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Electron-Spin Resonance Study of Mainstream and Sidestream Cigarette Smoke: Nature of the Free Radicals in Gas-Phase Smoke and in Cigarette Tar

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Radicals in the gas phase of both mainstream and sidestream cigarette smoke have been studied using electron-spin resonance ESR spin-trapping techniques with α-phenyl-*N*-*tert*-butyl nitrone (PBN) as the spin trap. The principal radicals we trap appear to be alkoxyl radicals. Mainstream and sidestream gas-phase smoke each have about the same concentration of radicals, about 1×10^{16} radicals per cigarette (or 5×10^{14} per puff). These radicals are reactive, yet they appear to be remarkably long-lived: they are still spin trapped from gas-phase smoke after more than 5 min. We propose that a steady-state concentration of reactive radicals exists in gas phase cigarette smoke. We suggest that this steady state is produced by the slow oxidation of nitric oxide (present in high concentrations in smoke and relatively unreactive) to the more reactive nitrogen dioxide, followed by the reaction of nitrogen dioxide with reactive organic molecules in smoke (such as olefins and dienes). Preliminary experiments reported here support this hypothesis.

Tar from both mainstream and sidestream smoke contains persistent free radicals that exhibit broad, single-line ESR spectra with *g* values of 2.003. The tar radical can be extracted into *tert*-butylbenzene and other organic solvents, and we have applied a variety of fractionation procedures to these solutions. Most of the radicals occur in the fractions that contain the phenolic tobacco leaf pigments. Treatment of alcoholic solutions of tar with base generates a new group of radicals that appear to be semiquinone radicals derived from the oxidation of the phenolic and polyphenolic species in tar.

Introduction

Free radicals were first detected in the condensate from whole cigarette smoke in 1958 by Lyons et al. using electron spin resonance spectroscopy (ESR) (*1*), and have since then also been shown to be present in unsmoked tobacco (*2*), chimney soot and automobile exhaust (*3*), and tobacco ash (*4*). The radicals in cigarette smoke consist of two populations: a transient, gas-phase population that can be studied directly by ESR only after condensation of the gas phase at low temperatures (*1, 5, 6*) or by spin trapping at ambient temperatures (*7–10*); and a stable, tar-phase population that persists at room temperature (*1, 3, 6*).

We have previously reported a study of the radicals in the gas phase of mainstream cigarette smoke using the ESR spin-trapping technique (*10*). Spin trapping (*11-13*) is based on the reaction shown in Eq. (1). Here, an unstable free radical is "spin-trapped" by reaction with a nitrone or nitroso compound (the spin trap) to produce a persistent nitroxide radical (the spin adduct).

$$\underset{\text{(Paramagnetic)}}{\text{Short-lived Radical}} + \underset{\text{(Diamagnetic)}}{\text{Spin Trap}} \longrightarrow \underset{\text{(Paramagnetic)}}{\text{Spin Adduct}} \quad (1)$$

The spin adduct is more conveniently studied by ESR than is the unstable radical and from the *g*

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value and the magnitude and multiplicity of the spin-adduct hyperfine splitting it is possible, in some cases, to identify the trapped radical (*14*).

In our previous report (*10*), we showed that bubbling filtered mainstream smoke through a solution of the spin trap α-phenyl-*N*-*tert*-butyl nitrone (PBN) results in an ESR spectrum consisting of three types of signals: a doublet of triplets consistent with a spin-trapped alkoxyl radical (Type I), a triplet consistent with no known spin adduct (Type II), and a triplet consistent with *N*-benzoyl-*N*-*tert*-butyl nitroxide (PBNOx) (Type III). In this communication, we wish to report our further progress in the study of mainstream smoke, as well as new data on sidestream smoke and on the radicals in the tar portions of the smoke.

Experimental

Materials

The spin trap used in this study, α-phenyl-*N*-*tert*-butyl nitrone (PBN), was obtained from Eastman Kodak Co. (Rochester, NY) and was used without further purification. *tert*-Butylbenzene (99%), used as solvent in some of these experiments, was purchased from Aldrich Chemical Co. (Milwaukee, WI) and was distilled prior to use. The cigarettes used were research grade 1R1 cigarettes supplied by the University of Kentucky Tobacco and Health Research Institute. All other chemicals were reagent grade and were used without further purification.

ESR Measurements

Our early ESR spectra were obtained with a JEOL model JES-3BS X-band spectrometer and our more recent work with a more sensitive Varian E-109 X-band spectrometer. With each, 100 Kc field modulation was employed. The *g* values were determined by comparison to 2,2-diphenyl-1-picrylhydrazyl (DPPH) (*15*) and to di-*tert*-butyl nitroxide (*14*).

Nuclear hyperfine splitting constants and spin-adduct concentration were determined by comparison of the unknown spin adduct to a spectrophotometrically standardized solution of di-*tert*-butyl nitroxide (λ_{max} 465 nm, ϵ = 8.9L/cm-1 mole) (*16*). The concentration of the radicals observed directly in the tar portion of the smoke was determined by comparison to solutions of DPPH >99% purity) in *tert*-butylbenzene (*15*). Spin-adduct half-lives were determined by positioning the magnetic field of the ESR spectrometer at the resonance position of either the maximum or minimum point of the most downfield spin-adduct peak and monitoring that peak height over a period of time.

