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Free Radicals in Pharmacology and Toxicology—Selected Topics

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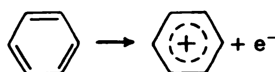
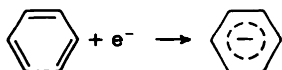
I. Introduction . . . 189
II. Novel free radical metabolites . . . 189
A. Semiquinoneimine free radicals . . . 189
B. Thiyl free radicals . . . 192
C. Nitrogen oxide free radicals . . . 194
D. Sulfur oxide free radicals . . . 195
E. Peroxy free radicals . . . 196
F. Ethanol free radicals . . . 196
G. Triarylmethane dye carbon-centered free radicals . . . 197
III. Free radical pathways in the metabolism of xenobiotics . . . 198
A. One-electron enzymatic reduction of xenobiotics . . . 198
B. One-electron enzymatic oxidation of xenobiotics . . . 201
IV. Prostaglandin synthetase and lipoxygenases: Examples of enzymatically controlled stereospecific lipid peroxidation . . . 201
V. Free radicals in drug-induced cutaneous photosensitization . . . 205
A. Halogenated salicylanilides . . . 206
B. Chlorpromazine . . . 206
C. Sulfanilamide . . . 207
VI. Conclusion . . . 208

I. Introduction

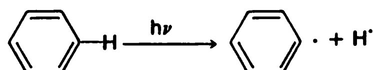
A FREE RADICAL is any molecule that has an odd number of electrons (12). Although most free radicals are organic, it is also possible to form inorganic free radicals, e.g. $\dot{O}_2^-$. A simple organic molecule like benzene may be transformed into a free radical by three different mechanisms. One-electron oxidation of benzene (removal of one of the pi-electrons) results in the formation of the benzene cation radical.



The addition of an electron, the one-electron reduction of benzene, generates the benzene anion radical.



Finally, the homolytic cleavage of one of the C—H bonds in benzene by ultraviolet or other radiation forms a hydrogen atom and the neutral phenyl radical.



Although extreme chemical reactions are required to form free radicals from benzene, many other molecules may be converted into radicals under relatively mild conditions including those that are encountered in biological systems. Thus it is not surprising that free radicals are found in many metabolic pathways involving both naturally occurring and foreign compounds.

In previous reviews, 14 different classes of free radical metabolites have been discussed (89, 90). In this review, seven additional novel free radical metabolites are described in detail (section II). This section is followed by a summary in tabular form (tables 2 and 3) of all known free radical pathways classified by chemical class and enzyme (section III). The next section (IV) describes in greater detail the free radicals formed by the action of prostaglandin synthetase and lipoxygenase on fatty acids. The final section (V) is concerned with the role of free radicals in drug-induced photosensitization. The technique of spin labeling, which employs stable nitroxide radicals as probes, will not be discussed in this review. The application of spin labeling to pharmacological and toxicological problems has already been reviewed recently (24).

II. Novel Free Radical Metabolites

A. Semiquinoneimine Free Radicals

The one-electron oxidation of phenylenediamine by the horseradish peroxidase/H_2O_2 system forms a cation radical. The same system also catalyzes the formation of benzosemiquinone from hydroquinone. Thus a free rad-

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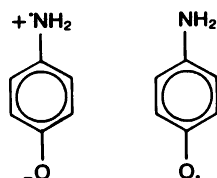
ical of aminophenol, a chemical hybrid of phenylenediamine and hydroquinone, should also be expected. Two reasonable resonance structures of the semiquinoneimine radical can be drawn: a nitrogen-centered zwitterion or an oxygen-centered neutral phenoxy radical. Since the



Hammett sigma value of the amino group is more negative than that of the hydroxyl group, the first resonance structure should be important. In aqueous solution, charge separation is possible, and many molecules exist as zwitterions. On the other hand, considerable electron spin density should also reside on the oxygen, and this radical could also be described as an aminophenoxy radical (106). Molecular orbital calculations may give an indication of the charge and spin distribution in this free radical. In any case, the oxidation of the *p*-aminophenol by the compound I form of peroxidase proceeds at a rate that is near the diffusion-controlled limit (71). Aminophenol is also a substrate for the one-electron oxidase, ceruloplasmin (170). The nephrotoxicity of *p*-aminophenol is thought to be due to the formation of either the semiquinoneimine or the quinoneimine. This oxidation occurs both nonenzymatically and by P-450-independent enzymatic pathways (17).

A serotonin-derived radical was observed by Borg in 1964 with electron spin resonance (ESR), but the ESR spectrum was not analyzed. The free radical was generated by permanganate oxidation. The biphasic decay kinetics of this spectrum suggested the presence of two free radical species (13). The importance of serotonin as a neurotransmitter prompted us to reinvestigate the ESR spectrum of its free radical (fig. 1). In addition, we have studied the ESR spectra of 5-hydroxytryptophan and 5-hydroxyindole with deuterium isotope substitution (120). The 6-hydroxytryptophan free radical has only a weak unresolved ESR spectrum. The weak signal is consistent with the fact that this radical cannot be resonance-stabilized by delocalization of the electron from the indole nitrogen to the 5-hydroxyl oxygen. Tryptophan and tryptamine, which contain the indole ring but not the 5-hydroxyl substituent, and melatonin and 5-methoxyserotonin, which have methoxy substituents, gave no detectable ESR spectra in alkaline solution, which indicated the importance of the 5-hydroxyl group to semiquinoneimine radical generation (table 1). In fact, these free radicals could be represented as neutral oxygen-centered phenoxy free radicals. The designation semiquinoneimine represents the resonance hybrid of the phenoxy radical with the resonance structure shown in figure 1, which is a nitrogen-centered zwitterion.

The *g*-values of the various 5-hydroxyindole radicals

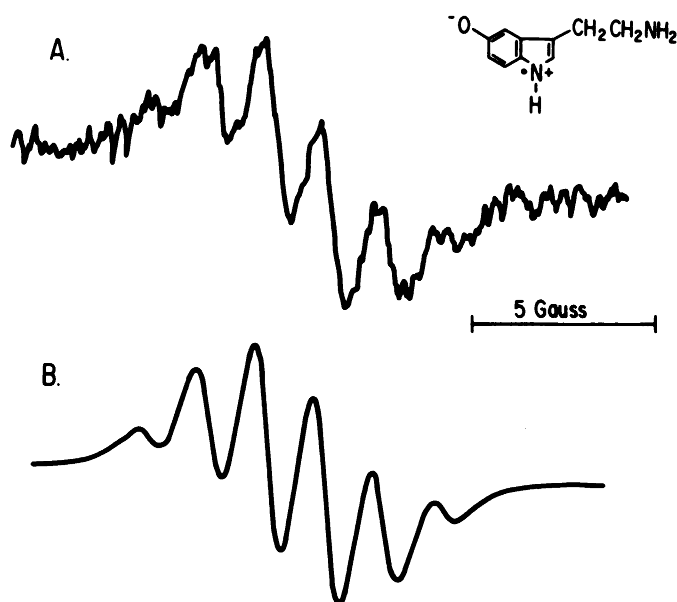


FIG. 1. A. The ESR spectrum of the serotonin semiquinoneimine radical. The radical was generated from 100 mM serotonin in 1 N NaOH. The spectrum was taken with a nominal microwave power of 10 mW, a calibrated modulation amplitude of 0.67 G, a scan time of 8 min and a time constant of 1 sec [From Perez-Reyes and Mason (120).]. B. A computer simulation of the spectrum shown in "A". The hyperfine splitting constants were:

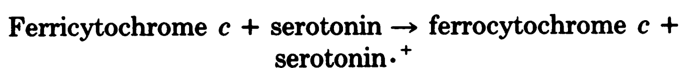
$$a^N = 1.6\ G,\ a^H_{NH} = 1.6\ G,\ a^H = 1.6\ G,\ \text{and}\ a^H = 1.4\ G.$$

These splittings are only approximate values, because the experimental spectrum had such poor resolution. The Lorentzian peak-to-peak line width was 0.9 G.

were measured (120). The similarity in *g*-values between these various 5-hydroxyindole free radicals suggests that they are all of the same chemical class. The *g*-values are consistent with their classification as semiquinoneimine free radicals, because these *g*-values are between those of the benzosemiquinone and the tetramethylphenylenediamine cation radical or Wurster salt (120). The semiquinoneimine can be thought of as a chemical hybrid of a semiquinone with a Wurster salt.

Secondary radicals are eventually formed because, with time, the ESR spectra of alkaline solutions of the 5-hydroxyindoles become asymmetric and more poorly resolved. Eventually, unresolved one-line spectra representative of polymer formation appear. These spectra may be related to the melanin-like spectra recently observed upon the incubation of serotonin and other 5-hydroxyindoles with microsomes and NADPH (160).

Semiquinoneimine free radicals, like benzosemiquinone, are very unstable at neutral pH, and 1 N NaOH is required to detect them with ESR. Therefore, we have tried to relate our ESR results to biochemistry at pH 7.4. The reduction of ferricytochrome *c* by serotonin must be a one-electron transfer and was proposed in 1964 to give a free radical of serotonin (1).



$$\text{Ferricytochrome } c + \text{serotonin} \rightarrow \text{ferrocytochrome } c + \text{serotonin}\cdot^{+}$$

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As expected, ferricytochrome *c* is reduced by all the 5-hydroxyindoles tested including serotonin. 6-Hydroxytryptophan is more active than any of these 5-hydroxyindoles. Neither indoles without hydroxyl substituents (tryptophan and tryptamine) nor indoles with methoxy substituents (melatonin and 5-methoxyserotonin) reduced ferricytochrome *c* significantly (table 1).

The reduction of ferricytochrome *c* by indoles at pH 7.4 correlates with the relative strength of the indole-derived ESR signals seen upon autoxidation in 1.0 N NaOH (table 1). The only exception is 6-hydroxytryptophan, which apparently can form a free radical (as indicated by ferricytochrome *c* reduction) that is unstable (as indicated by ESR). As noted previously, the instability of this free radical is expected from the absence of resonance stabilization.

A very similar structure-activity relationship has also been reported in the indole-stimulation of microsomal NADPH oxidation and oxygen consumption (table 1) (159). These reactions are inhibited by superoxide dismutase, which disproportionates superoxide. Because of this superoxide dismutase effect, Uemura et al. postulated that superoxide oxidizes serotonin and related compounds to a free radical and that oxygen and NADPH are consumed in some manner during cycling between serotonin and its free radical. This results in an unusually rapid utilization of the NADPH and O_2 cofactors. This superoxide-dependent, P-450-independent metabolism leads ultimately to the irreversible binding of serotonin metabolites to rat liver microsomal protein (159).

The scheme shown in figure 2 has been proposed previously, at least in part, where ferricytochrome *c* (1), ceruloplasmin (164, 10), or superoxide (159, 160) can oxidize serotonin to its one-electron oxidation product. In basic solutions, where the free radical is more stable, oxygen itself can oxidize the hydroxyindoles to their respective free radicals (120). The observation of interpretable ESR spectra of serotonin and of other 5-hydroxyindoles and the structure-activity relationships further support the idea that these free radicals are formed by the above biochemical oxidizing agents. The oxidation of NADPH by the serotonin radical has been proposed, and similar free radicals, such as the Wurster salts (164, 163) and the phenothiazine free radicals (87), have also been reported to oxidize NADPH. Nevertheless, conclusive evidence for this reaction is lacking. However, the reaction of the pyridinium nucleotide free radical with oxygen to form superoxide is well established (165, 84). Ultimately, this scheme predicts superoxide formation in the presence of NADPH.

Since superoxide can itself initiate the reaction sequence, a chain reaction is created that will consume NADPH and oxygen. This chain reaction is presumably responsible for the unusually rapid stimulation of

TABLE 1
Cytochrome c reduction by 5-hydroxyindoles and related compounds

	μ moles of Cytochrome *c* Reduced	Strength of ESR Signal	NADPH Oxidation	O_2 Consumption
None	0		36.2	56.9
Serotonin	9.7 ± 0.2	++	242.0	277.5
5-Hydroxytryptophan	13.7 ± 0.2	+++	215.7	289.3
5-Hydroxyindole	20.3 ± 0.7	+++	235.6	291.4
6-Hydroxytryptamine	40.6 ± 0.4	+	278.3	321.1
Tryptophan	0		36.2	53.6
Tryptamine	0.7 ± 0.1		27.6	41.8
Melatonin	0		35.8	48.2
5-Methoxyserotonin	0			

All incubations contained 2 mM substrate and 1.5 mg/ml of cytochrome *c* in a KCl-Tris-$MgCl_2$ (150 mM, 50 mM, 5 mM, pH 7.4, 37°C) buffer. Measurements were made at 5 minutes. NADPH oxidation and O_2 consumption data are taken from Uemura et al. (159) and reported as nanomoles/8 min/mg of protein. [From Perez-Reyes and Mason (120).]

