

VINYL CHLORIDE MUTAGENICITY AND CARCINOGENICITY VIA THE METABOLITES  
CHLOROOXIRANE AND CHLOROACETALDEHYDE MONOMER HYDRATE.

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SUMMARY

Mutagenicity tester strains of Bacillus and Salmonella were used to assay vinyl chloride in nutrient broth at a practical concentration level. Also screened without exogenous activation were seven potential metabolites of vinyl chloride in their pure forms as well as the related epichlorohydrin. Chlorooxirane, chloroacetaldehyde, chloroacetaldehyde monomer hydrate, chloroacetaldehyde dimer hydrate, chloroacetaldehyde trimer, and epichlorohydrin produced significant mutagenic activity in Salmonella typhimurium strains sensitive to base-pair mutation. A recombination repair deficient strain of Bacillus subtilis was inhibited in growth by these compounds, whereas excision repair deficient and wild type strains of Bacillus subtilis were relatively unaffected. On the basis of these assays a working hypothesis for the vinyl chloride carcinogenesis mechanism is proposed which involves chlorooxirane and chloroacetaldehyde monomer hydrate as the ultimate carcinogenic metabolites of vinyl chloride.

## INTRODUCTION

The carcinogenic potential of vinyl chloride monomer 1 was initially established by Viola et al. [1] and Maltoni et al. [2] with inhalation experiments using laboratory animals. Detection of angiosarcoma in polyvinyl chloride workers suggested a causal relationship between industrial exposure to vinyl chloride and the development of pathological conditions in humans. This contention was supported by epidemiological data which revealed an association between exposure to 1 and the onset of hepatic abnormalities including angiosarcoma [3,4]. A report by Hefner et al. [5] on the metabolic fate of 1 in rats indicated that 67% of the vinyl chloride inhaled by the rats was metabolized and excreted in the urine. The metabolic products identified were N-acetyl-S-(2-hydroxyethyl)cysteine and thiodiglycolic acid [5,6] which are sulfhydryl conjugates of chloroethanol 2 and chloroacetic acid 3 respectively. Chlorooxirane 4 and chloroacetaldehyde 5 were speculated to be the carcinogenic forms. We report herein the mutagenicity and carcinogenic potential of these compounds in their pure forms.

Several reports [7,8,9] have appeared recently using the Salmonella tester strains to test vinyl chloride and several of its supposed metabolic derivatives. Vinyl chloride and chloroethanol were found to be mutagenic after activation by liver homogenates [9]. Direct mutagenicity of vinyl chloride was also reported by McCann et al. [7] and Bartsch et al. [10] at 20% v/v in air (200,000 ppm). Since the solubility of vinyl chloride in water at 25°C and 1 atm has been determined to be  $7.79 \times 10^{-4}$  mole fraction [11] or 2,900 ppm, we have conducted further testing at this concentration level to secure a practical

dose-response comparison with its proximate metabolites. Regarding the latter, the exact chemical forms of the proximate metabolites previously tested are often questionable. Chloroacetaldehyde, like formaldehyde [12], dichloroacetaldehyde [13], and chloral [14], can exist in combinations of four forms depending on the history of sample preparations: the monomer  $\tilde{5}$ , the monomer hydrate  $\tilde{6}$ , the dimer hydrate  $\tilde{7}$ , and the trimer  $\tilde{8}$ . McCann et al. [7] used vacuum distilled chloroacetaldehyde without a follow-up analysis of its content. This distillate may have consisted of chloroacetaldehyde monomer  $\tilde{5}$  and its cyclic trimer  $\tilde{8}$  if water was totally absent, or it may have been a mixture of chloroacetaldehyde hydrates  $\tilde{6}$  and  $\tilde{7}$  in an aqueous medium. Bartsch et al. [10] tested a commercial aqueous chloroacetaldehyde solution which, according to our analysis reported herein, had an acidic pH and approximately equal concentrations of the two hydrates  $\tilde{6}$  and  $\tilde{7}$ . This solution was also contaminated by ethanol to the extent of 10%. We have therefore conducted individual assays of pure compounds, or assays of a known mixture of the specific forms of chloroacetaldehyde. Furthermore, the mutagenicity observed for chlorooxirane  $\tilde{4}$  [9] may be attributed to a chloroacetaldehyde hydrate rather than the chlorooxirane integrity. Under the 37°C aqueous testing conditions reported, chlorooxirane decomposed with a half life of 1.6 min [10] to chloroacetaldehyde. For this reason we have also screened epichlorohydrin  $\tilde{9}$  which is a stable chloro-epoxide homolog of  $\tilde{4}$  as a comparative assay to interpret the observations of the activity of chlorooxirane.

This investigation used the above-mentioned compounds  $\tilde{1} - \tilde{9}$  in mutagen assays without exogenous enzyme activation. A preliminary screen was performed using DNA repair-deficient mutants of Bacillus subtilis. This was followed by quantitative testing of the compounds for mutagenicity

with Salmonella typhimurium LT-2 strains [15]. Thus, a combination of these two screening procedures have led to information on the mutagenicity and carcinogenic potential of these compounds as well as their chemical mode of action.

#### MATERIALS AND METHODS

The bacterial strains used in the bioassays are shown in Table I. The Salmonella tester strains were designed to detect chemical carcinogens as mutagens [15]. The recombination and DNA repair-deficient Bacillus subtilis strains were used in the repair assays as an indirect test for mutagenicity [16,17]. Nutrient broth (Difco) and nutrient broth plus 0.5% NaCl was used for growth of stock cultures of Bacillus and Salmonella strains respectively. Nutrient agar (Difco) served as a solid medium for the growth of Bacillus strains in "repair-assays". The pour plates used with Salmonella strains consisted of molten (45°C) soft agar which contained 0.6% agar, 0.6% NaCl, 0.5mM biotin, and 0.5 mM histidine. The minimal agar plate was composed of Vogel-Bonner E medium [18], 1.5% agar, and 2% glucose.

Vinyl chloride gas was obtained from Matheson Scientific; aqueous chloroacetaldehyde (45% by wt.) from ICN Pharmaceuticals; epichlorohydrin from Matheson Coleman and Bell; and other chemicals from Aldrich Chemical Co.

#### Mutagenicity Assays

Salmonella - Vinyl chloride  $\overset{\sim}{1}$  was tested by the method of Ames [7]. A mixture of 0.1 ml of  $\overset{\sim}{1}$  in broth and 2 ml of top agar was added to 0.1 ml of cell culture. The solutions were then mixed and poured immediately onto the surface of a minimal medium plate. After incubation for 48 hrs at 25°C, colonies were counted and recorded. For compounds  $\overset{\sim}{2}$  -  $\overset{\sim}{9}$ , sample solutions of known concentrations

were prepared in dimethyl sulfoxide (DMSO). A 0.1 ml aliquot of the sample solution was admixed with 0.9 ml of the tester strain culture. Then, a 0.1 ml sample of this mixture was added to 2 ml of molten soft agar and applied to the surface of a minimal agar plate. Control plates for detection of the spontaneous reversion rates were prepared for each tester strain by omitting only the compounds tested. Pour plates were incubated for 48 hr at 37°C before revertant colonies to prototrophy were counted. For positive mutagenesis control, plates containing the mutagen 4-nitroquinoline-N-oxide were used.

