

Cigarette Smoke Radicals and the Role of Free Radicals in Chemical Carcinogenicity

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This article consists of two parts: a brief overview of the ways in which free radicals can be involved in chemical carcinogenesis, and a review of cigarette smoke chemistry. Carcinogenesis is generally agreed to involve at least three stages: initiation, promotion, and progression. It is suggested that radicals sometimes are involved in the initiation step, either in the oxidative activation of a procarcinogen (such as benzo[a]pyrene) to its carcinogenic form or in the binding of the carcinogenic species to DNA, or both. The fraction of initiation events that involve radicals, as opposed to two-electron steps, is not known, but radicals probably are involved in a substantial number, although probably not a majority, of cancer initiation reactions. Promotion always involves radicals, at least to some extent. Progression probably does not normally involve radicals. The second part of this article reviews the molecular mechanisms involved in cigarette-induced tumors, particularly by aqueous cigarette tar (ACT) extracts and by a model of these solutions, aged solutions of catechol. ACT solutions as well as aged solutions of catechol contain a quinone-hydroquinone-semiquinone system that can reduce oxygen to produce superoxide and hence hydrogen peroxide and the hydroxyl radical. Both the cigarette tar radical and the catechol-derived radical can penetrate viable cells, bind to DNA, and cause nicks. — *Environ Health Perspect* 105(Suppl 4):875-882 (1997)

Key words: adduct, cigarette, DNA, electron transfer, initiation, oxidation, promotion, radical, smoke, superoxide

Introduction

This review is divided into two parts. In the first, I suggest an overview and organizational scheme for the various ways in which free radicals may be involved in chemical carcinogenesis. In the second, I review some of the recent research on cigarette chemistry and biology done both in our laboratory and in others.

Mechanisms By Which Radicals Can Be Involved in Carcinogenicity

The involvement of radicals in chemical carcinogenesis seemed evident for many years, based on a number of generalities. First, since the activation of many procarcinogens involves an oxidation step, and since many oxidations involve radicals, it

seemed likely that radicals would be involved in carcinogenesis, at least some for some carcinogens (1,2). Second, a number of radical scavengers and/or antioxidants protect cells or animals from tumor formation, although individual antioxidants seem to protect against different carcinogens without a general pattern being clear (2-6). Third, a proinflammatory state, which can predispose tissue to tumor development, involves higher concentrations of endogenous radicals such as superoxide (7-10). Fourth, antioxidant defenses appear to be altered in procarcinogenic states (11-14). Fifth, it is clear that radical activity in the protumor state is increased regardless of whether the activity is measured by increased amounts of lipid (15,16) or DNA oxidation products (17-19).

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Abbreviations used: ACT, aqueous cigarette tar extracts; B[a]P, benzo[a]pyrene; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; ESR, electron spin resonance (also called electron paramagnetic resonance); PAH, polycyclic aromatic hydrocarbon; PAM, pulmonary alveolar macrophage; SOD, superoxide dismutase.

In recent years, the literature on the involvement of radicals in carcinogenesis has grown enormously. That can be seen quite strikingly in the comparison of the 1986 review by Kensler (7) and that by Frenkel (8) in 1992, which is about twice the length and includes more than three times the number of references.

Carcinogenesis and Radicals

Cancer is a multistep process, which generally is identified as three stages: initiation, promotion, and progression. The activation of many types of initiators can involve radicals. For example, the oxidation of polycyclic aromatic hydrocarbons (PAHs) to an electrophilic derivative that can attack and bind to DNA may involve radicals. Promotion almost certainly involves the production of higher levels of endogenous radicals such as superoxide. Once a tumor is established, progression may be controlled by genetic factors that probably only minimally involve the pathological reactions of radicals.

The various mechanisms for radical involvement are summarized in Table 1. In mechanism 1, radicals are involved in neither initiation nor promotion. Cases of this type certainly occur; the activation of a PAH such as benzo[a]pyrene (B[a]P) via a nonradical, P450-dependent process is an example, as is the chemistry involved in the binding of aflatoxin to DNA, although even this process can involve radicals (20). It is not known what fraction of all cancers is due to these and other nonradical tumorigenesis processes, but it generally is believed that a considerable fraction of cancers probably fits this category.

