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The Mechanism of Microsomal and Mitochondrial Nitroreductase. Electron Spin Resonance Evidence for Nitroaromatic Free Radical Intermediates†

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ABSTRACT: Electron spin resonance spectra are observed during the enzymatic reduction of many nitrophenyl derivatives by rat hepatic microsomes or mitochondria. The spectra indicate that nitroaromatic anion radicals are present and are freely rotating in aqueous solution at a steady-state concentration of 0.1–6 μM. The rate of formation of *p*-nitrobenzoate (NBzO) dianion radical in microsomal incubates is consistent with the radical being an obligate intermediate in the reduction of NBzO to *p*-aminobenzoic acid. A model system consisting of NBzO, NADPH, and FMN, but no heme-containing compounds, also reduced NBzO to the NBzO dianion free radical. The steady-state concentration of the anion radicals in microsomal systems is not altered by CO. This observation, together with the results from the model system, suggests that the formation of nitroaromatic anion radicals is mediated through a flavine and not cytochrome P-450. The oxidation of the anion radical intermediate by O_2 to the parent nitro compound is proposed to account for the well-known O_2 inhibition of microsomal nitroreductase.

Fouts and Brodie (1957) were the first to report the presence of nitroreductase in hepatic microsomes. This activity was found to be dependent on either NADH or NADPH, was inhibited by oxygen, and was markedly stimulated by the addition of flavines (Gillette, 1971; Weisburger and Weisburger, 1971). The system exhibited little substrate specificity, being able to reduce not only nitrobenzene and *p*-nitrobenzoate, but also a wide range of other nitro com-

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pounds such as nitroalkanes (Porter et al., 1972); the organophosphate, parathion (Hitchcock and Murphy, 1967); the antischistosomal, niridazole (Feller et al., 1971); and the antibiotic chloramphenicol (Fouts and Brodie, 1957).

The reduction entails a six-electron transfer from the pyridine nucleotide to the nitrobenzene moiety with the ultimate formation of the primary amine. On the basis of

$$Ph{-}NO_2 \xrightarrow{e^-} Ph{-}\dot{N}O_2^- \xrightarrow[2H^+]{e^-} Ph{-}NO \xrightarrow[4H^+]{4e^-} Ph{-}NH_2$$

their studies, Gillette et al. (1968) have suggested that initially two electrons are transferred so that the first intermediate in the reduction is the corresponding nitroso compound. Further reduction gives the phenylhydroxylamine, which accumulates during the formation of the amine product (Kato et al., 1969). On the basis of the inhibition by CO of the formation of the primary amine, Gillette et al. (1968) and Sasame and Gillette (1969) suggested that the reducing equivalents were transported through cytochrome P-450. Symms and Juchau (1972) extended these studies to a model system consisting of NADPH, FMN, and a wide variety of hemoproteins. They found that with the removal of any one of the components no amine was formed.

If the corresponding nitroso compound is truly the first intermediate in the reduction, then the first step must entail the transfer of two electrons to the nitrobenzene. Yet the microsomal flavines and cytochromes are almost invariably assumed to be one-electron donors (Kamin et al., 1965; Iyanagi and Yamazaki, 1969).

Hence one would anticipate that the first step in the reduction would be the formation of the nitrobenzene anion radical; and indeed, we have observed that such is the case. When microsomes, mitochondria or a flavine-containing model system are anaerobically incubated with an appropriate electron-donating cofactor and nitrobenzene in an electron spin resonance (ESR) spectrometer at room temperature, the characteristic spectrum (Geske and Maki, 1960) of the nitrobenzene anion radical is observed (Mason, 1974). This observation is facilitated, in part, by the long half-life of this resonance-stabilized anion radical (1–10 sec at pH 7–8) (Piette et al., 1962) which leads to steady-state concentrations of the radical of 0.1–6 μM.

Methods

Untreated, fed male, 180–200 g, CD rats obtained from Charles River Inc. were used in all experiments. Microsomes were prepared by homogenizing the livers in 3 volumes of KCl–Tris (150 mM, 50 mM, pH 7.4) and centrifuging the homogenate at 9000g for 15 min in an RC2-B centrifuge with an SS34 rotor. The supernatant was centrifuged at 165,000g for 38 min in a Model L2-65B centrifuge with a Ti 50 rotor. The 165,000g supernatant was used as the cytosol.

