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Mutagenicity of ethylene oxide and associated health hazard

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MUTAGENICITY OF ETHYLENE OXIDE AND ASSOCIATED HEALTH HAZARD

by

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DISSERTATION

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ABSTRACT

The mutagenicity of ethylene oxide, an important industrial chemical, was evaluated in various test systems as a preliminary attempt to define the genetic risk to the exposed human population. The test systems employed analysed the ability of ethylene oxide to produce mutations in bacterial cells and mammalian somatic and germinal cell lines.

The Salmonella typhimurium plate assay was used to screen not only the ability of ethylene oxide in producing mutations, but also its' major reaction and metabolic products: ethylene glycol, ethylene chlorohydrin, glyoxal, glycoaldehyde, glyoxalic acid and glycolic acid. This screening procedure showed ethylene oxide to be mutagenic in the strain of bacteria used through a base substitution mechanism. Of the reaction products, only ethylene chlorohydrin produced a significant increase in mutation frequency over the background level. Mechanistically, ethylene chlorohydrin also appeared to act through a base substitution event.

Metaphase cytogenetic studies on bone marrow samples from rats exposed to ethylene oxide at 250 ppm for several hours a day for three days showed a significant increase in chromosomal aberrations over the levels found in control animals. Total aberrations increased from 7/120 cells examined in the control group to 101/120 cells examined in the experimental group. Increases were seen in almost all types

of aberrations observed, including chromatoid gaps and breaks, isochromatid gaps and breaks, rearrangements and exchanges and dicentrics. No increases were seen in either double minutes or polyploidy nuclei.

In order to evaluate further the ability of ethylene oxide to produce chromosomal lesions in a dose-response relation, the micronucleus test was made on rats exposed to various air concentrations of ethylene oxide for four hour periods. The effects were observed following exposures to high concentrations, specifically to 50, 250 and 1000 ppm, and to low concentrations, specifically 10, 25 and 50 ppm. A significant increase in micronuclei, indicative of the production of chromosomal aberrations, was seen at all exposure levels of 50 ppm and above.

To examine the effect of ethylene oxide on germinal cells, a 10 week dominant-lethal study was conducted using male rats exposed to ethylene oxide at 1000 ppm for four hours. A significant increase in post-implantational fetal deaths was noted in the test group when compared to the negative control group. This increase occurred only during the first five weeks of the experiment, corresponding to germinal cells exposed to ethylene oxide after their meiotic division.

Using the dose-response relationship established with the micronucleus test a tentative risk estimation was established. Using the concept of "rad-equivalents", this

risk within the limitations of the modeling systems employed was set at 100 mrad equivalents/ppm h. Transferring this value to the industrial situation, it is clear that the TLV of 50 ppm would not adequately protect the worker exposed to ethylene oxide from unjustifiably high levels of genetic damage.

INTRODUCTION

A. Uses

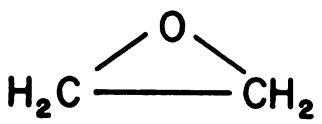
Approximately 2.5 billion pounds of ethylene oxide are produced and used in U.S. industry each year (Fishbein et al., 1970). Its principal application is in the manufacture of ethylene glycol and higher alcohols, which find important uses in automotive antifreeze, explosives, cellophane, polyester resins, synthetic fibers and rubbers, and hydraulic fluids. The higher alcohols such as di- and triethylene glycol are used as plasticizers, humectants, lubricants and solvents. Ethylene oxide is an important intermediate in the manufacture of glycol ether solvents, ethanolamines, and nonionic detergents. It is a raw material in the manufacture of acrylonitrile and hydroxyethylcellulose, thiodiethylene glycol, and ethylene halohydrins.

Ethylene oxide is one of the primary gas agents used in the sterilization of heat labile medical equipment and consumer goods. In the pharmaceutical industry ethylene oxide is used in the synthesis of choline, thiamine and as a sterilizing agent for antibiotics. The FDA allows three different foods to be sterilized with ethylene oxide: whole spices, black walnuts and cocoa.

B. Chemical Properties

Ethylene oxide is a colorless liquid having a molecular

weight of 44.053. It boils at 10.73°C and freezes at -12.5°. The solubility in water at 20°C is unlimited. Vapor pressure at 25°C is 7.3 psig. The flammable limits in air are 3-100%. Fifty ppm equals approximately 90 mg/m^3. The structure of ethylene oxide is shown below.



Ethylene oxide reacts with a wide range of compounds: 1) with water to form ethylene glycol or a polyglycol, 2) with hydrogen halides to give halohydrins, 3) with alcohols to form hydroxy ethers and monoethers of polyglycols, 4) with ammonia to give mono-, di-, and triethanolamines and morpholine. Many other reactions can also occur. Thermal decomposition yields ketene, acetaldehyde, carbon monoxide, ethane, propane, methane, and hydrogen.

C. Acute and Chronic Toxicity

The toxicology of ethylene oxide in experimental animals has been well reviewed and extended by Hollingsworth and his co-workers (1956). Work done prior to 1960 has been reviewed by Hine and Rowe (1962). The oral LD_{50} of an aqueous solution in rats is 330 mg/kg (Smyth et al., 1941). Given intraveneously to dogs the LD_{50} is 125 mg/kg (Hine and Rowe, 1962). Ethylene oxide when given to rats by inhalation for four hours shows an LC_{50} of 1460 ppm (Jacobson

et al., 1956). In guinea pigs, 0.3 to 8.5% ethylene oxide in air results in death. Air concentrations of from 0.06 to 0.13% ethylene oxide produce eye irritation, while below 0.025% no effects are observed (Waite et al., 1930). In the standard patch test of rabbits, 10 and 50% water solutions of ethylene oxide result in rapid hyperemia and edema (Hollingsworth et al., 1956). Industrial workers exposed chronically to levels of ethylene oxide in air averaging below 9 ppm showed no adverse symptoms (Joyner, 1964). However, Ehrenberg and his co-workers (1974) using Joyner's data found a leukocytosis and possibly a lymphocytosis in the workers.

Animals exposed to ethylene oxide on an acute basis respond as shown in Table 1. The physiological effect of ethylene oxide in mammals is primarily that of an irritant in low concentrations, and a vesicant in high concentrations. Generally, exposure of a skin surface to a water solution of ethylene oxide results in blistering. Acute exposures irritate eyes and lungs and give evidence of gross pathology in the kidneys.

The results of chronic exposure of cats to ethylene oxide are given in Table 2. Chronic low level exposure toxicity can partially be explained on the basis that inspired ethylene oxide is converted to the glycol and later to the diglycol which have a well known necrotic effect on kidneys and liver.

Table 1[1] Summary of Acute Response of Animals to Exposure to Ethylene Oxide[2,3,4,5]

PPM by Vol in air	Time (hrs.)	Animal	Results
250-800	8	Guinea pig	Slight Resp. change
	48	Guinea pig	Occasional death
560-600	7	Guinea pig, cat, dog	No death
	8	Guinea pig	Occasional death
	22	Guinea pig, cat	Death during or after
	22	Rabbit, dog	No death
710	4	Dog	0/3 in 14 days
1100	5	Guinea pig, rabbit	Mod. injury, no death
	8	Guinea pig, rabbit	Slt. injury, no death
	8	Rat, cat	Death w/in 24 hrs.
1300-1400	8	Guinea pig	Death in 1 to 8 dys.
	4	Dog	3/3 1st day
2200	1.5	Cat	Injury, no deaths
	3	Cat	Death w/in 24 hrs.
	4	Guinea pig	Injury, few deaths
	4	Rabbit	Injury, no deaths
	4	Dog	Death w/in 24 hrs.
3000	1	Guinea pig	No deaths
	3	Guinea pig	Death in 1 to 8 dys.
	8	Guinea pig	Death w/in 24 hrs.
7000	0.3	Guinea pig	No injury
	1	Guinea pig	Death in 1 to 8 dys.
	2.5	Guinea pig	Death w/in 24 hrs.
14000	10 min	Guinea pig	No injury
	20 min	Guinea pig	Death in 1 to 8 dys.
	60 min	Guinea pig	Death w/in 24 hrs.
51,000-64,000	5 min	Guinea pig	Death in 1 to 8 dys.
	10 min	Guinea pig	Death w/in 24 hrs.

