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Studies on Chemical Carcinogens and Mutagens. XXXIII.[1] Mutation Frequencies Induced by Combinations of Methylating and/or Ethylating Mutagens

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In order to study the mutagenic interaction between two chemicals, the mutation frequencies induced by simultaneous (combined) treatments of *Salmonella typhimurium* TA100 with two simple alkylating agents were compared with those induced by separate treatments with the two mutagens. The results indicated that ethylations produced by two ethylating agents, *N*-ethyl-*N*-nitrosourea (ENU) and ethyl methanesulfonate (EMS) may be recognized as equivalent mutational damage of deoxyribonucleic acid (DNA) by the host cell tested, and that methylation by *N*-methyl-*N*-nitrosourea and ethylation by either ENU or EMS may be recognized as partially but not entirely equivalent damage of DNA leading to mutation. In contrast, the isopropylation by *N*-isopropyl-*N*-nitrosourea appeared to be independent of either methylation or ethylation in inducing mutation. An analytical method for the classification of the mutagenic interactions as "equivalent" or "independent," is proposed.

Keywords——mutagenicity; alkylating agent; nitrosourea; methanesulfonate

It is of interest to study the mode in which one chemical mutagen interacts with another mutagen in inducing mutation. Only a few studies have been published along this line at the cellular level.[2] One of them indicated that the interactions between chemical mutagens and radiation were additive in some cases and slightly antagonistic in one case in inducing mitotic gene conversion of diploid yeast cells.[2] In this paper, we propose an analytical method applicable to possible modes of interactions. For the present study, we chose several direct-acting alkylating agents, which exhibit high dose–response rates in inducing mutation, *i.e.*, showing, in most cases, a slope of more than 3 units in the dose–response plots on a log–log scale. Thus, *N*-methyl-, *N*-ethyl-, and *N*-isopropyl-*N*-nitrosoureas and ethyl methanesulfonate were included. The mutation assay was carried out with *Salmonella typhimurium* TA100, which is deficient in the excision repair of deoxyribonucleic acid (DNA) damage,[3] for simplification of the analysis. This is because, in the mutation induction of excision repair-proficient strains, antagonistic or synergistic interactions might operate through the repair system in mutational processes induced by the simultaneous (combined) treatment with two mutagens.[4,5]

Calculation of the Mutation Frequencies Expected from Combined Treatment with Mutagen A and B without either Antagonistic or Synergistic Interaction (Refer to Fig. 1)

The calculations are based on the assumption that the dose–response plots are linear on a log–log scale. This assumption seems to be generally accepted in certain dose-ranges for usual assay systems and, in fact, the linearity was previously verified in all the experiments in the present study. The following notations are used.

C_A: Concentration of mutagen A.
C_B: Concentration of mutagen B.
MF_A: Mutation frequency (*MF*) induced by the separate treatment with mutagen A

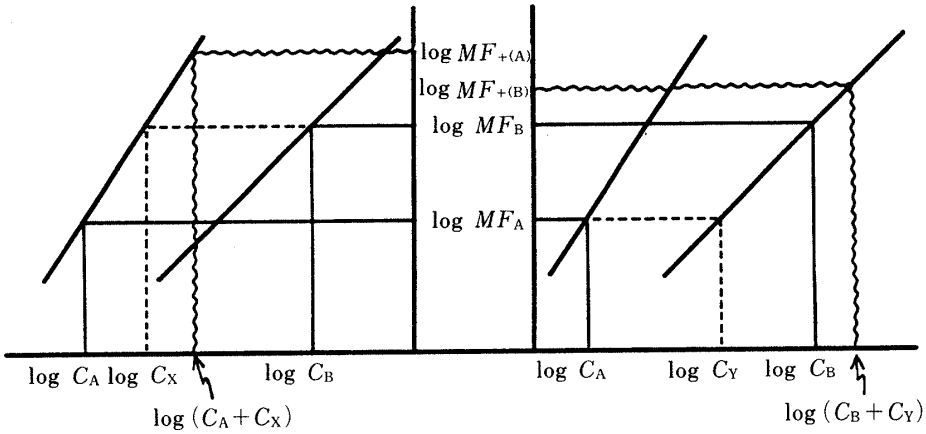

Fig. 1. Schematic Diagrams for Calculations of the Mutation Frequency Expected from Combined Treatment with Two Mutagens A and B

The terms n_A and n_B are the slopes of the dose–response lines in separate treatments with mutagens A and B, respectively. The left hand figure shows the case where the mutagenic contribution of mutagen B is dosimetrically converted to that of mutagen A. Conversely, the right hand figure shows the case where the mutagenic contribution of mutagen A is dosimetrically converted to that of mutagen B. C_A and C_B are the concentrations of mutagen A and B, respectively, and MF_A and MF_B are the mutation frequencies induced by the separate treatments at C_A of mutagen A and C_B of mutagen B, respectively. C_X is the concentration of mutagen A which induces the frequency of MF_B and C_Y is the concentration of mutagens B which is to induce the frequency of MF_A. $MF_{+(A)}$ and $MF_{+(B)}$ are the mutation frequencies to be induced at (C_A+C_X) of mutagen A and (C_B+C_Y) of mutagen B, respectively.

at a concentration of C_A.

