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Free-Radical Chemistry of Cigarette Smoke and Its Toxicological Implications

by Daniel F. Church* and William A. Pryor*†

Cigarette smoke contains two very different populations of free radicals, one in the tar and one in the gas phase. The tar phase contains several relatively stable free radicals; we have identified the principal radical as a quinone/hydroquinone (Q/QH_2) complex held in the tarry matrix. We suggest that this Q/QH_2 polymer is an active redox system that is capable of reducing molecular oxygen to produce superoxide, eventually leading to hydrogen peroxide and hydroxyl radicals. In addition, we have shown that the principal radical in tar reacts with DNA *in vitro*, possibly by covalent binding.

The gas phase of cigarette smoke contains small oxygen- and carbon-centered radicals that are much more reactive than are the tar-phase radicals. These gas-phase radicals do not arise in the flame, but rather are produced in a steady state by the oxidation of NO to NO_2, which then reacts with reactive species in smoke such as isoprene. We suggest that these radicals and the metastable products derived from these radical reactions may be responsible for the inactivation of α_1-proteinase inhibitor by fresh smoke.

Cigarette smoke oxidizes thiols to disulfides; we suggest the active oxidants are NO and NO_2. The effects of smoke on lipid peroxidation are complex, and this is discussed. We also discuss the toxicological implications for the radicals in smoke in terms of a number of radical-mediated disease processes, including emphysema and cancer.

Introduction

The periodic reports by the Surgeon General of the United States dramatically illustrate the research effort that has been expended to understand the health consequences of cigarette smoking, both to the smoker and to nearby nonsmokers (*1-3*). There is overwhelming evidence that smoking is, at least in part, responsible for such diverse and life-threatening diseases as emphysema, heart and blood vessel disease, and cancer.

Our research program has focused on the free-radical chemistry of smoke, particularly the radical chemistry that might be implicated in smoke toxicology (*4–15*). It has been known for many years that cigarette smoke contains free radicals, and it has been generally assumed that these radicals must somehow be involved in the pathology induced by smoking (*16-22*). However, until we began our studies, there had been no systematic investigation of the mechanisms by which radicals are produced in smoke, their structures and chemical reactivities, and possible biochemical and biological consequences of the exposure of biomolecules to smoke-borne radicals (*4–14*).

*Departments of Chemistry and Biochemistry, Louisiana State University, Baton Rouge, LA 70803.

†Author to whom correspondence and reprint requests should be addressed.

Two lines of evidence can be cited to support a role for smoke-borne radicals in smoking-induced pathology. Firstly, the evidence is becoming increasingly strong that free radicals are involved in many of the chronic diseases that are associated with smoking (*23–25*); in particular, radicals appear involved in emphysema (*26*) and chemical carcinogenesis (*27–31*). Secondly, the concentrations of radicals in smoke are so high (compared, for example, with smog) that radical-mediated reaction pathways appear certain to result from the exposure of tissue to smoke.

By convention in the smoking industry, two fractions of cigarette smoke are defined based on the use of a filter to separate the gas phase from the tar (*10*). The tar phase is defined as the material that is trapped when the smokestream is passed through a standard glass-fiber Cambridge filter that retains 99.9% of all particulate matter with a size greater than 0.1 μM (*32*). The gas phase is the material that passes through the Cambridge filter.

Cigarette tar contains several exceptionally stable radical species that can be observed directly by electron spin resonance (ESR) spectroscopy. Our data demonstrate that these radicals are not simple aromatic radical ions (*10,11*), as had been suggested by the original workers. The free radicals in the gas phase of cigarette smoke, in contrast to those in tar, cannot be observed

directly by ESR; however, they can be detected using spin trapping techniques (*4,10,12,14*). These gas-phase radicals appear to be relatively small alkoxyl and carbon-centered species (*14*); early workers assumed that these gas-phase radicals are formed in the flame, but we find this is not the case.

In this review, we summarize the data from our laboratory that provide insight into the structure and reactivities of the free radicals in both tar and gas-phase smoke. We also present our current viewpoint on what we believe is the relationship between these radicals and the health effects of smoking.

The Tar Radical(s) in Cigarette Smoke: ESR Studies

The first evidence for the presence of radicals in cigarette tar was obtained by Ingram's group in the late 1950s (*16,17*). Ingram et al. studied a variety of carbonaceous soots and tars, including cigarette tar, by ESR and observed a single, broad ESR line in each instance. All of the related tarry materials were hypothesized to contain a single type of radical consisting of an odd electron delocalized over a large aromatic hydrocarbon framework. This interpretation remained unchallanged until we began our investigations (*10,11*).

Figure 1*A* shows a typical ESR spectrum of the tar from three 1R1 research cigarettes on a Cambridge filter; there appears to be a major asymmetric line with a small shoulder on the low-field wing. The asymmetry of the principal line is more clearly evident in the second derivative spectrum shown in Figure 1*B*. The *g* value of the major line is 2.0029 in the spectrum shown here, while the smaller line at lower field occurs at 2.0089; the apparent linewidth of the principal line is 0.57 mT. However, the *g* value, linewidth, and saturation are all markedly power-dependent, indicating that there are several paramagnetic species contributing to the ESR spectra of solid cigarette tar.

Extraction of cigarette tar from the Cambridge filter with various solvents provides convincing evidence that there are indeed several radical species present in tar (see Fig. 2). Table 1 summarizes the *g* values of the lines observed when the tar radicals are extracted into homogeneous solutions in organic solvents. We have previously suggested (*11*) that the principal radical species (radical 3, Table 1), which has a *g* value of 2.0035-2.0038, consists of conjugated quinone (Q), semiquinone ($QH^{\cdot}$), and hydroquinone (QH_2) units in a polymeric, tarry matrix. That is, this paramagnetism is essentially

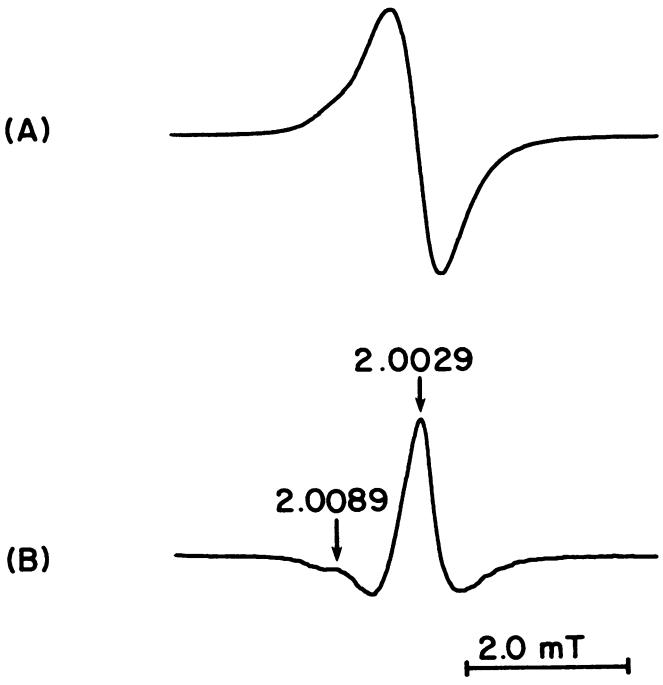


FIGURE 1. ESR spectrum of cigarette tar on a Cambridge filter: (*A*) first derivative spectrum; (*B*) second derivative spectrum obtained by differentiation of spectrum (*A*). The tar from three 1R1 cigarettes was collected on a Cambridge filter. The filter was then dried under vacuum, tightly rolled, and placed in an ESR tube. The spectrum shown was obtained by accumulating ten scans at 297 K using the following ESR settings: power, 2 mW; modulation, 0.16 mT at 100 kHz; gain, 8×10^4; time constant, 2 sec; center field, 349.40 mT; scan range, 6.0 mT; scan time, 200 sec.

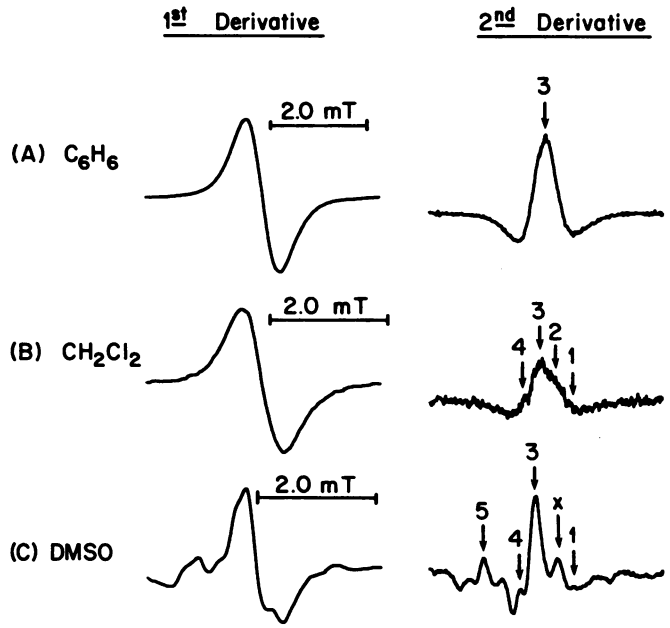


FIGURE 2. ESR spectra of cigarette tar extracted in various solvents. (*A*) The tar from 12 1R1 cigarettes in 1.5 mL benzene in a cylindrical ESR tube. ESR parameters: scans, 4; power, 20 mW; modulation, 0.125 at 100 kHz; gain, 1.25×10^5; time constant, 1 sec; scan range, 5.0 mT; scan time, 200 sec. This spectrum was obtained at 250°K, wereas the others were obtained at 297°K. The spectrum of the tar in frozen solution was identical to that obtained at higher temperature for the liquid. (*B*) The tar from three 1R1 cigarettes in dichloromethane in a flat cell. ESR parameters: scans, 20; power, 20 mW; modulation, 0.16 mT at 100 kHz; gain, 2×10^5; time constant, 1 sec; scan range, 4.0 mT; scan time, 100 sec. (*C*) The tar from one 1R1 cigarette in 10 mL DMSO in a flat cell. ESR parameters: scans, 10; power, 100 mW; modulation, 0.16 mT at 100 kHz; gain, 2.5×10^6; time constant, 10 sec; scan range, 4.0 mT; scan time, 200 sec. The peak marked X is due to an impurity in the quartz cell.

