Identification of Formaldehyde as the Metabolite Responsible for the Mutagenicity of Methyl *Tertiary*-Butyl Ether in the Activated Mouse Lymphoma Assay (44023)

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Abstract. **Methyl *tertiary*-butyl ether (MTBE), which is added to gasoline as an octane enhancer and to reduce automotive emissions, has been evaluated in numerous toxicological tests, including those for genotoxicity. MTBE did not show any mutagenic potential in the Ames bacterial assay or any clastogenicity in cytogenetic tests. However, it has been shown to be mutagenic in an *in vitro* gene mutation assay using mouse lymphoma cells when tested in the presence, but not in the absence, of a rat liver–derived metabolic activation system (S-9). In the present study, MTBE was tested to determine if formaldehyde, in the presence of the S-9, was responsible for the observed mutagenicity. A modification of the mouse lymphoma assay was employed which permits determination of whether a suspect material is mutagenic because it contains or is metabolized to formaldehyde. In the modified assay, the enzyme formaldehyde dehydrogenase (FDH) and its co-factor, NAD^+ are added in large excess during the exposure period so that any formaldehyde produced in the system is rapidly converted to formic acid which is not genotoxic. An MTBE dose-responsive increase in the frequency of mutants and in cytotoxicity occurred without FDH present, and this effect was greatly reduced in the presence of FDH+NAD^+. The findings clearly demonstrate that formaldehyde derived from MTBE is responsible for mutagenicity of MTBE in the activated mouse lymphoma assay. Furthermore, the results suggest that the lack of mutagenicity/clastogenicity seen with MTBE in other *in vitro* assays might have resulted from inadequacies in the test systems employed for those assays.** [P.S.E.B.M. 1996, Vol 212]

Methyl *tertiary*-butyl ether (MTBE) is an oxygen-containing organic compound which is added to motor gasoline to boost octane and to reduce emission of carbon monoxide (1). Large quantities of MTBE are produced for this purpose, and human exposures occur primarily through evaporation and spillage into the environment. In animals, most of an administered dose of MTBE is released unchanged in expired air; however, *tert*-butyl alcohol and methanol are produced *via* hydrolysis and are further metabolized (2). The metabolism of methanol leads to the production of formic acid *via* the intermediate formaldehyde (3–5). Formaldehyde is a genotoxic and carcinogenic agent in animal studies, but no toxicological effects have been directly linked to formaldehyde generation *via* MTBE metabolism.

MTBE has been tested in several genotoxicity tests and did not show any mutagenic effects in the Ames assay or in cultured mammalian cells (6). MTBE neither produced unscheduled DNA synthesis in cultured rat hepatocytes (6) nor caused chromosome aberrations in bone marrow cells of rats (7) or an increase in micronuclei in bone marrow cells of mice exposed

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by inhalation (8). MTBE also did not induce sex-linked recessive lethal mutations in Drosophila melanogaster (9). These results have led to the general consensus that MTBE is not genotoxic; however, in one test, the L5178Y mouse lymphoma assay, MTBE was mutagenic but only in the presence of an S-9 activation system. It was suggested that a metabolite, possibly formaldehyde, was responsible for the activity (10), and formaldehyde is known to be mutagenic to mouse lymphoma cells (11). Methanol is mutagenic in the mouse lymphoma assay with activation (12) but *tert*-butyl alcohol is nonmutagenic with and without activation (13).

In the present study, MTBE was evaluated in a mouse lymphoma assay modified to detect specifically the presence of formaldehyde generated *in situ*. This method involves the addition of formaldehyde dehydrogenase (FDH) + NAD^+ to oxidize formaldehyde to nonmutagenic formate (14, 15). Finding a substantial decrease in the observed mutant frequency with the FDH present compared with the frequency obtained without the FDH demonstrates that the mutagenicity resulted from formaldehyde.

Materials and Methods

Chemicals. Methyl *tertiary*-butyl ether, 99.7%, HPLC grade (CAS No. 163404-4), hexamethylphosphoramide (HMPA), 99% (CAS No. 680-31 -9), and 7,12-dimethylbenz[a]anthracene (DMBA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Formalin solution (37% formaldehyde solution stabilized with 10% methanol), ethyl methanesulfonate, formaldehyde dehydrogenase (FDH, EC 1.2.1.46), NAD^+ (sodium salt), isocitrate (tri-sodium salt), and trifluorothymidine (TFT) were purchased from Sigma Chemical Co. (St. Louis, MO). NADP was obtained from Pharmacia (Piscataway, NJ). Fisher's medium, sodium pyruvate and gentamicin were obtained from GIBCO (Gaithersburg, MD). Pluronic F127 was received from BASF Corporation (Parsippany, NJ). Horse serum was obtained from Sterile Systems. Noble agar was obtained from Difco Laboratories (Detroit, MI).