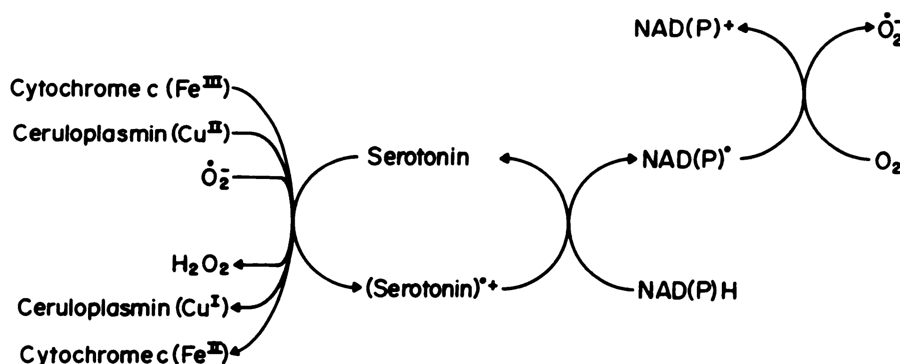

FIG. 2. Proposed mechanism for the stimulation of NAD(P)H oxidation and oxygen consumption by serotonin and other 5-hydroxyindoles. [From Perez-Reyes and Mason (120).]

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NADPH oxidation and oxygen consumption by 5-hydroxyindoles in microsomal incubations reported by Uemura et al. (table 1).

Ultimately, serotonin oxidation products form polymeric material that has melanin-like ESR signals in mitochondrial (162) and microsomal (160) incubations. The one-line ESR signal formed in microsomal incubations is abolished by the prior addition of superoxide dismutase. Free radical reactions of the serotonin semiquinoneimine free radical with itself, serotonin, and protein may be important in the formation of this melanin-like material. Whether serotonin-derived melanin actually forms in the brain is unknown, but these in vitro experiments clearly indicate that the one-electron oxidation of serotonin to a reactive free radical is possible under physiological conditions and not just in 1 N NaOH. The autoxidation of the neurotoxin 6-aminodopamine also leads to a species that can be described as a semiquinoneimine (119).

As in the case of benzosemiquinone formation, the semiquinoneimine free radical can also be generated by enzymatic reduction (90). The microsomal reduction of 2,6-dichloroindophenol is catalyzed by NADPH-cytochrome *c*(P-450) reductase (74, 94). In the past, this indophenol was considered to be a two-electron acceptor. Since this dye is reduced stepwise by ferrocyanide to form a free radical intermediate (34), the formation of this semiquinioneimine free radical in microsomal incubations containing NADPH is consistent with 2,6-dichloroindophenol being a one-electron acceptor (90). The metabolism of 2,6-dichloroindophenol to a free radical by *Escherichia coli* has been reported previously (65), but curiously no hyperfine structure was observed. While the structure of the free radical could not be determined, a semiquinoneimine structure seemed probable.

Since cytochrome P-450 is agreed to be a one-electron acceptor (124), it would appear that all of the known electron acceptors for NADPH-cytochrome *c*(P-450) reductase, including 2,6-dichloroindophenol, quinones (67), nitroaromatic compounds (91), and azoaromatic compounds (93) are one-electron acceptors. It then follows, unless a counterexample can be found, that NADPH-cytochrome *c*(P-450) reductase is an obligate one-electron donor.

An ESR spectrum of an unidentified free radical metabolite of the antitumor drug actinomycin D has been detected in microsomal incubations containing NADPH (8). Since actinomycin D contains a chromophore similar in structure to 2,6-dichloroindophenol, it has been proposed that this anticancer drug forms a semiquinoneimine metabolite upon enzymatic reduction (90). Semiquinoneimine free radicals are characterized by ESR spectra exhibiting significant hyperfine interaction with the nitrogen nucleus of what had been, in this case, the imine group (119, 120). Such nitrogens generally have attached protons that can easily be exchanged for deuterium by substitution of D_2O for H_2O in the buffer. The spectrum observed from a microsomal incubation of actinomycin D and NADPH in D_2O buffer consists of a five-line pattern with an intensity ratio of 1:2:3:2:1 (fig. 3). Computer simulation indicates that two nitrogen nuclei with hyperfine splitting constants of approximately 2.8 G are responsible for this hyperfine pattern. The ESR spectrum seen in H_2O indicates the presence of three protons with hyperfine splittings of 2.8 G in addition to the two nitrogens (fig. 3). If these three protons are attached to nitrogen as expected, then this would imply that the imine nitrogen has become protonated upon reduction, and that the 2-amino group has a nitrogen and two proton hyperfine interactions that are approximately equal to those observed for the half-reduced imine group. These exchangeable protons have been replaced by deuteriums in figure 3, as would occur in the D_2O buffer. The other nuclei of actinomycin D must have hyperfine interactions less than the line width, which is rather broad. The actinomycin D semiquinone structure is shown accordingly in the figure as a zwitterion. Microsomal oxygen consumption and superoxide formation are stimulated by actinomycin D (8, 149) and its spin-labeled derivatives (149), implying that actinomycin D semiquinone reduces molecular oxygen to superoxide, as do most semiquinones. It should be noted that the K_m for the stimulation of oxygen uptake by actinomycin D is 450 μg/ml (8), whereas the inhibition of RNA transcription occurs at a concentration of only 0.04 μg/ml (77, and references therein). It would appear that the free radical metabolism of actinomycin D is not responsible for its inhibition of RNA transcription or its other low dose biological effects.

B. *Thiyl Free Radicals*

Sulfhydryl groups are important in many natural biological processes, and are also thought to be responsible for the biological activities of sulfhydryl-containing compounds such as penicillamine and dithiothreitol. Unfortunately, even in chemical systems the investigation of thiyl free radicals ($RS^\cdot$) by ESR has been difficult. The direct detection of $RS^\cdot$ in solution is thought to be difficult, if not impossible (154). Thiyl spin adducts do not seem to be stable in chemical systems (68, 49), although a report of the trapping of thiyl radicals, formed by the Ce^{+4} oxidation of sulfhydryl-containing biochemicals, has appeared (51). In addition to Ce^{+4} oxidation (49), other transition metals can oxidize sulfhydryl compounds to form thiyl free radicals (100).

$$RSH + Me^{n} \rightarrow RS^\cdot + Me^{n-1} + H^+$$

For example, the reaction of Cu^{+2} with penicillamine to form Cu^{+1} implies that $RS^\cdot$ is formed (117, 169). The production of methemoglobin from oxyhemoglobin by thiols (151) is presumably analogous to the reaction of phenols, phenylhydrazine, or other oxidizable drugs with oxyhemoglobin (90).

$$HbO_2 + RSH \rightarrow metHb + RS^\cdot + H_2O_2$$

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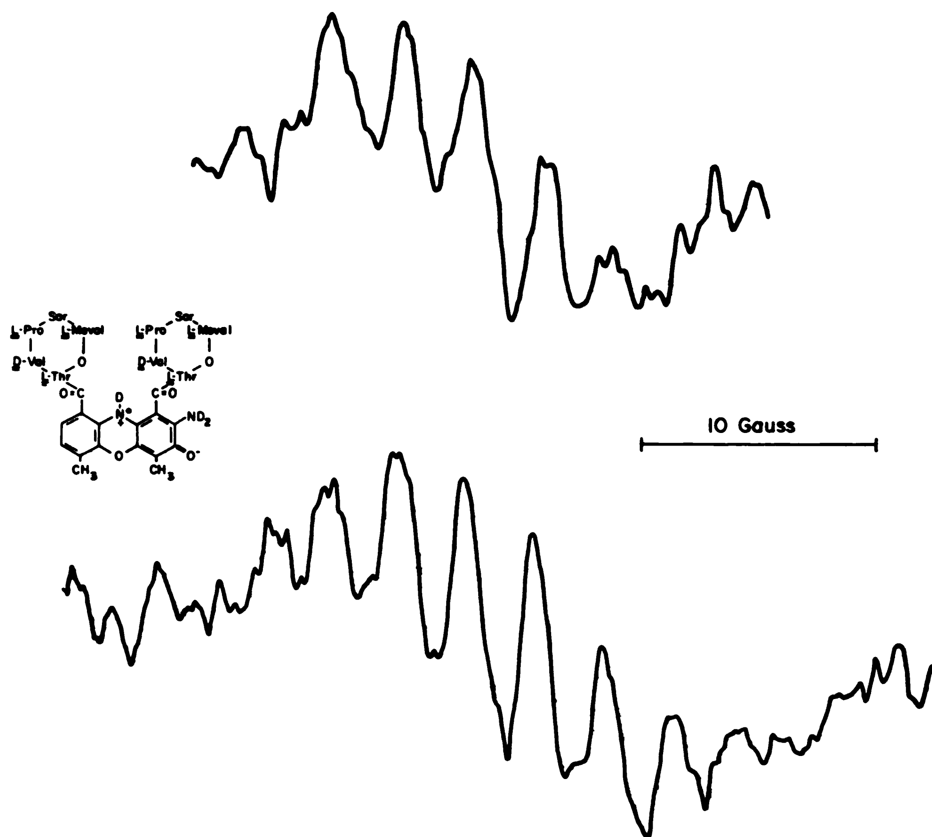
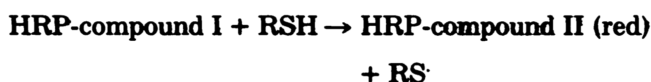


FIG. 3. The ESR spectrum of the actinomycin D free radical (upper) observed on incubation of 0.35 mM actinomycin D with 1 mg/ml of hepatic microsomes from male rats in KCl-Tris-$MgCl_2$-D_2O buffer (150 mM, 20 mM, 5 mM, and pD 7.4) containing 80 μM NADPH, 5.5 mM glucose-6-phosphate, and 2 units/ml of glucose-6-phosphate dehydrogenase. The lower spectrum is from an identical incubation where H_2O buffer was used instead of D_2O buffer.

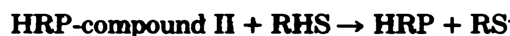
The chlorpromazine (110) and promazine cation radicals (88) are reduced by glutathione and penicillamine, respectively. Again, a one-electron transfer requires thiyl free radical formation if the parent phenothiazine is regenerated. Superoxide is also reduced by some thiol compounds (5) including cysteine (11). The sulfhydryl groups of papain (86) and glyceraldehyde-3-phosphate dehydrogenase (4) are prone to oxidation by superoxide due to activation of the sulfhydryl groups by the protein environment.

Evidence that thiyl free radicals can be generated enzymatically is, at present, indirect. Horseradish peroxidase/H_2O_2-catalyzed reactions are often, but not always, characterized by one-electron oxidation of the electron-donor substrate (166). Therefore, the oxidation of thiol compounds by this enzyme is expected to form thiyl free radicals.

$$\text{HRP} + H_2O_2 \rightarrow \text{HRP-compound I (green)} + H_2O$$



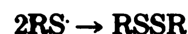
$$\text{HRP-compound I} + \text{RSH} \rightarrow \text{HRP-compound II (red)} + \text{RS}^\cdot$$



$$\text{HRP-compound II} + \text{RHS} \rightarrow \text{HRP} + \text{RS}^\cdot$$

The formation of compound II, the second peroxidase intermediate, during the oxidation of thiol compounds is a good indication that the thiyl free radical is being generated (111, 147). These reactions even occur with H_2S (107).

Dimerization of the resultant thiyl free radical

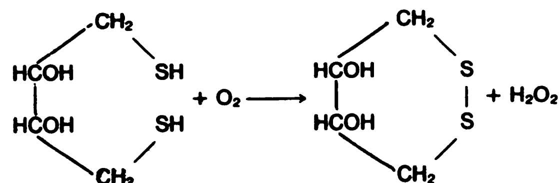


$$2\text{RS}^\cdot \rightarrow \text{RSSR}$$

is apparently responsible for the oxidation of the thiyl-containing nucleoside, 4-thiouridine, to bis(4,4'-dithiouridine) in the peroxidase/H_2O_2 system (147). The thiyl free radicals can also react with oxygen (144), to form

$$\text{RS}^\cdot + O_2 \rightarrow \text{RSO}_2^\cdot \rightarrow\rightarrow \text{RSO}_3\text{H}$$

sulfonic acids and other oxygen-containing oxidation products (86, 144). The peroxidase/O_2 oxidation of dithiothreitol produces oxidized dithiothreitol and hydrogen peroxide in an oxygen-consuming reaction (111).



