CHAPTER ONE

Metal-Independent Pathways of Chlorinated Phenol/Quinone Toxicity

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Abstract

Pentachlorophenol (PCP) was the most widely used chlorinated phenols, primarily for wood preservation. The genotoxicity of PCP has been attributed to its two major quinoid metabolites: tetrachlorohydroquinone and tetrachloro-1,4-benzoquinone (TCBQ). Although the redox cycling of PCP quinoid metabolites to generate reactive oxygen species is believed to play an important role, the exact molecular mechanism underlying PCP genotoxicity is not clear. Hydroxyl radical is one of the most highly reactive oxygen species produced in biological systems. Frequently, hydroxyl radical formation from hydrogen peroxide has been ascribed to the transition metal-catalyzed Fenton reaction. We found, however, that \( \cdot \text{OH} \) can be produced by PCP quinoid metabolite TCBQ and \( \text{H}_2\text{O}_2 \), but it is not dependent on the presence of either transition metal ions or its corresponding semiquinone radical. We propose that \( \cdot \text{OH} \) production by TCBQ and \( \text{H}_2\text{O}_2 \) is through a novel nucleophilic substitution coupled with homolytical decomposition pathway, which may partly explain the potential genotoxicity of PCP and other widely used polyhalogenated aromatic biocides.

1. Introduction

1.1. Chlorinated phenols

Chlorinated phenols constitute a series of 19 compounds composed of mono-, di-, tri-, and tetrachloroisomers and one pentachlorophenol (PCP). Although all of the possible structural isomers are available commercially, only four polychlorinated phenols (2,4-dichloro-, 2,4,5-trichloro-, 2,4,6-trichloro-, and PCP) have been of major industrial significance (Figure 1), primarily

![Chemical structures of the four representative chlorinated phenols.](image-url)
as pesticides and as intermediates in the synthesis of the widely used chlorophenoxy herbicides such as 2,4-dichlorophenoxyacetic acid (2,4-D) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T), the two major components of Agent Orange (1–7). The annual worldwide production of chlorinated phenols has been estimated to be about 200,000 tons, while PCP represents about 90,000 tons of that [1,2].

Chlorinated phenols have found wide use in pesticides, disinfectants, wood preservatives, personal care formulations, and many other products. Residues of chlorinated phenols have been found worldwide in soil, water, and air samples; food products; and human and animal tissues and body fluids. Environmental contamination with these chemicals occurs from industrial effluents, agricultural runoff, breakdown of chlorophenoxyacetic acid herbicides, and hexachlorobenzene, and also from spontaneous formation following chlorination of water for disinfection and deodorization during wood pulp bleaching with chlorine [1–3]. Chlorinated phenols are poorly biodegradable with a half-life in aerobic waters that can exceed 3 months and can exceed some years in organic sediments. The existing guidelines set the permissible chlorinated phenol level (depending on Cl content) in surface water at 0.06–4.4 mg/L and the permissible concentration in drinking water at 10 μg/L [4].

However, growing knowledge about the toxicities and environmental fates of specific chlorinated phenols has caused governments to regulate these compounds. Chlorinated phenols have been found in at least 166 of the 1467 National Priorities List sites, and 5 of them are listed by the U.S. Environmental Protection Agency (EPA) as priority pollutants, including the four chlorinated phenols mentioned above and 2-chlorophenol (2-CP), which are present in the environment in significant quantities [5]. For example, chlorinated phenols were recently found to occur at relatively high concentrations in some Chinese waters. 2,4-DCP and 2,4,6-TCP were more frequently detected at higher concentrations in the rivers of North China compared with those of South China. High-concentration sites of 2,4-DCP and 2,4,6-TCP mainly occurred in the Yellow River watershed, while PCP contamination mainly occurred in the Yangtze River watershed. PCP was the most ubiquitous chlorinated phenol being detected in 85.4% of samples; 2,4-DCP and 2,4,6-TCP were detected in 51.3% and 54.4%, respectively [6]. Thus, the ubiquitous nature of these substances coupled with their carcinogenicity in animal models has raised public awareness of the potential health risks posed by these chlorinated phenols.

It is known that toxicity of simple chlorinated phenols depends on the number and position of chlorine substitutions [7]. Some chlorinated phenols, such as PCP, are regarded as classic examples of oxidative uncoupling agents due to their hydrophobic (log Kow = 5.02) and weakly acidic nature (pK_a = 4.74) [8]. PCP is still used to protect timber from fungal
rot and wood-boring insects. PCP concentrations in groundwater can be 3–23 mg/L in wood-treatment areas and concentrations in milligram per liter can be found near industrial discharges.

The toxicity and bioaccumulation of chlorinated phenols increase with the degree of chlorine substitution [7]. The pKₐ values of chlorinated phenols decrease with the number of chlorine substitutions. *Ortho*-chlorinated phenols are more acidic than other isomers because of the large inductive effect of chlorine on the hydroxyl group in close proximity [9]. Increased chlorine substitution also increases the octanol–water partition coefficient (Kow) [10], which is positively correlated to the bioaccumulation potential of chlorinated phenols [11]. Thus, PCP with the largest Kow value is the most hydrophobic chlorinated phenol, which allows it to diffuse through cellular membranes. Highly chlorinated phenols undergo oxidation into the phenoxy radicals readily at physiological pH because the oxidation step involves the concerted loss of an electron and a proton and these chlorinated phenols exist as the phenolate form under physiological conditions.

Despite being banned in many countries and having its use severely restricted in others, PCP remains an important pesticide from a toxicological perspective [12]. It is a stable and persistent compound. In humans, it is readily absorbed by ingestion and inhalation but is less well absorbed dermally. Assessment of the toxicity of PCP is confounded by the presence of contaminants known to cause effects identical to those attributed to PCP. However, severe exposure by any route may result in an acute and occasionally fatal illness that bears all the hallmarks of being mediated by uncoupling of oxidative phosphorylation. Tachycardia, tachypnea, sweating, altered consciousness, hyperthermia, convulsions, and early onset of marked rigor (if death occurs) are the most notable features. Pulmonary edema, intravascular hemolysis, pancreatitis, jaundice, and acute renal failure have been reported. There are no antidote and no adequate data available to support the use of repeat-dose oral cholestyramine, forced diuresis, or urine alkalinization as effective methods of enhancing PCP elimination in poisoned humans. Supportive care and vigorous management of hyperthermia should produce a satisfactory outcome. Chronic occupational exposure to PCP may produce a syndrome similar to acute systemic poisoning, together with conjunctivitis and irritation of the upper respiratory and oral mucosae. Long-term exposure has also been reported to result in chronic fatigue or neuropsychiatric features in combination with skin infections (including chloracne), chronic respiratory symptoms, neuralgic pains in the legs, and impaired fertility and hypothyroidism secondary to endocrine disruption [13]. In a cross-sectional study on former sawmill workers exposed to PCP in New Zealand, McLean et al. found that PCP exposure was associated with a number of physical and neuropsychological health effects that persisted long after exposure had ceased [14].
1.2. Carcinogenesis of chlorinated phenols

It has been suggested that there might be an association between chlorinated phenols and Hodgkin’s disease, soft-tissue sarcoma, and acute leukemia [15]. Carcinogenicity of orally administered PCP has been tested in rats and mice. The purity of PCP has been considered as an important factor, since PCP is usually contaminated with chlorinated dibenzo-\(p\)-dioxins, some of which (2,3,7,8-tetrachlorodibenzo-\(p\)-dioxin, TCDD) are known animal carcinogens. Technical-grade PCP (90% pure) was tested in mice by The National Toxicology Program (NTP) [16]. Groups of male and female B6C3F1 mice were given diets containing 0, 100, or 200 parts per million (ppm) PCP. A significant increase over the male control incidence in tumors of the adrenal medulla and liver was found in PCP-treated males. Treated females displayed a significant increase over female controls with regard to the incidence of hemangiosarcomas of the spleen and liver. The occurrence of rare hemangiosarcomas was considered a carcinogenic response due to PCP exposure, although this study was limited because of the unusually low survival in the male control group [16]. Pure PCP (99% pure) was also tested by NTP for carcinogenicity in rats [17]. Groups of male and female F344 rats were given diets containing 0, 200, 400, or 600 ppm PCP for 105 days. Some evidence that purified PCP is carcinogenic to rats was detected in 2 years with malignant mesotheliomas and nasal squamous cell carcinomas being noted in some of the rats with high dose regimes that actually exceed the maximum tolerable dose. However, hepatocellular adenomas and carcinomas were not detected [17], which is different from the study with mice [16]. Overall, these choric animal bioassays suggested that the liver is a target organ for carcinogenesis in mice, but not in the rat. Based on the NTP data, a cancer potency factor of 0.12 mg/kg/day was calculated by the Integrated Risk Information Systems (IRIS) [18], which translates to an upper-bound unit risk level of \(9 \times 10^{-3}\) mg/kg/day for a cancer risk of 1 in 1000.

Cooper and Jones [19] reviewed currently available data to determine the extent to which recent studies assist in distinguishing the effect of PCP from that of its contaminants (e.g., dioxins and other chlorinated phenols). They performed a systematic review of published studies pertaining to cancer risk in relation to PCP exposure, focusing on results pertaining specifically to all cancer sites and specific hematopoietic cancers, and data pertaining to risks associated with other types of chlorinated phenols, dioxins, or furans. They found that the PCP studies presented considerable evidence pertaining to hematopoietic cancers, with strong associations seen in multiple studies, in different locations, and using different designs. There is little evidence of an association between these cancers and chlorinated phenols that contain fewer than four chlorines. The extension of a large cohort study of sawmill workers, with follow-up to 1995, provided
information about risks of relatively rare cancers (e.g., non-Hodgkin lymphoma, multiple myeloma), using a validated exposure assessment procedure that distinguishes between exposures to PCP and tetrachlorophenol. In contrast with dioxin, PCP exposure has not been associated with total cancer incidence or mortality. They concluded that the updated cohort study focusing on PCP provides increased statistical power and precision and demonstrates associations between hematopoietic cancer and PCP exposure not observed in earlier evaluations of this cohort. Contaminant confounding is an unlikely explanation for the risks seen with PCP exposure.