1. From Hine and Rowe, 1962
2. Hollingsworth et al., 1956
3. Jacobson et al., 1956
4. Waite et al., 1930
5. Flury and Zernick, 1931

Table 2. Response of Cats to Prolonged Repeated Exposure to Ethylene Oxide (Patty, 1949)

PPM	Exposure	Response
50	18 daily 3 hour exposures followed by-	Paralysis
100	2 daily 3 hour exposures, and 1 day of 1.5 hour exposure	Death (Day 22)
100	18 one hour exposures in 21 days	Death (Day 21+)
200	Two 1.5 hour exposures and one 1 hour exposure in four days	Death (Day 5)

In 1971, the Health Industries Association Sterile Disposable Device Committee established a study to investigate the toxicity and irritation capabilities of ethylene oxide, ethylene glycol and ethylene chlorohydrin. The final report of the study (Woodard, 1971) included data on the acute toxicity of the three compounds when given orally, intraperitoneally, and subcutaneously to several species. It also included observations of primary skin and eye irritation, of skin sensitization, and of irritation when given by intramuscular, subcutaneous and intracutaneous routes. A subacute safety evaluation study was also presented.

D. Threshold Limit Value

The toxic nature of ethylene oxide and its common use in industry made it apparent that workers needed to be protected from excessive exposure to this compound. As a result the Threshold Limit Value (TLV) for industrial exposure was set at 50 ppm. This implies that a worker may be exposed to 50 ppm ethylene oxide for 7 hours/day, 5 days/week for his working career without the development of ethylene oxide induced pathology.

E. Toxicology of Ethylene Oxide Sterilized Plastics

Ethylene oxide in various mixtures with either Freon^{R} or carbon monoxide is used in a pressurized system to steri-

lize numerous heat labile materials. These include drugs, food, plastic and surgical material. In the process of sterilization of plastic materials the ethylene oxide readily dissolves in the plastic. Levels as high as 2000 ppm of residual ethylene oxide remain in the material after the process is completed. For sterilized surgical material (e.g. catheter tubing, bone cement, polyethylene acetabulum, chest tubes, millipore filter units, etc.) a period of forced ventilation of at least 24 hours in air is applied to remove most of the remaining residual ethylene oxide prior to human use. In some cases the level of residual ethylene oxide remains elevated even after prolonged ventilation (Prieve, 1973).

The possible hazards of ethylene oxide sterilization of surgical material have been generally reviewed by Willson (1970). In one series of experiments, when sterilized plastic tubing containing 3 mg/g ethylene oxide residue was placed in tanks with guppies, they died within 70 minutes (O'Leary et al., 1969). Plastic aereated for 24 hours after sterilization produced no deaths in the guppy system. Twenty-four hours of aereation reduced residual levels below the part per thousand level (Anderson, 1971). Sterilized plastic tubing prior to aereation killed cell cultures; after the 24 hour aereation period no effect was seen (O'Leary et al., 1969). Hemolysis increased in static blood samples exposed to unaereated sterilized plastic tubings, whereas plastic aereated for 24 hours after

sterilization showed no increase in hemolysis over the control values (Hirose et al., 1963; O'Leary et al., 1969).

Sterilized plastic tubing inserted subcutaneously into rabbits invoked tissue reactions proportionate to the amount of residual ethylene oxide. Eight to 19 mg ethylene oxide/g plastic produced a severe tissue reaction, six to 9 mg/g a moderate, and 2-7 mg/g a mild tissue response. Levels of retained ethylene oxide below 4 mg/g produced no notable response (Anderson, 1971). In examining possible reasons for unexplained deaths in children following the use of heart-lung machines, the question of an adverse effect of residual ethylene oxide was considered. An increase in mortality was found in dogs when machines sterilized with ethylene oxide were used as compared to steam sterilized machines (Stanley et al., 1971).

F. Teratology and Reproductive Studies

In a search of the literature on ethylene oxide no reports were found that dealt with either teratogenic or reproductive effects of the compound in mammalian systems.

G. Mutagenicity

The value of ethylene oxide as the smallest monofunctional epoxide has led to its use in many experiments designed to investigate the mechanism of mutagenesis by alkylating agents. As a result there are many data available

showing the mutagenicity of ethylene oxide in a variety of systems including: Drosophilia melanogaster (Rapoport, 1948; Bird, 1952; Nakao and Auerbach, 1961; Rosival, 1970), Neurospora crassa (Kilmark and Westergaard, 1953; Kolmark and Giles, 1955; Kilbey and Kolmark, 1968; Kolmark and Kilbey, 1968), barley (Ehrenberg and Gustafsson, 1957; Ehrenberg, 1960; Lindgren and Sulovska, 1969; Sulovska et al., 1969), and Aspergillus (Morpurgo, 1963). In addition, ethylene oxide has been shown to produce chromosome aberrations in maize (Faberge, 1955), barley (Moutschen-Dahmen et al., 1968), and Vicia faba (Loveless, 1953). Only one paper has reported on a mutagenic effect of ethylene oxide in mammals. Strekalova (1971) found a significant increase in metaphase chromosome aberrations in bone marrow cells from rats given a single intraperitoneal injection of ethylene oxide in water.

Ethylene chlorohydrin, one of the expected reaction products of ethylene oxide in physiological solution, has been reported to be mutagenic in mutant strains of Salmonella typhimurium (Rosenkranz et al., 1974; Rosenkranz and Wlodkowski, 1974).

H. Carcinogenicity

Ethylene oxide is generally described as non-carcinogenic and, in view of the negative results of tests (Walpole, 1957; Weil et al., 1963; Van Duuren et al.,

1965; Van Duuren, 1970), it is probably not a strong carcinogen. From several points of view including the difficulty in performing carcinogenicity tests with gaseous compounds, the rapid evaporation of ethylene oxide in the skin painting test and the rapid absorption into the body from the sites of application the experimental evidence for the non-carcinogenicity is inconclusive. Structurally related compounds including propane-1, 3-epoxide, ethylene sulfide and ethyleneimine are definitely carcinogenic as are the less volatile homologs of ethylene oxide (Ehrenberg et al., 1974).

I. Pharmacokinetics

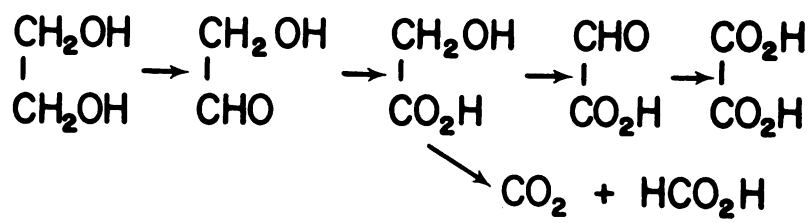
Ethylene oxide in water at neutral pH is a relatively slowly reacting compound with a half-life at 4°C of about 6 months. In reactions with DNA the rate constant in vitro is 0.9×10^{-4} l $(g \cdot DNA)^{-1}$ (Ehrenberg et al., 1974). This study also determined the tissue dose received from a given exposure to ethylene oxide, which for mice was calculated to be 0.58 uM h/ppm h. Their results agree with an absorption of all ethylene oxide in alveolar ventilation, a rapid distribution to all organs and a rapid excretion with a biological half-life of about 9 min.

Ethylene oxide as a strong nucleophil can be expected to have a high non-specific affinity in physiological solution for any electrophilic agent such as amines and

sulfhydryl groups, and a large proportion of the ethylene oxide in blood could be expected to react with miscellaneous organic molecules. From this information it could be assumed that all of the ethylene oxide would be hydrolysed or bound before it reached any target cells. On the contrary, the evidence on the mutagenic action of ethylene oxide in the various non-mammalian systems (usually involving a solution of ethylene oxide in water) and in the rat bone marrow work (Strekalova, 1971) indicates that ethylene oxide is, indeed, reaching target cells.

Any metabolism of ethylene oxide could be expected to play a very minor role in removing the ethylene oxide molecule from the system. It is much more likely that simple chemical reaction products would be the main route for disappearance of the chemical. The major degradation product in water is ethylene glycol. In the presence of free chloride ion another expected product would be ethylene chlorohydrin.

The production of ethylene glycol within the animal would lead to the formation of metabolic products which include glycoaldehyde, glycolic acid, glyoxalic acid and oxalate through the following pathway (Gessner et al., 1961):



J. Exposure

Industrial workers in the ethylene oxide industry are obviously the primary population exposed to the toxic hazards of ethylene oxide. Gas sterilization equipment operators are also exposed frequently to ethylene oxide and should be grouped with industrial workers. As mentioned previously the federally set standards for permissible exposure is 50 ppm. Acute human exposures due to accident have been reported (Ewert, 1937; Lundberg, 1938; Birath, 1940). Symptoms included nausea, vomiting and mental disoreintation. There appears to be a short latency period of a few hours before symptoms are noted. From an acute and chronic toxicological point of view the permissible levels if coupled with good occupational safety techniques are quite satisfactory.