These reaction products are not consistent with either dimerization of RS· or oxygen addition to RS·; therefore other reactions of the thiyl free radical must be involved.

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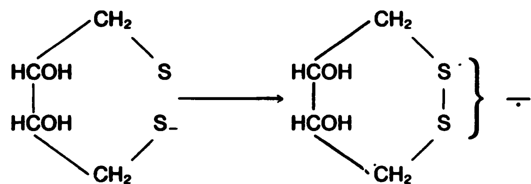
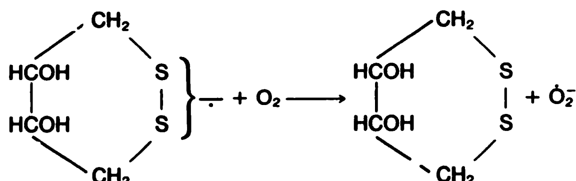
During chemical studies of the oxidation of dithiols, cyclic disulphide radical anions, including the lipoate disulphide anion, were detected at neutral pH with ESR and proposed to form from the thiyl free radical (49). By analogy the thiyl free radical of dithiothreitol would be expected to react as follows.

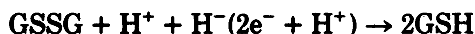


The electron transfer reaction of $RSSR^{\bar{\cdot}}$ with oxygen to form RSSR and superoxide is known and would account for the formation of the cyclic disulfide (144 and references therein),



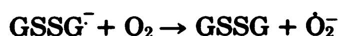
Furthermore, the disproportionation of the resultant superoxide would form the reported hydrogen peroxide. In fact, the generation of superoxide during the autoxidation of thiols has been reported (100). Since cyclic disulphide radical anions can be observed directly with ESR (49), their detection would provide good evidence for thiyl free radical formation in the peroxidase/H_2O_2 systems.

The reduction of oxidized glutathione by glutathione reductase is, to the authors' knowledge, oxygen-insensitive. This fact is good evidence that glutathione reductase catalyzes a two-electron reduction or a hydride reduction,



$$GSSG + H^+ + H^-(2e^- + H^+) \rightarrow 2GSH$$

because otherwise the reduction of oxygen by the radical intermediate would lead to oxygen inhibition of glutathione reduction through a futile cycle



$$GSSG^{\bar{\cdot}} + O_2 \rightarrow GSSG + \dot{O}_2^-$$

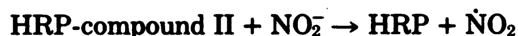
that would use NADPH, the glutathione cofactor, to reduce oxygen.

C. Nitrogen Oxide Free Radicals

In 1952 Chance reported that nitrite reacts with horseradish peroxidase-compound I by a one-electron transfer to form horseradish peroxidase-compound II (23). More recent work confirms that nitrite is oxidized by the general peroxidase mechanism (135),

$$HRP + H_2O_2 \rightarrow \text{HRP-compound I} + H_2O$$

$$\text{HRP-compound I} + NO_2^- \rightarrow \text{HRP-compound II} + \dot{N}O_2$$



$$\text{HRP-compound II} + NO_2^- \rightarrow HRP + \dot{N}O_2$$

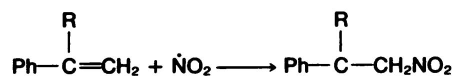
although the free radical $\dot{N}O_2$ has not been detected.

Even though the reaction of $\dot{N}O_2$ with water is rapid,

$$2\dot{N}O_2 + H_2O \rightarrow NO_3^- + NO_2^- + 2H^+$$

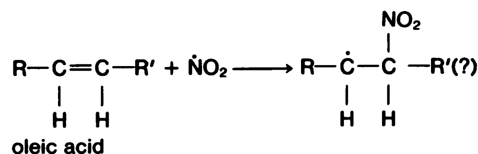
$\dot{N}O_2$ is thought to react with unsaturated lipids and thereby initiate lipid peroxidation (130, 98). Rat lung lipids are peroxidized by exposure of the animals to air containing as little as 1 ppm $\dot{N}O_2$ (156). Protection by dietary vitamin E suggests a free radical mechanism of toxicity (99).

Even in simple chemical systems, the reaction of $\dot{N}O_2$ with the double bond of olefins is very complex and invariably leads to nitroxide formation (153, 73). The first step in these reactions is thought to be the addition of $\dot{N}O_2$ across the carbon-carbon double bond. For example the reaction of $\dot{N}O_2$ with styrenes yields a β-nitroalkyl radical,

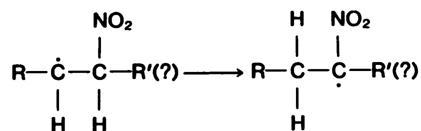


which has been spin-trapped with nitrosobenzene (73). Since carbon-centered free radicals are known to react with oxygen to form peroxy radicals, $\dot{N}O_2$ can initiate lipid peroxidation if it does, in fact, add across the carbon-carbon double bonds of unsaturated fatty acids in vivo.

Rowlands and coworkers (40, 140) have studied the reaction of $\dot{N}O_2$ with oleic acid in a continuous flow ESR system. The spectrum they observed during flow [see fig. 4A of Estefan et al. (40) or fig. 2 of Rowlands and Gause (140)] was attributed to a carbon-centered radical formed by the addition of $\dot{N}O_2$ across the double bond,



The ESR spectrum of this radical, simulated with the four hydrogen coupling constants reported by these workers, is shown in figure 4B. However, this spectrum is different from the observed spectrum, which can be better simulated with three hydrogen and one nitrogen coupling constants (fig. 4A), the magnitudes of which are typical of dialkylnitroxides. In these investigations, the species seen after stopping the flow of the reactants was attributed to a second type of carbon-centered free radical that was proposed to form as the result of a hydrogen atom transfer reaction.



This species, characterized by a nitrogen coupling constant of 7 Gauss, is an α-ketonitroxide (69),

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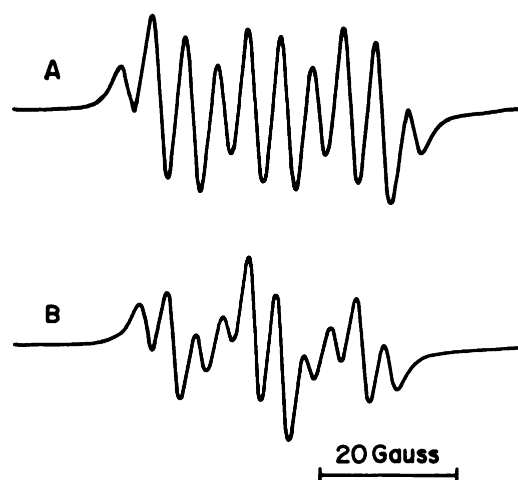


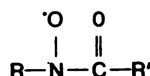
FIG. 4. A. Computer simulation of the radical produced under continuous flow conditions in the reaction of 20% (vol) oleic acid in dioxane with NO_2 in dimethoxyethane (40, 140). The hyperfine coupling constants were:

$$a^N = 14.0\text{ G} \quad \text{and} \quad a^H = a^H = a^H = 4.7\text{ G.}$$

The Lorentzian peak-to-peak line width was 2.3 G. B. Computer simulation with the hyperfine coupling constants reported in reference (40):

$$a^H = 16\text{ G}, a^H = 12\text{ G, and } a^H = a^H = 4\text{ G.}$$

The Lorentzian peak-to-peak line width was 2.3 G. This simulation is a poor representation of the experimental spectrum, which is very similar to the simulated spectrum in "A."



and not a second type of carbon-centered free radical as proposed. Aldehydes such as malonaldehyde are well known to form during lipid peroxidation, so the formation of such a species is not surprising. Recent work demonstrates that abstraction of allylic hydrogen by nitrogen dioxide predominates over addition to double bonds at nitrogen dioxide concentrations below 100 ppm (130).

In radiation chemistry, nitrous oxide is well known to be reduced by aqueous electrons.

$$N_2O + e^- \rightarrow HO\cdot + OH^- + N_2$$

On the basis of the enzymatic reduction of N_2O to N_2 by intestinal microflora, the above reaction has been proposed to occur during nitrous oxide anesthesia (62).

D. Sulfur Oxide Free Radicals

Near pH 7, SO_2 exists primarily as sulfite ($SO_3^{=}$) and bisulfite (HSO_3^-) and, to a minor extent, as hydrated SO_2 (122). The nomenclature (bi)sulfite is used when it is not known which species is involved in a reaction. Sulfite oxidase, a mitochondrial enzyme, is thought to be primarily responsible for the oxidation of (bi)sulfite to sulfate in vivo (131). If it is assumed that the fourth oxygen atom of sulfate is derived from water, this oxidation can be written either as a two-electron oxidation followed by hydrolysis,

$$SO_3^{=} \rightarrow SO_3 + 2e^-$$

$$SO_3 + H_2O \rightarrow SO_4^{=} + 2H^+$$

or as a one-electron oxidation followed by disproportionation.

$$SO_3^{=} \rightarrow \dot{S}O_3^- + e^-$$

$$2\dot{S}O_3^- + H_2O \rightarrow SO_4^{=} + SO_3^{=} + 2H^+$$

Sulfite oxidase is considered to be a two-electron acceptor of electrons from (bi)sulfite (28), but horseradish peroxidase, in the presence of hydrogen peroxide, forms compound I, which is reduced by (bi)sulfite in both one-electron and two-electron transfers (3). Although a peroxidase-(bi)sulfite intermediate is formed by reaction of peroxidase compound II with (bi)sulfite (3), reactions of the peroxidase intermediates, compound I and compound II, with (bi)sulfite should ultimately form $\dot{S}O_3^-$ in a catalytic cycle. In ESR investigations of this system the enzymatic formation of $\dot{S}O_3^-$ has been established and its identity determined from its *g*-value, which is unique for sulfur-oxygen radicals (102). The radical reacts with oxygen as indicated by the absence of an ESR spectrum under aerobic conditions. The ESR spectrum does not show evidence of a hyperfine interaction with a hydrogen nucleus, which indicates that the free radical is unprotonated. In the earlier literature, the (bi)sulfite-derived free radical was written both as $\dot{S}O_3^-$ and its acid conjugate, $H\dot{S}O_3$. The ESR spectrum indicates that the free radical exists primarily as the anion at pH 7.4 and above, which is in conformity with the chemical literature (112). At high concentrations, hydrogen peroxide alone oxidizes (bi)sulfite to the $\dot{S}O_3^-$ radical (43), but with micromolar concentrations of H_2O_2 the radical concentration is much higher in the presence of horseradish peroxidase (102).

Autoxidation of (bi)sulfite is also thought to form $\dot{S}O_3^-$, but direct ESR evidence is lacking. The $\dot{S}O_3^-$ radical formed in the autoxidation of (bi)sulfite has been proposed to be the causal agent responsible for the cleavage of DNA (59), the inactivation of bacterophage λ by impairment of its protein coat (82), and the oxidation of NADPH (158), tryptophan (168), methionine (167), and β-carotene (118).

The autoxidation of (bi)sulfite is quite rapid in systems that produce superoxide. For instance, either xanthine oxidase and xanthine [which generate superoxide directly (64)] or microsomes and NADPH [which generate superoxide by the air-oxidation of free radical metabolites of anticancer quinones (55), and triphenyltetrazolium (143)] will rapidly oxidize (bi)sulfite. In fact, the inhibition of (bi)sulfite autoxidation by superoxide dismutase was one of the earliest superoxide dismutase assays (96).

The $\dot{S}O_3^-$ radical (or $H\dot{S}O_3$) will add across the carbon-carbon double bonds of alkenes (43, 141), and (bi)sulfite

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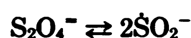
autoxidation will promote the peroxidation of corn oil (75) or rat liver homogenate (64). Presumably the carbon-centered lipid radical formed by the addition of the $\dot{S}O_3^-$ radical will react with oxygen to form a peroxy free radical that will initiate the lipid peroxidation chain reaction.

