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EPIDEMIOLOGY STUDIES
SCREENING FOR THE EARLY DETECTION OF DISEASE
IN INDIVIDUALS EXPOSED TO VINYL CHLORIDE

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SCREENING FOR THE EARLY DETECTION OF DISEASE IN INDIVIDUALS EXPOSED TO VINYL CHLORIDE

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DISCLAIMER

This project has been funded with Federal Funds from the Environmental Protection Agency under contract number 68-01-3859. The content of this publication does not necessarilyreflect the views or policies of the U.S. Environmental Protection Agency, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. Government.

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ABSTRACT

A prospective collaborative study was conducted to compare the effectiveness of four clinical techniques in the detection of liver damage due to vinyl chloride monomer exposure. A chemically exposed and medically monitored worker population was identified by histopathological and biochemical documentation. Three techniques were non-invasive: a) grey scale ultrasonography of the liver, b) microvascular skin capillary assessment, and c) urinary analysis of glycosaminoglycan excretion. The fourth technique was the standard ^{99m}Tc sulfur colloid radionucleotide liver spleen scan. The screening studies were performed on a randomly selected single cohort of chemical workers; some of whom were known to have disease. All four techniques were analyzed for their sensitivity and specificity as compared to results of the liver biopsy and biochemical blood test classification. Although all four screening techniques had a sensitivity and specificity sum greater than one, none were significantly better than could be explained by chance or the use of a biased coin. Reclassification of the population into those with more severe biochemical abnormalities improved the sensitivity of all screening tests, but only the sensitivity and specificity sum for the GAG test were statistically significant at the 0.05 level. There was no significant correlation between any pair of screening tests. None of the four screening tests agreed with the biopsy results better than might be obtained by biased coin or chance. These screening studies as presently constituted, do not provide sufficient sensitivity and specificity to warrant their use in community screening for subclinical asymptomatic hepatic injury due to chemical exposure.

INTRODUCTION

The initial reports of primary liver cancers (angiosarcoma) in rats exposed to vinyl chloride by Maltoni, et al (1) and the discovery of similar liver tumors in vinyl chloride polymerization workers by Johnson and Creech (2) has lead to considerable environmental concern regarding communities surrounding chemical industries which utilize potentially carcinogenic agents such as vinyl chloride. Several investigators have reported on various screening techniques as effective indicators of vinyl chloride chemical injury. Four such techniques - ultrasonography (3), radionucleotide scanning (4), nailbed capillary visualization (5), and glycosaminoglycan (GAG) (6) excretion - were reported to have some possible usefulness in detecting early chemical injury to the liver. In order to determine the useability of these techniques for community screening, the American Public Health Association (APHA) and Environmental Protection Agency (EPA) funded a multi-center collaborative study designed to determine the comparative sensitivity and specificity of these various techniques in detecting and identifying chemical related hepatic injury in asymptomatic individuals.

Materials and Methods

Population Selection

The chemical worker population consisted of 1,178 active (Group B, Figure 1) employees as of September 1, 1977, and 70 employees who had had liver biopsies regardless of current employment status (Group A, Figure 1); they were undergoing annual medical screening for the idertification of work-related disorders. The medical screening consisted of an annual or semi-annual (for those employees with 10 or more years of employment) comprehensive history and physical examinations, laboratory screening studies consisting of 35 biochemical tests, chest and abdominal X-rays, and radionucleotide liver-spleen scan.

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This population was selected for the collaborative study to determine the comparative effectiveness of four screening techniques in detecting liver damage as indicated by 1) past histopathological documentation of liver injury of 2) current hepatic dysfunction identified biochemically.

Three of the four techniques were included as potential non-invasive procedures suitable for determining the effects of vinyl chloride in a community population. These included 1) grey scale ultrasonography of the liver as developed by Taylor and colleagues (7, 13, 14), 2) a nailbed skin capillary evaluation of the middle and distal phalanges of the fingers as developed by Maricq and associates (8), and 3) urinary analysis of glycosaminoglycan excretions (GAG) as published by Kupchella and associates (9).

The fourth method included for comparison purposes was the standard radionucleotide liver-spleen scan utilizying ^{99}mTc colloid and interpreted by Whelan and associates (10).

The non-invasive screening studies were performed during a single week on a group randomly selected from all chemical company employees. The workers were selected on the basis of complete medical and work data for 1976-1977, and all those employees who had investigative liver biopsies performed during the screening program (1974-1977). The selection process for these workers is illustrated in Figure 1. The biochemical data and radioisotopic scans were part of the routine medical surveillance system for the employees. The pathological data was based on the last or most recent liver biopsy (s) which were performed for medical reasons, both related and not related to their work. Positive and negative results were determined as defined in Table 1 which list the technique, the evaluation or evaluators, and the criteria used. The employees targeted for examination were selected by simple random sampling from all available employees.

One hundred and twenty one of the targeted 170 employees (71 percent) participated. Twenty six declined to participate or could not be scheduled; 13 did not keep their scheduled appointment. After participation, nine employees were discovered to have been misclassified as biochemically abnormal. They had some biochemical abnormalities but not all (classified intermediate) and have been eliminated from the final analysis. Four biochemically abnormal individuals had abnormally low test values and they were included in the analysis.

Liver Biopsies

Liver biopsies were performed by the transjugular technique (11) and provided two to five biopsies from various areas of the liver. In addition, some individuals had second biopsies performed by the percutaneous or wedge biopsy via mini-laparotomy procedures. Pathological data was recorded in a computerized format identifying all histological abnormalities in a semi-quantitative fashion. Biopsies were read without knowledge of the individual's medical history or chemical exposure by two pathologists and a hepatologist with extensive experience in hepatic chemical injury. All biopsies were classified as 1) normal, 2) abnormal, a) chemical injury, and b) non-chemical injury.

The non-biopsy groups were drawn from those currently employed, and based on biochemical liver "function tests" individuals were sorted into positive, negative and indeterminate for hepatic disease. Only the positive and negative are included in this study.

The ultrasonic evaluation and its relative ability to identify hepatic damage due to vinyl chloride has been published elsewhere (3).

Microvascular techniques and the method of evaluation by Dr. Maricq and co-workers are also published in part (8).

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angiosarcoma and connective tissue damage of the liver has been published elsewhere (9). The effectiveness of radioisotopic (radionucleotide) scanning as a technique for identifying anatomical lesions in vinyl chloride workers is in preparation (12).

Method of Analysis

The biopsied group and the non-biopsied (biochemical) group were analyzed independently. For the biopsied group sensitivity and specificity were estimated for each screening test by assuming that the biopsy was correct. For the biochemical group the biochemical classification was assumed to be correct. For each analysis the data consisted of a simple cross classification. In a perfect screening test, the sum of sensitivity and specificity would be two. In a screening test which provided results no better than could be obtained by using a biased coin, the sum of sensitivity and specificity would be equal to one. We, therefore, estimated 95% confidence limits for the sum of sensitivity and specificity and observed whether or not one is included within these limits. As a test of statistical significance this is equivalent to the usual X^2 test for independent proportions.

Finally, in Table 4 we looked at the association (as measured by the phi coefficient) between each pair of screening tests. For these comparisons we used all employees who received both screening tests regardless of their biopsy status. In this case, we assumed both tests were subject to error and estimated the phi coefficient (r_ϕ) between them. Finally, for completeness, we give the biochemical classification for the 51 employees included in the biopsy group.

Results

Table 2 compares the histological and biochemical results for the 51 biopsied employees included in the study. Twenty-two of these employees had biochemical abnormalities as defined in Table 1. There was no significant

correlation between the biochemical and biopsy classification for the 29 employees with positive or negative biochemical classifications ($r_\phi = 0.21$; $X^2_1 = 0.20$). The biochemical studies used in this analysis were those determined at the time of this and not at the time the biopsy was performed. In all instances of disagreement, the biopsy was positive and the biochemical results negative ($P < 0.001$).

Figure 2 provides the sensitivity and specificity for each of the screening tests when compared to biopsy results. In no case is the sum significantly greater than one, indicating that the results are not statistically significantly better than could be obtained using the biased coin. With the exception of the GAG studies, similar results are obtained when comparing the sum of sensitivity and specificity in the biochemical group (Figure 3). The sum of sensitivity and specificity for the GAG studies are just statistically significant with 95 percen confidence limits of 1.06 to 1.52. Table 3 gives the frequency distribution of the results of the GAG test for employees with normal and abnormal biochemical results. The distributions differ in their spread (variance) and not in their location (means).

Finally, the correlation matrix for the four tests are given in Table 4. There is no significant correlation, as measured by r_ϕ, between any pair of the screening tests. This is also true when they are sorted by biopsy status.

After reviewing the results, a reclassification of the 51 employees who had biopsies was assessed in regard to whether there was chemically induced liver damage. Ultrasonographic evaluation was reclassified by Dr. Taylor and the liver biopsies by Drs. Tamburro and Popper. Table 5 gives the results of this additional analysis which demonstrates that there was no agreement that could not be explained by chance ($P = 0.60$).

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Discussion

The increasing industrialization in highly developed Western countries, such as the United States, continues to provide concern, not only for the health and safety of the industrial workers, but also for the surrounding communities in the areas of these industries. It is highly desirable to identify and validate the reliability of screening and diagnostic techniques which will identify the early development of injury due to the exposure of a variety of chemicals such as vinyl chloride. The assessment of newly developing techniques on a high-risk, exposed worker population, who have been carefully screened and prospectively followed, provide the most reliable method for determing both the sensitivity and specificity of these technical procedures in the asymptomatic subclinical high-risk exposed community population. The failure of such techniques to provide sufficient sensitivity in the presence of a required specificity is of critical clinical importance. This is especially so where the incidence of disease is relatively low and the population exposed large. Tests which provide a high sensitivity but of low specificity can and do medically stigmatize the population under surveillance leading to unnecessary anxiety and socioeconomic disturbances which can far outweigh the benefit of early detection of even serious disease in a smaller population.

Far too often screening techniques which have been developed in a highly diseased, clinically overt, hospitalized population are applied to an asymptomatic, clinically well-working populations without adequate determination of the sensitivity and specificity at this earlier stage of disease development.

In this study, all four techniques had, in the highly diseased hospitalized population, demonstrated either a sensitivity or specificity suggestive for the identification of underlying chemically related liver disease. Some techniques (Maricq and Kupchella) appeared ideal for community studies since they were non-invasive, relatively inexpensive, and provided a means of screening which would be highly accepted by a community.

This prospectively designed study has allowed us to estimate the ability of ultrasonography, nailbed capillary assessments, radioisotopic scanning, and glycosaminoglycan excretions to correctly predict the presence and absence of hepatic disease as documented by an exposed population. These studies clearly show that none of the four screening techniques sufficiently agree with either the biopsy, the biochemical results, or each other in a well defined population; they do not provide sufficient sensitivity or specificity to be useful as early indicators of chemical exposure injury.

The inclusion of nine individuals with intermediate biochemical results, who were originally misclassified as abnormal, would decrease the sum of sensitivity and specificity in all three screening tests. With their exclusion only the GAG tests provided results better than might be expected by chance ($P < 0.05$). Even the GAGs, from a pragmatic point of view, provide too high a false positive rate to be useful in its present stage. Possibly, with increased refinement and further study, this might provide a simple non-invasive technique for the identification of increased collagen changes related to chemical injury. More immediately, it should be duplicated to rule out chance.

Had we eliminated the four employees with an abnormally low biochemical test values, the sum of sensitivity and specificity for the GAGs and nailbed skin capillary screening tests would have been slightly reduced and that for the scan slightly increased. The GAG would still be of bordering significant ($X^2_1 = 3.73$), and the scan not significant ($X^2_1 = 1.46$).

Finally, the tests not only disagree with each other, but within the biopsy group there was no agreement between the biopsies and biochemical results. It should be noted, however, that the biochemical studies used in analysis were those chronologically closest to September, 1977, and not to the date of biopsy.

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The test determinations and the biopsies may have been as long as three years apart. An analysis of the biochemical tests value done at the time of the biopsy would have more accurrately reflected the liver status, as shown by histology (15). It has been shown, in previously published studies, that biochemical and histological findings each correlate with chemical injury and chemical exposure (16, 17).

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Table 1

CLASSIFICATION	DETERMINED BY	CRITERIA
Biopsy	1) Dr. Popper, Pathologist 2) Dr. Makk, Pathologist 3) Dr. Tamburro, Hepatologist	a) Positive by consensus agreement if medically significant pathology is present. Negative otherwise. b) Pathology is of chemical or non-chemical origin. (18)
Biochemical	Liver "Function" Tests Group 1: SGPT, ICG, SGOT, ALK. PHOSPHATASE Group 2: GGTP, BILIRUBIN	Positive if two or more Group tests were abnormal or if one Group 1 and both Group 2 tests were abnormal. Negative if all six tests were normal. Intermediate otherwise.
Ultrasound	Dr. Taylor	Defined as negative if the overall impression was normal. Positive otherwise.
Microvascular	Dr. Maricq	Defined as negative if there were no microvascular abnormalities. Positive otherwise.
Glycosaminoglycans	Uronic acid = UG Uronic Acid / MG Creatinine	Defined as positive for values less than 2.0 or greater than 4.8. Negative otherwise.
Liver Scan	Dr. Whelan	Defined as positive if any pathological defect was detected. Negative Otherwise.