Since PCP was the most well-studied chlorinated phenol, therefore, PCP will be used as an example for further review. We will focus on a novel mechanism for metal-independent decomposition of hydroperoxides by PCP quinoid metabolites and production of hydroxyl/alkoxyl radicals, which has been suggested as a potential mechanism for PCP-induced carcinogenicity.

## 2. Pentachlorophenol

### 2.1. PCP and its major carcinogenic quinoid metabolites

PCP is a major industrial and agricultural biocide that has been used primarily as a wood preservative [20,21]. The annual production of PCP has been estimated to be about 46 million pounds in the United States. Because of its efficiency, broad spectrum, and low cost, PCP has also been used as algaecide, bactericide, fungicide, herbicide, insecticide, and molluscicide [20–23]. In the USA, ~97% of PCP was used as a wood preservative [20,21]. The majority of U.S. use of PCP has been to preserve wooden poles for power transmission lines and other utilities. In China and other developing countries, PCP has also been used to kill snails to prevent snail fever (Schistosomiasis). Its worldwide usage and relative stability make PCP a ubiquitous environmental pollutant [20–23]. In fact, PCP has been detected in body fluids, such as human urine, serum, and milk, and tissues of people who are not occupationally exposed to it [20–23]. Extensive studies of PCP concentrations in body fluids (plasma or urine) of nonoccupationally exposed individuals have found average PCP concentrations of 40 parts per billion (ppb) (range: 0–1840 ppb). In contrast, in blood of occupationally exposed workers, the median level of PCP was found to be as high as 19,580 ppb (range: 6000–45,200 ppb). Postmortem analysis of serum, tissue, and urine samples from individuals who died from PCP intoxication showed tissue PCP concentrations of 20–140 ppm and urine concentrations of 28–96 ppm. The most likely source of exposure is PCP-treated wood products by way of the food chain. In groups of individuals who are not specifically exposed to PCP, net daily intake estimated in eight countries
varied from 5 to 37 µg. Net intake was between 51 and 157 µg/day in residents of homes made of PCP-treated logs [20]. In individuals occupationally exposed to PCP, net daily intake varied widely, from 35 to about 24,000 µg, depending on the type of work [20–23]. Human exposure to PCP can also originate, albeit to a minor extent, through metabolic formation from hexachlorobenzene or hexachlorocyclohexane, which are also ubiquitous environmental contaminants [20–23].

PCP is a potent carcinogen. Following chronic exposure of B6C3F1 mice to PCP, hepatocellular carcinomas or adenomas, hemangiosarcomas, and phaeochromocytomas were observed [24]. In a recent report, malignant mesothelioma and nasal squamous cell carcinomas were induced in F334/N rats [25]. In humans, malignant lymphoma and leukemia have been associated with occupational exposure to PCP [20–23]. PCP has been found in at least 313 of the 1585 National Priorities List sites, listed as a priority pollutant by the U.S. EPA, and classified as a group 2B (possibly carcinogenic to humans) environmental carcinogen by the International Association for Research on Cancer (IARC) [12,20,21]. While the precise mechanism of PCP’s genotoxicity remains to be elucidated, it has been suggested that its quinone and semiquinone metabolites play an important role (Figure 2).

PCP is oxidatively dechlorinated to produce tetrachlorohydroquinone (TCHQ) by liver microsomal cytochrome P450s from rats and humans in vitro [23,26] and by rodents in vivo [27–30]. About 20% of PCP was recovered in urine of PCP-treated B6C3F1 mice as TCHQ and its glucuronide and sulfate conjugates [28]. TCHQ can be further oxidized to tetrachloro-1,4-benzoquinone (TCBQ) via its corresponding semiquinone,

![Figure 2: PCP and its carcinogenic quinoid metabolites: TCHQ, tetrachlorohydroquinone; TCSQ*, tetrachlorosemiquinone radical; TCBQ, tetrachloro-1,4-benzoquinone.](image-url)
the tetrachlorosemiquinone radical (TCSQ•) [31]. TCBQ and its thiol and glucuronide conjugates were also found in animals and humans [26–30]. TCBQ has also been observed as a reactive oxidation intermediate or product in processes used to oxidize or destroy PCP and other polychlorinated persistent organic pollutants (POPs) in various chemical and enzymatic systems [23,32–35]. TCBQ itself has been widely used as a fungicide (Spergon) for treatment of seeds and foliage, and as an oxidizing or dehydrating agent in organic synthesis (often called p-chloranil [23]).

The Rappaport laboratory has developed an assay to simultaneously quantitate protein adducts of quinones and semiquinones following PCP administration to rats and mice [36]. They found that TCBQ is a Michael acceptor and forms adducts with cysteinyl residues of proteins both in vitro and in vivo [36]. TCBQ was found to retain its oxidized quinone structure following covalent attachment of cysteinyl residues with chloride displacement. The monoadduct continues to react with additional sulfhydryls leading to di- and tri-substituted adducts. This assay employs Raney nickel to selectively cleave the cysteinyl adducts, which generates quinones or tetrachlorophenols from semiquinone-derived adducts, and the latter products were only detected in vivo [36]. Therefore, this assay can be used to measure the extent of quinone versus semiquinone adduct formation during PCP metabolism. In this regard, administration of a single oral dose of PCP to Sprague–Dawley rats and B6C3F1 mice generated proportionally greater amounts of tetrachloro-1,2-semiquinone adducts in the livers of the rodents at low doses of PCP (<4–10 mg/kg body weight) that was 40-fold greater in rats than in mice. Production of TCBQ adducts was proportionally greater at high doses of PCP (460–230 mg/kg body weight) that was 2- to 11-fold greater in mice than in rats over the entire range of doses [36]. These results suggested that species differences in the metabolism of PCP to semiquinones and quinones were, in part, responsible for the production of liver tumors in mice but not in rats [36].

Redox cycling of compounds with a quinoid structure is a well-known phenomenon [37,38]. The cyclic (auto)oxidation and reduction reactions with the intermediary formation of semiquinone radicals can produce large amounts of reactive oxygen species (ROS) by reducing molecular oxygen to superoxide (O2•−), which in turn can induce oxidative stress [37,38]. However, it is generally accepted that O2•− itself is not directly attacking DNA, but only after its dismutation to hydrogen peroxide (H2O2) and subsequent metal-mediated cleavage to hydroxyl radical (•OH). This reaction sequence is called the Haber–Weiss reaction, or superoxide-driven Fenton reaction (M represents transition metals, especially iron and copper) [39]:

\[
\begin{align*}
2\text{O}_2\text{•}^- + 2\text{H}^+ & \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \\
\text{O}_2\text{•}^- + \text{M}^{n+} & \rightarrow \text{O}_2 + \text{M}^{(n-1)+} \\
\text{H}_2\text{O}_2 + \text{M}^{(n-1)+} & \rightarrow \text{•OH} + \text{OH}^- + \text{M}^{n+}
\end{align*}
\]
While PCP itself does not show any reactivity toward DNA, TCHQ was found to induce single-strand breaks in isolated DNA [30], a variety of cell lines [31,40–42], and liver of mice [43,44]. TCHQ also induced micronuclei and mutations at the HPRT locus of V79 cells [45,46], and the formation of 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in V79 cells [47] and B6C3F1 mice [43]. At low concentrations, TCHQ reduced colony-forming ability of human fibroblasts [40], and inhibited cell growth in CHO cells [42]. Recently, TCHQ was found to induce formation of direct adducts, apurinic/apyrimidinic (AP) sites, and oxidized bases in human HeLa S3 cells [48].

2.2. Molecular mechanisms of PCP quinoid metabolite-induced DNA damage

Both oxidative DNA damage and direct DNA adducts have been implicated in PCP-induced mouse liver carcinogenesis [23,42,44,48]. The most commonly analyzed biomarker of oxidative DNA damage, 8-OH-dG, was detected in the livers of B6C3F1 mice that received acute and subacute doses of PCP and TCHQ, and the levels were increased significantly over the corresponding controls [42]. Recent findings in Fischer 344 rats that had been administered PCP for 27 weeks also revealed a statistically significant increase in 8-OH-dG in hepatic DNA over control [49]. In addition to oxidized bases, it is likely that other types of oxidative DNA lesions are involved. Redox cycling of PCP quinoid metabolites to generate ROS is believed to play an important role in PCP genotoxicity.

Among ROS, *OH is regarded as the most reactive one produced in biological systems. DNA damage resulting from attack by *OH includes base oxidation, deoxyribose damage, strand breaks, and AP sites [39]. *OH can induce AP sites by direct hydrogen abstraction from the sugar moiety of DNA, resulting in 5'-nicked oxidized AP sites formation [50–52]. Another pathway for AP sites formation may involve depurination/depyrimidination of quinone–DNA adducts. TCBQ and its corresponding semiquinone radical are reactive electrophiles and therefore are also capable of alkylating DNA and forming DNA adducts [49,53,54]. Recently, Lin et al. [53] showed that low degree of oxidative and direct DNA damage was produced by high concentrations of TCBQ.

Comparison of the genotoxicity of TCHQ to H2O2 in human fibroblasts also revealed a greater genotoxic potential for TCHQ than for H2O2 [46]. DNA damage was determined after 1 h treatment with TCHQ or H2O2 by the comet assay (also called single cell gel electrophoresis assay). A distinct tail moment was noted for TCHQ at concentrations of 10 μM, whereas 60 μM H2O2 was required to produce the same extent of DNA damage. By monitoring the incorporation of [3H]-thymidine into DNA of nonreplicating cells (UDS), the extent of DNA repair was measured and
25 μM TCHQ was found to inhibit repair, while H$_2$O$_2$ continuously induced DNA repair up to 60 μM. In contrast to H$_2$O$_2$, TCHQ was also mutagenic in the HPRT locus of V79 cells with a mutant frequency of 75 and 151 mutants per 10$^6$ cloneable cells at nontoxic concentrations of 5 and 7 μM. It was suggested [46] that the TCSQ radical may react with DNA directly and cause AP sites which are transformed to strand breaks either by endonucleases or under the alkaline conditions of the comet assay. This would explain the ineffectiveness of dimethyl sulfoxide (DMSO) to quench TCHQ-induced damage in cellular DNA. However, an alternative explanation may stem from TCHQ-induced •OH production by a metal-independent process (see below) that takes place close to the DNA surface, precluding effective •OH scavenging by DMSO. The fact that TCHQ also inhibits DNA repair enzymes would provide a rationale for its potent genotoxicity and mutagenicity. In contrast, DNA damage by H$_2$O$_2$ would require transition metal ions for catalysis and DNA repair enzymes are not inhibited by H$_2$O$_2$ [46].

