Biochemical Pharmacology, Vol. 23, pp. 2907–2915. Pergamon Press, 1974. Printed in Great Britain.

HEPATOTOXICITY OF BROMOTRICHLOROMETHANE—BOND DISSOCIATION ENERGY AND LIPOPEROXIDATION*

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(*Received* 9 *October* 1973; *accepted* 19 *February* 1974)

Abstract—Bromotrichloromethane ($BrCCl_3$) is much more potent than CCl_4 or $CHCl_3$ as a liver poison. Bond dissociation energies for cleavage of $H—CCl_3$, $Cl—CCl_3$, and $Br—CCl_3$ are in the order $H—CCl_3 > Cl—CCl_3 > Br—CCl_3$. A low bond dissociation energy implies a greater tendency of the given bond to cleave homolytically. *In vitro*, $BrCCl_3$ is 200 times more potent than CCl_4 in promoting peroxidative decomposition of rat liver microsomal lipids. $CHCl_3$ has virtually no potency as a pro-oxidant *in vitro*. $BrCCl_3$ produces in rats about three times the degree of liver microsomal lipid peroxidation than does CCl_4, at equivalent doses. $CHCl_3$ does not produce lipid peroxidation *in vivo*. Administration of $BrCCl_3$ in very low doses (0·025 ml/kg of body weight) produces a dramatic fall in liver microsomal cytochrome P-450. Rats with low levels of cytochrome P-450 due to prior administration of $BrCCl_3$ are completely resistant to large, and otherwise lethal doses of CCl_4. A lethality study is presented which suggests that $BrCCl_3$ may have extrahepatic sites of action.

THE ACTION of bromotrichloromethane ($BrCCl_3$) on rat liver *in vivo* has been reported recently by Calligaro *et al.*,[1] as cited by Slater.[2] Changes in endoplasmic reticulum were seen within 15 min. In addition, these authors reported an increased electron spin resonance signal in liver samples from rats treated with $BrCCl_3$, and a lesser signal in samples from rats treated with CCl_4.

The view that metabolism of CCl_4 by the drug-metabolizing system is a prerequisite for the toxicity of this compound is supported by a wide array of evidence.[2,3] It was originally suggested by Butler[4] in 1961 that the key initial event in CCl_4 liver injury was probably the homolytic cleavage of the C–Cl bond, yielding trichlormethyl and chlorine free radicals.[4] Slater and Sawyer[5] more recently have noted that the bond dissociation energy for the homolytic cleavage of the C—Br bond of $BrCCl_3$ is considerably less than for the homolytic cleavage of the C—Cl bond of CCl_4, implying a greater tendency for free radical formation with $BrCCl_3$. The precise mechanism by which these highly reactive free radicals initiate and perpetuate their pathological effects is uncertain. Membrane proteins, particularly those containing functional sulfhydryl groups, and the membranous lipid components of the liver endoplasmic reticulum have been suggested as the site of the initial lesion.[2,6] In support of the latter suggestion, peroxidative decomposition of lipid components of the hepatic

* This work was supported by Grant AM-01489 from the National Institute of Arthritis, Metabolism and Digestive Diseases, and by Training Grant 5 T01 GM 00899 from the National Institutes of Health, United States Public Health Service. Send reprint requests to R. O. Recknagel.

† Submitted for partial fulfillment for requirements for the Ph.D. degree in Physiology.

microsomal membrane has been shown to be an early event in CCl_4 liver injury.[7] This destructive process, initiated by free radicals generated by CCl_4 metabolism, causes functional and structural derangement of liver cells.

On the basis of its increased tendency for free radical formation, it seems probable that $BrCCl_3$ would produce an increased pro-oxidant action *in vitro*. It would also be expected that liver microsomal lipid peroxidation *in vivo* due to $BrCCl_3$ would be increased when compared with a like dose of CCl_4. Furthermore, previous studies have shown that depression of the hepatic microsomal drug-metabolizing system by a small dose of CCl_4 rendered rats resistant to a subsequent and ordinarily lethal dose of the same liver poison.[8,9] A similar protection phenomenon with $BrCCl_3$ would also seem likely. This communication reports studies based on these predictions. In addition, a lethality study with $BrCCl_3$ is presented, the data of which suggest extra-hepatic sites of action for this haloalkane.

