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Project Summary

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Toxicological Testing of Organic Substances from Concentrated Drinking and Waste Waters

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The U.S. Environmental Protection Agency (EPA) has been given the responsibility for regulating the release of toxic chemicals into the environment. Accordingly, the EPA presently conducts an extensive and comprehensive research program to determine the adverse effects of environmental factors on human health. A considerable amount of this research activity is directed toward toxicological testing and test development. The Health Effects Research Laboratory of the EPA, in Cincinnati, Ohio, has specifically focussed on the task of determining, through the use of appropriate short-term assays, the potential health hazards of the complex mixtures of organic compounds found in drinking and waste waters. A battery testing approach is being employed by the EPA at Cincinnati for the toxicological testing of these waters, progressing from routine detection systems in microbial cells to more complex systems in mammalian cells and subsequently to short-term animal testing.

In support of the EPA's inhouse research and testing, a study was conducted employing a battery of five short-term assays for the toxicological testing of coded concentrated drinking and waste water samples. The five assays performed were: (1) the *in vivo* toxicity test, (2) *Salmonella typhimurium* mutagenesis assay, (3) Sencar mouse skin biossay, (4) rat liver focus assay, and (5) lung adenoma assay. The final report for this project gives the results of the testing of a total of 40 coded water samples in one or more of the above assays.

This Project Summary was developed by EPA's Health Effects Research Laboratory, Research Triangle Park, NC, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

The U.S. Environmental Protection Agency (EPA) has been given the responsibility for regulating the release of toxic chemicals into the environment under the Toxic Substances Control Act, Resource Conservation and Recovery Act, Federal Insecticide, Fungicide and Rodenticide Act, and the Clean Air and Water Acts. Along with the regulatory aspects of the above Acts, the EPA conducts an extensive and comprehensive research program to determine the adverse effects of environmental factors on human health. The purpose of these research programs is to provide information that is essential to formulate regulatory and compliance policies to protect public health and the environment.

With the advent of the Toxic Substances Control Act and evidence showing the relationship between environmental agents and adverse health effects (cancer, toxicity, developmental and genetic defects, pulmonary disease, etc.), the EPA has directed a con-

siderable amount of activity into toxicological testing and test development. Due to the large number of potentially hazardous chemicals being introduced into the environment each year, the backlog of untested chemicals already present, and the prohibitive cost and time necessary to assess chronic effects of these agents in long-term animal testing, the EPA has been particularly interested in the development, utilization, and evaluation of time- and cost-effective short-term *in vivo* and *in vitro* test systems.

In accordance with the above objectives, the Health Effects Research Laboratory of the EPA, in Cincinnati, Ohio, has specifically focussed on the task of determining, through the use of appropriate short-term assays, the potential health hazards of the complex mixtures of organic compounds found in drinking and waste waters. Preparation of these mixtures in sufficient quantity for toxicological testing has been made possible by the development of concentration (reverse osmosis) and isolation (using various macroreticular resins) techniques that concentrate water and/or waste water contaminants by 400- to 1000-fold. The identification, characterization, and possible interaction of these materials presents a formidable problem. Moreover, it is recognized that man is exposed to these compounds as complex mixtures and thus, that risk to man associated with these exposures can best be determined by toxicological testing in animals or *in vitro* testing at biological levels of organization that may simulate the response in man.

It has been proposed that testing of environmental agents proceed through a battery of well-defined and controlled short-term *in vivo* and *in vitro* assays that are reproducible between laboratories, possess a high degree of correlation with the chronic *in vivo* effects of various chemical compounds, and provide information relevant to effects observed in man. Ideally, these assays should generate no false-negative results and preferably only a low percentage of false-positives, be responsive to diverse classes of chemical agents, and lend themselves to quantitation. In addition, the assays should be relatively inexpensive, rapid and tehcnically simple. Such a battery approach is presently being employed by the EPA at Cincinnati for the toxicological testing of drinking/waste waters progressing from routine detection systems in microbial cells to more complex systems in mammalian cells and subsequently to short-term animal testing.

