

Quantitative Comparison of Genetic Effects of Ethylating Agents on the Basis of DNA Adduct Formation. Use of O⁶-Ethylguanine as Molecular Dosimeter for Extrapolation from Cells in Culture to the Mouse

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DNA-adduct formation and induction of gene mutations were determined simultaneously after treatment with the four ethylating agents, ethyl methanesulfonate (EMS), ethylnitrosourea (ENU), diethyl sulfate (DES), and *N*-ethyl-*N'*-nitro-*N*-nitrosoguanidine (ENNG). Both, in *E. coli* K-12 (NAL-resistance) and in V79 Chinese hamster cells in culture (HPRT-deficiency), the frequencies of mutation induction by all chemicals were the same when plotted against the amount of O⁶-ethylguanine formed in DNA, suggesting that this DNA adduct can be used as a common dosimeter for the comparisons of the frequencies of gene mutations induced by ethylating agents in various mutagenicity assay systems. Using ENU, such a comparison was performed between mutation induction in V79 cells *in vitro* and in the specific-locus assay in the mouse. The data indicate that at equal levels of O⁶-ethylguanine in the DNA of V79 cells and in testicular DNA from male mice treated with ENU, the frequencies of induced mutants in both assay systems were quite similar. These results support the concept that the determination of premutagenic DNA adducts *in vivo* can be used to monitor exposure to chemical mutagens and that genetic risk estimations may ultimately be performed on the basis of such measurements and of comparative mutagenesis *in vitro* and *in vivo*.

Introduction

Quantitative comparisons of genetic effects induced by a chemical in different organisms and assay systems are usually made on the basis of the exposure concentration of the chemical. Although such comparisons can be useful when closely related strains of the same microorganism are compared, or when different lines of mammalian cells are compared under identical treatment conditions, they do not take into account possible differences in penetration into the cells, in metabolism

of the chemical, or effects due to the distribution of the chemical or its activated form into different organs of a mammal. One way to estimate the possible effects of these various processes is the use of DNA adduct formation as parameter for comparative studies. However, this requires not only the development of methods to detect and quantify the different types of DNA adducts caused by the chemical under study, but it should also be known which of the DNA adducts are responsible for the type of genetic endpoint under study. Information concerning this last item may be obtained by comparing in one assay system the effects of different chemicals which cause similar types of DNA adducts but in different proportions (1-3).

We have investigated this concept using a number of ethylating agents and comparing their potency for the induction of gene mutations in *E. coli* and mammalian cells in culture (V79 Chinese hamster cells). These investigations showed that the frequency of O⁶-ethylguanine in DNA is a good indicator for mutation induction

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of these compounds in *E. coli* as well as in cultured mammalian cells. In addition mutation induction in the mouse has been quantified for ethylnitrosourea (ENU), using the mouse specific-locus assay, which determines the mutation rate at seven different loci in the offspring of male mice, treated with the chemical. Parallel to these genetic tests, adduct formation in DNA of different organs of the mouse, including the testis, has been quantified using the same exposure levels of the mutagen as used in the genetic tests. The results show that at equal frequencies of O⁶-ethylguanine in DNA of cultured mammalian cells and in testicular DNA of the mouse, mutation induction at the HPRT locus in cultured cells and the mean mutation frequency at the seven loci in the mouse specific-locus assay are very similar. These data suggest that the extent of DNA adduct formation and the degree of mutagenesis per DNA adduct formed in cultured mammalian cells can be used as a reliable dosimeter for the estimation of genetic effects induced in living animals.

