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Trihalomethanes as Initiators and Promoters of Carcinogenesis

by Michael A. Pereira,* Luan-Ho C. Lin,* John M. Lippitt* and Sydna L. Herren*

Chloroform and other trihalomethanes are contaminants of drinking water that have been demonstrated to be carcinogenic in laboratory animals. Determination of the mechanism of carcinogenicity of chloroform is required so that the animal data can be extrapolated to estimate the human health hazard. The extent of the binding of chloroform to rat liver and kidney DNA was approximately 0.1% the level of binding found for dimethylnitrosamine. Neither chloroform nor bromoform, in contrast to diethylnitrosamine-initiated GGTase-positive foci in either intact or partial hepatectomized rats, promoted with phenobarbital. Tumor-promoting activity of chloroform was indicated by the slight significant increase, compared to untreated controls, in the incidence of GGTase-positive foci in rats initiated with diethylnitrosamine (DENA) followed by the administration of chloroform twice weekly for a total of 15 doses. In this study, rats administered only the DENA or the chloroform did not contain an increased incidence of GGTase-positive foci compared to untreated controls. However, the incidence of foci in the group that received DENA followed by chloroform was not statistically different from that in either the group that received only the DENA or only the chloroform. In conclusion, we were unable to demonstrate tumor-initiating activity for chloroform, and the tumor-promoting activity of chloroform indicated by our results requires further confirmation.

Introduction

The chlorination of drinking water can represent a major exposure to humans of trihalomethanes including bromoform and chloroform (*1-3*). Chloroform induced hepatocellular carcinomas and kidney epithelial tumors in mice and rats, respectively (*4*). The extrapolation of these results from animals to the estimation of the carcinogenic risk in humans of chloroform in drinking water requires the determination of the mechanism of action for chloroform carcinogenicity.

Chemicals can increase the incidence of cancer by two distinct mechanisms: genetic and epigenetic (*5,6*). The proposed genetic mechanism of chemical carcinogenesis results from the covalent reaction of the carcinogen with DNA. This alteration could produce a somatic mutation resulting in the formation of a clone (focus) of transformed cells. Thus genetic carcinogens initiate the neoplastic progression. The other mechanism of action for carcinogens is epigenetic and involves an alteration in the control of cellular differentiation and replication. Tumor promoters are epigenetic carcinogens that decrease the time required for the appearance and increase the incidence of tumors that were either spontaneously or chemically initiated. Some carcinogens act by both genetic and epigenetic mechanisms. In this paper, we have attempted to determine whether chloroform increased the incidence of cancer in the NCI bioassay by genetic, epigenetic or both mechanisms.

Materials and Methods

Animals and Chemicals

Male (225 to 275 g) Sprague-Dawley rats and female B6C3/F1 mice (8 to 9 weeks old) were purchased from Charles River (Portage, Mich.) and used throughout these studies. The animals were maintained in accordance with the standards set

*Health Effects Research Laboratory, U.S. Environmental Protection Agency, Cincinnati, Ohio 45268.

forth by the National Research Council (*7*). Unless otherwise noted, Purina Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) and distilled water were provided *ad libitum*.

Chloroform (glass-distilled, non preservative) was purchased from Burdick and Jackson Laboratories, Inc. (Muskegon, Mich.), bromoform from Tridom Chemical Co. (Hauppauge, N.Y.), diethylnitrosamine (DENA) from Eastman Kodak Co., (Rochester, N.Y.), sodium barbital from Fisher Scientific Co. (Pittsburgh, Pa.), sodium phenobarbital from J.T. Baker Chemical Co. (Phillipsburg, N. J.) and Tricaprylin from ICN Nutritional Biochemicals (Cleveland, Oh.).

DNA Binding

Groups of mice and rats were administered intragastrically bromoform-^{14}C (2.7 mCi/mmole, ICN Pharmaceutical, Inc., Irvine, Calif.), or chloroform-^{14}C (15 mCi/mmole, ICN Pharmaceutical, Inc., Irvine, Calif.) dissolved in corn oil or dimethylnitrosamine-^{14}C (50.9 mCi/mmole, New England Nuclear, Boston, Mass.) in saline. The animals were sacrificed by cervical dislocation 16-18 hr later for the trihalomethanes and after 2 hr for dimethylnitrosamine (DMN). The liver and kidney were excised and stored at -80°C until the DNA was isolated.

