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Mutagenicity of Volatile Anesthetics:

Halothane

Jeffrey M. Baden, M.D., Michael Brinkenhoff, B.S.,† Robert S. Wharton, M.D.,‡ Ben A. Hitt, Ph.D.,§ Vincent F. Simmon, Ph.D.,¶ Richard I. Mazze, M.D.‖*

The mutagenicity of halothane was tested in an *in-vitro* microbial assay system employing two histidine-dependent mutants of *Salmonella typhimurium*, TA98 and TA100. Halothane in concentrations ranging from 0.1 to 30 per cent was incubated with bacteria in the presence or absence of a metabolic activation system prepared from either rat liver treated with Aroclor 1254 or human liver. Trifluoroacetic acid, a major metabolite of halothane, and urine from patients anesthetized with halothane also were tested. Halothane, trifluoroacetic acid, and patients' urines were not mutagenic. (Key words: Anesthetics, volatile, halothane; Biotransformation (drug), halothane; Bacteria, mutagenicity; Toxicity, mutagenicity.)

RECENT SURVEYS suggest an increased incidence of malignancies in operating room personnel[1,2]; the implication is that prolonged exposure to trace concentrations of anesthetic agents is the cause. One of the mechanisms by which environmental agents are thought to produce cancer involves mutation of DNA.[3] Since the DNA's of all organisms are chemically similar, a study of the mutagenic properties of anesthetic agents in bacteria was undertaken. Halothane was studied first because it is the most commonly used volatile inhalational anesthetic and also because its metabolism proceeds through reactive intermediates.[4]

Methods and Materials

BACTERIAL PREPARATION

Two histidine-dependent strains of *Salmonella typhimurium*, TA98 and TA100, were employed.[5] Prior to use they were stored in liquid nitrogen at −80 C. For each experiment, an inoculum from stock cultures was grown overnight at 37 C in a nutrient broth consisting of 1 per cent tryptone and 0.5 per cent yeast extract. After stationary overnight growth, the culture was shaken for 3 to 4 hours to ensure optimal growth and used within two hours, as described below.

METABOLIC ACTIVATION SYSTEM

Rat Liver. A metabolic activation system was prepared from the livers of male Sprague-Dawley rats treated five days prior to sacrifice with 500 mg/kg polychlorobiphenyls (PCB; Aroclor 1254). PCB was administered with a single intraperitoneal injection as a 20 per cent solution in corn oil. PCB is an inducer of the mixed-function oxidase system and stimulates halothane metabolism.** Rats were killed by decapitation and their livers removed in sterile fashion; subsequently, all procedures were conducted at 0–4 C. The liver was washed, minced in three volumes of cold, sterile 0.15 M KCl, and homogenized with a Potter-Elvehjem apparatus. The homogenate was centrifuged for 10 minutes

* Assistant Professor of Anesthesiology.
† Medical Student, University of Colorado School of Medicine, Denver, Colorado.
‡ Research Fellow of Anesthesiology.
§ Research Associate of Anesthesiology.
¶ Manager, Microbial Genetics Program, Stanford Research Institute.
‖ Associate Professor of Anesthesiology.

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Address reprint requests to: Jeffrey M. Baden, M.D., Anesthesiology Service (112A), VA Hospital, Palo Alto, California 94304.

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at 9,000 × *g*, and the supernatant divided into 2-ml portions and stored at −80 C. Immediately prior to use, the liver supernatant (2 ml) was thawed and 1.0 ml was added to 0.05 ml 1 M glucose-6-phosphate; 0.2 ml 1.65 M KCl; 0.2 ml 0.4 M $MgCl_2$; 0.4 ml 0.1 M NADP; 5.0 ml 0.2 M sodium phosphate buffer (*p*H 7.4); and sterile water; final volume was 10 ml.

Human Liver. Human liver was obtained from a 19-year-old male heart-transplant donor who had suffered irreversible brain damage following a head injury. Pulse and blood pressure were maintained at physiologic levels from the time of admission to the hospital until cardiectomy. Results of clinical and laboratory tests of liver function were within normal limits. Approximately an hour elapsed from cardiectomy until removal of the liver, which was then washed, minced, and placed in ice-cold saline solution. Following this, preparation of the metabolic activation system was identical to that used for rat liver.

