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Electron spin resonance studies of the free radical metabolites of toxic chemicals

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A free radical is any organic or inorganic molecule with an odd number of electrons. Even as simple an organic molecule as benzene can be transformed into three chemically distinct, highly reactive free radicals (Figure 1). One-electron oxidation, the removal of an electron from the pi-electrons, results in the formation of the benzene cation radical. The addition of an electron, that is, one-electron reduction of benzene, results in the formation of the benzene anion radical. The third free radical is formed by the homolytic cleavage of one of the C-H bonds by UV light or other radiation to form a hydrogen atom and the phenyl radical. Severe chemical conditions are not always necessary to form free radicals, as is the case in the formation of free radicals from benzene. In fact, many classes of free radicals are formed as a result of the metabolism of chemicals.

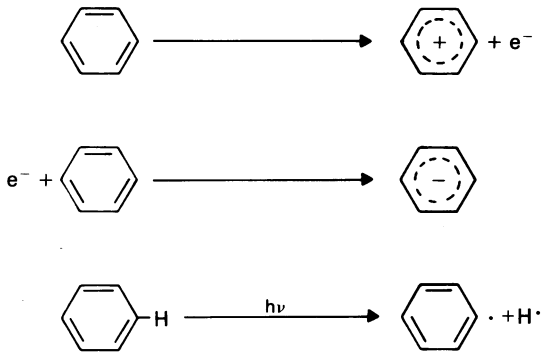

Figure 1 Free radicals from benzene by oxidation, reduction, and homolytic cleavage.

For a variety of reasons, the possibility of free radical metabolism has not received much attention in the past, although Michaelis of the Michaelis–Menton equation was interested in free radical metabolites and their importance in biochemistry in the 1940s (Michaelis, 1946). One reason for the late development of this area is that most biochemicals are not aromatic, as opposed to drugs and industrial chemicals which, in general, are aromatic. Aromaticity facilitates electron transfer and, thereby, metabolism through free radical intermediates. Cysteine and GSH are among the rather rare biochemicals that can form free radical metabolites (Harman *et al.*, 1984, 1986).

In our work we have taken the somewhat unorthodox approach of first searching for free radical metabolites with electron spin resonance (ESR) and then, on the basis of what is known about the free radical chemistry, investigating the biochemical and even the toxicological implications of these free radical reactions.

Our interests have concentrated on high-resolution ESR investigations of relatively stable aromatic radical cations and anions (Mason, 1979, 1982; Mason & Chignell, 1981). Many classes of free radical metabolites can be detected under physiological steady-state conditions for periods from several minutes to over an hour with the use of very simple procedures. In the steady-state condition, the rate of radical formation is equal to the rate of radical decay. Any strategy that will increase the rate of free radical formation or decrease the rate of radical decay will help achieve the 10^{-8}–10^{-7} M steady-state radical concentration necessary for detection.

In ESR the upper limit on the sample size, and therefore the sensitivity, is determined by the use of water as the solvent. High concentrations of cells, microsomes, or mitochondria should improve the sensitivity of the spectrometer to the extent that protein and membranes replace water. The use of packed cells or microsomal protein concentrations as high as 40 mg ml^{-1} will increase the rate of radical formation and, thus, the steady-state radical concentration. Any other approach that will increase the enzyme activity per unit volume, such as induction of cytochrome P-450, should also be employed.

Many free radical metabolites are aromatic cations and anions with decay constants that are pH-dependent. Radical cations, such as the benzidine cation radical, are more stable at acidic pH values, whereas radical anions, such as the metronidazole anion radical, are more stable at basic pH values. Orthosemiquinones, such as the catecholamine anion radicals, are stabilized by complex formation with Zn^{2+} (Kalyanaraman & Sealy, 1982).

The spin-trapping technique is a very effective approach to extending the lifetime of the free radicals (Mason, 1984*a*). Spin traps scavenge many reactive free radicals, even under physiological conditions, to form relatively stable nitroxide adducts. The formation of these secondary nitroxides enables the study of superoxide and other free radical metabolites that would otherwise be impossible to study with direct ESR under steady-state conditions.

A flat cell developed by Hyde (1972) has the optimum geometry for aqueous samples in ESR (Figure 2). We have developed a convenient technique to fill the flat cell by aspiration using a modified Gilford rapid sampler (Mason, 1984*b*). This approach has three major advantages. First, the position of the cell in the cavity is unchanged from one incubation to the next (Figure 3), hence the tuning of the spectrometer is unchanged for the biological controls. Second, the tedious adjustment of the flat cell in the cavity is done only once. Third, kinetic information with dead times of a few seconds is obtained easily.

Some free radicals are chemically too reactive to detect by direct ESR in static steady-state incubations. The fast-flow technique uses large quantities of enzymes and reagents, but is the only method of obtaining ESR spectra of highly reactive free radicals which cannot be spin trapped. The mixing jets allow millisecond observation times, but the enzymatic reaction must also be rapid. In practice, only peroxidases have turnover numbers high enough for the fast-flow technique. Presently this limits studies to horseradish peroxidase. Mammalian peroxidases are just too valuable to use in this fashion.

One approach to reducing the quantities of peroxidases necessary for fast flow experiments is to use a configuration with a smaller sample size. The loop-gap resonator developed

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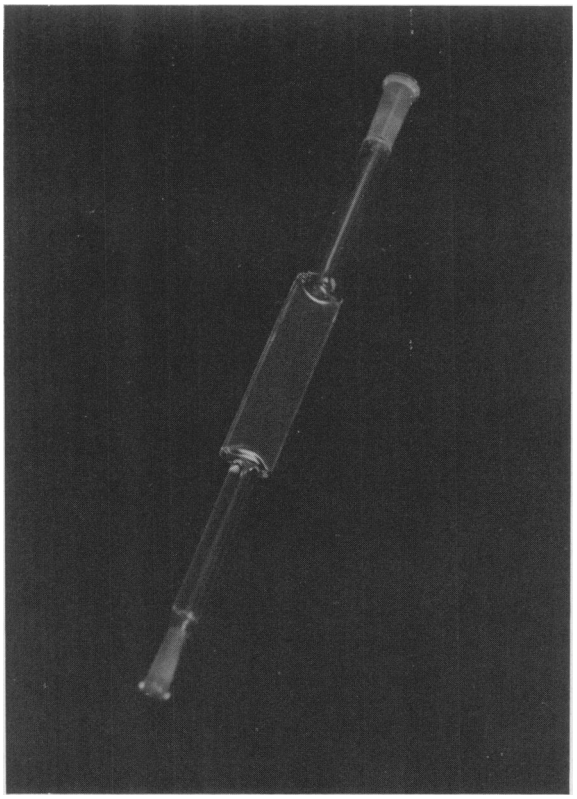


Figure 2 Aqueous ESR flat cell for TM_{110} cavity with 17 mm width.

