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Effect of Trihalomethanes on Cell Proliferation and DNA Methylation in Female B6C3F1 Mouse Liver

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Trihalomethanes (chloroform, bromodichloromethane, chlorodibromomethane, and bromoform) are regulated organic contaminants in chlorinated drinking water. In female B6C3F1 mouse liver, the 4 trihalomethanes have demonstrated carcinogenic activity when administered by oral gavage; however, chloroform was not carcinogenic when administered in drinking water. Female B6C3F1 mice were administered the trihalomethanes for 11 days by gavage at 2 dose levels or in the drinking water at ~75% saturation. When administered by gavage, the trihalomethanes were toxic to the liver, increased the liver:body weight (bw) ratio, and increased the proliferating cell nuclear antigen-labeling index (PCNA-LI). Chloroform and bromodichloromethane were the most toxic, and they increased the liver:bw ratio the most, while bromoform and chloroform increased the PCNA-LI the most. When administered in drinking water, the toxicity of the trihalomethanes was similar to their low gavage-dose. Furthermore, only chloroform significantly increased the liver:bw ratio and bromoform and chloroform increased the PCNA-LI. Chloroform and bromodichloromethane decreased the level of 5-methylcytosine in hepatic DNA. Methylation in the promoter region of the c-*myc* gene was reduced by the trihalomethanes. Chloroform administered by gavage was more efficacious than given in drinking water; the efficacy of the other trihalomethanes did not differ for the 2 routes. Thus, in mouse liver, the trihalomethanes administered by gavage enhanced cell proliferation and decreased the methylation of the c-*myc* gene, consistent with their carcinogenic activity. Furthermore, the more modest toxicity, enhancement of cell proliferation, and decreased methylation induced by chloroform administered in drinking water correlated with its lack of carcinogenic activity. Hence, the activity of the trihalomethanes was dependent on the rate of delivery, i.e. rapid by oral gavage and more slowly in drinking water.

Key Words: **bromodichloromethane, bromoform, cell proliferation, chlorodibromomethane, chloroform, c-*myc*, DNA methylation, hepatotoxicity, trihalomethanes.**

Trihalomethanes (THM), i.e., chloroform ($CHCl_3$), bromodichloromethane ($CHBrCl_2$), chlorodibromomethane ($CHClBr_2$), and bromoform ($CHBr_3$) are major organic by-products of drinking water chlorination, resulting from the reaction of chlorine with natural organic material and bromine in source waters (Uden and Miller, 1983; Coleman *et al*., 1984). The discovery of these by-products in drinking water has raised questions about their health hazards (Jolley *et al*., 1990, IARC, 1991). All four trihalomethanes, when administered by gavage, are carcinogenic in the livers of female B6C3F1 mice (NCI, 1976; NTP, 1985, 1987, 1989). The carcinogenic activity of the trihalomethanes has been proposed to be mediated through a nongenotoxic mechanism based on their weak activity in mutagenicity and genotoxicity assays (Le Cureiux *et al*., 1995; Reitz *et al*., 1990; Rosenthal *et al*., 1987). However, one trihalomethane, bromodichloromethane, has demonstrated mutagenic activity apparently though a glutathione (GSH) metabolite (Pegram *et al*., 1997).

The route of administration appears to play a significant role in the dosimetry and metabolism of the trihalomethanes and consequently in their carcinogenic and toxic activity. Although, chloroform was carcinogenic in mice when administered by gavage, it was not carcinogenic and did not promote liver cancer when administered in the drinking water (Jorgenson, *et al*., 1985; Klaunig *et al*., 1986; Pereira *et al*., 1985). Furthermore, cell proliferation was enhanced by chloroform administered by gavage but not given in drinking water (Larson *et al*., 1994; Pereira, 1994; Pereira and Grothaus, 1997). The trihalomethanes are metabolized to reactive dihalocarbonyl metabolites that react with nucleophilic compounds and macromolecules (Ammann *et al*., 1998; Lilly *et al*., 1997; Pankow *et al*., 1997; Pohl, 1977). Thus, the dihalocarbonyl metabolite of chloroform, phosgene, binds glutathione (GSH) in what appears to be a detoxifying mechanism (Ilett *et al*., 1973; Pohl, 1979). Administering the trihalomethanes as a bolus by gavage is expected to result in greater plasma and liver concentrations than when the same dose is delivered during the time the mice drink water. Thus, administering the trihalomethanes by gavage could overwhelm the ability of the liver to detoxify them, resulting in toxicity, regenerative hyperplasia, and promotion of cancer.

Regulation of cell proliferation is a critical facet of carcinogenesis; consequently, one mechanism proposed for nongenotoxic mouse liver carcinogens, including the trihalomethanes,

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is the enhancement of cell proliferation (Butterworth *et al*., 1992; Goodman *et al*., 1991; Roberts *et al*., 1997). Decreased methylation of DNA and protooncogenes could result in decreased regulation of cell proliferation (Counts and Goodman, 1995). 5-Methylcytosine (5-MeC) is a naturally occurring modification of eukaryotic DNA that plays a role in the control of gene expression (Baylin *et al*., 1998; Kegelmeye *et al*., 1997; Razin and Kafri, 1994; Wolffe *et al*., 1999). Decreased levels of 5-MeC in DNA and in specific genes are frequent early events in human and rodent tumors (Baylin *et al*., 1998; Bender *et al*., 1998; Bird *et al*., 1996; Pascale *et al*., 1993). In mouse liver, nongenotoxic carcinogens, including dichloroacetic acid (DCA), phenobarbital, trichloroacetic acid (TCA), and trichloroethylene, have been shown to decrease the methylation of DNA and protooncogenes (Counts and Goodman, 1995; Counts *et al*., 1996; Tao *et al*., 1998, 1999, 2000). Thus, DNA hypomethylation has been proposed as a mechanism for nongenotoxic carcinogens and tumor promoters (Counts and Goodman, 1995).

The protooncogene and cellular transcription factor, c-*myc*, plays a pivotal role in apoptosis, cell replication, and differentiation (Christensen *et al*., 1999; Holden *et al*., 1998). The expression of c-*myc* is increased in the liver during enhanced cell proliferation (Butterworth *et al*., 1994; Fausto and Shank, 1983; Sprankle *et al*., 1996; Wainfan and Poirier, 1992). Methylation of CpG sites in its promoter region regulates in part the expression of its mRNA (Jones and Buckley, 1990; Razin and Kafri, 1994; Wainfan and Poirer, 1992). The methylation of the c-*myc* gene is decreased by a diet deficient in choline and methionine (Wainfan and Poirer, 1992) and by DCA, TCA, and trichloroethylene (Tao *et al*., 1999, 2000). Thus, decreased methylation of the c-*myc* gene could be used as a biomarker for DNA hypomethylation induced by nongenotoxic carcinogens.