Mitochondria were prepared by homogenizing rat liver in 4 volumes of 0.25 M sucrose and centrifuging the homogenate at 600g for 5 min. The supernatant was centrifuged at 8000g for 10 min. The pellet was resuspended in 2 volumes of 0.25 M sucrose and recentrifuged. The resulting pellet was resuspended in 1 volume of sucrose and resedimented. The pellet was finally suspended to give 2 g-equiv of liver/ml (Holtzman and Seligman, 1973). Protein was determined by the method of Sutherland et al. (1949).

The incubation mixture (3 ml) containing substrate (2 mM) and a NADPH generating system consisting of NADP (0.7 mM), glucose 6-phosphate (10 mM), and 1.3 units/ml of glucose-6-phosphate dehydrogenase in KCl–Tris–$MgCl_2$ (150 mM, 20 mM, pH 7.4, and 5 mM) was placed in a 15-ml stoppered serum bottle and gassed for 15 min with N_2 or CO that had been deoxygenated by passage through a solution of 0.05% anthraquinone-2-sulfonate and 5% sodium dithionite in 0.1 N NaOH (Holtzman et al., 1968). Microsomes equivalent to 200 mg of liver were added and after further gassing, the outlet needle was lowered below the level of the mixture forcing the incubate into a Varian 248 aqueous flat cell. The flat cell was capped and placed in a Varian E-4 ESR spectrometer, and spectra were obtained at room temperature.

The magnetic field sweep of the E-4 spectrometer was calibrated with 10^{-4} M peroxylaminedisulfonate in 0.05 M K_2CO_3, using a nitrogen hyperfine splitting constant of 13.091 ± 0.004 G (Faber and Fraenkel, 1967). The concentration of the peroxylaminedisulfonate was determined spectrophotometrically at 248 nm using an extinction coefficient of 169.0 cm^{-1} M^{-1} (Murib and Ritter, 1952). The amplitude of the center line of the spectrum of peroxylaminedisulfonate maximized at a nominal power of 18 mW and a field modulation of 0.80 G, whereas the center line of the NBzO[1] dianion radical maximized at 5 mW and 0.63 G. Since the line width and saturation characteristics of the nitrobenzene anion radical and peroxylaminedisulfonate are quite similar, the relative amplitudes of the first derivative signals, which have been independently maximized with respect to saturation and field modulation, can be used to quantitate the nitroaromatic anion radical concentrations (Abragam, 1961). Account was taken of the differences in the multiplicity of hyperfine patterns of the various radicals in this quantitative determination of radical concentrations.

The rotational correlation time, τ_R, was calculated from a simplified expression for the line width variation of the three lines of greatest amplitude of p-nitrobenzoate dianion

$$\delta = A + BM_{^{14}N} + CM_{^{14}N}{}^2$$

radical (Figure 1a), where δ is the peak-to-peak derivative linewidth of the three lines and $M_{^{14}N}$ is the magnetic component of the ^{14}N nuclear spin (Goldman et al., 1972). The coefficients B and C are functions of the g tensor, the ^{14}N hyperfine tensor of the dianion radical, and the radical's rotational correlation time, τ_R. The ^{14}N hyperfine tensor and g tensor of the nitrobenzene anion radical in a crystalline matrix were used in the τ_R calculations (Mason, 1971). This calculation ignores possible complications due to the multiplicity of these three lines.

During the line width studies the magnetic field modulation amplitude and microwave power were reduced until they no longer broadened the intrinsic line width. Because the steady state radical concentration decayed slowly, the amplitudes used in computing relative line widths were the means of measurements made on successive upfield and downfield scans.

A flow system used for kinetic measurements was constructed from peristaltic pumps and a two-jet mixing flat cell (Wilmad Glass Co., Inc.). In order to obtain anaerobic conditions the entire apparatus was enclosed in a glove bag and purged with nitrogen. The radical formation rate was obtained by adjusting the magnetic field to maximize the steady-state signal of the center line of the 27-line ESR

[1] Abbreviation used is: NBzO, p-nitrobenzoate.

