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THE EFFECT OF SOIL ON THE MUTAGENIC PROPERTIES OF WASTE WATER

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ABSTRACT

The disposal of complex mixtures such as waste water on agricultural lands poses known and unknown environmental risks. Mutagens may be introduced into the eco-system and perhaps concentrated by crop plants or leached into ground water supplies. The purpose of this study was to determine the biological effect of a mutagenic waste water before and after application to soil. We used an XAD-8 methanol extract of waste water from the municipal sewage treatment facility at Sauget IL. This extract was a potent direct acting mutagen when assayed with the Salmonella typhimurium. 1 and 3 ml of extract were brought up to 10 ml volumes with water and added to 10 g of sterile or nonsterile native clay loam. These mixtures were placed in a shaking water bath at room temperature for 0, 24, and 48 h. After separation of solid and liquid portions by filtration, dichloromethane was added to extract the organic fractions from each component. Solvent extractions were evaporated to dryness under vacuum and brought up in DMSO. Tests for mutagenic activity were conducted using strain TA98. After addition to the soil for greater periods of time the mutagenic activity decreased. The solid component exhibited greater mutagenic activity than the liquid. The XAD-8 extract was also assayed using the yg2 assay in Zea mays and the micronucleus assay in Tradescantia. The extract did not induce mutation or chromosome aberrations in these assays. The sludge from the Sauget plant was chemically fractionated and assayed with S. typhimurium strains TA98 and TA100. The neutral fraction was the most mutagenic fraction followed by the weak acid, strong acid and basic fractions. These chemical fractions uncovered more mutagenic potency than was predicted by assaying a crude organic extract of the sludge.

KEYWORDS--genotoxin/ micronucleus/ mutagen/ mutation/ Salmonella typhimurium/ sludge/ soil/ Tradescantia/ waste water/ Zea mays

INTRODUCTION

There is substantial interest in the land treatment of municipal waste waters. U.S. Environmental Protection Agency guidelines require the examination of such systems as part of any Section 201 facility plans. As part of a recent evaluation of the health risks associated with slow-rate land treatment facilities, Uiga and Crites (1980) indicated that, "under well-maintained conditions, both conventional and land treatment systems provide a large measure of safety for public health." However, they observed that, "the health effects of trace metals are difficult to evaluate" and, "no estimate can be made on the health hazards resulting from the low level exposure to trace organics."

One health hazard from such organic contaminants is the ability of some compounds to affect the genetic material of organisms inducing mutation, cancer and birth defects. An environmental mutagen or genotoxin is an agent that is released or is present in the environment that can alter the genetic material (deoxyribonucleic acid, DNA) or alter the proper functioning of the genetic material. The presence of such genotoxic agents in the environment is a serious threat to the public health (Crow and Abrahamson, 1982). Depending upon the developmental stage of an individual, a genotoxin can exert teratogenic effects (birth defects) (Hemminki et al., 1980), precipitate coronary disease (Benditt, 1977), produce mutations involving germinal cells (Crow, 1973), or cause mutations in somatic cells that may become neoplastic (cancerous) (Ames, 1979).

It is widely believed that a majority of human cancers is due to the presence of carcinogens in the environment (Bergel, 1974; Epstein, 1974; de Serres, 1979; Ames, 1984). The somatic cell mutation theory of cancer revolves around the simple premise that cancer arises in humans and other organisms through damage to DNA, chromosomes or to mitotic recombination (Sorsa, 1980). During the last 15 years evidence has been reported that demonstrates that most, if not all, chemical carcinogens are mutagens.

Besides the induction of somatic cell mutations, environmental mutagens induce mutations in germinal cells. Genetic damage by environ-

mental mutagens to germinal cells has by far the most serious consequences with regard to the adverse long term effects upon our species (de Serres, 1979). With the induction of germinal cell mutations an exposed person has the possibility of deleterious effects as well as his progeny generation upon generation. The probability that environmental mutagens will increase the genetic load of our species through an increased mutation rate in germinal cells is the greatest danger posed by these genotoxic agents.

It has been found that among the effluents from conventional waste water treatment facilities in Illinois, many contain substantial mutagenic activity (Hopke et al., 1982; Plewa and Hopke, 1983; Plewa, 1985). If such effluents were applied to soils there would exist the possibility of infiltration through the soil column into ground water reserves. It may also be that many of the active compounds may adsorb on the soil particles and/or be deactivated by the action of soil bacteria so that land application may diminish the threat of mutagenic waste water effluents. The purpose of this project was to examine the fate of the mutagenic potency of agents found in a waste water when they are applied to soils. In addition, a detailed genetic analysis of the mutagenic properties of waste water and sludge from the Sauget Illinois Public Water Treatment Works was conducted.

The objectives of the original study were to analyze the effects of agriculturally important soil types on the mutagenicity of a chemically defined waste water, to determine the ability of different soil types to remove specific types of mutagens, and to predict the possible risks associated with the disposal of waste waters on agricultural lands that may result in the contamination of ground water supplies. Our attempts to obtain a suitable amount of a chemically defined waste water from the Sauget Illinois Public Water Treatment Works on a continual basis met with failure even with the intervention of the Illinois Environmental Protection Agency. We used two sources of agents to assay for the study. An XAD-8 methanol extract of waste water from the municipal treatment facility of Sauget was used. This extract had been chemically characterized in the laboratory (Ellis et al., 1982). The second sample was an analysis of the mutagenic potency of municipal sewage sludge from the Sauget treatment plant.

The influent to the Sauget Illinois Public Water Treatment Works (located near East St. Louis) is predominantly the wastes of a heavy chemical industrial plant, although the manufactures of alloys and metal tubing also contribute. The effluent flows into the Mississippi River and samples of this effluent have been demonstrated to be mutagenic. The chemical components of Sauget waste water encompass alkyl benzenes, chlorobenzenes, chloroanilines, nitroanilines, chloronitrobenzenes, hydrobenzoic acid, benzyl alcohol, various monoterpene derivatives, phenol, various chlorophenols and nitrophenols and their methyl esters. The adsorption of an acidified sample onto an XAD-8 column yielded the more highly substituted phenols; specifically the methyl esters of trichloro, tetrachloro-, and pentachlorophenol (Ellis et al., 1984). This extract demonstrated very potent direct acting mutagenic activity in Salmonella typhimurium. This direct acting mutagenicity was reduced after activation with mammalian hepatic microsomes.

In Part I of this research the Sauget XAD-8 methanol extract was analyzed for its mutagenic potency with S. typhimurium strain TA98 with and without rat hepatic microsomal activation. The waste water extract was incubated with soil for various times and the effect of this incubation on the mutagenicity of the extract was analyzed. The waste water extract was also analyzed for its ability to induce mutation at the yg2 locus in Zea mays and induce chromosome aberrations in Tradescantia. In Part II the sludge solid from the Sauget Illinois Public Water Treatment Works was analyzed for its mutagenicity using strains TA98 and TA100.

MATERIALS AND METHODS: PART I

BACTERIAL ASSAYS

All bacterial assays for mutagenic activity were conducted using S. typhimurium strain TA98, a histidine auxotroph (Maron and Ames, 1983). The standard plate overlay assay was conducted throughout the project.

Each extract was tested at a minimum of five different concentrations. All experiments were run in triplicate and repeated. The plates were incubated at 37°C for 48 h before scoring.

Within each experiment three controls were conducted, a spontaneous reversion control (plating the bacteria in buffer), a sterility control on all extracts and solvents used, a negative control (plating the bacteria and solvents together) and a positive control (ICR-191, an intercalating-alkylating mutagen).

XAD-8 EXTRACT/SOIL MIXTURE

In this study, 1 and 3 ml samples of the Sauget XAD-8 methanol extract were brought up to a 10 ml volume with deionized water and added to 10 g of sterile or nonsterile native clay loam (Figure 1). These mixtures were placed in a shaking water bath at ambient temperature for 0, 24, and 48 h. The solid and liquid portions were separated by centrifugation. Each portion was extracted with methylene chloride (6:1 v/v) and the organics partitioned in the methylene chloride. The soil/methylene chloride mixtures were mixed for 1 min in a VirTis 45 homogenizer and organic and aqueous fractions were separated in a separatory funnel. Each extraction was repeated once and the organic phase was pooled. The methylene chloride was taken to dryness on a Buchi Brinkman Roto-Vapor at 40° C. The organic extract was redissolved in 1 or 3 ml of dimethylsulfoxide (DMSO). Four methylene chloride extracts were derived from each experiment, 1) the extract from the aqueous component of a sterile soil mixture, 2) the extract from the solid component of a sterile soil mixture, 3) the extract from the aqueous component of a nonsterile soil mixture, and 4) the extract from the solid component of a nonsterile soil mixture. All tests for mutagenic activity were conducted using a defined concentration of the methylene chloride extract in DMSO. Strain TA98 was used to assay for mutagenic activity of the extracts from each experiment.

YG2 ASSAY

The maize kernels were generated in our genetics nursery at the South Farms of the Department of Agronomy at the University of Illinois. The heterozygous kernels were produced by crossing inbred lines of MGC

70-1245 yg2/yg2 by Yg2/Yg2 Early-Early Synthetic. The crossing of two inbreds insured that the F_1 progeny were highly isogenic.

The procedures of Plewa et al. (1984) were followed for the yg2 assays of the waste water extracts. Within each experiment Yg2/yg2 sibling kernels were used. Approximately 33 kernels per control and treatment group were used. The kernels were surface sterilized by a 5 min wash in a 0.5% sodium hypochlorite solution. The kernels were rinsed under running tap water for 10 min and placed in aerated distilled water for 72 h at 20°C. The temperature was regulated in a water bath by a Lauda K-2/R temperature control unit. During the soaking period the water was changed every 24 h. The kernels were randomly distributed into groups for treatment; each experiment had a concurrent negative and positive control.

Within each experiment, an equal number of kernels were distributed to each treatment group except for the positive control. In general 11 or 17 kernels and 15 ml of the test solution were placed into each thirty ml, wide mouth glass test tube. Each test tube was placed in a water bath at 20°C. A concurrent control of 15 ml of a 0.1 M phosphate buffer (pH 7.4) was conducted with each experiment. The treatment solutions were constantly aerated by bubbling with water-saturated compressed air during the 8 h treatment time. The treatment apparatus was kept in a vented chemical hood and appropriate safety precautions outlined in the "Guidelines for the Safe Handling of Chemical Carcinogens," Division of Health and Safety, University of Illinois, were enforced. After the treatment period, the solutions were disposed in a toxic waste container and the kernels were rinsed for 30 min under running tap water.

The kernels were planted by treatment group in a standard soil mixture of loam, peat moss and sand (4:2:1 v/v/v). Three kernels were planted in each 10 cm diameter plastic pot. The pots were labeled and placed into a plant growth chamber adjusted for a photoperiod of 17 h at an illumination of 300 $\mu E\ m^{-2}\ sec^{-1}$ PRR and temperature of 20°C. The plants were watered each day with deionized water or a liquid plant fertilizer.

The control and treatment groups were coded and in approximately 20 days, leaves four or five matured (identified by the presence of

the ligule) and were scored. A fluorescent light box (I^2R, Inc. Cheltenham PA) and a magnifying lens were used as aids in the analysis of the leaves. Only sectors that were yellow-green in color and greater than 1 mm in length were counted. The number of sectors per leaf scored was recorded. After the entire experiment was scored the groups were decoded.

TRADESCANTIA MICRONUCLEUS ASSAY

The Tradescantia paludosa micronucleus test measures chromosome damage in tetrads following meiosis of the pollen grain mother cells (Ma, 1981). A micronucleus may result from the induction of a multipolar nuclear division or by chromosome aberrations that result in acentric fragments. The fragment of broken chromosome can be observed as a separate micronucleus within the tetrad.

Inflorescences of Tradescantia clone 03 plants were used throughout this study. The use of a single clone insured that all of the inflorescences were isogenic. The procedures for exposing the inflorescences to waste water components and scoring the micronuclei have been published (Hopke et al., 1982; Plewa and Hopke, 1983).

STATISTICS

A one way analysis of variance was conducted for each set of aggregated data from the genetic assays within an experimental series. If a significant F value was calculated a pairwise comparison of all the treatment groups was conducted and the probability values for each relevant comparison was determined. A significant difference between a control and treatment group required a $P \leq 0.05$. Mean values were usually presented with the standard error of the mean.

RESULTS AND DISCUSSION: PART I

EFFECTS OF SOIL ON THE MUTAGENICITY IN SALMONELLA OF WASTE WATER XAD-8 EXTRACTS

The first experiment was a procedural control. The methylene chloride extraction procedure was calibrated for its ability to recover the mutagenic activity in the Sauget XAD-8 methanol extract sample. A sample of the Sauget XAD-8 methanol extract was roto-evaporated and redissolved in DMSO. This control was for the experimental protocol without the addition of the extract to the soil. The control consisted of a comparison of the mutagenic properties of equal amounts of the roto-evaporated Sauget XAD-8 methanol extract that was redissolved in DMSO with the "straight" XAD-8 methanol extract. The concentrations that were evaluated were 2, 4, 6, 8, and 10 µl of the "straight" Sauget XAD-8 methanol extract. The data for the roto-evaporated Sauget XAD-8 methanol extract redissolved in DMSO assayed with (●) and without (■) mammalian microsomal activation (S9), and the "straight" Sauget XAD-8 methanol extract assayed with (○) and without (□) S9 are illustrated in Figure 2. The samples were assayed with Salmonella strain TA98. The data clearly indicates that plating the roto-evaporated extract and plating the "straight" methanol extract give nearly identical results. Figure 2 also demonstrates that S9 mammalian microsomal activation decreases the otherwise very strong mutagenic potency of the Sauget waste water extract. Even with the reduction of the mutagenic response by S9, the Sauget waste water extract is a potent mutagen to Salmonella. However, because S9 did not activate the extract the remainder of the experiments using Salmonella were conducted without the addition of S9.