The studies reported here compared in mouse liver the ability of the trihalomethanes to induce toxicity, to increase cell proliferation, and to decrease the methylation of the c-*myc* protooncogene. We also report a comparison of the activity of the trihalomethanes administered by oral gavage to their activity when administered in drinking water.

MATERIALS AND METHODS

Chemicals and DNA probes. Bromodichloromethane, chlorodibromomethane, and bromoform were purchased from Aldrich Chemical Co., Inc. (Milwaukee, WI); chloroform, 3-3′-diaminobenzidine tablet set, monoclonal mouse anti-PCNA, proteinase K, and ribonuclease A type III-A were from Sigma Chemical Co. (St. Louis, MO); and TRIzol Reagent were from GIBCO BRL/Life Technologies, Inc. (Gaithersburg, MD). Vectastain ABC kit, peroxidase mouse IgG PK-4002 was purchased from Vector Laboratories (Burlingame, CA). Oligonucleotide probes for c-*myc* were obtained from Oncogene Research Products (Cambridge, MA). *Hpa II* and *Msp* I were from New England BioLabs (Beverly, Ma). Hybond™-N^+ nylon membranes, (α-^{32}P)-dCTP (6000 Ci/mmol), (γ-^{32}P)-ATP (5000 Ci/mmol), enhanced chemiluminescence reagents and T_4 polynucleotide kinase were obtained from Amersham Corp. (Arlington Heights, IL). Prime-a-Gene Labeling System was obtained from Promega Corp. (Madison, WI). All other chemicals were electrophoresis and HPLC grade or the highest purity available.

TABLE 1
Treatment Groups of Female B6C3F1 Mice Administered the Trihalomethanes by Oral Gavage or in the Drinking Water

Trihalomethanes	Gavage dose (mg/kg) or drinking water concentrations (mg/l)	Equivalent daily dose (mmol/kg)
Gavage		
Corn oil (4.0 ml/kg)	0.00	0.00
Chloroform	130	1.09
	260	2.18
Bromodichloromethane	150	0.92
	300	1.83
Chlorodibromomethane	100	0.48
	300	1.44
Bromoform	200	0.79
	500	1.98
Drinking water		
Control	0.00	0.00
Chloroform	1800	2.83
Bromodichloromethane	1000	0.85
Chlorodibromomethane	790	0.82
Bromoform	1000	1.19

Note. Nunber of mice in all groups = 10.

Animals. VAF (viral antibody-free) 6-week old female B6C3F1 mice were purchased from Charles River Breeding Laboratory (Portage, MI) and maintained in our AAALAC accredited laboratory-animal facility. The mice were housed in polycarbonate cages (4/cage) with stainless steel wire lids and absorbent corncob bedding (Andersons, Toledo, OH) in humidity and temperature controlled rooms with a 12-h light/dark cycle. Deionized filtered tap water with/without the trihalomethanes and Purina Rodent Diet (J&B Feed, Toledo, OH) were provided *ad libitum*.

Experimental design. At 7–8 weeks of age, the mice were weighed and randomly assigned to the different treatment groups (Table 1). The mice then started to receive the trihalomethanes either in their drinking water for 11 consecutive days or by oral gavage in corn oil administered daily for 5 days, off for 2 days, and then daily again for 4 days. The mice were sacrificed 24 h after the last gavage dose. The dose levels of the trihalomethanes administered by gavage in corn oil were selected so that the high dose had been previously demonstrated to be carcinogenic in female B6C3F1 mice (NCI, 1976; NTP, 1985, 1987, 1989). The gavage doses were formulated daily in corn oil. The concentrations of the trihalomethanes in the drinking water were chosen as approximately 75% saturation. Drinking water formulations were prepared every 3 days with deionized water and given to the mice in measured 200 ml aliquots so that consumption could be determined at each change of the water.

The mice were sacrificed by carbon dioxide asphyxiation. Body and liver weights were obtained. For consistency, pieces of the left liver lobe were fixed in buffered formalin for 24 h, transferred to 70% ethanol, processed, and embedded in paraffin for hemotoxylin and eosin staining and immunohistochemical analysis for the proliferating cell nuclear antigen (PCNA). The remaining liver was immediately frozen in liquid nitrogen and stored at –70°C.

Toxicity. Hemotoxylin and eosin-stained sections of liver were evaluated for toxicity using the semi-quantitative procedure described by Iijima *et al*. (1983), which was modified to incorporate a 0 to +4 grading of severity. Grade 1 consisted of mid lobular ballooning hepatocytes, Grade 2 of the mid lobular ballooning hepatocytes extending to the central vein, Grade 3 of centrilobular necrosis with ballooning hepatocytes, and Grade 4 of necrosis extending from the central vein to the mid lobule zone.

PCNA-labeling index. Paraffin sections were hydrated and placed in 2N HCl at 50°C for 20 min. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide for 30 min. The sections were blocked with diluted horse serum for 20 min and incubated with 100 μl monoclonal mouse anti-PCNA (1:300) at 4°C overnight. They were then washed and incubated with biotinylated anti-mouse IgG for 30 min at room temperature, followed by a 30-min incubation with Vectastain ABC reagent. Stain was developed using 3–3′-Diaminobenzidine followed by washing with deionized water and counter staining with hemotoxylin. The nuclei of PCNA-positive cells stained brown, while unlabeled nuclei were light blue. Approximately 1000 hepatocytes/mouse were evaluated and the PCNA-LI was determined to be the number of PCNA-positive cells divided by the total number of hepatocytes evaluated $\times$ 100.

