Chloroform Induction of Ornithine Decarboxylase Activity in Rats

by Russell E. Savage, Jr.,* Carol Westrich,* Charles Guion* and Michael A. Pereira*

Chloroform is a drinking water contaminant that has been demonstrated to be carcinogenic to mice and rats resulting in an increased incidence of liver and kidney tumors, respectively. The mechanism of chloroform carcinogenicity might be by tumor initiation and/or promotion. Since induction of ornithine decarboxylase (ODC) activity has been proposed as a molecular marker for tumor promoters, we have investigated the effect of chloroform on ODC activity in rats. Chloroform induced a dose-dependent increase of hepatic ODC with an apparent threshold at 100 mg/kg body weight. Female rats were two to four times more susceptible to chloroform. Upon daily dosing of chloroform for 7 days the liver became less susceptible, with the last dose of chloroform resulting in only 10% of the activity observed after a single dose. Nuclear RNA polymerase I activity was also induced by chloroform. Chloroform, rather than increasing the activity of renal ODC, resulted in a 35% reduction. The induction by chloroform of hepatic ODC activity might be associated with regenerative hyperplasia while the renal carcinogenicity of chloroform could not be demonstrated to be associated with ODC induction.

Introduction

Chloroform has been demonstrated in mice and rats to increase the incidence of hepatocellular carcinoma and epithelial tumors of the kidney, respectively (1). The initiation of carcinogenesis is believed to involve a somatic mutation; however, chloroform was nonmutagenic in the Ames Salmonella/microsome assay (2), in E. coli K 12 for basepair substitution (3), and in the 8-azaguanine locus in Chinese hamster lung fibroblast in cell culture (4). It also appeared that the amount of covalent binding of chloroform to rat liver and kidney DNA was very minimal (5). The negative evidence for chloroform mutagenicity and the low level of DNA interaction has resulted in the proposal that chloroform is not an initiator of carcinogenesis but is rather a tumor promoter.

Chloroform has long been known to be toxic in the liver and kidney, which are the organs in which an increased incidence of tumor formation has been found (1,6,7). The hepatotoxicity include fatty degeneration, glycogen depletion, vacuolation, swelling and necrosis. The severe tissue damage that resulted in regenerative growth of the liver (8) could stimulate the progression of preneoplastic cells to tumors. This proposed epigenetic stimulation of the neoplastic progression is called promotion.

Ornithine decarboxylase (ODC) induction has been associated with tumor promotion in skin (9) and liver (10), so that it might be a molecular marker for tumor promotion. ODC is the first and rate-limiting enzyme in polyamine biosynthesis (11) and has a very rapid turnover time of 10-20 min in liver (12,13). Drugs, hormones, and partial hepatectomy which increased cellular proliferation have been shown to increase ODC activity (14). In this communication, we report the effect of chloroform on ODC activity in rat liver and kidney.

Materials and Methods

Materials

Fischer 344 albino rats, weighing 175-200 g, were purchased from Charles River Breeding Laboratories (Wilmington, Mass.). Adrenalectomized rats were also purchased from Charles River. Purina
Laboratory Chow (Ralston Purina Co., St. Louis, Mo.) and distilled water were provided ad libitum.

DL-Ornithine $^{14}$C and uridine triphosphate-$^3$H were purchased from New England Nuclear (Boston, Mass.). Chloroform (glass distilled and without preservative), carbon tetrachloride and benzene were obtained from Burdick and Jackson Laboratories (Muskegon, Mass.), and Aroclor-1254 was purchased from Analab, Inc. (North Haven, Conn.).

