Investigation of Mutagenicity due to Compounds other than MX in Chlorinated Humic and Drinking Water.

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ABSTRACT

3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, i.e MX, is recognized as a principal mutagen contributing to (on an average) 30% of the mutagenicity of chlorinated drinking water in the Ames assay. Compounds with structural similarities to MX were suggested as probable mutagens which could account for the remaining 70% of the mutagenicity of chlorinated water. The theoretical basis for the formation and activity of these MX analogues was discussed. Three such candidate mutagens, red-MX, ox-MX and ox-EMX were synthesized, and their mutagenic activities determined (in the Ames assay, strain TA 100) to be 0.13 net revs./ng; 0.36 net revs./ng and 0.03 net revs./ng respectively. It would appear that the aldehyde group is a critical structural feature that governs the mutagenicity of MX. The concentration of MX in samples of chlorinated fulvic acids, humic and drinking water was determined. MX accounted for 36%, 32% and 17% of the overall mutagenicity of these samples, respectively. SIM mode GC/MS analyses on derivatized and underivatized extracts of the chlorinated waters revealed that red-MX and ox-MX were present in concentrations equal to or slightly greater than the amount of MX, while the concentration of ox-EMX was about 20-40

times higher. Due to their weak mutagenicities however, all three compounds were found to account for (individually) less than 1% of the overall activity in each sample.

Mutagenicity-directed HPLC fractionation was performed on a chlorinated solution of fulvic acids, selected because it was highly mutagenic but contained a low amount of MX (72% non-MX mutagenicity). After the second level of separation, all the activity had concentrated into a single sub-fraction (31 000 net revs./liter) which was free of MX. The responsible mutagens seem to be less polar compounds in comparison to MX.

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CONTENTS

LIST OF FIGURES.

LIST OF TABLES.

I: INTRODUCTION

Drinking water contains a complex mixture of organic chemicals, many of which are known to be potentially harmful to human health. Some of these compounds are introduced into source waters by industrial, agricultural and municipal effluents. Others however, are formed de novo by the very disinfection process meant to make our drinking water microbiologically safe. In fact, emphasis on the problem of organic micropollutants in drinking water has shifted markedly to the issue of these chlorination by-products.

In the last 15 years or so, great strides have been made in detecting these by-product compounds in drinking water (Rook,1974; Bellar, 1974; Symons et. al.,1975), recognizing their genotoxic and carcinogenic potential (Simmon and Tardiff, 1976; Nat. Cancer Inst., 1976; Loper,1978; Nestman et. al., 1979; Lang et. al., 1980; Cheh et. al., 1980; Kool et. al., 1985; Wilcox and Williamson, 1986) and understanding the mechanism of their formation (Cheh et. al., 1980; Christman et. al., 1983; Coleman et. al., 1984; Holmbom et. al., 1984; Kronberg et. al., 1985b; Meier et. al., 1986; Maruoka, 1986). From the regulatory standpoint in the United States, there is a need to identify

and examine the principal disinfection by-products in order to promulgate standards under Phase IV of the Revised National Primary Drinking Water Regulations (Cotruvo and Vogt, 1985). Thus, much of the recent research has concentrated on identifying and structurally characterizing the mutagens present in drinking water, particularly the non-volatile organohalides.

Although most of the mutagenic activity of drinking water can be attributed to organics in the non-volatile fraction, only a single major mutagen, 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone, i.e MX, has been identified so far (Kronberg et. al., 1987; Meier et. al., 1987b). On an average, this compound can account for about 30% of the total mutagenicity of the organic fraction. In order to get a more comprehensive understanding of the health hazards posed by the presence of mutagens in drinking water, chemical identities of compounds responsible for the residual two-thirds of the mutagenicity must be determined. This would allow syntheses of the pure compounds to be used in long-term animal toxicity and carcinogenicity studies as is being done with MX.

The objective of this research was to elucidate the structures and activities of compounds which could possibly be responsible for the yet uncharacterized mutagenicity (non-MX mutagenicity) of chlorinated drinking water. This objective was approached through two specific tasks:

(I) Investigation of specific candidate mutagens

i.e the MX analogues: Synthesize compounds with (critical) structural similarities to MX, determine their mutagenic potencies and subsequently search for these compounds in chlorinated fulvic acids, humic water and drinking water.

(II) Mutagenicity directed sub-fractionation: Chromatographic separation (with respect to mutagenicity) of a highly mutagenic extract where the amount of MX has been minimized, followed by GC/MS analysis of the ultimate active subfraction(s).

II: BACKGROUND.

2.1 : Chlorination By-Products in Drinking Water.

More than a decade of research has gone into understanding the formation and occurrence of these genotoxic compounds in drinking water. Some of the important aspects are discussed below.

2.1 (a) : Occurrence in Drinking Waters.

The initial concern about the presence of chlorination by-products was sparked by the discovery of trihalomethanes (THMs) and other volatile organohalides in drinking water in The Netherlands (Rook, 1974) and in the United States (Bellar et. al., 1974). The first of such chemicals identified were primarily the bromo- and chloro- substituted methanes. The widespread occurrence of these compounds in U.S. drinking waters was confirmed by the National Organics Reconnaissance Survey (Symons et. al., 1975). Since then, numerous other by-product compounds have been identified and detected in water. It is now known that a majority of these compounds are genotoxic/carcinogenic in nature, and that they are formed in the chlorination process during drinking water treatment.

Although volatile organic compounds were the center of attention initially, more recent research has shown that the non-volatile compounds may be of greater concern. About 90% of the dissolved organic content in drinking water is comprised of relatively non-volatile (high molecular weight, non-purgeable) organic compounds (NAS, 1977). These compounds are extractable with organic solvents or can be adsorbed onto XAD resin material. Since chlorine binds mainly to organics in the non-volatile fraction, most of the mutagenic activity can be attributed to non-volatile organohalides. Thus, organic extracts or XAD concentrates of chlorinated drinking water are most often mutagenic. Within this concentrate itself, one can distinguish between neutral, relatively non-polar compounds (which account for only 10% of the total mutagenicity), and polar, acidic compounds (accounting for the rest of the mutagenicity).

Almost all the compounds identified in water are present at concentrations of less than 1 ug/liter. These levels are far below those which would be required to account for the total observed mutagenic activity, based on known individual mutagenic potencies. Fielding and Horth (1986) demonstrated that 14 such compounds together could account for less than 10% of the mutagenicity of the extract itself (a mixture of 14 compounds, at 10 times the concentration typical in water could only equal the mutagenic response of a drinking water extract).

Against this background, it is easy to understand the importance of chlorinated hydroxyfuranones. 3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone i.e MX was the first single mutagen identified in drinking water which could account for a relatively large portion of the total (extracted) mutagenicity (Kronberg et. al., 1987; Meier et. al., 1987b). Although present at very low concentrations, MX contributes to about 5-60% of the total mutagenicity of drinking water. This is because MX is among the most potent known mutagens ever tested in the Ames TA 100 strain. The estimated activity of MX has ranged from 5600 to 13000 net revertants/nmole in TA 100 (Padmapriya et. al., 1985; Meier et. al., 1987a, 1987b; Kronberg et. al., 1987). In comparison, the most mutagenic among the THMs, i.e chlorodibromomethane, induces only about 0.004 net revertants/nmole in TA 100 (Simmon et. al., 1977). The concentration of MX in drinking waters varies considerably. Water samples from 23 cities in Finland had MX levels ranging from 5-67 ng/liter and accounting for 15-57% of the total mutagenicity (Kronberg, 1987); water samples from 3 cities in the U.S had 2-33 ng/liter, accounting for 15-34% of the total mutagenicity (Meier et. al., 1987b), while samples from a water treatment plant in the U.K had 2-10 ng/liter (Fielding and Horth, 1986). Another important mutagen identified is the geometric isomer of MX, E-2-chloro-3(dichloromethyl)-4-oxo-butenoic acid, i.e E-MX. This however is a weak mutagen (320 net revertants/nmole)

relative to MX, and accounts for only a few percent of the total mutagenicity (Kronberg et. al., 1987).

2.1 (b) : Formation During Chlorination.

Various investigators have shown that it is the interaction of aquatic organic matter with chlorine that leads to the formation of a mixture of halogenated and non-halogenated by-products. A variety of non-volatile aliphatic halogenated products can result from the exposure of aquatic humic and fulvic acid fractions to chlorine (Christman et. al., 1983). Dominant among them are the C_2 halogenated acids (principally di- and trichloroacetic acids), shown also to be present in chlorinated municipal drinking water (Uden and Miller, 1983). The mechanisms of by-product formation have been studied by employing isolated humic and fulvic acids (the fulvic acid fraction is a major part of aquatic humic substances) as well as some other model compounds resembling the complex molecules of natural humic matter. The general finding has been that the specific by-products formed will depend on the molecular structure of the substrate involved, its concentration, the carbon-to-chlorine ratio and the pH of chlorination, among other factors (NRC, 1987). Nevertheless , two categories of byproducts can be recognized : volatile hydrophobic and non-volatile hydrophilic compounds. The mutagenicity associated with chlorinated humic/fulvic acid solutions is however attributable primarily to the non-volatile compounds.

Working with extractions at different pH values and with solvents of different polarity, Kronberg et. al. (1985b) demonstrated that the principal mutagens, in a chlorinated humic acid solution, were relatively polar compounds. In support of this are other investigations showing that a majority of the mutagenicity of chlorinated humic and fulvic material resides in the strong acid fraction (Meir et. al., 1986) and that almost 65-75% of the recovered activity was due to polar mutagenic products (Maruoka, 1986). Among the commonly occurring mutagens, as resulting from humic and fulvic acid chlorination are chlorinated acetones, chlorinated propenals such as 1,3-dichloroacetone and 2-chloropropenal, dichloroacetonitrile, dichloro- and trichloroacetic acids, choral, and chloropicrin. Most of these significant by-products have been summarized in a review by Christman et. al. (1984). This review also discusses the involvement of the 1,3-dihydroxybenzene or resorcinol structures (found within the humic macrostructure) in the formation of the chlorination by-products. Another group of by-products of particular interest currently are the chlorinated hydroxyfuranones, which include MX and E-MX (Holmbom et. al., 1984).

Since organic nitrogenous compounds can account for a considerable part of the total organic carbon (TOC) in natural waters, it is also possible for mutagens to be formed due to exposure of such compounds to chlorine. Extracts of chlorinated amino-acids have been shown to

possess mutagenic activity, and some of the compounds identified in such extracts are haloacetonitriles (Trehy and Bieber, 1981), trichloroaldehyde (Trehy et. al., 1986) and MX and E-MX (Horth et. al., 1987). Other substrates implicated in the production of mutagenic chlorination by-products are proteins, peptides and phenolic compounds.

2.1 (c) : Stability of MX in Water

The stability of MX, and other possible acidic mutagens, in water is discussed at length by Kronberg and Christman (1988), Kronberg (1987), and Holmbom et. al. (1984). The pKa value of MX in aqueous solutions has been determined to be approximately 5.25, assuming that the hydration/dehydration reaction between the closed and protonated open form is much faster compared to the proton transfer. Thus, at pH values common in drinking water (pH 7.0-8.5), MX exists in its open form.

MX is stable in water at low pH (pH 2.0), but as the pH increases to about 7 or 8, it can partly convert to its geometric isomer E-MX, and partly undergo hydrolytic degradation reactions. The second reaction dominates at pH 8-9. At pH 8, the half-life of MX at 23 degrees celsius is about six days. Under neutral or alkaline conditions, E-MX does not isomerize to MX. However, if the pH is lowered to about 2.0, E-MX is quantitatively converted to MX (see Fig. 1, modified from Kronberg and Christman, 1988). These studies indicate that (a) the mutagens formed during

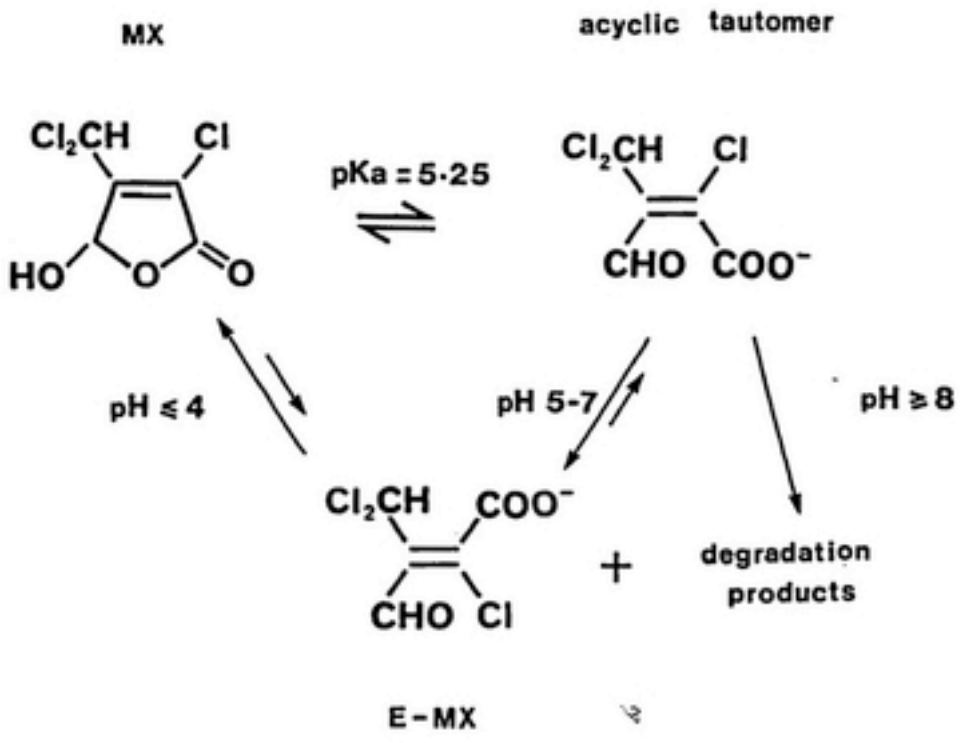


FIG.1 Stability of MX in Water.

chlorination are alkali-labile and (b) a majority of the MX formed will persist throughout most of the water distribution system. Theoretically, it may be possible for E-MX to isomerize to MX *in-vivo* as the drinking water passes through highly acidic conditions in the gastrointestinal tract. However, the rate of isomerization is too slow to cause any substantial increase in the mutagenicity of the consumed water (due to the more potent MX).

2.2 : Structure-Activity Relationships.

The high mutagenic potency of MX has prompted attempts to study and define the structural features (particularly in chlorofuranones) that are critical in rendering MX and related compounds mutagenic.

Both the type of substituent groups as well as their position(s) seem to be important. Compounds which differ from MX only in a substitution on the 5-position of the furanone ring (i.e 4-position of the acyclic tautomer) display mutagenic potencies almost equal to that of MX. Streicher (1987) explained the potencies of three such compounds by suggesting their susceptibility to hydrolyse to MX. However, a change in the 4-position substituent of the MX furanone ring can dramatically influence the potency of the compound. Replacing the dichloromethyl group of MX with a chlorine, a methyl group and a chloromethyl group reduced the mutagenicity by four, three and one order of magnitude respectively (Meier, Blazak and Knohl, 1987; Streicher

1987). It has been postulated that the dichloromethyl group of MX is in fact, the site of nucleophilic attack, and does not merely serve as an electronegative activator.

Another decisive factor governing the activity of MX is the *cis*_arrangement of the $CHCl^2$ and Cl substituents on the carbon-carbon double bond, which is present in both the furanone ring as well as the acyclic forms of MX (Ishiguro et. al., 1988). This fact is most evident in the mutagenicity displayed by E-MX, which has the Cl substituent *trans* to the dichloromethyl group. The activity of E-MX is, at the most, about 10% of that of MX (Kronberg et. al., 1988). Further, it is important for the dichloromethyl group to be alpha to the aldehyde group in the ring-opened form of MX. In this position, it is conceived to enhance the electrophilicity (and hence the mutagenicity) of the enone system through its electron withdrawing effect (Ishiguro et. al., 1988). Finally, there is the obvious contribution of the three chlorine substituents, as the furanone structure devoid of any chlorine is essentially non-mutagenic.

2.3 : Genotoxic Activity.

The current concern about chlorination by-products stems from the recognition that these compounds could pose a significant health hazard to humans. Numerous studies have compared the mutagenic and carcinogenic properties of treated drinking water with the raw source water. The one

common finding is that the chlorination process itself is responsible for the introduction of mutagens which are generally not present prior to this process (Cheh et. al., 1980; Loper et. al., 1985; Nestman et. al., 1979). The gravity of this problem came to light when a study by the National Cancer Institute implicated chloroform (one of the by-products formed) as being capable of inducing hepatocellular carcinoma in mice and renal tumors in rats.

The most extensively used tool for obtaining toxicity information on drinking water samples has been the Ames *Salmonella* assay. Almost all the strains recommended by Dr. Ames and co-workers (Ames, McCann and Yamasaki, 1975; Maron and Ames, 1983) for routine screening (TA 1535, TA 1538, TA 98, TA 97, TA 100, TA 102) have been used. TA 98 and TA 100 seem to be the most sensitive in their ability to detect the presence and potency of mutagenic compounds in drinking water (Meier, 1988) and the highest response is almost always in the absence of exogenous metabolizing enzymes, S9. The mutagens would thus appear to be direct acting frame-shift or base-pair substitution types.

2.3 (a) : Genotoxicity of Drinking Water/Concentrates.

Organic extracts and XAD resin concentrates of chlorinated fulvic acid solutions, humic water and drinking water have been shown to produce a dose-related increase in the reversion, well above the spontaneous rate, in the standard plate-incorporation Ames assay as well as the

modified fluctuation assay. Another genotoxic endpoint shown by drinking water concentrates in a microbial assay is the induction of SOS response in *E. coli* (Bourbigot et. al., 1986).

Drinking water concentrates have also been tested in some of the commonly used eukaryotic assay systems, considered by some to be more relevant to human health effects than microbial assays. Lang et. al. (1980) showed that drinking water concentrates were able to transform BALB/C3T3 cells *in-vitro*. When transplanted to athymic mice, these transformed cells could produce tumors *in-vivo* (Kurzepa et. al., 1984). This ability to transform a mouse fibroblast cell line (BALB/3T3) in a dose related manner was also shown by Loper (1978) using a reverse-osmosis concentrate which was earlier shown to be mutagenic in the Ames assay strains TA 98 and TA 100. Robinson et. al. (1981) working with a reverse-osmosis concentrate of drinking water from five cities, were able to demonstrate that the chemicals present were primarily initiators in the mouse skin assay rather than promoters or complete carcinogens. However, the duration of the experiment was probably not long enough to detect carcinogenicity, even at doses up to 30 mg of organic material per mouse.