Table 1. Mechanisms in which radicals are involved in either the initiation or promotion stages of carcinogenesis.^a

Mechanism no.	Initiation ^b	Promotion ^c	Example
1	No	No	P450/B[a]P
2	Yes	No	PGS/B[a]P
3	No	Yes	?
4	Yes	Yes	iso-P450/ B[a]P phenols
5	Radicals can be involved in mechanisms that do not involve binding of the carcinogen, e.g., superoxide-dependent DNA nicks.		

PGS, prostaglandin synthase. ^aAdapted from Pryor (2).

^bRadicals are involved in carcinogen activation.

^cRadicals involved in DNA binding of carcinogen.

Mechanism 2, in which initiation involves radicals but promotion does not, may be quite rare, since radical involvement in promotion is unquestionably important and common. Examples of radical-mediated activation of a procarcinogen include the prostaglandin synthase-dependent co-oxidation of a PAH (21–26), one-electron oxidation of PAHs to their cation-radicals that bind to DNA (27–33), and reactions involving radical attack on carcinogens (34). Other examples can involve the reaction of products produced by radical-mediated processes (such as malonaldehyde or 4-hydroxynonenal from lipid peroxidation), either with procarcinogens to activate them or directly with DNA (35–39).

The general perception is that carcinogens bind to DNA by even-electron (non-radical) pathway, and, to a certain extent, publications that argue the importance of a one-electron pathway for DNA binding are swimming upstream with the current against them. However, it is hard to determine whether this is because nature predominantly uses even-electron pathways for attacks on DNA, or because it is more or less a historical artifact, since the B[a]P-diol-epoxide pathway was elucidated very early, in studies by NIH authors (40–43), so that the known even-electron pathway dominated the thinking of many workers. I have always believed that the latter is true, at least in part.

Mechanism 3, in which radicals are involved in promotion but not initiation, may be common; but, as is true for mechanism 1, it is impossible to know what fraction of tumors results from this pathway. However, it is commonly accepted that the activation of procarcinogens to their carcinogenic state does not always involve radicals, whereas promotion most often involves a long-term, proinflammatory state with high radical fluxes in the affected tissues. Thus, it is likely that all initiation events—no matter whether radicals were involved or not—are followed by a radical-involving promotional state if a tumor is to result.

In mechanism 4, radicals are involved in both initiation and promotion, and probably are almost co-terminus with the mechanism in which radicals are involved in initiation alone.

In addition to these mechanisms, there is mechanism 5, in which radical-mediated damage is done to DNA without the formation of adducts (44–46). This mechanism may apply, for example, in high-energy radiation-initiated tumors.

Other Mechanisms

In addition to these somewhat obvious mechanisms by which radicals could be involved in carcinogenesis, there is a classification of “other mechanisms” that are more roundabout. Because this is a vague, catch-all category, many mechanistic types can fit it, and the examples given here do not include all the possibilities.

An example of the indirect effects radical reactions can have on cancer is the incompletely understood effects of oxidative stress on oncogenes. For example, an epidermal cell transfectant that overexpresses Cu/Zn superoxide dismutase (SOD) is more sensitive to oxidative stress, as monitored by increased *c-fos* message (11). Tumor cells may often be low in SOD activity (12).

Another example of these complex mechanisms involves the effects of β -carotene on gap junction regulation. β -Carotene strongly upregulates gap-junction communication (47), thus producing an antitumorigenic effect. Because β -carotene is often called an antioxidant, this effect might be ascribed to radical scavenging of tumor-promoting radicals (47).

Cancer and the Diet

The involvement of radical reactions in chemical carcinogenesis has recently been in the news, as it has become increasingly clear that the dietary intake of antioxidant nutrients such as vitamin E, vitamin C, and β -carotene can profoundly affect tumor susceptibilities of populations (4, 48–52). These concerns also have emphasized the need for a biomarker for the oxidative stress that can lead to cancer.

Were such a marker identified, it could be used as an end point in dietary trials, making them shorter and less expensive to perform (53–57).

The ways in which antioxidants might interfere with tumorigenesis are outlined in Figure 1. As can be seen, there are many possibilities.

Cigarette Smoke Chemistry

Cigarette smoke is operationally divided into gas-phase smoke and particulate matter (or tar). Tar is the material retained on a filter, whereas gas-phase smoke passes through the filter. (Typically, a Cambridge filter [University of Kentucky, Lexington, KY], which retains 99.9% of the particles $\geq 0.1 \mu\text{m}$, is used.) Both the tar and gas-phase smoke are very rich sources of radicals.

