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COMMENTARY

REACTIVITY AND TOXICITY AMONG HALOGENATED METHANES AND RELATED COMPOUNDS

A PHYSICOCHEMICAL CORRELATE WITH PREDICTIVE VALUE

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Interest in the hepatotoxic effects of halogenated methanes and alkanes appears to have stemmed from cases of human toxicity associated with the widespread use of $CHCl_3$ as a general anesthetic (introduced in 1847), and with the use of CCl_4 as an anthelmintic drug (introduced in 1921) and later as an industrial solvent (for reviews see Refs. 1–3). Early hypotheses regarding the toxicity of these compounds centered about their potential effects on lipid membranes by virtue of their solvent properties. In 1961, Butler [4] proposed that the toxicity of CCl_4, and possibly that of $CHCl_3$, was related to the ease of homolytic cleavage of the C—Cl bond, which increases in the series $Cl—CH_3 < Cl—CH_2Cl < Cl—CHCl_2 < Cl—CCl_3$. Such homolytic cleavages would produce chemically reactive free radical species (e.g. $Cl\cdot$ and $Cl_3C\cdot$) which could then interrupt normal biochemical processes in the cell. Although Butler was able to show that dogs given CCl_4 exhaled small amounts of $CHCl_3$, which could be rationalized as arising via $Cl_3C\cdot$ radicals, he did not suggest where the rather substantial energy required to dissociate a C—Cl bond (e.g. 68 kcal/mole for $Cl—CCl_3$) might be obtained *in vivo*.

Several years later Wirtschafter and Cronyn [5] extended this concept by relating the relative toxicities of hydrocarbons and their halogenated derivatives to another molecular property not unrelated to bond dissociation energies, namely, their relative propensity to react with "endogenous free radicals" produced as intermediates in normal biochemical pathways. Carbon tetrachloride and $CHCl_3$ were envisioned to react according to equations 1 and 2; the $Cl_3C\cdot$ radical thus generated could then react further with proteins and unsaturated lipids, thereby injuring the cell.

$$R\cdot + Cl—CCl_3 \longrightarrow R—Cl + Cl_3C\cdot \quad (1)$$

$$R\cdot + H—CCl_3 \longrightarrow R—H + Cl_3C\cdot \quad (2)$$

Shortly after this proposal, several groups reported evidence which linked the toxicity of CCl_4 to the peroxidative destruction of membrane lipids *in vitro* and *in vivo* (reviewed by Recknagel, [2]); both ^{36}Cl and ^{14}C were shown to become covalently bound to the microsomal lipids and proteins of rats treated *in vivo* with radiolabeled CCl_4 [6]; and $Cl_3C—CCl_3$ was isolated from the liver of rabbits fed CCl_4 [7]. These observations provided compelling support for the hypothesis that the $Cl_3C\cdot$ radical was a key intermediate in CCl_4 toxicity. They also set the stage for applying to the study of toxicological responses methods commonly used to investigate the mechanism of chemical reactions.

One method rather widely used in investigating chemical mechanisms is the perturbational approach, in which the structure of one of the reactants is varied, and the effect of this change on the course of the reaction is observed and ultimately analyzed in terms of a postulated reaction mechanism. The use of substituent effects and kinetic isotope effects are two important examples of this general approach. The observation of substituent effects on a chemical reaction or sequence of reactions provides a very useful way to probe the steric and/or electronic requirements for formation of the transition state in whatever portion of the overall sequence constitutes the *rate-limiting step*. However, there are two types of problems which can occur in the use of substituent effects to probe the mechanism of *biological* reactions. One is that substituent changes can drastically alter the absorption, metabolism, and distribution of a compound with respect to its presumed congeners. Nevertheless, this problem can often be avoided if the substituents are chosen carefully. A more fundamental problem is that changing the structure of a reactant *of necessity* changes the potential energy surface for the reaction. If this change is large enough, it may actually change the mechanism of the process and indeed may direct the entire course of the reaction toward a qualitatively different outcome. Isotopic substitution on the other hand does not alter the potential energy surface for the reaction but in general only primary kinetic *deuterium* isotope effects are large enough to be readily detectable given the relative standard deviations commonly associated with measurements based on biological endpoints. Further details and examples of substituent effects and isotope effects on toxicological responses may be found in Ref. 8.

Provided that a dose–response relationship exists, the critical rate-limiting step in the development of the biological or toxicological response will explicitly

involve the test compound or some closely related metabolite [8, 9]. Perturbation of the chemical properties and reactivity of the test compound should thus lead to alterations in the toxic response. In turn, this will reflect the nature of the critical interaction between toxin and cell. As discussed below, the use of substituent and isotope effects to probe the mechanism of toxicity of compounds containing a $—CCl_3$ group proved to be particularly illuminating.