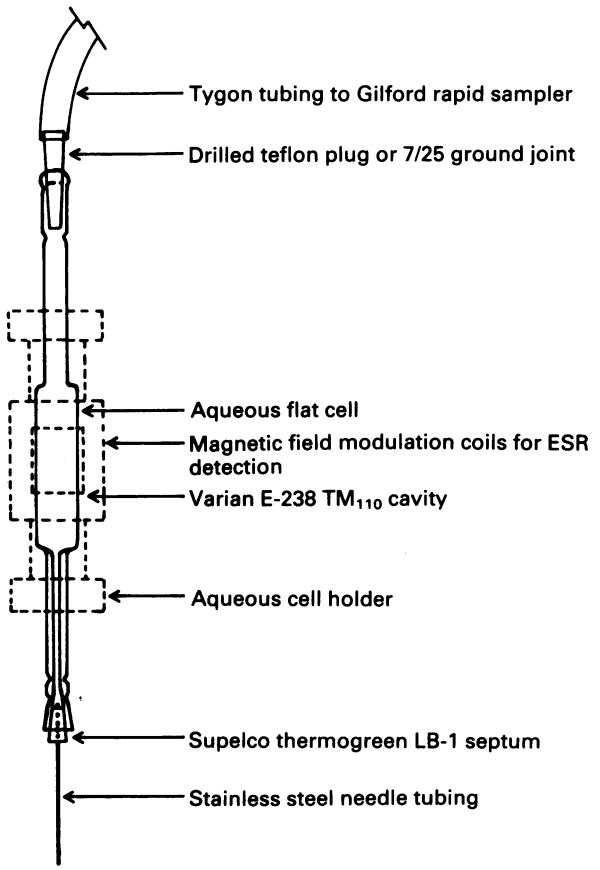


Figure 3 Apparatus for replacing an incubation in the flat cell *in situ* while still in the operate mode of the ESR spectrometer (from Mason, 1984*b*).

by Froncisz and Hyde (1982) has just become commercially available. Borg and Elmore (1967) have published work using a 35 GHz spectrometer, which also has the required smaller sample size.

Stolze and Mason (1987) have begun ESR studies using intercalating redox-active compounds, such as adriamycin and chlorpromazine. The DNA is oriented by capillary flow, and the orientation of the free radicals relative to the DNA helix is determined from the anisotropy of the ESR spectrum. In order to accomplish this, a capillary has been shaped like a flat cell (Figure 4). Orientation of the intercalated chlorpromazine cation radical perpendicular or parallel to the magnetic field can be obtained by simply twisting the cell in the cavity (Figure 5).

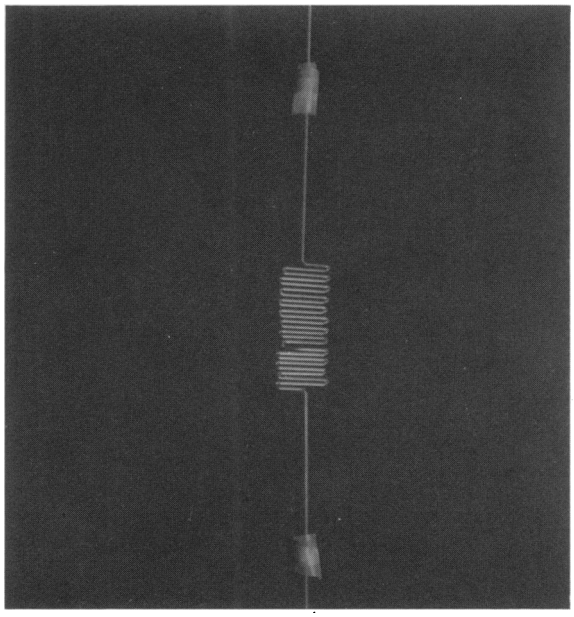


Figure 4 Photograph of folded capillary I.D. 0.25 mm, O.D. 1 mm made of pyrex glass. The width of the folded area is 17 mm, the length is 39 mm.

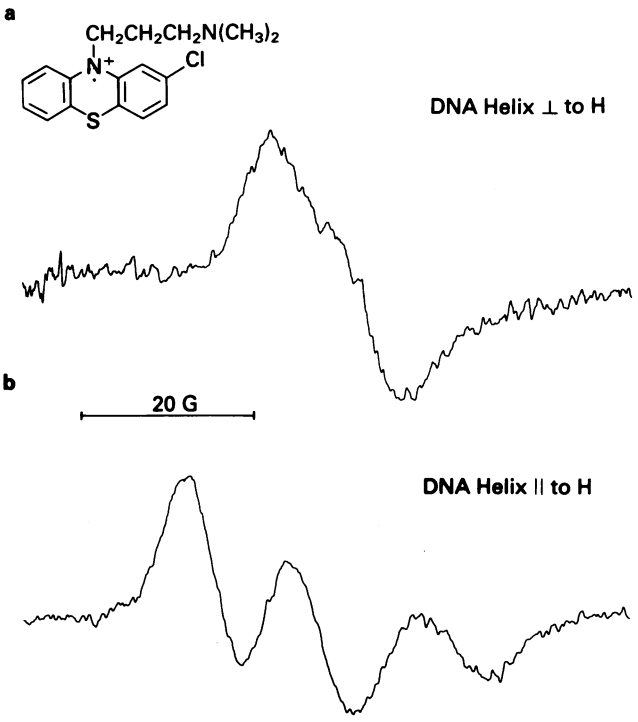


Figure 5 ESR spectra of the chlorpromazine cation radical intercalated into calf thymus DNA. (a) Flow perpendicular to the magnetic field; flow rate 3.5 ml min^{-1}. (b) Flow parallel to the magnetic field; flow rate 3.5 ml min^{-1}.