MF_B: MF induced by the separate treatment with mutagen B at a concentration of C_B.

MF_+: MF induced by the combined treatment with mutagen A and B at concentrations of C_A and C_B, respectively.

n_A and n_B: Slopes of the logarithmic dose–response lines of mutagens A and B, respectively.

The modes of interaction between two chemical mutagens are tentatively classified into the following three categories. Categories 1 and 2 are the cases where the combination of two mutagens does not produce any antagonistic or synergistic effect on mutation induction.

Category 1

When the DNA modifications produced by two mutagens are "independently" recognized as mutational damage by the host cell concerned, in other words, when the mutageneses by the two mutagens may be regarded as independent events, the mutation frequency induced by the combined treatment is expected to be the mathematical sum of the respective mutation frequencies induced by the separate treatments with the two mutagens.

$$MF_+ = MF_A + MF_B \tag{1}$$

Category 2

When the DNA modifications produced with two mutagens are "equivalently" recognized as mutational damage by the host cell, the mutation frequency induced by the combined treatment is expected to be that induced by the summed dose of the two mutagens, where the summed dose can be estimated by the dosimetric conversion of the dose of one mutagen to that of the other, as illustrated in Fig. 1. Thus, the calculation procedure involves first, estimation of the concentration of mutagen A (C_x) which induces the same mutation frequency (MF_B) as given by the separate treatment with mutagen B and then, calculation of the mutation frequency supposed to be induced after the treatment with mutagen A at the

concentration of $(C_A + C_x)$. The calculation is based on the following formulation, on the assumption of linearity of the dose–response plots.

$$MF_A = m_A C_A^{n_A} \quad \text{and} \quad MF_B = m_A C_X^{n_A}$$

$$MF_+ = m_A (C_A + C_X)^{n_A}$$

where m_A is the proportionality constant, which is probably related to the mutagenic efficiency of the DNA damage induced by mutagen A. Therefore,

$$MF_{+(A)} = (MF_A^{1/n_A} + MF_B^{1/n_A})^{n_A} \tag{2}$$

Conversely, when the calculation is done on the concentration of mutagen B (C_y) which induces the same mutation frequency (MF_A) as given by the separate treatment with mutagen A, and then on the mutation frequency expected by the treatment with mutagen B at the concentration of $(C_B + C_y)$, we have:

$$MF_{+(B)} = (MF_B^{1/n_B} + MF_A^{1/n_B})^{n_B} \tag{3}$$

Now, we would expect that the combined mutation frequency falls between $MF_{+(A)}$ and $MF_{+(B)}$, unless any antagonistic or synergistic interaction operates between the mutagenic actions of the two mutagens concerned.

In cases where the slopes, n_A and n_B, are the same, the combined frequency (MF_+) is simply formulated as follows.

$$MF_+ = (MF_A^{1/n} + MF_B^{1/n})^n$$

As can be seen from Eqs. 1 to 3, when the slope, n_A or n_B, is close to unity, the combined frequency estimated by Eq. 2 or 3 becomes close to that estimated by Eq. 1, in other words, the larger the slope, n, is the more clearly will the mode of interaction be distinguishable as "independent" (Category 1) or "equivalent" (Category 2).

Category 3 (Antagonistic or Synergistic Interaction)

When one mutagen exhibits an antagonistic or synergistic effect either chemically or biologically in the combined treatment with another mutagen, the mutation frequency induced by the combined treatment is expected to be less or more than those calculated by Eqs. 1 to 3.

Materials and Assay Method

Materials——The alkylating agents used here were prepared in our laboratory by reported methods.[6] Their purities were checked by nuclear magnetic resonance spectroscopy, thin-layer chromatography, and elementary analysis.

