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Mutagenicity Studies with Halothane in Drosophila melanogaster

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Halothane was investigated for its mutagenic effects in *Drosophila melanogaster*. The induction of sex-linked recessive lethals was used as an indicator of genetic damage. Adult male flies were exposed to halothane either for 14 days at 1,000 or 1,600 ppm (v/v) or for one or two days in 2,100 or 20,000 ppm. In several experiments slight increases of the mutation frequency were observed. For the pooled data of the 14 day-exposure experiments, the increase amounted to a doubling of the spontaneous rate, just reaching the level of 5 per cent significance. The authors consider this a borderline result, indicating, with a fair degree of probability, that halothane has weak mutagenic activity, under the conditions studied. (Key words: Anesthetics, volatile: halothane. Toxicity: mutagenicity.)

DURING THE PAST FEW YEARS several epidemiologic studies of operating room personnel have indicated the occurrence of relatively high rates of spontaneous abortions, and of congenital abnormalities in the progeny of exposed operating room workers. These effects have mainly been attributed to adverse effects of waste anesthetic gases and vapors. In unventilated operating rooms, halothane may reach concentrations of about 50 ppm.[1,2] Among the effects reported, the enhancement of congenital abnormalities in the children of male anesthetists[3,4] presents a special category, since this would indicate the induction of transmissible genetic damage in the germ line in the male, in contrast to the effects found in exposed females, which may result from genetic as well as nongenetic (*e.g.*, teratogenic) causes. Therefore, it was considered important to examine the effects of volatile anesthetics in a model system for mutagenicity. A second reason was the chemical similarity between some anesthetics and known mutagenic or carcinogenic agents, like vinylchloride, 1,2-dibromoethane and bischloromethylether.[5] In the present study, halothane was tested for its ability to induce sex-linked recessive lethal mutations in the fruit fly *Drosophila melanogaster*.

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The usefulness of *Drosophila* for mutagenicity testing has been extensively documented.[6,7] The main advantages of *Drosophila* are the availability of detailed information about its genetics and the fact that its metabolism of foreign compounds resembles that of mammals, in that it has the capacity to activate a large number of indirect mutagens (precarcinogens). The genetic test utilized in the present experiments, the sex-linked recessive lethal test, is a multi-purpose test in the sense that it will detect a number of different types of genetic damage, *i.e.*, point mutations, small deletions, and chromosomal aberrations. It is an efficient test, since it detects such damage at any site of the X chromosome capable of lethal induction, *i.e.*, it represents mutations at approximately 750 different genes. *Drosophila* is also a suitable organism for exposure to gaseous mutagens, since via the respiratory system gases will reach all tissues of the fly within a very short time.

Materials and Methods

One-day-old male flies of a wild-type strain (Oregon-K) were exposed to halothane-containing atmospheres, according to the method described by Verburgt and Vogel,[8] keeping the flies in air-tight sealed 1,160-ml flasks. Each flask contained about 8 g *Drosophila* food, and in the 14-day treatments, the flies were transferred every two to three days into new flasks with fresh food and were given fresh halothane. Liquid halothane was injected into the bottles through the rubber screw cap, using a 10-μl microsyringe. Halothane was obtained from Imperial Chemical Industries (England) as the standard material for anesthesia.

Halothane levels of 1,000–2,000 ppm (v/v) still allowed the flies to move around and to feed, although their activity was lessened. At 3,500 ppm (v/v) or more the flies lay down, motionless. Recovery depended on the duration of the treatment: all flies survived a few hours of anesthesia, while after 24 hours of treatment, recovery was less than 50 per cent. Consequently, experiments were carried out according to two exposure schemes: long-term treatment at non-anesthetizing levels (table 1) and short-term treatment with anesthesia (table 2). Gas chromatographic measurements[9] of the actual concentrations, which were made in Experiments 1, 2, 5, and 8, showed that

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the decreases of the halothane concentrations (possibly due to absorption by the food or the rubber cap) did not exceed 5 per cent per day.

The genetic mating scheme[10] begins with mating the treated males individually to three or four virgin females of the tester strain *Basc* (like man, *Drosophila* females have two X chromosomes, while males have an X chromosome and a Y chromosome). The F_1 daughters from this cross receive a potentially mutated X chromosome from the treated father and a *Basc* X chromosome from the mother. The *Basc* chromosome contains visible markers that distinguish it from the treated paternal X chromosome; it also contains a structural rearrangement that prevents crossing-over between the two X chromosomes. The scheme proceeds by individually mating F_1 females with their brothers, which carry a *Basc* X chromosome. In the F_2 generation, normally, males are of two classes, depending on whether they receive from their mother the treated, unmarked, X chromosome or the *Basc* X chromosome. A recessive lethal induced in a treated X chromosome will go undetected in the F_1 females (because the non-mutated *Basc* X chromosome "covers" the lethal effect), but will show in the F_2 culture by the absence of the class of males carrying the treated X chromosome (in males, which have only one X chromosome, the mutation expresses its lethal effect). In all lethal (or semilethal) cases, F_2 females carrying the treated X chromosome were mated with their brothers to confirm the effect.

