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Use of Biological Assay Systems to Assess the Relative Carcinogenic Hazards of Disinfection By-Products

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Other workers have clearly shown that most, if not all, drinking water in the U.S. contains chemicals that possess mutagenic and/or carcinogenic activity by using bacterial and *in vitro* methods. In the present work, increased numbers of tumors were observed with samples of organic material isolated from 5 U.S. cities administered as tumor initiators in mouse skin initiation/promotion studies. Only in one case was the result significantly different from control.

In studies designed to test whether disinfection practice contributes significantly to the tumor initiating activity found in drinking water mixed results have been obtained. In one experiment, water disinfected by chlorination, ozonation or combined chlorine resulted in a significantly greater number of papillomas when compared to nondisinfected water. In two subsequent experiments, where water was obtained from the Ohio River at different times of the year, no evidence of increased initiating activity was observed with any disinfectant. Analysis of water obtained at the comparable times of the year for total organic halogen, and trihalomethane formation revealed a substantial variation in the formation of these products.

Considering the problems such variability poses for estimating risks associated with disinfection by-products, a model system which makes use of commercially obtained humic acid as a substrate for chlorination was investigated using the Ames test. Humic and fulvic acids obtained from two surface waters as well as the commercially obtained humic acid were without activity in TA 1535, TA 1537, TA 1538, TA 98 or TA 100 strains of *S. typhimurium*. Following treatment with a 0.8 molar ratio of chlorine (based on carbon) significant mutagenic activity was observed with all humic and fulvic acid samples. Comparisons of the specific mutagenic activity of the chlorinated products suggests that the commercial material might provide a useful model for studying health hazards associated with disinfection reactions by-products.

Introduction

Development and widespread application of coupled gas chromatography and mass spectrometry has provided a much greater appreciation of the number and variety of organic chemicals that can be found in most drinking waters (*1*). For the most part, the large numbers of chemicals which are identifiable are present at very low concentrations, usually in fractions of a microgram per liter (*1*). However, in total, the amounts of organic material found in drinking waters derived from surface sources are often in the range of several milligrams per liter, hardly a negligible amount. It is now recognized that a very small fraction of the chemicals present in drinking water lend themselves readily to analysis even with today's modern analytical tools.

As a consequence of the above considerations, efforts were begun to obtain an estimate for the general magnitude of the problem without expending the huge sums of money to test individual chemicals as they were identified. A number of research groups around the world began to examine test mixtures of organic chemicals extracted from drinking waters. The test systems employed varied from the application of the *Salmonella*/microsome assay (*2–4*) for mutagenic chemicals

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developed by Ames et al. (*5*) to chronic studies conducted in rodents (*6–8*). In each of these cases, carcinogenic and/or mutagenic activity was detected in finished drinking water. In the U.S., Loper and co-workers (*2*) reported mutagenic activity of organic chemicals concentrated from the drinking water of first five, then six U.S. cities by a technique developed by Kopfler, et al. of our laboratory (*9*). Following up on these initial observations it was shown that these concentrates also contained chemicals capable of transforming BALB/3T3 cells (*10*) and a preliminary report demonstrated the ability of certain of the samples to initiate tumors in the mouse skin (*8*).

The work of Bellar and Lichtenberg (*11*) in the U.S. and Rook (*12*) in Europe changed the view of drinking water problems substantially. These workers documented that the organic chemicals most commonly found in drinking water and usually at the highest concentrations were produced by chlorination. These chemicals were, of course, the trihalomethanes. From this initial observation has come the gradual appreciation of a problem which is a direct result of currently accepted methods for producing a microbiologically safe drinking water. Before this, chemical hazards in drinking water had been viewed simply as one of industrial and/or agricultural contamination of the source water. Now any assessment of health hazards in drinking water must consider the qualitative changes in chemical composition that arise from water treatment—in particular disinfection.

Our laboratory has been concentrating on two problems. First, efforts have been directed towards determining if chemicals obtained from drinking water can be demonstrated to be carcinogenic *in vivo*. Second, an attempt is being made to develop a base of information which can be applied to a wide variety of water supplies. This latter effort requires that sufficient understanding of processes which contribute to the formation and occurrence of carcinogenic chemicals in drinking water be developed so that the risks involved can be minimized. It includes consideration of the carcinogenic activity of the products of four alternative disinfectants; chlorine, chlorine dioxide, ozone and chloramine.

With regard to disinfection by-products it has been established that a substantial fraction of the organic material found in surface water sources is of natural origin. A substantial portion of this natural background is composed of humic and fulvic material that has been shown to be a major precursor of trihalomethanes following their chlorination. Recovery of the humic material from water in quantity is difficult, but humic acid obtained from peat is commercially available. The question is whether differences in the chemical properties of humic material obtained from these sources result in the formation of clearly different toxic by-products. Therefore, we report here an initial attempt at determining whether humic acid provides a good model for the generation of biologically active by-products of disinfection for more comprehensive testing.

Methods

Sample Preparation

The five cities studied were selected as being representative of water supplies contaminated by various sources, or as having no known contamination by municipal, industrial or agricultural sources. Miami was chosen as representing ground water containing large quantities of natural organic chemicals; New Orleans, a surface water source impacted by industrial wastes; Ottumwa, a surface water contaminated by agricultural runoff; Philadelphia, a surface water impacted by municipal waste; and Seattle, a pristine surface water obtained from a protected watershed in the Cascade mountains. In all cases finished drinking water was used.