While little is known about direct interactions of TCSQ radical with DNA, the fully oxidized PCP quinoid metabolite TCBQ is known to react covalently with DNA to generate DNA adducts, as evidenced by the $^{32}$P-postlabeling assay [49,52]. Treatment of calf thymus (CT) DNA with 5 mM TCBQ generated four major and several minor adducts (3.5 adducts per 10$^5$ total nucleotides) [53]. These adducts were chemically stable and do not generate AP sites. In addition, increases in 8-oxo-dG and AP sites were observed that were ascribed to oxidative damage. These results demonstrated that PCP quinone and hydroquinone metabolites induce direct and oxidative base modifications as well as the formation of 5′-cleaved AP sites in genomic DNA [53]. Cell culture and in vivo studies have shown that PCP itself causes direct genotoxicity under certain conditions. Treatment of rat hepatocytes with a single dose of PCP (50 μM) generated 17 adducts per 10$^9$ total nucleotides. Chronic (60 mg/kg/day for 27 weeks), but not acute (60 mg/kg/day for 1 or 5 days), exposure of rat to PCP induced a twofold increase in 8-oxo-dG (1.8 vs. 0.91 × 10$^6$ in controls) and generated two major adducts, one derived from TCBQ, with relative $^{32}$P-postlabeling of 0.78 adducts per 10$^7$ total nucleotides [49]. The TCBQ-derived DNA adduct was also detected in mouse liver DNA following exposure to PCP at 8 adducts per 10$^7$ nucleotides which is 10-fold greater compared with the rat. The greater amounts of both oxidative and direct DNA damage, together with increased hepatotoxicity and cell proliferation, may provide the critical events necessary for hepatic carcinogenesis in the mouse. In contrast, the decreased amount of DNA damage and the lack of hepatotoxicity and cell proliferation in the rat do not result in such critical changes [49].

As mentioned above, DNA adducts attributable to TCBQ have been observed previously in vitro and detected in vivo. In addition, an unidentified adduct in these studies co-eluted with the product of the reaction of
deoxyguanosine (dG) and TCBQ. Sturla’s group [55] have synthesized, isolated, purified, and characterized the predominant adduct formed from the reaction of dG and TCBQ. They proposed that the adduct is a dichloro-benzoquinone nucleoside in which two chlorine atoms in TCBQ have been displaced by reaction at the 1 and N^2 positions of dG. Their results suggest that direct reactions between chlorophenols and DNA may play a role in the toxic effects of chlorinated phenols and indicate a potential difference in reactivity and biological influence between PCP and other less substituted chlorinated phenols. They further elucidated the structure of new agent-specific DNA adducts resulting from the reaction of dGuo, dCyd, and Thd with TCBQ [56]. Two dGuo adducts and one dCyd adduct resulting from the reaction of double-stranded DNA with TCBQ were identified. The results indicate that, in the structural context of DNA, TCBQ reacts most readily with dGuo compared to the other DNA bases and that the mode of TCBQ reactivity is dependent on the base structure, that is, multiple types of adducts are formed. DNA adducts consistent with TCBQ reactions were also observed when DNA or dGuo was treated with PCP and a peroxidase-based bioactivating system.

The association of chlorinated phenols to incidences of leukemia was also found to be consistent with the leukemogen activity of phenolic xenobiotics such as phenol and the phenolic anticancer drug etoposide [5]. Phenolic compounds are substrates for myeloperoxidase present in bone marrow and could be converted into phenoxyl radicals. Direct reactions of phenoxyl radicals and thiol radicals with biomolecules could also contribute to peroxidase-driven toxic effects of phenolic xenobiotics. With regard to direct reactions of phenoxyl radicals with DNA, an in vitro study by the Turesky’s group demonstrated that adduct levels (3679/10^5 nucleotides) by 0.1 mM PCP following activation by horseradish peroxidase (HRP)/H_2O_2 are 30-fold higher than levels induced by CYP450-containing microsomes and 10-fold higher than levels induced by TCBQ (5 mM) itself [57]. This suggested the possibility that the pentachlorophenoxyl radical generated by HRP/H_2O_2-mediated PCP oxidation might contribute to DNA adduct formation. Manderville’s group [58] found that treatment of PCP (0.1 mM) with HRP/H_2O_2 or myeloperoxidase (MPO/H_2O_2) with excess dG (2 mM) led to the isolation and identification of the oxygen-bonded C8-dG nucleoside adduct. The reaction was absolutely specific for dG; no detectable adduct(s) was observed from HRP/H_2O_2 and PCP in the presence of deoxyadenosine, deoxycytidine, or thymidine. Formation of the oxygen-bonded C8-dG nucleoside adduct was also specific for peroxidase activation that is known to oxidize PCP into its phenoxy radical. Treatment of PCP/dG with rat liver microsomes (RLM) failed to generate the adduct; instead, an adduct derived from the benzoquinone electrophile TCBQ was observed in the extracted ion chromatogram from the RLM/NADPH-treated PCP/dG sample. The oxygen-bonded
C8-dG nucleoside adduct is the first structurally characterized O-bonded phenolic DNA nucleoside adduct and highlights the ambident electrophilicity of phenoxy radicals (O vs. C) in reaction at C8 of dG. Given that PCP is known to induce DNA adduct formation in vivo and human exposure has been linked to incidences of leukemia, the adduct could play a key role in PCP-mediated carcinogenesis [58].

Then they expand their investigations on a wider range of chlorinated phenol substrates to establish their reactivity toward dG and duplex DNA (CT) following activation by HRP/H2O2, as a representative peroxidase system [59]. Their data showed that chlorophenoxyl radicals may either react directly with dG and CT-DNA to form C8-dG O-adducts in an irreversible process or couple to yield 1,4-BQ electrophiles that react with dG to afford adducts of the benzetheno variety. These results established the in vitro relevance of C8-dG O-adducts of phenolic toxins. The 1H NMR chemical shifts and reactivity of the benzetheno adducts favor 400'-hydroxy-1,N2'-benzetheno-dG adduct assignment, which is in contrast to the other literature which has assigned the 1,4-BQ-dG adduct as 300'-hydroxy-1,N2'-benzetheno-dG. Overall, their study has provided new insights into peroxidase-mediated activation of chlorinated phenol substrates and has strengthened the hypothesis that direct reactions of chlorophenoxyl radicals with DNA contribute to peroxidase-driven toxic effects of chlorophenolic xenobiotics.

2.3. The pro-oxidant versus antioxidant equilibrium

The pro-oxidant potential of biological systems and their antioxidant capacity usually are in an approximate equilibrium [39]. In normal cells, a primary defense against oxidative damage is provided by small molecule antioxidants such as glutathione (GSH) and ascorbate, which are present in millimolar concentrations. However, these defense mechanisms can be overwhelmed by xenobiotics such as PCP and its metabolites that induce the production of excessive ROS, which can result in damage to biological macromolecules such as DNA [37–39]. It should be noted that the concentration of ROS, including H2O2, in cells under normal physiological conditions is low, but ROS concentration may be significantly increased in cells that are subjected to oxidative stress conditions such as exposure to PCP metabolites, as we showed recently in NIH 3T3 cells [60]. TCHQ treatment was shown to cause more than 60% GSH depletion in liver tissues of mice, possibly by forming GSH-conjugates [30,37,38,44]. It was suggested that depletion of GSH and other antioxidants by PCP metabolites could abolish the protective ability of the cell against ROS and lead to DNA damage [45]. It is thus reasonable to hypothesize that PCP metabolite-induced DNA damage could be prevented if the levels of intracellular antioxidants were raised through supplementation of dietary antioxidants. Indeed, it has been demonstrated recently [61] that oral administration of
antioxidant vitamin E and diallyl sulfide 3 h before each PCP challenge significantly protected against elevation of hepatic 8-OH-dG levels in male B6C3F1 mice, while vitamin C, epigallocatechin gallate, and ellagic acid showed partial protection. These findings indicate that PCP-induced oxidative DNA damage in the target organ liver can be blocked by a number of dietary antioxidants.