Table I

CLASSIFICATION	DETERMINED BY	CRITERIA
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Biochemical	Liver "Function" Tests Group 1: SGPT, TCG, SGOT, ALK. PHOSPHATASE Group 2: GGTP, BILIRUBIN	Positive if two or more Group tests were abnormal or if one Group 1 and both Group 2 tests were abnormal. Negative if all six tests were normal. Intermediate otherwise.
Ultrasound	Dr. Taylor	Defined as negative if the overall impression was normal. Positive otherwise.
Microvascular	Dr. Maricq	Defined as negative if there were no microvascular abnormalities. Positive otherwise.
Glycosaminoglycans	$\text{Uronic acid} = \frac{\text{UG Uronic Acid}}{\text{MG Creatinine}}$	Defined as positive for values less than 2.0 or greater than 4.8. Negative otherwise.
Liver Scan	Dr. Whelan	Defined as positive if any pathological defect was detected. Negative Otherwise.

TABLE 2

COMPARISON OF BIOPSY AND BIOCHEMICAL DETERMINATIONS OF THE PRESENCE OF LIVER DISEASE

BIOPSY	BIOCHEMICAL		SUM	BIOCHEMICALLY INDETERMINATE	TOTAL
	POSITIVE	NEGATIVE			
POSITIVE	3	18	21	15	36
NEGATIVE	0	8	8	7	15
SUM	3	26	29	22	51

$r = 0.20966$ $\quad X_1^2 = 0.200$ N.S.

Matched $X_1^2 = (18 - 1)^2/18 = 16.1$ $\quad P < 0.001$

TABLE 3

FREQUENCY (F) AND RELATIVE FREQUENCY (R.F.)
OF GAGS FOR NORMAL AND ABNORMAL
BIOCHEMICAL RESULTS

	Normal		Abnormal	
GAG	f	R.F.	f	R.F.
< 2	4	0.111	6	0.250
2 < 3	16	0.444	7	0.292
3 < 4	13	0.361	6	0.250
4 < 5	1	0.028	1	0.042
5+	2	0.056	4	0.167
Sum	36	1.000	24	1.000
Mean	2.886		3.160	
Variance	0.746		1.776	

$t_{58} = 0.968$ N.S. T Test Independent Means

$F_{23,35} = 2.381$ $P < 0.05$ F Test Independent Variances (Two Taile

TABLE 3

FREQUENCY (F) AND RELATIVE FREQUENCY (R.F.)

OF GAGS FOR NORMAL AND ABNORMAL

BIOCHEMICAL RESULTS

GAG	Normal f	Normal R.F.	Abnormal f	Abnormal R.F.
< 2	4	0.111	6	0.250
2 < 3	16	0.444	7	0.292
3 < 4	13	0.361	6	0.250
4 < 5	1	0.028	1	0.042
5+	2	0.056	4	0.167
Sum	36	1.000	24	1.000
Mean	2.886		3.160	
Variance	0.746		1.776	

$t_{58} = 0.968$ N.S. T Test Independent Means

$F_{23,35} = 2.381$ $P < 0.05$ F Test Independent Variances (Two Taile

TABLE 4

CORRELATION MATRIX (Ro) BETWEEN FOUR SCREENING TESTS USED TO PREDICT THE PRESENCE OR ABSENCE OF LIVER DISEASE

	GAG ANALYSIS	CAPILLARY ASSESSMENT	ULTRASOUND STUDY	RADIOISOTOPIC SCAN
GAG ANALYSIS	.	0.08	-0.03	0.005
CAPILLARY ASSESSMENT	(113)	.	0.03	0.07
ULTRASOUND STUDY	(87)	(84)	.	0.06
RADIOISOTOPIC SCAN	(120)	(114)	(88)	.

None of the correlations are statistically significant (a = 0.05).
The correlations are given above the diagonal.
The sample size is given in parenthesis below the diagonal.

TABLE 5

RECLASSIFICATION OF BIOPSIED EMPLOYEES

FOR CHEMICALLY INDUCED ABNORMALITIES

Biopsy	Ultra Sound		SUM
	Chemical Abnormality	Other	
Chemical Abnormality	7	5	12
Other	26	13	39
SUM	33	18	51

Specificity = 13/39 = 0.333

Sensitivity = 7/12 = 0.583

Sum = 0.916

TABLE 5

RECLASSIFICATION OF BIOPSIED EMPLOYEES

FOR CHEMICALLY INDUCED ABNORMALITIES

Biopsy	Ultra Sound		SUM
	Chemical Abnormality	Other	
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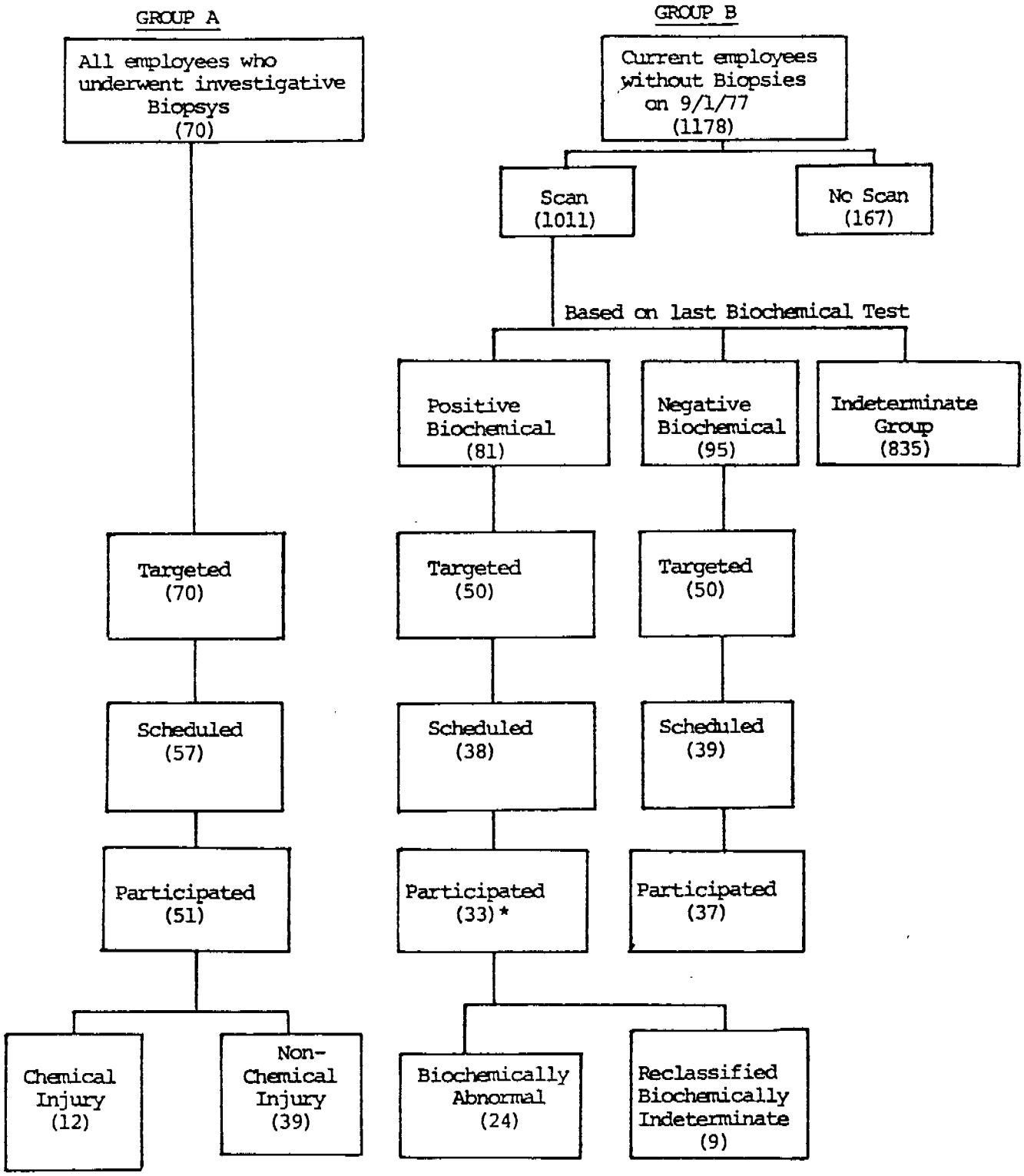
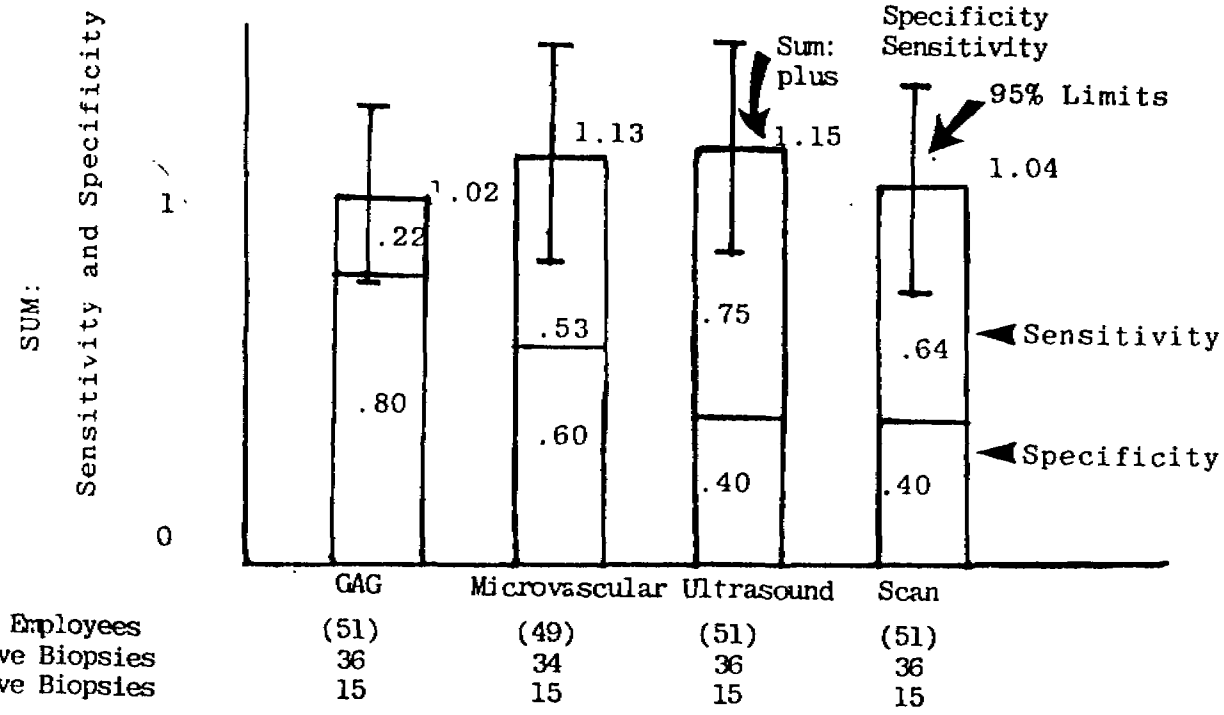
FIGURE 1