A smoker inhales gas-phase smoke (so called mainstream smoke) as well as particulates that penetrate whatever filter is being used. Both of these phases are highly oxidizing, putting an oxidative stress on the entire organism as well as on the lungs (58–60). For example, smokers have lower concentrations of vitamin C in their blood plasma and vitamin E in their lung lavage than do nonsmokers (13, 14, 61, 62). Some biomarkers of smoking are reduced by antioxidant vitamins (63). Some antioxidant enzymes (e.g., SOD) increase whereas others decrease in activity in the red blood cells of guinea pigs exposed to cigarette smoke (64). Cigarette smoke initiates lipid peroxidation in rat tracheal explants (65) as well as in other systems (66–72). Breath ethane, a measure of the oxidation of *n*-3 polyunsaturated fatty acids, is elevated in smokers (62, 73).

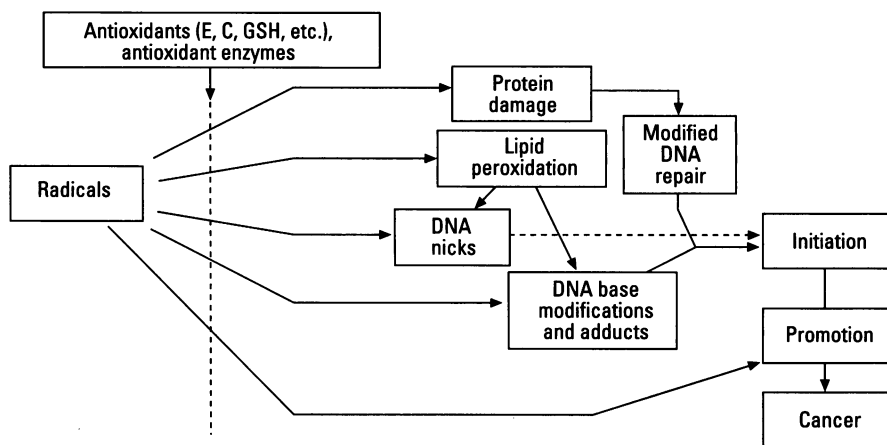


Figure 1. A schematic presentation of ways in which radicals may be involved, and antioxidants may block, tumorigenesis. Abbreviations: E, vitamin E; C, vitamin C; GSH, glutathione.

Gas-phase Smoke Radicals

Gas-phase smoke contains more than 10^{14} low-molecular-weight carbon- and oxygen-centered radicals per puff (74). In addition, smoke contains up to 500 ppm nitric oxide ($\cdot\text{NO}$), which slowly undergoes oxidation to nitrogen dioxide ($\cdot\text{NO}_2$) (75), and both these gases are, of course, radicals. The radicals in gas-phase smoke are too short lived to be detectable by direct electron spin resonance (ESR), but they can be studied by the indirect spin trap method (74,76,77).

The small organic radicals in gas-phase smoke are not produced in the flame: flame radicals are too short lived to pass through the cigarette. Rather, these organic radicals are produced in a steady state by the addition of nitrogen dioxide to isoprene and other similar compounds in the smoke to make carbon-centered radicals that react with oxygen to make oxyradicals (74,76,77). Because gas-phase smoke contains carbon- and oxygen-centered radicals and such high concentrations of nitric oxide (75), alkyl peroxy-nitrite and peroxy-nitrate esters also can be produced (78–82). Figure 2 shows a schematic outline of the isolation of gas-phase smoke and tar and the radicals that each phase produces.

Tar Radicals

In sharp contrast with the gas-phase radicals, the radical in tar is a long-lived semiquinone that can be studied directly by ESR on the filter or in organic solvents or aqueous extracts (83–85). Aqueous extracts of cigarette tar (ACT) contain a low-molecular-weight quinone–hydroquinone–semiquinone system ($\text{Q-QH}_2\text{-QH}\cdot$). The cigarette tar semiquinone radical, as is typical of such radicals, can reduce oxygen to produce superoxide, and hence hydrogen peroxide and the hydroxyl radical. Unlike the tar radical itself, the superoxide and hydroxyl radicals are too reactive and short lived to be observable by direct ESR; but they can be detected by the use of spin trap 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO) (78,86,87).

Figure 3 shows the ESR spectra obtained when tar on a Cambridge filter is placed in the ESR spectrometer; the g -value of 2.0039 is that of a semiquinone radical. We have developed a model for the cigarette tar radical consisting of aged (autoxidized) solutions of catechol. Figure 3 also shows the ESR spectrum produced when an aged solution of catechol is filtered; again the signal is due to a semiquinone (78).

