
	25	2.18×10^{-1}	3.06×10^{-6}
	15	3.76×10^{-2}	5.40×10^{-7}
N-Isopropyl-*N*-nitrosourea	37	4.66×10^{1}	1.47×10^{-4}
	25	8.29	1.33×10^{-5}
	15	1.77	1.47×10^{-6}
N-Butyl-*N*-nitrosourea	37	1.49	5.62×10^{-6}
	25	2.14×10^{-1}	1.02×10^{-6}
	15	3.51×10^{-2}	3.40×10^{-7}

a) Temperature at which the cells were treated with the mutagen. Mutation assay was done after the cells were freed from the mutagen and incubated on an SEM plate at 37 °C in all the experiments. *b*) Pseudo-first order rate constant for the disappearance of the mutagen in the assay system. *c*) Mutation frequency normalized in terms of the concentration (mM) – time (h) integrated dose (assuming that $f=1$): $MF\,(D=1)=MF_{obs}\{k/C_0(1-e^{-kt})\}^n$.

shown below, the standard deviations being given in parentheses.

methyl methanesulfonate	1.33 (±0.20)
ethyl methanesulfonate	3.34 (±0.53)
isopropyl methanesulfonate	1.09 (±0.02)
butyl methanesulfonate	0.98 (±0.08)
N-methyl-*N*-nitrosourea	1.71 (±0.14)
N-ethyl-*N*-nitrosourea	1.66 (±0.17)
N-isopropyl-*N*-nitrosourea	1.38 (±0.01)
N-butyl-*N*-nitrosourea	1.11 (±0.17)

When the cells were treated with the mutagen at 37, 25, and 15 °C for 1 h, the induced mutation frequency decreased in that order: the temperature-dependent dose-response plots of *N*-methyl-*N*-nitrosourea are shown in Fig. 1. From these data, the mutation frequencies normalized in terms of the concentration (mM)–time (h) integrated doses, $MF(D=1)$, were calculated by using Eq. 3, the data being summarized in Table I, where the apparent or averaged concentration is considered ($f=1$). The disappearance rates (h^{-1}) of the mutagens are included in the same table. The values of $(1/n)\cdot\log MF(D=1)$ are plotted *versus* $1/T$, as shown in Fig. 2, where the slope of the line gives $E_a/2.303\boldsymbol{R}$ for each mutagen. The ordinate of this figure is not numerically calibrated, because the normalized frequency includes the unevaluated constant terms which are considered to be common regardless of temperature variation for each mutagen. The apparent activation energies thus obtained are listed in Table II, together with the correlation coefficients (sample number = 3 in each case). The activation energies for the disappearance of the mutagens from the assay system are also given in the same table.

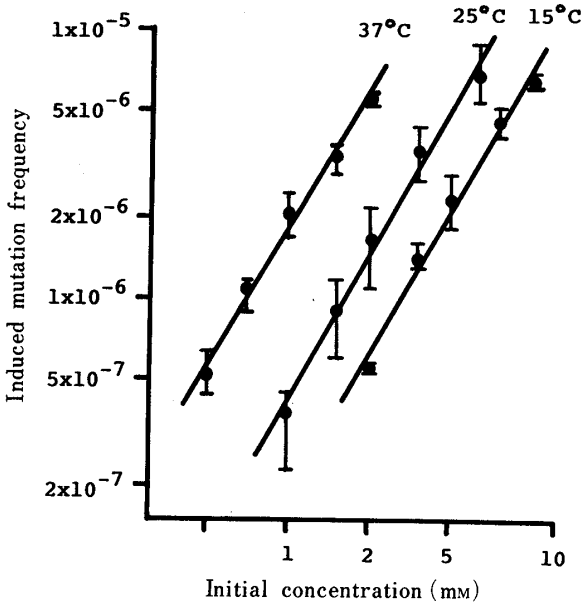

Fig. 1. Mutation Frequencies Induced by *N*-Methyl-*N*-nitrosourea at Three Different Reaction Temperatures