Bacillus - The "repair-assay" procedure was a modification of the "rec-assay" procedure of Kada et al. [19]. They were grown overnight in nutrient broth then diluted 10 fold in phosphate buffer (pH 7.0). Strains were streaked with pipettes onto nutrient agar plates. Filter paper discs (6 mm) saturated with test solution were placed upon the bacteria streaks. Following incubation for 24 hr at 37°C, growing bacteria were visible except in the inhibition zone. The lethality and mutagenic potential of compounds were assessed by comparison of inhibition zones between the 168 wild type strain and the DNA repair-deficient strain and the DNA repair-deficient strains. The control used was 4-nitroquinoline-N-oxide. Survival assays for  $\tilde{6}$  and  $\tilde{7}$  (cf. Fig. 3.) were made in MY-1 broth [17] solutions. Cultures were grown in tryptose blood agar base for 16 hrs, inoculated into MY-1 broth, and viable cell counts were done on TBAB agar plates.

#### Compound Synthesis and Purification

Chlorooxirane  $\tilde{4}$  prepared by the method of Walling and Frederick [20] was in higher purity (95% pure) than that by molecular chlorination [21] (50% pure). Thus, t-butyl hypochlorite and ethylene oxide at -10°C with 200 watt tungsten lamp irradiation yielded chlorooxirane  $\tilde{4}$ ; glpc (gas liquid phase chromatography)

$t_R = 1.2$  min at  $50^\circ\text{C}$ , infrared absorption ( $\nu\text{ cm}^{-1}$ ) 900, 1250, 1320, and 1710 as described previously [21], and PMR as shown in Table II. Derivatization of 4 with an excess of acidic 2,4-dinitrophenylhydrazine solution gave glyoxal-bis-dinitrophenylhydrazone, mp  $317\text{--}318^\circ\text{C}$  ( $317^\circ\text{C}$  reported [21]).

Chloroacetaldehyde monomer 5 was obtained in the purest form by cracking the chloroacetaldehyde trimer 8 at  $95^\circ\text{C}$  and distilling it into dry DMSO; glpc  $t_R = 2.25$  min at  $100^\circ\text{C}$  and PMR as shown in Table II.

Chloroacetaldehyde dimer hydrate 7 - A solution containing 49.5 ml of 38% hydrochloric acid, 30 ml of the 45% aqueous chloroacetaldehyde solution, and 55.5 ml of water was distilled over a 9 inch Vigreux fractionating column. The fraction (1/5 of the initial volume) collected from  $87\text{--}100^\circ\text{C}$  was redistilled. This second distillate at  $83\text{--}92^\circ\text{C}$  crystallized after 3 days at  $-15^\circ\text{C}$ . Upon sublimation of  $60^\circ\text{C}$  and 1 atm, white crystals of 7 were obtained; mp  $55^\circ\text{C}$  and PMR as shown in Table II.

Chloroacetaldehyde trimer 8 - Concentrated sulphuric acid (7.5 ml) was added to the 45% aqueous chloroacetaldehyde solution (5 ml) with vigorous stirring and external cooling ( $-5^\circ\text{C}$ ). The crystalline precipitate was filtered after standing overnight at  $-15^\circ\text{C}$ , washed with 5 ml of cold 20% aqueous methanol, and recrystallized 5 times from cold methanol; mp  $87\text{--}88^\circ\text{C}$ , corresponding to that reported by Natterer [13], and PMR as shown in Table II.

Quantitation of vinyl chloride in nutrient broth - The concentration of vinyl chloride 1 in the broth solution was determined by an extraction method in conjunction with glpc. This method involved (1) establishing the

linearity of the response of  $\lambda$  on glpc, (2) constructing a standard curve of vinyl chloride weight vs. peak area, and (3) extracting the broth with methylene chloride followed by glpc determination. (1) Linearity of response - A standard solution of  $\lambda$  was prepared by condensing it (bp  $-13.4^{\circ}\text{C}$ ) at  $-78^{\circ}\text{C}$  onto a known weight of methylene chloride in a 1 ml volumetric flask fitted with a serum cap. The condensed vinyl chloride was determined by weighing. A typical solution thus prepared was 0.2877 M and was subjected to glpc analysis by varying injection sizes from 1-9  $\mu\text{l}$ . The correlation of vinyl chloride weight and peak area was made by a least square computer routine: slope = 0.877, with an index of correlation of 0.972 (ideal 1.00) up to 6  $\mu\text{l}$  (0.1078 mg) of injection. (2) A standard linear curve using the above technique was established for 0.01-0.10 mg of vinyl chloride. (3) Extraction of broth - Vinyl chloride was allowed to bubble through the nutrient broth for 30 min at  $25^{\circ}\text{C}$ . A 1.0 ml broth sample was then extracted with 4 x 2 ml of methylene chloride; the extracts were combined and then made up to 10 ml in a volumetric flask with methylene chloride. Glpc analysis of this solution and application of the standard curve showed that there was 7.0 mg of  $\lambda$  in the 10 ml solution, or the concentration of  $\lambda$  in the broth was 0.0107 M. Repeated determination showed it to be 0.0105 M.

High Pressure Liquid Chromatographic (HPLC) Analysis of the Commercial 45% Aqueous Chloroacetaldehyde - Reverse phase HPLC on the commercial 45% chloroacetaldehyde solution (7 M, pH 2.6) resolved it into two components:  $t_{R1} = 6.3$  min and  $t_{R2} = 8.8$  min in a ratio of 40:60. The ratios of the two peaks on the chromatogram changed as the solution pH was varied by addition of 1 N NaOH at room temperature: 43:57 (pH 3.7), 44:56 (pH 5.0), 48:53 (pH 7.6),

and 56:44 (pH 8.2). The first eluted component at  $t_{R1} = 6.3$  min increased while the second at  $t_{R2} = 8.8$  min decreased at raised pH's. This indicated a retrograde aldol condensation type hydrolysis. The first peak was assigned to the monomer hydrate  $\tilde{6}$  and the second to the dimer hydrate  $\tilde{7}$ . When the 45% solution was diluted to 0.44 M at pH 3.6, refluxed for 1 hr, cooled, and chromatographed, the ratio of  $t_{R1}:t_{R2}$  became 33:67. This suggests an acid-catalyzed condensation of  $\tilde{6}$  to form the dimer hydrate  $\tilde{7}$ . When these two components were analyzed by glpc, both emerged at the same retention time ( $t_R = 2.8$  min at 60°C) as that of the monomer  $\tilde{5}$ . Dehydration of  $\tilde{6}$  and  $\tilde{7}$  must have occurred under the glpc conditions thereby reverting them to the monomer form. As shown in Table II, the PMR spectra of  $\tilde{6}$  and  $\tilde{7}$  are resolved from one another. Thus, the PMR spectrum of the 45% commercial solution also revealed the presence of both hydrates  $\tilde{6}$  and  $\tilde{7}$  with approximately the same integrals.

#### Instrumentation

High Pressure Liquid Chromatography (HPLC) - A Waters Associates model 600 pump combination (dual) with a model 440 differential 254 nm ultraviolet detector were used for the analysis of aqueous chloroacetaldehyde solutions. A Porasil Bondapak  $\mu C_{18}$  4 mm x 30 cm column was used with a 80:20 v/v 0.1 N  $NH_4H_2PO_4$ -MeOH isocratic eluant (pH 4.9) at 1 ml/min. These two components were also resolved on a Reeve Angel Partisil 10 ODS 4.6 mm x 25 cm column using the same eluant.

Gas Liquid Phase Chromatography (GLPC) - Analysis of vinyl chloride 1, chlorooxirane-4, and chloroacetaldehyde 5 were performed on a Carle model 9500 flame ionization gas chromatograph. A 10% SE-30 on Anakron 60/70 packed column was used at 30°C for vinyl chloride determination. A 20% Carbowax on chromosorb W column at 50°C and 40 ml/min of Helium was used for analysis of 4 and 5 unless specified otherwise. Both were 0.125 inch x 5 feet stainless steel columns.