DNA was isolated from the liver by a modification of the procedure described by Kirby (*8*). The liver was homogenized in a lysing medium containing 1% sodium triisopropylnaphthalenesulfonate, 6% isobutanol, 6% sodium *p*-aminosalicylate, 1% sodium chloride and 0.01% sodium deoxycholate and incubated with proteinase K for 1 hr. After extraction with Kirby's phenol solution, the DNA was precipitated with 2-ethoxyethanol. The DNA was then sequentially treated with RNase, α-amylase and pronase followed by a second phenol extraction. After precipitation with 2-ethoxyethanol, the DNA was dissolved in water. For determination of radioactivity, an aliquot of the DNA was hydrolyzed in 0.1*N* HCl at 70°C for 30 min, dissolved in ACS (American Corp., Arlington Heights, Ill.) and counted in a Beckman 9000 liquid scintillation counter (Beckman Instrument Co., Palo Alto, Calif.). DNA concentration was determined by the procedure of Kissane and Robins (*9*).

The DNA was further purified by CsCl isopyknic centrifugation. The DNA was dissolved in 1.3 ml of a 0.25% SDS solution and layered onto a 3.5 ml CsCl solution of an average density of 1.80 g/cm^3 so that the final average density was 1.59 g/cm^3. Centrifugation was performed in a Beckman SW 50.1 rotor (Beckman Instrument Co., Palo Alto) at 35,000 rpm and for 65 hr. Fractions were collected by puncturing the bottom of the centrifuge tube and were monitored for radioactivity, absorbance at 260 nm and density.

In attempts to isolate the adducts of chloroform covalently bound to DNA, the DNA was hydrolyzed in 0.1*N* HCl at 70°C for 30 min. The hydrolyzate was chromatographed on a high performance liquid chromatograph equipped with a semipreparative Partisil 10 SCX ion-exchange column (Whatman Inc., Clifton, N.J.). Elution was accomplished at a flow rate of 4 ml/min with 0.025*M* ammonium phosphate (pH 4.0) for 20 min, followed by a linear increase over 20 min in the concentration so that the final concentration was 0.25*M* ammonium phosphate (pH 4.0).

Initiation Assay

The rat liver foci bioassay (*10-12*) was used to distinguish the initiation and promotion activity of trihalomethanes. The protocols of the initiation and promotion assays are depicted in Figure 1. For the initiation assay, male rats received a 2/3 partial hepatectomy while under ether anesthesia. At 20-22 hr later, the rats were administered by gavage bromoform (0.8 mmole/kg body weight) or chloroform (1.5 mmole/kg body weight) in tricaprylin or diethylnitrosamine (0.5 mmole/kg body weight) in distilled water. The chemicals administered by gavage were given as 2 ml/kg body weight. Three days later, the rats started to receive 500 ppm sodium phenobarbital in their drinking water for a total of 47 days. Six days after the termination of the phenobarbital treatment the animals were sacrificed by decapitation and the right lateral liver lobe quickly excised. Four tissue blocks, each approximately 10 × 10 × 2 mm, were taken and frozen in O.C.T. compound (Fisher Scientific Co., Pittsburgh, Pa.) on dry ice. The slices were arranged so that the leading face of each block was not serially related to the leading face of another block. The tissue blocks were stored at -80°C until being sliced at -25°C into 6 μm sections. The sections were stained by the procedure of Rutenburg et al. (*13*) for γ-glutamyl transpeptidase (GGTase) activity and counterstained with hematoxylin. GGTase-positive foci that contained nine or more nuclei were counted. From each animal at least 2 cm^2 of tissue were scanned for foci. An increase incidence of GGTase-positive foci was indicative of carcinogenic activity.