The concentrated organics were prepared for EPA by Gulf South Research Institute by the procedure described by Kopfler et al. (*9*). The samples are concentrated from multiple 200 liter quantities of tap water by reverse osmosis using a cellulose acetate membrane (CA) with sufficient concentrated HCl added to maintain a pH of 5.5. The reject from the CA membrane is passed through a heat exchanger to maintain the water temperature at 15°C. Part of the reject stream is diverted through a Donnan softening unit to avoid salt precipitation. The CA permeate is adjusted to pH 10 and then concentrated by reverse osmosis using a nylon membrane. The nylon permeate is discarded. Both the CA and the nylon concentrates are then adjusted to neutral pH and extracted with pentane and methylene chloride. The aqueous phases are adjusted to a pH of 2 with HCl and again extracted with methylene chloride. For purposes of this study these fractions were combined and the solvent removed to produce the reverse osmosis extract sample (ROE).

The residual aqueous concentrate remaining after extraction with the organic solvents is passed through a column of purified XAD-2 resin at pH 2. Following the removal of metallic oxides and other inorganic agents by eluting with 1*M* HCl and distilled water the organics are then eluted from the column with 95% ethanol. The ethanol is removed from the eluate by vacuum distillation and eluates from both columns are combined to produce the XAD sample.

A series of six experiments has been initiated to examine the effects of seasonal variation on the

ability of different disinfectant treatments to produce carcinogenic by-products. The testing of the first three of these has progressed to a point where results can be reported. Routine analysis for general water parameters, however, are only available for the last three experiments. Consequently no parallels between biological and chemical results are as yet possible. In the first three studies, settled, coagulated and filtered Ohio river water was subjected to chlorine, chloramine, chlorine dioxide, or ozone disinfection. Disinfectant demands were determined on all samples prior to adding the disinfecting agents. The disinfectant contact times varied but were designed to result in residuals that were at a practical minimum. The doses of disinfectants amounted to 2.0-2.5 mg/l. chlorine, 1.0-3.0 mg/l. ozone, 2.0-3.0 mg/l. chlorine dioxide, and 2.0-3.0 mg/l. combined chlorine. A control consisted of filtered, but nondisinfected water. Waters and concentrates obtained in March, July and October of 1980 were assayed for various physical, chemical and microbiological parameters for quality control purposes (data not shown). All water samples were then concentrated by reverse osmosis using a cellulose acetate membrane. The May 1977 samples were concentrated 100- to 180-fold. Subsequent samples have all been concentrated to 400-fold, using freeze-drying to supplement reverse osmosis concentration where necessary.

Humic and fulvic acids were isolated from two natural lakes, Black Lake and Lake Drummond, by Christman and co-workers of the University of North Carolina as they have described previously (*13*). The major differences between these two lakes is the distribution of the total organic carbon between the recoverable humic and fulvic acid fractions. Black Lake contains a much higher proportion of its TOC as humic acid. Both lakes are remote from direct industrial contamination. Commercial humic acid was obtained from Fluka of Switzerland. These materials were prepared by adding 4 g of humic material to 500 ml deionized water. The pH was adjusted to 12 with NaOH and the solution was stirred overnight in the dark. The solution was then centrifuged at 16,000*g* for 20 min and the supernatant was brought to pH 3 by addition of HCl. The centrifugation was repeated. The supernatant was diluted to 1 liter, and the pH was adjusted to 7. The resulting total organic carbon (TOC) content was 2.5 g/l. Chlorination of the humic solution was accomplished by adding enough NaOCl (pH 12, 10 g/l.) to give a chlorine to carbon ratio of 0.8:1. After readjusting the pH to 6, the solution was diluted with sufficient deionized water to yield a TOC concentration of approximately 1 g/l. The solution was kept in a Teflon-capped bottle with no head space at room temperature for 5 days, at which time chlorine consumption was essentially complete. The final pH of this solution was 2.5 and the total chlorine residual was less than 1 ppm. The sample was then maintained at 4°C until time of assay for mutagenic activity. At the time of testing in Salmonella strains, the pH was adjusted to 7.

Analytical Methods

Measurements of trihalomethane (THM) concentrations, total organic halogen (TOX) and total organic carbon (TOC) were performed by Alan Stevens and his staff of the Drinking Water Research Division of the Municipal Environmental Research Laboratory, U.S. Environmental Protection Agency, Cincinnati or by Dr. Robert Lingg of the Health Effects Research Laboratory, U.S. Environmental Protection Agency, Cincinnati. Trihalomethane concentrations were determined by using the purge and trap method described by Bellar and Lichtenberg (*14*). Total organic halogen (TOX) was analyzed directly in ambient water samples by using a Dohrmann Total Organic Halogen Analyzer. In studies involving chlorination of humic and fulvic acids 0.2 ml of the sample was diluted to 50 ml before the analysis was performed. Total organic carbon (TOC) analyses were performed using the Dohrmann Total Organic Carbon Analyzer. Again ambient water samples were analyzed directly and chlorinated humic acid samples were diluted from 1.0 ml to 50 ml before analysis.

Mouse Skin Initiation/Promotion Assays

SENCAR mice were obtained from Oak Ridge National Laboratories, Oak Ridge, Tenn. The SENCAR mice are a strain of mice selected for their sensitivity to developing skin tumors in response to 7,12-dimethylbenz(a)anthracene (DMBA) (*15,16*). The animals were 6 to 9 weeks of age when started on study. The dorsal hair of each mouse was shaved with electric clippers three days before the initial dose of the test compound and then shaved once weekly during application of the promoting agent to facilitate the application and the observation of tumor occurrence. All animals received distilled water and Purina Laboratory Chow *ad libitum* throughout the study.

Experiments with the five cities concentrates utilized total doses of 150 mg/kg (4.5 mg/mouse) of combined ROE fractions and the XAD samples applied subcutaneously in the back of the mice in six injections over a 2 week period. The vehicle used was 0.1 ml Emulphor (a polyoxyethylated vegetable oil). Two weeks after the last injection, a

promotion schedule was begun involving the application to the shaved back of each mouse of 1 μg of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) 3 times weekly in 0.1 ml acetone. After 20 weeks of application, TPA treatment was suspended and the animals were held for observation for an additional 28 weeks.