Materials and Methods

Chemicals

(1-³H)Ethylmethane sulfonate was obtained from New England Nuclear at a specific activity of 4.8 Ci/mmol in diethyl ether. Stock solutions of 100 mM were prepared in PBS, pH = 7.2, as described earlier (4). The final specific activity ranged from 22 to 52 mCi/mmol. (2-³H)Ethylnitrosourea was obtained from New England Nuclear at a specific activity of 2.58 Ci/mmol in ethanol. Stock solutions of 25 to 50 mM were prepared in a phosphate buffer, pH = 6 (mouse experiments), or in a phosphate-citrate buffer, pH = 6 (experiments with *E. coli* and V79 cells), as described earlier (4). The final specific activity ranged from 20 to 80 mCi/mmol. (2-³H)Diethyl sulfate was obtained from New England Nuclear as a pure compound at a specific activity of 770 mCi/mmol. Stock solutions (60 mM) were prepared in 40% DMSO/PBS at a final specific activity of 260 to 280 mCi/mmol. *N*-(1-³H)-ethyl-*N'*-nitro-*N*-nitrosoguanidine was obtained from Amersham as a solid compound at a specific activity of 150 mCi/mmol. The compound was solubilized in pure DMSO which was subsequently diluted with PBS. The final stock solution was 5 mM in 10% DMSO/PBS with a specific activity of 150 mCi/mmol. The stock solutions for the genetic experiments were prepared as described for the ³H-labeled mutagens except that unlabeled mutagens were used. All stock solutions were prepared just before use.

E. coli

Escherichia coli, K-12 strain 343/113, was grown as described (4). Stationary cells were treated with the mutagens in PBS. Overnight cultures were washed with PBS and concentrated 40 times in PBS. A 1.9 mL portion of each concentrated suspension was mixed with

the stock solution of the test chemical and PBS was added to a final volume of 2.5 mL. The cells were then incubated for 1 hr (ENU, DES) or 2 hr (EMS) at 37°C. For the determination of genetic effects the cells were washed and diluted in medium to 5 × 10⁸ cells/mL and incubated overnight at 37°C in order to allow expression of induced mutations. Nalidixic acid resistance was determined by plating 10⁸ cells on agar plates containing 25 mg/mL of the selective agents (4). DNA adduct formation was determined in experiments where ³H-labeled mutagens were used, and DNA was isolated immediately after treatment of the cells.

V79 Chinese Hamster Cells

V79 cells were grown in Ham's F10 medium without hypoxanthine and thymidine and supplemented with 15% newborn calf serum and antibiotics. Cells were treated with mutagens in suspension in PBS at 37°C. Treatment with ENU, DES (1 hr) and EMS (2 hr) was at 10⁸ cells in 2.5 mL and with ENNG (1 hr) at 10⁸ cells in 5 mL. Following the treatment, the cells were washed, and mutation induction at the HPRT locus was determined as described earlier (5). DNA adduct formation was determined in experiments where ³H-labeled mutagens were used; the DNA was isolated immediately after treatment of the cells.

Mouse Specific-Locus Assay

The determination of the frequency of gene mutations at seven different genetic loci in the offspring of mice treated with ENU was carried out as described (6). (101 × C3H)F1 male mice, 10 to 12 weeks old, were injected IP with ENU stock solution. The exposure levels were 40, 80, 160, and 250 mg/kg. Immediately after treatment each male was caged separately with an untreated female homozygous for the following markers: a/a, b/b, c^{ch} p/c^{ch}p, d se/d se, s/s. The offspring were counted, sexed, and carefully examined externally at birth. The litters were examined again when cages were changed, the final examination being at weaning age. The classification based on the phenotype was frequently confirmed by an allelism test.

DNA adduct formation in several organs of the treated males was determined in experiments where ³H-ENU (45 mCi/mmol) was used. At 2 hr after injection of ³H-ENU, the liver, bone marrow, and testes were removed and frozen until DNA was extracted.