If the one-electron oxidation of (bi)sulfite forms a free radical, one might ask what the one-electron reduction of (bi)sulfite forms.

$$SO_3^= + 2H^+ + e^- \rightarrow \dot{S}O_2^- + H_2O$$

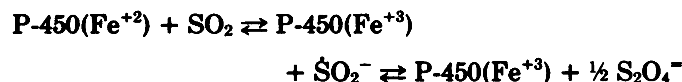
The sulfur dixoide radical anion ($\dot{S}O_2^-$) has been reported to form nonenzymatically in the presence of NADPH, NADH, or ascorbate, but we have not been able to duplicate these experiments (48). This radical is better known as the one-electron reducing species of dithionite,



$$S_2O_4^= \rightleftarrows 2\dot{S}O_2^-$$

which reduces free flavins, flavodoxin, methemoglobin (95), and cytochrome P-450 (61).

Conversely, (bi)sulfite is reduced to dithionite by reduced flavodoxins or a mixture of paraquat, H_2, and hydrogenase (95). Anaerobic microsomal incubations containing (bi)sulfite and NADPH form $\dot{S}O_2^-$, which has a different g-value than $\dot{S}O_3^-$ (103). If the N_2 atmosphere is replaced by CO, this ESR signal decreases by over 90%, implying that the well-known reduction of cytochrome P-450 by dithionite is reversible.



$$P\text{-}450(Fe^{+2}) + SO_2 \rightleftarrows P\text{-}450(Fe^{+3}) + \dot{S}O_2^- \rightleftarrows P\text{-}450(Fe^{+3}) + \tfrac{1}{2} S_2O_4^=$$

E. Peroxy Free Radicals

Peroxy free radicals ($RO_2\cdot$) have long been proposed as intermediates in both enzymatic lipid peroxidation (16) and prostaglandin biosynthesis (127), but only rarely have peroxy free radicals been observed with ESR even in biologically related systems. Protein peroxy radicals, formed by irradiation and subsequent exposure to oxygen, have been observed directly with ESR (35). The cumene peroxy radical, formed by the oxidation of cumene hydroperoxide by hematin, has been detected indirectly by the use of spin trapping (136). An adaptation of the usual free radical mechanistic scheme for peroxidases and hydrogen peroxide can be modified for catalase and hydroperoxides.

Catalase + ROOH → Catalase-compound I + ROH

Catalase-compound I + ROOH

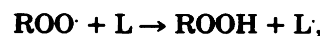
→ Catalase-compound II + ROO·

Catalase-compound II + ROOH

→ Catalase + ROO·

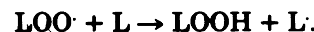
This would explain the one-electron reduction of the compound I form of catalase by methyl hydroperoxide (22). In contrast to biological systems, the peroxy free radicals have been detected in many purely chemical systems (63).

Once formed, peroxy free radicals clearly can initiate lipid peroxidation



$$ROO\cdot + L \rightarrow ROOH + L\cdot,$$

because this reaction is nothing more than a variation of the propagation reactions of lipid peroxidation,



$$LOO\cdot + L \rightarrow LOOH + L\cdot.$$

The participation of peroxy free radicals in the initiation reaction could be important in the promotion of lipid peroxidation by lipid hydroperoxides (157).

The self-reaction of peroxy radicals in chemical systems follows two routes depending on the structure of the peroxy radical (63). Tertiary peroxy radicals, such as the t-butylperoxy radical, react to release ground state triplet oxygen via tetroxide formation.

$$2\ t\text{-BuOO}\cdot \rightleftarrows t\text{-BuO}_4\text{-}t\text{-Bu} \rightarrow 2t\text{-BuO}\cdot + {}^3O_2$$

Secondary peroxy radicals, such as lipid hydroperoxy radicals, can self-react to give nonradical products (58).

$$2L(H)OO\cdot \rightleftarrows L(H)O_4L(H) \rightarrow L(H)OH + LO + {}^1O_2$$

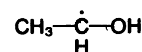
The Wigner spin-conservation rule requires that the oxygen so evolved be in an excited singlet state, if both the lipid alcohol and ketone are diamagnetic (63). This reaction is proposed to account for the formation of singlet oxygen during the oxidation of linoleic acid hydroperoxide by methemoglobin or hematin (58).

F. Ethanol Free Radicals

The extensive evidence of ethanol-induced lipid peroxidation in vivo has been reviewed elsewhere (134, 150). Although a free radical metabolite of ethanol is not thought to be responsible for the induced lipid peroxidation (134), reports that free radical scavengers attenuate the ethanol-induced increase in liver triglycerides have led to the suggestion that the ethoxy radical metabolite may be formed in the course of the oxidation of ethanol to acetaldehyde (150).

$$C_2H_5OH \xrightarrow{-H\cdot} C_2H_5O\cdot \xrightarrow{-H\cdot} C_2H_4O$$

The NADPH/liver microsome/Fe^{+2}/EDTA system forms the hydroxyl radical adduct with 5,5-dimethylpyrroline-N-oxide (DMPO) (83). When ethanol was added, a different radical adduct was detected and assigned as the DMPO-ethoxy radical adduct. This adduct was subsequently identified as the hydroxyethyl radical adduct (68).



$$CH_3\text{—}\dot{C}(H)\text{—}OH$$

Yet another possibility is suggested by the attempt to trap the cumyloxy radical in a chemical system with

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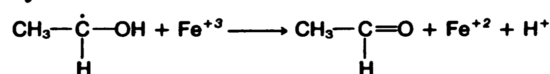
nitrosobenzene. Although the cumyloxy radical was believed to be formed and trapped, decomposition of the adduct led to the formation of nitrosobenzene cumyl adduct (155).

This decomposition may also be occurring in the metmyoglobin/cumene hydroperoxide and microsomal/cumene hydroperoxide systems, where the formation of the nitrosobenzene-cumyloxy (cumyl) radical adduct has been reported (53). The original author has since indicated that the cumyl radical is, in fact, the species trapped (6). The important point is that a similar decomposition of the DMPO-ethoxy adduct would form the DMPO-ethyl adduct, which would be difficult to distinguish from the DMPO-hydroxyethyl adduct. Therefore, yet another possibility must be considered.

The results of a series of investigations with free radical scavengers are also consistent with the formation of ethanol free radical metabolites. The microsomal oxidation of ethanol is specifically inhibited by DMPO (18). Several hydroxyl radical scavenging agents competitively inhibit acetaldehyde production in the NADPH/microsome/azide system (27). Hydrogen peroxide promotes this catalase-independent ethanol oxidation (19). Inhibition of acetaldehyde formation by hydroxy radical scavengers is also observed in reconstituted microsomal systems containing cytochrome P-450 and NADPH-cytochrome *c* reductase (109, 20). The xanthine/xanthine oxidase system also oxidizes ethanol to acetaldehyde, but this oxidation is inhibited by superoxide dismutase (109), which may imply a superoxide-dependent hydroxyl radical formation.

If the hydroxyethyl radical is in fact formed, it is a strong reducing agent and would be oxidized by ferrihemoproteins (161) or many other species to form acetaldehyde.



G. *Triarylmethane Dye Carbon-Centered Free Radicals*

Gentian violet, benzyl violet 4B, light green SF, and related triarylmethane dyes are used as biological stains, to dye wool and silk, as food dyes, and in cosmetics. Gentian violet is also used extensively in human and veterinary medicine. It is given internally to children and adults for pinworm, and is applied topically for fungus infections such as thrush. Several members of this class of compounds have been shown to be carcinogens and gentian violet causes DNA damage (7).

When gentian violet or a related compound is metabolized under nitrogen by rat hepatic microsomes supplemented with NADPH (57), an unresolved single line electron spin resonance spectrum is obtained (fig. 5). This ESR spectrum is that of the tri-N,N-dimethyl-anisyl methyl radical, which is the one-electron reduction product of gentian violet (85). The formation of this carbon-centered free radical is completely inhibited by oxygen and partially inhibited by carbon monoxide (57). Carbon monoxide inhibition indicates that this free radical forms (at least in part) by a one-electron transfer from reduced cytochrome P-450 (fig. 6).

The known chemistry of the triphenylmethyl radical suggests that the reactions of this species may be of toxicological significance. For instance, the triphenylmethyl carbon-centered free radical reacts with oxygen

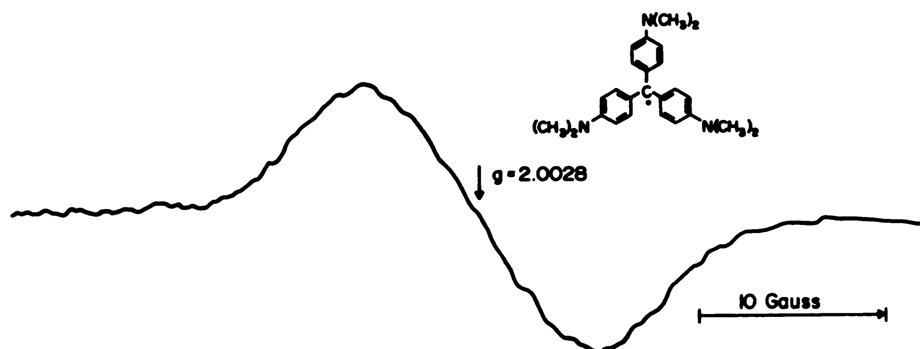


FIG. 5. The ESR spectrum of the tri-N,N-dimethyl-anisyl methyl radical observed on incubation of 1 mM gentian violet with 2 mg/ml of hepatic microsomes from male rats in KCl-Tris-$MgCl_2$ buffer (150 mM, 20 mM, and 5 mM, pH 7.4) containing 0.8 mM $NADP^+$, 11 mM glucose-6-phosphate, and 1.4 units/ml of glucose-6-phosphate dehydrogenase.

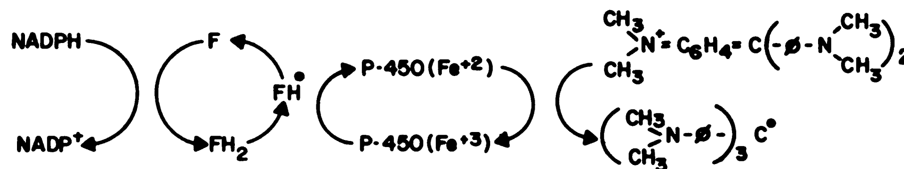


FIG. 6. The proposed mechanism of carbon-centered free radical formation by the hepatic microsomal reduction of gentian violet. The exact scheme of cytochrome P-450 reduction by NADPH-cytochrome P-450 reductase is unknown.

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to form a much more reactive peroxyl free radical (97), which should initiate lipid peroxidation.

The formation of a free radical metabolite of erythrosine B by the action of horseradish peroxidase and H_2O_2 has been proposed (44). Such a species must be a one-electron oxidation product of erythrosine B, possibly a *para*-substituted phenoxy radical (89) and, as such, is clearly a species distinct from the carbon-centered radicals of triarylmethane dyes.

III. Free Radical Pathways in the Metabolism of Xenobiotics

A. One-Electron Enzymatic Reduction of Xenobiotics

The free radical metabolites described in the previous section conclude an attempt to delineate the metabolic pathways by which a given class of free radicals may be formed. In earlier reviews, 14 different classes of free radical metabolites were described (89, 90), and that material has not been repeated here. Since a large number of enzymes are also involved in free radical xenobiotic metabolism, an overall view of this field is not obvious. In this section we will attempt to make some generalizations.

Yamazaki has classified some of these reactions as pure one-electron transfers, and others as combinations of one- and two-electron transfers that form both free radical and diamagnetic products (166). He has also provided an extensive discussion of the criteria necessary to prove the formation of a free radical metabolite. By definition, free radical metabolites must exist free of the enzyme, and, therefore, enzyme-xenobiotic transition states with free radical character are excluded. It follows that, if the free radical can be made quickly enough, it can be detected with electron spin resonance, and will have the same high resolution hyperfine pattern as the free radical made by purely chemical means.

If the discussion is limited to free radical metabolites of xenobiotics, two general features are clear. First, the xenobiotic must undergo either one-electron reduction or one-electron oxidation, if a free radical is to be formed. Two-electron transfers, such as hydride transfer between a substrate and an enzyme, obviously cannot create free radical metabolites. Most NAD(P)H dehydrogenases participate in apparent hydride transfer from NAD(P)H and to $NAD(P)^+$, but even here exceptions do exist (21). Second, the cofactor, which is the ultimate electron donor (or acceptor) in these enzymatic redox reactions, transfers two electrons to (or from) the enzyme in all known cases. In general, these cofactors are the physiological substrates for the enzymes, and since free radical metabolites are rare in the course of normal biochemistry, electron transfers involving cofactors do not generally lead to free radical intermediates. The major exception to this generalization is oxygen, which is known to be reduced to superoxide by many enzymes.