During the course of this and the following experiments the animals were weighed weekly and observed for tumor incidence. The incidence of both papillomas and carcinomas was charted weekly. Skin lesions were included in the cumulative papilloma count when they persisted for a minimum of 3 weeks. Following completion of the promotion period the animals are held for observation until they reach one year of age and then are sacrificed. Major organs and all grossly observed lesions are sectioned and fixed in a 10% buffered formaldehyde solution for subsequent histopathological diagnosis (histopathological confirmation made by Dr. John Orthoefer, HERL/Cincinnati).

In each of the three studies involving aqueous concentrates doses were administered subcutaneously in the shaved dorsal area of the mice. A total dose of 1.5 ml of the aqueous concentrates (6 × 0.25 ml) was given per mouse over a 2 week period. Negative controls received the same volumes of normal saline. As positive control either DMBA in doses of 7.5 or 25 μg/mouse or urethane at a dose of 9 mg/mouse was administered by the same routes. Both compounds were given subcutaneously in a 10% Emulphor vehicle. Two weeks following the last initiating dose, a tumor promotion schedule was begun wherein the mice received TPA topically to the shaved dorsum three times a week for 20 weeks, at a dose of 1.0 μg TPA in 0.2 ml acetone per application. It should be noted that in the first experiment (May 1977) a slightly different promotion protocol (than other experiments) was used. This involved application of 2.5 μg TPA/mouse three times weekly for 18 weeks. Due to the high sensitivity of the SENCAR mouse to the irritant effects of TPA at this dose, the dose was reduced in all subsequent experiments. For each group treated with TPA there was a corresponding group of 20 or 25 animals receiving acetone with no added TPA.

Assays for Mutagenic Activity

Salmonella typhimurium strains TA 1535, TA 1537, TA 1538, TA 98 and TA 100 were generously provided by Dr. Bruce Ames (University of California, Berkeley). Liver homogenate fractions (S9) were prepared from male Sprague-Dawley rats which had previously received a single IP injection of a PCB mixture (Aroclor 1254, Analabs, New Haven, Conn.) at a level of 500 mg/kg 4 days prior to sacrifice (*17*). Assay for mutagenic reversion of the test bacteria to histidine independence were performed according to the standard plate method of Ames (*5*). All assays were carried out in duplicate at three sample concentrations both in the absence and presence of S9 (25 μl/plate). Positive controls were assayed concomitantly with the test samples. For assays without S9 activation, these consisted of sodium azide for TA 1535 and TA 100; 2-nitrofluorene for TA 1538 and TA 98; and 9-aminoacridine for TA 1537. For assays with S9 activation, 2-aminoanthracene was used for all strains. Specific genetic markers (histidine dependence, ampicillin resistance, UV sensitivity and crystal violet sensitivity) were confirmed for each strain before use in assays.

Results

Five Cities Samples

The results obtained with the combined reverse osmosis extracts (ROE) from the five cities are shown in Figure 1. Although in all cases the tumor response was higher than in the control group, the sample obtained from Ottumwa stands out. It is notable that the increase in the development of tumors occurred after termination of the promotion schedule.

Results obtained with XAD fractions followed a similar course (Fig. 2). Here all samples again ended up at a higher tumor incidence than that observed in the control group. An excess in the total number of observed tumors per mouse can be used as evidence of carcinogenicity. No significant differences among proportions of animals with and without tumors for the ten test groups and control were found. This negative result might be due in part to the relatively low response rate (10–30%, $N = 40$ in each group). This type of data is called censured data, since both premature deaths and the end of the study precludes the full expression of the measured parameter in this case tumor incidence. Graphical comparisons did not reveal any gross differences in censoring patterns among the groups. An additional type of evidence for carcinogenicity would be a shorter time to first tumor distribution in exposed mice compared to control mice. Since the longest response times are observations that are censored the estimates of survival time (time without tumor) are truncated at the end of the study period. As a result, the mean time to first tumor will be underestimated, since very long times are not observed. However, if tumors developing very late in the study period may be due to other factors than exposure (e.g., age), then the time to tumor related to exposure may not be underestimated.