Desiccator Incubation Experiments

Fifty microliters of freshly grown bacteria at a concentration of approximately 10^9 organisms/ml, and 2 ml of molten top agar (43 C), containing 0.1 μmol histidine and 0.1 μmol biotin, were mixed and poured onto glucose-minimal medium petri plates and placed in a 9-liter airtight desiccator jar. Plates also were prepared with the above constituents and, in addition, 0.25 ml of rat liver metabolic activation system. Plates were randomly stacked without lids on a perforated horizontal shelf that partitioned the desiccator, and a known volume of liquid halothane or vinylidene chloride (positive control) was placed in an open glass dish attached to the underside of the shelf. Volumes of liquid were calculated to result in final halothane vapor concentrations of 0.01, 0.1, 1.0, 5.0 and 10 per cent; vinylidene chloride vapor concentration was 3 per cent. In other experiments, anesthetic concentrations were verified by gas chromatographic analysis of desiccator atmosphere and were found not to vary from predicted values by more than 10 per cent, nor to vary significantly with time. After addition of the test compound, the lid of the desiccator jar was immediately replaced and sealed with grease. A magnetic stirrer was placed on the floor of each desiccator jar to ensure even distribution of the vapor. Desiccator jars were incubated at 37 C. After eight hours of exposure to the anesthetic or positive control, the plates were removed from the desiccator and their lids replaced; incubation was then continued. After a total of two days of incubation, the colonies on each plate were counted. Replicate plates were prepared at each drug concentration, both with and without the metabolic activation system. The experiment was repeated once.

Liquid Incubation Experiments

Fifty microliters of freshly grown bacteria, 0.25 ml of the rat liver metabolic activation system, and various amounts of liquid halothane or positive control (vinylidene chloride for TA100 and 4-aminobiphenyl for TA98) were added to sterile 15-ml screw-top test tubes. Final halothane vapor concentrations in the test tubes were 0.1, 0.3, 1, 3, 10, and 30 per cent; vinylidene chloride vapor concentration was 3 or 10 per cent; 4-aminobiphenyl concentration in the reaction mixture was 0.75 mM. Vapor concentrations were verified by gas chromatographic analysis. The tubes were incubated on a rotator for one hour at 37 C, after which 2 ml of molten top agar were added to each tube, and the entire contents were poured onto glucose-minimal medium plates. Liquid incubation prior to plating increases the probability that reactive metabolites will come into contact with bacteria.[6] The plates were incubated at 37 C for two days and revertant colonies counted. Tubes also were prepared with all of the above constituents except that sodium phosphate buffer was substituted for the liver homogenate supernatant in the metabolic activation system. Replicate plates were prepared. In another experiment, human liver supernatant was substituted for rat liver supernatant in the metabolic activation system and the procedure carried out as above.

Urinary Metabolites

Twenty-four-hour urine collections were obtained, both before and immediately after operation, from seven surgical patients anesthetized with halothane. Urine, .25 ml, was added to test tubes containing 0.05 ml of

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TABLE 1. Desiccator Incubation Experiment

Strain	Metabolic Activation	Number of Revertant Colonies per Plate						
		Room Air	Per Cent Halothane					Vinylidene Cl 3 Per Cent
			0.01	0.1	1	5	10	
TA98 Mean	No	51.8	71.5	49.0	52.3	61.0	0.5*	79.0†
± SE		8.3	1.5	3.5	9.4	11.5	0.5	2.4
TA98 Mean	Yes	67.0	70.5	43.5	47.3	38.0	0.0*	174.8†
± SE		11.1	10.5	5.8	9.3	9.9	0.0	56.8
TA100 Mean	No	145.0	141.0	126.0	177.0	93.3	0.0*	228.0
± SE		22.2	33.9	30.6	1.0	26.7	0.0	65.0
TA100 Mean	Yes	161.3	173.3	162.0	157.5	106.5	0.0*	658.0†
± SE		22.4	27.2	23.8	20.1	21.3	0.0	76.6

* Less than room air control, $P < 0.05$.
† More than room air control, $P < 0.05$.

freshly grown bacteria, 0.25 ml of metabolic activation system, 65 units of beta-glucuronidase and 0.05 ml purified glusulase.†† Two ml of molten top agar were added to each tube, and the entire contents poured onto glucose-minimal medium plates. Plates were incubated at 37 C for two days and revertant colonies were counted.