*Not seen by Doctor Taylor

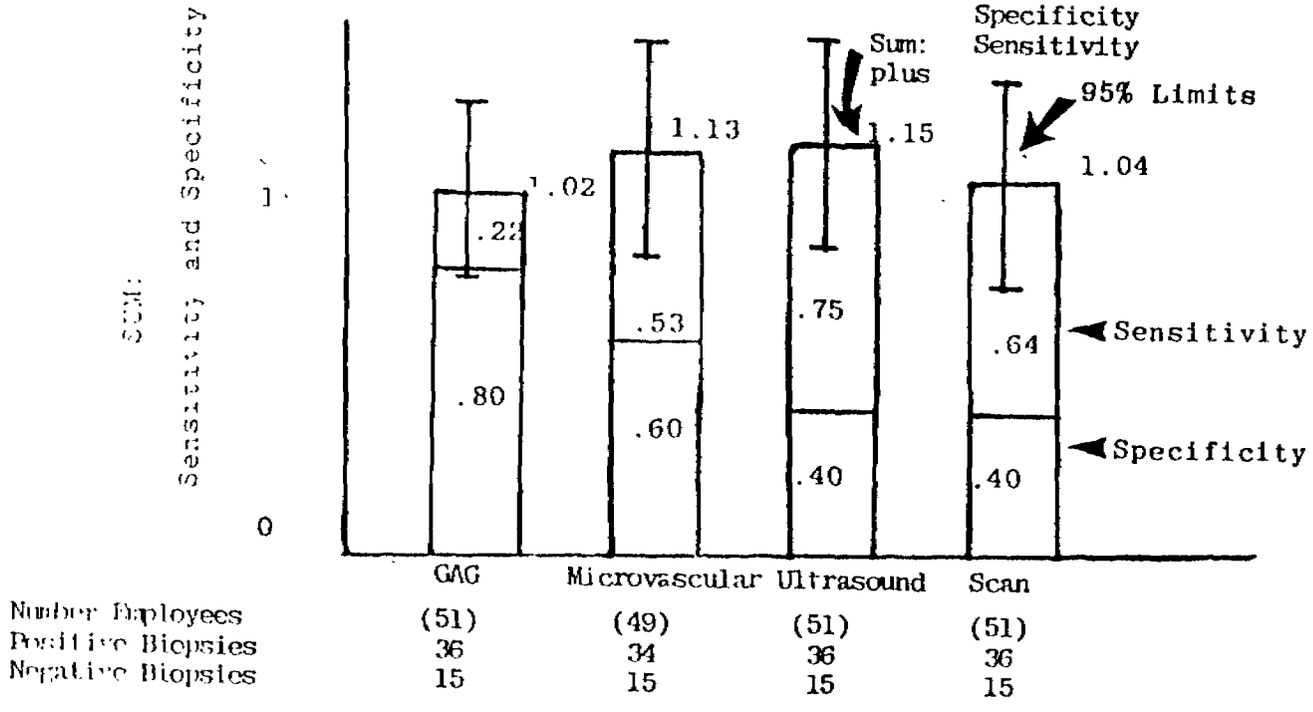
CMA 003716

SENSITIVITY AND SPECIFICITY FOR FOUR SCREENING TESTS FOR THE PREDICTION OF LIVER ABNORMALITIES (AS DETERMINED BY BIOPSY)



Number Employees	(51)	(49)	(51)	(51)
Positive Biopsies	36	34	36	36
Negative Biopsies	15	15	15	15

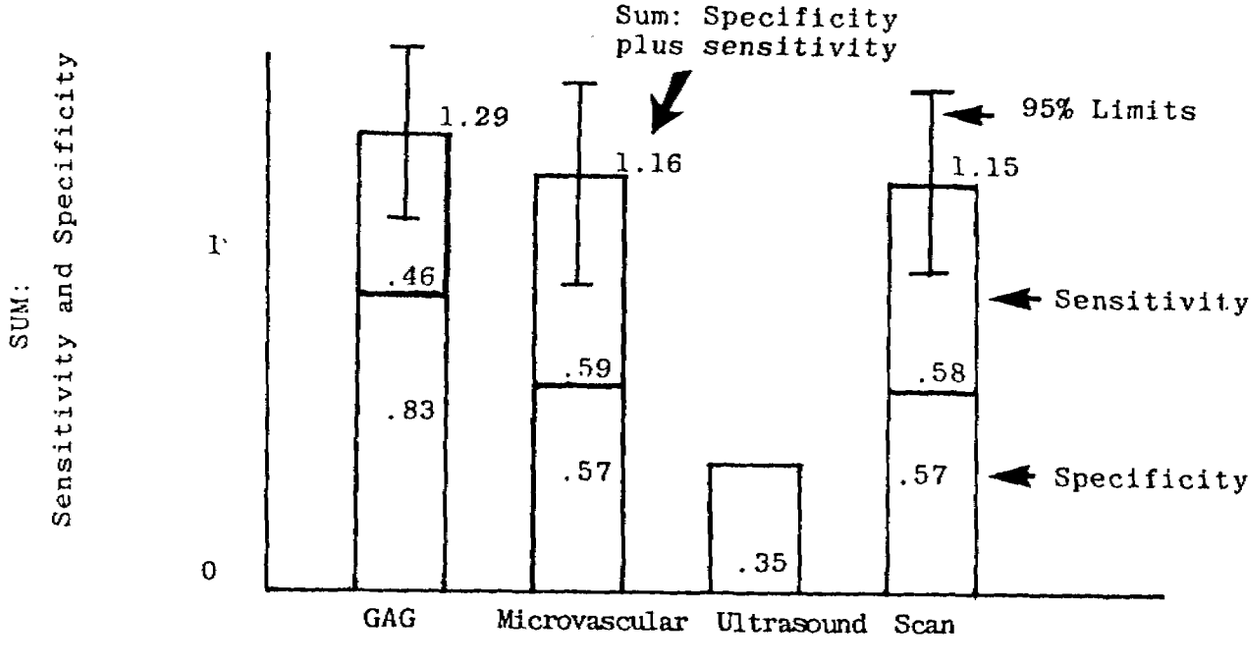
SENSITIVITY AND SPECIFICITY FOR FOUR SCREENING TESTS FOR THE PREDICTION OF LIVER ABNORMALITIES (AS DETERMINED BY BIOPSY)



Number Employees	(51)	(49)	(51)	(51)
Positive Biopsies	36	34	36	36
Negative Biopsies	15	15	15	15

Figure 3

SENSITIVITY AND SPECIFICITY FOR FOUR SCREENING TESTS FOR THE PREDICTION OF LIVER ABNORMALITIES (AS DETERMINED BY REVIEWED BIOCHEMICAL TESTS)



Number Employees	(60)	(57)	(37)	(61)
Positive Biochemical	24	22		24
Negative Biochemical	36	35		37

STUDIES ON THE MUTAGENICITY OF VINYL CHLORIDE METABOLITES AND RELATED CHEMICALS

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I. INTRODUCTION

The studies by Viola et al (26) and Maltoni et al (15) established the carcinogenic potential of vinyl chloride monomer. The detection of angiosarcoma in industrial workers exposed to polyvinyl chloride suggested a causal relationship between this chemical and the development of hepatic abnormalities (4,12). Hefner et al (7) have delineated the metabolic fate of inhaled vinyl chloride in rats and proposed that the epoxide, chlorooxirane, and chloroacetaldehyde were the carcinogenic intermediates. Their hypothesis has been supported by the work of several laboratories (3,14,16,19, Elmore, Wong, Laumbach and Streips, submitted for publication) using bacterial strains as mutagenic indicators.

In this communication we present additional data concerning the mutagenicity and the potential mechanisms of action of several vinyl chloride metabolites, including the previously unreported chloroacetadehyde monomer hydrate, chloroacetaldehyde dimer hydrate, and chloroacetaldehyde trimer. Epichlorohydrin, a mutagenic/carcinogenic (21,25) methylene homolog of chlorooxirane was also examined.

II. PROCEDURES AND MATERIALS USED

A. Bacterial Strains

The bacterial strains utilized in these studies are presented in Table I. The Bacillus subtilis strains were all maintained on AK agar (BBL). Salmonella typhimurium cultures were obtained from B. N. Ames (1) and were stored on Nutrient Agar (Difco) plus 5g NaCl per liter.

B. Mutagenicity Assays

The indirect assay utilized repair deficient strains of B. subtilis. The procedure was a modification of the "rec-assay" described by Kada et al (9). Cells were grown overnight in Nutrient Broth (Difco) at 37C in a rotary incubator shaker, then diluted tenfold in phosphate buffer (pH 7.0). The suspended cultures were streaked onto Nutrient Agar plates (Difco). Filter paper discs (6 mm) were saturated with the chemical solutions to be examined, then were placed onto the agar plates next to the streaked bacterial cultures. Following incubation at 37C overnight the plates were examined and the lethality and mutagenic potential of the test chemicals were assessed by comparing inhibition zones between the B. subtilis 168 wild type, a repair-capable strain and the various DNA repair-deficient strains. In all these studies 4-nitroquinoline-1-oxide (4NQO) was used as the positive mutagenic control.

Direct mutagenicity assays utilized the S. typhimurium tester strains described by Ames (1). The chemicals were examined by the methods of McCann et al (16). The cultures were grown in Nutrient Broth plus 0.5% NaCl overnight in a rotary incubator shaker at 37C. A mixture of the test chemical (0.1 ml) in dimethyl sulfoxide (DMSO) and 2 ml of soft agar (0.6% agar, 0.6% NaCl, 0.5 mM biotin, and 0.5 mM histidine) was added to 0.1 ml of the bacterial culture. The solutions were mixed thoroughly and overlaid onto minimal plates [Vogel-Bonner E medium (27), 1.5% agar, and 2% glucose]. Control samples were prepared by omitting the test chemicals. For the positive mutagenesis control, 4NQO was added to the mixtures in place of the test chemicals. All plates were incubated for 48 hr at 37C prior to the enumeration of revertant colonies.

C. Chemical Compounds

The chemical compounds utilized in these studies were prepared, purified, and analyzed by previously reported techniques (Elmore, Wong, Laumbach, and Streips, submitted for publication).

D. Preparation of DNA

Transforming DNA was isolated from B. subtilis cultures by the method described by Young and Wilson (29). In some of the experiments the cultures were pretreated for 15 min either with chloroacetaldehyde (16 mM) or epichlorohydrin (16 mM) prior to the extraction procedure. In alternate experiments S-9 liver homogenate mix was added to the compounds prior to addition to bacteria. The S-9 liver homogenate contains per ml, 0.3 ml of the S-9 fraction, 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-P, 4 mM NADP, and 100 mM sodium phosphate (pH 7.4). The DNA concentration in all lysates were assayed by the method of Richards (20).

E. Treatment of DNA In vitro with Chemicals

A sample of B. subtilis transforming DNA (0.9 ml) in standard saline citrate (SSC) (0.15 M NaCl-0.015 M trisodium citrate, pH 7.0) was combined with 0.1 ml chloroacetaldehyde (1.0 M in DMSO) or 0.1 ml

epichlorohydrin (1.0 M in DMSO). The mixture was allowed to react for 1 hr with occasional shaking. Following this treatment the treated DNA was dialyzed at 0C against three 500 ml changes of SSC for 24 hrs. In alternate experiments the DNA-chemical mixtures were placed in a dialysis bag and immersed in the S-9 liver homogenate mix. These samples were dialyzed in SSC as above.

F. Competent Cultures for Transformation Assays

The procedures for the development of competence were similar to those described (23). B. subtilis cells were grown in a modified Spizizen's minimal medium (GMI) (29) for 90 min at 37C after cessation of logarithmic growth in a rotary incubator shaker. The cells were then diluted tenfold into GMII medium (29) and incubated for an additional 60 min at 37C in the shaker. At this time the culture has attained maximum competence.

G. Transformation Procedures

A sample (0.1 ml) of extracted, treated or untreated DNA was added to 0.8 ml of the competent cultures and incubated at 37C for 30 min in the shaker. The reaction was terminated by the addition of 0.1 ml of deoxyribonuclease (500 µg/ml, Worthington Biochem. Corp.) for 15 min at 37C. The cells were plated on appropriate selective minimal media and incubated at 37C for 48 hrs.

III. RESULTS

A summary of preliminary mutagenesis screening experiments with potential vinyl chloride monomer metabolites and related compounds is presented in Table II. It is evident that chlorooxirane and chloroacetaldehyde are the ultimate mutagens in this system. These results agree with the published data (3,16). In addition, this table describes the mutagenicity of the other chemical forms of chloroacetaldehyde, notably a monomer hydrate, a dimer hydrate, and a trimer. The hydrate and dimer hydrate forms have been shown to form an equilibrium mixture by the spontaneous rearrangement of chloroacetaldehyde under physiological conditions (Elmore, Wong, Laumbach, and Streips, submitted for publication), and these hydrate forms must be regarded as potential metabolites of consequence. Purified dimer hydrate and trimer were synthesized under laboratory conditions. Neither acetaldehyde, chloroacetic acid, nor chloroethanol showed a significant level of mutagenicity in these assays. Other investigators have reported the mutagenicity of chloroethanol, however, either high concentrations or activation with microsomal enzymes was required for activity (3,16). Our results agree with those of McCann et al (16). These experiments suggest a molecular relationship involving the proximity of the chloride group to the aldehyde moiety for mutagenic activity. In this regard we are currently examining structurally analogous ketones, substituted with various halogens. Epichlorohydrin (1-chloro-2,3 epoxypropane) was also mutagenic in screens using the Salmonella tester strain TA100.

[illegible] Epichlorhydrin (1.0 M in DMSO). The mixture was allowed to react for 1 hr with occasional shaking. Following this treatment the treated DNA was dialyzed at 3C against three 500 ml changes of SSC for 24 hrs. In alternate experiments the DNA-chemical mixtures were placed in a dialysis bag and immersed in the S-9 liver homogenate mix. These samples were dialyzed in SSC as above.