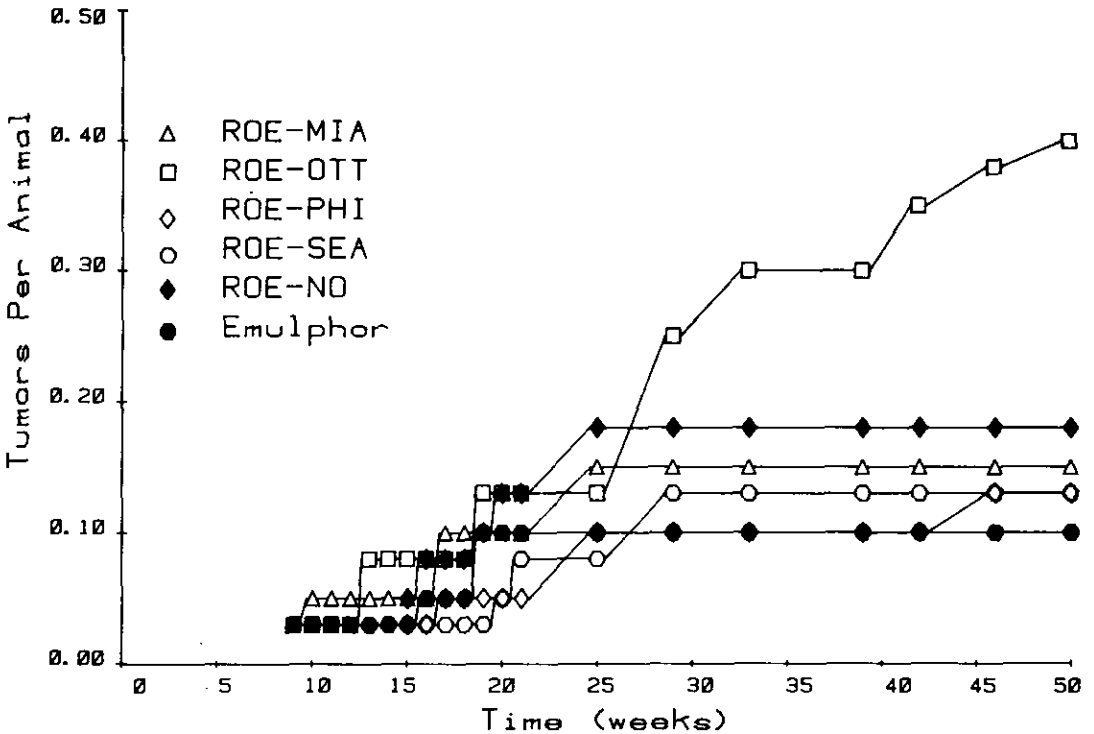

FIGURE 1. Tumor incidence through 50 weeks after an initiating dose of 150 mg/kg ROE (reverse osmosis extract) sample applied subcutaneously. Animals received 1 μg of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as a tumor promoter three times a week from weeks 0 to 20. Forty animals were included in each group. Emulphor was used as vehicle for ROE and the TPA was given in acetone.

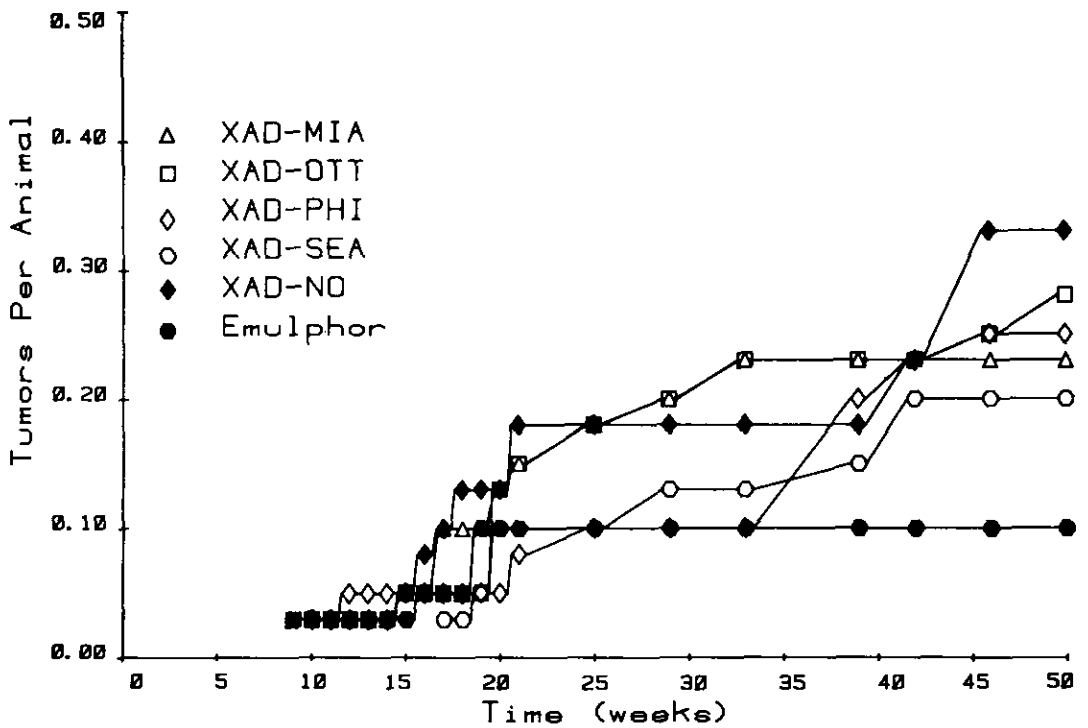

FIGURE 2. Tumor incidence through 50 weeks after an initiating dose of 150 mg/kg XAD (ethanol extract of XAD-2 resin containing residual ROE concentrate) sample applied subcutaneously. Animals received 1 μg of 12-*O*-tetradecanoylphorbol 13-acetate (TPA) as a tumor promoter three times a week from weeks 0 to 20. Forty animals were included in each group. Emulphor was used as vehicle for XAD and the TPA was given in acetone.

Table 1. Comparisons of mean time to first tumors distribution among the control and five cities samples.

City	Mean time to first tumor, weeks		
	ROE[a]	XAD[b]	Combined
Ottumwa	44.2	44.3	44.3
Miami	45.0	46.2	45.7
Seattle	46.1	48.6	47.4
Philadelphia	45.9	48.5	47.2
New Orleans	45.1	47.6	46.4
Vehicle control[c]	–	–	48.0

[a]ROE = combined reverse osmosis extract.
[b]XAD = residual aqueous concentrate from ROE is collected on XAD-2 resin column and then eluted with ethanol.
[c]Vehicle was 10% Emulphor (a polyoxyethylated vegetable oil applied in six injections of 0.25 ml each).

Table 2. Skin tumor initiation by reaction products of alternate disinfectants; study 1.[a]

Sample[b]	Concentration factor[b]	No. animals with tumors	Total tumors	Tumors/animal × 100
Nondisinfected	102	0/25	0	0
Chlorine	106	4/25	5	20
Chloramine	142	5/25	8	32
Chlorine dioxide	168	0/25	0	0
Ozone	186	7/25	9	36
Saline	—	1/25	1	4
DMBA	—	16/25	35	140

[a]Substrate was settled, coagulated, and filtered Ohio River water. Total dose 1.5 ml (6 × 0.25 ml) subcutaneously at the concentration factor indicated. Animals were subjected to a promotion schedule involving application of TPA at a dose of 2.5 μg/mouse, 3 times per week for 18 weeks.
[b]After treatment indicated water was subjected to reverse osmosis with cellulose acetate to the level indicated by the concentration factor (initial volume/final volume).