Table 1. Summary of radical species observed in various solvent extracts of cigarette tar.

Radical[a]	Extracting solvent			Suggested nature of the paramagnetic species
	Benzene	Dichloromethane	Dimethyl sulfoxide	
1	—	2.0009	2.0009	?
2	—	2.0028	—	?
3	2.0035	2.0037	2.0038	Q/QH_2 radical
4	—	2.0052	2.0053	Nitrogenous oxidized leaf material?
5	—	—	2.0089	Sulfur-centered radical?

[a] These numbers correspond to those shown in Figure 2. The peak marked X in spectrum C, Figure 2, is due to an impurity in the quartz.

that due to highly delocalized semiquinone radicals; rapid hydrogen atom transfer between the different quinone oxidation states may account for the lack of fine structure in the ESR line. The lower g value that we observe for this radical species from tar compared to those commonly observed for aqueous solutions of semiquinones may reflect the extensive conjugation of the radical from tar. (This could also account for the lack of observable fine structure.) Alternatively, the difference that we observe between the g value for the tar radical in organic solvents and the g values for semiquinones in water may be due, at least in part, to a solvent effect; the g values of semiquinones are known to be somewhat solvent-dependent (*33*). The data in Table 1 show a slight increase in g value from 2.0035 to 2.0038 as the polarity of the extracting solvent increases. In current work we find that the tar radical can be extracted into phosphate buffer, pH 7.4, and these aqueous solutions of the Q/QH_2 radical give a g value of 2.0045, in agreement with the values typically observed for semiquinones in water.

The identification of one of the principal radicals in cigarette tar as being derived from quinones and/or hydroquinones is reasonable. Cigarette tar is known to be rich in hydroquinones and quinones (*34*), especially catechol which occurs at levels up to 0.3 mg/cigarette (*32,35–37*). Thus, a rapid condensation of the quinones and hydroquinones present in tobacco smoke to give a Q/QH_2 polymer is a possible route to the material that gives the tar radical with $g = 2.0035$.

To test the hypothesis that the radical in cigarette tar with $g = 2.0035$ is due to a Q/QH_2 polymer, we have prepared several authenic such polymers by the condensation of either 1,7-naphthalenediol or 1,4-naphthoquinone (*11*). These synthetic polymers have ESR properties very similar to those of the radical in tar, including g values of 2.0035.

The possible contribution by the quinones and hydroquinones in cigarette smoke to the tar radical with $g = 2.0035$ was also demonstrated by experiments in which the cigarette tobacco was "spiked" with various compounds before smoking (*11*). Thus, the addition of either catechol or 1,4-naphthoquinone to the tobacco gave up to a 5-fold increase in the ESR signal at about $g = 2.0035$. By contrast, the addition of polynuclear aromatic hydrocarbons (PAH) such as pyrene or anthracene gave no increase. This result suggests that the tar radical may arise from reactions of the quinones or hydroquinones in the smokestream itself, and that the oxidation of PAH to quinones does not appear to be important in the generation of the Q/QH_2 tar radical.

We have not yet identified the other paramagnetic species in tar and their nature and origin therefore is speculative (see Table 1). Some suggestions, however, can be made. For example, a radical with a g value of 2.0052 is produced in tobacco when it is heated to about 300°C without combustion (W. A. Pryor, R. Saylor, and D. F. Church, unpublished data, this laboratory). We therefore suggest the higher g value radical in tar is formed from the pyrolysis of proteins or similar nitrogenous materials that occur in the tobacco leaf. Species 2 has a g value ($g = 2.0028$) that is consistent with an organic carbon-centered radical. We have preliminary evidence suggesting that the observation of this species depends on the nature of the sample preparation. Thus, if a solution of the benzene extract of tar is freeze-dried and then resuspended in benzene, the only signal observed is species 3 ($g = 2.0035$). On the other hand, the ESR spectrum of the original benzene extract shows both species 2 and 3. It is possible, therefore, that the two species are related to one another. As we have indicated in Table 1, the species with the g value of 2.0089 could be a sulfur-centered radical.

The Chemistry of the Cigarette Tar Radical(s)

Before describing our most recent studies of the nature and reactivity of the tar radical, a caveat is wise: Cigarette tar is an incredibly complex mixture; over 3000 compounds have been identified, while many more remain unknown (*18*). Since the tar radical(s) are part of this mixture, and since they have not been isolated and unambiguously identified, any conclusions concerning the chemistry or biochemistry of the tar radicals must be regarded as tentative at present.

We first began to suspect that the principal radicals in cigarette tar are not simple PAH radicals when we noticed a remarkable similarity between the properties of the tar radical and melanin radicals. Melanins are naturally occurring polymers containing quinone and hydroquinone groups that are ultimately derived from tyrosine via oxidation to dihydroxyphenylalanine (DOPA). A synthetic melanin can be prepared by enzymatic oxidation or by autoxidation of DOPA.

Table 2. Comparison of the tar radical to natural and synthetic melanins.

Property/test	Melanin	Autoxidized DOPA	Tar
ESR parameter			
g Value	2.001-2.005	2.003-2.004	2.0035
Linewidth, mT	0.5-1.2	0.4-0.8	0.5-0.6
Spins/gram	10^{16}-10^{19}	10^{17}-10^{18}	10^{16}-10^{17}
Effect on ESR signal			
+ NaOH	7-fold increase	—	7-fold increase
+ HCl	decrease	increase	2-fold increase
+ H_2O_2	signal disappears	—	33% decrease
+ Zn^{2+}	3-fold increase	7-fold increase	5-fold increase
+ Cu^{2+}	30% decrease	—	66% decrease
Reduction of Ag^+	silver mirror	silver mirror	silver mirror

Table 2 compares the ESR and chemical properties of melanins with those of an alcoholic extract of cigarette tar (*10*,*38*). The similarity between the ESR and chemical properties of the tar radical and those of natural and, especially, synthetic DOPA melanin is striking indeed. Many of these effects are regarded as characteristic of *o*-quinone and *o*-hydroquinone groups in melanins. For example, the increase in the melanin ESR signal in the presence of the diamagnetic metal ion Zn^{+2} has been suggested to be the result of complexation and stabilization of ortho semiquinone radicals by the metal (*39*). Similarly, the reduction of Ag^+ to form a silver mirror (Table 2), the classic histochemical test for melanins, is positive for *o*-semiquinones (*40*). We suggest that these tests indicate the presence of *ortho* Q/QH_2 groups in tar as well.

Thus, our current view of the principal paramagnetic species in tar (radical 3, Table 1) is that it is a polymer of modest molecular weight that contains quinone (Q), semiquinone ($QH^{\bullet}$), and hydroquinone (QH_2) moieties. These Q and QH_2 species can readily interconvert by hydrogen atom exchange, as schematically depicted in Figure 3. Presumably, this rapid hydrogen exchange, as well as extensive conjugation, produces the single-line spectrum without hyperfine splittings that is observed. Addition of metal ions, acids, bases, etc. (Table 2), presumably causes a shift in the equilibria shown in Figure 3 to either increase or decrease the number of semiquinone states in the system.

The identification of one of the radicals in cigarette tar as a Q/QH_2 polymer may have important implications for the toxicology of smoke. Synthetic Q/QH_2 polymers have been shown to be potent redox catalysts in organic chemistry (*41*–*44*). Even more important, redox cycling of Q and QH_2 compounds is extremely important in modulating oxy-radical levels in biological systems (*45*). We suggest that the Q/QH_2 polymer in cigarette tar may have the capability of altering oxy-radical levels in the lung.

Before describing our work with cigarette tar, it will be helpful first to review briefly what we believe is an analogous system, namely the melanins. There is a considerable body of evidence that melanins have the capacity to utilize molecular oxygen to generate active oxygen species such as superoxide and hydrogen peroxide. Felix et al. (*46*), using the change in the ESR saturation of the melanin radical, have demonstrated that photolyzed suspensions of melanin consume molecular oxygen. They found that the addition of superoxide dismutase (SOD) had almost no effect on the rate of oxygen utilization, while the addition of catalase cut the rate nearly in half. The apparent decrease in the rate of oxygen utilization with catalase undoubtedly reflects regeneration of oxygen via Eq. (1).

$$2\ H_2O_2 \xrightarrow{\text{catalase}} 2\ H_2O + O_2 \qquad (1)$$

Although SOD had little effect on oxygen consumption by melanins, superoxide was detected in aerated melanin solutions as its spin adduct with the spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO). The inference is

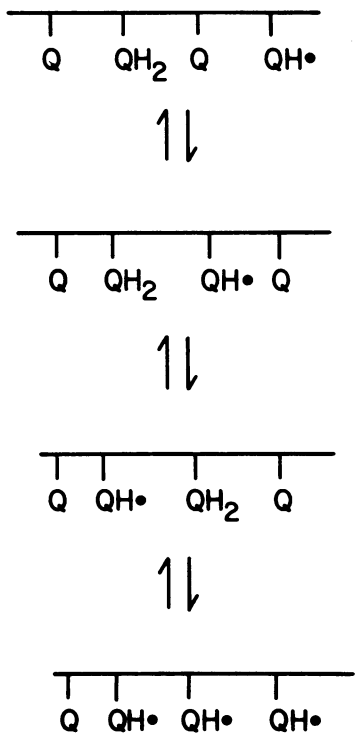


FIGURE 3. A schematic representation of the Q/QH/QH_2 equilibria in Q/QH_2 polymers such as melanins and cigarette tar.