The procedural control experiment indicated that all of the mutagenic activity of the Sauget XAD-8 methanol extract could be quantitatively recovered after roto-evaporation and redissolving in DMSO. The soil mixing experiments encompassed mixing known quantities of the Sauget XAD-8 methanol extract with a standard weight of clay loam, extracting the mixture and conducting a mutagenicity assay with TA98. The first series of soil mixture experiments were conducted with an incubation time

of zero. The Sauget XAD-8 methanol extract was added to the soil and immediately centrifuged and extracted with methylene chloride. In the first experiment (Figure 3) 1 ml of the Sauget XAD-8 methanol extract was added to 9 ml of deionized water and mixed with 10 g soil. The mixture was extracted with methylene chloride and roto-evaporated and each of the four components of the experiment (aqueous component of a sterile soil, solid component of a sterile soil, aqueous component of a nonsterile soil, solid component of a nonsterile soil) was dissolved in 1 ml of DMSO. The data are presented in Figure 3. For each extract of the four components, 10, 20, 30, 40, and 50 µl were separately plated. While all four components demonstrated a positive response, the sterile soil (□) and nonsterile soil (■), solid components induced more revertants per plate throughout the concentration range than did the aqueous components from the sterile soil (○) and nonsterile soil (●). Of the components of the experiment, the solid component from the sterile soil mixture contained the most extractable mutagenic material.

The data presented in Figures 2 and 3 indicate a significant decrease in the mutagenic potency of the Sauget XAD-8 methanol extract after exposure to soil. As illustrated in Figure 2, the addition of 10 µl of the methanol extract (without the addition of the S9 mix) induced over 1,200 revertants per plate. However, after the addition of the aqueous and solid component of either the sterile or nonsterile soil mixture the number of revertants per plate is markedly reduced. A four fold increase of the Sauget XAD-8 methanol extract only induced approximately 600 revertants per plate after being exposed to sterile soil (Figure 3).

In the next experimental series, the incubation time was still zero, however, 3 ml of the XAD-8 Sauget methanol extract were diluted to 10 ml with deionized water and added to either sterile or nonsterile soil. The purpose of this series was to determine the effect of adding more of the methanol extract of the soil. Again, 10, 20, 30, 40 and 50 µl of each of the methylene chloride extracted components was plated. The solid components from both the nonsterile and sterile soil mixtures (■ and □ Figure 4) induced more revertants per µl of methylene chloride extract plated. However, in these experiments, the solid component

from the nonsterile soil mixture appeared to be more mutagenic. The two aqueous components from the sterile (o) and nonsterile (•) mixtures yielded nearly identical results (Figure 4). The addition of more methanol extract to the soil resulted in a greater number of revertants induced per µl extract as was illustrated by the increased positive slopes in Figure 4 as compared with Figure 3.

In our final series of experiments we varied the time of incubation of the extract with the soil. The procedures were identical as previously discussed with the addition of a 24 h or 48 h incubation with soil. The purpose of these experiments was to determine the effect of increased incubation time on the mutagenic potency of the methanol extract.

The data presented in Figures 5-8 illustrate the results of the experiments in which 1 ml of the methanol extract was incubated for 0, 24, or 48 h with sterile or nonsterile soil. From the extract from each treatment time, 10, 20, 30, 40, and 50 µl were assayed with TA98 per plate. The extract from the solid component of the nonsterile soil mixture resulted in the greatest number of revertants per µl of extract (Figure 5). Figure 5 illustrates most dramatically the reduction in the mutagenic activity of the extract after 24 h (striped bars) and 48 h (white bars) as compared with the 0 h incubation time (black bars). The mutagenic activity of the extract from the solid component of the sterile soil mixture also decreased although not as dramatically as the solid component of the nonsterile mixture (Figure 6). The extract from the aqueous component of the nonsterile soil lost most of its activity after only 24 h of incubation (Figure 7), while the extract from the aqueous component of the sterile soil mixture lost some mutagenic activity after 24 h and more after 48 h incubation (Figure 8).

The same series of experiments were conducted with incubating 3 ml of the Sauget waste water methanol extract with sterile or nonsterile soil. The results were in general similar to the experiments in which 1 ml of the Sauget waste water methanol extract was used. The extract from the soil component of the sterile soil mixture demonstrated the greatest mutagenic activity which significantly decreased after 24 h and 48 h of incubation (Figure 9). The extract from the solid component of the nonsterile soil mixture significantly diminished after 24 and

48 h in the extracts from the aqueous components of both the nonsterile (Figure 11) and sterile soil mixture (Figure 12).

In summary, we simulated the exposure of a mutagenic complex mixture derived from a waste water to soil to determine its biological effect. We considered four variables; 1) sterile or nonsterile soil, 2) the addition of 1 or 3 ml of the methanol extract to the soil, 3) the aqueous or solid component of the mixtures, and 4) the incubation of 0, 24 and 48 h. Factors such as soil sterility and the addition of 1 or 3 ml of the methanol extract were not consistent for predicting the relative mutagenic activity of the extracted soil/complex mixture. These data suggest that the binding properties of the soil to the organic agents present in the complex mixture were responsible for the decrease in the mutagenic activity of the methanol extract after exposure to the soil. Also, these data suggest that soil may have a catalytic activity and degrade mutagens that bind with it. The lack of a consistent difference between the mutagenic activity of the extracts from the 1 or 3 ml additions of the Sauget extract indicate that the soil binding or degrading properties had not been saturated in these experiments.

In contrast to the two above factors, the aqueous or solid component of the mixtures and the incubation times of 0, 24, and 48 h, had a consistent and significant effect on the mutagenic activity exhibited by the Sauget methanol extract. In all the experiments, the soil component contained a greater mutagenic activity than the aqueous component. This phenomenon is consistent with the suggestions discussed above, that the soil forms a complex with the organic agents in the Sauget methanol extract. The experimental results also demonstrated that the mutagenic activity decreased over time.

THE EFFECT OF XAD-8 WASTE WATER CONCENTRATES ON MUTATION AT YG2 LOCUS IN MAIZE

Three experiments were conducted to determine the mutagenic potency of the Sauget XAD-8 methanol extract that was mutagenic in Salmonella. For experiments 4647 and 4649 the concentrations of the Sauget XAD-8 methanol extract ranged from 5% to 20% in a 0.1 M potassium phosphate

buffer, pH 7.6. In addition, negative controls consisting of buffer only, or of methanol mixed in buffer were used. A positive control of 10 mM ethylmethanesulfonate (EMS) in buffer was used in both experiments. The data for experiment 4647 are presented in Figure 13. Only one concentration of the Sauget XAD-8 methanol extract, 5%, was assayed. For leaf four the 5% Sauget XAD-8 methanol extract induced 0.07 yg2 sectors per leaf compared to the negative control value of 0.04. For leaf five the values were 0.11 and 0.04, respectively. The frequency of yg2 sectors for leaves 4 or 5 in the 5% Sauget XAD-8 methanol extract treatment group were not significantly different. The 10 mM EMS positive control induced yg2 sector per leaf frequencies of 4.80 and 1.2, for leaves 4 and 5, respectively. The data for experiment 4649 are presented in Figure 14. Three concentrations (10, 15, and 20%) of the Sauget XAD-8 methanol extract were assayed. The yg2 frequency for the leaf 4 control was 0.08 and the yg2 frequencies for the 10, 15, and 20% Sauget XAD-8 methanol extracts were 0.27, 0.28, and 0.10, respectively. The yg2 frequencies among the control and extracts were not significantly different. The yg2 frequencies for the leaf 5 control and 10, 15 and 20% Sauget XAD-8 methanol extracts were, 0.08, 0.18, 0.17, and 0.43, respectively. These values were not significantly different. The final experiment used DMSO as the solvent. The Sauget XAD-8 methanol extract was taken to dryness by flash evaporation and brought up in DMSO. Due to the scarcity of the Sauget XAD-8 methanol extract only one concentration was assayed. A 1% concentration of the XAD-8 extract in DMSO was analyzed with the yg2 assay. The control yg2 frequency for leaf 4 was 0.03 while the 1% XAD-8 extract induced a frequency of 0.03 yg2 sectors per leaf 4.

Thus the experiments using the yg2 assay of the Sauget XAD-8 methanol extract did not detect any mutagenic activity in the concentration range from 1 to 20%.

THE EFFECT OF XAD-8 WASTE WATER CONCENTRATES ON THE MICRONUCLEUS TEST IN TRADESCANTIA

Tradescantia inflorescences were exposed to concentrations of the Sauget XAD-8 methanol extract in Hoagland's solution that ranged from 0.5% to 10%. With each test negative controls consisting of Hoagland's solution or a solvent control were included. The treatment times were either 6 or 8 h and the inflorescences were cytologically fixed for 24 h in acetic acid:ethanol (1:3 v/v). The data for experiment WRCTRAD1 are presented in Figure 15. The micronucleus frequencies for control and the 5% methanol control were 7.14 and 8.39 micronuclei/100 tetrads. The frequencies for the 1, 5 and 10% Sauget XAD-8 methanol extract treatment groups were 5.52, 7.58 and 5.84 micronuclei/100 tetrads. An analysis of variance was conducted on the data and $F_{4,20} = 1.47$, indicating that no statistically significant difference existed among the groups in the experiment.

The treatment concentrations of the Sauget XAD-8 methanol extract for experiment WRCTRAD2 were 0.5, 1, and 5%. However, the exposure time was extended to 8 h. The data are presented in Figure 16. A significant difference was found between the 1% Sauget XAD-8 methanol extract and the negative control as well as with the 5% methanol solvent control. However, the 5% Sauget XAD-8 methanol extract treatment group did not induce an increased frequency of micronuclei.

To investigate the region around the 1% Sauget XAD-8 methanol extract concentration, experiment WRCTRAD3 was conducted. The concentrations of Sauget XAD-8 methanol extract included 0.5, 1, and 2.5%, however, the treatment time was reduced to 6 h to reduce toxicity (Figure 17). A concentration-dependent response was observed among the control and treatment groups. A significant difference ($P \geq 0.05$) was observed between the control and the 2.5% Sauget XAD-8 methanol extract. The data suggest that the Sauget XAD-8 methanol extract is a highly cytotoxic and moderately clastogenic agent in Tradescantia.

In order to overcome the toxicity problem, we removed the methanol from the Sauget XAD-8 methanol extract under vacuum and redissolved the extract in DMSO (experiment WRCTRAD4). The Sauget XAD-8 extract

in DMSO was assayed at three concentrations; 0.5, 1, and 5%. No significant difference was detected among the control and treatment groups (Figure 18).

CONCLUSIONS FROM THE YG2 AND MICRONUCLEUS TESTS

The data from the maize yg2 tests and the Tradescantia micronucleus tests indicate that the Sauget XAD-8 methanol extract is not a potent mutagen or clastogen in these two higher eukaryotic assays. The results from these experiments are indeed surprising and clearly indicate the need for further research. Are the mutagens in the Sauget XAD-8 methanol extract detected by the Salmonella assays primarily genotoxic only to prokaryote species? Or are the agents detoxified by maize and Tradescantia? The toxicity of the Sauget XAD-8 methanol extract could also confound the eukaryote assays in that the amount of mutagen in the complex mixture was below a detectable level due to the uptake of toxic substances. Nevertheless, the data suggest that the highly mutagenic agents detected by the Salmonella tests are not genotoxic to maize and Tradescantia.

MATERIALS AND METHODS: PART II

EXTRACTION OF SAUGET SEWAGE SLUDGE

Sauget waste water extracts were evaluated for their mutagenic properties in the studies discussed above. The waste water was the aqueous effluent and the sludge is the solid component in the waste water treatment facility at Sauget, IL. Since our supply of the Sauget XAD-8 methanol extract was extremely limited we analyzed Sauget sewage sludge and various fractions of the sludge for its mutagenicity in Salmonella strains TA98 and TA100 (Hopke et al., 1984). The Sauget waste water treatment plant uses a simple physical/chemical primary treatment. Lime is added to the sludge to improve coagulation, the sludge is vacuum filtered through cloth and landfilled.

The sludge was centrifuged at 25,400 xg for 1 h. The supernatant aqueous phase was discarded and the samples of sludge solids were subjected

to an extraction/fractionation scheme outlined in Figures 19 and 20. After lyophilization the sludge was extracted with chloroform:methanol. Lyophilization eliminated the sequestered water in the wet sludge solids.

The crude chloroform/methanol extract was then subjected to a further fractionation as outlined in Figure 20. These additional separation steps are useful to determine the presence or absence of mutagenic activity and to minimize synergistic and antagonistic effects of other compounds potentially present in these complex mixtures. The procedure separates the extracts into four general chemical classes: strongly acidic, weakly acidic, basic, and neutral compounds. The percent yields of the Sauget sludge were as follows, neutral 83.4%, basic 3.1%, weakly acidic 8.2%, and strongly acidic 5.3%.

BACTERIAL ASSAYS

Each of these fractions were examined for mutagenic activity using the Salmonella tester strains TA98 and TA100 with and without mammalian microsomal activation (S9) according to the methods of Hopke et al., 1983. The Sauget chloroform:methanol extract was very mutagenic in TA98 both with and without S9 mix. However, it was only mutagenic to TA100 with the addition of the S9 mix. At a doubling over control in the TA98 revertants per plate was induced by 139 µg per plate of Sauget crude extract residue in the absence and present of aroclor-induced S9. For TA100, only with S9 was a significant increase in the revertants per plate observed. The slopes of the responses indicated that the Sauget crude extract residue induced 270 TA98 revertants per mg without S9; 780 TA98 revertants per mg with aroclor-induced S9, and 137 TA100 revertants per mg with the aroclor-induced S9 (Table 1). The Sauget sludge extract is one of the most mutagenic sludges yet reported for both direct acting and activateable mutagenicity in Salmonella (Babish et al. 1983).