Promotion Assay

Male rats were administered by gavage an initiating dose of diethylnitrosamine (0.5 mmole/kg body weight) in distilled water (2 ml/kg body weight). Three days later, some of the rats received intragastrically chloroform (1.5 mmole/kg body weight)

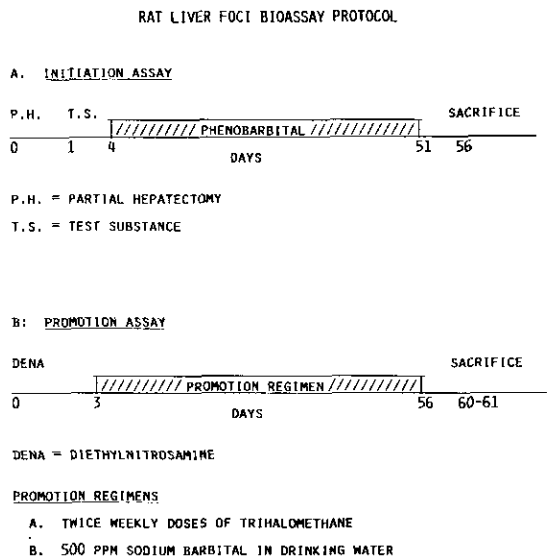

FIGURE 1. Schematic presentation of the initiation and promotion protocols of the rat liver foci bioassay.

in tricaprylin (2 ml/kg body weight) or tricaprylin (2 ml/kg body weight) twice weekly for a total of 53 days. Other rats received 500 ppm sodium barbital in their drinking water for the same length of time (positive control). Four to five days after the termination of the promotion regimens, the rats were sacrificed. Their liver were excised and scored for GGTase-positive foci as described above.

Results

DNA Binding

The DNA isolated by the phenol extraction procedure, even though it contained less than 3% protein, required further purification in order to demonstrate that the radioactivity was bound to DNA and not to the contaminating protein. Cesium chloride isopyknic centrifugation was used to further purify the liver and kidney DNA from the rats treated with either bromoform-^{14}C (1.5 mmole/kg body weight, 2.7 mCi/mmole), chloroform-^{14}C (0.4 mmole/kg body weight, 15 mCi/mmole), or DMN-^{14}C (0.1 mmole/kg body weight, 50.9 mCi/mmole) and from the liver of mice treated with chloroform-^{14}C (1 mmole/kg body weight, 15 mCi/mmole). In rat liver and kidney, a definite peak of radioactivity derived from chloroform was found associated with the ultraviolet-absorbing peak containing the DNA, whereas no association was found for chloroform in mouse liver and bromoform in rat liver (Fig. 2). The binding index of chloroform to rat liver and kidney DNA was 0.017 and 0.0055, respectively, which represents 0.05–0.15% the binding index for DMN (11.4, Table 1).

The radioactivity present in kidney DNA from rats treated with chloroform-^{14}C was demonstrated to represent the formation of adducts in contrast to incorporation during *de novo* DNA synthesis (Fig. 3). The kidney DNA was hydrolyzed in 0.1*N* HCl and the hydrolyzate chromatographed on a HPLC equipped with a semipreparative Partisil 10 SCX ion exchange column. Over 95% of the applied radioactivity was eluted in the early peak containing the pyrimidines and deoxyribosyl phosphate backbone of the DNA. There was no evidence for the incorporation of radioactivity into adenine and guanine. Since over 60% of the radioactivity was previously demonstrated to be bound to the DNA (Fig. 2*C*), it was unlikely that the radioactivity in the early peak represented contaminating protein or incorporation by *de novo* synthesis of the DNA constituents with exclusion of the purines. Therefore, chloroform appeared to bind DNA at the pyrimidines, or phosphate and/or to form dinucleotide linkage.

Initiation Assay

Bromoform (0.8 mmole/kg body weight) and chloroform (1.5 mmole/kg body weight) were tested in the initiation protocol of the rat liver foci bioassay (Fig. 1). The trihalomethanes did not increase the incidence of GGTase-positive foci when administered to either intact or partial hepatectomized rats (Table 2). DENA (0.5 mmole/kg body weight), the positive control, resulted in the expected positive response. The initiating activity, if any, of trihalomethanes was much less than DENA.

Promotion Assay

The promoting activity of chloroform was determined in the promotion protocol of the rat liver foci bioassay (Fig. 1). Chloroform (1.5 mmole/kg body weight) administered twice weekly for 53 days to non-initiated rats or DENA administered to non-promoted rats did not result in a statistically significant increased incidence of GGTase-positive foci (Table 3). A slight but significant increase in the incidence of GGTase-positive foci compared to untreated controls was observed in rats that received the initiating dose of DENA followed by the chloroform promotion regimen. The incidence of GGTase-positive foci in animals initiated with DENA and promoted with chloroform was not significantly different from the incidence in animals treated either only with DENA or with chloroform.