Detection of DNA Adducts

DNA from *E. coli* and V79 cells was isolated as described earlier (4). DNA from different organs of the mouse was isolated as described by Margison et al. (7). The total level of ethylations in DNA was determined as described (4). In part of the experiments (*E. coli* and V79 cells treated with EMS and ENU) the frequency of 7-ethylguanine and O⁶-ethylguanine was determined

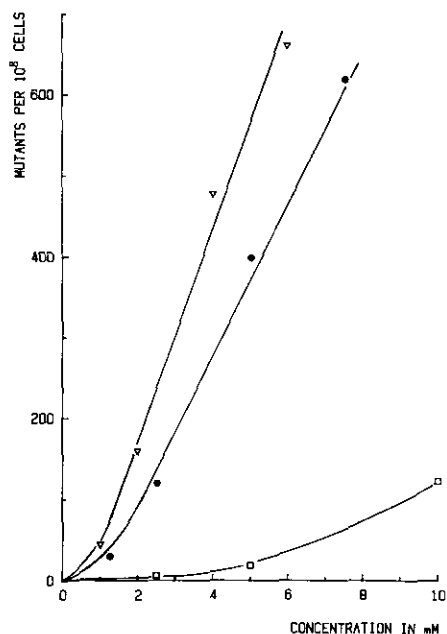


Figure 1. Induction of mutations (NAL^{res}) in *E. coli* by (□) EMS, (●) DES, and (▽) ENU.

after hydrolyzes of DNA in 0.1 M HCl overnight at 37°C and separation of the modified bases using Sephadex G10 chromatography (4). In all other experiments, the DNA was hydrolyzed by heating for 30 min at 100°C in 5 mM Bis-tris, pH = 7, followed by heating for 30 min at 70°C in 0.1 M HCl. The samples were cooled on ice, centrifuged and the supernatant was analyzed for the presence of O^6 -ethylguanine, 7-ethylguanine, 3-ethyladenine, and O^2 -ethylcytosine, using high-pressure liquid

chromatography (HPLC) with a Partisil 10 SCX column as described by Beranek et al. (8). The amount of DNA on the HPLC column was determined by measuring the surface of the adenine and guanine peaks in the chromatograms and calibration with known amounts of these two compounds.

Results

E. coli

Mutation induction, measured as NAL^{res} , after treatment with the three ethylating agents EMS, ENU, and DES, is shown in Figure 1. ENU is the strongest mutagen when compared on the basis of the exposure concentration, whereas EMS has the lowest mutagenic potency. The frequency of several DNA adducts has been determined immediately after treatment with 3H -labeled EMS, ENU, or DES. The data in Figure 2 are plotted on a log-log scale which means that if an adduct shows a proportional increase with the exposure concentration, the slope of the line should be 1. This is the case for most of the adducts shown, except for the frequency of O^6 -ethylguanine which shows an exponential increase in the case of EMS and ENU. The explanation for this probably lies in the presence of low levels of alkyltransferase (9) in these cells, which is able to remove alkyl groups from the O^6 -position of guanine. Since each protein molecule can do this only once, this repair process becomes probably saturated at higher exposure concentration. Although all three compounds used do induce similar type of DNA adducts, the exposure concentration is not a good parameter for comparing mu-