Smoking Conditions

The cigarette smoke used in this study was produced in two ways. Human smoking was mimicked by using the syringe-puffed system that we have described previously (*10*). Here, the cigarette was "smoked" by withdrawing the smoke through a Cambridge filter into 50-mL glass syringe and expelling the smoke into the spin-trap solution; the volume of smoke withdrawn was a 35-mL "puff" of 2-sec duration (*10,17*).

In experiments where it was not necessary to mimic human smoking, it was more convenient to use the apparatus shown in Figure 1. In this continuous-flow system, the smoke was aspirated from the cigarette, through the Cambridge filter, and directly into the spin-trap solution using a small electrical pump. The flow rate, which could be adjusted using a needle-valve downstream from the pump, was set at 1050 mL/min.

Spin-Trapping Radicals in Gas-Phase Smoke

For those experiments in which spin trapping was performed, 0.10*M* solutions of PBN were prepared in *tert*-butylbenzene. Both the syringe-puffed and the continuous-flow systems were used for producing the smoke. Filtered smoke was bubbled through the spin-trap solution, the solution was transferred to cylindrical, quartz ESR tubes, deoxygenated by flushing with argon for five minutes, and the ESR spectrum recorded.

Radicals in the sidestream smoke were spin trapped using the apparatus shown in Figure 2. The smoke that accumulates inside the three-necked flask is drawn through the Cambridge filter and into 1 mL of a 0.1*M* solution of PBN by using the electrical pump at a flow of 1500 mL/min. For this experiment, the sidestream smoke from three 1R1 cigarettes was produced by "smoking" each cigarette by aspirating the mainstream smoke using the syringe at the standard regimen of a 35-mL puff of 2-sec duration. The mainstream smoke was vented after each puff. After the last cigarette was smoked, the spin-trap solution was transferred to an ESR tube, flushed with argon for 5 min, and an ESR spectrum obtained.

The stability of the radicals in mainstream smoke was studied using the syringe-puffed system (*10*). A 35-mL puff of the filtered smoke was drawn into the syringe, held for *t* seconds, and then bubbled through 3.0 mL of a 0.1*M* solution of PBN in

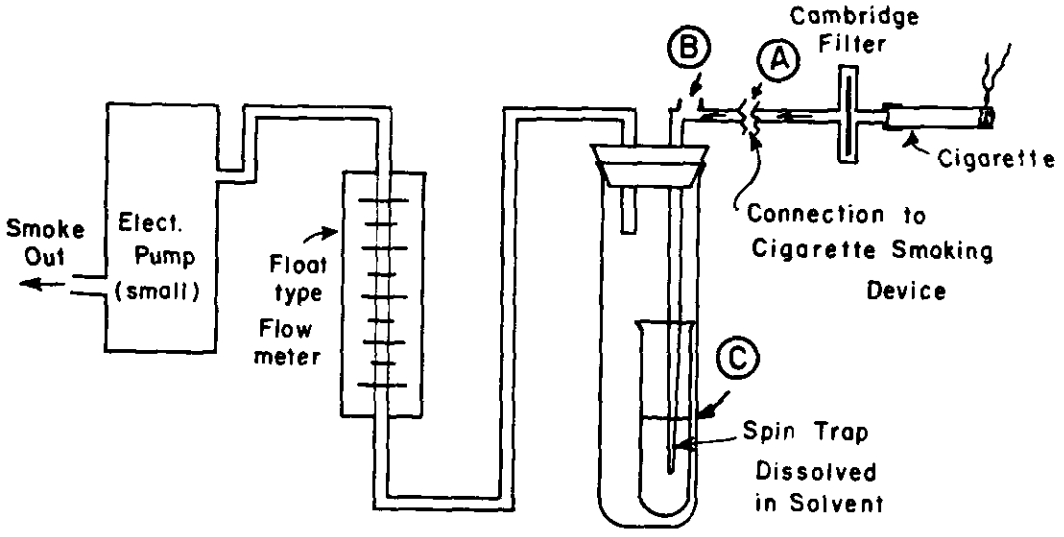


FIGURE 1. Continuous-flow system for collecting mainstream smoke.

tert-butylbenzene. This process was repeated five times, and 1 mL of the PBN solution transferred to an ESR tube, flushed with argon for 5 min, and an ESR spectrum obtained.

The effect of oxygen added to the mainstream smoke was determined by inserting a 200 mL/min stream of oxygen or nitrogen at point *B* of the continuous-flow apparatus (Fig. 1). The flow rate produced by the pump was adjusted to 1250 mL/min to compensate for the flow of the gases added to the smoke stream. Three 1R1 research cigarettes were then smoked in the usual manner and the smoke bubbled through 1.0 mL of a 0.1*M* solution of PBN in *tert*-butylbenzene. The PBN solution was then transferred to an ESR tube, the solution deoxygenated by flushing with argon, and an ESR spectrum obtained.

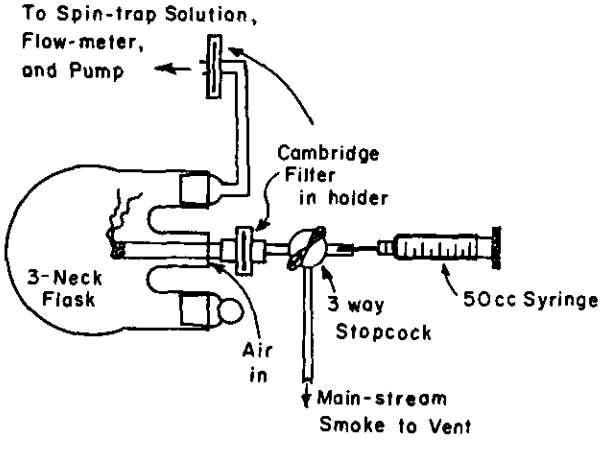


FIGURE 2. Sidestream smoke collection device.