The other population exposed to ethylene oxide is much larger, albeit, it is much more difficult to assess any toxic problems. This is that segment of the population which is exposed to ethylene oxide sterilized products and resultant residues of the gas contained in the material.

Of main concern in the latter category are those people exposed to sterilized surgical equipment. In this case the dissolved ethylene oxide residue leaches out into the respiratory system or into the various body cavities depending on the use of the sterilized material. It is quite

logical to assume that very high localized concentrations of residual ethylene oxide would be present at any tissue on which a sterilized piece of equipment is placed.

The reports in the literature indicate that the easily observable toxic manifestations such as tissue irritation assocated with the use of sterilized equipment are not apparent at residual levels below 4 mg/g. This level or lower can be reached with present day sterilization techniques and normal quality control. However, even with these present techniques, levels measurable in the parts per million or even parts per one hundred thousand can still remain at the time of use.

K. Statement of Problem

The standard toxicology associated with ethylene oxide is adequately worked out and present federal restrictions on ambient air levels in industry on this basis are satisfactory. With the realization that ethylene oxide residues remain within sterilized products, the hospital supply industry has implemented procedures to minimize exposure of patients to this hazard. With evaluation by normal toxicological procedures it is apparent that there are minimum toxic effects from the use of sterilized surgical material as now prepared.

Of more importance is the danger than ethylene oxide may be mutagenic in humans. With the large number of in-

dividuals that are exposed to the gas either through industry or through the use of sterilized articles, there exists a potential for deleterious genetic loading even at low exposure levels. The level of exposure through the use of ethylene oxide as a sterilant in contrast to industrial exposure is difficult to ascertain.

Other workers have reported ethylene oxide to be mutagenic in yeast, plants and insects. Also, from the Russian literature there is an indication that ethylene oxide is mutagenic in the rat. With this background it is an important toxicological problem to describe the mutagenic effect of ethylene oxide in various systems for extrapolation to the human situation. The most ominous result of a large population being exposed to a mutagen through a variety of routes is that deleterious changes in the gene pool at large might occur analogous to that due to ionizing radiation. The purpose of this present series of experiments is to attempt to define the hazard associated with ethylene oxide exposure within this context.

TESTING THEORY

The ultimate goal in describing the potential of a compound for inducing mutations in the genetic material of an organism within the framework of genetic toxicology in most cases, is to be able to extrapolate the information gained to the question of what happens when segments of the human population are exposed. The best modeling system would be to test human subjects with the compounds in question and monitor through several methods changes in genetic material. Since the hazards associated with exposure to mutagenic agents has not been fully evaluated, except for such cases as the induction of carcinogenic changes and birth defects, the use of human subjects is clearly not justifiable.

As a result the modeling system chosen from a number of possibilities to investigate the genetic toxicology of a compound, although not ideal, can be picked to give information that is as valuable as possible for later extrapolation. However, considering the costs inherent and the time involved in the numerous methods of mutagenic testing, in the end the choice of method is a trade off between the applicability of the method and the value of the data.

Each of the testing methods has certain advantages. For example, the bacterial plate assay is inexpensive, quick to run, and exposes a tremendous number of bacteria to the mutagen. It can also pinpoint the types of mutations

occurring. The metaphase cytogenetic assay gives precise information on macrochromosomal lesions. Along with the advantages, though, each test has disadvantages. The bacterial plate assay is not only not a mammalian cell test, but does not consider the effect of possible mammalian mutagenic activation of a compound. Metaphase cytogenetics is a very long and expensive procedure and does not evaluate the production of point or gene mutations.

It is generally recognized that a battery of tests is needed to fully assess the mutagenic potential of a compound. Thus it would be appropriate to begin the assessment with one of the simple screening tests, such as the bacterial plate assay, and if indicated continue with a host-mediated test and on to the assays that test mammalian somatic and germinal cell lines.

In testing ethylene oxide several different systems were used. These included the bacterial plate assay, metaphase cytogenetics, the micronucleus test and the dominant lethal assay. To properly define the results from these tests it is important to delineate the strengths and weaknesses of each test and to explain its position within the heirarchy of the entire evaluation.

A. Bacterial Plate Assay

The bacterial plate assay is one of the most simple, fastest and most inexpensive of the mutagenic tests. Strains

of Salmonella typhimurium have been developed (Ames, 1971; Ames et al., 1973) that because of specific mutations require histidine to grow and divide. Several strains are available, each with a different mutation causing the histidine requirement. For example, one strain has a base-pair substitution causing a defect in a specific gene. Another strain has a deletion of several base-pairs in a gene.

All of these strains have the ability to revert back to a normal non-histidine requiring strain by a specific mutation. This leads to the simple test of exposing the special strains to a compound and looking for reversion bacterial colonies indicating the test material was mutagenic. All of the strains have a low spontaneous reversion rate, but this is evaluated by including a concurrent negative control.

The specific strain of bacteria that is reverted gives a strong indication as to what type of mutagenic event is occurring. If the strain with originally a base-pair substitution is reverted then it can be assumed that the test compound causes base-pair changes. If the strain with a deletion is reverted, then the compound should be a frame-shift mutant.

The test is basically used as a screening test and beyond its simplicity its main advantage is that large numbers of bacteria (10^9) are exposed to the mutagen with the

result that even weak mutagens can be detected. The disadvantages of the test include the lack of consideration of a) metabolic activation which would cause a compound to be scored negative even if it was a mutagen in mammalian systems; and b) of the dissimilarities between bacterial and mammalian cells. The difficulty with lack of metabolic activation can be overcome by modifying the test to one described as a host-mediated assay where either *in vivo* or *in vitro* mammalian metabolic systems are incorporated into the test.

The bacterial plate assay was used in the ethylene oxide evaluation for several reasons. If ethylene oxide tested negative in this test and in a host-mediated assay then there would be no reason to further test the compound. Secondly, the testing, if positive, would indicate what type of mutagenic event was occuring. Since the chemical reaction products of ethylene oxide could be easily obtained, the assay without host-mediated modification was an easy way to assess the mutagenicity of these metabolites.

B. Cytogenetics

Cytogenetic studies can be carried out in almost all species including humans. It is the only procedure where the results of genetic damage can be directly observed through the light microscope. In animals both somatic and germinal cells can be analysed and in the *in vivo* situation

it provides a meaningful selective assay. In the mutagenic evaluation of ethylene oxide two different types of *in vivo* cytogenetic studies were performed; bone marrow metaphase studies and the micronucleus test.

1. Metaphase Analysis

The theory behind the value of metaphase cytogenetic studies in evaluating the mutagenic potential of a compound is quite simple. Mutagenic disruption of normal DNA will lead to morphological disruptions of the individual chromosomes that can be seen under a light microscope.

The actual procedure is just as simple, although of an exacting nature. Animals are exposed to the agent in question through any of the applicable routes of administration. After exposure a sample of bone marrow is taken and cultured with colcemide for several hours.

Colcemide, a spindle apparatus poison, effectively blocks dividing cells in the metaphase of division. This allows a large number of bone marrow cells to be captured in what is normally not a very prevalent stage. After incubation the cells are lysed and the nuclei spread on microscope slides. After further preparation and staining, large numbers of mitotic figures in metaphase can be seen under the light microscope. These are scanned and those that are smeared and stained well enough are analysed for chromosome aberrations.

This test has several advantages. Most importantly it

is an *in vivo* mammalian assay lending itself readily to extrapolation to human effects. Secondly, it gives precise data on macrochromosomal aberration production. The major disadvantage with the system is that the sample size is limited due to the expense and time necessary to evaluate individual mitotic figures.

After the initial experiment with a single exposure level to ethylene oxide, the time and money involved made it necessary to continue somatic cytogenetic analysis with a test system that, although it gives less precise information, is faster and less expensive to run. This was the micronucleus test.

2. Micronucleus Test

The principle behind the procedure is that mitotic cells with chromatin breaks or exchanges suffer from disturbances in the anaphase which causes bridge formation. The same is true if non-disjunction due to disturbances of the mitotic apparatus takes place. After telaphase, a sizable proportion of the displaced chromatin is not included in the nuclei of the daughter cells and can be detected in the cytoplasm of these cells by suitable staining technique.