Subcellular Fractionation

Animals were sacrificed by decapitation. The livers or kidneys were removed, rinsed, blotted, weighed and homogenized in 1.0 volume (1 ml/g tissue) of ODC homogenizing buffer (0.1M Na$_2$HPO$_4$, 0.8mM pyridoxal-5-phosphate, 2.0mM EDTA; pH 7.5) at 0°C with a glass homogenizer fitted with a Teflon pestle. The homogenate was centrifuged at 10,000g for 10 min at 4°C. The supernatant fraction was decanted off and further centrifuged at 144,000g for 60 min at 4°C. The 144,000g supernatant fraction was removed with a Pasteur pipet introduced below the surface to avoid the turbid lipid layer on top. This cytosol fraction was used as the enzyme source for the ornithine decarboxylase assay.

Liver nuclei used in the RNA polymerase I assay were prepared by the isolation procedure developed by Muramatsu et al. (15) and described by Yu and Feigelson (16).

Enzyme Assays

All animals for the ornithine decarboxylase assay were sacrificed between 9 and 10 AM. Cytoplasmic ODC activity was measured by determination of the release of $^{14}$CO$_2$ from ornithine (14-C) essentially as described by Bethell and Pegg (17). The cytoplasmic fraction (100 μl) was incubated with 900 μl of ODC incubation medium containing 56μM NaH$_2$PO$_4$, 3.8mM dithiothreitol, 2.3mM EDTA (pH 6.5) and 1.0 μCi of DL-ornithine (1-14C) (51.3 mCi/mmmole). After incubation for 30 min at 37°C the reaction was stopped by the addition of 0.3 ml of 5M H$_2$SO$_4$, and the $^{14}$CO$_2$ released was trapped on filter paper discs saturated with 0.25 ml of hyamine hydroxide. The production of $^{14}$CO$_2$ was linear with respect to the amount of enzyme protein added to each incubation. The discs were removed, placed in liquid scintillation vials containing 15.0 ml ACS (Amersham) and 0.41 ml of 0.5N HCl, and the radioactivity determined in a Beckman LS 8100 liquid scintillation counter (Beckman Instrument Inc., Palo Alto, Calif.). The background activity, determined with acid denatured cytosol, was subtracted in each case. Protein was measured by use of the Bio-Rad protein assay kit (Bio Rad, Rich-

mond, Calif.). Enzyme activity was expressed as CO$_2$ evolved/mg protein/30 min incubation.

RNA polymerase I activity was determined by using purified intact nuclei as the enzyme source as described by Roeder and Rutter (18). Isolated nuclei were incubated in a final volume of 1.0 ml containing 10 mg pyruvate kinase (400 units/mg protein), 23 μmole Tris-HCl (pH 7.9), 1.0 μmole MnCl$_2$, 4.0 μmole KCl, 3.0 μmole NaF, 2.0 μmole phosphoenol pyruvate, 0.8 μmole 2-mercaptoethanol. 0.3 μmole each GTP, CTP, ATP, 0.06 μmole unlabeled UTP, 0.002 μmole $^3$H-UTP (20.3 Ci/μmole), and 50 μmole ammonium sulfate and with and without 1mM α-amanitin (19). Following incubation for 10 min at 30°C, the reaction was terminated by the addition of 4.0 ml of 10% TCA. The samples were centrifuged at 3,000 rpm for 10 min in a Beckman TJ-6 table top centrifuge (Beckman Instrument Inc., Palo Alto). The supernatant was discarded and the pellet washed two times with 3.0 ml of 5% TCA. The pellet was resuspended in 3.0 ml of 5% TCA and collected under vacuum onto HA 0.45mM Millipore filters. The filters were washed with 10 ml of 5% TCA, followed by 5 ml of 60% ethanol and allowed to air-dry overnight. The next day they were oxidized in a Packard Model 306 sample oxidizer (Packard Instrument Co., Downers Grove, Ill.). Radioactivity was determined by liquid scintillation counting and protein measured as described above. The data were expressed as pica moles of $^3$H-UTP incorporated/10 min/mg protein.

Results

The effect of various carcinogens and tumor promoters on ornithine decarboxylase (ODC) activity in male rat liver was determined (Table 1). With the exception of benzene and saccharin, all of the compounds tested stimulated ODC. Chlorinated carcinogens and tumor promoters such as chloroform, carbon tetrachloride and the polychlorinated biphenyl, Aroclor-1254, caused the greatest increase in ODC activity.