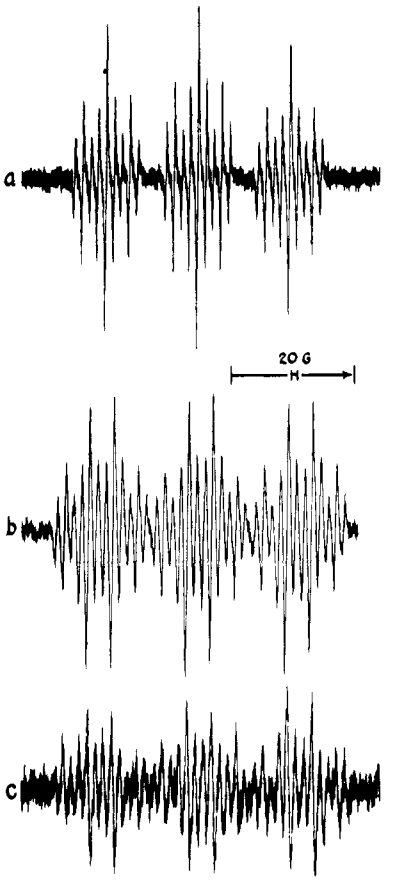

FIGURE 1: Spectrum a is of 1.1 μM *p*-nitrobenzoate dianion radical formed in the first microsomal incubate of Table II. Spectrum b is nitrobenzene anion radical under the same conditions as spectrum a. Spectrum c is of 0.2 μM nitrobenzene anion radical formed in the third mitochondrial incubate of Table II. For these spectra the nominal microwave power was 5 mW and the modulation amplitude was 0.63 G.

spectrum of *p*-nitrobenzoate dianion radical. After the zero base line was established, the flow (2.1 ml/sec) was initiated and then stopped. The rate was determined as the initial slope of the appearance of the ESR signal.

The amount of *p*-aminobenzoic acid formed at 23° was assayed according to the method of Bratton and Marshall (1939) as modified by Juchau (1969).

Results

The incubation of nitrobenzene and *p*-nitrobenzoic acid with the microsomal, mitochondrial, or 165,000*g* supernatant fractions in the presence of reduced pyridine nucleotides leads to a multi-line ESR spectrum (Figure 1a-c). The ESR spectra of nitro compounds which form nitro anion radicals have nearly unique hyperfine patterns, which in general enable an unambiguous identification of the radical structure (Forrester et al., 1968; Bowers, 1965). The hyperfine splitting constants resulting from the interaction of the delocalized unpaired electron with the nitrogen and ring hydrogens were determined from the observed ESR spectra and agree with the previously reported spectra (Table I).

When nitrobenzene was reduced by microsomes with either NADPH or NADH, the steady-state concentration of nitroaromatic anion radicals was present within seconds. The steady-state concentration decayed with a half-life of about 2 hr, apparently due to substrate depletion.

Omission of the NADP from the generating system or heating the microsomes on a steam bath for 10 min led to a total loss of activity (Table II). The NADPH generating system alone did not produce observable concentrations of the nitroaromatic anion radicals. In agreement with previous work on the cofactor requirements of microsomal nitroreductase (Gillette et al., 1968) NADPH was more effective than NADH as a source of reducing equivalents. The low steady-state concentration of the *p*-nitrobenzoate dianion obtained in the 165,000*g* supernatant in the presence of NADPH relative to the microsomal fraction is also consistent with previous results on nitroreductase (Gillette et al., 1968).

Mitochondria in the presence of the reduced pyridine nucleotides give an ESR signal that appears slowly and reaches a steady-state concentration which is only a fraction of the microsomal activity (Table II). This low activity could be due either to the low permeability of the mitochondrial membranes to the reduced pyridine nucleotides, or alternatively, to microsomal contamination. The mitochondrial activity observed with succinate and DL-β-hydroxybutyrate is clearly not due to microsomal contamination, as microsomes do not give detectable radical concentrations with these substrates. Since nitroreductase activity from mitochondria has not, to our knowledge, been previously reported in the absence of added flavines, the appearance of this ESR signal is surprising and would suggest that either the rate of the reaction is too slow to be detected by the usual chemical techniques, other species are necessary for complete reduction, previous authors did not wait long enough to find the primary amine when studying mitochondria, or the product is destroyed by the mitochondria.