3. Mechanism of Protection by the “Specific” Iron-Chelating Agent Desferrioxamine Against TCHQ-Induced DNA Damage

3.1. The protection by desferrioxamine against TCHQ-induced DNA damage was not due to its classic iron-chelating property, but rather to its scavenging of the reactive TCSQ.*

As discussed above, TCHQ has been identified as one of the main toxic metabolites of PCP. TCHQ can induce DNA single-strand breaks and has also been implicated in PCP-associated genotoxicity. The ability of TCHQ to induce DNA damage has been previously attributed to its ability to form \(^\cdot\)OH through the classic metal-dependent Fenton reaction (see above). This notion was based on the fact that TCHQ-induced DNA damage was completely prevented by desferrioxamine (DFO, also called Desferal® and deferoxamine). DFO has been used as an iron-chelating agent for the treatment of iron overload. This includes clinical cases of individuals who have ingested toxic oral doses of iron salts or require multiple blood transfusions, such as in the treatment of \(\beta\)-thalassemia. DFO is a linear trihydroxamic acid siderophore that forms a kinetically and thermodynamically stable complex with ferric iron, ferrioxamine (Figure 3). Its high binding constant (log \(\beta = 31\)) and its redox properties (\(E^0 = -0.45\) V) render the bound iron unreactive for the catalysis of oxygen radical production as has been implicated in a variety of biological processes. It has been classically assessed that prevention of damage by DFO was a sufficient proof for the role of loosely bound iron in the injurious processes. Although DFO has been repeatedly used to probe metal-catalyzed hydroxyl radical formation in biological systems, recent studies demonstrated the ability of this trihydroxamate compound to act as radical scavenger, in addition to and independent of its iron-binding properties. Diethylenetriaminepentaacetic acid (DTPA) is an analog of the widely used chelating agent ethylenediaminetetraacetic acid (EDTA). DTPA could also form a kinetically and thermodynamically stable complex with ferric iron (Figure 4; log \(\beta = 28\); \(E^0 = +0.03\) V). Both DFO and DTPA have been widely used to study the role of iron in various chemical and biological systems, and therefore
we employed these two structurally different but relatively specific iron-chelating agents to probe whether iron played any role in TCHQ-induced DNA damage. We found [62] that DFO protected against TCHQ-induced DNA single-strand breaks in isolated DNA, while other iron chelators such as DTPA did not. To better understand its underlying molecular mechanism, the auto-oxidation process of TCHQ yielding TCSQ* intermediate was studied in the presence and absence of these two iron-chelating agents.
We found that DFO led to a marked reduction in both the concentration and half-life of TCSQ\(^*\). Interestingly, the decay of TCSQ\(^*\) was accompanied by concurrent formation of DFO–nitroxide radicals (DFO\(^*\)), which contains the structural component \(-\text{CH}_2\text{–NO}^\bullet\text{–CO–}\) and gives a characteristic nine-line spectrum as a result of splitting of the nitroxide nitrogen coupling (\(d^N = 7.9 \text{ G}\)) by two protons [\(\sigma(2)\text{H} = 6.3 \text{ G}\)] from the neighboring \(\text{CH}_2\) group (Figure 5). These effects have been demonstrated both by UV–visible and electron spin resonance (ESR) spectral methods. In contrast, DTPA had no detectable effect on TCHQ auto-oxidation. These results suggest that the protection by DFO against TCHQ-induced DNA damage was not due to its binding of iron, but rather due to its scavenging of the reactive TCSQ\(^*\) [62].

Interestingly, we found that DFO could also dramatically enhance the hydrolysis (dechlorination) of TCHQ (and TCBQ) to form chloranilic acid (2,5-dichloro-3,6-dihydroxy-1,4-benzoquinone, DDBQ) [62]. The exact underlying molecular mechanism has recently been investigated in our lab, and to our surprise, a novel double Lossen rearrangement reaction was found to be involved in this process (see Section 5). Compared to TCBQ, the DDBQ molecule was considered to be more stable, less reactive, and much less toxic; therefore, the enhanced formation of DDBQ from TCBQ catalyzed by DFO also contributed to its reduction of the toxicity of TCHQ. Further, the enhanced conversion of TCHQ to DDBQ reduced the possibility of redox cycling between TCBQ and TCHQ in the cell, whereby repeatedly producing TCSQ\(^*\).

![Figure 5](image)

**Figure 5** The decay of tetrachlorosemiquinone radical (TCSQ\(^*\)) was accompanied by the concurrent formation of desferrioxamine (DFO) nitroxide radical, DFO\(^*\) (modified based on Ref. [62]).
3.2. Protection by DFO against TCHQ-induced cyto- and genotoxicity in human fibroblasts

Then we extended our study from isolated DNA to human fibroblasts, and from DFO to other hydroxamic acids. Cyto- and genotoxic effects of PCP metabolites were evaluated, respectively, by the MTT and “comet” assay (also called single cell gel electrophoresis). We found [63] that co-incubation of DFO provided marked protection against both the cyto- and genotoxicity induced by TCHQ. Pretreatment of the cells with DFO followed by washing also provided protection, although less efficiently compared to the simultaneous treatment. Similar patterns of protection were also observed for three other hydroxamic acids: aceto-, benzo-, and salicyl-hydroxamic acid (AHA, BHA, and SHA). Spectral studies showed that the three hydroxamic acids tested other than DFO also effectively scavenged the reactive TCSQ* and enhanced the formation of the less reactive and less toxic chloranilic acid (Figure 6). DMSO, an efficient *OH scavenger, provided partial protection only at high concentrations. The results of this study demonstrated that the protection provided by DFO and other hydroxamic acids against TCHQ-induced cyto- and genotoxicity

![Figure 6](image-url)  
*Figure 6* Inhibition of tetrachlorosemiquinone radical (TCSQ*) formation by desferrioxamine (DFO) and other hydroxamic acids monitored at 455 nm. AHA, aceto-hydroxamic acid; SHA, salicyl-hydroxamic acid; and BHA, benzo hydroxamic acid (modified based on Ref. [63]).
in human fibroblasts is mainly through scavenging of the observed reactive TCSQ and not through prevention of the Fenton reaction by the binding of iron in a redox-inactive form [63].

4. **Molecular Mechanism of PCP Quinoid Metabolite-Induced Genotoxicity**

4.1. **Metal-independent production of *OH by PCP quinoid metabolite and H₂O₂**

The above findings suggest that iron was not involved in TCHQ-induced DNA damage. In another word, TCHQ-induced DNA damage may not be due to the iron-mediated *OH production through the classic Fenton reaction. Then the question became what was the underlying molecular mechanism for PCP metabolites-mediated *OH production? To test whether *OH can be produced by PCP metabolites, we first employed the well-known salicylate hydroxylation method. HPLC with electrochemical detection was used to measure the levels of 2,3- and 2,5-dihydroxybenzoic acid (DHBA) formed when *OH reacts with salicylate. We found [64] that TCHQ and H₂O₂ could produce both 2,3- and 2,5-DHBA when incubated with salicylate. Their production was markedly inhibited by the *OH scavenging agents DMSO and ethanol. In contrast, their production was not affected by the nonhydroxamate iron chelators and the copper-specific chelator. Based on these results, we suggested that *OH was produced by TCHQ and H₂O₂, possibly through a metal-independent Fenton-like reaction [64].

Since the salicylate hydroxylation method cannot provide direct evidence for *OH formation, a more specific method, such as secondary radical ESR spin trapping with 5, 5-dimethyl-1-pyrroline N-oxide (DMPO), is needed to further substantiate and extend our previous observations. A typical DMPO/*OH signal, and DMPO/*CH₃ signal derived from *OH attack on DMSO, will be more conclusive evidence for *OH production from H₂O₂ and TCHQ or TCBQ [40] (Figure 7). We found [65] that when incubated with DMPO, TCBQ and H₂O₂ produced the DMPO/*OH adduct. In contrast, incubation of either compound alone did not cause *OH formation. The formation of DMPO/*OH was markedly inhibited by the *OH scavenging agents DMSO and formate, with the concomitant formation of the characteristic DMPO adducts with *CH₃ and *COO⁻, respectively (Figure 8). These secondary radical ESR spin-trapping results provided definitive evidence that *OH could indeed be produced by TCBQ and H₂O₂.

Then a critical question arose: Was the production of *OH by TCBQ and H₂O₂ metal-dependent or -independent? To answer this question, the
potential role of catalytic transition metals contaminating the DMPO/TCBQ/H$_2$O$_2$ reaction system was carefully examined by using several structurally different and relatively specific metal chelating agents for iron and copper [66–68]. Neither the DMPO/•OH signal nor the DMPO/•CH$_3$ signal produced by the DMPO/TCBQ/H$_2$O$_2$ system in the absence and presence, respectively, of DMSO was affected by the
addition of various nonhydroxamate iron-chelating agents, viz., bathophenanthroline disulfonate (BPS), ferrozine, and ferene, as well as the copper-specific chelating agent bathocuproine disulfonate (BCS). In addition, no significant decrease in the DMPO/*OH and DMPO/*CH₃ signal was observed by low concentrations (≤ 10 μM) of the trihydroxamate iron-chelating agent DFO. These DFO concentrations should be sufficient to chelate any trace amounts of iron contaminating the chelex-pretreated buffer. However, the formation of DMPO/*OH and DMPO/*CH₃ was abolished by high concentrations of DFO (≥ 1 mM), with the concurrent formation of the DFO•. As discussed before, the inhibition of *OH production by DFO was not due to its iron-binding capacity, but rather due to its ability to scavenge TCSQ•. Similar marked inhibition of DMPO/*OH and DMPO/*CH₃ formation was observed with another TCSQ• scavenger, BHA. In addition, even when trace amounts of iron (Fe(II), 0.5 μM) were added to the DMPO/TCPQ/H₂O₂ system, no increase in *OH production was observed.

In contrast, the formation of both DMPO/*OH and DMPO/*CH₃ by the DMPO/Fe(II)/H₂O₂ system in the absence and presence, respectively, of DMSO was almost completely inhibited by the nonhydroxamate iron-chelating agents BPS, ferrozine, and ferene, as well as the hydroxamate iron-chelating agent DFO. No concurrent formation of the DFO• was detected, indicating that DFO acted by chelating iron in this classic Fenton system. These results clearly demonstrated that the production of *OH by TCBQ and H₂O₂ is independent of transition metal ions.

It should be noted that the metal-independent production of *OH was not limited to TCBQ and H₂O₂, but was also observed in the presence of other halogenated quinones, that is, 2-chloro-, 2,5-dichloro-, 2,6-dichloro-, trichloro-, tetrafluoro-, and tetrabromo-1,4-benzoquinone (Figure 9). In contrast, no *OH production was detected from H₂O₂ and the nonhalogenated quinone, 1,4-benzoquinone, and the methyl-substituted quinones 2,6-dimethyl- and tetramethyl-1,4-benzoquinone [65].