Methylation of DNA. Liver tissue was homogenized in 0.75 ml TE buffer (10 mM Tris–HCl, pH 8.0, 0.1 M EDTA) containing 0.5% SDS, and the DNA isolated as previously described (Tao *et al.*, 1998). Briefly, the homogenate was treated with DNase-free RNase (400 μg/ml) and proteinase K (200 μg/ml) and then extracted 3 times with phenol, once with phenol:chloroform (1:1), and finally with chloroform. The DNA was precipitated with cold ethanol containing 10 M ammonium acetate (10% volume), washed twice with 70% ethanol, and hydrolyzed in 100 μl of 12 M perchloric acid at 100°C for 1 h. After the addition of 230 μl of 6 M KOH, the precipitate was removed by centrifugation. The supernatant filtered through a 0.2 μm polypropylene syringe filter (Whatman Inc., Clifton, NJ) and analyzed using a Waters Model 510 HPLC system (Milford, MA) equipped with a Whatman Partisphere C_{18} column (4.6 $\times$ 250mm, 5 μm particle). The column was eluted for 15 min with an isocratic mobile phase of 100 mM ammonium acetate (pH 4.25) containing 0.5% acetonitrile. The flow rate was 2 ml/min and the detection wavelength was 280 nm. The retention time for cytosine, 5-MeC and guanine were 5.0, 6.5, and 7.4 min, respectively, with thymine and adenine eluted later. The percentage of cytosine present as 5-MeC was calculated from the peak areas using the formula, 5-MeC/(5-MeC + cytosine) $\times$ 100.

Methylation of the promoter region of the c-myc gene. The methylation of the promoter region of the c-*myc* gene was evaluated using *Hpa* II restriction enzyme digestion followed by Southern blot analysis, as previously described (Tao *et al*., 1999, 2000). Briefly, isolated DNA was dissolved in TE buffer and digested overnight with *Hpa* II (10 U/μg DNA) at 37°C. *Hpa* II does not cut CCGG sites when the internal cytosine is methylated. The digested DNA was electrophoresed on a 1% agarose gel. Equal loading of the gel was indicated by equal ethidium bromide fluorescence. The gels were washed with 2X SSC and transferred to Hybond™-N^+ nylon membranes using a Model 785 vacuum blotter (Bio Rad Laboratories, Hercules, CA). The DNA was cross-linked by ultraviolet irradiation (UV Stratalinker Model 2400, Strategene, La Jolla, CA). The membranes were pre-hybridized at 42°C for 1 h in 20 ml of pre-hybridization solution (50% formamide, 5X Dehardt's Reagent, 6X SSPE, 10% dextran sulfate, 1% SDS, and 100 μg/ml denatured nonhomologous DNA). Random ^{32}P-labeled c-*myc* probe (65 μg) was added to the pre-hybridization solution and hybridization continued for 12 h at 42°C. After hybridization, the membranes at 20-min intervals were stringently washed 5 times with 4X SSC containing 0.5% SDS at 65°C, 3 times with 2X SSC containing 0.5% SDS at 37°C, and finally once with 2X SSC at 37°C. The membranes were dried, sealed in plastic bags, and autoradiography-processed at –70°C with Kodak Biomax MR X-ray film, Kodak intensifying screens, and a Kodak M35A automatic film processor. Optical density of the autoradiograms was measured with a Scion Image Analysis System (Scion Corp., Frederick, MD).

The c-*myc* probe was designed from the GeneBank database (GeneBank accession number, M1234) to contain the 1–1315 bp in the promoter region of the gene. The probe was produced by PCR amplification of mouse liver DNA using sense 5′-TCTAGAACCAATGCACAGAGCAAAAG-3′ and antisense 5′-GCCTCAGCCCGCAGTCCAGTACTCC-3′ primers.

Statistical evaluation. Statistical analysis was performed using SigmaStat software version 2.03 (Jandel Corp., San Rafeal, CA). Body and organ weights, liver:bw ratio, liver toxicity, PCNA-LI, and DNA methylation were analyzed for statistical significance by an ANOVA followed by a Tukey or Dunnett's test with a *p*-value < 0.05.

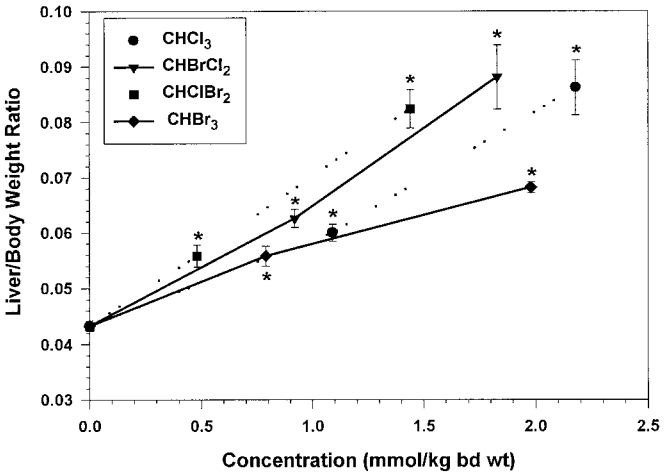

FIG. 1. Effect of trihalomethanes administered by gavage on the liver/body weight (bw) ratio. Female B6C3F1 mice were administered the trihalomethanes by oral gavage for 11 days. Results are mean ± SE for 10 animals/group. *Indicates a significant difference from the corn oil vehicle control group, *p*-value < 0.05.

RESULTS

Liver Weight and Toxicity

When administered by gavage, the trihalomethanes caused dose-dependent increases in the liver:bw ratio (Fig. 1). The order of efficacy was chlorodibromomethane > bromodichloromethane > chloroform > bromoform. When administered in drinking water, only chloroform significantly increased the liver:bw ratio (Fig. 2). Even though chloroform administered in drinking water resulted in a daily dose that was greater than the high-dose gavage (i.e., 2.83 and 2.18 mmol/kg, respectively), the increase in the liver:bw ratio was only similar to the low-dose gavage. Furthermore, the other trihalomethanes did not increase the liver:bw ratio, although the daily dose resulting from the drinking water exposure was at least equal to the low-dose gavage that significantly increased the ratio.