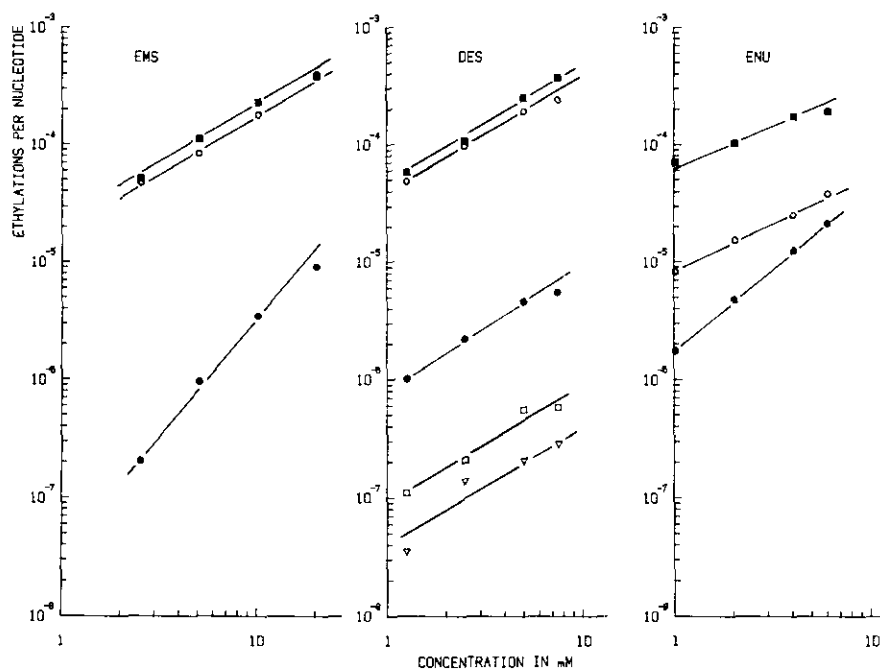


Figure 2. Frequency of several ethylation products in DNA of *E. coli* as a function of the exposure concentration of EMS, DES, or ENU: (■) total alkylations, (○) 7-ethylguanine, (●) O^6 -ethylguanine, (□) O^2 -ethylcytosine, and (▽) 3-ethyladenine.

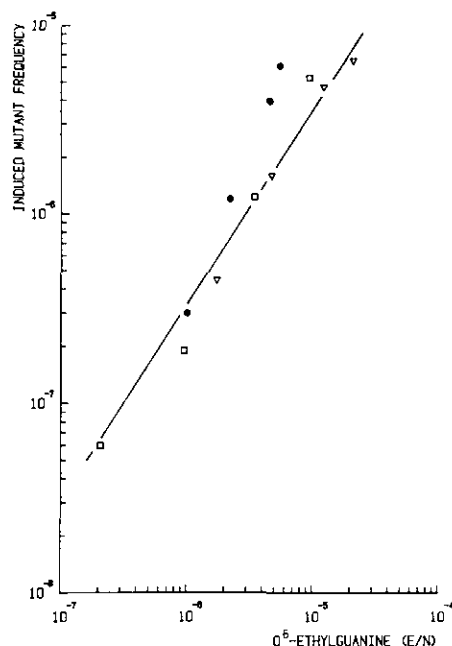


Figure 3. Induction of mutations (NAL^{res}) in *E. coli* as a function of the amount of O⁶-ethylguanine in DNA immediately after treatment with (□) EMS, (▽) ENU, or (●) DES.

tation induction, judging from the large differences in mutation frequency at equal exposure levels (Fig. 1). We compared, therefore, mutation induction by these compounds using each of the measured DNA adducts as parameter. The data in Figure 3 show that at equal frequency of O⁶-ethylguanine mutation induction in *E. coli* was very similar for the three compounds. This was not the case when the comparisons were made using the other measured DNA adducts as parameters. Therefore O⁶-ethylguanine might be responsible for mutation induction in *E. coli* by these chemicals and is at least a good indicator for mutation induction.

V79 Chinese Hamster Cells

Similar experiments were carried out with cultured V79 cells where mutation induction at the HPRT locus was measured as resistance to 6-thioguanine. The data on mutation induction, shown in Figure 4, indicate also in this case a large variation in mutagenic potency among the ethylating compounds tested. The ranking of the mutagenic potency compared on the basis of the exposure concentration was similar to the ranking found in *E. coli*. ENNG was by far the most powerful mutagen. This compound was already active in micromolar concentrations compared with millimolar concentrations for the other three chemicals. The frequency of several ethylation products found in DNA, immediately after treatment, as a function of the exposure concentration is plotted in Figure 5. In contrast with the results found in *E. coli*, the slopes of the lines for the frequency of O⁶-ethylguanine was close to 1, suggesting that there is no fast removal of some O⁶-ethylguanine at low ex-