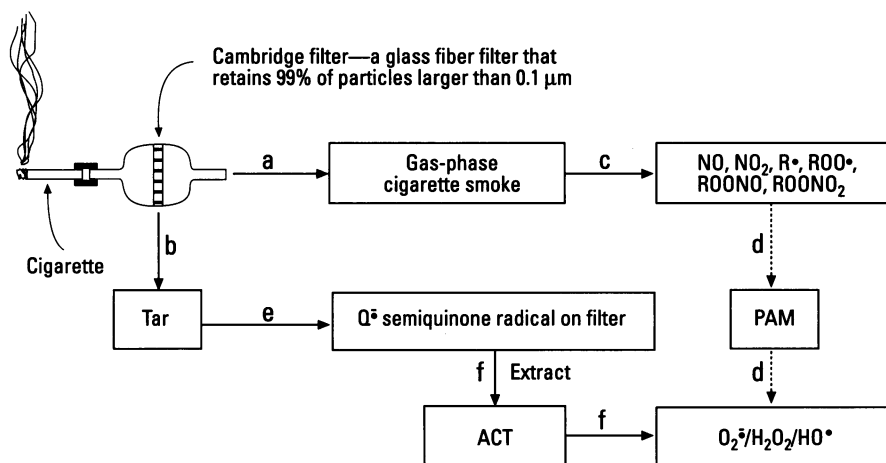


Figure 2. A scheme showing the isolation of gas-phase cigarette smoke and tar and the radicals each fraction produces (78). Using a Cambridge filter, whole smoke is separated into gas-phase smoke and tar (steps a, b). Gas-phase smoke, as shown in step c, contains a steady state level of nitrogen-, carbon-, and oxygen-centered radicals and produces esters and peroxyesters of nitrous and nitric acids (74,76,77). The gas phase also causes PAM to produce more active oxygen species (step d). The tar semiquinone radical (2,58,78,91,93,109,110) reduces oxygen and produces superoxide (step e) and this radical-producing component can be extracted into ACT extract solutions (step f). The tar radical, like other superoxide-producing systems, nicks DNA.

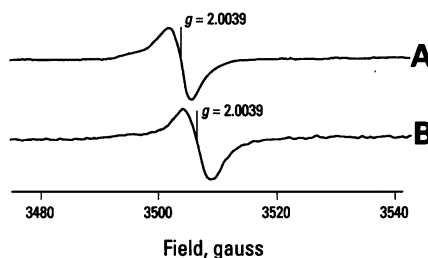


Figure 3. The electron spin resonance (ESR) spectra obtained when (A) a Cambridge filter is placed in the cavity of an ESR spectrometer after the smoke from a single cigarette has been pulled through the filter, and (B) an aged solution of catechol is filtered. Both spectra can be identified as being due to a semiquinone (78,92).

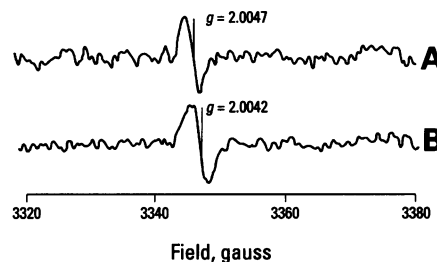


Figure 4. The electron spin resonance spectra of (A) a solution of ACT (10 mg tar/ml) and (B) a solution of catechol aged 1 week (33 mg/ml) (92).

The tar radical, a relatively stable semiquinone, is not reactive. However, ACT solutions produce superoxide, hydrogen peroxide, and the hydroxyl radical, and thus become potent oxidants. These ACT solutions can initiate lipid peroxidation, oxidize proteins such as the antiproteinase, α -1-protease inhibitor (88–90), and nick DNA (82,87). The quinone–hydroquinone–semiquinone system can penetrate viable mammalian cells, bind to, and nick cellular DNA (2,86,87,91,92). The nicks produced by the tar radical require multistep repair, suggesting a process that could be error prone (93). These ACT solutions also interfere with mitochondrial electron transport (94).