Cells. The cell line L5178Y $tk^{+/-}$ (subclone 3.7.2C) used in these assays was obtained from Dr. Donald Clive. Frozen stocks were maintained in liquid nitrogen, and were free of mycoplasma contamination. On the day before treatment, cells were subcultured to give a density equal to or less than 1×10^6 cells/ml. Stock cultures were exposed once to methotrexate (0.1 μg/ml) in the presence of thymidine (3 mg/ml), glycine (7.5 μg/ml), and hypoxanthine (5 μg/ml) within a week prior to treatment, to select against spontaneously arising $tk^{-/-}$ cells and to maintain a low background frequency of TFT-resistant cells.

Preparation of Dosing Solutions. MTBE and HMPA were added neat at appropriate target concentrations to each culture tube. The positive control formalin solution was prepared by adding 100 μl of 37% formalin to 9.9 ml Hanks' balanced salt solution and then administering 65 μl to each designated culture tube. The standard DMBA-positive control for the mouse lymphoma assay was prepared as a 0.5-mg/ml DMSO stock solution; 100 μl (for 5.0 μg/ml medium dose) or 50 μl (2.5 μg/ml medium/dose) was added to a culture tube containing S-9 metabolic activation mixture to verify the validity of the assay.

Media. Growth medium consisted of Fisher's medium, horse serum (heat inactivated, 10% by volume), sodium pyruvate (1 m*M*) and pluronic F127 (1 mg/ml). Serum was reduced to 6% in the treatment medium. Cloning medium included 20% serum, gentamicin (200, μg/ml), and 0.35% Noble agar. Selection medium was cloning medium with TFT (2.5 μg/ml).

S9 Mixture. Aroclor 1254 induced rat liver S-9 was purchased from Molecular Toxicology (Annapolis, MD). The co-factor mixture was prepared fresh for each use in chilled Fisher's medium. It was mixed with S-9 immediately prior to use, kept on ice, and added to the cell mixtures within 5 to 10 min. The final concentrations in activation treatment medium were 3.1 m*M* NADP, 17 m*M* isocitrate, and 10% S-9.

FDH+NAD^+ Activation Medium. NAD^+ was added to the co-factor mixture during its preparation. FDH was dissolved in cold Fisher's medium and added to the cultures at the time of treatment. The final concentrations in the FDH + NAD^+ activation medium were 8.1 m*M* NAD^+ and 0.09 units/ml FDH.

Toxicity Assay. Cytotoxicity assays were performed as follows. Because of the volatility of MTBE, all treatments were performed in glass vials with Teflon-lined screw caps. L5178Y mouse lymphoma cells at a density of 6×10^5 cells/ml in 10 ml of medium were exposed in the presence of activation mixture to MTBE for 3 hr at 37°C. At the end of the 3 hr incubation, cells were washed twice with culture medium and resuspended in 20 ml of growth medium and incubated. After 24 hr of incubation, cells in each culture were counted using a Coulter Counter Model ZM. Cell concentrations were adjusted to 3×10^5 cells/ml and were incubated at 37°C for an additional 24 hr. At the end of the 48-hr total incubation, cell numbers were determined. Relative suspension growth obtained from these data in which the cell concentration of the MTBE treatment was compared with the untreated control cultures provided an indication of the toxicity as an effect of treatment.

Mutation Assays. The procedures for performance of the assays are adapted from Clive *et al.* (16) and Turner *et al.* (17). All treatments were performed in glass vials with Teflon-lined screw caps. Treatment

consisted of exposing 6×10^5 cells/ml in activation treatment medium to one of several concentrations of MTBE or positive control chemicals for 3 hr at 37°C. Untreated cultures in activation medium served as the negative controls. At the end of the exposure, each culture was washed twice with medium to remove the treatment and resuspended at 3×10^5 cells/ml in growth medium.

Cells in each culture were counted approximately 24 hr after treatment. Cultures in which the cell density had reached 4×10^5 cells/ml or greater were reduced to 3×10^5 cells/ml by removing an appropriate amount of the culture medium and replacing it with fresh culture medium to allow maximum growth, recovery, and expression of the induced $tk^{-/-}$ phenotype. At 48 hr, the cells were counted and cloned in soft agar: 1×10^6 cells/plate in selective medium and 200 cells/plate in nonselective medium. Triplicate plates were used for each medium. The clone plates were incubated at 37°C for 11 or 12 days and the colonies counted on an Artek Automated Colony Counter. Representative plates were hand counted to ensure the accuracy of automated counts and to demonstrate that both large and small colonies were included.

The percentage relative growth, as described by Clive *et al.* (16), reflects both the 24- and 48-hr daily cell counts and the cloning efficiency at the time of selection in order to measure the overall effect on growth resulting from treatment.

The mutant frequency represents the number of TFT resistant cells per 10^{-4} surviving cells. The relative mutant frequency is the mutant frequency of treated cultures compared with the mutant frequency of concurrent controls.