Free Radicals in Tar

Tar samples for direct ESR study were prepared in three ways using the continuous-flow system: (1) trapping the tar on a Cambridge filter, (2) trapping the tar on a glass wool plug, and (3) leaching the trapped tar from either the Cambridge filter or glass wool plug into *tert*-butylbenzene. For example, after drawing the smoke from three 1R1 research cigarettes through a Cambridge filter, the filter was cut into 2-mm wide strips, the strips inserted into an ESR tube and argon passed through the tube for 5 min. The tube was then sealed and the ESR spectrum obtained.

Alternatively, three cigarettes were smoked without the Cambridge filter in the holder, and the smoke was passed through a glass capillary and into an ESR tube containing glass wool positioned in place of the spin-trap solution in Figure 1. Argon was then passed through the ESR tube for 5 min, the tube sealed, and an ESR spectrum obtained.

A solution of mainstream smoke tar was prepared by placing the Cambridge filter or glass wool plug through which the smoke from six 1R1 research cigarettes had been passed (and which contained about 0.6g of tar) into a 50-mL Erlenmeyer flask containing 25 mL of *tert*-butylbenzene. The suspension was stirred for 30 min and the filter or glass wool particles filtered out, leaving a reddish-brown solution of tar. A 1-mL portion of this solution was transferred to an ESR tube, flushed with argon for 5 min, the tube sealed, and an ESR spectrum obtained. Alternatively, a more concentrated solution of the tar from up to 30 1R1 research cigarettes was prepared in the same way and then fraction-

ated according to the method of Stedman with the use of *tert*-butylbenzene in place of diethyl ether (*18*).

The tar from mainstream smoke was also fractionated according to the procedure outlined in Figure 3. Tar from six 1R1 cigarettes was collected on a Cambridge filter using the continuous-flow apparatus (Fig. 1). The filter was placed in a 150-mL beaker and then washed by stirring with two successive 50-mL portions of distilled water at room temperature and exposed to room air. The water extracts were combined and the water removed by lyophilization, leaving a viscous, orange oil.

The residue remaining on the filter following the water wash was further extracted by stirring with four 25-mL portions of methylene chloride. The methylene chloride extracts were combined and subjected to ESR analysis. Following the methylene chloride wash, a light tan residue remained on the filter. This residue proved very resistent to further extraction; e.g., washing with dimethylformamide, dimethyl sulfoxide, ammonium hydroxide, or acetone did not remove any substantial amount of this residue, although washing with methanol and pyridine yielded very small amounts of ESR-detectable material (a weak, broad singlet with peak-to-peak linewidth of about 0.5 mT).

The methylene chloride extract was poured through a 2.5 × 7 cm glass column packed with silica gel. The material collected on the column was washed successively with 600-mL portions of propanol, methanol, and a 1:1:1 mixture of ammonium hydroxide, methanol, and ethanol. After the NH_4OH–CH_3OH–CH_3CH_2OH wash, there remained on the silica gel a straw-colored residue that was not removed by further extraction with methylene chloride, ethyl acetate, benzene, or methanol.

The solvent was evaporated from the propanol-soluble fraction under vacuum at room temperature and the residue resuspended in 2 mL of methanol. The suspension was mixed and centrifuged at 3000 rpm for 20 min. The supernatant is Fraction I (Fig. 3). The precipitate was dissolved in 2 mL of 33% methylene chloride/methanol and is Fraction II, (Fig. 3).

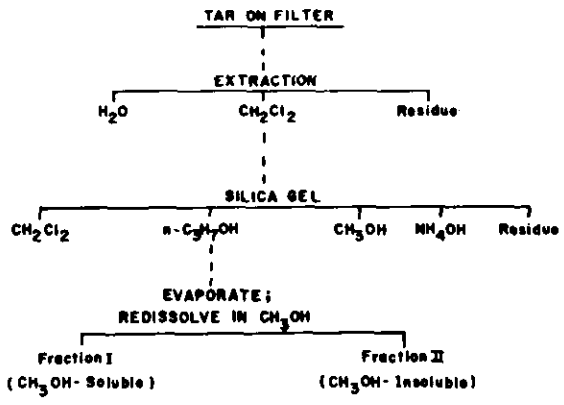


FIGURE 3. The fractionation of tar.

The effect of NaOH on the ESR signals of the two fractions was determined by adding sufficient powdered NaOH to either Fraction I or Fraction II to produce a 1*M* solution. The NaOH required 10 min to dissolve. The resulting solution was then subjected to ESR analysis immediately following deoxygenation with argon. The experiment was also performed using a solution of the methanol-soluble fraction (Fraction I) that had been deoxygenated prior to the addition of the NaOH.

The effect of added HCl was determined by adding sufficient concentrated HCl to either Fraction I and Fraction II to produce a 1*M* solution. The solution was subjected to ESR study both immediately and after 14 hr at 25°C.