Chloroform Time Course and Dose Response

The time course of chloroform induction of ODC in male rat liver was determined (Fig. 1). Enhancement was evident as early as 2 hr and slowly increased until 18 hr, at which time maximum enhancement of ODC activity occurred. The maximum enhancement of ODC activity was 43.5-fold control level and 74 pmole CO$_2$/30 min/mg protein. By 24 hr, the peak enhancement of ODC activity had decreased by 80%.
Table 1. Effect of suspected tumor promoters on rat hepatic ornithine decarboxylase activity.

| Compound          | Dose, mg/kg | ODC activity$^b$ | Enhancement$^c$ |
|-------------------|-------------|-------------------|-----------------|
| Aroclor-1254      | 800         | 38.1 ± 4.93$^d$  | 22.4            |
| Benzene           | 440         | 2.6 ± 0.69       | 1.5             |
| Carbon tetrachloride | 800     | 25.0 ± 4.30$^d$  | 14.7            |
| Chloroform        | 750         | 74.0 ± 11.6$^d$  | 43.5            |
| Methapyrile       | 10          | 9.3 ± 2.96$^d$   | 5.5             |
| Phenobarbital     | 100         | 9.6 ± 1.00$^d$   | 5.7             |
| Saccharin         | 1000        | 2.3 ± 0.31       | 1.4             |
| Testosterone      | 300         | 13.2 ± 4.00$^d$  | 7.8             |

$^a$Animals were injected intraperitoneally with the compound and sacrificed 18 hr later at 9:00 AM.
$^b$ODC activity was determined by measuring the release of $^{14}\text{CO}_2$ from DL-ornithine (1-$^{14}$C) and was expressed as pmole CO$_2$/30 min incubation/mg protein. Results are means from eight animals ± standard error.
$^c$Enhancement of ODC activity compared to the mean of eight saline-treated rats which was 1.7 ± 0.44.
$^d$Statistically greater than the activity in saline-treated rats by the Student t-test at the level of $p < 0.01$.

The administration of a daily dose of chloroform for 7 days resulted in a lower enhancement of ODC activity compared to a single dose (Fig. 2). After seven total doses of chloroform, the ODC activity was only 10% the activity observed after the first dose. The decreased responsiveness of the liver to chloroform was even apparent for a second dose of chloroform administered on the following day.

The dose-response relationship of ODC induction by chloroform in female and male rats was performed (Fig. 3). Female rats were two to four times as sensitive to chloroform as male rats. There was an apparent threshold in male rats for ODC induction below 100 mg/kg body weight. The ODC activity increased with dose of chloroform until 750 mg/kg body weight in both female and male rats. Higher doses of chloroform could not be tested in females due to a high incidence of lethality.

Renal ODC Activity

The ODC activity of male rat kidney was approximately 300-fold the activity in male rat liver (Table 2). The function of this high level of ODC activity in male kidney is unknown; however, after unilateral nephrectomy, a fourfold increase of ODC activity occurred 24 hr later in the regenerating kidney.

![Figure 1. Time course of chloroform induction of ODC in male rats. Male rats were injected intraperitoneally with 750 mg/kg and sacrificed after various intervals of time at 9:00 AM. All points represent the mean of a minimum of eight animals ± S.E.](image1)

![Figure 2. Effects of chronic chloroform treatment on rat hepatic ornithine decarboxylase activity. Male rats were injected intraperitoneally with 500 mg/kg CHCl$_3$ daily at 3:00 PM for a period of up to 7 days. Rats were sacrificed 18 hr after their last dose. All points represent the mean of a minimum of eight animals ± SE.](image2)
Compound CHCl₃ 750 18 min/mgprotein.

Saline CHCl₃ and min.