For purposes of comparison, other investigators have tested the same drinking water concentrates in more than one type of assay. Athanasiou and Kyrtopoulos (1983) reported that an XAD-2 extract of chlorinated drinking water from a

surface source was positive in the Ames assay and was able to induce SCE and chromosomal aberrations in CHO cells. Wilcox and Williamson (1986) tested the same XAD-2 concentrates of drinking water using *in-vitro* as well as *in-vivo* assays. While the concentrates were clastogenic in CHO cells and cultured human lymphocytes, they were not able to induce chromosomal aberrations in mouse bone-marrow cells following *in-vivo*-oral administration. The authors suggested that perhaps the clastogens were inactivated before they could reach the target cells in the bone-marrow This explanation is supported by *in-vitro* evidence as well.

Meier and Bull (1985) reported the ability of humic acids, chlorinated at pH conditions relevant to drinking water chlorination, to induce SCE in a mammalian cell line. However, these same samples could not increase the percent of micronucleated cells in the mouse micronucleus assay. They were negative in the mouse sperm morphology assay as well. A long term carcinogenicity study to address the effects of chronic exposure to chlorinated drinking water was done in The Netherlands. After 24 months of receiving organic concentrates (XAD-4/8) of drinking water (mutagenic in the Ames Assay) up to 68 times the estimated human exposure, the test rats displayed no increases in the incidence of tumors (Kool et. al., 1985)

2.3 (b) : Genotoxicity of MX

It is well established that among the identified chlorination by-products in water, MX is the single most potent mutagen, contributing significantly to the total mutagenicity of the extract. Thus, recent work on the mutagenicity and carcinogenicity of drinking water has focused on such properties of the pure compound (MX). Meier, Blazak and Knohl (1987) investigated the mutagenic and clastogenic properties of MX, and confirmed that its genotoxicity in-vitro is not restricted to bacterial cells alone. MX was found to induce a dose-related increase in the reversion rates of Ames *Salmonella* strains TA 1535, TA 1538, TA 92, TA 97, TA 98, TA 100, TA 102, all in the absence of S9. This would indicate that MX is a direct-acting mutagen capable of effecting both base-pair substitutions as well as frame-shift mutations. MX was also able to induce chromosomal aberrations (chromatid deletions and chromatid exchanges) in mammalian (CHO) cells. This work also described the acute toxicity of MX, the acute oral LD_{50} (to Swiss-Webster mice) being reported as 128 mg/kg/day.

Although MX can be considered a clastogen of comparatively high activity, it failed to induce micronuclei in mouse bone-marrow *in-vivo* even at the highest administered dose (70% of the LD_{50}, sacrificed after 72 hours). Thus, the authors concluded that "...MX fits into a fairly large category of chemicals that are clastogenic and

mutagenic *in-vitro* but are inactive for rodent bone-marrow *in-vivo*."

The genotoxic potential of MX was further confirmed by Brunborg et. al. (1988). They indicated that MX could induce SCE in cultured mammalian cells (V79) at very low levels (2-5 uM). Higher concentrations of MX blocked normal mitosis and therefore, gene mutations could not be registered. MX was also found to induce DNA damage in rat testicular cells.

Finally, the fate of MX *in-vivo* has been recently reported by Kopfler et. al. (1988). They concluded that MX was not active in the mouse micronucleus assay (in which the target cells are developing erythrocytes in the bone-marrow) probably because it is inactivated before reaching the target cells. MX is a very reactive electrophile and can be inactivated by reactions with nucleophiles like diethyldithiocarbamate and gluthathione. As for its absorption/distribution, most of the 8 mg gavage dose was excreted or expired. After sacrifice at 48 hours, the carcass and internal organs of the rats contained only about 8% of the dose (mainly in the kidney and liver). The gastrointestinal tract had nuclear anomalies following oral administration.

Based on a literature review, it would appear that although chlorinated drinking water contains compounds having mutagenic and clastogenic potential, their *in-vivo* effects have yet to be conclusively determined.

2.4 : Analytical Techniques.

2.4 (a) : Concentration and Extraction of Organics.

Organic compounds in drinking water are present at very low concentrations (1 ug/liter or less). Loper (1981) pointed out that at such typical concentrations, the mutagens are not likely to be detected by the Ames assay. Even the use of the modified microscale fluctuation assay which directly incorporates unconcentrated water samples, has proven unsuccessful in detecting mutagens (Foster et. al., 1983; Monarco et. al., 1985). Thus, to make compounds amenable to analytical and mutagenicity testing, they have to be isolated and concentrated from the water samples. Two very important features of the concentration method were outlined by Kronberg (1987); (a) the method should give high recoveries of a wide range of organics since the attempt is to "capture" compounds with unknown structures and properties, and (b) the organic solvent to be used in the concentration procedure should be compatible with both the intended bioassay and the analytical method (or be easily exchangeable to another solvent since the chemical as well as the mutagenicity analyses are to be done on the same concentrate).

Some of the methods which have been in common use are freeze-drying, reverse-osmosis, liquid-liquid extraction and binding to different solid-phase adsorbents. Concentration methods are selective in the types and amounts of compounds

recovered. Wilcox et. al. (1986) noted that differences in the types and levels of genotoxic activity are probably a reflection of this fact rather than due to real differences in the composition of the water samples. The most widely used concentration method seems to be adsorption of the organics onto macroreticular XAD resins (Kronberg, 1987; Meier, 1988). These non-ionic resins give efficient recoveries for both non-polar compounds (XAD-2 and XAD-4) and also compounds of intermediate polarity (XAD-7 and XAD-8). The adsorbed material is then eluted with an organic solvent which subsequently can be evaporated to obtain the extract. It appears that an eqi-weight mixture of XAD-4 and XAD-8 resins is the most effective in terms of recovering mutagenicity. Liquid-liquid extraction with diethyl ether is also considered as effective as using XAD. The pH of the water sample however seems to be critical in governing this efficiency (Kronberg, 1987).

Sample acidification prior to concentration (by XAD or liquid-liquid extraction) results in a higher recovery of organics and a higher mutagenic activity as well. This preferential isolation of mutagenicity (from chlorinated water) at acidic pH as compared to neutral pH suggests that acidic organic compounds are responsible for the activity. The ionization of acidic compounds is suppressed at low pH conditions, making their adsorption to XAD resins stronger. The adsorption of neutral compounds however occurs independent of pH. In liquid-liquid extractions, the

polarity of the solvent being used is also important in addition to pH. While acidic mutagens are recovered by polar solvents like diethyl ether, the neutral compounds are extractable with non-polar solvents like hexane.

These adsorption and extractability studies have indicated the existence of two distinct classes of mutagens in chlorinated drinking and humic waters, one with acidic and the other with neutral properties (Wigilius et. al., 1985; Maruoka, 1986; Kronberg, 1987; Ringhand et. al., 1987). A comparison of neutral and acidic concentrates indicates that the acidic fraction is about 7-10 times more mutagenic (Kronberg et. al., 1985; Monarca et. al., 1985; Meier et. al., 1986 Ringhand et. al., 1987). The identification of MX, an acidic chlorinated furanone, as a major contributor to the mutagenicity of drinking water has further confirmed this view.

2.4 (b) : GC/MS Analyses and Quantitation.

Non-volatile compounds are responsible for most of the mutagenic activity found in chlorinated water extracts. The earlier emphasis on the trihalomethanes (THMs) was partly due to the fact that these compounds were easily quantified by GC procedures. However, by comparing the total amount of organic halogen (TOX) produced in drinking water treatment to the levels of THMs formed, Oliver (1978) and Glaze et.al. (1980) found the amounts of TOX to be larger than the THMs. This suggested the need to consider the importance of the

non-volatile by-product compounds as well. Kopfler et. al. (1985) were able to show that most of the mutagenicity is not associated with compounds which can be identified by GC/MS analyses (at least not in their underivatized state). They were able to trap the gas stream exiting the GC system and show that it contained less than 10% of the mutagenicity in the original extract (of a chlorinated humic acid solution). Lyophilization of this same material however recovered about 90% of the mutagenic activity. This inability to recover most of the mutagenic activity following direct GC injection has also been reported by other investigators (Coleman et. al., 1984; Meier et. al., 1985; Kringstad et. al., 1983). Hence, in order to identify the mutagens by GC/MS analyses, properties such as their non-volatility, heat lability, polarity and acidic nature must be circumvented. One principal method used to overcome this problem has been solvent extraction followed by derivatization and then GC/MS (Norwood et. al., 1983). Suitable derivatives can be prepared which will provide enhanced volatility and thus convert the mutagens into gas chromatographable compounds. Some of the derivatization choices are acetylation of the hydroxyl groups and methylation of the free acids. A large number of non-volatile aliphatic halogenated by-products have been identified by such extraction-methylation-GC/MS analysis procedures. Christman and co-workers (Christman et. al., 1983; Johnson et. al., 1982 and Norwood et. al., 1983)

identified more than 100 different by-products (from the chlorination of isolated aquatic humic and fulvic acids) by GC/MS methods. The dominant compounds were chloroform, bromodichloromethane, chloral (trichloroethanal), dichloroacetic acid (DCA), trichloroacetic acid (TCA), dichlorosuccinic acid and dichloromalonic acid. A further example is the finding that the identification of MX in the mutagenic fractions required derivatization by methylation and analysis as the methyl ether (Holmbom et. al., 1984).

The use of GC/MS analyses to detect and quantify MX and E-MX in water has been well described in Kronberg's work (Kronberg et. al., 1987; Kronberg, 1987). Identification of MX and E-MX was carried out by SIM (selected ion monitoring) mode GC/MS analyses. The mass spectrometer was operated alternatively in the electron impact (EI) positive or negative ion chemical ionization modes. Presence of these compounds was confirmed by positive matching of the retention times of the ions and the methylated standard MX or E-MX (in the reconstructed ion chromatograms) as well as positive matching of the relative peak area ratios. Quantitation of MX and E-MX was done by reference to an internal standard, mucobromic acid (MBA), which was added to the extracts or fractions prior to methylation. It should be recognized here that even after derivatization of compounds to increase volatility, over 80% of the organic matter in drinking water is still not amenable to GC/MS for structural elucidation (Fawell and Fielding, 1985).

2.4 (c) : HPLC Separation-Bioassay (coupled) Procedure

In the preceding discussion, it was pointed out that drinking water contains a large portion of organic chemicals which are not amenable to GC-MS directly due to their insufficient volatily and thermal instability (Fawell and Fielding, 1985). High performance liquid chromatography (HPLC) is a separation technique which by operating at ambient temperatures with liquid mobile phases, overcomes this limitation. Since water contains a very large amount of organic compounds, it becomes necessary to separate the mutagenic compounds from the non-mutagenic compounds. HPLC separation of extracts of water provides fractions of interest (fractionated in semi-preparative or analytical columns) which can then be examined directly, or with derivatization by MS. HPLC has been widely used to fractionate drinking water organics (Crathorne et. al., 1979, 1984) and to obtain fractions of chlorinated drinking water for mutagenicity testing (Horth et. al., 1985).

An effective way of eliminating those compounds which are not mutagenic from a complex mixture is to fractionate the material with respect to mutagenicity using HPLC. This bioassay- directed separation can pinpoint a fraction containing only a few relatively potent compounds which, while being present at low concentrations, may still be the main contributors to the overall mutagenicity of the mixture. With respect to chlorinated waters, this procedure

generally starts with sorption/desorption on XAD resins, liquid-liquid extraction followed by repeated TLC and/or HPLC separations into smaller active subfractions. The structural determination of the mutagen(s) is then attempted with spectrometric studies of the mutagenic subfraction. Some of the major mutagens identified using this approach have been 3-(2-chloroethoxy)-1,2-dichloropropene in a chloroform extract of drinking water (Tabor and Loper, 1980), MX in kraft chlorination liquors (Holmbom et. al., 1984), and MX and E-MX in chlorinated humic water (Kronberg et. al., 1987). Thus, the HPLC technique provides a way to deal with the non-volatile compounds. Fielding and Horth (1986) pointed out that the mutagenic fractions obtained by their HPLC separation work did not contain the volatile mutagens (ex. chloral, chlorodibromomethane and chloroform) which had been identified earlier in extracts of the same drinking water by GC/MS techniques.

2.4 (d) : Mutagenicity Analysis: The Ames Assay.

The presence of genotoxic materials in drinking water may cause potentially adverse human health effects. Bull et. al. (1982) suggested that appropriate bioassays be used to screen raw and finished drinking water for genotoxic activity. This, used in addition to chemical analysis for known individual mutagens and carcinogens, allows for the complex mixture of chemicals (in water) to be characterized with respect to genotoxicity.

Information on the genotoxicity and mutagenicity of drinking water samples has been obtained, to date, primarily by the use of the Ames *Salmonella*/microsome mutagenicity assay (Kool et. al.,1983; Meier et. al., 1985; Meier, 1988). However, evidence from mammalian and eukaryotic assays indicates that the genotoxic activity is not restricted to just bacterial mutagenicity alone (Meier, 1988). The Ames assay has been used to demonstrate the mutagenicity produced by chlorination of drinking water, humic substances (Kronberg et. al., 1985) and pulping process effluents (Holmbom et. al., 1984). The common choice of the Ames assay in preliminary screening of unknowns is a reflection of its utility in being able to detect a range of chemicals quickly and economically. It may also be possible to use the exogenous xenobiotic metabolizing enzymes system (S9), to determine the effect of mammalian metabolic activation/inactivation on the mutagen. Initial validation of the Ames assay showed that it was considerably sensitive and specific (estimated at 85-90% and 74-87% respectively) in detecting carcinogens as being mutagens (Mccann and Ames, 1976; Sugimura et. al., 1976). Recent evidence however indicates a substantially lower correlation. Tennant et. al. (1987) evaluated four *in-vitro* short-term genotoxic assays (including the Ames assay) for their ability to predict carcinogenicity of a number of chemicals in rodents. The Ames assay alone had a positive predictive value of 62%, which was neither exceeded by any of the others, nor

improved upon by a battery of the tests together. However, it must be noted that certain known human carcinogens, as well as epigenetic carcinogens are not detected by the Ames assay.

The Ames assay utilizes *Salmonella typhimurium* strains which carry a specific mutation in certain genes that code for enzymes required in histidine production (histidine operon). Thus, the cells are unable to grow in the absence of histidine in the culture media. The assay essentially measures back mutation, wherein restoration of the normal gene function occurs. Such cells, regaining histidine-independence, are able to grow and form visible colonies on histidine-free media. Exposure of these constructed *Salmonella* strains to mutagenic agents induces an increase in the frequency of back-mutation, distinctly above the spontaneous rate. In addition to the histidine mutation, the standard tester strains also contain other mutations that greatly enhance their ability to detect mutagens (Maron and Ames, 1983).

There are a number of different standard tester strains of *Salmonella typhimurium* which respond to different types of changes in their genetic material. The strain TA 98 detects agents causing frame-shift mutations while TA 100 and TA 102, base-pair substitutions. TA 100 detects mutagens which effect base-pair substitution at the G-C pairs while TA 102 detects those affecting the A-T pairs. Some mutagens are known to react preferentially at the A-T

base-pair (Levin et. al., 1982). In addition, TA 102 can be used to identify mutagens which operate through oxidative damage. It is now recommended that TA 102, in addition to TA 98 and TA 100, be used for all routine screening. This is especially important in evaluating mutagenic by-products from the use of disinfectants other than chlorine (Meier, 1988).

III: APPROACH.

Non-MX mutagenicity is a term used here to describe the mutagenic activity of chlorinated fulvic acids, humic water or drinking water that is not accounted for by the identified mutagens. The most potent mutagen among the known compounds, MX, accounts for (on an average) about one-third of the total mutagenicity present in an organic extract of chlorinated water. The remaining identified compounds are relatively weak mutagens, and even collectively, account for barely a few percent of the mutagenic activity. Thus, the remaining two-thirds of the activity can be considered "non-MX mutagenicity", contributed by compounds yet to be identified.

Some of the responsible compounds may be similar to MX, particularly in having the structural configurations critical for high activity. It is conceivable that such MX analogues exist in water with MX. On the other hand, the unknown mutagens while not structurally related, may just share some of the physico-chemical properties of MX. Hence, it may be possible to separate and identify them using a scheme similar to that which was successful in identifying MX.

3.1: Formation of MX and MX Analogues during Chlorination.

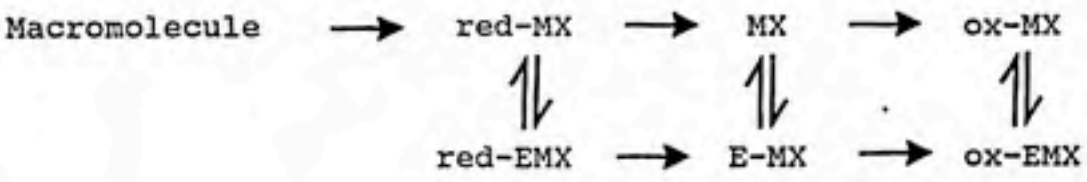
The activity of MX, like many other potent mutagens, is dictated by certain critical structural arrangements. Previous research has shown that the two most important features which bestow upon MX its extreme mutagenicity are the dichloromethyl group at the 3-position, which has to be alpha to the aldehyde group in the open form of MX and the *cis* arrangement of the $CHCl_2$ and Cl groups around the carbon-carbon double bond (see section 2.2). Considering its potency, it is conceivable that the unknown mutagens may be structurally similar to MX. The only compounds possibly fulfilling the structural requirements mentioned above are the MX analogues in which the aldehyde has been reduced to an alcohol group (red-MX), or oxidized to a carboxyl group (ox-MX).