Bacterial Tester Strains——*Salmonella typhimurium* TA100 strain, which had been subcultured from the stock at the laboratory of Professor Sohei Kondo of Osaka University, Medical School, was used. This particular stock was a gift from Professor Bruce N. Ames of the University of California.[3]

Assay for Mutagenicity——The tester cells were grown to the early stationary phase in liquid nutrient broth (0.8% Difco nutrient broth containing 0.5% NaCl) in an L-tube at 37 °C for 10—12 h. The cell culture containing 1.0×10^9 cells/ml was centrifuged and the cells were washed with 0.25 M phosphate buffer (pH 7.4) then resuspended in the same volume of the buffer. The culture was starved at 37 °C for 1.0—1.5 h. The starved cell culture (0.2 ml) was added to 0.75 ml of the buffer, then 0.05 ml of dimethylsulfoxide solution containing an appropriate amount of a test compound or two test compounds was added under ice-cooling. This "reaction mixture" was shaken at 37 °C for 60 min. 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 the diluted cell suspension was then further diluted with 4.0 ml of the phosphate buffer. Finally, 0.1 ml of the cell suspension was added to 2.5 ml of a solution, maintained at 45 °C, consisting of 0.7% Bacto-agar containing 0.6% NaCl and 1/10 volume of a solution of 0.5 mM L-histidine and 0.5 mM biotin. This was

immediately layered on a nutrient broth agar plate (prepared with 200 ml of deionized water containing 3 g of Bacto-agar, 1.6 g of nutrient broth and 1.0 g of NaCl) in an 86 mm plastic dish. The colonies obtained by incubation at 37 °C for 1 d were counted.

For the measurement of revertants, the cells in the residual fraction of the "reaction mixture" were collected, washed with the buffer, and resuspended in 0.5 ml of the buffer. They were mixed with 2.0 ml of a solution, maintained at 45 °C, consisting of 0.7% Bacto-agar containing 0.6% NaCl and 1/10 volume of a solution of 0.5 mM L-histidine and 0.5 mM biotin. This was immediately layered on a minimum glucose agar plate (prepared with 970 ml of deionized water containing 15 g of Bacto-agar and 20 ml of 50-fold concentrated medium E,[7] supplemented with 10 ml of 40% glucose) in an 86 mm plastic dish. The plate was incubated at 37 °C for 2 d and the revertant colonies formed were counted.

The mutation frequency was calculated at $(M-M_0)/N$, where M and M_0 are the numbers of revertants per 1.0 ml of the "reaction mixture" containing the test compound(s) and of the control dimethylsulfoxide, respectively, and N is the number of surviving cells per 1.0 ml of the "reaction mixture."

Results and Discussion

The linearity of the dose–response lines was experimentally confirmed in the dose ranges examined: up to 2, 10, 30, and 100 mM for MNU, ENU, isoPNU, and EMS, respectively. The slopes on a log–log scale of the agents used are shown below. The standard deviations and the numbers of experiments (n) are shown in parentheses.

N-methyl-*N*-nitrosourea (MNU)	3.30 ($\pm$0.92, $n=6$)
N-ethyl-*N*-nitrosourea (ENU)	3.30 ($\pm$0.27, $n=5$)
N-isopropyl-*N*-nitrosourea (isoPNU)	1.82 ($\pm$0.12, $n=5$)
ethyl methanesulfonate (EMS)	3.39 ($\pm$0.43, $n=5$)

Generally, the reproducibility of mutation experiments is rather poor because of the high sensitivity toward even indiscernible changes in experimental conditions, so that one set of experiments, *i.e.*, the separate and combined treatments, was always done at the same time with the same batches of preparations of the cells, reagents, and media. The results are summarized in Table I. The calculated values shown in Table I were obtained by means of Eqs. 1 to 3 from the mutation frequencies and the slopes of the dose–response plots observed in the separate treatments of the two mutagens concerned.

The results reveal some interesting features of the mutagenic interactions of different two mutagens. Thus, the mutation frequency observed by the combined treatment with the two ethylating agents, EMS and ENU, agreed with the frequency calculated in the "equivalent" manner by Eqs. 2 and 3, and differed greatly from that calculated in the "independent" manner by Eq. 1. It is, therefore, strongly suggested that the ethylations produced by these two types of ethylating agents are recognized as equivalent mutational damage of DNA by the host cell, although these agents are quite different from each other in their chemoselectivity for the alkylation of cellular DNA.[8-10]

Next, the combined treatment with a methylating agent and an ethylating agent showed an intermediate frequency, as seen in Table I. Thus, the mutation frequency observed by the combined treatment with either MNU and ENU or MNU and EMS is significantly larger than the mathematical sum of the frequencies induced by the separate treatments of the two mutagens, but not as large as the frequency calculated in the "equivalent" manner by using Eqs. 2 and 3. Although the details of the molecular mechanisms of mutational processes initiated by the alkylation of DNA are not well known,[11-13] this result may suggest that methylation and ethylation might be partially but not entirely equivalent as DNA damage leading to mutation.