Procedure

Four coded groups of concentrated drinking or waste water samples were screened for toxicological activity in one or more of the bioassays. One group - the Eight Sample Series - was composed of concentrated water samples. Two groups - the Nine Sample Series and Eighteen Sample Series - were composed of organic acetone samples. These latter samples were concentrated by removal of the acetone solvent followed by the addition of sterile water, to bring the volume to one-fifth that of the starting volume, and the addition of sterile 10% Emulphor* (1 volume to 4 volumes of sample). The remaining group - the Five Sample Series - consisted of wastewater concentrates dissolved in 2% Emulphor.

The *in vivo* toxicity test was used to evaluate the toxicity of the Eight Sample Series and the Five Sample Series in CD-1 mice. For each study, CD-1 mice (approximately 8 weeks of age) were exposed to test samples (or control) for a period of 30 days. Two dose groups, receiving 1X and 1/4X or 1/10X concentrations, were employed per test sample. The control group of mice received sterile deionized water or sterile 2% Emulphor. Each test or control group contained a total of 20 mice - 10 male and 10 female. For the Eight Sample Series, the test samples (or control) were administered in the drinking water, with only about a 3- or 4-day supply of water (approximately 35 mls/day/cage) placed in the bottle twice weekly. For the Five Sample Series, test samples (or control) were administered by gavage, 0.3 ml per mouse, on each Monday, Wednesday, and Friday of the 30-day treatment period (total of 13 doses).

A cage-side observation of each animal for overt signs of illness or death was performed twice daily and a detailed health status check was performed on each mouse once a week at the time of the weekly weighing. At the end of the 30-day treatment period, all mice were weighed, sacrificed and necropsied. Animals found dead or moribund during the treatment period were also necropsied. The liver, lungs, kidneys, spleen, brain, and gonads of each animal were examined, weighed, and preserved in individually labelled specimen bottles containing 10% buffered formalin.

The *Salmonella typhimurium* mutagenesis assay was used to evaluate the mutagenicity of the Nine, the Eighteen, and the Five Sample Series. In each experiment, a series of samples were tested in strains TA98 and TA100 at four sample doses in duplicate with and/or without S9 activation. In each experiment, samples were tested within an eight-fold range with the highest sample dose not exceeding 400 µl per plate. Although the actual amount of test sample added during individual experiments varied, the volume added to each test plate was held constant in each experiment by appropriate dilution of the samples with 2% Emulphor. Test plates were incubated in the dark at 37°C for 48 hours and revertants then counted using an automatic colony counter. The background lawn of the test plates was examined with a stereomicroscope to evaluate toxic effects.

The Sencar mouse skin bioassay was used to evaluate the tumor initiating potential of the Nine Sample Series. Six-to-eight-week-old female Sencar mice were dosed three times a week (M,W,F) for two weeks intragastrically with either 0.5 ml of the test article or 0.5 ml of the test article vehicle. Samples were tested undiluted with one exception (tested at 1/2X concentration). Positive controls received a single intragastric administration of urethane in deionized water (500 mg/kg b.w.) at the time when all other animals received the last dose of the test article or test article vehicle. Test and control groups contained 40 or 20 animals. Two weeks after the last initiating dose, TPA in acetone (1 µg/0.2 ml/animal) or acetone only (0.2 ml) was applied topically to the shaved backskin of the animals three times a week for 20 weeks. The positive control group receiving urethane was treated similarly but with TPA only.

Starting weekly (at the time of the first topical application of promoter or promoter vehicle), the number, type, and location of each backskin tumor was recorded on an individual animal basis. Such tumors were classified as papillomas or carcinomas, and whether or not they were cumulative tumors (persistent for three consecutive weeks) or actual tumors (present for any length of

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time). A necropsy was performed on each animal found dead or sacrificed during the course of the study. Gross observations on all external and internal lesions were recorded for each animal, including the number of skin papillomas and carcinomas observed, and the specified tissues were routinely examined and preserved in 10% neutral buffered formalin. Representative 5μ Hematoxylin and Eosin stained slides were produced from paraffin embedded blocks of each gross lesion found at necropsy and these slides were subsequently examined and evaluated by a veterinary pathologist.