RESULTS AND DISCUSSION: PART II

ANALYSIS OF SAUGET SEWAGE SLUDGE FOR MUTAGENICITY

The results of the mutation assays using TA98 and TA100 with each Sauget subfraction are presented in Table 2. All of the Sauget fractions induced a mutagenic response in TA98 and TA100. Two types of concentration-dependent responses induced by the Sauget fraction in the absence or presence of S9 are illustrated in Figures 21 and 22. In Figure 21, the neutral fraction results indicate the increased level of mutagenic activity in the presence of the S9 mix. In contrast, Figure 22 shows that for the weakly acidic fraction, the addition of S9 substantially deactivates the mutagens present. A similar deactivation pattern clearly holds for the strongly acidic fraction while the basic fraction gives a pattern indicative of toxicity.

The Sauget fractions demonstrated a different pattern of mutagenicity than the crude extract. The crude extract showed substantial activation for both TA98 and TA100. For the fractions, only the neutral fraction shows this pattern of activateable mutagenicity. The other fractions show deactivation particularly in TA100. However, since the neutral fraction has the vast majority of the mass, it apparently controls the properties of the crude extract.

On a revertant/mg of extract basis, the basic fraction was the most mutagenic to TA98 with a value of 1910 revertants/mg without S9 and 1650 revertants/mg with the S9 mix. The weak and strong acid fractions were most active in TA100 with values of 1680 and 1540 revertants/mg without S9, respectively. Again these values must be contrasted with the much lower apparent activity of the crude extract. This is particularly true for TA100 where the crude extract without S9 caused no mutagenicity while the basic and acid fractions show strong direct mutagenic activity. It appears that caution may be needed in interpreting low apparent activity of unfractionated extracts as indicated by the lack of mutagenicity in the sample.

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TABLE I. MUTAGENICITY OF SAUGET MUNICIPAL SEWAGE SLUDGE FRACTIONS WITH AND WITHOUT S-9

Sample	TA98[a] Equiv. vol. (µl)	Mass per Plate (µg)	Revertants per Plate -S-9	+S-9[c]	TA100[b] Equiv. vol. (µl)	Mass per Plate (µg)	Revertants per Plate -S-9	+S-9[c]
Neutral	0	0	19	50	0	0	148	174
	50	26.3	27	78*	50	26.3	138	194*
	100	52.5	45**	96**	1000	525.0	161	445**
	500	262.5	64**	197**	3000	1575.0	199**	690**
	1000	525.0	98**	282**	6000	3150.0	234**	988**
Bases	0	0	19	50	0	0	129	156
	50	1.0	20	59	50	1.0	152	150
	100	2.0	29	56	1000	19.7	98	132
	500	9.9	43*	64**	3000	59.1	283**	186
	1200	23.6	65**	93*	6000	118.2	155	124
Weak Acids	0	0	19	50	0	0	147	167
	50	2.6	24	75*	50	2.6	155	162
	100	5.2	26	52	1000	51.8	219**	182
	500	25.9	33	69*	3000	155.4	436**	232**
	1200	62.2	62**	77**	6000	310.8	659**	238**
Strong Acids	0	0	19	50	0	0	151	167
	50	1.7	20	44	50	1.7	119	177
	100	3.4	22	52	1000	33.6	259**	184
	500	16.8	31	56	3000	100.8	317**	190
	1200	40.3	55*	68**	6000	201.6	457**	206

[a] Results are from triplicate plates per treatment group.
[b] Results are from 6 plates per treatment group.
[c] Aroclor-induced S-9.
* Significantly greater than control, $p \leq 0.05$
** Significantly greater than control, $p \leq 0.01$

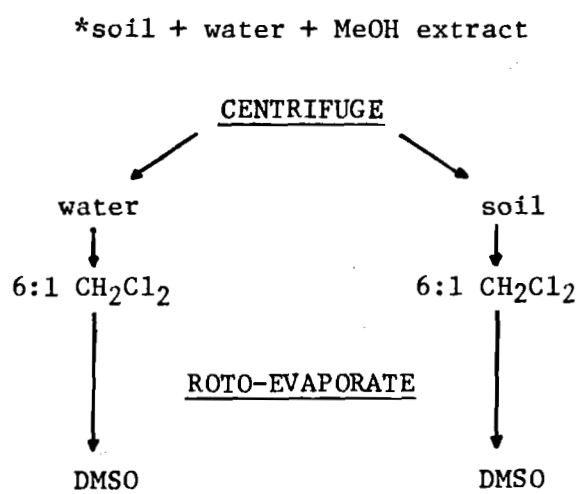

Figure 1. Flow Diagram Separation of the Aqueous and Solid Fractions from the Soil:Sauget XAD-8 Methanol Extract.

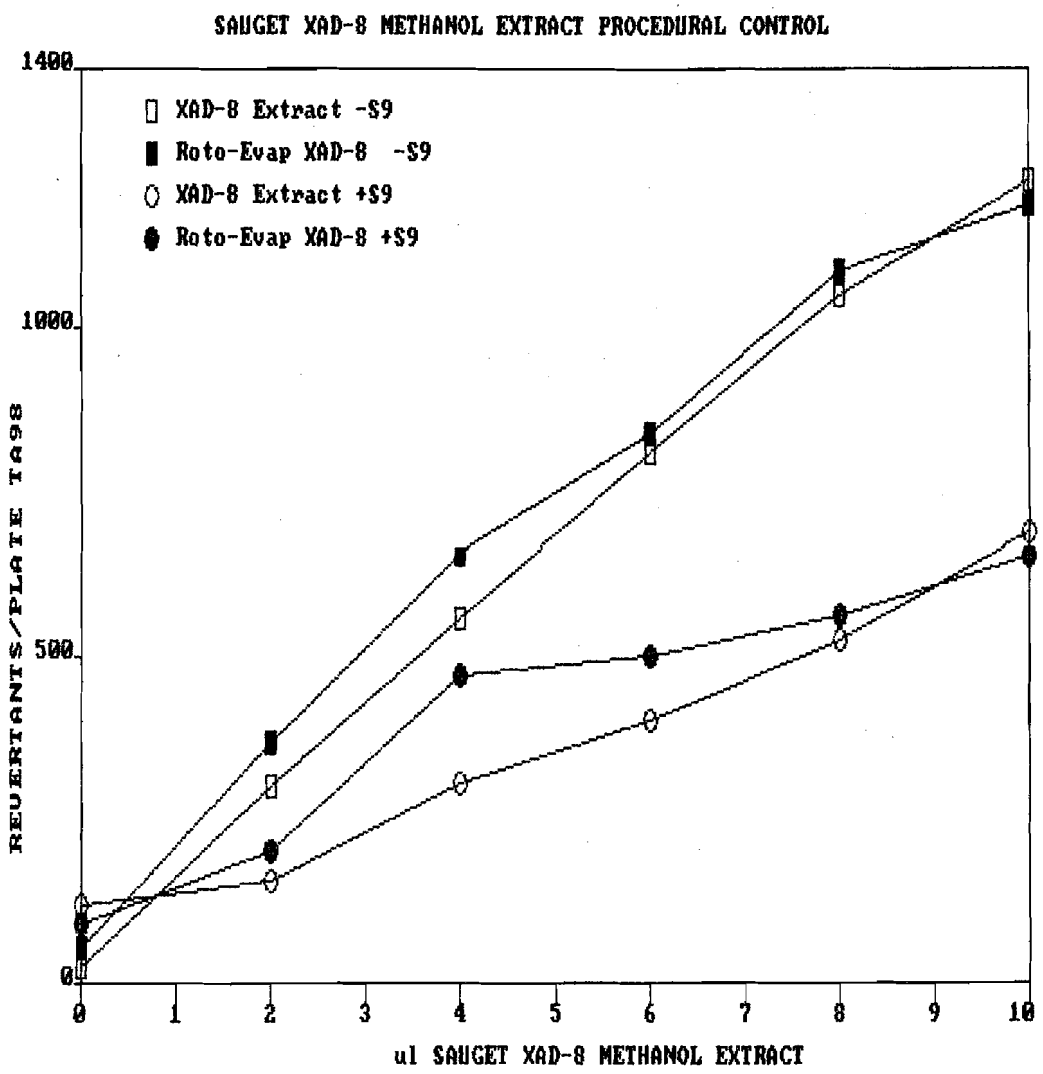

Figure 2. Sauget XAD-8 Methanol Extract Procedural Control.

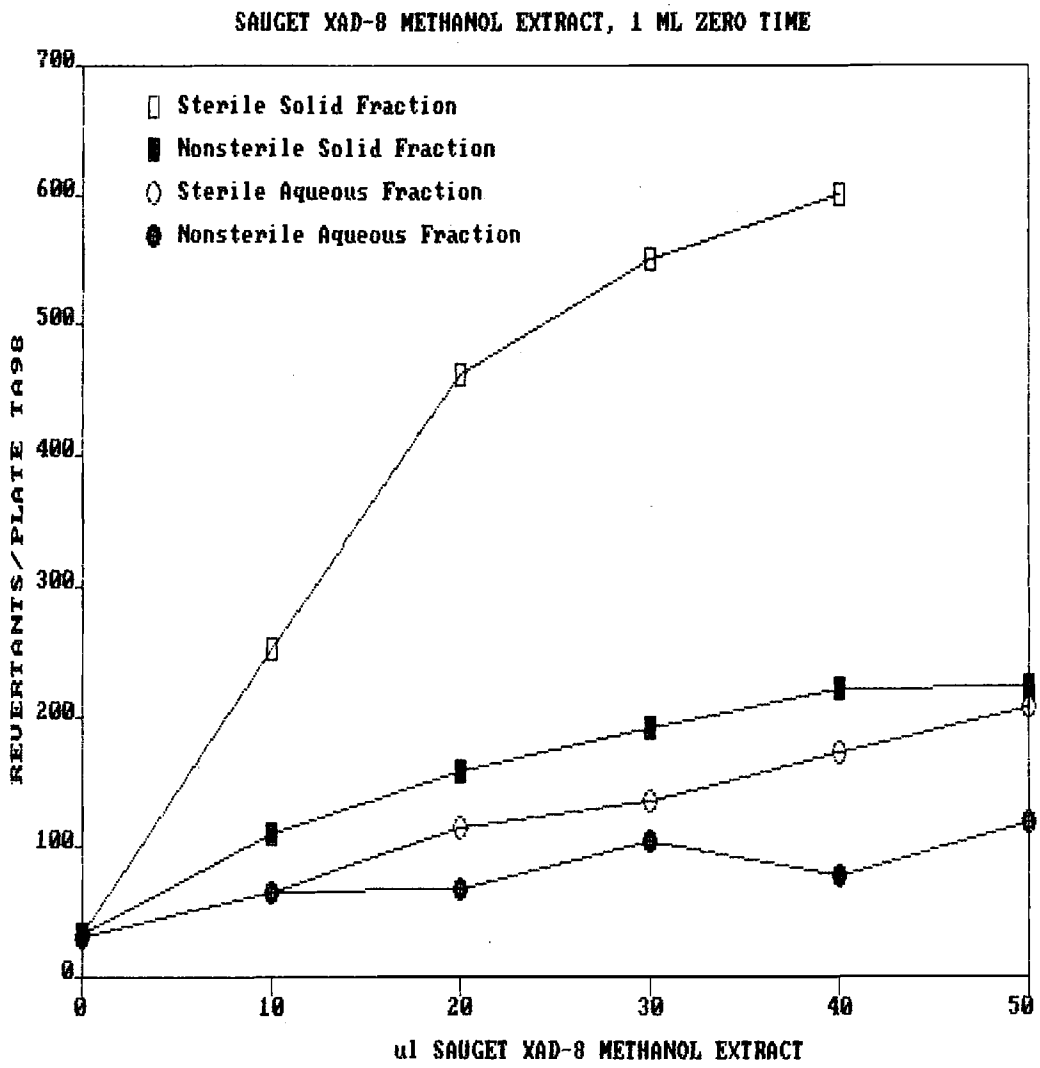

Figure 3. Mutagenicity of Sauget XAD-8 Methanol Extract 1 ml at Time Zero.