METHODS

Studies in vitro. The pro-oxidant action of $BrCCl_3$, CCl_4, and $CHCl_3$ *in vitro* was studied in a suitably supplemented microsome–supernatant system derived from rat liver. Rat liver tissue was homogenized at 0–4° in 0·155 M NaCl buffered with 0·05 M potassium phosphate at pH 6·5. The microsome–supernatant fraction was obtained by removal of the nuclei and mitochondria by centrifugation at 2700 g for 10 min. Incubation of the microsome–supernatant fraction was carried out aerobically for 45 min in a shaking water bath at 38°. Concentration of the microsome–supernatant fraction was 25 mg-equiv of the fraction per ml of total medium. A mg-equiv of subcellular fraction is the total yield of that fraction from 1 mg wet weight of liver. Other additions at their final concentrations were 0·1 mM NADP, 5·0 mM Mg^{2+} (as $MgCl_2$), 3·0 mM isocitrate, 2·5 mM nicotinamide and 1·0 mM reduced glutathione (GSH). The final incubation mixture of 8·0 ml also contained 2·5 mg isocitric dehydrogenase (Sigma, Type I). When added, the concentration of CCl_4 was 1 μl/ml of incubation mixture. $BrCCl_3$ was added as a solution in heptane. The maximum heptane addition was 1 μl heptane/ml of incubation mixture. In this system, heptane is inert. The degree of lipid peroxidation was determined by measuring malonic dialdehyde (MDA) production. MDA was measured with thiobarbituric acid after deproteinization at a final trichloroacetic acid concentration of 5 per cent.[10]

Treatment of animals. Male rats of the Sprague–Dawley strain were used. The animals were housed in wire-bottom cages and allowed free access to food (Wayne Lab Blox) and water except for various periods preceding and following treatment (see below). $BrCCl_3$ and CCl_4 were dissolved in mineral oil and administered by stomach tube under light ether anesthesia. Control animals received equal volumes of mineral oil.

Determination of lipid peroxidation in vivo. Rats were starved overnight prior to administration of various doses of $BrCCl_3$ or CCl_4 and refed, if not yet sacrificed, 6 hr after treatment. Animals were sacrificed 1, 3, 6 and 24 hr after treatment. The microsome fraction obtained by centrifugation of liver homogenates prepared in 0·25 M sucrose and 3 mM EDTA was assayed for the appearance of lipid-conjugated dienes.[10] Sedimented microsomes from 3 g whole liver were transferred quantitatively with 10 ml methanol to a graduated centrifuge tube, after which 20 ml $CHCl_3$ was

added with thorough mixing. After allowing the tubes to stand at room temperature for 15 min, 10 ml water was added and the contents of the tubes were mixed gently by inversion and then centrifuged. By means of aspiration, the upper methanol–water phase and protein between the two phases was removed. A 5-ml aliquot of the chloroform phase was transferred to a clean test tube and placed in a 40–50° water bath. The chloroform was removed under a stream of oxygen-free nitrogen. The chloroform-free lipid was then redissolved in 3 ml cyclohexane and placed in an ice bath until optical density from 300 to 220 nm was recorded against a cyclohexane blank. After u.v. measurements, 1·0-ml aliquots from each sample were assayed for total lipid content according to Chiang *et al.*[11] During u.v. spectral analysis, concentration of lipid in cyclohexane was approximately 1 mg/ml. All optical density measurements were then corrected to a uniform base of 1 mg lipid/ml of cyclohexane. Estimation of lipid peroxidation depends on determination of the mean difference spectrum at 233 nm between lipids from control animals and lipids from treated animals.[12] A minimum of four animals was used in each control group and in each experimental group.

Protection against CCl_4 *by prior administration of* $BrCCl_3$. Rats starved overnight were treated with 0·025 ml $BrCCl_3$/kg of body weight and refed 6 hr later. Food was again removed 16–18 hr prior to subsequent treatment. Forty-eight hr after administration of the original small dose of $BrCCl_3$, a challenging dose of 5 ml CCl_4/kg of body weight was given. Animals receiving only the challenging dose of CCl_4 served as controls. Rats were observed for at least 5 days to determine lethality.

Determination of $BrCCl_3$ *lethality.* Animals were fasted overnight before administration of various doses of $BrCCl_3$ and were refed 6 hr after treatment. Water was given *ad lib.* throughout the experiment. The animals were observed for 7 days to determine lethality.