The ESR and DNA binding and nicking behavior of the tar radical is very closely modeled by the semiquinone system

present in aged solutions of catechol (85,95,96). Figure 4 shows the similarity in the ESR spectra obtained for solutions of ACT and aged catechol (92). Thus, the tar radical is a low-molecular-weight semiquinone radical, which exists in equilibrium with its quinone and hydroquinone derivatives. This radical system can bind to DNA, produce superoxide, hydrogen peroxide, and the hydroxyl radical in the vicinity of the DNA, and nick DNA (2,78).

Figure 5 shows that the tar semiquinone radical signal becomes associated with DNA when ACT solutions are incubated with calf thymus DNA (82). Since the radical-producing system in ACT solutions is a quinone–hydroquinone–semiquinone system of low molecular weight, it can pass through cell membranes, diffuse to the

nucleus, and bind to DNA. Figure 6 shows the ESR spectra obtained when the DNA from rat alveolar macrophages that have been incubated either with ACT or aged catechol solutions are trapped and washed on polycarbonate filters (82). These ACT and aged catechol solutions bind to and then nick DNA. A plot of the amount of

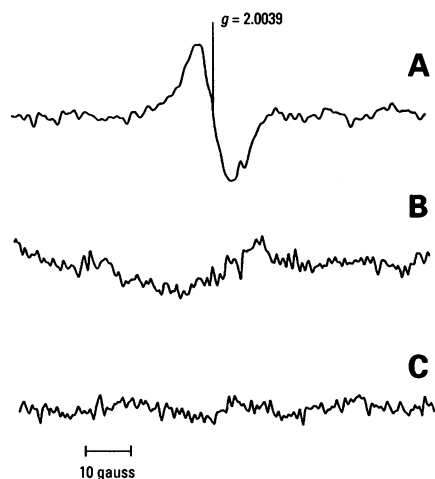


Figure 5. The electron spin resonance spectra of material remaining on polycarbonate filters after filtering the material from the following incubations: (A) ACT plus calf thymus DNA; (B) ACT solutions alone; (C) DNA alone (82).

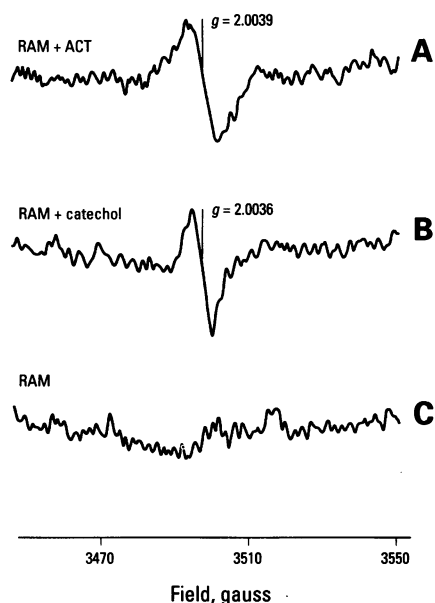


Figure 6. The electron spin resonance (ESR) spectra of the material obtained when the DNA from rat alveolar macrophages (RAM) that have been incubated either with (A) ACT or (B) aged catechol solutions and then isolated on polycarbonate filters (82). No ESR signal is obtained if the RAM are not exposed to ACT or catechol (C).

tar or of catechol used versus DNA nicks shows saturation behavior, suggesting that there might be certain sites in the DNA that are particularly prone to bind the tar radical; once these sites are saturated, binding decreases (78,82,92). Analyses of these binding data using Lineweaver-Burke or Eadie-Hofstee plots, or by computer curve fitting, give very similar binding constants and indicate that 0.24 cigarettes produce an ACT solution with the DNA nicking potency equivalent to a solution containing 254- $\mu\text{g}/\text{ml}$ catechol (82). This amount of catechol actually would be that contained in 0.3 cigarette (82).

Catalase, but not SOD, provides significant protection against ACT- and aged catechol-induced DNA nicks (82).

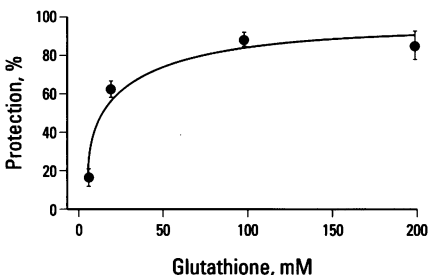


Figure 7. Protection against ACT-induced DNA nicks in rat alveolar macrophages provided by glutathione (82).

Deferoxamine provides little protection, suggesting that the iron required to convert hydrogen peroxide to the hydroxyl radical is tightly associated either with the ACT radical or with DNA or both (82). Glutathione also protects significantly, as shown in Figure 7.