In the past our work has focused on radical anion metabolites, which are formed by enzymatic one-electron reduction. These species are analogous to the benzene anion radical. Investigations of nitro, azo and quinone radical anion metabolite formation by NADPH cytochrome P-450 reductase have been described (Mason, 1982; Mason & Josephy, 1984). NADPH cytochrome P-450 reductase also reduces quinoneimines to free radicals, such as the quinoneimine metabolite of acetaminophen (Mason & Fischer, 1986) or the anticancer drug actinomycin D (Mason & Chignell, 1981). In some cases, cytochrome P-450 donates one electron to the chemical. For example, CCl_4, gentian violet (Harrelson & Mason, 1982), and sulfur dioxide (Mottley *et al.*, 1985) are reduced to free radicals by cytochrome P-450.

We have primarily used rat liver microsomes in our investigations of anion radical formation, but other cell fractions and purified microsomal enxymes have also been used. Morehouse and Mason (1987) are currently investigating the enzymatic one-electron reduction of porphyrins. Uroporphyrin I is a water soluble porphyrin which does not associate into dimers *in vitro* as most porphyrins do. Uroporphyrin I accumulates in body tissues of some congenital porphyria patients. The preliminary results with uroporphyrin I show porphyrin anion free radical formation by a rat hepatic microsomal reductase, with heat-denatured microsomes being inactive (Figure 6). This work is the enzymatic equivalent of the well-known photoreduction of porphyrins (Figure 6d), and even room light can form more anion radical than the microsomal reduction.

Investigation of free radical metabolite formation by living systems is progressing. Free radical formation by both pathogenic microbes (Docampo & Moreno, 1986) and lung cells (Horton *et al.*, 1986) has been demonstrated. Carbon tetrachloride, an insecticide, an antihelminthic, and a dry cleaner, is a classic hepatotoxicant. Carbon tetrachloride and related compounds, such as the fumigant ethylene dibromide (Tomasi *et al.*, 1983), are known to be reduced to carbon-centered free radicals by cytochrome P-450. Even DDT may undergo this one-electron reduction. Carbon-centered free radicals are too reactive to detect directly in biological systems, so the spin-trapping technique was used.

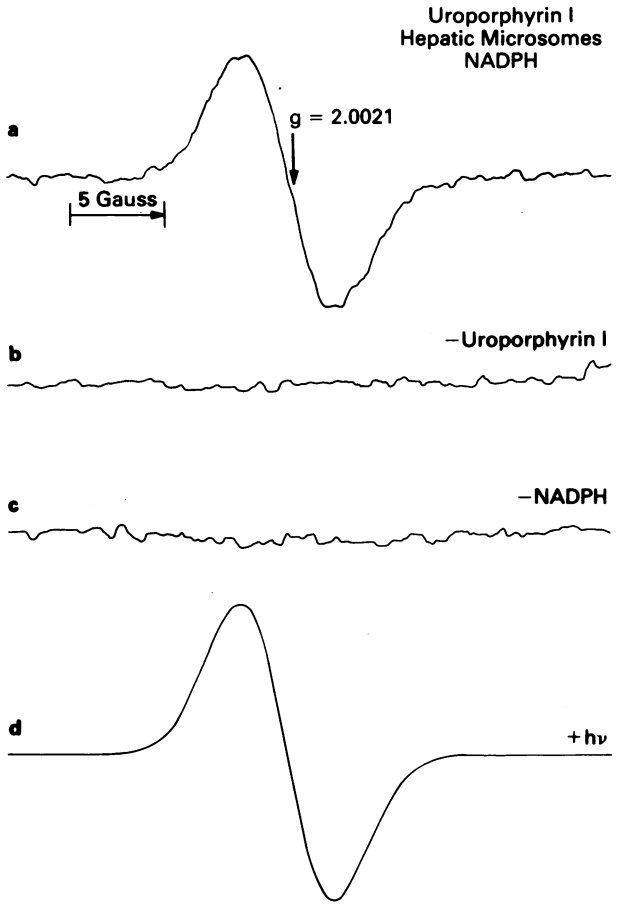
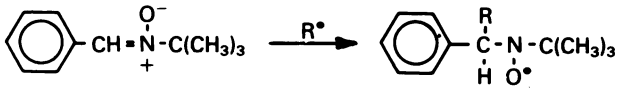


Figure 6 (a) Electron spin resonance spectrum of a rat hepatic microsomal incubation under nitrogen containing 1 mM uroporphyrin I, 1 mM NADPH in Tris buffer (pH 7.4) in the dark. (b) Same as (a), but in the absence of uroporphyrin I. (c) Same as (a), but in the absence of NADPH. (d) Same as (a), but in the presence of room light.

Spin trapping is a technique where a diamagnetic molecule (or spin trap) reacts with a free radical to produce a more stable radical (or spin adduct) which is readily detectable by ESR. Spin adducts are substituted nitroxide free radicals, which, for free radicals, are relatively stable. Some radical adducts are so stable that their structure has been determined by mass spectrometry and NMR.

The spin trap phenyl *N-t*-butylnitrone (PBN) has been used in previous investigations of carbon tetrachloride metabolism (Cheeseman *et al.*, 1985; Janzen *et al.*, 1985). The ESR spectra of its radical adducts are characterized by hyperfine couplings from the nitrogen of the nitroxide and the β-hydrogen, which give spectra with six lines.



The stability of radical adducts enables the detection of free radicals in biological systems, such as the perfused liver. The rat liver can be removed surgically while connected to a primitive heart-lung machine. Carbon tetrachloride was infused, bound to serum albumin, into the perfusion fluid by infusion pumps (Connor *et al.*, 1986). The effluent perfusion fluid was collected via a cannula in the vena cava. After flowing past an oxygen electrode, the perfusate was collected in polyethylene bottles for ESR analysis. It is possible to flow the perfusate directly into the ESR spectrometer.

Carbon tetrachloride is known to be dehalogenated reductively to the trichloromethyl free radical by cytochrome P-450. The trichloromethyl radical adduct was identified by others in microsomal incubations containing NADPH, carbon tetrachloride, and the spin trap PBN (Cheeseman *et al.*, 1985; Janzen *et al.*, 1985). These workers have studied the microsomal metabolism of carbon tetrachloride using [^{13}C]-carbon tetrachloride, where a twelve-line PBN/[^{13}C]-trichloromethyl radical adduct was detected. The PBN/[13C]-trichloromethyl radical adduct has also been reported *in vivo* where it was detected in liver extracts of rats given [^{13}C]-carbon tetrachloride and PBN orally.