Other methods. Microsomal cytochrome P-450 content was determined by the method of Omura and Sato.[13] Microsomal protein content was determined by the method of Lowry *et al.*[14]

RESULTS

Experiments in vitro. Table 1 summarizes Slater and Sawyer's[5] data *in vitro*, expressed as relative molar activity, and the data of our laboratory, expressed as pro-oxidant activity relative to CCl_4. The first column of figures indicates the bond dissociation energies for the reactions shown.* $BrCCl_3$ is approximately 200 times more potent a pro-oxidant than CCl_4. $CHCl_3$, which is known to be a relatively less potent hepatotoxin, produces virtually no pro-oxidant activity *in vitro*. Results of a typical experiment are shown in Fig. 1.

* At first glance the bond dissociation energies for $BrCCl_3$ and CCl_4 may not appear to differ greatly. However, the difference (19 kcal/mole) becomes greatly magnified when considering the Arrhenius equation:

$$k = A \exp(-E_{act}/RT) \quad (1)$$

This equation may be used to compute rates of the reactions shown in Table 1. Because only free radical products are formed, bond dissociation energies may be used in place of the energy of activation for this calculation. The pre-exponential factors (A) for two molecules as structually similar as CCl_4 and $BrCCl_3$ are virtually identical. The calculation for the relative reaction rate for the homolytic cleavage of $Br—CCl_3$ compared to $Cl—CCl_3$ follows: contd/

TABLE 1. RELATION OF BOND DISSOCIATION ENERGIES OF HALOALKANES TO THEIR EFFECTIVENESS AS PRO-OXIDANTS ON RAT LIVER MICROSOMAL LIPID PEROXIDATION

Reaction	Energy* (kcal/mole)	Relative molar activity†	Pro-oxidant activity relative to CCl_4‡
$CHCl_3 \rightarrow \cdot CCl_3 + \cdot H$	90	7	10
$CCl_4 \rightarrow \cdot CCl_3 + \cdot Cl$	68	100	100
$BrCCl_3 \rightarrow \cdot CCl_3 + \cdot Br$	49	3000	20,000

* Bond dissociation energy.

† Relative molar activity in stimulating production of malonaldehyde *in vitro*; data of T. F. Slater and B. C. Sawyer.

‡ Pro-oxidant activity was determined *in vitro* with a liver microsome–supernatant system supplemented with an NADPH generating system and glutathione (GSH). Chloroform was virtually inert. $BrCCl_3$ produced an equivalent yield of malonaldehyde at a concentration as little as 1/200 of that required for CCl_4.

The small open bar on the left shows MDA production in the complete system *in vitro*, but without added haloalkane. The tall open bar shows the increment in lipid peroxidation due to added CCl_4. The black columns show the effect of added $BrCCl_3$. Note that $BrCCl_3$ produces an effect equivalent to that of CCl_4, but in this experiment at 1/100 the concentration of CCl_4.

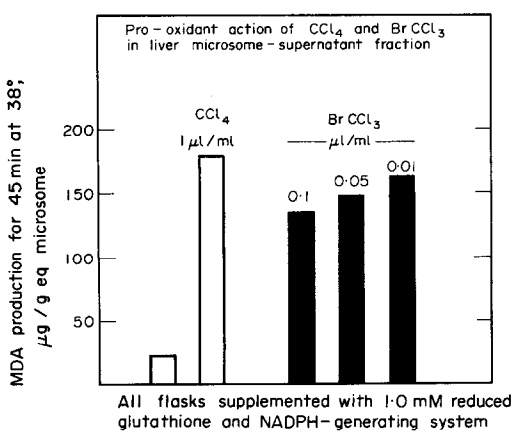

FIG. 1. Pro-oxidant action of CCl_4 and $BrCCl_3$ in microsome–supernatant fractions derived from rat liver.

(contd. from p. 2909)

$$\frac{k_{Br}}{k_{Cl}} = \frac{A \exp(-49 \text{ kcal mole}^{-1}/RT)}{A \exp(-68 \text{ kcal mole}^{-1}/RT)} \tag{2}$$

$$\ln \frac{k_{Br}}{k_{Cl}} = \frac{68 \text{ kcal/mole}}{RT} - \frac{49 \text{ kcal/mole}}{RT} \tag{3}$$

$$\log \frac{k_{Br}}{k_{Cl}} = \frac{1{\cdot}9 \times 10^4 \text{ cal/mole}}{1{\cdot}98 \text{ cal/deg mole} \times 298 \text{ deg} \times 2{\cdot}3} \cong 14 \tag{4}$$

Thus, the reaction rate, k_{Br}, for the homolytic cleavage of Br–CCl_3 is 10^{14} greater than the reaction rate k_{Cl} for the homolytic cleavage of Cl—CCl_3.