The rat liver focus assay was used to evaluate the tumor initiating potential of the Nine and the Five Sample Series. For each study, male Sprague-Dawley rats were subjected to two-thirds partial hepatectomies and then dosed once intragastrically with either 3 ml of test sample (undiluted), 3 ml of test sample vehicle (2% Emulphor), or positive control (diethylnitrosamine [DEN], 50 mg/kg body weight) precisely 24 hours after the hepatectomies. Each test or control group contained 10 animals. Seven to nine days after the partial hepatectomies, all rats received 0.05% phenobarbitol (PB) in their drinking water for a period of 8 weeks after which they were held for an additional week on drinking water without PB and sacrificed. Cryotomy sections from the livers of the animals were then stained and microscopically evaluated for: (1) the number of gamma glutamyl transpeptidase positive (GGT+) foci with nine or more nuclei per liver section; (2) the total number of morphometry points over areas of GGT+ Zone 1 induction; (3) the total number of morphometry points over the section of liver evaluated; and (4) the radius in cm of each GGT+ focus with nine or more nuclei.

The lung adenoma assay was used to evaluate the tumor initiation potential of the Nine and the Five Sample Series. For each study, Strain A/J mice (approximately 8 weeks of age) were dosed intragastrically with 0.25 ml of test sample or sample vehicle (2% Emulphor) 3X per week for 8 weeks. A positive control group received a single *per os* administration of urethane (10 mg/mouse). Two dose groups, receiving 1X and 1/2X or 1/2X and 1/4X sample concentrations, were employed per test sample. In addition to the vehicle and positive control groups, an untreated control group was also included to determine the incidence of spontaneous lung tumors. Each test or control group contained a total of 40 mice (20 male and 20 female).

At the end of the 24th experimental week (when mice were 32 weeks of age), all mice were weighed, sacrificed by CO_2 overdose, and subjected to gross necropsy. Any animals found moribund or dead were also necropsied, except for those animals where pathological examination was precluded due to severe postmortem autolysis. Lung adenoma counts were made for each animal necropsied. Lungs were perfused by tracheal cannulation with 10% buffered formalin and placed in separate labelled vials for subsequent counting of the lung adenomas. Individual lung lobes were observed under a dissecting microscope and adenoma counts recorded on forms depicting the individual lung lobes. Dual counts were made by two technicians working independently of each other's observations.

Differences between the two observers were reconciled by a third independent count. Randomly selected tumor-bearing lungs from each group (10%) were evaluated histologically for confirmation of adenomas and adenoma counts.

Results and Discussion

In Vivo *Toxicity Tests*

In both the Eight Sample and Five Sample Studies, data collected on the mean body weights and organ weights for the male and female mice in each test or control group indicated that none of the test samples produced any overt toxic effects in the coded sample groups, at either concentration, when compared to the control groups. In agreement with these findings, results of the detailed health status checks revealed no observable toxic effects.

Salmonella Typhimurium *Mutagenesis Assays*

The Nine Sample Series was tested in five separate experiments designed to determine the stability of the concentrated drinking water samples (in 2% Emulphor) during the concurrent testing in the animal assays (Sencar, rat liver focus, and lung adenoma). There were no significant differences in mutagenic response between drinking water samples concentrated and stored in 2% Emulphor at 4°C for 62 weeks, 33 weeks, 11 weeks, 3 weeks, and 2 weeks. The relative mutagenic potency of the five positive samples remained consistent; four of the nine samples were negative in all assays. It was also observed that the presence of the S9 activation system decreased or eliminated the positive responses observed without S9 addition.

Another experiment was designed to compare the stability of those samples stored in 2% Emulphor for 11 weeks and those freshly concentrated from original acetone samples stored at −20°C for the same period. Similar mutagenic responses were observed between the two series, thus indicating that the mutagenic compounds in these nine samples were stable under two different storage conditions.

The Eighteen Sample Series was evaluated for stability of mutagenic activity in two separate experiments. Results indicated that the samples positive in the first experiment lost some mutagenic activity over the 34-week storage period prior to retest, with a decrease from seven to four positive samples for TA98 and from five to four for TA100, even though the sample dose range was increased in the retest. Ten samples were negative in both experiments. Except for one sample, it was again observed that the presence of an S9 activating system decreased the positive responses.

The Five Sample Series was evaluated in a single experiment, designed to compare the mutagenic activity of these samples with activity observed in the animal bioassays (*in vivo* toxicity, rat liver focus, and lung adenoma). Three of the five samples demonstrated a positive mutagenic response at one or more sample doses. For all three positive samples, the presence of the S9 activating system decreased or negated the positive responses observed without S9 addition.