4.2. Molecular mechanism of metal-independent production of *OH by PCP quinoid metabolites and H₂O₂

Based on the above experimental results, we first proposed [64,65] that the production of *OH by TCBQ and H₂O₂ might be through a metal-independent semiquinone-mediated organic Fenton reaction:

\[
\text{TCSQ}^\bullet + \text{H}_2\text{O}_2 \rightarrow \bullet \text{H} + \text{OH}^- + \text{TCBQ}
\]

where TCSQ• substitutes for ferrous iron in the classic, metal-dependent Fenton reaction. This type of reaction between semiquinone radicals and
H$_2$O$_2$ has been previously proposed by Koppenol and Butler [69], who suggested that if a quinone/semiquinone couple has a reduction potential of between $\sim$330 and +460 mV, it can theoretically bring about a metal-independent Fenton reaction. It was suggested that such reactions are thermodynamically feasible and do not require metal ions for catalysis [69,70], which might be the case in this study, where the reduction potentials of the quinone/semiquinone couples for 2-chloro-, 2,5-dichloro-, tetrafluoro-, tetrabromo-, and tetrachloro-1,4-benzoquinone are $\sim$100, $\sim$60, $\sim$200, $\sim$240, and $\sim$250 mV, respectively [71]. These values are within the suggested range of $\sim$330 to +460 mV. In contrast, the reduction potentials for 2,6-dimethyl-1,4-benzoquinone and tetramethyl-1,4-benzoquinone are $\sim$430 and $\sim$600 mV, respectively [71], are outside this range, and indeed, no $^\bullet$OH formation could be detected.

If the above mechanism was correct, then the production of $^\bullet$OH from H$_2$O$_2$ and TCBQ should be dependent on the concentration of TCSQ$^\bullet$, that is, the higher the concentration of TCSQ$^\bullet$, the more $^\bullet$OH should be produced. Further, the main product of this reaction should be TCBQ. Using secondary radical ESR spin-trapping method, we found that DMPO/$^\bullet$CH$_3$ and DMPO/$^\bullet$OH adducts can be produced by H$_2$O$_2$ and TCBQ in the presence of the spin-trapping agent DMPO and $^\bullet$OH scavenger DMSO. However, no DMPO/$^\bullet$CH$_3$ and DMPO/$^\bullet$OH adducts were detected from H$_2$O$_2$ and TCHQ (the reduced form of TCBQ), although high concentrations of TCSQ$^\bullet$ could be produced during the auto-oxidation of TCHQ. Interestingly, if TCHQ was quickly oxidized to TCBQ with MPO, DMPO/$^\bullet$CH$_3$ and DMPO/$^\bullet$OH adducts could be detected again, similar to that produced by TCBQ (Figure 10).

Further, the formation of DMPO/$^\bullet$CH$_3$ and DMPO/$^\bullet$OH was found to be directly dependent on the concentrations of TCBQ and H$_2$O$_2$. These
results strongly suggest that TCBQ, but not its corresponding semiquinone radical TCSQ\(^*\), is essential for \(\cdot\text{OH}\) production. Therefore, the production of \(\cdot\text{OH}\) by TCBQ and \(\text{H}_2\text{O}_2\) appears not to occur through a semiquinone-dependent organic Fenton reaction.

To get more information on the mechanism of \(\cdot\text{OH}\) production by TCBQ/\(\text{H}_2\text{O}_2\), the time- and concentration-dependent production of DMPO/\(\cdot\text{OH}\) by TCBQ/\(\text{H}_2\text{O}_2\) was studied. Two distinct phases were observed: the first fast phase (about 30 s) and the second slower phase. This indicates that \(\cdot\text{OH}\) may be produced by two-step reactions between TCBQ and \(\text{H}_2\text{O}_2\). When TCBQ concentration was fixed at 0.1 mM, the rate of DMPO/\(\cdot\text{OH}\) production was dependent on \(\text{H}_2\text{O}_2\) concentration. It should be noted that DMPO/\(\cdot\text{OH}\) could be detected at \(\text{H}_2\text{O}_2\) concentration as low as 10 \(\mu\text{M}\). When \(\text{H}_2\text{O}_2\) concentration was fixed at 0.1 mM, DMPO/\(\cdot\text{OH}\) could be detected at TCBQ concentration as low as 5 \(\mu\text{M}\). Further, UV–visible spectral studies showed that there was a direct interaction between TCBQ and \(\text{H}_2\text{O}_2\), with the reaction mixture changing quickly from the original yellow color (\(\lambda_{\text{max}} = 292\) nm) to a characteristic purple color (\(\lambda_{\text{max}} = 295\) and 535 nm) in phosphate buffer (pH 7.4). The final reaction products between TCBQ and \(\text{H}_2\text{O}_2\) were then identified by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS). The mass spectrum of TCBQ is characterized by a
four-chlorine isotope cluster at \( m/z \) 246 and traces of a three-chlorine isotope cluster at \( m/z \) 227. The addition of \( \mathrm{H}_2\mathrm{O}_2 \) to TCBQ led to complete disappearance of the molecular ion peak clusters at \( m/z \) 246 and dramatic increase of the peak clusters at \( m/z \) 227. Tandem mass spectrometric analysis showed that the peak at \( m/z \) 227 could be fragmented to form the peak at \( m/z \) 197, which suggests that the peak at \( m/z \) 197 is solely derived from the peak at \( m/z \) 227. These results indicate that the major reaction product between TCBQ and \( \mathrm{H}_2\mathrm{O}_2 \) was probably the ionic form of trichloro-hydroxy-1,4-benzoquinone (\( \text{TrCBQ-OH} \)). This was further confirmed by comparing with the authentic TrCBQ-OH synthesized according to published method [73], which showed the same ESI-MS profile and the same retention time in HPLC.

To better understand the source and origin of the oxygen atom inserted into the reaction product TrCBQ-OH formed from the reaction between H\( \mathrm{H}_2\mathrm{O}_2 \) and TCBQ, TCBQ was incubated with oxygen-18-enriched H\( \mathrm{H}_2\mathrm{O}_2 \) (\( ^{18}\mathrm{O}-\mathrm{H}_2\mathrm{O}_2 \)). The mass spectra of the molecular ion region of deprotonated TrCBQ-OH, obtained with unlabeled and labeled \( \mathrm{H}_2\mathrm{O}_2 \), demonstrated the shift of the molecular ion isotope cluster peaks of the unlabeled compound with 2 mass units, as could be expected for the incorporation of \( ^{18}\mathrm{O} \). These results indicate that \( \mathrm{H}_2\mathrm{O}_2 \) is the source and origin of the oxygen atom inserted into the reaction product TrCBQ-OH.

It has also been shown [74] that both TCBQ and \( \mathrm{H}_2\mathrm{O}_2 \) were consumed with a stoichiometric ratio of about 1:1, and \( \mathrm{H}_2\mathrm{O}_2 \) accelerated the rate of TCBQ decomposition by two orders of magnitude with the loss of chloride. Thus, the metal-independent production of \( \cdot\mathrm{OH} \) by TCBQ and \( \mathrm{H}_2\mathrm{O}_2 \) may be not through a previously proposed semiquinone-mediated organic Fenton reaction. Based on the above experimental results and the fact that \( \mathrm{H}_2\mathrm{O}_2 \) is a better nucleophile than \( \mathrm{H}_2\mathrm{O} \) [75], we proposed a novel mechanism for \( \cdot\mathrm{OH} \) production by \( \mathrm{H}_2\mathrm{O}_2 \) and TCBQ [72] (Figure 11): a nucleophilic reaction may take place between TCBQ and \( \mathrm{H}_2\mathrm{O}_2 \), forming an unstable trichloro-hydroperoxy-1,4-benzoquinone (\( \text{TrCBQ-OOH} \)) intermediate, which can decompose homolytically to produce \( \cdot\mathrm{OH} \) and trichloro-hydroxy-1,4-benzoquinone radical (\( \text{TrCBQ-O}^* \)). TrCBQ-O\( ^* \) then may disproportionate to form the ionic form of trichloro-hydroxy-1,4-benzoquinone (\( \text{TrCBQ-O}^- \)). In the presence of excess of \( \mathrm{H}_2\mathrm{O}_2 \), TrCBQ-O\(^- \) may further react with \( \mathrm{H}_2\mathrm{O}_2 \) via similar pathway to produce another \( \cdot\mathrm{OH} \) (Figure 11).

Recently, we employed a previously developed photoelectrochemical DNA sensor to investigate whether DNA damage could be induced by tetrahalogenated quinones and \( \mathrm{H}_2\mathrm{O}_2 \) through the above metal-independent mechanism [76]. The sensor surface was composed of a double-stranded DNA film assembled on a \( \mathrm{SnO}_2 \) semiconductor electrode. A DNA intercalator, Ru(bpy)\(_2\)(dppz)\(^2+\), was allowed to bind to the DNA film and produce photocurrent upon light irradiation. After the DNA film was
exposed to 0.30 mM tetrafluoro-1,4-benzoquinone (TFBQ), the photocurrent dropped by 20%. In a mixture of 0.30 mM TFBQ and 2 mM H$_2$O$_2$, the signal dropped by 40%. The signal reduction indicates less binding of Ru(bpy)$_2$(dppz)$_2^{2+}$ due to structural damage of ds-DNA in the film. Similar results were obtained with TCBQ, although the signal was not reduced as much as TFBQ. Fluorescence measurement showed that TFBQ/H$_2$O$_2$ generated more *$\cdot$OH than TCBQ/H$_2$O$_2$. Gel electrophoresis proved that the two halogenated quinones produced DNA strand breaks together with H$_2$O$_2$, but not by themselves. These results indicate that DNA damage could be indeed induced by tetrahalogenated quinones and H$_2$O$_2$ through the above metal-independent mechanism for *$\cdot$OH production.

Direct evidence of *$\cdot$OH production in livers of *Carassius auratus* (one of the main economic fish species in Eastern China) exposed to PCP was found recently using ESR spin-trapping method [77]. A dose–effect relationship was obtained between *$\cdot$OH intensities and PCP exposure. It was observed that *$\cdot$OH was significantly induced by 0.001 mg/L (below the criteria for Chinese fishery water quality) of PCP exposure. A strong positive correlation ($r = 0.9581$, $p < 0.001$) was observed between PCP liver concentrations and *$\cdot$OH intensities within 7 days of PCP exposure, which suggests that *$\cdot$OH are mainly produced from PCP itself. However, no correlation was observed between PCP liver concentrations and *$\cdot$OH intensities after 7 days, and a higher intensity of OH could still be observed when the PCP liver concentrations decreased to a lower level, which suggests that other mechanisms may possibly contribute to *$\cdot$OH production after 7 days [77]. The glutathione/oxidized glutathione (GSH/GSSG)
ratio decreased below that of the control level during the entire period of PCP exposure (0.05 mg/L), which suggested oxidative stress occurred. It is not clear whether the production of $^*\text{OH}$ in this model system is metal independent or not. Further studies are needed to investigate this issue.