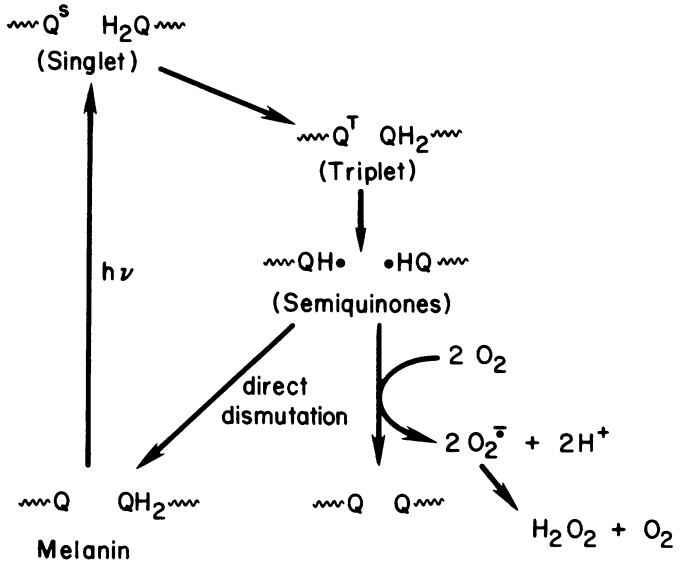


FIGURE 4. Scheme proposed to account for the photochemical reduction of molecular oxygen by Q/QH_2 polymers such as melanin.

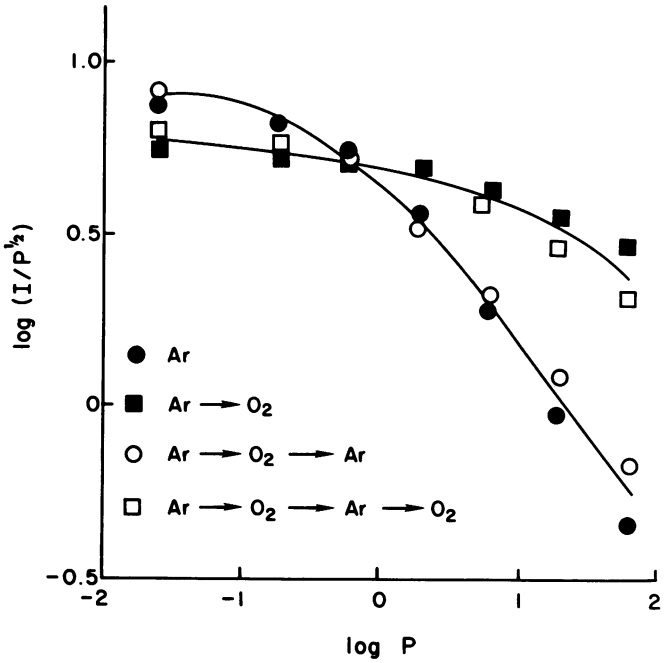


FIGURE 5. Effect of oxygen on the saturation behavior of the cigarette tar radical. The curves were obtained by alternatively bubbling argon or oxygen through a solution of the tar radical in benzene. The upper curve shows little saturation and was obtained when the tar solution was saturated with oxygen. The lower curve, showing strong power saturation, was obtained when the solution was saturated with argon. The symbol I represents the relative ESR signal strength of the tar radical, while the P represents the microwave power in milliwatts.

that superoxide is on the reaction pathway to hydrogen peroxide even though SOD had no effect. These results for melanins are rationalized in terms of the chemistry depicted in Figure 4. The concentration of semiquinone moieties is increased by photo-activation of the melanin Q/QH_2 polymer to a level higher than the equilibrium value. Equilibrium is then restored by electron transfer from a semiquinone to dioxygen to give superoxide. The ultimate product is hydrogen peroxide from the spontaneous dismutation of superoxide.

Melanins also have been shown to catalyze the reduction of dioxygen to hydrogen peroxide in the dark (*47–49*), although ultraviolet irradiation does markedly accelerate the process (*50*). In these oxidations, NADH can be the ultimate source of reducing equivalents. In these experiments, SOD inhibits the oxidation of NADH, while added metal ions (e.g., Cu^{+2}) accelerate the oxidation. We rationalize these results in terms of the reactions shown in Eqs. (2–8).

In Eq. (2), NADH reduces melanin quinone groups to the semiquinone, which then reduces dioxygen to superoxide [Eq. (3)]. The ultimate product, hydrogen peroxide, could then be formed either by dismutation of superoxide [Eq. (4)] or by the oxidation of another NADH molecule by superoxide [Eq. (5)]. This latter reaction would be inhibited by SOD. The acceleration by metals results from the Fenton-type cycling process [Eqs. (6) and (7)] that produces hydroxyl radicals capable of oxidizing NADH [Eq. (8)].

We suggest that the $g = 2.0035$ Q/QH_2 radical in tar may undergo many or all of these same processes. For example, we find that the intensity of the ESR signal that is observed at high microwave powers from solutions of cigarette tar decreases over a period of one to two days; however, the original signal intensity can be restored by aerating the solution. Figure 5 shows these

$$\text{NADH} + \sim\!\!\sim\text{Q} \longrightarrow \text{NAD}\bullet + \sim\!\!\sim\text{QH}\bullet \quad (2)$$

$$\sim\!\!\sim\text{QH}\bullet + \text{O}_2 \longrightarrow \sim\!\!\sim\text{Q} + \text{O}_2^{\bullet-} + \text{H}^+ \quad (3)$$

$$2\,\text{O}_2^{\bullet-} + 2\,\text{H}^+ \longrightarrow \text{H}_2\text{O}_2 + \text{O}_2 \quad (4)$$

$$\text{O}_2^{\bullet-} + \text{NADH} \longrightarrow \text{NAD}\bullet + \text{HO}_2^- \quad (5)$$

$$\text{M}^{+n} + \text{O}_2^{\bullet-} \longrightarrow \text{M}^{+(n-1)} + \text{O}_2 \quad (6)$$

$$\text{M}^{+(n-1)} + \text{H}_2\text{O}_2 \longrightarrow \text{M}^{+n} + \text{HO}\bullet + \text{HO}^- \quad (7)$$

$$\text{HO}\bullet + \text{NADH} \longrightarrow \text{NAD}\bullet + \text{H}_2\text{O} \quad (8)$$

effects; note that alternately purging a solution of tar with argon and oxygen reversibly changes the saturation behavior of the tar radical. The observation that the ESR intensity of spectra of the tar solutions de-

Table 3. Analyses of the ESR spectra shown in Figure 6.

	Spectrum	g	H_{p-p}, mT
(A)	Tar in benzene	2.0035	0.7
(B)	Poly(G) + tar[a]; no incubation	2.0040	0.7
(C)	Poly(G) + tar[a]	2.0040	0.9
(D)	Poly(A) + tar[a]	2.0038	0.9
(E)	Poly(U) + tar[a]	2.0042	0.9
(F)	Poly(C) + tar[a]	2.0041	0.8
(G)	Calf thymus DNA + tar[a]	2.0041	0.8

[a] Incubated for 1 day at 37°C.

creases with time can be explained as follows: the cigarette tar radical saturates much more easily in the absence of oxygen than in its presence. With time, the tar radical uses oxygen from the solution, saturates more easily, and exhibits a lower intensity at higher microwave powers. This saturation behavior of the tar radical with oxygen in solution indicates a significant interaction between the paramagetic center in tar and oxygen.

A recent communication by Nakayama et al. indicates that hydrogen peroxide is formed over a period of several hours when cigarette smoke is bubbled into water (*51*). Superoxide dismutase was found to inhibit hydrogen peroxide formation, indicating that superoxide is an intermediate. These results are consistent with the redox cycling of the tar Q/QH_2 radical that we have outlined above and are similar to effects observed with the melanins (J. P. Cosgrove, E. T. Borish, D. F. Church, and W. A. Pryor, submitted for publication).