The mechanisms of mutagen formation known so far indicate that MX is an intermediate of certain oxidation reactions in water. It is also known that MX results from the oxidation of humic macromolecular carbon, presumably through intermediate steps and compounds. Therefore, it is conceivable that red-MX could be present as an oxidized form of the humic macromolecule but as a reduced precursor of MX. Similarly, MX itself could be the reduced precursor of ox-MX

(oxidized MX). In water, this red-ox relationship could be described as

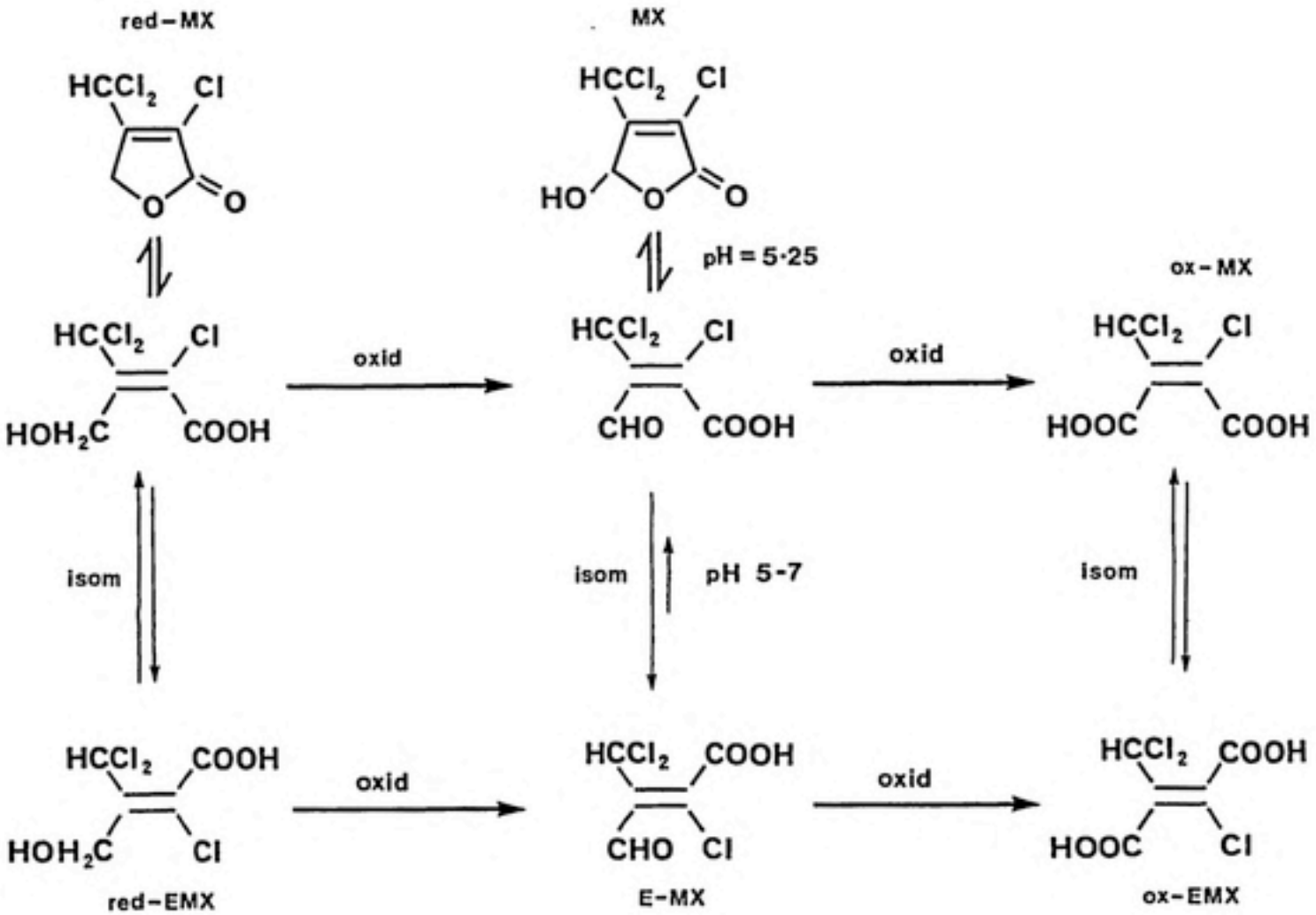
Macromolecule → red-MX → MX → ox-MX

Chlorine, being a good oxidant, would thus force the reaction to the right, forming red-MX, MX, and ox-MX among perhaps other intermediates. The actual humic and chlorine concentrations would affect the equilibrium conditions and at equilibrium, one or more of these compounds may be present in very small quantities. Another aspect of this equilibrium to consider is that when present in water, MX is always accompanied by its geometric isomer, E-MX. It is likely that the reduced and oxidized forms of E-MX (red-EMX and ox-EMX respectively) are also formed as intermediates. Further, since MX and E-MX do isomerize in aqueous solutions, red-MX and ox-MX can also be expected to transform into their respective E-isomers. The entire relationship, taking both red-ox and isomerization into consideration, can be written as



Thus, the structures which may be present in water are illustrated in Fig. 2. It must be borne in mind however, that there is little information on the kinetics involved,

FIG. 2 Formation of MX Analogues in Water.



and therefore, definitive reaction schemes cannot be written. Furthermore, we are very limited in our knowledge of the structure and influence of the precursor humic macromolecules, and their role in the formation of such by-product compounds.

3.2: Mutagenicity Directed Sub-fractionation.

Depending on the chlorination conditions and the TOC content, it is possible to obtain a chlorinated fulvic acid sample which is high in mutagenicity but (simultaneously) low in MX. A fulvic acid solution, with a high TOC content, will, upon chlorination, yield a comparatively large number of organo-halide compounds. If the concentration of MX is small in comparison to a relatively high overall mutagenicity, this would indicate that the extract contains many compounds (mutagens) of interest. The coupled HPLC-Bioassay procedure can thus be used to obtain active subfractions which are relatively free of MX as well as other non-mutagenic organic material. When sufficiently "pure", these active sub-fractions can be subjected to GC/MS analyses for structural determination.

IV: EXPERIMENTAL

4.1 : Materials.

For the Ames assay, *Salmonella typhimurium* strains TA 98, TA 100 and TA 102 were obtained from Dr. Bruce Ames, University of California at Berkeley. Sodium azide and 2-nitrofluorene were purchased from Aldrich (Milwaukee, WI), 2-anthramine and 1,8 dihydroxyanthraquinone (Danthron) from Sigma Chemical Co. (St. Louis, MO) and Daunomycin from Fluka Chemical Corp. (Ronkokoma, NY). Oxoid Nutrient Broth #2 was obtained from Oxoid, Ltd. (Basingstoke, Hants, England) and Bacto agar from Difco Laboratories (Detroit, MI). Molecular biology grade DMSO was purchased from Fisher Biotech. Chemicals for the VBME solution were all obtained from Fisher Scientific (Fairlawn, NJ). The NADP and Glucose-6-Phosphate were ordered from Boehringer Mannheim (West Germany). The S9 used was from the livers of Aroclor-1254-treated male Sprague-Dawley rats and was purchased from Moltox (College Park, MD).

HPLC grade acetonitrile and ethyl acetate were obtained from Fisher Scientific (Fairlawn, NJ). Ethyl ether was from Burdick & Jackson (Muskegon, MI). The sodium hypochlorite and potassium phosphate were also purchased from Fisher

Scientific (Pittsburgh, PA). The XAD resins were Amberlite (Rohm & Haas, Philadelphia).

The MX analogues were synthesized by Dr. Leif Kronberg using MX which in turn had been synthesized by the method of Padmapriya, et.al. (1985).

4.2 : Chlorination and Extraction of Samples.

All the chlorination was done with sodium hypochlorite. The concentration of the sodium hypochlorite solution was determined by the DPD Ferrous Titrimetric Method (Std. Methods, 408D) as was the residual chlorine in the chlorinated samples. The TOC content of the samples was analysed by the persulfate oxidation method using OI Corp. TOC700 TOC analyzer. The Cl_2/TOC ratios were on a weight to weight basis.

4.2 (a) : Humic Water Sample.

The humic water sample was natural freshwater with a high content of humic substances (TOC = 20 mg/L) collected from a marshy lake (Lake Savojaeri) in southwest Finland. The lake did not receive any municipal or industrial water effluents. The water was chlorinated at a 1:1 Cl_2/TOC ratio (on a weight to weight basis) while being buffered at pH 7.0 ±0.2 (with potassium phosphate buffer). The reaction was allowed to go on for 60 hours at ambient temperature in the dark, at the end of which the total chlorine residual was less than 0.1 mg/L. The water was then acidified to

pH 2.0 by adding 4N HCl. The mutagens were concentrated by passing the acidified humic water through a column of XAD-4 and XAD-8 resins (1:1 volume mixture) at a flow rate of one bedvolume/min (20 ml/min). Residual water remaining in the column was removed with a gentle stream of nitrogen. The absorbed organics were subsequently eluted in the reverse direction with 3 bedvolumes of ethyl acetate. The extract was then concentrated further by evaporation and its final volume adjusted such that 1 ml of ethyl acetate equalled 1 liter of original humic water. This extract of chlorinated humic water is referred to, in parts of this report, as HW.

4.2 (b) : Fulvic Acids Sample.

The fulvic acids were extracted from a highly colored natural lake water (Lake Drummond, VA) using the isolation method of Thurman and Malcolm (1981).

They were then dissolved in distilled water to give a working sample with a TOC of 2.5 g/liter. A portion of this sample was subdivided into 8 aliquots , 4 of which were adjusted to pH 7.0 using potassium phosphate buffer, while the other 4 were adjusted to pH 2.0 with 4N HCl. At each pH, the 4 aliquots were chlorinated (with sodium hypochlorite solution) at Cl_2/TOC ratios (wt./wt.) of 0.5, 1.0, 1.5, and 2.0 respectively. These were designated samples A, B, C, D for pH 7.0 and samples E, F, G, H for pH 2.0, in increasing Cl_2/TOC ratios. The reactions were

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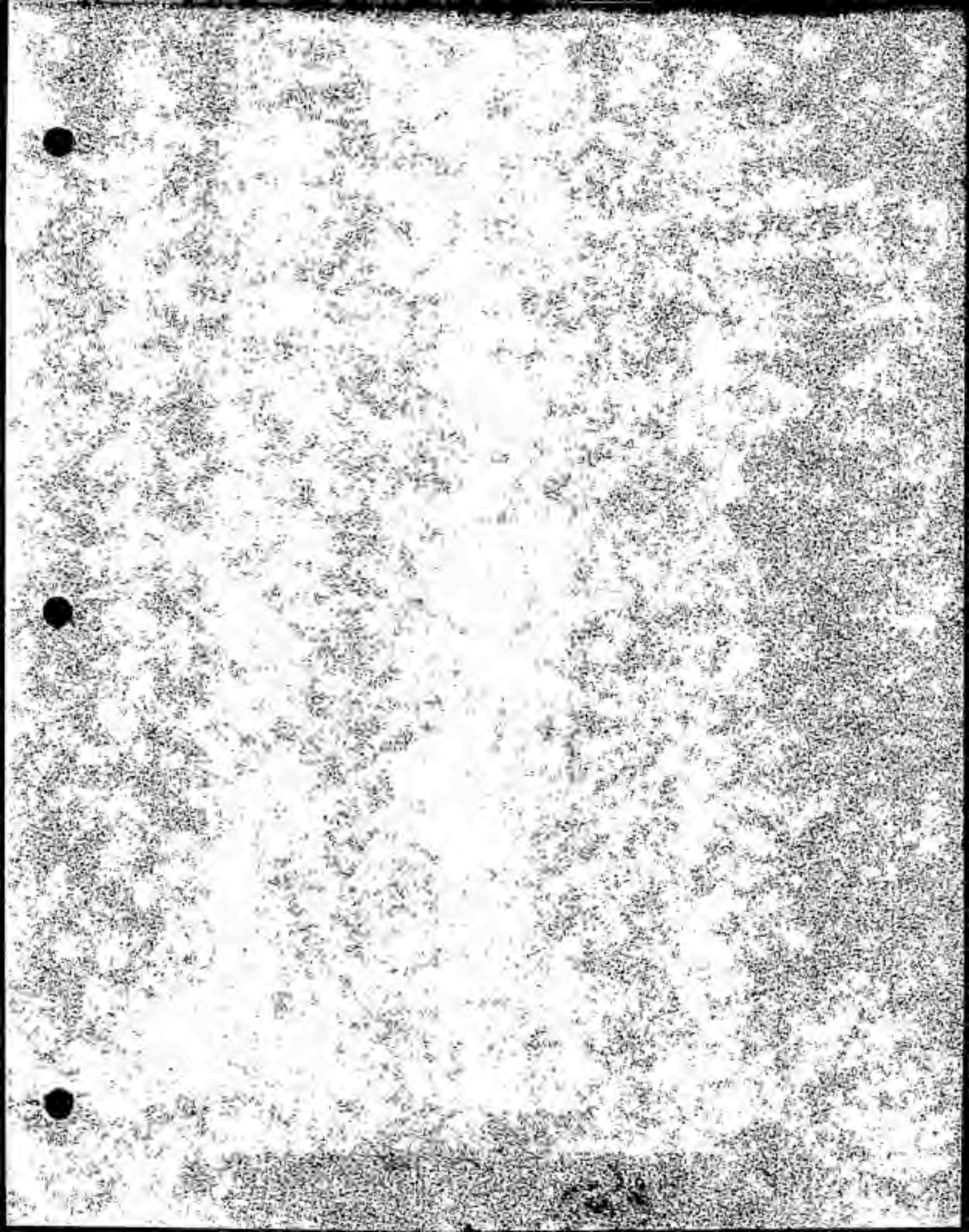
allowed to proceed at ambient temperatures in the dark for 60 hours. All the samples were then acidified to pH 2.0 with 4N HCl and extracted 3 times with diethyl ether. The extracts were combined, evaporated to dryness, and redissolved in ethyl acetate. The volume was adjusted such that 1 ml of ethyl acetate = 1 liter of water with a TOC = 20 mg/liter. The fulvic acid sample, chlorinated at pH 2.0 and at a Cl_2/TOC ratio of 2, is referred to as FA or as Sample H.

4.2 (c) : Drinking Water Sample.

The drinking water samples were collected directly from the distribution system (i.e., tap water) in Chapel Hill, North Carolina. The water treatment plant (OWASA) uses surface water with a TOC of approximately 5 mg/liter and chlorinates at approximately 5 mg Cl_2 per liter of raw water. The water samples were stored for 24 hours in a decanter glass in order to evaporate the residual chlorine. Subsequently, the pH of the water samples was adjusted to pH 2.0 with 4N HCl. The extracts were obtained by the procedure described for the humic water sample. This extract of drinking water is referred to hereafter as DrW.

4.3 : High Performance Liquid Chromatographic Separation.

First, aliquots of the aqueous sample H were adjusted to pH 6.0, 5.5, 5.0, 4.5, 4.0, 3.5, and 2.0. Each of these



were extracted with diethyl ether. The mutagenic activity of each extract was determined by the Ames assay, while the MX concentration was determined by GC/MS analysis.

The FA or sample H extract was fractionated using Reverse-Phase HPLC. Separation was achieved on a Varian LC-5000 (Palo Alto, CA) equipped with a six port Rheodyne injector. The first fractionation was done on a C_6 analytical column (Phase Sep. Spherisorb $5C_6$, 4.5 x 250 mm) fitted with a 4.5 x 50 mm pellicular ODS precolumn. The eluent system for this separation is described as Program I. The second fractionation was achieved on a C_{18} analytical column (Alltech, Econosphere $5C_{18}$, 4.5 x 250 mm) also fitted with a 4.5 x 50 mm pellicular ODS precolumn. The successful eluent system is described as Program II. HPLC effluent was monitored at 230 nm wavelength using a Perkin-Elmer LC-85B Spectrophotometric Detector with an LC-85 autocontrol (Norwalk, CT), and recorded on a Perkin-Elmer 561 recorder.

A suitable portion of the extract (equivalent to 1000-1500 mL of the original solution) was evaporated and redissolved in a 1:1 mixture of acetonitrile (ACN) and the 0.1 M potassium phosphate buffer. Then, about 330 ul was injected via a 500 μl loop. Chromatographic fractions were collected manually based on UV absorbance peaks obtained during a prior trial separation. Three portions of the extract (330 ul each) were injected and separated individually so as to avoid overloading the column. The corresponding fractions collected from each chromatographic

run were then combined. Each fraction was subsequently acidified to pH 2.0 with 4N HCl and repeatedly extracted using diethyl ether. The ether extracts were evaporated to dryness, the residue redissolved in ethyl acetate and refrigerated until further use.

Program I : Separation of Sample H.
C_6 analytical column; Flow: 1 ml/min
Isocratic elution:- 20% Acetonitrile (ACN) and 80% potassium phosphate buffer (0.1 M, pH 6.0)

Program II : Separation of H3.
C_{18} analytical column; Flow: 1 ml/min
Stepwise gradient elution with Acetonitrile (ACN) and 0.1 M potassium phosphate buffer (pH 6.0)

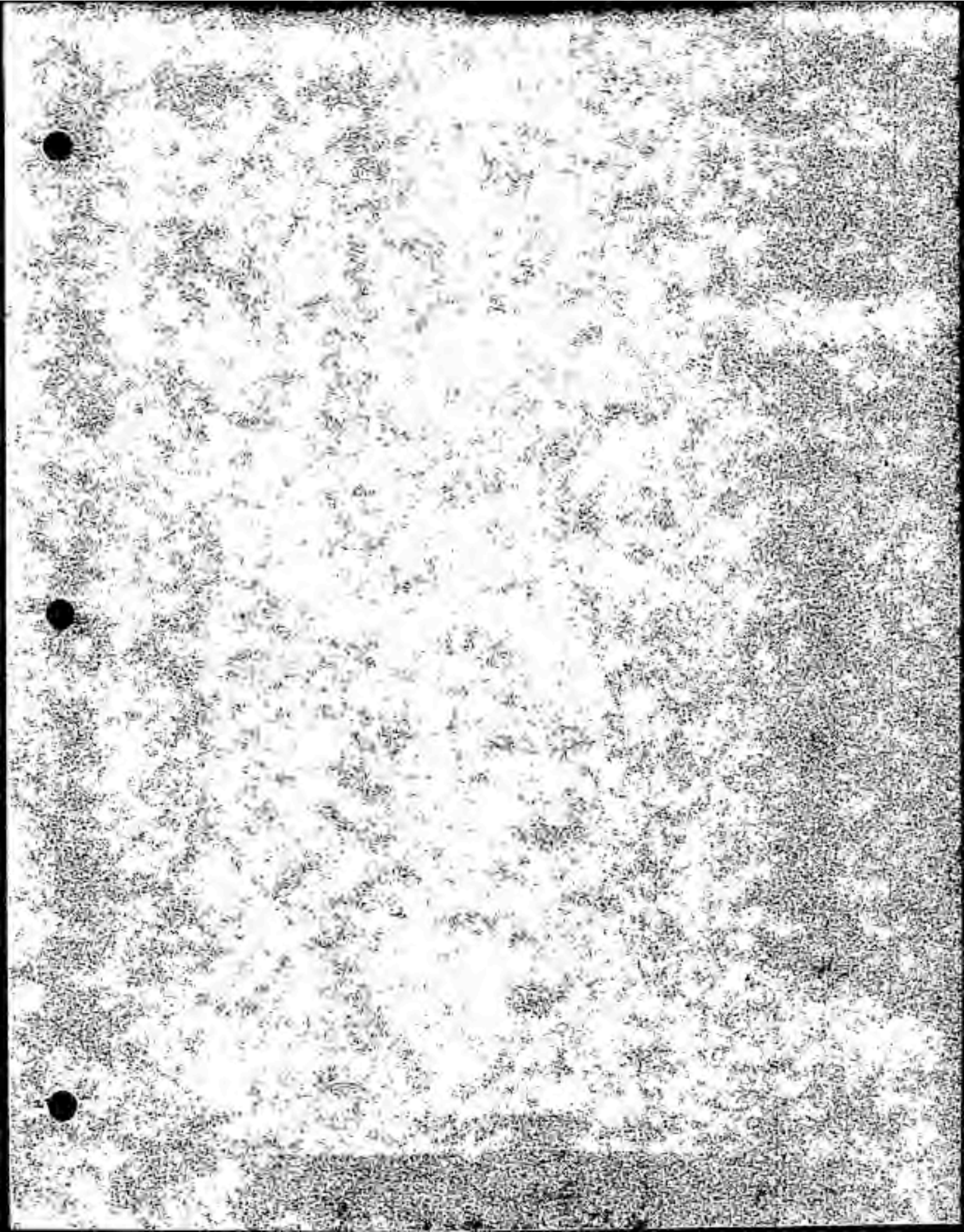
0-11 min:	100% buffer	
11-21 min:	90% buffer	10% ACN
22-32 min:	80% buffer	20% ACN
33-43 min:	70% buffer	30% ACN
44-54 min:	60% buffer	40% ACN
54-64 min:	60% buffer up to 100% buffer.	