Klaassen and Plaa [10] investigated the hepatotoxicity of a number of chlorinated methanes and ethanes including CCl_4, $CHCl_3$ and CH_3CCl_3. CCl_4 was somewhat more toxic than $CHCl_3$, and both produced similar histopathological changes in the liver (fatty infiltration and centrolobular necrosis), whereas CH_3CCl_3 was only weakly hepatotoxic. Surprisingly, CCl_4 was the *only* compound tested which led to significant amounts of lipid peroxidation. At first this was taken as evidence against the importance of lipid peroxidation in CCl_4 toxicity. However, the pronounced differences in the biological effects of these three compounds more likely suggests that, despite their superficial chemical similarity, the replacement of a chlorine in CCl_4 by either H or CH_3 completely changes the mechanism of interaction of the compound with the cell. The chemical basis for this "change of mechanism" will become apparent shortly.

Another revealing substituent effect study was carried out by Slater and Sawyer [11], who found that the relative activity of $Br—CCl_3$, $Cl—CCl_3$ and $F—CCl_3$ for enhancing the perodixation of microsomal lipids *in vitro* was *ca.* 100:3:1, which parallels the relative *in vivo* hepatotoxicity of these compounds [12–15]. Slater and Sawyer also obtained evidence that the enhancement of lipid peroxidation *in vitro* by CCl_4 required NADPH as well as molecular oxygen and viable microsomes. Because of the requirement for NADPH, a *reducing* agent, they proposed an "electron capture" process, represented by equation 3, as the basis for the activation of CCl_4 and its congeners.

$$CCl_4 + e^- \longrightarrow Cl^- + Cl_3C\cdot \qquad (3)$$

This was a great step forward, for it suggests the use of equations 4 and 5 as a basis for interpreting the failure of $HCCl_3$ and CH_3CCl_3 to induce lipid peroxidation, as well as the relative activities of $Br—CCl_3$, CCl_4 and $F—CCl_3$ in this regard.

$$X—CCl_3 + e^- \xrightarrow[X \neq H,CH_3]{X = Br > Cl > F} X^- + Cl_3C\cdot \qquad (4)$$

$$X—CCl_3 + e^- \xrightarrow[X \neq H,CH_3]{X = F,Br} Cl^- + XCl_2C\cdot \qquad (5)$$

These equations indicate clearly that the concept of "homolytic bond dissociations" is not a proper framework for interpreting toxicities of halogenated methanes and related compounds. They also provide a rationale for the divergent behaviour of CCl_4 on one hand and $CHCl_3$ and CH_3CCl_3 on the other, at least insofar as lipid peroxidation is concerned. Conversion of the latter to $Cl_3C\cdot$ would require the formation of H^- and CH_3^-, respectively, which would be most unfavorable energetically. Finally, and perhaps most significant, equations 4 and 5 find unambiguous justification in the electrochemical behavior of these compounds.

The polarographic reduction of chlorinated hydrocarbons was first investigated in 1949 by von Stackelberg and Stracke [16], and later by Wawzonek and Duty [17]. As summarized in a review by Fry [18], the ease of electrochemical reduction of the carbon—halogen (C—X) bond decreases in the order X = I > Br > Cl > F. Simple alkyl chlorides and fluorides are not reduced electrochemically, but allylic and benzylic chlorides and vicinal and geminal polyhalogenated compounds reduce much more readily than simply alkyl halides, e.g.

$$CCl_4 > CHCl_3 > CH_2Cl_2 > CH_3Cl$$

$$BrCH_2CH_2Br > CH_3CHBr_2 > CH_3CH_2Br$$

The half-wave potentials for these reactions are pH-independent, suggesting that the rate-limiting step (see equations 6–10) does not require preceding or concurrent proton transfer. Since each polarographic wave is a 2-electron process, the intermediate radical species ($R\cdot$) must be reduced more easily than R—X. Note, however, that while reduction of CH_3CHBr_2 gives CH_3CH_2Br, reduction of $BrCH_2CH_2Br$ gives ethylene ($CH_2{=}CH_2$) and two bromide ions. Nevertheless, the discrete existence of $R\cdot$ as a transient species has been clearly demonstrated, at least in some cases, through the isolation of chemical rearrangement products and/or R—R dimers formed near the electrode surface [18].