We have examined rat urine and effluent perfusate from perfused rat liver for PBN adducts formed during carbon tetrachloride metabolism (Connor *et al.*, 1986). In addition to detecting the trichloromethyl radical in extracts of liver and perfusate, a new free radical metabolite of carbon tetrachloride was discovered in the effluent liver perfusate and in rat urine. This new free radical intermediate is thought to be a product of the reaction of the trichloromethyl radical with oxygen.

As in the previous *in vivo* studies of carbon tetrachloride metabolism, ESR spectra were taken of chloroform-methanol extracts of the liver after perfusion with carbon tetrachloride and PBN (Figures 7a and 7b). Experiments utilizing carbon tetrachloride produced a stable six-line ESR spectrum due to the PBN radical adduct of the trichloromethyl radical. Confirmation of this spectral assignment was provided by the twelve-line ESR spectrum obtained from the organic extract of a liver into which [^{13}C]-carbon tetrachloride was infused. This spectrum exhibited an additional hyperfine coupling of 9.2 G attributable to the nuclear spin of ^{13}C.

ESR analysis of the aqueous layer of the extract of a liver exposed to carbon tetrachloride yielded a different six-line

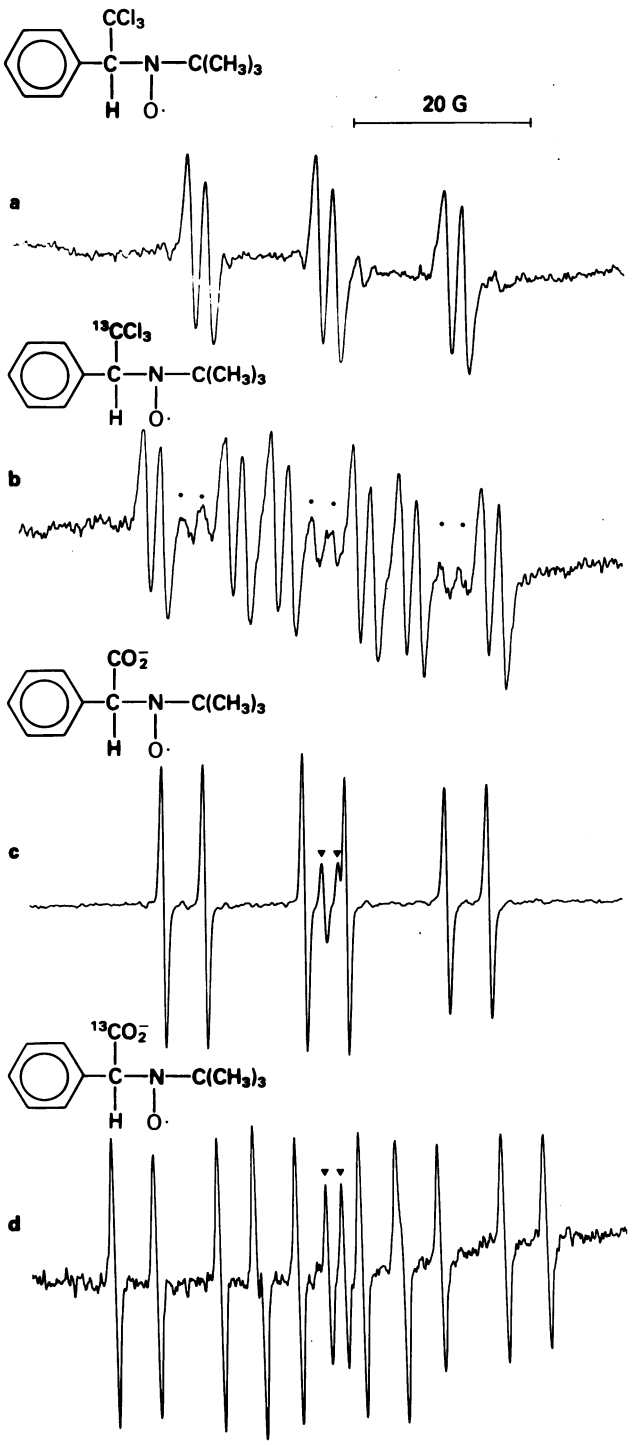


Figure 7 ESR spectra of liver extracts. (a) Spectrum of organic extract of liver after perfusion with PBN and CCl_4. (b) Spectrum of organic extract of liver after perfusion with PBN and $^{13}CCl_4$ (● = predominantly PBN/$^{12}CCl_3^{\cdot}$ spectrum). (c) Spectrum of aqueous extract of liver after perfusion with PBN and CCl_4 (▼ = ascorbate semidione radical spectrum). (d) Spectrum of aqueous extract of liver after perfusion with PBN and $^{13}CCl_4$ (▼ = ascorbate semidione radical spectrum). (From Connor *et al.*, 1986.)

spectrum (Figures 7c and 7d). Following infusion of [^{13}C]-carbon tetrachloride, the corresponding ESR spectrum also yielded a twelve-line spectrum where 2 lines are nearly superimposed. There was absolutely no evidence of this new radical in the organic phase. We assigned this new radical to the carbon dioxide anion radical adduct. A signal of the semidehydroascorbate is marked by the triangles.

ESR analysis of aqueous liver perfusate also yielded the spectra assigned to the carbon dioxide anion adduct from CCl_4 (Figure 8a) and $^{13}CCl_4$ (Figure 8b). It was actually in the perfusate that we first detected this signal.