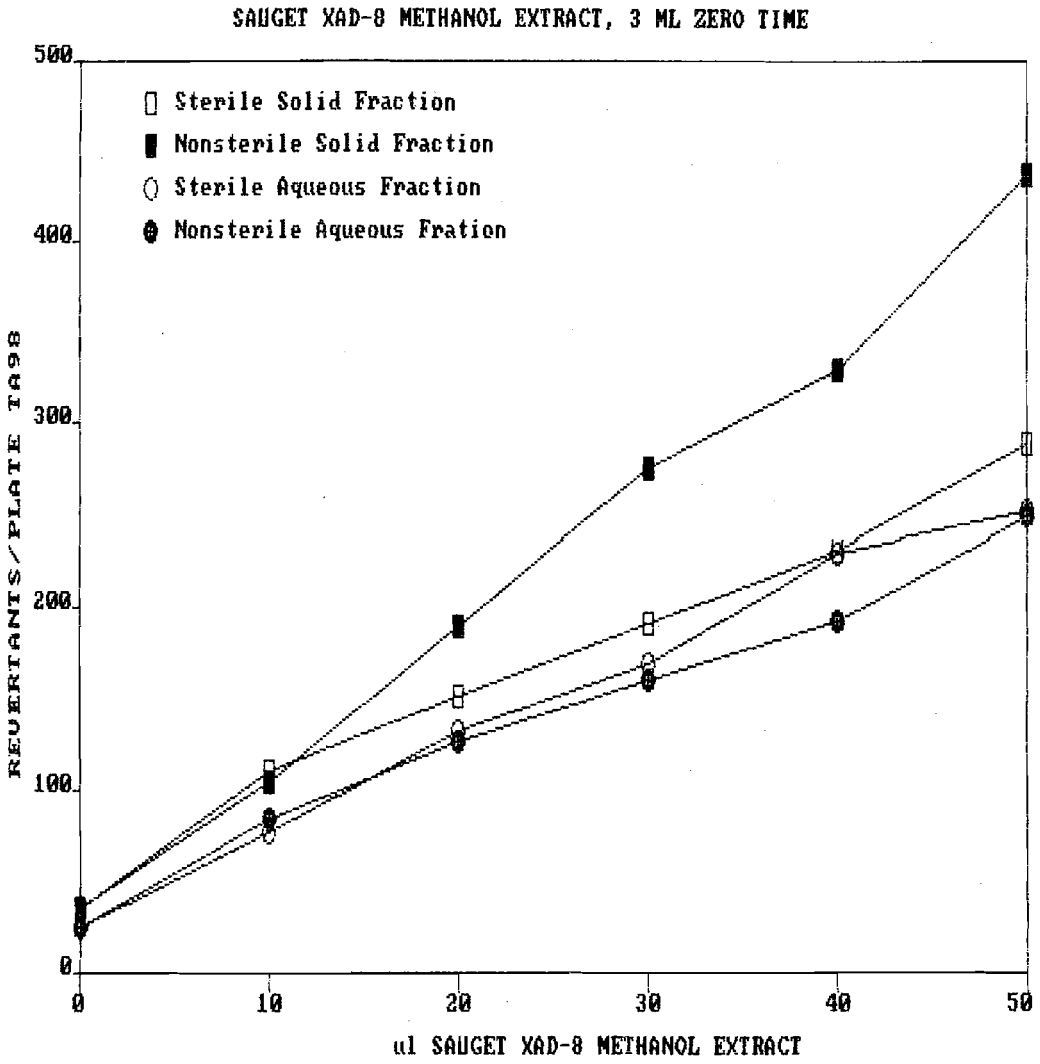

Figure 4. Mutagenicity of Sauget XAD-8 Methanol Extract 3 ml at Time Zero.

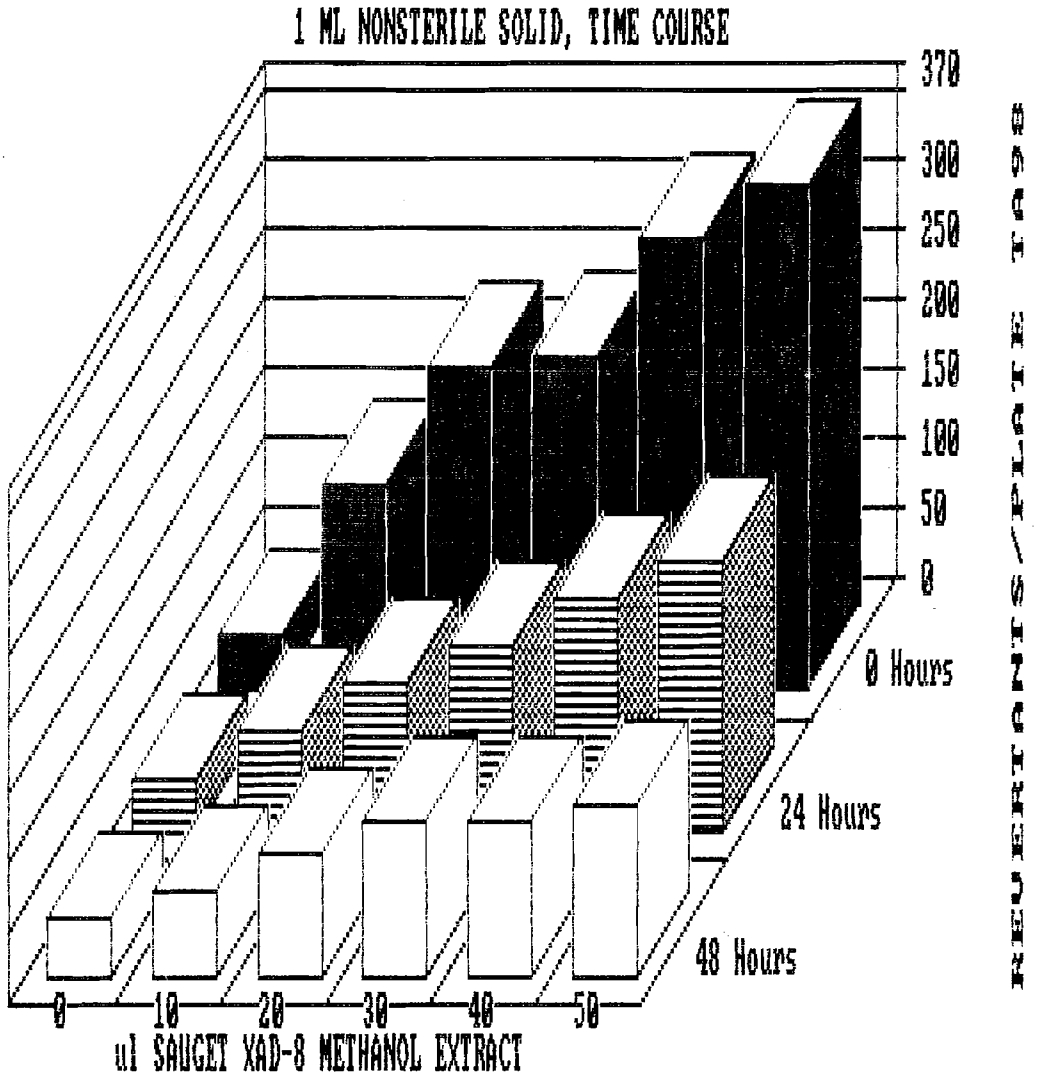

Figure 5. Time Course Mutagenic Analysis of 1 ml Nonsterile Solid Fraction of the Sauget XAD-8 Methanol Extract.

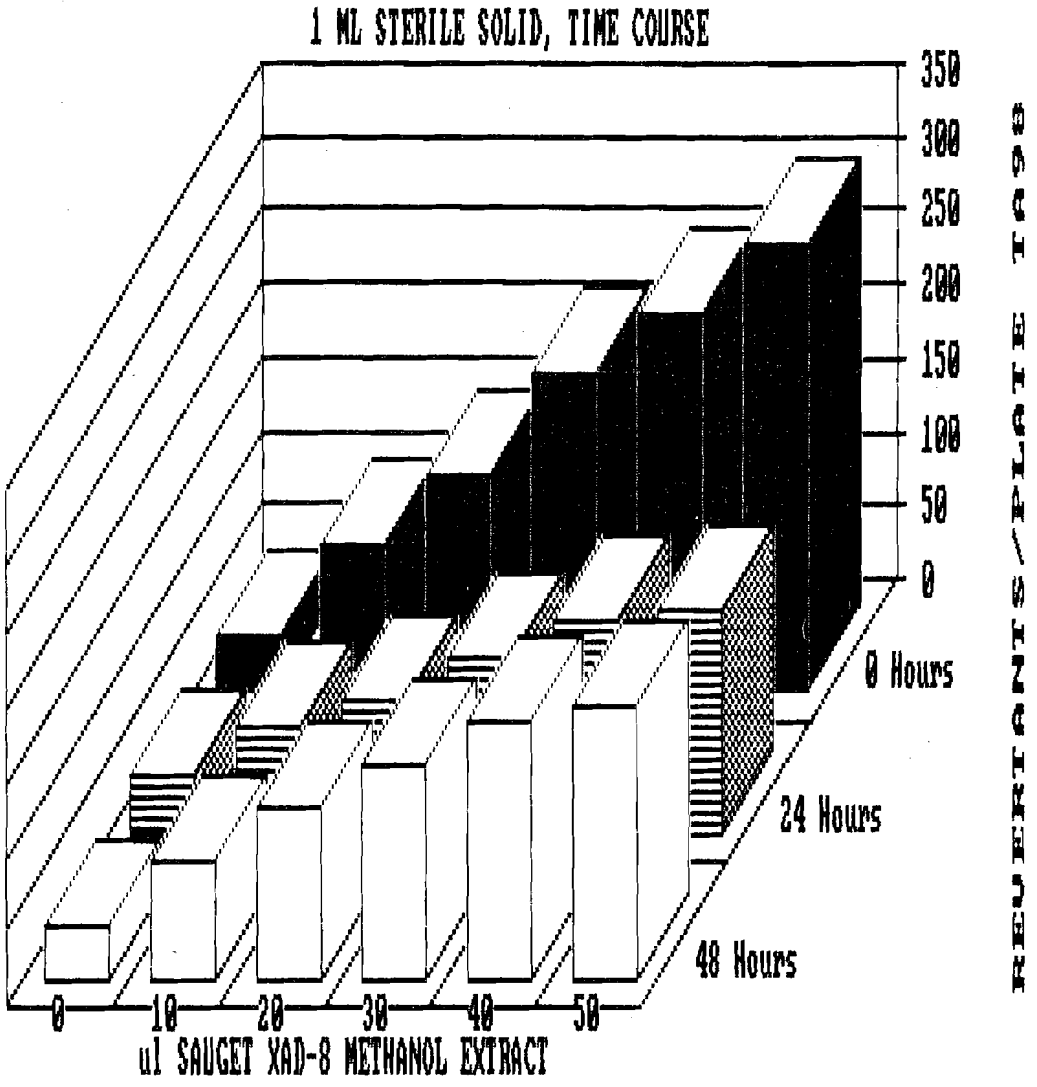

Figure 6. Time Course Mutagenic Analysis of 1 ml Sterile Solid Fraction of the Sauget XAD-8 Methanol Extract.

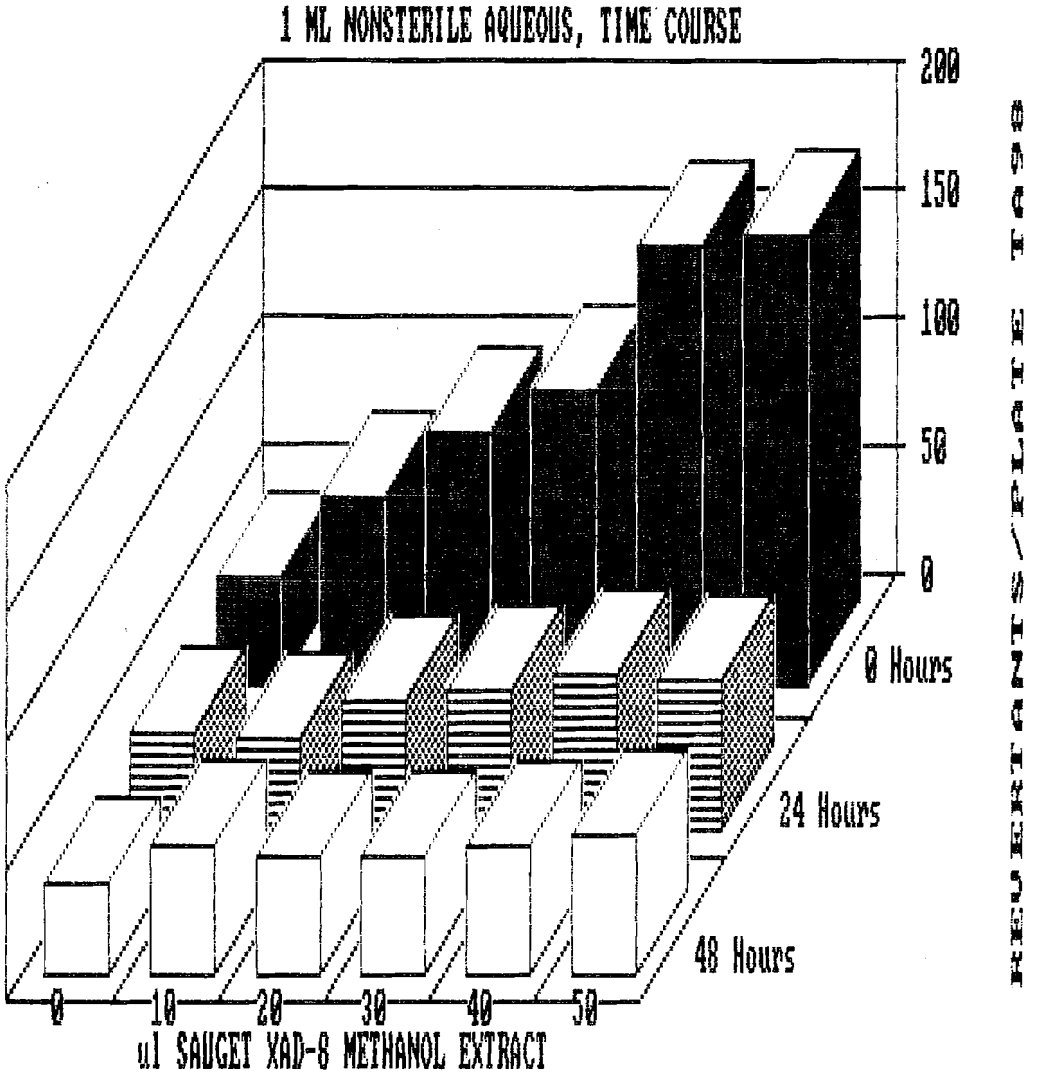

Figure 7. Time Course Mutagenic Analysis of 1 ml Nonsterile Aqueous Fraction of the Sauget XAD-8 Methanol Extract.

