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ABSTRACT

1,1,2-Trichloroethylene (TCE) is an industrial solvent that is widely used for degreasing metals; it is also used for adhesives, paint stripping, dry cleaning, and in the manufacture of pharmaceuticals and textiles. Because of its widespread use and often improper disposal, TCE has become a common environmental contaminant at many worksites. It is also found in ground and surface waters near most large urban areas and at many landfill locations. Public exposure occurs through contaminated waters that are ultimately used for human consumption. TCE produces hepatocellular carcinomas and lung tumors in the mouse and renal tubular adenomas and testicular tumors in the rat. However, two TCE metabolites, trichloroacetic acid (TCA) and dichloroacetic acid (DCA), which are especially potent hepatocarcinogens in mice, have been implicated in the carcinogenicity of TCE. Although these haloacetic acid metabolites of TCE are capable of forming covalent adducts with DNA, the mechanism of adduct formation is unknown. By means of spin-trapping techniques, we will test the hypothesis that TCA and DCA are converted in the liver to reactive free radical intermediates that are responsible for forming these DNA adducts. Using mouse liver hepatocytes, we will examine the formation of free radicals in two ways: 1) directly, by means of electron paramagnetic resonance spectroscopy (EPR) of the trapped radicals, and 2) indirectly, through the use of a ^{14}C-labeled spin trapping agent that will be converted to a stable derivative and quantitated by means of high-pressure liquid chromatography (HPLC). The rationale for the latter approach is based on recent evidence showing that viable cells have the ability to metabolize spin adducts to EPR-silent species, which could lead to a significant underestimate of radicals that have been trapped. We will also investigate the formation of DNA adducts by means of ^{32}P-postlabeling techniques, and we will determine the nature of these adducts by isolating the adducted DNA, digesting it with nucleases, and identifying the modified bases by means of HPLC and mass spectroscopy.

DESCRIPTION OF PROJECT

Epidemiologists estimate that a large percentage of all human cancer arises from chemical carcinogens that are present in the environment and taken up with the food, air, and water we consume (1,2). In 1992, Louisiana led the nation in toxic chemical releases to the environment (3). Drinking water derived from the Mississippi River basin is of particular concern because of the large number of industrial sites that release pollutants into the river and the elevated level of certain types of cancer in the surrounding region (4-6). Many of the pollutants are halogenated hydrocarbons and aromatic compounds that are solvents and manufacturing byproducts, and often they are contaminated with toxic metals (7,8). Additionally, there are fungicides, herbicides, insecticides, and chemical preservatives from agricultural and urban runoff, and pollutants from shipping accidents, discharges, and illegal dumping.

Of further concern are compounds that are generated when river water is treated with chlorine to eliminate harmful organisms prior to human consumption (9-13). This process can chlorinate unsaturated chemical centers, form unstable chloramines, and by means of chemical processes that are not fully understood, give rise to mutagenic agents from humic substances that are present in the water (14-17). Humic substances are decay products arising from the flora and fauna that live in the aquatic environment, and they may also be acquired from runoff containing decomposed natural products leached from the soil. The chlorination of humic substances can lead to the formation of a variety of harmful chlorinated products that include chloroform (18), the chlorinated furanone, MX (19), chlorinated acetonitriles (20-22), and dichloroacetate (DCA) and trichloroacetate (TCA) (23), which are the subject of the present proposal.

One of the most prevalent halogenated hydrocarbons in industry is 1,1,2-trichloroethylene (TCE), of which hundreds of millions of kg are produced each year (24). TCE is used extensively for degreasing metals, is an important solvent for adhesives, paint stripping, and dry cleaning, and is also used in the manufacture of pharmaceuticals and textiles. Because of its widespread use and often improper disposal, TCE has become a very common environmental contaminant of ground and surface waters and is among the 10 most cited chemicals at hazardous waste sites (25). Public exposure to TCE occurs by means of contaminated waters that are ultimately utilized for human consumption.

TCE produces hepatocellular carcinomas and lung tumors in the mouse and renal tubular adenomas and testicular tumors in the rat (24-28). A number of studies have shown that activated metabolites derived from TCE are capable of binding covalently to proteins, lipids, and DNA (29-36). Fig. 1 is a summary of the major metabolites known to be formed from TCE.

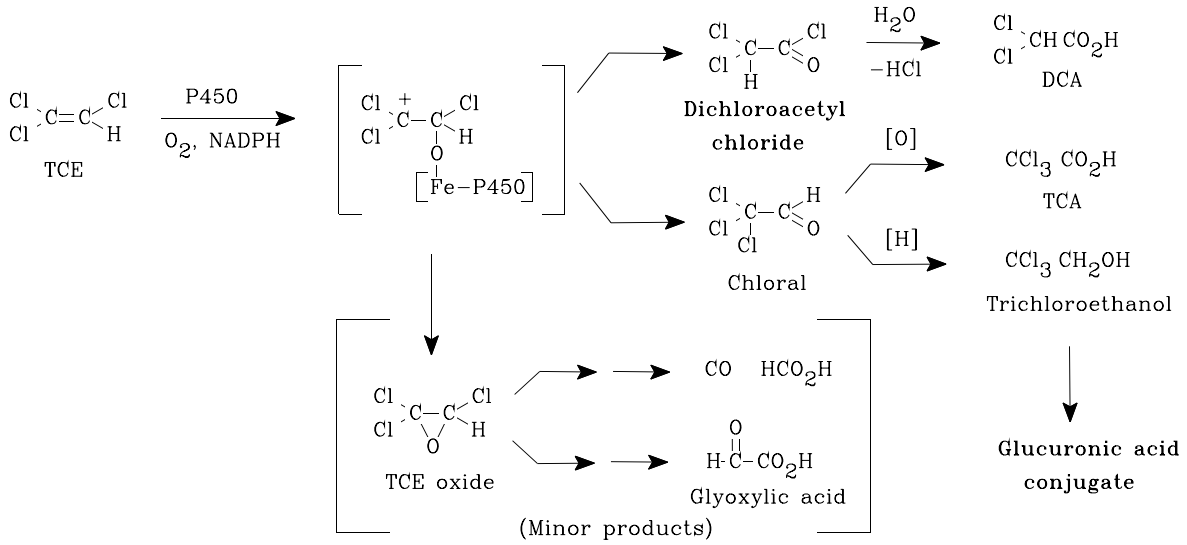

Figure 1. Summary of trichloroethylene (TCE) metabolism.