4.4 : Synthesis.

The MX analogues were synthesized, characterized (section 4.4), and quantified in the chlorinated samples (section 4.5) by Dr. Leif Kronberg, Åbo Akademi, Turku, Finland; visiting scholar (1988-1989), Department of Environmental Sciences and Engineering, University of North Carolina at Chapel Hill.

4.4 (a) : ox-MX.

(Z)-2-Chloro-3-(dichloromethyl)-butenedioic acid (ox-MX) was obtained by the oxidation of MX. 48 mg (185 μmol) of MX was treated with 2 ml of fuming nitric acid

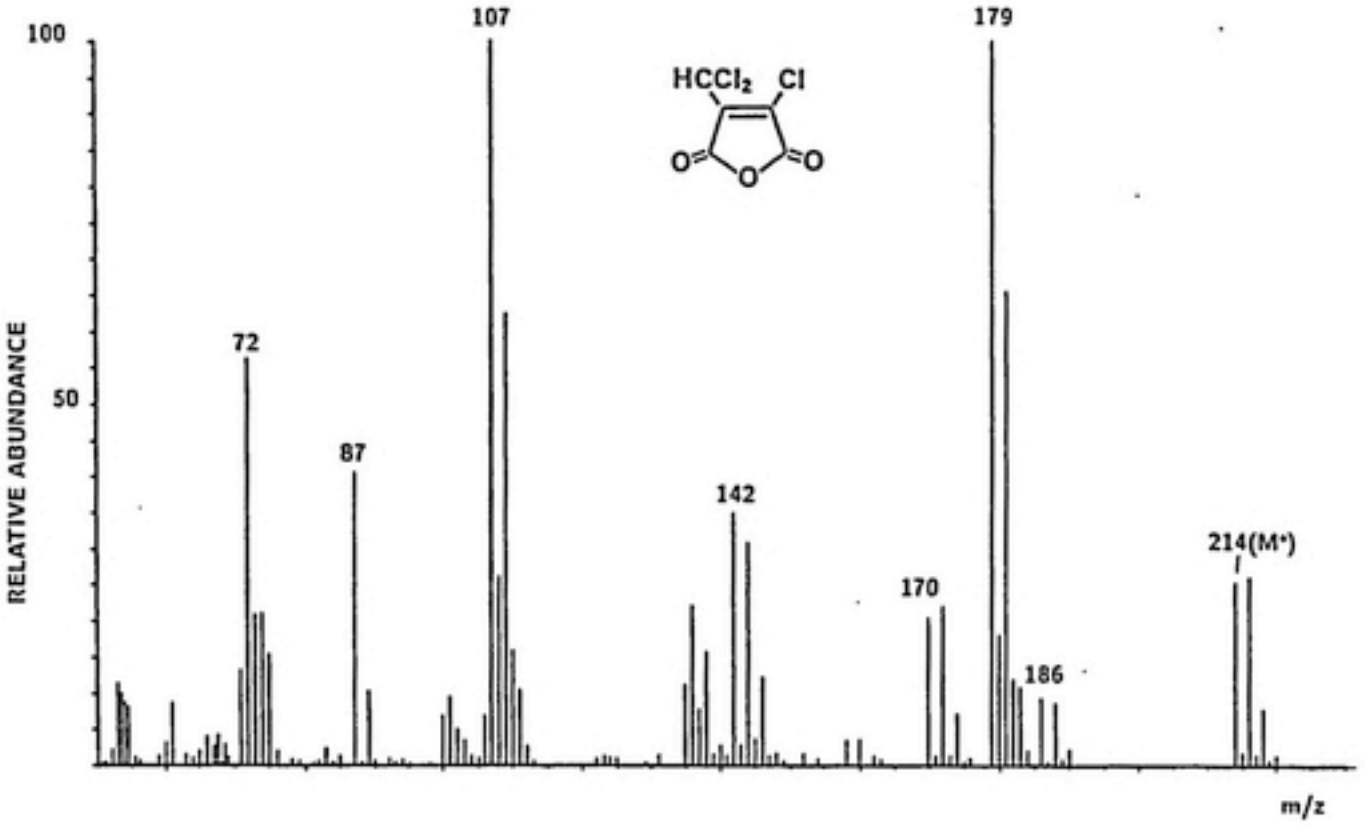


at 70°C for 24 hours. After cooling in ice and diluting it with 20 ml of ice-cold water, the reaction mixture was extracted 3 times with diethyl ether. The ether extracts were combined, washed with 0.01 M HCl and subsequently evaporated to dryness. Recrystallization from dichloromethane yielded the pure compound (9.1 mg, yield = 21%). The electron-impact mass spectrum of the compound is presented in Figure 3. This mass spectrum represents the anhydride of ox-MX since ox-MX loses a molecule of water upon heating in the MS inlet probe (or GC-injector). The ^{1}H NMR spectrum of the compound (obtained at 400 MHz, XL-400, Varian Associates, Palo Alto, CA) showed the resonance signal of the proton in the dichloromethyl group to appear at δ 6.2.

4.4 (b) : red-MX.

3-Chloro-4-(dichloromethyl)-2(5H)-furanone (red-MX) was obtained by the reduction of MX. 20 mg (93 μmol) of MX was treated with aluminum isopropoxide (225 μmol) in isopropanol (the Meerwein-Ponndorf reduction) for 2 hours at 70°C. The reaction was stopped by adding ice and 4N HCl. The acidified mixture was then heated to 50°C for a few minutes and then cooled again. Subsequently, it was extracted 3 times with diethyl ether. The extracts were combined, washed with 0.01 N HCl, and the ether evaporated to obtain the crude product. This was further purified on 6 g of SiO_2 using dichloromethane-hexane (1:1) as eluent. The yield of

FIG.3 Mass Spectrum of ox-MX anhydride



the pure compound was 17.3%. The electron-impact mass spectrum of red-MX is shown in Figure 4. The ^1H NMR resonance signal of the dichloromethyl group was observed at S 6.74 (1H) and of the protons in the lactone ring at δ 5.16 (2H).

4.4 (c) : ox-EMX.

(E)-2-Chloro-3-(dichloromethyl)-butenedioic acid (ox-EMX) was obtained from the oxidation of E-MX. 10 mg (46.3 μmol) of E-MX was treated with 5 mg (56 μmol) of $NaClO_2$ in water with resorcinol as chlorine scavenger. The reaction was allowed to proceed at pH 3.5 for 2.5 hours. Subsequently, the pH of the mixture was raised to 4.5 and the first diethyl-ether extraction was carried out. This ether extract was discarded, the pH of the solution was lowered to pH 2.0 and the diethyl ether extraction was repeated. This second extract was washed with 0.01 N HCl and, upon evaporation of the ether, the crude compound was recrystallized from dichloromethane. Finally, the crystals were washed with CCl_4. The yield of ox-EMX was 20%. The electron-impact mass spectrum of ox-EMX is given in Figure 5. The ^1H NMR resonance signal of the dichloromethyl group was observed at δ 5.6.

The attempts to synthesize (E)-2-Chloro-3-(dichloromethyl)-4-hydroxy-butenoic acid (red-EMX) were unsuccessful. Reduction of E-MX by treatment with aluminum

FIG.4 Mass Spectrum of red-MX

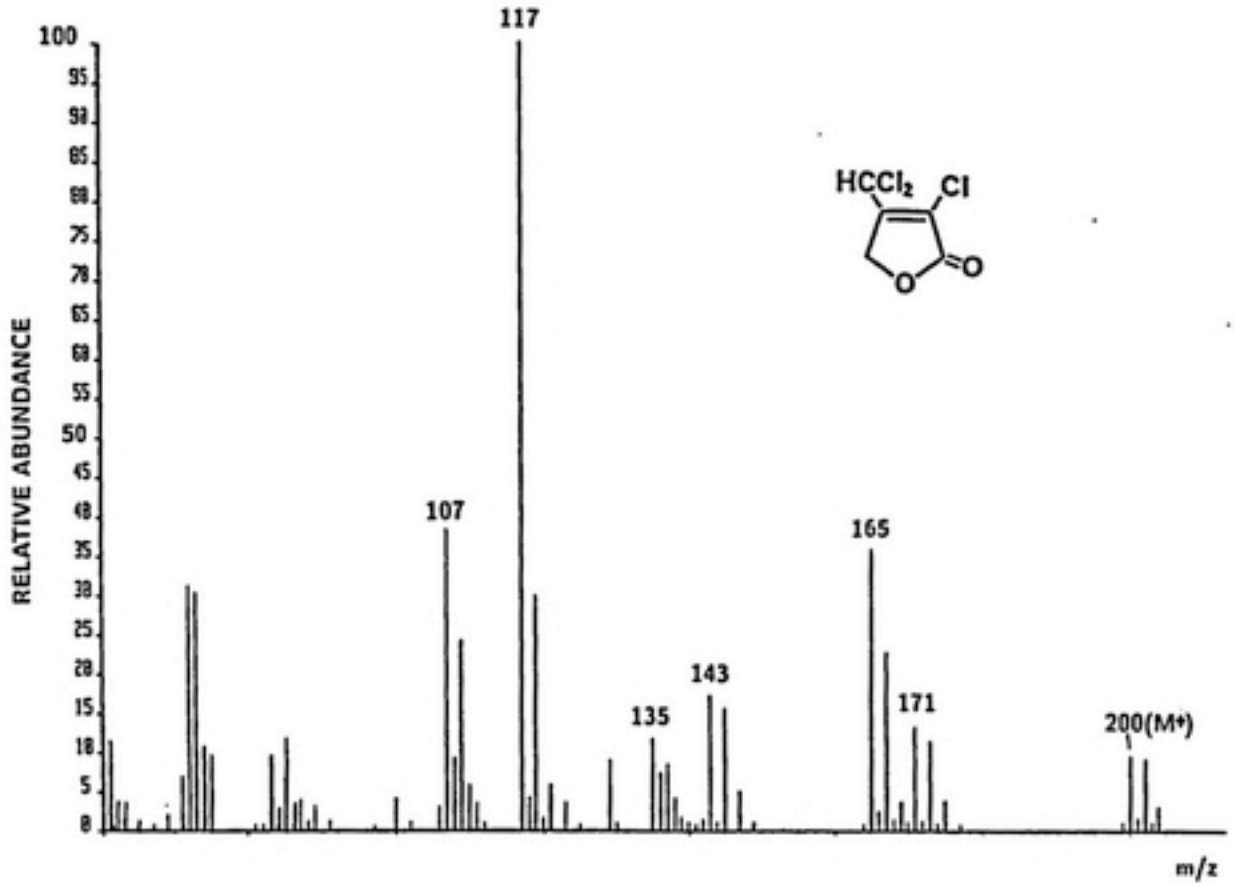
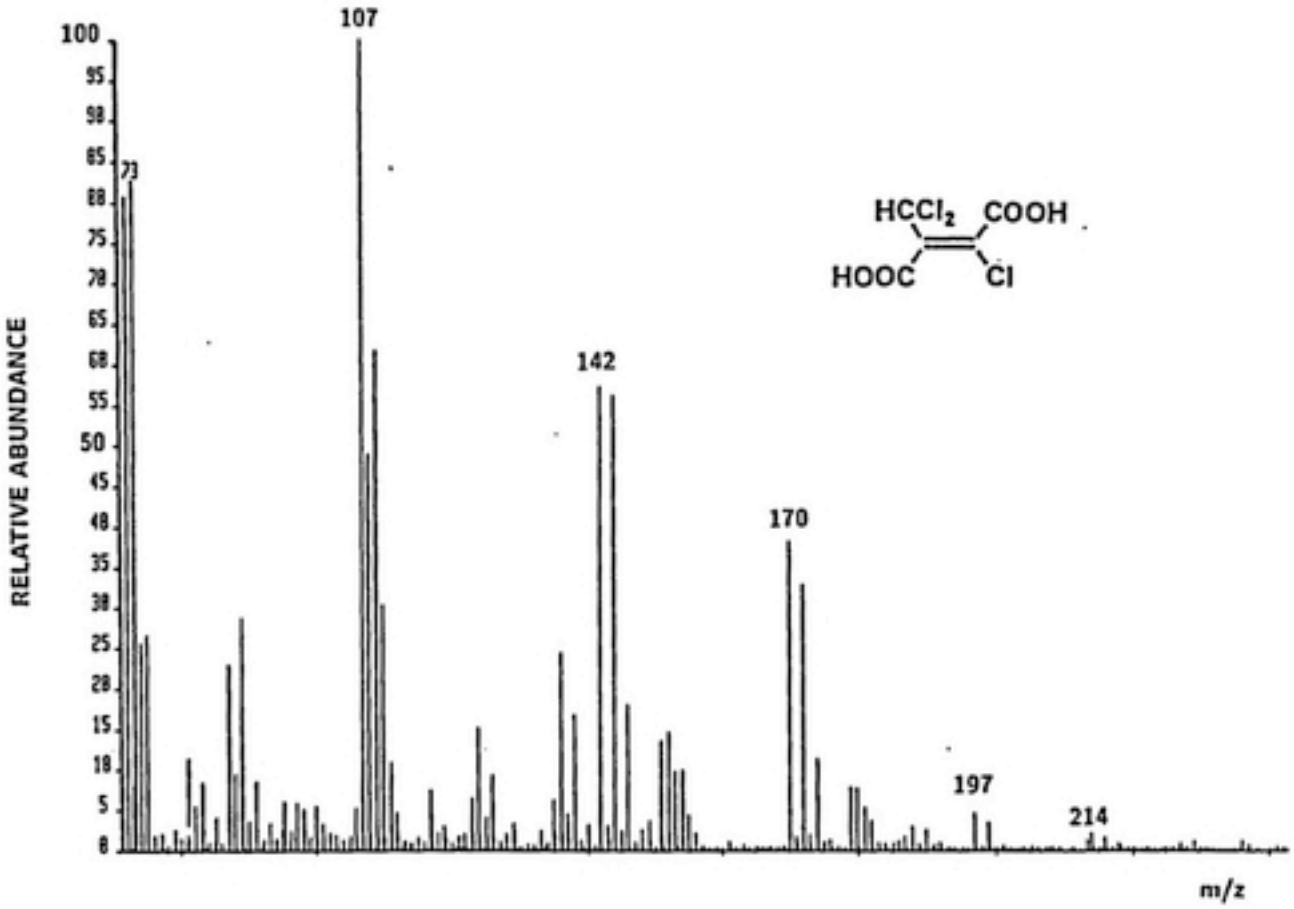


FIG.5 Mass Spectrum of ox-EMX



isopropoxide in isopropanol and with $NaBH_4$ in a mixture of isopropanol and water did not yield red-EMX as indicated by GC-MS analyses. The second reaction nevertheless produced a compound which could be an isomer of red-MX. It had an identical mass spectrum plus a chlorine ion cluster at mass/charge ratios of 121 and 123.

4.5 : Derivatization and Gas-Chromatography/Mass Spectrometry Analyses.

To prepare the chlorinated humic water, fulvic acid, and drinking water samples for GC/MS analyses, their extracts were evaporated to dryness and the residues were methylated using 250 μL of the respective methylation agent (see Table 1). In order to detect and quantify MX and E-MX by SIM mode GC/MS analysis, the extracts (ether or XAD extracts) were methylated using 2% (volume/volume)$\cdot H_2SO_4$ in methanol. The reaction was allowed to proceed for 1 hour at 70°C. The reaction mixture was neutralized by the addition of 2% aqueous $NaHCO_3$ and subsequently extracted twice using n-hexane (2 x 250 μL). For the determination of the synthesized ox-MX and ox-EMX in the samples, the extracts were methylated with BF_3 (12% by weight) in methanol. This reaction was allowed to go on for 12 hours at 70°C. The reaction mixture was similarly neutralized and extracted.

The hexane extracts, combined for each sample, were concentrated under a stream of nitrogen before being injected into the GC. Quantitative determination of the

compounds was carried out by reference to an internal standard, mucobromic acid (MBA), added to the extracts in known amounts.

The analysis of red-MX was carried out in unmethylated extracts using 2, 3-dibromo-2(5H)-furanone (red-MBA) as an internal standard. Attempts were also made to analyze ox-MX in unmethylated extracts, again, using red-MBA as the internal standard.

The GC/MS analyses of these extracts were performed on a Hewlett-Packard 5890 capillary gas chromatograph interfaced with a VG 70 - 250 SEQ mass spectrometer. The conditions for the analyses are given in Table 1. For qualitative and quantitative determination, the MS was operated in the selected ion monitoring (SIM) mode, and the ion peaks monitored are listed in Table 2. The SIM data was recorded and computed using the standard SIM routine of the VG 11-250J data system. Response factors for the ions were calculated versus the respective internal standard (Table 2) and the identification of the compounds in the extracts was based on positive matching of retention times and of relative ion peak area ratios.

For the synthesized compounds (reaction mixtures), the GC analysis was done on a Carlo-Erba HRGC 5160 capillary gas chromatograph equipped with a DB-1 fused silica capillary column (30 m long). Separation of ox-MX and ox-EMX was attempted on DB-1/30m, DB-17/30m, DB-1701/15m, SP-2340/30m, and DB-5/60m columns.