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Further experiments examined the effect of proposed metabolites on several different DNA repair deficient strains of B. subtilis. Chlorooxirane and the different forms of chloroacetaldehyde were all found to specifically inhibit the growth of strain MC-1, which lacks recombination repair (17) (Table III). Epichlorohydrin was capable of moderate reactivity only in the presence of the S-9 fraction.

Quantitative mutagenesis assays with Salmonella strain TA100, an indicator for base-pair substitution mutations, revealed that chloroacetaldehyde monomer had the highest mutagenic capacity of all the reactive metabolites (Table IV). The monomer-dimer hydrates, dimer hydrate, and trimer show progressively decreasing mutagenic efficiency as evidenced by the higher chemical concentration required for eliciting maximum reversion. All forms of chloroacetaldehyde were very toxic, thus the mutagenic response of each compound was limited to a narrow range of concentrations. However, epichlorohydrin, a weak mutagen by comparison, has a broad mutagenic spectrum and a corresponding low toxicity.

Since the mutagenic activity of the compounds constituted strong evidence that DNA was a primary target of attack, we examined the interaction of chloroacetaldehyde and epichlorohydrin with transforming DNA. It is known that the biological activity of transforming DNA can be altered by exposure to physical and chemical agents (8,22). Previous studies have shown that chloroacetaldehyde can bind to DNA in vitro (11). Accordingly, transforming DNA isolated from B. subtilis 168WT was treated with either chloroacetaldehyde or epichlorohydrin as described in Materials and Methods. The treated DNA was examined in transformation assays utilizing several different auxotrophic strains of B. subtilis as the recipients. Data presented in Table V reveals that in vitro treatment of DNA with either compound has little or no apparent effect on the biological activity of this DNA in transformation.

Since both chloroacetaldehyde and epichlorohydrin demonstrated mutagenic activity in the Salmonella TA100 strain, we examined the effect of these two compounds on B. subtilis DNA in vivo. Transforming DNA was isolated from B. subtilis following a 15 min exposure to the mutagenic chemicals. The DNA concentration was calculated from these samples, and levels equivalent to those used in the in vitro assays were added to competent cultures. The results of these transformation assays are shown in Table VI. Two major effects are evident with chloroacetaldehyde in vivo treated DNA. First, there was a major depression of the biological activity in the transforming DNA. Secondly, the depression showed genetic marker specificity. Moreover, the DNA segments containing genetic markers which have previously been shown to be associated to macromolecular structures such as the cell membrane (6,24,28) or the cell wall (Streips, Doyle, Sueoka, Brown, and Fan, submitted for publication) were selectively protected from attack by chloroacetaldehyde and epichlorohydrin. The activity of epichlorohydrin was less in these experiments, however, the patterns of specific marker inactivation are quite similar. The addition of the S-9 mix to the chemicals prior to addition to the cells, did not cause significant alteration in transformation efficiency (results not shown). In some samples there was an effect on the transforming DNA by DMSO, therefore all transformation values were corrected to account for this parameter.

IV. DISCUSSION

The major findings reported in this manuscript can be summarized: 1) We have confirmed the mutagenicity of chloroacetaldehyde and chlorooxirane, and extended it to include the additional potential metabolites, chloroacetaldehyde monomer hydrate, dimer hydrate and trimer, as well as the previously unreported chlorooxirane homolog, epichlorohydrin. 2) We have shown that recombination repair appears to be the mechanism for the correction of vinyl chloride metabolite elicited damage. 3) Chloroacetaldehyde causes a decrease in the biological activity of transforming DNA only if the cells are treated with the mutagen prior to the extraction of the DNA. In vitro studies showed no effect. 4) Epichlorohydrin apparently differs markedly from the vinyl chloride metabolites in mutagenic activity.

To understand the mutagenic potential of an environmental carcinogen, such as vinyl chloride and related chemicals,it is necessary to determine both its metabolic fate and probable mechanism of action for alteration of cellular processes. This report, as well as others, (3,14,16) has identified the potential active metabolites in vinyl chloride monomer mediated carcinogenesis. Furthermore, on the basis of a series of studies in microbial systems, we can postulate probable mechanisms of action of the vinyl chloride monomer metabolites and related chemicals. Understanding of these mechanisms is necessary for the development of possible blocking agents to the carcinogenic activity.

Recombination repair appears to be induced to correct DNA lesions caused by vinyl chloride monomer metabolites and epichlorohydrin. Salmonella strain TA100 which lacks excision repair (uvr$^-$), yet retains the capacity for recombination repair is capable of recovery and can express mutation following exposure. Furthermore, experiments with several repair-deficient B. subtilis mutants demonstrate that only the recombination repair mutant is specifically sensitive to the active metabolites, whereas the excision repair mutants and the wild type strain are relatively unaffected. The nature of the lesions may specifically evoke the recombination repair mechanism (10), or, alternatively, the chemical reactivity of the metabolites may directly suppress other repair. It is known that recombination repair is inducible, while other types of repair are mostly constitutive (5). Since chloroacetaldehyde has been shown to specifically interact with proteins containing -SH groups (J. Hoffman, personal communication), it is possible that the chemical could inactivate the constitutive repair enzymes leaving the repair to an inducible system.

The requirement for recombination repair of damage induced by these chemicals suggests the potential route of mutagenesis in bacteria. Recombination repair has been shown to be error prone (16). In this sense it resembles postreplication repair in mammalian cells (13). Thus, we can postulate that the analogous error prone repair pathway, postreplication repair, may function in mammalian cells in response to vinyl chloride metabolite elicited damage. A relationship between postreplication repair caused errors and somatic mutation and carcinogenesis has been suggested in patients with the skin disease, xeroderma pigmentosum (13).

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The increased inhibitory activity of the chloroacetaldehyde dimer and trimer forms for the other repair-deficient B. subtilis strains (Table III) may have been nonspecific killing of the cells, since all the strains other than MC-1 showed identical levels of inhibition. The necessity for metabolic activation of epichlorohydrin could reflect either a lack of permeability of the nonactivated compound or the requirement of a metabolite of this compound as the true mutagenic species.

Neither chloroacetaldehyde nor epichlorohydrin seemed to affect transforming DNA in vitro (Table V). Although several investigators have reported that CAA specifically modifies bases and causes mismatched base pairs (2,11), this reaction in vitro does not seem to affect the biological activity of the DNA. In contrast, DNA which was isolated from cells treated with either chloroacetaldehyde or epichlorohydrin (in vivo, Table VI) was severely affected. The overall biological activity of the transforming DNA is depressed, and it appears that the regions of the genome which are not protected by either the cell membrane or cell wall are most susceptible to attack and inactivation. It has also been postulated both in Escherichia coli and B. subtilis that the replication origin, terminus, and replication fork are all outer surface bound (18,24). Thus, these would be protected regions from chloroacetaldehyde attack and the nonreplicating DNA in the cytoplasm would be most susceptible. In this connection, recent experiments in our laboratory (Laumbach, Lee, Wong, and Streips, manuscript in preparation) have shown that chloroacetaldehyde causes enhanced mutation levels in cultures with nonreplicating genomes. This may imply that chloroacetaldehyde could be active in mammalian cells during growth stages where little DNA synthesis occurs.

The mode of action of epichlorohydrin, a known carcinogen (25), differs from that of the vinyl chloride monomer metabolites. Although epichlorohydrin causes similar base substitution mutations in Salmonella tester strain TA 100, it is a comparatively weaker alkylating agent based on quantitative assay. Epichlorohydrin also exhibits a lower toxicity level than vinyl chloride monomer metabolites, thus epichlorohydrin can demonstrate mutagenic activity through a wider range of concentrations. In addition, our laboratory has preliminary evidence that epichlorohydrin produces higher levels of mutation in Salmonella cultures which are actively replicating DNA than in cultures which have been arrested in DNA replication (Laumbach, Lee, Wong, and Streips, manuscript in preparation). The different activity spectra between chlorooxirane and its homolog epichlorohydrin points out the necessity for a multifaceted study of carcinogens.

V. SUMMARY

Our laboratories have utilized strains of B. subtilis and Salmonella typhimurium to investigate the mutagenicity of vinyl chloride metabolites and related compounds. The major findings reported in this manuscript are: 1) Confirmation of mutagenicity of chloroacetaldehyde and chlorooxirane. 2) Description of mutagenicity of additional potential metabolites of vinyl chloride, chloroacetaldehyde monomer hydrate, dimer hydrate, and trimer, as well as the mutagenic

carcinogenic chlorooxirane homolog, epichlorohydrin. 3) Recombination repair is postulated to be the mechanism for correcting vinyl chloride metabolite elicited damage. 4) Chloroacetaldehyde affects the transformation activity of DNA only if cells are treated with the mutagen prior to the extraction of the DNA. In vitro the chemical had no effect. 5) Epichlorohydrin differs from vinyl chloride metabolites in mode of action.

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Laumbach, A. D., Lee, S., Wong, J., and Streips, U. N.

Table I

Bacterial Strains

Bacillus subtilis	Genotype	Origin and Comments
RUB 783	purB6, leu-8, hisA1, metB10	U. Streips
BR 151	trpC2, lys-3, metB10	B. Reilly
BUL 709	ura-1, hisA1, leu-8, metB10	This laboratory
BUL 714	cysA, hisA1, leu-8, metB10	This laboratory
Hcr-9 (JB01-200)	trpC2	S. Okubo and W. Romig, hcr^-
MC-1	trpC2, recB2	S. Okubo and W. Romig, rec^-
FB-13	trpC2	C. Hadden, uvr^-
168WT	prototroph	A. Laumbach and I. Felkner

Salmonella typhimurium	Mutations in Strains				
	His^-	LPS	DNA Repair	R Factor	Mutation Detected
TA1535	hisB46	rfa	uvrB	-	base-pair substitution
TA100	hisB46	rfa	uvrB	pKM101	base-pair substitution
TA1537	hisC3076	rfa	uvrB	-	frameshift
TA1538	hisD3052	rfa	uvrB	-	frameshift
TA98	hisD3052	rfa	uvrB	pKM101	frameshift

STUDIES ON THE MUTAGENICITY OF VINYL CHLORIDE METABOLITES

Table II

Mutagenic Activity Assayed by Bacterial Test Systems

Compounds	Indirect Screen *B. subtilis* "Repair-Assay"	Direct Test[a] *S. typhimurium* Strain TA100
Acetaldehyde	NR[b]	NR
Chloroacetic Acid	NR	NR
Chloroethanol	NR	NR
Vinylidene Chloride	NR	NR
Vinyl Chloride	NR	NR
Chlorooxirane	+[c]	++[d]
Chloroacetaldehyde (monomer)	+++[e]	+++
Chloroacetaldehyde (monomer-dimer hydrates)	++	++
Chloroacetaldehyde (dimer hydrate)	+	+
Chloroacetaldehyde (trimer)	+	+
Epichlorohydrin	NR	+

[a] Experiments performed in absence of liver homogenate-mediated activation.

[b] NR no reaction detected

[c] + Reactive

[d] ++ Moderately reactive

[e] +++ Very reactive

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Chloroacetaldehyde (monomer-dimer hydrates)	++	++
Chloroacetaldehyde (dimer hydrate)	+	+
Chloroacetaldehyde (trimer)	+	+
Epichlorohydrin	NR	+

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Table III

"Repair-Assay" with Bacillus subtilis Strains

Compounds	Molar Concentration	Growth Inhibition in Millimeters[a]			
		168WT (hcr^+, rec^+)	MC-1 (hcr^+, rec^-)	Hcr-9 (hcr^-, rec^+)	FB-13 (uvr^+, rec^+)
Chloroacetaldehyde (monomer)	0.10	2.0	28.0	4.0	3.0
Chloroacetaldehyde (monomer-dimer hydrate)	0.115	NI[b]	23.0	NI	NI
Chloroacetaldehyde (dimer hydrate)	0.097	2.0	10.0	2.0	2.0
Chloroacetaldehyde (trimer)	0.096	7.0	15.0	6.0	7.0
Chlorooxirane	0.26	NI	10.0	NI	NI
Epichlorohydrin	0.997	NI	NI	NI	NI
Epichlorohydrin (plus liver homogenate)[c]	0.997	NI	3.0	NI	NI

[a]Average inhibition calculated from multiple experiments.

[b]No inhibition detected.

[c]9,000 x g supernatant (S-9) + NADPH generating system.

Table IV

Quantitative Mutagenicity Assay by Salmonella TA100 Reversion

Compound	Concentration in Soft Agar Layer mM/Plate[a]	Average Number Revertants/Plate[b]
Chloroacetaldehyde (monomer)	0.0004	265
Chloroacetaldehyde (monomer-dimer hydrate)	0.054	977
Chloroacetaldehyde (dimer hydrate)	0.490	311
Chloroacetaldehyde (trimer)	0.240	159
Epichlorohydrin	4.746	2856

[a]Highest effective non-toxic concentration for reversion.