SPOT TEST

Fifty microliters of freshly grown bacteria and 2 ml of molten top agar were mixed and poured onto glucose-minimal medium plates. After solidifying for 15 minutes, a sterile 6-mm-diameter paper disc was placed in the center of each plate and 0.1 ml of trifluoroacetic acid, a major halothane metabolite, or 2 μg of N-methyl-N'-nitro-N-nitrosoguanidine (positive control) were applied to the disc. Plates were incubated at 37 C for two days and observed for growth of revertant colonies.

ANALYSIS OF DATA

The numbers of revertant colonies in treated plates were compared with the numbers of spontaneous revertant colonies in plates exposed to room air. *t* tests were used for statistical analysis; $P < 0.05$ was considered significant.

CHEMICALS

Chemicals were obtained as follows: glusulase, Endo Laboratories (Garden City, New York); Aroclor 1254, Monsanto (St. Louis); halothane, Ayerst (New York); vinylidene chloride, Dupont (Wilmington, Delaware); 4-aminobiphenyl, Schuchardt (Munich); trifluoroacetic acid, Matheson, Coleman & Bell (Norwood, Ohio); histidine, Calbiochem (La Jolla, California); biotin, beta-glucuronidase, glucose-6-phosphate, and nicotinamide-adenosine-diphosphate, Sigma (St. Louis); n-methyl-N'-nitro-N-nitrosoguanidine, Starks Associates (Buffalo, New York).

Results

Table 1 shows the results of desiccator incubation of bacterial strains TA98 and TA100 with various concentrations of halothane and vinylidene chloride. There was no increase in reversion rate in any of the plates exposed to halothane. The highest concentration of halothane, 10 per cent, was toxic, as indicated by the decreased number

†† Glusulase is the intestinal juice of the snail, *Helix pomatia*; the mixture of enzymes includes sulfatase and glucuronidase.

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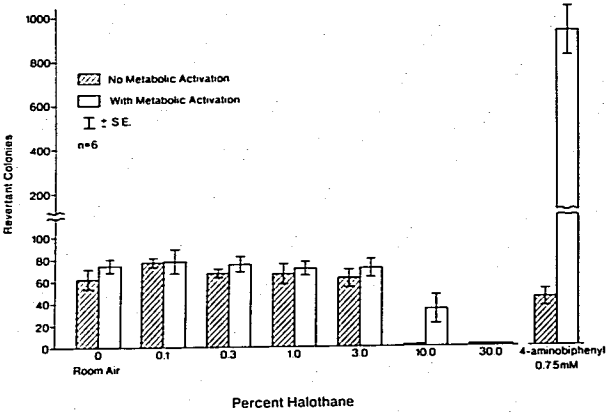

FIG. 1. Revertant colonies of *Salmonella typhimurium*, TA98, after liquid incubation with halothane or the positive control, 4-aminobiphenyl. Halothane, with or without metabolic activation, does not increase the number of revertant colonies to above the spontaneous reversion rate, but is toxic to bacteria in vapor concentrations of 10 and 30 per cent. In the presence of the complete metabolic activation system, 4-aminobiphenyl causes a significant increase in reversion rate.