TABLE 1. Derivatization Agents and GC Conditions for SIM Mode GC/MS Analyses

Compound	Internal Standard	Column	Temperature Program					Retention Time		Derivatization Agent
			Initial °C	Hold min	Rate °C/min	Final °C	Hold min	Compound	Min.	
MX EMX	MBA	DB-1/30m	115	3	6	165	1	MBA MX EMX	6:05 7:18 10:15	2% H_2SO_4 in MeOH
red-MX	red-MBA	DB-1/30m	100	3	6	135	1	red-MBA red-MX	6:57 7:46	
ox-MX	red-MBA	DB-1/30m	110	3	6	130		red-MBA ox-MX	5:42 4:35	
ox-MX ox-EMX	MBA	DB-5/60m	160	3	3	190	1	MBA ox-EMX ox-MX	8:12 10:02 10:07	BF_3-MeOH

TABLE 2. Ion Peaks Used and Relative Ratios Found for the SIM Mode GC/MS Analyses of MX and the MX Analogues in Chlorinated Water

Compound	Fragmention	M/Z	Relative Peak Area Ratios: Response Factor	Std	FA	HW	DW	DW Spike level 1	DW Spike level 2
MX	$M-OCH_3$	198.9120		0.58	0.43	0.62	0.39		
		200.9091	1.26	1.00	1.00	1.00	1.00		
		202.9061		0.60	0.72	0.60	0.45		
EMX	$M-OCH_3$	244.9537	3.36	1.00	1.00	1.00	1.00		
		246.9510		0.92	0.88	0.94	0.94		
red-MX	M-Cl	164.9510		1.52	1.49	1.60	2.15*	1.65	
		166.9481		1.00	1.00	1.00	1.00	1.00	
	M-CHO	170.9171		0.62	0.56	0.67	0.64	0.52	
		172.9142		0.59	0.48	0.58	0.58	0.50	
	M^+	199.9169		0.40	/*)	nm	0.34	0.28	
		201.9196		0.39	/*)	nm	0.37	0.31	
ox-MX (anhydride)	$M-CO_2$	169.9093		0.43	0.40	0.37	0.43	0.39	0.46
		171.9063		0.37	0.34	0.36	0.37	0.36	0.43
	M-Cl	178.9303	0.10	1.00	1.00	1.00	1.00	1.00	1.00
		180.9973		0.74	0.62	0.65	0.68	0.70	0.68
	M^+	213.8991		0.19	0.22	0.20	0.20	0.21	0.15
		215.8962		0.16	0.18	0.16	0.25	0.14	0.19
ox-MX (methylated)	$M-COOCH_3$	200.9277		0.76	3.79**	0.24**	1.16	0.78	0.78
		202.9247		0.65	2.79**	0.30**	0.62	0.89	0.73
	M-Cl	224.9722		1.18	0.42	0.60	1.75**	1.09	0.98
		226.9692		0.58	0.19	0.35	0.46	0.57	0.64
	$M-CH_3OH$	227.9148		0.97	1.03	1.23	1.01	0.95	0.98
		228.9226		1.08	1.41	0.96	0.69	1.16	1.07
	$M-OCH_3$	229.9118	0.60	1.00	1.00	1.00	1.00	1.00	1.00
		230.9197		1.01	1.57	1.13	*	0.88	0.86

Table 2 (Continued)

Relative Peak Area Ratios

Compound	Fragmention	M/Z	Response Factor	Std	FA	HW	DW	DW Spike Level 1	DW Spike Level 2
ox-EMX	M-$COOH_3$	200.9277		0.96	1.03	1.11	1.10		
		202.9247	0.50	1.00	1.00	1.00	1.00		
	M-OCH_3	224.9722		0.87	0.70	0.71	0.79		
		226.9692		0.39	0.43	0.48	0.40		
MBA	M-OCH_3	240.8325							
red-MBA	M-Br	160.9239							

*) Interference

nm= not measured

**) Interference from ox-EMX

***) Response factor= (Acomp x $Ccomp^{-1}$) x (Cstd. x $Astd.^{-1}$) where C=concentration and A=ion peak area ratio

4.6 : Mutagenicity (Ames) Assay.

The bacterial mutagenicity of red-MX, ox-MX, and ox-EMX was tested in the constructed *Salmonella typhimurium* strains TA 100, TA 98, and TA 102 according to the standard plate incorporation procedure of Maron and Ames (1983). The genotypes of these strains are as follows:

TA 100 - his G46, rfa, uvrB, pKM 101
TA 98 - his D3052, rfa, uvrB, pKM 101
TA 102 - his G428, rfa, pKM 101, pAQ1

The strains were kept in storage at -70°C, and from these master-plates were prepared and kept at 4°C. The presence of genetic markers as well as spontaneous reversion rates and positive control responses were verified for each master-plate before it was used to grow overnight-cultures of the strain. The positive control and spontaneous responses were also tested along with every experiment. Experiments giving values outside the historically acceptable range were rejected. The positive control chemicals and the amount added per plate were as follows:

TA 100 (-S9) : 1.5 µg Sodium Azide
TA 100 (+S9) : 0.5 µg 2-anthramine
TA 98 (-S9) : 3.0 µg 2-nitrofluorene
TA 98 (+S9) : 0.5 µg 2-anthramine
TA 102 (-S9) : 6.0 µg Daunomycin
TA 102 (+S9) : 30 µg 1,8 dihydroxyanthraquinone (Danthron)

The effect of exogenous xenobiotic metabolizing enzymes on the mutagenicity of the MX analogues was tested using Aroclor 1254 - induced rat liver homogenate fraction, S9 (Moltox). The S-9 was added at 0.3 mg protein/plate.

The synthesized compounds and the extracts were stored in ethyl acetate which, and at the time of testing, was evaporated under a stream of nitrogen or helium. The residues were then redissolved in the test solvent, DMSO. All experiments were done with a minimum of four doses using duplicate plates per dose. Each experiment was repeated at least once on a separate day. A linear dose-response above the background rate of spontaneous reversion was taken as indicative of positive mutagenicity. The mutagenic potency was determined from the slope of the line fitted by linear regression on the data points. The result was accepted only if the correlation factor r was equal to or more than 0.90.

In testing the mutagenicity of the chromatographic fractions only TA 100 (without metabolic activation) was used since TA 100 has been found to be the most sensitive towards extracts of chlorinated water and the mutagens MX and E-MX. The individual contribution of MX and its analogues to the overall mutagenicity of the chlorinated samples was determined on the basis of the mutagenic potency of the pure compound (net revertants/ng) and its concentration in solution.

V: RESULTS

5.1 : Mutagenicity of the MX Analogues

The synthesized MX analogues were found to be mutagenic (Table 3). The dose-response curves of the individual compounds (TA 100) are shown in Figs. 6, 7 and 8. Data for each curve is shown from one representative experiment with each compound. All the doses tested (for every compound) fell within the initial linear portion of the dose-response curves. The correlation coefficient, "r" was greater than 0.90 in all cases, and greater than 0.97 for most. In all the experiments, the Spontaneous and Positive Control revertants for each strain were within the historically acceptable range.

Revertants/Plate.

	TA 100	TA 98	TA 102
Spontaneous:	142 + 30	38 + 6	309 + 30
Pos. Control:	665 + 162	382 + 108	790 + 124

For the purpose of comparison, pure MX was tested in this same assay system, and it exhibited a mutagenic potency of 22 net revertants/ng (in the absence of S9). The slopes of the dose-

FIG.6 Mutagenic Response of ox-MX in TA 100 (-S9)

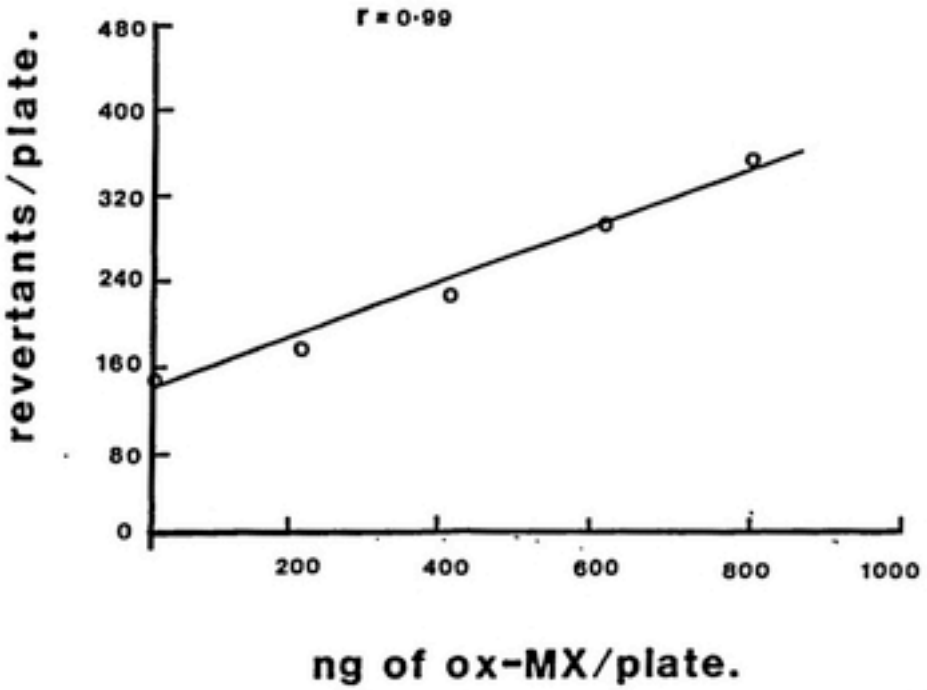


FIG.7 Mutagenic Response of red-MX in TA 100 (-S9)

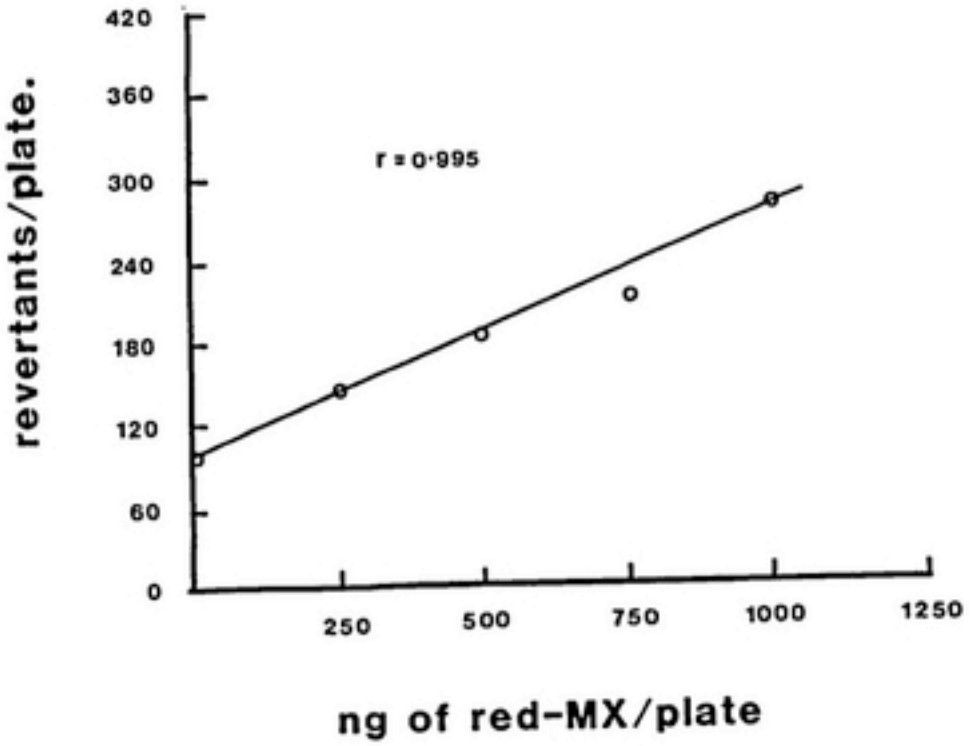


FIG. 8 Mutagenic Response of ox-EMX in TA 100 (-S9)

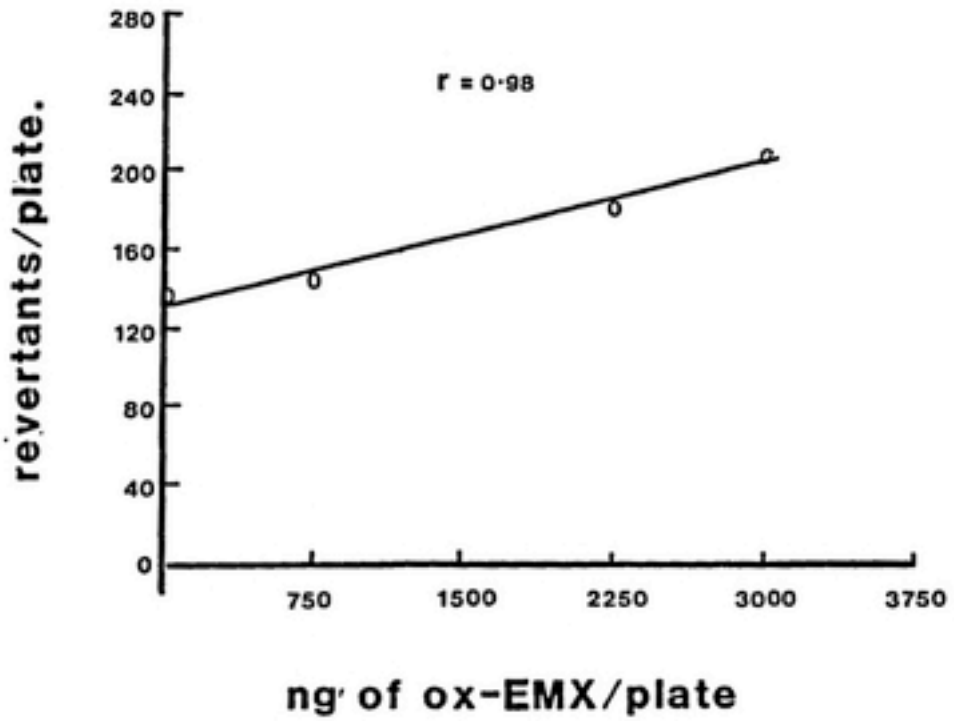


TABLE 3. Mutagenic Potencies of MX and the MX Analogues

Compound	Highest Dose Tested	Mutagenic Potency (net revertants/ng) TA 100 -S9	TA 100 +S9	TA 98 -S9	TA 98 +S9	TA 102 -S9	TA 102 +S9
red-MX (MW-200)	2000 ng	0.13 ± 0.03	0.045 ± 0.01	nm	nm	0.11±0.06	nm
ox-MX (MW-232)	1300 ng	0.36 ± 0.04	nm	nm	nm	nm	nm
ox-E-MX (MW=232)	3000 ng	0.03	nm	nm	nm	nm	nm
MX (MW=216)	16.4 ng	22 ± 2.1	nt	nt	nt	nt	nt

Note: nm = not mutagenic (approximately equal to the spontaneous reversion rate)
nt = not tested for mutagenicity

response curves indicated that the analogues were not very potent mutagens in comparison to MX or even E-MX.

The highest mutagenic activity was obtained in tests carried out with TA 100 in the absence of metabolic activation (S9). ox-MX and ox-EMX exhibited 1.4% (0.36 net revertants/ng) and 2% (0.03 net revertants/ng) of the mutagenicity of MX and E-MX respectively. None of the MX analogues elicited any mutagenic response in TA 98, even at the highest doses tested. The mutagenicity of red-MX in TA 102 (0.11 net revertants/ng) was about the same as its activity in TA 100 (0.13 net revertants/ng) while ox-MX and ox-EMX were found to be non-mutagenic in TA 102 (at the highest dose of 2000 ng/plate and 3000 ng/plate respectively). Addition of S9 rendered all the compounds, except red-MX, non-mutagenic. In the presence of S9, red-MX still induced 0.045 net revertants/ng which was less than half its activity without S9.

The mutagenic activities of all 3 compounds in strain TA 100 (without S9) are summarized below, both in terms of ng and nmole.

Compound	Mutagenic Activity in TA 100
red-MX	0.13 net rev./ng or 26 net rev./nmole
ox-MX	0.36 net rev./ng or 84 net rev./nmole
ox-EMX	0.03 net rev./ng or 7 net rev./nmole
MX	22 net rev./ng or 4680 net rev./nmole

5.2 : Quantitative Determination of the MX Analogues in the Mutagenic Extracts

The derivatization of ox-MX was attempted with BF_3 in methanol, with 2% H_2SO_4 in methanol and with diazomethane. The latter two were unsuccessful and hence ox-MX was methylated with BF_3 in methanol. The GC analyses done on 4 linearly increasing amounts of methylated ox-MX showed good correspondence and linearity. Analyses of underivatized pure ox-MX on the other hand did not give very convincing values, lacking in both linearity and reproducibility, particularly at the lower concentrations. Thus for the purpose of quantifying ox-MX and ox-EMX, the FA, HW, and DrW extracts were derivatized with BF_3 in methanol prior to SIM mode GC/MS analyses. The mass-spectra of methylated ox-MX and ox-EMX are presented in Figs. 9 and 10. In order to determine the amounts of MX and E-MX, the extracts were instead methylated with 2% H_2SO_4 in methanol. The determination of red-MX was carried out in underivatized samples.