[b]Spontaneous background revertants subtracted.

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[b]Spontaneous background revertants subtracted.

Table V

Effect of Chloroacetaldehyde and Epichlorohydrin of Transforming DNA *In vitro*

Recipient Strains [c]	Relative Transformation Efficiency[a] metB10	leu-8	cysA	hisA1	ura-1	trpC2	lys-8	purB6
Epichlorohydrin treated DNA								
BUL 714	.92	.97	.97	1.16				
RUB 783	.91	.60		.98				.77
BUL 709	.99	.95		1.02	.85			
BR 151	1.48					1.07	.62	
Chloroacetaldehyde treated DNA								
BUL 714	1.43	.92	.55	.91				
RUB 783	.93	1.45		.75				.89
BUL 709	.86	1.33		.90	.75			
BR 151						ND[b]	.77	

[a]Relative transformation efficiency calculated: $\frac{\text{number of transformants with treated DNA}}{\text{number of transformants with untreated DNA}}$

[b]Not determined.

[c]Conditions for competence and transformation as described in Materials and Methods.

Table VI

EFFECT OF CHLOROACETALDEHYDE AND EPICHLOROHYDRIN ON TRANSFORMING DNA *IN VIVO*

Recipient Strains[b]	Relative Transformation Efficiency[a] metB10	leu-8	cysA	hisA1	ura-1	trpC2	lys-3	purB16
Chloroacetaldehyde *in vivo* treated DNA								
BUL 714	.54	.09	.08	.36				
RUB 783	.33	.10		.35				.10
BUL 709	.53	.07		.32	.34			
BR 151	.37					.13	.11	
Epichlorohydrin *in vivo* treated DNA								
BUL 714	.55	.17	.25	.46				
RUB 783	.52	.15		.38				ND[c]
BUL 709	.59	.15		.50	.36			
BR 151	.37					.11	.19	

[a]Relative transformation efficiency calculated: $\frac{\text{number transformants with treated DNA}}{\text{number transformants with untreated DNA}}$

[b]Conditions for competence and transformation as described in Materials and Methods.

[c]Not determined.

Table VI

EFFECT OF CHLOROACETALDEHYDE AND EPICHLOROHYDRIN ON TRANSFORMING DNA IN VIVO

Recipient Strains[b]	Relative Transformation Efficiency[a] metB10	leu-8	cysA	hisA1	ura-1	trpC2	lys-3	purB16
Chloroacetaldehyde in vivo treated DNA								
BUL 714	.54	.09	.08	.36				
RUB 783	.33	.10		.35				.10
BUL 709	.53	.07		.32	.34			
BR 151	.37					.13	.11	
Epichlorohydrin in vivo treated DNA								
BUL 714	.55	.17	.25	.46				
RUB 783	.52	.15		.38				ND[c]
BUL 709	.59	.15		.50	.36			
BR 151	.37					.11	.19	

[a]Relative transformation efficiency calculated: $\frac{\text{number transformants with treated DNA}}{\text{number transformants with untreated DNA}}$

[b]Conditions for competence and transformation as described in Materials and Methods.

[c]Not determined.

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 7.79×10^{-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 5, the monomer hydrate 6, the dimer hydrate 7, and the trimer 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 5 and its cyclic trimer 8 if water was totally absent, or it may have been a mixture of chloroacetaldehyde hydrates 6 and 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 6 and 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 4 [9] may be attributed to a chloroacetaldehyde hydrate rather than the chlorooxirare 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 9 which is a stable chloro-epoxide homolog of 4 as a comparative assay to interpret the observations of the activity of chlorooxirane.

This investigation used the above-mentioned compounds 1 - 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

dose-response comparison with its proximate metabolites. Regarding the latter, the exact chemical forms of the proximate metabolites analyzed or 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 5, the monomer hydrate 6, the dimer hydrate 7, and the trimer 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 5 and its cyclic trimer 8 if water was totally absent, or it may have been a mixture of chloroacetaldehyde hydrates 6 and 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 6 and 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 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 9 which is a stable chloro-epoxide homolog of 4 as a comparative assay to interpret the observations of the activity of chlorooxirane.

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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 1 was tested by the method of Ames [7]. A mixture of 0.1 ml of 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 2 - 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 6 and 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 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 4; glpc (gas liquid phase chromatography)

t_R = 1.2 min at 50°C, infrared absorption (ν 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 317-318°C (317°C reported [21]).

Chloroacetaldehyde monomer 5 was obtained in the purest form by cracking the chloroactaldehyde trimer 8 at 95°C and distilling it into dry DMSO; glpc t_R = 2.25 min at 100°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 Vigreaux fractionating column. The fraction (1/5 of the initial volume) collected from 87-100°C was redistilled. This second distillate at 83-92°C crystallized after 3 days at -15°C. Upon sublimation of 60°C and 1 atm, white crystals of 7 were obtained; mp 55°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°C). The crystalline precipitate was filtered after standing overnight at -15°C, washed with 5 ml of cold 20% aqueous methanol, and recrystallized 5 times from cold methanol; mp 87-88°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 1 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 1 was prepared by condensing it (bp -13.4°C) at -78°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 μl. 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 μl (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°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 1 in the 10 ml solution, or the concentration of 1 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),

were prepared in dimethyl sulfoxide (DMSO). A 0.1 ml aliquot of the sample solution was admixed with 0.9 ml of one tester strain culture. Then, a 0.1 ml sterile portion 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.

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Tissue and Urinary Glycosaminoglycan Patterns Associated with a Fast, an Intermediate, and a Slow-growing Morris Hepatoma[1]

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ABSTRACT

The purpose of this investigation was to evaluate the glycosaminoglycans (GAG's) in different behavioral-histological types of i.m.-transplanted hepatomas and in the liver and urine of animals bearing these tumors. Groups of 10 Buffalo rats carrying fast-growing (7777), intermediate (5123tc), and slow-growing (9618A) Morris hepatomas were studied as the tumors reached 3 cm. Urinary and tissue GAG's were isolated by proteolysis, separated as cetylpyridinium complexes, and measured as uronic acid. The GAG's were further purified using anion-exchange chromatography and characterized with mucopolysaccharidases. Tissue GAG's were also evaluated histochemically using Alcian blue staining and mucopolysaccharidases. Tissue from fast-growing, intermediate, and slow-growing tumors exhibited greater GAG levels than did normal liver in the hyaluronic acid (0.4 M NaCl-soluble) fraction and in the chondroitin sulfate-heparan sulfate (1.2 M NaCl-soluble) fraction. The livers of tumor-bearing animals exhibited GAG levels similar to those of normal liver. Increased urinary GAG excretion was appreciated in animals bearing Tumors 5123tc and 9618A but not in those bearing Tumor 7777.

INTRODUCTION

An increasing number of reports cite the presence of comparatively high levels of GAG's[3] in both animal tumors (4, 7, 18, 22) and human tumors (5, 12, 16). There also have been reports citing qualitatively and quantitatively abnormal urinary GAG excretion in association with malignant tumors (6, 10, 11, 23–25). Although there have been attempts to establish any functional relationship between tumor GAG's and tumor cell properties (23, 25–27, 30, 31), the significance of elevated GAG's in malignant tumors remains obscure.

In view of the possibility that GAG's play an important role in the expression of one or more malignant cell properties, our purpose here was to evaluate the GAG patterns associated with transplantable hepatomas exhibiting different growth rates and metastatic properties. Because it has been suggested that tumor GAG may be contributed by normal host tissue in response to the presence of hepatic tumor (7), a secondary purpose was to evaluate the influence, if any, of "remote" hepatomas on the GAG's of the host liver; a third purpose was to evaluate the urinary GAG patterns in hepatoma-bearing animals.

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[3] The abbreviation used is: GAG, glycosaminoglycan.

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MATERIALS AND METHODS

Materials. Hyaluronic acid (umbilical cord) was purchased from Nutritional Biochemical Corp. (Cleveland, Ohio); chondroitin sulfate (whale and shark cartilage) and sodium heparin were purchased from Sigma Chemical Co. (St. Louis, Mo.). Authentic samples of heparin, chondroitin 4-sulfate, heparan sulfate, and hyaluronate were also kindly supplied by Dr. M. B. Matthews, University of Chicago. Bovine testicular hyaluronidase was purchased from ICN Pharmaceuticals (Cleveland, Ohio), *Streptomyces* hyaluronidase was obtained from Calbiochem (La Jolla, Calif.), and *Proteus vulgaris* chondroitinase ABC was purchased from Sigma.

Experimental Design. Forty male Buffalo rats were shipped from Lab Supply Company (Indianapolis, Ind.) to Washington, D. C., where 10 were inoculated bilaterally (thigh) with Tumor 7777, 10 were inoculated with Tumor 5123tc, and 10 were inoculated with Tumor 9618A. These were shipped to Louisville with 10 controls. Throughout the study, animals were provided free access to food and water even when placed in metabolic cages for urine collections.

Urine collections were made twice each week from animals bearing Tumor 7777 and once each week from animals bearing Tumors 5123tc and 9618A. Collections were made alternatively on one-half of the animals in each group, with 5 control animal collections made each time a collection was made from tumor-bearing animals.

Tumors. Line 5123tc is a tissue culture variant of a moderately differentiated trabecular hepatocellular carcinoma induced with dietary administration of *N*-2-fluorenylphthalamic acid. Tumors were received and studied here in the 165th generation.

Tumor line 7777 is a poorly differentiated hepatocellular carcinoma induced by dietary administration of *N*-2-fluorenylphthalamic acid. This line was studied in the 159th transplant generation.

Tumor line 9618A is a well-differentiated hepatocellular carcinoma induced by 2-(4'-methyl)benzoylaminofluorene. This tumor was studied in the 13th generation.

Characteristics of these 3 tumors observed in our laboratory and selected characteristics reported by Hruban *et al.* (13, 14) are summarized in Table 1 (see also Fig. 1).

Extraction and Purification of GAG's from Liver and Tumor Tissue. Dry defatted tissue was subjected to proteolysis, trichloroacetic acid precipitation, and dialysis, and th GAG's were separated as cetylpyridinium chloride complexes into 0.03 M NaCl-soluble, 0.4 M NaCl-soluble, 1.2 M NaCl-soluble, and 2.1 M NaCl-soluble fractions as described by Schiller *et al.* (28) and measured as uronic acid (see Schiller *et al.*) by the carbazole method of Bitter and Muir (1).

linearity of the response of 1 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 1 was prepared by condensing it (bp -13.4°C) at -78°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 µl. 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 µl (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°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 1 in the 10 ml solution, or the concentration of 1 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 6 and the second to the dimer hydrate 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 6 to form the dimer hydrate 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 5. Dehydration of 6 and 7 must have occurred under the glpc conditions thereby reverting them to the monomer form. As shown in Table II, the PMR spectra of 6 and 7 are resolved from one another. Thus, the PMR spectrum of the 45% commercial solution also revealed the presence of both hydrates 6 and 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_2O and DMSO-d_6 were used with 3-(trimethylsilyl)propanesulfonic acid sodium salt as internal reference. Tetramethylsilane was used as a reference in $CDCl_3$ and CCl_4. 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

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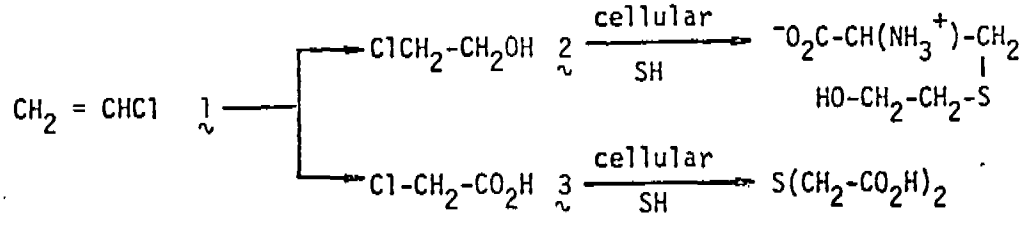
presaturated vinyl chloride broth solution at 25°C and 1 atm. The initial concentration of 0.022 M of vinyl chloride 1 rapidly decayed by 50% in 15 hr. Thereafter the escape of 1 from the broth slowed considerably. In the next 45 hr there was a further decline of only 18%. Thus the bacteria strains (B. subtilis MC-1 and Salmonella TA 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

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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_2O-DMSO-d_6 (80:20) mixture at pD 7.1 and 4°C by PMR technique showed that the rearrangement followed first order kinetics, k (sec^{-1}) = 2.5×10^{-4}. This translates to a half life of 46.2 min