The ability of the material in the two fractions to reduce silver nitrate was determined by adding 100 μL of either of the two fractions to 1.00 mL of 0.1*M* aqueous $AgNO_3$ in a 5-mL test tube. The resulting solution was mixed and allowed to stand at room temperature for 12 hr.

The effect of zinc(II) was studied by adding a sufficient amount of a 1*M* aqueous solution of zinc acetate to either Fraction I or Fraction II to produce a 0.02*M* solution.

The effect of copper(II) was determined by adding a sufficient amount of a 1*M* solution of copper sulfate to either of the two fractions to produce a 0.02*M* solution. The solution was mixed and subjected to ESR analysis after deoxygenation with argon.

The effect of hydrogen peroxide was determined by adding a sufficient amount of 30% H_2O_2 to either of the two fractions to produce a 0.125*M* solution. The ESR spectra were obtained immediately, after 2 hr and after 6 hr at room temperature.

Results and Discussion

Gas-Phase Smoke

Free radicals in the gas phase of cigarette smoke (the smoke that passes through a Cambridge filter) (*17*) were studied by spin trapping. As we have previously reported (*10*), a plot of the concentration of the spin adducts from mainstream smoke versus the length of tubing through which the smoke must travel before entering the spin-trap solution gives a curve with a maximum; that is, the yield of spin

adducts first increases as the path length from the cigarette is increased and then decreases at longer distances. We have probed this unexpected path length effect further by separating the distance effect (the distance the smoke must travel from the cigarette to the spin-trap solution) from the time effect (the time the smoke spends in the tubing during passage from the cigarette to the spin-trap solution) (W.H. Davis, this laboratory, unpublished data, 1977). We accomplished this by holding the smoke in the syringe that is part of the syringe-puffed apparatus described previously (*10*) for various periods of time t using a constant length of tubing. This system permits a study of the path length effect in the absence of possible complicating factors such as eddying, precipated tar gradients within the tubing, and mixing of puffs due to the discontinuous flow inherent in this type of smoking apparatus.

When t is plotted versus the peak heights of the spin-adduct signals, a curve similar to the original path length effect is obtained (*10*). In this case, the maximum peak height occurs when the smoke has been held in the syringe for about five minutes. The same results are obtained when the syringe is made of glass or plastic, when the tubing is Tygon or Teflon, or when the solvent is *tert*-butylbenzene or undecane. These results confirm our previously reported observation of a long-lived radical population in the gas phase of cigarette smoke (*10*).

We previously reported that the radicals spin trapped from mainstream smoke are primarily oxygen-centered (*10*). Carbon-centered radicals are rapidly scavenged by molecular oxygen (*20*) that enters the smoke through the pores of the paper (*21*) to give oxygen-centered radicals as shown in Eqs. (2) and (3).

$$\text{Tobacco} \longrightarrow R^{\cdot} \qquad (2)$$

$$R^{\cdot} + O_2 \longrightarrow \text{Oxygen-centered Radicals} \qquad (3)$$

We tested this hypothesis by adding additional oxygen to the smoke stream and studying its effect on the spin-trapped radicals. We find that when oxygen is added at point B of the continuous-flow system shown in Figure 1, the Type I spin-adduct signal (*10*) is increased by about 70% (compared to when nitrogen is added) while the Types II and III signals are not affected. This result confirms the involvement of oxygen in the production of the alkoxyl radicals that are spin trapped.

Small organic carbon- or oxygen-centered radicals of the types represented in Eqs. (2) and (3) under homogeneous gas phase conditions would not be expected to have the 5-min lifetimes that we observe. A possible resolution of this apparent paradox would be a system in which radicals are continuously produced and destroyed so that a steady-state level of radicals is maintained. We would like to suggest a mechanism by which this might occur and to present some preliminary data that support our hypothesis. Our suggestion involves the oxidation of nitric oxide (NO) to nitrogen dioxide (NO_2), and the reaction of NO_2 with reactive substances in the smoke stream (such as olefins and dienes) to give carbon-centered radicals.

Oxides of nitrogen occur in cigarette smoke at levels as high as 1400 micrograms per cigarette (*22*); the NO_x in fresh smoke is mainly nitric oxide. Nitric oxide is relatively unreactive toward organic materials (*23*); however, it is known to be oxidized to NO_2 in the smoke stream [Eq. (4)] (*24*), and nitrogen dioxide is quite reactive toward a number of types of organic substances (*25*). For example, smoke contains relatively high concentrations

$$\underset{\text{(Relatively) unreactive)}}{2\,NO + O_2} \longrightarrow \underset{\text{Reactive}}{2NO_2} \qquad (4)$$

of monoolefins and dienes (*17,26*), and these materials are known to react with NO_2 via allylic hydrogen abstraction [Eq. (5)] or addition [Eq. (6)] to give carbon-centered radicals (*27–29*). These carbon-centered radicals could then be scavenged

$$NO_2 + RH \longrightarrow HONO + R^{\cdot} \qquad (5)$$

$$NO_2 + \rangle C = C\langle \longrightarrow \underset{NO_2}{-\overset{|}{\underset{|}{C}}-\overset{|}{\underset{|}{C}}-} \qquad (6)$$

by oxygen [Eq. (7)] to produce peroxyl (*30*) radicals. The high nitric oxide concentrations in smoke would be expected to deoxygenate peroxyl radicals [Eq. (8)].