Results and Discussion

The data in Table I show a rise in mutant frequency and a reduction in total cell growth associated with MTBE treatment. Over a range of 1–4 μl/ml, MTBE induced a concentration-related increase in mutant frequency reaching a maximum of 18-fold above the untreated control, while reducing total growth to near zero at the highest concentration. Formalin (0.065 μl/ml) which was included as a positive control, induced more than a 5-fold increase in mutant frequency and reduced total cell growth to about 40% of the value for the untreated control (Table I).

In order to determine the effect of FDH on the mutagenicity and cytotoxicity of MTBE, two sets of mouse lymphoma cell cultures were treated with the same range of concentrations of MTBE in the presence of S-9 mix, with and without excess FDH and its co-factor (NAD^+). The results in Table II confirm the previous findings that MTBE is mutagenic in the presence of rat liver activation. In this study, the maximum increase in mutagenic frequency was about 12 times that seen in the untreated control. The addition of FDH and its co-factor to the cultures during the treatment period reduced the mutant frequencies to un-

Table I. Mutagenicity of MTBE in the Activated Mouse Lymphoma L5178Y $tk^{+/-}$ Assay

Test article	Conc.[a]	% Total growth[b]	Mutant frequency[c]
MTBE	4.0	0.3	4.00
	3.0	8.4	2.49
	2.0	25.5	2.01
	1.0	47.8	1.57
Untreated	—	102.5	0.18
	—	97.8	0.26
Formalin[d]	0.065	39.7	1.23
DMBA[e]	5.0	2.6	4.45
	2.5	49.3	1.98

[a] Concentrations are μl/ml culture medium for MTBE and formalin and μg/ml culture medium for DMBA.
[b] % Total Growth = (Relative Suspension Growth) × (Relative Cloning Efficiency) ÷ 100.
[c] Mutant Frequency = (Mean Number of TFT Colonies per Dish)/(Mean Number of VC Colonies per Dish) × (2×10^{-4}).
[d] Formalin is a 37% solution of formaldehyde in water, stabilized with 10% methanol.
[e] DMBA is 7,12-dimethylbenzanthracene, a compound which is converted to a mutagenic form by the S9 activation system. It is used as positive control.

Table II. Mutagenicity of MTBE in the Activated Mouse Lymphoma L5178Y $tk^{+/-}$ Assay with and without FDH

Test article	Conc. of medium (μl/ml)	FDH[a]	% Total growth[b]	Mutant frequency[c]
MTBE	4.0	−	9.2	1.97
	3.0	−	24.7	1.74
	2.0	−	48.0	1.45
	1.0	−	61.1	0.96
	4.0	+	73.9	0.19
	3.0	+	98.9	0.16
	2.0	+	103.3	0.17
	1.0	+	117.7	0.11
HMPA[d]	25.0	−	18.8	0.36
	22.5	−	31.9	0.32
	25.0	+	22.0	0.20
	22.5	+	26.5	0.20
Formalin[e]	0.065	−	55.2	0.98
	0.065	+	94.2	0.27
Control	—	−	100.0	0.16
	—	+	100.0	0.15

[a] FDH and NAD^+ were present (+) or absent (−) in the treatment medium.
[b] % Total Growth = (Relative Suspension Growth) × (Relative Cloning Efficiency) ÷ 100.
[c] Mutant Frequency = (Mean Number of TFT Colonies per Dish)/(Mean Number VC Colonies per Dish) × (2×10^{-4}).
[d] HMPA used as a positive control. It requires metabolic activation to release formaldehyde and is mutagenic to lymphoma cells in the activated assay.
[e] Formalin is a 37% solution of formaldehyde in water, stabilized with 10% methanol.

treated control levels. FDH and its co-factor also reduced cytotoxicity, as indicated by total growth levels near or at the corresponding untreated control levels. HMPA, a material that has been shown to be mutagenic in the activated mouse lymphoma assay due to metabolic-release of formaldehyde (11) was included in the experiment as an additional positive control. HMPA (Table II) produced about a 2-fold increase in the frequency of mutants in the activated assay in the absence of FDH and its co-factor, and near control mutant frequencies in the presence of FDH and its co-factor. Formalin produced about a 16-fold increase in the frequency of mutants in the absence of FDH, while in the presence of FDH and its co-factor the frequency was reduced to less than 2-fold above control (Table II).

Since Blackburn *et al.* (14, 15) have shown that mutagenicity, induced in mouse lymphoma cells by released formaldehyde, is ameliorated by FDH addition to test cultures, and since they have further established that mutagenicity unrelated to formaldehyde is unaffected by this modification, it is concluded that formaldehyde is responsible for the activity seen with MTBE in the activated mouse lymphoma assay.

The lack of mutagenicity reported by others (5–9) for MTBE in Ames bacterial tests may be due to technical difficulties in identifying volatile mutagens in plate incorporation assays (18, 19). Likewise, the conclusion for overall negative findings in the *in vitro* cytogenetic tests may also be attributed to difficulties in detecting clastogenicity with volatile compounds. Indeed, the negative findings for mutagenicity of MTBE in genotoxicity tests other than the mouse lymphoma assay may be due to inadequate test conditions.

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