5.2 (a) : Concentration and activity in Chlorinated Humic Water

The chlorinated lake humic water extract was determined to be mutagenic in TA 100, inducing 21000 net rev./liter (21 net rev./ml). SIM mode GC-MS analyses of the methylated extract indicated that all three of the MX analogues were present (Table 4). ox-EMX was most abundant, being present at a concentration of 5081 ng/liter. The other two analogues, red-

TABLE 4. Concentrations and Activities of MX and MX Analogues in Extracts of Chlorinated Water

Sample	Mutagen. net rev/mL	MX Conc. ng/L	MX Mutagen. Contr[a] %	EMX Conc. ng/L	EMX Mutagen. Contr[a] %	red-MX Conc. ng/L	red-MX Mutagen. Contr[b] %	ox-MX Conc. ng/L	ox-MX Mutagen. Contr[b] %	ox-EMX Conc. ng/L	ox-EMX Mutagen. Contr[b] %
FA	48	675	36	1204	4	643	0.3	961	0.7	26777	1.7
HW	21	260	32	526	4	370	0.4	306	0.5	5081	0.7
Dr.W.	2.04	13	17	20	2	41	0.4	53	0.9	251	0.4

[a]Calculated on the basis of 5600 and 320 net revertants/nmol specific MX and EMX mutagenicty, respectively

[b]Calculated on the basis of the mutagencity reported in Table 3

FIG.10 Mass Spectrum of Methylated ox-EMX

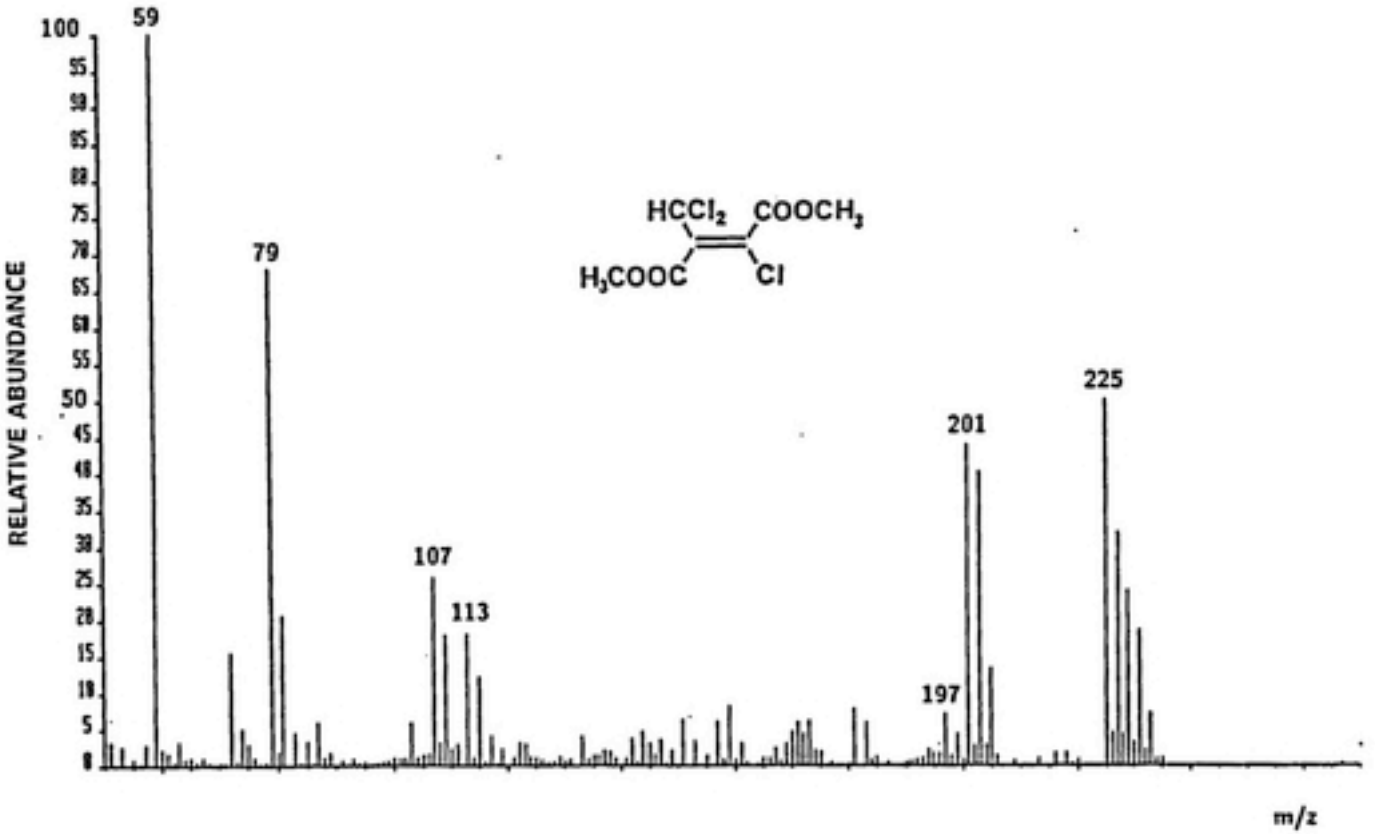
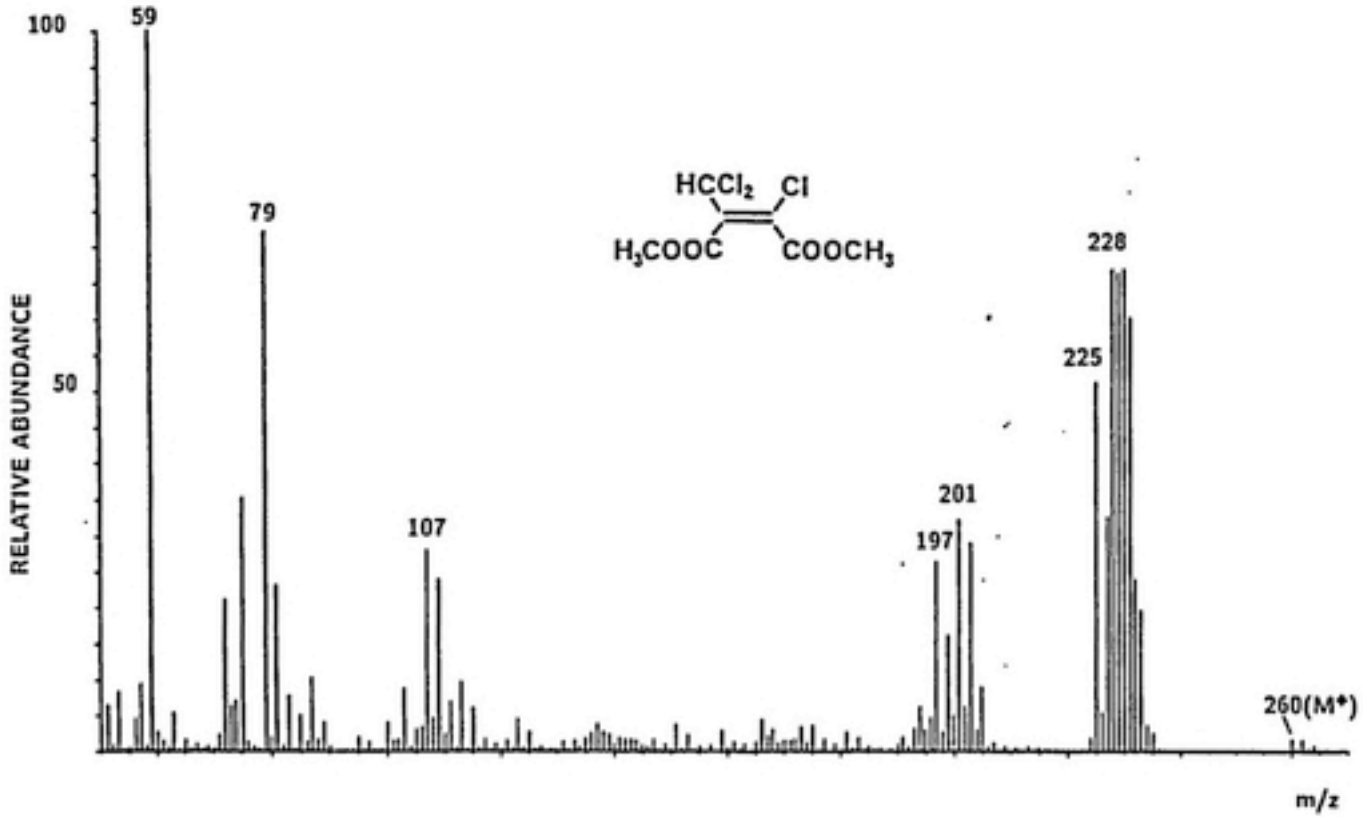


FIG.9 Mass Spectrum of Methylated ox-MX



MX and ox-MX, were present at concentrations just a little higher than the concentration of MX, i.e 370 ng/liter and 306 ng/liter respectively. On the basis of the individual mutagenic potencies reported in Table 3, and their concentration in the extract, it was possible to calculate the contribution of each MX analogue to the total activity of the extract. red-MX, ox-MX and ox-EMX accounted for 0.4%, 0.5% and 0.7%, respectively, of the overall 21000 net rev./liter.

5.2 (b) : Concentration and Activity in Chlorinated Fulvic Acids

The fulvic acid solution , chlorinated at pH 2 and at a Cl_2/TOC ratio of 2, was found to be very mutagenic in TA 100. This extract induced 48000 net revertants/liter (48 net rev./ml). All three MX analogues were present in the sample. SIM mode GC-MS analysis was used to determine their concentrations, which are shown in Table 4. Once again, red-MX and ox-MX were present in amounts not much greater than the amount of MX. However, the concentration of ox-EMX in this sample was significantly higher, i.e 26777 ng/liter. This amount was high enough for ox-EMX to account for 1.7% of the total activity (i.e inducing 816 net revertants/liter), while red-MX and ox-MX accounted for less than 1% individually. EMX was the second most abundant compound identified (1204 ng/liter, accounting for 4% of the total activity).

5.2 (c) : Concentration and Activity in Chlorinated

Drinking Water

The Chapel Hill drinking water sample comparatively had the lowest TOC. Hence the relatively lower mutagenic activity of the ether extract, i.e 2040 net revertants/liter, was reasonable. The amounts of MX and its analogues as determined by SIM mode GC-MS are shown in Table 4. The concentration of ox-EMX (251 ng/liter) was almost 20 times the concentration of MX (13 ng/liter) in this drinking water extract. However, it only accounted for 0.4% of the total mutagenicity. Next to ox-EMX, ox-MX was the most abundant identified compound (53 ng/liter) but it still accounted for less than 1% of the sample's activity.

It can be seen that only 20.7% of the mutagenic activity of the drinking water extract was accounted for by MX, E-MX and the three MX analogues. Within this , MX still remained the major contributor, accounting for a large 17% of the total activity (see Fig. 11).

5.3 : HPLC Fractionation of Chlorinated Fulvic Acid

5.3 (a) : Chlorination

Various chlorination conditions were attempted in order to obtain a chlorinated fulvic acid sample which would be highly mutagenic yet (simultaneously) have comparatively low levels of MX. The mutagenic activity as well as the concentrations of MX and E-MX were higher in samples chlorinated at pH 2 compared to corresponding samples chlorinated at pH 7 (Table 5 and Fig.12). At pH 2.0 (constant), the amount of MX, E-MX and the mutagenic

FIG. 11 Activities of Mutagens in Chapel Hill Drinking Water

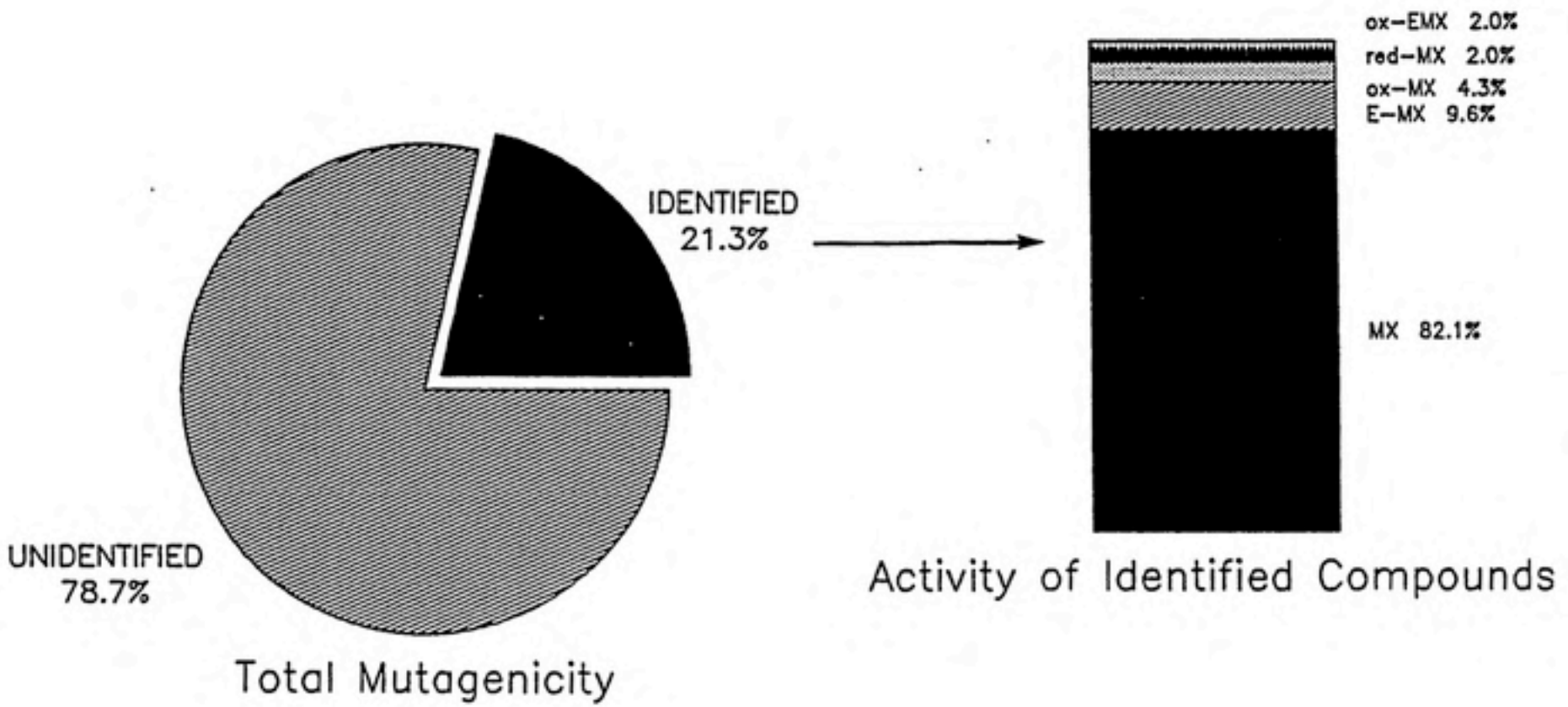
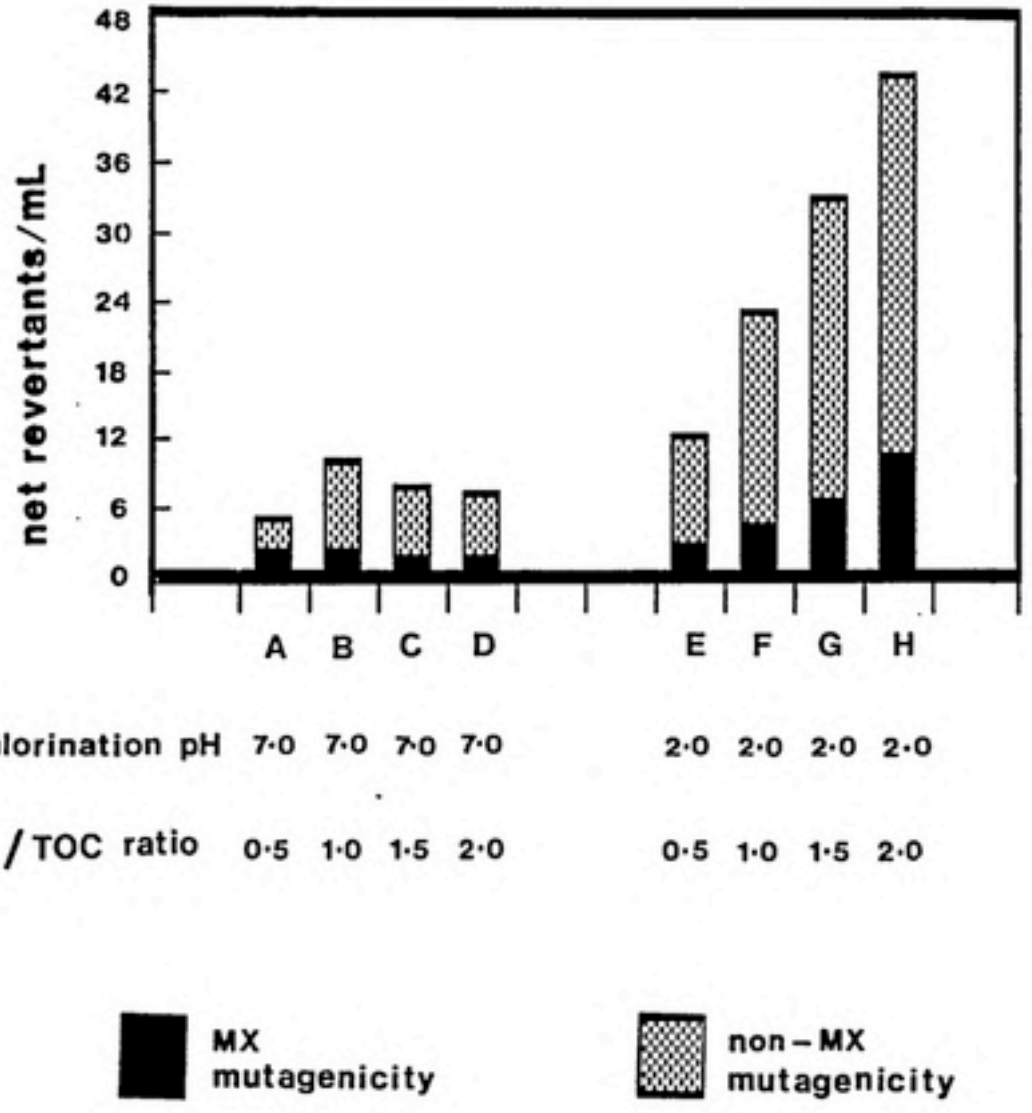


TABLE 5. Mutagenic Activity and MX, E-MX Concentrations in Aqueous Solutions of Chlorinated Fulvic Acids

Sample	pH[a]	Cl_2/TOC[b]	Mutagencity	Concentration ng/L		Mutagenicity Contribution				Total %
			net rev/mL	MX	EMX	MX[c] net rev/mL	%	E-MX[c] net rev/mL	%	Total %
A	7.0	0.5	5.1	77	171	2.0	40	0.3	6	46
B	7.0	1.0	10.1	86	235	2.2	22	0.3	3	25
C	7.0	1.5	7.6	71	130	1.8	24	0.2	3	27
D	7.0	2.0	7.4	69	136	1.8	24	0.2	3	27
E	2.0	0.5	12.0	107	328	2.8	24	0.5	4	28
F	2.0	1.0	22.0	180	703	4.7	21	1.1	5	26
G	2.0	1.5	33.0	261	851	6.8	21	1.3	4	25
H	2.0	2.0	43.5	377	658	10.3	24	0.9	2	26

a) chlorination pH
b) TOC of Aqueous Solution = 2.5 mg/mL, chlorination carried out at room temperature, in the dark.
c) Calculated on the basis of 5600 net rev./nmol and 320 net rev/nmol specific mutagenicity of MX and EMX, respectively.