The NADPH-microsomal reductase is apparently within

Table I: Hyperfine Coupling Constants (in gauss) of Nitrobenzene Anion Radicals.

Parent Compd	Method of Reduction	Solvent	a^N	a^H_{ortho}	a^H_{meta}	a^H_{para}
Nitrobenzene	Microsomal nitroreductase	Tris buffer (pH 7.4)	14.40	3.53	1.18	3.54
	Electrochemical[a]	0.1 *M* KCl in water	13.97	3.32	1.13	3.54
	Electrochemical[b]	*N,N*-Dimethylformamide	9.70	3.36	1.07	4.03
p-Nitrobenzoate anion	Microsomal nitroreductase	Tris buffer (pH 7.4)	13.11	3.33	1.13	
	Alkaline sodium dithionite[c]	Water	12.60	3.40	1.15	
	Electrochemical[d]	*N,N*-Dimethylformamide	9.56	3.28	1.06	
p-Nitrobenzene-sulfonate anion	Microsomal nitroreductase	Tris buffer (pH 7.4)	12.81	3.36	1.14	

[a] Piette et al., 1962. [b] Rieger and Fraenkel, 1963. [c] Kolker and Waters, 1964. [d] Allendoerfer and Rieger, 1967.

Table II: Intracellular Distribution of the Initial Steady State Concentration of the Nitro Anion Radical.

Fraction (Equivalent to mg of Liver)	Substrate[a]	Cofactor	Steady-State Concn (μM)
Mitochondria			
400 mg (1.6 mg of protein/ml)	Nitrobenzene	DL-β-Hydroxybutyrate (10 mM)	0.96[b]
400 mg (1.6 mg of protein/ml)	*p*-Nitrobenzoate	DL-β-Hydroxybutyrate (10 mM)	0.44[b]
400 mg	*p*-Nitrobenzoate	NADP (0.8 mM) and generating system	0.21[c]
400 mg	*p*-Nitrobenzoate	NADH (0.9 mM)	0.22[c]
800 mg	*p*-Nitrobenzoate	Succinate (10 mM)	0.14[c]
Microsomes			
400 mg, washed (1.5 mg of protein/ml)	*p*-Nitrobenzoate	NADP (0.8 mM) and generating system	2.6[b]
400 mg	*p*-Nitrobenzoate	Succinate (10 mM)	0.0[d]
400 mg	*p*-Nitrobenzoate	DL-β-Hydroxybutyrate (10 mM)	0.0
200 mg	*p*-Nitrobenzoate	None	0.0
200 mg	Nitrobenzene	Nitrosobenzene (saturated)	0.0
200 mg (heated over steam bath)	*p*-Nitrobenzoate	NADP (0.8 mM) and generating system	0.0
200 mg	*p*-Nitrobenzoate	NADH (0.9 mM)	0.65[b]
200 mg	*p*-Nitrobenzenesulfonate	NADP (0.8 mM) and generating system	6.4[b]
Cytosol (165,000g supernatant)			
83 mg, liver perfused (1.5 mg of protein/ml)	*p*-Nitrobenzoate	NADP (0.8 mM) and generating system	0.49[b]

[a] The substrate concentration was 2×10^{-3} M. [b] The initial steady-state concentration was measured within a few minutes. It was constant for approximately 30 min and then decayed with a half-life of about 2 hr, apparently due to substrate depletion. [c] The steady-state concentration was obtained only after 2 hr. [d] The lower limit of detectability is on the order of 5×10^{-8} M *p*-nitrobenzoate dianion radical.

a few ångstroms of the aqueous interface, because both NBzO (pK_a = 4.5) and *p*-nitrobenzenesulfonate, which are completely ionized at pH 7.4, and therefore cannot penetrate the membrane, are as readily reduced by the microsomal reductase as is nitrobenzene. ESR spectral evidence also suggests that the reductase is near the aqueous interface.