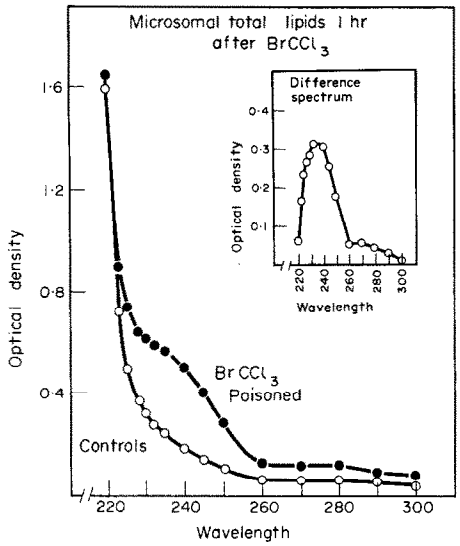

FIG. 2. Absorption spectra of liver microsomal lipids from control rats and from rats 1 hr after treatment with $BrCCl_3$. The data represent averages for four control rats and four rats treated with $BrCCl_3$. In the critical range from 220 to 260 nm, there was no overlap in experimental values compared with controls, at any given wavelength. Values plotted on the ordinate are the optical densities for the respective lipid samples, corrected to a uniform concentration of 1 mg/ml of cyclohexane.

Experiments in vivo. Marked conjugated diene absorption was detected in liver microsomal lipids 1 hr after intragastric administration of $BrCCl_3$ (1 ml/kg body wt, Fig. 2). The difference spectrum represents a typical conjugated diene peak.

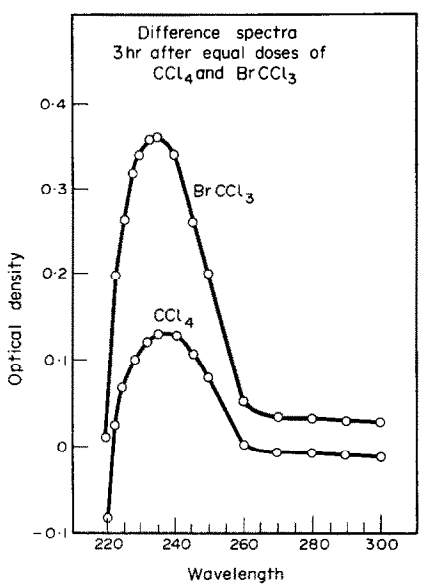

FIG. 3. Difference spectra between liver microsomal lipids of control rats and rats treated with either $BrCCl_3$ or CCl_4. Optical density values plotted on the ordinate have been corrected to a uniform concentration of 1 mg lipid/ml of cyclohexane. The difference spectra are for four rats treated with $BrCCl_3$ and four rats treated with CCl_4, compared respectively with that of four control rats.

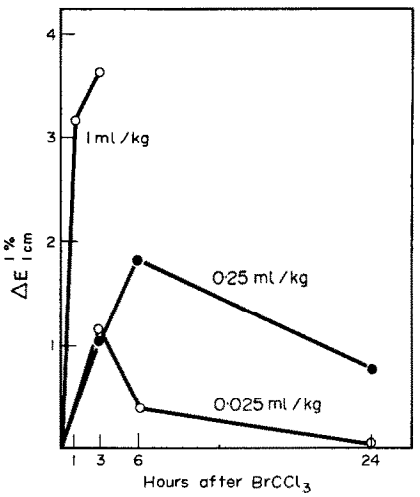

FIG. 4. Liver microsomal lipid peroxidation as a function of time after administration of various doses of $BrCCl_3$ to rats. To generate the $\Delta E_1{}^{1\%}_{1cm}$ values plotted on the ordinate, differences in optical densities at 233 nm between rats treated with $BrCCl_3$ and control rats were multiplied by 10 (see reference 12). Each point represents a mean $\Delta E_1{}^{1\%}_{1cm}$ value for four rats treated with the respective dose of $BrCCl_3$ and four control rats.