In an effort to gain an overall perspective we have constructed two matrices of free radical xenobiotic metabolism. Table 2 has been constructed by placing on the rows of the matrix all enzymes known to transfer one electron to a xenobiotic. Each column is a xenobiotic known to be enzymatically reduced to a free radical metabolite. For the purpose of this table, oxygen is considered to be a xenobiotic; however, several enzymes that have been reported to reduce oxygen to superoxide, but that have not, as yet, been reported to reduce xenobiotics to free radicals, have not been included in table 2.

This table of metabolites formed by one-electron reduction has several notable features. The most obvious is that there are many possibilities for enzymatic free radical metabolism that have not been investigated. Even with the limited amount of attention that free radical xenobiotic metabolism has received, it is clear that a wide variety of aromatic organic compounds are enzymatically reduced to form free radicals, which generally contain one more electron than their parent compounds. Some xenobiotics, including a wide variety of quinones and nitro compounds, will accept electrons from almost any redox flavoenzyme. The electron donor appears to be the flavin cofactor. In most cases these flavoenzymes normally reduce cytochromes, which have reduction potentials comparable to those of nitro and quinone compounds. Therefore, this free radical metabolism is a subversion of normal electron transport, where the xenobiotic competes with the endogenous cytochrome for reducing equivalents.

The microsomal reduction of nitroaromatic compounds, quinones, quinone-imines, some azoaromatic compounds, paraquat, or tetrazolium salts is catalyzed by NADPH-cytochrome *c* (P-450) reductase. One-electron transfer to these electron acceptors has been proved to be obligatory in the case of quinone and nitro compounds, and is probably obligatory in the other cases as well. Therefore, a reduction of an aromatic compound by NADPH-cytochrome *c* (P-450) reductase can probably be assumed to form a free radical metabolite, although a true two-electron acceptor for this enzyme is a theoretical possibility.

It is tempting to assume that each blank space in table 2 represents an undiscovered enzymatic free radical reaction. For instance, since xanthine oxidase can catalyze the reduction of oxygen, nitro, and quinone compounds to free radicals, one could think it safe to assume that the reduction of N-oxides by xanthine oxidase also occurs *via* a free radical intermediate (90). In fact, the oxygen of ^{18}O-labeled nicotinamide N-oxide is transferred to xanthine to form ^{18}O-labeled uric acid during the reduction of the N-oxide (104). This finding clearly indicates a two-electron transfer. Although nicotinamide N-oxide, like oxygen, also serves as a one-electron acceptor, this reaction is a minor pathway. This lack of predictive power is probably due to the fact that xanthine oxidase has two sites where reduction can occur, one containing molybdenum and the other containing flavin. Nevertheless,

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TABLE 2
Enzymatic free radical reduction of xenobiotics

	O_2	Quinones	Quinone-imines	Nitroaromatics	Azoaromatics	Carbon Tetrachloride	Bipyridyliums	Tetrazoliums	di-N-Oxides	Sulfur Oxides	Triphenylmethane Dyes
NADPH-cytochrome *c*(P-450) reductase (NADPH)	B† (7a, 53c)	A* (89, 90, 166)	B†,‡ (90)	A* (89, 90)	B† (89, 90)		B† (89, 90)	B† (89, 90)			
NADH-cytochrome b_5 reductase (NADH)		A* (89, 90, 166)		C§ (89, 90)							
Cytochrome b_5		D# (89, 90, 166)									
Cytochrome P-450	A* (160a)					C§ (89, 90)	B† (89, 90)			B†, ‖	B†,¶
NADH-dehydrogenase (NADH)		A* (89, 90, 166)		C§ (89, 90)							
Escherichia coli reductase (NADPH)	D† (90)	D# (58a)		B† (90)			D# (58a)		B† (90)		
Xanthine dehydrogenase (xanthine or NADH)		A* (166)									
Xanthine oxidase (xanthine or NADH)	A* (166)	A* (89, 90, 166)		D# (90)							
Lipoamide dehydrogenase (NADH)		A* (89, 90, 166)									
Aldehyde oxidase (aldehyde or NADH)	A* (166)			D# (90)							
Ferredoxin-NADP⁺ reductase (NADPH)		A* (89, 90, 166)									

* A. In these cases free radical formation has been quantified and compared to the total rate of metabolism.

† B. The enzymatic free radical formation has been demonstrated by electron spin resonance or through the use of visible spectroscopy, but the stoichiometry of radical formation to diamagnetic product formation has not been determined.

‡ See section II A of this review.

§ C. Enzymatic free radical formation has been demonstrated, but the enzyme involved is not conclusively proved.

D. Evidence for free radical formation is based only on oxygen inhibition of the reduction and/or stimulation of enzymatic superoxide formation or oxygen consumption by the xenobiotic.

‖ See section II D of this review.

¶ See section II G of this review.

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TABLE 3

Enzymatic free radical oxidation of xenobiotics

	Hydroquinones	Hydroamino-quinones	Aromatic Amines	Phenothiazines	Hydroxyaromatics	Hydroxyl-amines	Sulfhydryls	Hydrazines	Sulfur Oxide
Horseradish (or turnip) peroxidase (H_2O_2)	A* (89, 90, 166)	A* † (90)	B‡ (89, 90)	B‡ (89, 90)	A* (89, 90, 166)	A* (89, 90)	C§, ‖	B‡ (73a)	A*, ¶ (166)
Catalase (H_2O_2 or ROOH)			C§ (143a)	C§ (89, 90)					
Lactoperoxidase (H_2O_2)						B‡ (89, 90)			
Myeloperoxidase (H_2O_2)						B‡ (89, 90)			
Prostaglandin synthetase (ROOH or H_2O_2)			A* (84a)					B‡ (73a)	
Metmyoglobin (H_2O_2 or ROOH)			A* (53a,b)						
Methemoglobin (H_2O_2 or ROOH)					B‡ (89, 90)	B‡ (89, 90)			
Cytochrome P-450 (ROOH)			C§ (53a)						
Ceruloplasmin (O_2)	B‡ (89, 90)	C†, § (90)	B‡ (89, 90)	C§ (89, 90)					
Hemoglobin (O_2)					C§ (89, 90)	B‡ (89, 90)		B‡ (89, 90)	
Laccase (O_2)	A* (89, 90, 166)		B‡ (15a)						
Cytochrome oxidase (O_2)			C§ (89, 90)						

* A. In these cases free radical formation has been quantified and compared to the total rate of metabolism.

† See section II A of this review.

‡ B. The enzymatic free radical formation has been demonstrated by electron spin resonance, but the stoichiometry of radical formation to diamagnetic product formation has not been determined.

§ C. In most cases enzymatic free radical formation has been demonstrated by observing the optical spectrum of the radical, but in some cases only nonspecific evidence such as oxygen consumption is available.

‖ See section II B of this review.

¶ See section II D of this review.

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these matrices were constructed to indicate not only in what systems free radical metabolites have been found but in what systems they should be found.

B. One-Electron Enzymatic Oxidation of Xenobiotics

Horseradish peroxidase is by far the best studied enzyme that oxidizes xenobiotics to free radicals (table 3). Although only a few isolated cases of free radical formation by the mammalian peroxidases such as myeloperoxidase, lactoperoxidase, and prostaglandin synthetase/hydroperoxidase have been reported, it is fully expected that the horseradish peroxidase-catalyzed reactions are typical for this class of enzymes. Catalase is also a peroxidase that, in addition to the oxidation of hydrogen peroxide to oxygen, will oxidize some xenobiotics to free radicals (table 3). Even methemoglobin is a peroxidase. The lack of great interest in the action of peroxidases on drugs and other xenobiotics is difficult to justify, because hydrogen peroxide and hydroperoxides do exist in vivo. In fact, the peroxidase prostaglandin synthetase makes its own hydroperoxide, prostaglandin G_2.

Among the oxidases that form free radical metabolites, ceruloplasmin is the best studied (table 3). A note of caution is in order concerning the reports of oxyhemoglobin oxidizing xenobiotics to free radicals,

$$HbO_2(Fe^{+2}) + AH_2 \rightarrow metHb(Fe^{+3}) + \cdot AH + H_2O_2$$

because the reaction forms the methemoglobin-H_2O_2 system, which will produce the same xenobiotic free radical through a peroxidative activity. What is worse is that hydrogen peroxide (from the autoxidation of AH_2) and the ever present methemoglobin contamination of oxyhemoglobin could give the appearance of a reaction catalyzed by oxyhemoglobin, when it is catalyzed, in fact, by methemoglobin.

The number of enzymes reported to oxidize aromatic amines to reactive amino free radicals is most remarkable and may be related to metabolic activation of the carcinogenic aromatic amines.

IV. Prostaglandin Synthetase and Lipoxygenases: Examples of Enzymatically Controlled Stereospecific Lipid Peroxidation

The immediate precursor of the prostaglandins is the fatty acid arachidonic acid. Prostaglandin synthetase converts arachidonic acid to prostaglandin G_2, which is reduced to form prostaglandin H_2. All of the many other prostaglandins are formed from prostaglandin H_2 (fig. 7). The prostaglandin endoperoxides and related compounds have many important pharmacological properties, but these are outside the scope of this review. Lipoxygenase pathways are also important. They lead to 5-hydroperoxyarachidonic acid, a proposed intermediate in the biosynthesis of leukotrienes (15).

The prostaglandin synthetase-catalyzed oxidation of arachidonic acid and the lipoxygenase-catalyzed oxida-

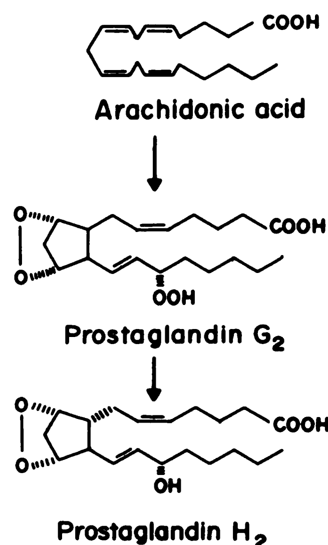

FIG. 7. Structures of the two prostaglandin endoperoxides.

tion of linoleic acid are specialized types of lipid peroxidation that have long been thought to form free radical intermediates (127, 33, 60 and references therein). Prostaglandin synthetase has two distinct activities, a fatty acid cyclo-oxygenase and a peroxidase. The cyclo-oxygenase bioxygenates fatty acids containing three or more double bonds to form cyclic endoperoxides containing a hydroperoxide. The bioxidation of arachidonic acid forms prostaglandin G_2. This endoperoxide hydroperoxide is reduced to an endoperoxide containing an alcohol, prostaglandin H_2 (fig. 7). Concomitant with this reduction a wide variety of substrates are oxidized. Some of these substrates have been shown to be oxidized to free radical intermediates (table 3). In time, all of the substrates known to be oxidized to free radicals by horseradish peroxidase (table 3) will probably be shown to be oxidized to free radicals by prostaglandin hydroperoxidase. The implications of such a statement are far-reaching, because, although many hemoproteins are known to have peroxidatic activity, only prostaglandin synthetase produces the necessary peroxide to drive the enzymatic reaction.