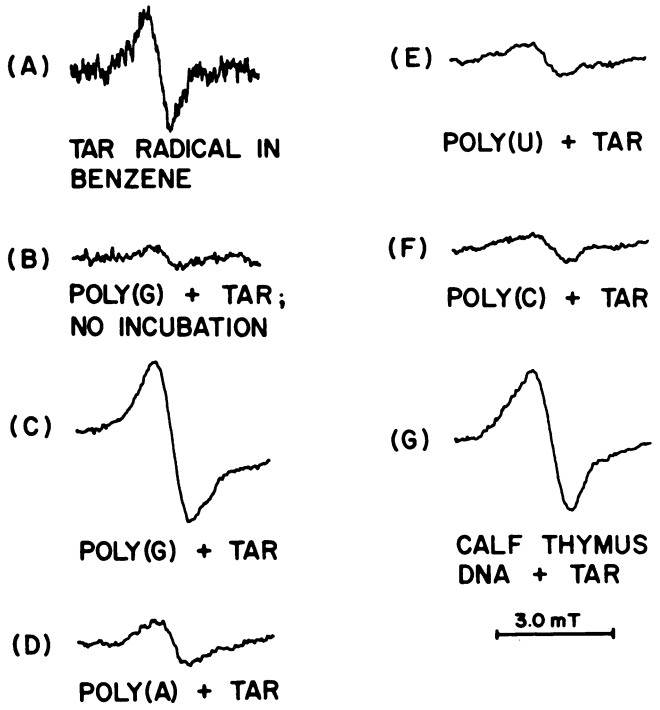


FIGURE 6. Radicals observed in polynucleotides and calf thymus DNA after incubation with cigarette tar. (*A*) The spectrum of tar in benzene for reference. In the following experiments, a solution of tar in 70% ethanol/water was added to a solution of the polynucleotide in water and the resulting mixture was then either separated immediately or incubated for 24 hr at 37°C and then separated. The mixture was first extracted with benzene to remove tar and the water was then removed from the polynucleotide or DNA under vacuum. (*B*) The spectrum of poly(G) plus tar that was separated immediately after mixing. (*C-G*) Spectra of substrates incubated 24 hr with tar: (*C*) poly(G) plus tar incubated for 24 hr; (*D*) poly(A) plus tar; (*E*) poly(U) plus tar; (*F*) poly(C) plus tar; (*G*) calf thymus DNA plus tar.

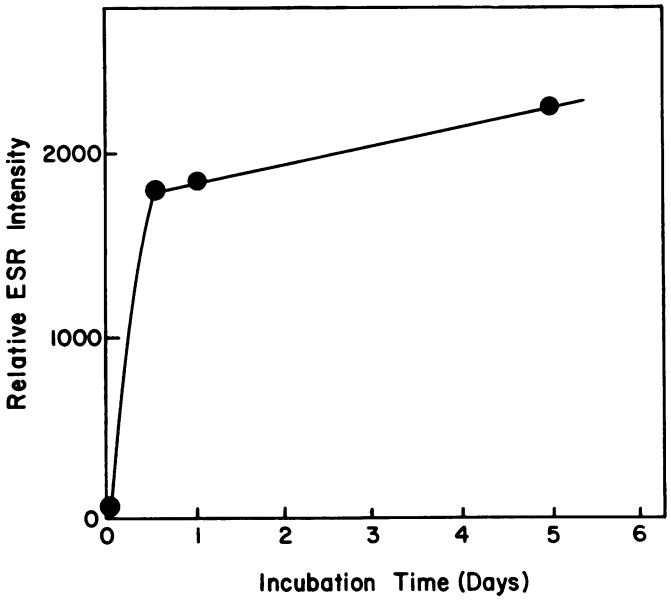


FIGURE 7. Plot of ESR signal intensity in poly(G) that has been incubated with tar at 37°C for various times.

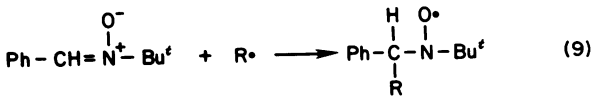
We have recently found (*13*) that when cigarette tar is incubated with polynucleotides or with calf thymus DNA a radical is observed in the recovered polynucleotide or DNA. Figure 6 shows the spectrum of the tar radical in benzene, the spectrum of poly(G) that has been mixed with a solution of the tar radical and immediately separated, and the spectra that result when the four polynucleotides or calf thymus DNA are mixed with the tar radical solution, incubated for 24 hr, and then separated. The ESR parameters corresponding to these spectra are presented in Table 3. Poly(G) and calf thymus DNA show the strongest signals after incubation, while the other polynucleotides show much weaker signals. When poly(G) and tar are mixed and then immediately separated without incubation (Fig. 6*B*), the signal in the recovered poly(G) is much weaker than the signal obtained after incubation for one day at 37°C (Fig. 6*C*). As Figure 7 shows, most of the signal intensity in the poly(G) has developed after approximately 12 hr of incubation (W. A. Pryor, K. Uehara, and D. F. Church, unpublished data, this laboratory). Thus, the development of the radical in the poly(G) appears to involve a relatively facile reaction between the polynucleotides and the tar radical. The ESR signals observed in the polynucleotides and DNA after incubation with tar have slightly larger g values and linewidths than does the tar radical itself (see Table 3). We suggest that the signal observed after incubation could be due to covalent binding of the tar radical itself to the polynucleotides and DNA. The larger g values then might arise from a slight

delocalization of the radical onto the nitrogenous bases.

In recent work we have shown that aqueous extracts of cigarette tar in phosphate buffers cause strand breaks in covalently closed circular pBR322 DNA (E. T. Borish, J. P. Cosgrove, D. F. Church, and W. A. Pryor, unpublished data). The time course for this reaction closely parallels that for the production of hydroxy radicals, as determined by spin-trapping techniques.

Radicals in Gas-Phase Cigarette Smoke: Spin Trapping Studies

Unlike the tar radical(s), which are long-lived and can be studied directly by ESR, the gas-phase radicals are both less stable and much lower in concentration. We have been able to study these gas-phase radicals using the ESR spin-trapping technique (*4,10,14*). This technique involves "trapping" unstable radicals with a compound such as phenyl-*tert*-butyl nitrone (PBN) to give a radical that is more stable than the radical that was trapped, and consequently can be more easily detected by ESR, as shown in Eq. (9).



The first application of this spin trapping method to the detection of the gas-phase radicals in cigarette smoke was reported by Bluhm et al. (*19*). They reported a poorly resolved spectrum that clearly indicated that nitroxide radicals had been formed, but little information about the nature of the radicals that had been trapped could be obtained from their data.

We have tested a variety of protocols for spin trapping the gas-phase radicals from cigarette smoke (*14*). Representative spectra are shown in Figure 8 *A–C*; analyses of these spectra are presented in Table 4. Spectra 8*A* and 8*C* were obtained by bubbling the smoke-stream through a solution of PBN in carbon tetrachloride or benzene,

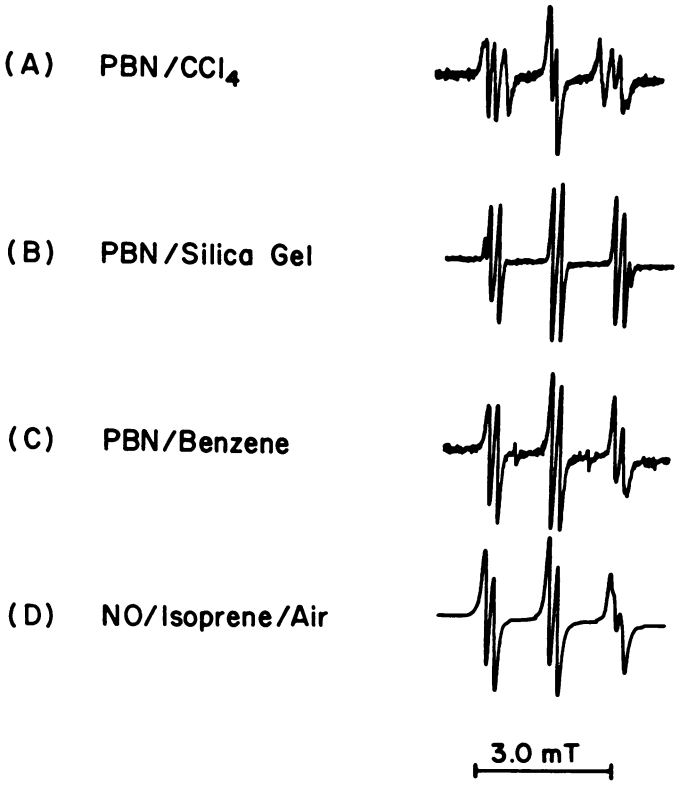


FIGURE 8. Spin-trapping of cigarette smoke under various conditions and of an NO/isoprene/air mixture. See the text and literature (*14*) for detailed descriptions of the protocols used. (*A*) The spectrum observed when the smoke from a 1R1 cigarette is bubbled through a solution of PBN in carbon tetrachloride. (*B*) The spectrum observed when the smoke from a 1R1 cigarette is passed through a silica gel column coated with PBN. The spectrum shown was obtained by eluting the column with benzene and obtaining the spectrum of the benzene solution. (*C*) The spectrum observed when the smoke from a 1R1 cigarette is bubbled through a solution of PBN in benzene. (*D*) The spectrum observed when a mixture of NO and isoprene in air is bubbled through a solution of PBN in benzene.

respectively. Of particular note is the spectrum (Fig. 8*B*), which was obtained by passing the smoke-stream through a short column of silica gel coated with 6% by weight of PBN, washing the silica gel with benzene, and obtaining the ESR spectrum of the benzene solution. This protocol

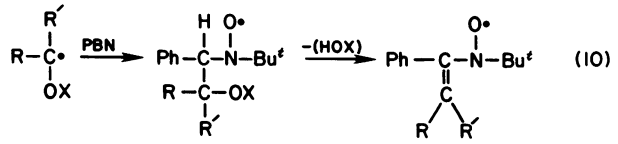
Table 4. Analysis of spin trapping of 1R1 cigarette smoke and NO/isoprene/air mixture.