FIGURE 3. Dose-response relationship of chloroform induction of hepatic ODC. Rats were injected intraperitoneally with the appropriate dose of CHCl₃ and sacrificed 18 hr later at 9:00 AM. The ODC activity in saline-treated control female and male rats was 1.7 ± 0.44 and 1.55 ± 0.38 enzyme units, respectively. All points represent the mean of a minimum of eight animals ± SE.

Table 2. Effect of CHCl₃ on rat renal ODC activity.

| Compound       | Dose, mg/kg ¹ | Time, hr | ODC activity ² |
|----------------|--------------|----------|----------------|
| Saline         | —            | 18       | 482.23 ± 24.23 |
| CHCl₃ 750      | 18           |          | 317.65 ± 33.55 |

¹Animals were injected intraperitoneally with either saline or CHCl₃ and the animals sacrificed 18 hr later at 9:00 AM.

²ODC activity was expressed as pmole CO₂ liberated/30 min/mg protein. Results are means of eight animals ± standard errors.

(20). Chloroform (750 mg/kg body weight), which resulted in a 43.5-fold enhancement of rat liver ODC activity (Table 1), caused a 35% reduction of kidney ODC activity (Table 2).

Hormonal Modulation of Chloroform-Induced ODC

Adrenalectomy prevented the increase in rat liver ODC activity that resulted from treatment with barbiturates (Table 3). The extent of chloroform induction of ODC activity was not statistically diminished by prior adrenalectomy. Therefore, unlike barbiturates, the enhancement of rat liver ODC by chloroform was not dependent on the adrenal gland. Pretreatment with propranolol (a beta adrenergic receptor blocker) or 17-α-hydroxyprogesterone (a corticosterone receptor blocker) also did not diminish the increased level of ODC induced by chloroform.

RNA Polymerase I Response to Chloroform

RNA polymerase I is another enzyme that has been demonstrated to increase during the hyperplastic response in rat liver (14). Chloroform induction of RNA polymerase I activity was determined

Table 3. Effects of hormonal manipulations on chloroform-induced hepatic ornithine decarboxylase.

| Compound                              | Dose, mg/kg ³ | Surgery ⁴ | ODC activity |
|---------------------------------------|--------------|------------|--------------|
| Saline                                | —            | —          | 1.7 ± 0.44⁵  |
| Saline                                | —            | Adrenalectomy | 3.2 ± 0.63⁵ |
| CHCl₃ 187.5                           | 18           | —          | 9.5 ± 5.00⁵  |
| CHCl₃ 187.5                           | 18           | Adrenalectomy | 13.5 ± 5.58⁵ |
| CHCl₃ 375.0                           | 375.0        | —          | 17.7 ± 4.71⁵ |
| CHCl₃ 375.0                           | 375.0        | Adrenalectomy | 12.6 ± 3.16⁵ |
| CHCl₃ 750                             | 750          | —          | 74.0 ± 11.6⁵ |
| CHCl₃ 750                             | 750.0        | Adrenalectomy | 47.5 ± 19.5⁵ |
| 17-Hydroxyprogesterone (HP) 20        | 20           | —          | 1.3 ± 0.36⁵  |
| 17-HP + CHCl₃ ²⁶                       | —            | —          | 110.1 ± 13.7⁵ |
| Propranolol 10                         | —            | —          | 1.7 ± 0.38⁵  |
| Propranolol + CHCl₃ ²⁶                 | —            | —          | 76.9 ± 15.6⁵ |
| Barbital 200                           | —            | —          | 13.1 ± 3.00⁵ |
| Barbital 200                           | —            | Adrenalectomy | 1.1 ± 0.52⁵  |
| Phenobarbital 100                      | —            | —          | 9.6 ± 1.00⁵  |
| Phenobarbital 100                      | —            | Adrenalectomy | 2.6 ± 0.89⁵  |

¹Animals were injected intraperitoneally and sacrificed 18 hr later. The 17α-hydroxyprogesterone and propranolol were injected 30 min prior to the chloroform and sacrificed 18 hr later.