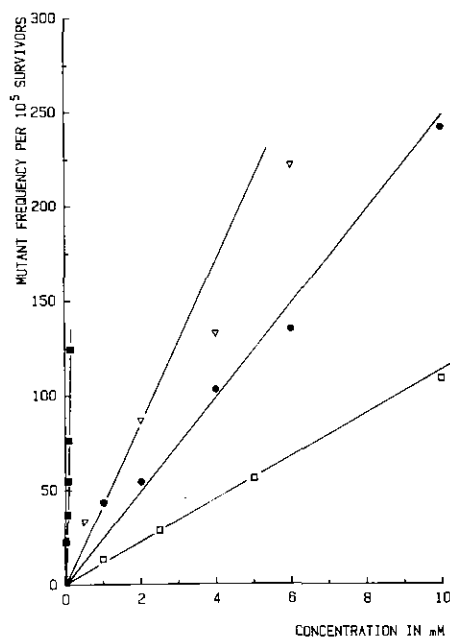


Figure 4. Induction of mutations (HPRT-deficiency) in V79 Chinese hamster cells by (□) EMS, (●) DES, (▽) ENU, and (■) ENNG.

posure concentrations in V79 cells. The frequency of 3-ethyladenine following treatment with DES was considerably higher in these cells than found in *E. coli* (Fig. 2), suggesting that this lesion is already removed in *E. coli* during the 1-hr treatment time with DES. A comparison of mutation induction using the frequency of O⁶-ethylguanine as the parameter shows that for all four ethylating compounds, the data points fall on a single straight line (Fig. 6). This indicates that in cultured mammalian cells, O⁶-ethylguanine is probably also responsible for the induction of gene mutations by these alkylating agents. Mutation induction did not correlate with any of the other measured DNA adducts.

Mouse-Specific Locus Assay

Mutation induction in the mouse-specific locus assay was determined using ENU as mutagenic compound. The data in Table 1 show that only mutations were observed in the offspring conceived 42 days or more after injection with ENU. This indicates that spermatogonia in the mouse are much more mutable than post-spermatogonial stages of spermatogenesis. Parallel to these genetic experiments, DNA adduct formation was determined in DNA from mouse testis as well as in different organs of the animals, 2 hr after injection with ³H-ENU. The data in Table 2 show that the formation of DNA adducts is considerably higher in DNA isolated from the liver than in DNA from testis or bone marrow. The ratio of the amounts of O⁶-ethylguanine and of 7-ethylguanine was furthermore considerably lower in the case of liver compared to DNA from testis or bone marrow. A comparison between the dose-response relationships of DNA adduct formation in testicular DNA