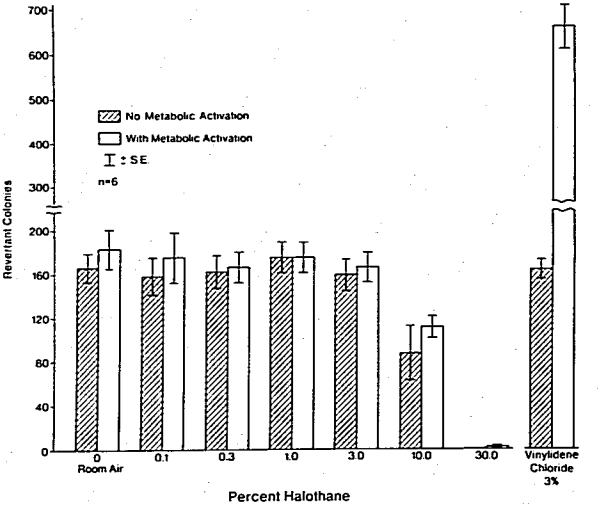

FIG. 2. Revertant colonies of *Salmonella typhimurium*, TA100, after liquid incubation with halothane or the positive control, vinylidene chloride. Halothane, with or without metabolic activation, does not increase the number of revertant colonies to above the spontaneous reversion rate, but is toxic in vapor concentrations of 10 and 30 per cent. In the presence of the complete metabolic activation system, vinylidene chloride causes a significant increase in reversion rate.

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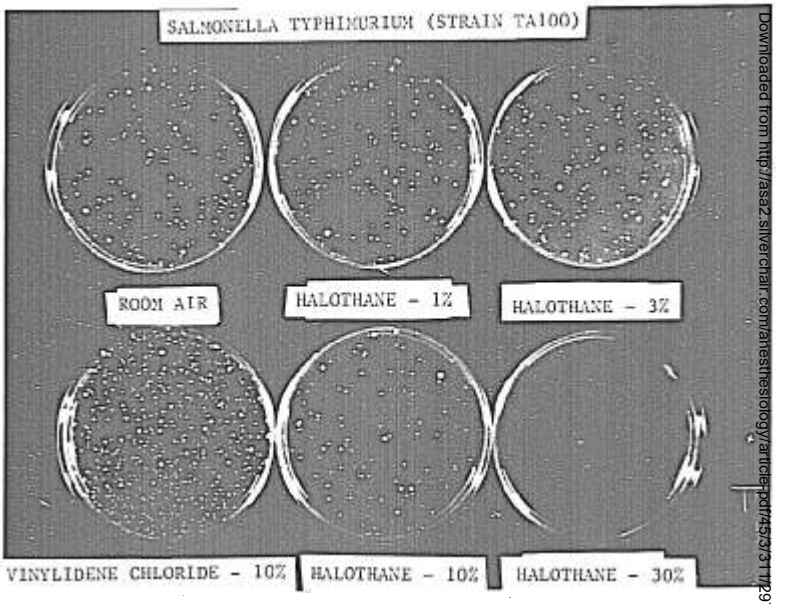

FIG. 3. Bacterial plates showing revertant colonies of *Salmonella typhimurium*, TA100, after liquid incubation in the presence of the human liver metabolic activation system. The number of colonies is not increased at any concentration of halothane. Complete killing of bacteria occurs at a halothane vapor concentration of 30 per cent. An increased number of colonies can be seen in the positive control plate (vinylidene chloride).

of revertant colonies. Both bacterial strains exposed to vinylidene chloride in the presence of the complete metabolic activation system showed significant increases in reversion rate. When liver homogenate supernatant was omitted from the metabolic activation system, there was a smaller increase in the number of revertant colonies.

Figures 1 and 2 show the results of liquid incubation of bacterial strains TA98 and TA100, using the rat liver metabolic activation system with various concentrations of halothane and the positive controls, 4-aminobiphenyl and vinylidene chloride. There was no increase in the number of revertant colonies in the bacteria exposed to any of the halothane concentrations compared with room-air controls. Bacteria exposed to 4-aminobiphenyl and vinylidene chloride, in the presence of the complete metabolic activation system, showed significant increases in the numbers of revertant colonies.

Figure 3 shows the results of liquid incubation of bacterial strain TA100 with the human liver metabolic activation system. No increase in the number of revertant colonies was observed when the bacteria were exposed to halothane. Positive controls were highly mutagenic when the metabolic activation system was added. Similar results were obtained with TA98.