²Adrenalectomy was performed by Charles River laboratory. The rats were maintained on saline for their drinking water and used 14 days after surgery.

³ODC activity was expressed as pmole CO₂/30 min incubation/mg protein.

⁴Results are means of eight animals ± standard errors.

⁵The dose of 17α-hydroxyprogesterone was 20 mg/kg body weight and of chloroform 750 mg/kg body weight.

⁶The dose of propranolol was 10 mg/kg body weight and of chloroform 750 mg/kg body weight.

⁷Statistically decreased from intact animal by the Student t-test at the level of significance of p < 0.01.
Discussion

The induction of hepatic ODC has been demonstrated to be associated with the hyperplastic and regenerative response to drugs, hormones and partial hepatectomy (21). Chloroform has been demonstrated to induce a regenerative hyperplasia in rat liver (8). Chloroform caused a dose-dependent enhancement of hepatic ODC that could be associated with the regenerative hyperplasia. An apparent threshold for chloroform induction of ODC activity was indicated below 100 mg/kg body weight which might indicate a threshold for regenerative hyperplasia. Female rats compared to males exhibited a 2- to 4-fold greater response of ODC induction. Chloroform also induced hepatic nuclear RNA polymerase I, another enzyme that has been associated with the hyperplastic response in liver (14). The induction of ODC and hyperplasia has been proposed as molecular markers for tumor promoters (9).

Tumor promotion appears to require the prolonged and repeated administration of the promoter (9). Upon daily administration of chloroform, the extent of the enhancement decreased so that after a total of seven doses, the ODC activity was only 10% the level after a single dose. This reduction in the effect of chloroform could have resulted from the inhibition of accumulated putrescine (22). The hyperplastic response of α-hexachlorobenzene in the liver as measured by the increase in DNA replication, was resistant to a second dose (23). The hyperplastic response of chloroform as indicated by the reduced enhancement of ODC might also be limited.

The kidney was the target organ of chloroform carcinogenicity in rats. Kidney ODC activity was not induced by chloroform but was rather reduced. The level of ODC activity in kidneys was approximately 300-fold the hepatic level. Even in presence of this high control level, unilateral nephrectomy was capable of inducing ODC in the regenerating kidney (24). Therefore, the carcinogenicity and possible tumor promoting activity of chloroform in rat kidney would appear not to be associated with the induction of ODC. In summary, the hepatic regenerative hyperplasia induced by chloroform in rats appeared to be associated with the induction of ODC while the renal carcinogenicity was not associated.

REFERENCES

1. Page, N. P., and Saffiotti, U. Report on Carcinogenesis Bioassay of Chloroform. U. S. NTIS, PB Rept.; ISS PB 264018/AS (1976).

2. Simmons, V. F., Kauhaven, K., and Tardiff, R. G. Mutagenic activity of chemicals found in drinking water. Dev. Toxicol. Environ. Sci. 2: 249-258 (1977).

3. Uehleke, H., Greim, H., Kraemer, M., and Werner, T. Covalent binding of halocalkaner to liver constituents, but absence of mutagenicity on bacteria in a metabolizing test system. Mutat. Res. 38: 114-132 (1976).

4. Sturrock, J. Mitosis in mammalian cells during exposure to anesthetics. Brit. J. Anaesth. 49: 207-210 (1977).

5. Pereira, M. A., Lin, H. C., Lippitt, J. M., and Herren, S. L. Trihalomethanes as initiators and promoters of carcinogene-

6. Klassen, C. D., and Plaa, G. Relative effects of various chlorinated hydrocarbons on liver and kidney function in mice. Toxicol. Appl. Pharmacol. 9: 139-153 (1966).