The initial step in the metabolism of TCE appears to involve its interaction with cytochrome P450 to yield an intermediate that contains a reactive carbonium center and is liganded by means of an oxygen atom (oxo bridge) to the P450 heme iron (Fig. 1). The major pathway for dissociation of the complex appears to involve migration of chlorine from the carbon atom bearing the oxo ligand to the carbon atom bearing the positive charge, leading to the formation of chloral. A second reaction pathway involves migration of hydrogen from the carbon atom bearing the oxo ligand to the carbon atom bearing the positive charge, leading to the formation of dichloroacetyl chloride, and a third reaction pathway involves the migration of oxygen from the P450 heme iron to the carbon atom bearing the positive charge, leading to the formation of an epoxide, TCE oxide (Fig. 1). Each of these three products undergoes further unique transformations.

Dichloroacetyl chloride undergoes rapid hydrolysis to yield dichloroacetic acid, DCA, which as an individual component appears to be the most genotoxic product formed (28,37). TCE oxide, which at one time was thought to be the primary intermediary metabolite of TCE (38,39), now appears to be formed in smaller quantities and to give rise to a variety of minor products that include carbon monoxide, formic acid, glyoxylic acid and oxalic acid (Fig. 1).

Chloral (trichloroacetaldehyde) appears to be the primary product of TCE oxidation by P450 and has several metabolic fates. Existing largely as the hydrate, chloral can be oxidized by aldehyde oxidase to trichloroacetic acid (TCA) and reduced by alcohol dehydrogenase to trichloroethanol, a reaction that would be influenced by the redox status (NAD^+/NADH ratio) of the liver. Trichloroethanol is ultimately converted to the glucuronic acid conjugate, the major excretory product of TCE

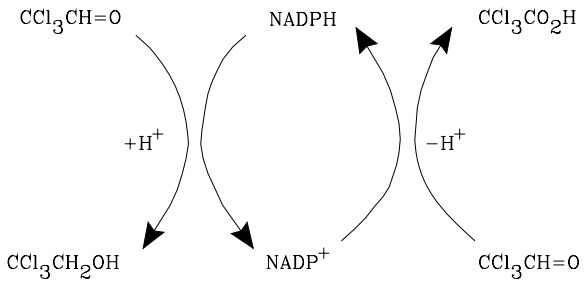

Figure 2. Aldehyde dismutation catalyzed by carbonyl reductase.

metabolism. TCA and trichloroethanol can also arise through the action of carbonyl reductase (40), an NADP-linked enzyme that catalyzes the dismutation of chloral as indicated in Fig. 2.

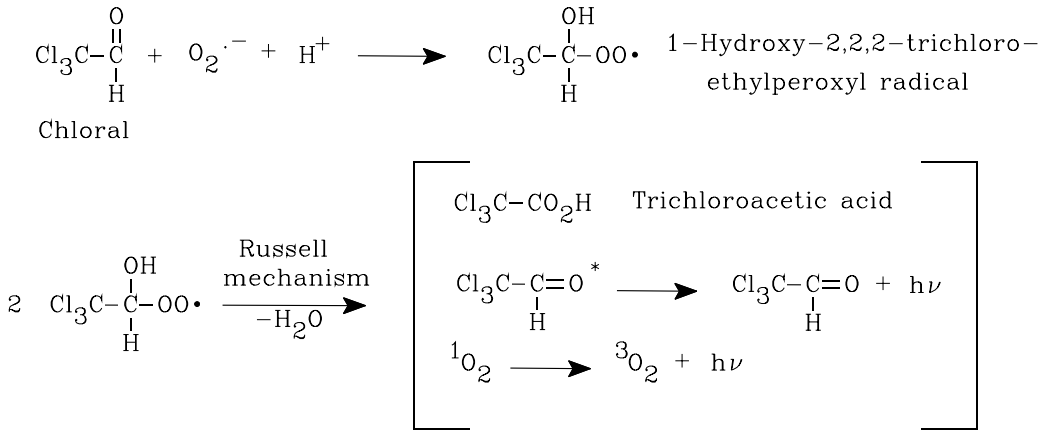

Figure 3. Formation and dismutation of the 1-hydroxy-2,2,2-trichloroethylperoxyl radical.

Chloral is a chemically reactive substance that can combine with superoxide ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2) to give unstable intermediates, and it can combine with the amino group of many cellular components to give stable derivatives. Both reactions can have cytotoxic consequences. The reaction of chloral with $O_2^{\cdot-}$ yields a 1-hydroxyperoxyl radical (Fig. 3) that could either abstract a hydrogen atom to form a peroxy acid (not shown) or undergo a Russell dismutation reaction (41,42) in which 2 mol of the peroxyl radical combine to form an intermediate tetroxide that decomposes to give an acid (TCA), an electronically-excited carbonyl (chloral*, Fig. 3), and singlet oxygen ($^1O_2$). Relaxation of the excited carbonyl to ground state is accompanied by the emission of light (hν) which may be observed as chemiluminescence (43,44). The singlet oxygen is a short-lived but strong oxidant that can react with cell constituents to yield hydroperoxides. Unreacted $^1O_2$ decays to its ground state triplet (normal oxygen, $^3O_2$) with the emission of light at wavelengths that are diagnostic for its presence and which can be measured with specialized infrared detectors (44-48).

The degree of chloral cytotoxicity that would arise from electronically excited species is strongly dependent on the quantity of $O_2^{\cdot-}$ that would be available for reaction. The latter would be a function of mitochondrial respiration which can yield $O_2^{\cdot-}$ under certain states, the activity of xanthine and aldehyde oxidases, and the presence of an inflammatory response in which activated neutrophils generate a large flux of $O_2^{\cdot-}$.

Another toxic effect of chloral would result from its condensation with amines such as lysine, adenosine, dopamine, and serotonin (note that chloral has sedative and narcotic properties) to give Schiff bases that are readily reduced to relatively stable secondary amines (Fig. 4). Modification of the above compounds could alter the structure of proteins, nucleic acids, and biogenic amines, respectively, and thus contribute to a disruption of normal metabolic activity.

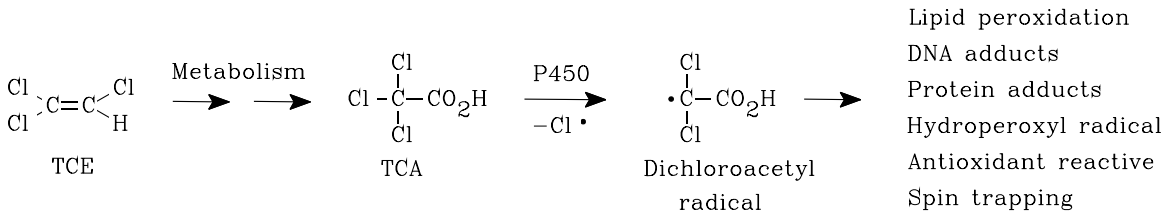

Figure 5. Postulated formation and fate of the radical species derived from trichloroacetic acid.