Equal doses of $BrCCl_3$ and CCl_4 (1 ml/kg of body weight) were administered to rats. Appearance of conjugated diene absorption was assayed at 3 hr. $BrCCl_3$ produced nearly three times as much lipid peroxidation as did CCl_4. At 233 nm, optical density values for animals treated with $BrCCl_3$ and CCl_4 were 0·362 and 0·132 respectively (Fig. 3). Lipid peroxidation, as indicated by the appearance of the conjugated double bond formation, was tracked with time for various doses of $BrCCl_3$ (Fig. 4). At the highest dose there was extremely rapid development of lipid peroxidation. Lesser doses showed a less rapid development followed by a gradual return toward control levels. At the highest dose, it was not possible to track liver

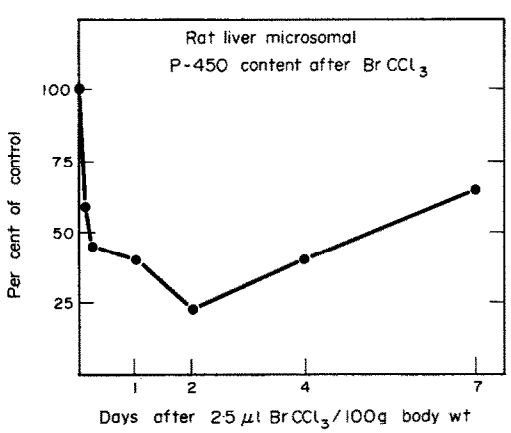

FIG. 5. Rapid loss of liver microsomal cytochrome P-450 content measured as per cent of control levels after a dose of 0·025 ml $BrCCl_3$/kg of body weight.

TABLE 2. PROTECTION AGAINST CCl_4-INDUCED LETHALITY BY PRIOR TREATMENT WITH $BrCCl_3$*

Dose of $BrCCl_3$ (ml/kg body weight)	No. of Animals	No. of Survivors	Survival %
0	8	0	0
0·025	7	7	100

* Conditions: $BrCCl_3$ in mineral oil or mineral oil alone was administered p.o. 48 hr before administration of CCl_4. CCl_4 was administered to all of the rats intragastrically in mineral oil under light ether anesthesia at a dose of 5 ml/kg of body weight. Note: 5 days after CCl_4 administration, the surviving rats were asymptomatic.

microsomal lipid peroxidation beyond about 3 hr because of the lethal potency of $BrCCl_3$ (see below).

Protection experiments. As previously reported, a small dose of CCl_4 (0·25 ml/kg), when administered 1, 2 or 3 days prior to an ordinarily lethal dose of the same liver poison, was found to protect rats from death.[8,9] This effect is believed to be due to a marked depression in activity of the liver drug-metabolizing system caused by the initial small dose. Data presented in Fig. 5 indicate the basis on which pretreatment with $BrCCl_3$ was used to obtain a similar protection phenomenon. Microsomal cytochrome content diminishes rapidly after administration of 0·025 ml $BrCCl_3$/kg of body weight. It reaches a minimum of 25 per cent of control levels by 48 hr, after which it gradually returns toward normal. An equivalent drop in cytochrome P-450 in the whole animal requires ten times as much CCl_4.[8] The results of challenging rats with a lethal dose of CCl_4 (5 ml/kg of body weight) 48 hr after being pretreated with $BrCCl_3$ (0·025 ml/kg of body weight) are shown in Table 2. All rats treated only with the challenging dose of CCl_4 died within 2 days, while all animals receiving the protective dose of $BrCCl_3$ 48 hr previously survived.

Lethality studies. Table 3 indicates the extreme lethality of $BrCCl_3$. As little as 0·3 ml $BrCCl_3$/kg of body weight was lethal within 1–2 days. Doses as low as 0·03 and 0·1 ml/kg of body weight killed some rats. A dose of 1 ml/kg of body weight killed all rats within 2–6 hr. Equivalent doses of CCl_4 are never lethal. In a subsequent study, rats which underwent 80–90 per cent surgical hepatectomy survived at least 24 hr. All

TABLE 3. LETHALITY OF $BrCCl_3$ IN RATS

Dose of $BrCCl_3$* (ml/kg body weight)	Interval between dosage and death	No. of rats	No. dead	Mortality %
0		5	0	0
0·01		5	0	0
0·03	2 days	5	1	20
0·1	2–3 days	5	2	40
0·3	1–2 days	5	5	100
1·0	2–6 hr	5	5	100

* Note: an LD_{95} for CCl_4 is about 5 ml/kg of body weight.

rats similarly hepatectomized which received 1 ml $BrCCl_3$/kg of body weight died within 12 hr.