In order to determine the combined effect of other alkylating agents, the combination with isoPNU was examined, although the slope of dose–response plot of this compound is

TABLE I. Mutation Frequencies Induced by Combinations of Methylating and/or Ethylating Mutagens[a] in *Salmonella typhimurium* TA100 and Those Calculated on the Basis of "Independently" and "Equivalently" Recognized DNA Alkylations

Observed frequency ($\times 10^5$)			Calculated frequency ($\times 10^5$)		Found/Calcd		
Separate treatment		Combined treatment	Independently recognized	Equivalently recognized	Independently recognized	Equivalently recognized	Remarks[b]
ENU	EMS						
0.73	0.72	8.90	1.43	7.16—7.62	6.22	1.24—1.17	
1.47	0.84	11.9	2.31	11.1—11.8	5.15	1.07—1.01	
1.74	2.15	12.6	3.89	19.1—20.3	3.24	0.66—0.62	
0.22	0.64	5.25	0.86	3.83—4.07	6.10	1.37—1.29	
				Average	5.18 (±1.36)	1.09 (±0.31)—1.02 (±0.29)	Equivalent
ENU	MNU						
0.72	2.89	4.50	3.61	15.3	1.25	0.30	
0.63	1.51	5.30	2.14	9.89	2.48	0.54	
1.47	1.30	5.57	2.77	13.6	2.01	0.41	
1.74	1.94	5.88	3.68	18.1	1.60	0.33	
1.66	3.23	5.57	4.89	23.2	1.14	0.24	
1.55	1.67	7.94	3.22	15.8	2.47	0.50	
				Average	1.83 (±0.56)	0.39 (±0.12)	Intermediate
EMS	MNU						
0.20	1.13	1.93	1.33	5.20—5.52	1.45	0.37—0.35	
1.00	1.75	3.76	2.75	13.2—14.0	1.37	0.29—0.27	
0.84	1.30	6.83	2.14	10.4—11.0	3.19	0.66—0.62	
2.15	1.94	8.67	4.09	20.1—21.4	2.12	0.43—0.41	
				Average	2.03 (±0.84)	0.44(±0.16)—0.42 (±0.15)	Intermediate
ENU	isoPNU						
0.67	0.95	1.66	1.65	2.90—8.07	1.01	0.57—0.21	
1.66	1.52	2.54	3.18	5.61—15.6	0.80	0.45—0.16	
1.55	1.25	2.29	2.80	4.93—13.7	0.82	0.47—0.17	
				Average	0.88 (±0.12)	0.50 (±0.06)—0.18 (±0.02)	Independent
MNU	isoPNU						
0.40	0.95	1.75	1.36	2.30—6.28	1.29	0.76—0.28	
1.67	1.25	1.80	2.92	5.13—14.3	0.62	0.35—0.13	
0.25	1.01	1.35	1.25	2.01—5.30	1.08	0.67—0.26	
0.29	0.52	0.94	0.81	1.40—3.86	1.16	0.67—0.24	
1.29	1.13	1.31	2.42	4.27—11.9	0.54	0.31—0.11	
				Average	0.94 (±0.34)	0.55 (±0.21)—0.20 (±0.08)	Independent

a) The concentrations of the alkylating agents used were approximately as follows. MNU, 0.8—1.0 mM; ENU, 2.0 mM; isoPNU, 5.0 mM; EMS, 15—20 mM. *b*) "Independent" and "equivalent" indicate that the found value on simultaneous treatment is proximate to the value calculated on the basis that the two types of alkylations are "independently" and "equivalently", respectively, recognized as mutational DNA damage.

considerably smaller than those of the other compounds examined in the present study. Here, the mutation frequency observed by the combined treatment with either isoPNU and MNU or isoPNU and ENU is consistent with the frequency calculated in the "independent" manner by Eq. 1, but not with that calculated in the "equivalent" manner by using Eqs. 2 and 3.

It is, however, worth noting that, in several experimental runs of the combination with isoPNU, the combined frequencies observed were smaller than even the mathematical sum of the frequencies induced by separate treatments with the mutagens; we found an extraordinary decrease of up to 20% of the mathematical sum. However, this apparent antagonistic effect was

poorly reproducible, so that the nature of the effect, *i.e.*, merely an experimental scattering or an antagonistic interaction, remains uncertain. In contrast, synergistic results, *i.e.*, inductions of significantly higher levels of mutation than would be expected, were never found in any experiments.

In conclusion, the analytical method proposed here may be useful for elucidation of the molecular processes involved in mutagenesis initiated by chemical modifications of DNA, and some tentative conclusions on the mutagenic interactions of simple alkylating agents have been presented.

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