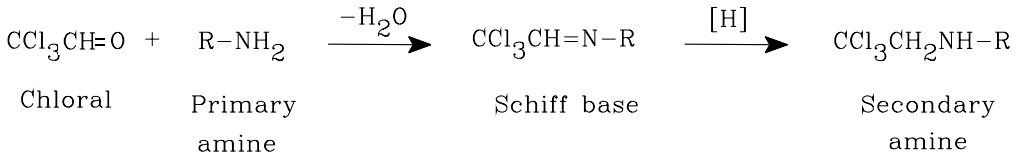

Figure 4. Formation and reduction of Schiff bases.

Although chloral is capable of toxic effects, its accumulation from TCE is likely to be minimal because of metabolic conversion by oxidation, reduction, and dismutation. Moreover, the genotoxicity of chloral is yet to be proved. However, two of its metabolites, dichloroacetic acid (DCA) and trichloroacetic (TCA) are especially potent hepatocarcinogens in mice (26,49-53), although the mechanism of carcinogenicity is unknown. Using electron paramagnetic resonance spectroscopy (EPR) and spin-trapping techniques, we will test the hypothesis that TCE, DCA, and TCA are converted in the liver to reactive free radical intermediates that are capable of forming adducts with DNA. We will also determine the nature of these adducts by isolating the adducted DNA, digesting it with nucleases, and identifying the modified bases.

In addition to being metabolites of trichloroethylene (24,25,54,55), DCA and TCA have also been found in municipal drinking water at concentrations of 34 to 161 μg/liter (23). DCA and TCA are formed from humic substances present in surface waters and are among the numerous chlorinated products that arise when these substances come in contact with the chlorine used for disinfection. The health hazard to humans from the consumption of water containing these compounds is not clear. However, male and female B6C3F1 mice that received 0.1% DCA or TCA in their drinking water eventually developed liver tumors (26,49,50). Although significant species differences exist in the response of rodents to DCA and TCA, their carcinogenicity in mice is well established (51-53).

Although DCA and TCA are potent hepatocarcinogens in mice, the mechanism by which they induce tumors is not known. By analogy with the routes of metabolic activation established for chloroform ($CHCl_3$) and carbon tetrachloride (CCl_4), we hypothesize that TCA is oxidized by cytochrome P450 to a free-radical intermediate that subsequently reacts to form injurious radicals and adducts that may be responsible for the hepatotoxicity and neoplasia. Thus, CCl_4 is metabolized to the trichloromethyl radical ($CCl_3^{\cdot}$) which subsequently combines with oxygen to form the trichloromethylperoxyl radical ($CCl_3OO^{\cdot}$) (56-58). The reaction of $CCl_3OO^{\cdot}$ with cell membranes and other cellular components is known to lead to lipid peroxidation and the formation of cytotoxic

adducts (59-62). That DCA and TCA may follow a similar course of action was made plausible by the recent work of Larson and Bull (52), who demonstrated an increase in lipid peroxidation in the livers of mice that were administered single oral doses of DCA and TCA. Lipid peroxidation, which is also a hallmark of CCl_4 toxicity (61-66), occurs when a metabolically generated carbon-centered free radical interacts with a fatty acid to abstract a hydrogen atom. The resulting lipid radical can combine with O_2 to yield a peroxyl radical which in turn can abstract a hydrogen atom from a second fatty acid, and so forth (67). Lipid hydroperoxides thus formed can damage the integrity of cell membranes and give rise to decomposition products that include malondialdehyde. The data of Larson and Bull (52) suggest that the same type of lipid peroxidation occurred in the livers of mice that were administered either DCA, TCA, or CCl_4.

We hypothesize that DCA and TCA are converted in the liver by the action of P450 to free radical species that are responsible for the cytotoxicity and carcinogenicity associated with these substances. Fig. 5 illustrates the formation and various fates of the dichloroacetyl radical. Using TCA as an example, we postulate that the chlorinated acid undergoes homolysis of a chlorine-to-carbon bond to yield chlorine radical ($Cl^{\cdot}$) and the carbon-centered dichloroacetyl radical (Fig. 5). Chlorine radical, which is essentially as energetic as the hydroxyl radical ($OH^{\cdot}$), will react at nearly the same rate at which it collides with other molecules and has a bimolecular rate constant of 10^9 M^{-1} sec^{-1} (Koppenol, W. H., personal communication). Therefore, $Cl^{\cdot}$ will abstract a hydrogen atom from its nearest neighbor to form HCl and at the same time initiate a new radical sequence. P450 itself is a potential target for $Cl^{\cdot}$ attack, leading to self destruction. Alternatively, as shown in Fig. 6, $Cl^{\cdot}$ can combine with Cl^{-} to form the chloride radical anion ($Cl_2^{\cdot -}$), which can also abstract a hydrogen atom and initiate lipid peroxidation. It should be pointed out that enzymic $Cl^{\cdot}$ formation has never been demonstrated and that alternative mechanisms exist for the P450-mediated activation of TCA, such as the scheme illustrated in Fig. 7 which involves the formation of hypochlorous acid (HOCl). The latter mechanism will be tested in our laboratory using the sensitive EPR technique we have developed for detection of HOCl (68).

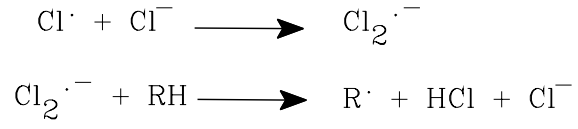

Figure 6. Formation and fate of the chloride radical anion ($Cl_2^{\cdot -}$).

The dichloroacetyl radical could react in two principal ways. First, hydrogen abstraction by the radical would yield DCA and at the same time initiate radical processes on the substrate from which the hydrogen atom was abstracted. Second, radical addition to an unsaturated center would form a radical adduct that would be stabilized either by single electron oxidation by a metal ion, or oxidation by molecular oxygen to a peroxyl radical followed by hydrogen abstraction and elimination of hydrogen peroxide. Examples of these processes are given in Figs. 8 and 9 below.

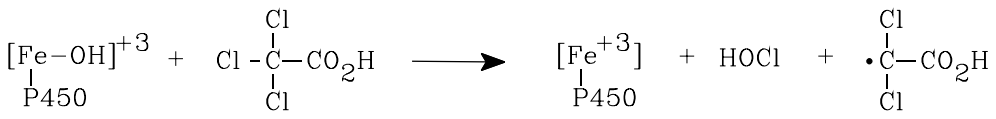

Figure 7. Hypothetical mechanism for formation of dichloroacetyl radical by reaction of trichloroacetic acid with P450 ferryl.