Table 2 shows the results of studies performed with urine of patients anesthetized with halothane. There was no increase in the reversion rates of strains TA98 and TA100.

Spot tests with trifluoroacetic acid were negative. The positive control, N-methyl-N'-nitro-N-nitrosoguanidine, was strongly mutagenic.

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TABLE 2. Urinary Mutagenesis Experiment

Strain	Metabolic Activation	Enzymes*	Revertant Colonies: Preanesthetic Mean (± SE)	Revertant Colonies: Postanesthetic Mean (± SE)†
TA98	–	–	27.3 (± 5.8)	24.3 (± 7.4)
	–	+	21.0 (± 4.1)	18.3 (± 6.4)
	+	–	25.6 (± 5.7)	24.3 (± 6.4)
	+	+	25.1 (± 5.0)	20.7 (± 4.5)
TA100	–	–	146.0 (± 21)	149.3 (± 20.4)
	–	+	132.0 (± 22)	130.3 (± 17.5)
	+	–	160.0 (± 20)	160.0 (± 27.2)
	+	+	157.0 (± 24)	165.0 (± 30.2)

* Glusulase and β-glucuronidase.
† No significant difference, preanesthetic *vs.* postanesthetic, all groups.

Discussion

There are compelling reasons why the oncogenic potential of anesthetic agents should be determined. A survey conducted by the American Society of Anesthesiologists[1] has demonstrated higher incidences of cancer and abortion among female anesthesia personnel than in a control group of female medical personnel who do not work in the operating room. Additional surveys from the United Kingdom,[7] the Soviet Union,[8] and the United States[2,9] support these findings. Anesthetic agents may be responsible for these effects. The carcinogen, bis(chloromethyl) ether is an alpha-halo ether[10,11]; methoxyflurane, enflurane and isoflurane are also alpha-halo ethers. In a preliminary study, Corbett *et al.*[12] suggested that isoflurane caused an increased incidence of hepatic tumors in mice. Several simple alkyl halides, such as iso-, secondary- and tertiary-butyl bromides, have been associated with increased incidences of tumors in the lungs of mice[13]; halothane is an alkyl halide. Vinyl chloride, a carcinogen in man,[14] is structurally similar to trichloroethylene, a halogenated alkene inhalation anesthetic. Hepatic tumors have been produced in mice chronically exposed to trichloroethylene.[15]

An established technique for determining the carcinogenic properties of chemicals employs lifetime exposure of laboratory animals to suspect compounds. Such *in-vivo* tests are expensive, time-consuming, and require the examination of large numbers of animals to obtain statistically significant results. Also, species differences in oncologic susceptibilities may require that several species be tested. The development of a system for testing mutagenesis in bacteria[2,5,16–18] has overcome many of the difficulties inherent in *in-vivo* animal studies. The test procedure is relatively inexpensive and rapid. Large numbers of organisms can be simultaneously exposed to identical test conditions. Furthermore, the conditions of the experiment may be selectively altered and each step examined independently.

The validity of the bacterial system for studying mutagenicity depends upon the fact that mutagens alter DNA. As all DNA's have the same double-helical structure and the same four nucleotides, any DNA-containing organism may be used as an indicator for mutagenesis.[3] Knowledge of the mutagenic properties of a chemical is of value in predicting whether it is a carcinogen. Eighty-five per cent of known carcinogens tested with bacterial assay systems have been mutagenic. The incidence of false positives is less than 10 per cent among non-carcinogens, many of which are closely related to carcinogens.[19]

The bacterial assay system used in this study has been developed by Ames *et al.*[2,5,16,17] Tester strains of *S. typhimurium* that are dependent upon exogenous histidine for growth by virtue of a mutation in the gene that codes for histidine synthesis are employed. A subsequent mutation that allows the organism to synthesize histidine is called a "reversion." A reversion is detected by the ability of bacteria to grow in a culture