	Conditions	a_N, mT	a_H, mT	%	Radical
(A)	1R1 cigarette	1.38	0.18	51	RO-PBN
	PBN/CCl_4	1.45	0.33	14	R-PBN
		1.04	a	35	b
(B)	1R1 cigarette	1.37	0.20	75	RO-PBN
	PBN/silica gel	1.43	0.32	25	R-PBN
		1.36	0.19	67	RO-PBN
(C)	1R1 cigarette	1.44	0.20	30	R'-PBN
	PBN/benzene	0.80	—	3	PBNOx
(D)	NO/isoprene/air	1.38	0.21	42	RO-PBN
	PBN/benzene	1.42	0.21	55	R'-PBN
		0.79	—	3	PBNOx

[a] There are four unresolved lines due to long-range hydrogen splitting(s); a_H ca. 0.04–0.06 mT.
[b] Possibly a vinyl nitroxide; see text.

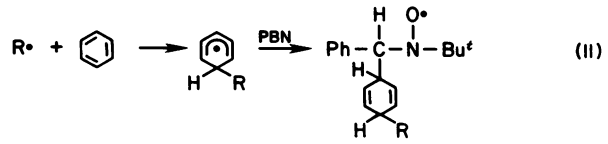
gives an exceptionally clean spectrum, presumably due to the fact that interactions with other smoke components are minimized. To our knowledge, this is the first time that such a "solid-state" spin-trapping protocol has been applied to an environmental problem. In view of the clean spectrum obtained, we believe that this method may be an important technique for trapping other environmentally important radicals.

As the analyses in Table 4 show, the three protocols in Figure 8 all give alkoxyl radical spin adducts as the major species detected. Protocols (A) and (B) also show substantial amounts of alkyl (or similar carbon-centered radical) spin adducts. Using PBN in carbon tetrachloride, we also detect what we believe is a vinyl nitroxide that we have suggested (*14*) is produced from the species that is initially trapped, as shown in Eq. (10).



(10)

The ESR spectrum obtained using PBN in benzene shows a nitrogen hyperfine splitting constant (hfsc) characteristic of an alkyl spin adduct, but with a much smaller hydrogen hfsc than is normally observed. We have suggested that this adduct can be rationalized as being due to a cyclohexadienyl radical that is formed when gas-phase alkyl radicals are intercepted by the aromatic solvent before they are spin-trapped by the PBN [Eq. (11)].



(11)

Steady-State Hypothesis for Gas-Phase Smoke Radicals

A remarkable feature of the gas-phase cigarette smoke radicals is that they have amazingly long lifetimes in the gas phase. The solid line in Figure 9 shows a plot of spin adduct concentrations observed after aging the smoke for the indicated times before trapping. Not only are the radicals apparently long-lived, but maximum spin adduct yields are not observed until the smoke is at least one minute old. These long lifetimes are clearly inconsistent with the known stabilities of oxygen- and carbon-centered radicals of the type that we observe; these radicals would have lifetimes of much less than a second in smoke (*4,10*).

One conclusion from these results is that the small organic free radicals that we spin-trap from gas-phase smoke can not be those that are formed during the combustion process. While radicals are known to be formed by the combustion of tobacco (as well as other types of organic materials), these short-lived radicals must undergo termination reactions while flowing down the tobacco column in the cigarette and do not reach the spin-trap solution (or the lungs of a smoker).

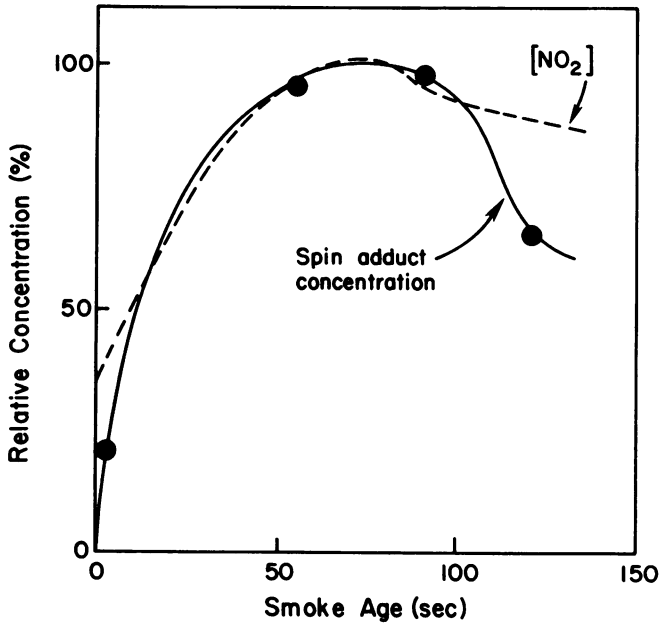
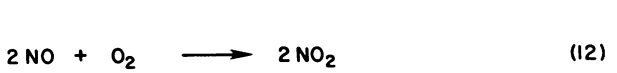


FIGURE 9. The effect of aging cigarette smoke: (—) spin adduct concentrations observed after the smoke was aged for the indicated times before bubbling through a solution of PBN in benzene; (- -) nitrogen dioxide concentration in smoke as a function of time. Both curves have been scaled to a relative intensity scale with a maximum intensity of 100%.

To rationalize the apparent contradiction between the known short lifetime of the types of radicals that we spin-trap and the apparent lifetimes indicated by data such as presented in Figure 9, we have proposed a steady-state hypothesis for radical formation in cigarette smoke. That is, we have suggested that radicals are being continuously formed and destroyed as the result of gas-phase radical reactions.

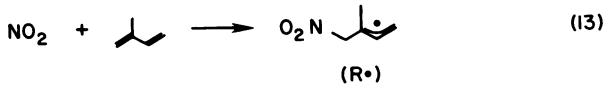
One mechanism by which radicals could be continuously formed in smoke involves reactions of nitogen oxides with other smoke constituents. The dashed curve in Figure 9 shows the relative change in the nitrogen dioxide (NO_2) concentration in aging cigarette smoke. Notice that the NO_2 concentration follows a time course remarkably similar to that for the formation of organic radicals as measured by the appearance of spin adducts.

The radicals that we spin-trap might arise out of the NO_2 chemistry in smoke in the following manner: Fresh smoke contains little or no NO_2; rather, it contains primarily nitric oxide (NO) as the principal nitrogen oxide. The NO levels in smoke are extremely high relative to the values in smog, typically being on the order of 300–500 ppm (*32*). Nitric oxide is relatively unreactive with most organic species, but does undergo slow oxidation in air to the much more reactive NO_2, [Eq. (12)].



(12)

Nitrogen dioxide can undergo facile reactions with many of the species in smoke. As one likely candidate, we have concentrated on isoprene, a reactive diene that occurs at high levels in smoke (*32*). The reactions that occur in smoke in this NO-isoprene steady-state mechanism are shown in Eqs. (13)–(15). Nitrogen dioxide is known to add readily to dienes like isoprene to generate carbon-centered radicals [Eq. (13)].



(13)

These carbon-centered radicals would then be rapidly scavenged by oxygen in smoke to give peroxyl radicals [Eq. (14)].

(14)

Finally, peroxyl radicals are rapidly deoxygenated to alkoxyl radicals by NO [Eq. (15)].

(15)

These reactions can produce the alkoxyl and alkyl radicals that we spin-trap. However, we do not observe spin adducts of peroxyl radicals, probably because their rapid deoxygenation by NO [Eq. (15)] keeps their concentrations low. Possible gas-phase termination reactions are shown in Eqs. (16)–(18); some of these termination products could have toxicological importance as we shall discuss below.

(16)

(17)

(18)

To test whether this mechanism could be occurring in cigarette smoke and lead to the spin adducts that we observe, we have prepared synthetic models of cigarette smoke involving NO and isoprene in air (*14*). When this mixture is bubbled through a solution of PBN in benzene, we observe spin adducts that are very similar to what we observe from cigarette smoke (see Fig. 8 *D*) and Table 4); the two major spin adducts observed are those due to an alkoxyl radical and to a radical we tentatively identify as the cyclohexadienyl radical resulting from addition of smoke-borne alkyl radicals to the benzene solvent.

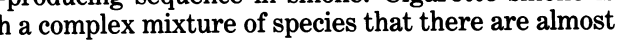
The results from our NO/air/isoprene model smoke are very encouraging in their similarity to cigarette smoke, establishing the reality of the steady-state model and the likelihood of a pathway involving NO and a reactive organic compound contributing to radical production (*14*). However, the chemistry outlined in Eqs. (12)–(18) very likely represents only one possible radical-producing sequence in smoke. Cigarette smoke is such a complex mixture of species that there are almost certainly other reaction pathways that could be responsible for radical production. Nevertheless, the concept of a steady-state and continuous radical production through the reaction of metastable species would have to be similar to that demonstrated for the NO/air/isoprene system.

Inactivation of α_1-Proteinase Inhibitor by Cigarette Smoke

α_1-Proteinase inhibitor (a1PI) is the major serum antiprotease in humans, accounting for more than 90% of the functional anti-elastase activity in the bronchoalveolar lavage fluid of normal individuals (*52*). In 1963, Laurell and Eriksson (*53*) published their now-classic report that an early-onset form of emphysema is associated with an inheritable deficiency of a1PI. This observation led to the protease-antiprotease balance theory, in which lung connective tissues are proposed to be protected from leukocyte proteases by endogenous protease inhibitors. This theory suggests a mechanism for the development of pulmonary emphysema in which a1PI, the major regulator of polymorphonuclear neutrophil (PMN) elastase in the lower respiratory tract of humans, plays a pivotal role. Although Laurell and Eriksson's discovery was of enormous importance in terms of understanding the pathogenesis of emphysema, homozygous a1PI deficiency accounts for only about 1% of patients with severe obstructive airways disease (*52*). Instead, cigarette smoking is recognized as the major risk factor in virtually all emphysema patients (*54*).