All experiments were carried out at 25 C. In Experiments 2, 3, 4, 8, and 9 the males were repeatedly mated with virgins for two or three successive periods ("broods"). After short-term treatment this "brooding" procedure allows separate sampling of germ cells that were at different developmental stages at the time of treatment. Broods A, B, and C roughly correspond to treated sperm, spermatids, and early spermatids–spermatocytes, respectively. In general, these stages may differ in their sensitivities to mutagens.[7,10] On long-term exposure the sampled germ cells were treated during the major part of their development from spermatogonia to mature sperm, which lasts approximately 12 days. Consequently, one may expect accumulation of damage in the treated cells, but the separate sampling of germ cells treated in successive stages is not possible.

Halothane contains thymol, 0.01 per cent, as a stabilizer. To investigate the possibility that this compound was responsible for the mutagenic effect, it was tested separately. A 3-mM solution of thymol in 1:100 ethanol in water containing sucrose, 5 per cent, was fed to adult males for 12 days at 25 C, using the technique of Vogel and Lüers[11]; the flies were kept in gas-tight 9-liter containers and were given fresh solutions every three to four days.

Results

In the long-term treatments, there was a slight increase in mutation frequency over the control values (table 1). The pooled data of the 1,000-ppm experiments (Experiments 1, 2, and 4) as well as those of all long-term treatments (including also Experiment 3) provide a statistically significant increase over the control, at the 5 per cent level.[12] Although the inhomogeneity in the data may render this pooling procedure questionable, that the increase was found in three of the four experiments supports the notion that the effect was real.

It was unexpected that with the greatest exposure (Experiment 3, 1,600 ppm) the lowest mutation frequency was found. On the other hand, a lack of an exposure–effect relationship has been found in *Drosophila* for several mutagens that need metabolic activation, *e.g.*, vinyl chloride[8]; it has been tentatively explained by saturation of metabolizing enzymes,[7] so that increased substrate concentration would not enhance the amount of product formed.

In the long-term treatments where some of the sampled germ cells are treated during premeiotic stages, when they undergo mitotic division, "clusters" may occur, *i.e.*, a group of lethals derived from one originally mutated cell.[10] In the procedure followed by us of individually mating treated males, a cluster can be recognized by the appearance of a group of lethals among the offspring of a single treated male. In our experiments (table 1) only one such group appeared, consisting of two lethals only. Correction for this does not change the statistical significance.

In the short-term treatments (table 2) a tendency towards an increase was apparent only in Brood A of the 10,000-ppm tests (Experiments 6, 8, and 9). The pooled frequency of 0.38 per cent does not provide a statistically significant increase over control.[12] These data may be of limited significance, since treating flies during anesthesia represents a rather unphysiologic condition; moreover, the flies may have closed down their spiracles, thus drastically reducing their exposure. The deaths of a large proportion of flies during this treatment may have been caused by halothane, as well as (more likely) by starvation.

With thymol, altogether two lethals were found in 742 tested chromosomes (0.27 per cent). Therefore, it seems unlikely that exposure to thymol, which was much less in the halothane experiments than in the thymol test, caused the increased mutation frequencies found in the long-term treatments.

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TABLE 1. Percentages of Sex-linked Recessive Lethals after 14 Days of Treatment of Male *Drosophila melanogaster* with Halothane

	Treatment Concentration in ppm (v/v)	Mating Scheme (Days)*	Brood A: Number of Lethals Detected/Number of Chromosomes Tested	Brood A: Per Cent Lethals Calculated	Brood B: Number of Lethals Detected/Number of Chromosomes Tested	Brood B: Per Cent Lethals Calculated	Brood C: Number of Lethals Detected/Number of Chromosomes Tested	Brood C: Per Cent Lethals Calculated
Experiment 1	1,000†	6	5/983‡	0.51				
	Control	6	1/994	0.10				
Experiment 2	1,000†	2–3	5/1,607	0.31	4/1137	0.35		
	Control	2–3	2/1,133	0.18	0/649	0		
Experiment 3	1,600	1–2	3/1,152	0.26	3/1159	0.26		
	Control	1–2	3/577	0.52	1/566	0.18		
Experiment 4	1,000	1–2–3	2/930	0.22	7/897	0.78	0/455	0
	Control	1–2–3	0/1,088	0	2/763	0.26	0/432	0
Experiments 1–4	Treatments A–C		29/8,320	0.35				
	Controls A–C		9/6,202	0.15				

* This column indicates the lengths in days of the successive mating periods; for example, "2–3" means that the males were mated for two successive periods 2 and 3 days long, respectively, receiving virgins for every mating. The mating periods are referred to as broods.