The mean time to first tumor for each animal was used as the response variable in the following analysis. Comparisons of the distribution of these values for the XAD and ROE concentrates were made within each of the five city groups, using both the generalized Savage and the generalized Wilcoxon Statistic. Since no significant differences were found, the XAD and ROE groups were combined for each city to provide larger groups for the comparison of cities and control. No significant differences were found among the time to first tumor distributions of the five cities and the control using both test statistics ($p > 0.20$). However, all cities did have a shorter mean time to first tumor (Kaplan-Meier product limit estimates) than the control with Miami and Ottumwa being over two and three weeks shorter, respectively (Table 1). An alternative technique to analyze this type of data is the proportional hazards (or Cox regression) model. In this analysis, both concentrates from Ottumwa showed regression coefficients significantly different from zero, indicating a shorter mean time to first tumor in the Ottumwa group than in the controls. However, no other city concentrate group shows a significant difference from the control. It is notable, however, that all the ROE fractions resulted in shorter mean times to first tumor, ranging from a decrease of 1.9 weeks with the Seattle sample to a decrease of 3.8 weeks with Ottumwa. The XAD samples on the other hand, were much closer to the mean time to first tumor for the control animals with the exception of Ottumwa.

Alternate Disinfectant Reaction Products

The results of the first experiment testing aqueous concentrates from alternatively disinfected drinking waters are shown in Table 2. The overall chi-square analysis comparing the proportion of mice with tumors in the disinfectant groups (saline excluded) was highly significant ($p < 0.001$). It was found that most of the tumors (classified as papillomas by gross observation) were observed in groups of which 16, 20 and 28% of the animals treated were found to have tumors, respectively. When the overall chi-square was partitioned, these treatments combined were all significantly different from the combined responses observed with the nondisinfected water (0%) and chlorine dioxide treated water (0%). Although there were small differences in the degree to which these samples were concentrated by reverse

Table 3. Summary of macroscopically observed lesions at autopsy in alternate disinfectant study 1.[a]

	Skin tumors[b]		Systemic tumors[c]		
	Papillomas	Squamous carcinomas	Lung adenoma	Mammary tumors	Stomach tumors
Nondisinfected	0	1	0	0	0
Chlorine dioxide	0	0	4 (3)[d]	3	1
Chloramine	1	2	5 (4)[d]	0	0
Chlorine	1	3	2	0	0
Ozone	2	2	1	0	0
Saline	0	1	1	2	0
DMBA	8 (6)[d]	1	0	0	0

[a]Sample collected in May 1977.
[b]Skin tumors include only those animals promote with TPA (25 mice/group).
[c]Systemic tumors include both promoted (TPA) and nonpromoted animals (50 mice/group).
[d]Number of animals when not same as tumor count.

Table 4. Incidence of papillomas in alternate disinfectant studies.[a]

	Animals with papillomas, %					
Sample	Control[b]	Non-disinfected	Ozone	Chlorine	Chlorine dioxide	Chloramine
May 77	4	0	28	16	0	20
Nov 78	15	20	13	10	18	23
Mar 80	13	20	8	18	15	15
Average	11[c]	13	16	15	11	19

[a]Results in SENCAR mice after 1 year on study (40 mice/exposure group).
[b]Control animals injected with 0.9% saline.
[c]Historical control incidence is 8 ± 3% based upon six experiments with a total of 190 animals.

osmosis, there was no correlation between tumor response and degree of concentration ($r = 0.4$, $p \leq 0.50$). Consequently, we concluded that the differences were probably due to the reactions between disinfectants and trace organic material present in the water.

Papillomas are nonmalignant tumors and in themselves are not life-threatening. Burns and Albert (*18*) have shown that although carcinomas can arise from or independently of papillomas, the number of papillomas which arise are predictive of later carcinoma development. In Table 3 it can be seen that greater development of carcinomas did occur generally in the groups having more papillomas even though the incidences do not parallel precisely. Most of the carcinomas are to be found in the chloramine, chlorine and ozone-treated water groups. However, the response level of malignant tumors was too low to be statistically significant ($p = 0.36$).

When the numbers of systemic tumors that were observed on termination of this study were examined (Table 3) it was found that eight systemic tumors were found in the chlorine dioxide group. This included four lung adenomas (in three animals), three mammary gland tumors and one stomach tumor in the 50 animals treated with this sample (this includes 25 animals which did not receive topical TPA applications). Increased numbers of lung adenomas (5) were also observed with animals that received the chloramine-treated water. These tumors were not observed at all with the nondisinfected water. Only one lung adenoma and two mammary tumors were observed with the 50 animals injected with saline. The overall chi-square analysis comparing the number of animals with systemic tumors for this experiment is significant at $p = 0.05$ and the chlorine dioxide group is significantly different from the combined nondisinfected and saline controls at $p = 0.01$. Other treatments were not significant different statistically.

It was obvious that a single experiment of this type provides far too little information upon which to generalize concerning the relative safety of alternate disinfectant reaction products. Consequently, a series of five additional experiments has been initiated to examine the repeatability of the first experiment over the course of several seasons. Two of these five experiments have progressed to the point where the development of skin papillomas can be analyzed. Table 4 summarizes the results obtained from the experiment just described and these two

Table 5. Seasonal variations in the production of total organic halogen (TOX) and trihalomethane (THM) with alternate disinfectants using Ohio River water.