In vivo exposure to cigarette smoke has been shown to bring about a decrease in the elastase inhibitory capacity (EIC) in the serum of rats (*55*) as well as in pulmonary lavage fluids in humans (*26,56*), although these last reports have been the subject of recent controversy (*57*). The decreased EIC in these cases is not the result of a decreased level of a1PI itself, but rather the result of the formation of an inactive form of the protein containing oxidized methionine residues (*26*). At least one methionine residue in a1PI is at the active site and is essential for the elastase binding ability of the protein (*58*).

The role of cigarette smoke in mediating this oxidation is not fully understood but could proceed by a number of pathways. One major pathway involves oxidation of a1PI by reactive oxygen species released by lung polymorphonuclear leukocytes (PMN). Chronic cigarette smoking not only increases the number of PMN in lung fluid (*59*) but also activates these cells metabolically (*60*). Activated PMN undergo a "respiratory burst" characterized by increased oxygen consumption. Superoxide is a product of this respiratory burst, being formed from the single-electron reduction of molecular oxygen catalyzed by a cyanide-insensitive, membrane-bound NADPH-dependent oxidoreductase (*61*) as shown in Eq. (19).



(19)

The superoxide produced in Eq. (19) can react directly to inactivate a1PI or react further to generate more powerful activated oxygen species such as hydrogen peroxide [Eq. (20)] or hydroxyl radical [Eq. (21)].

$$2O_2^{\cdot-} + 2H^+ \longrightarrow H_2O_2 + O_2 \quad (20)$$

$$O_2^{\cdot-} + H_2O_2 \longrightarrow HO\cdot + HO^- + O_2 \quad (21)$$

[Note that Eq. (21) is not a simple process and requires catalysis by iron chelates (*62*).] The hydrogen peroxide that is produced in Eq. (20) could act as a substrate for the myeloperoxidase system of PMN to form, in the presence of chloride anions, hypochlorous acid, a powerful oxidizing agent (*63*). *In vitro* studies have shown that methionines in a1PI are oxidized and the a1PI is inactivated *in vitro* by all of these oxidants: stimulated PMN preparations (*64*), a combination of H_2O_2 and $O_2^{\cdot-}$ generated by xanthine oxidase (*65*), and the myeloperoxidase system (*66,67*).

A second major pathway for cigarette smoke-induced inactivation of a1PI is the direct oxidation of a1PI by components in the smoke itself. Incubation of a1PI with previously prepared aqueous solutions of cigarette smoke have been shown to result in the loss of EIC of the a1PI solutions (*68–70*), and this inactivation has been attributed to free radicals (*68*). However, as indicated in the section above, we have recently shown that the small organic oxygen- and carbon-centered radicals in gas-phase smoke are too reactive to survive long enough in solution to be responsible for the inactivation observed in those experiments.

To probe the nature of the inactivating species in cigarette smoke, we recently reported (*15*) the results of experiments in which we compared the inactivation of a1PI exposed to aqueous smoke extracts to the inactivation caused by direct exposure of a1PI to gas-phase cigarette smoke. The latter protocol is one in which free radicals would be present and also more closely mimics the conditions in smoker's lungs. The results are shown in Figure 10*A*; the circles represent the direct exposure while the squares show the inactivation by the aqueous smoke extract. The direct exposure protocol gives an initial rapid decrease in EIC that is not seen when a1PI is exposed to the extract. Both protocols, however, cause a similar slow inactivation that occurs over many hours following the initial exposure. Moreover, as shown in Figure 11, the rapid inactivation process caused by direct exposure to gas-phase smoke is a function of the age of the smoke. Maximum inactivation of a1PI occurs with smoke that is aged for 40–60 sec before it is bubbled into the a1PI solution. This time course is nearly identical to what was observed in the spin trapping experiments described above and suggests that the inactivation is related either to the free radicals in smoke or to the levels of NO_2.

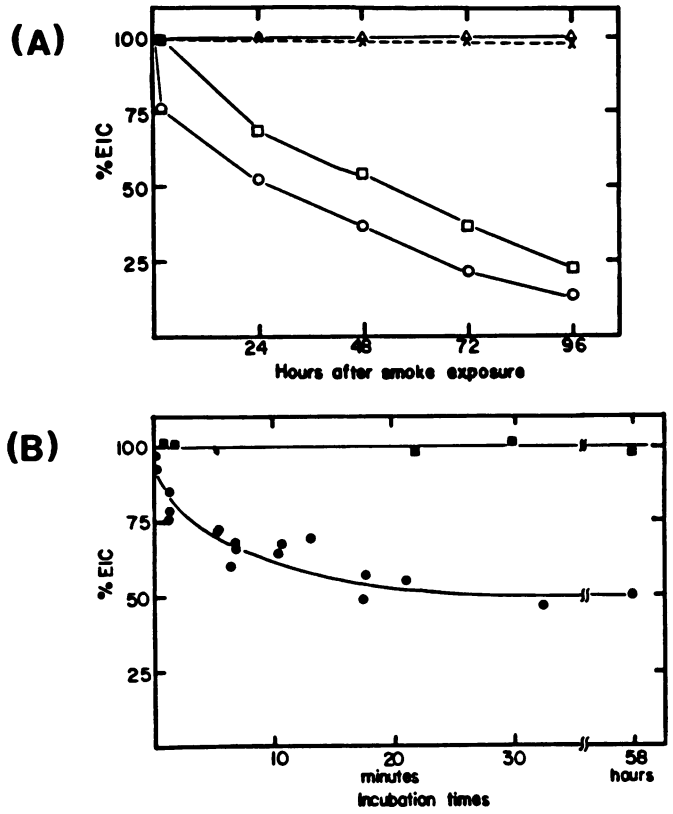


FIGURE 10. Plot of (*A*) effect of gas-phase cigarette smoke on the EIC of a1PI. The a1PI was exposed to the gas-phase smoke from one cigarette and assayed as described in the literature (*15*): (Δ) control a1PI (untreated); (×) air control (direct exposure to 10 "puffs" of air); (□) aqueous extract exposure; (○) direct smoke exposure. (*B*) Inactivation of a1PI by *tert*-butyl peroxynitrate: (●) 50 nmol *tert*-butyl peroxynitrate or (■) 700 nmole *tert*-butyl nitrate as a control in 1 μL $CHCl_3$ added to 0.5 mL a1PI solutions (0.25 mg/mL). EIC was determined at various times following peroxynitrate or nitrate addition.

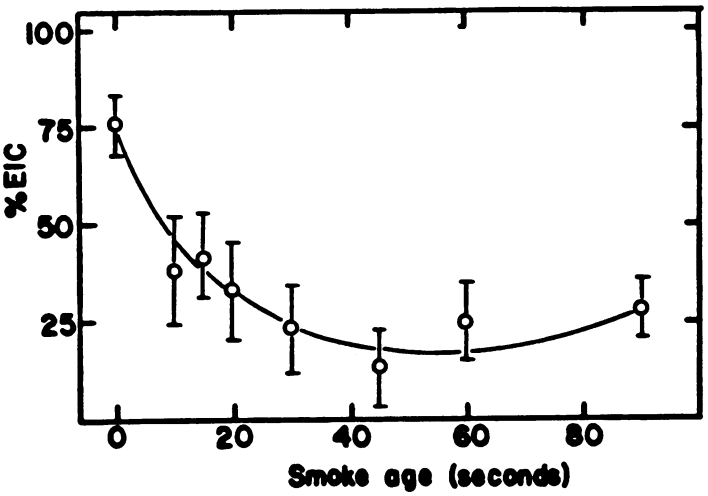


FIGURE 11. Effect of smoke age on the short-term loss of EIC. The a1PI was exposed directly to the gas-phase smoke from one cigarette. The smoke was bubbled through the a1PI solutions either immediately after being drawn or after being allowed to age the indicated length of time in the syringe (*15*). Assays for EIC were carried out immediately following smoke exposure. Results are expressed as the mean EIC ± 1 standard deviation.

We have shown (*9*) that NO_2 itself does not cause a1PI to lose EIC and H_2O_2 alone also causes no inactivation (*9,65*). However, inactivation does occur when NO_2 is bubbled into an a1PI solution containing 1 mM H_2O_2. We found that a1PI could be partially protected against this inactivation by either mannitol or SOD and concluded from these results that $O_2^{\cdot-}$ and $HO^{\cdot}$ are involved in the inactivation process. Hydroxyl radicals may be generated by a displacement reaction as shown in Eq. (22), a process known to occur in the gas-phase (*71*), although not reported in solution.

$$NO_2 + H_2O_2 \longrightarrow HONO_2 + HO\cdot \qquad (22)$$

One possibility for the generation of $O_2^{\cdot-}$ is an electron transfer reaction as shown in Eq. (23).

$$NO_2 + H_2O_2 \longrightarrow NO_2^- + O_2^{\cdot-} + 2H^+ \qquad (23)$$

The hypothesis we had tested was that NO_2 in smoke could react with H_2O_2 to produce $HO^{\cdot}$ as described above; we had envisioned that the H_2O_2 might arise from activated PMN's. However, Nakayama et al. have reported that H_2O_2 is formed in smoke itself or in aqueous smoke solutions (*51*), and we have shown that aqueous solutions of cigarette tar produce hydrogen peroxide (J. P. Cosgrove, E. T. Borish, D. F. Church, and W. A. Pryor, submitted for publication). This suggests that NO_2 may react with the hydrogen peroxide that is produced from the reduction of dioxygen by smoke and/or tar, rather than just with hydrogen peroxide produced from PMN's.