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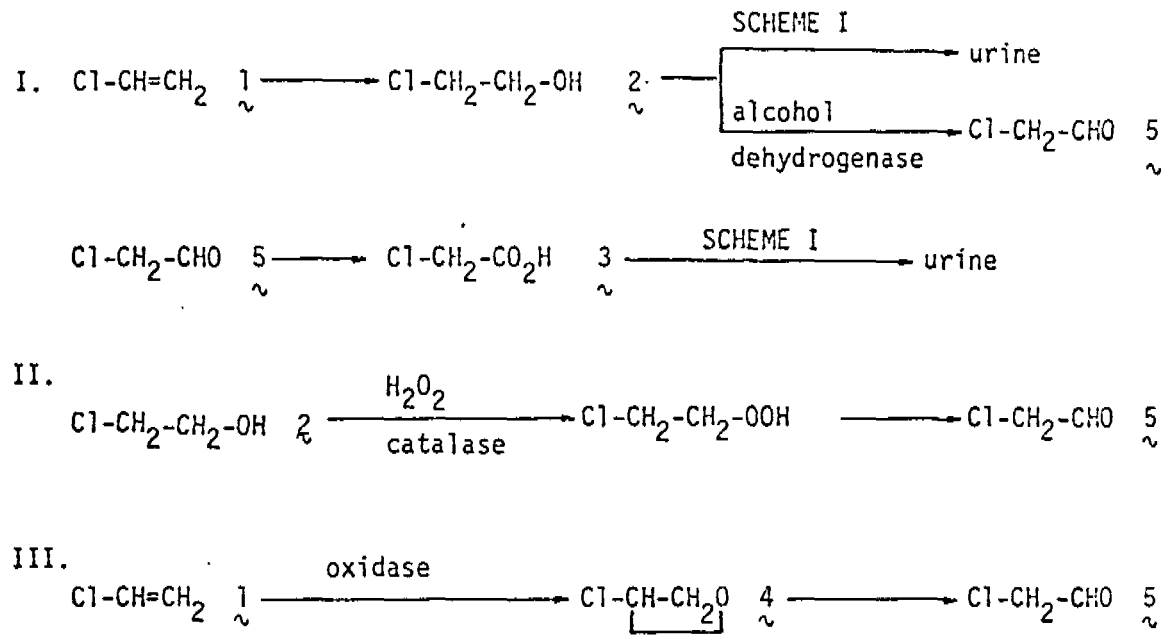
at 4°C as compared to 1.6 min at 37°C [9]. Such instability allows only limited testing in the cold as well as interpretation of the results. On the other hand, epichlorohydrin 9, which can be considered the epoxidation metabolite of allyl chloride, is a methylene homolog of 4. Both 4 and 9 are structurally 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⁻ 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 rec$^-$ phenotype (MC-1) was immediately inactivated. All the 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

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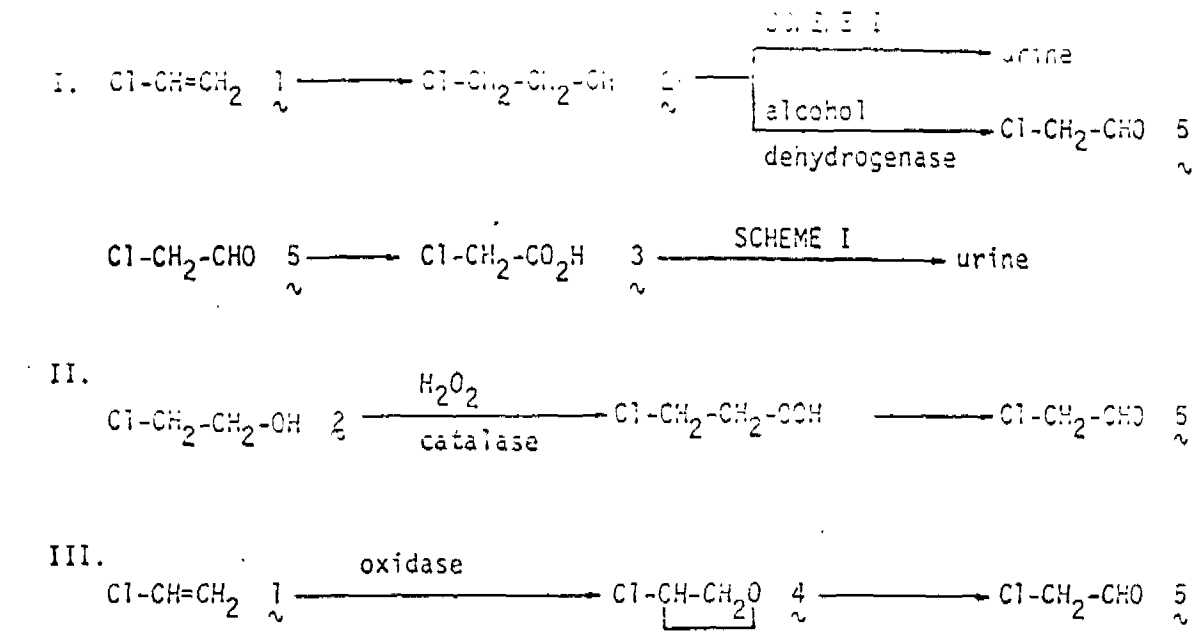

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has been suggested [24]. Cells from patients with the skin disease xeroderma pigmentosum lack the ability to excise pyrimidine dimers and must rely on postreplication repair to remove these lesions [24]. It is believed that this error prone process 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^1 and N^6 nitrogens of adenosine and N^3 and N^4 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 chloroxirane 4 compared to 6 may reflect the unstable nature of chlorooxirane as an α-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 $\dot{C}lCH\text{-}CH_2\dot{O}$. 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.

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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 ClH_2CCHO) 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 ClH_2CCHO) 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^-, rec^+ (✣—✣); Hcr-9 hcr^-, rec^+ (◇—◇); 168M wild type (○—○); and MC-1 uvr^+, rec^- (▷—▷).

Fig. 4. Dose response curves with Salmonella typhimurium TA100. Sample solution 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 ClH_2CCHO) 6 and 7 (◇—◇); chloroacetaldehyde dimer hydrate 7 (▷—▷); chloroacetaldehyde trimer 8 (✕—✕); and epichlorohydrin 9 (□—□).

LEGENDS TO FIGURES

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

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 ClH_2CCHO) 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.

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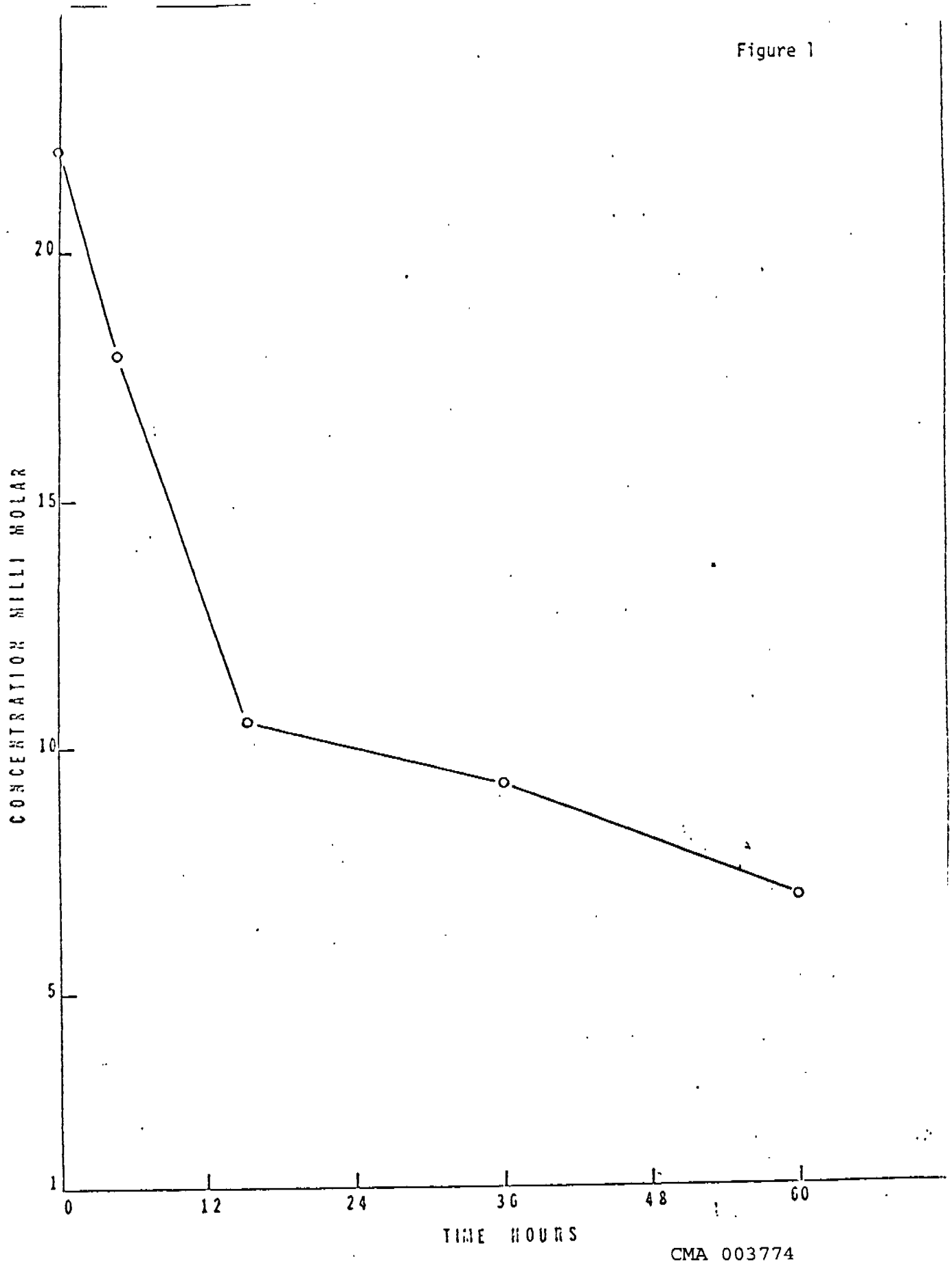
Figure 1