The magnitudes of the nitrogen hyperfine splitting constants of these anion radicals are sensitive to the water content of the environment. For example, in dimethylformamide–H_2O mixtures the nitrogen hyperfine constant increases asymptotically with the percentage of H_2O (Ludwig et al., 1964). The magnitudes of the nitrogen hyperfine splittings correspond to that reported for nitroaromatic anions in pure aqueous solution and are independent of the source of nitroreductase (Table I).

The observed nitrophenyl anion radicals have well resolved hyperfine structure, which implies that the radicals are freely rotating and not attached to any macromolecule. More precisely, an isotropic rotational correlation time, τ_R, was calculated to be $3\text{–}4 \times 10^{-12}$ sec, and was also found to be essentially independent of the source of nitroreductase. Using the Stokes–Einstein relation $\tau_R = 4\pi\eta r^3/3kT$, where r is the hydrodynamic radius, k the Boltzmann constant, T in °K, and η the viscosity (taken to be that of water at 23°), the calculated hydrodynamic radius of 3.1–3.4 Å is that of a small molecule. These results would suggest that the observed radical is freely rotating in an aqueous phase. Although the evidence that the detectable nitroaromatic anion radicals are freely rotating in the aqueous phase is consistent with the reductase being very near the aqueous interface, the few seconds necessary for the appearance of the radical after initiation of the reaction are sufficient for the hydrophilic anion radical to diffuse several microns.

The presence of nitrophenyl anion radical in the incubate does not, in itself, mitigate against the initial formation of nitrosobenzene by a two-electron transfer since the nitrosobenzene could conceivably comproportionate with nitrobenzene to give two molecules of the anion radical. Yet this seems unlikely since substitution of nitrosobenzene for pyridine nucleotides in the presence of microsomes and nitrobenzene gave no nitrobenzene anion radical (Table II).

If the nitrophenyl anion radical is in fact an intermediate in the nitroreductase pathway, then the rate of formation of the radical should be equal to or greater than the formation of the aniline. The stoichiometry would depend on whether the subsequent reducing equivalents are derived from the microsomal electron chain, the reduced pyridine nucleotides, or, as has been suggested from chemical studies (Kastening, 1964; Corvaja et al., 1966), by disproportionation of the anion radical and the rapid subsequent reduction by the radical of the intermediate thus formed. Hence the apparent 6.4:1 stoichiometry (Table III) between the radical formation and the aniline production might suggest that the radical donates all six of the reducing equivalents necessary to form the aniline. Alternatively, an intermediate such as the hydroxylamine could accumulate along with the aniline so that only a portion of the radical formed would become fully reduced. In support of this latter possibility, Kato et al. (1969) observed that even during the 30–60-min interval when the rate of aniline formation is maximal, the concentration of the *p*-hydroxyaminobenzoic acid was approximately equal to half that of the aniline. If the formation rate of the *p*-hydroxyaminobenzoic acid is one-half as great as the *p*-aminobenzoic acid formation, the stoichiometry would then imply that the dianion radical provides the first four electrons required for the formation of the hydroxylamine, as solutions of the pure nitrophenyl anion radicals

Table III: Kinetic Results of Microsomal Nitroreduction of *p*-Nitrobenzoate.[a]

	nmol per min per mg of Protein
p-Nitrobenzoate dianion radical	3.31 ± 0.34[b]
p-Aminobenzoic acid[c]	0.517 ± 0.021

[a] The incubate mixtures contained *p*-nitrobenzoate (2.2 m*M*), microsomal protein (1.5 mg/ml), and an NADPH generating system consisting of NADP (0.3 m*M*), glucose 6-phosphate (5 m*M*), and 0.67 unit/ml of glucose-6-phosphate dehydrogenase in KCl–Tris–$MgCl_2$ (150, 20, and 5 m*M*; pH 7.37). [b] Average ± maximum deviation. [c] As reported by Kato et al. (1969) the rate of formation of *p*-aminobenzoic acid increased up to 30 min and decreased after 60 min. The reported rate is for the 30–60-min time interval when the rate is maximal.