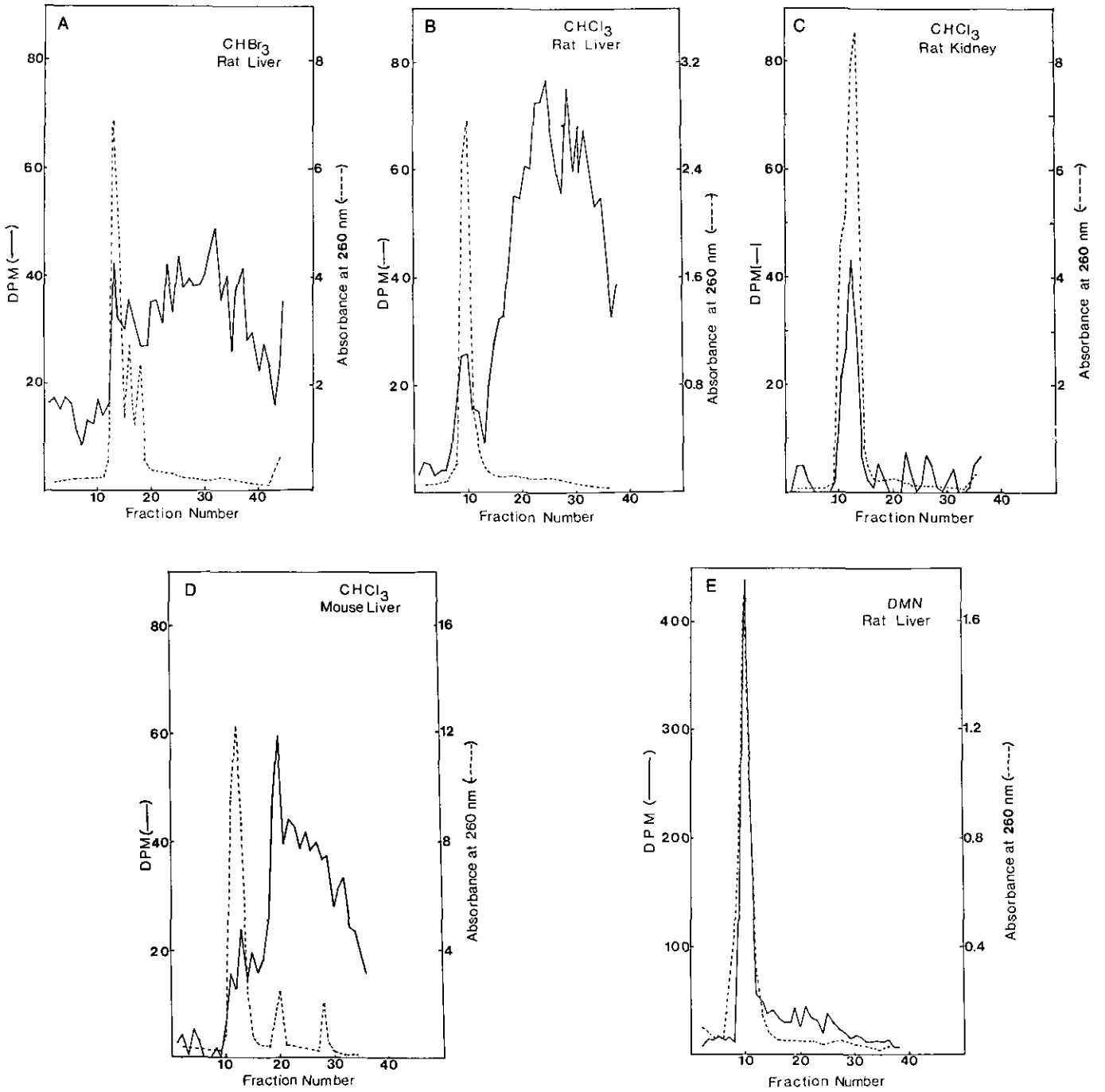

FIGURE 2. Cesium chloride isopyknic centrifugation of DNA. The DNA was dissolved in 0.25% SDS and layered on a CsCl solution (1.80 g/cm^3) so that the final density was 1.59 g/cm^3. Centrifugation was performed in a Beckman SW 50.1 rotor at 35,0000 rpm for 65 hr. Fractions were collected by puncturing the bottom of the centrifuge tube and were monitored for (———) radioactivity (dpm) (—) absorbance at 260 nm and density: (*A*) liver DNA from bromoform treated rats, amount applied 1.3 mg, and 1990 dpm; (*B*) liver DNA from chloroform-treated rats, amount applied 0.46 mg and 1475 dpm; (*C*) kidney DNA from chloroform-treated rats, amount applied 2.9 mg and 438 dpm; (*D*) liver DNA from chloroform-treated mice, amount applied 2.8 mg and 875 dpm; (*E*) liver DNA from dimethylnitrosamine-treated rats, amount applied 0.32 mg and 1890 dpm.

Table 1. Binding of trihalomethanes to DNA.

Chemical	Species	Organ	Binding index CsCl[a]
Chloroform	Rat	Liver	0.017
	Rat	Kidney	0.0055
	Mouse	Liver	< 0.0012
Bromoform	Rat	Liver	< 0.015
DMN[b]	Rat	Liver	11.4

[a]Binding index = pmole bound per mg DNA/μmole kg body weight.
[b]DMN = dimethylnitrosamine.

Discussion

Chloroform is an environmental carcinogen found in drinking water (*3,4*). Estimation of the human health hazard as a consequence of this exposure to chloroform requires extrapolation of the animal carcinogenicity data to humans. Models for the extrapolation of animal data to humans are being developed. Two possible mechanisms of action for the carcinogenicity of chloroform are genetic (initiation) and/or epigenetic (promotion). The determination of the contribution of these two mechanisms of action to the carcinogenicity of chloroform is required prior to the adaptation of the appropriate extrapolation model.

The possible initiating activity of chloroform was investigated by determination of the binding to DNA and the ability to initiate an increased incidence of GGTase-positive foci in the rat liver foci bioassay (*10-12*). Chloroform was demonstrated to bind rat liver and kidney DNA but there was no evidence for binding to mouse liver DNA within the sensitivity of the assay. The total binding of chloroform or bromoform to rate liver or kidney DNA was less than 0.15% the binding of DMN to rat liver DNA. The low level of DNA binding by bromoform and chloroform indicated that the contribution of the genetic or initiating component to the carcinogenicity of the trihalomethanes was much less than the genetic component of DMN. In the rat liver foci bioassay for initiating activity, bromoform and chloroform when administrated to either partial hepa-