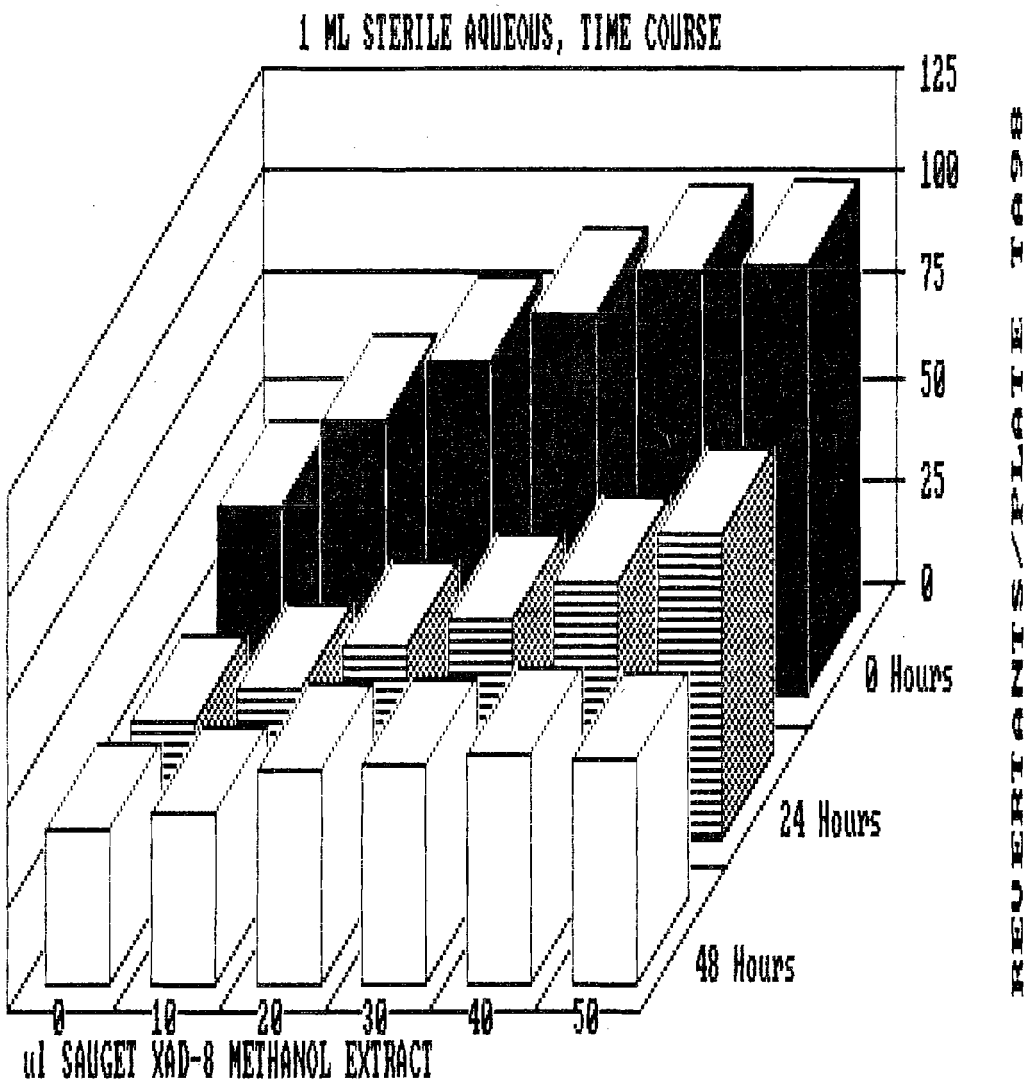

Figure 8. Time Course Mutagenic Analysis of 1 ml Sterile Aqueous Fraction of the Sauget XAD-8 Methanol Extract.

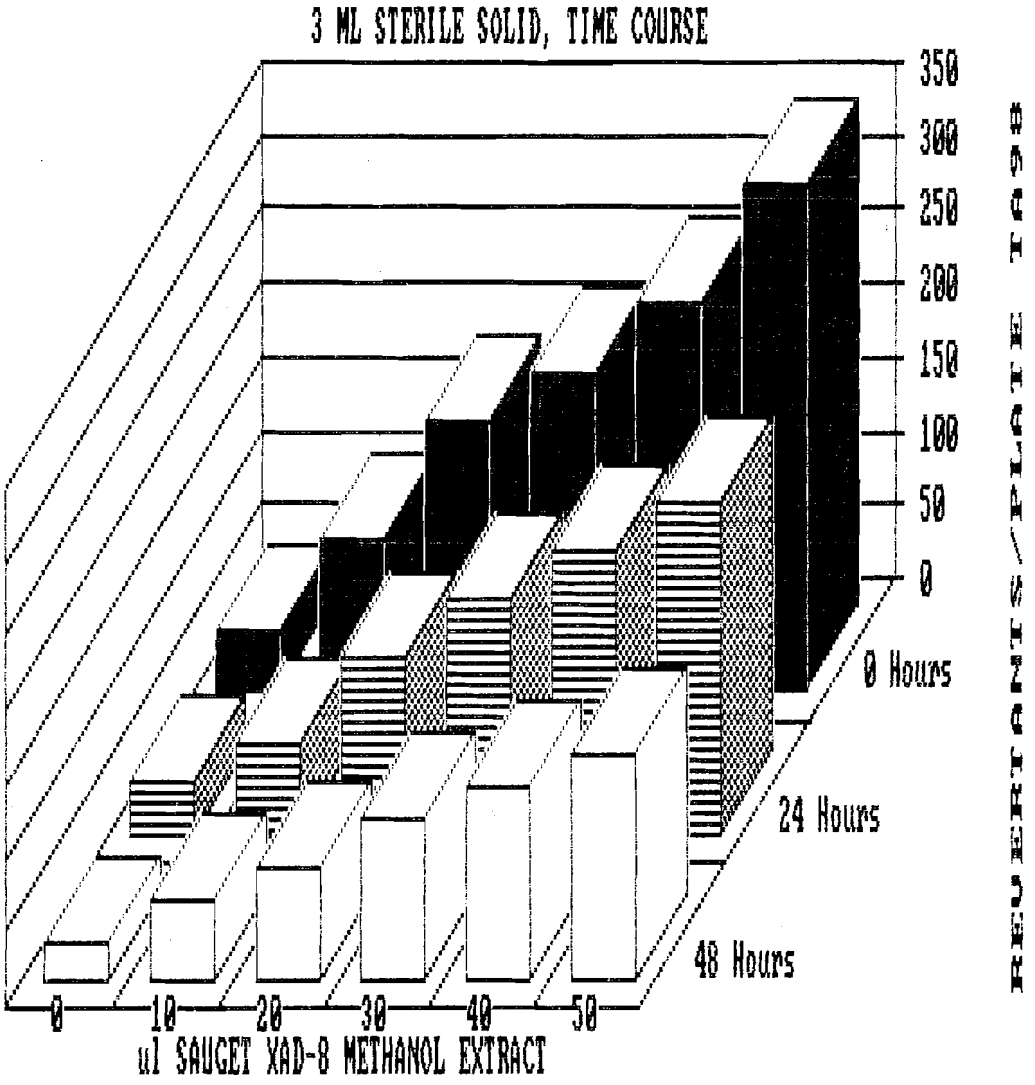

Figure 9. Time Course Mutagenic Analysis of 3 ml Sterile Solid Fraction of the Sauget XAD-8 Methanol Extract.

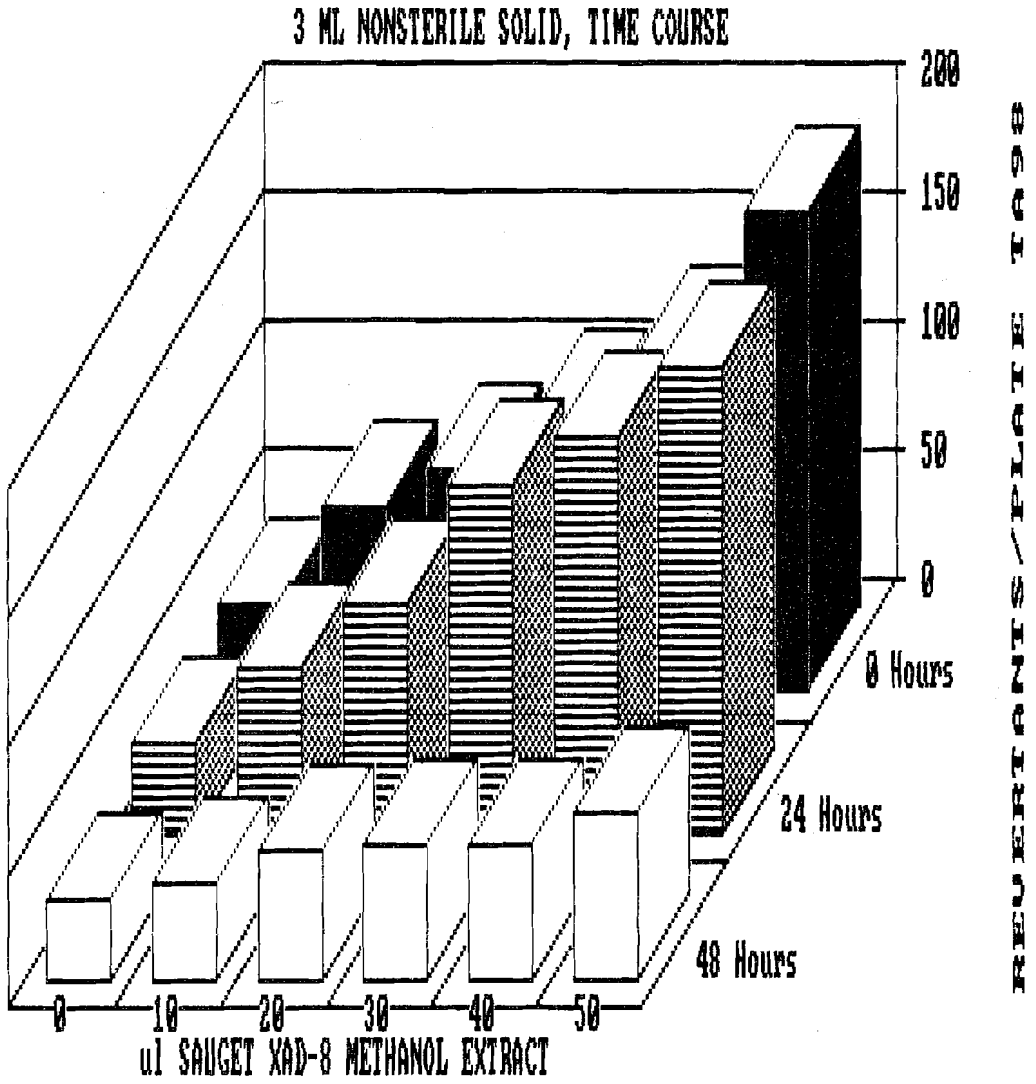

Figure 10. Time Course Mutagenic Analysis of 3 ml Nonsterile Solid Fraction of the Sauget XAD-8 Methanol Extract.

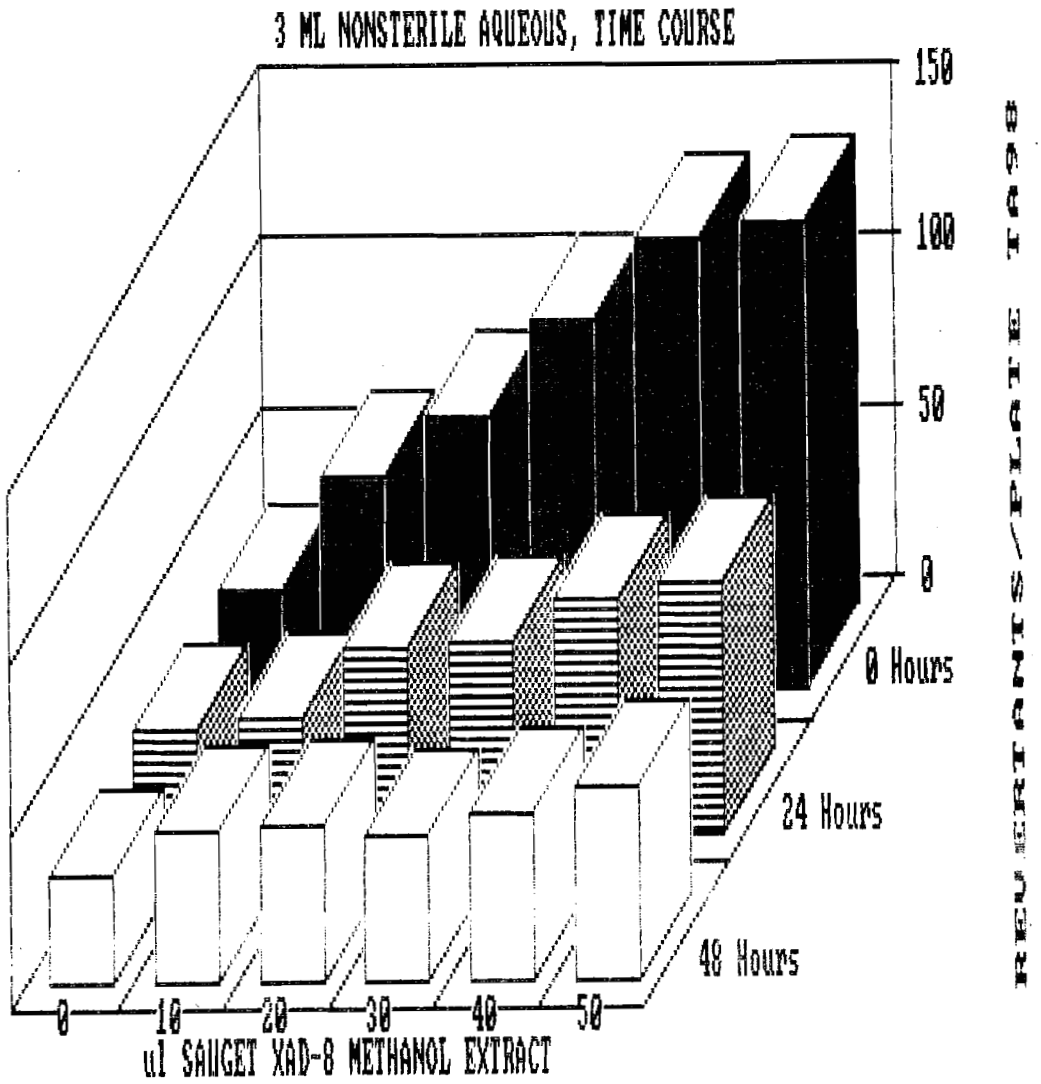

Figure 11. Time Course Mutagenic Analysis of 3 ml Nonsterile Aqueous Fraction of the Sauget XAD-8 Methanol Extract.