In biochemical systems, the availability of electrons is not unlimited as it effectively is at the surface of an electrode; rather, electrons are available in discrete stoichiometry, frequently in single-electron steps. This will have the effect of enhancing the chance that the radical $R\cdot$ will escape from its site of formation prior to undergoing another reduction step as shown in equation 8. Evidence in support of this concept

$$R—X + e^- \xrightarrow[\text{slow}]{} [R—X]^- \qquad (6)$$

$$[R—X]^+ \longrightarrow X^- + R\cdot \qquad (7)$$

$$R\cdot + e^- \longrightarrow R^- \qquad (8)$$

$$R^- + H_2O \rightarrow RH + HO^- \qquad (9)$$

$$\text{net: } R—X + 2e^- + H_2O \rightarrow R—H + X^- + HO^- \qquad (10)$$

has been provided recently by Kubic and Anders [19], who found that reduction of CCl_4 to chloroform by microsomes in D_2O leads to $CHCl_3$ and not $CDCl_3$; presumably the hydrogen atom came from reaction of $Cl_3C\cdot$ with lipids present in the microsomes (see below). The same authors also found that CF_3CH_2Cl containing no D from D_2O was produced by microsomal reduction of halothane, again implying the intermediacy of the $CF_3CHCl\cdot$ radical and its abstraction of a hydrogen atom from microsomal lipids. In contrast, *electrochemical* reduction of 1,2-dihalides generally leads to the formation of an olefin via a 2-electron reduction because the intermediate radical is reduced more easily than its precursor halide, and because the physical electrode,

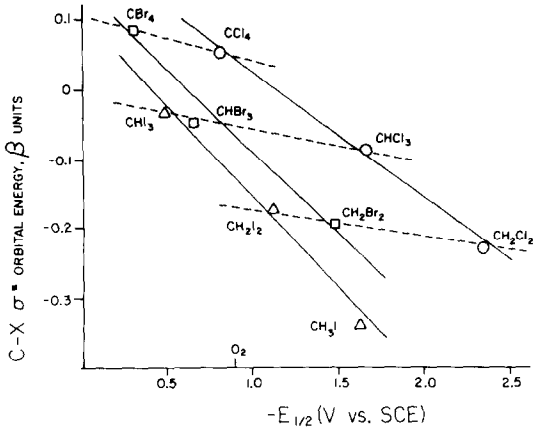

Fig. 1. Plot of the polarographic half-wave reduction potential of various halogenated methanes versus the energy of their lowest unoccupied molecular orbital. Data from Refs. 16 and 20.

in contrast to biological electron transport sources, has a virtually unlimited supply of electrons in both a kinetic and a stoichiometric sense.

The effects of substituents on the reduction potential of various halogenated methanes was elegantly explained in 1963 by Fukui *et al.* [20], who compared their polarographic $E_{1/2}$ values with the calculated energy of their lowest unoccupied molecular orbital (LUMO). This orbital is a C—X σ^* or antibonding orbital, and placing an electron into it corresponds to the reaction shown in equation 6. The energy of this orbital, and hence the energy required to put an electron into it, is lowered by successive halogenation of the central carbon as shown by the data in Fig. 1. Note that the compounds can be divided into three groups according to either the *type* of halogen present (dotted lines) or the *number* of halogens present (solid lines), and that either way there are striking parallels between the groups.

Figure 1 offers plausible explanations for several aspects of the toxicity of CCl_4 and related compounds. First, the failure of $CHCl_3$ to initiate lipid peroxidation is attributable to its reluctance to undergo 1-electron reduction ($E_{1/2} = -1.6$ V); probably no biochemical reducing agent sufficiently powerful to reduce $CHCl_3$ (or CH_3CCl_3) can exist in the presence of other oxidants such as H^+ or O_2. Molecular oxygen is, of course, required for the *propagation* of lipid peroxidation reactions. It has long been known that raising or lowering the concentration of oxygen in the atmosphere significantly blocks or potentiates, respectively, the hepatotoxicity of CCl_4 [21–23] and, *in vitro*, the covalent binding of CCl_4 is enhanced under anaerobic conditions [24]. It has been suggested that molecular oxygen may compete with CCl_4 for the electron needed to form the $Cl_3C\cdot$ radical [25]. Some indication of the delicacy with which this competition may be poised *in vivo* is given by comparison of the reduction potentials of CCl_4 and oxygen as shown in Fig. 1. Since far more lipid is consumed in the chain propagation phase of lipid peroxidation, even slight changes in the frequency of *initiation* events stemming from $Cl_3C\cdot$ production would be amplified many-fold, with corresponding alterations in the toxic manifestations of the lipid peroxidation process.