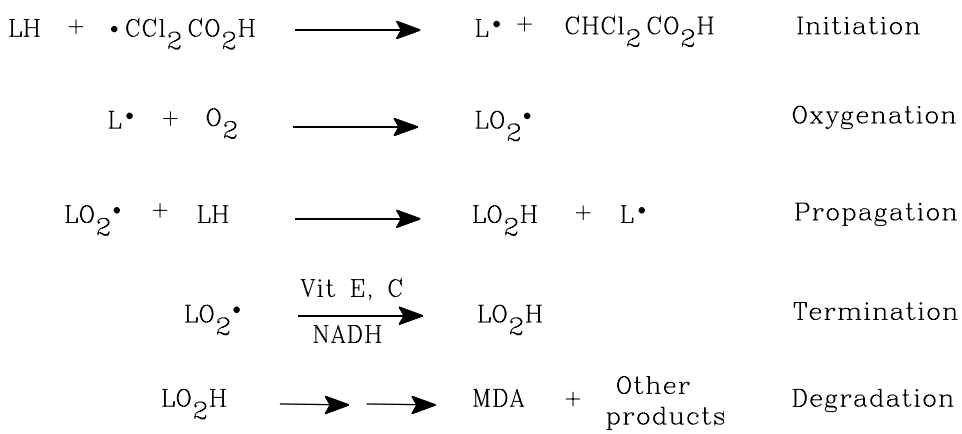

Figure 8. Lipid peroxidation. L = unsaturated lipid, L· = L radical, LO_2· = L peroxyl radical, LO_2H = L hydroperoxide, and MDA = malondialdehyde.

The initiation of lipid peroxidation by an alkyl radical such as the dichloroacetyl radical (Fig. 8) is a significant mechanism of cytotoxicity because it can lead to disruption of membrane structure and function. Damage to the mitochondrial membrane can result in a loss of ATP production, and damage to the cell membrane can result in a loss of ionic regulation. Such injuries can lead to cell death. As indicated in Fig. 8, the antioxidant vitamins E and C working together can interrupt chain propagation while the NAD-linked enzyme dehydroascorbate reductase will maintain vitamin C in its reduced state. Lipid peroxide degradation products such as malondialdehyde (MDA), pentane, and ethane are ultimately excreted in the urine or exhaled in breath (69). Also possible, but not shown, is the addition of dichloroacetyl radical to an unsaturated lipid to yield a lipid adduct with modified properties.

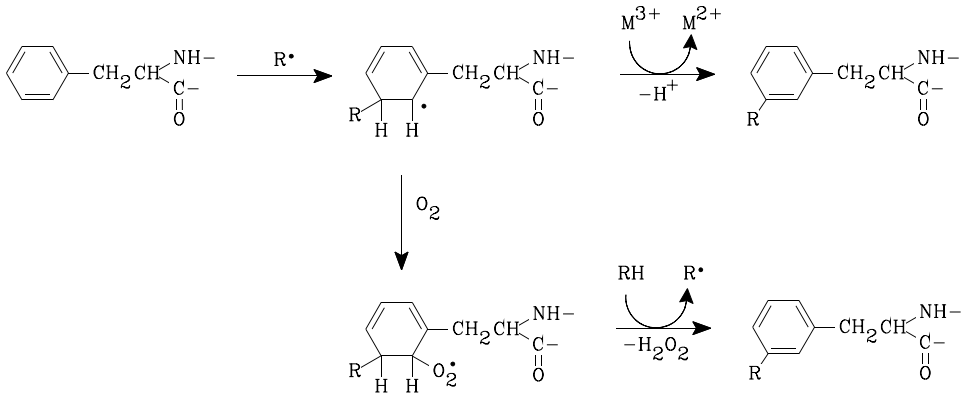

Figure 9. Radical addition to an aromatic center (here, phenylalanine) to give a stable adduct.

Radical addition to an aromatic center is illustrated in Fig. 9. Phenylalanine is used as an example but similar reactions would occur with nucleic acid bases and other unsaturated compounds. The radical intermediate could be oxidized by a metal ion to yield the stable adducted product with termination of the chain reaction. Alternatively, the radical intermediate could be oxidized by molecular oxygen to form a peroxyl radical that would abstract a hydrogen atom to form

a hydroperoxide with propagation of the radical chain. Elimination of hydrogen peroxide from the hydroperoxide intermediate would yield the same adducted product obtained through metal ion oxidation. Formation of a protein adduct can alter its function and have a detrimental structural or metabolic consequence to the cell whereas formation of a nucleotide adduct can have a mutagenic and/or carcinogenic consequence. Both types of adduct formation appear to occur in the livers of B6C3F1 mice. In Fig. 9, R· represents the dichloroacetyl radical but can just as well represent other radicals including the Cl· and OH· radical.

In the work that is proposed we will attempt to directly demonstrate the formation of the dichloroacetyl radical from TCA and the chloroacetyl radical from DCA using EPR techniques and two different spin-trapping agents. The more polar spin trap, 5,5-dimethyl-1-pyrroline 1-oxide (DMPO), will partition into both polar and non-polar phases, whereas the more lipophilic spin trap, α-phenyl-*N*-*tert*-butylnitrone (PBN), will partition largely into non-polar phases. Fig. 10 illustrates the adducts that would be expected from reaction of these spin-trapping agents with the radical formed from TCA.

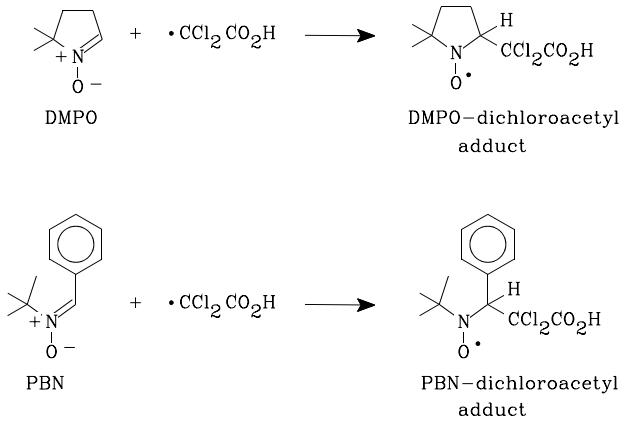

Figure 10. Spin trapping of the dichloroacetyl radical.