$$R^{\cdot} + O_2 \longrightarrow RO^{\cdot} \qquad (7)$$

$$RO^{\cdot} + NO \longrightarrow RO^{\cdot} + NO_2 \qquad (8)$$

We have performed preliminary experiments to test whether this mechanism for radical production is possible. We have modeled the gas phase of cigarette smoke by mixing various olefins with either NO or NO_2 in air, followed by passing these mixtures through a solution of PBN. We find that, when NO_2 alone is passed through a solution of PBN, the only spectrum observed is that due to PBNOx, confirming our earlier suggestion (*10*) that the "Type III" signal we observe may arise from the one-electron oxidation of PBN by NO_2. With mixtures of 200 ppm NO_2 and either monoolefins (such as ethylene or propylene) or a diene (such as

isoprene or butadiene) in air, alkoxyl radicals are trapped by PBN. The signal is particularly intense when dienes are used, perhaps reflecting the fact that dienes add NO_2 faster than either ethylene or propylene (*31*, *32*). When NO (5-5000 ppm) is mixed with isoprene (3900 ppm) in air, the alkoxyl radical is again trapped by PBN (*32*). In this system, the PBNOx signal is observed only with high ratios of NO to olefin.

The effect of added oxygen, described above, also is consistent with this hypothesis. Oxygen enters the scheme for radical production at two places, [Eqs. (3) and (4)]; it both converts carbon radicals to oxy-radicals and oxidizes nitric oxide to nitrogen dioxide.

We have previously reported that the radicals spin trapped from mainstream smoke are primarily alkoxyl radicals (*10*). We have now studied the radicals in sidestream smoke using the apparatus shown in Figure 2. Using PBN as the spin trap, we observe the spectrum shown in Figure 4. This spectrum consists of a doublet of triplets with $a(N) = 1.34$ and $a(H) = 0.18$ mT, values that are consistent with the PBN spin adducts of oxy-radicals such as alkoxyl (*14*). The yield of spin adduct produced is about 6×10^{15} spins per cigarette smoked. This is a lower limit for the radicals in smoke, since spin trapping certainly is less than 100% efficient. Under the same conditions, we spin trap 8×10^{15} free radicals per cigarette from mainstream smoke; thus, the radical concentrations in the two smoke streams appear to be essentially identical as probed by the spin trap method. The spin adducts from both smoke streams appear to be similar in stability; the half-lives of the spin adducts from mainstream and sidestream smoke are the same (39 and 40 min, respectively).

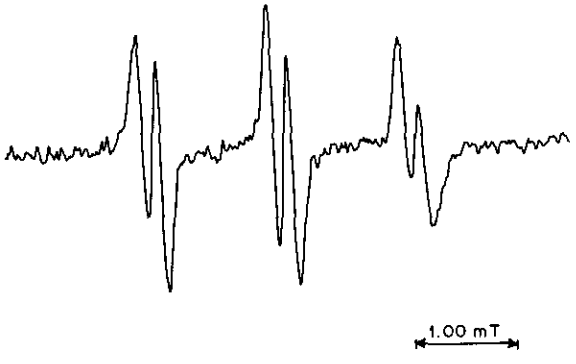


FIGURE 4. Sidestream smoke from 3 1R1 research cigarettes bubbled through PBBN in *tert*-butylbenzene. Field = 333.54 mT; scan range = 10.0 mT; modulation amplitude = 0.063 mT; gain = 8×10^3; scan time = 4 min; power + 1mW.

Radicals in Tar

The free radicals in mainstream tar were studied directly (without spin traps). Figure 5 shows the ESR spectrum of the persistent radical that is present in tar collected on a Cambridge filter. The spectrum consists of a broad single line with no indication of fine structure. The linewidth is generally 0.5 to 0.6 mT, although failure to thoroughly deoxygenate the sample causes some additional broadening. The g value is near 2.003.

Sidestream smoke tar also contains a persistent radical. Figure 6 shows the ESR spectrum of the radicals that is present in tar from sidestream smoke collected on the Cambridge filter in the apparatus shown in Figure 2. As with mainstream smoke, the spectrum of the radicals in sidestream tar collected on filters consists of a broad singlet with a linewidth of 0.5 to 0.6 mT and $g = 2.003$. This spectrum appears slightly less symmetrical than the spectrum from mainstream tar. The ESR signals from both mainstream and sidestream smoke remain unchanged after several days exposure to air at room temperature.

Solutions of the Tar Radical

For the first time, we also have been able to extract the tar into solution in benzene or *tert*-butylbenzene and study the tar radical under homogeneous solution-phase conditions. When the tar from a filter is extracted by using *tert*-butylbenzene, 90-97% of the radicals go into solution, and the yield

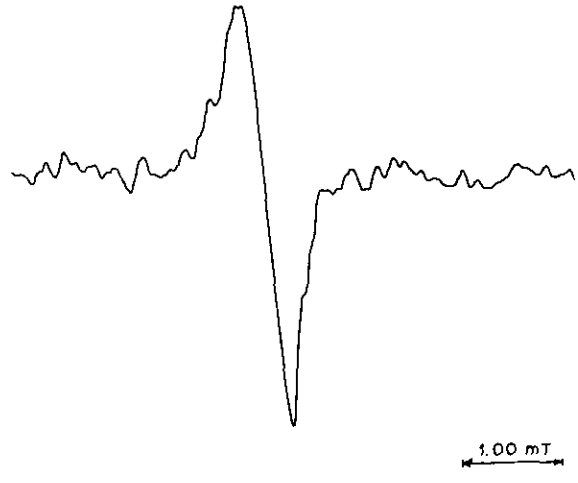


FIGURE 5. Tar radical from mainstream smoke of 3 1R1 research cigarettes collected on a Cambridge filter. Field = 335.10 mT; scan range = 20.0 mT; modulation amplitude = 0.063 mT; gain = 2.5×10^3; time constant = 0.5 sec; scan time = 4 min; power = 1 mW.