Proton Magnetic Resonance (PMR) - Spectra were obtained using a Varian A-60 A and a Perkin Elmer R-12 spectrometer. Solutions of D<sub>2</sub>O and DMSO-d<sub>6</sub> were used with 3-(trimethylsilyl)propanesulfonic acid sodium salt as internal reference. Tetramethylsilane was used as a reference in CDCl<sub>3</sub> and CCl<sub>4</sub>. Probe temperature was 38 °C.

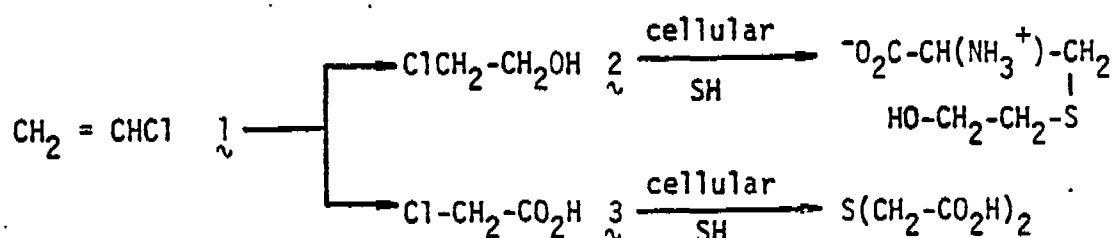
## RESULTS AND DISCUSSION

Mutagenicity assays with Bacillus and Salmonella for compounds 1-9 are summarized in Table III. They are grouped into three categories in subsequent discussion. Further testing data are included in Tables IV-VI and Figures 2 - 4.

Nonmutagenicity of vinyl chloride, chloroethanol, and chloroacetic acid - Only high concentrations of vinyl chloride (20% v/v in air) have produced mutagenic action in previous assays with the Salmonella tester strains [9,10]. We have found that tests with both the Salmonella and the Bacillus cultures were negative within the practical solubility range of vinyl chloride in the nutrient broth under ambient conditions. Figure 1 shows the stability of a

presaturated vinyl chloride broth solution at 25°C and 1 atm. The initial concentration of 0.022 M of vinyl chloride 1 rapidly decayed by 50% in 15 hr. Thereafter the escape of 1 from the broth slowed considerably. In the next 45 hr there was a further decline of only 18%. Thus the bacteria strains (8, subtilis MC-1 and Salmonella JA 100) were exposed to the stabilized solution of 0.0106 M (723 ppm) of vinyl chloride in the nutrient broth. The negative activity observed was not unexpected since vinyl chloride lacks the electrophilic character common to many mutagens [23]. Although the direct mutagenicity of vinyl chloride at 200,000 ppm (20% v/v in air) observed previously [10] may be real, the chronic human exposure problem most likely requires metabolic activation of vinyl chloride to an electrophilic reactive form [8]. Also tested were chloroethanol 2 and chloroacetic acid 3 which probably are metabolic intermediates as shown in Scheme I. Both S-(2-hydroxyethyl)cysteine and

SCHEME I: Vinyl Chloride Metabolites



thiodiglycolic acid in Scheme I have been identified [6] as the urinary metabolites of vinyl chloride. Neither chloroethanol 2 nor chloroacetic acid 3 exhibited any mutagenic effects at 1 mM concentration in our mutagenicity assays (cf. Fig. 2 ). Although Bartsch et al, [10] found considerable mutagenic

activity with chloroethanol 2 for TA 1530 strain in the absence of microsomal activation, our observations corroborate with those of McCann et al. [7] who showed that 2 was weakly mutagenic directly even at high concentrations for the sensitive TA 100 and that it showed only trace activity for TA 1535. The enhanced activity of 2 after microsomal activation observed by these groups suggests that chloroacetaldehyde derivatives were being formed from chloroethanol. We have found potent mutagenicity and lethality with the various forms of chloroacetaldehyde as shown in Figures 2 - 4 and Tables IV-V.

Mutagenesis of chloroacetaldehyde, the monomer hydrate, dimer hydrate, and the trimer - When chloroacetaldehyde 5 was distilled into distilled water, a mixture of the monomer hydrate 6 and the dimer hydrate 7 was formed instantly as determined by HPLC and PMR spectroscopy. Analysis of the commercial 45% aqueous chloroacetaldehyde solution with the same techniques showed a 50:50 mixture of the two hydrates. Upon standing under dry conditions, the monomer 5 cyclized to form trimer 8. The trimer was sparingly soluble in water, but was disproportionated upon heating in water to form the hydrates 6 and 7.

Purified samples of chloroacetaldehyde 5, the commercial 45% chloroacetaldehyde solution containing a 50:50 mixture of the hydrates 6 and 7, the dimer hydrate 7, and the trimer 8, in DMSO solutions were tested for mutagenic potential. The data in Table IV summarizes the results of the repair assays with B. subtilis. Chloroacetaldehyde 5 and the monomer hydrate 6 specifically inhibited the growth of B. subtilis MC-1, a mutant lacking recombination repair of DNA. These unrepaired DNA lesions then led to cell death. However, compounds 5 and 6 did not inhibit the wild type B. subtilis or those mutant

strains (Hcr-9, FB-13) having the capacity for recombination repair. In contrast, purified samples of the dimer hydrate 7 and trimer 8 inhibited all of the mutants as well as the wild type, although MC-1 again demonstrated the most sensitivity. It is unlikely that 7 and 8 caused DNA lesions that are different from those by 5 and 6 because the excision repair mutants and the wild type were inhibited to an equivalent extent. Inhibition of these strains by 7 and 8 may result from a metabolic poisoning or cellular damage similar to that observed in Escherichia coli after exposure to vinyl chloride waste [24].

Viability assays on B. subtilis strains during short term exposure to the chloroacetaldehyde 5 revealed that recombination repair was essential for recovery (Figure 2). The B. subtilis strains with recombination repair capabilities displayed repair kinetics as evidenced by the shoulders in curves in Figure 3. The sensitive MC-1 strain lacking recombination repair yet having excision repair was rapidly killed following the exposure. We surmise that these DNA lesions caused by the chloroacetaldehydes were repaired solely by the recombination repair mechanism.

The direct mutagenic potential of the samples 4-9 was determined by the mutations expressed in Salmonella strain TA 100. The dose response relationships of these compounds (cf. Table V), and the dose-response curves (cf. Figure 4) were determined with strain TA 100. At high concentrations the dose response curves for all compounds became nonlinear because the toxicity of the chemicals reduced the number of potential revertants on the plates. The reduction of the cell population by high concentrations was confirmed by viable counts and observations of decreased background lawn on the test plates.