	Sampling date		
	3/80	8/80	11/80
TOC, mg/l.			
Nondisinfected	1.7	2.7	2.9
Ozone	1.7	2.4	3.2
Chlorine	1.8	2.4	2.4
Chlorine dioxide	1.7	2.6	2.5
Chloramine	2.0	2.7	3.1
TOX, mg/l.			
Nondisinfected	0.013	0.021	0.020
Ozone	0.011	0.023	0.041
Chlorine	0.053	0.237	0.169
Chlorine dioxide	0.017	0.060	0.042
Chloramine	0.026	0.064	0.065
THM, mg/l.			
Nondisinfected	0.0006	N.D.	0.0001
Ozone	N.D.	N.D.	0.0001
Chlorine	0.0083	0.080	0.065
Chlorine dioxide	N.D.	N.D.	0.0001
Chloramine	N.D.	0.0001	N.D.

subsequent experiments. It is clear that these latter two experiments are providing a different picture than that seen in the first experiment. A major difference is that a response rate greater than that observed in the control group is being observed in both experiments for the nondisinfected water. This might be the result of the greater concentration factor involved in these experiments compared to the first. All samples were uniformly concentrated 400-fold by a process initially involving reverse osmosis, but followed by freeze-drying. Whatever the reason, it is clear that the carcinogenic activity associated with alternatively disinfected samples did not significantly exceed that in the nondisinfected samples in these two experiments.

It should be pointed out that if taken in total and against historical controls, the average tumor incidence in the chloramine group is almost double the simultaneous control incidence and more than twice the historical control response. In addition, this increased incidence has been consistent across all three experiments. Although the differences are not statistically significant when compared to the simultaneous controls, if this trend is maintained through the remaining three experiments statistical significance may well be achieved.

There has been insufficient time since the completion of the latter two studies to confirm the gross pathological findings at terminal sacrifice. Consequently, comparison of the systemic tumors between experimental groups is not yet possible.

Data obtained from analyses of the nonconcentrated water used in subsequent experiments provides some insight into the variability of these results (Table 5). The organic chemical content of the Ohio River tends to be at a minimum during late winter and early spring and increases 60-80% with the lower flow of later summer and fall. These changes result in even more dramatic effects upon disinfection by-products. For example, total organic halogen (TOX) concentration is increased much more dramatically by chlorine, chlorine dioxide and combined chlorine in late summer than in the sample collected in March. In the case of chlorination, the trihalomethane (THM) portion of the TOX is 10-fold that observed in March. It should also be noted that under the conditions of late fall, ozone is capable of significantly increasing the TOX, whereas no significant alterations are observed in March or August.

Mutagenesis Assays

No net increase in the number of revertants was observed with *Salmonella* strains TA 1535, TA 1537 or TA 1538 treated with commercially available humic acid either before or after the addition of chlorine. As shown in Figure 3 the sample of humic material prior to the addition of chlorine was also without activity in strains TA 98 and TA 100 in the presence or absence of rat liver S9 fraction. However, if the sample is treated with chlorine it gives a clear cut response with both TA 98 and TA 100. A linear dose-response curve was observed in both strains. This activity is apparently destroyed by the incorporation of Aroclor 1254-induced rat liver S9 fraction.

In Table 6 the activity of the humic and fulvic acid fractions from two natural lakes (Lake Drum-

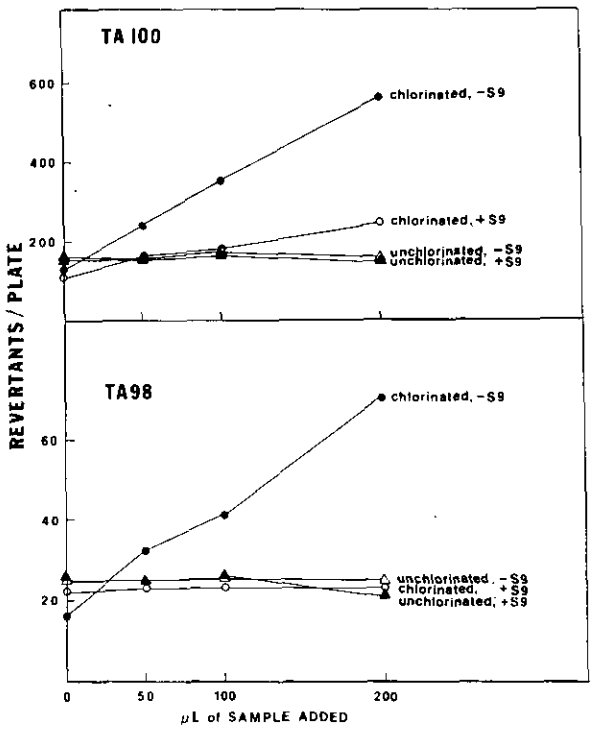

FIGURE 3. Effect of chlorination on the production of mutagenic activity in solutions prepared with commercially available humic acid. The Ames Salmonella/microsome assay and chlorination of samples were performed as described in Methods. Assays were carried out both with and without S-9 added. Each point represents the average of duplicate determinations.

mond and Black Lake) along with that obtained using humic acid obtained commercially from Fluka are shown. In all cases involving the humic acid fraction, the same clear dose-response relationship is seen with the chlorinated material. The unreacted material was without activity as indicated earlier for the commercial humic acid. In all cases the activity of the chlorinated material was decreased by the incorporation of rat liver S9 fraction. It is notable in Table 7 that the degree of chlorine incorporation appears to be quite similar. The TOX/TOC ratio was 0.34 with Black Lake humic, 0.34 with Lake Drummond fulvic, 0.36 with Black Lake fulvic, 0.38 with Fluka humic and 0.44 with Lake Drummond humic. It appears that the specific mutagenic activity relative to either TOC or TOX is virtually identical for TA 100 with humic and fulvic acids from all three sources. In the case of TA 98, however, the specific activities observed ranged from no measurable mutagenic activity with the chlorinated Lake Drummond fulvic acid to a maximum specific activity with the chlorinated Lake Drummond humic acid. Chlorinated fulvic acid from Black Lake gave approximately 21 to 58% of the specific mutagenic activity in TA 98 when compared to the three sources of humic acid treated with chlorine.