4.3. Metal-independent decomposition of organic hydroperoxides and formation of alkoxyl radicals by halogenated quinones

Organic hydroperoxides (ROOH) can be formed both nonenzymatically by reaction of free radicals with polyunsaturated fatty acids and enzymatically by lipoxygenase- or cyclooxygenase-catalyzed oxidation of linoleic acid and arachidonic acid [39,78,79]. It has been shown that organic hydroperoxides can undergo transition metal ion-catalyzed decomposition to alkoxyl radicals, which may initiate de novo lipid peroxidation or further decompose to $\alpha,\beta$-unsaturated aldehydes that can react with and damage DNA and other biological macromolecules [39,78,79].

\[
\text{ROOH} + \text{Me}^{(n-1)+} \rightarrow \text{RO}^* + \text{OH}^- + \text{Me}^{n+}
\]

In previous studies, using the salicylate hydroxylation assay and ESR spin-trapping methods, we found that $^*\text{OH}$ can be produced from $\text{H}_2\text{O}_2$ by TCBQ and other halogenated quinones independent of transition metal ions [65,66]. However, it is not clear whether halogenated quinones react in a similar fashion with organic hydroperoxides to produce alkoxyl radicals independent of transition metal ions.

Using 2,5-dichloro-1,4-benzoquinone (DCBQ) as a model halogenated quinone, and tert-butylhydroperoxide ($t$-BuOOH) as a model short-chain organic hydroperoxide, we found [81] that DCBQ could markedly enhance the decomposition of $t$-BuOOH, leading to the formation of the DMPO adducts with $t$-butoxyl radicals ($t$-BuO*) and methyl radicals ($t$-CH$_3$) (Figure 12). The formation of DMPO/$t$-BuO* and DMPO/$t$-CH$_3$ from DCBQ and $t$-BuOOH was dose dependent with respect to both DCBQ and $t$-BuOOH and was not affected by iron-specific or copper-specific metal chelators. Comparison of the data obtained with DCBQ and $t$-BuOOH with those obtained in a parallel study with ferrous iron and $t$-BuOOH strongly suggested that $t$-BuO* was produced by DCBQ and $t$-BuOOH through a metal-independent mechanism. Other halogenated quinones such as TCBQ were also found to enhance the decomposition of $t$-BuOOH and other organic hydroperoxides such as cumene hydroperoxide, leading to the formation of the respective organic alkoxyl radicals in a metal-independent fashion.

UV–visible spectral studies showed that there was a direct interaction between DCBQ and $t$-BuOOH, with the reaction mixture changing
quickly from the original yellow color ($\lambda_{\text{max}} = 272 \text{ nm}$) to a characteristic purple color ($\lambda_{\text{max}} = 278$ and $515 \text{ nm}$) in phosphate buffer (pH 7.4). The reaction intermediate and final products between DCBQ and $t$-BuOOH were identified by ESI–MS. These MS results indicate that the major reaction intermediate between DCBQ and $t$-BuOOH was probably chloro-$t$-butylperoxyl-1,4-benzoquinone (CBQ-OO-$t$-Bu), and the major reaction product between DCBQ and $t$-BuOOH was probably the ionic form of 2-chloro-5-hydroxy-1,4-benzoquinone (CBQ-OH; peak clusters at $m/z$ 157).

Based on the above experimental results, a novel mechanism can be proposed [79] for DCBQ-mediated $t$-BuOOH decomposition and formation of $t$-BuO$^*$ and $\cdot$CH$_3$: a nucleophilic reaction may take place between DCBQ and $t$-BuOOH, forming a CBQ-OO-$t$-Bu intermediate, which can decompose homolytically to produce $t$-BuO$^*$ and 2-chloro-5-hydroxy-1,4-benzoquinone radical (CBQ-O$^*$). CBQ-O$^*$ then disproportionates to form the ionic form of 2-chloro-5-hydroxy-1,4-benzoquinone (CBQ-O$^-$), and $\cdot$CH$_3$ can be produced through $\beta$-scission of $t$-BuO$^*$ (Figure 13). It should be noted that CBQ-O$^*$ could not be detected under current experimental conditions. The reason may be that either its half-life span is too short, or its steady-state concentration is too low.

These findings suggest that the chlorinated quinones may react with lipid hydroperoxides and exert toxic effects through enhanced production of...
alkoxyl radicals, and hence increased lipid peroxidation. Additional work is needed to investigate whether these reactions occur and are relevant under physiological conditions or in vivo.

4.4. Detection and identification of a key quinone ketoxy radical intermediate

In the above studies, we found that halogenated quinones could enhance the decomposition of hydroperoxides and formation of alkoxy/hydroxyl radicals through a metal-independent mechanism [72,79]. However, neither the major reaction products nor the proposed quinone–peroxide reaction intermediate CBQ-OO-\textit{t}-Bu and quinone enoxy radical CBQ-O\textit{t}-Bu were unambiguously identified.

Therefore, in the following study, we employed semi-preparative HPLC method in order to quickly isolate and purify the proposed unstable quinone–peroxide reaction intermediate and the major products from the reaction between DCBQ and \textit{t}-BuOOH [80]. Two major compounds were isolated and purified. As expected, one of them was identified as CBQ-OH. The other compound was first assumed to be the quinone–peroxide reaction intermediate CBQ-OO-\textit{t}-Bu (MW 230), since this compound was characterized by ESI-Q-TOF-MS with one-chlorine isotope clusters at \(m/z\) 229,
and tandem mass spectrometric analysis showed that this peak at $m/z$ 229 could be readily fragmented to form a peak at $m/z$ 172. Further, detailed studies on its chemical structure suggested, however, that this compound was not the expected quinone–peroxide reaction intermediate.

The NMR and IR spectral studies demonstrated that this compound may contain a hydroxyl group based on the following lines of evidence: (1) $D_2O$ exchange experiment showed that the chemical shift at 10.74 vanished instantly after adding $D_2O$; (2) its IR spectra showed one broad band at 3318 cm$^{-1}$, which is a distinct vibration mode for an O–H bond; and (3) it showed a characteristic purple color ($\lambda_{max} = 551$ nm) in phosphate buffer (pH 7.4), which is the typical color for hydroxylated quinones. In addition, this compound was found to be stable for at least 1 h in the reaction solution.

Based on the above analysis and other experimental data, this compound was finally identified, unexpectedly, as 2-hydroxy-3-$t$-butoxy-5-chloro-1,4-benzoquinone (CBQ(OH)-O-$t$-Bu), which is the rearranged isomer of the proposed unstable quinone–peroxide reaction intermediate CBQ-OO-$t$-Bu (for its chemical structure, see Figure 13).

This surprising finding raised two new questions: (1) Is the new reaction product CBQ(OH)-O-$t$-Bu derived directly from the unstable quinone–peroxide reaction intermediate CBQ-OO-$t$-Bu? (2) If so, what is the underlying molecular mechanism? To answer these questions, we hypothesized that the homolytical decomposition of the unstable reaction intermediate CBQ-OO-$t$-Bu could lead to the production of $t$-BuO$^\cdot$ and CBQ-O$^\cdot$. The oxygen–centered quinone enoxy radical CBQ-O$^\cdot$ might be isomerized spontaneously to form its corresponding carbon–centered quinone ketoxy radical ($\bullet$CBQ = O), which might be more stable because it is stabilized by the resonant delocalization of the unpaired electron over the adjacent $\pi$ system which contains two conjugated carbonyl groups [81]. $\bullet$CBQ = O then may couple with $t$-BuO$^\cdot$ to produce the final reaction product CBQ(OH)-O-$t$-Bu via keto–enol tautomerization (Figure 13).

If the above hypothesis was correct, DMPO should compete with $\bullet$CBQ = O to trap $t$-BuO$^\cdot$ since we have shown in our previous study that DMPO can trap alkoxyl radicals [79]. This will reduce the chance of $t$-BuO$^\cdot$ coupling to $\bullet$CBQ = O and therefore inhibit the formation of CBQ(OH)-O-$t$-Bu accordingly. This was found to be exactly the case: The formation of CBQ(OH)-O-$t$-Bu was indeed inhibited by DMPO in a concentration-dependent manner.

Interestingly and unexpectedly, in the presence of DMPO, a new peak was observed from the reaction between DCBQ and $t$-BuOOH. This new compound was characterized by ESI-Q-TOF-MS with one-chlorine isotope peak clusters at $m/z$ 268 (ESI negative) or at $m/z$ 270 (ESI positive). These data indicate that the new compound might be a DMPO adduct with either the proposed quinone enoxy CBQ-O$^\cdot$ or ketoxy $\bullet$CBQ = O radical (MW 157) (for simplicity, this DMPO adduct was referred as DMPO-157).
To get more accurate molecular weight, elemental composition, and structural information of the DMPO radical adducts, Fourier transform ion cyclotron resonance (FTICR) mass spectrometry was used for further studies since it is one of the techniques that can provide high mass accuracy and high mass resolution [82,83]. DMPO-157 was characterized by FTICR/MS with one-chlorine isotope peak clusters at m/z 268.0383, which corresponds to the deprotonated molecule of the oxidized nitrone form (theoretical mass 268.0377) of the DMPO-157 nitroxide radical adduct. The same DMPO-157 adduct was also observed when t-BuOOH was substituted by other hydroperoxides such as H_2O_2 and cumene hydroperoxide.

Interestingly, DMPO adducts with the corresponding quinone radical could also be detected by FTICR/MS when DCBQ was substituted by certain halogenated quinones such as 2,5-dibromo-1,4-benzoquinone (2,5-DBrBQ, characterized with one-bromine isotope peak clusters at m/z 312) and trichloro-1,4-benzoquinone (TrCBQ, characterized with two-chlorine isotope peak clusters at m/z 302), but not by other halogenated quinones such as 2,3-dichloro-, tetrachloro-, and tetrabromo-1,4-benzoquinone.