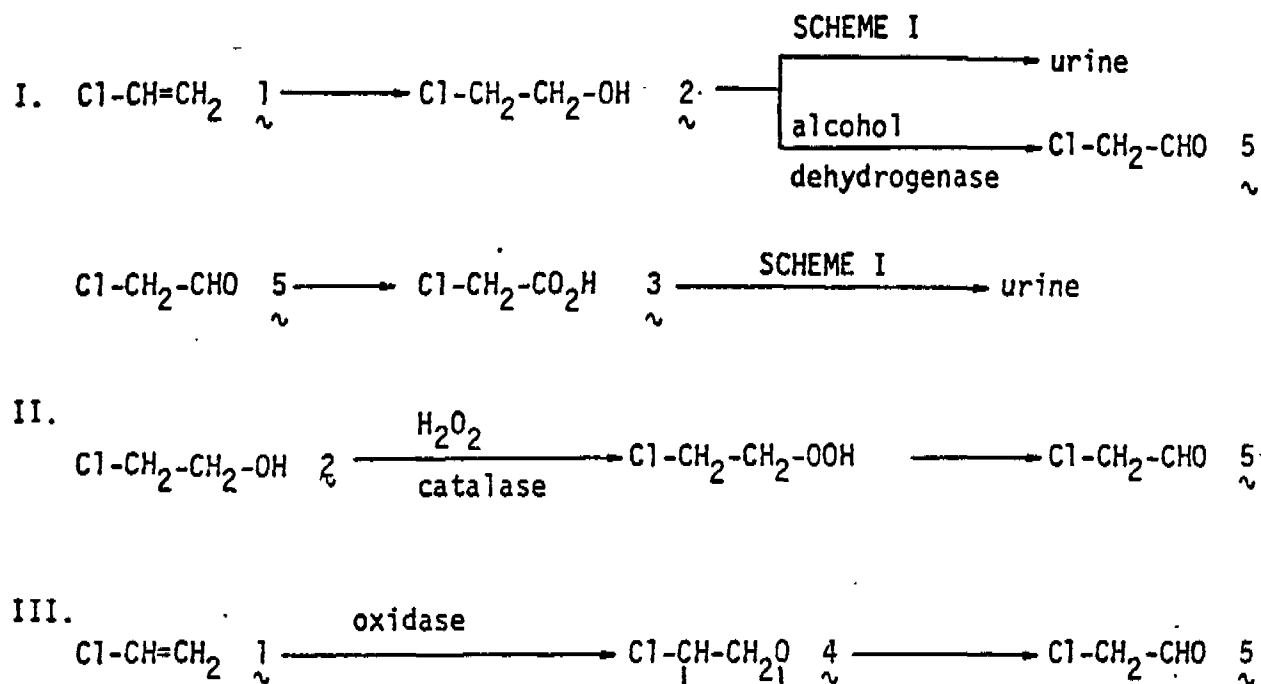
Data from the dose response curves (Figure 4) showed that chloroacetaldehyde 5 and the monomer hydrate 6 were more active than 7 and 8. The monomer 5 was the most mutagenic as predicted because its electrophilic carbonyl group remains intact. The restoration of the carbonyl character to the monomer hydrate 6 can be projected in hydrophobic environment of the cell membrane by loss of water, hence its mutagenicity is also explicable. The substantial activity of the dimer hydrate 7 can be attributed to its ready equilibrium with the monomer hydrate 6 in aqueous medium. The relationship of the activity of the cyclic trimer 8 to the chloroacetaldehyde action cannot be deduced on the basis of the curves in Figure 4. Nevertheless, these mutations were the base-substitution type and were expressed in Salmonella strains TA 100 and TA 1535. The mutagenic response in TA 1535 was very weak as compared to TA 100. It should be noted that the latter contains an "R" factor that enhances mutation by an error-prone recombination repair mechanism following DNA damage [7].

Comparison of mutagenic response of chlorooxirane with its methylene homolog epichlorohydrin - The prevailing opinion [5] is that chlorooxirane 4 is the primary metabolite of vinyl chloride and is derived from the action of the microsomal mixed function oxidase. This compound, prepared independently from chlorination of ethylene oxide, was found to rearrange readily to chloroacetaldehyde in aqueous or DMSO solution at ambient temperatures. A kinetic study of a 0.15 M solution of 4 in a D<sub>2</sub>O-DMSO-d<sub>6</sub> (80:20) mixture at pH 7.1 and 4°C by PMR technique showed that the rearrangement followed first order kinetics,  $k \text{ (sec}^{-1}\text{)} = 2.5 \times 10^{-4}$ . This translates to a half life of 46.2 min

at 4°C as compared to 1.6 min at 37°C [9]. Such instability allows only limited testing in the cold as well as interpretation of the results. On the other hand, epichlorohydrin 9, which can be considered the epoxidation metabolite of allyl chloride, is a methylene homolog of 4. Both 4 and 9 are structurally bis-alkylating agents, hence comparing their mutagenic activities will lend insight into the mode of action of chlorooxirane [25,26]. Table IV tabulates the mutagenic response of these two compounds in the Bacillus repair assay and Table VI the Salmonella TA 100 reversion. Due to the instability of chlorooxirane at 37°C, the assays shown in Table VI were preincubated at 3°C in solution with the Salmonella TA 100 strain before plating to insure that chlorooxirane could reach the cells intact. Mutant strains of B. subtilis were not inhibited when exposed to high concentrations of epichlorohydrin 9. In contrast, chlorooxirane 4 selectively inhibited the rec<sup>-</sup> strain MC-1 in a manner similar to chloroacetaldehyde hydrate 6. Tests with Salmonella showed that strain TA 100 was very susceptible to the mutagenic action of both epoxides 4 and 9. However 9 was less toxic to the tester strains than 4. The low toxic effects of 9 indicate a different type of DNA lesion compared to that caused by chlorooxirane 4. It is possible that 9 may react with DNA by a mechanism which does not cause potential lethal strand-scissions. On the other hand, chlorooxirane 4 may act on the bacteria via a NIH shift [27,28] to form chloroacetaldehyde 5 or 6. Conceivably, chlorooxirane can also behave as a diradical intermediate rather than a conventional  $S_N1$  or  $S_N2$  type alkylating agent in its reaction with DNA.

Vinyl chloride carcinogenesis mechanism hypothesis - Among the compounds tested in the metabolic Scheme II, chloroacetaldehyde 5 and chlorooxirane 4

## SCHEME II: The metabolic pathways of vinyl chloride [5].



were the most mutagenic with the lowest toxic side effects. Hence, they may qualify to be the active carcinogenic derivatives of vinyl chloride. Considering the aqueous milieu of the metabolic environment, however, the chloroacetaldehyde monomer hydrate 6 is a more realistic choice as an ultimate carcinogen than the monomer 5 which reacts immediately with water. Viability assays following exposure of the *B. subtilis* mutants to 6 indicated that the compound induced recombination repair. The strain with the  $\text{rec}^-$  phenotype (MC-1) was immediately inactivated. All the  $\text{rec}^+$  strains showed survival ability and repair kinetics of similar nature. It has been shown [27] that mammalian cells have postreplication repair of DNA which has many similar features to the recombination repair in bacteria. In addition, a relationship between this mammalian postreplication repair process and mutation as well as carcinogenesis

has been suggested [24]. Cells from patients with the skin disease xeroderma pigmentosum lack the ability to excise pyrimidine dimers and must rely on postreplication repair to remove these lesions [24]. It is believed that this error prone process of postreplication repair is responsible for the production of somatic mutations and cancer in patients with this disease. Since chloroacetaldehyde monomer hydrate 6 induces recombination repair in bacteria responding to lesions, it may also be capable of activating the error prone postreplication repair in exposed mammalian cells. At the molecular level, chloroacetaldehyde is known to react with N<sup>1</sup> and N<sup>6</sup> nitrogens of adenosine and N<sup>3</sup> and N<sup>4</sup> nitrogens of cytidine in single-stranded DNA [28,29]. It therefore appears that chloroacetaldehyde monomer hydrate 6 should merit our consideration as an ultimate carcinogenic metabolite of vinyl chloride.