Discussion

The present work adds to growing evidence that chemicals with carcinogenic activity are found in

Table 6. Comparison of mutagenic activities in chlorinated humic and fulvic solutions derived from a commercial source and two natural lakes.

Source	Volume added per plate, μl	Total revertants/plate[a] TA 98 –S9	TA 98 +S9	TA 100 –S9	TA 100 +S9
	No addition (A)	15 (6)	25 (6)	152 (4)	127 (8)
	(B)	18 (2)	29 (2)	160 (18)	170 (27)
Fluka humic	50	32 (3)	23 (5)	237 (3)	161 (10)
	100	41 (13)	23 (1)	354 (9)	169 (28)
	200	70 (5)	23 (1)	559 (27)	264 (63)
Black Lake humic	50	45 (17)	32 (13)	299 (17)	203 (34)
	100	66 (11)	54 (3)	448 (20)	281 (49)
	200	110 (1)	73 (33)	653 (73)	343 (5)
Lake Drummond humic[b]	50	19 (10)	28 (10)	203 (15)	154 (11)
	100	31 (4)	33 (11)	233 (36)	152 (6)
	200	59 (9)	32 (20)	309 (20)	203 (0)
Black Lake fulvic	50	21 (1)	18 (8)	226 (42)	145 (18)
	100	21 (13)	26 (5)	375 (126)	281 (97)
	200	44 (1)	25 (13)	561 (16)	215 (42)
Lake Drummond fulvic	50	19 (9)	23 (3)	328 (5)	286 (24)
	100	20 (2)	22 (10)	395 (24)	303 (6)
	200	25 (7)	26 (9)	540 (63)	409 (11)

[a]Values are mean values of duplicate place counts with range values in parentheses. Humic and fulvic samples were assayed in separate experiments with control values indicated by (A) and (B), respectively.

[b]Significant precipitation of material occurred during acidification step in sample preparation. This resulted in a TOC concentration approximately 25% of other humic acid samples (see Table 7).

Table 7. Specific mutagenic activities of chlorinated humic and fulvic acids.

Source	TOC, mg/ml[a]	TOX, mg/ml[b]	Net revertants/mg TOC[c]		Net revertants/mg TOX[c]	
			TA 98	TA 100	TA 98	TA 100
Fluka humic	0.92	0.35	309	2323	811	6106
Black Lake fulvic	1.01	0.34	450	2382	1335	7076
Lake Drummond humic	0.69	0.25	181	3003	508	8288
Lake Drummond humic	0.25	0.11	857	2694	1963	6173
Lake Drummond fulvic	0.76	0.26	ND[d]	2367	ND	6919

[a]Total organic content; analysis conducted by R. Lingg and R. Kaylor, HERL, USEPA, Cincinnati.
[b]Total organic halogen content; analysis provided by A. Stevens of MERL, USEPA, Cincinnati.
[c]Values are based on dose-response data from assays without S9 added.
[d]ND = no detectable mutagenic activity.

drinking water. Data showing an increased tumor incidence and a reduction of the mean time to first tumor in mouse skin initiation/promotion assays demonstrates the presence of tumor initiating activity in samples obtained from at least one city. Although not statistically significant, increases in tumor incidence and reduced mean times to tumor were observed in the other four cities studied. These data provide confirmation of the presence of chemicals with carcinogenic activity in the same samples which produced mutagenic affects in Salmonella strains TA 98 and TA 100 (*2*) and increased transformation in BALB/3T3 cells (*10*).

The importance of these results lies strictly in the fact that a tumorigenic effect can be seen in an intact animal model. These data should not be understood as demonstrating that cancer hazards are any greater in Ottumwa than the other cities studied. Nor are there implications of a specific level of risk attributable to cancer causing agents in any of these waters. At least two factors militate against the use of mouse skin initiation/promotion studies for assessing relative risks. First, the system responds selectively to different carcinogen classes, giving rise to a large number of false negative results (*19,20* and Bull et al., unpublished data). Secondly, the utilization of a promoting agent greatly enhances the tumorigenic effect of tumor initiators. In certain instances (e.g., topical ethyl carbamate application) it is virtually impossible to produce tumors in the absence of promotion.

Given the fact that a hazard of undefined proportions can be seen in drinking waters two very important questions must be addressed. The first of these is, "what is the source of this carcinogenic material?" The second is, "what is the magnitude of the risk to the consuming population?"

Several authors have shown that drinking water treatment including chlorination substantially increases the level of direct acting mutagens (*3,21–23*). Organic chemicals resulting from the treatment of wood pulp with chlorine (*24,25*) also result in production of direct acting mutagens in Salmonella tester strains. Ozonation of municipal wastewater has been shown to increase both the direct and indirect mutagenic activity present (*26*). Kool et al. (*22*) demonstrated that chlorine dioxide also resulted in increased levels of direct acting mutagens in strain TA 98. Consequently, it is clear that material present in drinking water sources is capable of reacting with disinfectants to produce biologically active products.