The fatty acid cyclo-oxygenase system itself can be thought of as a series of sequential reactions (127), the first of which appears to be very similar to the first step of the lipoxygenase reaction (fig. 8). Tritium-labeling studies have shown that the 13-L hydrogen of arachidonic acid was removed in the cyclo-oxygenation (108, 54), and that this isotopic substitution significantly decreased the rate of prostaglandin formation. Clearly the equivalency of the two methylene hydrogens is removed in the enzyme-arachidonic acid complex. Deuterium substitution at the 11-L hydrogen of linoleic acid decreases the rate of hydroperoxide formation by soybean lipoxygenase ninefold (37). These isotope effects indicate that the removal of the 13-L hydrogen is the rate-limiting step of prostaglandin fatty acid cyclo-oxygenase catalysis, and

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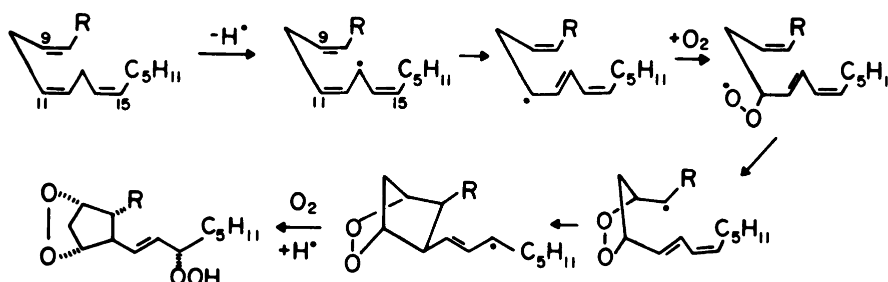


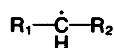
FIG. 8. Adapted from figure 2 in Porter (127) with permission, where R is $CH_2CH{=}CH(CH_2)_3COOH$. Note that the second cyclization must be very fast or this C-8 carbon-centered free radical must be inaccessible to oxygen, because oxygenated products of this kind are not observed.

that the removal of the 11-L hydrogen is the rate-limiting step of lipoxygenase catalysis. It is probable that hydrogen abstraction is also the first step in these enzymatic lipid peroxidations (127), as it is in the autoxidation of many organic chemicals (63), including even egg lecithin (phosphatidylcholine) (9). However, the idea that hydrogen abstraction is the first step in the mechanism of prostaglandin synthetase is not accepted universally (123).

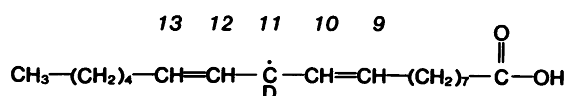
The earliest evidence that this hydrogen abstraction formed a fatty acid free radical arose from the observation that, if oxygen was limited, lipoxygenase formed fatty acid dimers of linoleic acid. Because the formation of dimers often occurs as the result of the self-termination of two free radicals, the formation of a linoleic acid free radical was proposed (47). In the first application of spin trapping to a biological problem, de Groot et al. showed that soybean lipoxygenase forms linoleic acid free radical, as indicated by its trapping with 2-methyl-2-nitrosopropanol (33). The spectrum consisted of a triplet of doublets

$$(a^N = 16.0, \quad a^H_\beta = 2.0 \text{ G}),$$

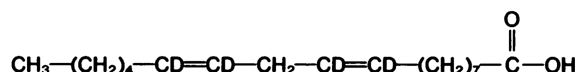
indicating that a carbon-centered free radical had been trapped. Before trapping, the linoleic acid radical must have had the structure,



where the identity of R_1 and R_2 is not ascertainable spectroscopically. An identical spectrum was obtained from cis,cis-9,12[11,11-D]linoleic acid, indicating that the spin adduct was not derived from the carbon-centered radical formed by the loss of one of the two deuteriums at C-11. Note that the abstraction of the 11-L hydrogen is generally thought to be the initial step of soybean lipoxygenase catalysis (37).

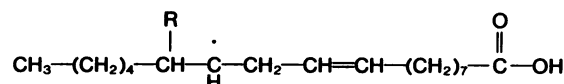


However, deuteration at carbons 9, 10, 12, and 13 did change the spectrum to one consisting of only a nitrogen triplet, indicating that the radical trapped had a deuterium attached to the carbon center. This would imply that the carbon-centered radical lies at one of these four positions.



Two conflicting interpretations of this result are in the literature. De Groot et al. (33) suggested that their results were consistent with the nitroso compound reacting with a linoleic acid radical at C-13 and/or C-9. In subsequent work Sekiya et al. (146) assumed that hydrogen abstraction at C-11 by potato lipoxygenase was followed by isomerization of the *cis* double bond between C-9 and C-10 to a *trans* double bond between C-10 and C-11, thus forming a carbon-centered free radical at C-9. The addition of oxygen to this conjugated dienyl free radical will form a 9-peroxy free radical, whereas the addition of a nitroso compound will form a nitroxide attached to C-9. In these studies the site of nitroxide formation was assumed to be the same as the site of oxygen addition, but the origins and the implications of this assumption were not discussed (146).

This interpretation of the spin trapping results with lipoxygenases and linoleic acid was recently criticized (41, 68). The spin trapping results with deuterated linoleic acid reported by de Groot et al. showed no evidence of linoleic acid having trapped a C-11-centered radical. It was argued that the radical centered at C-11, which is in conjugation with two double bonds, must be of equivalent reactivity to the free radicals centered at C-9 and C-13. Hence, nitroxides derived from linoleic acid radicals from all three carbon sites (C-9, C-11, and C-13) were predicted. Since the deuteration studies clearly indicated that the C-11-centered radical was not trapped, it was argued that a different species with the radical center at carbons 9, 10, 12, or 13 must be proposed (41, 68). It was further proposed that the species trapped was created by the addition reaction of some unknown radical to one of the two double bonds of linoleic acid,



where R is perhaps an alkylperoxy or alkoxy radical (68). It should be noted that such an intermediate is not consistent with the biosynthetic pathways to hydroper-

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oxides, and the trapped radical must, in this case, represent a minor side reaction.

The site of carbon radical trapping can be proved by experiments with specifically labeled deuterated linoleic acid, where only hydrogen on a single carbon is replaced by deuterium (i.e., C-9 for potato lipoxygenase and C-13 for soybean lipoxygenase). Alternatively, the adducts could be isolated and their positional and configurational isomers separated and determined. The criticism of the spin trapping investigations of the soybean lipoxygenase mechanism is not convincing for two reasons. First, the free radical autoxidation of linoleic acid forms only 9- and 13-hydroperoxide, and no 11-hydroperoxide is detected (128). If the three reaction sites of the pentadienyl-type radical of linoleic acid (C-9, C-11, and C-13) are of equal reactivity, nonkinetic factors must determine which positional isomers are formed. If nitroso compounds react in a manner similar to oxygen, as expected, then not even during autoxidation will nitroxides form attached at C-11.

The second argument is based on the fact that the site of oxygenation of linoleic acid by lipoxygenases is both positionally and configurationally specific (37). When oxygen or, presumably, a spin trap such as nitroso *t*-butyl (2-methyl-2-nitrosopropane) reacts with the carbon-centered linoleic acid free radical, the free radical is bound to the enzyme, and the enzyme allows oxygen or, presumably, a spin trap to react with only one of the three chemically reactive sites. Certainly these enzymes form only one stereoisomer when oxygen is the "spin trap." Since the mechanism of soybean lipoxygenase is formally similar to that of prostaglandin fatty acid cyclo-oxygenase, it is natural to try to use spin trapping to detect any arachidonic acid free radical in the prostaglandin cyclo-oxygenase-catalyzed conversion of arachidonic acid to prostaglandin G_2 (92).

The electron spin resonance spectrum of the spin adduct observed in an incubation mixture containing ram seminal vesicle microsomes, a source of prostaglandin cyclo-oxygenase, and arachidonic acid clearly showed an unpaired electron interaction with the nitroxide nitrogen and the attached β-proton (fig. 9). The hyperfine couplings

$$(a^N = 15.7\ G,\ a^H_\beta = 2.5\ G)$$

are similar to the values reported for the carbon-centered linoleic acid radical adduct

$$(a^N = 16.0\ G,\ a^H_\beta = 2.0\ G).$$

Indomethacin, a classic synthetase inhibitor, decreased the electron spin resonance signal intensity at a concentration of 100 μM. No signal was detected at a concentration of 400 μM indomethacin. The presence of a small ESR signal from a mixture of microsomes and nitroso *t*-butyl alone was due presumably to endogenous unsaturated fatty acids present in the microsomes. In the absence of microsomes, no electron spin resonance signal was observed.

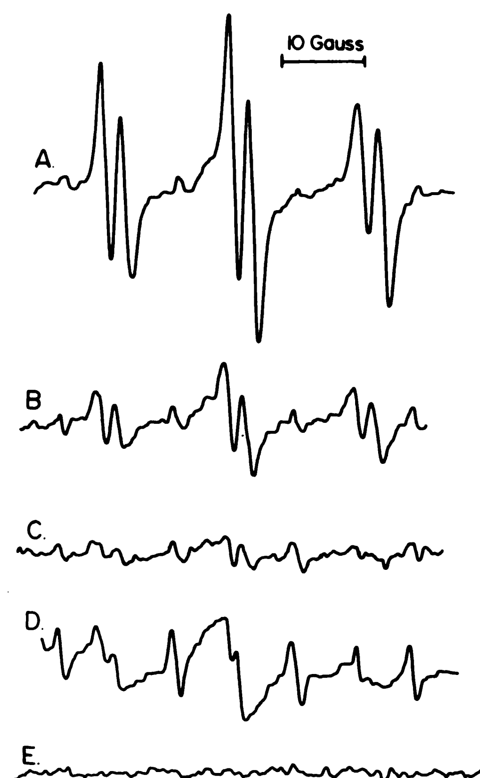

FIG. 9. ESR spectra of prostaglandin synthesis incubations. A. ESR spectrum of the carbon-centered arachidonic acid-nitroso *t*-butyl radical adduct obtained upon the addition of 400 μM arachidonic acid to an incubation mixture (0.25% ethanol) containing 2.0 mg/ml of ram seminal vesicle microsomal protein in a 1-mg/ml nitroso *t*-butyl solution of Tris buffer (pH 7.5). B. ESR spectrum obtained from an incubation mixture (0.3% ethanol) like that described above, but which had been preincubated for 2 minutes with 100 μM indomethacin. C. ESR spectrum obtained from an incubation mixture (0.5% ethanol) like that described for "A," but which had been preincubated for 2 minutes with 400 μM indomethacin. D. ESR spectrum obtained from the incubation of 2.0 mg/ml of ram seminal vesicle microsomal protein in the nitroso *t*-butyl solution of "A." E. Base line obtained upon mixing 400 μM arachidonic acid with a 1 mg/ml nitroso *t*-butyl solution of Tris buffer (pH 7.5, 0.25% ethanol). [From Mason et al (92)].

Concomitant with the appearance of the electron spin resonance signal, there was an increased rate of oxygen uptake upon the addition of arachidonic acid to the incubation mixture containing ram seminal vesicle microsomes (fig. 10). Oxygenation of arachidonic acid by these microsomes has been found to be stoichiometric with prostaglandin formation (36), therefore oxygen uptake is considered a good index of prostaglandin synthetase activity. Arachidonic acid-initiated oxygen consumption was inhibited by indomethacin (fig. 10), with a dose dependence similar to that observed for the carbon-centered arachidonic acid radical adduct. Note that the incorporation of oxygen into endogenous unsaturated fatty acids present in the microsomes corresponded to the presence of a small electron spin resonance signal from a mixture of microsomes and nitroso *t*-butyl alone.

After a prolonged time, or at 200-fold higher concentrations of arachidonic acid, a chemical reaction between

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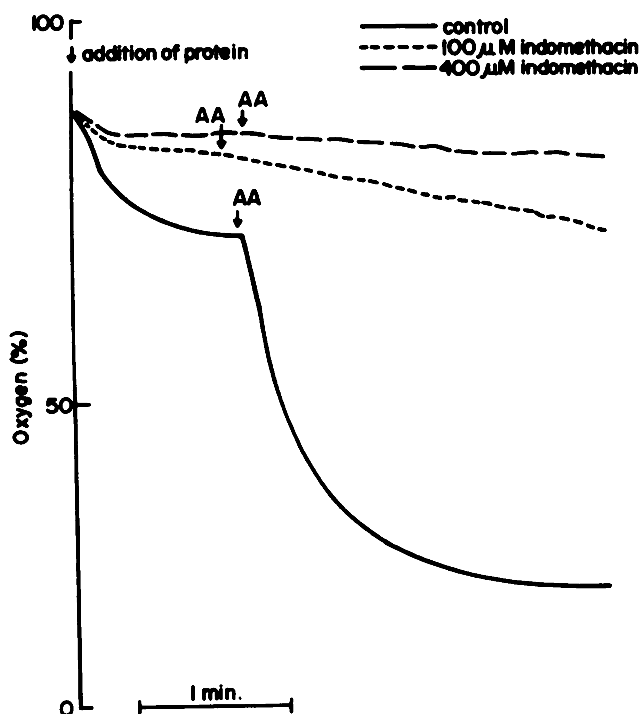

FIG. 10. Oxygen consumption tracings corresponding to figure 9A (——, after the addition of arachidonic acid), figure 9B (-----), figure 9C (— —), and figure 9D (——, before the addition of arachidonic acid). [From Mason et al (92).]