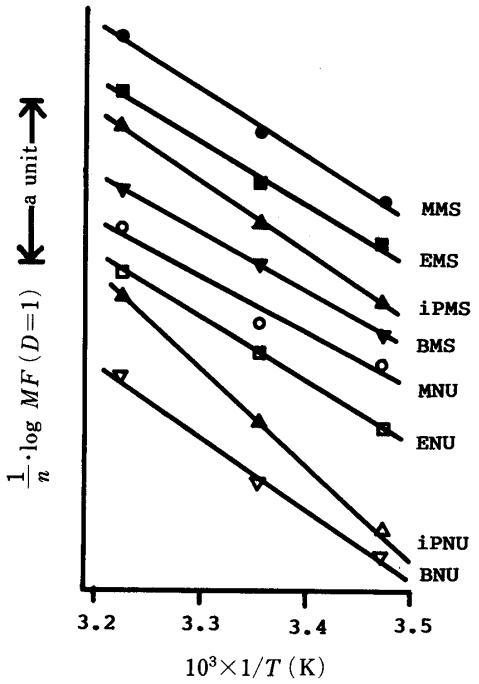

Fig. 2. Arrhenius-like Plots of $(1/n)\cdot\log MF(D=1)$, *i.e.*, $(1/n)\cdot\log[MF_{obs}\{k/C_0(1-e^{-kt})\}^n]$ *versus* $1/T$(K)

Since the normalized frequency includes unevaluated constant terms for each mutagen, the ordinate can not be numerically calibrated. The slope has a physical meaning, corresponding to $E_a/2.303\boldsymbol{R}$.

TABLE II. Apparent Activation Energies for Mutagenic Modifications Induced in *E. coli* B Hs30R by Alkyl Methanesulfonates and *N*-Alkyl-*N*-nitrosoureas. Comparison with Those for Hydrolysis

	r of Arrhenius plot[a)]	E_a for mutagenic modification (A) (kcal/mol)	E_a for hydrolysis (B) (kcal/mol)	A/B
Methyl methanesulfonate	−0.998	18.8	22.8	0.82
Ethyl methanesulfonate	−0.997	17.4	21.5	0.81
Isopropyl methanesulfonate	−0.999	19.8	24.4	0.81
Butyl methanesulfonate	−1.000	16.3	22.6	0.72
N-Methyl-*N*-nitrosourea	−0.982	15.6	27.8	0.56
N-Ethyl-*N*-nitrosourea	−1.000	17.8	29.0	0.61
N-Isopropyl-*N*-nitrosourea	−1.000	25.4	26.3	0.97
N-Butyl-*N*-nitrosourea	−0.996	20.5	29.8	0.69

a) Correlation coefficient; sample number = 3.

Discussion

In the present study, we attempted to quantitatively correlate the mutation frequency by some direct-acting alkylating agents with the mutagenic injury caused by these mutagens on the basis of several assumptions. The results suggest that the initial process leading to mutation can be treated as a chemical process, to which basic chemical kinetics can be applied. Thus, satisfactory linearity of the Arrhenius-like plot of the mutation frequency normalized in terms of the integrated dose seems to support the validity of the procedures and assumptions used in this study. Naturally, the activation energy obtained in this way is just an apparent one, since the mutagenic modification processes may involve serial reactions/equilibria or may consist of two or more competitive reaction processes leading to mutation with different mutagenic efficiencies.[8-10,22-24)]

In conclusion, it is worth emphasizing that the activation energy estimated from the mutagenicity may cast light on the chemical characteristics of the induced chemical event leading to the biological end-point of mutation. Further, such an approach might be useful in relation to the characteristics of the chemical events leading to other biological end-points, *e.g.*, lethality in the same organism, or mutagenicity/carcinogenicity in other organisms, including mammalian cells, if the activation energy could be estimated for the chemical events leading to these biological end-points by a method similar to that described in this paper.

As far as the mutagens examined here are concerned, all the apparent activation energies estimated for the mutagenic modifications are more or less smaller than those of the hydrolyses of the mutagens in the reaction medium. The activation energies for mutagenic modification by alkyl methanesulfonates are all close (*ca.* 80%) to those for the hydrolyses of these mutagens, whereas those in the case of alkyl nitrosoureas vary from *ca.* 50 to 100% of those of their hydrolyses.[12,25)] It would be of interest to compare these values with the activation parameters for the *in vitro* modifications of the sites suspected as genotoxic target sites in mutagenesis and/or carcinogenesis, such as O^6-guanine, N^7-guanine, *etc.*[2-10,26,27)] A chemical approach along this line is now being pursued using isotope-labeled mutagens.

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