Sencar Mouse Skin Bioassay: Initiation/Promotion

The Nine Sample Series was tested in three separate and independent experiments. Survival and body weight records indicated that the test compounds did not cause acute or delayed toxicity resulting in death or altered body weights.

The gross scoring of skin tumors conducted during these studies proved to be quite accurate both in terms of the ability to detect a tumor and to classify them as papillomas or carcinomas. Overall, histopathological evaluations

revealed only seven cases where skin lesions were scored incorrectly.

During these studies, a total of only six carcinomas were detected. One was detected grossly at week 30 and the remaining five were detected microscopically. The extremely small number of detected carcinomas and their late appearance eliminated the use of skin carcinoma formation as a parameter for evaluating the initiating potential of the test articles. Time until a 50% incidence of cumulative tumors was also eliminated as a valid test parameter, since the test article groups never attained higher than a 33% incidence.

Control groups receiving test article initiation followed by acetone promotion or initiator and promotor vehicles (2% Emulphor and acetone) showed no skin tumor formation.

Animals in the positive control groups (receiving urethane with TPA promotion) showed cumulative tumor incidences after 30 experimental weeks of 65% to 70%. Animals in the control groups receiving initiator vehicle (2% Emulphor) and TPA promotion demonstrated a weaker response for cumulative tumor incidences at 30 weeks (9% to 36%).

Initiation with any of the nine test articles followed by promotion with TPA yielded tumor incidences in animals (cumulative skin tumors by 30 experimental weeks) that were not significantly different from the vehicle/TPA controls (range of 8% to 33%).

When the average latency period for the appearance of all cumulative tumors on all tumor bearing animals within a group was calculated, it was determined that none of the test articles caused a reduced latency compared to the TPA-only controls.

Rat Liver Focus Assays

Results in both the Nine Sample and Five Sample Studies demonstrated a significant difference between the positive control and vehicle control groups for three designated test parameters (number of GGTase positive foci/cm^2 of liver evaluated, number of GGTase positive foci/cm^3 of liver evaluated, and percentage of liver evaluated exhibiting GGTase positive foci). When, however, the test groups in each study were compared to the vehicle control groups, there was no significant difference between any of the groups and the vehicle control.

The use and significance of the percentage of liver evaluated exhibiting GGTase positive zonal induction (ZI) as a parameter to measure initiation by the test articles was questionable in both studies, since there were no significant differences in ZI between the positive and vehicle control groups in either study. In the Nine Sample Study, however, there were significant differences in ZI between the vehicle control and some test groups and between the positive control and some test groups. In the Five Sample Study, none of the test groups showed a significant increase in the amount of GGTase positive zonal induction. Results of both studies suggested that the ability of a compound to initiate GGTase positive foci does not correlate with its ability to initiate production of GGTase positive zonal induction.

In the Nine Sample Study, the percent of deaths within one week of the intragastric administrations of the test articles indicated possible acute toxicities for five of the compounds. No sample toxicity was observed in the Five Sample Study.

Lung Adenoma Assays

With the Nine Sample Series, the incidence of lung adenomas was 100% for both the male and female positive control groups, with cumulative lung nodule indexes of 11.78 and 11.66, for males and females, respectively. Animals in the untreated and vehicle control groups showed a much weaker response, with tumor incidences ranging from 5 to 10% and cumulative lung nodule indexes from 0.05 to 0.10.

The incidence of lung adenomas in animals treated with the nine coded samples varied from 0 to 30% among males and 0 to 28% among females while the average number of tumors per animal ranged from 0 to 0.35 in the males and 0 to 0.33 in the females. When these responses were compared to those observed in the untreated and vehicle control groups, there were no indications that any of these samples were initiators of lung adenomas.

With the Five Sample Series, the incidence of lung adenomas was 30% for the male positive control group and 35% for the female group with a cumulative lung nodule index of 0.40 for both males and females. These responses were much lower than those observed in the Nine Sample Study. Animals in the untreated and vehicle control groups yielded tumor incidences ranging from 5.6 to 21% and cumulative lung nodule indexes from 0.06 to 0.42.