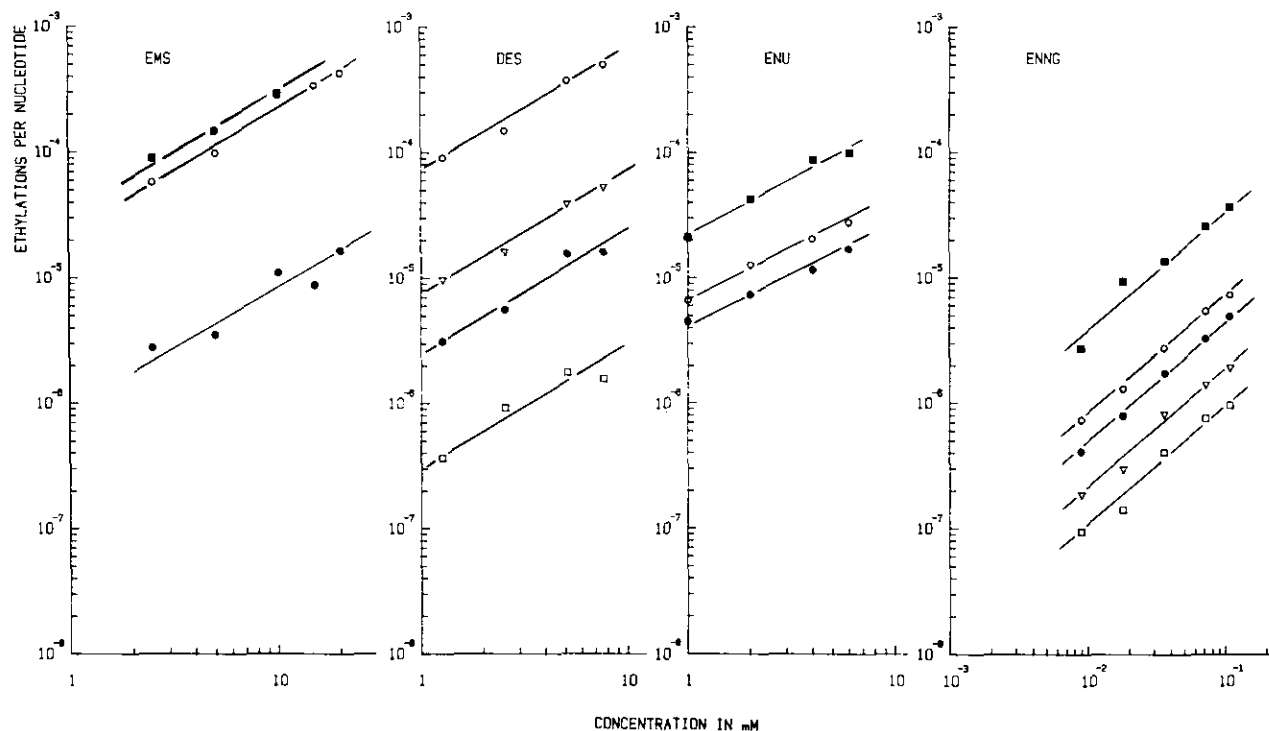


Figure 5. Frequency of several ethylation products in DNA of V79 Chinese hamster cells as a function of the exposure concentration of EMS, DES, ENU, or ENNG: (■) total alkylations, (○) 7-ethylguanine, (●) O^6 -ethylguanine, (□) O^2 -ethylcytosine, and (▽) 3-ethyladenine.

and mutation induction in spermatogonia is made in Figure 7. DNA adduct formation is not linear with the exposure of ENU, suggesting a threshold at low exposure levels. The line drawn in Figure 7B through the data points for mutation induction was fitted by eye. However, a statistical analysis of the data does not exclude the possibility that the line should be drawn through the origin. In that case there would be no threshold for mutation induction by ENU in the mouse.

Discussion

Mutation induction in *E. coli* and in V79 Chinese hamster cells, as well as the formation of several ethylation products in their DNA were determined following exposure to a series of ethylating agents. The data show that the ranking of the mutagenic potency of these compounds was similar in both test systems, namely ENNG > ENU > DES > EMS. In both assay systems, the data points of mutants induced plotted against the amount of O^6 -ethylguanine formed were situated on a single line, indicating that O^6 -ethylguanine may be responsible for mutation induction by these chemicals in these assay systems. At least O^6 -ethylguanine is a good parameter for the prediction of mutation induction by an ethylating agent. A comparison of the absolute values of the frequency of gene mutations in *E. coli* and V79 cells, made at equal levels of O^6 -ethylguanine shows that

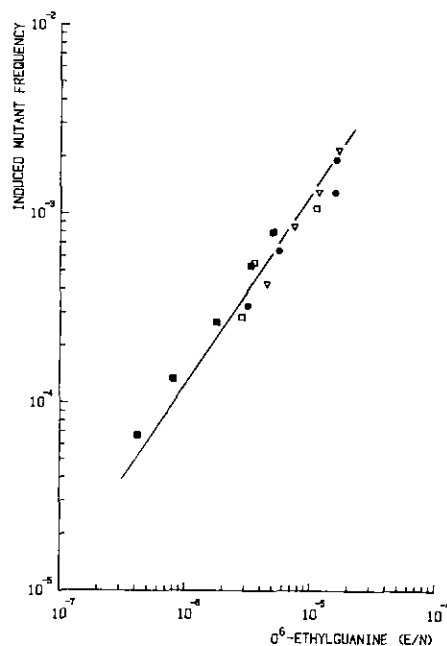


Figure 6. Induced mutations (HPRT-deficiency) in V79 Chinese hamster cells as a function of the amount of O^6 -ethylguanine in DNA immediately after treatment with (□) EMS, (▽) ENU, (●) DES, or (■) ENNG.

Table 1. Induction of specific-locus mutations in male mice by ethylnitrosourea.