The lower mutagenic activity of chlorooxirane 4 compared to 6 may reflect the unstable nature of chlorooxirane as an  $\alpha$ -chloroether. While the carcinogenic chloromethyl methyl ether is a bifunctional alkylating agent [30], the mutagenic activity of chlorooxirane cannot be so categorized, especially when it is compared with epichlorohydrin 9. One mode of action of chlorooxirane is a rearrangement to chloroacetaldehyde via the NIH shift [27,28]. Another is a homolytic ring cleavage to yield a stabilized diradical intermediate  $\text{Cl}\dot{\text{C}}\text{H}-\text{CH}_2\dot{\text{O}}$ . Both are capable of reacting with DNA, thereby accounting for the mutagenicity of 4. In mammalian cells, chlorooxirane, being a reactive epoxide, could be trapped by a glutathione epoxide transferase [31] at a faster rate than the detoxification of chloroacetaldehyde via the less active aldehyde dehydrogenase [32]. Such detoxification of chlorooxirane could

explain the reported decrease in sulfhydryl level in liver cells during metabolism of short chain halo hydrocarbons including vinyl chloride [31]. Perhaps the lower mutagenicity of chlorooxirane 4 in these bacterial assays, compared to the chloroacetaldehydes, is also attributable to its being detoxified faster. We therefore consider both chlorooxirane 4 and the chloroacetaldehyde monomer hydrate 6 to be the ultimate carcinogenic metabolites of vinyl chloride. Their reactions with DNA causing mutations in Bacillus and Salmonella suggest a causal relationship with vinyl chloride carcinogenesis in human and laboratory animals.

#### Acknowledgements

This work was supported by grants from the B. F. Goodrich Co. This grant program was initiated and administered by the Cancer Center of the University of Louisville. The Salmonella tester strains were kindly provided by Dr. B. N. Ames of the University of California, Berkeley. We also thank George D. Stratton, Jr. and S. E. Yen for their able assistance.

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## LEGENDS TO FIGURES

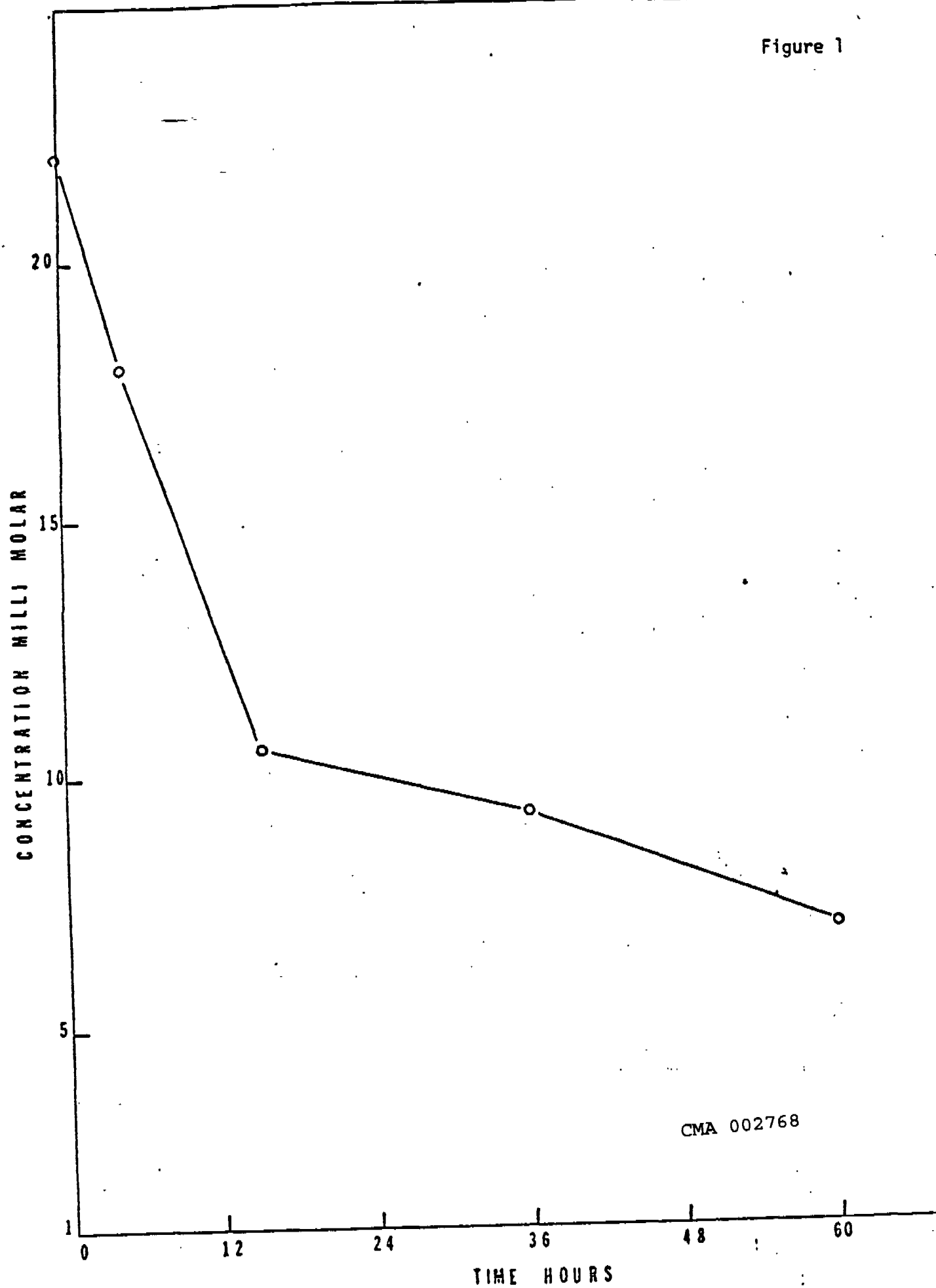
Fig. 1. Stability of vinyl chloride in nutrient broth at 25°C, 1 atm.

Fig. 2. Survival of *Bacillus subtilis* MC-1 after incubation with: 1.0 mM chloroethanol 2 (+-+); 1.0 mM chloroacetic acid 3 (□-□); 5.76 mM (concentration based on  $\text{ClH}_2\text{CCHO}$ ) chloroacetaldehyde (45% aqueous solution) 6 and 7 (▷-▷); 0.1 mM 4-nitroquinoline-N-oxide (◇-◇); and untreated control cells (○-○). Mid-logarithmic cultures grown in MY-1 broth were incubated with compounds at 37°C. Samples were plated at the times indicated from which viable cell counts were recorded. The mutagen 4-nitroquinoline-N-oxide served as a positive mutagenicity control.

Fig. 3. Survival of *Bacillus subtilis* strains in the presence of 5.0 mM (concentration based on  $\text{ClH}_2\text{CCHO}$ ) chloroacetaldehyde (45% aqueous solution) 6 and 7. Cultures were grown to mid-logarithmic phase in MY-1 broth and then incubated with the chloroacetaldehyde solution at 37°C. Samples were plated at the times indicated from which the percent survival was determined. FB-13 *uvr*<sup>-</sup>, *rec*<sup>+</sup> (+-+); Hcr-9 *hcr*<sup>-</sup>, *rec*<sup>+</sup> (◇-◇); 168M wild type (○-○); and MC-1 *uvr*<sup>+</sup>, *rec*<sup>-</sup> (▷-▷).