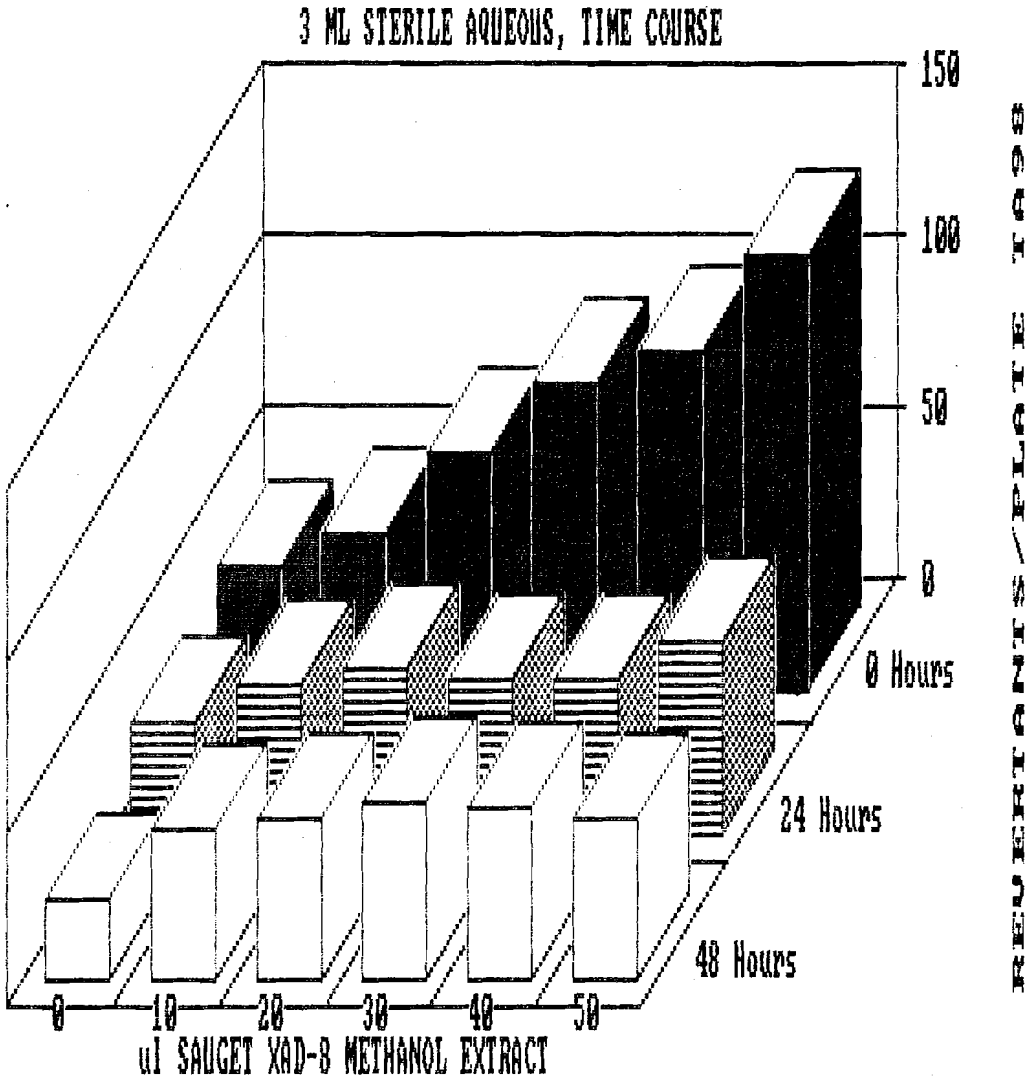

Figure 12. Time Course Mutagenic Analysis of 3 ml Sterile Aqueous Fraction of the Sauget XAD-8 Methanol Extract.

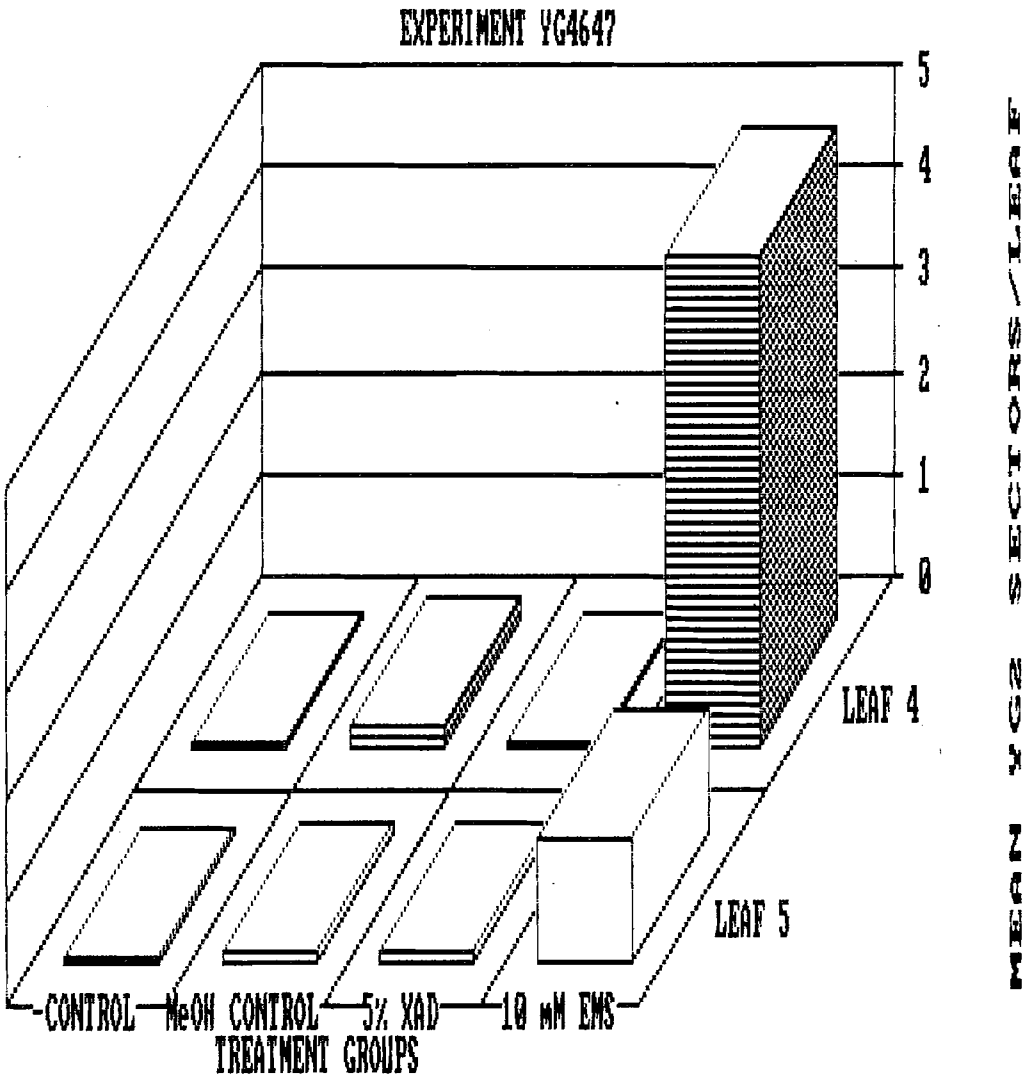

Figure 13. Maize yg2 Assay of the Sauget XAD-8 Methanol Extract.

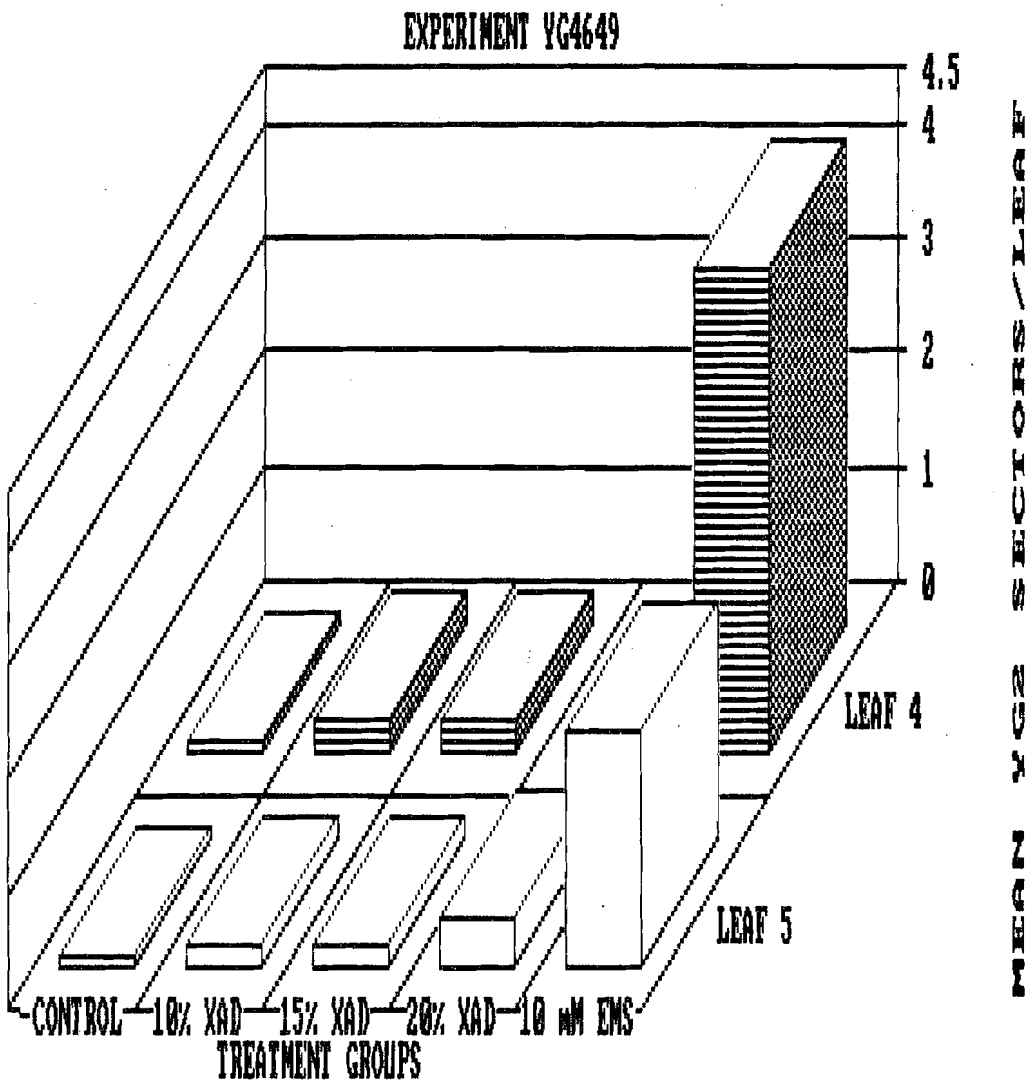

Figure 14. Maize yg2 Assay of the Sauget XAD-8 Methanol Extract.

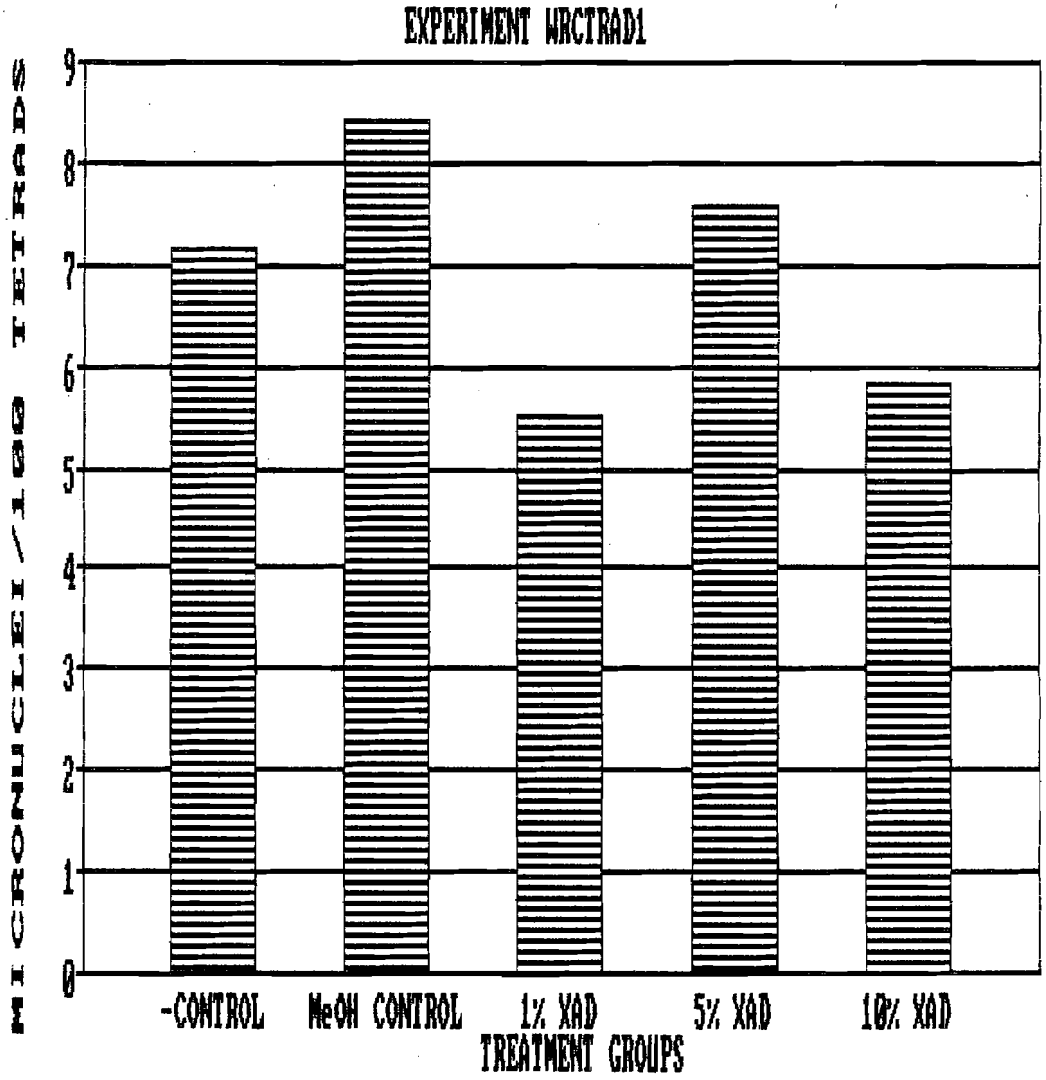

Figure 15. Tradescantia Micronucleus Assay of Sauget XAD-8 Methanol Extract.