Although the [^{13}C]-carbon tetrachloride experiment proves that the PBN/carbon dioxide anion radical adduct is carbon tetrachloride-derived, a rigorous proof of radical adduct structure is necessary. A Fenton system containing formate was used to generate the PBN/carbon dioxide anion radical adduct. This is an independent synthesis of the radical adduct. The hydroxyl radical produced by the reduction of hydrogen peroxide by ferrous iron abstracts the hydrogen atom from the formate ion, producing the carbon dioxide anion free radical, which was then trapped by PBN.

$$Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + OH^- + {}^{\bullet}OH$$

$$H-\underset{\underset{O}{\|}}{C}-O^- + {}^{\bullet}OH \longrightarrow {}^{\bullet}\underset{\underset{O}{\|}}{C}-O^- + H_2O$$

$$^{\bullet}\underset{\underset{O}{\|}}{C}-O^- + \underset{H\ O^-}{\overset{CO_2^-}{\underset{|}{\overset{|}{C}}}}=N^+-C(CH_3)_3 \longrightarrow \underset{H\ O^{\bullet}}{\overset{CO_2^-}{\underset{|}{\overset{|}{C}}}}-N-C(CH_3)_3$$

The pH-dependence of the ESR hyperfine couplings for the PBN/carbon dioxide anion radical adduct was measured (Figure 9). Identification of the pH at the midpoint of the pH-sensitive region of the hyperfine couplings gave a pK_a value of 2.85 for this radical adduct produced from either the Fenton system or effluent liver perfusate.

The ESR hyperfine coupling constants for the radical adduct in effluent perfusate correspond closely to values reported for the PBN/carbon dioxide anion radical adduct generated photochemically (Aurian-Blajeni *et al.*, 1982).

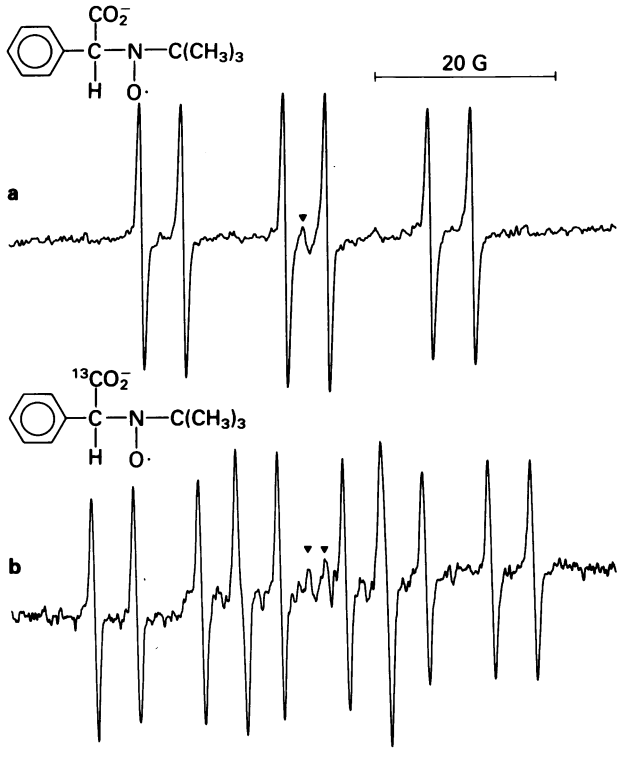


Figure 8 ESR spectra of effluent perfusate. (a) Spectrum of effluent perfusate from a liver perfused with PBN and CCl_4 (▼ = ascorbate semidione radical spectrum). (b) Spectrum of effluent perfusate from a liver perfused with PBN and $^{13}CCl_4$. (▼ = ascorbate semidione radical spectrum). (From Connor *et al.*, 1986).

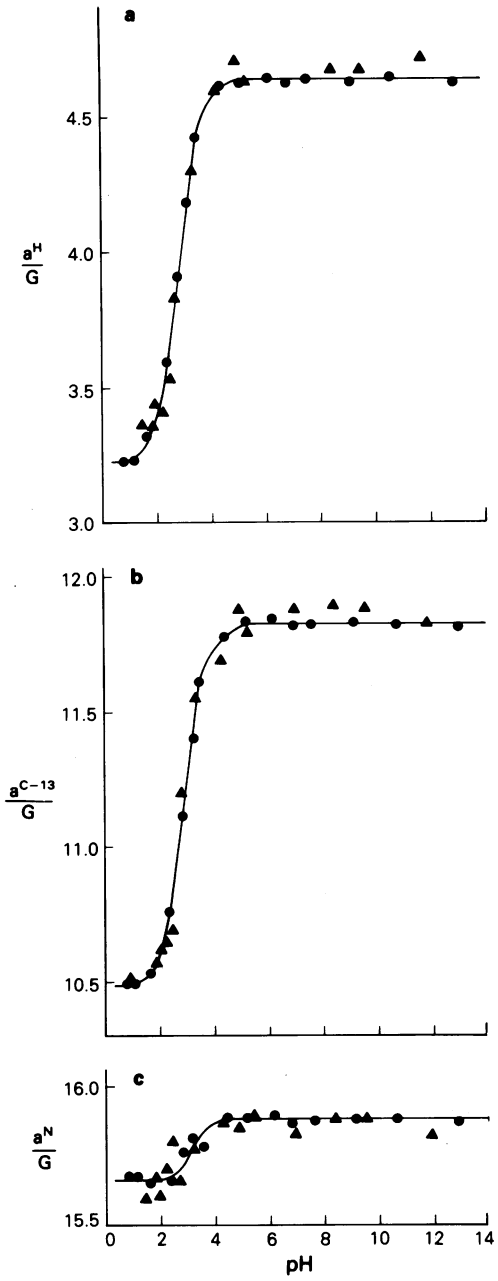


Figure 9 pH variation of ESR hyperfine coupling constants of PBN/$^{13}CO_2^{\bullet-}$. (a) pH variation of ESR hyperfine coupling for β-hydrogen of PBN/$^{13}CO_2^{\bullet-}$ radical adduct. (b) pH variation of ESR hyperfine coupling for β-carbon-13 of PBN/$^{13}CO_2^{\bullet-}$ radical adduct. (c) pH variation of ESR hyperfine coupling for nitrogen of PBN/$^{13}C_2^{\bullet-}$ radical adduct ($\triangle$ = liver perfusion, ● = Fenton system). (From Connor *et al.*, 1986).

Furthermore, the radical detected in the effluent perfusate had hyperfine couplings identical to those of the PBN/carbon dioxide anion radical adduct produced from a Fenton system containing formate (Table I). It is concluded, therefore, that this new species is the PBN/carbon dioxide anion radical adduct. This conclusion is supported by the studies of the effect of pH on the ESR hyperfine coupling constants (Figure 9).

Most previous ESR studies utilized organic extracts of liver to study radicals formed during carbon tetrachloride metabolism. Since the pK_a of the PBN/carbon dioxide anion radical is less than 3, it is ionized at physiological pH. Since this species is charged, it does not appear in organic extracts, but is observed in the effluent perfusate. This ionic character may explain, in part, why the PBN/carbon dioxide anion radical adduct has been overlooked in past studies.