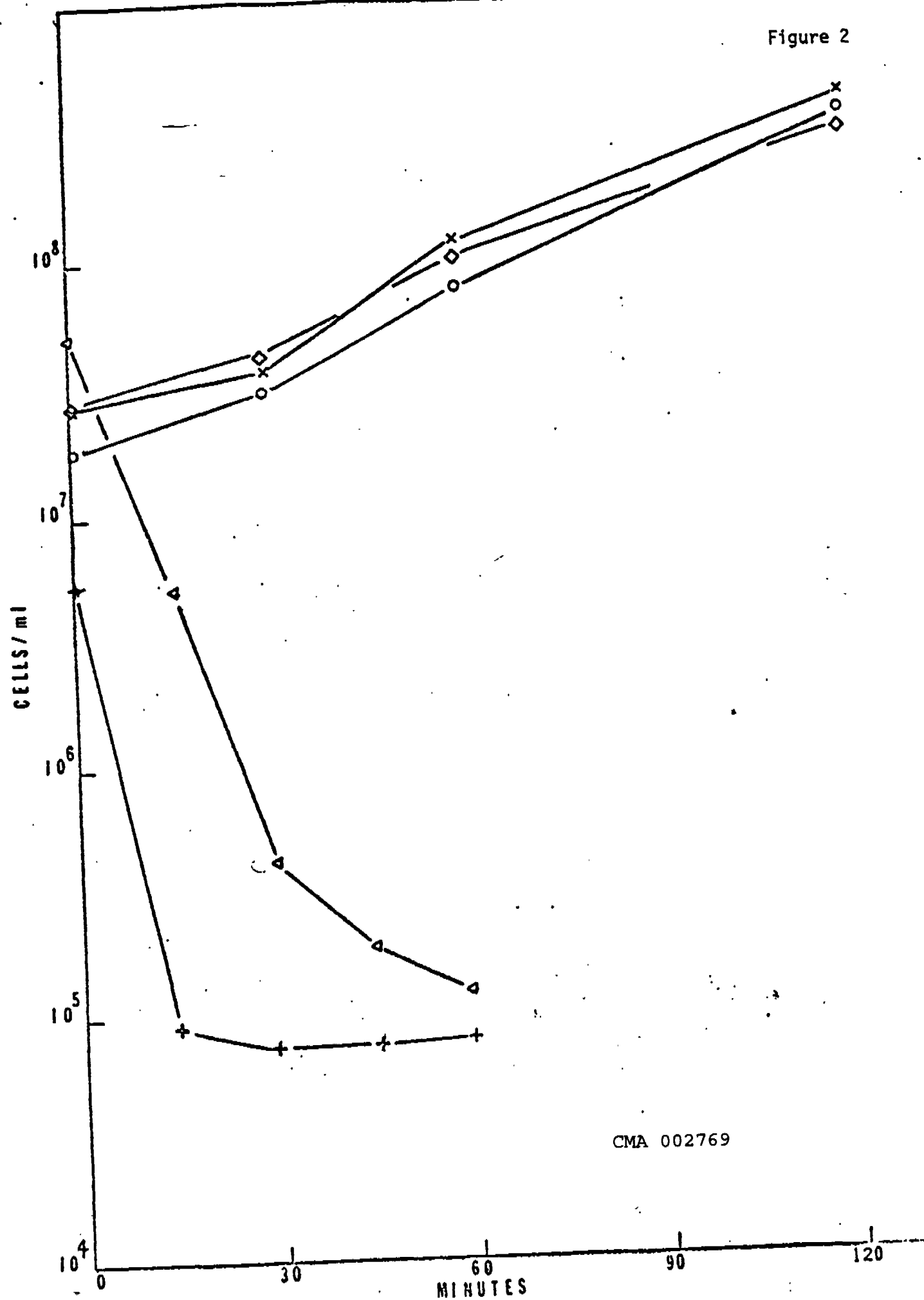
Fig. 4. Dose response curves with *Salmonella typhimurium* TA100. Sample solutions of known concentrations prepared in DMSO were mixed with tester strain culture and soft agar. Plates were poured, incubated at 37°C for 48 hrs, and then scored for revertant colonies to prototrophy. Chloroacetaldehyde monomer 5 (○-○); chloroacetaldehyde (45% aqueous solution-concentration based on  $\text{ClH}_2\text{CCHO}$ ) 6 and 7 (◇-◇); chloroacetaldehyde dimer hydrate 7 (▷-▷); chloroacetaldehyde trimer 8 (x-x); and epichlorohydrin 9 (□-□).