One cell population that is extremely suitable for this assay is the polychromatic erythrocyte. In mammals erythrocyte precursors are nucleated. After several divisions the nucleus is expelled from the cell. If pieces of chromatin have been lost from the nucleus by the mechansism

described above, they will stay in what is now called a polychromatic erythrocyte rather than being expelled. Upon suitable staining the pieces of chromatin stand out sharply against the cytoplasm of the cell and are termed micronuclei.

The exposure of the reticulocyte population *in vivo* to mutagenic agents causes chromosomal aberrations that lead to micronuclei production. Examination of smears of stained bone marrow from animals exposed to mutagens will show an increase in micronuclei compared to control smears.

This assay system when compared to the standard metaphase analysis system is more sensitive and yields a response over a greater range of concentrations (Weber and Legator, 1974). It is also easier to run and less expensive.

C. Dominant-Lethal Assay

The dominant-lethal assay is an *in vivo* test of mutagenesis in germinal cell lines. As implied by its name it is an assay of dominant genetic change that is incompatible with the survival of the conceptus. In this procedure male animals are exposed to the test compound. They are then mated sequentially with groups of untreated females for the duration of the spermatogenic cycle. Several days prior to term the females are sacrificed and

scored for corpora lutea and implants comprising early deaths and living fetuses respectively. The sequential mating essentially provides a means of sampling all stages of the spermatogenic cycle. By noting in which weeks the greatest number of dominant lethalities occur, an accurate estimate can be made as to which sperm stages are affected. An increase in early fetal deaths at any of the times corresponding to specific cell stages when compared to the control animals at the same cell stage indicates an increase in dominant-lethal mutations.

The importance of the dominant-lethal test lies in the fact that it is an *in vivo* germinal cell assay. Except for possible links to carcinogenic changes, the most important aspects of exposure to mutagens is the adverse effect upon the population gene pool. The assay is easy to run, but rather expensive in terms of animals and animal care costs. A single one dose assay with controls can use over 700 animals if it is to detect a simple doubling of the rate of dominant-lethal mutation production.

MATERIALS AND METHODS

A. Bacterial Plate Assay

The bacterial plate assay as developed by Ames (1971) and his co-workers (1973) was used to screen ethylene oxide and its major probable reaction products for mutagenicity.

1. Compounds

In addition to ethylene oxide the following possible chemical reaction products of ethylene oxide were tested: ethylene glycol, 2-chloroethanol (MCB, Manufacturing Chemists); glyoxal, glyoxalic acid hydrate, glycolic acid and glycoaldehyde (Aldrich).

2. Bacterial Strains

The TA 1535 set of Salmonella typhimurium histidine auxotrophs (Dr. Bruce Ames, Biochem. Dept., UC Berkeley, 94720) was used in the bacterial plate assay. This set which consists of four tester strains differing in the type of mutation required to revert to the wild type, all contain a rfa mutation for the polysaccaride coat resulting in an increase in coat permeability. All four strains also contain a deletion through uvrB which eliminates the excision repair system for DNA. These two mutations not only increase the sensitivity of the bacteria to mutagenic agents, but also make it non-pathogenic.

The four tester strains within the TA 1535 set revert to the wild type through four different mutagenic mechanisms. TA 1535 is a coat and repair variant of HisG46, a histidine requiring mutant of Salmonella typhimurium LT-1, which has a base substitution altering one codon in the mRNA from the gene coding for the first enzyme of histidine. This strain reverts either by direct mutation or by a variety of suppressor mutations. It is reverted by a variety of alkylating agents, by 2-aminopurine and by nitrogen mustards.

TA 1536, a variant of HisC207, is a frameshift mutant of LT-2 that is apparently missing one or two base pairs in the aminotransferase gene of the histidine operon. Reverse mutation can be caused by a variety of agents such as acridine compounds that can add a base to the DNA in the region of the missing base pairs. This strain was not included in this analysis because of the insensitivity to compounds other than ICR 191.

TA 1537 is a variant of HisC3076, which contains an added base pair in the aminotransferase gene. Agents capable of causing a single deletion such as acridine and quinicrin will cause reversion.

TA 1538 contains the HisD3052 frameshift mutation that reverts to the wild type by a deletion of two specific base pairs. Reversion occurs after exposure to various aromatic nitroso derivatives of amine and by 2-nitroso-

fluorene.

3. Method

A modified version of the bacterial plate assay was used. In this method the various mutants were grown over night in nutrient broth (Appendix 1) at 37°C with shaking. After this incubation the cultures could be stored up to several weeks in a refrigerator prior to use.

Minimal glucose agar plates (Appendix 1) were prepared and allowed to stand inverted for at least three days to remove excess moisture. A second layer of top agar (Appendix 1) containing histidine and biotin in quantitites just sufficient to allow a slight background lawn of bacteria to grow (2 or 3 divisions) and the bacterial strain to be tested was then added over the top of the minimal glucose agar. After the layer had hardened a small amount of the material to be tested was placed in the center of the plate and the plates were incubated at 37°C for 48 hours.

The procedure for testing ethylene oxide differed alsightly since it is a gas at room temperature. Small pieces of Tygon^{R} tubing were placed in a flask containing ethylene oxide gas. After one hour the pieces of tubing which now contained dissolved ethylene oxide were removed and tapped gently into the layer of top agar. The leaching of ethylene oxide out of the tubing then exposed the

plate to this agent.

Each tester strain was run with concurrent positive and negative controls. The negative controls were sterile distilled water and untreated Tygon^{R}. The positive controls are shown below:

Strain	+Control
TA 1535	N-methyl-N'-nitro-N-nitrosoguanidine
TA 1537	Quinicrin
TA 1538	2-Nitrosofluorene

After the incubation period each plate was examined for revertant colonies against the faint background lawn.

B. Cytogenetic Testing

1. Metaphase Chromosome Analysis

a. Animals. Six male Long-Evans rats (Simonson, Gilroy, Ca.) within the weight range of 120-150 g were used in this experiment. The rats were received five days prior to the start of the experiment and were housed at a constant temperature of 23^{o} in stainless steel cages with sawdust bedding while acclimating. Food and water were presented ad libitum.

b. Exposure. The rats were exposed to ethylene oxide in air while running free in a chamber of 60 liter volume. The chamber was a plexiglass box with dimensions of 38.5x38.5x30 cm. A screen supported several centimeters off the base of the chamber provided the flooring

for the animals. The gas inlet was a vertical pipe in which a series of holes had been drilled extending from the base almost to the top of the chamber. The outlet was at the bottom of the chamber in the opposite corner from the inlet pipe. A 20x20 cm door with latch was located in the front of the chamber for loading and unloading the animals. The entire chamber was located inside a hood for safety.

A laboratory compressed air outlet supplied the air for the chamber after the air was filtered through a 5u cellulose filter to remove moisture, oil and large particles. The air before entering the chamber was also filtered through a 1.25 l container of silica gel to dry the air further. Line pressure was controlled by an air regulator and final flow was controlled by a rotameter.

Ethylene oxide was supplied from a commercial cylinder (Matheson) with final flow regulated by a micrometer valve metered by a small rotameter. The ethylene oxide and air lines converged in a small mixing bulb before entry into the chamber. The exhaust was released into the hood flue. Air flow remained constant at 60 l/min giving a total volume sufficient to displace the chamber volume in one minute. With the microvalve the ethylene oxide flow was regulated to give the level of gas concentration desired.

For the metaphase cytogenetic experiment the six rats

were placed in the chamber at 250 ppm ethylene oxide for 7 hours/day for three days.

c. Air Analysis. The concentration of ethylene oxide within the chamber was monitored every 20 minutes during exposure by gas chromatography analysis of 1 cc grab samples. The GC (Aerograph Hy-Fi. Model 600-C) was equipped with a three foot Porapak Q (80/100) column. Oven temperature was 150^{o}C and gas flows were for air, nitrogen and hydrogen, 17.2, 12 and 26 psi, respectively. The equipment was calibrated by injecting known concentrations of ethylene oxide prepared by dilution of pure ethylene oxide with room air.

d. Animal Sampling. Each of the six rats served as its own control for the experiment. Five days before the start of the exposure each rat was biopsied for a femur bone marrow sample using the method described in the next sub-section. On the fourth day after the start of the three day exposure a second femur bone marrow sample was taken. Immediately after sampling, slides were made of the bone marrow for later analysis.

e. Bone Marrow Sampling and Slide Preparation. Bone marrow samples were taken using the biopsy technique of Nemenzo (1975). The rat was immobilized by wrapping the upper 2/3 of the body with a towel held in place by a hemostat.