Based on this type of analysis one would predict that halomethanes which reduce more easily than CCl_4 should all initiate lipid peroxidation and produce hepatotoxicity similar to that of CCl_4. Those which reduce much less easily than CCl_4 or O_2, including $CHCl_3$, are predicted not to initiate lipid peroxidation and their hepatotoxicity, if any, must be explained by another mechanism. Relatively little information could be found to substantiate these "predictions". However, Sell and Reynolds [26] demonstrated that iodoform, CHI_3, causes hepatic centrolobular necrosis in rats, and that lipid peroxidation is involved. They commented that "the close cytochemical and morphological similarities between the cellular injury produced in the liver by iodoform and that produced by carbon tetrachloride suggest common pathogenetic mechanisms associated with damage to membranes." Bromoform is also reported to be hepatotoxic in rats [27], yet like CHI_3 and CCl_4 it does not deplete hepatic glutathione, whereas $CHCl_3$ does. These observations also support a commonality of mechanism and toxicity among reductively metabolized halomethanes on one hand, and $CHCl_3$, CH_2Cl_2, and related compounds on the other.

The hepatotoxicity of both CCl_4 and $CHCl_3$ is increased by pretreating animals with inducers of the cytochrome P-450 system, especially phenobarbital. An early observation [27] that *deuterio*chloroform ($DCCl_3$) was only one-third to one-half as toxic as $CHCl_3$ provided a valuable clue to the mechanism of its toxicity, since kinetic deuterium isotope effects of this magnitude are commonly observed for P-450 dependent *hydroxylations*, but would not be expected for reductive processes. Trapping experiments quickly confirmed that phosgene (Cl_2CO), presumably formed by spontaneous decomposition of HO—CCl_3, was the major if not sole metabolite of $CHCl_3$ [3]. This accounts for the fact that metabolism-dependent covalent binding of $CHCl_3$ to cellular constituents leads to incorporation of ^{14}C but *not* of ^{3}H or ^{36}Cl [28], whereas covalent binding of CCl_4 leads to incorporation of both ^{14}C and ^{36}Cl [6]. The formation of phosgene as a metabolite of CCl_4 has been demonstrated recently [29, 30], and conclusive evidence for the intermediacy of $Cl_3C\cdot$ has been obtained from spin-trapping experiments and e.s.r. spectroscopy [31, 32]; the Cl_3C—O—O$\cdot$ radical can be trapped in chemical systems but is evidently too reactive to be spin-trapped in biological systems [32].

Figure 2 compares the metabolic activation processes for CCl_4 and $CHCl_3$ relative to their covalent binding (and presumably their toxicity). It can also serve as a paradigm for halogenated alkanes in general. For example, polyhalogenated ethanes still possessing C—H bonds, such as halothane or Cl_2CH—CH_2Cl, may be able to undergo a combination of both oxidative and reductive bioactivation pathways *in parallel*. Reductive pathways in these cases may lead to free radicals and lipid peroxidation, but may also lead via dehalogenation reactions to halogenated ethanes and/or ethylenes as products. Because of their redox potentials, the latter may not

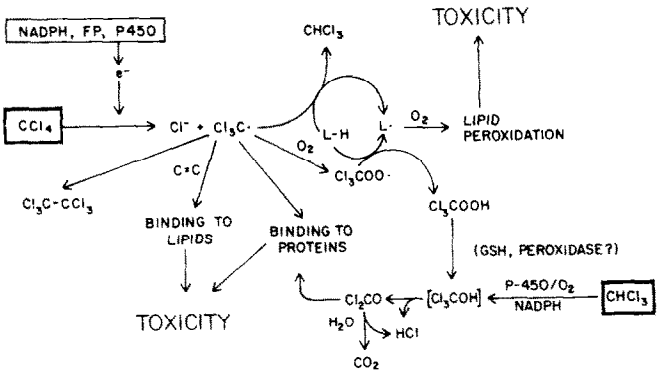

Fig. 2. Composite diagram relating oxidative and reductive metabolism of carbon tetrachloride and chloroform to their conversion to chemically reactive metabolites ($Cl_3C\cdot$, $Cl_3COO\cdot$, and Cl_2CO). L—H and C=C represent the pertinent chemical features of unsaturated membrane lipids. See text for details.

be subject to further reductive metabolism in biological systems. Consequently, they are eliminated unchanged, or are metabolized by glutathione conjugation or by oxidative pathways involving the formation of other forms of chemically reactive metabolites such as epoxides and their rearrangement products, the acyl halides [33].

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