Figure 1



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Figure 2



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Figure 3

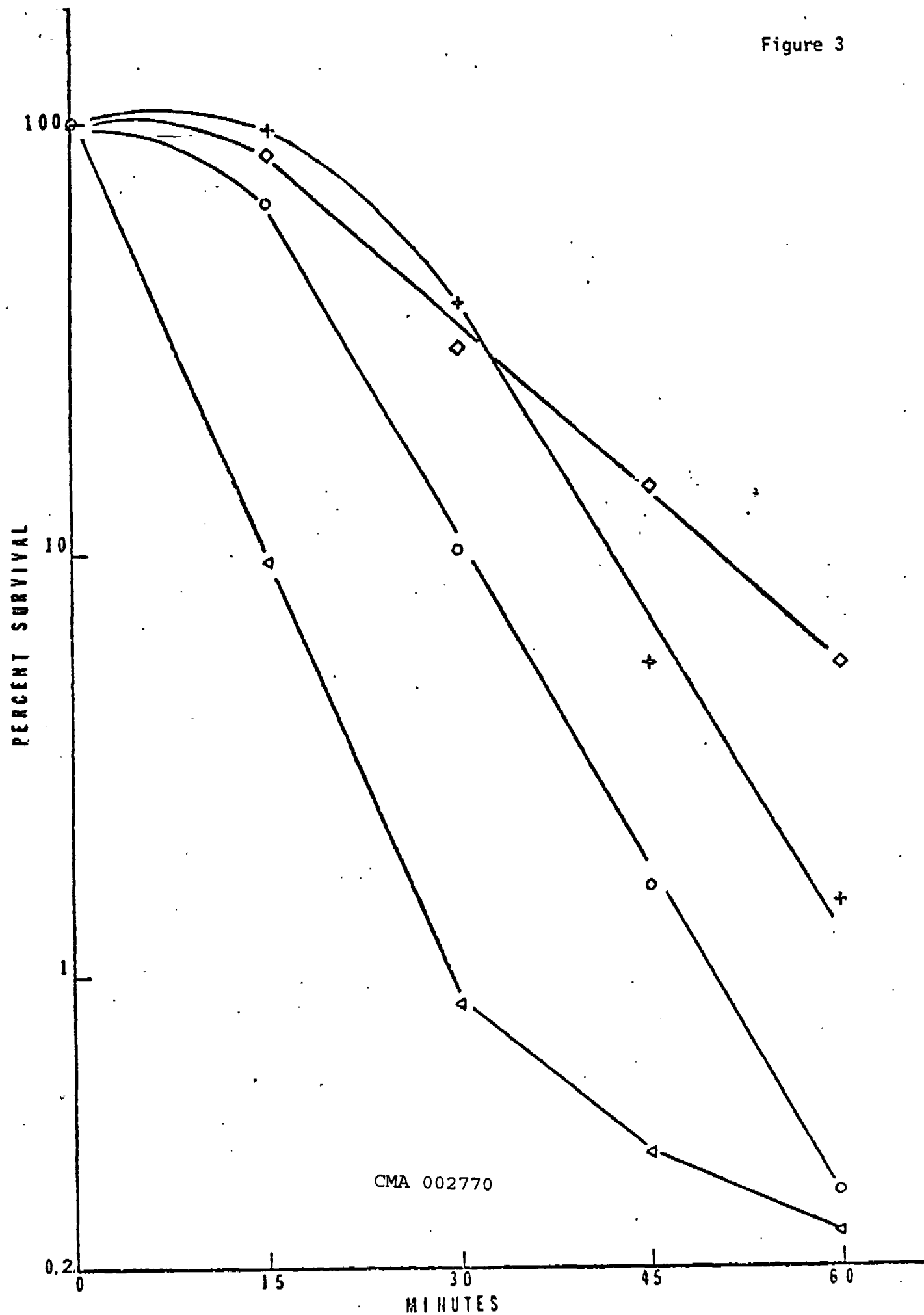


Figure 4

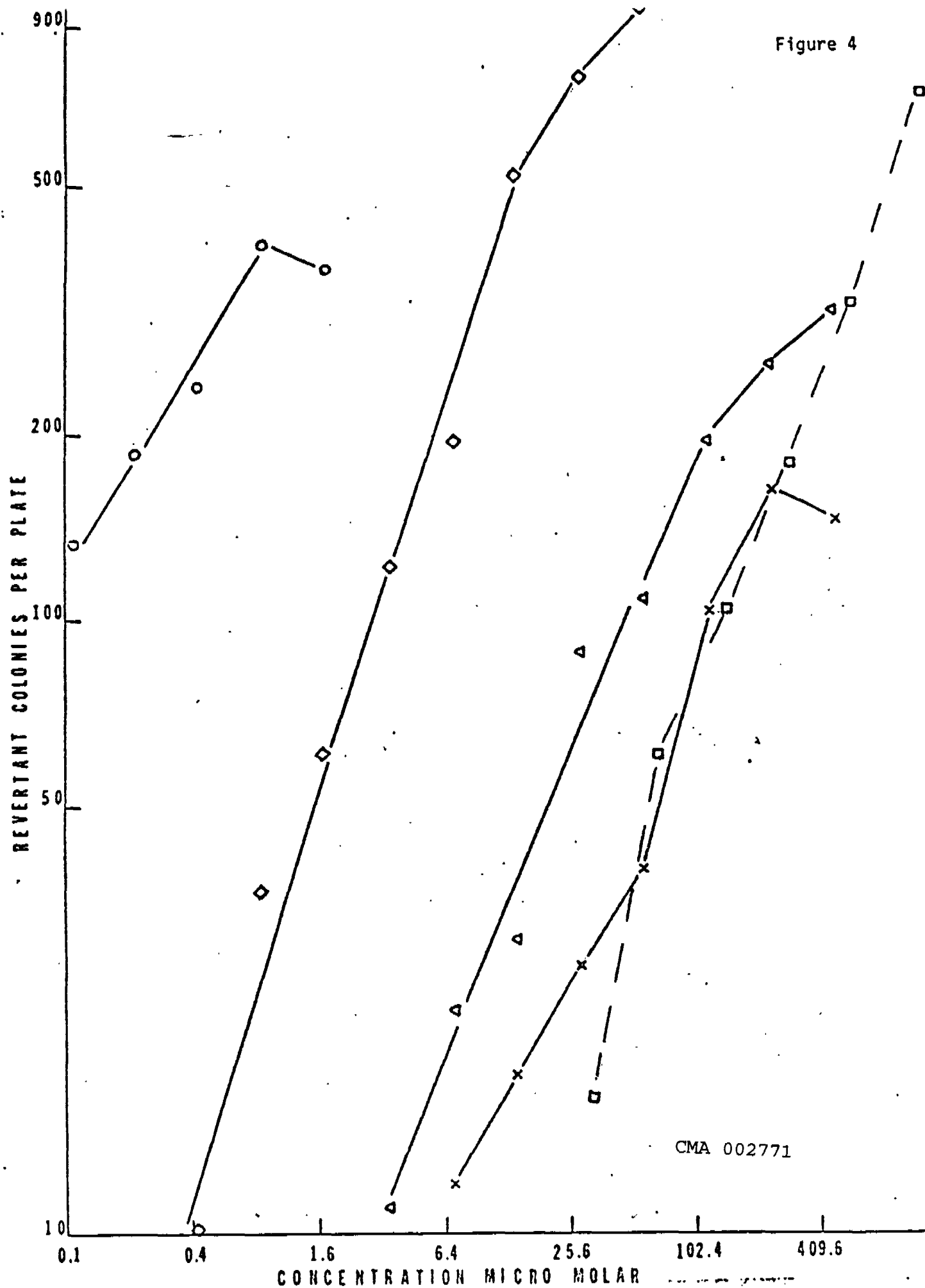


TABLE I: Bacteria Tester Strains

A. Salmonella typhimurium LT-2 Tester Strains<sup>a</sup>

<sup>a</sup>All tester strains contain *uvrB* repair mutations which eliminate the excision repair system; mutations in the histidine operon; and *rfa* mutations which alter the cell wall by increasing permeability and eliminating pathogenicity.

<sup>b</sup>The resistance transfer factor, "R" factor, enhances the error-prone recombination repair system thus making the strains more susceptible to mutation [15].

<sup>c</sup>The strains susceptible to base-pair substitution contain mutations in the histidine G46 operon and those susceptible to frameshift mutation contain mutations in the histidine operon C 3076 (TA 1537) or D3052 (TA 1538, TA 98).

| Strains | "R" factor <sup>b</sup> | Mutation detected <sup>c</sup> |
|---------|-------------------------|--------------------------------|
| TA 1535 | -                       | base-pair substitution         |
| TA 100  | +                       | base-pair substitution         |
| TA 1537 | -                       | frameshift                     |
| TA 1538 | -                       | frameshift                     |
| TA 98   | +                       | frameshift                     |

B. Bacillus subtilis Tester Strains

<sup>a</sup>*Trp*<sup>-</sup> denotes a requirement for tryptophan; *Mit-S* denotes sensitivity to mitomycin C.

<sup>b</sup>*hcr*<sup>+</sup> denotes a host-cell reactivation DNA repair capacity; *hcr*<sup>-</sup> lacks a host-cell reactivation DNA repair capacity; *rec*<sup>+</sup> denotes a recombination DNA repair capacity; *rec*<sup>-</sup> lacks a recombination DNA repair capacity; *uvr*<sup>-</sup> is sensitive to ultraviolet-induced DNA damage.

| Strains | Phenotype <sup>a</sup>                 | DNA Repair <sup>b</sup>                           |
|---------|----------------------------------------|---------------------------------------------------|
| 168 M   | Prototroph (wild type)                 | <i>hcr</i> <sup>+</sup> , <i>rec</i> <sup>+</sup> |
| Hcr-9   | <i>Trp</i> <sup>-</sup>                | <i>hcr</i> <sup>-</sup> , <i>rec</i> <sup>+</sup> |
| FB-13   | <i>Trp</i> <sup>-</sup>                | <i>uvr</i> <sup>-</sup> , <i>rec</i> <sup>+</sup> |
| MC-1    | <i>Trp</i> <sup>-</sup> , <i>Mit-S</i> | <i>hcr</i> <sup>+</sup> , <i>rec</i> <sup>-</sup> |

TABLE II. PMR Spectra of Vinyl Chloride Derivatives

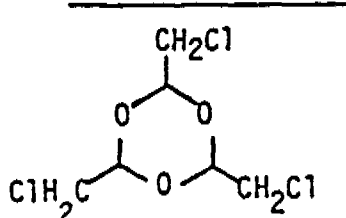
| Compounds                                                                                        | Solvent                                                    | Spectra, $\delta$ TMS=0 (J=Hz)                                         |
|--------------------------------------------------------------------------------------------------|------------------------------------------------------------|------------------------------------------------------------------------|
| $\text{ClHC}-\underset{\text{Cl}}{\text{CH}_2}\text{O}$ <div>4</div>                             | $\text{CCl}_4$                                             | 2.75 (q, CH, J=1.5)<br>2.85 (q, CH, J=2.4)<br>4.90 (q, CH, J=2.4, 1.5) |
| $\text{ClH}_2\text{C}-\text{CHO}$ <div>5</div>                                                   | $\text{CCl}_4$                                             | 4.00 (d, CH, J=2.2)<br>9.57 (t, $\text{CH}_2$ , J=2.2)                 |
|                                                                                                  | $\text{DMSO}-d_6$                                          | 3.50 (d, CH)<br>9.60 (t, $\text{CH}_2$ )                               |
| $\text{ClH}_2\text{C}-\text{CH}(\text{OH})_2$ <div>6</div>                                       | $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 1:8<br>(pD 0.1)  | 3.60 (d, $\text{CH}_2$ , J=5)<br>4.60 (t, CH, J=5)                     |
| $\text{ClH}_2\text{C}-\underset{\text{O}}{\text{CH}}-\text{OH}$ <div>7</div>                     | $\text{DMSO}-D_6$                                          | 3.55 (d, $\text{CH}_2$ , J=4.9)<br>5.05 (t, CH, J=4.9)                 |
| $\text{ClH}_2\text{C}-\underset{\text{O}}{\text{CH}}-\text{OH}$ <div>7</div>                     | $\text{CD}_3\text{OD}-\text{D}_2\text{O}$ 1:8<br>(pD 20.1) | 3.60 (d, $\text{CH}_2$ , J=4.5)<br>4.83 (t, CH, J=4.5)                 |
|  <div>8</div> | $\text{CCl}_4$                                             | 3.52 (d, $\text{CH}_2$ , J=4.7)<br>5.08 (t, CH, J=4.7)                 |
|                                                                                                  | $\text{DMSO}-d_6$                                          | 3.75 (d, $\text{CH}_2$ , J=4.1)<br>5.45 (t, CH, J=4.1)                 |