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medium deficient in histidine. Tester strain TA98 is susceptible to reversion by mutagens that add or delete one or more base pairs (frame-shift mutations); TA100 is reverted by substances that cause substitution of an incorrect base pair for the correct one (base-pair mutation). Both strains have known spontaneous rates of reversion. Mutagens increase these rates. The tester strains have been made more susceptible to mutation due to deletions in the gene involved in repair of damaged DNA and by virtue of a mutational defect in the lipopolysaccharide coat that increases permeability to large molecules. Finally, introduction of the R factor plasmid, which codes for an error-prone DNA repair system, has enhanced the susceptibilities of both strains to mutagens.[5]

In Ames' original assay system, the suspect mutagen and bacteria were combined and poured on an agar plate[16]; no metabolic activation was used. Although this system was capable of detecting the mutagenic effects of many chemicals, it could not identify promutagens, *i.e.*, compounds that require metabolic activation before they are mutagenic. Therefore, metabolically active cell fractions, either liver postmitochondrial supernatant[6,17] or liver microsomes[20] were combined with bacterial tester strains. *In-vitro* systems prepared with liver microsomes have permitted detection of procarcinogens, such as dimethylnitrosamine.[6,20] The ability to activate dimethylnitrosamine paralleled the content of cytochrome P-450 in the microsomal preparations.[20,21] The reaction was inhibited by carbon monoxide and the process reversed by monochromatic light at 450 nm.[22] These observations indicate that some carcinogens are activated to mutagenic derivatives by the nonspecific cytochrome P-450-dependent enzyme system.

Metabolism of chemicals *in vivo* may generate mutagenic products not produced in *in-vitro* metabolic activation systems. These may be detected in urine. However, some of these products may be inactivated by conjugation prior to excretion. Treatment of urine with enzymes, such as glucuronidase and glusulase, hydrolyzes these conjugates, permitting mutagenic metabolites to be detected.[23,24] It also is possible that mutagenic metabolites are formed *in vivo* but further degradation prior to excretion renders these products non-mutagenic.

In the present study, halothane, its major metabolite, trifluoroacetic acid, and urine from patients anesthetized with halothane were not mutagenic. In every experiment, positive controls ensured that the test system was sensitive to mutation. There are a number of possible explanations for this negative result. First, halothane may not be mutagenic under any condition. Second, it is possible that the bacterial assay system is not sensitive enough to detect weak mutagens. Although it is true that 85 per cent of known carcinogens are mutagens, most of these compounds are potent carcinogens.[19] Many weaker carcinogens probably are not detected because they also are weak mutagens. Based on epidemiologic studies[1,2] it is likely that anesthetic agents are only weakly carcinogenic, if at all. Third, it is possible that the *in-vitro* metabolic activation system did not generate metabolites in amounts sufficient to interact with the bacterial DNA. Although many of the metabolites of halothane are known to be highly reactive,[4] when produced in small quantities, it is unlikely that they would reach bacterial chromosomes before reacting with other molecules. Finally, it is possible that halothane is mutagenic *in vivo*, *i.e.*, metabolism *in vivo* may differ qualitatively and quantitatively from that which occurs *in vitro* using hepatic enzymes. The lack of mutagenesis in studies employing urine obtained from patients anesthetized with halothane suggests that this is not the case, although it is possible that mutagenic metabolites are formed but are further degraded to non-mutagenic products prior to excretion.

In summary, halothane was not mutagenic in the bacterial assay system used in this study. These results do not exclude the possibility that halothane is carcinogenic to chronically exposed operating room personnel.

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Erratum

The editorial by R. I. Mazze and B. A. Hitt, Methoxyflurane Metabolism (ANESTHESIOLOGY 44:369–371, 1976) inadvertently erred when commenting on the paper by L. Adler *et al.*, in the same issue. The editorial was based on the original copy of the paper by Adler *et al.*, which was sent to Drs. Mazze and Hitt rather than the revised copy that was published. Thus, the statement in the editorial that Adler *et al.* did not derive K_m values is in error. K_m values appear in table 2 of the Adler paper.

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