All 4 trihalomethanes were toxic to the liver; however, the toxicity of bromodichloromethane was different from the other 3. Thus, chloroform, chlorodibromomethane, and bromoform produced liver toxicity that progressed from mid-lobular ballooning hepatocytes to necrosis extending from the central vein to the mid lobular zone, i.e., Grades 1–4, as described in Material and Methods. In contrast, the low dose of bromodichloromethane induced hydropic degeneration bridging between the central veins, and the high dose-induced necrosis and fibrosis with calcification and giant-cell reaction bridging between the central veins (Fig. 3). NTP (1987) has reported that a low dose of bromodichloromethane induced degeneration and a higher dose induced necrosis, fibrosis, and microgranul-

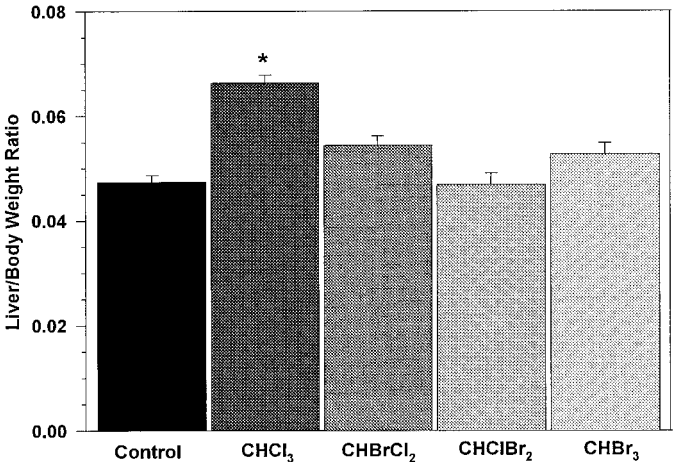

FIG. 2. Effect of trihalomethanes administered in the drinking water on the liver/bw ratio. Female B6C3F1 mice were administered the trihalomethanes for 11 days. Results are mean ± SE for 6–10 animals/group. *Indicates a significant difference from the drinking water vehicle control group, p-value < 0.05.

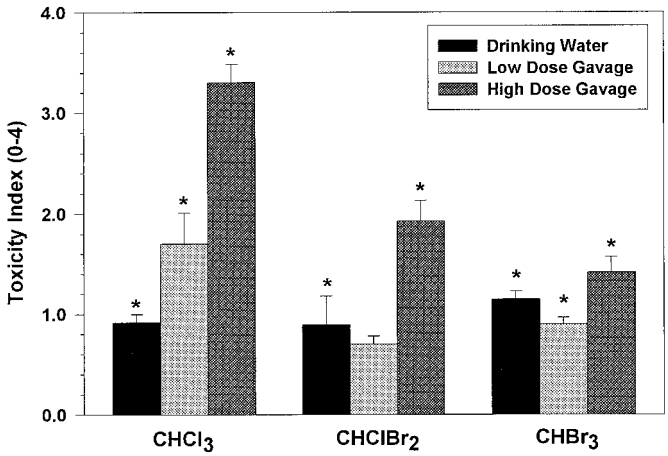

FIG. 4. Ability of trihalomethanes to induce liver toxicity. Liver sections were stained with hematoxylin and eosin and toxicity graded from 0 to +4. Results are means ± SE. *Indicates significant difference from the vehicle control group, p-value < 0.05.

omas at the central vein. The 2 dose levels of the NTP (1987) study were similar to those used in our study.

The relative toxicity of the 3 other trihalomethanes is presented in Figure 4, using the 1–4 grading system. Bromodichloromethane is not included because of the different pathogenesis of its hepatotoxicity. The toxicity of the low dose of the 3 trihalomethanes consisted of mainly mid-lobular ballooning hepatocytes (Grade 1). The high doses of chlorodibromomethane and bromoform resulted in mid- lobular ballooning hepatocytes extending to the central vein (Grade 2), and the high dose of chloroform resulted in necrosis at the central vein (Grades 3 and 4). Hence, necrosis was apparent only in mice administered the high dose of chloroform and bromodichloromethane, so that these 2 trihalomethanes were more toxic than chlorodibromomethane and bromoform.

The toxicity resulting from the 3 trihalomethanes other than bromodichloromethane, when administered in the drinking water, is also presented in Figure 4. The toxicity of chlorodibromomethane and bromoform administered in drinking water did

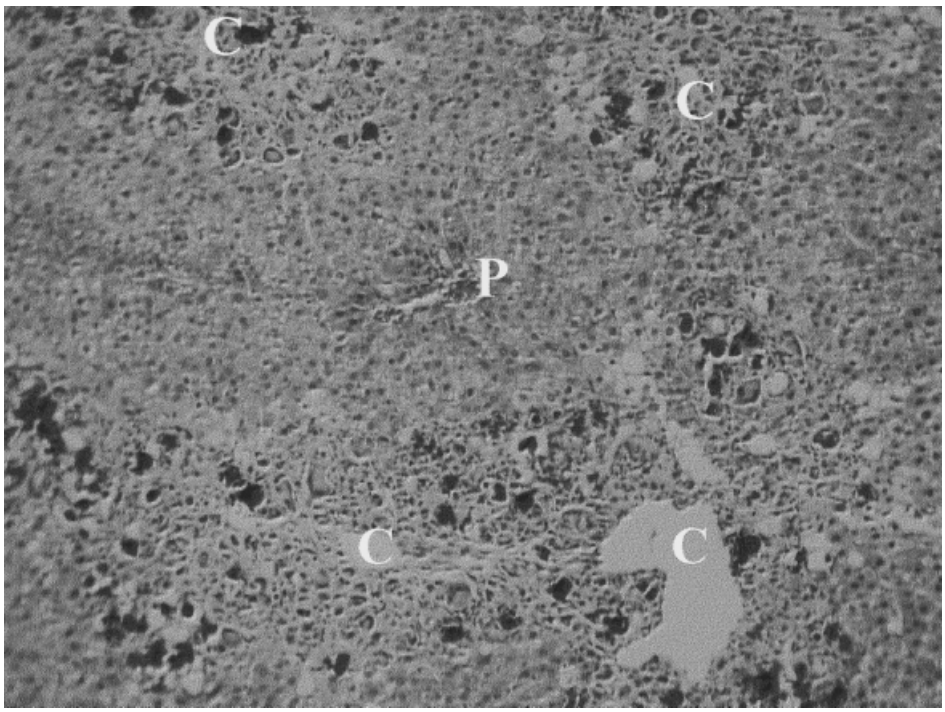

FIG. 3. Centrilobular necrosis and fibrosis produced by the high dose of bromodichloromethane administered by oral gavage. The liver section was stained with hematoxylin and eosin. Extensive calcification and giant cell reaction are apparent. C indicates the central vein and P the periportal region. Magnification ×8.