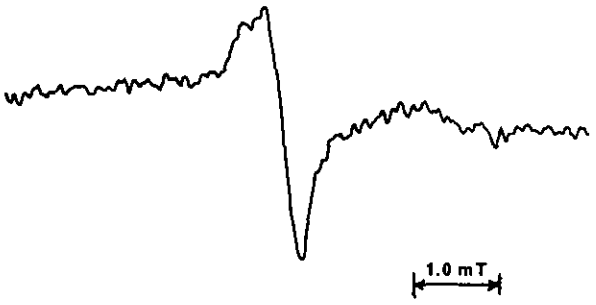


FIGURE 6. Tar radical from sidestream smoke collected on a Cambridge filter. Field = 335.40 mT; scan range = 20.0mT; modulation amplitude = 0.5 mT; gain = 5×10^3; time constand = 0.5 sec, scan time = 4 min; power = 1 mW.

of radicals in solution is about 9×10^{16} radicals/g tar, or approximately 4×10^{14} per puff. The ESR spectrum of the solution of the tar radical is similar to that of the radicals on the filter shown in Figure 5.

Our success in detecting an ESR signal from a *tert*-butylbenzene solution of tar prompted us to attempt to fractionate the tar. Extracting a *tert*-butylbenzene solution of the tar radical with 1*M* sodium hydroxide appears to partition the tar radical between the aqueous and organic layers. (It is not possible to quantify radical concentrations in this experiment, since as we shall discuss further below, treatment of tar extracts with base produces a marked increase in the intensity of the free radical signal.) Either acidification of the aqueous layer to pH 6 followed by extraction with *tert*-butylbenzene or shaking the organic layer with 1*M* HCl results in the formation of a precipitate that is not soluble in *tert*-butylbenzene. Although the precipitate from either fraction contains a paramagnetic species, a major portion of the radicals remain in solution.

The formation of a precipitate upon treatment of the tar solutions with dilute acid suggested that the tar radical is associated with the phenolic pigment fraction of tar. Tobacco leaf pigments are produced from plant phenols by an enzymatic oxidation process much like that responsible for the production of the animal melanin pigments (*19,33-35*). We therefore have compared the properties of tar to those of natural and synthetic melanins, and we find striking similarities (see Table 1).

The radical ESR signal in melanins is associated with quinoidal groups (*19*). Tar also is very rich in quinoidal groups, with, for example, 0.3 mg catechol per cigarette (*17*). We therefore suggest that the tar free radical is similar to the free radical present in melanins. This model for the tar radical is quite different from what has been proposed. Usually the tar radical is depicted as being a "radical in a hole" (*1,2,5*); that is, an electron delocalized in a hydrocarbon molecule that may either be a polycyclic aromatic hydrocarbon (PAH) or a graphitelike species. To test whether the tar radical has similarities to a melanin-type paramagnetic system, we have applied a number of diagnostic tests that have been designed by Sealy et al. (*19*) to our tar radical extracts (Table 1).

We first fractionated the tar (Fig. 3) and then applied the tests for melanins to the resulting fractions. The ESR spectra taken of the water and methylene chloride washes of the Cambridge filter (Fig. 3) reveal that the greater portion of the free-radical signal initially on the filter (>95%) appears in the methylene chloride-soluble fraction, while the water-soluble fraction contains less than 2% of the radical signal. The residue that remains on the filter also exhibits a weak ESR signal (about 3–4% of the original signal), but it is not possible to elute this remaining radical into solution. The methylene chloride run-off from the silica gel column gives no ESR signal while the propanol-, methanol- and NH_4OH–MeOH–EtOH-soluble fractions exhibit similar broad singlets of roughly equal intensity. When the propanol was removed and the residue then resuspended in methanol, only part of the residue was soluble in methanol alone (Fraction I). The remainder was redissolved in a mixture of methanol and methylene chloride; this fraction is what we

Table 1. Comparison of tar radical to melanins.

Property	Tar radical	Melanins[a]	Autoxidized DOPA[a]
Description	Dark brown granules	Brown/black/yellow/white granules	
Solubility	Insoluble in benzene, *tert*-butylbenzene, and water	Insoluble in most solvents	Insoluble in most solvents
Spins/gram	$10^{16} - 10^{17}$[b]	$10^{16} - 10^{19}$	$10^{17} - 10^{18}$
g-value	2.003[b]	2.001 – 2.005	2.003 – 2.004
Linewidth, mT	0.5 – 0.6[b]	0.5 – 1.2	0.4 – 0.8

[a]Data of Sealy (*19*).
[b]The parameters were measured for the tar radical that was initially soluble in *tert*-butylbenzene. The insoluble precipitate that formed on acid treatment has similar properties.