While the leg was held in adduction by the thumb and middle finger, the index finger was used to palpate for

the epiphyseal joint. A heparinized needle (19 or 20 g) with syringe (5 ml plastic disposable) was inserted into the center of the epiphysis. As the needle penetrated the periosteum and entered the femoral cavity, resistance was lost. Negative pressure was then applied and sustained until a brownish-red substance entered the syringe.

The collected material was then flushed into a heparinized centrifuge tube of approximately 10 ml capacity to which 5 ml of minimum essential medium containing 10 ug/cc Colcemid^{R} had been added. The marrow was immediately suspended by gently flicking the lower end of the tube and the tubes were incubated for 2-3 hours at 37^{o}.

After incubation the cell-media suspension was centrifuged for five min at 1200 rpm. The supernatant was removed leaving the compacted cells undisturbed. The cells were then resuspended in 37^{o} 0.075 M KCl and left at room temperature for 15-20 min.

After centrifugation at 800 rpm for five min followed by decanting, 4-5 ml of fixative (1:3 glacial acetic acid-methanol) was added and the cells resuspended by gently bubbling with a Pasteur pipette. The centrifugation was repeated and a new volume of fixative added after decanting.

The preparation was again centrifuged at 800 rpm followed by decanting and 2 ml of fixative was added. The cells were gently resuspended by bubbling with a Pasteur

pipette.

A smear of the suspended cells was made by adding three drops of the suspension to a pre-cleaned slide pre-immersed in distilled water. The slide was air dried and stained with giemsa stain (Appendix 2) for 10-15 min. After drying, the slides were cleaned with xylene and mounted.

f. Analysis of Metaphase Chromosomes. With a light microscope slides for each rat were examined for nuclei in metaphase. These were carefully analyzed for a variety of possible chromosome aberrations and photographed under oil immersion.

Twenty metaphases were examined for each rat for each sampling period. The photographs were analysed using the notes from the initial microscopic examination. Each metaphase was then scored for each of the possible aberrations. Any discrepency between the notes and the photographs was re-examined by relocating the metaphase of interest on the original slide.

2. Micronucleus Test

a. Animals. Male rats of the Long-Evans strain (Simonson) within the weight range of 120-130 g were used in this series of experiments. The rats were received seven days prior to the start of exposure to allow time for acclimatization to the new situation and were housed in stainless steel cages with sawdust bedding. Temper-

ature was maintained at approximately 23^{o} during this period. Food and water were presented ad libitum.

b. Exposure. Two separate experiments were involved in investigating the production of micronuclei after ethylene oxide exposure. The first experiment exposed rats in groups of five to 1000, 250 and 50 ppm for four hours. A negative control and positive control (Triethylene melamine (TEM), 0.25 mg/kg, intraperitoneally, Bioclinical Laboratory, Bohemia, N.Y.) were also run.

The second experiment was designed to test lower levels of ethylene oxide and the rats in groups of five were exposed to either 50, 25 or 10 ppm for four hours. Again postive (TEM, 0.25 mg/kg ip) and negative controls were run.

The method of exposing the rats to ethylene oxide gas and the analysis of chamber concentration was the same as that described in the metaphase cytogenetic section (Material and Methods, B-1-b and B-1-c).

After ethylene oxide exposure the rats were returned to their cages for 24 hours before sampling. The negative control rats were placed in the chamber without ethylene oxide exposure for four hours and were also sampled 24 hours later. The positive controls were injected intraperitoneally with TEM 24 hours prior to sampling.

c. Bone Marrow Sampling and Slide Preparation. Bone marrow samples were obtained bioptically as described

under metaphase cytogenetics (Materials and Methods, B-1-d). The samples were then prepared as described by Schmid (1975).

The bone marrow sample was placed in a centrifuge tube containing 7 ml of fetal calf serum and centrifuged at 1000 rpm for 5 min. The supernatant was removed and the sediment mixed using a Pasteur pipette and gentle aspiration.

One drop of the sediment was transferred to the end of a clean slide and spread behind a polished cover slip held at a 45^{o} angle. The smear was then allowed to air dry.

The smears were stained within 24 hours. This consisted of a three minute stain with undiluted May-Gruenwald solution (Appendix 2) followed by a two minute immersion in 1:1 May-Gruenwald solution and distilled water. After a brief rinse in distilled water the slides were stained with giemsa stain (Appendix 2) diluted with water 1:6. The slides were then rinsed and blotted dry with filter paper. Mounting of the slides after they were dry was preceeded by a five minute clearing in xylene.

d. Analysis of Slides. Under a light microscope with oil immersion lens an appropriate area of each slide was chosen for analysis based on the excellence of the smear. Several slides for each rat were prepared to insure that at least one area of smear would be suitable for analysis.

For each rat 2000 polychromatic erythrocytes were examined for presence of micronuclei. The total number of polychromatics with micronuclei was recorded.

C. Dominant-Lethal Assay

1. Animals

Male and female Long-Evans rats (Simonson) approximately 12 weeks old were used in this experiment. Since a new group of females is needed each week for mating, a new batch was supplied weekly, four days before their mating period.

2. Exposure

The male rats were divided into three groups. One group (n=15) was exposed to ethylene oxide at 1000 ppm for four hours. The method of chamber air analysis and exposure was the same as described in the metaphase cytogenetics section (Materials and Methods, B-1-b and B-1-c).

The second group (n=10) served as positive control and was treated with an intraperitoneal dose of TEM (0.25 mg/kg). The final group was the negative control. This group in addition to being handled in the same manner as the ethylene oxide group, received olive oil intraperitoneally so that they could also be used as negative controls for another concurrent dominant-lethal study.

lethal study.

3. Mating

The procedure involved in the dominant-lethal assay was modified from Epstein (1973). After treatment each male rat was placed with two virgin females. At the end of one week, each male was placed with a new pair of females; this practice was continued for 10 weeks. Total adult rats involved in the experiment numbered 735.

4. Sampling

On day 17 after the first day to mating, the females were necropsied and scored for corpora lutea, late fetal deaths, early fetal deaths and total implants. This method scores pregnancies that at the minimum were 10 days and at the maximum, 17 days into term.

D. Data Analysis

1. Bacterial Plate Assay

The raw data from the bacterial plate assay consists of total counts of bacterial colonies appearing on individual plates. For any one compound and tester strain examined the various colony counts were combined and a mean with standard deviation computed. Student's T-test was then used to compare means and establish confidence levels for differences between means.

2. Metaphase Cytogenetics

The analysis of individual metaphase nuclei gave

data that was a tabulation of the appearance of chromosome aberrations. Within either the control or test group, the total number of each specific type of aberration were calculated. Differences between the control and test groups were then analysed using the Chi-square technique.

3. Micronucleus Test

The initial data from the micronucleus test consisted of the total number of polychromatic erythrocytes containing micronuclei out of the 2000 examined. This datum was obtained for each rat. Within exposure groups, means and standard deviations were computed and differences between groups were analysed using the Student T-test.

4. Dominant-Lethal Assay

Data from the dominant-lethal assay was tabulated and statistically analysed using a computer program available at the Stanford Research Institute (Menlo Park, Ca). The mean data for total and dead implants in pregnant females, were analysed using the Student's T-test. Preimplantation loss defined as corpora lutea minus total implants per pregnant female was analysed using the T-test on the Freeman-Tukey transformed data. The Chi-square technique was used to detect differences in the fertility index which is defined as the number of females pregnant/ number mated.

Five one way analyses of variance were performed on

the control group data. The variables were number of pregnant females, number of implantations per pregnant female, pre-implantation loss per pregnant female, number of dead implants per pregnant female and the ratio of dead implants to the total implants per pregnant female.

RESULTS

A. Bacterial Plate Assay

The results of the Salmonella typhimurium bacterial plate assay appear in Table 3. Each of the colony count figures are means ($\bar{x}$) computed from a number of plates. The total plates (n) run for any one bacterial strain and compound are indicated within the brackets. The standard deviation (SD) around the mean is indicated after the mean value. As asterisk indicates a value significantly higher than the control value using a confidence level of 95% and the Student's T-test. All of the compounds were tested using TA 1535. Only ethylene oxide, ethylene glycol and ethylene chlorohydrin were tested on the 1537 and 1538 strains.

In several instances the number of colonies on a plate was so great that a solid ring of bacterial colonies was present. In that case the total number of colonies was listed as being greater than 1000. No standard deviation could be calculated for these figures. The positive controls, for each of the strains used, all had colony counts greater than 1000.

The results on the test compounds using strains TA 1537 and 1538 were negative. None of the compounds showed any ability to cause reversion mutation in these frameshift mutant strains.