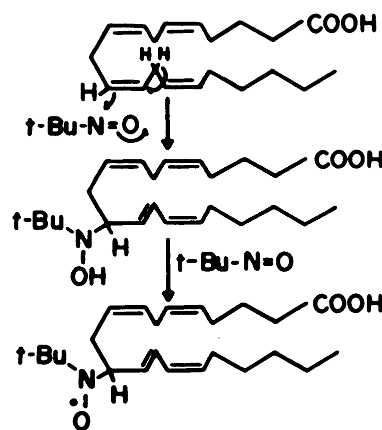

FIG. 11. The addition of nitroso *t*-butyl to the C-11 double bond of arachidonic acid by an ene mechanism. [From Mason et al (92).]

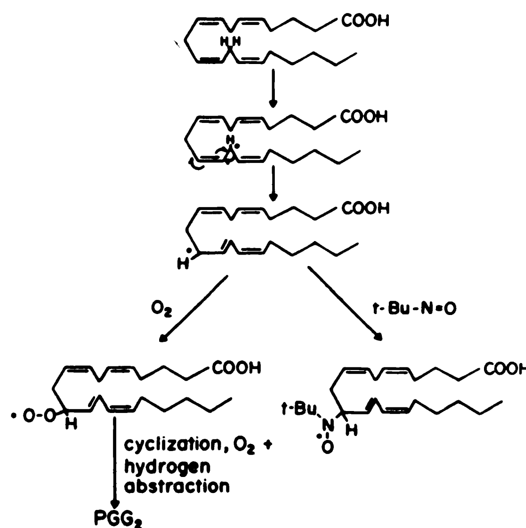

FIG. 12. The mechanism of the first phase of prostaglandin biosynthesis showing the incorporation of the first molecule of oxygen and how nitroso *t*-butyl would react with the C-11 carbon-centered free radical. [From Mason et al (92).]

arachidonic acid and nitroso *t*-butyl occurs, giving a similar spectrum. The electron spin resonance spectrum

$$(a^N = 15.5\ G,\ a^H_\beta = 2.0\ G)$$

of this species appears under either anaerobic or aerobic conditions and, therefore, cannot be due to the trapping of a free radical formed by arachidonic acid autoxidation. Therefore, this nitroxide adduct must arise by a different mechanism. A stable nitroxide can be synthesized by reacting nitrosobenzene with an olefin through a pseudo-Diels-Alder or ene reaction (152). A similar reaction occurs between 2-nitrosofluorene and oleic acid (45). A related reaction between nitroso *t*-butyl and arachidonic acid could give an arachidonic acid-*t*-butyl nitroxide identical with that thought to be formed by the enzymatic reaction (fig. 11). Considering the addition reaction that appears to be taking place, up to eight radical adducts are possible where both β-substituents are C_4 or longer. Either one radical adduct predominates, or the radical adducts formed have indistinguishable electron spin resonance spectra. This chemical reaction appears to provide a convenient method of independently synthesizing unsaturated lipid-*t*-butyl nitroso radical adducts.

The investigators of the reaction mechanism of the oxygenation of linoleic acid by potato tuber lipoxygenase and soybean lipoxygenase have proposed that the initial carbon-centered free radical isomerizes to a *cis-trans* conjugated diene prior to the oxygenation. A similar mechanism was adopted in the present work, differing mainly in that hydrogen abstraction takes place at the 13-C position (fig. 12). Isomerization of the 11 *cis* double bond to a 12 *trans* double bond will form a carbon-centered free radical at 11-C. Oxygenation of this conjugated dienyl radical then occurs. This is consistent with the 11-peroxy arachidonic acid radical being the first oxygen-containing intermediate in the conversion of arachidonic acid to prostaglandin G_2, as proposed by Samuelsson, Nugteren, and others (127). It should be noted that the 11-hydroperoxide arachidonic acid (11-hydroperoxyeicosa-5,8,12,14 tetraenoic acid) has been shown not to be an intermediate in prostaglandin G_2 formation (129).

Again, positional deuteration of arachidonic acid is necessary to identify spectroscopically an arachidonic acid free radical as a particular secondary carbon-centered free radical. For example, the peroxy radical at C-11 will, upon cyclization, form an oxygen-containing secondary carbon-centered free radical at C-15 that can react with either a second molecule of oxygen or a nitroso spin trap (fig. 8). We believe that, if arachidonic acid

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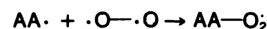
were specifically labeled at C-11 and C-15, a three-line spectrum would be observed, because deuteration will remove the doublet splitting due to the β-hydrogen.

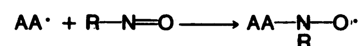
A less direct argument is based on oxygen consumption studies (92). The site of oxygenation is under both stereospecific and carbon-atom specific control by prostaglandin synthetase. As illustrated (fig. 12), the presence of nitroso spin traps should inhibit the free radical oxygenation of arachidonic acid if the nitroso compound is acting as an oxygen substitute. The strongest evidence that the carbon-centered free radical of arachidonic acid is an obligatory intermediate in the formation of prostaglandin G_2 is that nitroso *t*-butyl is an inhibitor of the oxygenation of arachidonic acid. This would imply that the reaction with nitroso compounds is also stereospecific and carbon-atom specific. We find that nitrosobenzene is a much stronger inhibitor of the oxygenation of arachidonic acid than is nitroso *t*-butyl. That nitrosobenzene is better than nitroso *t*-butyl as an inhibitor of oxygen uptake is consistent with the known faster trapping rates of nitrosobenzene. The ratio of the rate constants for the entrapment of oxygen vs. nitrosobenzene by the carbon-centered linoleic acid free radical has been determined to be 50 in the soybean lipoxygenase-catalyzed reaction (2). These electron spin resonance results have been criticized, because no heat-inactivated enzyme control was reported. This control is necessary to rule out a chemical ene reaction of the linoleic acid with the reactive enophilic nitrosobenzene (121). In any case, nitroso compounds inhibit the oxygenation of arachidonic acid by ram seminal vesicles nearly as well as do aspirin or indomethacin, but the well-known toxicity of these compounds must preclude their use as anti-inflammatory agents (92). It should be noted that many explanations of inhibition experiments are usually possible. In this case, inhibition by nitrosobenzene, which is a good electron acceptor, may be related to the reduction of nitroblue tetrazolium during arachidonic acid peroxidation (132).

The well-resolved hyperfine spectrum of the enzymatically formed nitroso *t*-butyl arachidonic radical adduct is indistinguishable from that of the radical formed chemically in solution (92). If the arachidonic acid adduct were bound to the enzyme, the electron spin resonance spectrum would be very different and would be similar to those of nitroxide-labeled fatty acids bound to enzymes or to membranes (24). The oxygen consumption studies imply that the nitroso compound reacts with the arachidonic acid carbon-centered radical on the surface of the enzyme; however, the electron spin resonance spectrum of the arachidonic acid adduct is that of a nitroxide dissociated from the enzyme and free in solution. Electron spin resonance signals of free radicals bound to the enzyme will, in general, have much poorer signal-to-noise ratios, for a variety of technical reasons, but, in any case, no such species has yet been detected.

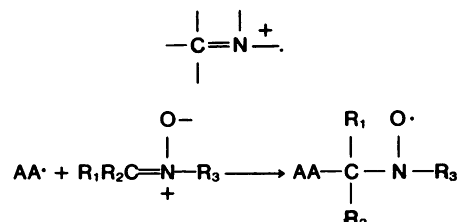
The fatty acid carbon-centered radicals generated by prostaglandin cyclooxygenase, plant lipoxygenases and, presumably, mammalian lipoxygenases can react with a variety of double bonds. This may have important consequences for xenobiotic metabolism. The reaction of the arachidonic acid free radical, AA·, with oxygen is the normal biochemical trapping reaction.



Reactions with nitrosobenzene and nitroso *t*-butyl are additions across the nitroso double bond, —N=O.

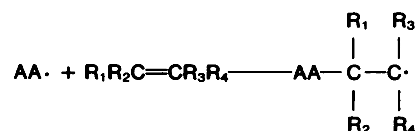


Evidence for these two reactions has been discussed, and many analogous reactions are possible. Reactions with the commercial nitrone spin traps α-phenyl-*tert*-butyl nitrone or 5,5-dimethylpyrroline-N-oxide are additions across the carbon-nitrogen double bond,



Librium is an example of a drug containing a nitrone functional group that could form this type of fatty acid adduct.

The formation of linoleic acid dimers by soybean lipoxygenase under anaerobic conditions (47) could be an example of addition across the carbon-carbon double bond followed by hydrogen abstraction.



All of the above reactions (except the first) can form fatty acid xenobiotic free radical metabolites that, upon hydrogen abstraction or one-electron reduction, will form the corresponding diamagnetic compounds. If such xenobiotic metabolites form in vivo as well as in vitro, they would represent an entirely new pathway for the metabolism of xenobiotics.

V. Free Radicals in Drug-Induced Cutaneous Photosensitization

Light is known to interact with chemical agents in exposed tissues such as the skin and eyes to produce photosensitization (113). The chemical agent may be endogenous (protoporphyrin in erythropoietic protoporphyria), a drug (sulfonamide, declomycin, chlorpromazine), a topical agent (*p*-aminobenzoic acid and its esters in sunscreens; halogenated salicylanilides in soaps), or an environmental agent (polycyclic aromatic hydrocarbons

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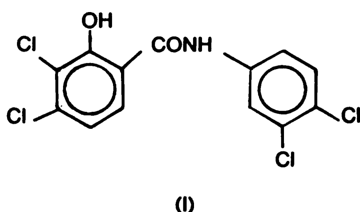
in coal tar; amyl esters of *o*-aminobenzoic acid in printer's ink) (38, 79). Photosensitization may take the form of an exaggerated sunburn (phototoxicity) or may involve a delayed hypersensitivity reaction (photoallergy). While the initial step in both phenomena must be the absorption of light by the chemical or its metabolites, the precise mechanisms of photosensitivity are, for the most part, unknown. The close similarity between phototoxicity and the normal erythemal response to excessive sunlight exposure suggests that the mechanisms are probably the same or very similar (113). In photoallergy the agent is converted to a chemically reactive species (hapten), which then combines with a protein, or other macromolecule (carrier), to form an allergen. The mechanism of the subsequent immunological response is, presumably, similar to other types of delayed hypersensitivity (56).

In this section the possibility that light-induced free radicals may be implicated in both phototoxic and photoallergic reactions will be examined.

A. Halogenated Salicylanilides

3,5,3′,4′-Tetrachlorosalicylanilide (TCSA) (I) and other halogenated salicylanilides are known to cause



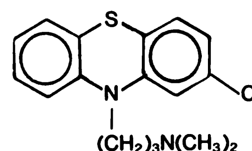
(I)

both phototoxic and photoallergic responses in some people (80, 79). These chemicals have been used primarily as antibacterial agents in creams, anti-acne topical preparations, deodorant soaps, and as antifungal preparations. Coxon et al. (30) have shown that irradiation of the anions of halogenated salicylanilides with near ultraviolet light causes the halogen atoms substituted in the 3- or 5-positions to be replaced by hydrogen. With 3,5-disubstituted salicylanilides the halogen in the 3-position is lost; 5-substituted bromosalicylanilides lose the 5-bromosubstituent. Since Jenkins et al. (70) have shown that ultraviolet irradiation of solid TCSA produces free radicals, it is possible that the salicylanilides lose their halogen by homolytic fission of the carbon-halogen bond(s). Additional evidence for the appearance of free radicals during this reaction was provided by the observation that iodine was released when potassium iodide was added to either air- or nitrogen-saturated solutions of salicylanilides during ultraviolet irradiation.

In order to produce an allergic response it is necessary for the hapten to modify a protein covalently to produce an antigen. Since albumin is a soluble protein present in skin, it is a likely candidate for the carrier portion of the antigen. Kochevar and Harber (80) have shown that under irradiation TCSA forms a covalent complex with human serum albumin but not γ-globulin. In addition it was found that TCSA photosensitizes the oxidation of histidines in albumin. Thus, it is possible that the photoallergic response induced by TCSA is due to the photoinduced covalent modification of albumin or some other protein in the skin. Although it has yet to be proved conclusively, it seems likely that the structurally related photoallergens 2,2′-thiobis-(4,6-dichlorophenol) (bithionol) and *n*-butyl-4-chlorosalicylamide (Jadit) have a similar toxicity mechanism.