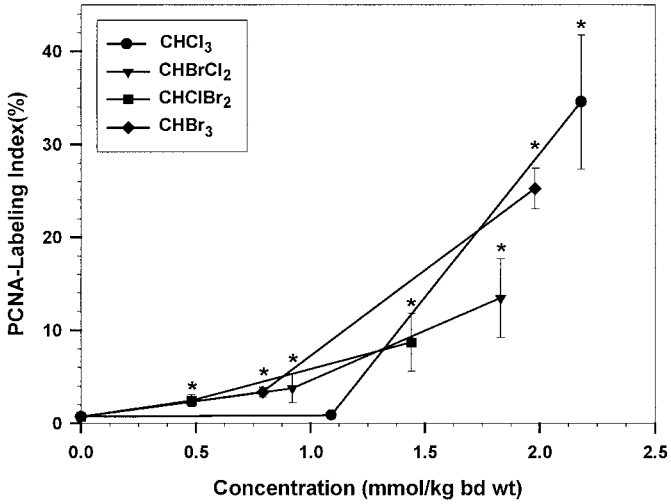

FIG. 5. Effect of the trihalomethanes administered by oral gavage on the PCNA-LI. Female B6C3F1 mice were administered the trihalomethanes by oral gavage. Liver sections were stained with monoclonal mouse anti-PCNA and approximately 1000 hepatocytes were evaluated for PCNA labeling. The PCNA-LI was determined and the results are mean ± SE. *Indicates a significant difference from the corn oil vehicle control group, p-value < 0.05.

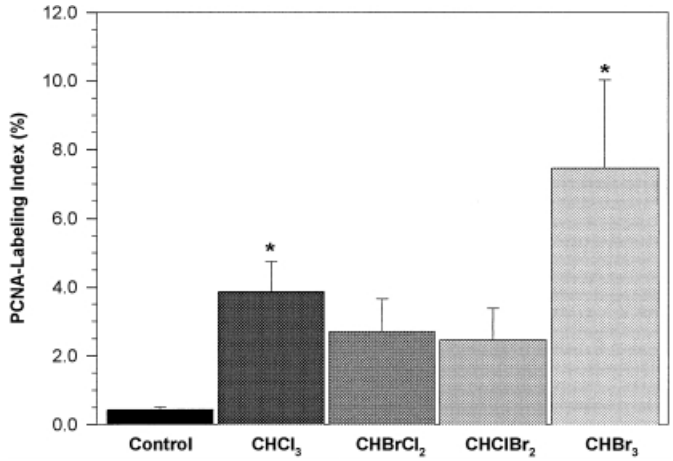

FIG. 6. Effect of the trihalomethanes administered in drinking water on the PCNA-LI. Female B6C3F1 mice were administered the trihalomethanes in the drinking water. Liver sections were stained with monoclonal mouse anti-PCNA and approximately 1000 hepatocytes were evaluated for PCNA labeling. The PCNA-LI was determined and the results are mean ± SE. *Indicates a significant difference from the corn oil vehicle control group, p-value < 0.05.

not differ from the low-dose gavage. Although the daily dose resulting from chloroform administered in drinking water was greater than the high-dose gavage, it was less toxic than even the low-dose gavage. The toxicity of bromodichloromethane administered in drinking water was similar to the low-dose gavage, inducing hydropic degeneration at the central vein that in some cases was bridging between central veins.

PCNA-LI

The trihalomethanes, when administered by gavage, caused a dose-dependent increase in the PCNA-LI (Fig. 5). The high-dose level of chloroform and bromoform resulted in the greatest increase in the PCNA-LI, followed by bromodichloromethane and chlorodibromomethane. The low-dose gavage of the trihalomethanes, with the exception of chloroform, also increased the PCNA-LI. When administered in drinking water, the trihalomethanes increased the PCNA-LI to an extent similar to their low gavage dose (Fig. 6). However, the increase was only statistically significant for chloroform and bromoform.

Methylation of DNA and the c-myc Gene

Before determining whether the trihalomethanes decreased the methylation of the c-*myc* gene, the ability of the high gavage dose of chloroform and bromodichloromethane to decrease global DNA methylation was determined. Both trihalomethanes decreased global DNA methylation by about 40% (Fig. 7). Since 2 trihalomethanes demonstrated the ability to decrease DNA methylation, it seemed reasonable to determine the ability of the trihalomethanes to decrease the methylation of a specific gene, i.e., the promoter region for the c-*myc* gene. Southern blots of *Hpa* II-digested liver DNA from mice administered the trihalomethanes contained bands of 2.7, 2.2, 1.0, 0.5, and 0.2 Kb when probed for the c-*myc* promoter region (Figs. 8 and 9). These bands were absent when the DNA was not digested with *Hpa* II and when DNA isolated from vehicle (corn oil and drinking water) control mice was digested with *Hpa* II. Thus, in vehicle control animals the internal cytosine of CCGG sites appears to be methylated, thereby preventing digestion by *Hpa* II.

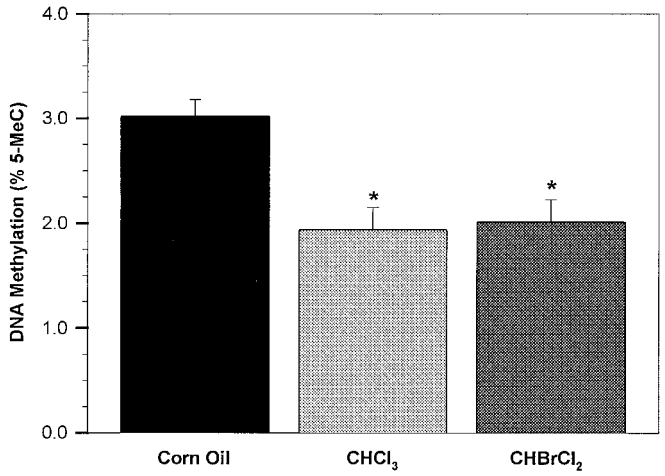

FIG. 7. Effect of chloroform and $CHBrCl_2$ administered by gavage on global DNA methylation. Chloroform (2.18 mmol/kg) and $CHBrCl_2$ (1.83 mmol/kg) were administered in corn oil by gavage for 11 days. Liver DNA was isolated, hydrolyzed with 12 M perchloric acid, and analyzed on a Waters Model 510 HPLC equipped with a Whatman Partisphere C_{18} column. Results are mean ± SE. *Indicates significant difference from the corn oil vehicle control group, p-value < 0.05.