Table 3. Mutagenic Effect of Ethylene Oxide and Reaction Products on the Salmonella typhimurium Plate Assay. Means and Standard Deviations of Colony Counts.

Chemical	TA 1535			TA 1537			TA 1538		
	N	X	SD	N	X	SD	N	X	SD
Negative control	(12)	21.0	2.3	(5)	8.6	1.4	(5)	15.8	2.8
Ethylene oxide	(20)	1000*		(10)	10.3	2.6	(10)	13.7	3.5
Ethylene glycol	(10)	20.4	4.9	(5)	8.2	1.9	(5)	11.6	4.1
Ethylene chlorohydrin	(10)	32.3*	5.1	(5)	7.2	2.4	(5)	14.0	1.6
Glyoxal	(4)	17.3	5.7						
Glycoaldehyde	(4)	23.5	4.3						
Glyoxalic acid	(4)	25.3	7.2						
Glycolic acid	(4)	19.8	6.7						
N-methyl-N'-nitro-N-nitrosoguanidine	(5)	1000*							
Quinicrin				(5)	1000*				
2-Nitrosofluorine							(5)	1000*	

* $P < 0.05$

Ethylene oxide tested with the TA 1535 strain showed a strong ability to cause reversion mutations. All of the plates tested had greater than 1000 colonies. Ethylene chlorohydrin also showed positive mutagenic results in this strain; the increase in colony counts was, however, only slightly above the background rate. None of the other tested reaction products showed any significant increase in colony counts.

B. Cytogenetics

1. Bone Marrow Metaphase

A tabulation of results from the metaphase analysis is given in Table 4. For each of the various aberration types the information for both the control and the ethylene oxide groups is broken up into two categories. The first category consists of the enumeration of the total cells and animals that showed the aberration only once within a single metaphase. The second records the total instances of animals and cells where an aberration appears two or more times within a single metaphase plate.

The last line of the table gives a summation of the various aberrations and a total of metaphases showing a variance from the normal chromosome count of 42. For both the single aberration and summation figures, significant differences at a 95% confidence level between the control and treated groups are indicated by an asterisk.

Table 4. Metaphase Cytogenetic Analysis of Bone Marrow Samples from Rats Exposed to 250 PPM Ethylene Oxide for 7 Hours/Day for 3 Days

	CONTROL				ETHYLENE OXIDE			
Aberration Type	Animals	Cells(1)	Animals	Cells(2)	Animals	Cells(1)	Animals	Cells(2)
Chromatid Gap	3/6	3/120	- - -	- - -	6/6	30/120*	6/6	15/120*
Isochromatid Gap	1/6	1/120	- - -	- - -	3/6	4/120*	- -	- - -
Chromatid Break	1/6	1/120			6/6	32/120*	6/6	31/120
Isochromatid Break	- -	- - -			5/6	12/120*	- -	- - -
Double Minutes					1/6	1/120		
Rearrangements and Exchanges					5/6	10/120*	5/6	7/120*
Dicentrics					3/6	5/120*		
Rings					4/6	6/120*		
Polyploidy	1/6	1/120			1/6	1/120		
Cells with more than one aberration type per plate	1/6	1/120			6/6	50/120*		
Total Aberrations		7/120			6/6	101/120*		
Chromosome count different from 42	1/6	2/120	3/6	4/120	6/6	10/120*	4/6	9/120*

*$P < .05$

The bone marrow metaphases from rats treated with ethylene oxide showed a large and significant increase in total aberrations. A total of 101 out of 120 metaphases examined showed some sort of abnormal chromosome structure. The control sample had a total of only seven metaphases showing aberrations out of 120 examined. This difference is well outlined in an examination of individual aberration types.

All aberrations scored except for double minutes and polyploidy figures showed a significant increase in the treated group. The control group had no single metaphase with more than one of a single aberration type. The ethylene oxide group had 38 metaphases with duplicate aberrations. Cells with more than one aberration type were, except for one, absent from the control group. The ethylene oxide group had 50 cells with multiple aberrations.

Rearrangements, exchanges, ring structures and dicentric aberrations were not seen in the control group, while the test group had approximately 20 cells with one of these types of aberrations.

2. Micronucleus Test

Table 5 gives the results from the micronucleus test for rats exposed to various levels of ethylene oxide, to TEM, the positive control and to uncontaminated air, the negative control group. Since they were exposed at different times the high exposure level and low exposure level are recorded separately, although the overlapping

Table 5. Micronuclei in Polychromatic Erythrocytes from Long-Evans Rats after Four Hour Exposure to Ethylene Oxide.

High Exposure Levels

Group	Animals	Treatment	Micronuclei	SD
1	5	Control	0.12%	0.13
2	5	50 ppm	0.42*	0.22
3	5	250 ppm	0.52*	0.19
4	5	1000 ppm	1.64*	0.48
5	5	TEM, 0.25 mg/kg	5.50*	0.97

Low Exposure Levels

1	5	Control	0.09	0.08
2	5	10 ppm	0.12	0.06
3	5	25 ppm	0.20	0.07
4	5	50 ppm	0.43*	0.09
5	5	TEM, 0.25 mg/kg	3.41*	1.01

*$P < 0.05$

values give good agreement.

In the high exposure series, 0.12% of the polychromatic erythrocytes in the negative control group contained micronuclei. In the positive control group (TEM treated), 5.5% of these cells had micronuclei. The ethylene oxide-exposed rats for exposures of 50 ppm and above had values intermediate between these two, ranging from 0.42% for the 50 ppm exposure to 1.64% for the 1000 ppm exposure. The lowest value at 50 ppm was significantly higher than the negative control value.

In the low exposure group, which tested rats down to 10 ppm ethylene oxide for four hours, the negative control group had 0.09% of its polychromatic erythrocytes with micronuclei. The positive control had 3.41%. At 50 ppm the response (0.43%) was close to that seen in the high exposure group. This was significantly different from the control value.

The rats tested at 25 and 10 ppm had 0.2 and 0.12% of the polychromatic erythrocytes with micronuclei. These values were not significantly different from the control values.

C. Dominant-Lethal Assay

Following the four hour 1000 ppm ethylene oxide exposure, all of the exposed rats showed slight signs of ethylene oxide toxicity. These included central depres-

sion, diarrhea, and occular and respiratory irritation. Within 24 hours, however, all rats appeared normal and no deaths were recorded during the experiment. The TEM and negative control groups also sustained no deaths during the experiment. One male within the control group produced no pregnancies for the full 10 weeks of the experiment. Before analysis of the results, all data pertaining to this animal and the females caged with him were discarded.

The compilation of scored data and generated statistics for each of the three groups for the 10 week duration of the experiment appear in Table 6. No significant differences between groups were seen in the last 5 weeks of the assay. In comparing the ethylene oxide data with the negative control data, significant differences were apparent during the first weeks of the experiment in both the fertility index and the indices dealing with post-implantational losses.

The fertility index or the number of rats pregnant/number mated shows a significant decrease in the ethylene oxide test group in the third and fourth weeks. Other significant changes were seen in the total implants/pregnant female (decreased, second week), in dead implants/pregnant female (increased second, third and fifth weeks) and in dead implants/total implants (increased weeks one, two, three and five). The latter is expressed in the table

Table 6. Summary and Analysis of Dominant-Lethal Data from Rats Exposed to 1000 PPM Ethylene Oxide for Four Hours (Weeks 1-5)

Group	Week No.	Pregnancy MTD	Pregnancy PREG.	Fertility Index	Implants Live	Implants Dead	Total Implants/ Preg. Female	Dead Implants/ Preg. Female	Mutagenic Index (Dead Implants/ Total Implants X 100	Average Pre-implantation Loss/Pregnant Females
Control	1	18	12	0.67	138	3	11.75	0.25	2.	0.33
	2	18	15	0.83	153	17	11.33	1.13	10.	0.60
	3	18	17	0.94	187	8	11.47	0.47	4.	0.59
	4	18	18	1.00	168	15	10.17	0.83	8.	0.61
	5	18	17	0.94	181	8	11.12	0.47	4.	1.00
Ethylene Oxide	1	30	14	0.47	122	17	9.93	1.21	12.*	1.07
	2	30	16	0.53	104	44	9.25*	2.75*	30.*	0.75
	3	30	16	0.53*	114	48	10.12	3.00*	30.*	0.81
	4	30	16	0.53*	135	13	9.25	0.81	9.	0.75
	5	30	20	0.67	172	20	9.60	1.00*	10.*	1.20
TEM 0/25 mg/ kg,ip	1	20	16	0.80	144	28	10.25	1.75*	16.*	0.88
	2	20	19	0.95	23	138	8.47*	7.26*	86.*	1.11
	3	20	16	0.80	18	118	8.50*	7.38*	87.*	1.38
	4	20	18	0.90	4	102	5.89*	5.67*	96.*	1.11*
	5	20	16	0.80	117	60	11.06	3.75	34.*	0.81