Table 2. Tests for melanin applied to fractionated tar.[a]

Test	Result obtained with melanin[b]	Tar Fraction I	Tar Fraction II
Reduction of $AgNO_3$	Silver mirror	Silver mirror	Silver mirror
ESR Signal in NaOH	7-Fold increase	160-Fold increase	7-Fold increase
ESR Signal in HCl	Increases or decreases[c]	4-Fold increase	2-Fold increase
ESR Signal in H_2O_2	Signal disappears	No effect	33% decrease
ESR Signal in Zn^{2+} solution	3- to 7-Fold increase	9-Fold increase	5-Fold increase
ESR Signal in Cu^{2+} solution	Signal	Slight decrease	66% decrease

[a]P. Premovic (this laboratory), unpublished data, 1981.
[b]Data of Sealy et al (*19*).
[c]Natural melamines decrease about 50% in ESR intensity but synthetic melanins increase.

refer to below as the methanol-insoluble fraction (Fraction II).

Table 2 lists the tests that we applied to the methanol-soluble and the methanol-insoluble fractions (Fractions I and II) from the propanol extract, including the results obtained on melanins (*19*). Although both fractions produce positive results for the reduction of silver nitrate, which is a classical histochemical test for melanins (*19*), neither fraction gives positive results for the entire group of tests. Variations of this sort are not unexpected in view of the very complex mixture of compounds in both melanins and tar. The increase in the ESR signal observed when a diamagnetic metal ion such as Zn^{2+} is added to the mixture strongly implies the presence of the ortho-semiquinone functionality (*19*). The decrease in the signal when the paramagnetic ion Cu^{2+} is added also is evidence that the radical species in melanins and the tar radical are structurally similar.

The reaction of the propanol eluate with sodium hydroxide is of particular interest (P. I. Premovic, this laboratory, unpublished work, 1982). In the absence of air, the addition of 1*M* sodium hydroxide causes about a ten-fold reduction in the intensity of the tar radical singlet. Upon exposure to air, there is a rapid increase in the signal intensity, and a five-line spectrum appears (Fig. 7*A*); the intensity of the five-line spectrum is more than 100 times greater than that of the original tar radical. The intensity ratio of the five lines is 1:4:6:4:1, the hyperfine splitting constant is 0.236 mT, and the *g* value is 2.0045 (Fig. 7*A*). Over a period of several hours, this five-line spectrum disappears and is replaced by a series of more complex intermediate spectra (Fig. 7*B*). Ultimately, the complex structure gives way to a single line spectrum that is very similar in shape and line-width to the spectrum of the initial radical extracted form tar. However, the *g* value (2.004) is slightly greater.

The five-line spectrum has ESR parameters consistent with literature data for the *p*-benzenesemiquinone radical anion (*36,37*). Since it appears after base treatment of the propanol extract in the presence of air, the semiquinone probably is formed by base-catalyzed oxidation of hydroquinone (*38*) which

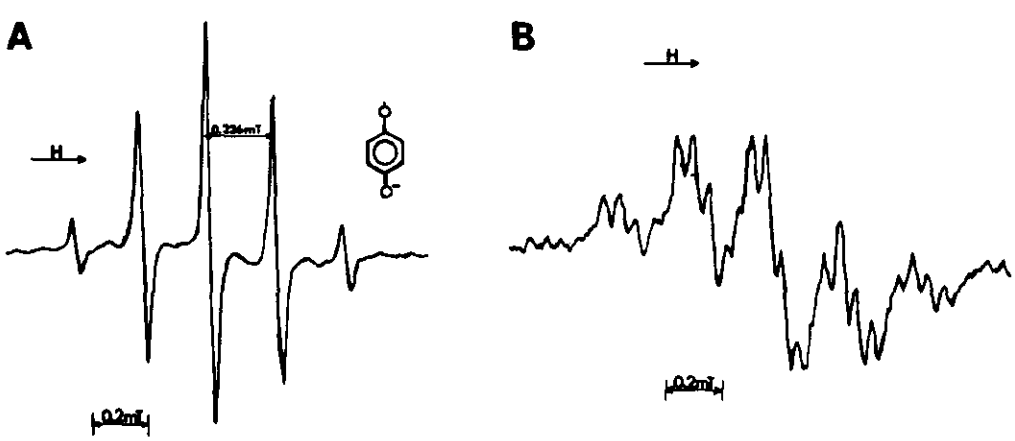
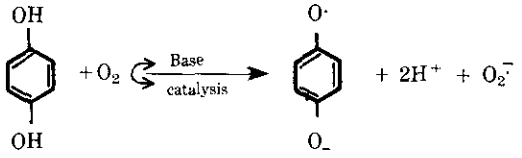


FIGURE 7. (A) The five-line spectrum observed when alcoholic extracts of cigarette tar are treated with sodium hydroxide in the presence of air. (B) The Above sample after aging for about 1 hr.

is known to be present in tar in large amounts (*39*) [Eq. (9)]. The other radicals with fine structure



(9)

that appear following base treatment are probably also semiquinones formed by condensation reactions of benzenesemiquinone and from the more complex polyphenols that are present in tar (*17*). The nature of the final species has not yet been determined.

The benzenesemiquinone radical is less stable in the tar mixture than under the conditions where it is usually observed (*36,37*). This may be due to the reducing nature of tar, as illustrated by the silver-mirror test reported in Table 2. This ability of cigarette tar to behave as a reducing agent has previously been demonstrated both with an indophenol dye and potentiometrically (*40*), as well as by its effect on nitroxide radicals (discussed below).