Table I Hyperfine coupling constants of carbon tetrachloride-derived PBN/carbon dioxide anion radical adduct

		Hyperfine splittings (Gauss)		
Source	*Structure*	a_β^H	a^N	$\alpha_\beta^{Cl^{13}}$
Effluent perfusate $^{13}CCl_4$ liver perfusion	PBN/$^{13}CO_2^{\bullet-}$	4.6	15.8	11.7
Fenton system containing $H^{13}COONa$	PBN/$^{13}CO_2^{\bullet-}$	4.6	15.8	11.7
Photochemical system	PBN/$CO_2^{\bullet-}$	4.6	15.9	

The absence of any mention of the PBN/carbon dioxide anion radical adduct in previous spin-trapping studies of carbon tetrachloride metabolism *in vivo* led to the conclusion that this species does not remain in the liver, the focus of previous work, but rather moves into the bloodstream and eventually is excreted in the urine. Indeed, the radical adduct was observed in rat urine collected two hours after the rat had been treated with PBN and [^{13}C]-carbon tetrachloride (Connor *et al.*, 1986). The FDA and EPA require corporations to determine the urinary metabolites formed from drugs and pesticides. This requirement could now be extended to the free radical metabolites.

The reaction sequence most likely responsible for PBN/carbon dioxide radical adduct formation involves the trichloromethyl peroxyl radical (Figure 10). The trichloromethyl peroxyl radical is converted to the trichloromethoxy radical by a two-electron reduction. The trichloromethoxy radical then reacts to produce the chlorocarbonyl radical, which reacts with PBN. Upon contact with water, the PBN chlorocarbonyl radical adduct would hydrolyze to give the PBN/carbon dioxide anion radical adduct.

It is also possible that the carbon dioxide anion free radical is generated by direct hydrolysis of the chlorocarbonyl radical (Figure 10). The carbon dioxide anion

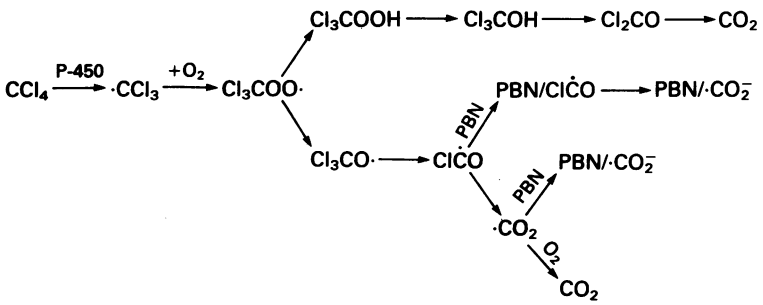
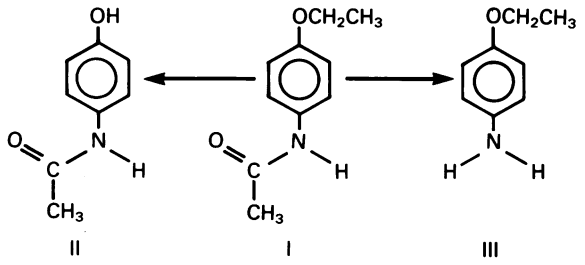


Figure 10 Although PBN/$CO_2^{\bullet-}$ is clearly $CCl_3^{\bullet}$-derived and presumably $CCl_3OO^{\bullet}$-derived, other aspects of the proposed mechanism(s) are speculative. (From Connor *et al.*, 1986.)

radical would then react with PBN to form the PBN/carbon dioxide anion radical adduct. The carbon dioxide anion radical is known to reduce oxygen to superoxide with a nearly diffusion-limited rate. This reaction forms carbon dioxide, the known final product of carbon tetrachloride metabolism.

One-electron oxidation of foreign compounds can also result in free radical metabolism. The earliest work of this type was done with horseradish peroxidase by Yamazaki *et al.* (1959). A variety of mammalian peroxidases, such as lactoperoxidase or prostaglandin hydroperoxidase, also catalyze the one-electron oxidation of many chemicals. Using purified peroxidases and inhibitors of prostaglandin H synthase, free radical metabolites of a wide variety of chemicals have been studied (Mason & Chignell, 1981; Mason, 1982; Eling *et al.*, 1983). Many of these chemicals are well-recognized electron donors, and their oxidation to free radicals by enzymes is not really surprising.

Phenacetin, an analgesic and antipyretic drug, causes toxic effects such as renal necrosis and tumours to the kidney and the lower urinary tract. Phenacetin (I) is metabolized extensively *in vivo* and forms acetaminophen (II) and *para*-phenetidine (III) as major metabolites.



Using the fast-flow technique we were able to observe the ESR spectrum of the acetaminophen phenoxyl free radical in a system consisting of lactoperoxidase, hydrogen peroxide and acetaminophen (West *et al.*, 1984; Fischer *et al.*, 1986). No signal could be found if either hydrogen peroxide, lactoperoxidase, or acetaminophen was omitted from the reaction mixture (Figure 11). Lactoperoxidase has a soret optical spectrum which is similar to that of thyroid peroxidase, intestinal peroxidase, uterine peroxidase, eosinophil peroxidase, and prostaglandin H synthase. As such, lactoperoxidase appears to be a useful prototype for most mammalian hemoprotein peroxidases.

An improved, highly-resolved spectrum of the acetaminophen phenoxyl free radical was obtained from the reaction of acetaminophen with horseradish peroxidase and hydrogen peroxide (Figure 12). The simulated spectrum contains hyperfine splitting constants from all nuclei with spin in acetaminophen and is an unambiguous proof of radical structure (Fischer *et al.*, 1986).

The possible free radical formation from the other phenacetin metabolite, *para*-phenetidine, was investigated in a similar manner (Fischer *et al.*, 1986). Rapidly mixing *para*-phenetidine and hydrogen peroxide with horseradish peroxidase at pH 7.5 resulted in a highly-resolved ESR spectrum (Figure 13a) which was analyzed (Figure 13b). The formation of the free radical is dependent upon the presence of hydrogen peroxide, horseradish peroxidase, and *para*-phenetidine (Figure 13 c-e).