* $P < .05$

Table 6. (con't). Summary and Analysis of Dominant-Lethal Data from Rats Exposed to 1000 PPM Ethylene Oxide for Four Hours (Weeks 6-10)

Group	Week No.	Pregnancy MTD	Pregnancy PREG	Fertility Index	Implants Live	Implants Dead	Total Implants/ Preg. Female	Dead Implants/ Preg. Female	Mutagenic Index (Dead Implants/ Total Implants X 100	Average Pre-implantation Loss/Pregnant Females
Control	6	18	16	0.89	162	2	10.75	0.63	6.	0.38
	7	18	15	0.83	172	11	12.20	0.73	6.	0.20
	8	18	17	0.94	168	12	10.59	0.71	7.	0.59
	9	18	15	0.83	143	11	10.27	0.73	7.	0.81
	10	18	17	0.94	149	19	9.88	1.12	11.	0.53
Ethylene Oxide	6	30	28	0.93	279	34	11.18	1.21	11.	0.11
	7	30	24	0.80	251	24	11.12	0.67	6.	0.13
	8	30	26	0.87	173	36	11.88	1.38*	12.	0.12
	9	30	28	0.92	283	25	11.00	0.89	8.	0.32
	10	30	27	0.90	262	25	10.63	0.93	9.	0.56
TEM 0.25 mg/ kg/ip	6	20	17	0.94	167	22	11.12	1.29	12.	0.35
	7	20	18	0.90	180	11	10.61	0.61	6	0.94
	8	20	17	0.94	167	14	10.65	0.82	8	0.71
	9	20	20	1.00	183	23	10.30	1.15	11.	0.55
	10	20	17	0.85	176	8	10.82	0.47	4.	0.47

* $P < .05$

as the mutagenic index (X100).

The TEM positive control group, when compared to the negative control, also showed significant changes in total implants/pregnant female (decreased weeks two, three and four), in dead implants/pregnant female (increased each week up to fifth) and in the mutagenic index (increased each week up to fifth). Except for week four in the TEM group, neither ethylene oxide nor TEM resulted in significant changes in average pre-implantation loss/pregnant female. TEM did not significantly change the fertility index.

The results of the control group analysis of variance on the variables included showed no significant differences between or within males over the various weeks of the experiment.

DISCUSSION

It is becoming evident that as the field of medicine increases its efficacy in decreasing the incidence and severity of bacterial, viral and parasitic disease states, impairments in man resulting from gene and chromosomal changes are causing an increasingly serious impact on the health and economy of the human community. Moreover, the evidence available indicates that many man-made chemicals are capable of producing mutations in sub-human systems, posing the possibility that these chemicals may constitute a genetic hazard for the human population.

A mutation can be defined as any heriditary alteration in the informational content or in the distribution of the hereditary material in an organism (Freeze, 1971). This definition excludes changes due to segregation or recombination. A mutation may be the result of a chemical alteration of a single nucleotide (point mutation) or it may involve an increase, decrease or rearrangement of an entire or a part of a chromosome (chromosomal mutation). Mutagenic events can occur in germinal cell lines in which case it can be passed on to resultatnt progeny and in somatic cells where they will die out with the organism. Mutations can result in biochemical or morphological changes that can have a variety of effects on the developing organism. A mutation is generally accepted to be detri-

mental in varying degrees to the health status of the individual or to its ability to have normal offspring, but it would not be unusual to find a mutation that elicited no definable effect. Mutations that are advantageous to the carrier are extremely rare.

It is the province of genetic toxicology to assay the ability of chemicals of interest to produce mutational events. The research efforts reported in this dissertation were designed to make a preliminary evaluation on the mutagenicity of ethylene oxide, so that more information would be available for assessing the degree of risk to the exposed human population.

A. Bacterial Plate Assay

The prinicpal value of the bacterial plate assay is as a screening test and as an indicator of the type of mutation occurring. The results from this assay with ethylene oxide clearly indicated that ethylene oxide is a consistently strong mutagen in the TA 1535 strain of S. typhimurium. The ability of ethylene oxide to revert this strain indicates that this compound is mutagenic through a base substitution event.

This preliminary datum on ethylene oxide, in clear agreement with previous positive mutagenic work done in other sub-mammalian species, made it evident that the compound should be investigated using mammalian somatic and

germinal cell systems.

The bacterial plate assay is generally incorporated into either a host or liver homogenate activation system to investigate the role of metabolites in inducing mutations. Since ethylene oxide was a mutagen without mammalian enzyme activation, investigation of metabolites with the mediated system was not feasible. However, all of the probable reaction products could be obtained through chemical supply houses and were tested individually with the simple plate assay

Only one of the reaction products gave positive results with this assay. This was ethylene chlorohydrin which had already been reported to be mutagenic in a similar system (Rosenkranz et al., 1974; Rosenkranz and Wlodkowski, 1974). Since the assay system was set up on a purely qualitative basis employing spot testing, no dose-response relation was attempted.

B. Cytogenetics

Cytogenetic analysis of either bone marrow or blood samples has become a common assay for detecting the mutagenic potential of a compound. In genetic toxicology the ultimate goal is to amass data that provides for the best possible extrapolation to the human condition with good sensitivity. Cytogenetic studies in addition to being a sensitive test of chemical-induced clastogenic changes in animal studies, is the only currently employed in vivo

test that can be used on humans exposed inadvertently to the agent in question.

The significance of visible chromosome aberrations and their genetic implication, according to Grice (1974) involves more speculation than fact. Although chromosome lesions can be heritable and are significant in the human population, the important question is whether or not their appearance serves as an indication that point mutations are also occurring. Available evidence indicates that there is a high correlation between the ability of a mutagen to produce chromosome lesions and the production of point mutations.

1. Metaphase cytogenetics

The microscopic examination of bone marrow nuclei in metaphase from rats exposed to 250 ppm ethylene oxide for three seven hour days showed a large significant increase in most types of chromosome abnormalities over control levels. The high percentage of abnormalities discovered clearly indicates that ethylene oxide is a strongly clastogenic agent.

Ethylene oxide exposure, however, did not produce a change in the number of double minutes found. This type of aberration, sometimes termed interstitial deletions (Evans and O'Riordan, 1975), has been associated with neoplastic conditions, (Lubs and Salmon, 1965; Mark, 1967; Levan et al, 1969; Zankl and Zang, 1971).

Normally, cells from a proliferating tissue system containing a radiation or chemically induced chromosome aberration are selected against, but in some cases have been found to persist for long periods of time (Puck, 1958; Bender and Gooch, 1963). The crucial period for a cell containing a chromosome abnormality occurs at cell division when three possible fates have been hypothesized (Carrano and Heddle, 1973). The aberration lacking a centremere could either 1) be lost to both daughter nuclei, 2) be split with incorporation into one or both of the nuclei or 3) be incorporated into one daughter nucleus. Available information (Sasaki and Norman, 1967) indicates that the second possibility occurs only rarely. This implies that fragment transmission is essentially an all or none process with the fragment either being lost or going to a single daughter nucleus.

2. Micronucleus Test

The micronucleus test was employed in this study to provide a dose response relation for the ability of ethylene oxide to produce clastogenic events. The information obtainable with the micronucleus test is similar to that obtained from metaphase cytogenetics except that no breakdown is available on the specific aberrations produced. Several strong alkylating agents including trenimon, mitomycin C and TEM, have been evaluated with results comparable to those obtained from metaphase cytogenetics.

Weber and Legator (1974) compared trimethylphosphate in the micronucleus test and metaphase cytogenetics and found the micronucleus to be more sensitive and to yield a response over a greater range of concentrations than the standard metaphase analysis.

The results of the micronucleus test show a clear dose response relation for four hour exposures with varying levels of ethylene oxide. Detectable changes in the number of micronuclei produced were not seen below 50 ppm. It must be kept in mind, however, that the lack of significant change below 50 ppm does not constitute a no effect level. Rather at 10 and 25 ppm the production of micronuclei is closely approaching the level of spontaneously induced micronuclei formation such that the sample sizes employed within the framework of the statistics applied cannot detect a difference.