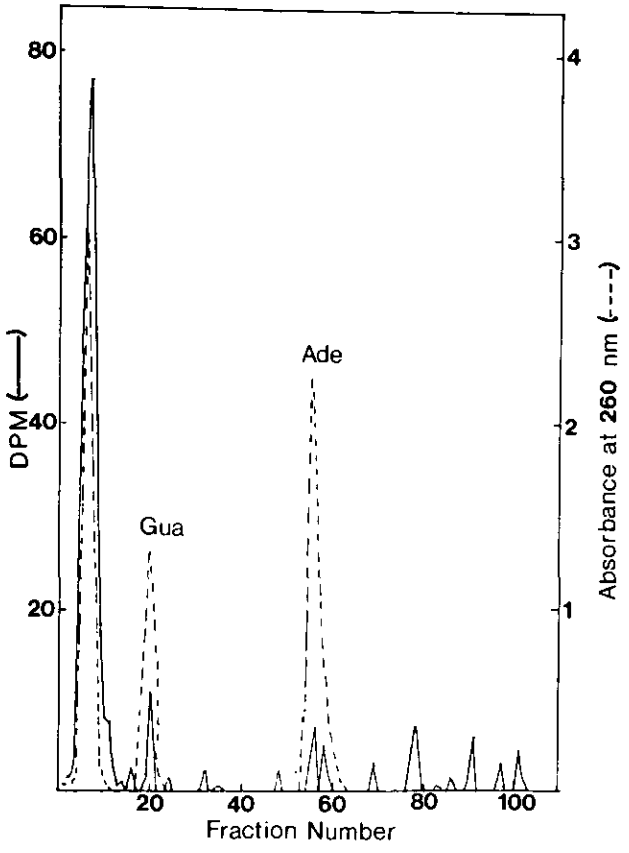

FIGURE 3. HPLC elution profile of acid-hydrolyzed DNA. Kidney DNA from chloroform treated rats was hydrolyzed in 0.1*N* HCl at 70°C for 30 min. The hydrolyzate was applied to a high performance liquid chromatograph equipped with a semipreparative Partisil 10 SCX ion exchange column. The column was eluted at a flow rate of 4 ml/min with 0.025*M* ammonium phosphate (pH 4.0) for 20 min, followed by a linear increase over 20 min in the concentration to 0.25*M* ammonium phosphate (pH 4.0). The eluate was monitored for absorbance at 260 nm and 2 ml fractions collected for determination of radioactivity.

Table 2. Initiation assay of trihalomethanes.

Group	*N*	Treatment: Initiation	Treatment: Partial hepatectomy	Treatment: Promotion[a]	GGTase foci, foci/cm^2
A	12	Chloroform	+	+	1.08 ± 0.28[b]
B	12	Chloroform	–	+	0.40 ± 0.21
C	12	Bromoform	+	+	0.48 ± 0.12
D	12	Bromoform	–	+	0.26 ± 0.18
E	12	Tricaprylin	+	+	1.07 ± 0.24
F	12	Tricaprylin	–	+	0.13 ± 0.07
G	12	DENA	+	+	15.8 ± 1.9

[a]Promotion was accomplished with 500 ppm sodium phenobarbital in drinking water for 47 days.
[b]Results are means ± standard errors.

Table 3. Promotion assay of chloroform.

Group	N	Treatment		Weight, g[a]		Organ body weight × 100[a]	GGTase foci, foci/cm^2[a]
		Initiation	Promotion	Body	Liver		
A	15	Water	Tricaprylin	498 ± 12[b]	15.1 ± 0.5	3.0 ± 0.06	0.45 ± 0.14
B	16	DENA	Tricaprylin	497 ± 10	15.4 ± 0.5	3.0 ± 0.13	0.90 ± 0.31
C	15	Water	Chloroform	466 ± 8.6	15.0 ± 0.5	3.2 ± 0.05	1.42 ± 0.44
D	16	DENA	Chloroform	466 ± 9.4	14.6 ± 0.5	3.1 ± 0.08	2.22 ± 0.63[c]
E	10	DENA	Barbital	—	—	—	3.02 ± 1.10[c]

[a]Results are means ± standard errors.
[b]The initial body weights of the groups were 260-268.
[c]Different from group A by a nonparametric test with $p < 0.05$.

tectomized or intact rats and followed by promotion with phenobarbital did not initiate GGTase-positive foci. Initiation of GGTase-positive foci was demonstrated for DENA (the positive control). The low level of DNA binding and the failure to demonstrate initiating activity in the rat liver foci bioassay would indicate that any initiating activity of chloroform is substantially lower than nitrosamines.

Chloroform is toxic to the liver and kidney (*14, 15*) which are the target organs of carcinogenesis in rodents. The hepatic response to chloroform in rats included regenerative hyperplasia (*16*) and induction of ornithine carboxylase (*17*). Regenerative hyperplasia and induction of ornithine decarboxylase are properties possessed by hepatic tumor promoters (*18, 19*). We attempted to obtain evidence in the rat liver foci bioassay for the tumor promoting activity of chloroform. In rats initiated with DENA, subsequent treatment with chloroform increased the incidence of GGTase-positive foci when compared to untreated rats. However, when compared to rats that received only the DENA or chloroform the increased incidence of foci was not significant. Therefore, further confirmatory studies are required to demonstrate the tumor promoting activity of chloroform. In summary, chloroform would appear to possess (1) very little if any tumor-initiating activity and (2) as yet unproven tumor-promoting activity.