FIG.12 MX and non-MX Mutagenicity in Solutions of Chlorinated Fulvic Acids

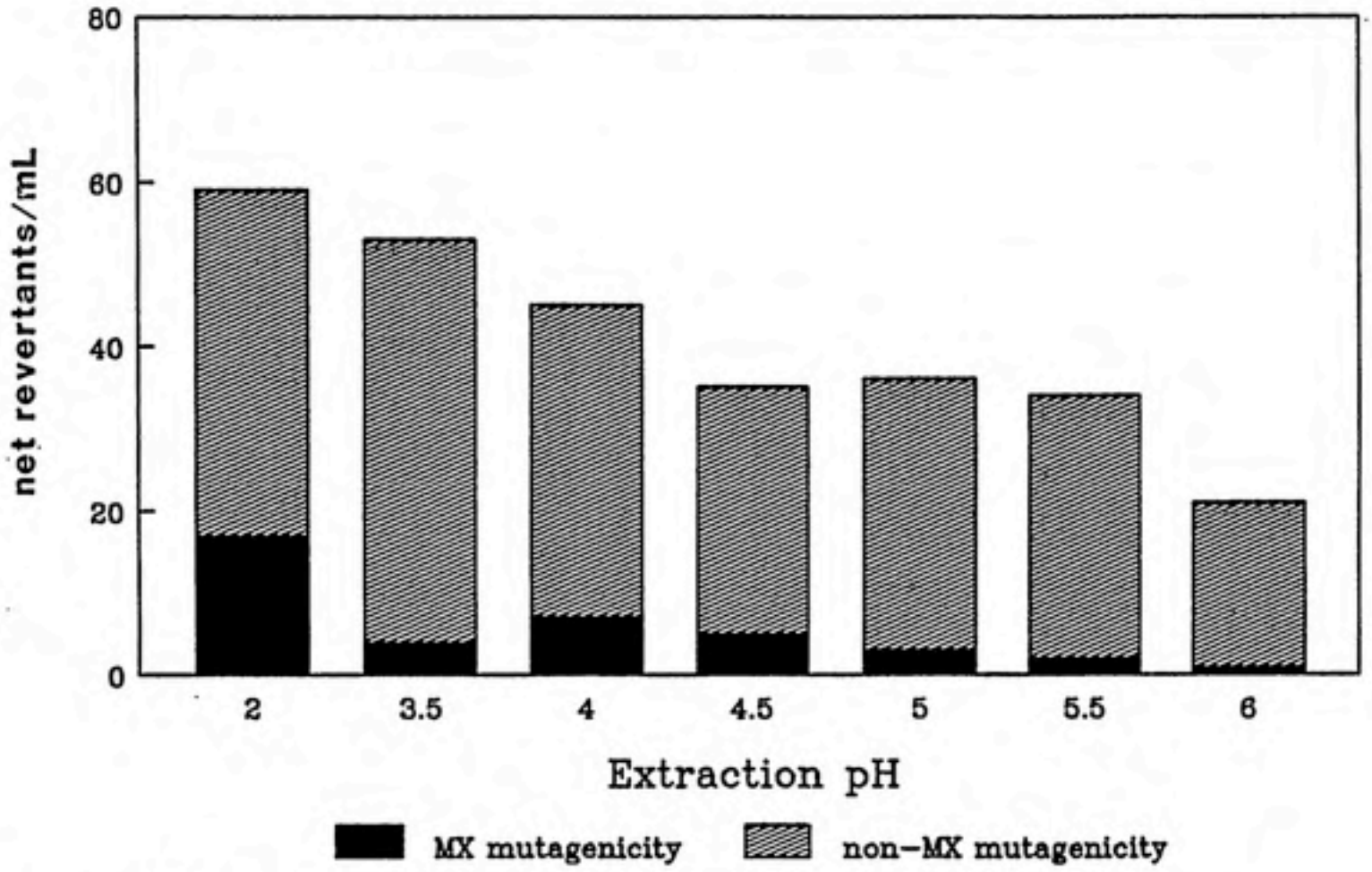


activity all increased with increasing doses of chlorine (higher Cl_2/TOC ratio, upto 2.0). The highest absolute yield of mutagenic activity not due to MX was found in Sample H, chlorinated at pH 2.0 and a Cl_2/TOC ratio of 2.0 (Fig. 12). The overall mutagenic activity in this sample was 43500 net revertants/liter, of which only 24% was due to MX, present at a concentration of 377 ng/liter and only 2% was due to E-MX, (present at a concentration of 658 ng/liter. Thus, Sample H contained sufficient amounts of non-MX mutagenicity which could be fractionated in an attempt to identify the principal mutagens besides MX and E-MX.

5.3 (b) : Separation and Activity of Fractions

First, the polarity and acidic properties of mutagens in the chlorinated fulvic acids (aqueous solution) was investigated. Diethyl ether (liquid-liquid) extraction of the solution, adjusted to seven different pH conditions, indicated an almost constant increase in the extracted/extractable mutagenicity with decreasing pH values (Table 6, Fig. 13). It would seem that the solution contains a range of mutagenic compounds having various pKa values. However, Table 6 and Fig. 13 also show an increase in the amount of MX that is extracted as the pH is lowered. At pH 2.0, the extracted mutagenic activity is equivalent to 59000 net revertants/liter, of which 29% is contributed by MX, present at a concentration of 0.68 ng/liter. This concentration is almost 4 to 6 times higher than the amount of MX extracted at pH 4.5 and pH 5.0 respectively.

FIG.13 Effect of pH on the Extractability of Mutagenic Activity from Solutions of Chlorinated Fulvic Acids



net revs. of MX based on concentration

TABLE 6. Total Activity and MX, E-MX Concentrations in Sample H Extracted at Various pH Values

pH	Mutagenicity net rev/mL	Concentrations ng/mL MX	E-MX	Mutagenicity Contribution MX net rev/mL	%
2.0	59	0.68	1.03	17	29
3.5	53	0.15	0.66	4	8
4.0	45	0.26	0.21	7	16
4.5	35	0.18	0.07	5	14
5.0	36	0.11	0.03	3	8
5.5	34	0.09	--	2	6
6.0	21	0.03	--	1	5

Since MX has a pKa value of 5.25 (i.e at pH 5.25, there are equal amounts of the protonated and non-protonated forms of MX), the amount of its cyclic, non-ionized isomer should not vary significantly after about two pH units below 5.25. At pH 3.25, more than 99% of MX should be in the cyclic non-ionized form. Consequently, the amount of MX extractable with diethyl ether cannot be significantly different at pH values below 3.25. Therefore, it is unlikely that the extractability is based solely on its acidic properties.

The scheme used in the separation of Sample H (mutagenic extract) is shown in Fig. 14. The first separation of the chlorinated fulvic acid solution was accomplished on a C_6 column with RP-HPLC, using Program 1. Four fractions were collected and these are referred to as fractions H1 (2-5 mins.), H2 (5-8.25 mins.), H3 (8.25-22.3 mins.), and H4 (22.3-30 mins.). The chromatogram for this separation is shown in Fig. 15. A large portion of the material (compounds) eluted early in the program, indicating the presence of mainly polar compounds. Of the four fractions collected, only the first three were mutagenic in TA 100, in the absence of S9. The amount of MX found in each fraction was also determined (Table 7 and Fig. 16). Although fraction H2 was the most mutagenic (25000 net revertants/liter), it was not considered suitable for further fractionation due to a comparatively higher concentration of MX (205 ng/liter). Instead, fraction H3 which had the most non-MX mutagenicity (its MX concentration being the lowest, 120 ng/liter) was fractionated further. An MX

FIG. 14 Scheme for the Separation and Purification of Sample H

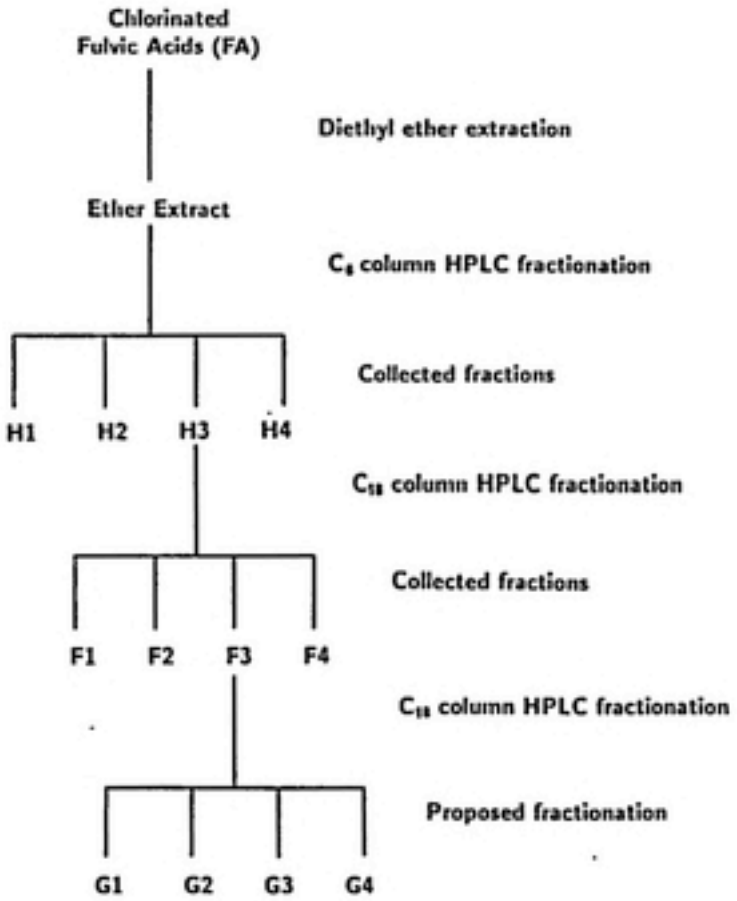


FIG. 15 HPLC Profile of Sample H Separation

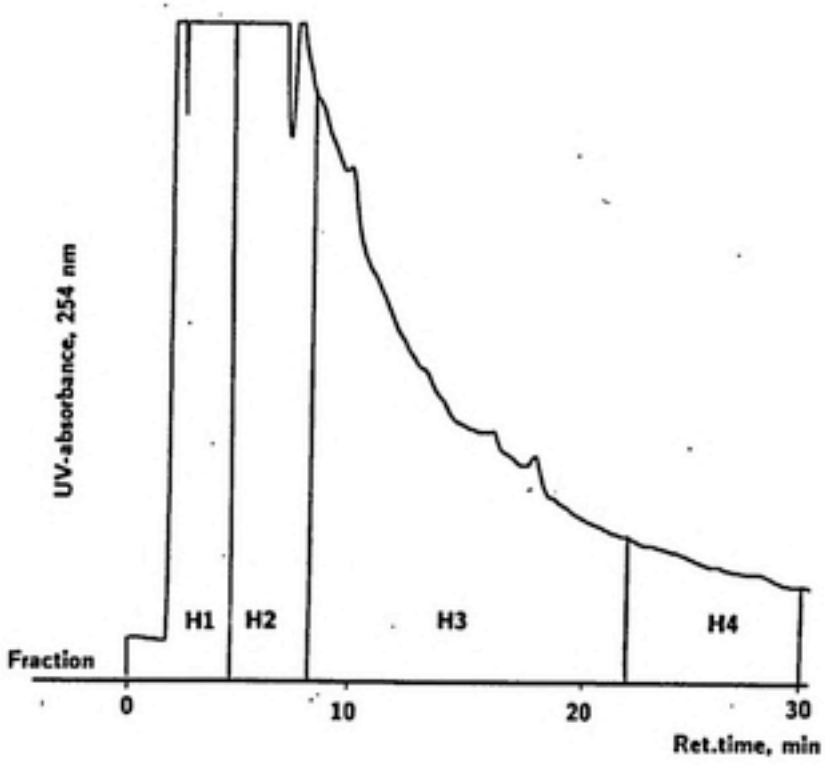


FIG.16 MX and non-MX Mutagenicity in Sample H Sub-fractions

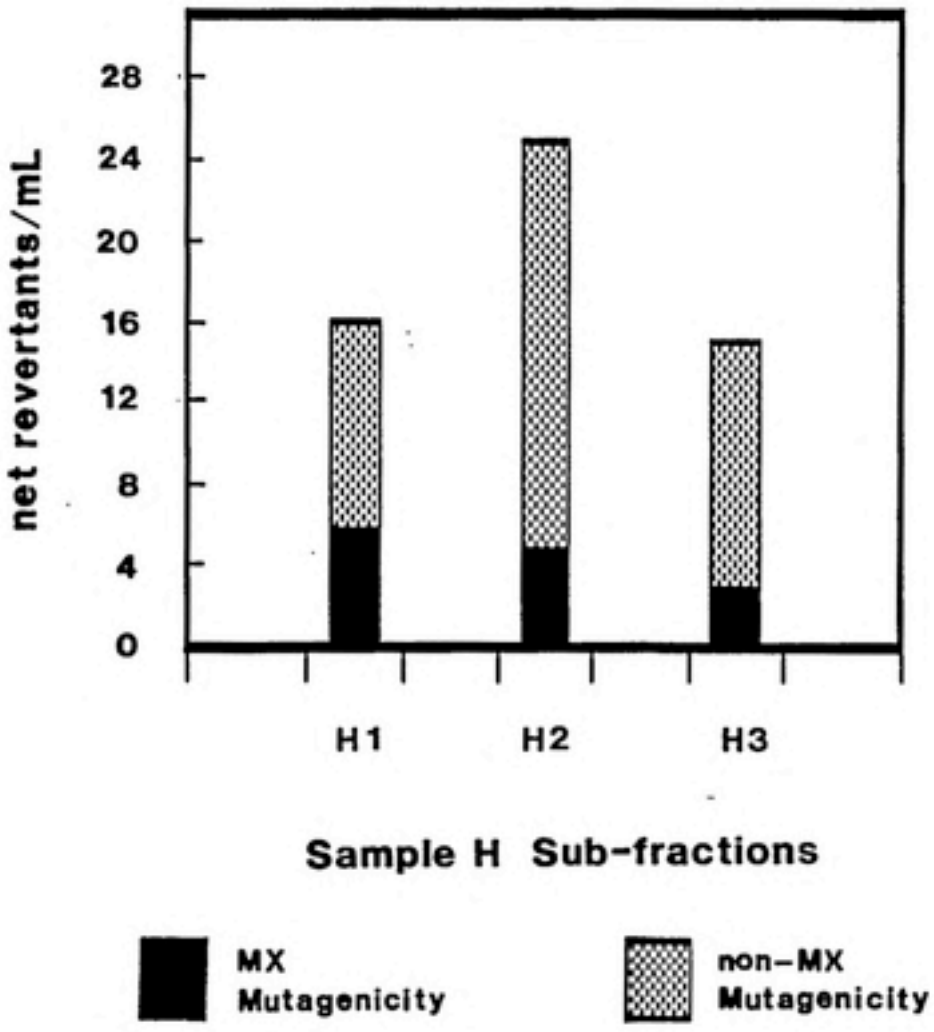


FIG.17 HPLC Profile of Fraction H1 Separation

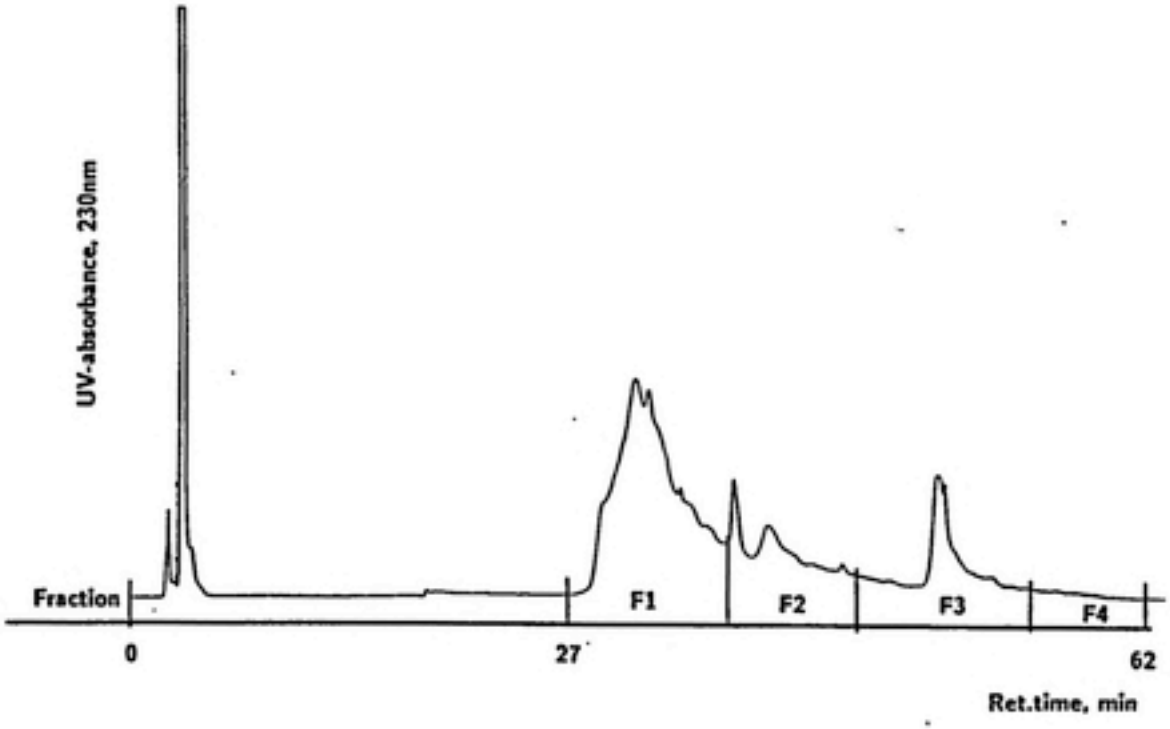


TABLE 7. Total Activity and MX Concentration in HPLC Sub-fractions of Sample H

Fraction	Mutagencity net rev/mL	MX Conc. ng/L	MX Mutag. Contr. % a)
H1	16	188	31
H2	25	205	21
H3	15	120	21
H4	nonm.	-	-
H3/F1	nonm.	na	
H3/F2	nonm.	na b)	
H3/F3	31	na	
H3/F4	nonm.	na	
H3/F1 - F4 c)	33	na	

na - not analysed
nonm. - not-mutagenic
a) based on a specific MX mutagenicity of 5600 net rev/nmol
b) Pure MX analysed at similar chromatographic concitions was found to elute in the beginning of fraction H3/F2
c) Following work-up the fractions were re-combined and re-tested for mutagenicity

standard (in solution) was also subjected to Program 1 under the same conditions. The pure compound was found to elute at 6.59 minutes, indicating that almost all the MX in the fulvic acid solution should have collected in fraction H2. This however was not the case. It is possible that the "spread" of MX over a broad area of the chromatogram may have been due to an overloading of the column (considering that the chlorinated solution was very concentrated w.r.t TOC and TOX).

The second separation was achieved by subjecting fraction H3 to Program 2 on a C_{18} column. Again, four fractions were collected during the stepwise gradient elution (chromatogram shown in Fig. 17) and these are referred to as F1 (27-37 mins.), F2 (37-45 mins.), F3 (45-55 mins.) and F4 (55-70 mins.). All the mutagenicity was recovered in fraction F3 (31000 net revertants/liter in TA 100), while F1, F2 and F4 exhibited no mutagenic activity at all. This fraction (F3) is, in all probability, free of MX as the small amount in the parent fraction H3 would have eluted in fraction F1 (when the mobile phase was 20% ACN). The mutagenic activity seems to be additive since the recombined fractions (F1+F2+F3+F4) induced 33000 net revs./liter.