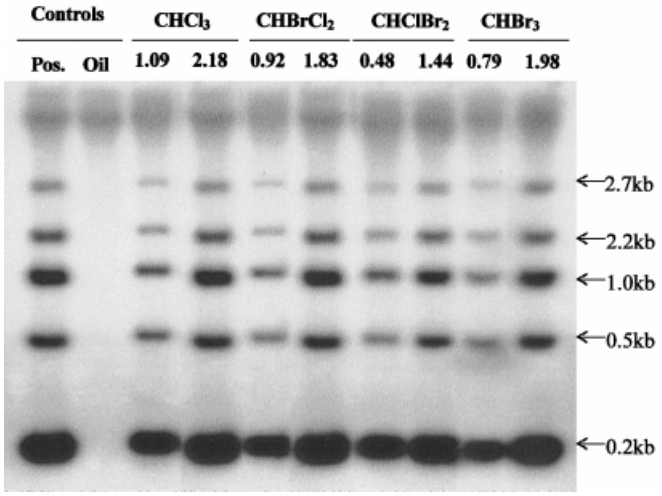

FIG. 8. Effect of the trihalomethanes administered by gavage on the methylation of the promoter region of the c-*myc* gene. Isolated DNA (30 μg) was digested with *Hpa* II, electrophoresed in a 1% agarose gel, transferred to a Hybond™-N^+ membrane, hybridized to a ^{32}P-labeled probe for the c-*myc* promoter, and visualized by autoradiography. Lane 1 (Pos.) is a positive control for hypomethylated DNA containing a standardized mixture of *Hpa* II-digested DNA from chloroform and $CHBrCl_2$-treated mice that has previously been shown to be hypomethylated. Lane 2 (Oil) contains *Hpa* II-digested DNA from a mouse administered corn oil (Vehicle control). Lanes 3–10 contain *Hpa* II digested DNA from mice administered 1.09 and 2.18 mmol/kg chloroform, 0.92 and 1.83 mmol/kg $CHBrCl_2$, 0.48, and 1.44 mmol/kg $CHClBr_2$, or 0.79 and 1.98 mmol/kg bromoform, respectively. The arrows in the right margin indicate the size of the bands.

To demonstrate that the bands present in *Hpa* II digested DNA from trihalomethane-treated mice resulted from decreased methylation at CCGG sites, DNA was digested with *Msp* I. *Msp* I cuts DNA irrespective of the methylation status of the internal C of CCGG sites. *Msp* I-digested liver DNA from control mice, when probed for c-*myc*, resulted in a smear of radioactivity in the 100–600 bp range (data not presented). This smear of radioactivity is likely the result of cuts at the 12 CCGG sites in the area probed. Since the smear of radioactivity was not present after the DNA was digested with *Hpa* II, these sites must be methylated in control mice. Furthermore, the 2.7- and 2.2-Kb bands were demonstrated to result from *Hpa* II cutting of the DNA at a CCGG site in the promoter region and at a site downstream from the probe. When DNA from trihalomethane-treated mice was digested with both *Hpa* II and *Eco*0109 I, the larger bands were lost with the appearance of a 0.7-Kb band. *Eco*0109 I is a methylation-insensitive restriction enzyme that cuts PuGGNCCPy sites between the GG. The 0.7-Kb band corresponded to the distance between a CCGG site and the *Eco* 0109 I site in the promoter region. Thus, 0.7 Kb of the larger bands was demonstrated to be within the promoter region of the c-*myc* gene, with the rest of the band downstream from the *Eco* 0109 I site.

The intensity of the bands present after *Hpa* II digestion was determined using a Scion Image Analysis System (Scion Corp., Frederick, MD). Methylation of the c-*myc* promoter decreased with the increasing dose of the trihalomethane administered by gavage. The 5 bands after *Hpa* II digestion exhibited similar dose-response curves. Therefore, Figure 10 contains the dose-response curves for band 3 (1.0Kb), chosen as representative since it is the middle band in size and the second darkest in intensity. The dose-response curves for chloroform and bromodichloromethane increased sharply between the low- and high-dose levels, while the relationships for bromoform and chlorodibromomethane appeared convex. Furthermore, chloroform and bromodichloromethane reduced the methylation of the c-*myc* gene more than the 2 other trihalomethanes.

The intensities of the 5 bands after *Hpa* II digestion of DNA from mice administered the trihalomethanes in drinking water were compared, using the equivalent mmol/kg body weight dose to the dose-response relationship obtained when they were administered by gavage. Because similar results were obtained for the 5 bands, only the comparison for band 3 is presented (Fig. 10). The intensity of band 3 from mice exposed via their drinking water to the trihalomethanes, except for chloroform, was not different from that predicted by the dose-response relationship. In contrast, the intensity of band 3 resulting from chloroform was much less than predicted. The 4 other bands in *Hpa* II-digested DNA from mice administered chloroform in drinking water also had intensities that were, at

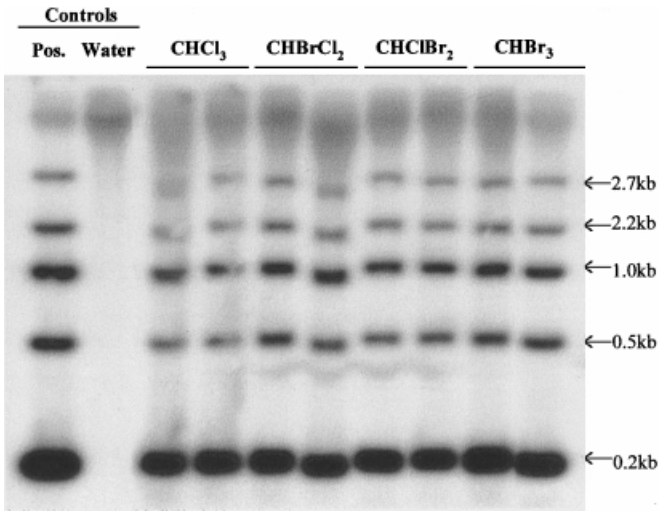

FIG. 9. Effect of the trihalomethanes administered in the drinking water on the methylation of the promoter region of the c-*myc* gene. Isolated DNA (30 μg) was digested with *Hpa* II, electrophoresed in a 1% agarose gel, transferred to a Hybond™-N^+ membrane, hybridized to a ^{32}P-labeled probe for the c-*myc* promoter, and visualized by autoradiography. Lane 1 (Pos.) is a positive control for hypomethylated DNA containing a standardized mixture of *Hpa* II-digested DNA from $CHCl_3$- and $CHBrCl_2$-treated mice. Lane 2 (Water) is the vehicle control and contains *Hpa* II-digested DNA from a drinking water-control mouse. Lanes 3–10 contain *Hpa* II-digested DNA. Lanes 3 and 4 ($CHCl_3$) are from mice administered 15.07 mmol/l chloroform, Lanes 5 and 6 ($CHBrCl_2$) are from mice administered 6.10 mmol/l $CHBrCl_2$, Lanes 7 and 8 ($CHClBr_2$) are from mice administered 3.80 mmol/l $CHClBr_2$, and Lanes 9 and 10 ($CHBr_3$) are from mice administered 3.95 mmol/l bromoform. The arrows in the right margin indicate the size of the bands.