Exposure, mg/kg	Germ cell stage treated	Mutations per locus	No. of mutants ^a	No. of F1 offspring
40	Post-spermatogonia	0	0	5028
80		0	0	4660
160		0	0	4416
250		0	0	3360
40	Spermatogonia	3.8×10^{-5}	3	11410
80		21.5×10^{-5}	20	13274
160		52.8×10^{-5}	35(32)	8658
250		83.4×10^{-5}	64(57)	9766
Historical control		0.84×10^{-5}	19(13)	222153

^aMutants were scored at seven different loci. Numbers in parentheses are independent events (corrected for clusters).

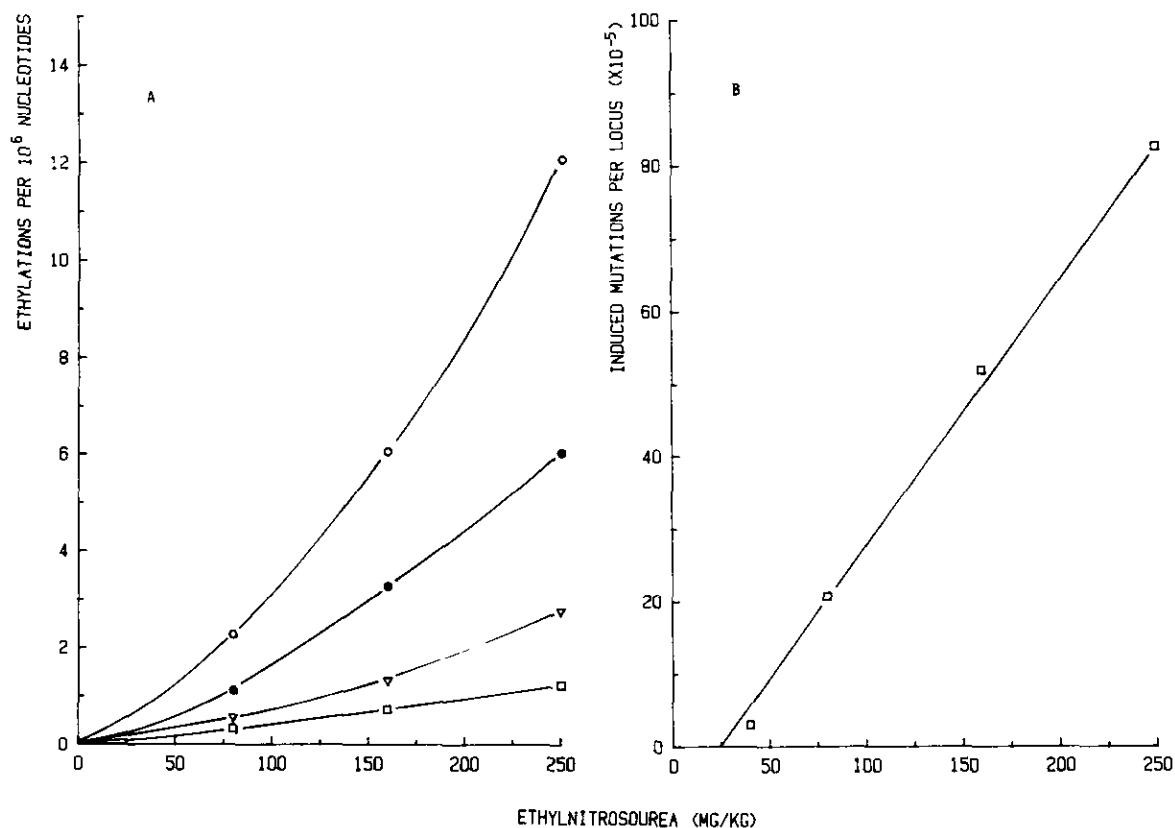


Figure 7. Plots of (A) the frequency of several ethylation products in testicular DNA of the mouse as a function of the exposure to ENU. (○) 7-ethylguanine, (●) O⁶-ethylguanine, (▽) 3-ethyladenine, and (□) O²-ethylcytosine; (B) induced mutations (mean of seven loci) in the mouse as a function of the exposure to ENU.

mutation induction in *E. coli* is about 50 times lower than in V79 cells. Both genetic markers used are forward mutations. Therefore mutation data obtained in this *E. coli* system cannot be used to predict absolute

values of induced frequencies of gene mutations in cultured mammalian cells.