In initial experiments, DCA and TCA will be incubated with mouse liver microsomes in the presence of the spin-trapping agent, and the reaction mixtures will be examined by EPR for the presence of trapped radicals. The expected adducts from TCA would be derived from dichloroacetyl and peroxydichloroacetyl radicals, respectively. The participation of cytochrome P450 in this process will be determined by using specific inhibitors (*ie.*, CO and SKF525A) and examining the requirement for NADPH, O_2, and P450 reductase. In addition, microsomes from mice chronically fed DCA and TCA will be tested for induction of cytochrome P450 activity, and the livers of these animals will be examined by means of the thiobarbituric acid assay for the formation of lipid peroxides. The incorporation of DCA and TCA into proteins and nucleic acids will be determined by culturing mouse hepatocytes with ^{14}C-labeled DCA and TCA and assaying the isolated protein and DNA fractions for radioactivity. The formation of radioactive metabolites will be determined by high pressure-liquid chromatographic (HPLC) analysis of extracts, and sensitive ^{32}P-postlabeling techniques will be employed to detect the formation of DNA adducts. The formation of a stable adduct of DCA and TCA with DNA would be consistent with the mechanism of action of other carcinogenic substances and would provide a rationale for the ability of DCA and TCA to induce tumors in mice.

Preparation of Microsomes

B6C3F1 mice (Charles River) will be fasted overnight, anesthetized with ether, sacrificed by exanguination, and the liver will be removed, chilled, and perfused with 0.15 M KCl to reduce contamination with hemoglobin. The liver will then be homogenized and fractionated in 0.25 M sucrose by conventional differential centrifugation according to Hogeboom (70) as modified by Levin *et al.* (71). The microsomal fraction will be obtained by sedimentation at 100,000 x g for 1 hr, and the pellet washed twice and resuspended (20-50 mg/ml) in 0.1 M K_2HPO_4-KH_2PO_4, pH 7.4, and 0.1 mM EDTA and either used immediately or stored at -70 °C in small aliquots. Preparation time will be substantially reduced by using a rotor that can develop very high gravitational force. Comparative studies will also be conducted using microsomes prepared from rat liver.

Spin-Trapping Experiments

Incubations will be conducted at 37 °C in stirred suspensions of microsomes, 1 mg/ml, containing 50 mM Tris-chloride, pH 7.5, 5 mM $MgCl_2$, 20 mM spin-trapping agent (DMPO or PBN), NADPH, and various concentrations of DCA and TCA. At timed intervals, a sample will be removed, transferred to a flat EPR cell, and analyzed for the presence of trapped radicals with a Bruker ESP-300 spectrometer. Controls will consist of omitting each component of the incubation mixture singly and in combination. Boiled microsomes will serve as a control for enzyme activity. Incubations will also be conducted in the absence of spin-trapping agent to permit the accumulation of intermediates; the spin trap will then be added and the reaction mixture assayed for trapped radicals. Spin trapping is perhaps the the most useful modern tool available for the detection and identification of short-lived free radicals in biological systems (72,73).

To examine the requirement for O_2, the twin-compartment anaerobic apparatus described by Janzen (74) will be employed. Microsomes will be placed in one arm, the remaining components in the second arm, and the apparatus flushed with argon in a manner that will permit excess gas to escape through an inverted, attached EPR cell. The reaction will be started by tipping the contents of one arm into the other. Following an incubation period, the apparatus will be inverted to deliver its contents into the EPR cell for analysis. The same apparatus will be used to study the effect of carbon monoxide, a cytochrome P450 inhibitor (75-77), on the formation of trapped radicals.

Some controversy has existed regarding the interpretation of EPR spectra from microsomal preparations treated with CCl_4 (78). This has occurred, in part, because of the presence of mixed signals arising from oxygen-centered lipid peroxyl radicals and carbon-centered radicals derived from CCl_4 (57,67). Careful control over conditions will help avoid similar confusion. In the case of DCA and TCA, it will be possible to obtain unambiguous identification of carbon-centered radicals by using ^{13}C-labeled DCA and TCA as substrates. Conversion of these substances to a carbon-centered radical will result in a nitroxyl adduct having a distinctive EPR spectrum with additional lines indicating attachment of a ^{13}C atom *alpha* to the nitroxyl function (79,80).

An important consideration that is unique to the use of spin-trapping agents in the presence of viable cells relates to the fact that adducts can interact with intracellular redox processes and be converted to EPR-silent species. When this occurs, it can lead to a gross underestimation and even failure to detect radical formation. For example, several investigators have pointed out that decomposition of the spin-trapped superoxide radical, DMPO-O_2H, leads to various unidentified EPR-silent products (81-83), and in our own (unpublished) work we have also observed the rapid disappearance of nitroxyl species in the presence of viable cells. In general, spin adducts are subject to the same metabolic processes that convert the more stable nitroxide spin labels to EPR-silent species (84-97). While the complexity of these reactions makes the interpretation of spin-trapping experiments difficult and may limit the usefulness of some EPR studies, we believe that the ability of spin-trapping agents to interact with cellular components can be an important asset for the study of metabolic reactions that proceed by single-electron transfer. What is required are additional analytical tools for determining how much of a spin-trapped adduct has been reduced and how much oxidized. The principal EPR-silent species that can be formed from spin-trapped radicals include the products of single-electron reduction (*i.e.*, hydroxylamines), single-electron oxidation (*i.e.*, oxoammonium salts), and dimeric derivatives.

Until recently, existence of the oxoammonium salts was not widely recognized because the compounds had been described primarily in the Russian literature [see Golubev *et al.* (98)]. However, oxoammonium salts apparently participate in a major way in the dismutation of spin-trapped adducts, and their analysis is necessary for any complete assessment of trapped radicals. Thus, in our proposed work with hepatocytes, we will assume that nitroxyl adducts representing trapped radicals will be subject to metabolic conversion to EPR-silent species as illustrated with DMPO in Fig. 11. Alternative methods will be needed to quantitate these EPR-silent spin adducts.

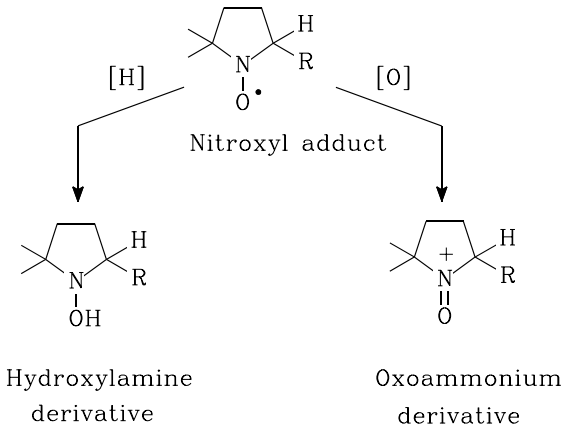

Figure 11. Conversion of nitroxyl adduct (DMPO-R) to EPR-silent species.