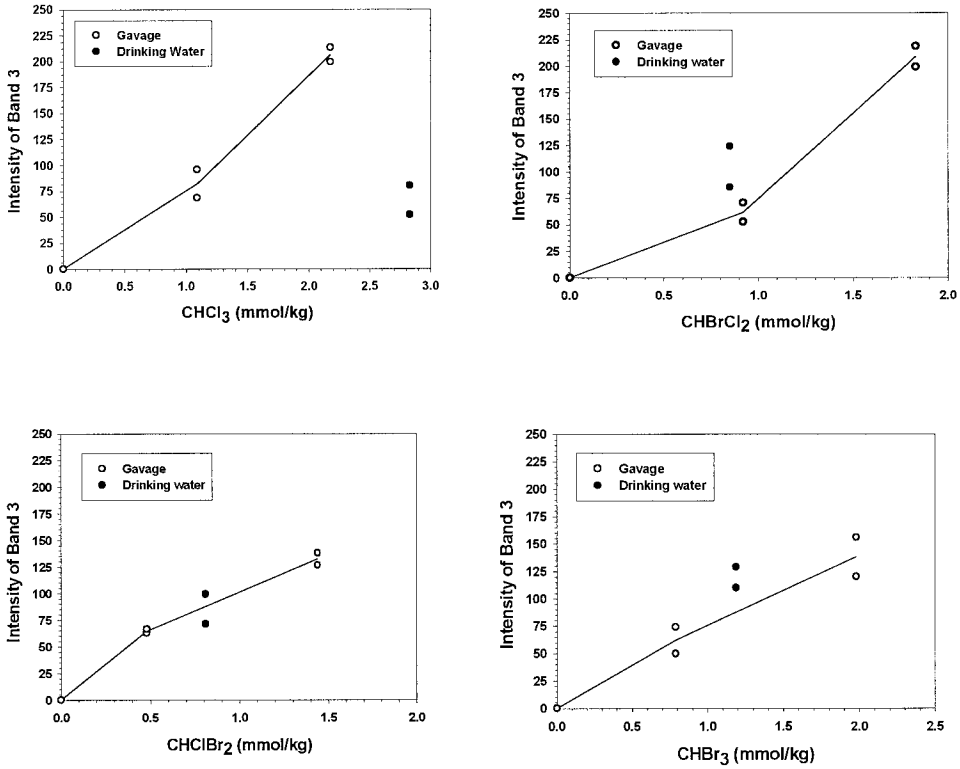

FIG. 10. Effect of the trihalomethanes on the intensity of the 1.0 Kb band of *Hpa* II-digested DNA. The intensity of the 1.0-Kb band of *Hpa* II-digested and c-*myc*-probed DNA (determined using the Scion Image Analysis System) is presented.

most, similar to the low gavage dose (data not presented). Hence, although the dose resulting from chloroform in drinking water was greater than the high gavage dose, the intensity of the *Hpa* II digestion bands were, at most, similar to the low gavage dose. Thus, chloroform administered in drinking water had much less effect than when administered by gavage.

DISCUSSION

The trihalomethanes are found in finished drinking water as by-products of chlorination (Uden and Miller, 1983). The U.S. Environmental Protection Agency (1979, 1994) has set a standard for trihalomethanes in drinking water, in part because of their carcinogenic activity in laboratory animals, including liver cancer in female B6C3F1 mice (NCI, 1976; NTP, 1985, 1987, 1989). However, the relevance to humans of the carcinogenic activity in mouse liver of the trihalomethanes, especially for chloroform, has been questioned (Pohl *et al*., 1979; U.S. EPA, 1979; Reitz *et al*., 1990). It was argued that the very weak genotoxic activity of chloroform indicates a nongenotoxic mechanism resulting from regenerative hyperplasia in response to toxicity (Butterworth *et al*., 1992; Reitz *et al*., 1990; Rosenthal, 1987).

Toxicity, cell proliferation, and DNA methylation are all possible components of a nongenotoxic mechanism for the trihalomethanes. DNA methylation can control the expression of genes including those associated with cell proliferation. The expression of c-*myc* has been reported to be increased by chloroform administered by gavage to B6C3F1 mice (Sprankle *et al*., 1996). We have also found increased expression of c-*myc* in mice administered chloroform by gavage as well as in the drinking water (data not presented). Chloroform also increased the expression of c-*fos* (Sprankle *et al*., 1996) and 4 genes identified by the differential-display technique (Kegelmeyer *et al*., 1997). These genes could also be hypomethylated, as suggested by the significant decrease (~40%) in global DNA methylation, indicating that many genes besides c-*myc* are hypomethylated. Thus, the hypomethylation of c-*myc* could be an indicator of possible hypomethylation of other genes.

A possible mechanism for DNA hypomethylation by the trihalomethanes is to prevent, after DNA replication, the methylation of the daughter strands of DNA. Under normal conditions, there is very little cell proliferation in the liver. Therefore, this mechanism requires that the trihalomethanes enhance cell proliferation in order to produce unmethylated daughter strands of DNA. All four trihalomethanes did enhance cell proliferation. The trihalomethanes could then prevent the methylation of the daughter strands of DNA by inhibiting DNA methyltransferase (DNA MTase) or by reducing the availability of S-adenosyl methionine (SAM). DNA MTase activity is increased in most tumors, including liver tumors, in the presence of DNA hypomethylation (Baylin *et al*., 1998; Bird *et al*., 1996; Jones and Buckley, 1990; Wolffe *et al*., 1999). Also dichloroacetic acid and trichloroacetic acids in mouse liver decreased the methylation of the c-*myc* gene without decreas-

ing DNA MTase activity (Tao *et al*., 2000b). Therefore, it is unlikely that decreased activity of DNA MTase is the mechanism for DNA hypomethylation. The level of SAM could be reduced by liver toxicity that reduces the level of ATP required for the synthesis of SAM or by reducing the level of GSH as proposed by Lertratanangkoon *et al*. (1997). Depletion of GSH induces its synthesis, utilizing SAM for the synthesis of cysteine. The prevention of dichloroacetic acid- and trichloroacetic acid-induced hypomethylation of c-*jun* and c-*myc* genes by methionine supports this hypothesis (Tao *et al*., 2000a). Presumably, methionine prevented the decrease in methylation by maintaining the availability of SAM. Hence, it is proposed that the trihalomethanes decreased DNA methylation by enhancing cell proliferation, possibly as a regenerative response to their toxicity, and then preventing the methylation of the newly synthesized daughter strands of DNA.