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REFERENCES

1. Bellar, T. A., Lichtenberg, J. J., and Kroner, R. C. The occurrence of organohalides in chlorinated drinking water. J. Am. Water Works Assoc. 66: 703-706 (1974).
2. Rook, J. J. Formation of haloforms during chlorination of natural water. Water Treatment Exam. 23: 234-243 (1974).
3. Symons, J. M., Bellar, T. A., Carswell, J. K., DeMarco, J., Kropp, K. L., Robeck, G. G., Seeger, D. R., Slocum, C. J., Smith, B. L., and Stevens, A. A. National organics reconnaissance survey for halogenated organics. J. Am. Water Works Assoc. 67: 634-647 (1975); J. Am. Water Works Assoc. 67: 708-709 (1975).
4. National Cancer Institute. Carcinogenesis bioassay of chloroform. Natl. Tech. Inform. Service No. PB 264018/AS, Washington, D. C. (1976).
5. Boutwell, R. K. Function and mechanism of promoters of carcinogenesis. CRC Crit. Rev. Toxicol. 2: 419-443 (1974).
6. Boutwell, R. K. Biochemical mechanism of tumor promotion. In: Carcinogenesis, Vol. 2. Mechanisms of Tumor Promotion and Cocarcinogenesis, T. J. Slaga, A. Sivak and R. K. Boutwell, Eds., Raven Press, New York, 1978, pp. 49-58.
7. National Research Council. Guide for the Care and Use of Laboratory Animals. Institute of Laboratory Animal Resources, NIH Publ. No. 80-23, Washington D. C., 1980.
8. Kirby, K. S. Isolation of nucleic acids with phenolic solvents. In: Methods in Enzymology. Vol. 12, Part B, L. Grossman and K. Moldare (Eds.), Academic Press, New York, 1967, pp. 87-99.
9. Kissane, J. M., and Robins, E. The fluorometric measurement of deoxyribonucleic acid in animal tissues with special reference to the central nervous system. J. Biol. Chem. 233: 184-188 (1958).
10. Ford, J. O. and Pereira, M. A. Short-term *in vivo initiation/promotion bioassay for hepatocarcinogens*. J. Environ. Pathol. Toxicol. 4: 39-46 (1980).
11. Pereira, M. A. Rat liver foci bioassay. J. Am. Coll. Toxicol. 1: 101-118 (1982).
12. Pitot, H. C., and Sirica, A. E. The stages of initiation and promotion in hepatocarcinogenesis. Biochim. Biophys. Acta 605: 191-215 (1980).
13. Rutenburg, A. M., Kim, H., Fishbein, J. W., Hanker, S. S., Wassenburg, H. L. and Seligman, A. M. Histochemical and ultrastructural demonstration of gamma-glutamyl transpeptidase activity. J. Histochem. Cytochem. 17: 517-526 (1969).
14. Pohl, L. R. *Biochemical toxicology of chloroform*. Revs. Biochem. Toxicol. 1: 79-107 (1979).
15. Klassen, C. D., and Plaa, G. L. Relative effects of various chlorinated hydrocarbons on liver and kidney function in mice. Toxicol. Appl. Pharmacol. 9: 139-153 (1966).
16. Reitz, R. H., Quast, J. F., Scott, W. T., Watanabe, P. G. and Gehring, P. J. Pharmacokinetics and macromolecular effects of chloroform in rats and mice: Implication for carcinogenic risk estimation. In: Water Chlorination: Environmental Impact and Health Effects, Vol. 3, R. L. Jolley, W. A. Brungs, R. B. Cumming, and V. A. Jacobs (Eds.), Ann Arbor Science Publishers, Ann Arbor, Mich., 1980, pp. 983-994.
17. Savage, R. E., Westrich, C., Guion, C., and Pereira, M. A. Chloroform induction of ornithine decarboxylase in rat liver. Environ. Health Perspect. 46: 157-162 (1982).
18. Pereira, M. A., Savage, R. E., Jr., Herren, S. L., and Guion, C. W. Comparison of tumor promotion and induction of ornithine decarboxylase in rat liver by barbiturates. Carcinogenesis 3: 147-150 (1982).
19. Schulte-Hermann, R., Hoffman, V., Parzefall, W., Kallenbach, M., Gerhardt, A., and Schuppler, J. Adaptive response of rat liver to the gestagen and anti-androgen cyproterone acetate and other inducers. II. Induction of growth. Chem.-Biol. Interactions 31: 287-300 (1980).