One approach to detecting EPR-silent species would be to incubate hepatocytes (or microsomes) with ^{36}Cl- or ^{14}C-labeled DCA and TCA and the spin-trapping agent and then separate the labeled components by means of HPLC. However, DCA and TCA are converted to a variety of products in addition to forming adducts with several types of biomolecules. Thus, low levels of spin adducts would be difficult to identify and quantitate by this method. As an alternative, we propose to synthesize and use ^{14}C-labeled spin-trapping agents in reaction mixtures. Following incubation, we will convert all nitroxyl adducts and oxoammonium salts to their stable hydroxylamine derivatives with sodium cyanoborohydride ($NaBH_3CN$) and then isolate the derivatives by means of solvent extraction and separate the labeled components by means of HPLC. Fractions will be collected and assayed for radioactivity by scintillation counting, and metabolites will be identified by comparison with standards that have been characterized by mass spectroscopy and NMR.

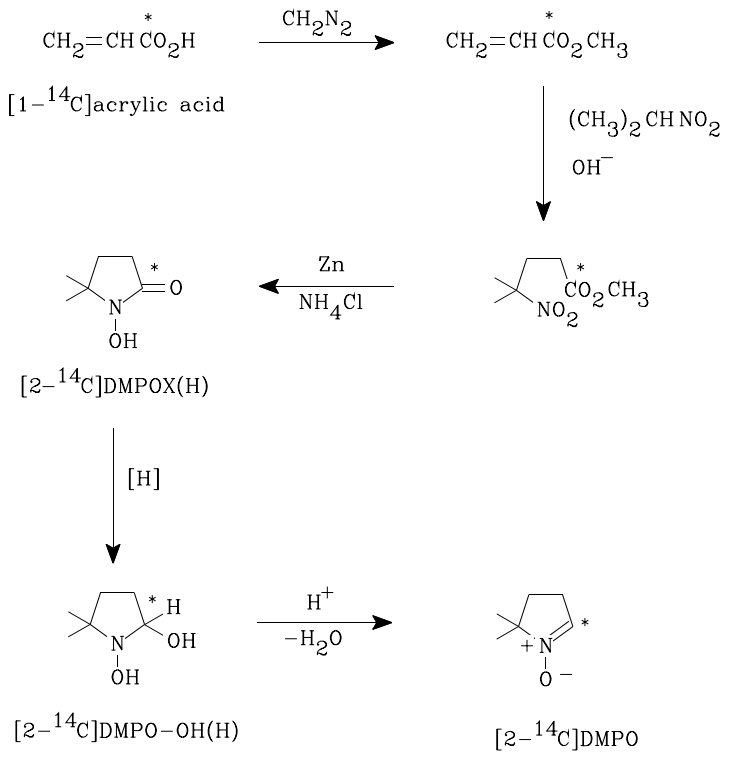

Figure 12. Proposed synthesis of [2-^{14}C]DMPO.

Initially, [2-^{14}C]DMPO will be used to study trapped radicals that are subsequently metabolized to EPR-silent species, and if this approach is successful we will also consider the use of radiolabeled PBN. DMPO was selected because of our previous experience with synthesis of the hydroxylamine derivative of 5,5-dimethyl-2-pyrrolidone 1-oxyl, DMPOX(H), and similar compounds (68,99). The synthesis outlined in Fig. 12 represents a novel pathway for the formation of radiolabeled DMPO. Commercially available [1-^{14}C]acrylic acid will be used as the starting material (Fig. 12), and the final product will be purified by preparative thin-layer chromatography. In preliminary experiments using non-radioactive materials, mass spectroscopy of the final product has verified the formation of DMPO.

Experiments with Hepatocytes

For many purposes, it is desirable to use hepatocytes in place of intact animals to study metabolic processes specific to the liver (39,60,100-103). To prepare hepatocytes, we will follow the procedure of Berry (104), as modified by Klaunig *et al.* (105). B6C3F1 mice will be anesthetized with Nembutal and the livers perfused with oxygenated Hanks' Buffered Saline Solution supplemented with 0.05% collagenase and 0.1% hyaluronidase. The treated livers will be mechanically dispersed, and the hepatocytes will be isolated by centrifugation and sieving and placed into culture using Leibovitz's L-15 medium (Grand Island).

Hepatocytes will be incubated with spin-trapping agents (DMPO, PBN, and [2-^{14}C]DMPO) in the absence and presence of DCA and TCA and examined by EPR for the formation of trapped radicals. The cells will also be cultured for up to 72 hr with various levels of the chlorinated acetates and examined again for the formation of radicals. In addition, hepatocytes will be examined for

viability and the formation of lipid peroxides. Hepatocyte viability will be determined by dye exclusion assay (106) and by the cellular content of ATP. The assay of ATP will be based on the luciferase-luciferin reaction as currently practiced in this laboratory (107).

For studies employing [2-^{14}C]DMPO, incubation mixtures will be treated with $NaBH_3CN$, extracted with ethyl acetate, and analyzed by HPLC using a reversed phase column. Fractions will be collected and assayed for radioactivity by scintillation counting, and isolated peaks will be identified by comparison with standards that will be characterized by mass spectroscopy and NMR. All data will be related to the protein content of the original samples.

Lipid Peroxidation

Hepatocytes cultured in the presence of various levels of DCA and TCA will be examined for lipid peroxidation by means of the thiobarbituric acid assay (108,109). Concentrated cell suspensions will be mixed with 6 vol of 1% phosphoric acid and 2 vol of 0.6% thiobarbituric acid and heated for 1 hr in a boiling water bath. The cooled samples will be extracted with 8 vol of n-butanol and the colored reaction products assayed at 532 nm. Although the thiobarbituric acid assay is not specific for malondialdehyde, it provides a useful measure (*i.e.*, thiobarbituric acid reacting substances, TBARS) of lipid peroxidation. More specific, and elaborate, methods are also available (110-112) for quantitating lipid peroxidation and will be used if the results with TBARS appear promising. The extent of lipid peroxidation will be correlated with the data obtained by means of spin trapping.

DNA Adducts

Recent advances in the ^{32}P-postlabeling of 3′-deoxynucleotides have made it possible to identify adducts at a sensitivity of about 1 adduct per 10^9 bases using microgram samples of DNA (113). In this technique, the DNA of treated cells is digested with nucleases to yield mixtures of normal 3′-deoxynucleotides and adducted 3′-deoxynucleotides. The latter have an increased hydrophobicity and are selectively extracted into water-saturated n-butanol containing tetrabutylammonium chloride. The adducted nucleotides are then labeled in the 5′-position with ^{32}P using polynucleotide kinase, and the ^{32}P-labeled 3′,5′-deoxynucleotides separated by multidimensional chromatography on PEI cellulose plates and assayed by autoradiography.