There is interest in the possible reactions of the acetaminophen phenoxyl free radical metabolite with biochemicals (Figure 14). We were able to clearly exclude a reaction of the acetaminophen phenoxyl radical with oxygen which would form superoxide. Superoxide was not detectable using different approaches, and we were able to demonstrate that the report of superoxide formation is dubious (Fischer *et al.*, 1985*a*, *b*; Mason & Fischer, 1986).

There is some discussion in the literature as to whether GSH will reduce the acetaminophen phenoxyl free radicals,

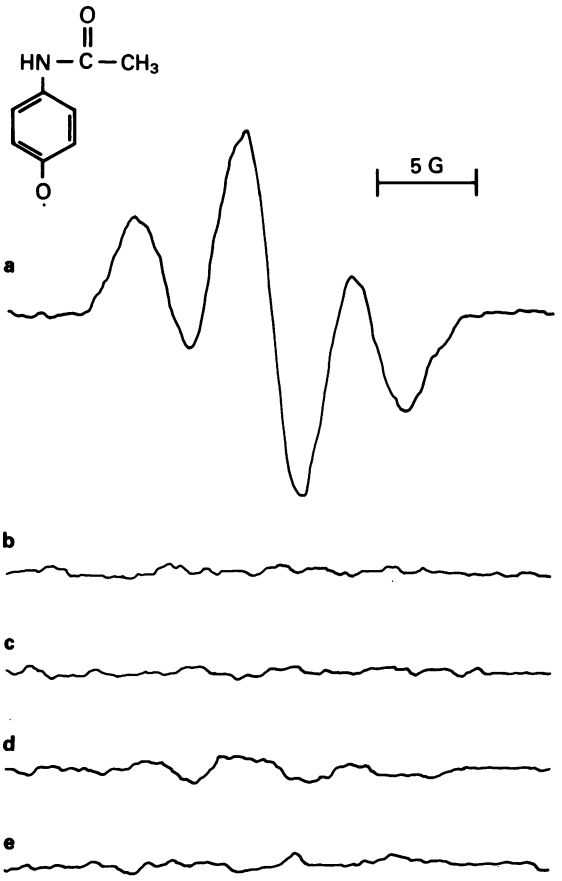


Figure 11 The ESR fast-flow spectra of the acetaminophen phenoxyl free radical produced by the reaction of acetaminophen, lactoperoxidase and hydrogen peroxide. Equal volumes of acetaminophen/H_2O_2 and lactoperoxidase in pH 7.5 phosphate buffer were mixed milliseconds prior to entering the flat cell at a total flow rate of 100 ml min^{-1}. (a) Complete system with acetaminophen, H_2O_2, and lactoperoxidase. (b) Same as in (a), but no H_2O_2. (c) Same as in (a), but no lactoperoxidase. (d) Same as in A, but with heat-denatured lactoperoxidase. (e) Same as in A, but no acetaminophen. (From Fischer *et al.*, 1986.)

thus regenerating acetaminophen and forming a thiyl radical (Figure 14). Ross *et al.* (1984) reported spin trapping the GS thiyl radical in incubations of acetaminophen, horseradish peroxidase, H_2O_2, and GSH, whereas Bisby *et al.* (1985) have reported that this reaction is slow ($k \leqq 10^6\ M^{-1}\ s^{-1}$) relative to the reduction of the acetaminophen phenoxyl free radical by ascorbate ($1.2 \times 10^8\ M^{-1}\ s^{-1}$). We have recently investigated these reactions using fast-flow ESR spectroscopy (unpublished data).

We have detected an increasing variety of free radical metabolites with ESR. Thiyl free radicals of L-cysteine and glutathione have been spin-trapped (Harman *et al.*, 1984, 1986). The acetaminophen phenoxyl and the nitrogen-centered cation radical of *para*-phenetidine have been detected (Fischer *et al.*, 1986). Inorganic chemicals such as azide (Kalyanaraman *et al.*, 1985) and sulfite (Mottley *et al.*, 1982, 1985) are also substrates for free radical formation. Many other free radical metabolites have been detected for the first time.

Each of these free radicals has its own chemistry. Generally we can make use of the chemical literature to anticipate the reactions of these radicals. Under physiological conditions we have shown dimerization of acetaminophen and *para*-phenetidine free radicals (Fischer *et al.*, 1986), and stabilization of the azo anion free radical metabolite of a Ca^{2+} indicator by metal complex formation (Docampo *et al.*, 1983).

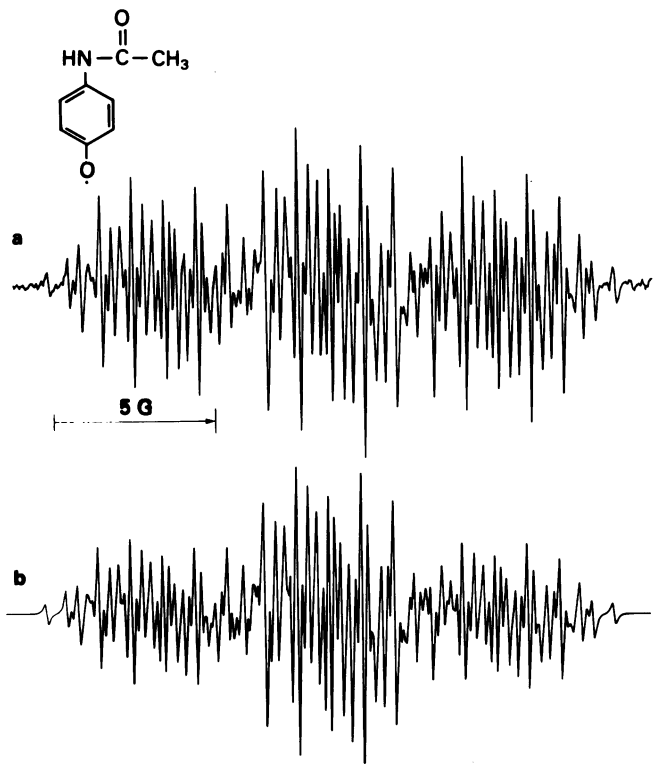


Figure 12 The high-resolution fast-flow ESR spectrum of the acetaminophen phenoxyl free radical produced by horseradish peroxidase and hydrogen peroxide. (a) Equal volumes of acetaminophen/H_2O_2 and horseradish peroxidase in pH 7.5 phosphate buffer were mixed milliseconds prior to entering the flat cell at a total flow rate of 100 ml min^{-1}. (b) Computer simulation. Hyperfine splitting constants were $a^N = 0.81_5$ G, $a^H_{NH} = 1.35$ G, $a^H_{meta} = 0.64$ G, $a^H_{ortho} = 5.11$ G and $a^H_{C(O)CH_3} = 1.01$ G. (From Fischer *et al.*, 1986.)