7. Pohl, L. R. Biochemical toxicology of chloroform. Revs. Biochem. Toxicol. 1: 79-107 (1979).

8. 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: Environ-

9. Boutwell, R. K. Biochemical mechanisms of tumor promotor. In: Carcinogenesis: Mechanisms of Tumor Promotion and Cocarcinogenesis, Vol. 2, T. J. Slaga, A. Sivak, and R. K. Boutwell, Eds., Raven Press, New York, 1978, pp. 49-58.

10. Olson, J. W. and Russell, D. H. Prolonged ornithine decarboxylase induction in regenerating carcinogen-treated liver. Cancer Res. 40: 4373-4380 (1980).

11. Raina, A., Papula, R.-L., and Eloranta, T. A rapid assay method for spermidine and spermine synthetases, distribution of polyamine-synthesizing enzymes and methionine adenosyl transferase in rat tissues. FEBS Letters 67: 252-255 (1976).

12. Russell, D. H., Snyder, S. H., and Medina, V. J. Growth hormone induction of ornithine decarboxylase in rat liver. Endocrinology 86: 1414-1419 (1970).

13. Russell, D. H. and Snyder, S. H. Amine synthesis in regenerating rat liver: Extremely rapid turnover of ornithine decarboxylase. Molecular Pharm. 5: 253-262 (1969).

Table 4. Effect of CHCl₃ on rat hepatic RNA polymerase I.

| Compound | Dose, mg/kg * | Time, hr * | RNA polymerase I activity ‡ |
|----------|---------------|------------|----------------------------|
| Saline   | —             | 18.5       | 138.03 ± 16.02‡            |
| CHCl₃    | 750           | 18.5       | 319.40 ± 36.14‡            |

*Animals were injected intraperitoneally with either saline (1 mg/kg body weight) or CHCl₃.

†The animals were sacrificed 18.5 hr after treatment at 9:00 a.m.

‡RNA polymerase I activity was calculated in the presence of 1mM α-amanitin and was expressed as pmole UTP incorporated/10 min/mg protein.

‡Results are means of eight animals ± standard errors.
14. Russell, D. H., Byus, C. V. and Manen, C.-A. Proposed model of major sequential biochemical events of a trophic response. Life Sci. 19: 1297-1306 (1975).
15. Muramatsu, M., Hodnett, J. L., Steele, W. J., and Busch, H. Synthesis of 28-S ribonucleic acid in nucleolus. Biochim. Biophys. Acta 123: 116-125 (1966).
16. Yu, F.-L. and Feigelson, P. Cortisone stimulation of nucleolar RNA polymerase activity. Proc. Natl. Acad. Sci. (U.S.) 68: 2177-2180 (1971).
17. Bethell, D. R., and Pegg, A. E. Effects of diamines on ornithine decarboxylase activity in control and virally transformed mouse fibroblasts. Biochem. J. 180: 87-94 (1979).
18. Roeder, R. G., and Rutter, W. J. Multiple forms of DNA-dependent RNA polymerase in eukaryotic organisms. Nature 224: 234-237 (1969).
19. Duceman, B. W., and Jacob, S. T. Transcriptionally active RNA polymerases form Morris hepatomas and rat liver. Biochem. J. 190: 781-789 (1980).
20. Sochor, M., and McClean, P. Changes in ornithine decarboxylase activity in experimental diabetes. Correlation with severity of diabetes and effects of unilateral nephrectomy. Enzyme 25: 289-296 (1980).
21. Janne, J., Pusó, H., and Raina, A. Polyamines in rapid growth and cancer. Biochim. Biophys. Acta 473: 241-293 (1978).
22. Pett, D. M., and Ginsburg, H. S. Metabolism of polyamines in KB cells. Fed. Proc. 27: 615 (1968).
23. Schulte-Hermann, R., Hoffman, V., Parzefall, W., Kallenbach, M., Gerhardt, A., and Schuppier, 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).
24. Brandt, J. T., Pierce, D. F., and Fausto, N. Ornithine decarboxylase activity and polyamine synthesis during kidney hypertrophy. Biochim. Biophys. Acta 279: 184-193 (1972).