DISCUSSION

Early efforts to define principles governing solvent toxicity attempted to relate physicochemical properties of chlorinated methanes with their physiological actions. These efforts were not successful.[15] This study confirms and extends the more recent contributions of Wirtschafter and Cronyn[16] and of Slater and Sawyer[5] in approaching this problem. In 1964 it was suggested that the mechanism by which the entire range of hydrocarbon and halogenated hydrocarbon solvents interact with biological systems is via free radical processes.[16] Wirtschafter and Cronyn[16] were the first to indicate that the thermodynamic value for a homolytic bond cleavage may be an important factor in producing solvent toxicity. Subsequently, Slater and Sawyer[5] measured the relative peroxidative activities *in vitro* of a homologous halomethane series. Coupling of their experimental results with the bond dissociation energies of respective haloalkanes indicates a positive correlation between ease of free radical formation and lipoperoxidation *in vitro*. Consistent with these findings, our results show $BrCCl_3$ to be 200 times more active than CCl_4 as a pro-oxidant *in vitro*. $CHCl_3$, with a higher bond dissociation energy, i.e. a lesser tendency for homolytic bond cleavage, elicits virtually no pro-oxidant response *in vitro*, Data *in vivo* reported here parallel the action of $BrCCl_3$ *in vitro*. Liver microsomal lipoperoxidation occurs rapidly *in vivo* after administration of $BrCCl_3$.

At equivalent doses, $BrCCl_3$ produced three times as much lipoperoxidation as did CCl_4. Again consistent with its low tendency for free radical formation, $CHCl_3$ is incapable of initiating lipoperoxidation *in vivo*.[17] Rapid appearance of conjugated diene absorption in rats treated with lethal doses of $BrCCl_3$ suggests a positive correlation between massive lipoperoxidation of the hepatic endoplasmic reticulum and early death of the rat. Animals treated with nonlethal doses (e.g. 0·025 ml/kg of body weight) show a somewhat slower development of appearance of conjugated diene absorption and a smaller absorption maximum followed by a gradual recovery. Similar reversibility has been demonstrated in regard to hepatic fat accumulation, serum glutamic oxaloacetic transaminase (SGOT) and liver weight in rats treated with sublethal doses of CCl_4.[18]

The protection phenomenon, described by Dambrauskas and Cornish,[19] Ugazio *et al.*[9] and Glende,[8] is characterized by a remarkable ability of rats to survive an ordinarily lethal dose of CCl_4 administered after prior treatment with a small dose of the same haloalkane. As shown by Glende,[8] development of resistance is directly related to depression of NADPH-linked microsomal drug metabolism. If $BrCCl_3$ is acting by the same chemical mechanism as CCl_4, a similar protective phenomenon would be expected to occur. It was found that reduction in liver microsomal cytochrome P-450 content by pretreatment with a small dose of $BrCCl_3$ renders rats resistant to a subsequent and ordinarily lethal dose of CCl_4. Contrary to this pattern, however, pretreatment with a small dose of $BrCCl_3$ or CCl_4 did not provide protection from death against $BrCCl_3$ (R. R. Koch, unpublished observations). This lack of resistance to lethal doses of $BrCCl_3$ suggests that this toxic agent may have extrahepatic sites of action. Supporting this possibility is the rapid death (within 2–6 hr) of rats given 1 ml $BrCCl_3$/kg of body weight. It seems unlikely that such a rapid

death is due to even massive liver failure. The possibility that toxic metabolites produced in the liver by $BrCCl_3$ may exit that organ to do damage elsewhere has not been ruled out. However, the death of partially hepatectomized rats within 12 hr after administration of $BrCCl_3$ argues against this possibility. It seems unlikely that the small remaining portion of liver could be capable of producing sufficient amounts of toxic metabolites to account for so rapid a death. The answer to the question of what ultimately kills the rat after administration of $BrCCl_3$ or CCl_4 is far from clear, and deserves further study. It is gratifying to realize that study of the molecular pathobiology of at least a small homologous series of halogenated hydrocarbon solvents has reached a degree of sophistication such that production of effects both *in vitro* and *in vivo* can be verified by suitable experiments based on a chemical property as fundamental as the bond dissociation energy.

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