Another pathway for the direct inactivation of a1PI by free radicals formed in gas-phase cigarette smoke could involve the reaction of NO_2 and alkenes as described above. In fact, we have found (W. A. Pryor, M. M. Dooley, and D. F. Church, submitted for publication) that a1PI is inactivated by the NO/air/isoprene model system in a way indistinguishable experimentally from the inactivation by gas-phase cigarette smoke. That is, there is a rapid inactivation of a1PI that occurs when the NO/air/isoprene mixture is bubbled directly through the a1PI solution, plus a slow inactivation that occurs following either direct exposure or exposure to an aqueous extract. The rapid inactivation is dependent on the age of the NO/air/isoprene mixtures, suggesting that the NO must be oxidized to NO_2, consistent with Eqs. (12) to (18).

In control experiments we find that small alkyl and alkoxyl radicals, such as would be formed in the NO/air/isoprene reaction scheme by Eqs. (12) to (18), do not cause inactivation of a1PI. While we cannot yet conclusively exclude the possibility that the direct reaction of oxy radicals causes inactivation of a1PI, it appears that they may be too short-lived or too reactive in an aqueous environment to cause much specific damage. We have therefore concluded that a metastable species present in smoke and dependent on NO_2 concentration is involved in the inactivation of a1PI. A possible candidate is an alkyl peroxynitrate, $ROONO_2$, formed in Eq. (16). Pernitrates had not previously been suggested as important constituents of cigarette smoke, although they are known to be present in smog.

We find that pernitrates do inactivate a1PI. We have reported (*15*) the inactivation of a1PI by *tert*-butyl peroxynitrate; the results are shown in figure 10*B*. Inactivation is essentially complete after 20 min, a time course consistent with what we have observed for the rapid inactivation by cigarette smoke. Peroxynitrates are quite unstable in water due to their hydrolysis followed by the rapid disproportionation of NO_2 to nitrous and nitric acids. This shifts the equilibrium of Eq. (16) to the left and depletes the peroxynitrate concentration (*72,73*). Therefore, if peroxynitrates are involved in the inactivation of a1PI, they must be the short-lived, fast-reacting oxidant; peroxynitrates are too short-lived to be a factor in the slow inactivation that occurs over several days. Our experiments, however, do not allow us to determine the actual oxidant that attacks a1PI. It could be either the peroxyl radicals formed as Eq. (16) shifts to the left, or it could be the peroxynitrate itself.

Cigarette smoke condensate (tar) causes inactivation of a1PI (*74*), and we have confirmed that result (W. A. Pryor, M. M. Dooley, and D. F. Church, unpublished data, this laboratory). However, the inactivation of a1PI by cigarette tar is a slow process similar to the slower inactivation caused by gas-phase smoke; tar does not cause the rapid inactivation of the type seen when a1PI is exposed to gas-phase smoke directly. It is possible that the hydrogen peroxide produced from redox cycling of the Q/QH_2 radical in tar is responsible for this slow inactivation of a1PI. We have found that aqueous solutions of tar produce hydrogen peroxide and the hydroxyl radical (J. P. Cosgrove, E. T. Borish, D. F. Church, and W. A. Pryor, submitted for publication).

Whereas gas-phase smoke is oxidizing, the tar fraction is reducing overall (*34,75*) and might, therefore, be expected to quench radical reactions. For example, we have shown that tar reacts with stable radicals such as nitroxides and DPPH to quench them, probably by transferring hydrogen atoms from the tar QH_2 molecules to the radical (*4,10*). In this regard, it is interesting to note that, although the presence of tar does not affect the slow inactivation when a1PI is exposed to whole smoke rather than to the gas phase alone, there is a slight decrease in the magnitude of the *rapid* inactivation process (W. A. Pryor and M. M. Dooley, submitted for publication). This result may suggest an important difference in the mechanisms involved in the two inactivation stages. It also demonstrates that whole smoke is slightly less oxidizing than is filtered smoke, a finding with obvious toxicological implications for smokers.

Oxidation of Thiols

We have reported (*7*) that both NO and NO_2 oxidize

cysteine and glutathione to the corresponding disulfides; the reaction with NO_2 is much more rapid than is the reaction with NO. This suggests that cigarette smoke, with its high levels of both NO and NO_2, could cause damage by the oxidation of sulfhydryl groups in key enzymes or in a protective species such as glutathione.

There is substantial evidence that smoke does indeed do damage to thiols. An unidentified factor in cigarette smoke has been shown to oxidize thiols (*76*), and the inactivation of alveolar macrophages by smoke is prevented by added glutathione and cysteine (*77*). Smoke inactivates glucose 6-phosphate dehydrogenase and other thiol-containing enzymes (*78–81*). Leuchtenberger et al. (*82*) have shown that both cytotoxicity and malignant transformations in hamster lung cell cultures correlate with the reactivity of smoke towards thiols rather than with, for example, tar or nicotine. Their data suggest that an important agent for thiol destruction is the NO in the gas phase of smoke. It has been shown that the *in vivo* response to the oxidative threat by smoke is an increased generation of glutathione (*83*).

Mutagenesis and Cancer

In recent years, it has become clear that free radicals are involved in many of the biological processes that occur when chemicals transform cells (*84*). Since cigarette smoking increases the concentrations of radicals in the lungs (by increasing macrophage-derived superoxide/hydrogen peroxide, by the nitrogen oxide-driven reactions described above, and by depositing the radical-rich tar with its metastable paramagnetic species), it appears reasonable to assume that some of the tumorigenicity of smoke derives from the free radicals it contains or causes to be produced in the lung.

Evidence of a number of types can be adduced to support the involvement of radicals in chemical carcinogenesis. In particular, six facts implicate radicals:

- Antioxidants protect against carcinogenesis
- Promotion involves radicals
- Prostaglandin (PG) synthetase causes xenobiotic oxidation
- Tumor cells have anomolous rates of lipid peroxidation
- Superoxide itself causes DNA damage
- PAH metabolism and P450 activity differ in various tissues

(1) It has been known for many years that a number of types of antioxidants protect a variety of experimental animals against the effects of many types of chemical carcinogens (*28,85,86*). The protection in many cases is impressive; nevertheless, it should not be inferred either that all radical scavenger drugs are anticarcinogens or that those antioxidants that do show anti-tumorigenic properties necessarily do so because of their antioxidant activity (*87*).

(2) In the usual mouse skin test, tumorigenesis can be divided into two stages, initiation and promotion; in addition, promotion appears to involve at least two stages (*88*). While the mechanism of promotion remains elusive, the involvement of radicals in this extremely important processes is indicated by many lines of evidence (*89*). For example, structure–activity relationships in the phorbol esters show that those compounds that cause the greatest production of superoxide are the strongest promoters. Also, many radical-producing compounds, such as benzoyl peroxide, lauroyl peroxide, and cumene hydroperoxide, are themselves promoters (*30*).

(3) The prostaglandin sythetase (PGS) system of enzymes contains a peroxidase component, as Marnett originally showed (*90*). This peroxidase, with either endogenous hydroperoxides (such as PGG or HPETEs) or exogenous hydroperoxides (such as *tert*-butyl hydroperoxide), causes the co-oxidation of compounds present during the oxidation of arachidonate to prostaglandins and leukotrienes. In particular, xenobiotics such as the 7,8-diol of benzo(a)pyrene (BaP) are converted to the 7,8-diol-9,10-epoxide, the ultimate carcinogen from BaP (*90*). Since the oxidation of arachidonate by PGS involves radical intermediates and produces peroxidic species, this PGS-mediated xenobiotic co-oxidation represents a mechanism for the conversion of pro-carcinogens to carcinogenic compounds by radical-mediated processes (*90–93*).

(4) Slater and Ingold have recently shown that tumorous tissue autoxidizes at an anomolously slow rate relative to matched normal controls (*22,94*). The reasons for this appear to be a lower content of polyunsaturated fatty acids (PUFA) in the tumor, higher concentrations of antioxidants such as tocopherols (perhaps because of their being spared by the lower rates of autoxidation), and a P-450 activity that does not express the usual chelated-iron activity.

(5) As originally shown by Birnboim some years ago, superoxide (released by superoxide-generating systems such as neutrophils) itself causes DNA strand scission. The importance of this pathway *in vivo* is not known, but it is suspected to be significant particularly in nonnuclear DNA (*95*).

(6) Finally, it should be mentioned that the process by which PAH such as BaP are oxidized by P-450-dependent processes to diol-epoxides cannot rationalize the carcinogenicity of all PAH in all tissues of all animals. Some chemical carcinogenicity appears to be the result of oxidation of PAH to phenols, diols, and quinones, and these oxidations are now accepted to involve radicals (*96*).

Several mechanisms can be suggested that invoke the free radical activity of smoke or tar to rationalize the known tumorigenicity of cigarette smoking. Firstly, as we have seen, gas-phase smoke itself contains a strongly oxidizing component (demonstrated using a1PI as the substrate), and this component could cause the oxidation and activation of procarcinogens (*5,6*). Secondly, tar produces superoxide/hydrogen peroxide (*51*) and $HO^{\cdot}$ radicals (J. P. Cosgrove, E. T. Borish, D. F. Church, and W. A. Pryor, submitted), and these species are implicated in carcinogenesis. And thirdly, the paramagnetic species in tar could bind to DNA and produce biological consequences, one of which might be transformation (*13*).