As mentioned above, the same DMPO-157 adduct could also be observed when t-BuOOH was substituted by H_2O_2 to react with DCBQ. According to the above hypothesis, the source and origin of the oxygen atom inserted into DMPO-157 adduct should be from hydroperoxide. To test whether this is the case, DCBQ was incubated with oxygen-17-labeled H_2O_2 ([^{17}O]-H_2O_2). The mass spectra of the molecular ion region of deprotonated DMPO-157, obtained with unlabeled and labeled H_2O_2, demonstrated the shift of the molecular ion isotope cluster peaks of the unlabeled compound with 1 mass unit, as could be expected for the incorporation of ^{17}O. These results indicate that H_2O_2 is the source and origin of the oxygen atom inserted into DMPO-157 adduct.

In our previous study on DCBQ/t-BuOOH/DMPO system, beside the strong and predominant ESR signals for DMPO/t-BuO^· and DMPO/CH_3, a minor DMPO/radical adduct with weak ESR signals was also observed, but remained unidentified [79] (Figure 12). With the new FTICR/MS data, we realized that this unidentified radical adduct might be the DMPO adduct either with the proposed oxygen-centered quinone enoxy radical CBQ-O^· or with its carbon-centered ketoxy spin isomer •CBQ = O. However, due to the interference by the strong DMPO/t-BuO^· and DMPO/CH_3 signals, only a weak four-line ESR signal with equal intensity could be observed. Thus, it is not clear just from these results whether the quinone radical trapped by DMPO is an oxygen-centered or a carbon-centered radical.

According to our previously proposed mechanism for halogenated quinone-enhanced decomposition of hydroperoxides and formation of alkoxyl/hydroxyl radicals [72,79], we expect that, in the presence of DMPO, the reaction between DCBQ and H_2O_2 should produce the
same DMPO-157 radical adduct with the same FTICR/MS and ESR characteristics as from the reaction between DCBQ and t-BuOOH. To get rid of the interference of the strong ESR signals of DMPO/t-BuO• and DMPO/•CH3 and obtain more simple and clear ESR spectra for DMPO-157 radical adduct, we studied DCBQ/H2O2/DMPO system. As expected, a six-line ESR signal with equal intensity ($a_H = 28.8$ G; $a_N = 17.0$ G; $a_N/a_H = 0.59$) could be clearly observed in addition to the DMPO/•OH signal (Figure 14). These parameters are characteristic of the spin trapping of a carbon-centered quinone ketoxy radical •CBQ = O, rather than an oxygen-centered enoxy radical CBQ-O• [84]. It is interesting to note that the total width of the spectrum of DMPO-quinone ketoxy radical is unusually wide for a DMPO spin adduct. The larger $a_N$ and $a_H$ values of this novel spin adduct is probably due to an increase in spin density on nitrogen and a decrease in β-CH dihedral angle caused by intramolecular H-bonding from the hydroxyl hydrogen on the quinone ring to the nitrooxide oxygen [85].

Similar six-line ESR signals for DMPO/carbon-centered quinone ketoxy radical adducts were also observed when DCBQ was substituted by 2,5-dibromo- and trichloro-1,4-benzoquinone, but not by 2,3-DCBQ, tetrachloro-, and tetrabromo-1,4-benzoquinone. These ESR spin-trapping results are in good agreement with the above FTICR/MS results, which suggest that in order to observe the carbon-centered quinone ketoxy radical, it is important for the halogenated quinone to contain one hydrogen atom at the ortho-position of halogen atom on the quinone ring.

To get more definitive evidence, the DMPO-157 nitrone adduct was isolated and purified by semi-preparative HPLC, and characterized by 1H NMR. The downfield signals at 6.26 and 6.82 ppm, corresponding to the single protons at the C3′ position on CBQ-OH and C2 position on DMPO,

![Figure 14](image-url) DMPO nitroxide adduct with the carbon-centered quinone ketoxy radical.
respectively, was clearly missing in the spectrum of DMPO-157 nitrene adduct presumably due to the formation of a new covalent bond between DMPO and CBQ-OH. The formation of this covalent bond also caused significant chemical shift changes of the neighboring proton signals, namely, the protons at the C3 position, but had little or no effect on the proton signals farther away, that is, 5,5-dimethyl protons and the protons at the C4 and C6' positions. Therefore, our NMR data provide more direct evidence to support the structural assignments of the DMPO-157 nitrene adduct. To the best of our knowledge, this is the first report that a carbon-centered quinone ketoxy radical was detected and identified by the complementary application of ESR spin trapping, NMR, and FTICR/MS methods.

It should be noted, however, that although we can isolate DMPO-157 adduct with semi-preparative HPLC, no corresponding six-line ESR signal was observed with the collected fraction. The reason might be that either the DMPO-157 nitroxide radical adduct may not be stable enough to pass through the HPLC column and decayed to its ESR-silent nitrene form during the separation process, or the concentration of the radical adduct in the collected fraction was just too low to be detected by ESR.

The detection and identification of a carbon-centered quinone ketoxy radical as one of the major radical intermediates, together with the identification of CBQ(OH)-O-t-Bu as one of the major reaction products from the reaction between DCBQ and t-BuOOH, strongly support the possible existence of its corresponding isomer, the proposed quinone–peroxide reaction intermediate CBQ-OO-t-Bu. However, we could not isolate and purify its nature by traditional separation methods due to its unstable nature. These results together with the identification of CBQ-OH as another major reaction product also support the possible existence of the proposed oxygen-centered quinone enoxy radical CBQ-O, although we still could not detect it directly by ESR, either due to its short half-life or due to its low steady-state concentration.

Taken together, these new results provided direct experimental evidence for the involvement of the carbon-centered quinone ketoxy radicals during metal-independent decomposition of hydroperoxides and formation of alkoxyl/hydroxyl radicals by halogenated quinones. Therefore, the previously proposed molecular mechanism was further expanded to incorporate all of our new findings with previous experimental data [80]: A nucleophilic reaction may take place between DCBQ and t-BuOOH, forming a quinone–peroxide reaction intermediate CBQ-OO-t-Bu, which can decompose homolytically to produce t-BuO and CBQ-0. CBQ-0 then either disproportionate to form one of the major reaction product CBQ-OH or isomerizes to form the carbon-centered quinone ketoxy radical •CBQ = O, which then coupled with t-BuO to produce another major reaction product CBQ(OH)-O-t-Bu via keto-enol tautomerization (Figure 13).
Our observation that not only DCBQ but also other halogented quinones can react with both organic hydroperoxides and hydrogen peroxide to produce carbon-centered quinone ketoxy radicals in addition to alkoxyl/hydroxyl radicals in a metal-independent manner may also have interesting biological implications. Our data suggest that these halogenated quinones may react with hydroperoxides and exert toxic effects not only through enhanced production of alkoxyl/hydroxyl radicals but also through the formation of carbon-centered quinone ketoxy radicals which may react directly with critical biological macromolecules such as DNA, protein, and lipids.

Recently, \( \text{H}_2\text{O}_2 \) has been increasingly favored as an environmentally safe oxidant for remediation of the environmental pollutants such as chlorinated phenols [33–35]. In these “environmentally green” systems, \( \text{H}_2\text{O}_2 \) is often used at millimolar levels. One recent study showed 2,6-DCBQ could be detected within the first minutes and then undergone further transformations during the oxidative mineralization of 2,4-6-TCP by \( \text{H}_2\text{O}_2 \) with the catalysis of iron complexes [35]. Another study showed [74] that \( \text{H}_2\text{O}_2 \) could accelerate the rate of TCBQ decomposition by two orders of magnitude, and the rate of this reaction was too fast to measure except at acidic pH. It was suggested that peroxide-dependent decomposition pathway for halogenated quinones may be important in systems where hydroperoxide is either used or produced. However, the exact molecular mechanisms underlying such further transformations are not clear. Our new findings may provide a new perspective to better understand such transformation mechanisms during wastewater treatment or remediation process in which halogenated quinones are formed.

### 5. Detoxifying Carcinogenic Polyhalogenated Quinones by Hydroxamic Acids via an Unusually Mild and Facile Double Lossen Rearrangement Mechanism

Hydroxamic acids have attracted considerable interest recently because of their capacity to inhibit a variety of enzymes such as metallo-proteases and lipoxygenase, and transition metal-mediated oxidative stress. Some hydroxamates, such as suberoylanilide hydroxamic acid and DFO, have been used clinically for the treatment of cancer or iron-overload diseases [86–89]. Much of the activities of these hydroxamic acids were thought to be due to their metal chelating properties.

In our previous work, we found that DFO and other hydroxamic acids, but not other classic iron chelators such as DTPA, provided strong protection against PCP quinoid metabolite-induced cyto- and genotoxicity in human fibroblasts [62,63]. During our recent studies on metal-independent
decomposition of hydroperoxides by halogenated quinones, we showed that these hydroxamic acids could also markedly inhibit TCBQ-mediated hydroperoxide decomposition and hydroxyl/alkoxyl radical formation [64,65,72,79,80]. Interestingly, we found that the protection or inhibition by these hydroxamic acids was not due to their iron-chelating properties, but possibly due to their effective scavenging of the reactive TCSQ• and (or) their remarkable acceleration of TCBQ hydrolysis to the much less reactive and almost nontoxic DDBQ (also called chloranilic acid) [62,63].

It was a great surprise for us to find that hydroxamic acids could markedly accelerate the conversion of TCBQ to DDBQ, since we could not find a reasonable explanation based on our chemical knowledge at the time. Although we first observed the above interesting phenomenon a decade ago [62], its underlying molecular mechanism remained a puzzle. Recently, the reactions between TCBQ and hydroxamic acids were carefully reexamined, and the reaction products were isolated, purified, and unambiguously identified by HPLC/ESI–MS, NMR, and oxygen-18 isotope-labeling methods. We found, unexpectedly, that a novel double Lossen rearrangement was responsible for this unusual reaction [90].