† In these experiments the concentrations were checked by gas chromatography. Mean concentrations were 980 and 970 ppm (v/v) in Experiments 1 and 2, respectively.

‡ Among these five lethals two were found in the progeny of one male and may thus be of common origin (see text).

Discussion

The data reported here, especially those from the long-term treatments, present a borderline result, indicating, with a fair degree of probability, that halothane has weak mutagenic activity, under the conditions applied. These conditions include the use of concentrations that are 20 or more times greater than those present in the operating room environment. It should be realized, however, that submammalian tests, although considered appropriate qualitative indicators of genetic damage, cannot serve as a basis for making quantitative assessments of risk for man.

Mutation induction by halothane has also been studied by Uehleke *et al.*[13] and by Baden *et al.*[14] using various microsome-mediated bacterial assays, all with negative results. In a study for the induction of gene mutations in Chinese hamster cells *in vitro*,[15] the incorporation of a liver microsomal fraction was omitted, which may explain the negative results obtained. The finding of a weak mutagenic effect in *Drosophila* in the absence of such an effect in bacteria is not unexpected: the reaction of halothane with cellular macromolecules presumably occurs via short-lived reactive radicals generated by microsomal enzymes.[13,16] In *Drosophila* these metabolizing enzymes are closer to the target DNA, namely, they are present in the germ cells,[7] while in bacterial mutagenicity tests the microsomal enzymes—added as rat liver preparations—are extracellular.

Other effects of halothane related to mutagenesis

TABLE 2. Percentages of Sex-linked Recessive Lethals after One or Two Days of Treatment of Male

	Treatment Time, Concentration in ppm (v/v)	Mating Scheme (Days)*	Brood A: Number of Lethals Detected/Number of Chromosomes Tested	Brood A: Per Cent Lethals Calculated	Brood B: Number of Lethals Detected/Number of Chromosomes Tested	Brood B: Per Cent Lethals Calculated
Experiment 5†	2 days, 2100‡	3	0/830	0		
Experiment 6†	24 h, 10,000	5	3/475	0.63		
Experiment 7†	24 h, 20,000	5	0/114	0		
Experiment 8	24 h, 10,000‡	2–3–3	2/779	0.26	0/605	0
Experiment 9	24 h, 10,000	3–2–3	1/311	0.32	1/1,342	0.07
Experiments 6, 8, and 9	Treatments		6/1,565	0.38	1/1,947	0.05
Laboratory controls		3–3–3	4/3,136	0.13	7/2,997	0.23

* This column indicates the lengths in days of the successive mating periods; for example, "2–3" means that the males were mated for two successive periods 2 and 3 days long, respectively, receiving virgins for every mating. The mating periods are referred to as broods.

† These were preliminary experiments.

‡ In these tests the concentrations were checked by gas chromatography. Mean concentrations were 2,100 and 9,600 ppm (v/v) in experiments 5 and 8, respectively.

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include prolongation of the mitotic cycle in Chinese hamster fibroblasts[17] and effects on mitosis in *Vicia faba* root-tip cells, resulting in breaks and aneuploidy.[18,19] This disturbing effect on cell division may be relevant to an explanation of the observed epidemiologic phenomena where females are exposed. In general, spontaneous abortions frequently show aneuploidy, probably resulting from meiotic nondisjunction,[20] while disturbing mitosis during early embryogenesis may cause developmental disorders.[21]

In conclusion, a further evaluation of mutagenic effects (including interaction with cell division) of halothane, but also of related anesthetics, seems appropriate. Recent studies by Baden *et al.*[22] and Waskell[23] are examples of such comparative investigations using the Ames' *Salmonella* test. The former investigators showed fluroxene to be clearly active, while in both studies a number of other anesthetics were nonmutagenic. Extension of this approach may lead to the selection of nonmutagenic (and otherwise acceptable) anesthetics, for the benefit of both operating room personnel and patients.