A similar comparison was made between the data obtained with mammalian cells in culture and the mouse

Table 2. Induction of DNA adducts in different organs of mice by ethylnitrosourea.^a

Organ	DNA adduct	Ethylation per nucleotide at various ENU exposures		
		80 mg/kg	160 mg/kg	250 mg/kg
Testis	7-ethylguanine	2.27×10^{-6}	6.06×10^{-6}	12.10×10^{-6}
	O ⁶ -ethylguanine	1.12×10^{-6}	3.27×10^{-6}	6.04×10^{-6}
	3-ethyladenine	0.57×10^{-6}	1.33×10^{-6}	2.77×10^{-6}
	O ² -ethylcytosine	0.33×10^{-6}	0.73×10^{-6}	1.24×10^{-6}
Bone marrow	7-ethylguanine	3.00×10^{-6}	5.36×10^{-6}	11.0×10^{-6}
	O ⁶ -ethylguanine	2.26×10^{-6}	4.86×10^{-6}	6.96×10^{-6}
	3-ethyladenine	1.07×10^{-6}	2.16×10^{-6}	3.19×10^{-6}
	O ² -ethylcytosine	0.42×10^{-6}	1.01×10^{-6}	1.57×10^{-6}
Liver	7-ethylguanine	17.4×10^{-6}	36.8×10^{-6}	57.0×10^{-6}
	O ⁶ -ethylguanine	7.14×10^{-6}	17.1×10^{-6}	25.6×10^{-6}
	3-ethyladenine	3.03×10^{-6}	9.06×10^{-6}	13.5×10^{-6}
	O ² -ethylcytosine	2.39×10^{-6}	5.29×10^{-6}	7.79×10^{-6}

^aDNA was isolated 2 hr after IP injection of ³H-ENU.

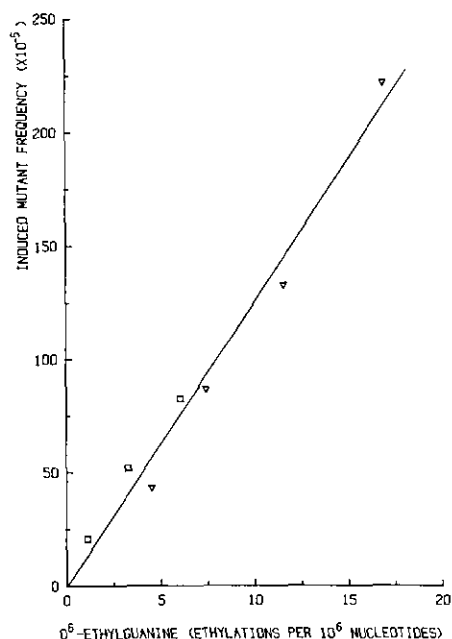


Figure 8. Induced mutations in (∇) V79 Chinese hamster cells and (□) in the mouse specific-locus assay as a function of the amount of O⁶-ethylguanine in DNA of V79 cells and mouse testis, respectively.

specific locus assay (Fig. 8). The results show that when O⁶-ethylguanine is used as parameter for the comparison, very similar mutation frequencies per locus are observed. Apparently, it may be possible to extrapolate genetic data obtained with cultured mammalian cells to genetic effects to be expected in whole animals, molecular dosimetry being used to generate the parameters for the extrapolation (10). The data in Figure 7 are not conclusive concerning the question whether there exists a no-effect level in the mouse at low exposure levels of ENU. Experiments are presently under way to measure the formation of DNA adducts in testicular DNA after ENU exposures of 10 to 40 mg/kg.

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