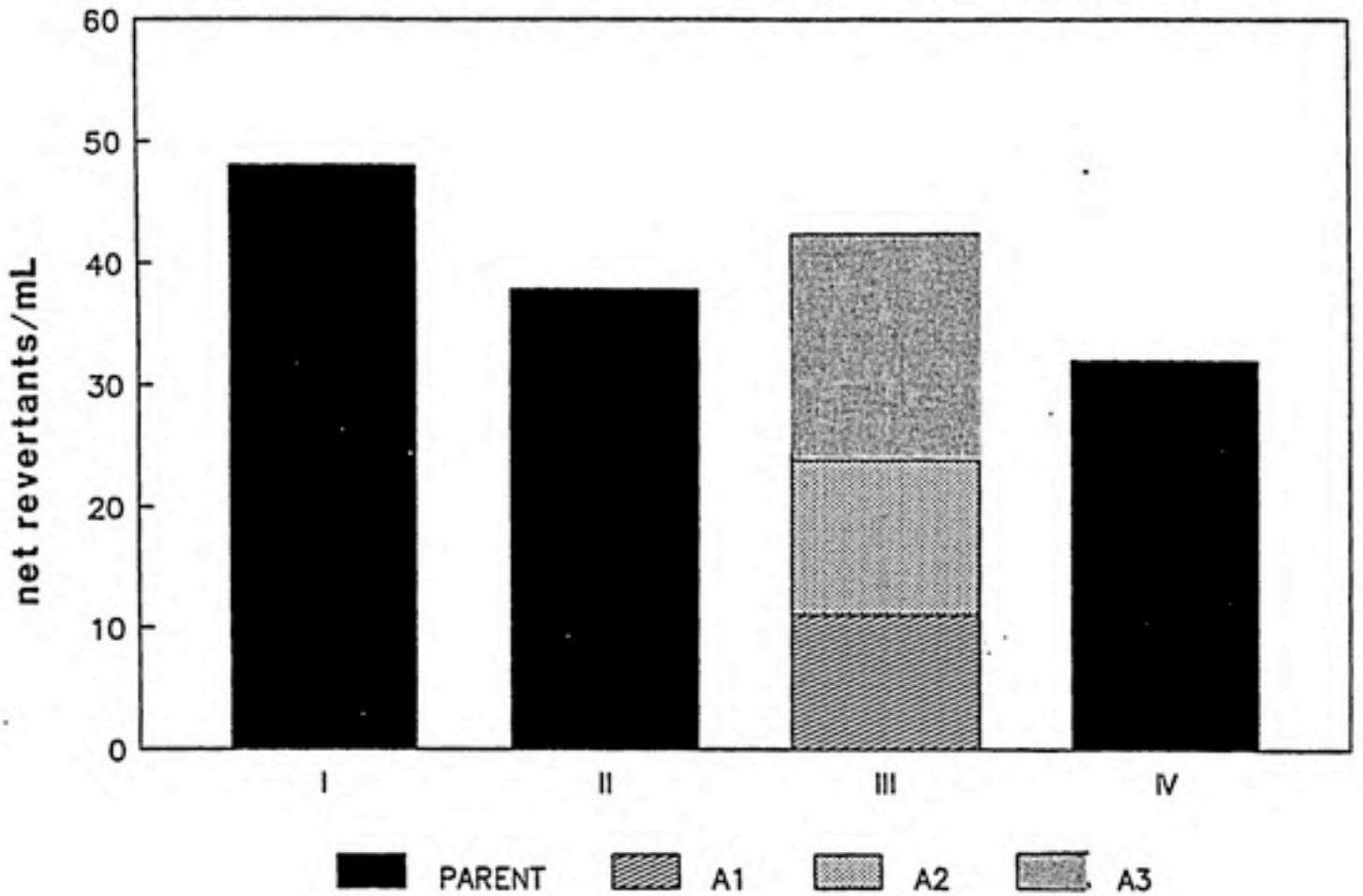
5.3 (c) : Additive Effect of the Mutagenic Activity

In order to confirm the additive effect of the mutagenic activity, an ether extract of Sample H (chlorinated at pH 2.0 and Cl_2/TOC ratio of 2.0) was tested for mutagenicity, then separated (on RP-HPLC, C_6 column) into 3 fractions called A1,

TABLE 8. Additive Effect of the Mutagenic Activity present in Sample H (FA)

Test Component	Mutagenic Activity
Sample H ether extract (parent extract)	48.1 net revs./ml
F_A (parent extract injected & collected off the column as a single fraction, F_A)	37.83 net revs./ml
Parent extract injected & collected as 3 separate fractions, i.e A1, A2 & A3	
Individual mutagenicity of A1	11.05 net revs./ml
Individual mutagenicity of A2	12.85 net revs./ml
Individual mutagenicity of A3	18.53 net revs./ml
Sum of individual mutagenicities, i.e A1+A2+A3	42.43 net revs./ml
A1+A2+A3 composite (the individual fractions A1, A2 & A3 combined and the composite tested for mutagenicity)	31.86 net revs./ml

FIG. 18 Additive Effect of the Mutagenic Activity present in Sample H (FA)



I. Sample H
II. Single Fraction
III. Sum of Individual Activities
IV. Combined Fractions

A2, and A3, all of which were subsequently assayed. The results, as shown in Table 8 and Fig. 18, indicate that although a certain amount of mutagenic activity was lost during HPLC fractionation, most of it was retained and it is additive in nature. The individual mutagenic activities of the three fractions collected separately (A1, A2 and A3) added up to 42.43 net revertants/ml. In comparison, the entire injected material (i.e parent Sample H) collected off the column as a single fraction induced 37.83 net revertants/ml. These values, given intra-assay variability and losses during fractionation (some mutagenicity may be retained on the column), and during transfer of samples, did not differ meaningfully from the activity of the parent Sample H, i.e 48.1 net revertants/ml.

VI: DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

6.1 : Discussion

This study was based on the assumption that some of the non-MX mutagenicity in chlorinated water could be due to certain candidate mutagens, the MX analogues. The theoretical basis for the existence of red-MX, ox-MX and ox-EMX can be justified by the findings of this research. However, it is merely the presence of these compounds that's established, and we cannot be certain that the mechanisms of their formation are exactly as hypothesized. The concentration of MX in the chlorinated fulvic acids solution, humic water and drinking water was such that it accounted for 36%, 32% and 17% respectively of the overall activity displayed by these samples. For a substantial part of the remaining mutagenicity to be explained by the MX analogues, they would have had to be either highly mutagenic or weak mutagens present in very large amounts.

In comparison to MX (the parent compound), red-MX, ox-MX and ox-EMX were weak mutagens, inducing only 0.13 net revs./ng; 0.36 net revs./ng and 0.03 net revs./ng respectively in the Ames TA 100 strain. red-MX and ox-MX are compounds in which the dichloromethyl group as well as the *cis* arrangement between the $CHCl_2$ and Cl substituents

have been retained. Their low mutagenicity relative to MX indicates that the aldehyde group is yet another critical structural feature that determines the potency of MX. The oxidation of this group seems to reduce the activity of the compound by almost 98% as ox-MX and ox-EMX exhibited only 1.4% and 2% of the mutagenicity of MX and E-MX respectively. Reduction of the aldehyde group on the other hand, decreases the mutagenicity to an even greater extent since red-MX was found to exhibit only 0.5% of the activity of MX. It has been shown that a *trans* arrangement between the Cl and $CHCl_2$ substituents reduces the activity considerably as E-MX exhibits only one-tenth the mutagenicity of MX. Therefore, as would be expected, ox-EMX exhibited the lowest mutagenicity among the three MX analogues since it lacked both the aldehyde group and the *cis* arrangement between the $CHCl_2$ and Cl substituents. Attempts to synthesize and detect red-EMX were unsuccessful in this study. However, since red-EMX would have had the unfavoured *trans* arrangement in addition to its aldehyde group reduced to an alcohol, its activity is predicted to be lower than that of ox-EMX.

The low activities of the MX analogues no doubt illustrate the importance of the aldehyde group. In fact, Streicher (1987) compared different carbonyl groups in alpha-chloro, alpha, beta-unsaturated systems (w.r.t. mutagenicity) and ranked them as Aldehyde > Ketones > Esters > Carboxylic acids. Ishiguro et. al. (1987) noted that

replacing the aldehyde substituent with a carboxyl group completely eliminated the mutagenicity of 2-chloropropenal. Elsewhere, Ishiguro et. al. (1988) concluded that an aldehyde attached to a rigid skeleton which also possesses strong electron withdrawing substituents is related to high mutagenicity. There can be, however, another perspective on the low mutagenicity of the MX analogues. A compound's uptake, transport and retention *in-vivo* is often determined by its lipophilicity vs. its hydrophilicity. Compounds with the carboxylic acid group are more polar due to the acid's high hydrophilicity. The more polar a compound is, the less likely it is to be taken up by the bacterial cells (in the Ames assay) and the more likely it is to be excreted. Thus, such a compound will give a low mutagenic response in the assay simply because it may be inaccessible. It can be seen that all three MX analogues have the COOH group, making them comparatively polar. Furthermore, ox-MX and ox-EMX cannot cyclize into a closed form. Information on MX has revealed that although the COOH group makes the compound polar, it nevertheless can convert to its cyclic, non-ionized tautomer as the pH is lowered. Chlorinated hydroxyfuranones are found to be more mutagenic in their cyclic forms, presumably because the closed form can pass through the bacterial cell membranes with greater ease (compared to the polar open form). Under the Ames assay test conditions (pH 7.0), the mutagenicity of MX is probably due mainly to the closed form which is present in equilibrium with the open form (the open

form being less likely to be taken up). As the pH is lowered, more of the cyclic tautomer is formed, explaining the higher mutagenic response of MX at pH 6.0 than at pH 7.0 (Meier et. al., 1987a). With this in mind, it seems conceivable that the low mutagenic responses of ox-MX and ox-EMX may have been due to the fact that they are polar (hydrophilic) compounds which cannot form cyclic tautomers, and thus are not taken up by the bacteria like MX (i.e they remain polar). To support or refute this, the bacterial test strains could have been incubated with these compounds, and compounds in which the COOH groups had been derivatized into their esters (decrease the polarity), and subsequently the amounts of uptake determined. An immediate counter-argument to the above is that red-MX, which <u>can</u> form a cyclic tautomer still exhibits a low mutagenic response.

The positive response of the MX analogues in strain TA 100 (without S9) suggests that like MX, they are direct-acting mutagens, operating primarily through base-pair substitution at the G-C pairs. In strain TA 98, red-MX, ox-MX and ox-EMX, even at the highest doses tested (1000 ng, 2000 ng and 3000 ng respectively) did not elicit any mutagenic responses. It appears that these compounds do not operate as frame-shift mutagens, at least not at these concentrations. The mutagenicity of red-MX in the novel strain TA 102 was about the same as its response in TA 100, while ox-MX and ox-EMX were not mutagenic in TA 102. These results are in agreement with the fact that strain TA 100 is

the most sensitive towards the mutagenicity of chlorinated water extracts or individual chlorination by-products.

It is well recognized that certain compounds can be procarcinogens which can be transformed into electrophiles (which then react with DNA) by the action of the cytochrome P-450 enzyme system within cells and tissues. The exogenous xenobiotic metabolizing enzyme system, S9, is used in the Ames assay to determine if such a transformation could occur with the test compound. A concern about the activity of red-MX was that perhaps its CH_2OH group could be oxidized by the P-450 enzymes to a CHO group, transforming the compound into the more potent MX. The assay results however indicated that the mutagenicity of red-MX is not increased in the presence of S9. It may be that *in-vivo* oxidation of red-MX (upon consumption of drinking water) is unlikely. On the other hand, it is also important to consider the possibility that the structural configuration of red-MX may not have permitted the CH_2OH group to fit into the active sites of the S9 enzymes. Perhaps allowing the compound to react *in-vitro* with a single enzyme, alcohol dehydrogenase, might have revealed whether or not the oxidation to MX can take place. The S9 mix reduced or eliminated the activity of all three compounds. The mechanism by which S9 exerts this effect is not entirely known. Some investigators have suggested metabolism to non-mutagenic products, non-specific protein binding, reaction with reduced glutathione or other

sulfhydryl groups (in the S9) and other possible enzymatic reactions (Ishiguro et. al., 1988).

As shown in Table 4, red-MX and ox-MX were found in the chlorinated waters at concentrations almost equal to or slightly greater than the amount of MX in corresponding samples. ox-EMX on the other hand, was present at relatively large concentrations, about 20-40 times higher compared to MX. Because of their weak mutagenic potencies however, none of the three compounds contributed to more than 1% of the total activity in each sample. The only exception was ox-EMX in the chlorinated fulvic acids solution, where it accounted for 1.7% of the total mutagenicity. Even if considered collectively, the mutagenicities of these compounds still leave a very major portion of the total activity of each chlorinated sample unaccounted for by any identified compounds. This is best illustrated by considering the Chapel Hill drinking water sample. In an ether extract of this chlorinated water (non-volatile organic portion), MX and its analogues including E-MX, accounted for only 21% of the total activity. It should be a matter of concern that more than three-quarters of the mutagenicity exhibited by the drinking water sample was due to unidentified compounds.

E-MX, ox-MX, red-MX and ox-EMX have been shown to be significantly less mutagenic than MX. The question therefore arises that could the residual non-MX mutagenicity be due to compounds not so closely related to MX in

structure. So far, the evidence regarding the structural features of importance favors this possibility. Only the COOH group in the open form of MX has not been altered in any of the MX analogues studied here (including E-MX). However, Streicher (1987) working with a different type of MX analogue (MX with the CHO group changed to a $CHCl_2$), noted that when the COOH group in this compound was changed to its amide, the mutagenicity was reduced by a factor of 5000. This would mean that for a compound to be as mutagenic as MX (or even close), all the critical structural features mentioned earlier, including the COOH group, must be present.

The extractability data (Fig. 13 and Table 6) revealed that the amount of mutagenicity which can be extracted increases as the pH of the chlorinated solution is lowered. This would suggest that the solution contained numerous, mainly acidic, mutagenic compounds with various pKa values. On the other hand, it was noted that the decrease in pH also led to an increase in the amount of MX (pKa = 5.25) recovered. Thus, the extractability of such mutagens may be based on factors other than their acidic properties (see section 5.3b.). One possible explanation could be that the low molecular weight mutagens are associated with high molecular weight organic material, and the extractability of the mutagenicity is in fact dependent on the pKa of the organic material.

Another indicator of the properties of the unidentified compounds comes from the chromatographic characteristics of the active subfractions. Fractionation of the mutagenic fulvic acids solution (Sample H) by RP-HPLC achieved substantial separation of the organic material. By the second level of separation, all the activity had concentrated in a single fraction, F3, which was virtually free of MX since the pure compound elutes much earlier (in F2) under the same mobile phase. However, fraction F3 was not analysed for the presence of MX. Further, no GC/MS structural identification work can be done on the fraction yet since it is still a relatively complex mixture. Attempts to separate F3 further have been unsuccessful. It appears that the mutagens may be slowly decomposing upon long storage and thus altering their chromatographic characteristics. Even though the fractionation of non-volatile mutagens in drinking water is best achieved by HPLC, loss of mutagenicity during repeated fractionation and the complexity of even highly purified fractions still remain a problem. Considering their retention times, the compounds in the active subfractions appear to be less polar than to MX. This information is still insufficient to predict even a general category to which the mutagens might belong to.

The break-down of the total mutagenic activity displayed by chlorinated waters into parts accounted for by individual compounds assumes that the mutagenicity is

additive in nature. The results of the fractionation-bioassay procedures done on Sample H support this assumption (Table 8). The activities of the individually collected subfractions add up to the mutagenicity displayed by the unseparated (parent) sample, and the combined-fractions sample, taking into account intra-assay variability and loss of mutagenicity during fractionation due to some mutagens being retained on the column. This additive effect of mutagenic responses (of compounds in drinking water) have been reported previously by Fielding and Horth (1986), who also indicated that there was no synergism or antagonism between the separated mutagenic fractions.

6.2 : Conclusions

This research has lead to the following conclusions:

(a). 3-Chloro-4-(dichloromethyl)-2(5H)-furanone, i.e red-MX; (Z)-2-Chloro-3-(dichloromethyl)-butenedioic acid, i.e ox-MX; and (E)-2-Chloro-3-(dichloromethyl)-butenedioic acid, i.e ox-EMX, are three other non-volatile compounds which result from the chlorination of humic and fulvic material in water. All three compounds are direct-acting mutagens in the Ames assay strain TA 100.

(b). While being structurally very similar to MX (MX analogues), these compounds nevertheless exhibited significantly lower mutagenic activities.

(c). The aldehyde substituent in MX is a structural feature critical in determining the mutagenic potency of MX.

All the MX analogues identified in chlorinated water so far have been weak mutagens.

(d). ox-MX and ox-EMX are acyclic compounds having COOH groups in their structure, which makes them relatively polar. Their weak mutagenic activities as determined by the Ames assay may just be due to the fact that these compounds are not taken up by the bacteria as easily as cyclic or non-polar compounds.

(e). The concentration of red-MX and ox-MX in chlorinated water was about the same as MX, but the concentration of ox-EMX was about 20-40 times higher. Due to their weak mutagenicities and such typical concentrations however, these compounds (collectively) accounted for only about 2% of the total activity exhibited by chlorinated water. The mutagenic activities of the individual components were determined to be additive in nature.

(f). A fulvic acids solution (TOC = 2.5 g/liter) chlorinated at pH 2.0 and a Cl_2/TOC ratio of 2.0 exhibited a high mutagenic activity (43500 net revertants/liter), of which 76% was "non-MX mutagenicity". HPLC fractionation of this material allowed MX to be separated out, and a highly mutagenic fraction containing unidentified mutagens (free of MX) was obtained. Working with this fraction, it will be possible to identify the principal mutagens besides MX in chlorinated water.

(g). 3-Chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone or MX still remains the only major mutagen

identified in chlorinated drinking water. In the drinking water tested during this study, MX and its analogues accounted for only 20.7% of the total mutagenicity. The results also indicated that the residual mutagenicity is due to compounds which are acidic in nature, comparatively less polar than MX and probably not closely related to MX in structure.

The use of chlorine as the primary disinfectant in drinking water treatment in the U.S will probably continue for some time to come. In terms of the associated health risks, there is sufficient current evidence pointing to the fact that the importance of the trihalomethanes may have been overestimated. The non-volatile by-products of humic and fulvic acid chlorination may be more important than previously believed. The Safe Drinking Water Committee's Subcommittee on Disinfectants and Disinfectant By-Products (NRC, 1987) has stated that

"There is a larger risk associated with the unidentifiable by-products of water disinfection, ...(a risk) high enough to warrant additional effort to determine its qualitative source and quantitative magnitude".

It is hoped that this work has contributed to a better understanding of the non-MX or residual mutagenicity of chlorinated drinking water. Further research is still needed to fully characterize this residual mutagenicity. To this end, some areas of research are suggested below.

6.3 : Recommendations for Future Work.

(1). Fraction F3 presumably contains unidentified but comparatively potent mutagens. This fraction needs to be fractioned further prior to structural elucidation by GC/MS.

(2). As shown in Fig. 13, in addition to fraction H3, H1 and H2 also exhibit a considerable amount of mutagenicity that is not due to MX. Bioassay-directed HPLC fractionation should be done on these samples in an attempt to identify the principal mutagens apart from MX.

(3). It should be investigated whether the low mutagenic activities of ox-MX and ox-EMX are in fact due to their high hydrophilicity, which may be preventing bacterial uptake of these compounds.

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