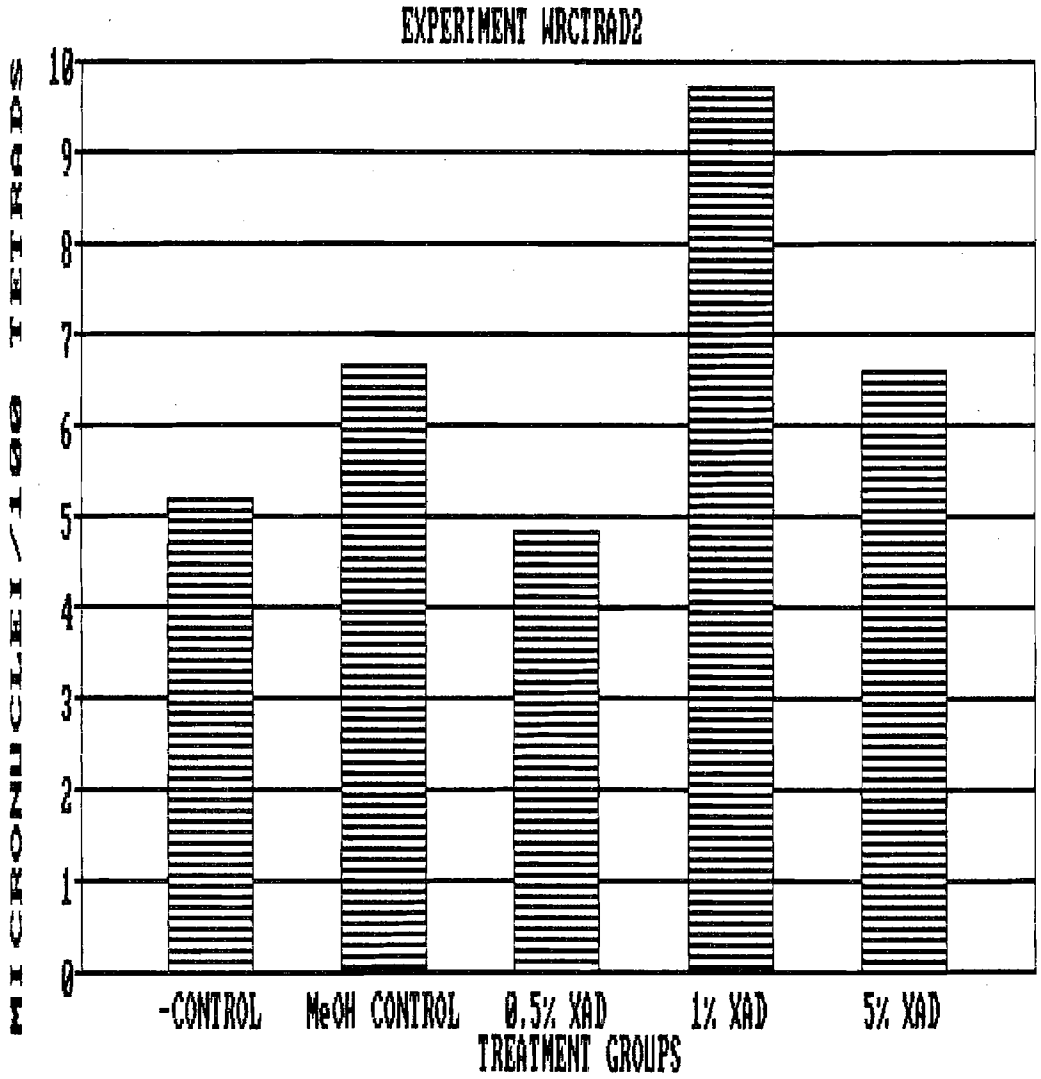

Figure 16. Tradescantia Micronucleus Assay of Sauget XAD-8 Methanol Extract.

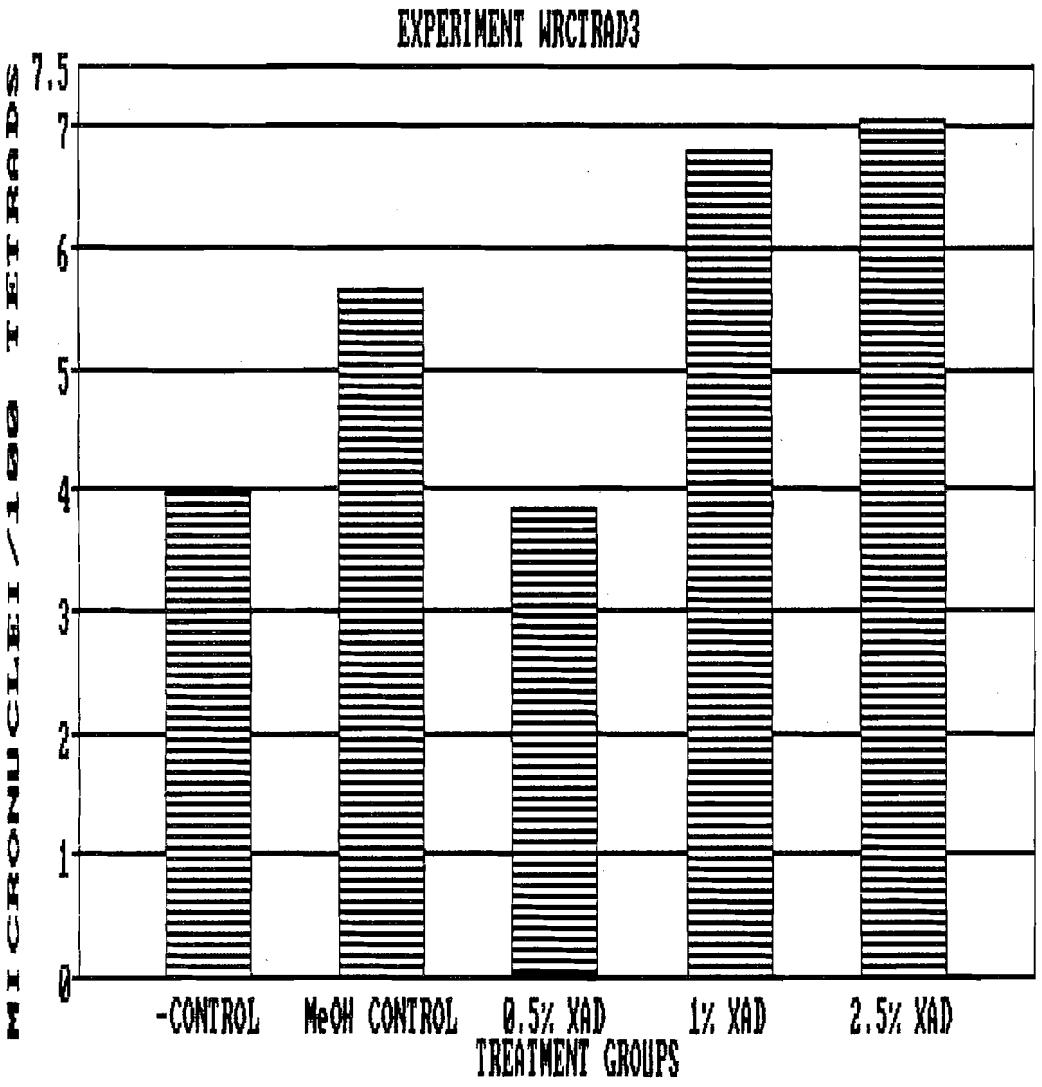

Figure 17. Tradescantia Micronucleus Assay of Sauget XAD-8 Methanol Extract.

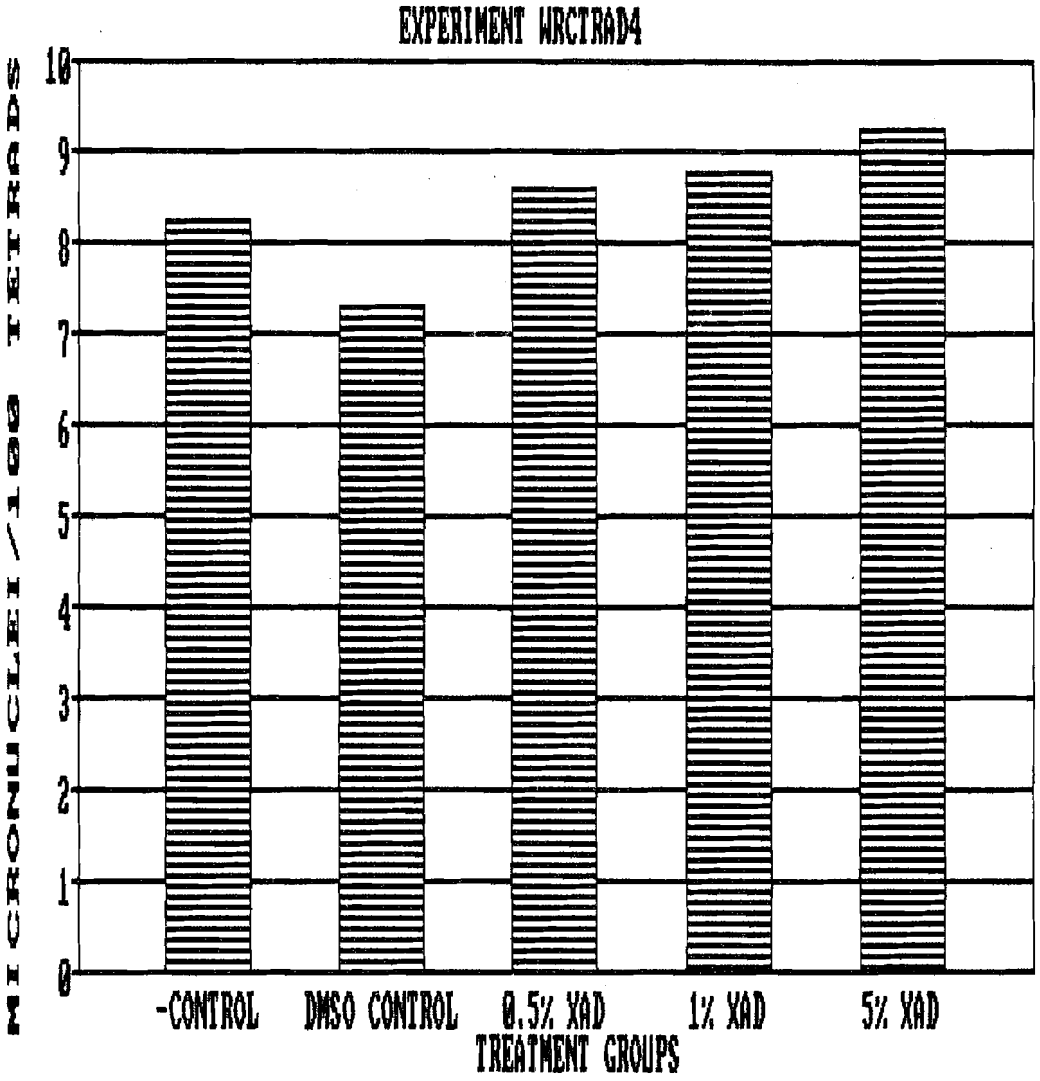

Figure 18. Tradescantia Micronucleus Assay of Sauget XAD-8 Methanol Extract.