Table IV: The Effect of Gassing on the Steady-State Concentration of the *p*-Nitrobenzoate Dianion Radical.[a]

Purging Gas	Steady-State Concn (*M*)
N_2	1.08×10^{-6} [b]
CO	1.10×10^{-6}
Air	0.0

[a] The reaction mixtures contained NADP (0.8 m*M*), generating system, *p*-nitrobenzoate (2.0 m*M*), and microsomes equivalent to 200 mg of liver. [b] The steady-state concentration was determined after 0.25 hr.

are known to do (Kastening, 1964; Corvaja et al., 1966). On the basis of this and CO inhibition studies (Gillette et al., 1968) the subsequent reducing equivalents are most likely derived from cytochrome P-450.

Although the reduction of *p*-nitrobenzoate to *p*-aminobenzoic acid by liver microsomes is strongly inhibited by CO (Gillette et al., 1968), as shown in Table IV, CO did not affect the steady-state concentration of the *p*-nitrobenzoate dianion radical, indicating that either cytochrome P-450 has no role in the formation or decay of the first intermediate or that possibly both the formation and the decay of the free radical are equally inhibited by CO. Preliminary kinetic studies suggest that the former is the case.

If the incubate is not completely deoxygenated, the nitroaromatic radicals cannot be detected, and both hydroxylamine (Kato et al., 1969) and aniline formation (Fouts and Brodie, 1957; Gillette et al., 1968) are almost completely inhibited. From their studies Gillette et al. (1968, 1971) have proposed that oxygen may inhibit nitroreductase either by the rapid reoxidation of the phenylhydroxylamine intermediates to nitrosobenzene or by competing with nitro compounds for the reduced form of cytochrome P-450.

Our results would suggest a third, and we feel more likely, mechanism for this inhibition which is the rapid oxidation of the nitroaromatic anion radicals by O_2 regenerating the nitroaromatic compound and forming the superoxide anion (Kastening, 1964; Russell and Bemis, 1967). This reaction is not only quantitative (Russell and Bemis, 1967) but nearly diffusion-limited (Greenstock and Dunlop, 1973). Hence the absence of further products of reduction under aerobic conditions does not imply that the nitroaromatic anion radicals have not been formed but only that they are rapidly oxidized by O_2 reforming the parent nitroaromatic compound and superoxide. The latter is in turn destroyed by superoxide dismutase and catalase to give O_2 and water.

Table V: Nitrobenzoate Dianion Radical Steady-State Concentration in a Model System.[a]

Catalysts	Steady-State Concn (*M*)
5×10^{-4} *M* FMN[b] and 1.0×10^{-4} *M* cytochrome *c*	1.4×10^{-7} [c]
5×10^{-4} *M* FMN	1.6×10^{-7}
	0.0[d]

[a] Symms and Juchau, 1972. [b] The reaction mixtures also contained NADPH (2.0×10^{-3} *M*) and *p*-nitrobenzoate (3.0×10^{-3} *M*) in phosphate buffer (pH 7.4) unless otherwise stated. [c] The steady-state signal decayed with a half-life of about 1 hr. The radical concentration was determined about 0.5 hr after the adding of NADPH. [d] The lower limit of detectability is on the order of 5×10^{-6} *M p*-nitrobenzoate dianion radical.