Figure 8 depicts the binding of the ACT (or catechol) radical to DNA, the reduction of oxygen by the semiquinone radical to produce superoxide, the dismutation of superoxide to give hydrogen peroxide, and the reduction of hydrogen peroxide by iron ions (probably associated with DNA, the radical, or both) to the hydroxyl radical.

Randerath and associates (97-106) and others (107,108) have used the phosphorous postlabeling method to demonstrate that cigarette smoke and tar lead to the formation of DNA adducts both *in vitro* and *in vivo*. Radicals need not be involved in the binding of cigarette tar components, presumably PAH derivatives, to DNA. However, it is striking that glutathione appears to protect DNA very similarly against adduct formation, as observed by Randerath and associates (97) and the DNA nicks that we observe (Figure 7) (82).

Recently, we fractionated ACT solutions into 120 fractions and compared the strength of the ESR signal of the tar radical in each fraction with the ability of these

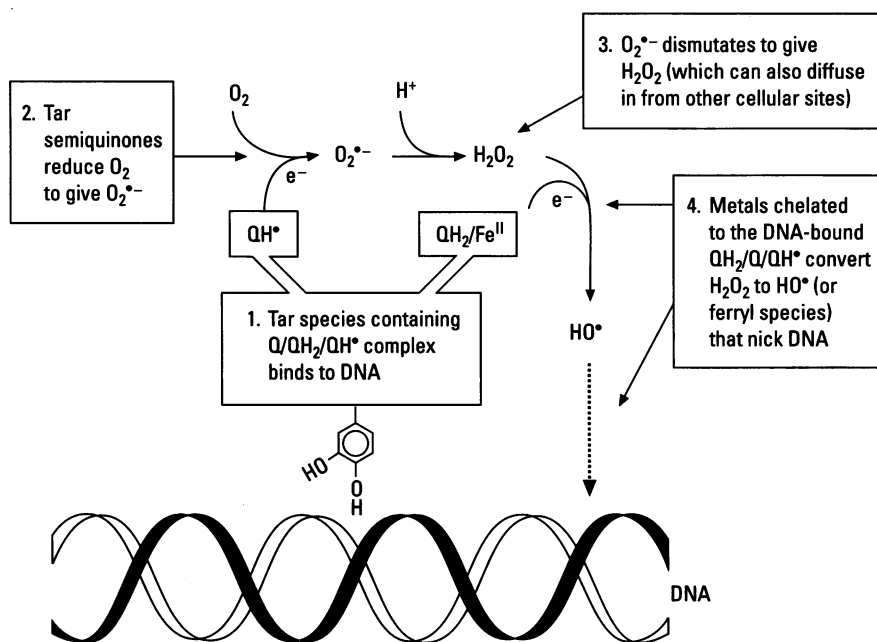


Figure 8. Graphic representation showing 1) the binding of the ACT (or catechol) radical to DNA, 2) the reduction of oxygen by the semiquinone radical to produce superoxide, 3) the dismutation of superoxide to give hydrogen peroxide, and 4) the reduction of hydrogen peroxide by iron ions (probably associated with DNA, the radical, or both) to the hydroxyl radical or a ferryl species that nicks DNA (78,82,92).