CMA 003774

Figure 2

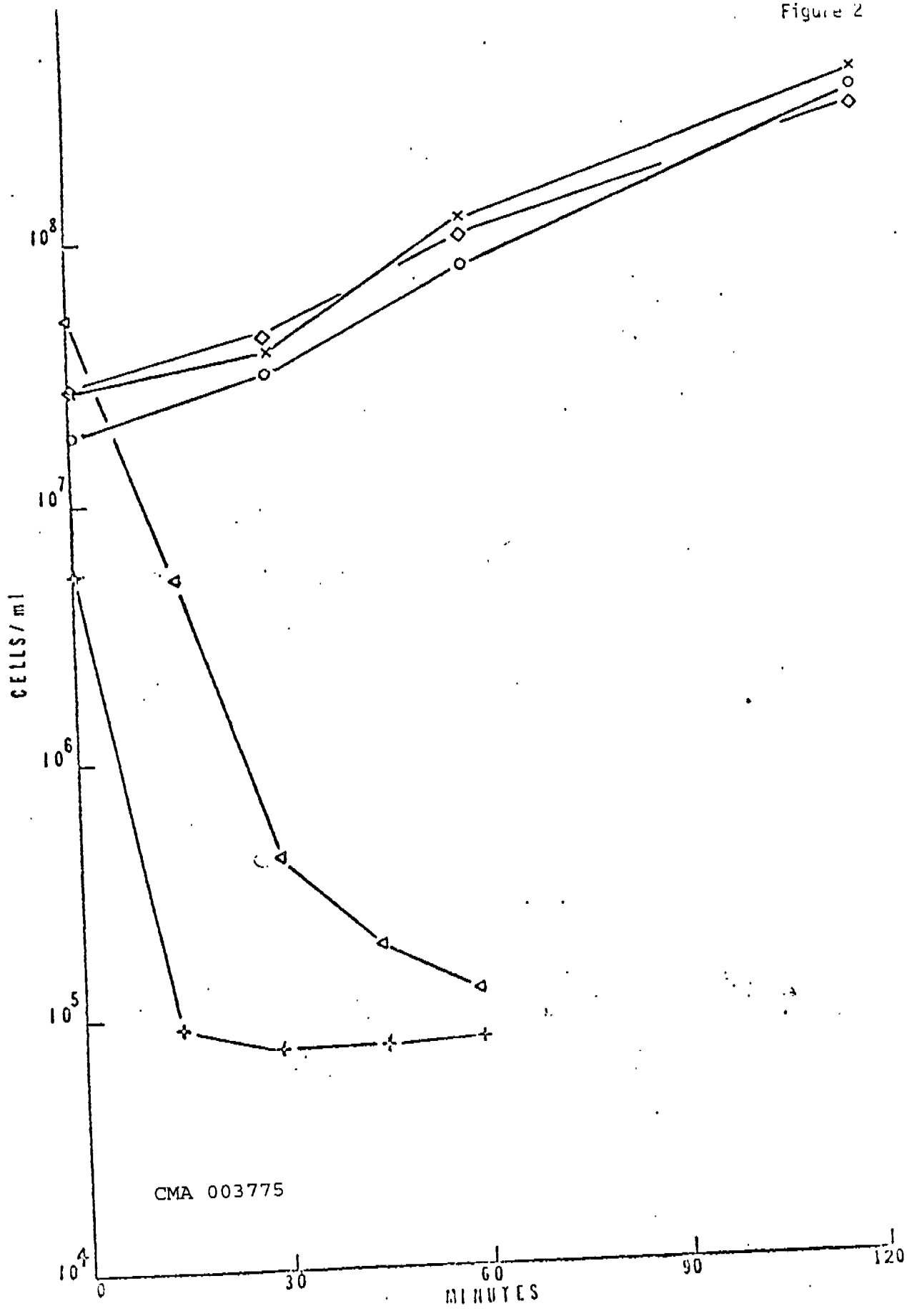
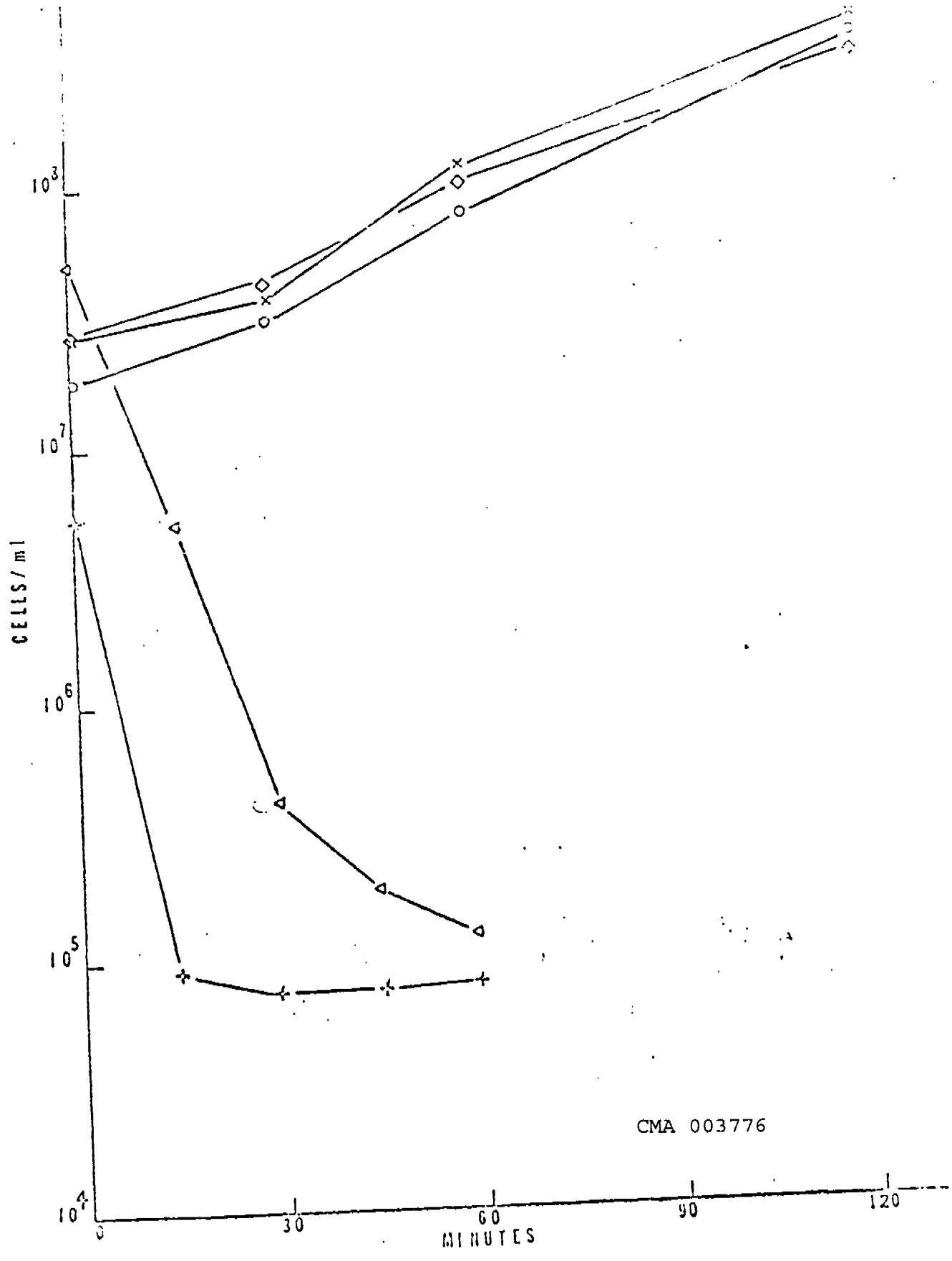


Figure -



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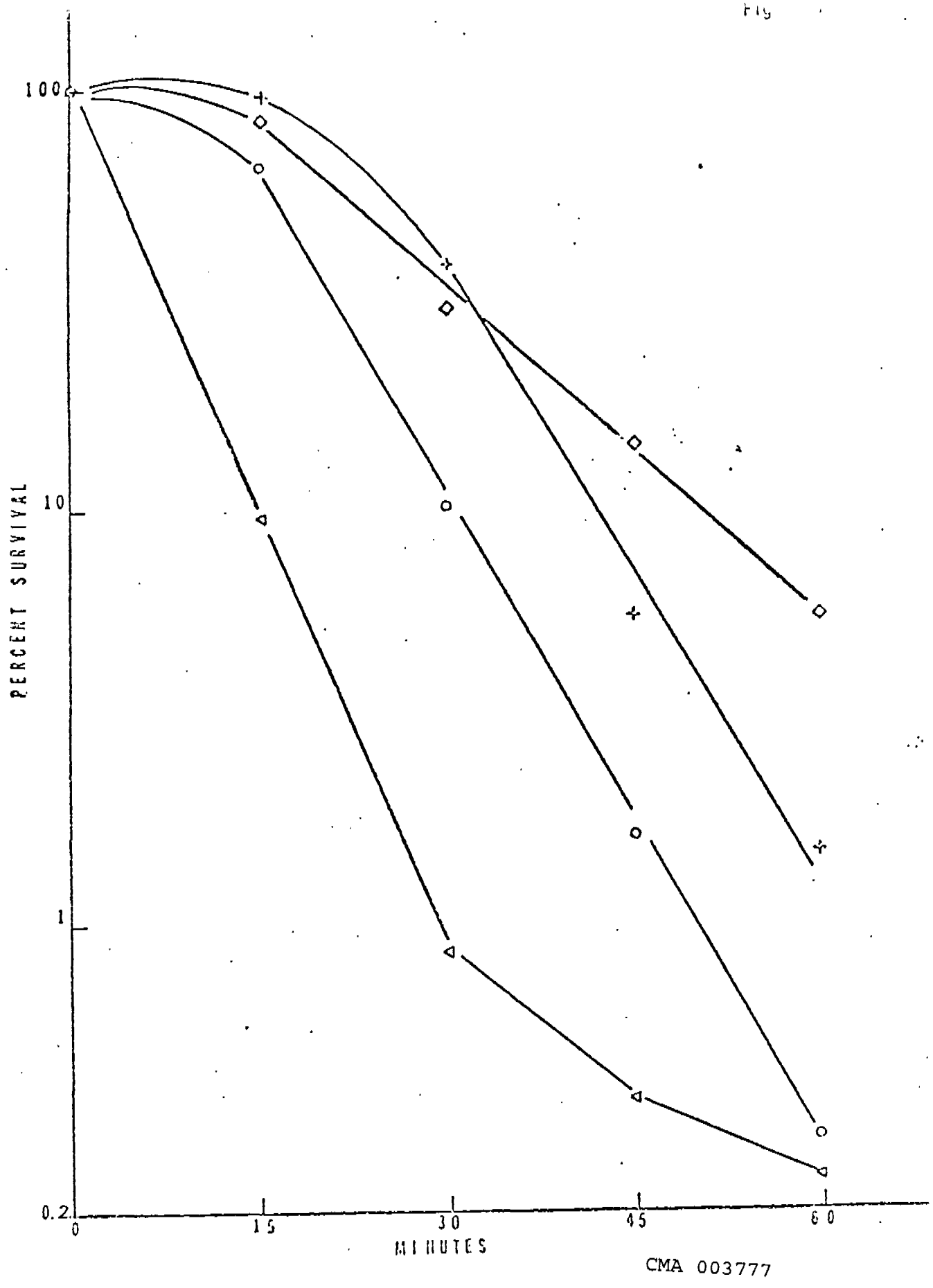

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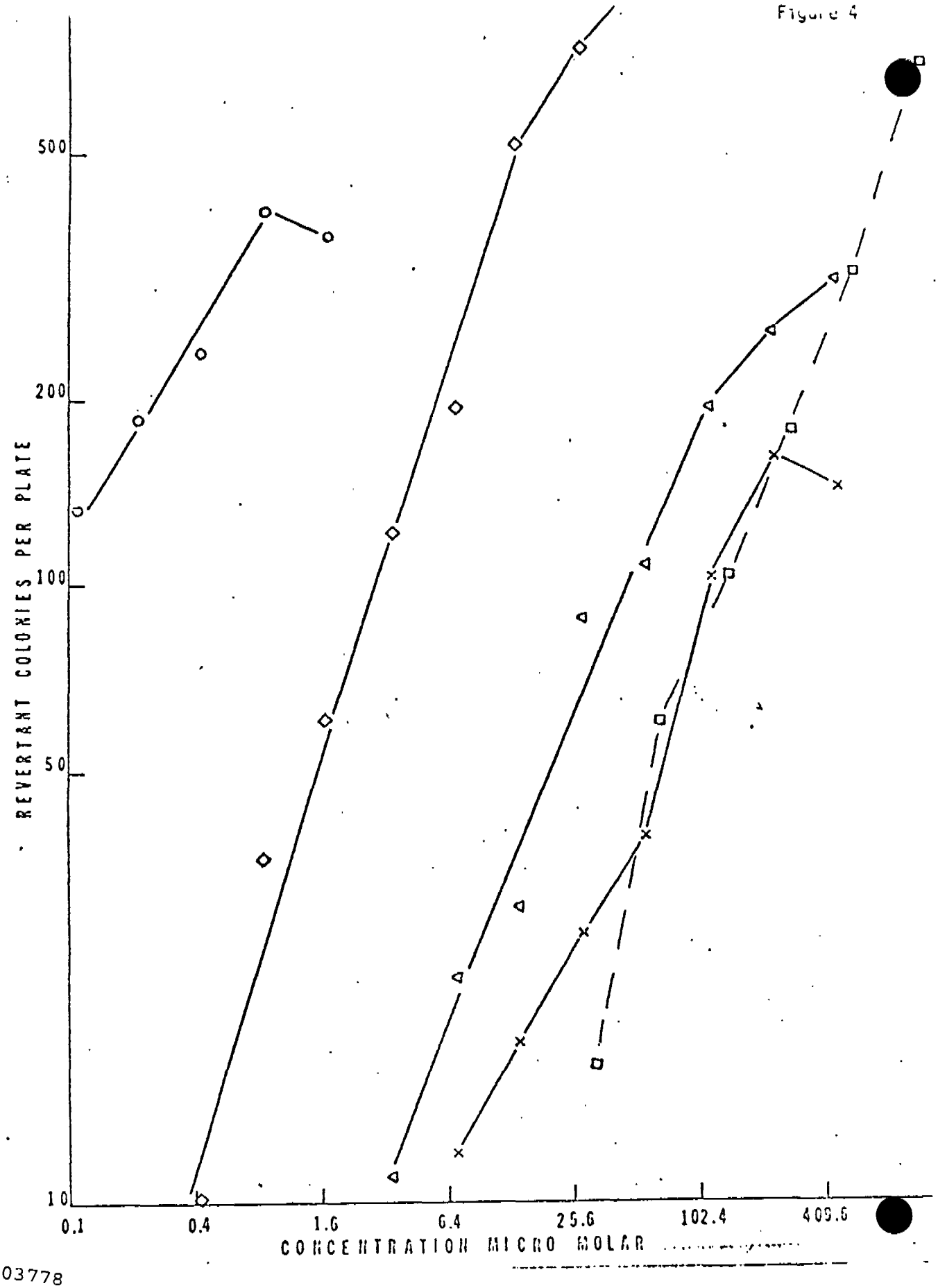
Figure 4