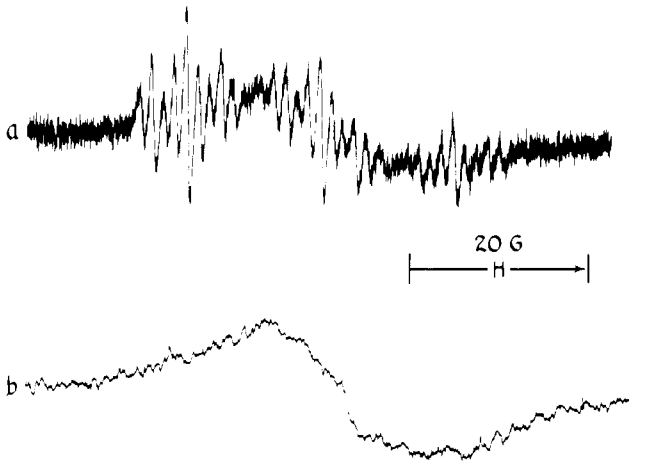

FIGURE 2: In spectrum a the signal of the *p*-nitrobenzoate dianion radical is superimposed on that of the flavine semiquinone. After 2 hr the *p*-nitrobenzoate dianion radical has decayed away leaving spectrum b of the flavine semiquinone. The reaction mixture contained FMN (1×10^{-2} *M*), NADPH (2×10^{-2} *M*), and *p*-nitrobenzoate (5×10^{-2} *M*). The spectrometer settings were the same as in Figure 1 except that the modulation amplitude was increased to 2.0 G for spectra b.

Symms and Juchau (1972) have reported the reduction of *p*-nitrobenzoic acid to *p*-aminobenzoic acid by a CO-sensitive model system consisting of NADPH (10^{-3} *M*), FMN (5×10^{-4} *M*), and any of several heme-containing compounds. Deletion of any one component from the complete system resulted in a total loss of activity. In our studies this model system also produced a steady-state concentration of the *p*-nitrobenzoate dianion radical (Table V). In agreement with the failure of CO to alter the radical concentration in the microsomal system, the absence of heme did not affect the steady-state concentration of *p*-nitrobenzoate dianion radical, implying that the heme is not involved in either the formation or reduction of the radical (Table V). The steady-state concentration was unchanged in the dark, precluding a photochemical reduction of NBzO to its dianion radical. In the absence of heme, $FMNH_2$ reduces *p*-nitrobenzoic acid to the corresponding hydroxylamine (Voshall and Carr, 1973). Projecting these results to the microsomal system would suggest that cytochrome P-450, the CO-sensitive component, donates electrons to some intermediates after the anion radical, most likely the hydroxylamine.

At higher concentrations of flavine the spectrum of the *p*-nitrobenzoate dianion radical is superimposed on that of FMNH· (Figure 2a), the one-electron oxidation product of

$FMNH_2$, implying that the formation of the flavine semiquinone radical occurs during the formation of *p*-nitrobenzoate dianion radical by a one-electron transfer. Fox and

$$FMNH_2 + {}^{-}O_2C{-}C_6H_4{-}NO_2 \rightleftharpoons FMNH\cdot + {}^{-}O_2C{-}C_6H_4{-}\dot{N}O_2^{-} + H^+$$

Tollin (1966a,b) have shown that FMNH· forms in this way during the one-electron reduction of the nitroprusside ion by the NADPH-FMN system.

Discussion

These studies indicate that the first intermediate in the reduction of both nitrobenzene and *p*-nitrobenzoic acid is probably the nitroaromatic anion radical, and further would suggest that this reaction can be anticipated whenever reduced flavine is present. In agreement with this, other flavoproteins such as xanthine oxidase (Taylor et al., 1951; Morita et al., 1971), liver aldehyde oxidase (Wolpert et al., 1973), and NADPH-cytochrome *c* reductase (Feller et al., 1971) have been reported to reduce many nitro compounds to their corresponding hydroxylamines.

These flavoproteins have been shown to donate only a single electron to potential two-electron acceptors such as quinones and oxygen (Iyanagi and Yamazaki, 1970), although in some cases two-electron donation can also be important. Therefore, the reduction of nitroaromatics by these flavoproteins is expected to proceed, at least in part, through the formation of the nitroaromatic anion radical intermediate. In particular, the microsomal flavoenzymes, NADPH-cytochrome *c* reductase and NADH-cytochrome b_5 reductase, both of which catalyze the reduction of quinones exclusively to their semiquinones (Iyanagi and Yamazaki, 1969), are probably the microsomal enzymes responsible for the reduction of nitroaromatics to their anion radicals.