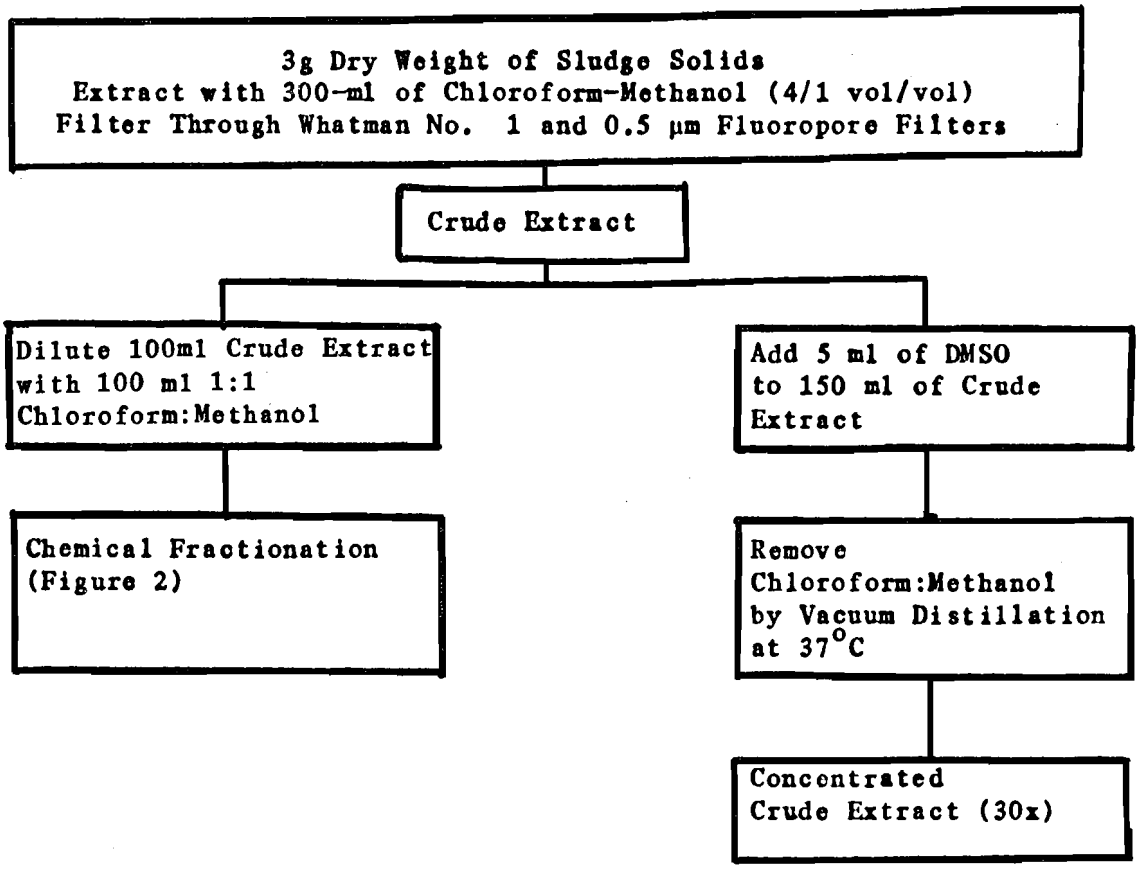

Figure 19. Flow Diagram of the Preparation of the Crude Chloroform:Methanol Extract of the Sauget Sludge.

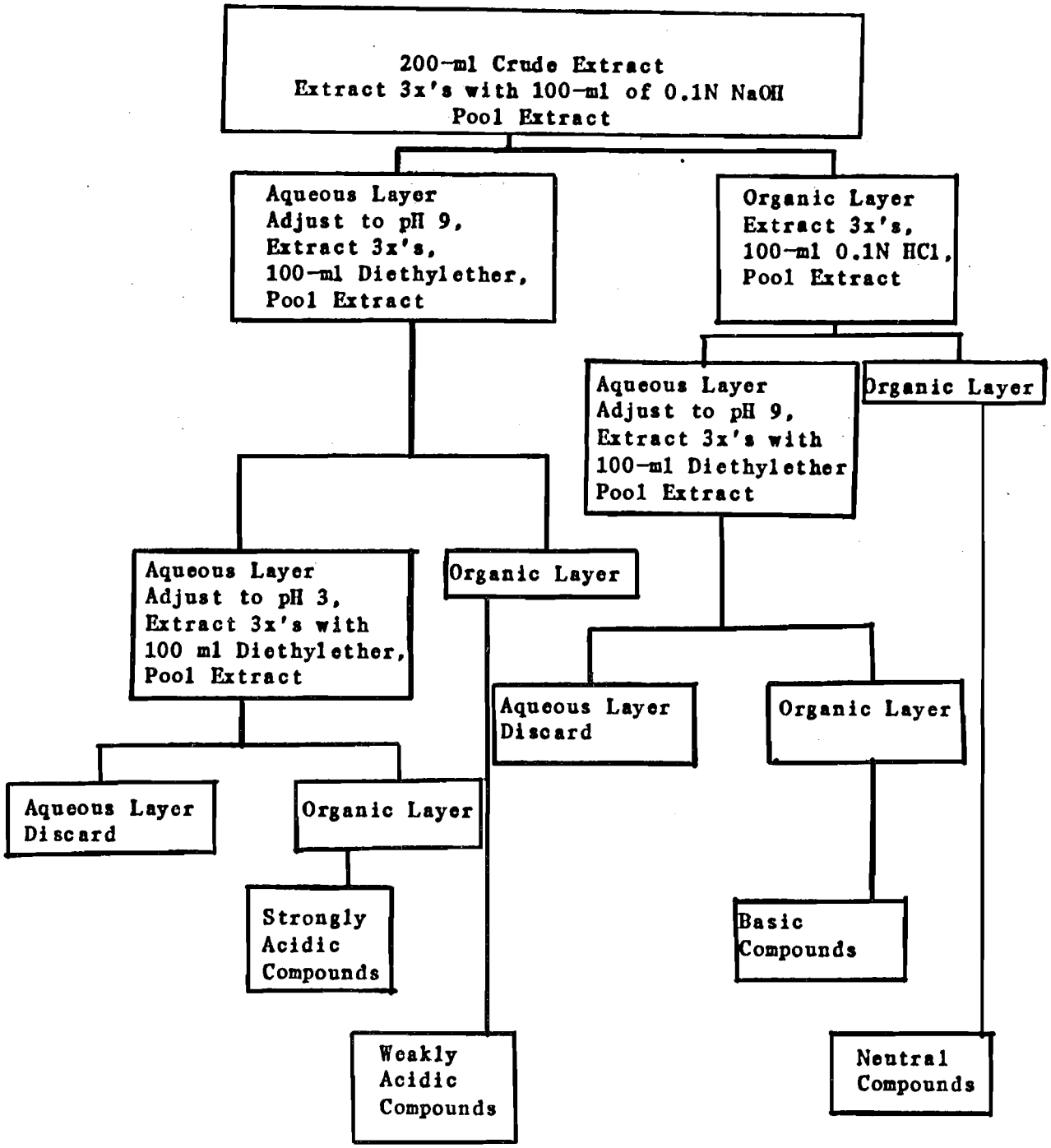

Figure 20. Flow Diagram of the Preparation of the Chemical Fractionation of the Sauget Sludge.

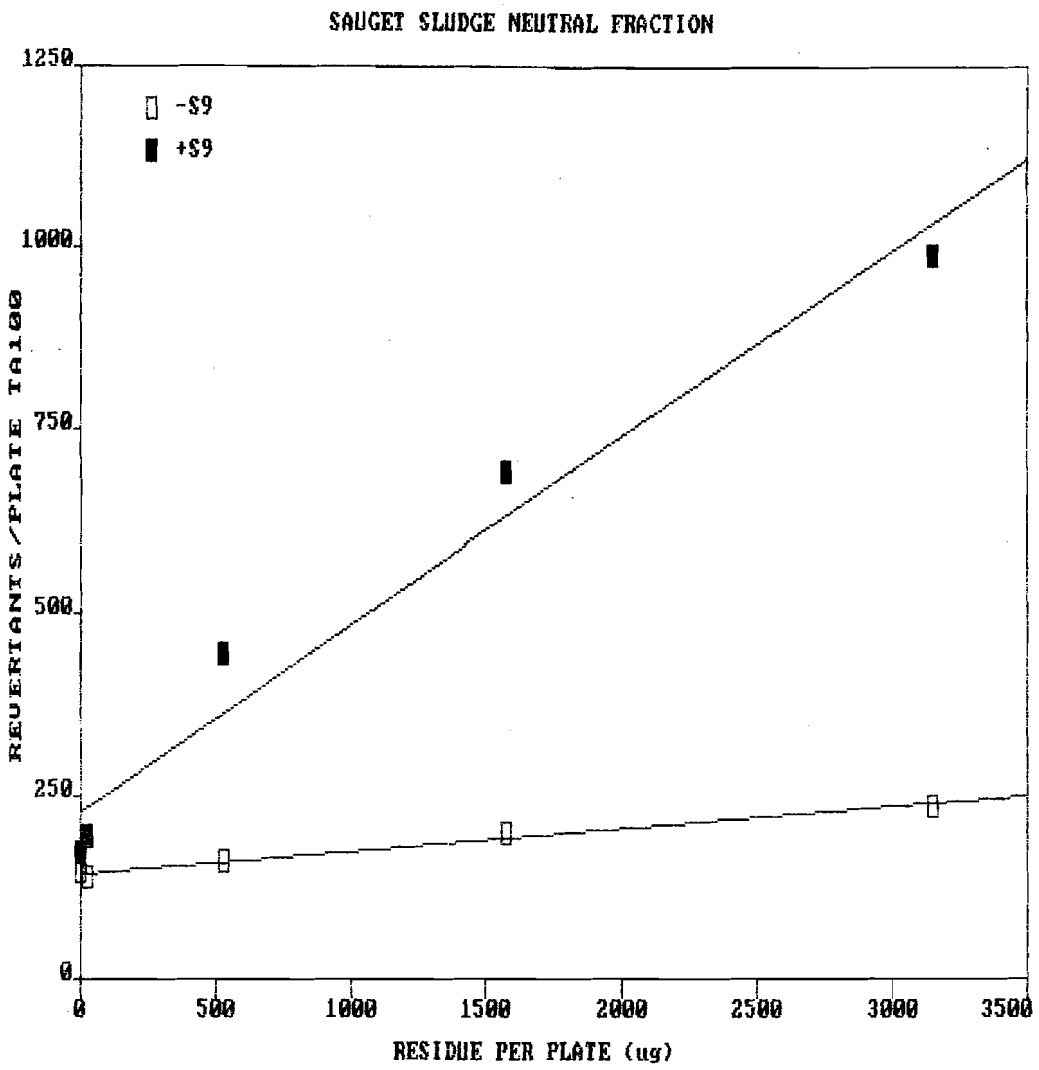

Figure 21. Mutagenicity of the Sauget Sludge Neutral Fraction.

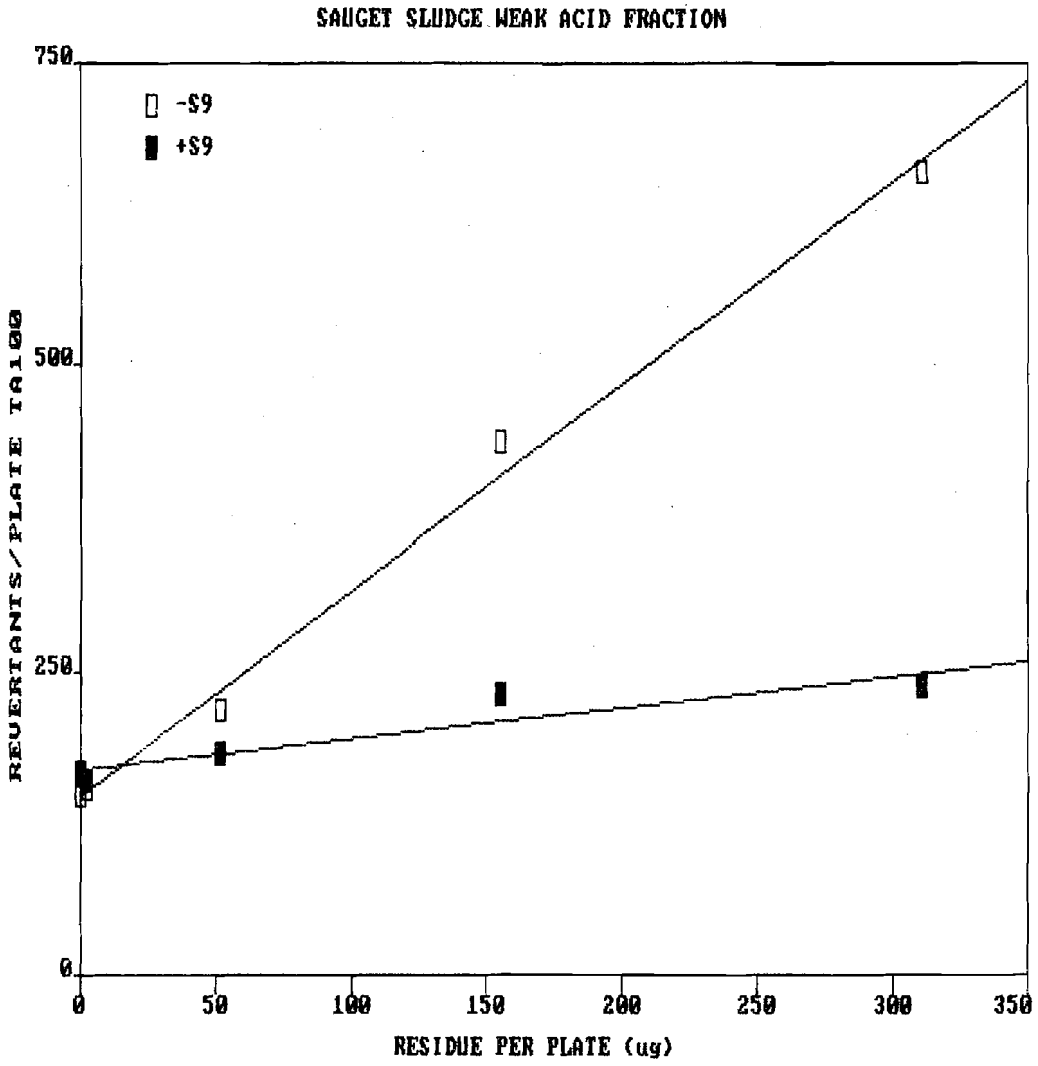

Figure 22. Mutagenicity of the Sauget Sludge Weak Acid Fraction.