For the proposed studies, DNA will be isolated from control and exposed hepatocytes and from the livers of B6C3F1 mice that have been chronically fed DCA and TCA in their drinking water. To prepare DNA, the protein and RNA will be removed by incubating detergent-lysed cells with buffer-saturated phenol, and the DNA further purified by extraction with chloroform/isoamyl alcohol (113). The DNA will then be digested with micrococcal nuclease and spleen phosphodiesterase and the nucleotide adducts extracted as described above. The n-butanol layer will be washed with water to remove normal (non-adducted) nucleotides and the adducted nucleotides concentrated by centrifugal lyophylization. The adduct fraction will then be labeled with [γ-^{32}P]ATP using T_4 polynucleotide kinase (113). Excess ATP will be removed with potato apyrase, and the reaction mixture will be resolved on PEI cellulose, first to separate non-adducted ^{32}P-labeled material from slowly-migrating adducts, then to separate the ^{32}P-labeled adducts from each other. Chromatography in a second dimension using a different solvent system will then produce a fingerprint pattern that is characteristic of the adducting agent (114).

To visualize adducts, the dried chromatograms will be placed in contact with Kodak X-omat AR X-ray film in a cassette equipped with an intensifying screen and kept at -70 °C for up to 2 days. The exposed film will then be developed to yield the fingerprint pattern. Computerized images of the autoradiographs will be obtained, annotated, and stored on disk for future comparative analysis.

Gupta and Randerath (113) have summarized a wide variety of mutagenic agents that form adducts with DNA in the tissues of exposed animals. These agents yield characteristic patterns in the ^{32}P-postlabeling assay, and we anticipate that specific adducts will also be formed when animals or isolated hepatocytes in culture are exposed to DCA and TCA.

Isolated microsomes will also be studied for their ability to form adducts. For these experiments, microsomes will be incubated with exogenous, purified calf thymus DNA and ^{14}C-labeled TCE, DCA, and TCA. The DNA will be precipitated and purified as described by Kozumbo *et al.* (115) and examined by scintillation counting for the incorporation of radioactivity. This will be a direct test of the ability of mouse liver microsomes to convert the above chlorinated species into reactive derivatives capable of forming adducts. Alternatively, microsomes and DNA will be incubated with non-radioactive TCE, DCA, and TCA and the DNA isolated and subjected to the ^{32}P-postlabeling procedure. Adducts thus formed will also be compared with those obtained from the exposure of hepatocytes to TCE, DCA, and TCA in culture.

The above experiments will be varied and modified to obtain optimal conditions for adduct formation, and once this has been established we will prepare larger quantities of DNA from both hepatocytes and the livers of animals treated with ^{14}C-labeled DCA and TCA. The object of these studies will be to identify the base or bases that have been modified. The isolated DNA will be purified by phenolic extraction (116), hydrolyzed to deoxynucleoside 3′-monophosphates with micrococcal nuclease and spleen phosphodiesterase, and the adducts will be assayed by chromatography as described by Ji and Marnett (117). This approach is similar to the procedure used to identify the guanosine adduct of benzo[a]pyrene metabolites in rodent, bovine, and human cells (118,119).

REFERENCES

(119 References – Not Shown)

CURRICULUM VITAE

(Not Shown)

DETAILED BUDGET

(Not Shown)

Originally submitted for the May 15, 1994 deadline. Note that, following the initial review, the budget was increased to approximately $250,000 at the request of the Air Force program director. This is reflected on the cover page to the response to reviewers' remarks below.

FACILITIES AND EQUIPMENT

The proposed work will be conducted in two modern laboratory rooms equipped with fume hoods, balances, pH meter, incubator, water bath, and the normal array of glassware and small equipment items. Specialized equipment includes an IBM high pressure liquid chromatograph, Virtis lyophylizer, Hewlett-Packard 8451A spectrophotometer, Bio-Orbit luminometer, refrigerated fraction collection facility with ISCO components, Beckman LS-100C scintillation counter, refrigerated centrifuges, and an all glass still. Other resources available to the principal investigator are a 500 MHz NMR spectrometer (GE 500 PSG) and Kratos Concept 1-H mass spectrometer, which are operated by the Coordinated Instrumentation Facility of Tulane University. In addition, an IBM 200D EPR spectrometer located at the Southern Regional Research Center in New Orleans and a newly acquired Bruker ESP-300 EPR spectrometer that is being installed at Tulane Medical Center, will be available to support the proposed research.

HUMAN SUBJECTS

The proposed work will use blood from normal human donors recruited from the student population of Tulane Medical Center. An informed consent form approved by the Human Subjects Committee will be obtained from each potential donor. Approximately 25 donations of 100-ml each will be used per 12-month period, and neutrophils will be prepared by standard procedures established in this laboratory. Blood will be drawn in the Medical Center's Hematology Research Laboratory by a trained technician under the supervision of a staff physician. A letter of support from Dr. Cindy Leissinger, Director of the Hematology Research Laboratory, is attached.

ADDENDUM TO PROPOSAL SUBMITTED TO
AIR FORCE OFFICE OF SCIENTIFIC RESEARCH
BOLLING AFB DC, 20332-6448

Name of Institution:	Tulane University School of Medicine
Proposal Number:	94-NL-219
Proposal Title:	Radical Intermediates of Haloacetic Acids
Principal Investigator:	Carl Bernofsky, Ph.D.
Mailing Address:	Department of Biochemistry 1430 Tulane Avenue New Orleans, Louisiana 70112
Proposed Period:	2 years, 04/01/95 to 03/31/97
Total Amount Requested:	$250,876

OBJECTIVE

The widely-used industrial solvent 1,1,2-trichloroethylene (TCE) is a prevalent environmental pollutant that causes tumors in rodents. We will test the hypothesis that trichloracetic acid (TCA) and dichloroacetic acid (DCA), two TCE metabolites believed responsible for these tumors, are converted in the liver to reactive free radical intermediates that form DNA adducts that may account for their carcinigenic potential.

SCIENTIFIC APPROACH

Spin-trapping agents will be used to detect the formation of TCA and DCA radicals by mouse liver hepatocytes and isolated microsomes. Spin adducts will be examined directly, by means of electron paramagnetic resonance spectroscopy (EPR), or indirectly through the use of a ^{14}C-labeled spin-trapping agent that will be converted to a stable derivative and quantitated by means of high pressure liquid chromatography (HPLC). DNA adducts will be investigated by measuring the incorporation of ^{14}C-labeled TCA and DCA into DNA and by employing sensitive ^{32}P-postlabeling techniques. The nature of the adducts will be examined by isolating the DNA, digesting it with nucleases, and identifying the modified bases by means of HPLC and mass spectroscopy.