Lipid Peroxidation

Experimental evidence for lipid peroxidation by cigarette smoke is mixed and contradictory. Lentz and DiLuzio (*20*) exposed rabbit pulmonary alveolar macrophages (PAM) to a filtered aqueous extract of cigarette smoke; they monitored lipid peroxidation by the TBA test and found increased levels of TBA-reactive materials (TBARM) in the macrophages that were exposed to the smoke. Opposite results were obtained by Chow (*83*), who exposed vitamin E-deficient and vitamin E-sufficient rats to whole cigarette smoke and monitored peroxidation in lung lipids. For the E-deficient group, Chow found 13% lower TBARM from the lung lipids of rats exposed to whole (unfiltered) smoke compared to controls; no change in the TBARM was found in an E-deficient group exposed to gaseous (filtered) smoke. For the E-sufficient group he observed no effect. Admittedly the protocols of Lentz and DiLuzio and of Chow are very different. In the former, the smoke was clearly "aged" in the form of the extract and was filtered so that there would have been little particulate (i.e., tar). The latter protocol is probably more realistic in terms of actual smoking habits; however, the implication that cigarette tar protects against lipid peroxidation requires more direct confirmation.

We suggest that these contradictory results may at least partially be rationalized in terms of the very different natures of the gas and tar phases of cigarette smoke. The gas phase, with its NO_2 and small organic oxygen-centered radicals, should be highly oxidizing and capable of initiating peroxidation. In fact, we find that the NO/air/isoprene model system for gas-phase smoke described above initiates lipid peroxidation (e.g., of neat ethyl linolenate), whereas cigarette tar inhibits autoxidation (T. J. Burkey, D. F. Church, and W. A. Pryor, unpublished data). The tar, however, is highly reducing. We have found that tar posesses much more reducing power (measured by the reduction of stable nitroxide or DPPH free radicals) compared with the number of paramagnetic centers in tar itself (*5,6*). We interpret this to mean that the Q/QH_2 system that is the predominent radical in tar contains far more hydroquinone QH_2 functionality (with its known reducing power) than it does semiquinone $QH^{\cdot}$ groups (with its ESR-active radical).

Most of the available evidence to date suggests that it is the reducing character of the tar phase that predominates in whole smoke. For example, Benedict et al. (*75*) used redox indicators to show that whole smoke is reducing; Schmeltz et al. (*34*) confirmed this finding on whole smoke using an electrochemical method. Bilimoria et al. shows that whole smoke inhibits the polymerization of vinyl acetate (*97*) and the autoxidation of ascorbate (*98*). However, a recent report of Cohen and James (*69*) conflicts with these results; they found, using either a redox indicator or the inactivation of a1PI, that whole smoke is oxidizing and that the oxidizing power resides mainly in the tar or particulate fraction!

An Overview of the Toxicology of the Cigarette Smoke Free Radicals

Cigarette smoke is a complex chemical system and there are many potential pathways for these species to interact with one another and with biopolymers in a smoker's lung (*99*). We believe that the evidence that free radical processes play a significant role in cigarette smoke toxicology is becoming increasingly strong, and we would like to try to place some possibilities in perspective. Figure 12 shows an overview of the free radical chemistry of cigarette smoke and the pathological consequences it could imply.

We view cigarette smoke radicals as arising out of three pathways. Firstly, combustion itself is known to be a radical process and both oxygen- and carbon-centered radicals would be expected to be produced in the cigarette flame. However, as we have already discussed, these radicals are too reactive and short-lived to play a role in the toxicology of smoke. The other two pathways involve the formation of the stable free radicals in tar (one of which we have tentatively identified as a Q/QH_2 radical), and the oxidation of NO to the much more reactive NO_2 followed by reaction of NO_2 with smoke constituents as shown in Eqs. (12)–(15).

Figure 12 attempts to summarize the biological effects of gas-phase cigarette smoke in tar in terms of the chemical mechanisms that have been discussed in this article. Figure 12*A* describe the effects of gas-phase smoke. Reaction a illustrates the activation of pulmonary alveolar macrophages by smoke; this process produces superoxide and hydrogen peroxide, and these species (in iron-catalyzed reactions) can directly cause biological damage. The scheme also shows nitric oxide and various reactive organic molecules (such as isoprene) being produced in the smoke stream in reaction b. Nitric oxide is quite unreactive, but it undergoes oxidation to form nitrogen dioxide (reaction c). Nitrogen dioxide can react with hydrogen peroxide (reaction d) to produce species that we have shown inactivate a1PI. From the protection observed with various scavengers, we conclude that these species include superoxide and the hydroxyl radical. In addition, nitrogen dioxide can react with other gaseous components of cigarette smoke (such as isoprene) to form alkyl radicals, as is shown in reaction e. These carbon-centered radicals react with oxygen to become peroxidized (reaction f). These peroxyl radicals can react with either NO or NO_2 to form either pernitrite or pernitrate esters (reaction g). We have shown that pernitrate esters are able to inactivate a1PI, reaction l. The peroxyl radicals also undergo deoxygenation by nitric oxide, reaction h, to produce alkoxyl radicals. Alkoxyl radicals are known to be extremely reactive and would be expected to cause a variety of pathological changes in the lung (reaction m). Finally, we have shown that both NO and NO_2 (reaction i) oxidize thiols to disulfides, and this could oxidize glutathione in the lung, changing the thiol/disulfide oxidation–reduction balance and causing extra-pulmonary changes in blood chemistry (reaction k).

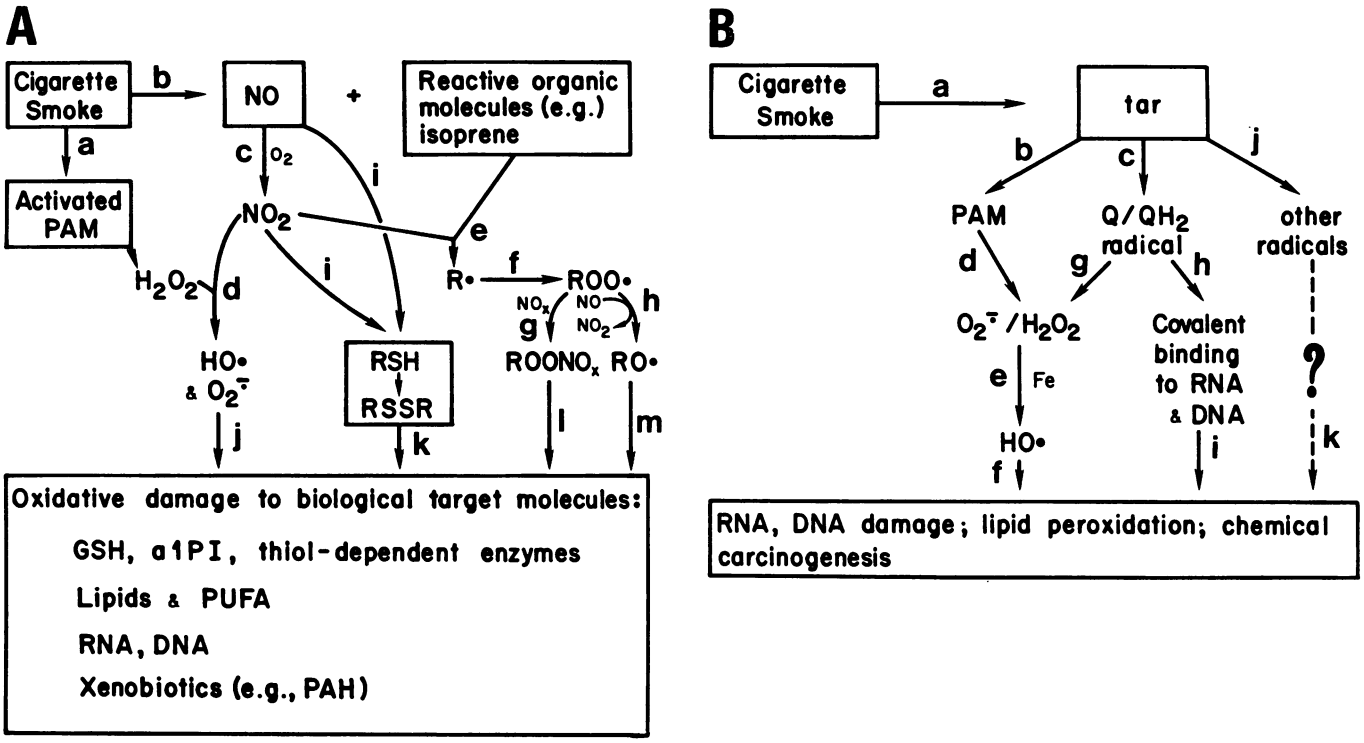


FIGURE 12. Schemes summarizing (*A*) the free radical chemistry of gas-phase cigarette smoke and possible biological consequences; (*B*) chemical reactions and possible biological consequences of the radicals associated with cigarette tar.

Figure 12*B* shows the processes we have discussed for tar. Tar activates PAM, equation b, which produce superoxide and hydrogen peroxide and, through iron-catalyzed reactions, produce the hydroxyl radical (reaction e). The hydroxyl radical, of course, can cause biological damage by a number of processes (reaction f), as is well known. We also have shown the principal paramagnetic species in tar, the semiquinone radical, produces hydrogen peroxide (reaction g). Again, this could lead to the very damaging hydroxyl radical. This Q/QH_2 radical also appears to bind to DNA (reaction h). The biological consequences of this (reaction i) can only be speculated upon at present. Finally, it must be stressed that we have just studied the reactions of the Q/QH_2 radical, but there are other paramagnetic species present in tar as well. These species also might have biological effects, as is outlined in equation j and k.

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