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Drosophila melanogaster with Halothane

Brood C		Broods A–C	
Number of Lethals Detected/Number of Chromosomes Tested	Per Cent Lethals Calculated	Number of Lethals Detected/Number of Chromosomes Tested	Per Cent Lethals Calculated
1/636	0.16	3/2,020	0.15
2/777	0.26	4/2,430	0.16
3/1,413	0.21	10/4,925	0.20
4/3,118	0.13	15/9,251	0.16

References

1. Whitcher CE, Cohen EN, Trudell JR: Chronic exposure to anesthetic gases in the operating room. ANESTHESIOLOGY 35: 348–353, 1971
2. Burm AGL, Spierdijk Joh, Rejger V: Concentrations of anesthetic agents in the air in operating rooms, Anesthesia and Pharmacology. Edited by Spierdijk Joh, Feldman SA, Mattie H. Leiden, University Press, 1976, pp 263–274
3. ASA Ad Hoc Committee: Occupational disease among operating room personnel. ANESTHESIOLOGY 41:321–340, 1974
4. Knill-Jones RP, Rodrigues LV, Moir DD, et al: Anaesthetic practice and pregnancy: Controlled survey of male anaesthetists in the United Kingdom. Lancet 2:807–809, 1975
5. Fishbein L: Industrial mutagens and potential mutagens. I. Halogenated aliphatic derivatives. Mutation Res 32:267–308, 1976
6. Lee WR: Chemical mutagenesis, The Genetics and Biology of Drosophila. Edited by Ashburner M, Novitski E. London, Academic Press, 1976, pp 1299–1342
7. Vogel E, Sobels FH: The function of Drosophila in genetic toxicology testing, Chemical Mutagens. Volume 4. Edited by Hollaender A. New York, Plenum, 1976, pp 93–142
8. Verburgt FG, Vogel E: Vinyl chloride mutagenesis in *Drosophila melanogaster*. Mutation Res 48:327–336, 1977
9. Beneken Kolmer HH, Burm AG, Cramers CA, et al. The uptake and elimination of halothane in dogs. I. Gas chromatographic determination of halothane in blood and in inspired and end-tidal gases. Br J Anaesth 47:1049–1052, 1975
10. Wurgler FE, Sobels FH, Vogel E: Drosophila as assay system for detecting genetic changes, Handbook of Mutagenicity Test Procedures. Edited by Kilbey BJ, Legator MS, Nichols WW, et al. Amsterdam, Elsevier, 1977, pp 336–373
11. Vogel E, Luers H: A comparison of adult feeding to injection in *Drosophila melanogaster*. Drosophila Inform Serv 51:113–114, 1974
12. Kastenbaum MA, Bowman KO: Tables for determining the statistical significance of mutation frequencies. Mutation Res 9:527–549, 1970
13. Uehleke H, Greim H, Krämer M, et al: Covalent binding of haloalkanes to liver constituents, but absence of mutagenicity on bacteria in a metabolizing test system. Mutation Res 38:114, 1976
14. Baden JM, Brinkenhoff M, Wharton RS, et al: Mutagenicity of volatile anesthetics: Halothane. ANESTHESIOLOGY 45: 311–318, 1976
15. Sturrock JE: Lack of mutagenic effect of halothane or chloroform on cultured cells using the azaguanine test system. Br J Anaesth 49:207–210, 1977
16. Uehleke H, Hellmer KH, Tabarelli-Poplawski S: Metabolic activation of halothane and its covalent binding to liver endoplasmic proteins *in vitro*. Arch Pharmacol 279:39–52, 1973
17. Sturrock JE, Nunn JF: Mitosis in mammalian cells during exposure to anesthetics. ANESTHESIOLOGY 43:21–32, 1975
18. Grant CJ, Powell JN, Radford SG: Effects of halothane on DNA synthesis and mitosis in root tip meristems of *Vicia faba*. Br J Anaesth 46:653–657, 1974
19. Grant CJ, Powell JN, Radford SG: The induction of chromosomal abnormalities by inhalational anaesthetics. Mutation Res 46:177–184, 1977
20. Jacobs PA: Epidemiology of chromosome abnormalities in man. Am J Epidemiol 105:180–191, 1977
21. Wilson JG: Environment and birth defects. New York, Academic Press, 1973
22. Baden JM, Kelley M, Wharton RS, et al: Mutagenicity of halogenated anesthetics. ANESTHESIOLOGY 46:346–350, 1977
23. Waskell L: A study of the mutagenicity of anesthetics and their metabolites. Mutation Res 57:141–153, 1978

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