CMA 003778

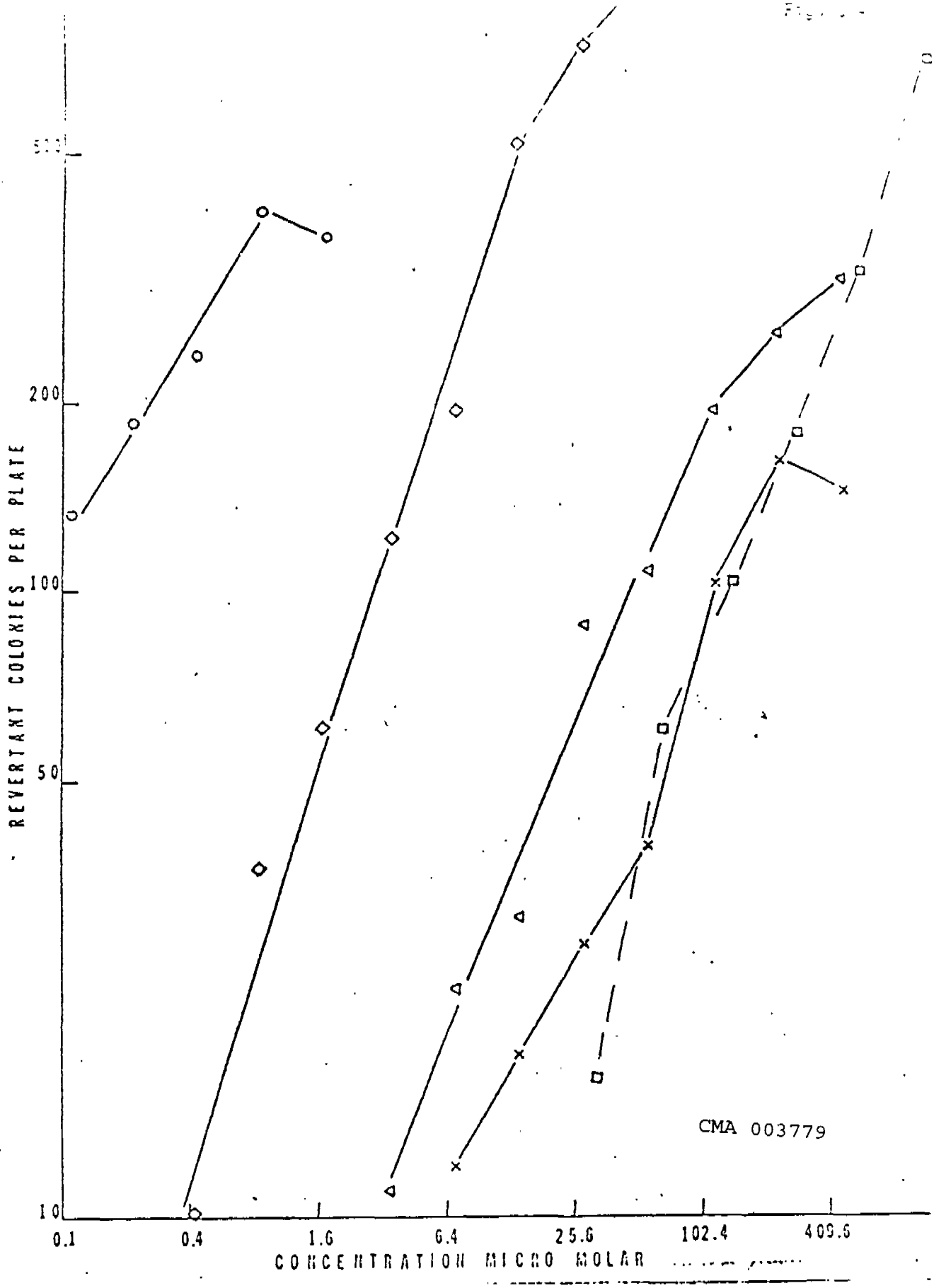

REVERTANT COLONIES PER PLATE
200
100
50
10
0.1
0.4
1.6
6.4
25.6
102.4
409.6
CONCENTRATION MICRO MOLAR
CMA 003779

TABLE I: Bacteria Tester Strains

A. *Salmonella typhimurium* LT-2 Tester Strains[a]

[a]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.

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

[c]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[b]	Mutation detected[c]
TA 1535	-	base-pair substitution
TA 100	+	base-pair substitution
TA 1537	-	frameshift
TA 1538	-	frameshift
TA 98	+	frameshift

B. *Bacillus subtilis* Tester Strains

[a]Trp^- denotes a requirement for tryptophan; Mit-S denotes sensitivity to mitomycin C.

[b]hcr^+ denotes a host-cell reactivation DNA repair capacity; hcr^- lacks a host-cell reactivation DNA repair capacity; rec^+ denotes a recombination DNA repair capacity; rec^- lacks a recombination DNA repair capacity; uvr^- is sensitive to ultraviolet-induced DNA damage.

Strains	Phenotype[a]	DNA Repair[b]
168 M	Prototroph (wild type)	hcr^+, rec^+
Hcr-9	Trp^-	hcr^-, rec^+
FB-13	Trp^-	uvr^-, rec^+
MC-1	Trp^-, Mit-S	hcr^+, rec^-

TABLE II. PMR Spectra of Vinyl Chloride Derivatives

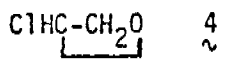
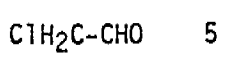
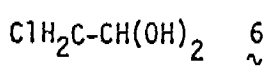
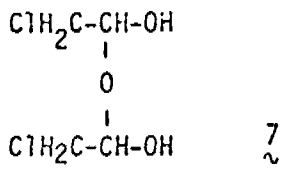
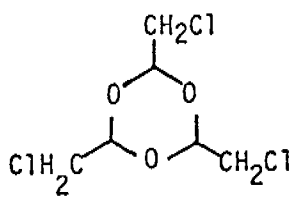
Compounds		Solvent	Spectra, δTMS=0 (J=Hz)
	4	CCl_4	2.75 (q, CH, J=1.5) 2.85 (q, CH, J=2.4) 4.90 (q, CH, J=2.4,1.5)
	5	CCl_4	4.00 (d, CH, J=2.2) 9.57 (t, CH_2, J=2.2)
		DMSO-d_6	3.50 (d, CH) 9.60 (t, CH_2)
	6	CD_3OD-D_2O 1:8 (pD 0.1)	3.60 (d, CH_2, J=5) 4.60 (t, CH, J=5)
	7	DMSO-D_6	3.55 (d, CH_2, J=4.9) 5.05 (t, CH, J=4.9)
		CD_3OD-D_2O 1:8 (pD 0.1)	3.60 (d, CH_2, J=4.5) 4.83 (t, CH, J=4.5)
	8	CCl_4	3.52 (d, CH_2, J=4.7) 5.08 (t, CH, J=4.7)
		DMSO-d_6	3.75 (d, CH_2, J=4.1) 5.45 (t, CH, J=4.1)

TABLE II. PMR Spectra of Vinyl Chloride Derivatives

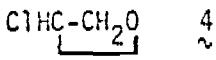
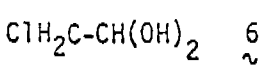
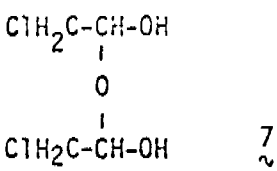
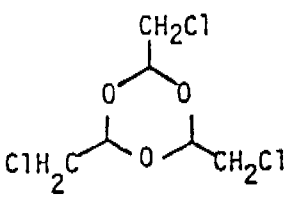
Compounds		Solvent	Spectra, δT, δ=0 (Int.
	4	CCl_4	2.75 (q, CH, J=1.5) 2.85 (q, CH, J=2.4) 4.90 (q, CH, J=2.4,1.5)
	5	CCl_4	4.00 (d, CH, J=2.2) 9.57 (t, CH_2, J=2.2)
		DMSO-d_6	3.50 (d, CH) 9.60 (t, CH_2)
	6	CD_3OD-D_2O 1:8 (pD 0.1)	3.60 (d, CH_2, J=5) 4.60 (t, CH, J=5)
	7	DMSO-D_6	3.55 (d, CH_2, J=4.9) 5.05 (t, CH, J=4.9)
		CD_3OD-D_2O 1:8 (pD 2 0.1)	3.60 (d, CH_2, J=4.5) 4.83 (t, CH, J=4.5)
	8	CCl_4	3.52 (d, CH_2, J=4.7) 5.08 (t, CH, J=4.7)
		DMSO-d_6	3.75 (d, CH_2, J=4.1) 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	Bacillus subtilis Repair Assay	Salmonella typhimurium Reversion Assay
Control: 4-nitroquinoline-N-oxide	++	++
1 $H_2C=CHCl$	NI	NR
2 ClH_2C-CH_2OH	NI	NR
3 $ClH_2C-COOH$	NI	NR
4 $ClHC-CH_2-O$	+	++
5 ClH_2C-CHO	++	++
6 $ClH_2C-CH(OH)_2$	++	++
7 $ClH_2C-CHOH-O-CHOH-CH_2Cl$	++	++
8 $(ClH_2C-\overset{|}{C}HO-)_3$	++	+
9 $ClH_2C-CH-CH_2O$	NI	++

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

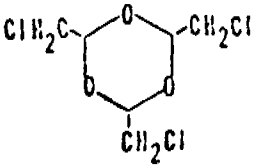
Mutagen	Molarity	168M	MC-1	Hcr-9	FB-13
ClH_2C-CHO 5	0.100	2.0	27.7	3.7	2.7
Chloroacetaldehyde (45% aqueous solution) 6 & 7	0.115	NI	22.5	NI	NI
ClH_2C-CH-OH O ClH_2C-CH-OH 7	0.097	1.5	9.5	1.5	1.5
 8	0.096	7.0	14.5	6.0	[illegible]
ClHC-CH_2O 4	0.260	NI	10.0	NI	NI
ClH_2C-CH-CH_2O 9	0.113	NI	NI	NI	NI
4-Nitroquinoline-N-oxide (control)	0.001	10.0	18.0	15.0	15.0

TABLE [illegible]

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

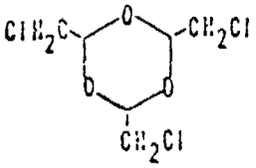
Mutagen	Molarity	168M	MC-1	Hcr-9	FB-1[illegible]
ClH_2C-CHO 5	0.100	2.0	27.7	3.7	2.7
Chloroacetaldehyde 5 & 7 (45% aqueous solution)	0.115	NI	22.5	NI	NI
$ClH_2C-CH-OH$ O $ClH_2C-CH-OH$ 7	0.097	1.5	9.5	1.5	1.5
 8	0.096	7.0	14.5	6.0	7.0
$ClHC-CH_2O$ 4	0.260	NI	10.0	NI	NI
$ClH_2C-CH-CH_2O$ 9	0.113	NI	NI	NI	NI
4-Nitroquinoline-N-oxide (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 6 : 7 50:50		Monomer 6		Dimer Hydrate 7		Trimer 8	
Molarity	Revertants	Molarity	Revertants	Molarity	Revertants	Molarity	Revertants
5.3×10^{-5}	977	1.3×10^{-5}	18	4.8×10^{-4}	311	4.8×10^{-4}	144
2.7×10^{-5}	723	6.7×10^{-6}	68	2.4×10^{-4}	259	2.4×10^{-4}	159
1.4×10^{-5}	512	3.3×10^{-6}	88	1.2×10^{-4}	193	1.2×10^{-4}	101
6.9×10^{-6}	194	1.7×10^{-6}	361	6.0×10^{-5}	107	6.0×10^{-5}	39
3.4×10^{-6}	120	8.4×10^{-7}	404	3.0×10^{-5}	88	3.0×10^{-5}	27
1.7×10^{-6}	61	4.2×10^{-7}	238	1.5×10^{-5}	30	1.5×10^{-5}	18
8.6×10^{-7}	36	2.1×10^{-7}	185	7.5×10^{-6}	23	7.4×10^{-6}	12
4.3×10^{-7}	10	1.1×10^{-7}	131	3.8×10^{-6}	11	3.7×10^{-6}	-0

TABLE VI: Reversion of S.typhimurium 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 Time	Epichlorohydrin 9 (1.0 mM)	Chlorooxirane 4 (0.26 mM)
0 hr	186	31
1 hr	204	4
2 hr	297	6
4 hr	202	114
6 hr	154	44

TABLE VI: Reversion of Strain 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 Time	Epichlorohydrin 9 (1.0 mM)	Chlorooxirane 4 (0.26 mM)
0 hr	186	31
1 hr	204	4
2 hr	297	6
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6 hr	154	44