RESPONSE TO CRITIQUES

The literature on the cytotoxicity of trichloroethylene (TCE) has treated chloral primarily as a transient metabolic intermediate with little or no intrinsic genotoxic potential except as a precursor to dichloroacetic acid (DCA) and trichloroacetic acid (TCA), which are considered to be the major genotoxic products derived from TCE. The other product derived from chloral, trichloroethanol, is widely believed to be responsible for the sedative properties of chloral and is rapidly eliminated as the glucuronide excretory product. Despite the apparently transient involvement of chloral, both reviewers have raised provocative questions about the possible role of chloral as a cytotoxin, and an examination of current literature suggests that there is a basis for their concern.

First, recent studies with children (1) indicate that while the greater portion of a chloral bolus is quickly cleared from the blood, the remainder persists for hours, possibly in bound form. Moreover, the concentration of chloral in rabbit brain was found to persist for hours after administration of the drug by stomach tube (2). The persistence in brain is especially interesting because chloral has hypnotic and sedative effects and is widely used as a pediatric anesthetic (3). As suggested by Revewer 1, the condensation reactions of chloral may be highly significant; an example would be the condensation of chloral with biogenic amines such as dopamine (4).

In addition, chloral has recently come under close scrutiny for direct genotoxic effects, and it now appears that chloral inhibits normal spindle formation and causes chromosomal abnormalities in a variety of test systems (5-11). In particular, chloral has been found to be aneugenic in mouse spermatocytes (12,13), where it may exert its genotoxic effect through the induction of DNA strand breaks (14). Interestingly, similar effects were not observed in mouse oocytes (15).

The above observations suggest that more attention should be given to understanding the toxic effects of chloral. Presently, little is known about its biochemical reactivity. The hypothetical scheme given by the principal investigator (Fig. 3) is based on known reactions of aldehydes with $O_2^{\cdot-}$. As pointed out by Reviewer 1, this could be a significant mechanism of superoxide toxicity because the reaction product, a peroxyl radical, is a likely candidate for initiating lipid peroxidation. The rationale for the reactions indicated in Fig. 3 is based on studies of acetaldehyde metabolism in heart mitochondria (16) in which the formation of electronically-excited species was measured by means of chemiluminescence (see enclosed reprint). Briefly, we observed that metabolic conditions that favored the formation of $O_2^{\cdot-}$ by mitochondria caused bursts of light emission which we attributed to the formation of excited carbonyls and/or singlet oxygen, $^1O_2$, in accordance with the Russell mechanism. However, the formation of $^1O_2$ was entirely speculative as we did not have access to an appropriate infrared detector.

As implied by Reviewer 1, the above type of reaction is of sufficient interest to warrant further study with chloral. Accordingly, we will investigate the reaction of chloral in a model system in which $O_2^{\cdot-}$ will be generated with hypoxanthine and xanthine oxidase in the presence of the iron chelator diethylenetriaminepentaacetic acid (DPTA) to suppress $OH^{\cdot}$ radical formation. The rate of $O_2^{\cdot-}$ generation will be controlled by varying the concentration of xanthine oxidase, and the formation of electronically-excited products will be monitored by means of chemiluminescence. Assuming that these studies will be positive, we will then determine the ability of isolated hepatocytes to generate electronically-excited species from chloral and TCE. We will also seek collaboration to determine if the chemiluminescent emission has a $^1O_2$ component. The contribution of peroxyl radicals will be tested by using spin-trapping agents to inhibit the light emission, and we will attempt to correlate this inhibition with EPR studies of the spin-trapped adducts. It should be noted that, even though $O_2^{\cdot-}$ and chloral may react as predicted in a model system, the actual level of $O_2^{\cdot-}$ and TCE-derived chloral in the hepatocyte may be insufficient to permit detection of a reaction product.

DCA and TCA are persistent end products that accumulate as a result of chloral metabolism and, therefore, are more likely to be responsible for cytotoxic effects. As indicated by Reviewer 1, the carbon-centered free radical metabolites of DCA and TCA would quickly combine with oxygen to yield peroxyl radicals in the manner that has been established for the carbon-centered free radical metabolite of carbon tetrachloride and chloroform. The significance of this reaction lies in the ability of peroxyl radicals to abstract hydrogen atoms from fatty acids, thereby initiating the cytotoxic process of lipid peroxidation. Reviewer 2 suggested that differences in polarity between carbon tetrachloride and TCA may lessen the analogy between the cytotoxic effects of their respective peroxyl radical derivatives. Unlike carbon tetrachloride, TCA is very soluble in water. Nevertheless, TCA is able to partition into non-aqueous phases. For example, extraction into diethylether is a common procedure for removing TCA from TCA-treated cell extracts. Thus, it is possible that TCA-derived peroxyl radicals could initiate lipid peroxidation. Moreover, because TCA-derived peroxyl radicals are so polar, they could penetrate the aqueous regions of a cell and have access to a wide variety of components that could be damaged through hydrogen abstraction.

With regard to lipid peroxidation, Reviewer 2 pointed out the advantage of measuring lipid peroxides directly instead of assaying for malondialdehyde which could arise from several sources. Several excellent methods based on the original triiodide assay for peroxides (17,18) have been described for the determination of total lipid hydroperoxides in a variety of sample types (19-23). We will utilize these methods to directly measure the appearance of lipid hydroperoxides in solvent-extracted samples of hepatacytes and microsomal preparations treated with TCA, DCA, and TCE under various experimental conditions.

Reviewer 1 stressed the importance of controlling oxygen in our proposed spin-trapping studies because oxygen can compete with spin-trapping agents in reactions with carbon-centered radicals. We are aware of this circumstance and have proposed using the device described by Janzen (24) to create an anaerobic atmosphere for spin-trapping studies. However, it is clear that a more versatile arrangement is warranted, and we have devised a 3-chamber attachment for the standard flat cell that will allow us to control the gas phase of a reaction mixture and add the spin-trapping agent at any time without disturbing the integrity of the internal atmosphere. Ideally, we would like to have a stopped flow/chemical quench accessory for our EPR spectrometer that would allow us to measure the kinetics of rapid radical reactions under conditions of controlled atmosphere. In the absence of this sophisticated equipment, we plan to utilize motor-driven syringes to obtain kinetic information about radical reactions whose rates are not excessively fast.

Lastly, Reviewer 2 commented on the time frame needed to examine DNA adducts by means of ^{32}P-postlabeling techniques. It should be noted that we are already familiar with several aspects of this procedure; namely, the thin layer chromatography of nucleotides on PEI cellulose plates (25), the exposure and development of separated ^{32}P-labeled nucleotides on X-ray film (26), and the labeling and nuclease digestion of nucleic acids (26). In addition, local expertise and specialized reagent kits will provide assistance with the isolation of DNA from tissues.

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