All four trihalomethanes were toxic to the liver. The toxicity of the trihalomethanes, except for bromodichloromethane, started with ballooning hepatocytes in the mid-zone of the lobule and progressed to centrilobular necrosis. However, in contrast to the other trihalomethanes, the toxicity induced by bromodichloromethane was mainly confined to the central lobular zone and included hydropic degeneration that progressed to centrilobular bridging necrosis, fibrosis and calcification. Similar toxicity for bromodichloromethane has been reported by NTP (1987); the low dose used in their bioassay resulted in degeneration, and the high dose resulted in necrosis, fibrosis, and the presence of microgranulomas at the central vein. The two dose levels of their bioassay were similar to those reported here. Bromodichloromethane has also been reported to differ from the other trihalomethanes by demonstrating mutagenic activity that required activation with GSH (Pegram *et al*., 1997). Thus, the genotoxicity and toxicity of bromodichloromethane distinguishes it from the other trihalomethanes.

Comparison of the toxicity of the trihalomethanes to their ability to enhance cell proliferation indicated a lack of correlation. All four trihalomethanes, when administered by gavage, increased the liver:bw ratio, induced liver toxicity, and enhanced cell proliferation, i.e., the PCNA-LI. Bromodichloromethane and chloroform were more toxic than chlorodibromomethane and bromoform. However, chlorodibromomethane was the most potent in increasing the liver:bw ratio followed by bromodichloromethane $>$ chloroform $>$ bromoform. With respect to the PCNA-LI, chloroform and bromoform were the most efficacious. Thus, the high dose of bromoform increased the PCNA-LI similarly to the high dose of chloroform although, of the four trihalomethanes, it was the weakest in increasing the liver:bw ratio and inducing toxicity. Although both the high dose of bromoform and the low dose of chloroform were minimally toxic (Grade $\sim$1.5), only bromoform increased the PCNA-LI, i.e., 25.3 ± 2.2 and 0.87 ± 0.22 for the high dose of bromoform and the low dose of chloroform, respectively. Thus, the ability of a trihalomethane to increase cell proliferation did not correlate with its toxicity.

The effect of the trihalomethanes on the PCNA-LI also did not correlate with their effect on the liver:bw ratio. The large increase in the PCNA-LI induced by bromoform did not correlate with its limited increase in the liver/bw ratio, which could be due to its very weak toxicity, i.e. inducing only mid-zonal ballooning hepatocytes. In contrast, along with their enhancement of cell proliferation, the more extensive toxicity of chloroform and bromodichloromethane could contribute to their greater increase in the liver:bw ratio. Furthermore, the extensive toxicity of chloroform and bromodichloromethane is consistent with their enhancement of cell proliferation resulting from regenerative hyperplasia, while the minimal toxicity of bromoform and chlorodibromomethane indicate that they increase cell proliferation by another mechanism.

For the most part, the dose-response relationships for the increase in the PCNA-LI and the decrease in the methylation of the c-*myc* gene by the trihalomethanes were similar. The PCNA-LI increased sharply between the low and high doses of chloroform, bromodichloromethane, and bromoform with the dose-response curve for chloroform suggesting a threshold. The dose-response curves for the ability of chloroform and bromodichloromethane to reduce the methylation of the c-*myc* gene also increased sharply with dose. Thus, the dose-response curves for the enhancement of the PCNA-LI and for the reduction in the methylation of the c-*myc* gene suggested that chloroform and bromodichloromethane have to overcome detoxifying mechanisms prior to exerting full activity.

Chloroform administered in drinking water affected the liver:bw ratio, toxicity, cell proliferation, and methylation of the c-*myc* gene much less than when administered by gavage. The daily dose of chloroform administered in drinking water was greater than its high gavage-dose. Furthermore, drinking water exposure was for seven days a week, while gavage treatment was only five days a week. Still, the ability of chloroform in drinking water to increase the liver:bw ratio, induce toxicity, increase the PCNA-LI, and reduce the methylation of the c-*myc* gene was at most only as efficacious as its low gavage dose. This is consistent with the previously reported weaker ability of chloroform administered in the drinking water to induce toxicity and to enhance cell proliferation, compared to chloroform administered by oral gavage (Larson *et al*., 1994; Pereira, 1994; Pereira and Grothaus, 1997). It was also consistent with the inability of chloroform to promote liver tumors in mice when administered in drinking water at daily dose levels similar to its carcinogenic dose when administered by gavage (Jorgenson *et al*., 1985; Klaunig *et al.*, 1986, Pereira *et al*., 1985). The weaker activity of chloroform administered in drinking water could result from its incremental delivery each time the mouse drinks, which should result in a lower liver concentration than obtained from the bolus delivered by oral gavage. The lower liver concentration of chloroform could be insufficient to overcome GSH and other detoxification mechanisms.

Drinking water exposure to the other three trihalomethanes resulted in daily dose levels that were similar to or slightly higher than the low dose administered by oral gavage (the greatest difference was for bromoform, i.e., 1.19 and 0.79 mmol/kg/day by drinking water and gavage, respectively). Drinking water exposure affected liver:bw ratio, liver toxicity, PCNA-LI, and c-*myc* methylation, to a degree similar for the most part to their low gavage dose. However, in contrast to chloroform, the evaluation of the other trihalomethanes in drinking water at concentrations that were equivalent to their high gavage dose was prevented by their limited solubility.

In conclusion, the trihalomethanes administered by gavage increased cell proliferation and decreased DNA methylation, supporting a nongenotoxic mechanism for their carcinogenic activity in mouse liver. The dose-response curves of the trihalomethanes, especially chloroform and bromodichloromethane, suggested the need to overcome detoxification mechanisms prior to exerting full activity, which could explain their weak activity, especially that of chloroform, when administered in drinking water. The slower rate of delivery by drinking water is expected to result in a lower liver concentration that should increase the opportunity for detoxification. Hence, the activity of the trihalomethanes appears to be dependent on their rate of delivery, i.e., rapidly by oral gavage and more slowly in drinking water.

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