3. Dominant-Lethal Test

A dominant-lethal mutation is defined as a genetic event that results in death to the individual which carries its heterozygously. The genetic basis for dominant-lethality is believed to be the formation of structural and numerical chromosome anomalies (Epstein, 1973). In itself, the appearance of dominant-lethal mutations is not significant in increasing the deleterious genetic load, since it is by definition self-limiting. As a screening method, however, there is good reason to believe that those agents capable of causing dominant-

lethal mutations are also capable of inducing the more serious point or gene mutations. Data comparing the effects of known mutagens in the dominant-lethal assay and the specific locus test seem to bear out this point (Grice, 1973).

In the dominant-lethal assay, a significant increase in the incidence of post-implantational early fetal deaths may be of genetic origin (Bateman and Epstein, 1971) and may indicate that damage occurred in gametes prior to fertilization (Bateman, 1966). In studies with known chemical mutagens (triethylene phosphoramide and methyltriethylenephosphoramide), early fetal deaths clearly afforded the most convenient as well as a quantitatively unequivocal parameter of mutagenicity (Epstein et al., 1970). On the other hand, pre-implantational egg losses as a measure of mutagenicity can only be taken with certain reservations.

As an indirect measure of dominant-lethality, the pre-implantation losses failed to show any significant increase in the ethylene oxide group. It has been reported by Green and Springer (1973) that the number of corpora lutea as the basis for the pre-implantation loss is extremely variable. This variability, which we also observed, could effectively mask any actual increase in pre-implantation loss with the sample size used.

The significant increase in post-implantational early

fetal deaths found in both the dead implants/pregnant female and the mutagenic index for the ethylene oxide group as compared to the negative control group gives strong evidence that ethylene oxide at 1000 ppm for four hours is able to induce dominant-lethal mutations. The timing of the dominant-lethal effect in only the first 5 weeks of the study indicates that, like most mutagens, ethylene oxide is able to induce the effect, so that it persists only in sperm stages that are exposed to ethylene oxide after meiotic division.

The significant decrease in the fertility index in the third and fourth weeks after ethylene oxide exposure is probably attributable either to a decrease in sexual vigor of the males, a spermicidal effect of the ethylene oxide or another manifestation of the mutagenic effect of the compound.

The results on the TEM positive control rats indicate that the assay system was operative and capable of picking up dominant-lethal mutations.

D. Genetic Hazard Determination

The establishment of risk to the human population associated with exposure to chemical mutagens is a difficult problem. Considering that ionizing radiation is at present the sole environmental factor that has been subjected to quantitated estimates of the risks of cancer

and mutation, radiation hazard philosophy is suggested for use as a frame of reference (Rohrborn, 1970). An ultimate aim of genetic toxicology would be to express genetic risk from various chemicals in one unit, namely the "rad-equivalent" (Bridges, 1973). This would facilitate the comparisons of risks and form a basis for realistic exposure limits.

The first effort in assigning a rad-equivalent value to ethylene oxide exposure has been done by Ehrenberg et al., (1974). Their work was based on the degree of alkylation of protein by ethylene oxide over time to establish tissue doses which were then compared to mutations produced in a barley system by both radiation and ethylene oxide. They calculated that a tissue dose of 1mM·h of ethylene oxide is comparable to 80 rad of low LET radiation. This means that workers exposed to 5 ppm ethylene oxide (0.1·TLV) would receive a weekly gonad dose amounting to about 4 rad-equivalents. The maximum permissible weekly dose of ionizing radiation to radiological workers is 0.1 rad/week.

From the work presented in this dissertation a wide estimate of rad-equivalence can be calculated. Using the dose-response relationship produced with the micronucleus test, it can be seen that a doubling of the background mutagenic rate occurs between 100 and 200 ppm·h. This is a conservative estimate. The radiation dose necessary to

double the mutagenic rate is reported to be between 10 and 100 rads and is often set at 20 rads (Casarett, 1968). Conservatively, a 200 ppm·h exposure is then equivalent to 20 rads in its ability to produce mutations, or the genetic risk is equal to 100 mrad-equivalences/ppm·h. The risk reported by Ehrenberg and his co-workers (1974) was equal to 20 mrad-equivalences/ppm·h.

It should be emphasized that with the data available, the calcualtion of risk in terms of rad-equivalents is only a gross estimate. Considering the obvious differences in the way that Ehrenberg et al. (1974) calculated his risk figures as compared to the one presented here, the values show a surprizing closeness.

At this time the estimation of risk in rad-equivalents is difficult because of a lack of standardization as to which radiation effect levels are to be used. There will be an obvious difference when comparing acute exposures to chronic ones. Other variances appear when rate of dosage is considered. It will not be possible to assign accurate risk estimates until the field of genetic toxicology moves to better define the term rad-equivalents in real values.

Although there is likely a low confidence level associated with the presented risk of 100 mrad equivalents/ppm·h, the figure does provide a preliminary starting point. Assuming that the value is approximately correct

and that chemical industry workers should be afforded the same degree of protection from mutagenic events as radiation workers, then the TLV for ethylene oxide is set too high. For equal protection the TLV would need to be set at less than 1 ppm.

Before any strong suggestion can be made, however, about what the TLV should be for ethylene oxide exposure, it will be imperative to better define the genetic risk at low dose levels under chronic exposure conditions. The dose-response relation under these conditions will be most important in setting a rad-equivalent risk estimate. It is, however, obvious that the present TLV of 50 ppm is too high.

At this time it is impossible to calculate the risk to the general population arising from the use of ethylene oxide sterilized consumer goods. An unknown quantity also exists for those people undergoing medical treatment with ethylene oxide sterilized surgical material.

It should be safe to assume that the total dose of ethylene oxide received would be quite low in these instances and that the extra degree of risk associated with any exposure to ethylene oxide would be acceptable in light of the benefits. There is, however, every reason to believe that the mutagenic effects of ethylene oxide would be additive to those produced by other widespread environmental mutagens and that the total body burden of mutagens should be controlled.

SUMMARY

This work has shown ethylene oxide to be mutagenic in bacterial and mammalian somatic and germinal cell systems. The mammalian work using rats exposed to various levels of ethylene oxide gas showed a significant increase in chromosomal aberrations and dominant lethal mutations. The micronucleus test was used to establish a dose-response relation between the exposure to ethylene oxide and production of micronuclei. These results were then used to assign a risk presented in terms of rad-equivalents which indicated that the TLV for ethylene oxide of 50 ppm is too high to afford ethylene oxide workers the same degree of protection as that available to radiation workers. Further evaluation is needed before a reasonable TLV can be formulated.

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Appendix 1. Bacterial Plate Assay Solutions and Media

VB Salts (Vogel and Bonner, 1956)

warm distilled H_2O (45°C)	670 ml
magnesium sulfate ($MgSO_4$ $7H_2O$)	10 g
citric acid monohydrate	100 g
potassium phosphate dibasic, anhydrous (K_2HPO_4)	500 g
sodium ammonium phosphate ($NaNH_4HPO_4$ $4H_2O$)	175 g
	1000 ml

Add salts in the order given. Allow each salt to dissolve completely before adding the next. When the salts are all dissolved, allow the solution to cool to room temperature. Add 5 ml chloroform per liter of solution and store at room termperature.

Nutrient Broth

Bacto Nutrient Broth (Difco)	0.8 g
sodium chloride	0.5 g
distilled water	100 ml

Dissolve the ingredients and dispense into capped closure tubes. Autoclave and store at room temperature.

Minimal Glucose Agar Plates

A.	VB salts	20 ml
	distilled water	500 ml
B.	agar (Difco)	15 g
	distilled water	500 ml
c.	40% glucose solution	50 ml

Autoclave solutions A, B and C in separate containers While still hot, mix together and pour plates (30 ml/plate).

Top Agar

agar (Difco)	0.6 g
sodium chloride	0.5 g
distilled water	100 ml

Dissolve and autoclave. Maintain at 45°C. Add 10 ml/ 100 ml histidine-biotin solution.

Appendix 1 (Continued)

Histidine-Biotin Solution

L-histidine (0.5 mM) and biotin (0.5mM) in distilled water. Sterilize by filtration.

Appendix 2. Biological Stains

Giemsa Stain

Giemsa dye, 1 g, mixed in 66 ml of glycerin. Heat to 60°C for 2 hours followed by cooling. Add 66 ml of methanol, stir for 24 hours and filter.

May-Gruenwald Stain

Mix 0.3 g of May-Gruenwald dye in 100 ml of methanol. Warm to 50°C then cool to ambient temperature. Let stand with occasional shaking for 24 hours. Filter.