The incidence of lung adenomas in animals treated with the five coded samples varied from 10 to 35% among males and 0 to 25% among females while the average number of tumors per animal ranged from 0.10 to 0.35 in the males and 0 to 0.30 in the females. When these responses were compared to those observed in the untreated and vehicle groups, there were no indications that any of these samples were initiators of lung adenomas.

Correlation between gross adenoma counts and histological confirmation was 89% in both studies. None of the test samples in either series exhibited any overt toxicity, as indicated by changes in body weight or mortality data.

Conclusions and Recommendations

Four coded groups of concentrated drinking or waste water samples, identified as the Eight Sample Series, the Nine Sample Series, the Eighteen Sample Series, and the Five Sample Series, were screened for toxicological activity in one or more of the following bioassays: (1) *in vivo* toxicity test, (2) *Salmonella typhimurium* mutagenesis assay, (3) Sencar mouse skin bioassay, (4) rat liver focus assay, and (5) lung adenoma assay.

Positive results were obtained with the *Salmonella* screen (strains TA100 and TA98). Several mutagenic samples were present in each of the three sample groups - Nine, Eighteen, and Five Sample Series - tested with this assay. However, it was observed that the relative activity of these samples, at the concentrations tested, was somewhat low as compared to most known mutagenic compounds. It was also observed that the presence of the S9 activation system decreased or eliminated the positive responses observed without S9 addition. Experimental results indicated that some of the concentrated water samples lost activity during storage at 4°C in the Emulphor vehicle (Eighteen Sample Series) while other samples retained their activity (Nine Sample Series). It was also determined that the mutagenic compounds in the Nine Sample Series were stable under different storage conditions, i.e., concentrated and stored in 2% Emulphor for 11 weeks at 4°C versus freshly concentrated from original acetone samples stored at −20°C for the same period.

No carcinogenic activity was observed with any of the coded samples tested in the Sencar, rat liver focus, or lung adenoma assays, nor was any toxic activity observed with coded samples tested in the *in vivo* toxicity test. The weak activity of samples positive in the *Salmonella* assay suggests that the concentrations of mutagens/carcinogens in the samples might have been too low for detection in the animal bioassays. However, in view of the toxic responses observed in the rat liver focus assay, additional concentration, using the same methods, might not be productive. Rather, it might be necessary to fractionate the sample concentrates, in an attempt to separate the toxic components of these complex mixtures from their mutagenic/carcinogenic components. Sensitive short-term tests such as the *Salmonella* screen might be useful in guiding these fractionation procedures.

In lieu of fractionation and additional concentration, potentially more sensitive *in vitro* and *in vivo* systems may be employed for identifying the toxic/mutagenic/carcinogenic compounds in water samples. Such systems for detecting cell transformation *in vitro* include: the hamster embryo focus or colony assay, the mouse fibroblast C3H10T1/2 CL8 assay, and the Syrian hamster embryo/SA7 enhancement assay. For measurement of toxic activity *in vitro*, inhibition of growth of pig aortic endothelial cells may be used. Some *in vivo* systems, which may prove to be more sensitive for measurement of activity in concentrated drinking and waste waters, include: the sex-linked recessive lethal test in *Drosophila*, the detection of sister chromatid exchanges (SCEs) in the liver, lung or other major organs of mice, and the rat neonatal survival assay.

The inclusion of this latter *in vivo* assay is particularly important, since developmental toxicity can result from nonmutagenic/noncarcinogenic mechanisms. Moreover, the test parameters in the rat neonatal survival assay are not limited to the assessment of gross abnormalities in structural development but also include endpoints such as adult fertility, stillbirths, pre- and post-natal growth retardation and functional deficits in the offspring.

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***Robert G. Miller** is the EPA Project Officer (see below).*
The complete report, entitled "Toxicological Testing of Organic Substances from Concentrated Drinking and Waste Waters," (Order No. PB 86-219 136/AS; Cost: $16.95, subject to change) will be available only from:
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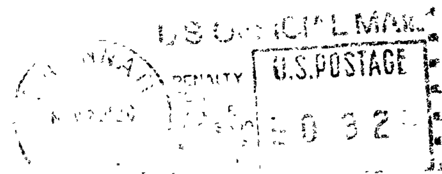

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