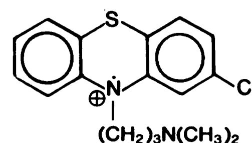
B. Chlorpromazine

Chlorpromazine and several other phenothiazine tranquilizers cause both phototoxic and photoallergic reac-



tions in patients receiving low doses of these drugs (171). High dosage and prolonged treatment can produce severe dermatitis that is frequently accompanied by darkening of the skin due to the deposition of melanin in lower layers of the dermis. Such patients may also suffer retinal damage, ocular opacity, and loss of vision (171).

Oxidation of chlorpromazine in acid solution is known to produce the corresponding red cation radical (14, 126).



The same radical is also produced during oxidation by horseradish peroxidase and hydrogen peroxide (125).

Forrest et al. (46) have reported the formation of a colorless free radical from chlorpromazine during the exposure of dilute aqueous solutions of the drug to a sunlamp for three hours. The free radical character of the photoproduct was inferred from electron spin resonance spectra taken on solid samples of a 2,4-dinitrophenylhydrazine derivative. Borg and Cotzias (14) failed to detect free radicals in aqueous solutions of chlorpromazine irradiated with ultraviolet light. They have suggested that the ESR spectrum of the solid derivatives isolated by Forrest et al. (46) was due to the cation radical formed during derivatization. Subsequently Piette and Forrest (126) reported the generation of a purple/blue substance by photooxidation of chlorpromazine. The electron spin resonance spectrum of this photoproduct was not the same as that of the red chlorpromazine radical cation formed under strong acidic conditions.

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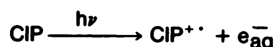
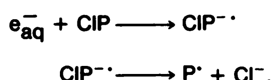
Flash photolysis studies of either anaerobic or aerobic aqueous solutions of chlorpromazine (66, 105) have provided evidence for the photoionization of the drug to give the chlorpromazine cation radical ($ClP^{+\cdot}$) and the aqueous electron (e_{aq}^-).



$$ClP \xrightarrow{h\nu} ClP^{+\cdot} + e_{aq}^-$$

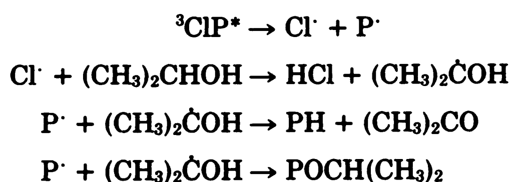
Felmeister and Discher (42) also detected the chlorpromazine cation radical in aqueous acidic solutions of the drug with a low pressure mercury arc lamp. Pulse radiolysis of aqueous chlorpromazine solutions have shown (31) that the aqueous electron may react with chlorpromazine to form the radical anion ($ClP^{-\cdot}$), which can then eliminate a chloride ion,



$$e_{aq}^- + ClP \longrightarrow ClP^{-\cdot}$$
$$ClP^{-\cdot} \longrightarrow P^{\cdot} + Cl^-.$$

Grant and Greene (52) have found that aqueous solutions of chlorpromazine exposed to sunlight, in the absence of oxygen, were converted to promazine and 2-hydroxypromazine. They proposed that the chlorpromazine triplet underwent direct homolytic fission to give the chloride atom ($Cl^{\cdot}$) and the promazine radical ($P^{\cdot}$), which reacted with solvent to give the observed products.

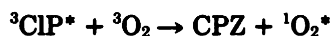
In oxygen-free *iso*propanol chlorpromazine undergoes homolytic carbon-chlorine bond fission to form a neutral radical ($P^{\cdot}$), which then reacts with the solvent to form promazine (PH), 2-*iso*propoxypromazine, HCl, and acetone.



$$^3ClP^* \rightarrow Cl^{\cdot} + P^{\cdot}$$
$$Cl^{\cdot} + (CH_3)_2CHOH \rightarrow HCl + (CH_3)_2\dot{C}OH$$
$$P^{\cdot} + (CH_3)_2\dot{C}OH \rightarrow PH + (CH_3)_2CO$$
$$P^{\cdot} + (CH_3)_2\dot{C}OH \rightarrow POCH(CH_3)_2$$

A similar mechanism can be proposed to explain the production of promazine and 2-substituted promazine when chlorpromazine is irradiated in other solvents including methanol, ethanol, and aqueous dimethylamine (52, 137).

In oxygen-saturated *iso*propanol, energy transfer occurs from triplet state chlorpromazine to molecular oxygen (32) yielding singlet oxygen ($^1O_2^*$).



$$^3ClP^* + {}^3O_2 \rightarrow CPZ + {}^1O_2^*$$

Thus, under aerobic conditions, in organic solvents, there is no photodegradation of chlorpromazine. This has been confirmed recently by Moore and Tamat (101), who have shown that the production of chloride ion by photoirradiation of a methanol solution of chlorpromazine is inhibited almost completely when the solution is saturated with oxygen. In aqueous solution oxygen causes only partial reduction in the rate of chloride ion release, presumably because it is still possible to produce the chloride ion from the chlorpromazine anion (vide supra).

Chlorpromazine is known to have both phototoxic and photoallergic properties. The mechanism of chlorpromazine phototoxicity has been studied by a number of workers (81, 72, 29). Kochevar and Lamola (81) have reported that red cells are lysed when irradiated in the presence of chlorpromazine. Oxygen caused only a small increase in hemolysis, suggesting that it is not an important factor. Similar results have been described by Johnson (72). However, Copeland et al. (29) have found that oxygen is necessary for the disruption of liposomes by light in the presence of chlorpromazine. Kochevar and Lamola (8) also found that red cells were lysed in the dark by incubation with chlorpromazine solutions that had been previously irradiated in the absence of oxygen. This suggests that chlorpromazine photoproducts may be responsible for the phototoxic effect of this drug, although the identity of these photoproducts is not known.

The photoallergic effect of chlorpromazine must be due to covalent modification of proteins or other macromolecules by photoproducts. Davies et al. (32) have suggested that the promazine radical ($P^{\cdot}$) may be the reactive species that generates the antigen in vivo.

C. Sulfanilamide

Epstein (39) was the first to recognize that sulfanilamide can give rise to both phototoxicity and photoallergy. The photolysis of sulfanilamide in aqueous and organic solvents has been shown to produce a wide variety of photodegradation products including $(NH_4)_2SO_4$, 4-hydroxylaminobenzenesulfonamide, aniline, 4,4′-disulfamoylbenzene, 4-nitrobenzenesulfonamide, benzidine, SO_2, and sulfanilic acid (138, 148, 133, 116, 115, 114, 142). Although it was proposed initially that 4-hydroxylaminobenzenesulfonamide was the causative agent in sulfanilamide photoallergy (145), more recent studies have shown that not all sensitive patients cross-react to this substance (139).

Direct evidence for the production of free radicals during the irradiation of sulfanilamide has been provided recently by Chignell et al. (25, 26), who used the technique of spin trapping (68, 121). In this procedure diamagnetic organic molecules ("spin traps") that react with free radicals to produce a stable free radical ("spin adduct") were employed. Studies with 2-methyl-2-nitrosopropane and 5,5-dimethyl-1-pyrroline-1-oxide showed that when aqueous solutions of sulfanilamide were irradiated with a xenon arc lamp the following reactions occurred:

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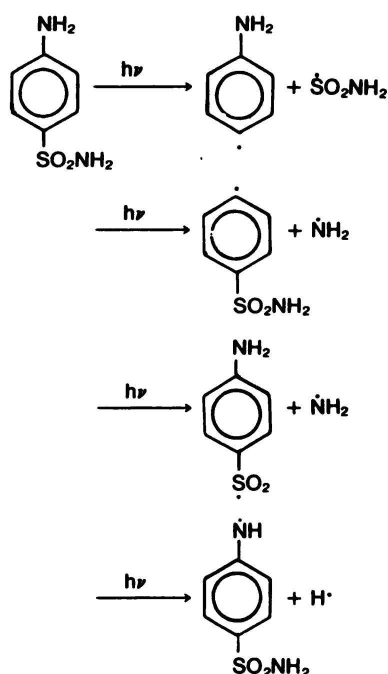
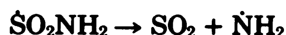
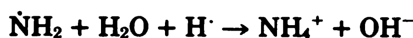


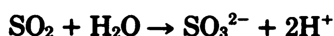
The primary radicals then undergo a series of further reactions:



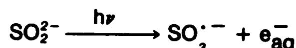
$$\dot{S}O_2NH_2 \rightarrow SO_2 + \dot{N}H_2$$



$$\dot{N}H_2 + H_2O + H^{\cdot} \rightarrow NH_4^+ + OH^-$$

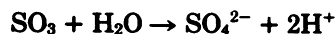


$$SO_2 + H_2O \rightarrow SO_3^{2-} + 2H^+$$

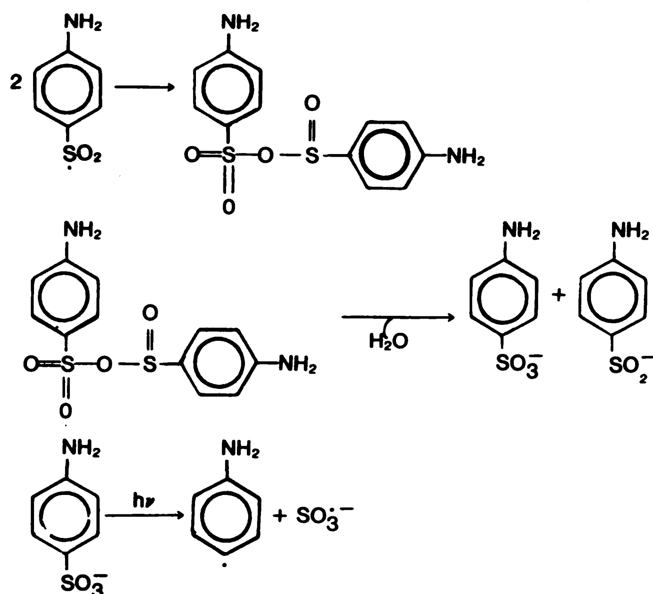


$$SO_3^{2-} \xrightarrow{h\nu} SO_3^{\cdot -} + e^-_{aq}$$

$$2SO_3^{\cdot -} \rightarrow SO_3 + SO_3^{2-}$$



$$SO_3 + H_2O \rightarrow SO_4^{2-} + 2H^+$$



It may be clearly seen that, with the possible exception of 4-hydroxylaminobenzenesulfonamide, it is possible to derive all of the known photoproducts of sulfanilamide from the primary free radicals or their decomposition products.

At the present time it is not possible to determine which of the sulfonamide photoproducts is responsible for the cutaneous photosensitization caused by this drug. It is certainly possible that the free radicals may initiate lipid peroxidation. For example, aromatic sulfonyl radicals are known to add to olefins to form carbon-centered radicals (78). Gilbert and Marriott (50) have shown that the ammoniumyl radical cation ($\dot{N}H_3^+$) reacts readily with thiols to produce a variety of oxidation products. The generation of SO_2 may also be involved in the phototoxic reaction to sulfanilamide. The photoallergic response to sulfanilamide probably results from covalent modification of protein by the photogenerated radicals.

VI. Conclusion

In conclusion, free radicals can, in principle, be generated from any aromatic organic chemical. For a variety of reasons, the possibility of free radical xenobiotic metabolism has not received much attention in the past, although Michaelis was interested in free radical metabolites and their importance in biochemistry in the 1930s. One reason for the late development of this area is that most biochemicals are not aromatic; those that are aromatic are not metabolized easily through free radical intermediates. In contrast, many synthetic aromatic organic compounds, and some closely related natural products, are metabolized readily to reactive free radicals. In fact, the enzymatic formation of free radical metabolites of synthetic aromatic compounds is the rule and not the exception.

The role of free radicals in photoinduced toxicity has been neglected also, in part because of the prominence of singlet oxygen in the proposed mechanism of phototoxicity. Regardless of the significance of singlet oxygen, free radicals can be studied directly with electron spin resonance, whereas evidence for singlet oxygen usually depends upon inhibition studies.

Electron spin resonance investigations have been, and will continue to be, obligatory to the understanding of the roles of free radicals in biological systems. Although this spectroscopy is inherently more difficult to understand than most other spectroscopies, its specificity for free radicals and the low abundance of biochemical species contributing to the room temperature background signal make this spectroscopy very rewarding.

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