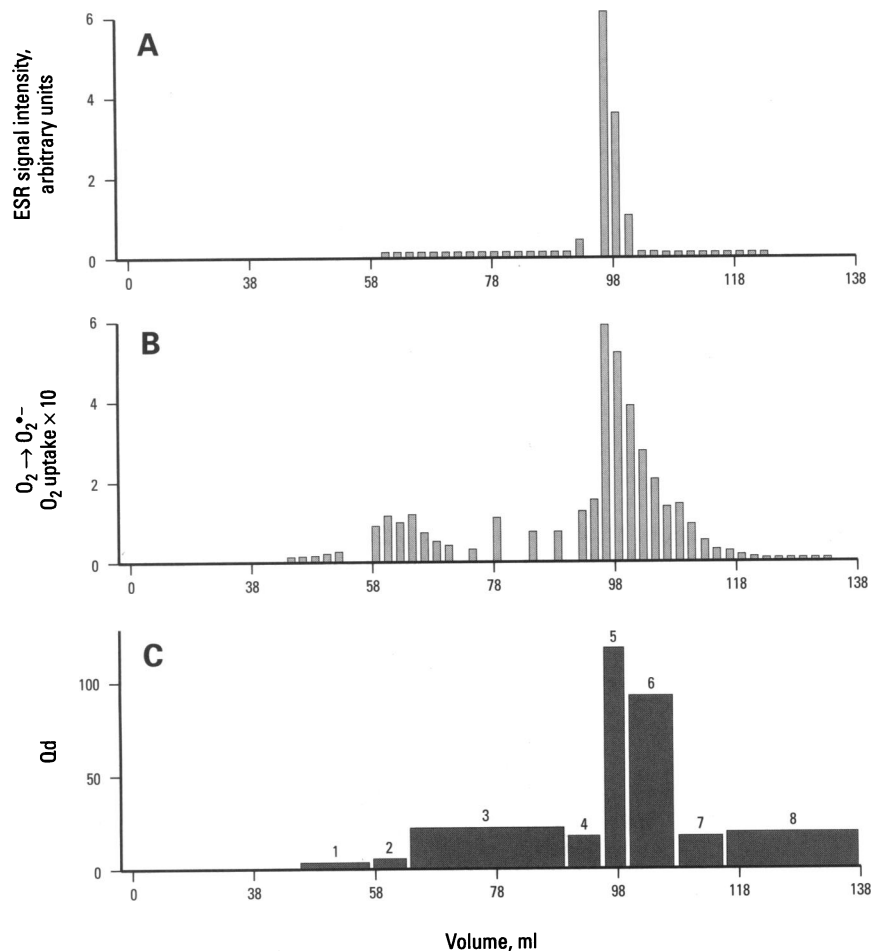


Figure 9. Comparison of fractions of ACT solutions. (A) Signal strength of the electron spin resonance (ESR) signal due to the tar radical; as can be seen, the radical primarily is contained in fractions that elute at 94 to 106 ml. (B) Ability of these fractions to take up oxygen and reduce it to hydrogen peroxide. The activity is largely confined to the same fractions. (C) Ability of these fractions to cause nicks in rat alveolar macrophage DNA, expressed as the damage quotient number (Qd). The DNA nicking activity is mainly confined to those fractions that (A) contain the tar radical and that (B) reduce O_2 to superoxide. Data from Zang et al. (96).

fractions to take up and reduce oxygen to hydrogen peroxide and to nick DNA. If the cigarette tar radical is the species that reduces oxygen, ultimately producing

hydrogen peroxide, and if this cigarette tar radical system is responsible for nicking DNA, then all three of these characteristics of the ACT fractions should be proportional.

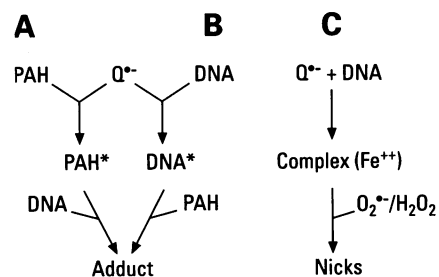


Figure 10. Possible ways for the cigarette tar radical, $Q^{\cdot-}$ to produce DNA damage. The tar radical could complex with DNA, bind iron, and cause nicks, as shown at the right side of the figure (C) and also in more detail in Figure 8. In (A) the tar radical is proposed to interact with a procarcinogen (such as a PAH) to produce an activated PAH, PAH^* which can bind to DNA; this activated species, PAH^* might for example be a cation-radical or a phenoxyl radical of the PAH. In (B), the tar radical is proposed to interact with DNA to activate a base (DNA^*) that can then bind a carcinogen.

Indeed, Figure 9 shows that this is the case. This is the strongest evidence we have discovered so far that it is the tar radical that is responsible for binding to and nicking DNA (96).

Figure 10 suggests possible ways for the cigarette tar radical, $Q^{\cdot-}$, to produce DNA damage. Mechanisms A and B show adduct formation and mechanism C shows DNA nicking. In Figure 10A the tar radical is proposed to interact with a procarcinogen (such as a PAH) to produce an activated PAH, PAH^* which can bind to DNA. In Figure 10B, the tar radical is proposed to activate DNA to a form that can then bind a carcinogen. The tar radical could interact with DNA, as shown in Figure 8; this is abbreviated as shown in Figure 10C. We have not yet compared the ability of ACT fractions to produce adducts, as demonstrated by Randerath et al. (97-102), but that clearly is the next step in our efforts to demonstrate that the cigarette tar radical is involved in the harmful effects of smoking.

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