Testing of aqueous concentrates of alternately disinfected water as tumor initiators in the mouse skin illustrates many of the problems that arise in hazard evaluations of complex mixtures. Eventually such evaluations must deal with the individual processes that impact the chemical composition of the mixture to which man is exposed. In the first experiment a rather straightforward conclusion that reactions of ozone, chlorine, chloramine and chlorine dioxide resulted in a clear increase in the carcinogenic activity relative to that in the source water seemed possible. Subsequent experiments, however, failed to confirm this pattern. A major difference was that the nondisinfected water appears to possess activity not apparent in the earlier experiment. If one can assume that the series of analyses of water performed in 1980 reflects gross seasonal variations in the organic content and the reactivity of this material with disinfectants, it is clear that the variability suggested by the tumorigenesis data is clearly possible. Therefore, it becomes clear that consideration must be given to the variability in the source on the interpretation of results.

A second aspect of this variability is that disinfectants are very reactive chemically. Although the measures supplied in Table 4 concentrate on one aspect of disinfectant reactions, halogen substitution, all the alternate disinfectants are strong oxidants. Given the presence of biologically active chemicals in a source water it is not unlikely that

such chemicals will be destroyed by disinfectants as easily as new chemicals with biological activity might be produced.

A third major source of variability is that testing is obviously being conducted at the limits of the sensitivity of this assay. This makes it predictably difficult to differentially determine the contribution of disinfectant to formation of carcinogenic products from the variations of the source contribution. Bioassays based on toxicological effects do not distinguish among the responsible chemical species. Therefore, it becomes very difficult to determine what strategy is necessary to reduce hazards or even to arrive at a circumstance which results in the lowest overall risk because of an inability to develop the data for systematic decision making.

Contributing to the lack of systematic or predictive information is the selective responsiveness of certain bioassays to certain classes of chemical carcinogens mentioned above. The mouse skin certainly suffers from this difficulty (*20*). Consequently, future work must take an approach which comprehensively handles the question of relative carcinogenic hazards irrespective of the chemical classes present. An approach involving the use of a matrix of testing systems to define relative carcinogenic hazards has been previously described (*19*).

The above described difficulties argue for a better fundamental understanding of the chemical reactions that give rise to mutagenic, carcinogenic and otherwise toxic chemicals during disinfection of drinking water. It has been fairly well established that the organic substrates producing trihalomethanes and other reaction products of chlorination are quite varied. Perhaps the most widespread chemicals in surface sources of drinking water are a poorly characterized group of natural substances referred to as humic and fulvic acids. It is well established that chlorine, ozone and chlorine dioxide react with this class of chemicals to reduce color or ultraviolet absorbance present in many surface sources of drinking water. In particular, reactions of these chemicals with chlorine have been studied extensively (*13*). It is clear that they can serve as a source of trihalomethanes (*27*) and it is known that many other reaction by-products are formed which are yet to be identified. It is important to realize that these reactions include oxidative processes as well as chlorine substitution reactions.

From the use of the rather gross parameter TOX, a general case can be made that reaction of chemicals in drinking water results in the formation of both trihalomethanes and non-trihalomethane TOX. Choosing conditions which maximize chlorine substitution reactions (pH < 7) we have demonstrated virtually equivalent levels of chlorine substitution in commercially obtainable humic acids and humic and fulvic material isolated from surface water sources.

In a broad sense there is also a similarity in the level and type of mutagenic activity produced by chlorination of commercially available humic acid and comparable material isolated from surface water. In no case were any of these materials mutagenic without being chlorinated. Virtually identical specific activities of directly effective mutagens were observed in Salmonella strain TA 100 in all cases following treatment with chlorine. Incorporation of Aroclor 1254-induced rat liver microsomes reduces the mutagenic activity. These observations are consistent with the types of activity which are observed upon chlorination of water (*21–23*). Comparisons of mutagenic activity in Salmonella strain TA 98 was more variable. Fulvic acids isolated from water gave rise to nondetectable to low mutagenic activity in TA 98 following treatment with chlorine. Chlorinated humic acids derived from the same waters gave the highest specific activities in TA 98. Commercially obtained humic acid upon chlorine treatment produced the median specific mutagenic activity in TA 98.

On the basis of these results it would appear reasonable to utilize commercially available humic acids as models for studying hazards associated with disinfection by-products. Recovery of sufficient material from surface sources to perform life time carcinogenesis bioassays would be prohibitively expensive. However, this is the only method acceptable for estimating the magnitude of risks to human populations. Consequently, the use of such a model system seems essential to provide guidance to the study of disinfectant reaction products at ambient concentrations. The results of these studies can form the basis for identifying important disinfectant by-products by providing clues to how these materials might be isolated from drinking water. Obviously a substantial effort will be necessary to compare the chemistry of disinfection products produced at the high concentrations used here and the much lower concentrations involved in drinking water disinfection. Understanding of the chemistry involved will form the basis of determining how directly experiments conducted with commercially available humic acids can be applied to estimating risks arising from disinfection by-products. Even if it is not possible to make a direct comparison at the very least the use of the commercially available material should allow the design of methods for recovering and detecting the active chemicals from actual drinking waters. This in itself will be a major contribution to arriving at estimates of risk posed by disinfection by-products.

In summary, it seems much too early to decide the relative hazards associated with alternative disinfectants. Of all the issues involved the question

of disinfection reaction by-products is perhaps the most difficult. Depending upon the variety of water characteristics which exist in the real world, production of by-products might be quite diverse. At alkaline pH, considerable portions of the organic chlorine substitution appears to be in the trihalomethanes (27). At lower pH, such as those used in the present study, much more of the organic chlorine substitution is in the non-THM fraction of organically bound chlorine. It is only after a body of data has been established that has enough depth of information to allow generalizations to be made confidently can the problem of disinfection by-products be considered solved. Therefore, the greatest need is to provide the basic core of information that can be modified systematically and logically to fit local conditions. The development of a basic experimental model and a means of field validating the results and predictions of the model under a variety of circumstances seems to be an essential step in developing a workable solution to the problem.

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