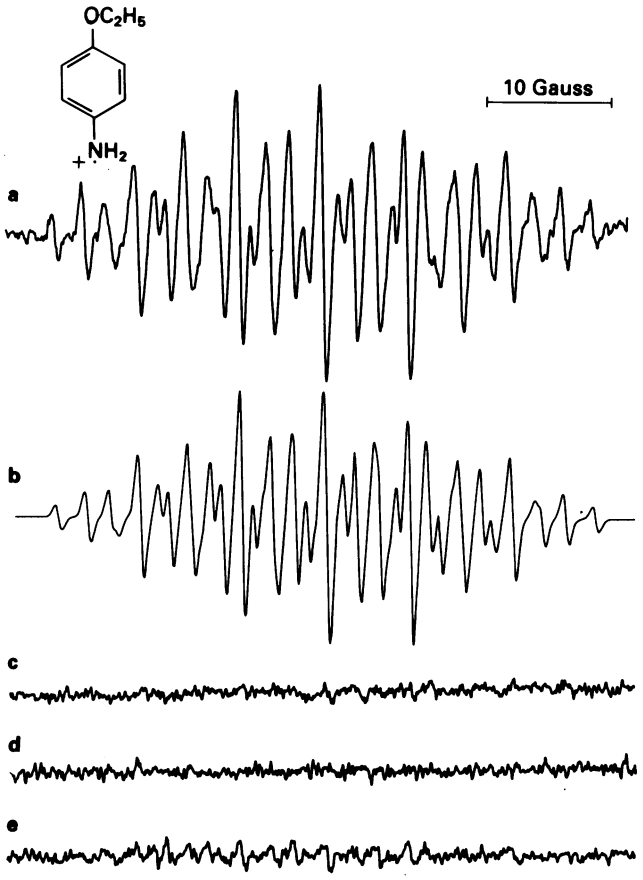


Figure 13 The ESR fast-flow spectrum of the 4-ethoxyaniline cation free radical produced in a system of *p*-phenetidine, horseradish peroxidase and hydrogen peroxide. *p*-Phenetidine and hydrogen peroxide were flowed against an equal volume of horseradish peroxidase. (a) Complete system with *p*-phenetidine, hydrogen peroxide and horseradish peroxidase. (b) Computer simulation. Hyperfine splitting constants were $a^N = 6.75$ G, $a^H_{NH_2} = 8.20$ G, $a^H_{ortho} = 4.25$ G and $a^H_{OCH_2} = 2.37$ G. (c) Same as in (a), but no H_2O_2. (d) Same as in (a) but no horseradish peroxidase. (e) Same as in (a) but no *p*-phenetidine.

Our ultimate goal is to understand the biochemical and toxicological consequences of free radical metabolism. The azidyl radical formation by catalase and hydrogen peroxide is certainly something that biochemists who use azide as a catalase inhibitor should be aware of (Kalyanaraman *et al.*, 1985). Glutathione peroxidase does not form a thiyl free radical from GSH (Harman *et al.*, 1986), which is in line with the thesis that normal biochemistry does not form free radical metabolites. We have also shown that glutathione may be a physiological electron-donor for the reduction of prostaglandin G_2 to prostaglandin H_2 (Eling *et al.*, 1986). We have recently proposed a mechanism for the co-carcinogenicity of SO_2 with benzo(a)pyrene, based on the epoxidation of (±)-7,8-dihydroxy-7,8-dihydrobenzo(*a*)pyrene by a sulfite-derived peroxyl free radical (Reed *et al.*, 1986). In summary, free radical metabolites are being investigated with ESR in a great number of chemico-biological systems, but many experiments remain before the significance of free radical metabolites in biochemistry, pharmacology, and toxicology will be fully comprehended.

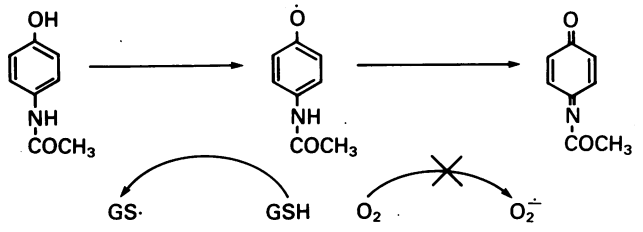


Figure 14 The acetaminophen phenoxyl free radical reacts with GSH, but not with oxygen.

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Discussion

Asmus: Five or ten years ago we always thought that thiyl radicals, once they were formed, could only undergo recombination to the disulphides. In the meantime we have learned that thiyl radicals do not only undergo this recombination but are also relatively good oxidants. They also add on to double bonds and hetero atoms which provide free electron pairs. Particularly the last aspect means that sulphur-centered radicals can associate themselves with other molecules or atoms thereby establishing equilibria. Of course, unless you know all the kinetic and thermodynamic data of these equilibria – which are very often interconnected – then you just cannot fully understand the possible reaction of the radicals. In addition to that, there are particular problems when you change over from homogenous aqueous solutions to a system of more biological relevance. The two complimentary techniques that you have mentioned, namely ESR and pulse radiolysis have provided excellent data so far, but I think there is still a lot of work ahead.

Wardman: Concerning radiation chemists helping out ESR people, I am happy to provide an estimate for the rate constant of the repair of the acetaminophen phenoxy radical by glutathione. This was obtained by my colleague Dr Ian Wilson from gamma radiolysis studies of dose rate effects and glutathione depletion in systems where the phenoxy radical was produced and numerical simulation of the reaction scheme, the estimate of 3×10^4, this reaction rate constant is too slow to be obtained by conventional pulse radiolysis.

Butler: It worries me that you actually saw the azide radical in the absence of hydroxyl radicals.

Mason: No, peroxidases do not form the hydroxyl radical.

Butler: So you get the azide radical from the H_2O_2 without $^{\bullet}OH$ being formed.