TABLE III: Summary of Mutagen Activity in Microbial Systems

NI = No inhibition of growth detected in Bacillus subtilis MC-1;  
 NR = No increase of revertants in Salmonella typhimurium TA 100  
 compared to control; + = active; ++ = very active. Acetaldehyde, a  
 potential metabolite of 1, and allyl chloride, the parent olefin of  
 9, were negative in these two systems.

| Compounds Tested                                                               | <u>Bacillus subtilis</u><br>Repair Assay | <u>Salmonella typhimurium</u><br>Reversion Assay |
|--------------------------------------------------------------------------------|------------------------------------------|--------------------------------------------------|
| Control: 4-nitroquinoline-N-oxide                                              | ++                                       | ++                                               |
| 1 $\text{H}_2\text{C}=\text{CHCl}$                                             | NI                                       | NR                                               |
| 2 $\text{ClH}_2\text{C}-\text{CH}_2\text{OH}$                                  | NI                                       | NR                                               |
| 3 $\text{ClH}_2\text{C}-\text{COOH}$                                           | NI                                       | NR                                               |
| 4 $\text{ClHC}-\text{CH}_2-\text{O}$                                           | +                                        | ++                                               |
| 5 $\text{ClH}_2\text{C}-\text{CHO}$                                            | ++                                       | ++                                               |
| 6 $\text{ClH}_2\text{C}-\text{CH}(\text{OH})_2$                                | ++                                       | ++                                               |
| 7 $\text{ClH}_2\text{C}-\text{CHOH}-\text{O}-\text{CHOH}-\text{CH}_2\text{Cl}$ | ++                                       | ++                                               |
| 8 $(\text{ClH}_2\text{C}-\text{CHO}-)_3$                                       | ++                                       | +                                                |
| 9 $\text{ClH}_2\text{C}-\text{CH}-\text{CH}_2\text{O}$                         | NI                                       | ++                                               |

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-TABLE IV: Growth Inhibition of Bacillus subtilis Strains.

Inhibition was measured in mm after 24 hr at 37°C as described in text;  
NI denotes no inhibition

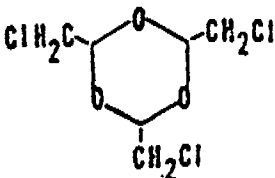
| Mutagen                                                                             |            | Molarity | 168M | MC-1 | Hcr-9 | FB-13 |
|-------------------------------------------------------------------------------------|------------|----------|------|------|-------|-------|
| $\text{ClH}_2\text{C-CHO}$                                                          | 5<br>~     | 0.100    | 2.0  | 27.7 | 3.7   | 2.7   |
| Chloroacetaldehyde<br>(45% aqueous solution)                                        | 6 & 7<br>~ | 0.115    | NI   | 22.5 | NI    | NI    |
| $\text{ClH}_2\text{C-CH-OH}$<br> <br>O<br> <br>$\text{ClH}_2\text{C-CH-OH}$         | 7<br>~     | 0.097    | 1.5  | 9.5  | 1.5   | 1.5   |
|  | 8<br>~     | 0.096    | 7.0  | 14.5 | 6.0   | 7.0   |
| $\text{ClHC-CH}_2\text{O}$<br> <br>CH <sub>2</sub>                                  | 4<br>~     | 0.260    | NI   | 10.0 | NI    | NI    |
| $\text{ClH}_2\text{C-CH-CH}_2\text{O}$<br> <br>CH <sub>2</sub>                      | 9<br>~     | 0.113    | NI   | NI   | NI    | NI    |
| 4-Nitroquinoline-N-oxide<br>(control)                                               |            | 0.001    | 10.0 | 18.0 | 15.0  | 15.0  |

TABLE V. Relative Mutagenicity of the Four Forms of Chloroacetaldehyde  
with S. typhimurium TA 100

| 45% Aqueous Soln<br>6 : 7 50:50<br>~ ~ |            | Monomer 5<br>~       |            | Dimer Hydrate 7<br>~ |            | Trimer 8<br>~        |            |
|----------------------------------------|------------|----------------------|------------|----------------------|------------|----------------------|------------|
| Molarity                               | Revertants | Molarity             | Revertants | Molarity             | Revertants | Molarity             | Revertants |
| $5.3 \times 10^{-5}$                   | 977        | $1.3 \times 10^{-5}$ | 18         | $4.8 \times 10^{-4}$ | 311        | $4.8 \times 10^{-4}$ | 144        |
| $2.7 \times 10^{-5}$                   | 723        | $6.7 \times 10^{-6}$ | 68         | $2.4 \times 10^{-4}$ | 259        | $2.4 \times 10^{-4}$ | 159        |
| $1.4 \times 10^{-5}$                   | 512        | $3.3 \times 10^{-6}$ | 88         | $1.2 \times 10^{-4}$ | 193        | $1.2 \times 10^{-4}$ | 101        |
| $6.9 \times 10^{-6}$                   | 194        | $1.7 \times 10^{-6}$ | 361        | $6.0 \times 10^{-5}$ | 107        | $6.0 \times 10^{-5}$ | 39         |
| $3.4 \times 10^{-6}$                   | 120        | $8.4 \times 10^{-7}$ | 404        | $3.0 \times 10^{-5}$ | 88         | $3.0 \times 10^{-5}$ | 27         |
| $1.7 \times 10^{-6}$                   | 61         | $4.2 \times 10^{-7}$ | 238        | $1.5 \times 10^{-5}$ | 30         | $1.5 \times 10^{-5}$ | 18         |
| $8.6 \times 10^{-7}$                   | 36         | $2.1 \times 10^{-7}$ | 185        | $7.5 \times 10^{-6}$ | 23         | $7.4 \times 10^{-6}$ | 12         |
| $4.3 \times 10^{-7}$                   | 10         | $1.1 \times 10^{-7}$ | 131        | $3.8 \times 10^{-6}$ | 11         | $3.7 \times 10^{-6}$ | -0         |

TABLE VI: Reversion of *Styphimurium* TA100 by Chlorooxirane 4  
and Epichlorohydrin 9

Broth solutions of 4 or 9 with TA100 were preincubated at 3°C before plating. Duplicate plates were evaluated after 48 hrs at 37°C. The average number of revertant colonies per plate minus the number of spontaneous reversions were recorded.

| Preincubation<br>Time | Epichlorohydrin 9<br>(1.0 mM) | Chlorooxirane 4<br>(0.26 mM) |
|-----------------------|-------------------------------|------------------------------|
| 0 hr                  | 186                           | 31                           |
| 1 hr                  | 204                           | 4                            |
| 2 hr                  | 297                           | 6                            |
| 4 hr                  | 202                           | 114                          |
| 6 hr                  | 154                           | 44                           |