We found that hydroxamic acids could markedly accelerate TCBQ hydrolysis to the much less toxic DDBQ (also called chloranilic acid), and among the five hydroxamic acids tested, the most effective one was BHA, with rate accelerations of up to 150,000-fold (Figure 15). In contrast, no enhancing effect was observed with O-methyl BHA, which clearly indicates that the free benzohydroxamate anion is essential for the dramatic acceleration of TCBQ hydrolysis to occur [90].

Analogous results were observed when TCBQ was substituted with other tetrahalogenated quinones, including tetrabromo- and tetrachloro-1,4-benzoquinones and their corresponding hydroquinone forms, as well as tetrabromo- and tetrachloro-1,2-benzoquinones, and when BHA was substituted with other hydroxamic acids such as suberoylanilide hydroxamic acid and DFO. These findings suggest that this is a general reaction between hydroxamic acids and tetrahalogenated quinoid compounds.

Since the rate of TCBQ hydrolysis in the presence of BHA is much faster than that of its spontaneous hydrolysis, it appeared that BHA could catalyze this process. However, we found that BHA was consumed during its reaction with TCBQ, with the concurrent formation of a new compound with m/z at 255. This suggested that the hydroxamic acid is not a true catalyst in the TCBQ hydrolysis reaction. To better understand the underlying molecular mechanism for this reaction, the final products of BHA after reacting with TCBQ were then isolated, purified, and identified by HPLC–ESI–MS and NMR. Interestingly, O-phenylcarbamyl benzo-hydroxamate (I) was identified as the major reaction product of BHA, while N, N′-diphenylurea (II) as a minor product.
Then the question is: How are these products (I and II) formed? It has been shown [91,92] that product I could be formed through nucleophilic addition of phenyl isocyanate with a molecule of BHA and product II could be formed through the hydrolysis of phenyl isocyanate to aniline, followed by reaction of aniline with another molecule of phenyl isocyanate (Figure 16). The above analysis strongly suggests that phenyl isocyanate (ArN = C = O) should be formed as an initial unstable product during the reaction of BHA and TCBQ.

This leads to a new question: In what way could phenyl isocyanate be produced from BHA? It has been shown [91,92] that product I could be formed through the classic Lossen rearrangement, a well-known reaction which describes the transformation of an O-activated hydroxamic acid (RC(O)NHOX) into the corresponding isocyanate [92,93]. The rate-limiting step

**Figure 15**  (A) TCBQ conversion to DDBQ was dramatically enhanced by benzohydroxamic acid (BHA) and other hydroxamic acids (HAs); (B) the chemical structures of HAs (modified based on Ref. [90]).
of this reaction is the activation of the hydroxamic acid by various agents (i.e., sulfonyl and benzoyl chloride, etc.; \(X = \text{SO}_2\text{R}, \text{C(O)R}\)):
The above analysis suggested that the reaction between BHA and TCBQ might proceed through an analogous, but previously unknown Lossen-type rearrangement pathway.

Based on the above experimental results and earlier research on Lossen rearrangement [91–94], as well as the fact that the benzohydroxamate anion is a particularly effective \( \alpha \)-nucleophile [91], a novel mechanism for BHA-accelerated TCBQ hydrolysis was proposed (Figure 16). According to the mechanism, a nucleophilic reaction takes place between the benzohydroxamate anion (\( \text{ArC(O)NHO}^- \)) and TCBQ, first forming an unstable transient intermediate \( \text{ArC(O)NHOTrCBQ} \). Following loss of a proton from nitrogen to form the anionic \( \text{ArC(O)N}^- \text{OTrCBQ} \) intermediate, a spontaneous Lossen-type rearrangement leads to the formation of \( \text{TrCBQ-O}^- \) (at low BHA/TCBQ molar ratios) and phenyl isocyanate. When BHA is in excess, \( \text{TrCBQ-O}^- \) further reacts with BHA, through a similar reaction intermediate, and a second-step spontaneous Lossen-type rearrangement reaction yields DDBQ and another molecule of phenyl isocyanate [90]. As mentioned above, phenyl isocyanate could react with another molecule of BHA to yield the major reaction product I.

If the above proposed double Lossen rearrangement mechanism is correct, then we expect that the source and origin of the oxygen atom in DDBQ and product I from the reaction of TCBQ (or TrCBQ-OH) with BHA should be from the hydroxyl group of BHA, not from water. To test whether this is the case, TCBQ (or TrCBQ-OH) was incubated with BHA in buffer solution prepared with oxygen-18-labeled H\(_2\)O (\([^{18}\text{O}]\text{H}_2\text{O}\)) as solvent. If water is the source of the oxygen atom in any of the products, the mass spectra of the molecular ion region of DDBQ and product I, obtained with unlabeled and labeled H\(_2\)O, should show the shift of the molecular ion isotope cluster peaks of the unlabeled compounds with 2 or 4 mass units, as could be expected for the incorporation of \(^{18}\text{O}\). However, no \(^{18}\text{O}\) was found to be incorporated in any of these products. These isotope-labeling data provide strong experimental evidence to further support the above proposed mechanism.

Most of the previously reported Lossen rearrangement reactions take place only under alkaline conditions and/or through heating to a requisite temperature [91–94]. In the present study, we found that the reaction between BHA and TCBQ could occur at room temperature and under neutral or even weakly acidic pH. This is possibly due to the unusually rapid and facile rearrangement of the postulated reaction intermediate, the \( O^-\text{trichloroquinonated BHA} \).

We found that this unusual double rearrangement reaction mechanism is not only limited to TCBQ and BHA, but it is also a general mechanism for all tetrahalogenated quinonoid compounds and hydroxamic acids. Therefore, our findings may have interesting biological and environmental implications: Many widely used polyhalogenated aromatic compounds can be
metabolized in vivo [23,37,95–97], or dehalogenated chemically and enzymatically [33–35], to their corresponding quinones. Polychlorinated quinoid compounds were also found in discharges from pulp and paper mills [20–23]. More recently, several polyhalogenated quinones, which are suspected bladder carcinogens, were identified as new chlorination disinfection byproducts in drinking water [98]. These polyhalogenated quinones not only cause oxidative damage to DNA and other macromolecules but also form protein and DNA adducts both in vitro and in vivo [20–23,37]. Thus, these molecules are potential mammalian carcinogens, which render their destruction or remediation under mild conditions of critical importance.

Of particular interest in this regard is the fact that two hydroxamic acids are already approved for clinical applications, DFO for iron overload and suberoylanilide hydroxamic acid (also called Vorinostat), recently approved for cutaneous T-cell lymphoma [86–89]. As demonstrated in the present and our previous studies [62–65,72,76,79,80], these compounds, in addition to BHA, might be especially suited for detoxication of polyhalogenated quinones. It is worth noting that hydroxamic acids can also efficiently inhibit hexachlorobenzene-induced porphyria [99] and detoxify chemical warfare agents such as the nerve gases Sarin and Soman (which are fluorinated organophosphonates) [92,93,100], as well as nitrogen mustard [101] in animal models, possibly through an analogous Lossen rearrangement mechanism. Therefore, suicidal nucleophilic attack coupled with spontaneous Lossen rearrangement may serve as a general, but previously unrecognized new detoxication mechanism for the widely used hydroxamic acids. Therefore, our findings may have broad chemical, biological, and environmental implications for future research on polyhalogenated aromatic pollutants and hydroxamic acids, which are two important classes of compounds of major environmental and biomedical concern that have been attracting the attention of both academic researchers, and the broader general public.

6. Conclusions and Future Research

The above findings represent a novel mechanism of *OH and alkoxyl radical formation not requiring the involvement of redox-active transition metal ions, and may partly explain the potential carcinogenicity of not only PCP, but also other widely used polyhalogenated aromatic compounds such as 2,4,6- and 2,4,5-TCP, hexachlorobenzene, Agent Orange (the mixture of 2,4,5-T and 2,4-D) and the brominated flame-retardant 3,3′,5,5′-tetrabromobisphenol A (TBBPA), since these compounds can be metabolized in vivo [20–23,95,99,102–105], or dechlorinated chemically to tetra-, di- or mono-halogenated quinones. Our data suggest that TCBQ and other
halogenated quinones may react with hydroperoxides and exert toxic effects through enhanced production of hydroxyl/alkoxyl radicals, and hence increased DNA, protein, and lipid oxidation. We also found that hydroxamic acids could detoxify TCBQ and other polyhalogenated quinoid carcinogens via a novel two-step Lossen rearrangement mechanism. The two clinically used hydroxyamic acids (DFO and suberoylanilide hydroxyamic acid (Vorinostat)) might be used as prophylactics for the prevention or treatment of human diseases such as liver and bladder cancer associated with the toxicity of polyhalogenated quinoid carcinogens.

It should be noted, however, that many questions still need to be addressed, especially regarding the biological relevance of the reactions under study. For example, “How does the reaction of the quinone with $H_2O_2$ compare kinetically with reactions with other good nucleophiles such as GSH and other thiols which are present at high concentrations in vivo?” What is the rate constant for the reaction between TCBQ and $H_2O_2$, and could it be able to compete with the classic Fenton reaction? Could $*OH$ produced by this pathway be detected in cell culture or even in an animal model? Could a more stable carbon-centered quinone ketoxy radical adduct with the corresponding clean nitroxide ESR signal be isolated and purified using other spin-trapping agents? Could the proposed oxygen-centered quinone enoxy radical and the quinone–peroxide intermediate be detected by a freeze-quenching technique? Could the carbon-centered quinone ketoxy radical react with macromolecules such as DNA, protein, and lipids? Could hydroxamic acids provide protection against PCP-induced genotoxicity in animal models?

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REFERENCES


[16] NTP, NTP technical report on the toxicology and carcinogenesis studies of two pentachlorophenol technical grade mixtures (CAS no. 87-86-5) in B6C3F1 mice (feed studies). National Toxicology Program, Research Triangle Park, NC, 1989 NTP TR 349, NIH publication no. 89-2804.


Metal-Independent Pathways of Chlorinated Phenol/Quinone Toxicity