A metabolite of nitro reduction is usually thought to be responsible for the toxicity of nitroaromatic compounds. Reductive activation is thought to be important in the mechanism of the covalent binding of nitroaromatic compounds to protein (McCalla et al., 1971; Ings et al., 1974). The carcinogenicity of highly conjugated 5-nitrofurans (Cohen et al., 1973), 4-nitroquinoline *N*-oxide (Kato et al., 1970), and nitroarenes (Poirier and Weisburger, 1974) may also require reduction of the nitro group. In the past, the responsible reactive metabolite has been thought to be the phenylhydroxylamine. Although the toxicity of the nitroaromatic anion radicals has not been demonstrated and little is known of their chemical behavior, this free radical may be one proximate toxin of nitroaromatic compounds.

Finally, it may well be that the microsomal azo reduction, which is also mediated by NADPH-cytochrome *c* reductase (Hernandez et al., 1967), stimulated by flavines, inhibited by oxygen (Fouts et al., 1957), and stimulated by 5-nitrofurans (Smith and Van Loom, 1969), is mediated through a similar free-radical mechanism.

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Acid-Volatile Selenium Formation Catalyzed by Glutathione Reductase†

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ABSTRACT: The production of acid-volatile selenide (apparently H_2Se) was catalyzed by glutathione reductase in an anaerobic system containing 20 m*M* glutathione, 0.05 m*M* sodium selenite, a TPNH-generating system, and microgram quantities of highly purified yeast glutathione reductase. H_2Se production in this system was proportional to glutathione reductase concentration and was maximal at pH 7. Significant nonenzymic H_2Se production occurred in the system lacking glutathione reductase and TPNH. A concentration of arsenite (0.1 m*M*) which does not inhibit glutathione reductase inhibited selenide volatilization, as did bovine serum albumin (1.67 mg/ml). Both appear to inhibit Se volatilization by reacting with the selenide product(s). The selenotrisulfide derivative of glutathione (GSSeSG) was readily converted to H_2Se by glutathione reductase and TPNH without the addition of glutathione. These results suggest that GSSeSG formed nonenzymically from glutathione and selenite undergoes stepwise reduction by glutathione reductase (or excess GSH) to GSSeH and finally to H_2Se. The same pathway operates when glutathione is used as the reducing agent but to a lesser extent.

Studies on the metabolism of sodium selenite show that selenite undergoes reduction in animals. Selenite is a weak oxidizing agent and reacts with thiol compounds such as cysteine, coenzyme A, 2-mercaptoethanol, glutathione (Ganther, 1968), or reduced pancreatic ribonuclease (Ganther and Corcoran, 1969) as proposed by Painter (1941):

$$H_2SeO_3 + 4RSH \longrightarrow RSSeSR + RSSR + 3H_2O \quad (1)$$

The reaction between selenite and glutathione is particularly interesting because glutathione is the most abundant thiol compound in animal tissues. Moreover, glutathione is specifically required, along with TPNH, for the synthesis of dimethyl selenide from selenite in a cell-free liver system (Ganther, 1966). Selenodiglutathione (GSSeSG[1]) is the first stable intermediate formed in the reaction of selenite and glutathione (Ganther, 1968; Sandholm and Sipponen, 1973). GSSeSG can be further reduced to a labile selenopersulfide (GSSeH) by glutathione reductase (reaction 2) or by excess glutathione (Ganther, 1971):

$$GSSeSG + TPNH + H^+ \xrightarrow{\text{glutathione reductase}} GSSeH + GSH + TPN^+ \quad (2)$$

Diplock et al. (1971) observed that acid-volatile selenium was released by adding strong acid to liver homogenates prepared from rats which had previously received selenite. It was noted that this acid-volatile selenium, believed to be H_2Se, underwent oxidation very easily and was difficult to study. Later these investigators developed a technique for quantitatively trapping H_2Se produced from selenite, zinc dust, and strong acid, in 0.1 *N* $AgNO_3$ (Diplock et al., 1973). Using a similar technique, it was observed in this laboratory that monothiols (glutathione and 2-mercaptoethanol) or dithiols formed H_2Se from selenite. Rhead and Schrauzer (1974) have provided kinetic evidence for such a reaction. This report presents evidence that H_2Se

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[1] Abbreviations used are: GSSeSG, selenodiglutathione; GSSeH, selenopersulfide; GSH, reduced glutathione.