The Path Length Effect

One possible explanation for the path length effect, as remarked before (*10*), is the quenching by a smoke component such as tar of the gas phase radicals or of their nitroxide spin adducts. Tar gradually precipitates from the gas phase as the smoke flows down a long tube (*10*); thus less tar reaches the spin-trap solution as the distance from the cigarette increases. If tar entering the spin-trap solution quenches either the radicals being trapped or their spin adducts, then (since less of the tar enters the solution at long path lengths) less of the radicals or nitroxide would be destroyed at longer path lengths. We have tested this explanation for the path length effect by studying the reactions of tar from mainstream cigarette smoke with nitroxides. In a typical experiment, a solution of a nitroxide was mixed with a solution of tar and the disappearance of the nitroxide signal was followed by ESR. The nitroxides that were studied included the *tert*-butoxyl, benzoyloxyl and phenyl adducts of PBN, all prepared independently by known procedures (*14*). The ESR signals from all three nitroxides were found to be quenched by tar at room temperature using solutions of 1 g tar/100 mL *tert*-butylbenzene. The order of reactions of the nitroxides with tar is: PhCOO-PBN· > *t*-BuO-PBN· > Ph-PBN· (K. Terauchi and W.H. Davis, this laboratory, unpublished data, 1977). The disappearance of the nitroxide signal follows a first-order decay. A typical rate constant for the reaction of BzO-PBN· with tar at 25°C is 10^{-3} L/g-sec.

Several types of organic molecules were studied in an effort to model the reaction of tar with nitroxides. It was found that strongly reducing species such as thiols and phenols react rapidly with the three nitroxides whereas polynuclear aromatic hydrocarbons (such as phenanthrene) and benzyl alcohol and benzoic acid react much more slowly. The reaction of thiols with nitroxides is first order in nitroxide (under conditions where the thiol is in excess); the rate constant for the reaction of butanethiol with PhCOO-PBN·, for example has a value of $10^{-3}/M$ sec at 25°C in benzene as solvent.

The kinetic isotope effect was measured for the thiol/nitroxide reaction. Qualitatively, the isotope effects for aliphatic thiols appear to follow the curve for isotope effect versus heat of reaction (*41*), indicating that the reaction is a hydrogen-atom transfer. The products of the reaction were not examined, but other workers have reported products that can be rationalized as dimerization and combination products from thiyl and nitroxide radicals (*42*). Qualitatively, thiophenol appears to have a much smaller isotope effect, suggesting the possibility of a rate-limiting electron-transfer reaction (K. Terauchi and W. H. Davis, this laboratory, unpublished data, 1977). Thus, the reaction of cigarette tar with nitroxyl radicals and spin adducts could consist of a hydrogen-atom transfer or an electron transfer reaction.

Conclusions

The slow, continuous oxidation of NO to NO_2 in gas phase smoke followed by the reaction of NO_2 with reactive smoke constituents (such as olefins and dienes) could produce a steady-state of reactive radicals in gas-phase cigarette smoke, explaining the apparently long lifetimes observed for the radicals in mainstream and sidestream smoke. The effect of added oxygen on the radicals spin trapped from mainstream smoke and the preliminary spin-trapping results we have obtained from the reactions of olefins with NO and NO_2 in the gas phase are evidence in favor of this hypothesis.

Radicals are also spin trapped from the gas phase of sidestream smoke and the concentration of these radicals is nearly the same as that of mainstream smoke radicals. Thus, nonsmokers in the vicinity of smokers are exposed to gas phase free radicals much as are the smokers themselves.

The tar radical can be extracted into benzene or *tert*-butylbenzene. A part of the tar radical is weakly acidic and extractable with base, characteristic of a polyphenol, and a part of the tar radical remains in benzene solution. The appearance of a radical signal in the basic extract may be a result of the oxidation

of polyphenols and phenols to radical species that were not originally present in the tar. The polyphenolic nature of the tar radical also may be responsible for the similarities we find in the behavior of the tar radical and melanins, since melanin pigments are polyphenols. Many of the tests of Sealy et al. for melanins (*19*) probe the redox properties of melanins; as remarked above, cigarette tar has the ability to quench spin adducts, undoubtedly by a redox reaction.

The phenolic and polyphenolic fraction in cigarette smoke is known to be the most potent cocarcinogen in smoke (*39*). This effect is critical in understanding the biological effects of smoke, since polynuclear aromatic hydrocarbons (PAH), the principal carcinogens in smoke, are present at concentrations that are too low to explain the carcinogenicity of smoke. The chemistry and biology of promotion and cocarcinogenicity are not well understood, but many systems that activate PAH involve promoters that are readily oxidizable (*43*). Therefore, we wish to suggest that the cocarcinogenicity of the polyphenolic fraction of cigarette tar involves the ease of oxidation of this fraction and the initiation of the oxidation of PAH (both enzymatically and nonenzymatically) by the cooxidation of the polyphenolic fraction. There is substantial evidence that the oxidation of compounds like hydroquinone results in the production of superoxide [Eq. (*9*)], a reaction that can be mediated by reductase enzymes (*38*) or can occur spontaneously on exposure to oxygen. The superoxide thus produced may be involved in the activation of PAH to carcinogens (*44,45*). Finally, semiquinones are known to bind covalently to DNA bases, especially guanine (*46*), suggesting that the oxidation of the polyphenolic fraction may be involved in its carcinogenicity (*39*).

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